Distribution Agreement

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

_____ _____

Dan B. Swartzlander Date

Regulation of Base Excision Repair in Response to Genotoxic Stress

By
Dan B. Swartzlander
Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
Genetics and Molecular Biology

_____

Paul W. Doetsch, Ph.D.
Advisor

_____

Anita H. Corbett, Ph.D.
Committee Member

_____

Gray F. Crouse, Ph.D.
Committee Member

_____

Yoke Wah Kow, Ph.D.
Committee Member

_____

Ichiro Matsumura, Ph.D.
Committee Member

Accepted:

_____

Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____

Date

Regulation of Base Excision Repair in Response to Genotoxic Stress

By

Dan B. Swartzlander
B.S., Carnegie Mellon University, 2004

Advisor: Paul W. Doetsch, Ph.D.

An abstract of
A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in Graduate Division of Biological and Biomedical Sciences
Genetics and Molecular Biology
2012

Abstract

Regulation of Base Excision Repair in Response to Genotoxic Stress

By Dan B. Swartzlander

Numerous human pathologies are the result of unrepaired oxidative DNA damage. Oxidative DNA damage is abundantly present in cells due to oxidative stress caused by environmental exposures and cellular metabolism. The base excision repair (BER) pathway initiated by N-glycosylase apurinic/apyrimidinic lyase proteins is primarily responsible for repair of oxidative DNA damage in both nuclear and mitochondrial genomes. Despite the importance of BER in maintaining nuclear and mitochondrial genomic stability, knowledge concerning the regulation of this evolutionarily conserved repair pathway is almost nonexistent. The *Saccharomyces cerevisiae* BER protein, Ntg1, was used as a model protein in order to elucidate mechanisms of BER regulation. Two separate but possibly related pathways for regulating BER were discovered, including dynamic localization and sumoylation of the model protein Ntg1. The regulation of BER by dynamic localization was elucidated through investigation of the intracellular localization of Ntg1 in response to nuclear and mitochondrial oxidative stress. These experiments revealed that Ntg1 is dynamically localized to nuclei and mitochondria and that Ntg1 localization is likely dependent on the oxidative DNA damage status of the organelle. In an effort to mechanistically define the regulatory components required for dynamic localization, the elements necessary for nuclear and mitochondrial localization of Ntg1 were identified. These elements included a bipartite classical nuclear localization signal, a mitochondrial matrix targeting sequence and the classical nuclear protein import machinery. Loss of these components compromises Ntg1 dynamic localization, and confers a mutator phenotype sensitizing cells to the cytotoxic effects of DNA damage. Furthermore, in an endeavor to characterize the regulation of BER by sumoylation, the mechanism of Ntg1 sumoylation was determined. Our results show that sumoylation of Ntg1 increases in response to oxidative stress, that it is associated with nuclear localization, and that it requires the E3 ligases Siz1/Siz2 to generate both monosumoylated and multisumoylated Ntg1. Mutational analysis of putative Ntg1 sumoylation sites reveals that Ntg1 is predominantly sumoylated at five distinct consensus sumoylation sites that cluster at both termini, where K396 is the major site of monosumoylation. Collectively, these results detail two biological pathways initiated by oxidative stress signaling, and concluding with the dynamic localization and the post-translation modification of a key BER protein. Our study provides insights into important mechanisms of BER regulation and into the pleiotropic effects of reactive oxygen species.

Regulation of Base Excision Repair in Response to Genotoxic Stress

By

Dan B. Swartzlander
B.S., Carnegie Mellon University, 2004

Advisor: Paul W. Doetsch, Ph.D.

A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in Graduate Division of Biological and Biomedical Sciences
Genetics and Molecular Biology
2012

Table of Contents

Figure and Tables

Chapter 1

General Introduction

Many human ailments are the result of unrepaired nuclear and mitochondrial oxidative DNA damage generated through normal cellular metabolism [1-12]. The base excision repair (BER) pathway is the primary pathway responsible for the repair of oxidative DNA damage and is highly conserved between *Saccharomyces cerevisiae* and humans [33]. Despite the importance of BER in maintaining nuclear and mitochondrial genomic stability, knowledge concerning the regulation of this pathway is almost nonexistent. In this dissertation *S. cerevisiae* is used as a model organism to examine the mechanisms underlying the regulation of BER in response to oxidative stress.

1. DNA Damage and Oxidative Stress

A plethora of agents can damage DNA. These factors can come from outside the cellular environment (exogenous) or from inside the cell itself (endogenous). Exogenous sources of DNA damage include ionizing radiation, UV radiation, and different chemical agents. Endogenous sources of DNA damage include alkylation, errors in DNA replication, spontaneous hydrolysis, and oxidative stress. Oxidative stress can be induced directly by reactive oxygen species (ROS) that are generated through normal cellular metabolism [1-4]. Some common ROS are superoxide radicals ($O_2^{\bullet -}$), hydroxyl radicals ($HO^{\bullet}$), and hydrogen peroxide (H_2O_2). Oxidative stress may also be induced indirectly through exposure to UV light, ozone, ionizing radiations, metals, pesticides, air pollutants or pharmaceutical drugs [1-4]. Every source of DNA damage generates a spectrum of damage, which ranges from single base lesions and complex distorting damages to single and double strand breaks [5]. Oxidative DNA damage has been linked to, and is perhaps causative of, aging and human diseases such as cancer and neurodegenerative diseases

[6-10]. Examples of oxidative DNA damages include thymine glycol, 5-hydroxycytosine, 5-hydroxy-5,6-dihydrothymine, and 8-oxoguanine (Figure 1). Oxidative DNA damage is thought to be one of the most frequently occurring spontaneous DNA damages. It is estimated that more than 90,000 oxidative lesions arise in a mammalian cell on a per day basis [9, 11]. Oxidative DNA damage that is left unrepaired may contribute to human disease through the generation of mutations and lethal blocks to replication and transcription [12].

2. DNA Damage in Organelles

Eukaryotic cells have DNA in two organelles, the nucleus and the mitochondria. Interestingly, mitochondrial DNA damage frequencies are estimated to be 10 – 70 times greater than nuclear DNA damage frequencies [13]. This observation may be caused by a number of important differences between the nuclei and the mitochondria. First, nuclear DNA is structurally organized into chromatin by histones that provide some protection from the elements [14]. Mitochondrial DNA lacks the organizational structure provided by chromatin; however, mitochondria have their own DNA binding proteins that may offer some level of protection [15]. Second, mitochondria are limited in their repair capacity compared to nuclei, because mitochondria contain fewer repair enzymes [16]. Finally, mitochondria are responsible for oxidative phosphorylation and subsequent energy production. This process leads to the creation of ROS in close proximity to the mitochondrial DNA [17]. For these reasons mitochondrial DNA is more vulnerable to DNA damage from oxidative stress and other agents than is the nuclear DNA. This concept was tested in a study that quantified the levels of single base lesions that result

from either ROS generated by normal metabolism (spontaneous) or by exogenous ROS (induced) sources. Spontaneous frequencies of 8-oxoguanine (8OG) in rat liver are estimated to be 1:130,000 in the nucleus and 1:8,000 in the mitochondria [18]. Induced levels of DNA damage are estimated to be roughly two- to three-fold higher in mitochondria than in nuclei, both in humans and in yeast [18].

In order to manage the high frequencies of DNA damage in the nucleus and the even higher frequencies in the mitochondria, all organisms have evolved mechanisms to repair DNA in both organelles. Historically however, DNA repair has been studied with a more nuclear or perhaps prokaryotic point of view. The result is the characterization of six DNA maintenance pathways with well-defined nuclear modes of action (Figure 2) [19, 20]. They are nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), recombination repair (RR), translesion synthesis (TLS), and direct reversal [19, 20]. Direct reversal, NER, BER, and MMR, lead to the removal of damaged bases and restoration of the original DNA. NER primarily removes bulky, helix distorting lesions from the DNA. BER primarily removes single base lesions and processes apurinic/apyrimidinic (AP) sites. MMR repairs mismatches that arise during DNA replication [19]. Direct reversal is a process where a DNA lesion is reversed on the DNA, and is a process possessed at different levels by eukaryotes such as *Saccharomyces cerevisiae* and humans [21]. During TLS and RR, DNA damage is not removed and repaired; instead, it is tolerated. TLS allows the replication machinery to replicate past DNA lesions, and RER utilizes homologous sequences to bypass a lesion during replication [19]. All of these pathways (except NER) are present not only in the nucleus but also to some extent in the mitochondria where they maintain the mitochondrial

genome (Figure 3) [19]. Several of the mitochondrial repair pathways are limited in their ability to maintain the mitochondrial genome, because not all of the proteins involved in nuclear repair are present in the mitochondria. However, mitochondrial BER is fully competent for the repair of oxidative DNA damage, because most BER enzymes that are active in the nucleus are also active in the mitochondria (Table 1) [16, 19, 22-26].

3. Base Excision Repair

BER is the primary pathway for the repair of single base lesions including oxidative, alkylation damages, and AP sites. These lesions can arise spontaneously or can be caused by environmental sources and metabolic processes (Figure 4) [27, 28]. The first step in BER is recognition of a damaged base by different *N*-glycosylases that remove the damaged base from the DNA and create an AP site [29, 30]. Following removal of the damaged base, AP sites are processed by either an AP lyase or an AP endonuclease. These enzymes cleave the sugar-phosphate DNA backbone of AP sites on the 3' or 5' side respectively [31]. *N*-glycosylases can be subdivided into categories that depend upon their substrate specificities and their *N*-glycosylase and/or AP lyase activities (mono- or bifunctional). Monofunctional *N*-glycosylases recognize and remove damaged bases from DNA leaving behind an intact AP site [32]. Bifunctional *N*-glycosylases are AP lyases in addition to *N*-glycosylases. This dual function allows them to subsequently process the AP site to a 3' unsaturated aldehyde blocking group [32]. This blocking group is then processed by a 3' phosphodiesterase to generate the 3' end that is necessary for subsequent polymerization and repair [32]. The single nucleotide

gaps are then filled in by DNA polymerases, and finally DNA ligases restore the covalent integrity of the DNA [33].

A number of studies in humans and in the model system, *S. cerevisiae,* highlight the importance of the BER pathway for biological fitness [19, 20,34]. In contrast, the loss of function of BER, NER, TLS, or RR has little effect on viability in *S. cerevisiae* [34]. The only pathway that can support cell viability as the sole repair pathway (meaning three out of four pathways have been lost), is the BER pathway [34]. Isolated NER, TLS, and RR pathways cannot maintain cellular viability [34]. These results show the critical importance of the BER pathway in the maintenance of cell viability. Furthermore, these results suggest that the inability to repair spontaneously occurring base lesions, not other DNA damages, is lethal, because the inability to repair spontaneously forming bulky adducts, single strand breaks, and double strand breaks does not make cells inviable. There are also many instances in primary cancers where BER protein functions have been altered or lost [35-43]. These instances suggest that the BER pathway is important in cancer prevention. For example, altered function of human thymine glycol-DNA-glycosylase (NTHL1), human 8-oxoguanine DNA glycosylase (OGG1), thymine DNA glycosylase (TDG), uracil DNA glycosylase (UNG), and hMYH have all been associated with different types of cancers [35-40]. Lastly, the observation that Alzheimer's patients have decreased activity of both UNG and OGG1 implicates BER in neurodegenerative disorders [41-43]. The above examples represent a fraction of the evidence regarding the importance of BER for the fitness of eukaryotic cells. These examples also illustrate the need to gain a better understanding of BER regulation under both stress and non-stress conditions.

4. *Saccharomyces cerevisiae* as a Model System

The budding yeast, *S. cerevisiae* also referred to as baker's yeast, provides an excellent model organism for the study of the BER pathway. *S. cerevisiae* is a single celled eukaryotic organism that can exist in both diploid and haploid states; furthermore, the fundamental biological pathways in budding yeast are highly similar to the pathways found in humans [27]. The diploid and haploid states of the *S. cerevisiae* genome make it a very convenient organism for genetic manipulation and subsequent biological study. Researchers have utilized this model extensively to examine the mechanisms underlying the DNA damage management pathways [27, 44, 45]. Moreover, the rapid rate of cell growth and array of techniques developed for use in *S. cerevisiae* allows for informative and accurate interrogation of biochemical activities, protein localization, nuclear and mitochondrial mutagenesis, as well as the contributions that individual BER proteins have on BER function [44]. Unlike most eukaryotic organisms, *S. cerevisiae* survives without functional mitochondria. This fact makes *S. cerevisiae* an ideal model system for the study of BER in mitochondria [44, 46, 47].

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

BER is initiated by a number of different *N*-glycosylases. The *S. cerevisiae* Ntg1 and Ntg2 proteins belong to a class of endonuclease III/thymine glycol-DNA-glycosylases (Table 1). These two proteins are encoded on chromosomes I and XV, respectively [48]. Ntg1 and Ntg2 are closely related; they share significant sequence similarity/identity to one another (41% identity, 63% similarity), and to *Escherichia coli*

Nth (Ntg1: 24% identity, 46% similarity; Ntg2: 25% identity, 51% similarity) [49]. The catalytic domains of both proteins are almost identical and are comprised of helix-hairpin-helix motifs [45, 49]. Both Ntg1 and Ntg2 proteins are bi-functional *N*-glycosylase/AP lyase enzymes. They are also homologs of the human thymine glycol-DNA-glycosylase (NTHL1) protein. These two glycosylases recognize and excise the following substrates: 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-OHUra), 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) [50, 51]. As Ntg1 and Ntg2 have AP lyase activity, they both contribute to the repair of AP sites that are generated either spontaneously or through *N*-glycosylase activity [52, 53]. Even though Ntg1 and Ntg2 have very similar substrate specificities, their localization patterns differ significantly [54]. Ntg1 localizes to both nuclei and to mitochondria [54]. Ntg1 contains both a bipartite classical nuclear localization signal (cNLS) and a mitochondrial matrix targeting sequence (MTS) [54]. In contrast, Ntg2 has a strictly nuclear localization and contains only a putative cNLS [54]. Ntg2 has an iron sulfur center while Ntg1 does not, which suggests that during the evolution of *S. cerevisiae* it became counterproductive for Ntg1 to contain an iron sulfur center [49, 50]. An iron sulfur center could be disadvantageous in the mitochondria, where the high levels of ROS may be further increased by the Fenton reaction, which would cause additional DNA damage [55, 56].

In the context of BER, Ntg1 and Ntg2 each play a significant role in the repair of damaged DNA by protecting both nuclear and mitochondrial DNA from oxidative stress and other genotoxic insults [57]. However, there is significant redundancy in DNA repair; therefore, the loss of function of Ntg1, Ntg2, and the primary *S. cerevisiae* AP endonuclease, Apn1, is required to confer compromised BER function [57]. This resulting triple mutant has increased nuclear mutation rates, and has increased chromosomal instability, highlighting the importance of BER for the removal and repair of spontaneously occurring nuclear oxidative lesions [57, 58]. A fourth deletion that disrupts NER, through the loss of function of Rad1, is required for genomic integrity to be substantially compromised. This quadruple mutant renders these cells both NER and BER-deficient [57]. NER partially compensates for the loss of BER, which illustrates the redundancy that exists in DNA management pathways. The nuclear phenotypes described above are understandable given the nuclear localization of Ntg1 and Ntg2. However, unlike Ntg2, Ntg1 can also localize to the mitochondria where it is one of very few BER proteins present. As one of a few BER proteins in the mitochondria, Ntg1 plays an important role in mitochondrial DNA repair (Table 1). Loss of Ntg1 by itself leads to an increase of mitochondrial oxidative DNA damage as neither Ntg2 nor NER can compensate for loss of Ntg1 [22, 24]. The human homologue of Ntg1, NTHL1, is also important for genomic stability. Loss of NTHL1 function is associated with lung and gastric cancers [36, 40, 59]. These observations suggest NTHL1 mutations in contribute to a mutator phenotype perhaps driving carcinogenesis and metastasis.

6. Regulation of Base Excision Repair: Current Concepts and Observations

The activities of BER proteins must be tightly regulated, as loss of BER activity perhaps leads to or further contributes to the development primary cancers and neurodegenerative disorders. Regulation of base excision repair has been proposed to occur through a variety of different mechanisms including transcriptional regulation, post-translational modification, regulation of protein localization, and protein-protein interactions (Table 1) [60]. Each of these mechanisms may diversely regulate BER and alter DNA repair capacity.

Protein localization is perhaps the most fundamental form of regulation in eukaryotic BER, as repair proteins must be given access to the genomic DNA to function and to remove DNA damage. All BER proteins have mechanisms for nuclear localization. Most of these proteins likely do so by using the classical nuclear import pathway (Table 1) [61]. Classical nuclear import operates through the recognition of a cNLS by importin α. Importin α binds the cargo protein in the cytoplasm and then imports the cargo into the nucleus through nuclear pores in complex with importin β [62]. Functional mitochondria are also important for cell survival and fitness making the maintenance of mitochondrial DNA by BER proteins critical. The sequences of most BER proteins that localize to the mitochondria contain mitochondrial matrix targeting sequences (MTS). These sequences are usually located in the N-terminal region of a protein and consist of 10-80 amino acids and form amphipathic α-helices that are recognized by the mitochondrial outer membrane translocase [63, 64]. Proteins with an MTS enter the mitochondrial matrix by passing through the outer membrane translocase and then through the inner membrane translocase [65-67]. Protein localization can also

be controlled through protein-protein interactions and post-translational modifications [68, 69].

For some BER proteins their steady-state transcript levels are regulated in a cell cycle-dependent manner, while for other BER proteins steady-state transcript levels change in response to oxidative stress (Table 1) [70]. For example, changes in transcript levels have been documented for *N*-methylpurine DNA glycosylase (MPG), NTHL1, UNG, and human AP site-specific endonuclease (APEX1) in a cell cycle-dependent manner, increasing 2.5- to 3.5-fold during G_1 and decreasing to basal levels following mitosis [71]. On the other hand, the mRNA levels of NTHL1, OGG1, and APEX1 increase by as much as three-fold when cells are exposed to hydrogen peroxide or to other reactive oxygen species (ROS) [72-74].

Protein-protein interactions increase the efficacy of repair in mammals and link BER to different pathways, such as NER or mismatch repair (MMR) (Table 1) [75]. For example, several human DNA glycosylases have increased base excision activity in the presence of APEX1 (Table 1) [75]. Physical interactions also occur; for example, to enhance base excision activity specific glycosylases associate with X-ray repair cross complementing group 1, (XRCC1) (Table 1) [76]. Many BER proteins interact with proliferating cellular nuclear antigen, (PCNA), and/or replication protein A, (RPA), which suggests that they are involved in post-replication BER responses (Table 1) [77-80].

Known post-translational modifications of BER proteins include phosphorylation, acetylation, nitrosylation, ubiquitination, and sumoylation (Table 1). Phosphorylation of

human OGG1, A/G-specific adenine DNA glycosylase (MUTYH), and UNG affects enzyme excision activity in different ways (Table 1) [81-83]. Acetylation by the histone acetyltransferase, p300, regulates OGG1, thymine DNA glycosylase (TDG), endonuclease VIII-like 2 (NEIL2), polymerase beta (polβ), and APEX1 each in a unique manner (Table 1) [70]. For instance, acetylation of OGG1 causes a decrease in its affinity for AP sites and increases the rate of OGG1 turn over through stimulation by APEX1. In contrast, acetylation of NEIL2 significantly reduces glycosylase and AP lyase activities of this enzyme [84, 85]. Nitrosylation modifies the activity of APEX1 and OGG1, inhibiting repair activity, by triggering nuclear export or by inhibiting excision activity, respectively [86-88]. Ubiquitination typically leads to degradation and thus provides a way to control steady-state protein levels, as is reported for Polβ [89]. Ubiquitination of APEX1, however, does not affect protein degradation, and could play additional roles in APEX1 regulation that remain unknown [90]. Lastly, there are examples of sumoylation of a few BER proteins including human TDG, Ntg1, and Ntg2 [91, 92]. TDG sumoylation is the best understood case, where the small ubiquitin related modifier (SUMO), of sumoylated TDG competes with the regulatory domain of TDG for DNA binding. This competition allows TDG to overcome product inhibition and ultimately increases enzyme turnover [91, 93].

Overall, the regulation of BER is only understood to a limited extent and includes only a handful of examples. Our limited knowledge of BER regulation and its potential role in cancer pathology and neurodegenerative disorders makes it important to elucidate new mechanisms of BER regulation in response to cellular stress.

7. Sumoylation as a Means of Protein Regulation

Sumoylation is a pathway which covalently attaches the small SUMO protein to a substrate protein by formation of an isopeptide bond in a monosumoylated (one SUMO), multisumoylated (many SUMO at different sites), or polysumoylated (polymeric SUMO at one site) manner (Figure 5) [94-97]. This pathway is closely related to ubiquitination, and it involves a cascade of conjugation catalyzed by an E1, an E2, and E3 ligases. Sumoylation is very a dynamic process that is readily reversible and modifies protein function many different ways. In *S. cerevisiae* the E1 is a heterodimeric complex that is made up of Uba2 and Aos1. It forms a thioester linkage between an active cysteine on the E1 and the C-terminal glycine of SUMO in an ATP-dependent process [98]. The active SUMO protein is then transferred to the E2 conjugating enzyme Ubc9 by another thioester linkage. This is an intermediate step that occurs before conjugating SUMO to a specific lysine residue on the substrate via the SUMO C-terminal di-glycine motif [99, 100]. E1 and E2 ligases can sumoylate a protein *in vitro*; however, *in vivo*, SUMO conjugation to a substrate is mediated and often enhanced by one of four E3 ligases that allow regulated conjugation [101]. These ligases are Siz1, Siz2, Zip3, and MMS21. Siz1 and Siz2 contribute to global sumoylation levels and have partially overlapping substrate specificities [102]. Mms21 is part of the Smc5/6 complex in *S. cerevisiae*; this complex aids in the repair of double stranded breaks via homologous recombination. Mms21 sumoylates Ku70 and Smc5 while part of the Smc5/6 complex [103, 104]. Zip3 is responsible for initiation of assembly of the synaptonemal complex, and it is also responsible for the formation of polymeric SUMO chains along meiotic chromosomes [95].

Sumoylation is a reversible process; desumoylation is carried out by the two SUMO proteases Ulp1 and Ulp2 [105, 106]. Ulp1 is an essential protein required for the G_2 to M transition. Ulp1 localizes to the nuclear pore complex where it functions in SUMO maturation and as the major desumoylation enzyme [105, 107, 108]. Ulp2 is not essential for viability, and it localizes predominantly to the nucleoplasm. Ulp2 is primarily responsible for desumoylation of polymeric SUMO chains [96].

A large number of proteins are sumoylated in yeast and in humans [109, 110]. The majority of these proteins localize to the nucleus, hence the nuclear-centric nature of the sumoylation pathway. Approximately two-thirds of known SUMO substrates contain at least one consensus sumoylation motif Ψ-K-x-D/E (where Ψ is a hydrophobic residue, K is the lysine conjugated to SUMO, x is any amino acid, and D/E is an acidic residue). Additionally, between one-third to one-half of all human proteins sequences contain this motif. This observation suggests that many SUMO substrates have not been identified [110, 111]. Resolved crystal structures of Ubc9 (E2) and their SUMO substrates show that consensus motifs adopt an extended conformation in which the substrate acceptor lysine fits into the hydrophobic grove of Ubc9. These results indicate that SUMO consensus motifs are more likely to associate with Ubc9 when they arise in extended loops or intrinsically disordered regions [112, 113]. SUMO E3 ligases are thought to catalyze SUMO transfer through two mechanisms. First, E3 ligases may bind the E2-SUMO thioester. This interaction facilitates a more productive orientation for catalysis and enhances SUMO conjugation [114]. Second, E3 ligases may directly interact with the substrate and recruit the E2-SUMO thioester into a complex that facilitates conjugation [114].

The regulation of sumoylation and desumoylation is a complex process that is subject to redox-regulation [115-121]. Redox-regulation of sumoylation primarily depends on the redox state of cysteine residues in the active sites of sumoylation proteins. Cysteine residues can be reversibly oxidized to SOH groups and irreversibly oxidized to SO_2 and SO_3 groups [122]. The active sites of both the E1 and E2 conjugating enzymes require reduced cysteine residues. For example, oxidation of E1 and E2 by hydrogen peroxide inhibits sumoylation of both c-Fos and c-Jun [123-125]. The active sites of all human and *S. cerevisiae* SUMO proteases, except Ulp2, also have cysteine residues. Oxidation of the Ulp1 active site by exposure to hydrogen peroxide leads to a global increase in the levels of sumoylated proteins [126]. Since both sumoylation and desumoylation are redox-regulated, substrate-specific SUMO modification must be characterized on a case by case basis

The sumoylation pathway has many diverse targets that range from transcription factors and proteins comprising ion channels, to transport and DNA maintenance proteins [127-151]. Transcriptional regulation is strongly modified by sumoylation. Most of the known sumoylation events that regulate transcription lead to the suppression of transcription. For example, sumoylation can augment the function of a transcriptional repressor, or it can inhibit the function of a transcriptional activator [127-131]. However, there are a growing number of cases where sumoylation has been shown to enhance transcriptional activity [132-135]. Sumoylation alters transcription factor activity by changing protein localization and/or folding.

Genomic integrity is yet another area in which protein sumoylation plays a significant role. For instance, proliferating cell nuclear antigen, (PCNA), is sumoylated

during S-phase and inhibits recombination [136-138]. Rad52 is sumoylated in response to DNA damage. The Mre11-Rad50-Xrs2 (MRX) complex initiates this sumoylation event and protects Rad52 from proteasomal degradation, which increases homologous recombination [137, 139]. As previously mentioned, sumoylation of the BER protein, TDG, and the E3 ligase, Mms21, increases genomic stability.

Sumoylation is also important in nucleocytoplasmic trafficking. RanGAP1, an important protein in nucleocytoplasmic trafficking, needs to be sumoylated in order to localize to the nuclear pore complex (NPC) [140-143]. Once at the NPC, RanGAP1 activates the small GTPase, Ran, which is essential for the translocation of proteins through the NPC [140-143]. Furthermore, sumoylation of ribosomal precursor particles in the nucleus and desumoylation of these particles at NPC by Ulp1 is required for efficient ribosome biogenesis and export of these particles to the cytoplasm [116, 144, 145].

In addition to nucleocytoplasmic trafficking, sumoylation is also implicated in directing protein localization. For example, sumoylation directs the subnuclear localization of proteins to PML nuclear bodies, polycomb group bodies, DNA damage foci, Cajal bodies, centrosomes, and centromeres [146-149]. Moreover, sumoylation is involved in nuclear export of proteins. MEK1 for example is exported from the nucleus once it is sumoylated [150]. Lastly, sumoylation has a role in subcytoplasmic and plasma membrane targeting. Sumoylation of DRP1, a GTPase required for mitochondrial fission, promotes its recruitment from the cytosol to the mitochondrial outer membrane [151].

Sumoylation is also involved in protein longevity where it has two opposing roles. First, SUMO can stabilize proteins by antagonizing ubiquitination. For example, sumoylation of IκBα, Smad4, and Huntingtin proteins prevents ubiquitin modification and subsequent degradation [152-154]. At the same time, sumoylation can destabilize proteins because polymeric SUMO chains often serve as a signal for protein ubiquitination and subsequent degradation (Figure 6) [155-159]. SUMO-targeted ubiquitination occurs through the E3 ubiquitin ligase heterodimer, which consists of the Slx5 and Slx8 proteins. These proteins contain SUMO interacting motifs (SIM), which target them to proteins containing polymeric SUMO chains. Lastly, protein sumoylation is not only able to antagonize ubiquitination, but it can also block protein acetylation on lysine residues. For instance, sumoylation of the transcription factor, MEF2, inhibits its acetylation and consequently blocks its transactivator function [160, 161].

The de-regulation of sumoylation contributes to a wide range of disorders including cancer, cardiovascular development, neurodegenerative diseases, pathogen-host interactions, and type I diabetes [162]. The clinical implications of SUMO de-regulation emphasize the importance of understanding the function and regulation of sumoylated proteins. Furthermore, there are a large number of pathways that involve SUMO modifications. These pathways are diverse and the role that sumoylation plays in their regulation is not well understood.

8. Summary of Project Objectives

This research project examines the regulation of BER in response to oxidative stress. Specifically, the *S. cerevisiae* protein Ntg1 was used as a model for studying the

regulation of BER protein functions in response to oxidative stress. Our studies reveal two mechanisms of Ntg1 regulation and address their impact on DNA repair capacity through the analysis of Ntg1 localization and post-translational modification as well as cellular mutation rates, cytotoxicity, and enzyme activity. Ntg1 dynamically relocalizes to nuclei or mitochondria in response to organelle-specific oxidative stress, which is induced by either hydrogen peroxide (nuclear) or hydrogen peroxide and antimycin A (mitochondrial). Dynamic localization of Ntg1 requires its bipartite classical nuclear localization signal, its mitochondrial matrix targeting sequence, and the classical nuclear import machinery. Furthermore, we find that Ntg1 is sumoylated in response to oxidative stress. Sumoylation of Ntg1 is associated with the nucleus and occurs at any of five different sumoylation consensus sequences within Ntg1. A lysine at position 396 is the primary site of monosumoylation. We determine that Ntg1 is sumoylated primarily by the E3 ligase, Siz1, and desumoylated by the SUMO protease, Ulp1. Lastly, we determine that sumoylation of Ntg1 is a multifaceted and potentially ordered process that has distinct patterns of sumoylation depending upon the lysines available. Collectively, these results detail two possibly linked methods of regulating the BER pathway, both of which commence with oxidative stress signaling. The first concludes with the trafficking of a key BER protein to organelles containing high levels of oxidative DNA damage. The second concludes with the post-translation modification of the protein by sumoylation. These novel discoveries provide insight into not just important mechanisms of regulating BER, but also into the broad roles of protein localization and sumoylation themselves.

9. References

1. Finkel, T. and N.J. Holbrook, *Oxidants, oxidative stress and the biology of ageing.* Nature, 2000. **408**(6809): p. 239-47.
2. Turrens, J.F., *Mitochondrial formation of reactive oxygen species.* J Physiol, 2003. **552**(Pt 2): p. 335-44.
3. Limon-Pacheco, J. and M.E. Gonsebatt, *The role of antioxidants and antioxidant-related enzymes in protective responses to environmentally induced oxidative stress.* Mutation research, 2009. **674**(1-2): p. 137-47.
4. Lindahl, T., *Instability and decay of the primary structure of DNA.* Nature, 1993. **362**(6422): p. 709-15.
5. Hoeijmakers, J.H., *DNA damage, aging, and cancer.* N Engl J Med, 2009. **361**(15): p. 1475-85.
6. Wei, Y.H., *Oxidative stress and mitochondrial DNA mutations in human aging.* Proc Soc Exp Biol Med, 1998. **217**(1): p. 53-63.
7. Wallace, D.C., *Diseases of the mitochondrial DNA.* Annu Rev Biochem, 1992. **61**: p. 1175-212.
8. Cooke, M.S., et al., *Oxidative DNA damage: mechanisms, mutation, and disease.* FASEB J, 2003. **17**(10): p. 1195-214.
9. Beckman, K.B. and B.N. Ames, *Oxidative decay of DNA.* The Journal of biological chemistry, 1997. **272**(32): p. 19633-6.
10. Lenaz, G., *Role of mitochondria in oxidative stress and ageing.* Biochimica et biophysica acta, 1998. **1366**(1-2): p. 53-67.

11. Fraga, C.G., et al., *Oxidative damage to DNA during aging: 8-hydroxy-2'-deoxyguanosine in rat organ DNA and urine.* Proc Natl Acad Sci U S A, 1990. **87**(12): p. 4533-7.

12. Ide, H., Y.W. Kow, and S.S. Wallace, *Thymine glycols and urea residues in M13 DNA constitute replicative blocks in vitro.* Nucleic acids research, 1985. **13**(22): p. 8035-52.

13. Haag-Liautard, C., et al., *Direct estimation of the mitochondrial DNA mutation rate in Drosophila melanogaster.* PLoS Biol, 2008. **6**(8): p. e204.

14. Enright, H.U., W.J. Miller, and R.P. Hebbel, *Nucleosomal histone protein protects DNA from iron-mediated damage.* Nucleic acids research, 1992. **20**(13): p. 3341-6.

15. Caron, F., C. Jacq, and J. Rouviere-Yaniv, *Characterization of a histone-like protein extracted from yeast mitochondria.* Proc Natl Acad Sci U S A, 1979. **76**(9): p. 4265-9.

16. Bohr, V.A., *Repair of oxidative DNA damage in nuclear and mitochondrial DNA, and some changes with aging in mammalian cells.* Free radical biology & medicine, 2002. **32**(9): p. 804-12.

17. Miquel, J., *An integrated theory of aging as the result of mitochondrial-DNA mutation in differentiated cells.* Arch Gerontol Geriatr, 1991. **12**(2-3): p. 99-117.

18. Richter, C., J.W. Park, and B.N. Ames, *Normal oxidative damage to mitochondrial and nuclear DNA is extensive.* Proc Natl Acad Sci U S A, 1988. **85**(17): p. 6465-7.

19. Boesch, P., et al., *DNA repair in organelles: Pathways, organization, regulation, relevance in disease and aging.* Biochimica et biophysica acta, 2011. **1813**(1): p. 186-200.

20. Larsen, N.B., M. Rasmussen, and L.J. Rasmussen, *Nuclear and mitochondrial DNA repair: similar pathways?* Mitochondrion, 2005. **5**(2): p. 89-108.

21. Mishina, Y., E.M. Duguid, and C. He, *Direct reversal of DNA alkylation damage.* Chem Rev, 2006. **106**(2): p. 215-32.

22. Doudican, N.A., et al., *Oxidative DNA damage causes mitochondrial genomic instability in Saccharomyces cerevisiae.* Molecular and cellular biology, 2005. **25**(12): p. 5196-204.

23. Mandavilli, B.S., J.H. Santos, and B. Van Houten, *Mitochondrial DNA repair and aging.* Mutation research, 2002. **509**(1-2): p. 127-51.

24. O'Rourke, T.W., et al., *Mitochondrial dysfunction due to oxidative mitochondrial DNA damage is reduced through cooperative actions of diverse proteins.* Molecular and cellular biology, 2002. **22**(12): p. 4086-93.

25. Pinz, K.G. and D.F. Bogenhagen, *Efficient repair of abasic sites in DNA by mitochondrial enzymes.* Molecular and cellular biology, 1998. **18**(3): p. 1257-65.

26. Singh, K.K., et al., *Inactivation of Saccharomyces cerevisiae OGG1 DNA repair gene leads to an increased frequency of mitochondrial mutants.* Nucleic acids research, 2001. **29**(6): p. 1381-8.

27. Memisoglu, A. and L. Samson, *Base excision repair in yeast and mammals.* Mutation research, 2000. **451**(1-2): p. 39-51.

28. Nilsen, H. and H.E. Krokan, *Base excision repair in a network of defence and tolerance.* Carcinogenesis, 2001. **22**(7): p. 987-98.

29. Lindahl, T., *New class of enzymes acting on damaged DNA.* Nature, 1976. **259**(5538): p. 64-6.

30. Lindahl, T., *DNA glycosylases, endonucleases for apurinic/apyrimidinic sites, and base excision-repair.* Prog Nucleic Acid Res Mol Biol, 1979. **22**: p. 135-92.

31. Barnes, D.E., T. Lindahl, and B. Sedgwick, *DNA repair.* Curr Opin Cell Biol, 1993. **5**(3): p. 424-33.

32. Marenstein, D.R., et al., *Substrate specificity of human endonuclease III (hNTH1). Effect of human APE1 on hNTH1 activity.* The Journal of biological chemistry, 2003. **278**(11): p. 9005-12.

33. Boiteux, S. and M. Guillet, *Abasic sites in DNA: repair and biological consequences in Saccharomyces cerevisiae.* DNA repair, 2004. **3**(1): p. 1-12.

34. Morey, N.J., P.W. Doetsch, and S. Jinks-Robertson, *Delineating the requirements for spontaneous DNA damage resistance pathways in genome maintenance and viability in Saccharomyces cerevisiae.* Genetics, 2003. **164**(2): p. 443-55.

35. Radak, Z., et al., *Lung cancer in smoking patients inversely alters the activity of hOGG1 and hNTH1.* Cancer letters, 2005. **219**(2): p. 191-5.

36. Karahalil, B., V.A. Bohr, and N.C. De Souza-Pinto, *Base excision repair activities differ in human lung cancer cells and corresponding normal controls.* Anticancer research, 2010. **30**(12): p. 4963-71.

37. Sweasy, J.B., T. Lang, and D. DiMaio, *Is base excision repair a tumor suppressor mechanism?* Cell Cycle, 2006. **5**(3): p. 250-9.

38. Al-Tassan, N., et al., *Inherited variants of MYH associated with somatic G:C-->T:A mutations in colorectal tumors.* Nat Genet, 2002. **30**(2): p. 227-32.

39. Sieber, O.M., et al., *Multiple colorectal adenomas, classic adenomatous polyposis, and germ-line mutations in MYH.* N Engl J Med, 2003. **348**(9): p. 791-9.

40. Goto, M., et al., *Altered expression of the human base excision repair gene NTH1 in gastric cancer.* Carcinogenesis, 2009. **30**(8): p. 1345-52.

41. Coppede, F. and L. Migliore, *DNA damage and repair in Alzheimer's disease.* Current Alzheimer research, 2009. **6**(1): p. 36-47.

42. Jeppesen, D.K., V.A. Bohr, and T. Stevnsner, *DNA repair deficiency in neurodegeneration.* Progress in neurobiology, 2011. **94**(2): p. 166-200.

43. Weissman, L., et al., *Defective DNA base excision repair in brain from individuals with Alzheimer's disease and amnestic mild cognitive impairment.* Nucleic acids research, 2007. **35**(16): p. 5545-55.

44. Sherman, F., *Getting started with yeast.* Methods Enzymol, 2002. **350**: p. 3-41.

45. Eide, L., et al., *Base excision of oxidative purine and pyrimidine DNA damage in Saccharomyces cerevisiae by a DNA glycosylase with sequence similarity to endonuclease III from Escherichia coli.* Proceedings of the National Academy of Sciences of the United States of America, 1996. **93**(20): p. 10735-40.

46. Ferguson, L.R. and R.C. von Borstel, *Induction of the cytoplasmic 'petite' mutation by chemical and physical agents in Saccharomyces cerevisiae.* Mutation research, 1992. **265**(1): p. 103-48.

47. Ephrussi, B., H. de Margerie-Hottinguer, and H. Roman, *Suppressiveness: A New Factor in the Genetic Determinism of the Synthesis of Respiratory Enzymes in Yeast.* Proc Natl Acad Sci U S A, 1955. **41**(12): p. 1065-71.

48. Barton, A.B. and D.B. Kaback, *Molecular cloning of chromosome I DNA from Saccharomyces cerevisiae: analysis of the genes in the FUN38-MAK16-SPO7 region.* J Bacteriol, 1994. **176**(7): p. 1872-80.

49. Augeri, L., et al., *Purification, characterization, gene cloning, and expression of Saccharomyces cerevisiae redoxyendonuclease, a homolog of Escherichia coli endonuclease III.* Biochemistry, 1997. **36**(4): p. 721-9.

50. You, H.J., R.L. Swanson, and P.W. Doetsch, *Saccharomyces cerevisiae possesses two functional homologues of Escherichia coli endonuclease III.* Biochemistry, 1998. **37**(17): p. 6033-40.

51. Senturker, S., et al., *Substrate specificities of the ntg1 and ntg2 proteins of Saccharomyces cerevisiae for oxidized DNA bases are not identical.* Nucleic acids research, 1998. **26**(23): p. 5270-6.

52. Hanna, M., et al., *Involvement of two endonuclease III homologs in the base excision repair pathway for the processing of DNA alkylation damage in Saccharomyces cerevisiae.* DNA repair, 2004. **3**(1): p. 51-9.

53. Meadows, K.L., B. Song, and P.W. Doetsch, *Characterization of AP lyase activities of Saccharomyces cerevisiae Ntg1p and Ntg2p: implications for biological function.* Nucleic acids research, 2003. **31**(19): p. 5560-7.

54. Swartzlander, D.B., et al., *Regulation of base excision repair: Ntg1 nuclear and mitochondrial dynamic localization in response to genotoxic stress.* Nucleic acids research, 2010. **38**(12): p. 3963-74.

55. You, H.J., et al., *Saccharomyces cerevisiae Ntg1p and Ntg2p: broad specificity N-glycosylases for the repair of oxidative DNA damage in the nucleus and mitochondria.* Biochemistry, 1999. **38**(35): p. 11298-306.

56. Cadet, J., et al., *Hydroxyl radicals and DNA base damage.* Mutation research, 1999. **424**(1-2): p. 9-21.

57. Swanson, R.L., et al., *Overlapping specificities of base excision repair, nucleotide excision repair, recombination, and translesion synthesis pathways for DNA base damage in Saccharomyces cerevisiae.* Molecular and cellular biology, 1999. **19**(4): p. 2929-35.

58. Degtyareva, N.P., et al., *Chronic oxidative DNA damage due to DNA repair defects causes chromosomal instability in Saccharomyces cerevisiae.* Molecular and cellular biology, 2008. **28**(17): p. 5432-45.

59. Koketsu, S., T. Watanabe, and H. Nagawa, *Expression of DNA repair protein: MYH, NTH1, and MTH1 in colorectal cancer.* Hepato-gastroenterology, 2004. **51**(57): p. 638-42.

60. Saha, T., et al., *Transcriptional regulation of the base excision repair pathway by BRCA1.* The Journal of biological chemistry, 2010. **285**(25): p. 19092-105.

61. Knudsen, N.O., et al., *Nuclear translocation contributes to regulation of DNA excision repair activities.* DNA repair, 2009. **8**(6): p. 682-9.

62. Gorlich, D., et al., *Two different subunits of importin cooperate to recognize nuclear localization signals and bind them to the nuclear envelope.* Current biology : CB, 1995. **5**(4): p. 383-92.

63. Neupert, W. and J.M. Herrmann, *Translocation of proteins into mitochondria.* Annu Rev Biochem, 2007. **76**: p. 723-49.

64. Bruch, M.D. and D.W. Hoyt, *Conformational analysis of a mitochondrial presequence derived from the F1-ATPase beta-subunit by CD and NMR spectroscopy.* Biochimica et biophysica acta, 1992. **1159**(1): p. 81-93.

65. Wang, Y. and H. Weiner, *Evaluation of electrostatic and hydrophobic effects on the interaction of mitochondrial signal sequences with phospholipid bilayers.* Biochemistry, 1994. **33**(43): p. 12860-7.

66. Dekker, P.J., et al., *The Tim core complex defines the number of mitochondrial translocation contact sites and can hold arrested preproteins in the absence of matrix Hsp70-Tim44.* The EMBO journal, 1997. **16**(17): p. 5408-19.

67. Dietmeier, K., et al., *Tom5 functionally links mitochondrial preprotein receptors to the general import pore.* Nature, 1997. **388**(6638): p. 195-200.

68. Liu, W., et al., *Nuclear transport of human DDB protein induced by ultraviolet light.* The Journal of biological chemistry, 2000. **275**(28): p. 21429-34.

69. Fitch, M.E., I.V. Cross, and J.M. Ford, *p53 responsive nucleotide excision repair gene products p48 and XPC, but not p53, localize to sites of UV-irradiation-induced DNA damage, in vivo.* Carcinogenesis, 2003. **24**(5): p. 843-50.

70. Tudek, B., *Base excision repair modulation as a risk factor for human cancers.* Molecular aspects of medicine, 2007. **28**(3-4): p. 258-75.

71. Bouziane, M., et al., *Promoter structure and cell cycle dependent expression of the human methylpurine-DNA glycosylase gene.* Mutation research, 2000. **461**(1): p. 15-29.

72. Meyer, F., E. Fiala, and J. Westendorf, *Induction of 8-oxo-dGTPase activity in human lymphoid cells and normal fibroblasts by oxidative stress.* Toxicology, 2000. **146**(2-3): p. 83-92.

