

# Regulation of base excision repair: Ntg1 nuclear and mitochondrial dynamic localization in response to genotoxic stress

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## 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 nonexistent. The *Saccharomyces cerevisiae* BER protein, Ntg1, relocates 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.**

## 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 *Saccharomyces cerevisiae* BER protein, Ntg1. Ntg1 is a bifunctional DNA glycosylase with associated apurinic/aprimidinic (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

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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  $\alpha$ , which binds the cargo protein in the cytoplasm and imports the cargo into the nucleus through nuclear pores in complex with importin  $\beta$  (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.

## MATERIALS AND METHODS

### Strains, plasmids and media

All haploid *S. cerevisiae* strains and plasmids used in this study are listed in Supplementary Table S1. 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 green fluorescent protein (GFP) expression vector (2  $\mu$ m, *URA3*), pPS904 (32) was employed for generation

of the C-terminally tagged Ntg1-GFP fusion protein. The *S. cerevisiae* haploid deletion mutant  $\Delta$ 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 (Supplementary Table S1).

A vector expressing tandem GFP (pAC1069) was employed for generation of C-terminally tagged NLS1<sub>NTG1</sub>-GFP<sub>2</sub>, NLS2<sub>NTG1</sub>-GFP<sub>2</sub>, NLS1/2<sub>NTG1</sub>-GFP<sub>2</sub> and MTS<sub>NTG1</sub>-GFP<sub>2</sub> fusion proteins (34), creating the plasmids pD0386 – pD0389 (Supplementary Table S1). The plasmids were introduced into  $\Delta$ NTG1 cells.

To express recombinant Ntg1, the *NTG1* open reading frame was cloned into pET-15b (Invitrogen) to generate C-terminal His<sub>6</sub> epitope tagged Ntg1-His<sub>6</sub> (pD0390), (Supplementary Table S1). Site-directed mutagenesis of Ntg1-His<sub>6</sub> was performed to create Ntg1<sub>nls1</sub>-His<sub>6</sub> (pD0391), Ntg1<sub>nls2</sub>-His<sub>6</sub> (pD0392), Ntg1<sub>mts</sub>-His<sub>6</sub> (pD0393) and Ntg1<sub>catalytic</sub>-His<sub>6</sub> (pD0394) (Supplementary Table S1). Expression vectors were transformed into DE3 cells.

