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Harry R. Powers 4/18/2011

The Regulation of Base Excision Repair by Sumoylation in Saccharomyces cerevisiae

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Abstract

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Oxidative stress caused by reactive oxygen species is one of the main sources of DNA damage in the cell. Oxidative DNA damage can be cytotoxic or mutagenic and is also know to contribute to the development of cancer, neurodegenerative disorders, and aging in humans. In order to combat these deleterious effects, cells have evolved DNA repair pathways. The main repair pathway for oxidative DNA damage is the base excision repair (BER) pathway. In this project, we are investigating the Saccharomyces cerevisiae BER protein, Ntg1 which is a bifunctional N-glycosylase and AP lyase. Ntg1 is know to be modified by the small ubiquitin-like modifier (SUMO) in response to oxidative stress. We hypothesize that sumoylation is regulating the function of Ntg1 to repair nuclear DNA in response to oxidative stress. In order to determine the function of the sumoylation of Ntg1, it was first necessary to identify the sites of sumoylation of Ntg1. Putative sumoylation sites were identified using SUMO site prediction software. Site-directed mutagenesis was used to generate Ntg1 with lysine to arginine substitutions at the putative sumoylation sites. Immunbloting was then employed to determine the sumoylation status of the Ntg1 mutants. Our results indicated that Ntg1 is sumoylated at least five residues. The C-terminus is the main site of SUMO modification of Ntg1 with K396 being the principle sumoylation site followed by K376 and K388. Additional, tertiary sites of sumoylation are located at K20 and K38, both on the N-terminus. From this research, a non-sumoylatable Ntg1 has been created that can be used in functional studies to determine the manner in which sumoylation regulates the function of Ntg1.

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Background

Cellular DNA is frequently damaged at a rate of 20,000 DNA adducts per cell per day (*1, 2*). This damage is caused by a number of sources that can be either endogenously or exogenously produced such as ionizing radiation, UV light, reactive oxygen species, and alkylating agents (*3-5*). DNA damage if left unrepaired can be mutagenic or lead to arrest of transcription and replication (*6*). In human health, mutations caused by DNA damage are one of the driving forces in tumorigenesis (*7*). In order to protect their genomes, cells have evolved DNA repair pathways that such as the base excision repair (BER) pathway and the nucleotide excision Repair (NER) pathway that remove and replace damage bases (*3*). The importance of these repair pathways to human health can be seen in many hereditary cancer syndromes whose cause are defects in DNA repair, such as xeroderma pigmentosum where an inherited defect in NER leads to the developing of skin cancers at a very earlier age (*8*).

Oxidative stress caused by reactive oxygen species (ROS) is one of the main sources for DNA damage in the cell. ROS can either be produced from exogenous or endogenous sources. Exogenous sources of ROS include ionizing radiation and chemical oxidants in the environment (*9*). Endogenous sources of ROS include the inflammatory response and normal cellular metabolism that uses oxygen such as oxidative phosphorylation in the mitochondria (*9*). ROS can cause numerous types of DNA damage especially DNA base alterations such as 8-oxo-gaunine (*10*). Oxidative DNA damage has been implicated in the development of cancer, neurodegenerative disorders, and the ageing process (*9, 11*).

Cells have evolved different pathways to deal with the threat of oxidative damage. Cells attempt to prevent oxidative damage through free radical scavenging enzymes such as superoxide dismutase and catalase as well as antioxidant molecules like glutathione, which act to

neutralize ROS (*12*). If these methods fail to defend the DNA against the effects of ROS, evolutionarily conserved DNA repair pathways have evolved in order to correct the damage.

However, if the amount of DNA damage overwhelms the DNA repair capacity of the cell, then there are two options for the cellular response: translesion synthesis or apoptosis. The cell can attempt to replicate past the DNA damage lesion using special DNA polymerases in a process called translesion synthesis (TLS) (*4*). However, these DNA polymerases are extremely error-prone, and thus TLS is a mutagenic process (*4*). The second, in multicellular organism, is the cell can enter into the apoptotic pathway and commit programmed cell death (*13*).

The main DNA repair pathway that is responsible for the repair of oxidative DNA damage is the base excision repair (BER) pathway (*14*). BER is a highly conserved pathway between yeast and humans, allowing *S. cerevisiae* to be exploited as a model organism to study human BER repair (*15, 16*). Deficiencies in BER are known to contribute to genomic instability and cancer (*2, 17*). The BER pathway begins with the recognition and removal of the damaged base by an *N*-glycosylase, resulting in the formation of an apurinic/ apyrimidic (AP) site (*18*). The DNA backbone is then cleaved either on the 5' side of the AP site by an AP endonuclease or to the 3' side by an AP lyase (*18*). Then after processing of the blocking group which is either a 5'- deoxyribosephosphate (5'-dRP) or 3'-dRP, the excised bases are replaced by DNA polymerase, and DNA ligase acts to rejoin the strand (*18*). While the enzymology of BER is understood in some detail, the manner by which BER is regulated in the face of numerous types of DNA damage occurring in different cellular locations is largely unknown. However, recent work with the yeast BER protein Ntg1 has provided some insight into the regulation of BER in response to oxidative stress (*19*).