73. Kim, H.N., et al., *Changes in DNA 8-hydroxyguanine levels, 8-hydroxyguanine repair activity, and hOGG1 and hMTH1 mRNA expression in human lung alveolar epithelial cells induced by crocidolite asbestos.* Carcinogenesis, 2001. **22**(2): p. 265-9.

74. Pines, A., et al., *Activation of APE1/Ref-1 is dependent on reactive oxygen species generated after purinergic receptor stimulation by ATP.* Nucleic acids research, 2005. **33**(14): p. 4379-94.

75. Fan, J. and D.M. Wilson, 3rd, *Protein-protein interactions and posttranslational modifications in mammalian base excision repair.* Free radical biology & medicine, 2005. **38**(9): p. 1121-38.

76. Marsin, S., et al., *Role of XRCC1 in the coordination and stimulation of oxidative DNA damage repair initiated by the DNA glycosylase hOGG1.* The Journal of biological chemistry, 2003. **278**(45): p. 44068-74.

77. Parker, A., et al., *Human homolog of the MutY repair protein (hMYH) physically interacts with proteins involved in long patch DNA base excision repair.* The Journal of biological chemistry, 2001. **276**(8): p. 5547-55.

78. Morland, I., et al., *Human DNA glycosylases of the bacterial Fpg/MutM superfamily: an alternative pathway for the repair of 8-oxoguanine and other oxidation products in DNA.* Nucleic acids research, 2002. **30**(22): p. 4926-36.

79. Nagelhus, T.A., et al., *A sequence in the N-terminal region of human uracil-DNA glycosylase with homology to XPA interacts with the C-terminal part of the 34-kDa subunit of replication protein A.* The Journal of biological chemistry, 1997. **272**(10): p. 6561-6.

80. Otterlei, M., et al., *Post-replicative base excision repair in replication foci.* The EMBO journal, 1999. **18**(13): p. 3834-44.

81. Hu, J., et al., *Phosphorylation of human oxoguanine DNA glycosylase (alpha-OGG1) modulates its function.* Nucleic acids research, 2005. **33**(10): p. 3271-82.

82. Parker, A.R., et al., *Defective human MutY phosphorylation exists in colorectal cancer cell lines with wild-type MutY alleles.* The Journal of biological chemistry, 2003. **278**(48): p. 47937-45.

83. Lu, X., et al., *The p53-induced oncogenic phosphatase PPM1D interacts with uracil DNA glycosylase and suppresses base excision repair.* Molecular cell, 2004. **15**(4): p. 621-34.

84. Bhakat, K.K., T.K. Hazra, and S. Mitra, *Acetylation of the human DNA glycosylase NEIL2 and inhibition of its activity.* Nucleic acids research, 2004. **32**(10): p. 3033-9.

85. Bhakat, K.K., et al., *Acetylation of human 8-oxoguanine-DNA glycosylase by p300 and its role in 8-oxoguanine repair in vivo.* Molecular and cellular biology, 2006. **26**(5): p. 1654-65.

86. Qu, J., et al., *Nitric oxide controls nuclear export of APE1/Ref-1 through S-nitrosation of cysteines 93 and 310.* Nucleic acids research, 2007. **35**(8): p. 2522-32.

87. Hussain, S.P., L.J. Hofseth, and C.C. Harris, *Radical causes of cancer.* Nature reviews. Cancer, 2003. **3**(4): p. 276-85.

88. Tang, C.H., W. Wei, and L. Liu, *Regulation of DNA repair by S-nitrosylation.* Biochimica et biophysica acta, 2011.

89. Dianov, G.L., C. Meisenberg, and J.L. Parsons, *Regulation of DNA repair by ubiquitylation.* Biochemistry. Biokhimiia, 2011. **76**(1): p. 69-79.

90. Busso, C.S., M.W. Lake, and T. Izumi, *Posttranslational modification of mammalian AP endonuclease (APE1).* Cellular and molecular life sciences : CMLS, 2010. **67**(21): p. 3609-20.

91. Hardeland, U., et al., *Modification of the human thymine-DNA glycosylase by ubiquitin-like proteins facilitates enzymatic turnover.* The EMBO journal, 2002. **21**(6): p. 1456-64.

92. Griffiths, L.M., et al., *Dynamic compartmentalization of base excision repair proteins in response to nuclear and mitochondrial oxidative stress.* Molecular and cellular biology, 2009. **29**(3): p. 794-807.

93. Smet-Nocca, C., et al., *SUMO-1 regulates the conformational dynamics of thymine-DNA Glycosylase regulatory domain and competes with its DNA binding activity.* BMC Biochem, 2011. **12**: p. 4.

94. Tatham, M.H., et al., *Polymeric chains of SUMO-2 and SUMO-3 are conjugated to protein substrates by SAE1/SAE2 and Ubc9.* The Journal of biological chemistry, 2001. **276**(38): p. 35368-74.

95. Cheng, C.H., et al., *SUMO modifications control assembly of synaptonemal complex and polycomplex in meiosis of Saccharomyces cerevisiae.* Genes Dev, 2006. **20**(15): p. 2067-81.

96. Bylebyl, G.R., I. Belichenko, and E.S. Johnson, *The SUMO isopeptidase Ulp2 prevents accumulation of SUMO chains in yeast.* The Journal of biological chemistry, 2003. **278**(45): p. 44113-20.

97. Mukhopadhyay, D., et al., *SUSP1 antagonizes formation of highly SUMO2/3-conjugated species.* J Cell Biol, 2006. **174**(7): p. 939-49.

98. Johnson, E.S. and G. Blobel, *Ubc9p is the conjugating enzyme for the ubiquitin-like protein Smt3p.* The Journal of biological chemistry, 1997. **272**(43): p. 26799-802.

99. Sampson, D.A., M. Wang, and M.J. Matunis, *The small ubiquitin-like modifier-1 (SUMO-1) consensus sequence mediates Ubc9 binding and is essential for SUMO-1 modification.* The Journal of biological chemistry, 2001. **276**(24): p. 21664-9.

100. Song, J., et al., *Identification of a SUMO-binding motif that recognizes SUMO-modified proteins.* Proc Natl Acad Sci U S A, 2004. **101**(40): p. 14373-8.

101. Johnson, E.S. and A.A. Gupta, *An E3-like factor that promotes SUMO conjugation to the yeast septins.* Cell, 2001. **106**(6): p. 735-44.

102. Reindle, A., et al., *Multiple domains in Siz SUMO ligases contribute to substrate selectivity.* J Cell Sci, 2006. **119**(Pt 22): p. 4749-57.

103. Zhao, X. and G. Blobel, *A SUMO ligase is part of a nuclear multiprotein complex that affects DNA repair and chromosomal organization.* Proc Natl Acad Sci U S A, 2005. **102**(13): p. 4777-82.

104. Jackson, S.P. and J. Bartek, *The DNA-damage response in human biology and disease.* Nature, 2009. **461**(7267): p. 1071-8.

105. Li, S.J. and M. Hochstrasser, *A new protease required for cell-cycle progression in yeast.* Nature, 1999. **398**(6724): p. 246-51.

106. Li, S.J. and M. Hochstrasser, *The yeast ULP2 (SMT4) gene encodes a novel protease specific for the ubiquitin-like Smt3 protein.* Molecular and cellular biology, 2000. **20**(7): p. 2367-77.

107. Panse, V.G., et al., *Unconventional tethering of Ulp1 to the transport channel of the nuclear pore complex by karyopherins.* Nat Cell Biol, 2003. **5**(1): p. 21-7.

108. Takahashi, Y., et al., *Yeast Ulp1, an Smt3-specific protease, associates with nucleoporins.* Journal of biochemistry, 2000. **128**(5): p. 723-5.

109. Wohlschlegel, J.A., et al., *Global analysis of protein sumoylation in Saccharomyces cerevisiae.* The Journal of biological chemistry, 2004. **279**(44): p. 45662-8.

110. Vertegaal, A.C., et al., *Distinct and overlapping sets of SUMO-1 and SUMO-2 target proteins revealed by quantitative proteomics.* Mol Cell Proteomics, 2006. **5**(12): p. 2298-310.

111. Rodriguez, M.S., C. Dargemont, and R.T. Hay, *SUMO-1 conjugation in vivo requires both a consensus modification motif and nuclear targeting.* The Journal of biological chemistry, 2001. **276**(16): p. 12654-9.

112. Bernier-Villamor, V., et al., *Structural basis for E2-mediated SUMO conjugation revealed by a complex between ubiquitin-conjugating enzyme Ubc9 and RanGAP1.* Cell, 2002. **108**(3): p. 345-56.

113. Lin, D., et al., *Identification of a substrate recognition site on Ubc9.* The Journal of biological chemistry, 2002. **277**(24): p. 21740-8.

114. Gareau, J.R. and C.D. Lima, *The SUMO pathway: emerging mechanisms that shape specificity, conjugation and recognition.* Nat Rev Mol Cell Biol, 2010. **11**(12): p. 861-71.

115. Vertegaal, A.C., et al., *A proteomic study of SUMO-2 target proteins.* The Journal of biological chemistry, 2004. **279**(32): p. 33791-8.

116. Panse, V.G., et al., *A proteome-wide approach identifies sumoylated substrate proteins in yeast.* The Journal of biological chemistry, 2004. **279**(40): p. 41346-51.

117. Manza, L.L., et al., *Global shifts in protein sumoylation in response to electrophile and oxidative stress.* Chem Res Toxicol, 2004. **17**(12): p. 1706-15.

118. Mabb, A.M., S.M. Wuerzberger-Davis, and S. Miyamoto, *PIASy mediates NEMO sumoylation and NF-kappaB activation in response to genotoxic stress.* Nat Cell Biol, 2006. **8**(9): p. 986-93.

119. Denison, C., et al., *A proteomic strategy for gaining insights into protein sumoylation in yeast.* Mol Cell Proteomics, 2005. **4**(3): p. 246-54.

120. Hayakawa, F. and M.L. Privalsky, *Phosphorylation of PML by mitogen-activated protein kinases plays a key role in arsenic trioxide-mediated apoptosis.* Cancer Cell, 2004. **5**(4): p. 389-401.

121. Li, T., et al., *Sumoylation of heterogeneous nuclear ribonucleoproteins, zinc finger proteins, and nuclear pore complex proteins: a proteomic analysis.* Proc Natl Acad Sci U S A, 2004. **101**(23): p. 8551-6.

122. Barford, D., *The role of cysteine residues as redox-sensitive regulatory switches.* Curr Opin Struct Biol, 2004. **14**(6): p. 679-86.

123. Tatham, M.H., Y. Chen, and R.T. Hay, *Role of two residues proximal to the active site of Ubc9 in substrate recognition by the Ubc9.SUMO-1 thiolester complex.* Biochemistry, 2003. **42**(11): p. 3168-79.

124. Desterro, J.M., J. Thomson, and R.T. Hay, *Ubch9 conjugates SUMO but not ubiquitin.* FEBS Lett, 1997. **417**(3): p. 297-300.

125. Bossis, G. and F. Melchior, *Regulation of SUMOylation by reversible oxidation of SUMO conjugating enzymes.* Molecular cell, 2006. **21**(3): p. 349-57.

126. Zhou, W., J.J. Ryan, and H. Zhou, *Global analyses of sumoylated proteins in Saccharomyces cerevisiae. Induction of protein sumoylation by cellular stresses.* The Journal of biological chemistry, 2004. **279**(31): p. 32262-8.

127. Sachdev, S., et al., *PIASy, a nuclear matrix-associated SUMO E3 ligase, represses LEF1 activity by sequestration into nuclear bodies.* Genes Dev, 2001. **15**(23): p. 3088-103.

128. Poukka, H., et al., *Covalent modification of the androgen receptor by small ubiquitin-like modifier 1 (SUMO-1).* Proc Natl Acad Sci U S A, 2000. **97**(26): p. 14145-50.

129. Bies, J., J. Markus, and L. Wolff, *Covalent attachment of the SUMO-1 protein to the negative regulatory domain of the c-Myb transcription factor modifies its stability and transactivation capacity.* The Journal of biological chemistry, 2002. **277**(11): p. 8999-9009.

130. Long, J., et al., *Repression of Smad4 transcriptional activity by SUMO modification.* Biochem J, 2004. **379**(Pt 1): p. 23-9.

131. Muller, S., et al., *c-Jun and p53 activity is modulated by SUMO-1 modification.* The Journal of biological chemistry, 2000. **275**(18): p. 13321-9.

132. Terui, Y., et al., *Dual role of sumoylation in the nuclear localization and transcriptional activation of NFAT1.* The Journal of biological chemistry, 2004. **279**(27): p. 28257-65.

133. Goodson, M.L., et al., *Sumo-1 modification regulates the DNA binding activity of heat shock transcription factor 2, a promyelocytic leukemia nuclear body associated transcription factor.* The Journal of biological chemistry, 2001. **276**(21): p. 18513-8.

134. Hong, Y., et al., *Regulation of heat shock transcription factor 1 by stress-induced SUMO-1 modification.* The Journal of biological chemistry, 2001. **276**(43): p. 40263-7.

135. Rodriguez, M.S., et al., *SUMO-1 modification activates the transcriptional response of p53.* The EMBO journal, 1999. **18**(22): p. 6455-61.

136. Pfander, B., et al., *SUMO-modified PCNA recruits Srs2 to prevent recombination during S phase.* Nature, 2005. **436**(7049): p. 428-33.

137. Heun, P., *SUMOrganization of the nucleus.* Curr Opin Cell Biol, 2007. **19**(3): p. 350-5.

138. Papouli, E., et al., *Crosstalk between SUMO and ubiquitin on PCNA is mediated by recruitment of the helicase Srs2p.* Molecular cell, 2005. **19**(1): p. 123-33.

139. Sacher, M., et al., *Control of Rad52 recombination activity by double-strand break-induced SUMO modification.* Nat Cell Biol, 2006. **8**(11): p. 1284-90.

140. Matunis, M.J., J. Wu, and G. Blobel, *SUMO-1 modification and its role in targeting the Ran GTPase-activating protein, RanGAP1, to the nuclear pore complex.* J Cell Biol, 1998. **140**(3): p. 499-509.

141. Mahajan, R., et al., *A small ubiquitin-related polypeptide involved in targeting RanGAP1 to nuclear pore complex protein RanBP2.* Cell, 1997. **88**(1): p. 97-107.

142. Matunis, M.J., E. Coutavas, and G. Blobel, *A novel ubiquitin-like modification modulates the partitioning of the Ran-GTPase-activating protein RanGAP1 between the cytosol and the nuclear pore complex.* J Cell Biol, 1996. **135**(6 Pt 1): p. 1457-70.

143. Mahajan, R., L. Gerace, and F. Melchior, *Molecular characterization of the SUMO-1 modification of RanGAP1 and its role in nuclear envelope association.* J Cell Biol, 1998. **140**(2): p. 259-70.

144. Haindl, M., et al., *The nucleolar SUMO-specific protease SENP3 reverses SUMO modification of nucleophosmin and is required for rRNA processing.* EMBO Rep, 2008. **9**(3): p. 273-9.

145. Rosas-Acosta, G., et al., *A universal strategy for proteomic studies of SUMO and other ubiquitin-like modifiers.* Mol Cell Proteomics, 2005. **4**(1): p. 56-72.

146. Zhao, J., *Sumoylation regulates diverse biological processes.* Cellular and molecular life sciences : CMLS, 2007. **64**(23): p. 3017-33.

147. Orlando, V., *Polycomb, epigenomes, and control of cell identity.* Cell, 2003. **112**(5): p. 599-606.

148. Muller, S., A. Ledl, and D. Schmidt, *SUMO: a regulator of gene expression and genome integrity.* Oncogene, 2004. **23**(11): p. 1998-2008.

149. Watts, F.Z., *The role of SUMO in chromosome segregation.* Chromosoma, 2007. **116**(1): p. 15-20.

150. Sobko, A., H. Ma, and R.A. Firtel, *Regulated SUMOylation and ubiquitination of DdMEK1 is required for proper chemotaxis.* Dev Cell, 2002. **2**(6): p. 745-56.

151. Harder, Z., R. Zunino, and H. McBride, *Sumo1 conjugates mitochondrial substrates and participates in mitochondrial fission.* Current biology : CB, 2004. **14**(4): p. 340-5.

152. Desterro, J.M., M.S. Rodriguez, and R.T. Hay, *SUMO-1 modification of IkappaBalpha inhibits NF-kappaB activation.* Molecular cell, 1998. **2**(2): p. 233-9.

153. Lin, X., et al., *SUMO-1/Ubc9 promotes nuclear accumulation and metabolic stability of tumor suppressor Smad4.* The Journal of biological chemistry, 2003. **278**(33): p. 31043-8.

154. Steffan, J.S., et al., *SUMO modification of Huntingtin and Huntington's disease pathology.* Science, 2004. **304**(5667): p. 100-4.

155. Yang, L., J.R. Mullen, and S.J. Brill, *Purification of the yeast Slx5-Slx8 protein complex and characterization of its DNA-binding activity.* Nucleic acids research, 2006. **34**(19): p. 5541-51.

156. Wang, Z., G.M. Jones, and G. Prelich, *Genetic analysis connects SLX5 and SLX8 to the SUMO pathway in Saccharomyces cerevisiae.* Genetics, 2006. **172**(3): p. 1499-509.

157. Uzunova, K., et al., *Ubiquitin-dependent proteolytic control of SUMO conjugates.* The Journal of biological chemistry, 2007. **282**(47): p. 34167-75.

158. Xie, Y., et al., *The yeast Hex3.Slx8 heterodimer is a ubiquitin ligase stimulated by substrate sumoylation.* The Journal of biological chemistry, 2007. **282**(47): p. 34176-84.

159. Mullen, J.R. and S.J. Brill, *Activation of the Slx5-Slx8 ubiquitin ligase by poly-small ubiquitin-like modifier conjugates.* The Journal of biological chemistry, 2008. **283**(29): p. 19912-21.

160. Gregoire, S. and X.J. Yang, *Association with class IIa histone deacetylases upregulates the sumoylation of MEF2 transcription factors.* Molecular and cellular biology, 2005. **25**(6): p. 2273-87.

161. Zhao, X., et al., *Regulation of MEF2 by histone deacetylase 4- and SIRT1 deacetylase-mediated lysine modifications.* Molecular and cellular biology, 2005. **25**(19): p. 8456-64.

162. Xu, Z., et al., *SUMO proteases: redox regulation and biological consequences.* Antioxid Redox Signal, 2009. **11**(6): p. 1453-84.

163. Johnston, L.H. and A.L. Johnson, *The DNA repair genes RAD54 and UNG1 are cell cycle regulated in budding yeast but MCB promoter elements have no essential role in the DNA damage response.* Nucleic acids research, 1995. **23**(12): p. 2147-52.

164. Chatterjee, A. and K.K. Singh, *Uracil-DNA glycosylase-deficient yeast exhibit a mitochondrial mutator phenotype.* Nucleic acids research, 2001. **29**(24): p. 4935-40.

165. Parikh, S.S., et al., *Base excision repair initiation revealed by crystal structures and binding kinetics of human uracil-DNA glycosylase with DNA.* The EMBO journal, 1998. **17**(17): p. 5214-26.

166. Wu, X., et al., *Processing of branched DNA intermediates by a complex of human FEN-1 and PCNA.* Nucleic acids research, 1996. **24**(11): p. 2036-43.

167. Slupphaug, G., et al., *Cell cycle regulation and in vitro hybrid arrest analysis of the major human uracil-DNA glycosylase.* Nucleic acids research, 1991. **19**(19): p. 5131-7.

168. Akbari, M., et al., *Different organization of base excision repair of uracil in DNA in nuclei and mitochondria and selective upregulation of mitochondrial uracil-DNA glycosylase after oxidative stress.* Neuroscience, 2007. **145**(4): p. 1201-12.

169. Nilsen, H., et al., *Nuclear and mitochondrial uracil-DNA glycosylases are generated by alternative splicing and transcription from different positions in the UNG gene.* Nucleic acids research, 1997. **25**(4): p. 750-5.

170. Kavli, B., et al., *hUNG2 is the major repair enzyme for removal of uracil from U:A matches, U:G mismatches, and U in single-stranded DNA, with hSMUG1 as*

a broad specificity backup. The Journal of biological chemistry, 2002. **277**(42): p. 39926-36.

171. Haushalter, K.A., et al., *Identification of a new uracil-DNA glycosylase family by expression cloning using synthetic inhibitors.* Current biology : CB, 1999. **9**(4): p. 174-85.

172. Chen, J., B. Derfler, and L. Samson, *Saccharomyces cerevisiae 3-methyladenine DNA glycosylase has homology to the AlkA glycosylase of E. coli and is induced in response to DNA alkylation damage.* The EMBO journal, 1990. **9**(13): p. 4569-75.

173. Chen, J. and L. Samson, *Induction of S.cerevisiae MAG 3-methyladenine DNA glycosylase transcript levels in response to DNA damage.* Nucleic acids research, 1991. **19**(23): p. 6427-32.

174. Huh, W.K., et al., *Global analysis of protein localization in budding yeast.* Nature, 2003. **425**(6959): p. 686-91.

175. Likhite, V.S., et al., *Interaction of estrogen receptor alpha with 3-methyladenine DNA glycosylase modulates transcription and DNA repair.* The Journal of biological chemistry, 2004. **279**(16): p. 16875-82.

176. Miao, F., et al., *3-Methyladenine-DNA glycosylase (MPG protein) interacts with human RAD23 proteins.* The Journal of biological chemistry, 2000. **275**(37): p. 28433-8.

177. Campalans, A., et al., *XRCC1 interactions with multiple DNA glycosylases: a model for its recruitment to base excision repair.* DNA repair, 2005. **4**(7): p. 826-35.

178. Engelward, B.P., et al., *Cloning and characterization of a mouse 3-methyladenine/7-methyl-guanine/3-methylguanine DNA glycosylase cDNA whose gene maps to chromosome 11.* Carcinogenesis, 1993. **14**(2): p. 175-81.

179. Dherin, C., et al., *Characterization of a highly conserved binding site of Mlh1 required for exonuclease I-dependent mismatch repair.* Molecular and cellular biology, 2009. **29**(3): p. 907-18.

180. Gellon, L., M. Werner, and S. Boiteux, *Ntg2p, a Saccharomyces cerevisiae DNA N-glycosylase/apurinic or apyrimidinic lyase involved in base excision repair of oxidative DNA damage, interacts with the DNA mismatch repair protein Mlh1p. Identification of a Mlh1p binding motif.* The Journal of biological chemistry, 2002. **277**(33): p. 29963-72.

181. Klungland, A., et al., *Base excision repair of oxidative DNA damage activated by XPG protein.* Molecular cell, 1999. **3**(1): p. 33-42.

182. Bessho, T., *Nucleotide excision repair 3' endonuclease XPG stimulates the activity of base excision repairenzyme thymine glycol DNA glycosylase.* Nucleic acids research, 1999. **27**(4): p. 979-83.

183. Marenstein, D.R., et al., *Stimulation of human endonuclease III by Y box-binding protein 1 (DNA-binding protein B). Interaction between a base excision repair enzyme and a transcription factor.* The Journal of biological chemistry, 2001. **276**(24): p. 21242-9.

184. Luna, L., et al., *Cell-cycle regulation, intracellular sorting and induced overexpression of the human NTH1 DNA glycosylase involved in removal of*

formamidopyrimidine residues from DNA. Mutation research, 2000. **460**(2): p. 95-104.

185. Ikeda, S., et al., *Differential intracellular localization of the human and mouse endonuclease III homologs and analysis of the sorting signals.* DNA repair, 2002. **1**(10): p. 847-54.

186. Takao, M., et al., *Mitochondrial targeting of human DNA glycosylases for repair of oxidative DNA damage.* Nucleic acids research, 1998. **26**(12): p. 2917-22.

187. Wiederhold, L., et al., *AP endonuclease-independent DNA base excision repair in human cells.* Molecular cell, 2004. **15**(2): p. 209-20.

188. Hegde, M.L., et al., *Physical and functional interaction between human oxidized base-specific DNA glycosylase NEIL1 and flap endonuclease 1.* The Journal of biological chemistry, 2008. **283**(40): p. 27028-37.

189. Hazra, T.K., et al., *Identification and characterization of a human DNA glycosylase for repair of modified bases in oxidatively damaged DNA.* Proceedings of the National Academy of Sciences of the United States of America, 2002. **99**(6): p. 3523-8.

190. Hu, J., et al., *Repair of formamidopyrimidines in DNA involves different glycosylases: role of the OGG1, NTH1, and NEIL1 enzymes.* The Journal of biological chemistry, 2005. **280**(49): p. 40544-51.

191. Hazra, T.K., et al., *Identification and characterization of a novel human DNA glycosylase for repair of cytosine-derived lesions.* The Journal of biological chemistry, 2002. **277**(34): p. 30417-20.

192. Torisu, K., et al., *Hematopoietic tissue-specific expression of mouse Neil3 for endonuclease VIII-like protein.* Journal of biochemistry, 2005. **138**(6): p. 763-72.

193. Luna, L., et al., *Dynamic relocalization of hOGG1 during the cell cycle is disrupted in cells harbouring the hOGG1-Cys326 polymorphic variant.* Nucleic acids research, 2005. **33**(6): p. 1813-24.

194. Jaiswal, M., et al., *Human Ogg1, a protein involved in the repair of 8-oxoguanine, is inhibited by nitric oxide.* Cancer research, 2001. **61**(17): p. 6388-93.

195. Hill, J.W., et al., *Stimulation of human 8-oxoguanine-DNA glycosylase by AP-endonuclease: potential coordination of the initial steps in base excision repair.* Nucleic acids research, 2001. **29**(2): p. 430-8.

196. Whitehouse, C.J., et al., *XRCC1 stimulates human polynucleotide kinase activity at damaged DNA termini and accelerates DNA single-strand break repair.* Cell, 2001. **104**(1): p. 107-17.

197. Nakabeppu, Y., *Regulation of intracellular localization of human MTH1, OGG1, and MYH proteins for repair of oxidative DNA damage.* Prog Nucleic Acid Res Mol Biol, 2001. **68**: p. 75-94.

198. Kundu, S., et al., *Ser 524 is a phosphorylation site in MUTYH and Ser 524 mutations alter 8-oxoguanine (OG): a mismatch recognition.* DNA repair, 2010. **9**(10): p. 1026-37.

199. Gu, Y., et al., *Human MutY homolog, a DNA glycosylase involved in base excision repair, physically and functionally interacts with mismatch repair proteins human MutS homolog 2/human MutS homolog 6.* The Journal of biological chemistry, 2002. **277**(13): p. 11135-42.

200. Luncsford, P.J., et al., *A structural hinge in eukaryotic MutY homologues mediates catalytic activity and Rad9-Rad1-Hus1 checkpoint complex interactions.* J Mol Biol, 2010. **403**(3): p. 351-70.

201. Boldogh, I., et al., *hMYH cell cycle-dependent expression, subcellular localization and association with replication foci: evidence suggesting replication-coupled repair of adenine:8-oxoguanine mispairs.* Nucleic acids research, 2001. **29**(13): p. 2802-9.

202. Ohtsubo, T., et al., *Identification of human MutY homolog (hMYH) as a repair enzyme for 2-hydroxyadenine in DNA and detection of multiple forms of hMYH located in nuclei and mitochondria.* Nucleic acids research, 2000. **28**(6): p. 1355-64.

203. Boland, M.J. and J.K. Christman, *Characterization of Dnmt3b:thymine-DNA glycosylase interaction and stimulation of thymine glycosylase-mediated repair by DNA methyltransferase(s) and RNA.* J Mol Biol, 2008. **379**(3): p. 492-504.

204. Bellacosa, A., et al., *MED1, a novel human methyl-CpG-binding endonuclease, interacts with DNA mismatch repair protein MLH1.* Proceedings of the National Academy of Sciences of the United States of America, 1999. **96**(7): p. 3969-74.

205. Kondo, E., et al., *The thymine DNA glycosylase MBD4 represses transcription and is associated with methylated p16(INK4a) and hMLH1 genes.* Molecular and cellular biology, 2005. **25**(11): p. 4388-96.

206. Hendrich, B. and A. Bird, *Identification and characterization of a family of mammalian methyl-CpG binding proteins.* Molecular and cellular biology, 1998. **18**(11): p. 6538-47.

207. Tini, M., et al., *Association of CBP/p300 acetylase and thymine DNA glycosylase links DNA repair and transcription.* Molecular cell, 2002. **9**(2): p. 265-77.

208. Steinacher, R. and P. Schar, *Functionality of human thymine DNA glycosylase requires SUMO-regulated changes in protein conformation.* Current biology : CB, 2005. **15**(7): p. 616-23.

209. Waters, T.R., et al., *Human thymine DNA glycosylase binds to apurinic sites in DNA but is displaced by human apurinic endonuclease 1.* The Journal of biological chemistry, 1999. **274**(1): p. 67-74.

210. Privezentzev, C.V., M. Saparbaev, and J. Laval, *The HAP1 protein stimulates the turnover of human mismatch-specific thymine-DNA-glycosylase to process 3,N(4)-ethenocytosine residues.* Mutation research, 2001. **480-481**: p. 277-84.

211. Shimizu, Y., et al., *Xeroderma pigmentosum group C protein interacts physically and functionally with thymine DNA glycosylase.* The EMBO journal, 2003. **22**(1): p. 164-73.

212. Li, Y.Q., et al., *Association of Dnmt3a and thymine DNA glycosylase links DNA methylation with base-excision repair.* Nucleic acids research, 2007. **35**(2): p. 390-400.

213. Um, S., et al., *Retinoic acid receptors interact physically and functionally with the T:G mismatch-specific thymine-DNA glycosylase.* The Journal of biological chemistry, 1998. **273**(33): p. 20728-36.

214. Lucey, M.J., et al., *T:G mismatch-specific thymine-DNA glycosylase (TDG) as a coregulator of transcription interacts with SRC1 family members through a novel tyrosine repeat motif.* Nucleic acids research, 2005. **33**(19): p. 6393-404.

215. Kim, E.J. and S.J. Um, *Thymine-DNA glycosylase interacts with and functions as a coactivator of p53 family proteins.* Biochem Biophys Res Commun, 2008. **377**(3): p. 838-42.

216. Guan, X., et al., *The human checkpoint sensor Rad9-Rad1-Hus1 interacts with and stimulates DNA repair enzyme TDG glycosylase.* Nucleic acids research, 2007. **35**(18): p. 6207-18.

217. Chen, D., et al., *T:G mismatch-specific thymine-DNA glycosylase potentiates transcription of estrogen-regulated genes through direct interaction with estrogen receptor alpha.* The Journal of biological chemistry, 2003. **278**(40): p. 38586-92.

218. Mohan, R.D., et al., *Opposing regulatory roles of phosphorylation and acetylation in DNA mispair processing by thymine DNA glycosylase.* Nucleic acids research, 2010. **38**(4): p. 1135-48.

219. Vongsamphanh, R., P.K. Fortier, and D. Ramotar, *Pir1p mediates translocation of the yeast Apn1p endonuclease into the mitochondria to maintain genomic stability.* Molecular and cellular biology, 2001. **21**(5): p. 1647-55.

220. Ramotar, D., et al., *Intracellular localization of the Apn1 DNA repair enzyme of Saccharomyces cerevisiae. Nuclear transport signals and biological role.* The Journal of biological chemistry, 1993. **268**(27): p. 20533-9.

221. Unk, I., et al., *Stimulation of 3'-->5' exonuclease and 3'-phosphodiesterase activities of yeast apn2 by proliferating cell nuclear antigen.* Molecular and cellular biology, 2002. **22**(18): p. 6480-6.

222. Bennett, R.A., *The Saccharomyces cerevisiae ETH1 gene, an inducible homolog of exonuclease III that provides resistance to DNA-damaging agents and limits spontaneous mutagenesis.* Molecular and cellular biology, 1999. **19**(3): p. 1800-9.

223. Bhakat, K.K., et al., *Role of acetylated human AP-endonuclease (APE1/Ref-1) in regulation of the parathyroid hormone gene.* The EMBO journal, 2003. **22**(23): p. 6299-309.

224. Busso, C.S., T. Iwakuma, and T. Izumi, *Ubiquitination of mammalian AP endonuclease (APE1) regulated by the p53-MDM2 signaling pathway.* Oncogene, 2009. **28**(13): p. 1616-25.

225. Vidal, A.E., et al., *XRCC1 coordinates the initial and late stages of DNA abasic site repair through protein-protein interactions.* The EMBO journal, 2001. **20**(22): p. 6530-9.

226. Ahn, B., et al., *Regulation of WRN helicase activity in human base excision repair.* The Journal of biological chemistry, 2004. **279**(51): p. 53465-74.

227. La Belle, M., S. Linn, and L.H. Thompson, *Apurinic/apyrimidinic endonuclease activities appear normal in the CHO-cell ethyl methanesulfonate-sensitive mutant, EM9.* Mutation research, 1984. **141**(1): p. 41-4.

228. Gaiddon, C., N.C. Moorthy, and C. Prives, *Ref-1 regulates the transactivation and pro-apoptotic functions of p53 in vivo.* The EMBO journal, 1999. **18**(20): p. 5609-21.

229. Dianova, II, V.A. Bohr, and G.L. Dianov, *Interaction of human AP endonuclease 1 with flap endonuclease 1 and proliferating cell nuclear antigen involved in long-patch base excision repair.* Biochemistry, 2001. **40**(42): p. 12639-44.

230. Ranalli, T.A., S. Tom, and R.A. Bambara, *AP endonuclease 1 coordinates flap endonuclease 1 and DNA ligase I activity in long patch base excision repair.* The Journal of biological chemistry, 2002. **277**(44): p. 41715-24.

231. Fung, H., et al., *Asbestos increases mammalian AP-endonuclease gene expression, protein levels, and enzyme activity in mesothelial cells.* Cancer research, 1998. **58**(2): p. 189-94.

232. Tell, G., et al., *Mitochondrial localization of APE/Ref-1 in thyroid cells.* Mutation research, 2001. **485**(2): p. 143-52.

233. Tomkinson, A.E., R.T. Bonk, and S. Linn, *Mitochondrial endonuclease activities specific for apurinic/apyrimidinic sites in DNA from mouse cells.* The Journal of biological chemistry, 1988. **263**(25): p. 12532-7.

234. Warbrick, E., et al., *Homologous regions of Fen1 and p21Cip1 compete for binding to the same site on PCNA: a potential mechanism to co-ordinate DNA replication and repair.* Oncogene, 1997. **14**(19): p. 2313-21.

235. Tsuchimoto, D., et al., *Human APE2 protein is mostly localized in the nuclei and to some extent in the mitochondria, while nuclear APE2 is partly associated with proliferating cell nuclear antigen.* Nucleic acids research, 2001. **29**(11): p. 2349-60.

236. Li, X., et al., *Lagging strand DNA synthesis at the eukaryotic replication fork involves binding and stimulation of FEN-1 by proliferating cell nuclear antigen.* The Journal of biological chemistry, 1995. **270**(38): p. 22109-12.

237. Gary, R., et al., *The DNA repair endonuclease XPG binds to proliferating cell nuclear antigen (PCNA) and shares sequence elements with the PCNA-binding*

regions of FEN-1 and cyclin-dependent kinase inhibitor p21. The Journal of biological chemistry, 1997. **272**(39): p. 24522-9.

238. Loizou, J.I., et al., *The protein kinase CK2 facilitates repair of chromosomal DNA single-strand breaks.* Cell, 2004. **117**(1): p. 17-28.

239. Luo, H., et al., *A new XRCC1-containing complex and its role in cellular survival of methyl methanesulfonate treatment.* Molecular and cellular biology, 2004. **24**(19): p. 8356-65.

240. Marintchev, A., M.R. Gryk, and G.P. Mullen, *Site-directed mutagenesis analysis of the structural interaction of the single-strand-break repair protein, X-ray cross-complementing group 1, with DNA polymerase beta.* Nucleic acids research, 2003. **31**(2): p. 580-8.

241. Marintchev, A., et al., *Solution structure of the single-strand break repair protein XRCC1 N-terminal domain.* Nature structural biology, 1999. **6**(9): p. 884-93.

242. Marintchev, A., et al., *Domain specific interaction in the XRCC1-DNA polymerase beta complex.* Nucleic acids research, 2000. **28**(10): p. 2049-59.

243. Gryk, M.R., et al., *Mapping of the interaction interface of DNA polymerase beta with XRCC1.* Structure, 2002. **10**(12): p. 1709-20.

244. Wei, Y.F., et al., *Molecular cloning and expression of human cDNAs encoding a novel DNA ligase IV and DNA ligase III, an enzyme active in DNA repair and recombination.* Molecular and cellular biology, 1995. **15**(6): p. 3206-16.

245. Nash, R.A., et al., *XRCC1 protein interacts with one of two distinct forms of DNA ligase III.* Biochemistry, 1997. **36**(17): p. 5207-11.

246. Caldecott, K.W., et al., *An interaction between the mammalian DNA repair protein XRCC1 and DNA ligase III.* Molecular and cellular biology, 1994. **14**(1): p. 68-76.

247. Caldecott, K.W., et al., *Characterization of the XRCC1-DNA ligase III complex in vitro and its absence from mutant hamster cells.* Nucleic acids research, 1995. **23**(23): p. 4836-43.

248. Masson, M., et al., *XRCC1 is specifically associated with poly(ADP-ribose) polymerase and negatively regulates its activity following DNA damage.* Molecular and cellular biology, 1998. **18**(6): p. 3563-71.

249. Schreiber, V., et al., *Poly(ADP-ribose) polymerase-2 (PARP-2) is required for efficient base excision DNA repair in association with PARP-1 and XRCC1.* The Journal of biological chemistry, 2002. **277**(25): p. 23028-36.

250. Plo, I., et al., *Association of XRCC1 and tyrosyl DNA phosphodiesterase (Tdp1) for the repair of topoisomerase I-mediated DNA lesions.* DNA repair, 2003. **2**(10): p. 1087-100.

251. Caldecott, K.W., *DNA single-strand break repair and spinocerebellar ataxia.* Cell, 2003. **112**(1): p. 7-10.

252. Fan, J., et al., *XRCC1 co-localizes and physically interacts with PCNA.* Nucleic acids research, 2004. **32**(7): p. 2193-201.

253. Brosh, R.M., Jr., et al., *Werner syndrome protein interacts with human flap endonuclease 1 and stimulates its cleavage activity.* The EMBO journal, 2001. **20**(20): p. 5791-801.

254. Caldecott, K.W., et al., *XRCC1 polypeptide interacts with DNA polymerase beta and possibly poly (ADP-ribose) polymerase, and DNA ligase III is a novel molecular 'nick-sensor' in vitro.* Nucleic acids research, 1996. **24**(22): p. 4387-94.

255. Leppard, J.B., et al., *Physical and functional interaction between DNA ligase IIIalpha and poly(ADP-Ribose) polymerase 1 in DNA single-strand break repair.* Molecular and cellular biology, 2003. **23**(16): p. 5919-27.

256. Messner, S., et al., *Sumoylation of poly(ADP-ribose) polymerase 1 inhibits its acetylation and restrains transcriptional coactivator function.* FASEB J, 2009. **23**(11): p. 3978-89.

257. Hassa, P.O., et al., *Acetylation of poly(ADP-ribose) polymerase-1 by p300/CREB-binding protein regulates coactivation of NF-kappaB-dependent transcription.* The Journal of biological chemistry, 2005. **280**(49): p. 40450-64.

258. Hassa, P.O. and M.O. Hottiger, *The diverse biological roles of mammalian PARPS, a small but powerful family of poly-ADP-ribose polymerases.* Front Biosci, 2008. **13**: p. 3046-82.

259. Tseng, H.M. and A.E. Tomkinson, *Processing and joining of DNA ends coordinated by interactions among Dnl4/Lif1, Pol4, and FEN-1.* The Journal of biological chemistry, 2004. **279**(46): p. 47580-8.

260. Vallen, E.A. and F.R. Cross, *Mutations in RAD27 define a potential link between G1 cyclins and DNA replication.* Molecular and cellular biology, 1995. **15**(8): p. 4291-302.

261. Kalifa, L., et al., *Evidence for a role of FEN1 in maintaining mitochondrial DNA integrity.* DNA repair, 2009. **8**(10): p. 1242-9.

262. Henneke, G., S. Koundrioukoff, and U. Hubscher, *Phosphorylation of human Fen1 by cyclin-dependent kinase modulates its role in replication fork regulation.* Oncogene, 2003. **22**(28): p. 4301-13.

263. Hasan, S., et al., *Regulation of human flap endonuclease-1 activity by acetylation through the transcriptional coactivator p300.* Molecular cell, 2001. **7**(6): p. 1221-31.

264. Guo, Z., et al., *Methylation of FEN1 suppresses nearby phosphorylation and facilitates PCNA binding.* Nature chemical biology, 2010. **6**(10): p. 766-73.

265. Pascal, J.M., et al., *Human DNA ligase I completely encircles and partially unwinds nicked DNA.* Nature, 2004. **432**(7016): p. 473-8.

266. Kim, I.S., *Down-regulation of human FEN-1 gene expression during differentiation of promyelocytic leukemia cells.* Exp Mol Med, 1998. **30**(4): p. 252-6.

267. Qiu, J., et al., *Cell cycle-dependent and DNA damage-inducible nuclear localization of FEN-1 nuclease is consistent with its dual functions in DNA replication and repair.* The Journal of biological chemistry, 2001. **276**(7): p. 4901-8.

268. Liu, P., et al., *Removal of oxidative DNA damage via FEN1-dependent long-patch base excision repair in human cell mitochondria.* Molecular and cellular biology, 2008. **28**(16): p. 4975-87.

269. Szczesny, B., et al., *Long patch base excision repair in mammalian mitochondrial genomes.* The Journal of biological chemistry, 2008. **283**(39): p. 26349-56.

270. Hoege, C., et al., *RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO.* Nature, 2002. **419**(6903): p. 135-41.

271. Bauer, G.A. and P.M. Burgers, *Molecular cloning, structure and expression of the yeast proliferating cell nuclear antigen gene.* Nucleic acids research, 1990. **18**(2): p. 261-5.

272. Miller, A., et al., *Proliferating cell nuclear antigen (PCNA) is required for cell cycle-regulated silent chromatin on replicated and nonreplicated genes.* The Journal of biological chemistry, 2010. **285**(45): p. 35142-54.

273. Lee, K.Y. and K. Myung, *PCNA modifications for regulation of post-replication repair pathways.* Mol Cells, 2008. **26**(1): p. 5-11.

274. Naryzhny, S.N. and H. Lee, *The post-translational modifications of proliferating cell nuclear antigen: acetylation, not phosphorylation, plays an important role in the regulation of its function.* The Journal of biological chemistry, 2004. **279**(19): p. 20194-9.

275. Bergink, S. and S. Jentsch, *Principles of ubiquitin and SUMO modifications in DNA repair.* Nature, 2009. **458**(7237): p. 461-7.

276. Kedar, P.S., et al., *Direct interaction between mammalian DNA polymerase beta and proliferating cell nuclear antigen.* The Journal of biological chemistry, 2002. **277**(34): p. 31115-23.

277. Jonsson, Z.O., R. Hindges, and U. Hubscher, *Regulation of DNA replication and repair proteins through interaction with the front side of proliferating cell nuclear antigen.* The EMBO journal, 1998. **17**(8): p. 2412-25.

278. Levin, D.S., et al., *An interaction between DNA ligase I and proliferating cell nuclear antigen: implications for Okazaki fragment synthesis and joining.* Proceedings of the National Academy of Sciences of the United States of America, 1997. **94**(24): p. 12863-8.

279. Levin, D.S., et al., *Interaction between PCNA and DNA ligase I is critical for joining of Okazaki fragments and long-patch base-excision repair.* Current biology : CB, 2000. **10**(15): p. 919-22.

280. Montecucco, A., et al., *DNA ligase I is recruited to sites of DNA replication by an interaction with proliferating cell nuclear antigen: identification of a common targeting mechanism for the assembly of replication factories.* The EMBO journal, 1998. **17**(13): p. 3786-95.

281. Waga, S. and B. Stillman, *Cyclin-dependent kinase inhibitor p21 modulates the DNA primer-template recognition complex.* Molecular and cellular biology, 1998. **18**(7): p. 4177-87.