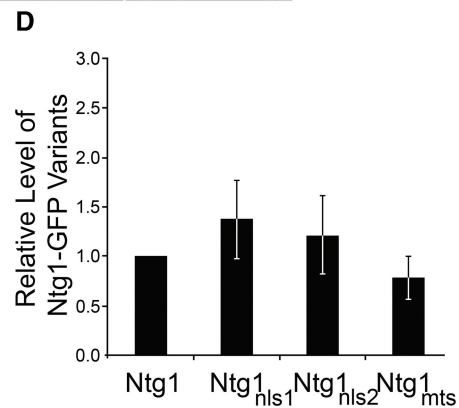
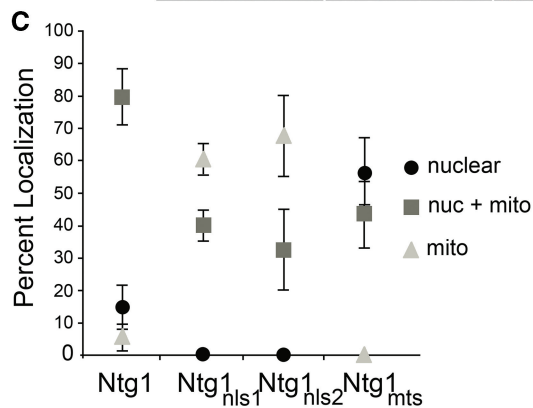
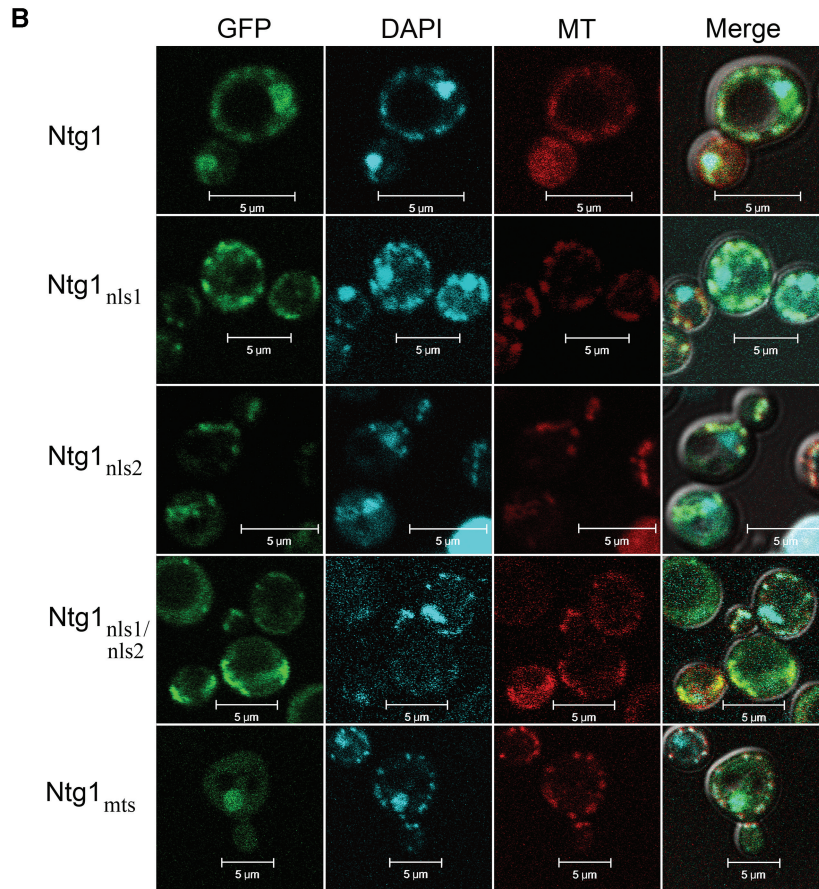
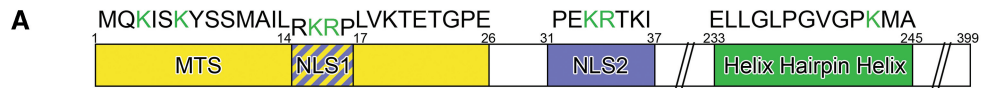
*Saccharomyces cerevisiae* haploid wild-type (DSC0367) and BER<sup>-</sup>/NER<sup>-</sup> (DSC369) cells were utilized to examine H<sub>2</sub>O<sub>2</sub> and MMS sensitivity studies and H<sub>2</sub>O<sub>2</sub> mutation frequency studies. Site-directed mutagenesis at the *NTG1* locus of the wild-type (DSC0367) parent was performed via *delitto perfetto* protocol (35) in order to generate Ntg1<sub>nls1</sub>, Ntg1<sub>nls2</sub>, Ntg1<sub>mts</sub> and Ntg1<sub>catalytic</sub> encoded at the endogenous *NTG1* locus. The resulting mutants were then crossed with the BER<sup>-</sup>/NER<sup>-</sup> mutant (LAR023), creating diploids which were then dissected to identify cells with the each Ntg1 variant BER<sup>\*</sup>/NER<sup>-</sup> strain, which were selected for functional studies of Ntg1 (Supplementary Table S1).

### Exposure to DNA damaging agents

Cells were grown in 5 mL YPD or SD -URA media to  $5 \times 10^7$  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<sub>2</sub>O<sub>2</sub> (Sigma); 1–55 mM MMS (Sigma); or 10  $\mu$ g/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.

### 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  $\mu$ g/ml DAPI for 5 min to visualize DNA. Cells were then analyzed by direct fluorescence confocal microscopy, employing a Zeiss LSM510 META



**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 (Supplementary Table S1) was assessed via direct fluorescence microscopy. GFP (green), DAPI (blue), Mitotracker (red) and merged images of cells expressing wild-type Ntg1 (WT), Ntg1<sub>nls1</sub>, Ntg1<sub>nls2</sub>, Ntg1<sub>nls1/2</sub> and Ntg1<sub>mts</sub> 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.

**Table 1.** Ntg1 localization motifs

Ntg1 localization sequence name	Residue number	Wild-type amino acid sequence	Mutant amino acid sequence <sup>a</sup>	Mutant name
cNLS1	14–17	RKRP	<b>RAAP</b>	Ntg1 <sub>nls1</sub>
cNLS2	31–37	PEKRTKI	<b>PEAATKI</b>	Ntg1 <sub>nls2</sub>
MTS	1–26	MQKISKYSSMAILRKR PLVKTTETGPE	<b>MQEISEYSSMAIL</b> <b>RKRPLVKTTETGPE</b>	Ntg1 <sub>mts</sub>
Catalytic	233–245	ELLGLPGVGPQMA	<b>ELLGLPGVGPQMA</b>	Ntg1 <sub>cat</sub>

<sup>a</sup>Mutant amino acids substitutions shown in bold type.