Ntg1 is an evolutionarily conserved BER protein that has both N-glycosylase and AP lyase activities (*14, 20, 21*). Ntg1is highly conserved with the *E. coli* homologue endonuclease III and the human homologue hNth1 (Figure 1). As an N-glycosylase, Ntg1 recognizes a wide range of chemically altered DNA bases including oxidative DNA damage (*14*). Ntg1 contains both a mitochondrial targeting sequence and a bipartite nuclear localization sequence and repairs DNA in both the mitochondria and the nucleus (*14, 21*). Ntg1 has been found to dynamically localize to either the mitochondria or the nucleus in response to oxidative stress in the appropriate organelle (*19*). This dynamic localization is hypothesized to be a major regulatory mechanism of Ntg1 upon oxidative insult (*19, 22*). The potential importance of dynamic localization can be seen in the human homologue hNth1, whose subcellular localization is misregulated in a subset of gastric cancers (*17*). Also upon oxidative insult, Ntg1 is posttranslationally modified by the small ubiquitin-like modifier (SUMO) which suggests that SUMO may also regulate Ntg1 function (*19*).

SUMO is a small protein with a tertiary structure that resembles ubiquitin and serves as a posttranslational modification (*23*). The first SUMO protein was discovered in 1995 in *S. cerevisiae* and named Smt3 (*24, 25*). Since then sumoylation has been shown to be a highly conserved process that is also present in mammals including humans (*26*). Sumoylation occurs via a highly conserved three enzyme pathway that is very similar to the ubiquitin pathway (*27*). First, the immature SUMO protein is cleaved by Ulp1 sumo protease to reveal the C-terminal glycine glycine motif. Then, the SUMO protein is conjugated to the heterodimer Uba2/ Aos1, the E1 Sumo activating protein through a thioester bond (*25*). The SUMO protein is then transferred over to Ubc 9, the E2 Sumo conjugating protein, again though a thioester bond (*25*). The E2 protein together with the E3 SUMO ligase attaches the SUMO protein to the substrate through

the epsilon amine on lysine (*25*). Unlike ubiquitin where there are numerous E3 ligases that create substrate specificity, there are only a limited number of E3 SUMO ligases that have been identified (*27-29*). Siz1, Siz2, Mms21, and Zip3 are the E3 ligases that have been discovered in *S. cerevisiae* (*27*). Also, unlike ubiquitin, it has been shown that the E2 protein can attach SUMO to the substrate in the absence of the E3 ligase which adds another level of complexity to the sumoylation process (*27, 29*). Sumoylation can be reversed through the action of the SUMO proteases Ulp1 or Ulp2 (*27*). Ulp1 is associated with the removal of monosumoylated sites, whereas Ulp2 usually removes SUMOs that are a part of a polySUMO chain (*30*). The SUMO protein is often attached at the SUMO consensus site ΨKXE/D (where Ψ is a bulky aliphatic residue and X stands for any amino acid) (*30*).

There are a couple of different ways in which proteins can be sumoylated. They can be sumoylated at only one residue, at multiple residues, or it can be modified with a SUMO chain(*30*). The SUMO chain is formed through the attachment of the SUMO protein to a lysine on another SUMO protein. The yeast SUMO protein, Smt3, has three lysines with a SUMO consensus sequence at residues K11, K15, and K19 (*30*). All three of these sites are used for the construction of SUMO chains in *S. cerevisiae* (*30, 31*).

Sumoylation regulates a diverse range of protein functions, including DNA repair, nuclear localization, and protein stability (*25, 27*). Sumoylation is believed to regulate protein function through modifying the protein's interactions with its binding partners (*23*). There are numerous DNA repair proteins that are regulated by sumoylation. For example, in homologous recombination, Rad52 sumoylation is induced by the MRX complex in response to double stand DNA breaks, and increases the stability of Rad52 by competing for ubiquitin modification and preventing its proteasomal degradation (*25, 32, 33*). In BER, sumoylation regulates the function

of human Thymine DNA glycosylase (TDG) by causing a conformational change in TDG that facilitates its release from the DNA after processing of the aberrant thymine (*25, 34-36*).

As previously mentioned, sumoylation of Ntg1 is induced by oxidative stress (*19*). Interestingly, sumoylated Ntg1 is found only in the nucleus (*19*). This compartmentalization seems to indicate a role for sumoylation in the response of Ntg1 to nuclear oxidative stress (*19*). Sumoylation in response to oxidative stress is not novel to Ntg1, and appears to be common method of regulating protein function in response to oxidative stress (*25, 37-40*). One cause of the increase in sumoylation in response to oxidative stress seems to be the oxidation of cysteine residues on the SUMO proteases, including Ulp1, which leads to their dimerization and inactivation (*41*). The exact mechanism by which oxidative stress leads to Ntg1 sumoylation is currently unknown, but it is hypothesized to be a part of a nuclear oxidative DNA damage signals (NODDS) that act to recruit BER proteins to the nucleus in response to oxidative DNA damage (*19*).

Adding support to this hypothesis, it has been shown that the disruption of the predicted SUMO site at residue K364 in Ntg1 results in a reduction in the dynamic localization of Ntg1 to the nucleus in response to oxidative stress, as well as decrease cell survival in H_2O_2 (*19*). However, Ntg1 with the lysine to arginine amino acid substitution at residue 364 is still sumoylated, which indicates that there are other sites of sumoylation on Ntg1 that could play a role in the functional regulation of Ntg1.

Our hypothesis is that sumoylation regulates the response of Ntg1 to oxidative DNA damage. First, in order to determine the biological effect of sumoylation, a non-sumoylatable form of Ntg1 is required. The best way to achieve this goal is to identify the sites of sumoylation

on Ntg1so that they can be eliminated. There are five sumoylation consensus sites on Ntg1 as well as other lysines that could serve as possible sumoylation sites. In order to identify the sites of sumoylation, Ntg1 mutants that have lysine to arginine amino acid substitution at putative sumoylation sites have been created using site-directed mutagenesis. These mutants have then been evaluated for their sumoylation status. In an effort to determine the functional importance of Ntg1 sumoylation, we have carried out preliminary experiments on the effect of the Ntg1 mutants on nuclear mutation rates and cytotoxicity to hydrogen peroxide. In this study, we have shown that Ntg1 is sumoylated at its five sumoylation consensus sites, and have made an Ntg1 mutant that is non-sumoylatable in order to investigate the effect of sumoylation on the function of Ntg1.