282. Naryzhny, S.N., *Proliferating cell nuclear antigen: a proteomics view.* Cellular and molecular life sciences : CMLS, 2008. **65**(23): p. 3789-808.

283. Bravo, R., et al., *Identification of a nuclear and of a cytoplasmic polypeptide whose relative proportions are sensitive to changes in the rate of cell proliferation.* Exp Cell Res, 1981. **136**(2): p. 311-9.

284. Bravo, R. and J.E. Celis, *A search for differential polypeptide synthesis throughout the cell cycle of HeLa cells.* J Cell Biol, 1980. **84**(3): p. 795-802.

285. Slupphaug, G., B. Kavli, and H.E. Krokan, *The interacting pathways for prevention and repair of oxidative DNA damage.* Mutat Res, 2003. **531**(1-2): p. 231-51.

286. Davidsen, T. and T. Tonjum, *Meningococcal genome dynamics.* Nat Rev Microbiol, 2006. **4**(1): p. 11-22.

287. Miteva, M., et al., *Sumoylation as a Signal for Polyubiquitylation and Proteasomal Degradation Conjugation and Deconjugation of Ubiquitin Family Modifiers*, M. Groettrup, Editor. 2010, Springer New York. p. 195-214.

10. Tables

Table 1: Eukaryotic Base Excision Repair Proteins and Mechanisms of their Regulation

Protein	Organism	Gene	Level of Regulation			
			PTM	Protein Interactions	Transcript Level	Localization
Uracil DNA glycosylase	*S. cerevisiae*	*UNG1*	-	-	↑ in G_1-phase [163]	Nuc and Mito [164]
	Human	*UNG*	**Phos** (Thr6, Thr126) [83]	APEX1 ↑ Activity [165] Associated with RPA, XRCC1, and PCNA [79, 80, 166]	↑ in G_1- and S-phase [71, 167] Mitochondrial form ↑ in response to ROS [168]	Nuc (UNG2) and Mito (UNG1) Forms [168, 169]
		SMUG1	-	APEX1 ↑ Activity [170]	-	Nuc [171]
3-methyl adenine DNA glycosylase	*S. cerevisiae*	*MAG1*	-	-	↑ in response to alkylating agents and UV [172, 173]	Nuc [174]
	Human	*MPG*	**Acetyl** [175]	Associated with hHR23, XRCC1, and ERα [175-177]	↑ in G_1-phase [71]	Nuc [178]
Endonuclease III/thymine glycol DNA glycosylase	*S. cerevisiae*	*NTG1*	**SUMO** [92]	-	↑ in response to menadione [45, 50]	Nuc and Mito [55, 92]
		NTG2	**SUMO** [92]	Associated with MLH1 [179, 180]	-	Nuc [55, 92]
	Human	*NTHL1*	-	APEX1 ↑ Activity [32] Associated with XPG, XRCC1, and YB-1 [177, 181-183]	↑ in G_1- and S-phase [71, 184]	Nuc and Mito [184-186]
Endonuclease VIII-like	Human	*NEIL1*	-	Associated with FEN1 and XRCC1 [187, 188]	↑ in S-phase [189]	Nuc and Mito [190]
		NEIL2	**Acetyl** (Lys49, Lys153) [84]	Associated with p300 and XRCC1 [84, 177]	No cell cycle dependence [191]	Nuc [191]
		NEIL3	-	Associated with RPA[78]	-	Nuc [192]
8-oxoguanine DNA glycosylase	*S. cerevisiae*	*OGG1*	-	-	-	Nuc and Mito [26]
	Human	*hOGG1*	**Phos** (Ser326) [81, 193] **Acetyl** (Lys41, Lys338) [85] **Nitro** [194]	APEX1 ↑ Activity [195, 196] Associated with XRCC1 [76]	No cell cycle dependence [71] ↑ in response to ROS [73]	Nuc and Mito [186, 197]
A-G-mismatch DNA glycosylase	Human	*MUTYH*	**Phos** (Ser524) [82, 198]	Associated with APEX1, PCNA, RPA, RAD9-RAD1-HUS1 complex, and MSH6 [77, 199, 200]	↑ in S-phase [201]	Nuc and Mito [186, 197, 201, 202]
G-T-mismatch DNA glycosylase	Human	*MBD4*	-	Associated with MLH1, SIN3A, and HDAC1 [203-205]	-	Nuc [206]
		TDG	**Acetyl** [207] **SUMO** [208]	APEX1 ↑ Activity [209, 210] Associated with APEX1, CPB, p300, DMNT3A, DMNT3B, RXR, RAR, SCR1, p53, p73α, p63γ, ERα, RAD9, and XPC [203, 207, 211-217]	No cell cycle dependence [71]	Nuc [218]

Protein	Organism	Gene	Level of Regulation			
			PTM	Protein Interactions	Transcript Level	Localization
AP-endonuclease	*S. cerevisiae*	*APN1*	-	Associates with PIR1 [219]	-	Nuc and Mito [219, 220]
		APN2	-	Associates with POL30 [221]	↑ in response to alkylating agents [222]	Nuc [174]
	Human	*APEX1*	**Acetyl** by p300 [223] **Phos** [75] **Ub** (K24, K25, K27) [224] **Nitro** [86, 88]	↑ glycosylase activity of UNG1, SMUG, NTHL1, OGG1, and TDG [32, 165, 170, 195, 196] Associated with MUTYH,TDG, XRCC1, HSP70, p53, PCNA, LIG1, FEN1, and WRN [77, 207, 225-230]	↑ in G_1-phase [71] ↑ in response to ROS [74]	Nuc and Mito [231-233]
		APEX2	-	Associated with PCNA [234, 235]	-	Nuc and Mito [234-237]
X-ray repair cross complementing group 1	Human	*XRCC1*	**Phos** [238, 239]	Associated with OGG1, NTHL1, NEIL1, NEIL2, MPG, POL*β,* LIG3, PARP1, PARP2, UNG2, PNK, CK2, TDP1, ATPX, PCNA, and APEX1 [76, 166, 177, 187, 196, 225, 238-252]	↑ in S-phase [245]	Nuc [229, 246, 247, 253]
Poly (ADP-ribose) polymerase	Human	*PARP1*	**Phos** [254] **SUMO** (K486) [255, 256] **Acetyl** (K498, K505, K508, K521, K524) [257]	Activity ↑ in response to ROS [258] Associated with LIG3, XRCC1, POL*β,* and numerous non-BER proteins [248, 249, 254, 255, 258]	-	Nuc [244]
Flap endonuclease	*S. cerevisiae*	*RAD27*	-	Associated with POL4 and DNL4/LIF1 [259]	↑ in G_1-phase ↑ in response to alkylating agents [260]	Nuc and Mito [261]
	Human	*FEN1*	**Phos (**Ser187) [262] **Acetyl** [263] **Methyl** (R192) [264]	Associated with PCNA, WRN, CDK1, CDK2 NEIL1, LIG1, p300, and APEX1 [166, 188, 229, 234, 236, 237, 253, 262, 263, 265]	↑ in mitotic cycling cells ↓in stationary cells [266]	Nuc and Mito [267-269]
Proliferating cell nuclear antigen	*S. cerevisiae*	*POL30*	**Ub** (K164)[270] **SUMO** (K127, K164) [270]	Associates with APN2 [221]	↑ in G_1- and S-phase [271]	Nuc [272]
	Human	*PCNA*	**Ub** (K164) [273] **Acetyl** [274] **SUMO** (K127, K164) [270, 275]	Associated with MUTYH, p21, POLβ, XRCC1, FEN1, LIG1, APEX1, and numerous non-BER proteins [77, 166, 229, 234, 236, 237, 252, 276-282]	↑ in S phase [283, 284]	Nuc [235]

11. Figures

Figure 1

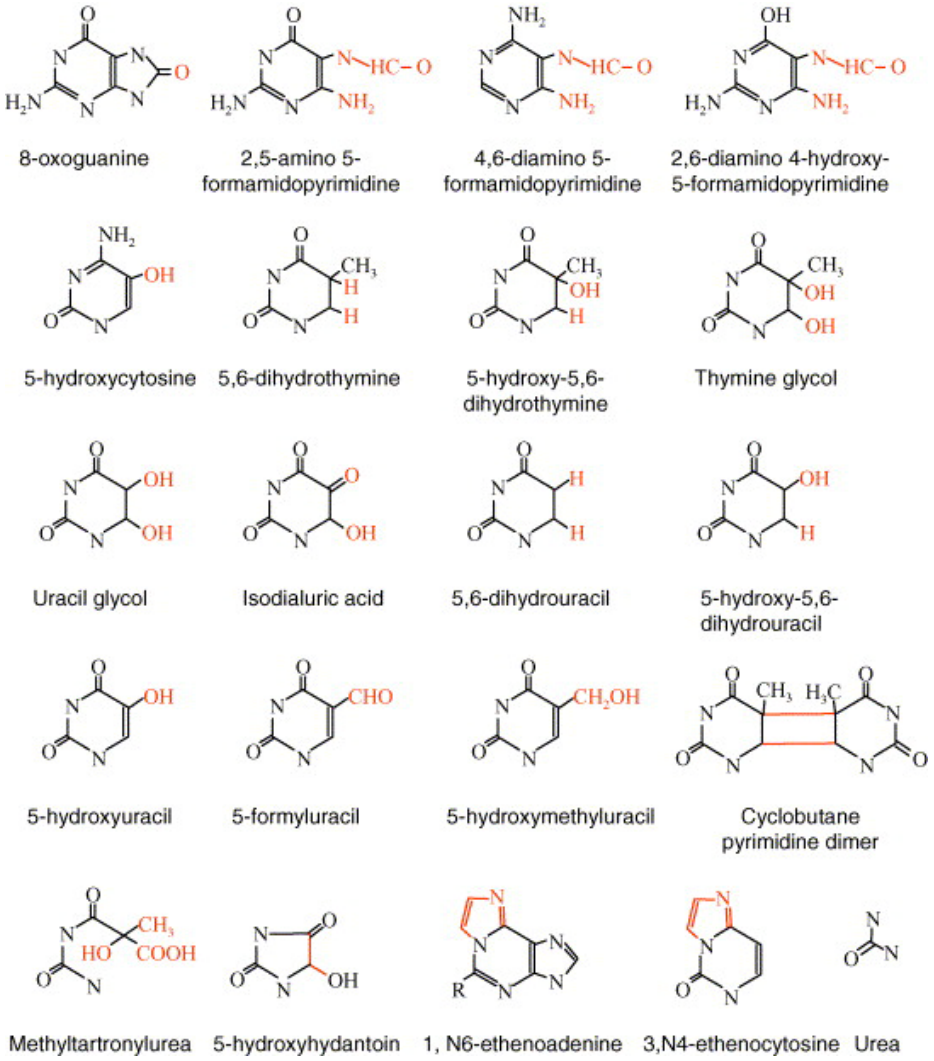


Figure 1 Legend: Examples of Oxidative DNA Lesions. A number of oxidized DNA bases can result from deleterious interactions with endogenous reactive oxygen species, produced during cellular metabolism, or by exogenous ionizing or UV radiation. The lesions listed include ring opened forms and oxidized aromatic derivatives and represent only a subset of the 40 to 60 known oxidative lesions. This figure was adapted from Slupphaug et al. Mutation Research, 2003 [285].

Figure 2

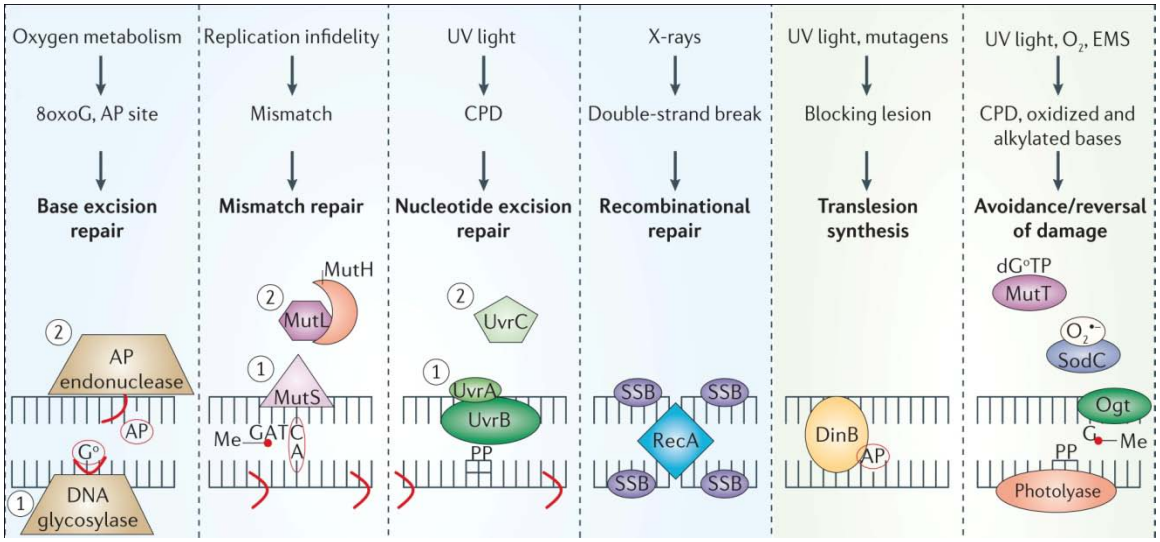


Figure 2 Legend: DNA Repair Pathways. The six DNA management pathways are outlined. In base excision repair (BER), a single or a short stretch of nucleotides is removed and replaced with the correct nucleotides. Mismatch repair (MMR) recognizes and repairs erroneous insertions, deletions and mis-incorporated bases that may arise during DNA replication. In nucleotide excision repair (NER), stretches of 25 - 30 nucleotides are removed in order to excise large helix distorting lesions from the DNA. Recombination repair (RER) uses homologous sequence in a sister chromosome to bypass lesions encountered during replication. Translesion synthesis (TLS) uses specific polymerases to bypass lesions during DNA replication in a mutagenic manner. Avoidance/reversal of damage, often referred to as direct reversal (DR) is a process where DNA lesions are directly reversed on the DNA. This figure was adapted from Davidsen et al. Nature Reviews Microbiology, 2006 [286].

Figure 3

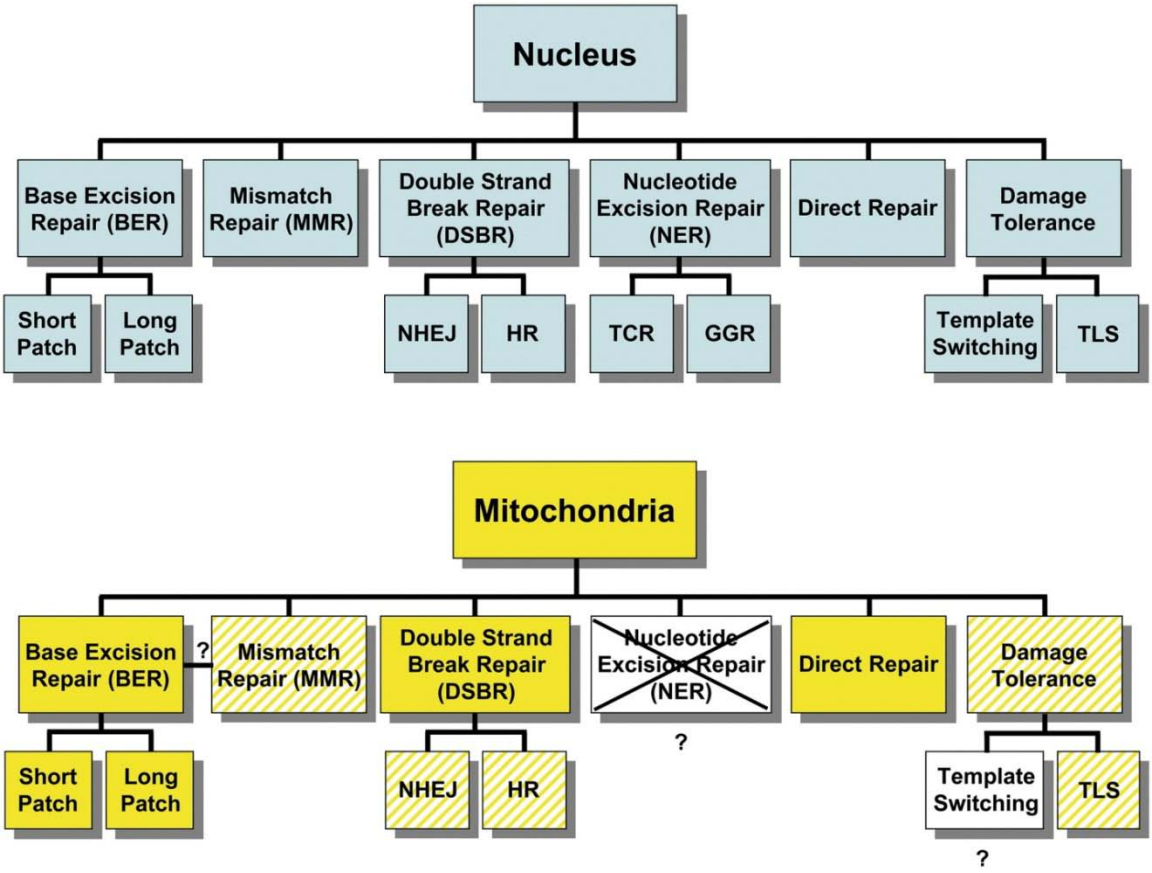


Figure 3 Legend: Nuclear and Mitochondrial DNA Repair Pathways. The figure displays the DNA repair pathways which can be found in the nucleus and the mitochondria. Established pathways are shown with a full color background, whereas documented pathways which need further confirmation are on a hatched background. The mitochondria are considered to have no NER pathway. Abbreviations: NHEJ, non-homologous end joining; HR, homologous recombination; TCR, transcription-coupled repair; GGR, global genome repair; TLS, translesion synthesis. This figure was adapted from Boesch et al. Biochimica et biophysica acta, 2011 [19].

Figure 4

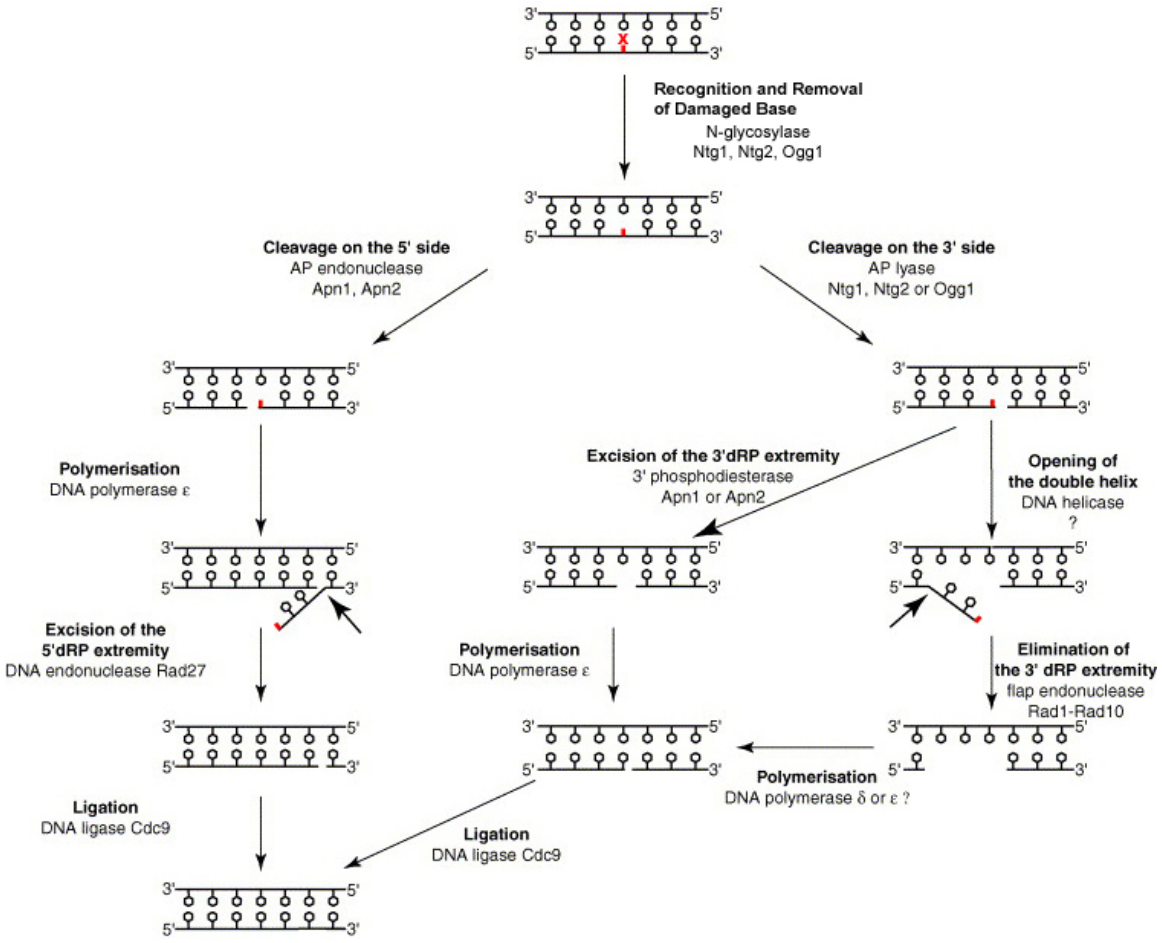


Figure 4 Legend: Base Excision Repair Pathway in *Saccharomyces cerevisiae*. Base excision repair is initiated through recognition of a damaged base by *N*-glycosylases, which removes the damaged base from the DNA and results in the formation of an AP site. Following removal of the damaged base, AP sites may be further processed by either an AP lyase or and AP endonuclease, which cleave the sugar-phosphate DNA backbone on the 3' or 5' side respectively. The subsequent single nucleotide gaps are then filled in by DNA polymerases and the integrity of the DNA is restored by DNA ligases. The figure was adapted from Boiteux et al. DNA Repair, 2004 [33].

Figure 5

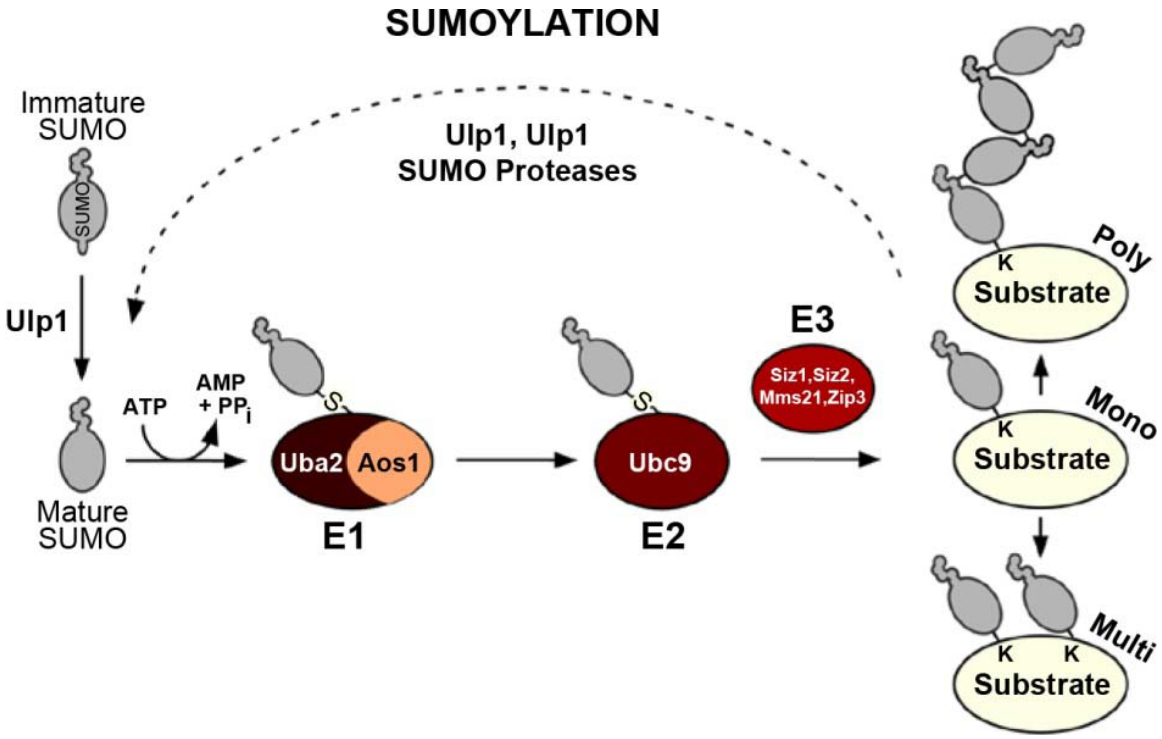


Figure 5: The Sumoylation Pathway. The immature SUMO is processed by the SUMO protease Ulp1 to its mature form where it is activated by the E1 Uba2/Aos1 and subsequently conjugated to a substrate by complexes of Ubc9 and one of four E3 ligases (including Siz1, Siz2, Mms21, and Zip3) in a mono-, multi-, or polysumoylated manner. Desumoylation is carried out by the SUMO proteases Ulp1 and Ulp2. This figure was adapted from Miteva et al. Subcellular Biochemistry, 2010 [287].

Figure 6

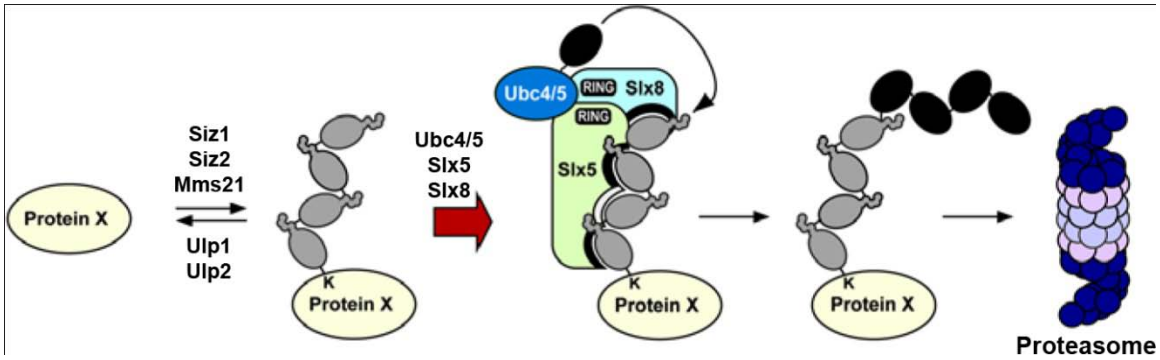


Figure 6 Legend: Protein degradation by SUMO-targeted ubiquitin ligases. Sumoylation and polysumoylation in particular may trigger ubiquitination by the SUMO-targeted ubiquitin ligases (STUbLs), Slx5 and Slx8. Slx5 and Slx8 are E3 ubiquitin ligases that contain SUMO-interacting motifs (SIMs) which allow them to recognize sumoylated substrates and in conjunction with the E2 Ubc4/5 ubiquitinate certain proteins, targeting them for subsequent degradation by the proteasome. This figure was adapted from Miteva et al. Subcellular Biochemistry, 2010 [287].

Chapter 2:

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

Lyra M. Griffiths[1,2], Dan Swartzlander[1,2], Kellen L. Meadows[1,6], Keith D. Wilkinson[1], Anita H. Corbett[1], Paul W. Doetsch*[1,3,4,5]

From the Department of Biochemistry[1], Graduate Program in Genetics and Molecular Biology[2], Emory Winship Cancer Institute[3], and Department of Radiation Oncology[4] and Hematology and Medical Oncology[5], Emory University School of Medicine, Atlanta, GA

[6]Current address: Duke Clinical Research Institute, Durham, NC 27705

The contribution by Dan Swartzlander includes partial written contributions, image creation, and data presented in Figures 1,7, and 8.

Published in Molecular and Cellular Biology (2009 Feb;29(3):794-807. Epub 2008 Nov 24).

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 [1-5]. Reactive oxygen species (ROS) are a byproduct of normal cellular metabolic processes that can cause oxidative damage to DNA, lipids, and proteins [6]. Unrepaired oxidative DNA lesions can result in mutations and lead to arrest of both DNA replication and transcription [7]. In order to combat such continuous insults to the genome, cells have evolved DNA repair and DNA damage tolerance pathways [8].

Base excision repair (BER) is the primary process by which oxidative DNA damage is repaired [9, 10]. BER is initiated by the recognition and excision of a base lesion by an *N*-glycosylase resulting in an apurinic/apyrimidinic (AP) site [11, 12]. 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 [13]. Subsequent processing involving DNA repair polymerases replaces the excised nucleotides, and DNA ligase completes the repair process [14].

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 [15-18]. 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 [19]. 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 [20]. Human DJ-1 protein, mutations in which are implicated in Parkinson's disease [21], translocates to mitochondria following oxidative stress in order to protect against cytotoxicity [22, 23]. 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 [19, 24, 25]. 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 [26, 27]. Several proteins involved in DNA repair and maintenance are sumoylated, conferring a range of functions [28]. For example, sumoylation of human thymine DNA glycosylase affects its glycosylase activity and localization to sub-nuclear regions [29, 30]. 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 [31-34]. Sumoylated HSF1 colocalizes with nuclear stress granules, facilitating transcription of specific heat-shock genes [34]. 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 [31]. 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 [35, 36]. 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 [37-40]. 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) [39, 41], Ntg1 is found in both the nucleus and mitochondria [10, 42]. In contrast, Ntg2, which contains only a putative NLS but no MTS, is localized exclusively to the nucleus [10, 42].

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 [43].

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 [44]. The *S. cerevisiae* haploid strain FY86 was utilized for all localization studies [45]. 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 [46], 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 [47]. 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 [48]. 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 [49], at the C-terminus of *SMT3* and *NTG1* or *NTG2* in the haploid strain ACY737 [50]. ACY737 contains mutations in the sumoylation deconjugating enzymes, Ulp1 and Ulp2, which can aid in the isolation of sumoylated proteins [50].

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 2×10^7 cells/mL. Dihydroethidium (DHEt) was added to the YPD to a concentration of 160 μM to detect cellular superoxide [51]; 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 5×10^7 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 [52] 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 [53]. Following this procedure, mitochondrial fractions were further purified using sucrose gradient centrifugation [54]. 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 [55]. 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 [56]. 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 [57]. 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) [58]. 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 [10, 42]. 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 [56]. 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* [59-61]. 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 [51, 62, 63]. 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 [63]. In addition, cells were exposed to H_2O_2 plus antimycin to increase mitochondrial oxidative stress. Antimycin blocks oxidative phosphorylation [64], and exposure of cells to H_2O_2 plus antimycin increases oxidative stress, leading to induced oxidative DNA damage in yeast mitochondria [47]. 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 [65]; whereas, MitoSox accumulates in the mitochondrial matrix, allowing determination of superoxide levels specifically in mitochondria [66]. 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 [51, 62, 63]. 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 <u>nuclear only</u> or <u>nuclear plus mitochondrial</u> 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 [47]. 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 [10, 42]. 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 [63] 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 [67]. 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 [47] 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 [31-34]. 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 [68, 69]. 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 monosumoylated Ntg1 and Ntg2 [10]. In addition, a recent study cataloging sumoylated yeast proteins reported that Ntg1 interacts with Smt3 [70], which encodes the yeast SUMO [71-73]; 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 monosumoylated Ntg1-TAP (70 kDa) as well as Ntg2-TAP (46 kDa) and monosumoylated 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 [56], 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 [68], 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 [74]. 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 [57]. These BER-/NER- defective cells are severely compromised for the repair of oxidative DNA damage and are highly sensitive to H_2O_2 [57]. 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

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.

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 [75, 76]. Mitochondrial oxidative stress is increased when cellular oxidative phosphorylation activity is particularly high or disrupted [6]. Furthermore, aging and various diseases have been associated with increased nuclear and mitochondrial ROS levels [1-4]. Under conditions where nuclear oxidative stress is high, nuclear oxidative DNA damage is elevated [62, 77]. Likewise, conditions that increase mitochondrial oxidative stress are associated with high levels of mitochondrial oxidative DNA damage [6, 47]. 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 [15-18], 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 [78-81], 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 [19, 78-83], 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 [28], and sumoylation has been implicated in the nuclear localization of a number of proteins [84]. 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 [85]. 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 [29, 85]. In this case, sumoylated TDG promotes a single event whose consequences persist after de-sumoylation [29]. 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 [84]. 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 [26, 27]. 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 [86]. 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 [57]. Yeast mitochondria do not contain Ntg2 or NER proteins, leaving mitochondrial DNA vulnerable in the absence of Ntg1 [87]. 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.

Acknowledgments

This work was supported by NIH Grants ES 011163 (P.W.D.), GM 066355 (K.D.W.), and GM 58728 (A.H.C.).

We would like to thank the microscope core facility at the Winship Cancer Institute, Emory University School of Medicine, especially Katherine Hales and Adam Marcus. We thank the flow cytometry core facility at Emory University School of

Medicine, especially Robert E. Karaffa, II. We would like to also thank the members of the Doetsch Lab for helpful suggestions regarding the writing of the manuscript.

References

1. Cooke, M.S., et al., *Oxidative DNA damage: mechanisms, mutation, and disease.* Faseb J, 2003. **17**(10): p. 1195-214.

2. Wallace, D.C., *Diseases of the mitochondrial DNA.* Annu Rev Biochem, 1992. **61**: p. 1175-212.

3. Lenaz, G., *Role of mitochondria in oxidative stress and ageing.* Biochim Biophys Acta, 1998. **1366**(1-2): p. 53-67.

4. Wei, Y.H., *Oxidative stress and mitochondrial DNA mutations in human aging.* Proc Soc Exp Biol Med, 1998. **217**(1): p. 53-63.

5. Beckman, K.B. and B.N. Ames, *Oxidative decay of DNA.* J Biol Chem, 1997. **272**(32): p. 19633-6.

6. Valentine, J.S., et al., *The dark side of dioxygen biochemistry.* Curr Opin Chem Biol, 1998. **2**(2): p. 253-62.

7. Ide, H., Y.W. Kow, and S.S. Wallace, *Thymine glycols and urea residues in M13 DNA constitute replicative blocks in vitro.* Nucleic Acids Res, 1985. **13**(22): p. 8035-52.

8. Aravind, L., D.R. Walker, and E.V. Koonin, *Conserved domains in DNA repair proteins and evolution of repair systems.* Nucl. Acids Res., 1999. **27**(5): p. 1223-1242.

9. Slupphaug, G., B. Kavli, and H.E. Krokan, *The interacting pathways for prevention and repair of oxidative DNA damage.* Mutat Res, 2003. **531**(1-2): p. 231-51.

10. You, H.J., et al., *Saccharomyces cerevisiae Ntg1p and Ntg2p: broad specificity N-glycosylases for the repair of oxidative DNA damage in the nucleus and mitochondria.* Biochemistry, 1999. **38**(35): p. 11298-306.

11. Lindahl, T., *New class of enzymes acting on damaged DNA.* Nature, 1976. **259**(5538): p. 64-6.

12. Lindahl, T., *DNA glycosylases, endonucleases for apurinic/apyrimidinic sites, and base excision-repair.* Prog Nucleic Acid Res Mol Biol, 1979. **22**: p. 135-92.

13. Barnes, D.E., T. Lindahl, and B. Sedgwick, *DNA repair.* Curr Opin Cell Biol, 1993. **5**(3): p. 424-33.

14. Boiteux, S. and M. Guillet, *Abasic sites in DNA: repair and biological consequences in Saccharomyces cerevisiae.* DNA Repair (Amst), 2004. **3**(1): p. 1-12.

15. Jans, D.A., C. Xiao, M. H. C. Lam, *Nuclear targeting signal recognition: a key control point in nuclear transport?* BioEssays, 2000. **22**(6): p. 532-544.

16. Nigg, E.A., *Nucleocytoplasmic transport: signals, mechanisms and regulation.* Nature, 1997. **386**(6627): p. 779-787.

17. Gorlich, D., *Transport into and out of the cell nucleus.* Embo J, 1998. **17**(10): p. 2721-7.

18. Hartl, F.-U., et al., *Mitochondrial protein import.* Biochimica et Biophysica Acta (BBA) - Reviews on Biomembranes, 1989. **988**(1): p. 1-45.

19. Yoshida, K. and Y. Miki, *Enabling death by the Abl tyrosine kinase: mechanisms for nuclear shuttling of c-Abl in response to DNA damage.* Cell Cycle, 2005. **4**(6): p. 777-9.

20. Kuge, S., et al., *Regulation of the Yeast Yap1p Nuclear Export Signal Is Mediated by Redox Signal-Induced Reversible Disulfide Bond Formation.* Mol. Cell. Biol., 2001. **21**(18): p. 6139-6150.

21. Bonifati, V., et al., *Mutations in the DJ-1 Gene Associated with Autosomal Recessive Early-Onset Parkinsonism.* Science, 2003. **299**(5604): p. 256-259.

22. Canet-Avilés, R.M., et al., *The Parkinson's disease protein DJ-1 is neuroprotective due to cysteine-sulfinic acid-driven mitochondrial localization.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(24): p. 9103-9108.

23. Li, H.M., et al., *Association of DJ-1 with chaperones and enhanced association and colocalization with mitochondrial Hsp70 by oxidative stress.* Free Radical Research, 2005. **39**(10): p. 1091 - 1099.

24. Calnan, D.R. and A. Brunet, *The FoxO code.* Oncogene. **27**(16): p. 2276-2288.

25. Scoumanne, A., K.L. Harms, and X. Chen, *Structural basis for gene activation by p53 family members.* Cancer Biol Ther, 2005. **4**(11): p. 1178-85.

26. Gill, G., *SUMO and ubiquitin in the nucleus: different functions, similar mechanisms?* Genes Dev., 2004. **18**(17): p. 2046-2059.

27. Heun, P., *SUMOrganization of the nucleus.* Current Opinion in Cell Biology, 2007. **19**(3): p. 350-355.

28. Muller, S., A. Ledl, and D. Schmidt, *SUMO: a regulator of gene expression and genome integrity.* Oncogene. **23**(11): p. 1998-2008.

29. Hardeland, U., et al., *Modification of the human thymine-DNA glycosylase by ubiquitin-like proteins facilitates enzymatic turnover.* Embo J, 2002. **21**(6): p. 1456-64.

30. Mohan, R.D., et al., *SUMO-1-dependent allosteric regulation of thymine DNA glycosylase alters subnuclear localization and CBP/p300 recruitment.* Mol Cell Biol, 2007. **27**(1): p. 229-43.

31. Hanson, C.A., L. D. Wood, S. W. Hiebert, *Cellular stress triggers TEL nuclear export via two genetically separable pathways.* Journal of Cellular Biochemistry, 2008. **104**(2): p. 488-498.

32. Wang, Y., et al., *The Small Ubiquitin-Like Modifier (SUMO) and SUMO-Conjugating System of Chlamydomonas reinhardtii.* Genetics, 2008. **179**(1): p. 177-192.

33. Kono, K., et al., *The mobility of Bach2 nuclear foci is regulated by SUMO-1 modification.* Exp Cell Res, 2008. **314**(4): p. 903-13.

34. Hong, Y., et al., *Regulation of Heat Shock Transcription Factor 1 by Stress-induced SUMO-1 Modification.* J. Biol. Chem., 2001. **276**(43): p. 40263-40267.

35. Memisoglu, A. and L. Samson, *Base excision repair in yeast and mammals.* Mutat Res, 2000. **451**(1-2): p. 39-51.

36. Eisen, J.A. and P.C. Hanawalt, *A phylogenomic study of DNA repair genes, proteins, and processes.* Mutat Res, 1999. **435**(3): p. 171-213.

37. Augeri, L., et al., *[10] Purification and properties of yeast redoxyendonuclease,* in *Methods in Enzymology*. 1994, Academic Press. p. 102-115.

38. Senturker, S., et al., *Substrate specificities of the Ntg1 and Ntg2 proteins of Saccharomyces cerevisiae for oxidized DNA bases are not identical.* Nucleic Acids Res, 1998. **26**(23): p. 5270-6.

39. You, H.J., R.L. Swanson, and P.W. Doetsch, *Saccharomyces cerevisiae Possesses Two Functional Homologues of Escherichia coli Endonuclease III.* Biochemistry, 1998. **37**(17): p. 6033-6040.

40. Hanna, M., et al., *Involvement of two endonuclease III homologs in the base excision repair pathway for the processing of DNA alkylation damage in Saccharomyces cerevisiae.* DNA Repair, 2004. **3**(1): p. 51-59.

41. Augeri, L., et al., *Purification, Characterization, Gene Cloning, and Expression of Saccharomyces cerevisiae Redoxyendonuclease, a Homolog of Escherichia coli Endonuclease III.* Biochemistry, 1997. **36**(4): p. 721-729.

42. Alseth, I., et al., *The Saccharomyces cerevisiae homologues of endonuclease III from Escherichia coli, Ntg1 and Ntg2, are both required for efficient repair of spontaneous and induced oxidative DNA damage in yeast.* Mol Cell Biol, 1999. **19**(5): p. 3779-87.

43. Ito, H., et al., *Transformation of intact yeast cells treated with alkali cations.* J Bacteriol, 1983. **153**(1): p. 163-8.

44. Kahana, J.A., B.J. Schnapp, and P.A. Silver, *Kinetics of Spindle Pole Body Separation in Budding Yeast.* Proceedings of the National Academy of Sciences, 1995. **92**(21): p. 9707-9711.

45. Winston, F., C. Dollard, and S.L. Ricupero-Hovasse, *Construction of a set of convenient Saccharomyces cerevisiae strains that are isogenic to S288C.* Yeast, 1995. **11**(1): p. 53-5.

46. Wach, A., A. Brachat, R. Pöhlmann, P. Philippsen, *New heterologous modules for classical or PCR-based gene disruptions in Saccharomyces cerevisiae.* Yeast, 1994. **10**(13): p. 1793-1808.

47. Doudican, N.A., et al., *Oxidative DNA damage causes mitochondrial genomic instability in Saccharomyces cerevisiae.* Mol Cell Biol, 2005. **25**(12): p. 5196-204.

48. Belli, G., et al., *Functional analysis of yeast essential genes using a promoter-substitution cassette and the tetracycline-regulatable dual expression system.* Yeast, 1998. **14**(12): p. 1127-38.

49. Longtine, M.S., et al., *Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae.* Yeast, 1998. **14**(10): p. 953-61.

50. Schwienhorst, I., E.S. Johnson, and R.J. Dohmen, *SUMO conjugation and deconjugation.* Mol Gen Genet, 2000. **263**(5): p. 771-86.

51. Rowe, L.A., N. Degtyareva, and P.W. Doetsch, *DNA damage-induced reactive oxygen species (ROS) stress response in Saccharomyces cerevisiae.* Free Radical Biology and Medicine, 2008. **45**(8): p. 1167-1177.

52. Rigaut, G., et al., *A generic protein purification method for protein complex characterization and proteome exploration.* Nat Biotech, 1999. **17**(10): p. 1030-1032.

53. Diekert, K., et al., *Isolation and subfractionation of mitochondria from the yeast Saccharomyces cerevisiae.* Methods Cell Biol, 2001. **65**: p. 37-51.

54. Querol, A. and E. Barrio, *A rapid and simple method for the preparation of yeast mitochondrial DNA.* Nucleic Acids Res, 1990. **18**(6): p. 1657.

55. Rout, M.P. and J.V. Kilmartin, *Components of the yeast spindle and spindle pole body.* J Cell Biol, 1990. **111**(5 Pt 1): p. 1913-27.

56. Rieder, S.E. and S.D. Emr, *Overview of subcellular fractionation procedures for the yeast Saccharomyces cerevisiae.* Curr Protoc Cell Biol, 2001. **Chapter 3**: p. Unit 3 7.

57. Swanson, R.L., et al., *Overlapping specificities of base excision repair, nucleotide excision repair, recombination, and translesion synthesis pathways for DNA base damage in Saccharomyces cerevisiae.* Mol Cell Biol, 1999. **19**(4): p. 2929-35.

58. Seedorf, M., et al., *Interactions between a nuclear transporter and a subset of nuclear pore complex proteins depend on Ran GTPase.* Mol Cell Biol, 1999. **19**(2): p. 1547-57.