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.

### 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: (1:5000 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.

### 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<sub>6</sub> plasmids were grown to an OD<sub>600</sub> of 0.5–1.0 and expression induced for 4 h at 25°C. Cells were lysed via sonication and the supernatant was applied to Ni + affinity chromatography (Qiagen) to purify the Ntg1-His<sub>6</sub> variant, which was further purified to apparent homogeneity by Mono S FPLC (Pharmacia).

### In vitro binding assay

To assess the interaction between Ntg1 and importin- $\alpha$ , we employed a truncated form of importin- $\alpha$  (pAC1338), which lacks the auto-inhibitory N-terminal importin- $\beta$  binding domain ( $\Delta$ IBB-importin- $\alpha$ ), GST- $\Delta$ IBB-importin- $\alpha$  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- $\Delta$ IBB-importin- $\alpha$  was further purified to apparent homogeneity on a Superdex S-200 gel-filtration column as previously described (41). For binding assays, 4  $\mu$ g of purified Ntg1-His<sub>6</sub> or 4  $\mu$ g of Nab2-His<sub>6</sub> (pAC785) were incubated for 2 h at 4°C with 12  $\mu$ g GST- $\Delta$ IBB-importin- $\alpha$  or 12  $\mu$ g GST alone, with glutathione Sepharose beads in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na<sub>2</sub>HPO<sub>4</sub>, and 1.47 mM KH<sub>2</sub>PO<sub>4</sub>) 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 three times in PBS for 10 min at 4°C, and the bound fraction was eluted with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) buffer (50% glycerol, 10% SDS, 10%  $\beta$ -ME, 100 mM 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.

### 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, USA). A complementary strand containing a guanine opposite the DHU position was obtained from Eurofins MWG/Operon (Huntsville, AL, USA). The DHU-31mer was 5'-end-labeled with [ $\gamma$ -<sup>32</sup>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  $\mu$ l) 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  $\mu$ l 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).

### 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<sub>2</sub>O<sub>2</sub> 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).

## RESULTS

### 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 and 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 (ELLGLPGVGPCKMA), 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 GFP fusion proteins. cNLS1 (RKRP, residues 14–17) was changed to RAAP, creating Ntg1<sub>nls1</sub>-GFP, while cNLS2 (PEKRTKI, residues 31–37) was changed to PEAATK creating Ntg1<sub>nls2</sub>-GFP. The localization of both Ntg1<sub>nls1</sub>-GFP and Ntg1<sub>nls2</sub>-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<sub>nls1/2</sub>-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.

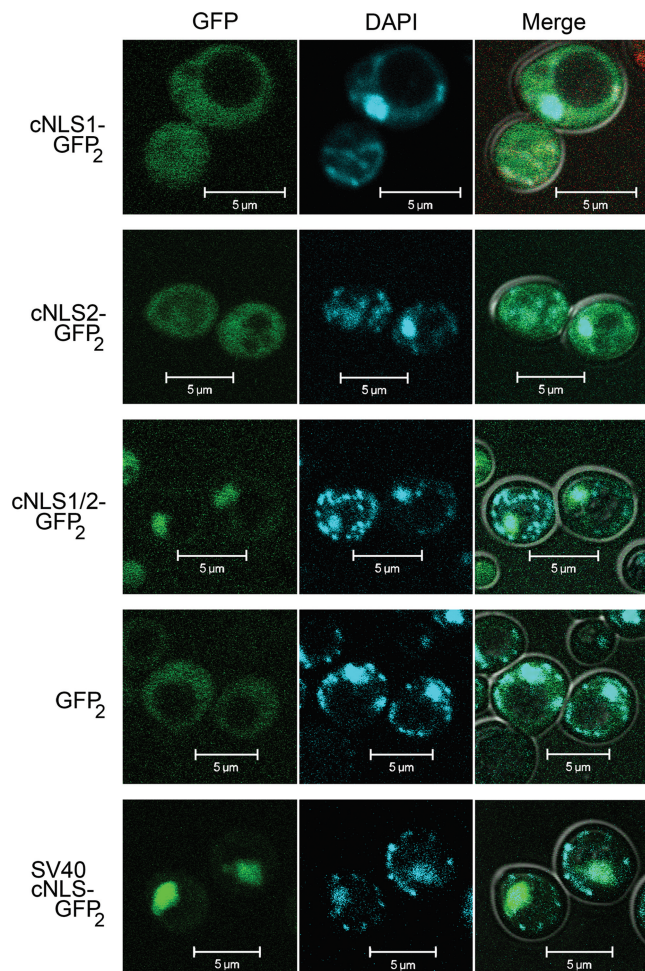
To experimentally define the MTS in Ntg1, we changed the third and sixth basic amino acids in the sequence KISK to glutamic acid (EISE) to reverse the charge and eliminate the potential to form the amphipathic  $\alpha$ -helix required for mitochondrial entry (28). Localization of the resulting Ntg1<sub>mts</sub>-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<sub>nls1</sub>-GFP, Ntg1<sub>nls2</sub>-GFP or Ntg1<sub>mts</sub>-GFP (Figure 1C). Ntg1<sub>nls1</sub>-GFP and Ntg1<sub>nls2</sub>-GFP localization to nuclei was reduced ~60% compared to wild-type Ntg1-GFP, while Ntg1<sub>mts</sub>-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<sub>2</sub>) (see 'Materials and Methods' section) 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<sub>2</sub> nor cNLS2-GFP<sub>2</sub> localized to the nucleus, while cNLS1/2-GFP<sub>2</sub> was sufficient to target GFP<sub>2</sub> 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, suggesting that Ntg1 interacts with the classical nuclear protein import machinery to access the nucleus.