Materials and Methods

Strains, and Media

Three haploid strains of *Saccharomyces cerevisiae* were used in the project. For a list of strains and their genotypes see Table 1. All yeast strains were grown on synthetic dropout (SD) media, which is limited media containing glucose, yeast nitrogen base, ammonium sulfate, and supplemented with either a full drop out mix for SD complete , – uracil drop out mix for SD – URA, or – arginine with 60 mg/ml L-canavanine for SD CAN, and agar for plates. YPD, which is rich media contained glucose, peptone, yeast extract, and agar for plates. All media was supplemented with .0005% adenine sulfate.

NTG1 Expression Plasmid

All the experiments in this project used a plasmid based NTG1 expression system. The NTG1 plasmid was constructed from pRS316 (Sikorski and Hieter, 1989). On the plasmid,

NTG1 had a tetracycline-off overexpression promoter and a C-terminus tandem affinity purification (TAP) tag. The plasmid possesses a centromeric sequence and both an amphicillin and uracil selectable marker.

Site-directed Mutagenesis

Site-directed mutagenesis was used to create lysine to arginine point mutations at putative sumoylation sites on Ntg1. Lysine to arginine point mutations were used because the SUMO protein cannot be conjugated to arginine, but since arginine and lysine are both basic amino acid, there should be a minimal effect on the tertiary structure of Ntg1. Site-directed mutagenesis was performed using the Quikchange 2XL site-directed mutagenesis kit (Stratagene). The Ntg1 mutant plasmids were transformed into Δ*ntg1*, BER/NER deficient, and Ulp1*ts*1 backgrounds.

Cell Growth and Treatment

Frozen stock strains were plated on SD plates and grown at 30^{o}C for around 48 hours. The yeast strains were then inoculated in 5 ml of SD media and grown up overnight at 30^{o}C. Then, the yeast strains were inoculated in fresh SD media and grown overnight to saturation (5 x 10^7 cells/ ml) at 30^{o}C. The cells were washed in 10 ml of H_2O and then resuspended in 25 ml of H_2O. The cells were either treated with 20 mM H_2O_2 and incubated at 30^{o} C for 1 hour or incubated at 37^{o} C for 1 hour.

Whole Cell Extracts

After treatment with H_2O_2, the cells were washed in 10 ml of H_2O and then resuspended in .5 ml of PBS-T protease inhibitor cocktail (Complete Mini, Roche). The cells incubated at 37^{o}

C were spun down and resuspended directly into .5 ml of the PBS-T protease inhibitor cocktail. The cells were then lysed following the glass beads protocol.

Immunoblotting

Immunobloting was performed to assess the steady-state levels of Ntg1 and sumoylated Ntg1. Immunobloting was performed according to standard protocols. The primary antibodies used were Rabbit anti-TAP (1:3333 dilution) (Open Biosystems) to detect Ntg1 and Mouse anti-PGK (1:5000 dilution) (Invitrogen) to detect PGK, a loading control. The secondary antibodies used for developing with the typhoon imager were, Cy3 anti-Rabbit (1:5000 dilution) (GE life sciences) and Cy 5 anti-Mouse (1:5000) (GE life sciences) were used. The Typhoon images were quantified using Imagequant TL.

Mutation Rates and Cytotoxicity Assay

In order to assess the biological effect of the sumoylation site mutation on the function of Ntg1, a mutation rates assay and cytotoxicity assay were performed on the Ntg1 mutants in a DNA repair deficient strain.

In order to measure the nuclear mutation rates, an L-canavanine resistance assay was performed (*42*). The cells grown in liquid media for two days were plated on SD CAN plates in order to identify mutations in the *CAN1* locus and on YPD plates. The colonies were counted and compared to determine nuclear mutation rates. The YPD plates were replica plated onto SD – URA plates in order to determine the rate of plasmid retention.

A cytotoxicity assay was performed to determine the sensitivity of the Ntg1 mutants to hydrogen peroxide(*19*). After growing up for two days, the cells were treated with 4 mM H_2O_2

for 1 hour and then plated on SD media. The resulting colonies were then counted and compared to the non treated cells in order to determine the percent survival.

Results

Multiple putative SUMO Modification sites on Ntg1:

In order to identify putative sites of SUMO modification on Ntg1, five different web-based SUMO site prediction programs, SUMOSp 1.0, SUMOSp 2.0, PCL SUMO, SUMO Plot, and SUMOPre were employed. Each of these programs gave each lysine a score based on its potential for SUMO modification. The scores for each lysine were averaged together to generate a list of the top ten most likely sumoylation sites present on Ntg1 (Table 2). The top five ranked sites all contain the ΨKXE/D (where Ψ is a bulky aliphatic residue and X stands for any amino acid) SUMO site consensus sequence. The top two ranked sites are both located on the N-terminus near the bipartite NLS (Figure 2). Five of the top ten are all clustered in the C-terminus (Figure 2). The top ten sites were used as the target for site-directed mutagenesis in this project.