59. Zinser, E. and G. Daum, *Isolation and biochemical characterization of organelles from the yeast, Saccharomyces cerevisiae.* Yeast, 1995. **11**(6): p. 493-536.

60. Rout, M.P. and G. Blobel, *Isolation of the yeast nuclear pore complex.* J Cell Biol, 1993. **123**(4): p. 771-83.

61. Nathanson, L., T. Xia, and M.P. Deutscher, *Nuclear protein synthesis: a re-evaluation.* Rna, 2003. **9**(1): p. 9-13.

62. Evert, B.A., et al., *Spontaneous DNA damage in Saccharomyces cerevisiae elicits phenotypic properties similar to cancer cells.* J Biol Chem, 2004. **279**(21): p. 22585-94.

63. Salmon, T.B., et al., *Biological consequences of oxidative stress-induced DNA damage in Saccharomyces cerevisiae.* Nucleic Acids Res, 2004. **32**(12): p. 3712-23.

64. Potter, V.R. and A.E. Reif, *Inhibition of an electron transport component by antimycin A.* J Biol Chem, 1952. **194**(1): p. 287-97.

65. Trushina, E., et al., *Mutant huntingtin impairs axonal trafficking in mammalian neurons in vivo and in vitro.* Mol Cell Biol, 2004. **24**(18): p. 8195-209.

66. Johnson-Cadwell, L.I., et al., *'Mild Uncoupling' does not decrease mitochondrial superoxide levels in cultured cerebellar granule neurons but decreases spare respiratory capacity and increases toxicity to glutamate and oxidative stress.* J Neurochem, 2007. **101**(6): p. 1619-31.

67. Ferguson, L.R. and R.C. von Borstel, *Induction of the cytoplasmic 'petite' mutation by chemical and physical agents in Saccharomyces cerevisiae.* Mutat Res, 1992. **265**(1): p. 103-48.

68. Xue, Y., et al., *SUMOsp: a web server for sumoylation site prediction.* Nucl. Acids Res., 2006. **34**(suppl_2): p. W254-257.

69. Rodriguez, M.S., C. Dargemont, and R.T. Hay, *SUMO-1 Conjugation in Vivo Requires Both a Consensus Modification Motif and Nuclear Targeting.* J. Biol. Chem., 2001. **276**(16): p. 12654-12659.

70. Hannich, J.T., et al., *Defining the SUMO-modified Proteome by Multiple Approaches in Saccharomyces cerevisiae.* J. Biol. Chem., 2005. **280**(6): p. 4102-4110.

71. Meluh, P.B. and D. Koshland, *Evidence that the MIF2 gene of Saccharomyces cerevisiae encodes a centromere protein with homology to the mammalian centromere protein CENP-C.* Mol. Biol. Cell, 1995. **6**(7): p. 793-807.

72. Johnson, E.S., et al., *The ubiquitin-like protein Smt3p is activated for conjugation to other proteins by an Aos1p/Uba2p heterodimer*. Embo J, 1997. **16**(18): p. 5509-19.

73. Dohmen, R.J., et al., *An Essential Yeast Gene Encoding a Homolog of Ubiquitin-activating Enzyme.* J. Biol. Chem., 1995. **270**(30): p. 18099-18109.

74. Hoege, C., et al., *RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO.* Nature, 2002. **419**(6903): p. 135-141.

75. Jamieson, D.J., *Oxidative stress responses of the yeast Saccharomyces cerevisiae.* Yeast, 1998. **14**(16): p. 1511-27.

76. Liu, X.F., et al., *Yeast lacking superoxide dismutase. Isolation of genetic suppressors.* J Biol Chem, 1992. **267**(26): p. 18298-302.

77. Yakes, F.M. and B. Van Houten, *Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress.* Proc Natl Acad Sci U S A, 1997. **94**(2): p. 514-9.

78. Tell, G., et al., *TSH controls Ref-1 nuclear translocation in thyroid cells.* J Mol Endocrinol, 2000. **24**(3): p. 383-90.

79. Tell, G., et al., *An 'environment to nucleus' signaling system operates in B lymphocytes: redox status modulates BSAP/Pax-5 activation through Ref-1 nuclear translocation.* Nucleic Acids Res, 2000. **28**(5): p. 1099-105.

80. Frossi, B., et al., *H_2O_2 induces translocation of APE/Ref-1 to mitochondria in the Raji B-cell line.* J Cell Physiol, 2002. **193**(2): p. 180-6.

81. Ding, S.Z., et al., *Helicobacter pylori and H_2O_2 increase AP endonuclease-1/redox factor-1 expression in human gastric epithelial cells.* Gastroenterology, 2004. **127**(3): p. 845-58.

82. Liu, W., et al., *Nuclear transport of human DDB protein induced by ultraviolet light.* J Biol Chem, 2000. **275**(28): p. 21429-34.

83. Yoon, J.H., et al., *The retinitis pigmentosa-mutated RP2 protein exhibits exonuclease activity and translocates to the nucleus in response to DNA damage.* Exp Cell Res, 2006. **312**(8): p. 1323-34.

84. Palancade, B. and V. Doye, *Sumoylating and desumoylating enzymes at nuclear pores: underpinning their unexpected duties?* Trends Cell Biol, 2008. **18**(4): p. 174-83.

85. Johnson, E.S., *Protein modification by SUMO.* Annu Rev Biochem, 2004. **73**: p. 355-82.

86. Santos, J.H., B.S. Mandavilli, and B. Van Houten, *Measuring oxidative mtDNA damage and repair using quantitative PCR.* Methods Mol Biol, 2002. **197**: p. 159-76.

87. Clayton, D.A., J.N. Doda, and E.C. Friedberg, *The absence of a pyrimidine dimer repair mechanism in mammalian mitochondria.* Proc Natl Acad Sci U S A, 1974. **71**(7): p. 2777-81.

88. Goldstein, A.L. and J.H. McCusker, *Three new dominant drug resistance cassettes for gene disruption in Saccharomyces cerevisiae.* Yeast, 1999. **15**(14): p. 1541-53.

89. Nakai, K. and P. Horton, *PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization.* Trends in Biochemical Sciences, 1999. **24**(1): p. 34-35.

90. Hilbert, T.P., et al., *Purification of a mammalian homologue of Escherichia coli endonuclease III: identification of a bovine pyrimidine hydrate-thymine glycol DNAse/AP lyase by irreversible cross linking to a thymine glycol-containing oligoxynucleotide.* Biochemistry, 1996. **35**(8): p. 2505-11.

91. Kuo, C.F., et al., *Atomic structure of the DNA repair [4Fe-4S] enzyme endonuclease III.* Science, 1992. **258**(5081): p. 434-40.

Table 1: Strains and Plasmids Used in this Study

Strain or Plasmid	Description	References
FY86 (ACY193)	*MATa ura3-52 leu2Δ1 his3Δ200*	[10]
ACY737	*MATa ulp1ts ulp2Δ lys2 trp1*	[50]
SJR751 (DSC0025)	*MATα ade2-101$_{oc}$ his3Δ200 ura3ΔNco lys2ΔBgl leu2-R*	[57]
SJR1101 (DSC0051)	*MATα ade2-101$_{oc}$ his3Δ200 ura3ΔNco lys2ΔBgl leu2-R ntg1Δ::LEU2 ntg2Δ::hisG apn1Δ::HIS3 rad1Δ::hisG*	[57]
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	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open

(DSC0313)		Biosystems
pNTG1-GFP	NTG1-GFP, *2μ, URA3, AMP^R*	[10, 44, 45]
pNTG2-GFP	NTG2-GFP, *2μ, URA3, AMP^R*	[10, 44, 45]
pNTG1K364R-GFP	NTG1 K364R-GFP, *2μ, URA3, AMP^R*	This study
pFA-KMX4	*KANAMYCIN^R, CEN, AMP^R*	[46]
pAG32	*Hygromycin B phosphor transferase MX4^R, AMP^R*	[88]
pCM225	tet0$_7$, *CEN, Kan MX4^R, AMP^R*	[48]
p1375	3HA, *CEN, TRP1, AMP^R*	[49]
p2245	pGAL1-GST, *CEN, TRP1, AMP^R*	[49]

Figure 1

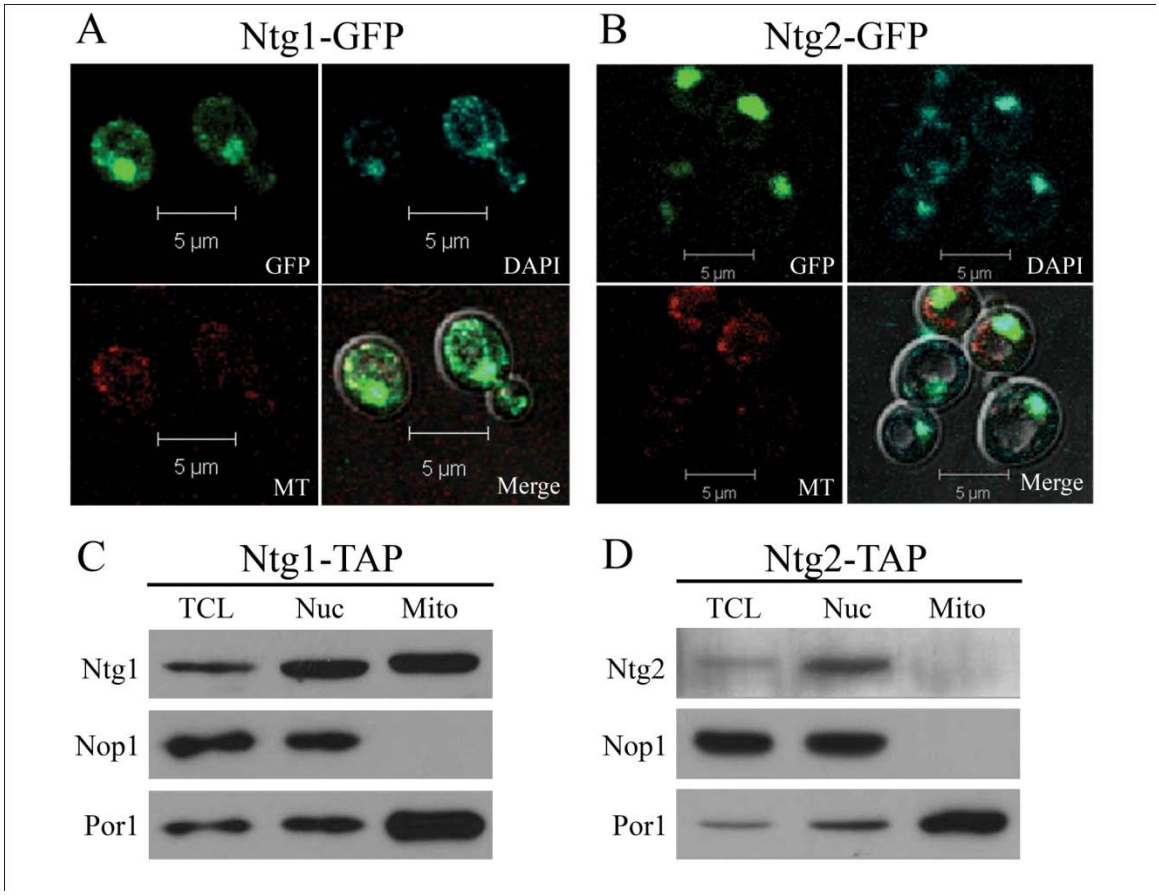


Figure 1: Subcellular localization of Ntg1 and Ntg2 under normal growth conditions. A, B. Localization of GFP-tagged protein was assessed via direct fluorescence microscopy. GFP, DAPI, Mitotracker (MT), and merged images of cells expressing Ntg1-GFP or Ntg2-GFP are displayed. **C, D.** Sucrose gradient 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

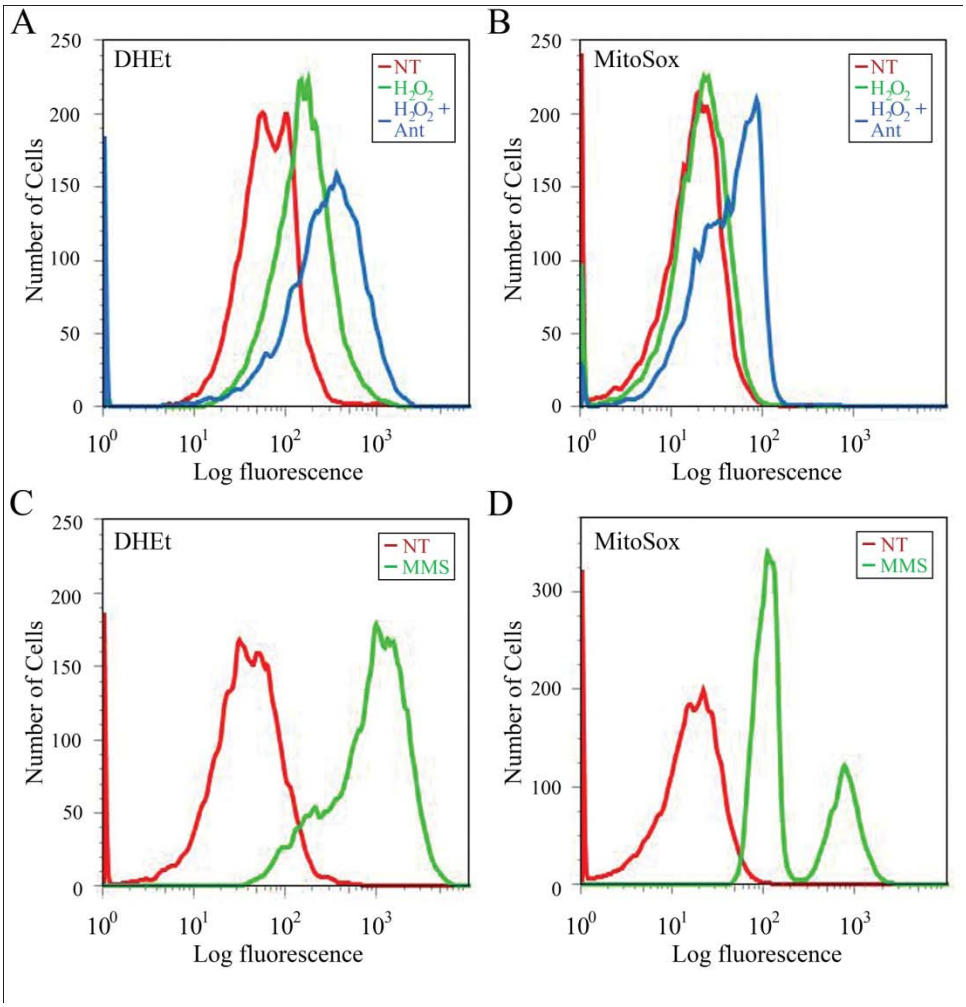


Figure 2: Flow cytometric analysis of cells to determine intracellular ROS levels following nuclear or mitochondrial oxidative stress. A, B. Cells were left untreated (red, NT) or exposed to 20 mM H_2O_2 (green) or 20 mM H_2O_2 plus 10 μg/mL antimycin (blue) and incubated with dihydroethidium (DHEt) 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

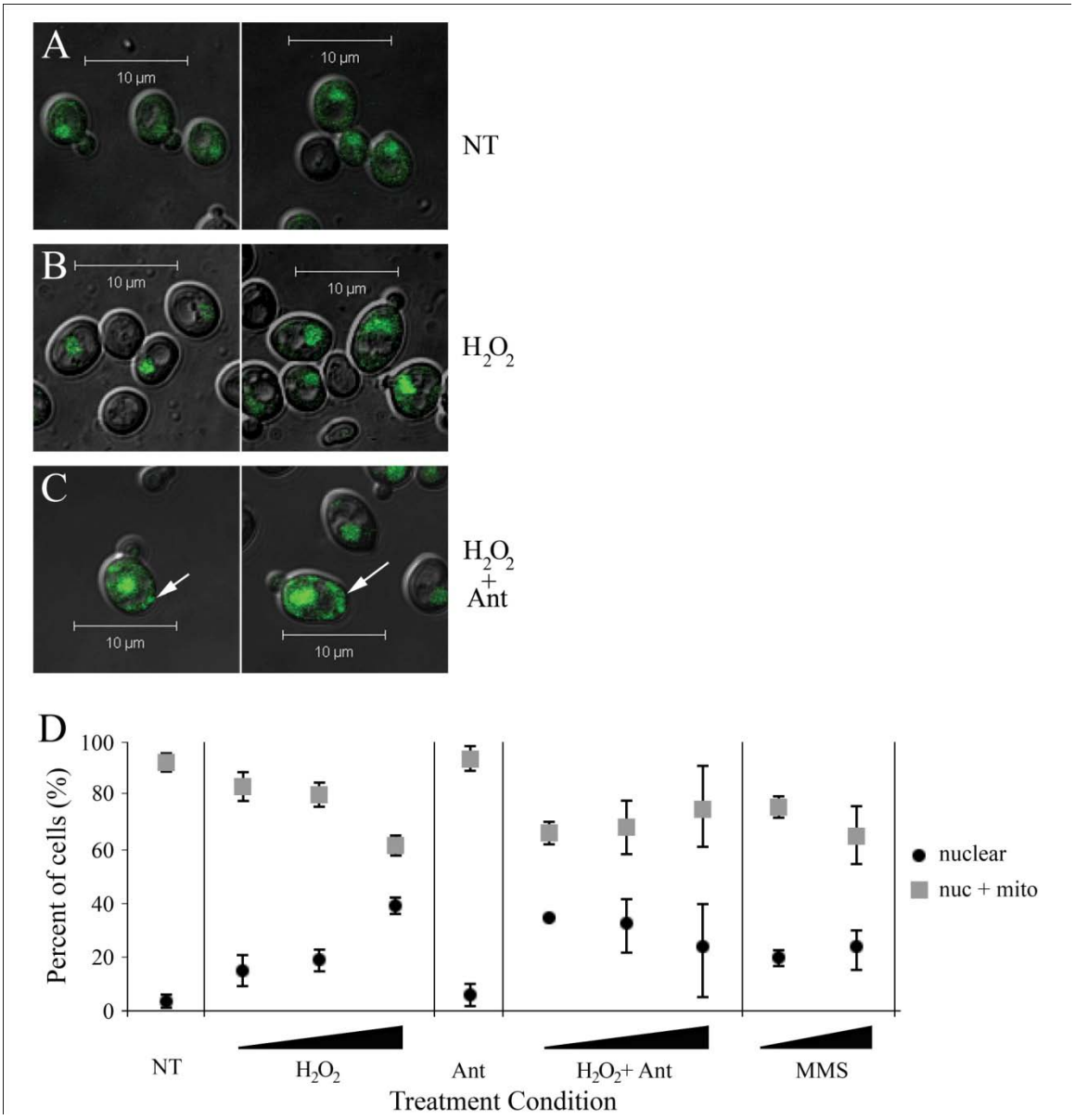


Figure 3: Subcellular localization of Ntg1 following exposure to nuclear and mitochondrial oxidative stress. Localization of GFP-tagged Ntg1 was assessed via direct fluorescence microscopy following exposure to the indicated oxidative stress agent for 1 hour. **A**. GFP images of untreated cells expressing 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

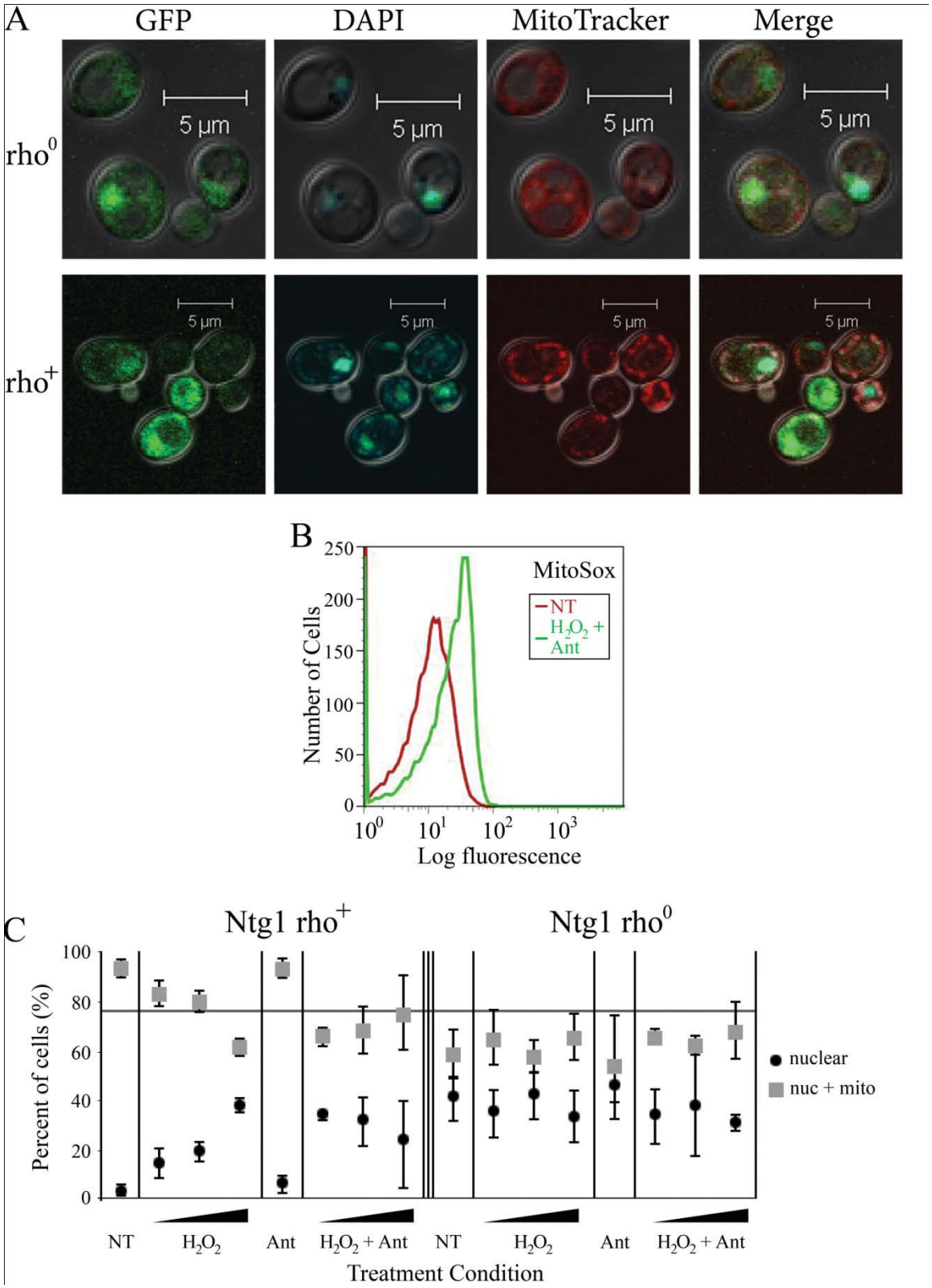

A
GFP
DAPI
MitoTracker
Merge
rho0
rho+
5 µm
B
MitoSox
NT
H2O2 + Ant
Number of Cells
Log fluorescence
C
Ntg1 rho+
Ntg1 rho0
Percent of cells (%)
nuclear
nuc + mito
NT
H2O2
Ant
H2O2 + Ant
Treatment Condition

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

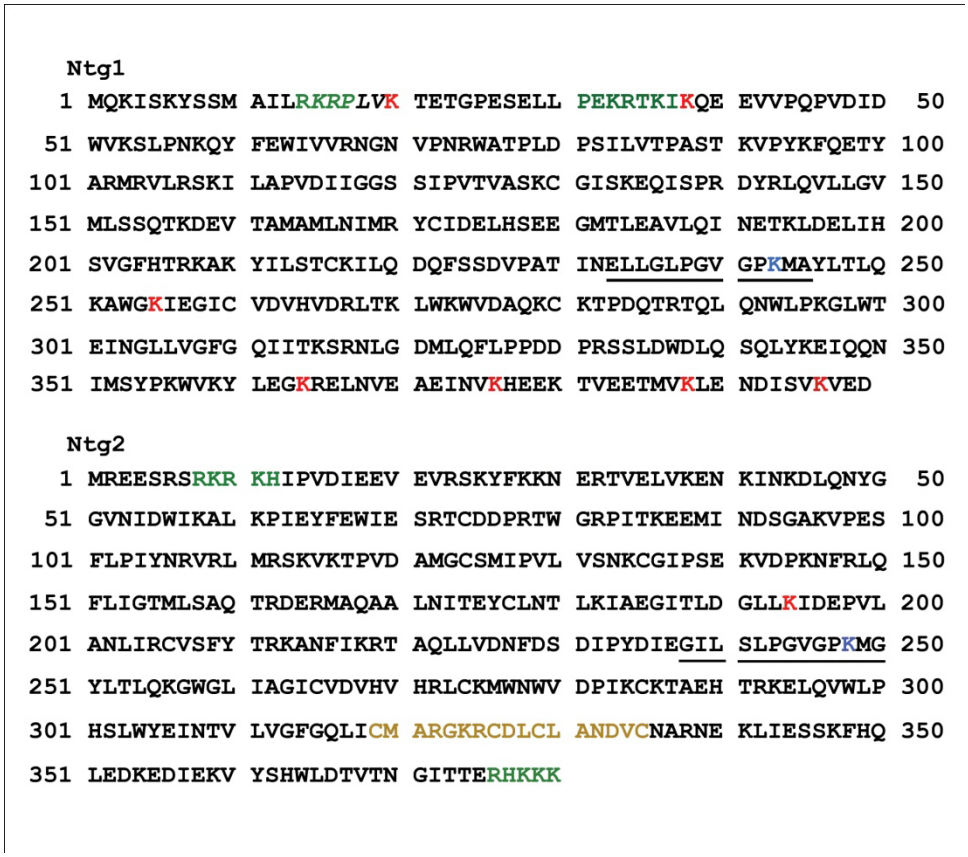


Figure 5: **Amino acid sequences of Ntg1 and Ntg2.** The amino acid sequences of Ntg1 (top) and Ntg2 (bottom) are shown. The following domain structures are indicated: potentially sumoylated lysines with a [Hydrophobic] K x [ED] motif (red) as predicted by the SUMOsp 1.0 program [68], predicted nuclear localization sequence (green) as determined with the NUCDISC subprogram of PSORTII [89], predicted mitochondrial targeting sequence (bold, italicized) as determined with the MITDISC subprogram of PSORTII [89], predicted helix hairpin helix active site region (underlined) and active site lysine (blue) determined due to significant homology with endonuclease III and its homologs [41, 90], and the [4Fe-2S]-cluster (brown) characterized by the sequence, C-X_6-C-X-X-C-X_5-C [91].

Figure 6

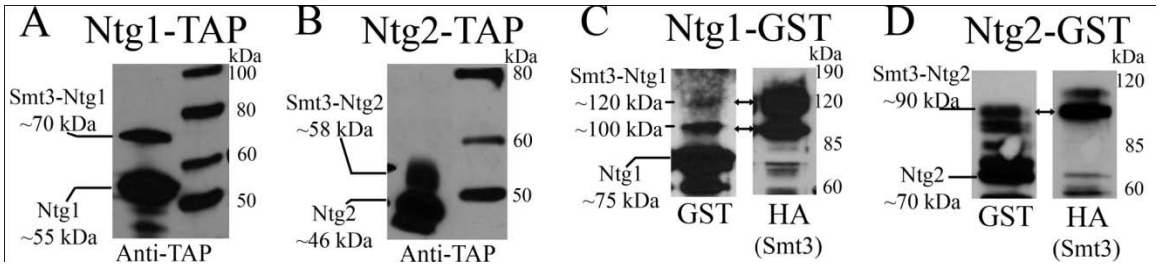


Figure 6: Post-translational modification of Ntg1 and Ntg2 by SUMO. A, B. Western analysis of TAP-purified Ntg1-TAP and Ntg2-TAP utilizing an anti-calmodulin TAP antibody. Non-sumoylated and sumoylated species of Ntg1 and Ntg2 are indicated. Protein sizes are indicated in right margins. **C, D.** Western 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

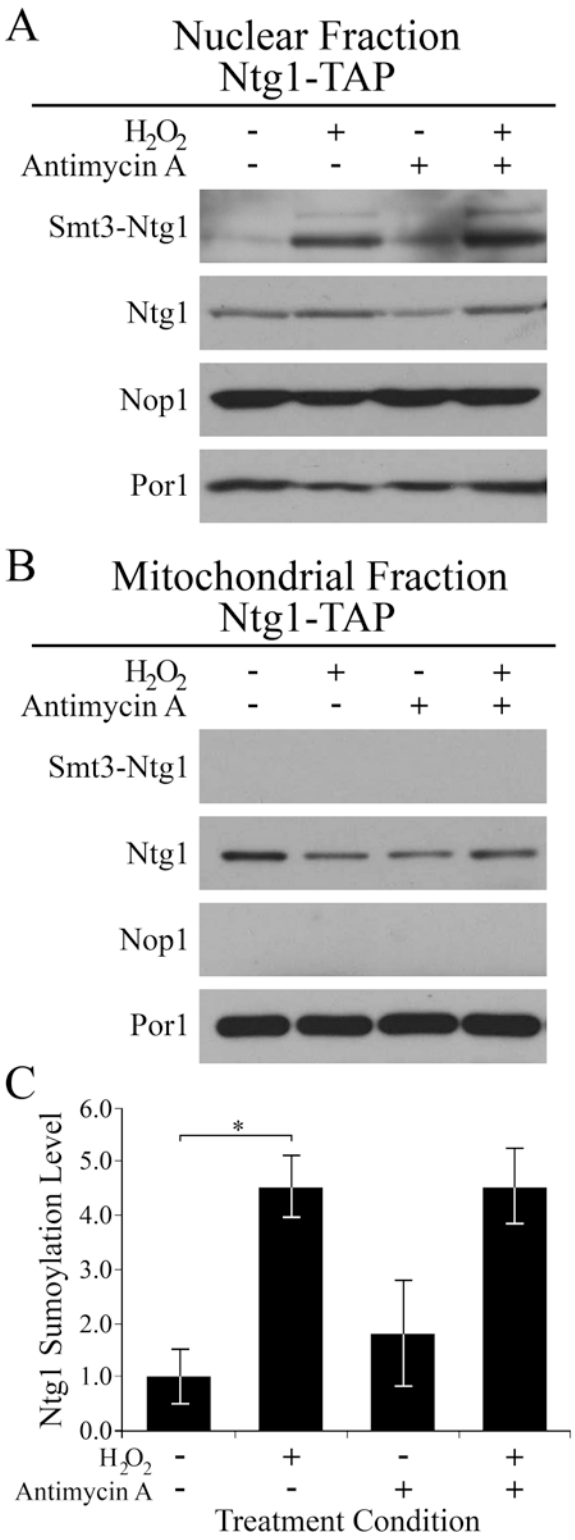

A
Nuclear Fraction
Ntg1-TAP
H2O2 - + - +
Antimycin A - - + +
Smt3-Ntg1
Ntg1
Nop1
Por1
B
Mitochondrial Fraction
Ntg1-TAP
H2O2 - + - +
Antimycin A - - + +
Smt3-Ntg1
Ntg1
Nop1
Por1
C
Ntg1 Sumoylation Level
6.0
5.0
4.0
3.0
2.0
1.0
0.0
*
H2O2 - + - +
Antimycin A - - + +
Treatment Condition

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

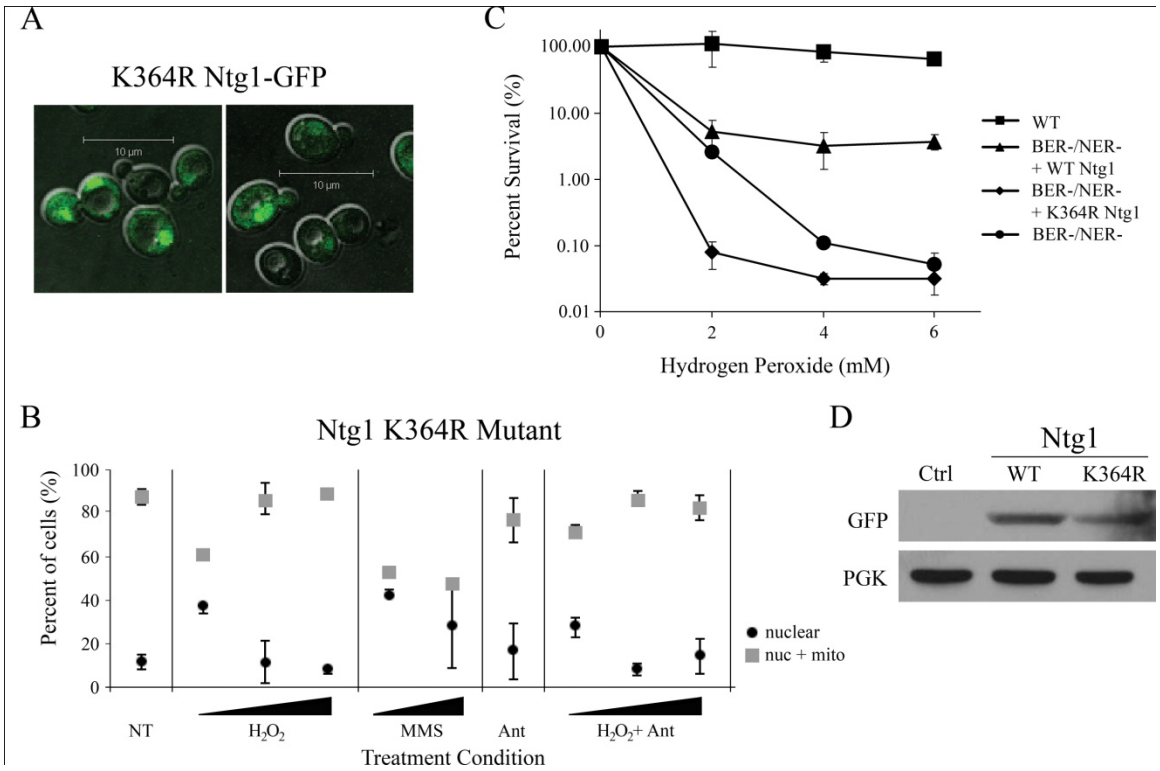


Figure 8: Subcellular localization and function of the Ntg1 K364R mutant. A. GFP image of cells expressing Ntg1 K364R-GFP. **B.** Quantification of Ntg1 K364R-GFP localization. Cells were not treated (NT) or were exposed to the indicated oxidative stress inducing agent for 1 hour (Materials and Methods). 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

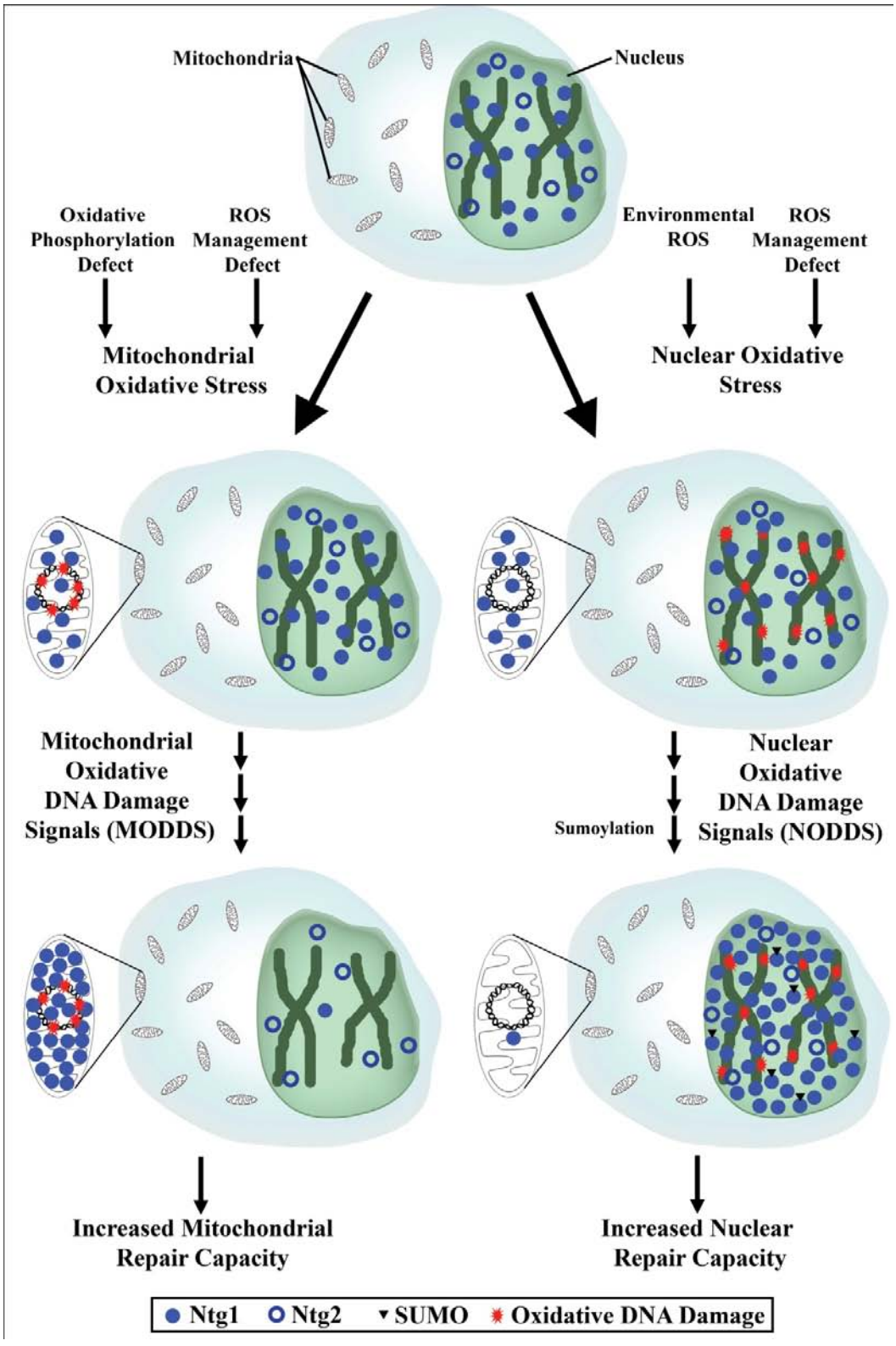

Mitochondria
Nucleus
Oxidative Phosphorylation Defect
ROS Management Defect
Environmental ROS
ROS Management Defect
Mitochondrial Oxidative Stress
Nuclear Oxidative Stress
Mitochondrial Oxidative DNA Damage Signals (MODDS)
Sumoylation
Nuclear Oxidative DNA Damage Signals (NODDS)
Increased Mitochondrial Repair Capacity
Increased Nuclear Repair Capacity
Ntg1
Ntg2
SUMO
Oxidative DNA Damage

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.

Chapter 3

Regulation of Base Excision Repair: Ntg1 Nuclear and Mitochondrial Dynamic Localization in Response to Genotoxic Stress.

Dan B. Swartzlander[§1,2], Lyra M. Griffiths[§1,2], Joan Lee[3,4], Natalya P. Degtyareva[1,5], Paul W. Doetsch[‡1,5,6,7], and Anita H. Corbett*[‡1,5]

From the Department of Biochemistry[1], Graduate Program in Genetics and Molecular Biology[2], Graduate Program in Biochemistry, Cell, and Developmental Biology[3], Department of Pharmacology[4], Winship Cancer Institute[5], and Department of Radiation Oncology[6] and Hematology and Medical Oncology[7], Emory University School of Medicine, Atlanta, GA 30322 USA

[§]The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

The contribution by Dan Swartzlander includes significant written contribution, image creation, and data presented in Figures 1,3,5,6, and 7.

Published in Nucleic Acids Research (2010 Jul;38(12):3963-74. Epub 2010 Mar 1.).

1. Abstract

Numerous human pathologies result from unrepaired oxidative DNA damage. Base excision repair (BER) is responsible for the repair of oxidative DNA damage that occurs in both nuclei and mitochondria. Despite the importance of BER in maintaining genomic stability, knowledge concerning the regulation of this evolutionarily conserved repair pathway is almost non-existent. The *Saccharomyces cerevisiae* BER protein, Ntg1, relocalizes to organelles containing elevated oxidative DNA damage, indicating a novel mechanism of regulation for BER. We propose that dynamic localization of BER proteins is modulated by constituents of stress response pathways. In an effort to mechanistically define these regulatory components, the elements necessary for nuclear and mitochondrial localization of Ntg1 were identified, including a bipartite classical nuclear localization signal, a mitochondrial matrix targeting sequence, and the classical nuclear protein import machinery. Our results define a major regulatory system for BER which when compromised, confers a mutator phenotype and sensitizes cells to the cytotoxic effects of DNA damage.

2. Introduction

DNA that is damaged and left unrepaired in either nuclei or mitochondria is linked to cancer, aging, and various degenerative diseases [1-3]. Oxidative DNA damage occurs frequently in both nuclear and mitochondrial genomes and is primarily repaired by the base excision repair (BER) pathway [4, 5]. While the components of BER have been extensively studied, the regulatory mechanisms that ensure optimal deployment of BER proteins are virtually unknown.

In order to investigate new modes of regulation of BER, we focused on the *S. cerevisiae* BER protein, Ntg1. Ntg1 is a bi-functional 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 [6-9]. 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 [10-13]. As functional homologs are expressed from bacteria to human, and the BER pathways are conserved between these species, the elucidation of BER mechanisms should have broad implications for all eukaryotic organisms.

Ntg1 and hNTH1 are localized to both nuclei and mitochondria [11, 14-16], where they repair oxidative lesions and maintain genomic stability of nuclear and mitochondrial DNA [17-19]. Recently, it was reported that nuclear and mitochondrial oxidative stress is associated with dynamic localization of Ntg1 to these two organelles [15]. Relocalization of proteins in response to DNA damage and oxidative stress has been previously reported [20-22], suggesting that dynamic localization may be a general mode of regulation in response to genotoxic and other stress events. Thus, the mechanistic components identified in this process are likely to represent factors that mediate regulation of DNA repair and other genotoxic stress responses that prevent the mutagenic and cytotoxic effects of DNA damage.

In order to determine the mechanism for Ntg1 dynamic localization, it was first necessary to delineate the basic mechanisms 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 [15, 23]. These putative sequences include two predicted 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) [24]. 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 [25]. MTSs are typically 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 [26-28]. Proteins with MTSs enter the mitochondrial matrix after being passed from the outer membrane translocase to the inner membrane translocase [29, 30].

In this study, we functionally defined the 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 identified the import pathway required for nuclear localization of Ntg1. In the absence of either the cNLS or MTS, dynamic localization of Ntg1 does not occur, resulting in increased nuclear and mitochondrial mutagenesis. 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 and provide the framework for a general model for the regulation of BER.

3. Materials and Methods

3.1 Strains, Plasmids, and Media. All haploid *S. cerevisiae* strains and plasmids used in this study are listed in Supplemental 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 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 [31].

A GFP expression vector (2 micron, *URA3*), pPS904 [32] was employed for generation of the C-terminally tagged Ntg1-GFP fusion protein. 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 [15, 33]. 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 (Supplemental Table 1).

A vector expressing tandem GFP (pAC1069) 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 (Supplemental 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 C-terminal His_6 epitope tagged Ntg1-His_6 (pD0390), (Supplemental 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) (Supplemental Table 1). Expression vectors were transformed into DE3 cells.

S. cerevisiae haploid wild type (DSC0367) and BER^{-}/NER^{-} (DSC369) cells were utilized to examine H_2O_2 and MMS sensitivity studies and H_2O_2 mutation frequency studies. Site directed mutagenesis at the *NTG1* locus of the wild type (DSC0367) parent was performed via *delitto perfetto* protocol [35] in order to generate $Ntg1_{nls1}$, $Ntg1_{nls2}$, $Ntg1_{mts}$, and $Ntg1_{catalytic}$ encoded at the endogenous *NTG1* locus. The resulting mutants were then crossed with the BER^{-}/NER^{-} mutant (LAR023), creating diploids which were then dissected to identify cells with the each Ntg1 variant BER^{*}/NER^{-} strain, which were selected for functional studies of Ntg1 (Supplemental Table 1).