### 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 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<sub>cNLS1</sub>-GFP<sub>2</sub>, Ntg1<sub>cNLS2</sub>-GFP<sub>2</sub>, Ntg1<sub>cNLS1/2</sub>-GFP<sub>2</sub> and two proteins, the negative control GFP<sub>2</sub> alone (empty vector) and the bipartite positive control SV40<sub>cNLS</sub>-GFP<sub>2</sub> (34).

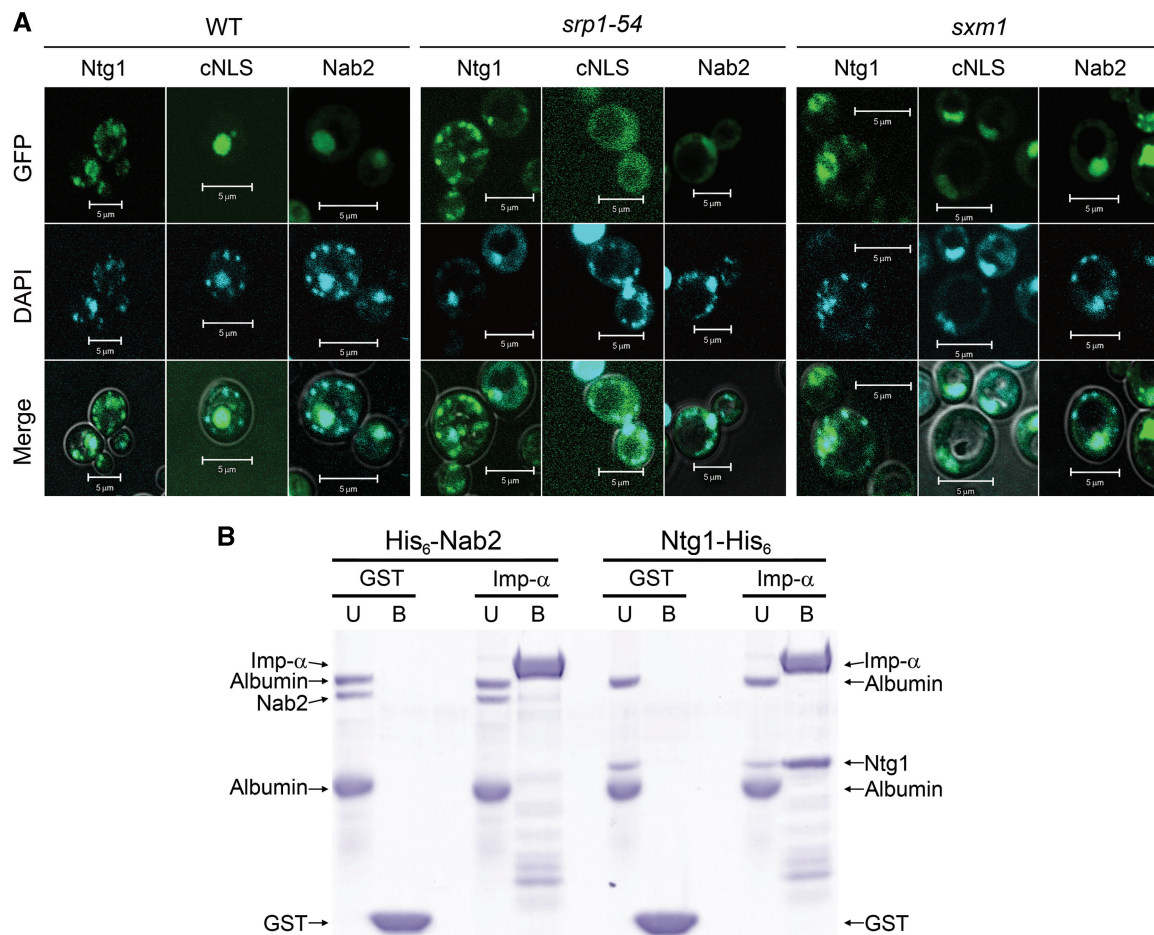
(Figures 1 and 2), we tested whether the classical nuclear protein import pathway is responsible for Ntg1 nuclear localization. The classical nuclear protein import pathway relies on the cNLS receptor subunit, importin  $\alpha$ , and the nuclear pore targeting subunit, importin  $\beta$  (25). Both importin  $\alpha$  and  $\beta$  are essential for classical nuclear localization, so we assessed the localization of Ntg1 in conditional mutants of importin  $\alpha$  (*srp1-54*) and  $\beta$  (*rsf-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 (30°C) and the nonpermissive (37°C) 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  $\alpha$  mutant cells following a shift to the nonpermissive temperature (Figure 3A), indicating that importin  $\alpha$  is required for proper nuclear localization of Ntg1. Similarly, Ntg1 and the SV40 bipartite cNLS