Ntg1 is SUMO modified at at least 5 sites

Immunoblotting was used as a screening tool to assess the SUMO modification status of the Ntg1 SUMO site mutants. First, Ntg1 mutants for each of the ten putative sumoylation sites were created through site-directed mutagenesis (Table 3). These mutants were transformed into a Δ*ntg1* cells. These mutants were exposed to H_2O_2 to induce sumoylation and then immunoblotting was performed. Each of the ten Ntg1 single amino acid substitution mutants is SUMO modified (Figure 3). This indicates that Ntg1 is most likely sumoylated at multiple residues.

Due to our finding that Ntg1 is sumoylated on multiple lysine residues, Ntg1 mutants harboring multiple sumoylation site mutations were created. The combination of sumoylation sites to mutate was based on the SUMO site priority list and according to the proximity of the SUMO sites to one another on the primary sequence of Ntg1. To determine whether any one SUMO site mutation was impacting the level of Ntg1 sumoylation, the Typhoon imager was employed to provide quick and accurate quantification of all Ntg1 bands. However, the level of SUMO modification induced by 20 mM H_2O_2 was difficult to detect with the Typhoon imager (Figure 4). To enhance the steady-state level of sumoylation of Ntg1 and facilitate quantification of the SUMO modification of Ntg1, the Ntg1 mutants were transformed into an Ulp1*ts* mutant background which has been shown to increase the steady state level of sumoylated Ntg1 at the non-permissive temperature (D. Swartzlander, unpublished results). The Ulp1, a sumo protease, removes the SUMO protein from the substrate. Following incubating at the non-permissive temperature, the conditional Ulp1 mutant can no longer remove SUMO from Ntg1.

Results of this approach revealed that Ntg1 strains expressing all of the single SUMO site mutations, as well as K20,38R, K376-396R, and K359-396R can be sumoylated (Figure 5). Interestingly, the K20,38R mutant appears to be missing the second higher molecular weight band. Quantification of the scanned images provides insight into the effects of lysine to arginine substitution on sumoylation (Figure 6). In Ulp1*ts* mutants incubated at the permissive temperature, 3.7% of Ntg1 is sumoylated. Following shift to the non-permissive temperature of 37^{o}C, 7.7% of Ntg1 was sumoylated. Of all the Ntg1 mutants analyzed, K396R shows the lowest level of sumoylation at 3.6% (Figure 6). The K20,38R double mutant does not show a marked decrease in total sumoylation, but the second higher molecular weight band is not detectable by quantification either. This suggests that bisumoylated Ntg1 is not present when these two sites

are eliminated. The triple C-terminus mutant, K376-396R, show a marked decrease in sumoylation with only 1.3% of the total Ntg1 sumoylated (Figure 6). Consistent with this finding, the quintuple C-terminus mutant, K359-396R, also shows a very similar decline in Ntg1 sumoylation with only 1.3% of Ntg1 sumoylated (Figure 6).

Further combination mutants were generated to identify all SUMO modification sites. The K20,38,376R and K20,38,376,388R mutants still showed sumoylation (Figure 6). Quantification showed that both of these mutants had similar levels of sumoylated Ntg1 (5.0% for K20,38,376R and 5.1% for K20,38,376,388R) (Figure 6). These values do not markedly differ from the control plasmids or single amino acid substitutions on the previous Western blot (Figure 5). SUMO modification of the K20,38,359-396R mutant could not be detected (Figure 6 and 7) suggesting that only these seven SUMO sites can be sumoylated.

Preliminary Analysis of the functional importance of SUMO modification of Ntg1

While none the of the single sumo site mutants showed any statistical reduction in the level of Ntg1 sumoylation, it is possible that any one of those sumoylation sites influences the function of Ntg1. To assess the impact of the single sumoylation site substitutions on Ntg-mediated repair of nuclear DNA, we examined the nuclear mutation rates and cytotoxicity in H_2O_2 in a DNA repair deficient background. The background used was deficient in BER and NER through disruption of *APN*1, *NTG*2 and *NTG*1for BER and *RAD*1 for NER (*42*). The BER/ NER (Δapn1Δ ntg2Δ ntg1 Δrad1) deficient cells have DNA repair capacity that is severely compromised (*42*). Transforming the Ntg1 mutants into the BER-/NER- strain allowed us to assess the function of the Ntg1 mutants (*22*).

The mutation rate assay was performed in order to test the Ntg1-mediated repair of nuclear DNA (*42*). In the one mutation rates assay that was performed, the control with wildtype DNA repair capacity showed a mutation rate of 1.97×10^{-6} per colony forming unit whereas the BER-/NER- strain showed a much higher mutation rate of 4.91×10^{-4} per colony forming unit (Table 4). The BER-/NER- strain expressing wildtype Ntg1 had a mutation rate of 3.89×10^{-5} per colony forming unit. With the exception of K194R, all of the mutation rates of the SUMO site mutants did not show a marked difference from wildtype Ntg1. The K194R showed a marked increase in mutation rates with 1.06×10^{-4} per colony forming unit. However, the reason for the increased mutation rate is not clear since we have not yet determined whether K194R is a sumoylation site.

The cytotocixity assay was performed to assess the Ntg1-mediated repair of nuclear DNA in response to oxidative stress (*43*).The control strain with wildtype DNA repair capacity treated with 4 mM H_2O_2, showed around 40% survival, whereas the BER-/NER- deficient strain showed only 1% survival (Figure 8). The BER/NER deficient strain expressing wildtype Ntg1 had a survival of around 20%. The survival for the SUMO site mutants was extremely varied with K20R, K38R, K157R, and K359R having less survival than wildtype Ntg1 and the rest of the mutants having equal or greater survival than wildtype Ntg1 (Figure 8). In fact, K255R, K388R, and K376-396R mutations had greater survival than even the parental strain. The results are preliminary, and they need to be optimized and repeated before conclusions can be made.