3.2 Exposure to DNA Damaging Agents. Cells were grown in 5 mL YPD or SD -URA media to 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); 1-55 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 colony counting to determine the number of colony forming units.

3.3 Fluorescence Microscopy. For all experiments, cultures were grown and either left untreated or exposed to DNA damaging agent(s) as previously described [15]. During exposure to DNA damaging agents, cultures were also incubated with 25 nM MitoTracker in order to visualize mitochondria. Cells were incubated with 1

μg/mL DAPI for 5 minutes to visualize DNA. Cells were then analyzed by direct fluorescence confocal microscopy, employing a Zeiss LSM510 META microscope and Carl Zeiss LSM Image Browser software. For quantification 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 two different polyclonal anti-GFP antibodies: (1:5,000 dilution) [36]; (1:10,000 dilution, Synaptic Systems). Either an anti-3-phosphoglycerate (PGK) (1:10,000 dilution; Invitrogen) or an anti-Nop1 (1:25,000 dilution, EnCor) antibody 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 [37]. Briefly, *E.coli* BL21 (DE3) cells containing each variant Ntg1-His_6 plasmids were grown to an OD600 of 0.5–1.0 and expression induced for 4 hours at 25°C. Cells were lysed via sonication and the supernatant was applied to Ni+ affinity chromatography (Qiagen) to purify the Ntg1-His_6 variant, which was further purified to apparent homogeneity by Mono S FPLC (Pharmacia).

3.6 In Vitro Binding Assay. To assess the interaction between Ntg1 and importin-α, we employed a truncated form of importin-α (pAC1338) which lacks the auto-inhibitory N-terminal importin-β binding domain (ΔIBB-importin-α), GST-ΔIBB-

importin-α and a GST alone (pAC736) control [38, 39]. The GST fusion proteins were batch purified as previously described [40] using glutathione-Sepharose 4B (GE Healthcare). GST-ΔIBB-importin-α was further purified to apparent homogeneity on a Superdex S-200 gel-filtration column as previously described [41]. For binding assays, 4 μg of purified Ntg1-His_6 or 4 μg of Nab2-His_6 (pAC785) was incubated for 2 hrs at 4^{o}C with 12 μg GST-ΔIBB-importin-α or 12 μg GST alone, with glutathione sepharose beads in phosphate-buffered saline (PBST) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, and 0.5% Triton X-100). As a non-specific competitor, all binding reactions also contained 0.1 mg/mL chicken egg albumin. Beads were collected, washed 3 times in PBST for 10 min at 4°C, and the bound fraction was eluted with SDS-PAGE buffer (50% glycerol, 10% SDS, 10% β-ME, 100mM Tris-HCl, and 0.1% bromophenol blue). The unbound and bound fractions were analyzed via 10% SDS-PAGE, and the gel was stained with Coomassie Blue.

3.7 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 [37]. 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 [42]. 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).

3.8 Functional Analysis of Ntg1 in vivo. To assess the biological function of the Ntg1, survival and mutagenesis experiments were carried out using cells that express each Ntg1 variant encoded at the endogenous *NTG1* locus. The survival of cells expressing wild type Ntg1 and either repair-compromised or Ntg1 localization mutants were assessed by examining the sensitivity of cells to treatment with H_2O_2 or MMS as previously described [15]. In order to assess the frequency of nuclear DNA mutation, an L-canavanine (CAN) resistance assay was employed [43]. Wild type and repair-compromised cells were grown in 5 mL SD complete media or SD -URA media to saturation. Dilutions of cells were plated onto YPD or CAN-containing medium (SD -ARG 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 and 95% confidence limits determination [44, 45]. In order to assess the frequency of mitochondrial DNA mutation, an erythromycin resistance assay was employed. Erythromycin resistance assay conditions were adapted from [46]. Cells were grown in 5 mL YPG and plated onto YPG and YPG plus 1 mg/mL erythromycin (Sigma) as previously described [18].

Mutation frequencies were calculated as follows: number of erythromycin-resistant colonies/total number of colonies. Average frequencies were calculated from 16-20 independent cultures, and 95% confidence limits determined [45].

4. Results

4.1 Regulatory Targeting Signals in Ntg1. Ntg1 is important for maintenance of both nuclear and mitochondrial genomes [11, 18]. Consistent with this dual role, Ntg1 is localized to both nuclei and mitochondria (Figure 1A, B) [11, 14]. 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) [47]. The first predicted cNLS consists of amino acids 14-17 (RKRP) and the second consists of amino acids 31-37 (PEKRTKI) [15]. Ntg1 also contains a putative MTS identified by the MitoProt II program as the first 26 amino acids [48]. The putative MTS contains basic amino acids thought to be responsible for proper amphipathic alpha helix formation, including K3 and K6 (Figure 1A) [26]. 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 [15, 23].

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 (Table 1) and examined the localization of the

resulting proteins by creating C-terminal green fluorescent protein (GFP) fusion proteins. cNLS1 (RKRP, residues 14-17) was changed to RAAP, creating $Ntg1_{nls1}$-GFP, while cNLS2 (PEKRTKI, residues 31-37) was changed to PEAATK creating $Ntg1_{nls2}$-GFP. 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 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). Collectively, these results suggest that Ntg1 contains a bipartite cNLS with a longer linker sequence (16 amino acids) than most conventional bipartite cNLS motifs [24] that is necessary for proper nuclear localization of Ntg1.

To experimentally define the MTS in Ntg1, we changed the third and sixth basic amino acids in the sequence KISK to glutamic acid (EISE) to reverse the charge and eliminate the potential to form the amphipathic alpha helix required for mitochondrial entry [28]. 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 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 ~60% compared to wild type Ntg1-GFP, while $Ntg1_{mts}$-GFP localization to mitochondria was reduced ~40%

compared to wild type Ntg1-GFP. This quantitative analysis confirmed that cNLS1 and cNLS2, are components of a bipartite cNLS, and 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 other mechanisms since we observe low levels of residual localization even when these targeting signals are altered. In order to verify that the altered 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 not significantly different compared to wild type Ntg1-GFP (Figure 1D).

To assess whether the nuclear targeting motifs identified within Ntg1 function as a bipartite cNLS, each cluster was fused separately (cNLS1 or cNLS2) or in combination (cNLS1/2) 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 [49]. Direct fluorescence microscopy revealed that neither cNLS1-GFP_2 nor cNLS2-GFP_2 localized to the nucleus, while cNLS1/2-GFP_2 was sufficient to target GFP_2 to the nucleus (Figure 2), indicating that both cNLSs are required and confirming that cNLS1 and cNLS2, together, comprise a bipartite cNLS. These data indicate that the bipartite cNLS is a major determinant for the nuclear localization of Ntg1 and suggests that Ntg1 interacts with the classical nuclear protein import machinery to access the nucleus.

4.2 Regulation of Ntg1 Functions via the Classical Nuclear Import Machinery.

As Ntg1 contains a bipartite cNLS that is both necessary and sufficient for nuclear

protein import of Ntg1 (Figure 1, 2), we tested whether the classical nuclear protein import pathway is responsible for Ntg1 nuclear localization. The classical nuclear protein import pathway relies on the cNLS receptor subunit, importin α, and the nuclear pore targeting subunit, importin β [25]. 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*) [50, 51]. As controls, we also evaluated the localization of the previously characterized SV40 bipartite cNLS [34] and a non-cNLS containing protein, Nab2 [52], in these mutant cells. All proteins examined localized to the nucleus in wild type cells, both at the permissive (30ºC) and the non-permissive (37ºC) temperatures (Figure 3A), 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 3A), 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 (Supplemental Figure 1). The control, Nab2-GFP, which is imported to the nucleus in an importin α-independent manner [52], 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 3A). As a further control, Ntg1-GFP, remained localized to the nucleus in Δ*sxm1* cells (Figure 3A), which are missing a transport receptor that facilitates non-classical nuclear protein import [53], indicating that Ntg1 is not mislocalized in all transport receptor mutant cells.

In order to determine whether Ntg1 binds directly to the cNLS receptor, importin α, we performed a direct binding assay using purified recombinant proteins. These studies employed an N-terminally truncated importin α, GST-ΔIBB-importin α, that lacks the auto-inhibitory importin β binding (IBB) domain [54]. The truncated form of importin α mimics the import complex that forms when importin β binds the IBB domain of importin α to prevent competition for the cNLS binding pocket of importin α [54, 55] and hence binds more tightly to cNLS cargo than full length importin α. As controls, we employed two proteins, a GST control, which does not interact with Ntg1, and His_6-Nab2 [38, 39]. Results of the *in vitro* binding assay (Figure 3B) reveal that Ntg1 interacts directly with GST-ΔIBB-importin α, but not with GST alone. The control Nab2 did not bind either GST alone or GST-ΔIBB-importin α, demonstrating that the interaction between Ntg1 and importin α is specific (Figure 3B).

4.3 Regulatory 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 [15]. In order to determine whether mutants of Ntg1 defective for 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) to directly increase nuclear oxidative stress, H_2O_2 plus antimycin to increase mitochondrial oxidative stress, or methylmethane sulfonate (MMS) an alkylating agent which indirectly increases oxidative stress in both the nucleus and mitochondria [15, 56, 57]. While nuclear or mitochondrial localization of wild type

Ntg1-GFP increased in response to either nuclear (H_2O_2) or mitochondrial (H_2O_2 plus antimycin) oxidative stress, respectively [15], the localization of neither $Ntg1_{nls2}$-GFP nor $Ntg1_{mts}$-GFP was changed 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 utilized a strain that is deficient in both BER through disruption of three genes with overlapping DNA repair capacities *apn1*, *ntg2*, and *ntg1* (BER^-) and nucleotide excision repair (NER) through disruption of the single gene *rad1* (NER^-) yielding *apn1 ntg2 ntg1 rad1* (BER^-/NER^-) cells [43]. BER^-/NER^- cells are severely compromised for the repair of DNA damage [43] allowing for the individual contribution of each Ntg1 variant to DNA repair to be assessed *in vivo*. In order to analyze Ntg1 function, the endogenous locus of *NTG1* was mutated in *apn1 ntg2* (BER*) *rad1* (NER^-) cells to create BER^*/NER^- mutants expressing each of the Ntg1 variants analyzed in this study. The decrease in BER capacity of strains harboring Ntg1 mutations could result in two quantifiable biological endpoints: increased DNA mutation rates and/or decreased survival following exposure to DNA damaging agents [58]. To determine whether repair of oxidative DNA damage by Ntg1 plays a role in the prevention of nuclear and/or mitochondrial DNA mutations, we assessed nuclear and mitochondrial mutations [43, 59] in the wild type, BER^-/NER^-, and all Ntg1 BER^*/NER^- variants.

BER^-/NER^- and BER^*_{cat}/NER^- (*apn1 ntg2 ntg1*$_{cat}$ *rad1*) cells displayed higher nuclear mutation rates compared to both wild type and BER^*_{wt}/NER^- (*apn1 ntg2 NTG1*

rad1) cells (Table 2), indicating that Ntg1 plays an important role in preventing nuclear mutations. In order to assess the impact of dynamic localization of Ntg1 on nuclear and mitochondrial mutations, BER*$_{nls1}$/NER^{-} (*apn1 ntg2 ntg1$_{nls1}$ rad1*), BER*$_{nls2}$/NER^{-} (*apn1 ntg2 ntg1$_{nls2}$ rad1*) and BER*$_{mts}$/NER^{-} (*apn1 ntg2 ntg1$_{mts}$ rad1*) cells were analyzed. Nuclear mutation rates of both BER*$_{nls1}$/NER^{-} and BER*$_{nls2}$/NER^{-} cells were significantly higher than the mutation rates of BER*$_{wt}$/NER^{-} cells (Table 2), indicating that the nuclear localization of Ntg1 is important to prevent nuclear DNA mutations.

BER^{-}/NER^{-} and BER*$_{cat}$/NER^{-} cells displayed higher mitochondrial mutation frequencies compared to both wild type and BER*$_{wt}$/NER^{-} cells (Table 2). Neither BER*$_{nls1}$/NER^{-} nor BER*$_{nls2}$/NER^{-} had higher mitochondrial mutation frequencies than BER*$_{wt}$/NER^{-} cells; however, BER*$_{mts}$/NER^{-} cells had significantly elevated mitochondrial mutation frequencies compared to both wild type and BER*$_{wt}$/NER^{-} cells (Table 2). These results suggest that mitochondrial localization of Ntg1 plays an important role in reducing the number of spontaneous mitochondrial mutations.

DNA damaging agents induce a variety of base lesions that are substrates for Ntg1, and as different lesions possess varying capacities for the induction of cytotoxic effects, different cytotoxicity profiles may result [7]. In order to determine whether Ntg1 is important for cellular survival in the presence of different DNA damaging agents, the same isogenic strains were assessed for survival following exposure to either H_2O_2 or MMS. Survival of BER*$_{wt}$/NER^{-} mutants exposed to either H_2O_2 or MMS was greater than that of BER^{-}/NER^{-} cells, but less than that of WT cells (Figure 5A, B), indicating that Ntg1 partially rescues sensitivity to H_2O_2 and MMS. The impact of dynamic localization of Ntg1 was assessed utilizing BER*$_{nls1}$/NER^{-}, BER*$_{nls2}$/NER^{-} and

BER*$_{mts}$/NER^{-} cells. Survival of these cells following H_2O_2 treatment was not statistically different from BER*$_{wt}$/NER^{-} cells (Figure 5A). However, the survival of BER*$_{nls1}$/NER^{-} and BER*$_{nls2}$/NER^{-} cells treated with MMS was significantly reduced compared to BER*$_{wt}$/NER^{-} cells, while the survival of BER*$_{mts}$/NER^{-} cells was similar to BER*$_{wt}$/NER^{-} cells (Figure 5B). These results suggest that nuclear dynamic localization of Ntg1 in response to DNA damage is required to prevent a subset of lesions from becoming cytotoxic and suggests roles for Ntg1 dynamic relocalization in the elimination of mutagenic and cytotoxic base damages, respectively.

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 [8]. 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$_6$ (Figure 6). To confirm that the cleavage activity detected is due to the combined N-glycosylase/AP lyase activities of Ntg1, we created a K243Q amino acid substitution within a key predicted catalytic residue [15, 23]. As shown in Figure 6, cleavage of the DHU-containing oligonucleotide was not detected with the Ntg1 catalytic mutant. 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

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. Very little is known about the mechanisms that regulate BER in eukaryotes. 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. Importantly, we demonstrated 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 reveals how the cell counter deleterious consequences that result from oxidative DNA damage. Taken together, this data supports a new paradigm for the processes involved in the regulation of BER.

We propose 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) [15]. This mode of BER regulation can be further delineated 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 (Figure 4). The inability to mobilize additional 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) which are likely to include nuclear import factors, post translational modifications, and chaperones become activated to interact with or modify Ntg1 in order to recruit it to the appropriate organelle to repair oxidative DNA damage [15]. 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 (Figure 7). Results from this study suggest that the classical nuclear protein import proteins, importin α/β, are novel DNA damage responders. 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.

We speculate that dynamic localization in response to DNA damage signals may be a general mode of regulation for BER. Several BER proteins localize to both mitochondria and nuclei [60], all of which are candidates for regulation by dynamic localization. One particularly interesting candidate is the multifunctional human AP endonuclease 1 (hAPE1), which relocalizes from the cytoplasm to nuclei and mitochondria following exposure to oxidative stress (H_2O_2) [20, 61]. Other potential candidates can be identified by sequence homology to Ntg1, such as the *S. cerevisiae*

BER protein uracil DNA glycosylase, Ung1, which contains predicted bipartite cNLS and MTS signals tantalizingly similar to that of Ntg1 [62]. In addition, both human hNTH1 and mouse mNTH1 contain bipartite cNLS and MTS signals that target these proteins to both nuclei and mitochondria [16, 63], signifying 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.

The discovery of mechanisms underlying the stress-induced relocalization of an initiating protein for BER has implications not only for general regulation of BER but also for regulation of other stress components. It is conceivable that specialized, stress-activated nuclear import factors could orchestrate the mobilization and delivery of components that mediate responses such as changes in transcription programs and activation of checkpoints. A key example of such a responder is Yap1, a transcription factor that continuously cycles between the nucleus and cytoplasm under non-stress conditions, but whose nuclear export is blocked under oxidative stress conditions allowing it to upregulate genes that protect against cell stress-induced damage [64]. The activation of a nuclear transport “stressome” could provide a central clearinghouse to mount a coordinated stress response that synchronizes multiple distinct nuclear activities. Our findings provide the first direct evidence for this type of integrated response to cellular stress, with strong implications regarding the DNA damage response.

Dynamic localization of Ntg1 protects nuclear and mitochondrial DNA from mutation (Table 2), suggesting that dynamic localization plays an important role in the regulation of genomic stability. As the accumulation of DNA mutations is associated

with nuclear genomic stability [18, 65], these results indicate that regulation of BER through dynamic localization of Ntg1 plays a key role in maintaining the integrity of the nuclear and mitochondrial genomes. Interestingly, nuclear dynamic localization of Ntg1 guards against cytotoxicity induced by MMS, but not H_2O_2. These two agents generate distinct DNA lesions which are repaired by Ntg1 with differing efficiencies [11]. Altering key localization signals may not preclude Ntg1 from entering nuclei via alternate import pathways. In the case of H_2O_2-induced DNA base damage, residual Ntg1 levels are sufficient to repair cytotoxic lesions (Figure 5A). However, for MMS-induced DNA damage a large fraction of base lesions would be expected to be converted into AP sites, exceeding the capacity of the remaining Ntg1 to mediate repair of such toxic damage (Figure 5B).

The fact that dynamic localization is important for thwarting mutagenesis and DNA damage induced cytotoxicity emphasizes the impact that this mode of regulation may have on disease etiology. In this regard, the human homologue of Ntg1 (hNth1) displays predominantly cytoplasmic localization in a significant percentage of gastric and colorectal cancer tissues, suggesting that corruption of dynamic organelle targeting of BER proteins may be a characteristic of certain tumors [66, 67].

6. References

1. Polyak, K., et al., *Somatic mutations of the mitochondrial genome in human colorectal tumours.* Nat Genet, 1998. **20**(3): p. 291-3.

2. Kujoth, G.C., et al., *Mitochondrial DNA mutations, oxidative stress, and apoptosis in mammalian aging.* Science, 2005. **309**(5733): p. 481-4.

3. Altieri, F., et al., *DNA damage and repair: from molecular mechanisms to health implications.* Antioxid Redox Signal, 2008. **10**(5): p. 891-937.

4. Slupphaug, G., B. Kavli, and H.E. Krokan, *The interacting pathways for prevention and repair of oxidative DNA damage.* Mutat Res, 2003. **531**(1-2): p. 231-51.

5. Richter, C., J.W. Park, and B.N. Ames, *Normal oxidative damage to mitochondrial and nuclear DNA is extensive.* Proc Natl Acad Sci U S A, 1988. **85**(17): p. 6465-7.

6. Augeri, L., et al., *[10] Purification and properties of yeast redoxyendonuclease*, in *Methods in Enzymology*. 1994, Academic Press. p. 102-115.

7. Senturker, S., et al., *Substrate specificities of the Ntg1 and Ntg2 proteins of Saccharomyces cerevisiae for oxidized DNA bases are not identical.* Nucleic Acids Res, 1998. **26**(23): p. 5270-6.

8. You, H.J., R.L. Swanson, and P.W. Doetsch, *Saccharomyces cerevisiae Possesses Two Functional Homologues of Escherichia coli Endonuclease III.* Biochemistry, 1998. **37**(17): p. 6033-6040.

9. Hanna, M., et al., *Involvement of two endonuclease III homologs in the base excision repair pathway for the processing of DNA alkylation damage in Saccharomyces cerevisiae.* DNA Repair, 2004. **3**(1): p. 51-59.

10. Aspinwall, R., et al., *Cloning and characterization of a functional human homolog of Escherichia coli endonuclease III.* Proc Natl Acad Sci U S A, 1997. **94**(1): p. 109-14.

11. You, H.J., et al., *Saccharomyces cerevisiae Ntg1p and Ntg2p: broad specificity N-glycosylases for the repair of oxidative DNA damage in the nucleus and mitochondria.* Biochemistry, 1999. **38**(35): p. 11298-306.

12. Gossett, J., et al., *Yeast redoxyendonuclease, a DNA repair enzyme similar to Escherichia coli endonuclease III.* Biochemistry, 1988. **27**(7): p. 2629-34.

13. Hilbert, T.P., et al., *Cloning and expression of the cDNA encoding the human homologue of the DNA repair enzyme, Escherichia coli endonuclease III.* J Biol Chem, 1997. **272**(10): p. 6733-40.

14. Alseth, I., et al., *The Saccharomyces cerevisiae homologues of endonuclease III from Escherichia coli, Ntg1 and Ntg2, are both required for efficient repair of spontaneous and induced oxidative DNA damage in yeast.* Mol Cell Biol, 1999. **19**(5): p. 3779-87.

15. Griffiths, L.M., et al., *Dynamic compartmentalization of base excision repair proteins in response to nuclear and mitochondrial oxidative stress.* Mol Cell Biol, 2009. **29**(3): p. 794-807.

16. Ikeda, S., et al., *Differential intracellular localization of the human and mouse endonuclease III homologs and analysis of the sorting signals.* DNA Repair (Amst), 2002. **1**(10): p. 847-54.

17. Evert, B.A., et al., *Spontaneous DNA damage in Saccharomyces cerevisiae elicits phenotypic properties similar to cancer cells.* J Biol Chem, 2004. **279**(21): p. 22585-94.

18. Doudican, N.A., et al., *Oxidative DNA damage causes mitochondrial genomic instability in Saccharomyces cerevisiae.* Mol Cell Biol, 2005. **25**(12): p. 5196-204.

19. Ide, H. and M. Kotera, *Human DNA glycosylases involved in the repair of oxidatively damaged DNA.* Biol Pharm Bull, 2004. **27**(4): p. 480-5.

20. Frossi, B., et al., *H_2O_2 induces translocation of APE/Ref-1 to mitochondria in the Raji B-cell line.* J Cell Physiol, 2002. **193**(2): p. 180-6.

21. Yoon, J.H., et al., *The retinitis pigmentosa-mutated RP2 protein exhibits exonuclease activity and translocates to the nucleus in response to DNA damage.* Exp Cell Res, 2006. **312**(8): p. 1323-34.

22. Liu, W., et al., *Nuclear transport of human DDB protein induced by ultraviolet light.* J Biol Chem, 2000. **275**(28): p. 21429-34.

23. Augeri, L., et al., *Purification, Characterization, Gene Cloning, and Expression of Saccharomyces cerevisiae Redoxyendonuclease, a Homolog of Escherichia coli Endonuclease III.* Biochemistry, 1997. **36**(4): p. 721-729.

24. Lange, A., et al., *Classical nuclear localization signals: definition, function, and interaction with importin alpha.* J Biol Chem, 2007. **282**(8): p. 5101-5.

25. Görlich, D., et al., *Two different subunits of importin cooperate to recognize nuclear localization signals and bind them to the nuclear envelope.* Current Biology, 1995. **5**(4): p. 383-392.

26. Neupert, W. and J.M. Herrmann, *Translocation of Proteins into Mitochondria.* Annual Review of Biochemistry, 2007. **76**(1): p. 723-749.

27. Bruch, M.D. and D.W. Hoyt, *Conformational analysis of a mitochondrial presequence derived from the F1-ATPase [beta]-subunit by CD and NMR spectroscopy.* Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology, 1992. **1159**(1): p. 81-93.

28. Wang, Y. and H. Weiner, *Evaluation of electrostatic and hydrophobic effects on the interaction of mitochondrial signal sequences with phospholipid bilayers.* Biochemistry, 1994. **33**(43): p. 12860-12867.

29. Dekker, P.J., et al., *The Tim core complex defines the number of mitochondrial translocation contact sites and can hold arrested preproteins in the absence of matrix Hsp70-Tim44.* Embo J, 1997. **16**(17): p. 5408-19.

30. Dietmeier, K., et al., *Tom5 functionally links mitochondrial preprotein receptors to the general import pore.* Nature, 1997. **388**(6638): p. 195-200.

31. Ito, H., et al., *Transformation of intact yeast cells treated with alkali cations.* J Bacteriol, 1983. **153**(1): p. 163-8.

32. Kahana, J.A., B.J. Schnapp, and P.A. Silver, *Kinetics of Spindle Pole Body Separation in Budding Yeast.* Proceedings of the National Academy of Sciences, 1995. **92**(21): p. 9707-9711.

33. Winston, F., C. Dollard, and S.L. Ricupero-Hovasse, *Construction of a set of convenient Saccharomyces cerevisiae strains that are isogenic to S288C.* Yeast, 1995. **11**(1): p. 53-5.

34. Hodel, A.E., et al., *Nuclear localization signal receptor affinity correlates with in vivo localization in Saccharomyces cerevisiae.* J Biol Chem, 2006. **281**(33): p. 23545-56.

35. Storici, F. and M.A. Resnick, *The delitto perfetto approach to in vivo site-directed mutagenesis and chromosome rearrangements with synthetic oligonucleotides in yeast.* Methods Enzymol, 2006. **409**: p. 329-45.

36. Seedorf, M., et al., *Interactions between a nuclear transporter and a subset of nuclear pore complex proteins depend on Ran GTPase.* Mol Cell Biol, 1999. **19**(2): p. 1547-57.

37. Meadows, K.L., B. Song, and P.W. Doetsch, *Characterization of AP lyase activities of Saccharomyces cerevisiae Ntg1p and Ntg2p: implications for biological function.* Nucl. Acids Res., 2003. **31**(19): p. 5560-5567.

38. Leung, S.W., et al., *Dissection of the Karyopherin {alpha} Nuclear Localization Signal (NLS)-binding Groove: FUNCTIONAL REQUIREMENTS FOR NLS BINDING.* J. Biol. Chem., 2003. **278**(43): p. 41947-41953.

39. Gruss, O.J., et al., *Ran Induces Spindle Assembly by Reversing the Inhibitory Effect of Importin [alpha] on TPX2 Activity.* Cell, 2001. **104**(1): p. 83-93.

40. Fasken, M.B., M. Stewart, and A.H. Corbett, *Functional significance of the interaction between the mRNA-binding protein, Nab2, and the nuclear pore-associated protein, Mlp1, in mRNA export.* J Biol Chem, 2008. **283**(40): p. 27130-43.

41. Kelly, S.M., et al., *Recognition of polyadenosine RNA by zinc finger proteins.* Proc Natl Acad Sci U S A, 2007. **104**(30): p. 12306-11.

42. Venkhataraman, R., et al., *Enzymatic processing of DNA containing tandem dihydrouracil by endonucleases III and VIII.* Nucleic Acids Res, 2001. **29**(2): p. 407-14.

43. Swanson, R.L., et al., *Overlapping specificities of base excision repair, nucleotide excision repair, recombination, and translesion synthesis pathways for DNA base damage in Saccharomyces cerevisiae.* Mol Cell Biol, 1999. **19**(4): p. 2929-35.

44. Drake, J.W., *Mutation rates.* Bioessays, 1992. **14**(2): p. 137-40.

45. Wierdl, M., et al., *Destabilization of simple repetitive DNA sequences by transcription in yeast.* Genetics, 1996. **143**(2): p. 713-21.

46. Chi, N.W. and R.D. Kolodner, *Purification and characterization of MSH1, a yeast mitochondrial protein that binds to DNA mismatches.* J Biol Chem, 1994. **269**(47): p. 29984-92.

47. Nakai, K. and P. Horton, *PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization.* Trends in Biochemical Sciences, 1999. **24**(1): p. 34-35.

48. Claros, M.G. and P. Vincens, *Computational method to predict mitochondrially imported proteins and their targeting sequences.* Eur J Biochem, 1996. **241**(3): p. 779-86.

49. Paine, P.L., L.C. Moore, and S.B. Horowitz, *Nuclear envelope permeability.* Nature, 1975. **254**(5496): p. 109-14.

50. Koepp, D.M., et al., *Dynamic localization of the nuclear import receptor and its interactions with transport factors.* J Cell Biol, 1996. **133**(6): p. 1163-76.

51. Yano, R., et al., *Cloning and characterization of SRP1, a suppressor of temperature-sensitive RNA polymerase I mutations, in Saccharomyces cerevisiae.* Mol Cell Biol, 1992. **12**(12): p. 5640-51.

52. Aitchison, J.D., G. Blobel, and M.P. Rout, *Kap104p: A Karyopherin Involved in the Nuclear Transport of Messenger RNA Binding Proteins.* Science, 1996. **274**(5287): p. 624-627.

53. Rosenblum, J.S., L.F. Pemberton, and G. Blobel, *A Nuclear Import Pathway for a Protein Involved in tRNA Maturation.* J. Cell Biol., 1997. **139**(7): p. 1655-1661.

54. Fanara, P., et al., *Quantitative Analysis of Nuclear Localization Signal (NLS)-Importin alpha Interaction through Fluorescence Depolarization. EVIDENCE FOR AUTO-INHIBITORY REGULATION OF NLS BINDING.* J. Biol. Chem., 2000. **275**(28): p. 21218-21223.

55. Kobe, B., *Autoinhibition by an internal nuclear localization signal revealed by the crystal structure of mammalian importin alpha.* Nat Struct Biol, 1999. **6**(4): p. 388-97.

56. Salmon, T.B., et al., *Biological consequences of oxidative stress-induced DNA damage in Saccharomyces cerevisiae.* Nucleic Acids Res, 2004. **32**(12): p. 3712-23.

57. Rowe, L.A., N. Degtyareva, and P.W. Doetsch, *DNA damage-induced reactive oxygen species (ROS) stress response in Saccharomyces cerevisiae.* Free Radic Biol Med, 2008. **45**(8): p. 1167-77.

58. Morey, N.J., P.W. Doetsch, and S. Jinks-Robertson, *Delineating the requirements for spontaneous DNA damage resistance pathways in genome maintenance and viability in Saccharomyces cerevisiae.* Genetics, 2003. **164**(2): p. 443-55.

59. Netter, P., et al., *MITOCHONDRIAL GENETICS VII. ALLELISM AND MAPPING STUDIES OF RIBOSOMAL MUTANTS RESISTANT TO CHLORAMPHENICOL, ERYTHROMYCIN AND SPIRAMYCIN IN S. CEREVISIAE.* Genetics, 1974. **78**(4): p. 1063-1100.

60. Larsen, N.B., M. Rasmussen, and L.J. Rasmussen, *Nuclear and mitochondrial DNA repair: similar pathways?* Mitochondrion, 2005. **5**(2): p. 89-108.

61. Tell, G., et al., *An 'environment to nucleus' signaling system operates in B lymphocytes: redox status modulates BSAP/Pax-5 activation through Ref-1 nuclear translocation.* Nucleic Acids Res, 2000. **28**(5): p. 1099-105.

62. Chatterjee, A. and K.K. Singh, *Uracil-DNA glycosylase-deficient yeast exhibit a mitochondrial mutator phenotype.* Nucleic Acids Res, 2001. **29**(24): p. 4935-40.

63. Takao, M., et al., *Mitochondrial targeting of human DNA glycosylases for repair of oxidative DNA damage.* Nucleic Acids Res, 1998. **26**(12): p. 2917-22.

64. Kuge, S., N. Jones, and A. Nomoto, *Regulation of yAP-1 nuclear localization in response to oxidative stress.* Embo J, 1997. **16**(7): p. 1710-20.

65. Degtyareva, N.P., et al., *Chronic oxidative DNA damage due to DNA repair defects causes chromosomal instability in Saccharomyces cerevisiae.* Mol Cell Biol, 2008. **28**(17): p. 5432-45.

66. Goto, M., et al., *Altered expression of the human base excision repair gene NTH1 in gastric cancer.* Carcinogenesis, 2009. **30**(8): p. 1345-52.

67. Koketsu, S., T. Watanabe, and H. Nagawa, *Expression of DNA repair protein: MYH, NTH1, and MTH1 in colorectal cancer.* Hepatogastroenterology, 2004. **51**(57): p. 638-42.

68. Green, D.M., et al., *Nab2p is required for poly(A) RNA export in Saccharomyces cerevisiae and is regulated by arginine methylation via Hmt1p.* J Biol Chem, 2002. **277**(10): p. 7752-60.

69. Seedorf, M. and P.A. Silver, *Importin/karyopherin protein family members required for mRNA export from the nucleus.* Proc Natl Acad Sci U S A, 1997. **94**(16): p. 8590-5.

70. Yano, R., et al., *Yeast Srp1p has homology to armadillo/plakoglobin/beta-catenin and participates in apparently multiple nuclear functions including the maintenance of the nucleolar structure.* Proc Natl Acad Sci U S A, 1994. **91**(15): p. 6880-4.

71. Harreman, M.T., et al., *The auto-inhibitory function of importin alpha is essential in vivo.* J Biol Chem, 2003. **278**(8): p. 5854-63.

Table 1: Ntg1 Localization Motifs

Ntg1 localization sequence name	Residue number	Wild type amino acid sequence	Mutant amino acid sequence[a]	Mutant Name
cNLS1	14-17	RKRP	R**AA**P	$Ntg1_{nls1}$
cNLS2	31-37	PEKRTKI	PE**AA**TKI	$Ntg1_{nls2}$
MTS	1-26	MQKISKYSSMAILR KRPLVKTETGPE	MQ**E**IS**E**YSSMAIL RKRPLVKTETGPE	$Ntg1_{mts}$
Catalytic	233-245	ELLGLPGVGPKMA	ELLGLPGVGP**Q**MA	$Ntg1_{cat}$

[a] Mutant amino acids substitutions shown in bold type.

Table 2: Nuclear and Mitochondrial Mutations Rates in Cells with Different DNA Excision Repair Capacities

DNA Repair Background	Median Nuclear Mutation Rate[a] (μ) (10^{-7}) (95% Confidence Limits[c])	Fold Change	Median Mitochondrial Mutation Frequency[b] (*f*) (10^{-8}) (95% Confidence Limits[c])	Fold Change
WT	6 (1 - 43)	1	2.0 (1.0 - 4.7)	1
BER^{-}/NER^{-} (*apn1 ntg2 ntg1 rad1*)	413 (212 - 623)	68	53.6 (28.9 - 117)	28
BER^{*}_{wt}/NER^{-} (*apn1 ntg2 NTG1 rad1*)	44 (29 - 58)	7	5.3 (3.5 - 9.3)	3
BER^{*}_{nls1}/NER^{-} (*apn1 ntg2 ntg1$_{nls1}$ rad1*)	156 (103 - 684)	26	11.3 (7.1 - 20.8)	6
BER^{*}_{nls2}/NER^{-} (*apn1 ntg2 ntg1$_{nls2}$ rad1*)	83 (60 – 175)	14	5.6 (4.0 - 14.3)	3
BER^{*}_{mts}/NER^{-} (*apn1 ntg2 ntg1$_{mts}$ rad1*)	37 (25 – 59)	6	15.0 (10.7 – 16.9)	8
BER^{*}_{cat}/NER^{-} (*apn1 ntg2 ntg1$_{cat}$ rad1*)	274 (117 – 1190)	45	13.1 (10.4 – 18.1)	7

[a] Nuclear mutation rates were assessed via the *CAN1* locus [43].

[b] Mitochondria mutation frequencies were assessed by an erythromycin resistance assay [59].

[c] Confidence limits were calculated as previously described [45].

Figure 1

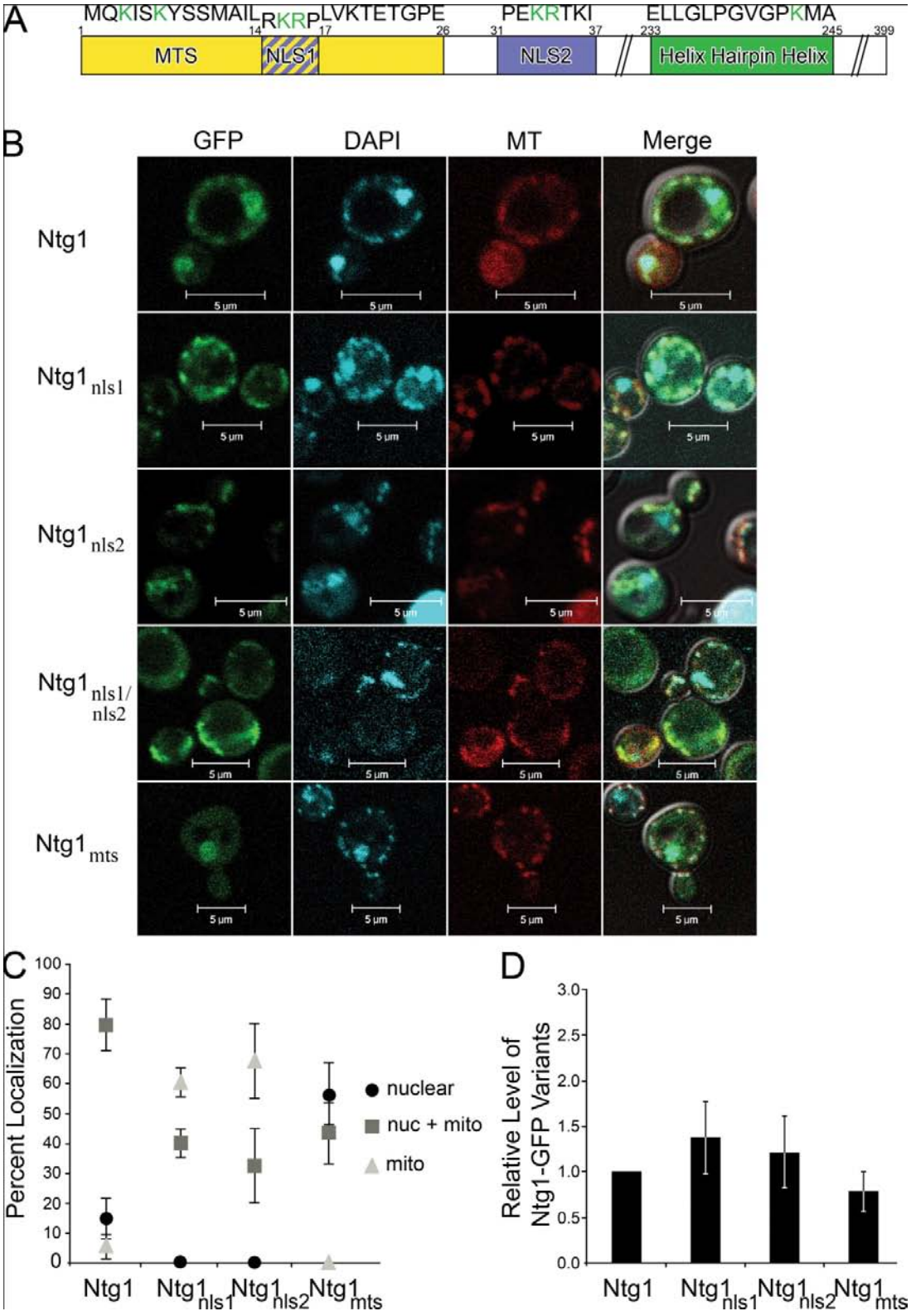

A
MQKISKYSSMAILRKRPLVKTETGPE
PEKRTKI
ELLGLPGVGPKMA
1
14
17
26
31
37
233
245
399
MTS
NLS1
NLS2
Helix Hairpin Helix
B
GFP
DAPI
MT
Merge
Ntg1
Ntg1 nls1
Ntg1 nls2
Ntg1 nls1/ nls2
Ntg1 mts
5 µm
C
Percent Localization
nuclear
nuc + mito
mito
Ntg1
Ntg1 nls1
Ntg1 nls2
Ntg1 mts
D
Relative Level of Ntg1-GFP Variants
Ntg1
Ntg1 nls1
Ntg1 nls2
Ntg1 mts

Figure 1: Definition of functional intracellular targeting signals within Ntg1. A. Schematic of Ntg1. Predicted critical residues for nuclear and mitochondrial localization and catalytic activity of Ntg1 are indicated, including, the putative MTS (residues 1-26), two putative cNLSs (residues 14-16 and 31-37), and the putative active site (residues 233-245). Amino acids depicted in green were altered in this study in order to examine Ntg1 function (Table 1). **B.** The localization of GFP-tagged Ntg1 proteins (Supplemental Table 1) was assessed via direct fluorescence microscopy. GFP (green), DAPI (blue), Mitotracker (red), and merged images of cells expressing wild-type Ntg1 (WT), $Ntg1_{nls1}$, $Ntg1_{nls2}$, $Ntg1_{nls1/2}$, and $Ntg1_{mts}$ variants of Ntg1-GFP are shown. **C.** Quantification of localization of Ntg1-GFP variants 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 for at least 200 cells per variant. Error bars represent standard deviation. **D.** Quantification of steady state expression levels of Ntg1 variants by immunoblotting and densitometry. Five separate experiments were quantified. The expression of Ntg1-GFP was normalized and the mean level of wild-type Ntg1-GFP was set to 1.0. Error bars represent standard deviation.

Figure 2

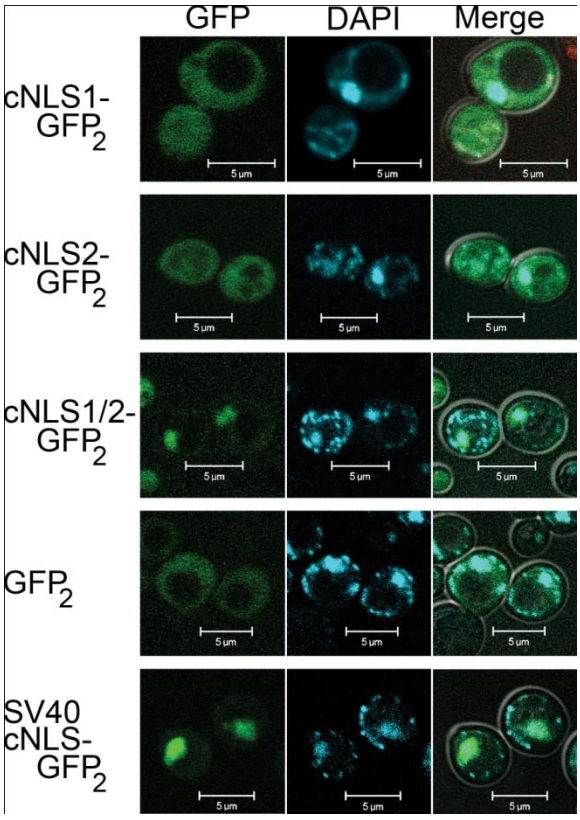


Figure 2: The bipartite cNLS of Ntg1 is sufficient to direct nuclear localization of Ntg1. GFP (green), DAPI (blue), and merged images of untreated cells expressing $Ntg1_{cNLS1}$-GFP_2, $Ntg1_{cNLS2}$-GFP_2, $Ntg1_{cNLS1/2}$-GFP_2, and two proteins, the negative control GFP_2 alone (empty vector) and the bipartite positive control $SV40_{cNLS}$-GFP_2 [34].