were mislocalized in importin  $\beta$  mutant cells at the nonpermissive temperature (Supplementary Figure S1). The control, Nab2-GFP, which is imported to the nucleus in an importin  $\alpha$ -independent manner (52), was localized to the nucleus of importin  $\alpha$  mutant cells at both the permissive and nonpermissive 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  $\Delta$ *sxm1* cells (Figure 3A), which are missing a transport receptor that facilitates nonclassical 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  $\alpha$ , we performed a direct binding assay using purified recombinant proteins. These studies employed an N-terminally truncated importin  $\alpha$ , GST- $\Delta$ IBB-importin  $\alpha$ , that lacks the auto-inhibitory importin  $\beta$  binding (IBB) domain (54). The truncated form of importin  $\alpha$  mimics the import complex that forms when importin  $\beta$  binds the IBB domain of importin  $\alpha$  to prevent competition for the cNLS binding pocket of importin  $\alpha$  (54,55) and hence binds more tightly to cNLS cargo than full-length importin  $\alpha$ . As controls, we employed two proteins, a GST control, which does not interact with Ntg1, and His<sub>6</sub>-Nab2 (38,39). The results of the *in vitro* binding assay (Figure 3B) reveal that Ntg1 interacts directly with GST- $\Delta$ IBB-importin  $\alpha$ , but not with GST alone. The control Nab2 did not bind either GST alone or GST- $\Delta$ IBB-importin  $\alpha$ , demonstrating that the interaction between Ntg1 and importin  $\alpha$  is specific (Figure 3B).

### 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<sub>2</sub>O<sub>2</sub>) to directly increase nuclear oxidative stress, H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub>) or mitochondrial (H<sub>2</sub>O<sub>2</sub> plus antimycin) oxidative stress, respectively (15), the localization of neither Ntg1<sub>nls2</sub>-GFP nor Ntg1<sub>mts</sub>-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.



**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  $\alpha$  (*srp1-54*), and control *sxm1* mutant cells are shown at the nonpermissive temperature, 37°C. See also Supplementary Figure S1. (B) An *in vitro* binding assay reveals direct binding of Ntg1 to the cNLS receptor, importin- $\alpha$ . Either GST- $\Delta$ IBB-importin- $\alpha$  (Imp- $\alpha$ ) or GST alone (GST) as a control was incubated with Ntg1-His<sub>6</sub> or a His-tagged control protein Nab2-His<sub>6</sub> as described in 'Materials and Methods' section. 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 nonspecific protein competitor, is present in the unbound fractions.

### 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<sup>-</sup>) and nucleotide excision repair (NER) through disruption of the single gene *rad1* (NER<sup>-</sup>) yielding *apn1 ntg2 ntg1 rad1* (BER<sup>-</sup>/NER<sup>-</sup>) cells (43). BER<sup>-</sup>/NER<sup>-</sup> 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<sup>\*</sup>) *rad1* (NER<sup>-</sup>) cells to create BER<sup>\*</sup>/NER<sup>-</sup> 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<sup>-</sup>/NER<sup>-</sup> and all Ntg1 BER<sup>\*</sup>/NER<sup>-</sup> variants.