Discussion

This project sought to understand the role of SUMO modification in the regulation of the BER protein Ntg1. To determine this, we first needed to identify the sites of sumoylation of Ntg1

so that they could be eliminated. We have identified 5 sites of SUMO modification on Ntg1 and have created a non-sumoylatable Ntg1 that can now be used in future studies to assess the role of SUMO modification on the function of Ntg1.

Ntg1 can be sumoylated at multiple sites

According to SUMO site prediction analysis, Ntg1 contains five SUMO site consensus sequences, and at least five other lysines that could serve as possible sumoylation sites. Our results indicate that there is no single lysine to arginine amino acid substitution that leads to a complete abrogation of sumoylation. This finding provided evidence that Ntg1 is being sumoylated at multiple residues. This result is consistent with previous work showing that proteins with multiple sumo consensus sequences have been shown to have multiple residues where sumoylation does occur (*44, 45*). Quantification revealed that Ntg1with K396R had a decrease in the amount of sumoylation which strongly suggest that K396 is a sumoylation site. The partial C-terminus mutant showed an even greater reduction in sumoylation, which identified either K376, K388, or both as additional sumoylation sites indicating that the primary location of sumoylation on Ntg1 is likely on the C-terminus. The full C-terminus mutant, K359-396R did not show any additional loss of sumoylation, which indicates that K359 and K364 are not important sites of sumoylation. This finding is interesting because it indicates that the phenotype associated with the amino acid substitution of argnine for lysine at K364 is not likely due to altered sumoylation (*19*). The C-terminus on Ntg1 is a non-conserved region that is predicted to be highly disorganized and outside of globular fold (Swartzlander, unpublished). The finding that sumoylation occurs primarily in the unstructured C-terminal makes sense because sumoylation occurs mainly in unorganized regions, outside of the globular fold (*27*).

Ntg1 having a cluster of sumoylation sites on the C-terminus is not unusual since proteins having clusters of sumoylation sites in specific regions have been reported(*46*).

Our results indicate that complete abrogation of sumoylation occurs in the K20,38, 359-396R mutants. This finding indicates that in addition to sumoylation sites on the C-terminus, K20 and K38 are also sites of sumoylation. They, however, appear to be secondary sites of sumoylation, because the K20,38R mutant does not affect sumoylation levels unless the C-terminus mutants are also present. The K20,38R double mutant though is missing the second higher molecular weight band. This indicates that when Ntg1 is bi-sumoylated, one of the sumo modifications must occur on either K20 or K38. However, this requirement does not appear to exist for any other form of sumoylated Ntg1.

Interestingly, it appears that the five sites of sumoylation of that have been identified in Ntg1 all posses the SUMO site consensus sequence. Since this is the case, these results provides further evidence that this consensus sequence plays a major role in the attaching of the SUMO protein to the substrate by the E2 and E3 proteins.

Model of Ntg1 Sumoylation

From the above research and unpublished work by graduate student Dan Swartzlander, a model for Ntg1 sumoylation has been developed (Figure 9). SUMO appears to be conjugated to Ntg1 mainly through the activity of the E2 Ubc9 and the E3 ligase Siz1 (Swartzlander, unpublished). SUMO is preferentially attached to K396 and then to either K376 or K388 on the C-terminus. If the C-terminal sites are unavailable or if forming the bi-sumoylated form, SUMO is attached to K20 or K38 on the N-terminus. Furthermore, once SUMO is attached to a lysine

residue, a polySUMO chain can be built (Swartzlander, unpublished). Desumoylation of Ntg1 then can occur primarily through the Ulp1 SUMO protease (Swartzlander, unpublished).

Relevance of sumoylation to Ntg1 function

The goal of my project was to lay the groundwork for the functional analysis of sumoylation of Ntg1. I have now identified the sites of SUMO modification of Ntg1 and created a mutant that cannot be sumoylated. Going forward, this mutant can be used for functional studies. There are at least three different ways by which sumoylation could regulate Ntg1 function. First, sumoylation could be regulating the steady-state localization of Ntg1 to the nucleus through increasing the association of Ntg1 with proteins involved in nuclear import or decreasing interactions with proteins involved in nuclear export (*19, 47*). By enhancing nuclear localization of Ntg1, sumoylation would thus increase the nuclear repair capacity of cells exposed to elevated levels of oxidative DNA damage (*19*). Sumoylation could prevent the polyubiquination and proteasomal degradation of Ntg1. The prevention of polyubiquination is a well characterized function of sumoylation since the two modification compete for same target lysines (*25, 48*). By increasing the steady-state levels of Ntg1, sumoylation could generally increase the repair capacity of the cell in response to oxidative stress with no preference for nuclear or mitochondrial repair. Finally, another possibility is that sumoylation is altering the substrate specificity of Ntg1. Sumoylation could increase the affinity of Ntg1 to repair oxidative DNA damage by modulating the substrate specificity of Ntg1 while lowing Ntg1-mediated repair of non-oxidative lesions like AP sites (*14*). The increased repair of oxidative lesions would increase the again generally increase the potential of Ntg1 to response to oxidative stress.

Ntg1 is not the only BER protein that could be regulated by sumoylation. Ntg2, another homolog of endonuclease III in *S. cerevisiae*, is also SUMO modified (*19*). In addition, the human orthologue of Ntg1, hNth1, contains SUMO consensus sites and also could be regulated by sumoylation. Furthermore, other BER proteins show potential for similar regulation (*49, 50*).