Figure 3

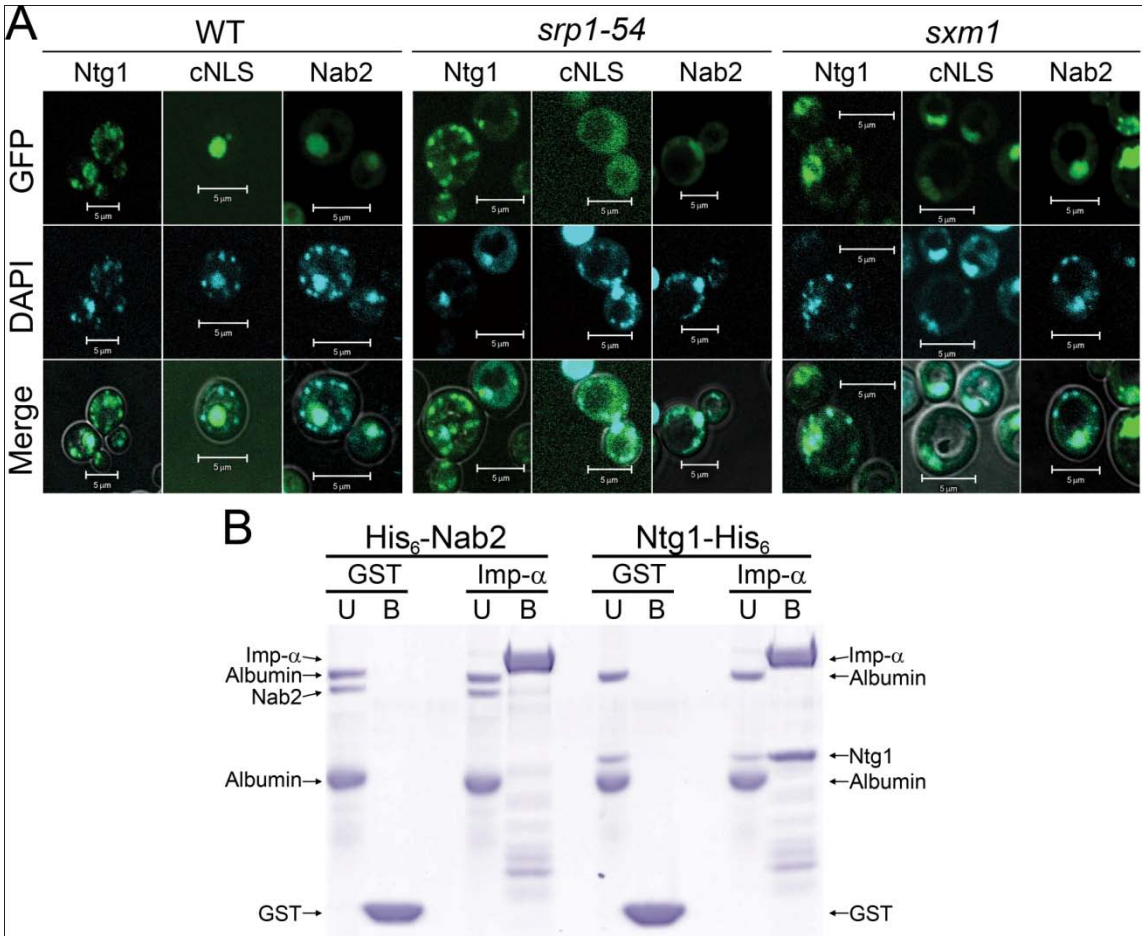


Figure 3: The classical nuclear protein import pathway is required for nuclear localization of Ntg1. A. Localization of Ntg1-GFP (Ntg1), and two control proteins, a cNLS cargo, SV40 bipartite cNLS (cNLS) [34], and a non-cNLS cargo [68], Nab2-GFP (Nab2), was assessed via direct fluorescence microscopy in untreated cells. GFP (green), DAPI (blue), and merged images of wild type (WT), importin α (*srp1-54)*, and control *sxm1Δ* mutant cells are shown at the non-permissive temperature, 37ºC. See also Supplemental Figure 1. **B.** An *in vitro* binding assay reveals direct binding of Ntg1 to the cNLS receptor, importin-α. Either GST-ΔIBB-importin-α (Imp-α) or GST alone (GST) as a control was incubated with Ntg1-His_6 or a His-tagged control protein Nab2-

His_6 as described in experimental procedures. Both the unbound (U) and bound (B) fractions were resolved by SDS-PAGE and then stained with Coomassie Blue. Albumin, which was used as a non-specific protein competitor is present in the unbound fractions.

Figure 4

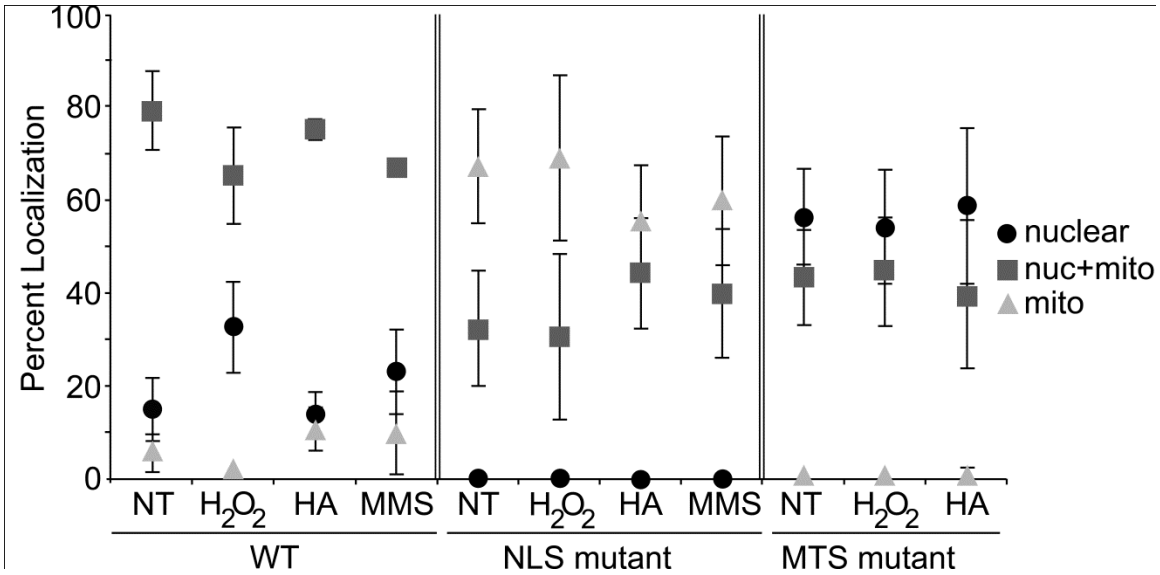


Figure 4: Functional intracellular targeting signals are required for dynamic localization of Ntg1 in response to oxidative DNA damage. Quantification of Ntg1-GFP, $Ntg1_{nls2}$-GFP, and $Ntg1_{mts}$-GFP localization following cellular stress. Cells were not treated (NT) or were exposed to 20 mM H_2O_2, 55 mM MMS, or 20 mM H_2O_2 plus 10 µg/mL antimycin (HA). 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. WT-HA and WT-MMS nuc+mito standard deviations are small and are obscured by the data point symbols

Figure 5

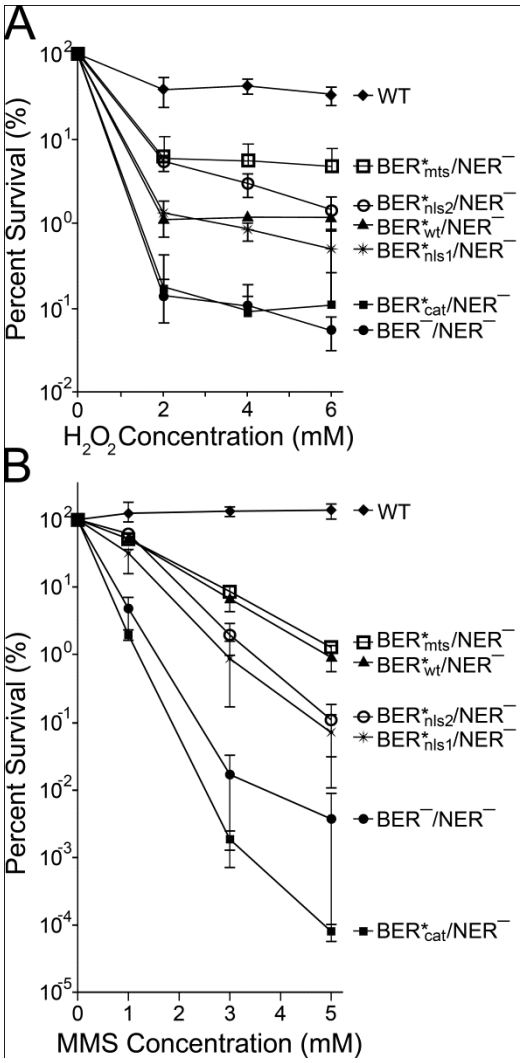


Figure 5: Functional analysis of the dynamic localization of Ntg1. A. The H_2O_2 sensitivity of wild type (WT), *apn1 ntg2 ntg1 rad1* (BER^-/NER^-), *apn1 ntg2 NTG1 rad1* (BER^*_{wt}/NER^-) and *apn1 ntg2 ntg1$_{mutant}$ rad1* (BER^*_{mutant}/NER^-) cells were assessed. The percent survival was set to 100% for untreated samples and was determined for 0, 2, 4, and 6 mM H_2O_2 doses. **B.** The MMS sensitivity of wild type (WT), (BER^-/NER^-), (BER^*_{wt}/NER^-) and (BER^*_{mutant}/NER^-) cells were assessed. The percent survival was

set to 100% for untreated samples and was determined for 0, 1, 3, and 5 mM MMS doses. Error bars indicate standard deviations in data.

Figure 6

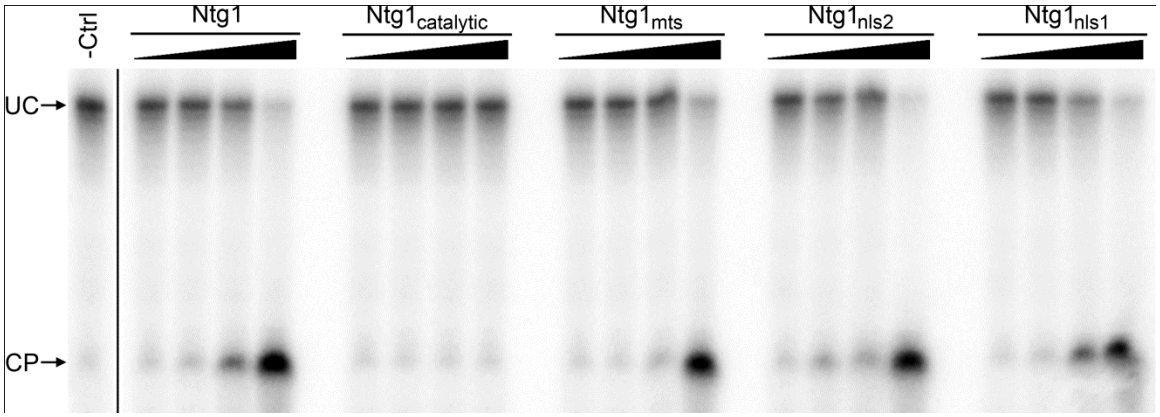


Figure 6: Amino acid substitutions within intracellular targeting signals do not affect the catalytic activity of Ntg1. Ntg1 DNA glycosylase/AP lyase activity was assessed by monitoring cleavage of a ^{32}P 5′-end-labeled oligonucleotide (31mer) containing dihydrouracil by the Ntg1 variant proteins, 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, the black line represents lanes that were cropped from the image.

Figure 7

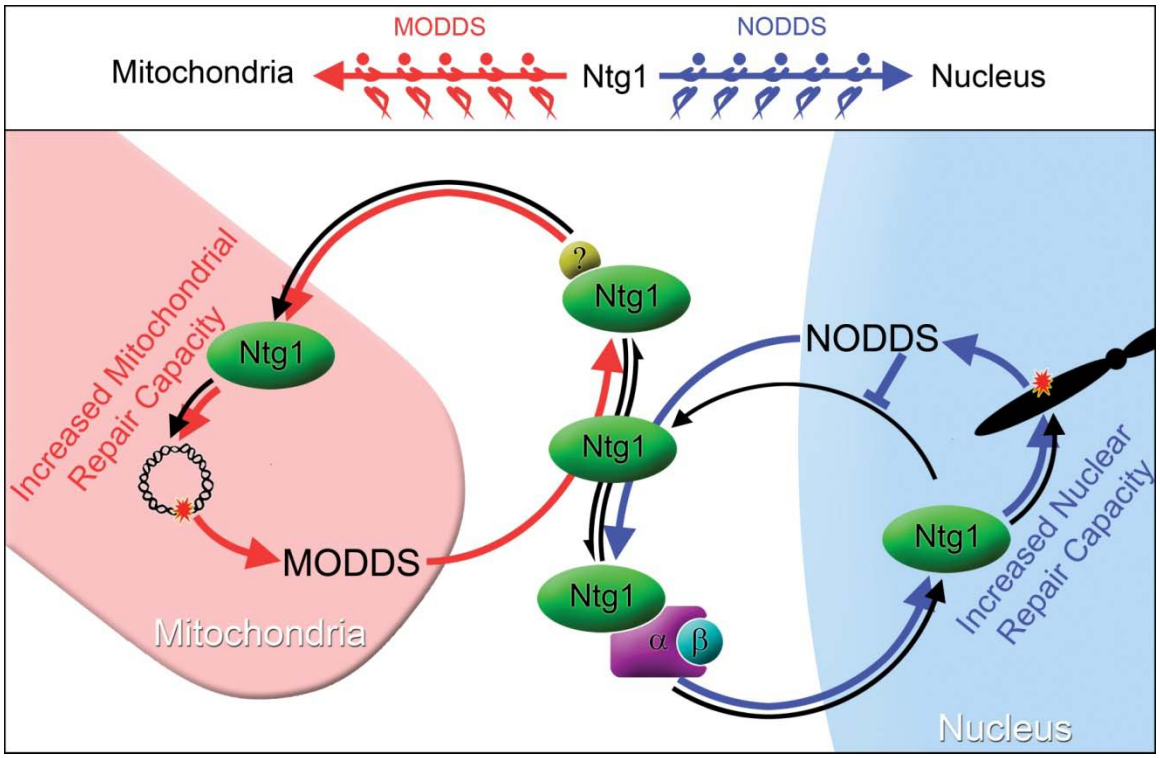
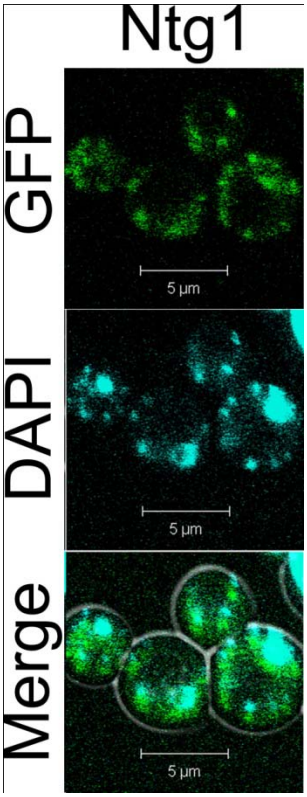


Figure 7: Model of Ntg1 dynamic localization in response to nuclear and mitochondrial oxidative DNA damage. Nuclear oxidative DNA damage signals (NODDS) and mitochondrial oxidative DNA damage signals (MODDS) compete for the recruitment of Ntg1 to sites of oxidative DNA damage from the cellular pool of 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.

7. Supplemental Information

7.1 Supplemental Figure 1 (Related to Figure 3A)



Supplemental Figure 1 Legend: Importin β is required for nuclear localization of Ntg1. Localization of Ntg1-GFP (Ntg1) was assessed via direct fluorescence microscopy. GFP (green), DAPI (blue) to indicate the position of the nucleus and merged images of importin β (*rsl1-1*) mutant cells [50] are shown.

Supplemental Table 1: Strains and Plasmids Used in this Study

Strain or Plasmid	Description	References
FY86 (ACY193)	*MATα ura3-52 leu2Δ1 his3Δ200*	[11]
PSY883 (ACY208)	*MATα ura3-52 trp1Δ63 leu□Δ□□his3-11 ade2-1 rsl1Δ::HIS3*	[50]
SJR751 (DSC0025)	*MATα ade2-101$_{oc}$ his3Δ200 ura3ΔNco lys2ΔBgl leu2-R*	[43]
SJR1101 (DSC0051)	*MATα ade2-101$_{oc}$ his3Δ200 ura3ΔNco lys2ΔBgl leu2-R ntg1Δ::LEU2 ntg2Δ::hisG apn1Δ::HIS3 rad1Δ::hisG*	[43]
ACY443	*MATa leu2Δ1 his3Δ200 trp1Δ63 sxm1 ΔHIS3*	[69]
ACY1563	*MATa ura3 leu2 his3 trp1 ade2 can1 srp1–54*	[70]
DSC0282	*MATa ura3-52 leu2Δ1 his3Δ200 ntg1::KANAMYCIN bar1::HYG*	[15]
DSC0367	*MATa his7- lys2Δ3′::LEU-lys2Δ5′ ade5-1 trp1-289 ura3-52*	[65]
DSC0369	*MATa his7-1 lys2Δ3′::LEU-lys2Δ5′ ade5-1 ura3-52 ntg1::hphMX4 ntg2::BSD apn1::TRP1 rad1::kanMX*	[65]
DSC0371	*MATa his7-1 lys2Δ3′::LEU-lys2Δ5′ ade5-1 ura3-52 ntg2::BSD apn1::TRP1 rad1::kanMX*	[65]
DSC0430	*MATa his7-1 lys2Δ3′::LEU-lys2Δ5′ ade5-1 ura3-52 ntg2::BSD apn1::TRP1 rad1::kanMX NTG1$_{nls1}$*	This study
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*	[65]
pAC719	*NAB2-GFP, 2μ, URA3*	[68]
pAC891	*SRP1-c-myc* (3X), *CEN, URA3, amp^R*	[71]
pAC960	Δ*IBB-SRP1-c-myc* (3X), *CEN, URA3, amp^R*	[71]
pAC1056	*BPSV40-NLS-GFP$_2$, CEN, URA3, amp^R*	[34]
pAC1069	*GFP$_2$, CEN, URA3, amp^R*	[34]
pAC736	pGEX-4T-3 *amp^R*	Pharmacia
pAC1338	pGEX-4T *GST-ΔIBB-SRP1, amp^R*	This study
pAC785	pET28a *His$_6$-NAB2, kan^R*	[68]
pNTG1-GFP pAC2669	*NTG1-GFP, 2μ, URA3, amp^R*	[11, 32, 33]
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

Supplemental Table 1 Legend: Complete list of the relevant genotypes of all strains and plasmids used in this study.

Chapter 4

Sumoylation of Ntg1 and the regulation of base excision repair in *Saccharomyces cerevisiae*

Dan B. Swartzlander, Harry R. Powers, Anita H. Corbett, and Paul W. Doetsch

The contribution by Dan Swartzlander includes all written portions, figure generation, and most of the data presented in Figures 1-7. Harry Powers contributed significantly to the data presented in Figure 5 and 6.

1. **Abstract**

Oxidative DNA damage, often caused by reactive oxygen species, is the most frequent type of DNA damage. These damaging molecules occur naturally in cells due to environmental exposures and cellular metabolism. The base excision repair (BER) pathway initiated by N-glycosylase apurinic/apyrimidinic (AP) lyase proteins is primarily responsible for repair of oxidative DNA damage in both nuclear and mitochondrial genomes. The proteins that comprise the BER pathway are highly conserved across eukaryotes. Even so, little is known about the effect that post-translational modifications have on BER protein function. The *Saccharomyces cerevisiae* BER protein, Ntg1, is sumoylated (small ubiquitin-like modifier). We used Ntg1 as a model system to ascertain the effects of sumoylation on the regulation of BER and to study the dynamics of Ntg1 sumoylation in response to cell stress. Our results show that Ntg1 sumoylation increases in response to oxidative stress. We also found that the E3 SUMO ligases Siz1/Siz2 are responsible for generating both monosumoylated and multisumoylated Ntg1. Interestingly, we determine that sumoylation of Ntg1 is associated with its nuclear localization. Furthermore, mutational analysis of putative Ntg1 sumoylation sites revealed that Ntg1 is predominantly sumoylated at five distinct consensus sumoylation sites. These sites cluster at both termini of the protein, with K396 being the major site of monosumoylation. Collectively, these results help us understand this dynamic biological pathway, which is responsible for regulating a key BER protein; in addition, they help us better understand sumoylation.

2. **Introduction**

Oxidative DNA lesions occur in both the nuclei and mitochondria. There are upwards of 90,000 oxidative lesions and 200,000 apurinic/apyrimidinic (AP) sites generated per human cell per day [1, 2]. When left unrepaired, these lesions have been linked to numerous human pathologies including cancer, aging, and neurodegenerative disorders [3-7]. The base excision repair (BER) pathway is the primary pathway responsible for the removal and subsequent repair of these oxidative lesions [8, 9]. The regulatory pathways for both nucleotide excision repair (NER) and double strand break repair are well understood. In contrast, little is known about BER regulation and the role these regulatory mechanisms play in the efficient and accurate repair of oxidative DNA lesions in the nuclear and mitochondrial genomes.

Current research shows that the regulation of BER occurs in several different ways including through regulation of transcript levels, post-translational modifications, protein-protein interactions, and protein localization [10]. For instance, transcript level regulation of *N*-methylpurine–DNA glycosylase (MPG), uracil DNA glycosylase (UNG), and human AP site-specific endonuclease (APEX1) occurs in a cell cycle–dependent manner, increasing 2.5 – 3.5-fold during G_1 and returning to basal levels following mitosis [11]. Protein-protein interactions involving BER proteins are numerous and have varied effects such as increasing the glycosylase activities of UNG, single-strand-selective mono-functional uracil–DNA glycosylase 1 (SMUG1), human thymine glycol–DNA glycosylase (NTHL1), 8-oxoguanine DNA glycosylase (OGG1), and thymine DNA glycosylase (TDG), through association with the APEX1 protein [12-16]. Targeting protein localization in response to cellular stimuli is a critical form of regulation: for

example, oxidative stress affects the nuclear and mitochondrial localization of both the human APEX1 and Cockayne syndrome complementation group B protein (CSB) [17-19]. Finally, post-translational regulation of BER proteins can also occur through different types of modifications including phosphorylation, acetylation, nitrosylation, ubiquitination, and sumoylation [20-33]. The small ubiquitin related modifier (SUMO) is of particular interest in BER regulation as it modulates protein function in a variety of fashions. The best known example of sumoylation in BER is with the human TDG, where the SUMO modification competes with the regulatory domain of TDG for DNA binding. After TDG removes a DNA lesion, sumoylation of TDG allows it to release the DNA; thus, sumoylation increases enzyme turnover [20, 21]. Regrettably, little is known about the regulation of BER proteins beyond these examples, and even less is known concerning the role that oxidative stress plays in this process. It is the goal of this project to define major regulatory pathways that govern the regulation of BER in response to oxidative stress.

In order to investigate the complex role that oxidative stress plays in the regulation of BER, we focused on the *S. cerevisiae* thymine glycol–DNA glycosylase, (Ntg1). Ntg1 is a functional genetic homolog of the *Escherichia coli* BER protein, endonuclease III, (Nth), and the human BER protein, thymine glycol–DNA glycosylase, (NTHL1). All of these enzymes are critical for the repair of oxidative DNA damage [34-37]. These endonuclease III homologs are bi-functional as they have both DNA *N*-glycosylase and apurinic/apyrimidinic (AP) lyase function. These activities allow Ntg1 and NTHL1 to recognize oxidative DNA damage, to then create an AP site by removing

the lesion from the DNA strand, and to then nick the DNA backbone on the 3′ side of the AP site [38-41].

Ntg1 provides an excellent model for the study of BER regulation in response to oxidative stress, because oxidative stress affects Ntg1 in two distinct but possibly related manners. First, oxidative stress alters the localization pattern of Ntg1, and second, it results in the post-translational modification of Ntg1 by SUMO [22]. The effect on the localization of Ntg1 is the first and best-understood of these two responses. Ntg1 dynamically localizes to either nuclei or mitochondria in response to organelle-specific oxidative stress, dependent upon the inherent localization signals within Ntg1 [22, 42]. On the other hand, the mechanism and the consequence(s) of Ntg1 sumoylation are poorly understood; however, we anticipate that they represent novel pathways through which BER proteins are regulated in response to oxidative stress.

The general sumoylation process involves a series of conjugations that, in *S. cerevisiae*, are catalyzed by the E1 (Uba2/Aos1 heterodimer), the E2 (Ubc9), and one of four E3 (Siz1, Siz2, MMS21, and Zip3) ligases. These ligases attach the SUMO protein to a substrate through formation of an isopeptide bond [43-51]. Sumoylation is a very dynamic process that is readily reversible by the *S. cerevisiae* SUMO proteases Ulp1 and Ulp2 and that modifies protein function in diverse manners [52, 53]. Sumoylation predominately occurs at SUMO consensus sequences [54, 55]. More than two-thirds of known SUMO substrates contain at least one consensus sumoylation motif Ψ-K-x-D/E (where Ψ is a hydrophobic residue, K is the lysine conjugated to SUMO, x is any amino acid, and D/E is an acidic residue) [54, 55].

In this study, we identify the necessary components for the sumoylation of Ntg1, the stimuli responsible for Ntg1 sumoylation, and the sites required for Ntg1 sumoylation. We also elucidate the complex mechanism by which Ntg1 is sumoylated at multiple lysines. In the absence of all consensus sumoylation sites or in the absence of the E3 ligases Siz1 and Siz2, Ntg1 ceases to be sumoylated at a quantifiable level. Collectively, our results detail a biological pathway commencing with oxidative stress signaling and concluding in the post-translation modification of a key BER protein. Our study provides insight into an important mechanism of BER regulation and into the dynamics of sumoylation.

3. **Materials and Methods**

3.1 Strains, Plasmids, and Media. All haploid *S. cerevisiae* strains and plasmids used in this study are listed in Table 1. Yeast cells were cultured at 25°C, 30°C, 37°C, or 42°C in YPD medium (1% yeast extract, 2% peptone, 2% dextrose, 0.005% adenine sulfate, and 2% agar for plates) or SD medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate, 2% dextrose, 0.5% adenine sulfate, and 2% agar for plates). In order to introduce plasmids, yeast cells were transformed by a modified lithium acetate method [56].

A centromeric vector (*CEN*, *URA3*), pRS316 [57] was employed as the backbone for the generation of a construct overexpressing C-terminally tagged Ntg1-TAP fusion protein (pD0436). The insert was amplified using the primers listed in Table 2 and inserted at the NotI restriction site of pRS316. The insert includes the tetracycline repressible promoter (Tet-Off) and the C-terminally tagged NTG1-TAP fusion from the

DSC0295 strain [22]. The *S. cerevisiae* haploid deletion mutant *ntg1Δ* (DSC0470) generated from wild type cells (hDNP19), and the SUMO pathway mutant collection (DSC0527 - DSC0530 and - DSC0534 - DSC0536) were utilized to assess the level of sumoylated wild type and mutant Ntg1 [15,33,78]. All amino acid substitutions were performed using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) using the primers listed in Table 2. The resulting plasmids were sequenced to ensure the introduction of the desired mutation and the absence of any additional mutations (Table 1).

3.2 Exposure to DNA Damaging Agents. Cells were grown in 25 – 35 mL YPD or SD -URA media to 5 x 10^7 cells/mL, centrifuged, and washed with water. Cells were then resuspended in 25 – 35 mL water containing the appropriate agent: 1 – 20 mM H_2O_2 (Sigma); 500 – 10,000 µM menadione bisulfate (Sigma); 150 – 2000 µM paraquat (Sigma); 10 – 100 µM arsenic trioxide (Sigma); 10% ethanol (Deconlabs); 5% SDS (Sigma); or 10% fetal bovine serum (HyClone). Cells were exposed to agents for one - two hours at 30°C. The cytotoxicities of agents were evaluated by incubating cells in agent, washing cells with water, plating cells, and colony counting to determine the number of colony forming units.

3.3 Immunoblotting. The steady-state level of each Ntg1-TAP fusion protein variant was assessed by immunoblotting whole cell lysates with the rabbit polyclonal anti-TAP antibody (1:3,333 dilution, Open Biosystems) to determine the relative level of differentially sumoylated Ntg1 products. An anti-3-phosphoglycerate (PGK) antibody (1:10,000 dilution; Invitrogen) was utilized to determine the relative level of protein lysate loaded into each lane.

The analysis of immunoblots was performed utilizing the ECL Plex western blotting detection system (Amersham), the Typhoon Trio variable mode imager (GE Healthcare), and the ImageQuant TL software package (GE Healthcare). The ratio of SUMO modified Ntg1 (mono, di, tri) to total Ntg1 was determined for wild type Ntg1 and each lysine to arginine amino acid substitution mutant of Ntg1. Standard error of the mean was calculated for each. The two-sample t-test was employed to test for significance (α=0.05).

4. **Results**

4.1 Ntg1 is post-translationally modified by SUMO primarily in response to oxidative stress. In order to understand the effect of Ntg1 sumoylation, it was first necessary to define which stimuli result in the sumoylation of Ntg1. Previously, it was reported that hydrogen peroxide is able to increase the level of sumoylated Ntg1 in nuclear fractions obtained from subcellular fractionation [22]. This increase in nuclear sumoylated Ntg1 can be explained two ways: by a global increase of sumoylated Ntg1 in the cell, and/or by a movement of sumoylated Ntg1 into the nucleus. To better understand the increase of sumoylated Ntg1 in response to hydrogen peroxide treatment, we performed a quantitative analysis of this response. Our results showed that there is a positive correlation between global sumoylated Ntg1 levels and the dose of hydrogen peroxide treatment (Figure 1A). This led us to conclude that global levels of sumoylated Ntg1 increase in response to hydrogen peroxide treatment. Furthermore, during this experiment we detected the presence of multiple higher molecular weight forms of Ntg1, which suggests that Ntg1 can be multiply sumoylated (Figure 1A).

To test the effect of other stress agents on Ntg1 sumoylation, we treated cells with the oxidative stress agents menadione bisulfite and paraquat; the osmotic stress agents sodium dodecyl sulfate (SDS) and ethanol; and fetal bovine serum (FBS) which induces a hyphal growth state (Figure 1C). None of these agents induced the high levels of sumoylated Ntg1 that were observed after hydrogen peroxide treatment. Menadione, paraquat, ethanol, and FBS induced sumoylation; however, at very low levels (Figure 1C). These results suggest that the reactive oxygen species hydrogen peroxide specifically induces the sumoylation of Ntg1 and that induction by other agents occurs through the generation of hydrogen peroxide.

To better understand the dynamics of Ntg1 sumoylation by acute hydrogen peroxide treatment, a time course experiment was performed. Monosumoylated Ntg1 was detected in as little as fifteen minutes after exposure, while higher molecular weight products did not appear until one hour after treatment. Ntg1 sumoylation levels peaked two hours after treatment and began to slowly decline three hours after treatment (Figure 1B). These data indicate that hydrogen peroxide is a potent inducer of Ntg1 sumoylation, causing significant levels of sumoylated Ntg1 to persist for long periods of time.

4.2 Genetic analysis of the Ntg1 sumoylation pathway. In order to determine which elements of the sumoylation pathway play a role in the sumoylation of Ntg1, we evaluated the sumoylation status of over-expressed Ntg1-TAP in a SUMO pathway mutant collection. This collection includes the E3 ligases Siz1 and Siz2, and the SUMO proteases Ulp1 and Ulp2 [52, 53]. We quantified Ntg1 sumoylation levels in these mutants under two different conditions: one in which Ntg1 sumoylation was induced by hydrogen peroxide (Figure 2B,D), and the other in which Ntg1 sumoylation was not

induced (Figure 2A,C). We used *siz1Δ*, *siz2Δ*, and *siz1Δsiz2Δ* knock-out strains to determine which E3 ligase is responsible for the induction of Ntg1 sumoylation by hydrogen peroxide. We transformed these strains with a Tet-Off Ntg1-TAP construct and exposed them to hydrogen peroxide (Figure 2B). The *siz1Δ* strain had greatly reduced levels of Ntg1 sumoylation (1.45%) compared to wild type (4.73%). The *siz2Δ* mutant was mostly unchanged (3.24%), and the *siz1Δsiz2Δ* double mutant had no observable sumoylation (Figure 2B,D). These results demonstrate that Siz1 is the primary E3 ligase responsible for hydrogen peroxide–induced sumoylation of Ntg1, while Siz2 has a minor role in Ntg1 sumoylation.

Sumoylation is a dynamic process, where only a very small percent of sumoylated product is present at any given time. Therefore, it was important to determine which if any SUMO proteases desumoylate Ntg1 and to quantify their contribution to desumoylation. To do so we examined Ntg1 sumoylation in the *ulp1-ts* and the *ulp2Δ* strains. Loss of Ulp1 resulted in a dramatic increase in sumoylated Ntg1 with 15.38% of total Ntg1 being monosumoylated and as much as 2.54% being multiply modified. On the other hand, loss of Ulp2 had no impact on Ntg1 sumoylation levels (Figure 2A,C). Furthermore, the levels of hydrogen peroxide–induced Ntg1 sumoylation in the *ulp1-ts* strain were significantly higher than in the wild-type strain. The *ulp1-ts* strain had 10.91% monosumoylated Ntg1 and 1.14% multiply sumoylated Ntg1, contrasting with the 4.73% monosumoylated Ntg1 and 0.05% multiply sumoylated Ntg1 observed in the wild-type strain. Hydrogen peroxide treatment had a negligible effect on sumoylation in the *ulp2Δ* strain (Figure 2D). These results show that the loss of Ulp1 function leads to the overall increase of multiply sumoylated protein. This observation led us to conclude

that the higher molecular weight products of Ntg1 are the result of multiple additions of SUMO. We also concluded that these multisumoylated forms of Ntg1 result from the addition of the multiple SUMO proteins to different residues within Ntg1 rather than from the formation of polymeric SUMO chains (polysumoylated). We arrived at this conclusion, because the *ulp2Δ* strain has the same level of sumoylation as the wild type strain and because the primary function of Ulp2 is to desumoylate polysumoylated proteins [45].

We used a mutant form of the yeast SUMO (*smt3allR*) to test the hypothesis that multiple sumoylation occurs on multiple different residues of Ntg1. The *smt3allR* background is a strain in which all lysines within Smt3 have been conservatively substituted to arginine. Therefore, this form of Smt3 is unsumoylatable and polymeric SUMO chains cannot arise [58]. Consequently, this strain allows us to determination if a protein that is multiply sumoylated is polysumoylated or if it is monosumoylated at multiple residues simultaneously. In both the control and the *smt3allR* background strains Ntg1 had distinct and quantifiable di and trisumoylated bands (Figure 3AB). Since we did not see the loss of di- and trisumoylated forms of Ntg1 in the *smt3allR* background, we concluded that Ntg1 is multisumoylated.

4.3 Insights into location and function of Ntg1 sumoylation. Ntg1 can dynamically localize to either nuclei or mitochondria depending upon the oxidative stress status of the organelle. Furthermore, oxidative stress can also increase levels of sumoylated Ntg1 in the nucleus but not in the mitochondria [22]. This previous data suggest that there could be a link between sumoylation and the dynamic localization of Ntg1. Our first step to address this hypothesis was to determine where in the cell Ntg1

sumoylation takes place. To accomplish this goal, we performed site-directed mutagenesis on Ntg1-TAP and disrupted the classical nuclear localization signal (cNLS) of Ntg1. The resulting Ntg1 had reduced nuclear localization capacity [42]. We tested this Ntg1$_{nls}$-TAP mutant for Ntg1 sumoylation and compared its sumoylation in three different Ntg1 sumoylation-induction backgrounds. Two of these backgrounds rely on hydrogen peroxide, and the third relies upon the loss of function of Ulp1 (Figure 4AB). In every background, the percent of sumoylated Ntg1 appears to decrease for Ntg1$_{nls}$-TAP compared to Ntg1-TAP with the *ulp1-ts* background having the least pronounced decrease. These results indicate that nuclear localization may be required for Ntg1 sumoylation and suggest that Ntg1 is sumoylated within the nucleus (Figure 4B). Moreover, these data hint that Ntg1 is less stable within the nucleus and that Ntg1 sumoylation itself reduces Ntg1 stability. The former point is supported by the observation that the total levels of Ntg1 are consistently increased in the cNLS mutant (Figure 4C). Alternatively, the decrease in percent sumoylated Ntg1 observed with Ntg1$_{nls}$-TAP mutant could be the result of the increased Ntg1 levels. Increased Ntg1 levels may result in lower Ntg1 sumoylation if the sumoylation machinery is the rate limiting step in Ntg1 sumoylation. This possibility makes further experiments that titrate the expression of Ntg1 necessary. In order to conclusively determine the role of Ntg1 sumoylation in Ntg1 function, we needed to create and study an unsumoylatable form of Ntg1.

4.4 Determination of Ntg1 sumoylation sites. In order to better understand the function of Ntg1 sumoylation, it was necessary for us to define where sumoylation occurs on Ntg1. Ntg1 contains ten putative sumoylation sites which were identified using a

conglomeration of the five prediction programs SUMOsp 1.0, SUMOsp 2.0, SUMOplot, SUMOpre, and PCI-SUMO (Figure 5AB) [59-61]. We prioritized the ten putative sumoylation sites using the predicted SUMO-site strength, which is based upon a cumulative sum of all programs scores with weights of 0 (none), 1 (weak), or 2 (strong). These weights are assigned to each Ntg1 lysine based upon the respective output of each program; thus, the resulting lysines had cumulative predictive strengths ranging from 0 to 10 (Figure 5B). Of the ten sites, the top five (K20, K38, K376, K388, and K396R) are consensus sumoylation sites, while the lower five (K364, K157, K255, K194, and K359) are not. In order to derive a form of Ntg1 that is non-sumoylatable, we utilized site-directed mutagenesis to make conservative amino acid substitutions of putative sumoylation site lysines to arginines. These substitutions were made in order of predicted site strength for all single sites, in series of predicted site strength (beginning with a single substitution and proceeding with double, triple, quadruple, etc. substitutions), and spatially based upon both primary sequence and homology modeling of Ntg1 to include either the N-terminus, the C-terminus, or both the termini at once. We then analyzed the sumoylation status of all the resulting mutants of Ntg1-TAP using the *ulp1-ts* strain. We used this strain for two reasons: first, because it is capable of the highest percent Ntg1 sumoylation, and second, to standardize the cellular conditions and to remove confounding factors from the analysis.

The single substitutions were tested first, and the results showed that all single mutants are sumoylated (Figure 6A). Quantification of the single substitution data showed that only substitution at K396 results in a sizeable drop of monosumoylated Ntg1, thus signifying that K396 might be the primary site of monosumoylation (Figure

6C). These data support earlier data suggesting that Ntg1 is able to be sumoylated at multiple lysines simultaneously. This means that multiple substitutions are required to render Ntg1 unsumoylatable. Next, we tested multiple substitution mutants for Ntg1 sumoylation. The double and triple mutants $Ntg1_{K20,38R}$-TAP and $Ntg1_{K20,38,376R}$-TAP involving the N- and C-termini are both still monosumoylated; interestingly however, they have completely lost the disumoylated form but retain the trisumoylated form (Figure 6B,C). The quadruple mutant, $Ntg1_{K20,38,376,388}$-TAP, has complete loss of all SUMO except for monosumoylation; while additional loss of K396, the primary site of monosumoylation, leads to the complete loss of all quantifiable sumoylated Ntg1 (Figure 6B,C). These results demonstrate that Ntg1 is sumoylated at any of five consensus sumoylation sites and that all five sites must be mutated in order to lose Ntg1 sumoylation. This conclusion is further supported by the data from the triple mutants $Ntg1_{K376,388,396R}$-TAP and $Ntg1_{K20,38,396R}$-TAP. Both these mutants are sumoylated, albeit to a lesser extent than wild type Ntg1. These results show that when either K20 and K38, or K376 and K388 are present Ntg1 is still sumoylated at quantifiable levels (Figure 6D,E). Interestingly, in the absence of all five consensus sumoylation sites on Ntg1, monosumoylation still occurs, though at negligible levels. This result suggests that the sumoylation machinery is able to sumoylate Ntg1 at a non-preferred site in the absence of the preferred sumoylation sites, but does so less efficiently (Figure 6D). In accordance with this hypothesis, when we extended the site substitution in Ntg1 to nine out of ten sites the resulting mutant had barely detectable monosumoylation (Figure 6E). We present a summary of all the substitution mutations made to date and their relative effect on monosumoylation in (Figure 7).

5. Discussion

5.1 The regulation of BER through sumoylation. BER is a critical process required for the maintenance of both mitochondrial and nuclear genomes; and, it plays a crucial role in the prevention of human disease [3-7]. Little is known about the effect that post-translational modifications have on BER proteins and whether these modifications occur in response to cellular stress. Collectively, the data in this study elucidate new details concerning the regulation of BER by defining a biological pathway commencing with oxidative stress signaling and concluding in the post-translation modification of a key BER protein. This study provides insight not just into an important mechanism regulating BER, but also into the dynamics of sumoylation. Specifically, our results show that sumoylation of Ntg1 increases primarily in response to oxidative stress; that sumoylation is associated with nuclear localization; and that the E3 ligases Siz1/Siz2 are required to generate both monosumoylated and multisumoylated Ntg1. Furthermore, mutational analysis of putative Ntg1 sumoylation sites revealed that Ntg1 is predominantly sumoylated at five distinct consensus sumoylation sites that cluster at both termini and that K396 is the major site of monosumoylation.

5.2 Illuminating the Ntg1 sumoylation response. Ntg1 is regulated by, and able to respond to, nuclear and mitochondrial oxidative DNA damaging signals (NODDS and MODDS, respectively). This regulation and response allows Ntg1 to dynamically localize to the organelle with the higher level of oxidative stress [22]. Little is known concerning how Ntg1 dynamically localizes to the nucleus in response to NODDS other than that it requires the classical nuclear import pathway and the classical bipartite

nuclear localization signal of Ntg1 [42]. The goal of this study was to better understand the observation that there is an increase in nuclear sumoylated Ntg1 after oxidative stress. To do so we started out by defining the mechanism and extent of its sumoylation. We propose a model where hydrogen peroxide specifically induces the intricate monosumoylation and multisumoylation of Ntg1 (in the nucleus) at five consensus sumoylation sites, which culminates in an improved Ntg1 nuclear repair response against oxidative stress (Figure 8).

The induction of Ntg1 sumoylation in response to oxidative stress, particularly to hydrogen peroxide, is supported by a number of observations. First, the data showing that only high levels of hydrogen peroxide and not other oxidative stress agents induce large levels of Ntg1 sumoylation (Figure 1C). Second, the SUMO protease Ulp1, which is critical for the desumoylation of Ntg1, is sensitive to oxidative stress. Its active site cysteine can become irreversibly oxidized by hydrogen peroxide which renders it unable to remove SUMO from a substrate [62]. Lastly, the generation of oxidative stress in response to DNA damage is a well-characterized phenomenon that acts as a signal to increase Ntg1 protein sumoylation [63, 64].

The hypothesis that Ntg1 sumoylation takes place in the nucleus is supported by the fact that most of the sumoylation pathway, including Siz1, Siz2, and Ulp1, is concentrated within the nucleus [65, 66]. This hypothesis is further supported by biochemical subcellular fractionation studies where sumoylated Ntg1 was found in nuclei, but not in mitochondria [22]. In addition, the low levels of sumoylated Ntg1 observed in the $Ntg1_{nls}$-TAP mutant provides the strongest evidence that Ntg1 sumoylation occurs within the nucleus. We observed that the $Ntg1_{nls}$-TAP mutant

significantly reduces but does not eliminate nuclear localization of Ntg1 (Figure 4A,B) [42]. The latter observation provides a plausible explanation as to why sumoylated Ntg1 is not completely absent in this mutant. These data collectively signify that sumoylation of Ntg1 is important to the nuclear function of Ntg1 in response to threats to genome integrity.

5.3 Ntg1 and sumoylation dynamics. The dynamics of Ntg1 sumoylation help us understand the different sumoylation states of Ntg1 that exist, and they provide a greater understanding of protein sumoylation as a whole. Ntg1 is sumoylated at any of five consensus sumoylation site lysines, including two at the N-terminus (K20 and K38) and three at the C-terminus (K376, K388, and K396). K396 is the most strongly monosumoylated of the five consensus sites, as is established by both the single substitution $Ntg1_{K396R}$-TAP mutant Ntg1 levels and the quadruple substitution $Ntg1_{K20,38,376,388R}$-TAP Ntg1 levels (Figure 6C). The amino acid substitution analysis additionally supports the earlier hypothesis derived from the *ulp2Δ* mutant and the *smt3allR* mutant data that Ntg1 is multisumoylated not polysumoylated (Figure 2B-D and 3A,B). This hypothesis is further supported by the results from the quadruple substitution $Ntg1_{K20,38,376,388R}$-TAP mutant (which has high levels of monosumoylation but no detectable multiply sumoylated bands), and from the quantification of $Ntg1_{K20R}$-TAP and $Ntg1_{K38R}$-TAP where reduced levels of multiply sumoylated Ntg1 are detected (Figure 6B-D).