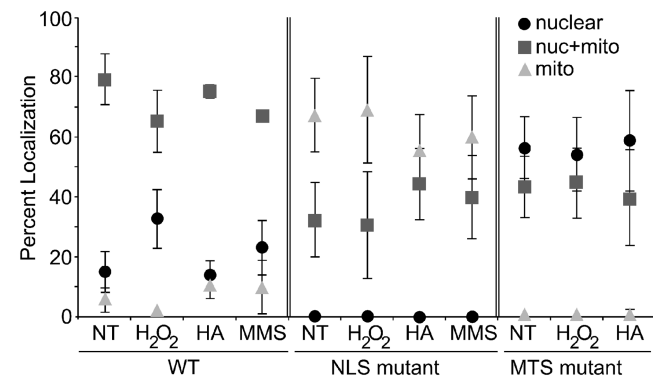
BER<sup>-</sup>/NER<sup>-</sup> and BER<sup>\*</sup><sub>cat</sub>/NER<sup>-</sup> (*apn1 ntg2 ntg1<sub>cat</sub> rad1*) cells displayed higher nuclear mutation rates compared to both wild-type and BER<sup>\*</sup><sub>wt</sub>/NER<sup>-</sup> (*apn1 ntg2 NTG1 rad1*) cells (Table 2), indicating that 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<sup>\*</sup><sub>nls1</sub>/NER<sup>-</sup> (*apn1 ntg2 ntg1<sub>nls1</sub> rad1*), BER<sup>\*</sup><sub>nls2</sub>/NER<sup>-</sup> (*apn1 ntg2 ntg1<sub>nls2</sub> rad1*) and BER<sup>\*</sup><sub>mts</sub>/NER<sup>-</sup> (*apn1 ntg2 ntg1<sub>mts</sub> rad1*) cells were analyzed. Nuclear mutation rates of both BER<sup>\*</sup><sub>nls1</sub>/NER<sup>-</sup> and BER<sup>\*</sup><sub>nls2</sub>/NER<sup>-</sup> cells were significantly higher than the mutation rates of

BER\*<sub>wt</sub>/NER<sup>-</sup> cells (Table 2), indicating that the nuclear localization of Ntg1 is important to prevent nuclear DNA mutations.

BER<sup>-</sup>/NER<sup>-</sup> and BER\*<sub>cat</sub>/NER<sup>-</sup> cells displayed higher mitochondrial mutation frequencies compared to both wild-type and BER\*<sub>wt</sub>/NER<sup>-</sup> cells (Table 2). Neither BER\*<sub>nls1</sub>/NER<sup>-</sup> nor BER\*<sub>nls2</sub>/NER<sup>-</sup> had higher mitochondrial mutation frequencies than BER\*<sub>wt</sub>/NER<sup>-</sup> cells; however, BER\*<sub>mts</sub>/NER<sup>-</sup> cells had significantly elevated mitochondrial mutation frequencies compared to both wild-type and BER\*<sub>wt</sub>/NER<sup>-</sup> 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<sub>2</sub>O<sub>2</sub> or MMS. The survival of BER\*<sub>wt</sub>/NER<sup>-</sup> mutants exposed to either H<sub>2</sub>O<sub>2</sub> or MMS was greater than that of BER<sup>-</sup>/NER<sup>-</sup> cells, but less than that of WT cells (Figure 5A and B), indicating that Ntg1 partially rescues sensitivity to H<sub>2</sub>O<sub>2</sub> and MMS. The impact of dynamic localization of Ntg1 was assessed utilizing BER\*<sub>nls1</sub>/NER<sup>-</sup>, BER\*<sub>nls2</sub>/NER<sup>-</sup> and BER\*<sub>mts</sub>/NER<sup>-</sup> cells. The survival of these cells following H<sub>2</sub>O<sub>2</sub> treatment was not statistically different from BER\*<sub>wt</sub>/NER<sup>-</sup> cells (Figure 5A). However, the survival of BER\*<sub>nls1</sub>/NER<sup>-</sup> and BER\*<sub>nls2</sub>/NER<sup>-</sup> cells treated with MMS was significantly reduced compared to BER\*<sub>wt</sub>/NER<sup>-</sup> cells, while the survival of BER\*<sub>mts</sub>/NER<sup>-</sup> cells was similar to BER\*<sub>wt</sub>/NER<sup>-</sup> 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 relocation in the elimination of mutagenic and cytotoxic base damages, respectively.