Issues with Cytotoxicity and Mutation Rates Assays

In order to assess the functional importance of the sumoylation of Ntg1, we examined both cytotoxicity and nuclear mutation rates in DNA repair deficient cells expressing Ntg1 mutants as the only copy of Ntg1. We wished to determine if the mutations lead to a change in Ntg-mediated repair of nuclear DNA. However, the results of our initial experiments were difficult to interpret for several reasons. First, the BER/NER deficient cells did not show the expected level of cell death in 4 mM H_2O_2 (*22*). Also, the survival of the Ntg1 mutants did not fall into the dynamic range between the control strain with wildtype DNA repair capacity and the BER/ NER deficient strain. This may have been due to the fact that Ntg1 was located on an overexpression plasmid. Now that we have created a non-sumoylatable Ntg1, it can be inserted into the backbone of the yeast genome under its endogenous promoter. This will allow for more straight forward functional analysis without the complications of plasmid loss or overexpression of the protein.

Overall this study represents one of the most in depth investigations of sumoylation of a protein with multiple sumoylation consensus sites. While it has been previously demonstrated that sumoylation can occur at multiple sites on a single protein (*30, 39, 45, 46, 51*), we have shown that it is possible for a protein to be sumoylated at five different sites. This shows the great flexibility of the sumoylation machinery in placing a SUMO modification on to a substrate

as well as the dynamic ability of SUMO to potentially regulate a protein's function by targeting many different residues. From an evolutionary perspective, this flexibility as well as the redundancy in sumoylation consensus sites points to the great importance that sumoylation has on the regulation of many proteins' function.

Future Directions

Previously before this study, the sites of sumoylation of Ntg1 were unknown. Now, K20, K38, K376, K388, and K396 have all been identified as sites of SUMO modification. An Ntg1 with K20R, K38R, K376R, K388R, and K396R substitutions needs to be generated to confirm that these residues are the only sites of sumoylation. Mass spectroscopy of purified Ntg1 should also be employed to independently confirm these residues are sites of sumoylation *in vivo*. Also, the sumoylation of the K20R, K38R, K376R, K388R, and K396R mutant should be assess in hydrogen peroxide to determine if the sites of sumoylation are the same when Ntg1 is sumoylated in response to oxidative stress.

Now that we have identified the sites of sumoylation of Ntg1, and created a non-sumoylatable Ntg1, we can move ahead with a major goal of this project to determine the functional importance of the sumoylation of Ntg1. The non-sumoylatable Ntg1 should be used in the mutation rates assay and the cytotoxicity assay to determine the effect of sumoylation on Ntg1-mediated repair of nuclear DNA. Also, the non-sumoylatable Ntg1 should be used with a GFP tag for assessing cellular localiztion to determine the effect of sumoylation on the dynamic localization of Ntg1 to the nucleus.