Perhaps the most curious of the results from the mutation analysis is that whenever both K20 and K38 are mutated, the disumoylated form of Ntg1 is lost while the trisumoylated form increases over that of wild type Ntg1 (Figure 6B-D). This

observation demonstrates a number of things about Ntg1 sumoylation: (1) In order for Ntg1 disumoylation to occur, the second SUMO addition requires an intact sumoylation site on the N-terminus of the protein. This conclusion is further supported by the decrease in disumoylated Ntg1 seen with the $Ntg1_{K20R}$-TAP and $Ntg1_{K38R}$-TAP single substitution mutants (Figure 6C). (2) Trisumoylated Ntg1 utilizes the three C-terminal sites (K376, K388, and K396) and arises independently from disumoylated Ntg1. These observations indicate that each multisumoylated form of Ntg1 may have a separate function (Figure 6B-D). (3) The trisumoylated form of Ntg1 increases in abundance which suggests that the absence of sumoylation on the N-terminus signals for trisumoylation (Figure 6B-D). (4) Neither K376 nor K396 are required for trisumoylation indicating that either the third monosumoylation event occurs at a non-consensus sumoylation site, or that trisumoylation requires polysumoylation at one of the available consensus sites on the C-terminus. Taken as a whole, these Ntg1 sumoylation results constitute one of the most detailed genetic analyses of complex protein sumoylation making it a very informative system to study sumoylation dynamics. Furthermore, these results demonstrate how redundant the protein sumoylation machinery may be as well as how specific and intricate multisumoylation is.

5.4 BER proteins regulated by sumoylation. There are many other proteins in the BER pathway that are sumoylated. These proteins include the Ntg1 paralog Ntg2, the human proteins thymine DNA glycosylase (TDG), Poly (ADP-ribose) polymerase (PARP), and proliferating cell nuclear antigen (PCNA) [67-71]. The number of sumoylated BER proteins suggests that protein sumoylation, triggered by DNA damage signals, is a global DNA damage response which regulates BER. This hypothesis is

supported by a recent systematic screen of sumoylation in DNA replication and repair proteins. This study found that the four BER proteins AP-endonuclease (Apn1), 8-oxoguanine DNA glycosylase (Ogg1), 3-methyl adenine DNA glycosylase (Mag1), and Ntg1 undergo sumoylation under DNA damage induced replication stress [72]. However, it is important to note that this screen did not identify Ntg2 as being sumoylated, which indicates that the sumoylation response profile of each BER protein is unique, and indicates that other BER proteins, such as uracil DNA glycosylase (Ung1), may be sumoylated under other conditions.

5.5 Functional role of protein sumoylation in regulating BER. Regulation of BER by protein sumoylation may function through one of many distinct mechanisms and is currently an area of intense interest in our lab. One likely mechanism is through directing BER protein localization. This localization may allow BER proteins to concentrate in the nucleus in response to increased nuclear oxidative DNA damage signals; it may also be acting on a subnuclear level by allowing BER proteins to localize specifically to DNA damage foci. This type of subnuclear localization is known to occur with Rad52 [73, 74]. SUMO modifications could also regulating Ntg1 by increasing enzyme turnover. The human BER protein TDG is regulated by this mechanism, where SUMO competes with the regulatory domain of TDG for DNA binding, which allows TDG to overcome product inhibition [20, 21]. We further hypothesis that sumoylation of bi-functional glycosylases may alter substrate specificity for specific lesions or AP sites. One final mode of action by which sumoylation regulates BER could be by facilitating crosstalk between the different repair pathways. Sumoylation could potentially trigger the recruitment of BER proteins to sites of complex DNA damage that require nucleotide

excision repair and/or double-strand break repair. There are many others mechanisms by which sumoylation could modulate BER; these include protein stability, enzymatic turnover, nucleocytoplasmic trafficking, and transcriptional regulation.

The fact that Ntg1 has evolved five different sumoylation consensus sequences, and that it is sumoylated in such an extensive and complex manner in response to hydrogen peroxide suggests that sumoylation has an important role in regulating Ntg1 function. The role of hydrogen peroxide in the Ntg1 sumoylation response also has crucial implications in the DNA damage response. Our observations suggest that oxidative stress acts as a signaling agent responsible for altering BER function and maintaining genomic stability. In these regards, our studies have detailed a major pathway responsible for regulating a large proportion of BER and provided insight into the dynamics of the sumoylation response itself.

6. References

1. Fraga, C.G., et al., *Oxidative damage to DNA during aging: 8-hydroxy-2'-deoxyguanosine in rat organ DNA and urine.* Proc Natl Acad Sci U S A, 1990. **87**(12): p. 4533-7.
2. Nakamura, J. and J.A. Swenberg, *Endogenous apurinic/apyrimidinic sites in genomic DNA of mammalian tissues.* Cancer research, 1999. **59**(11): p. 2522-6.
3. Wei, Y.H., *Oxidative stress and mitochondrial DNA mutations in human aging.* Proc Soc Exp Biol Med, 1998. **217**(1): p. 53-63.
4. Wallace, D.C., *Diseases of the mitochondrial DNA.* Annu Rev Biochem, 1992. **61**: p. 1175-212.
5. Cooke, M.S., et al., *Oxidative DNA damage: mechanisms, mutation, and disease.* FASEB J, 2003. **17**(10): p. 1195-214.
6. Beckman, K.B. and B.N. Ames, *Oxidative decay of DNA.* The Journal of biological chemistry, 1997. **272**(32): p. 19633-6.
7. Lenaz, G., *Role of mitochondria in oxidative stress and ageing.* Biochimica et biophysica acta, 1998. **1366**(1-2): p. 53-67.
8. Memisoglu, A. and L. Samson, *Base excision repair in yeast and mammals.* Mutation research, 2000. **451**(1-2): p. 39-51.
9. Nilsen, H. and H.E. Krokan, *Base excision repair in a network of defence and tolerance.* Carcinogenesis, 2001. **22**(7): p. 987-98.
10. Saha, T., et al., *Transcriptional regulation of the base excision repair pathway by BRCA1*. The Journal of biological chemistry, 2010. **285**(25): p. 19092-105.

11. Bouziane, M., et al., *Promoter structure and cell cycle dependent expression of the human methylpurine-DNA glycosylase gene.* Mutation research, 2000. **461**(1): p. 15-29.

12. Hill, J.W., et al., *Stimulation of human 8-oxoguanine-DNA glycosylase by AP-endonuclease: potential coordination of the initial steps in base excision repair.* Nucleic acids research, 2001. **29**(2): p. 430-8.

13. Whitehouse, C.J., et al., *XRCC1 stimulates human polynucleotide kinase activity at damaged DNA termini and accelerates DNA single-strand break repair.* Cell, 2001. **104**(1): p. 107-17.

14. Marenstein, D.R., et al., *Substrate specificity of human endonuclease III (hNTH1). Effect of human APE1 on hNTH1 activity.* The Journal of biological chemistry, 2003. **278**(11): p. 9005-12.

15. Parikh, S.S., et al., *Base excision repair initiation revealed by crystal structures and binding kinetics of human uracil-DNA glycosylase with DNA.* The EMBO journal, 1998. **17**(17): p. 5214-26.

16. Kavli, B., et al., *hUNG2 is the major repair enzyme for removal of uracil from U:A matches, U:G mismatches, and U in single-stranded DNA, with hSMUG1 as a broad specificity backup.* The Journal of biological chemistry, 2002. **277**(42): p. 39926-36.

17. Aamann, M.D., et al., *Cockayne syndrome group B protein promotes mitochondrial DNA stability by supporting the DNA repair association with the mitochondrial membrane.* FASEB J, 2010. **24**(7): p. 2334-46.

18. Frossi, B., et al., *H(2)O(2) induces translocation of APE/Ref-1 to mitochondria in the Raji B-cell line.* J Cell Physiol, 2002. **193**(2): p. 180-6.

19. Tell, G., et al., *An 'environment to nucleus' signaling system operates in B lymphocytes: redox status modulates BSAP/Pax-5 activation through Ref-1 nuclear translocation.* Nucleic Acids Res, 2000. **28**(5): p. 1099-105.

20. Hardeland, U., et al., *Modification of the human thymine-DNA glycosylase by ubiquitin-like proteins facilitates enzymatic turnover.* The EMBO journal, 2002. **21**(6): p. 1456-64.

21. Smet-Nocca, C., et al., *SUMO-1 regulates the conformational dynamics of thymine-DNA Glycosylase regulatory domain and competes with its DNA binding activity.* BMC Biochem, 2011. **12**: p. 4.

22. Griffiths, L.M., et al., *Dynamic compartmentalization of base excision repair proteins in response to nuclear and mitochondrial oxidative stress.* Molecular and cellular biology, 2009. **29**(3): p. 794-807.

23. Hu, J., et al., *Phosphorylation of human oxoguanine DNA glycosylase (alpha-OGG1) modulates its function.* Nucleic acids research, 2005. **33**(10): p. 3271-82.

24. Parker, A.R., et al., *Defective human MutY phosphorylation exists in colorectal cancer cell lines with wild-type MutY alleles.* The Journal of biological chemistry, 2003. **278**(48): p. 47937-45.

25. Lu, X., et al., *The p53-induced oncogenic phosphatase PPM1D interacts with uracil DNA glycosylase and suppresses base excision repair.* Molecular cell, 2004. **15**(4): p. 621-34.

26. Tudek, B., *Base excision repair modulation as a risk factor for human cancers.* Molecular aspects of medicine, 2007. **28**(3-4): p. 258-75.

27. Bhakat, K.K., T.K. Hazra, and S. Mitra, *Acetylation of the human DNA glycosylase NEIL2 and inhibition of its activity.* Nucleic acids research, 2004. **32**(10): p. 3033-9.

28. Bhakat, K.K., et al., *Acetylation of human 8-oxoguanine-DNA glycosylase by p300 and its role in 8-oxoguanine repair in vivo.* Molecular and cellular biology, 2006. **26**(5): p. 1654-65.

29. Qu, J., et al., *Nitric oxide controls nuclear export of APE1/Ref-1 through S-nitrosation of cysteines 93 and 310.* Nucleic acids research, 2007. **35**(8): p. 2522-32.

30. Busso, C.S., M.W. Lake, and T. Izumi, *Posttranslational modification of mammalian AP endonuclease (APE1).* Cellular and molecular life sciences : CMLS, 2010. **67**(21): p. 3609-20.

31. Dianov, G.L., C. Meisenberg, and J.L. Parsons, *Regulation of DNA repair by ubiquitylation.* Biochemistry. Biokhimiia, 2011. **76**(1): p. 69-79.

32. Hussain, S.P., L.J. Hofseth, and C.C. Harris, *Radical causes of cancer.* Nature reviews. Cancer, 2003. **3**(4): p. 276-85.

33. Tang, C.H., W. Wei, and L. Liu, *Regulation of DNA repair by S-nitrosylation.* Biochimica et biophysica acta, 2011.

34. Aspinwall, R., et al., *Cloning and characterization of a functional human homolog of Escherichia coli endonuclease III.* Proc Natl Acad Sci U S A, 1997. **94**(1): p. 109-14.

35. Gossett, J., et al., *Yeast redoxyendonuclease, a DNA repair enzyme similar to Escherichia coli endonuclease III.* Biochemistry, 1988. **27**(7): p. 2629-34.

36. Hilbert, T.P., et al., *Cloning and expression of the cDNA encoding the human homologue of the DNA repair enzyme, Escherichia coli endonuclease III.* J Biol Chem, 1997. **272**(10): p. 6733-40.

37. You, H.J., et al., *Saccharomyces cerevisiae Ntg1p and Ntg2p: broad specificity N-glycosylases for the repair of oxidative DNA damage in the nucleus and mitochondria.* Biochemistry, 1999. **38**(35): p. 11298-306.

38. Augeri, L., et al., *Purification, characterization, gene cloning, and expression of Saccharomyces cerevisiae redoxyendonuclease, a homolog of Escherichia coli endonuclease III.* Biochemistry, 1997. **36**(4): p. 721-9.

39. Hanna, M., et al., *Involvement of two endonuclease III homologs in the base excision repair pathway for the processing of DNA alkylation damage in Saccharomyces cerevisiae.* DNA repair, 2004. **3**(1): p. 51-9.

40. Senturker, S., et al., *Substrate specificities of the ntg1 and ntg2 proteins of Saccharomyces cerevisiae for oxidized DNA bases are not identical.* Nucleic acids research, 1998. **26**(23): p. 5270-6.

41. You, H.J., R.L. Swanson, and P.W. Doetsch, *Saccharomyces cerevisiae possesses two functional homologues of Escherichia coli endonuclease III.* Biochemistry, 1998. **37**(17): p. 6033-40.

42. Swartzlander, D.B., et al., *Regulation of base excision repair: Ntg1 nuclear and mitochondrial dynamic localization in response to genotoxic stress.* Nucleic acids research, 2010. **38**(12): p. 3963-74.

43. Tatham, M.H., et al., *Polymeric chains of SUMO-2 and SUMO-3 are conjugated to protein substrates by SAE1/SAE2 and Ubc9.* The Journal of biological chemistry, 2001. **276**(38): p. 35368-74.

44. Cheng, C.H., et al., *SUMO modifications control assembly of synaptonemal complex and polycomplex in meiosis of Saccharomyces cerevisiae.* Genes Dev, 2006. **20**(15): p. 2067-81.

45. Bylebyl, G.R., I. Belichenko, and E.S. Johnson, *The SUMO isopeptidase Ulp2 prevents accumulation of SUMO chains in yeast.* The Journal of biological chemistry, 2003. **278**(45): p. 44113-20.

46. Mukhopadhyay, D., et al., *SUSP1 antagonizes formation of highly SUMO2/3-conjugated species.* J Cell Biol, 2006. **174**(7): p. 939-49.

47. Johnson, E.S. and G. Blobel, *Ubc9p is the conjugating enzyme for the ubiquitin-like protein Smt3p.* The Journal of biological chemistry, 1997. **272**(43): p. 26799-802.

48. Johnson, E.S. and A.A. Gupta, *An E3-like factor that promotes SUMO conjugation to the yeast septins.* Cell, 2001. **106**(6): p. 735-44.

49. Reindle, A., et al., *Multiple domains in Siz SUMO ligases contribute to substrate selectivity.* J Cell Sci, 2006. **119**(Pt 22): p. 4749-57.

50. Sampson, D.A., M. Wang, and M.J. Matunis, *The small ubiquitin-like modifier-1 (SUMO-1) consensus sequence mediates Ubc9 binding and is essential for SUMO-1 modification.* The Journal of biological chemistry, 2001. **276**(24): p. 21664-9.

51. Song, J., et al., *Identification of a SUMO-binding motif that recognizes SUMO-modified proteins.* Proc Natl Acad Sci U S A, 2004. **101**(40): p. 14373-8.

52. Li, S.J. and M. Hochstrasser, *A new protease required for cell-cycle progression in yeast.* Nature, 1999. **398**(6724): p. 246-51.

53. Li, S.J. and M. Hochstrasser, *The yeast ULP2 (SMT4) gene encodes a novel protease specific for the ubiquitin-like Smt3 protein.* Molecular and cellular biology, 2000. **20**(7): p. 2367-77.

54. Vertegaal, A.C., et al., *Distinct and overlapping sets of SUMO-1 and SUMO-2 target proteins revealed by quantitative proteomics.* Mol Cell Proteomics, 2006. **5**(12): p. 2298-310.

55. Rodriguez, M.S., C. Dargemont, and R.T. Hay, *SUMO-1 conjugation in vivo requires both a consensus modification motif and nuclear targeting.* The Journal of biological chemistry, 2001. **276**(16): p. 12654-9.

56. Ito, H., et al., *Transformation of intact yeast cells treated with alkali cations.* J Bacteriol, 1983. **153**(1): p. 163-8.

57. Sikorski, R.S. and P. Hieter, *A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae.* Genetics, 1989. **122**(1): p. 19-27.

58. Takahashi, Y., E.A. Toh, and Y. Kikuchi, *Comparative analysis of yeast PIAS-type SUMO ligases in vivo and in vitro.* J Biochem, 2003. **133**(4): p. 415-22.

59. Ren, J., et al., *Systematic study of protein sumoylation: Development of a site-specific predictor of SUMOsp 2.0.* Proteomics, 2009. **9**(12): p. 3409-3412.

60. Xue, Y., et al., *SUMOsp: a web server for sumoylation site prediction.* Nucleic Acids Res, 2006. **34**(Web Server issue): p. W254-7.

61. Xu, J., et al., *A novel method for high accuracy sumoylation site prediction from protein sequences.* BMC Bioinformatics, 2008. **9**: p. 8.

62. Zhou, W., J.J. Ryan, and H. Zhou, *Global analyses of sumoylated proteins in Saccharomyces cerevisiae. Induction of protein sumoylation by cellular stresses.* The Journal of biological chemistry, 2004. **279**(31): p. 32262-8.

63. Rowe, L.A., N. Degtyareva, and P.W. Doetsch, *DNA damage-induced reactive oxygen species (ROS) stress response in Saccharomyces cerevisiae.* Free Radic Biol Med, 2008. **45**(8): p. 1167-77.

64. Evert, B.A., et al., *Spontaneous DNA damage in Saccharomyces cerevisiae elicits phenotypic properties similar to cancer cells.* J Biol Chem, 2004. **279**(21): p. 22585-94.

65. Ghaemmaghami, S., et al., *Global analysis of protein expression in yeast.* Nature, 2003. **425**(6959): p. 737-41.

66. Huh, W.K., et al., *Global analysis of protein localization in budding yeast.* Nature, 2003. **425**(6959): p. 686-91.

67. Hoege, C., et al., *RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO.* Nature, 2002. **419**(6903): p. 135-41.

68. Bergink, S. and S. Jentsch, *Principles of ubiquitin and SUMO modifications in DNA repair.* Nature, 2009. **458**(7237): p. 461-7.

69. Leppard, J.B., et al., *Physical and functional interaction between DNA ligase IIIalpha and poly(ADP-Ribose) polymerase 1 in DNA single-strand break repair.* Molecular and cellular biology, 2003. **23**(16): p. 5919-27.

70. Messner, S., et al., *Sumoylation of poly(ADP-ribose) polymerase 1 inhibits its acetylation and restrains transcriptional coactivator function.* FASEB J, 2009. **23**(11): p. 3978-89.

71. Steinacher, R. and P. Schar, *Functionality of human thymine DNA glycosylase requires SUMO-regulated changes in protein conformation.* Current biology : CB, 2005. **15**(7): p. 616-23.

72. Cremona, C.A., et al., *Extensive DNA Damage-Induced Sumoylation Contributes to Replication and Repair and Acts in Addition to the Mec1 Checkpoint.* Molecular cell, 2012.

73. Altmannova, V., et al., *Rad52 SUMOylation affects the efficiency of the DNA repair.* Nucleic Acids Res, 2010. **38**(14): p. 4708-21.

74. Ohuchi, T., et al., *Rad52 sumoylation and its involvement in the efficient induction of homologous recombination.* DNA Repair (Amst), 2008. **7**(6): p. 879-89.

75. Chen, X.L., A. Reindle, and E.S. Johnson, *Misregulation of 2 microm circle copy number in a SUMO pathway mutant.* Mol Cell Biol, 2005. **25**(10): p. 4311-20.

76. Chen, X.L., et al., *Topoisomerase I-dependent viability loss in saccharomyces cerevisiae mutants defective in both SUMO conjugation and DNA repair.* Genetics, 2007. **177**(1): p. 17-30.

77. Bylebyl, G.R., I. Belichenko, and E.S. Johnson, *The SUMO isopeptidase Ulp2 prevents accumulation of SUMO chains in yeast.* J Biol Chem, 2003. **278**(45): p. 44113-20.

78. Degtyareva, N.P., et al., *Chronic oxidative DNA damage due to DNA repair defects causes chromosomal instability in Saccharomyces cerevisiae.* Mol Cell Biol, 2008. **28**(17): p. 5432-45.

79. Kelley, L.A. and M.J. Sternberg, *Protein structure prediction on the Web: a case study using the Phyre server.* Nat Protoc, 2009. **4**(3): p. 363-71.

80. Sheng, W. and X. Liao, *Solution structure of a yeast ubiquitin-like protein Smt3: the role of structurally less defined sequences in protein-protein recognitions.* Protein Sci, 2002. **11**(6): p. 1482-91.

7. Tables

Table 1: Strains and Plasmids Used in this Study

Strain or Plasmid	Description	References
DSC0295	*MAT***a***his3Δ1 leu2Δ0 met 15Δ0 ura3Δ0*; Tet-Off C-terminally TAP-tagged Ntg1	[22]
YSC1178-7499106 (DSC0297)	*MAT***a***his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*; C-terminally TAP-tagged Ntg1	Open Biosystems
BY4147 (DSC0313)	*MAT***a***his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
DSC0470	*MAT***a** *ntg1::hphMX4, his7-1, lys2Δ5′::LEU-lys2Δ3′, ade5-1, trp1-289, ura3-52*	This study
EJY341 (DSC0527)	*MAT***a** *trp1-Δ1 ura3-52 his3-Δ200 leu2-3,112 lys2-801* [cir°]	[75]
EJY342 (DSC0528)	*MAT***a** *trp1-Δ1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 siz1Δ::LEU2* [cir°]	[75]
EJY343 (DSC0529)	*MAT***a** *trp1-Δ1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 siz2Δ::TRP1* [cir°]	[75]
EJY344 (DSC0530)	*MAT***a** *trp1-Δ1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 siz1Δ::LEU2 siz2Δ::TRP1* [cir°]	[75]
MHY1488 (DSC0534)	*MAT***a** *ulp1Δ::HIS3 LEU2::ulp1-333*	[52]
EJY447 (DSC0535)	*MAT***a** *trp1-Δ1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 ulp2Δ::kanMX* [cir°]	[76]
GBY5 (DSC0536)	*MAT***a** *smt3-allR::TRP1*	[77]
DSC0537	*MAT***a** *ntg1::hphMX4, his7-1, lys2Δ5′::LEU-lys2Δ3′, ade5-1, trp1-289, ura3-52*, pD0436	This study
DSC0538	*MAT***a** *trp1-Δ1 ura3-52 his3-Δ200 leu2-3,112 lys2-801* [cir°], pD0436	This study
DSC0539	*MAT***a** *smt3-allR::TRP1*, pD0436	This study
DSC0540	*MAT***a** *ulp1Δ::HIS3 LEU2::ulp1-333*, pD0436	This study
hDNP19	*MAT***a***/MATα rad1*::*kanMX/RAD1 ntg1*::*hphMX4/NTG1 ntg2*::*BSD/NTG2 apn1*::*TRP1/APN1 DSF1*::*URA3/DSF1 his7-1/his7-1 lys2Δ5′*::*LEU-lys2Δ3′*/*lys2Δ5′*::*LEU-lys2Δ3′ ade5-1/ade5-1 trp1-289/trp1-289 ura3-52/ura3-52*	[78]
pD0436	Tet-Off NTG1-TAP, CEN, URA3, amp^R	This study
pD0437	Tet-Off $ntg1_{K20R}$-TAP, CEN, URA3, amp^R	This study
pD0438	Tet-Off $ntg1_{K38R}$-TAP, CEN, URA3, amp^R	This study
pD0439	Tet-Off $ntg1_{K157R}$-TAP, CEN, URA3, amp^R	This study

pD0440	Tet-Off ntg1$_{K194R}$-TAP, CEN, URA3, amp^{R}	This study
pD0441	Tet-Off ntg1$_{K255R}$-TAP, CEN, URA3, amp^{R}	This study
pD0442	Tet-Off ntg1$_{K359R}$-TAP, CEN, URA3, amp^{R}	This study
pD0443	Tet-Off ntg1$_{K364R}$-TAP, CEN, URA3, amp^{R}	This study
pD0444	Tet-Off ntg1$_{K376R}$-TAP, CEN, URA3, amp^{R}	This study
pD0445	Tet-Off ntg1$_{K388R}$-TAP, CEN, URA3, amp^{R}	This study
pD0446	Tet-Off ntg1$_{K396R}$-TAP, CEN, URA3, amp^{R}	This study
pD0447	Tet-Off ntg1$_{K20,38R}$-TAP, CEN, URA3, amp^{R}	This study
pD0448	Tet-Off ntg1$_{K20,38,376R}$-TAP, CEN, URA3, amp^{R}	This study
pD0449	Tet-Off ntg1$_{K20,38,396R}$-TAP, CEN, URA3, amp^{R}	This study
pD0450	Tet-Off ntg1$_{K20,38,376,388R}$-TAP, CEN, URA3, amp^{R}	This study
pD0451	Tet-Off ntg1$_{K20,38,376,388,396R}$-TAP, CEN, URA3, amp^{R}	This study
pD0452	Tet-Off ntg1$_{K376,388,396R}$-TAP, CEN, URA3, amp^{R}	This study
pD0453	Tet-Off ntg1$_{K359,364,376,388,396R}$-TAP, CEN, URA3, amp^{R}	This study
pD0454	Tet-Off ntg1$_{K20,38,359,364,376,388,396R}$-TAP, CEN, URA3, amp^{R}	This study
pD0455	Tet-Off ntg1$_{K20,38,157,359,364,376,388,396R}$-TAP, CEN, URA3, amp^{R}	This study
pD0456	Tet-Off ntg1$_{K20,38,194,359,364,376,388,396R}$-TAP, CEN, URA3, amp^{R}	This study
pD0457	Tet-Off ntg1$_{K20,38,255,359,364,376,388,396R}$-TAP, CEN, URA3, amp^{R}	This study
pD0458	Tet-Off ntg1$_{K20,38,157,194,359,364,376,388,396R}$-TAP, CEN, URA3, amp^{R}	This study
pD0459	Tet-Off ntg1$_{K20,38,157,255,359,364,376,388,396R}$-TAP, CEN, URA3, amp^{R}	This study
pD0460	Tet-Off ntg1$_{K20,38,194,255,359,364,376,388,396R}$-TAP, CEN, URA3, amp^{R}	This study
pD0461	Tet-Off ntg1$_{nls}$-TAP, CEN, URA3, amp^{R}	This study

Table 2: Plasmid Construction Primers

Primer Purpose	Primer Name	Sequence (5' - 3')
pD0436	tetNtg1Cla-F1	GAATCGATTGCAGTTTCATTTGATGCTCGATGAG
	His-Ntg1Cla-R1	GAATCGATGTATTCTGGGCCTCCATGTCGC
K20R	K20R2-F	CAATTCTGAGGAAAAGACCGCTGGTAAGGACTGAAACTGG
	K20R2-R	CCAGTTTCAGTCCTTACCAGCGGTCTTTTCCTCAGAATTG
K38R	K38R-F	GGACCAAAATCAGACAAGAAGAGGTTGTCCCTCAACCCGTG
	K38R-R	CACGGGTTGAGGGACAACCTCTTCTTGTCTGATTTTGGTCC
K157R	K157R-F	GATGCTATCATCGCAAACAAGAGATGAAGTTACCGCAATGGC
	K157R-R	GCCATTGCGGTAACTTCATCTCTTGTTTGCGATGATAGCATC
K194R	K194R-F	CCGTTTTACAAATCAATGAGACCAGATTAGACGAATTGATTCATTCAG
	K194R-R	CTGAATGAATCAATTCGTCTAATCTGGTCTCATTGATTTGTAAAACGG
K255R	K255R-F	CATTACAAAAGGCATGGGGCAGGATTGAAGGTATCTGCGTTGACG
	K255R-R	CGTCAACGCAGATACCTTCAATCCTGCCCCATGCCTTTTGTAATG
K359R	K359R-F	GCAAAATATCATGAGTTATCCAAAGTGGGTGAGATACCTGGAAGG
	K359R-R	CCTTCCAGGTATCTCACCCACTTTGGATAACTCATGATATTTTGC
K364R	K364R-F	TACCTGGAAGGAAGACGTGAACTGAACGTGGAGGCGG
	K364R-R	CCGCCTCCACGTTCAGTTCACGTCTTCCTTCCAGGTA
K376R	K376R-F	CGTGGAGGCGGAAATCAATGTTAGACACGAGGAGAAAACAG
	K376R-R	CTGTTTTCTCCTCGTGTCTAACATTGATTTCCGCCTCCACG
K388R	K388R-F	CGAGGAGAAAACAGTTGAAGAAACTATGGTCAGACTGGAAAATG
	K388R-R	CATTTTCCAGTCTGACCATAGTTTCTTCAACTGTTTTCTCCTCG
K396R	K396R-F	GGAAAATGATATTTCTGTTAGAGTAGAGGACGGTCGACGG
	K396R-R	CCGTCGACCGTCCTCTACTCTAACAGAAATATCATTTTCC
pD0461	NTG1-NLS2-F	GAATCAGAACTCCTACCGGAGGCAGCGACCAAAATCAAACAAG
	NTG1-NLS2-R	CTTGTTTGATTTTGGTCGCTGCCTCCGGTAGGAGTTCTGATTC

8. **Figures**

Figure 1

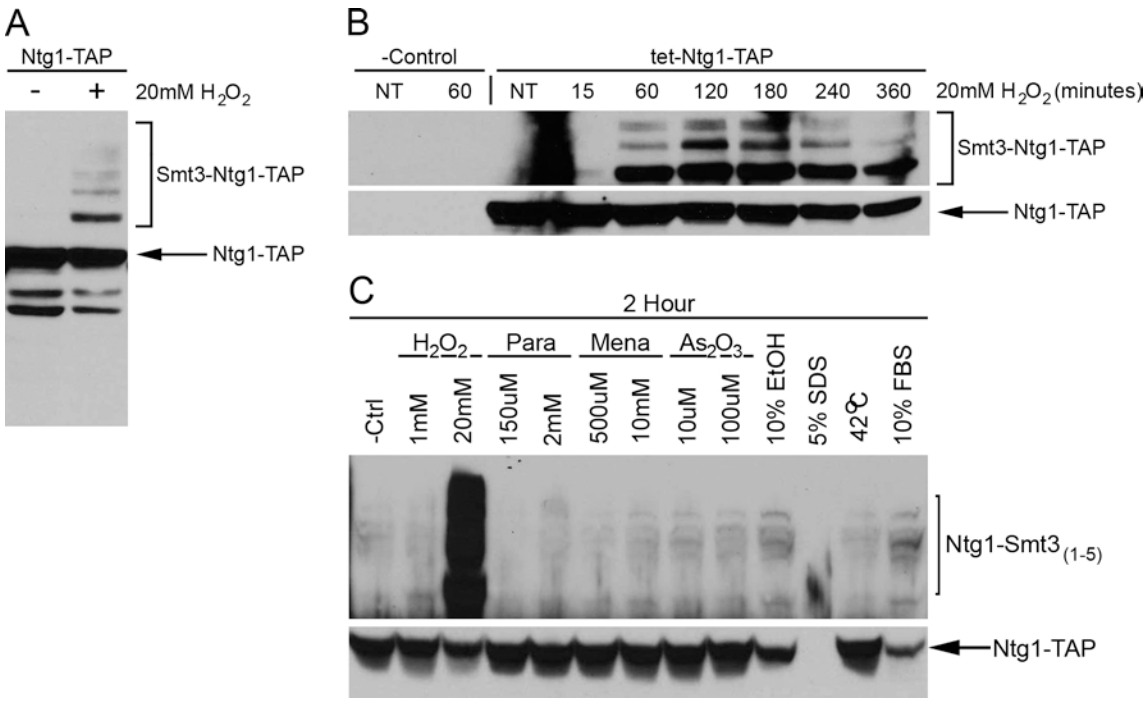


Figure 1 Legend: Induction of Ntg1 sumoylation. Immunoblot analyses of Ntg1-TAP (A) and Tet-Off Ntg1-TAP (B and C) strains utilizing an antibody to the calmodulin domain of TAP. **A.** Cells were either exposed to 20 mM H_2O_2 or water for two hours in DSC0297. Ntg1-TAP indicates unmodified Ntg1 while Smt3-Ntg1-TAP indicates sumoylated products. **B.** A time course where tet-Ntg1-TAP (DSC0295) or -Control (DSC0313) cells were exposed to 20 mM H_2O_2 for either 0, 15, 60, 120, 180, 240, or 360 minutes. Ntg1-TAP indicates unmodified Ntg1 while Smt3-Ntg1-TAP indicates sumoylated products. **C**. The ability of different agents to induce Ntg1 sumoylation was examined. Cell were treated for two hours with the indicated agents and concentration. Abbreviations: -Ctrl, negative control; H_2O_2, hydrogen peroxide; Para, paraquat; Mena, menadione bisulfite; As_2O_3, arsenic trioxide; EtOH, ethanol; SDS, sodium dodecyl sulfate; FBS, fetal bovine serum; and NT, no treatment.

Figure 2

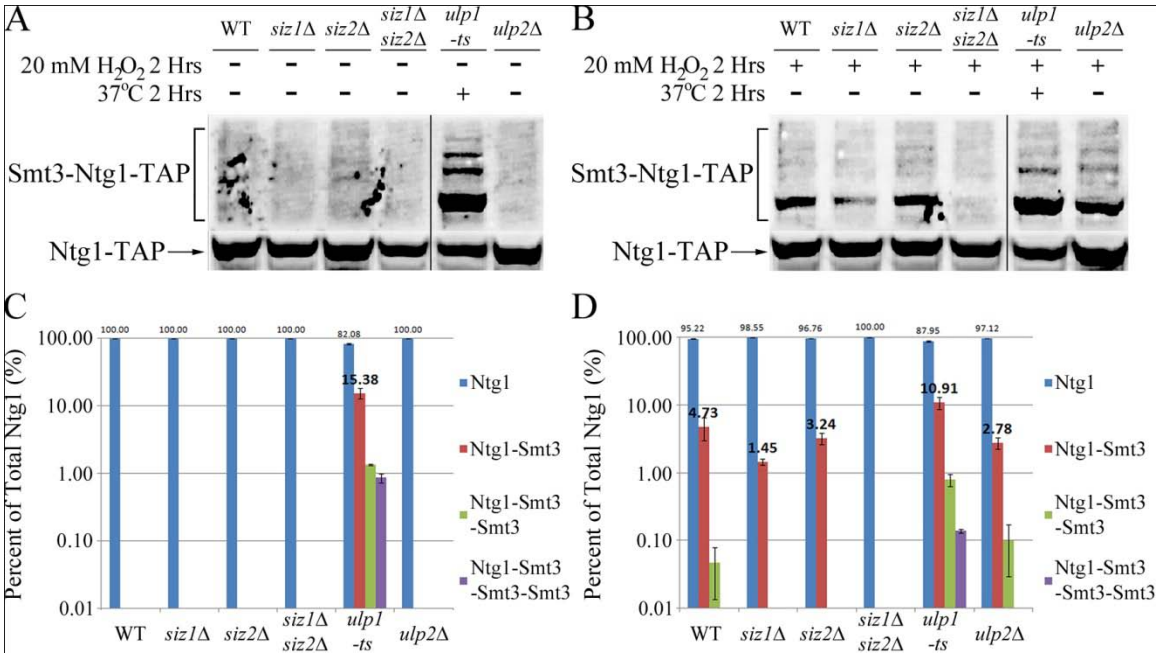


Figure 2 Legend: The sumoylation pathway and Ntg1. Immunoblot analysis of overexpressed Ntg1-TAP utilizing an antibody to the calmodulin domain of TAP in the indicated background strain. **A.** The effect of each SUMO protease (*ulp1-ts* and *ulp2Δ*) mutant on Ntg1 sumoylation was examined. **B.** The effect the E3 ligases Siz1 and Siz2 have on the induction of Ntg1 sumoylation by hydrogen peroxide was determined. Smt3-Ntg1-TAP indicates sumoylated Ntg1, while Ntg1-TAP indicates unsumoylated Ntg1. The upper and lower portions of the blot at the same immunoblots show at different white levels. **C.** Quantitation of each Ntg1 band from part A as a percent of total Ntg1. **D.** Quantitation of each Ntg1 band from part B as a percent of total Ntg1. Ntg1 indicates unsumoylated Ntg1, Ntg1-Smt3 indicates monosumoylated Ntg1, Ntg1-Smt3-Smt3 indicates disumoylated Ntg1, and Ntg1-Smt3-Smt3-Smt3 indicates trisumoylated Ntg1.

Figure 3

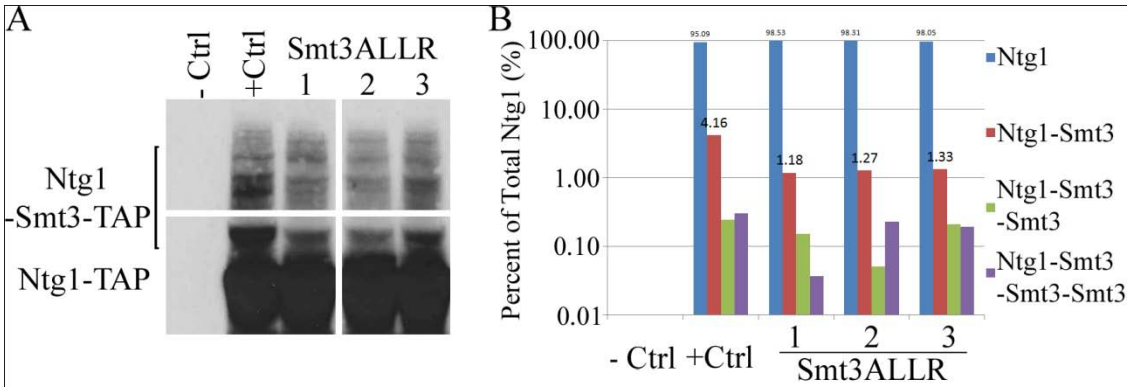


Figure 3 Legend: Examination of multiply sumoylated Ntg1. A. Immunoblot analysis of overexpressed Ntg1-TAP utilizing an antibody to the calmodulin domain of TAP in -Ctrl (DSC0527), +Ctrl (DSC0538), and three different isolates (1,2 and 3) of the *smt3allR* (DSC0539) polysumolation incompetent mutant background. Ntg1-Smt3-TAP indicates sumoylated Ntg1, while Ntg1-TAP indicates unsumoylated Ntg1. The upper and lower portions of the blot are the same immunoblots show at different white levels. **B.** Quantitation of each Ntg1 band from part A as a percent of total Ntg1.

Figure 4

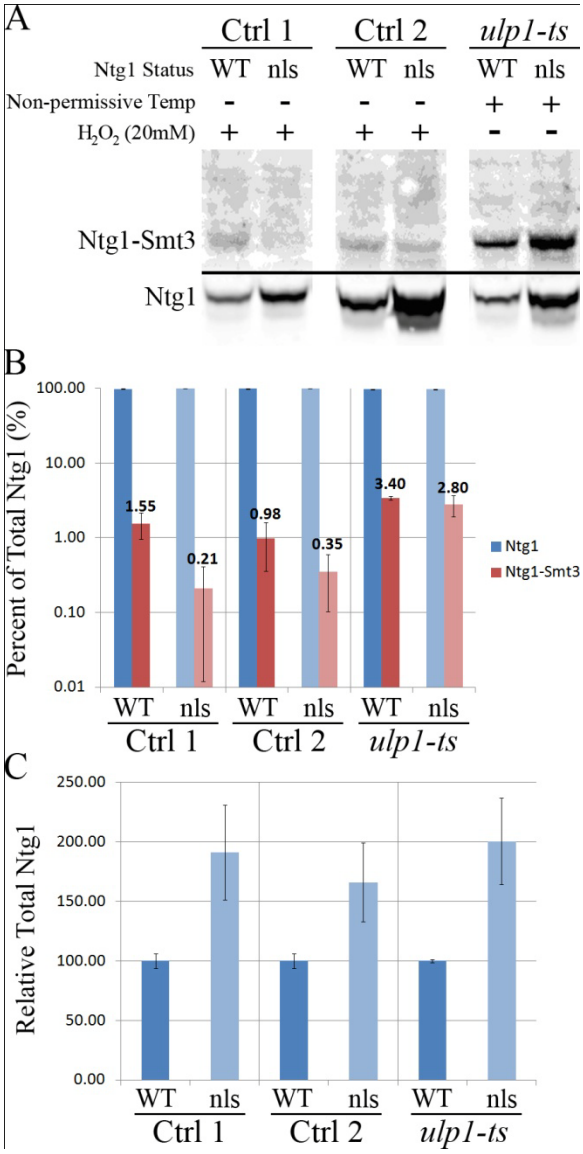


Figure 4 Legend: Importance of Ntg1 localization on sumoylation. A. Immunoblot analysis of overexpressed Ntg1-TAP utilizing an antibody to the calmodulin domain of TAP in three strains, two testing the induction of Ntg1 sumoylation by hydrogen peroxide Ctrl 1 (DSC0537) and Ctrl 2 (DSC0538), and one tested in the *ulp1-ts* background

(DSC0540). Ntg1-smt3 represents monosumoylated Ntg1 while Ntg1 represents unsumoylated Ntg1. **B.** Quantitation of each Ntg1 band from part A as a percent of total Ntg1. **C.** Quantitation of total Ntg1 for each condition in part A normalized to a loading control and presented as a percent of total Ntg1 for wild type Ntg1 compared to cNLS deficient Ntg1. Abbreviations: wild type Ntg1 (WT) and nuclear localization sequence mutant Ntg1 (nls).

Figure 5

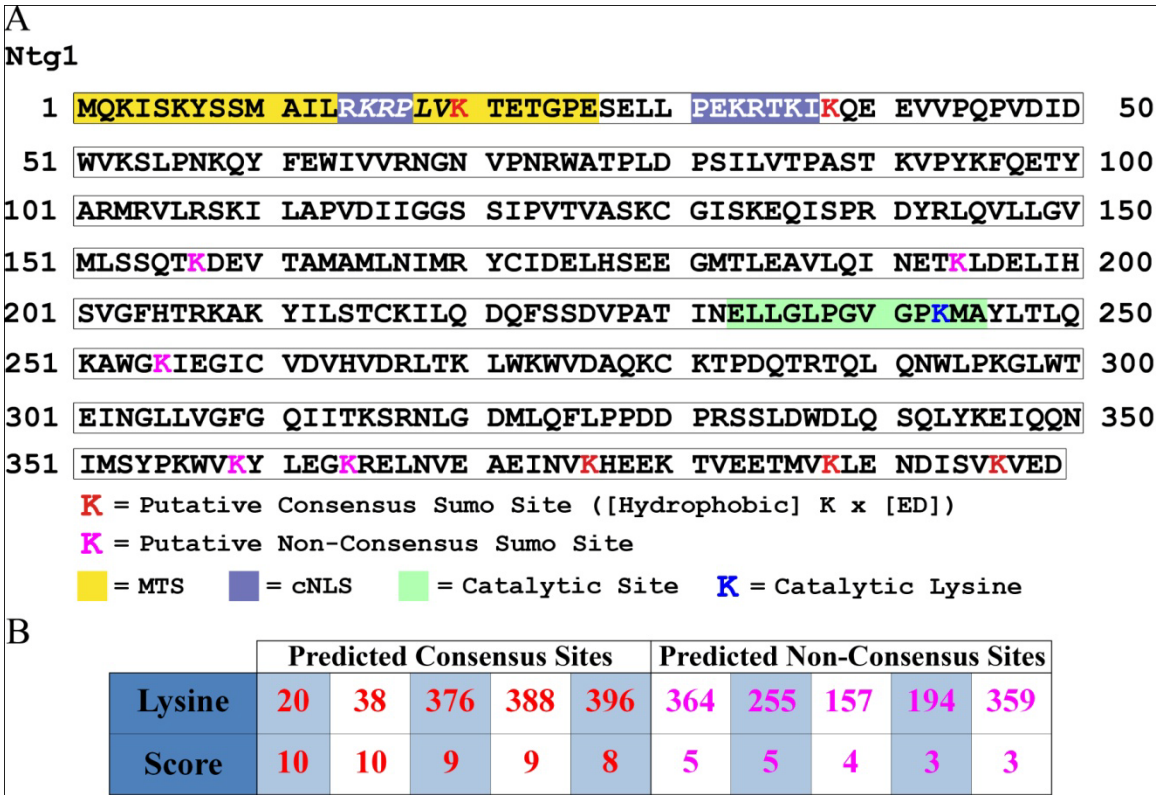


Figure 5 Legend: Predicted Ntg1 sumoylation sites. A. Schematic representation of Ntg1 features. **B.** Putative sumoylation sites identified using a conglomeration of the five prediction programs (SUMOsp 1.0, SUMOsp 2.0, SUMOplot, SUMOpre, and PCI-SUMO), ranked according to a cumulative sum of all program scores with weights of 0 (none), 1 (weak), or 2 (strong) assigned to each Ntg1 lysine based upon the respective output of each program [59-61].