**Figure 4.** Functional intracellular targeting signals are required for dynamic localization of Ntg1 in response to oxidative DNA damage. Quantification of Ntg1-GFP, Ntg1<sub>nls2</sub>-GFP and Ntg1<sub>mts</sub>-GFP localization following cellular stress. Cells were not treated (NT) or were exposed to 20mM H<sub>2</sub>O<sub>2</sub>, 55mM MMS, or 20mM H<sub>2</sub>O<sub>2</sub> 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.

#### 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<sub>6</sub> (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<sub>nls1</sub>-His<sub>6</sub>, Ntg1<sub>nls2</sub>-His<sub>6</sub> and Ntg1<sub>mts</sub>-His<sub>6</sub> all exhibited robust enzymatic activity that is comparable to wild-type Ntg1-His<sub>6</sub> (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.

**Table 2.** Nuclear and mitochondrial mutations rates in cells with different DNA excision repair capacities

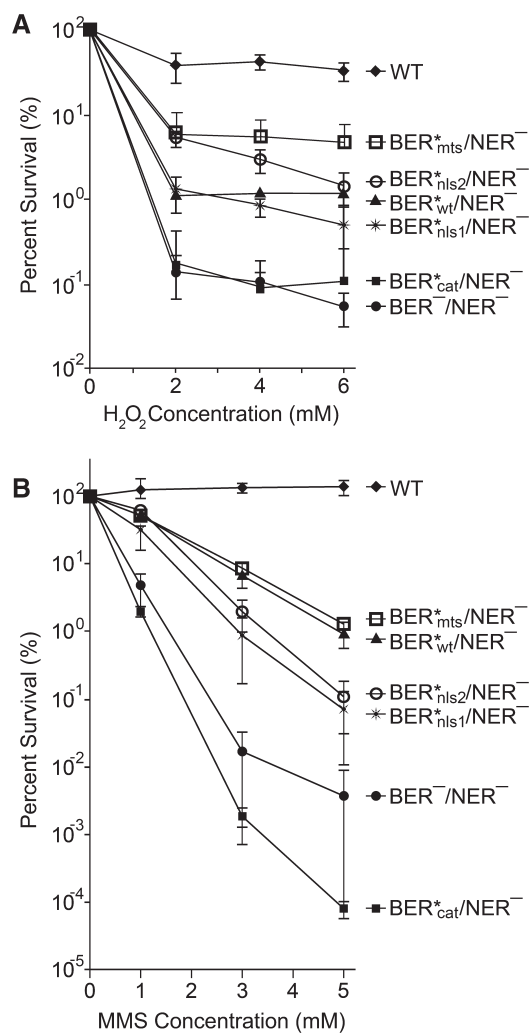
DNA repair background	Median nuclear mutation rate <sup>a</sup> ( $\mu$ ) ( $10^{-7}$ ) (95% confidence limits <sup>c</sup> )	Fold change	Median mitochondrial mutation frequency <sup>b</sup> ( $f$ ) ( $10^{-8}$ ) (95% confidence limits <sup>c</sup> )	Fold change
WT	6 (1–43)	1	2.0 (1.0–4.7)	1
BER <sup>-</sup> /NER <sup>-</sup> ( <i>apn1 ntg2 ntg1 rad1</i> )	413 (212–623)	68	53.6 (28.9–117)	28
BER* <sub>wt</sub> /NER <sup>-</sup> ( <i>apn1 ntg2 NTG1 rad1</i> )	44 (29–58)	7	5.3 (3.5–9.3)	3
BER* <sub>nls1</sub> /NER <sup>-</sup> ( <i>apn1 ntg2 ntg1<sub>nls1</sub> rad1</i> )	156 (103–684)	26	11.3 (7.1–20.8)	6
BER* <sub>nls2</sub> /NER <sup>-</sup> ( <i>apn1 ntg2 ntg1<sub>nls2</sub> rad1</i> )	83 (60–175)	14	5.6 (4.0–14.3)	3
BER* <sub>mts</sub> /NER <sup>-</sup> ( <i>apn1 ntg2 ntg1<sub>mts</sub> rad1</i> )	37 (25–59)	6	15.0 (10.7–16.9)	8
BER* <sub>cat</sub> /NER <sup>-</sup> ( <i>apn1 ntg2 ntg1<sub>cat</sub> rad1</i> )	274 (117–1190)	45	13.1 (10.4–18.1)	7

<sup>a</sup>Nuclear mutation rates were assessed via the *CAN1* locus (43).

<sup>b</sup>Mitochondria mutation frequencies were assessed by an erythromycin resistance assay (59).

<sup>c</sup>Confidence limits were calculated as previously described (45).