Figures

Figure 1

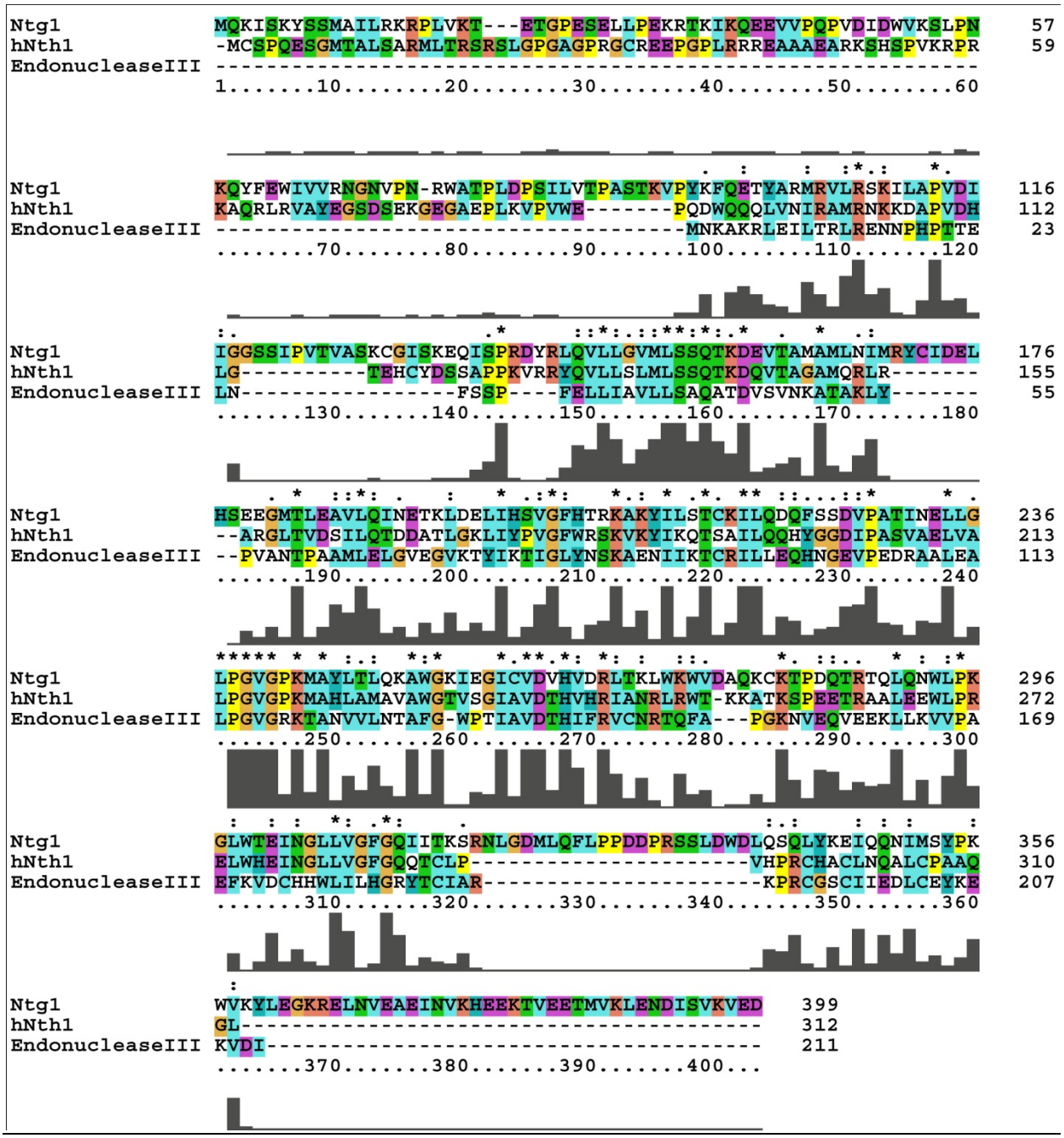


Figure 2

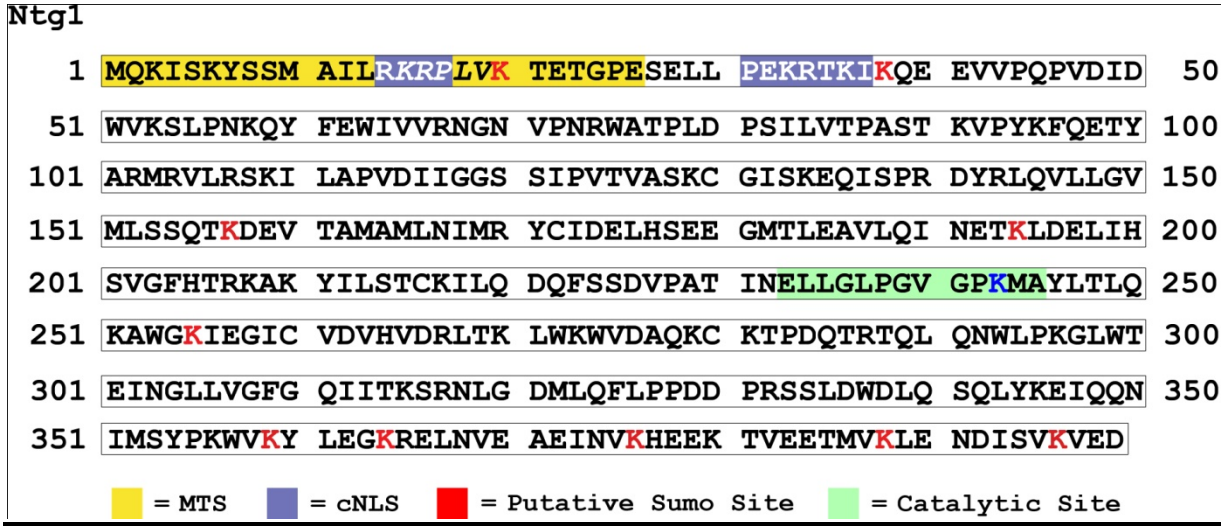

Ntg1
1 MQKISKYSSM AILRKRPLVK TETGPESELL PEKRTKIKQE EVVPQPVDID 50
51 WVKSLPNKQY FEWIVVRNGN VPNRWATPLD PSILVTPAST KVPYKFQETY 100
101 ARMRVLRSKI LAPVDIIGGS SIPVTVASKC GISKEQISPR DYRLQVLLGV 150
151 MLSSQTKDEV TAMAMLNIMR YCIDELHSEE GMTLEAVLQI NETKLDELIH 200
201 SVGFHTRKAK YILSTCKILQ DQFSSDVPAT INELLGLPGV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