Figure 6

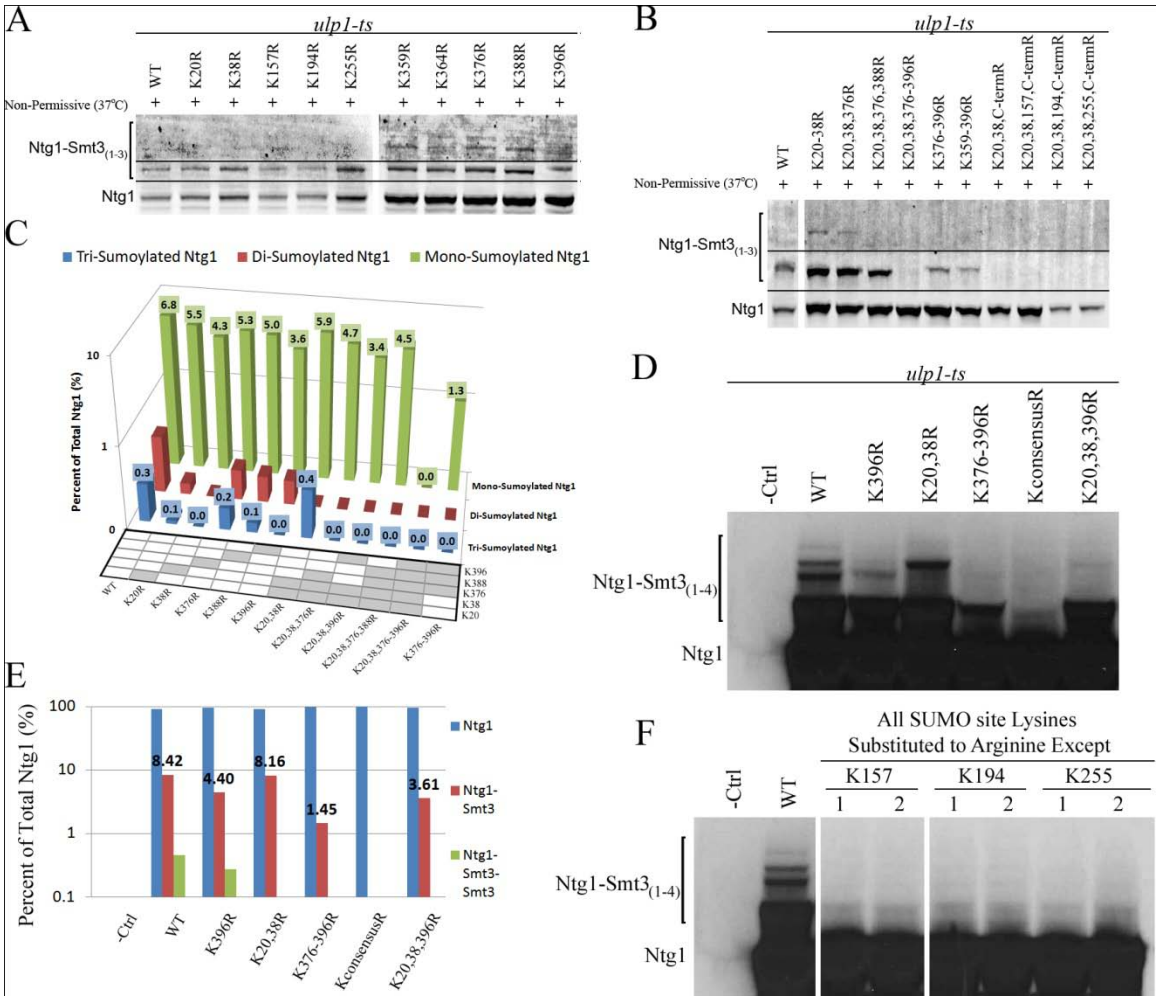


Figure 6 Legend: Ntg1 sumoylation dynamics. Immunoblot analyses utilizing an antibody to the calmodulin domain of TAP and their quantifications using overexpressed Ntg1-TAP and Ntg1-TAP lysine to arginine substitution mutants in the *ulp1-ts* background. Ntg1-Smt3 indicates sumoylated Ntg1, while Ntg1 indicates unsumoylated Ntg1. **A.** Ntg1 wild type (WT) and Ntg1 single lysine to arginine substitution mutants. **B.** Ntg1 wild type (WT) and Ntg1 with multiple lysine to arginine substitution mutations. **C.** Quantification of select Ntg1-TAP mutants from part A and B indicating the percent of mono-, di-, and trisumoylated Ntg1 compared to total. **D.** Immunoblot showing the loss

of disumoylation in both the $Ntg1_{K20,38R}$-TAP and $Ntg1_{K20,38,396R}$-TAP mutants. **E.** Quantification of part D. **F.** Immunoblot showing the barely detectable level of monosumoylation in Ntg1-TAP with lysine to arginine amino acid substitutions made at nine out of ten putative sumoylation sites.

Figure 7

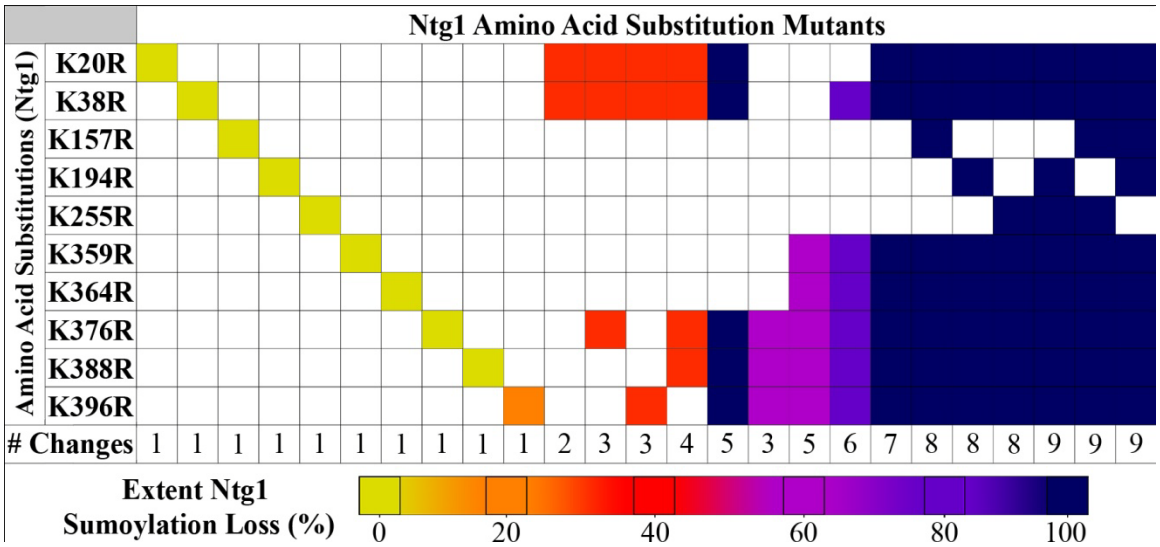


Figure 7 Legend: Summary of all Ntg1 amino acid substitutions. Schematic representation of quantitative monosumoylation analysis of Ntg1 amino acid substitution mutants. Colored squares in a column correspond to the extent loss of Ntg1 monosumoylation (0 – 100%) for individual Ntg1 lysine to arginine amino acid substitution mutants with each row representing a putative Ntg1 sumoylation site and its mutation status (colored or not).

Figure 8

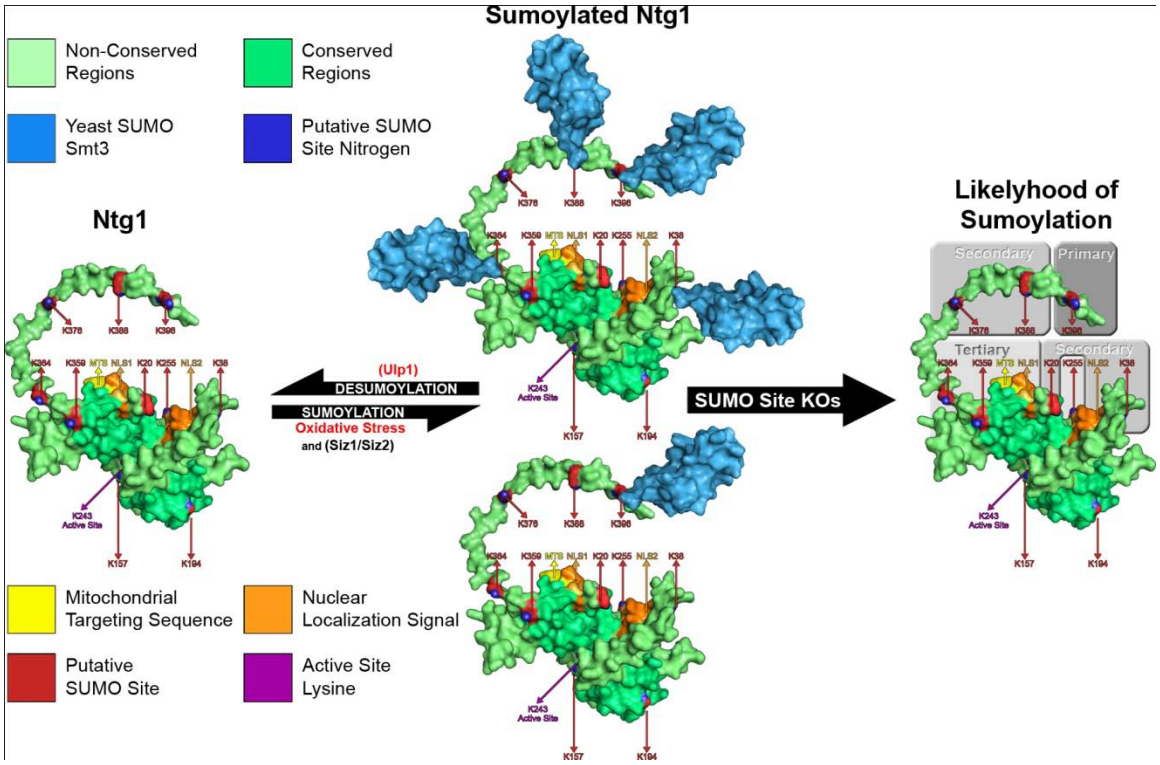


Figure 8 Legend: Model of Ntg1 sumoylation. A space filled homology model of Ntg1 (green) is depicted representing the first 359 amino acids (protein body). Amino acids 360 – 399 (protein tail) were artificially added as an unstructured domain based upon predictive protein folding (Phyre 2.0) [79]. A space filled crystal structure of smt3 is shown (light blue) [80]. Briefly, Ntg1 is sumoylated by the E3 ligases Siz1 and Siz2 in response to oxidative stress in either a mono- (middle bottom), or multisumoylated (middle top) manner. Desumoylation is carried out by the SUMO protease Ulp1. In the absence of Ulp1 steady-state levels of sumoylated Ntg1 increase. Amino acid substitution of Ntg1 sumoylation sites indicates that K396 is the primary site of monosumoylation with K20, K38, K376, and K388 also being major secondary sites of

sumoylation. K157, K194, K255, K359 and K364 are tertiary sites only becoming sumoylated when the primary and secondary sites are absent.

Chapter 5

Conclusions and Future Directions

Reactive oxygen species (ROS) are generated under normal cellular conditions as a byproduct of cellular metabolism. They are also created by exogenous sources such as UV light, ozone, ionizing radiations, metals, pesticides, air pollutants or pharmaceutical drugs [1-4]. ROS can induce a state of oxidative stress by damaging cellular macromolecules including lipids, proteins, RNA, and the focus of this body of work, DNA [5]. Oxidative DNA damage is thought to be one of the most frequent types of spontaneous DNA damage. More than 90,000 oxidative lesions are estimated to arise per mammalian cell per day [6, 7]. Unrepaired oxidative DNA lesions cause mutations and blocks to replication, which ultimately lead to cell death. Consequently, they are associated with aging and numerous human pathologies, including cancer and other degenerative disorders [6, 8-12]. The primary responsibility of the base excision repair pathway (BER) is to efficiently repair such oxidative lesions in order to maintain genome integrity and prevent disease [13, 14].

Eukaryotic DNA is located in two distinct compartments of the cell, the nucleus and the mitochondria. Each of these organelles has potentially different levels of DNA damage that are generated by their unique oxidative environments [15]. The BER pathway is the only pathway present in the mitochondria capable of repairing oxidative lesions, because the nucleotide excision repair (NER) pathway is absent from the mitochondria altogether [16]. BER has to be tightly regulated in order to coordinate the localization of BER proteins, a process which is necessary for the maintenance of genomic stability. Despite the importance of BER in this role, very little was known prior to these studies concerning BER regulation.

The studies presented in this dissertation address the role of oxidative stress as it relates to the regulation of the *Saccharomyces cerevisiae* BER protein, Ntg1. The findings fall into one of two distinct but possibly related pathways for regulation, which are dynamic localization and the post-translational modification, sumoylation. Specifically, dynamic localization of Ntg1 leads to the accumulation of Ntg1 in either the nucleus or the mitochondria in response to oxidative stress within an organelle [17]. Dynamic localization to the mitochondria, in addition to oxidative stress, relies upon the presence of mitochondrial DNA [17]. This result suggested that oxidative DNA damage is necessary to trigger dynamic localization of Ntg1 to either the mitochondria or the nucleus. Beyond the requirements of oxidative stress and the presence of DNA, we found that the following sequences within Ntg1 are necessary for dynamic localization: the classical bipartite nuclear localization sequence (cNLS) and the mitochondrial matrix targeting sequence (MTS) [18]. Furthermore, the classical nuclear import proteins importin α and β are necessary responders for nuclear dynamic localization of Ntg1 [18]. Finally, we found that the nucleus and the mitochondria compete for the pool of Ntg1. More specifically, oxidative stress alters the localization equilibrium of Ntg1, and leads to an organelle-specific increased repair capacity in order to maintain genomic stability [18].

Sumoylation is the second mechanism of Ntg1 regulation that we examined in this study. We found that Ntg1 is sumoylated in response to oxidative stress by the E3 ligases Siz1 and Siz2, and that it is desumoylated by Ulp1 [17]. This event occurs on any of five sumoylation consensus sequences present at either the N- or C-termini of the protein. Sumoylation of these five sites occurs in a complex manner with specific

multisumoylation products that require particular sumoylation sites. Interestingly, Ntg1 sumoylation is associated with nuclear localization of Ntg1. This observation suggests that sumoylation may be linked to nuclear dynamic localization in response to oxidative stress. These two separate mechanisms of BER regulation that result from oxidative stress, provide insight into our understanding of BER regulation as a whole. We hypothesize that these are features of general BER regulation.

Identification of novel means of regulating BER. The data in Chapter 2 of this dissertation identifies two separate but possibly related means of BER regulation: dynamic localization and sumoylation. Oxidative stress appears to play a key role in the regulation of these responses, which suggests that it is central to the regulation of BER as a whole. When there is an increase of nuclear oxidative stress that leads to an increase in nuclear oxidative DNA damage, Ntg1 dynamically localizes to the nucleus, and Ntg1 is extensively sumoylated (Chapter 2, Figures 3 and 7).

In Chapter 2 the focus was on the dynamic localization of Ntg1, and on the origin of this dynamic localization response. To address this, rho^0 cells were employed and it was established that the presence of mitochondrial DNA is required for mitochondrial dynamic localization. These results suggest that the DNA damage is also required for nuclear dynamic localization (Chapter 2, Figure 4) [17]. We also hypothesize that nuclear and mitochondrial oxidative DNA damage signals, (NODDS) and (MODDS) respectively, are responsible for mediating dynamic localization. Two questions arise from this hypothesis. First, “how do proteins respond to NODDS and MODDS?”, and second, “what is the overall impact that dynamic localization has on genomic stability?”

Characterization of the Ntg1 dynamic localization response and its components. As a follow-up to the prior work, Chapter 3 focuses on defining the elements driving dynamic localization and illustrates its importance. In the first part of this study we investigated the manner by which Ntg1 is targeted for dynamic localization by NODDS and MODDS. Here, we identified the functional cis-acting bipartite cNLS and the MTS and the trans-acting nuclear import factors importin α and β (Chapter 3, Figures 1 and 3) [18]. These components are all required for dynamic localization, which suggests that dynamic localization uses the inherent import pathway of a protein. Furthermore, importin α and β are critical responders to NODDS and MODDS [18]. This finding signifies that nuclear dynamic localization of Ntg1 functions either by increasing the rate of Ntg1 classical nuclear import, or that it functions by decreasing nuclear export. We further studied the biological significance of dynamic localization by using mutant Ntg1 proteins. These mutants have altered cNLS or MTS sequences. These mutants were expressed in a repair compromised background as the sole copy of Ntg1 allowing us to determine the contribution of dynamic localization to genomic stability. When either the cNLS or MTS was lost, Ntg1 no longer responded to NODDS and MODDS (respectively), which caused an increase in nuclear mutation rates or mitochondrial mutation frequencies as well as an increase in the cytotoxicity of DNA damaging agents (Chapter 3, Figure 5 and Table 2) [18]. These data show that dynamic localization is critical to genome integrity.

Overall these findings suggest that there are many other factors involved in the dynamic localization process, which indicates that many responders to NODDS and

MODDS have not yet been identified. One possible way to determine which proteins are responders to NODDS and MODDS is to identify Ntg1 interacting factors comparing control and oxidative conditions. Furthermore, this approach should employ either total cell lysates or fractionated nuclear, mitochondrial, or cytoplasmic lysates

In addition to the responders, the NODDS and MODDS signal transducers have not been identified. There are two non-mutually exclusive hypotheses concerning the identity of NODDS and MODDS, both of which are consistent with the idea that the presence of DNA is required. The first hypothesis is that DNA lesions themselves are initiators of NODDS and MODDS. This concept relies upon a central transducer protein that recognizes abasic sites. The hypothesized transducer protein will translate the detected abasic site into a DNA repair signal that recruits other BER proteins. The second hypothesis is that ROS are part of NODDS and MODDS. This notion relies upon the observation that oxidative DNA damage results in the production of ROS [19, 20]. There are several examples that support the latter hypothesis. In these examples, post-translational modifications to proteins are driven by ROS, consequently causing changes in activity, abundance, localization, and interactions with other proteins [21, 22]. This hypothesis hinges upon unique redox sensor proteins, associated with either nuclei or mitochondria. These proteins transform the ROS signal and recruit BER proteins in an organelle-specific manner.

Characterization of the Ntg1 sumoylation response mechanism. The data presented at the end of Chapter 2 provides a possible link between SUMO modification

and the dynamic localization of Ntg1 observed in response to oxidative stress. The conclusions drawn in this section come from the observation that a mutation in the putative sumoylation site, K364, alters the Ntg1 dynamic localization pattern. This data strongly suggests that sumoylation is required for proper nuclear dynamic localization (Chapter 2, Figure 8) [17]. As a follow up of this observation, Chapter 4 defines the mechanism and sites of Ntg1 sumoylation and in doing so uncovers intriguing details about sumoylation dynamics.

Surprisingly, when we investigated potential sumoylation sites, we discovered that K364 is not the sole site of Ntg1 sumoylation in response to oxidative stress, nor is it even a primary site, as Ntg1 is still monosumoylated when K364 is altered to a non-sumoylatable arginine (Chapter 4, Figure 7). This discovery however, does not rule out the possibility that multisumoylation is required for nuclear dynamic localization of Ntg1. The latter point requires additional investigation. In an effort to completely understand Ntg1 sumoylation, we further characterized Ntg1 sumoylation sites. In doing so, we identified K20, K38, K376, K388 and K396, with K396 as the primary sites of monosumoylation (Chapter 4, Figure 6B,C). Multisumoylated Ntg1 is created by the addition of extra SUMO modifications to different sumoylation sites. Interestingly, disumoylation of Ntg1 requires either K20 or K38 while trisumoylation does not (Chapter 4, Figure 6B-E). Tandem mass spectrometry analysis of purified mono-, di-, and trisumoylated Ntg1 is necessary for us to better understand the complexities of Ntg1 sumoylation. Use of mass spectrometry would allow us to identify the different populations of post-translationally modified Ntg1 *in vivo*. This technique will further provide valuable information about the sumoylation attachment sites and about the

presence and location of polymeric SUMO chains. As, this experiment is technically challenging, we also consider other approaches that could be employed to address these questions.

Structural studies are another important approach we can use to further our understanding of how sumoylation alters the function of Ntg1. Protein homology modeling and predictive protein folding algorithms are two methods we have used to construct predicted Ntg1 protein structures (Figure 1). Both of these methods have advantages and disadvantages. The protein homology model was made by aligning Ntg1 to the crystal structure of the structurally related MutY protein from *Geobacillus stearothermophilus*. This method provided a model of the first 359 amino acids of Ntg1. The core of this amino acid sequence aligns well with crystal structures of endonuclease III. The major drawback of this model is that it does not predict the structure of the C-terminus. This makes it impossible to guess how the C-terminus interacts with the rest of the protein and ultimately how C-terminal sumoylation alters Ntg1 function.

We generated the predictive protein folding model using the Phyre 2.0 server. In the resulting model 77% of the amino acids were modeled with greater than 90% confidence [23]. However, the remaining 23% of amino acids, which have very low confidence values, primarily comprise both the N- and C-termini, the regions that are of greatest interest for localization and sumoylation. This modeling produced a structure that at its core closely resembles crystal structures of other glycosylases, but with unstructured termini. The notion that Ntg1 termini are unstructured supports our observations that these termini are regulatory regions, because unstructured domains are often associated with regulatory regions of a protein [24]. Consistent with this

observation, Ntg1 has four regulatory regions at the N-terminus, the MTS, the bipartite cNLS, and two different sumoylation sites. Furthermore, Ntg1 contains three regulatory regions at the C-terminus in the form of three sumoylation sites. All five consensus sumoylation sequences fall within these predicted unstructured regions, which appears appropriate given that the sumoylation machinery prefers less rigid protein structures [25, 26]. These models of Ntg1 lead us to hypothesize that Ntg1 sumoylation does one of three things: it interferes with protein binding to the Ntg1 intracellular targeting signals; it creates new protein-protein interactions; or it changes how Ntg1 interacts with DNA.

The interaction between Ntg1 and the sumoylation machinery is one of the focuses of Chapter 4. In these studies we discovered that two E3 ligases, Siz1 and Siz2, are predominantly responsible for Ntg1 sumoylation. We also discovered that Ulp1 is responsible for Ntg1 desumoylation (Chapter 4, Figure 2A-D). In addition to identifying the main players in Ntg1 sumoylation, we also uncovered the role of oxidative stress in the induction of Ntg1 sumoylation. In these studies we used the following agents: hydrogen peroxide; the oxidative stress agents menadione bisulfate and paraquat; the osmotic stress agents sodium dodecyl sulfate (SDS) and ethanol; and the hyphal stress agent fetal bovine serum (FBS) (Chapter 4, Figure 1C). Only hydrogen peroxide induced high levels of Ntg1 sumoylation, while menadione bisulfite, paraquat, ethanol, and FBS induced low levels of sumoylation. These results suggest that hydrogen peroxide specifically triggers the sumoylation of Ntg1, and that other agents could indirectly cause sumoylation. We hypothesize that these agents do so because they induce cell stress which in turn leads to a secondary ROS response [19, 20]. One potential mechanism of hydrogen peroxide induced Ntg1 sumoylation is through the inactivation of Ulp1 by

oxidation of the Ulp1 active site cysteine [27]. This notion is supported by the data from the *ulp1-ts* background where in the absence of Ulp1 function, Ntg1 sumoylation levels are equal to or higher than the maximal hydrogen peroxide induced sumoylation (Chapter 4, Figure 2A-D). Other inducers ofNtg1 sumoylation may also exist. These could include other stress agents or certain growth conditions. In one recent study, investigators observed such a finding. In that study, Ntg1 sumoylation was induced by treating exponentially growing cells with methyl methanesulfonate (MMS), which causes replication stress [28]. The sumoylation response was observed using immunoblot assays with purified protein extracts [28]. Furthermore, that study identified three other yeast BER proteins, Apn1, Ogg1, and Mag1 that undergo protein sumoylation under the same conditions, thus supporting the idea that BER as a whole is regulated by sumoylation [28].

The mechanism by which sumoylation regulates BER proteins is unknown. However, we have now identified the sites of Ntg1 sumoylation, which is critical for future studies addressing how sumoylation regulates BER. One obvious future direction is to create Ntg1 SUMO mutant constructs that are integrated into the genome or that are tagged with GFP. These integrated constructs should be assayed for nuclear mutation rates, mitochondria mutation frequencies, and agent induced cytotoxicity in a repair compromised background to help us understand the contributions of Ntg1 sumoylation on the critical cellular roles of Ntg1. The GFP constructs will allow for any link between sumoylation and dynamic localization to be found. Since Ntg1 is 399 amino acids in length and K396 is the major site of monosumoylation, an artificial Ntg1 C-terminal

SUMO fusion construct could help in determining whether Ntg1 has different cellular localization or enzymatic activity when it is sumoylated.

Generality of regulatory mechanisms in BER. The work presented in this dissertation focuses on Ntg1 as a model for BER regulation. In this work we identified two possibly related mechanisms of regulation. However, further investigation is required to determine whether dynamic localization and/or sumoylation function as general BER regulatory mechanisms. We used a candidate approach in an effort to identify further yeast BER proteins that are regulated in a similar manner to Ntg1. For this approach we used predictive programs to determine whether any other BER proteins contain putative cNLSs, MTSs, or sumoylation sites in a pattern similar to that of Ntg1. Only Ung1 and Ogg1 have a similar pattern. Ung1, like Ntg1, has partially overlapping nuclear and mitochondrial localization sequences, and both Ung1 and Ogg1 have multiple putative sumoylation sites. These finding make Ung1 and Ogg1 prime candidates for future work. The paralog of Ntg1, Ntg2 localizes only to the nucleus and is not subject to dynamic localization (Chapter 2, Figure 1) [17]. Interestingly however, Ntg2 is also sumoylated, which suggests that sumoylation and dynamic localization are distinct mechanism for BER regulation (Chapter 2, Figure 6) [17]. The human homolog of Ntg1 and Ntg2, human thymine glycol-DNA-glycosylase (NTHL1), localizes to both nuclei and mitochondria, thus making it a leading candidate for translating this work into a human system [29-31].

Significance of BER regulation to human disease. There are many examples in primary cancers where the activity, expression, or localization of specific BER proteins is altered or lost [32-38]. These observations suggest that misregulation of BER has significant biological consequences. For example, in a subset of human lung cancer tumors, nuclei show increased NTHL1 and human 8-oxoguanine DNA glycosylase (OGG1) repair activity, while this activity is reduced in their mitochondria [32, 33]. In gastric cancers, the enzyme NTHL1 is often lost in the nucleus, which is the result of global down-regulation of steady-state protein levels, or abnormal localization of NTHL1 to the cytoplasm [34, 35]. The latter is observed in a significant number of primary gastric cancers [34]. There are also examples of BER dysfunction in neurodegenerative disorders [36, 37]. For example, the activities of the human uracil DNA glycosylase, (UNG), and of OGG1 are decreased in Alzheimer's patients [38]. These examples exemplify the importance of regulating both the function and localization of repair proteins in order to maintain genomic stability because when repair capacity decreases in either the nucleus or the mitochondria disease is often the result. Dynamic localization and sumoylation are two mechanisms of BER regulation that need to be further investigated in order to understand the entirety of their impact on genomic stability in both yeast and in humans.

Integrated model of BER regulatory mechanisms. Many details about the regulation of BER remain unknown. However, the work presented in this dissertation provides novel insight into some of these details. More specifically in this work we elucidate the role that dynamic localization and sumoylation play in BER regulation. Our model places oxidative stress at the forefront of BER regulation, since it triggers dynamic

localization and sumoylation (Figure 2). In the case of dynamic localization, oxidative stress causes DNA damage and is generated in response to DNA damage, leading to the formation of either NODDS or MODDS. NODDS and MODDS function through different responder proteins, and they lead to an increase in nuclear or mitochondrial BER protein levels. NODDS specifically, lead to an increased nuclear concentration of BER proteins; it does so by increasing nuclear import, decreasing mitochondrial import, or decreasing nuclear export. MODDS characteristically lead to increased mitochondrial BER protein concentration by facilitating mitochondrial import or by decreasing nuclear import. In both cases, changes in localization are the result of post-translational modifications (PTMs), such as oxidation or sumoylation. These modifications drive or impede interactions of the import machinery with either cNLSs or MTSs. In the case of sumoylation, oxidative stress directly oxidizes proteins, consequently promoting a sumoylation response. Sumoylated proteins are associated with the nucleus and may be directly related to dynamic localization. On the other hand, sumoylated proteins may alter protein function by changing enzymatic activity, substrate specificity, protein longevity, nucleocytoplasmic trafficking, transcriptional regulation, and/or subnuclear localization. Deregulation of either pathway results in increased genomic instability, promoting cancer and other neurodegenerative diseases.

References

1. Finkel, T. and N.J. Holbrook, *Oxidants, oxidative stress and the biology of ageing.* Nature, 2000. **408**(6809): p. 239-47.
2. Turrens, J.F., *Mitochondrial formation of reactive oxygen species.* J Physiol, 2003. **552**(Pt 2): p. 335-44.
3. Limon-Pacheco, J. and M.E. Gonsebatt, *The role of antioxidants and antioxidant-related enzymes in protective responses to environmentally induced oxidative stress.* Mutation research, 2009. **674**(1-2): p. 137-47.
4. Lindahl, T., *Instability and decay of the primary structure of DNA.* Nature, 1993. **362**(6422): p. 709-15.
5. Sies, H. and E. Cadenas, *Oxidative stress: damage to intact cells and organs.* Philos Trans R Soc Lond B Biol Sci, 1985. **311**(1152): p. 617-31.
6. Beckman, K.B. and B.N. Ames, *Oxidative decay of DNA.* The Journal of biological chemistry, 1997. **272**(32): p. 19633-6.
7. Fraga, C.G., et al., *Oxidative damage to DNA during aging: 8-hydroxy-2'-deoxyguanosine in rat organ DNA and urine.* Proc Natl Acad Sci U S A, 1990. **87**(12): p. 4533-7.
8. Wei, Y.H., *Oxidative stress and mitochondrial DNA mutations in human aging.* Proc Soc Exp Biol Med, 1998. **217**(1): p. 53-63.
9. Wallace, D.C., *Diseases of the mitochondrial DNA.* Annu Rev Biochem, 1992. **61**: p. 1175-212.
10. Cooke, M.S., et al., *Oxidative DNA damage: mechanisms, mutation, and disease.* FASEB J, 2003. **17**(10): p. 1195-214.

11. Lenaz, G., *Role of mitochondria in oxidative stress and ageing.* Biochimica et biophysica acta, 1998. **1366**(1-2): p. 53-67.

12. Ide, H., Y.W. Kow, and S.S. Wallace, *Thymine glycols and urea residues in M13 DNA constitute replicative blocks in vitro.* Nucleic acids research, 1985. **13**(22): p. 8035-52.

13. Memisoglu, A. and L. Samson, *Base excision repair in yeast and mammals.* Mutation research, 2000. **451**(1-2): p. 39-51.

14. Nilsen, H. and H.E. Krokan, *Base excision repair in a network of defence and tolerance.* Carcinogenesis, 2001. **22**(7): p. 987-98.

15. Haag-Liautard, C., et al., *Direct estimation of the mitochondrial DNA mutation rate in Drosophila melanogaster.* PLoS Biol, 2008. **6**(8): p. e204.

16. Boesch, P., et al., *DNA repair in organelles: Pathways, organization, regulation, relevance in disease and aging.* Biochimica et biophysica acta, 2011. **1813**(1): p. 186-200.

17. Griffiths, L.M., et al., *Dynamic compartmentalization of base excision repair proteins in response to nuclear and mitochondrial oxidative stress.* Molecular and cellular biology, 2009. **29**(3): p. 794-807.

18. Swartzlander, D.B., et al., *Regulation of base excision repair: Ntg1 nuclear and mitochondrial dynamic localization in response to genotoxic stress.* Nucleic acids research, 2010. **38**(12): p. 3963-74.

19. Rowe, L.A., N. Degtyareva, and P.W. Doetsch, *DNA damage-induced reactive oxygen species (ROS) stress response in Saccharomyces cerevisiae.* Free Radic Biol Med, 2008. **45**(8): p. 1167-77.

20. Evert, B.A., et al., *Spontaneous DNA damage in Saccharomyces cerevisiae elicits phenotypic properties similar to cancer cells.* J Biol Chem, 2004. **279**(21): p. 22585-94.

21. Ma, Q., *Transcriptional responses to oxidative stress: pathological and toxicological implications.* Pharmacol Ther, 2010. **125**(3): p. 376-93.

22. Wang, Y., J. Yang, and J. Yi, *Redox sensing by proteins: oxidative modifications on cysteines and the consequent events.* Antioxid Redox Signal, 2011.

23. Kelley, L.A. and M.J. Sternberg, *Protein structure prediction on the Web: a case study using the Phyre server.* Nat Protoc, 2009. **4**(3): p. 363-71.

24. Dyson, H.J. and P.E. Wright, *Intrinsically unstructured proteins and their functions.* Nat Rev Mol Cell Biol, 2005. **6**(3): p. 197-208.

25. Bernier-Villamor, V., et al., *Structural basis for E2-mediated SUMO conjugation revealed by a complex between ubiquitin-conjugating enzyme Ubc9 and RanGAP1.* Cell, 2002. **108**(3): p. 345-56.

26. Lin, D., et al., *Identification of a substrate recognition site on Ubc9.* The Journal of biological chemistry, 2002. **277**(24): p. 21740-8.

27. Zhou, W., J.J. Ryan, and H. Zhou, *Global analyses of sumoylated proteins in Saccharomyces cerevisiae. Induction of protein sumoylation by cellular stresses.* The Journal of biological chemistry, 2004. **279**(31): p. 32262-8.

28. Cremona, C.A., et al., *Extensive DNA Damage-Induced Sumoylation Contributes to Replication and Repair and Acts in Addition to the Mec1 Checkpoint.* Molecular cell, 2012.

29. Ikeda, S., et al., *Differential intracellular localization of the human and mouse endonuclease III homologs and analysis of the sorting signals.* DNA repair, 2002. **1**(10): p. 847-54.

30. Takao, M., et al., *Mitochondrial targeting of human DNA glycosylases for repair of oxidative DNA damage.* Nucleic acids research, 1998. **26**(12): p. 2917-22.

31. Luna, L., et al., *Cell-cycle regulation, intracellular sorting and induced overexpression of the human NTH1 DNA glycosylase involved in removal of formamidopyrimidine residues from DNA.* Mutation research, 2000. **460**(2): p. 95-104.

32. Karahalil, B., V.A. Bohr, and N.C. De Souza-Pinto, *Base excision repair activities differ in human lung cancer cells and corresponding normal controls.* Anticancer research, 2010. **30**(12): p. 4963-71.

33. Radak, Z., et al., *Lung cancer in smoking patients inversely alters the activity of hOGG1 and hNTH1*. Cancer letters, 2005. **219**(2): p. 191-5.

34. Goto, M., et al., *Altered expression of the human base excision repair gene NTH1 in gastric cancer.* Carcinogenesis, 2009. **30**(8): p. 1345-52.

35. Koketsu, S., T. Watanabe, and H. Nagawa, *Expression of DNA repair protein: MYH, NTH1, and MTH1 in colorectal cancer.* Hepato-gastroenterology, 2004. **51**(57): p. 638-42.

36. Coppede, F. and L. Migliore, *DNA damage and repair in Alzheimer's disease.* Current Alzheimer research, 2009. **6**(1): p. 36-47.

37. Jeppesen, D.K., V.A. Bohr, and T. Stevnsner, *DNA repair deficiency in neurodegeneration.* Progress in neurobiology, 2011. **94**(2): p. 166-200.

38. Weissman, L., et al., *Defective DNA base excision repair in brain from individuals with Alzheimer's disease and amnestic mild cognitive impairment.* Nucleic acids research, 2007. **35**(16): p. 5545-55.

39. Fromme, J.C., et al., *Structural basis for removal of adenine mispaired with 8-oxoguanine by MutY adenine DNA glycosylase.* Nature, 2004. **427**(6975): p. 652-6.

Figure 1

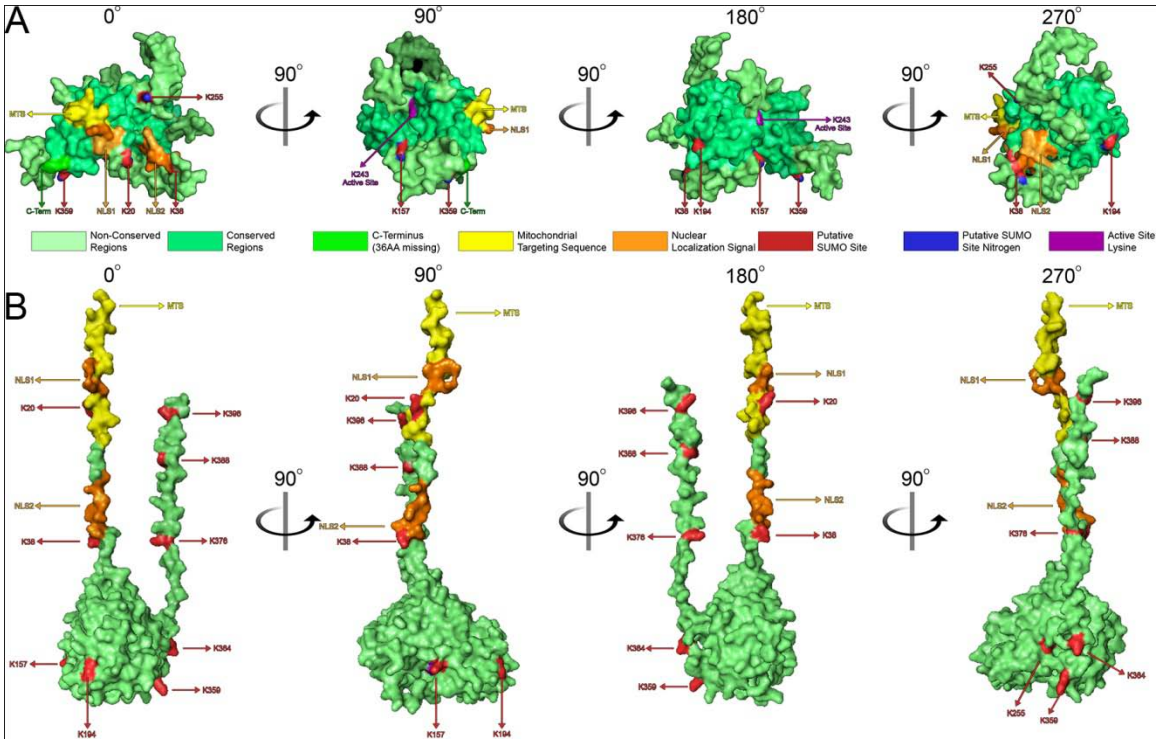


Figure 1 Legend: Protein models of Ntg1. A. Protein homology model containing the first 359 amino acids of Ntg1, made by aligning Ntg1 to the crystal structure of MutY from *Geobacillus stearothermophilus* [39]. **B.** Predictive protein folding model containing all 396 amino acids of Ntg1 made using the Phyre 2.0 server [23]. 77% of the amino acids were modeled with greater than 90% confidence while the remaining 23% of amino acids are predicted with very low confidence comprising both the N- and C-termini.

Figure 2

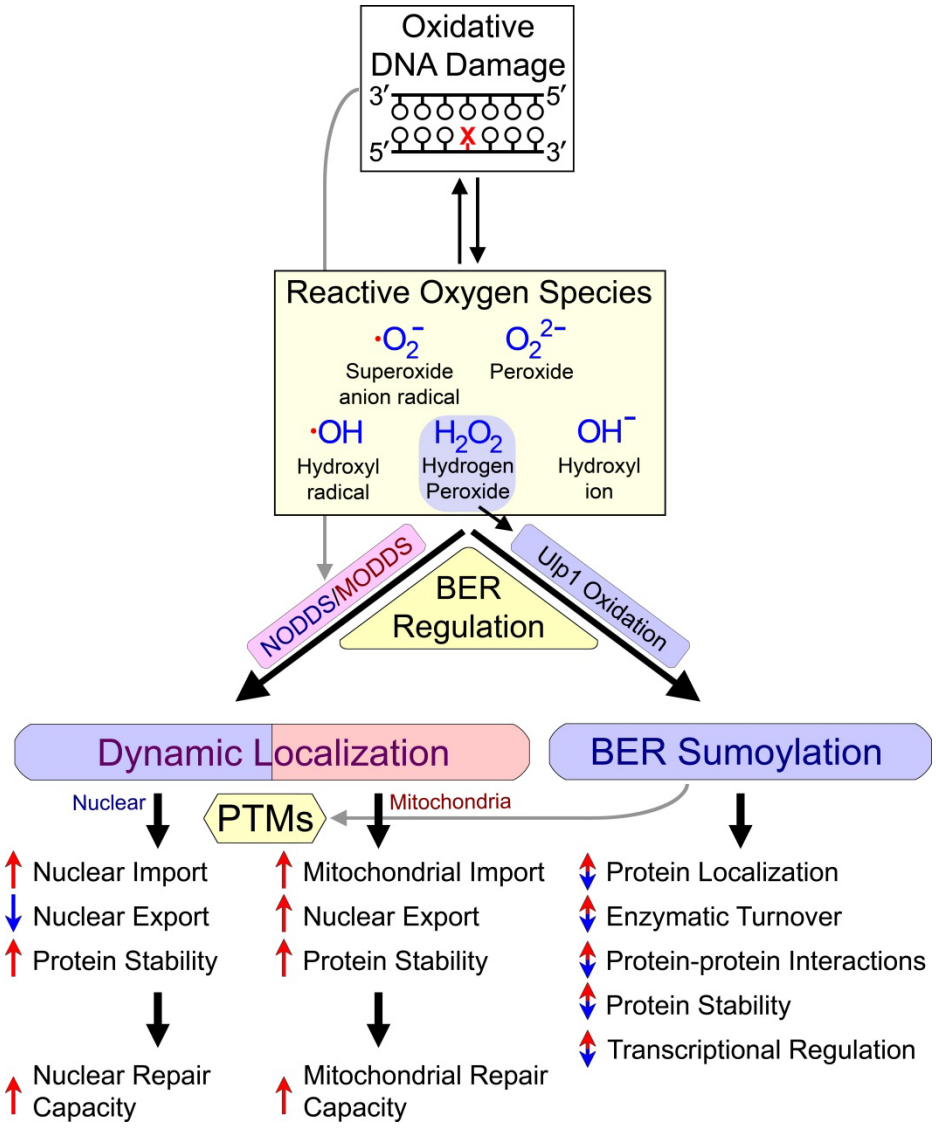


Figure 2 Legend: Model of Novel BER Regulatory Mechanisms. Oxidative DNA damage causes the production of reactive oxygen species (ROS) which in turn oxidatively damage DNA. ROS function as a signaling molecule in order to regulate BER in one of two manners. (1) By triggering the oxidation of Ulp1 leading to the sumoylation of BER proteins modulating protein localization, enzymatic turnover, protein-protein interactions, protein stability, or transcriptional activity. (2) By

generating Nuclear or Mitochondrial Oxidative DNA Damage Signals (NODDS or MODDS, respectively), driving dynamic localization of BER proteins. Dynamic localization increases nuclear or mitochondria DNA repair capacity through post-translational modifications (PTM) of BER proteins themselves, or to NODDS and MODDS responders such as importin α/β. Oxidative DNA damage may directly generate NODDS and MODDS. Sumoylation may act as PTMs to drive nuclear dynamic localization.