**Figure 5.** Functional analysis of the dynamic localization of Ntg1. (A) The H<sub>2</sub>O<sub>2</sub> sensitivity of wild type (WT), *apn1 ntg2 ntg1 rad1* (BER<sup>-</sup>/NER<sup>-</sup>), *apn1 ntg2 NTG1 rad1* (BER\*<sub>wt</sub>/NER<sup>-</sup>) and *apn1 ntg2 ntg1<sub>mutant</sub> rad1* (BER\*<sub>mutant</sub>/NER<sup>-</sup>) cells were assessed. The percent survival was set to 100% for untreated samples and was determined for 0, 2, 4 and 6 mM H<sub>2</sub>O<sub>2</sub> doses. (B) The MMS sensitivity of wild type (WT), (BER<sup>-</sup>/NER<sup>-</sup>), (BER\*<sub>wt</sub>/NER<sup>-</sup>) and (BER\*<sub>mutant</sub>/NER<sup>-</sup>) 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.

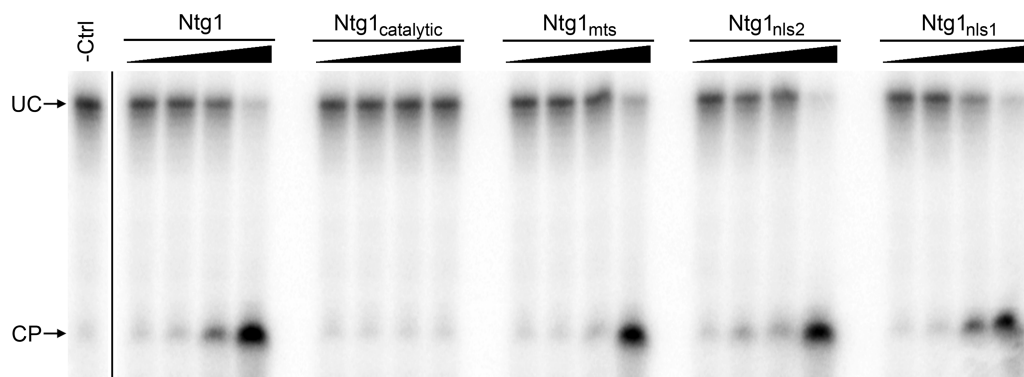
## DISCUSSION

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

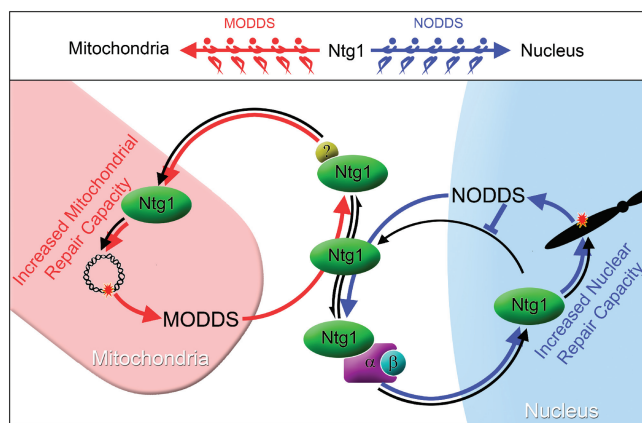
counters deleterious consequences that result from oxidative DNA damage. Taken together, these data support a new paradigm for the processes involved in the regulation of BER.

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 suggest 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 capable of not only facilitating recruitment, but also preventing localization to the opposing organelle by interfering with crucial localization signals (Figure 7). The results from this study suggest that the classical nuclear protein import proteins, importin  $\alpha/\beta$ , 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 relocates from the cytoplasm to nuclei and mitochondria following exposure to oxidative stress (H<sub>2</sub>O<sub>2</sub>) (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.



**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  $^{32}\text{P}$  5'-end-labeled oligonucleotide (31mer) containing dihydrouracil by the Ntg1 variant proteins, Ntg1, Ntg1<sub>nls1</sub>, Ntg1<sub>nls2</sub>, Ntg1<sub>mts</sub> or Ntg1<sub>catalytic</sub>. The positions of the uncleaved 31-mer oligonucleotide (UC) and the cleaved 13-mer 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/ $\mu\text{l}$ , 5.5 ng/ $\mu\text{l}$ , 16.6 ng/ $\mu\text{l}$  and 50 ng/ $\mu\text{l}$ . All lanes are from the same gel at the same exposure, the black line represents lanes that were cropped from the image.



**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  $\alpha/\beta$ , 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.

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 nonstress 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  $\text{H}_2\text{O}_2$ . 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  $\text{H}_2\text{O}_2$ -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 homolog of Ntg1 (hNth1) predominantly displays 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).

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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