Figure 3

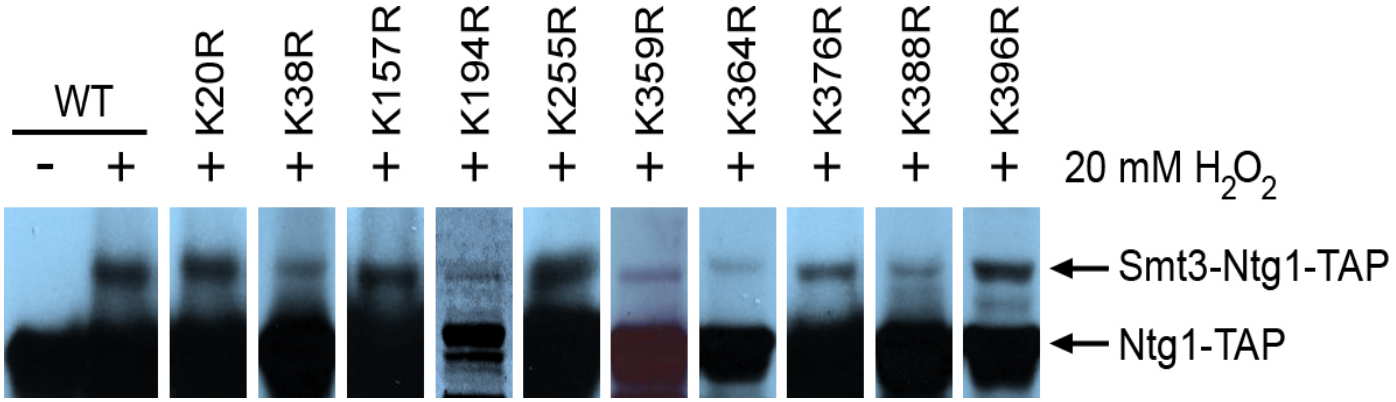

WT
- +
+ K20R
+ K38R
+ K157R
+ K194R
+ K255R
+ K359R
+ K364R
+ K376R
+ K388R
+ K396R
20 mM H_2O_2
Smt3-Ntg1-TAP
Ntg1-TAP

Figure 4

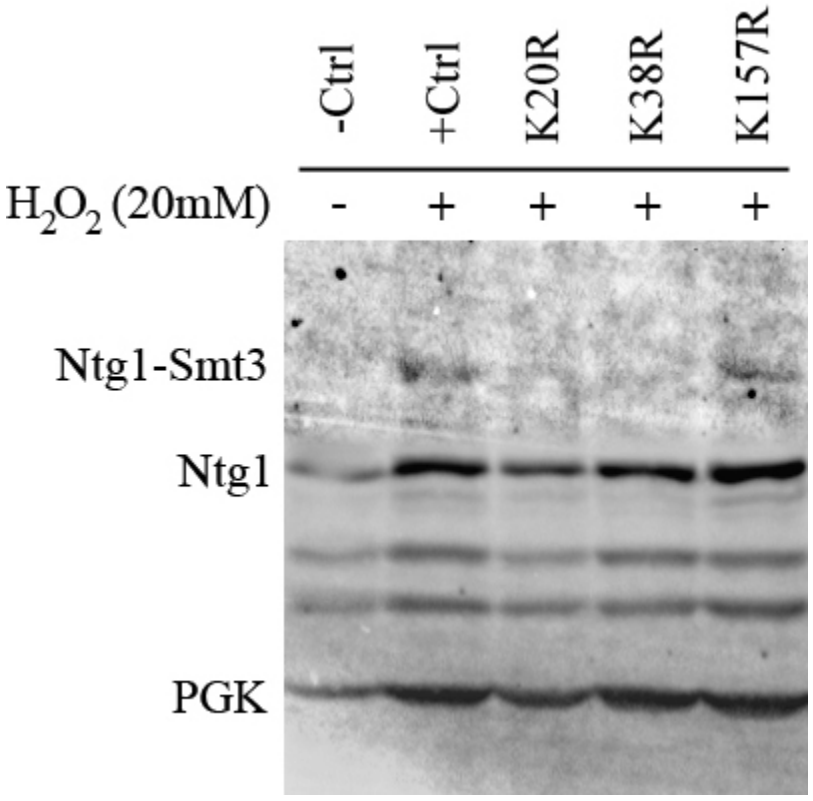


Figure 5

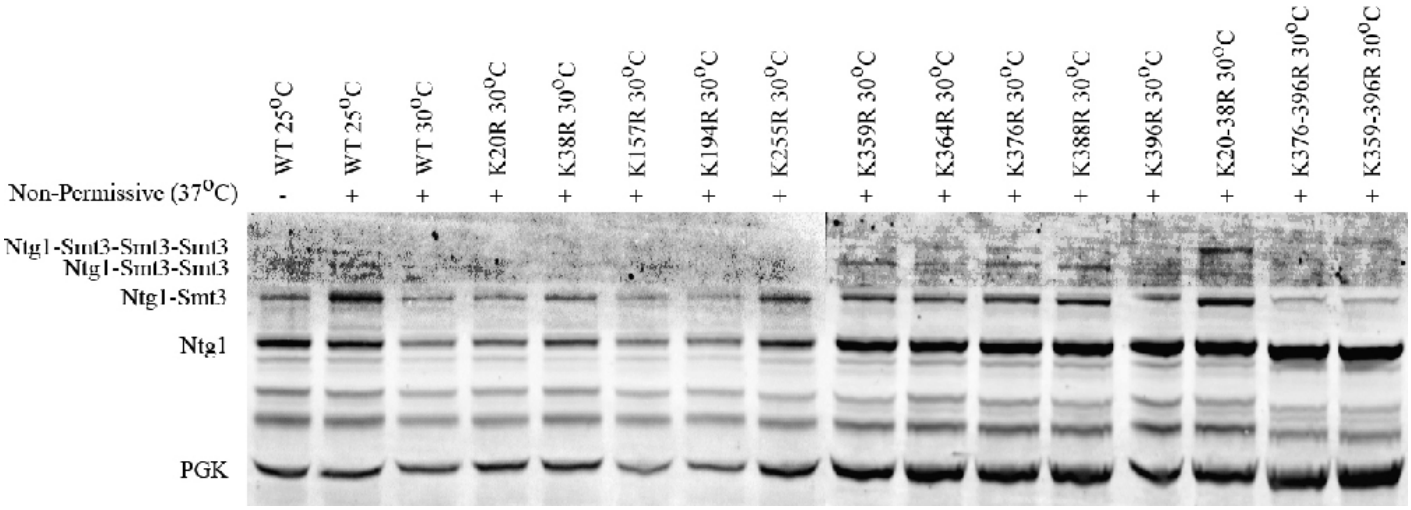

WT 25°C
WT 25°C
WT 30°C
K20R 30°C
K38R 30°C
K157R 30°C
K194R 30°C
K255R 30°C
K359R 30°C
K364R 30°C
K376R 30°C
K388R 30°C
K396R 30°C
K20-38R 30°C
K376-396R 30°C
K359-396R 30°C
Non-Permissive (37°C) - + + + + + + + + + + + + + + +
Ntg1-Smt3-Smt3-Smt3
Ntg1-Smt3-Smt3
Ntg1-Smt3
Ntg1
PGK

Figure 6

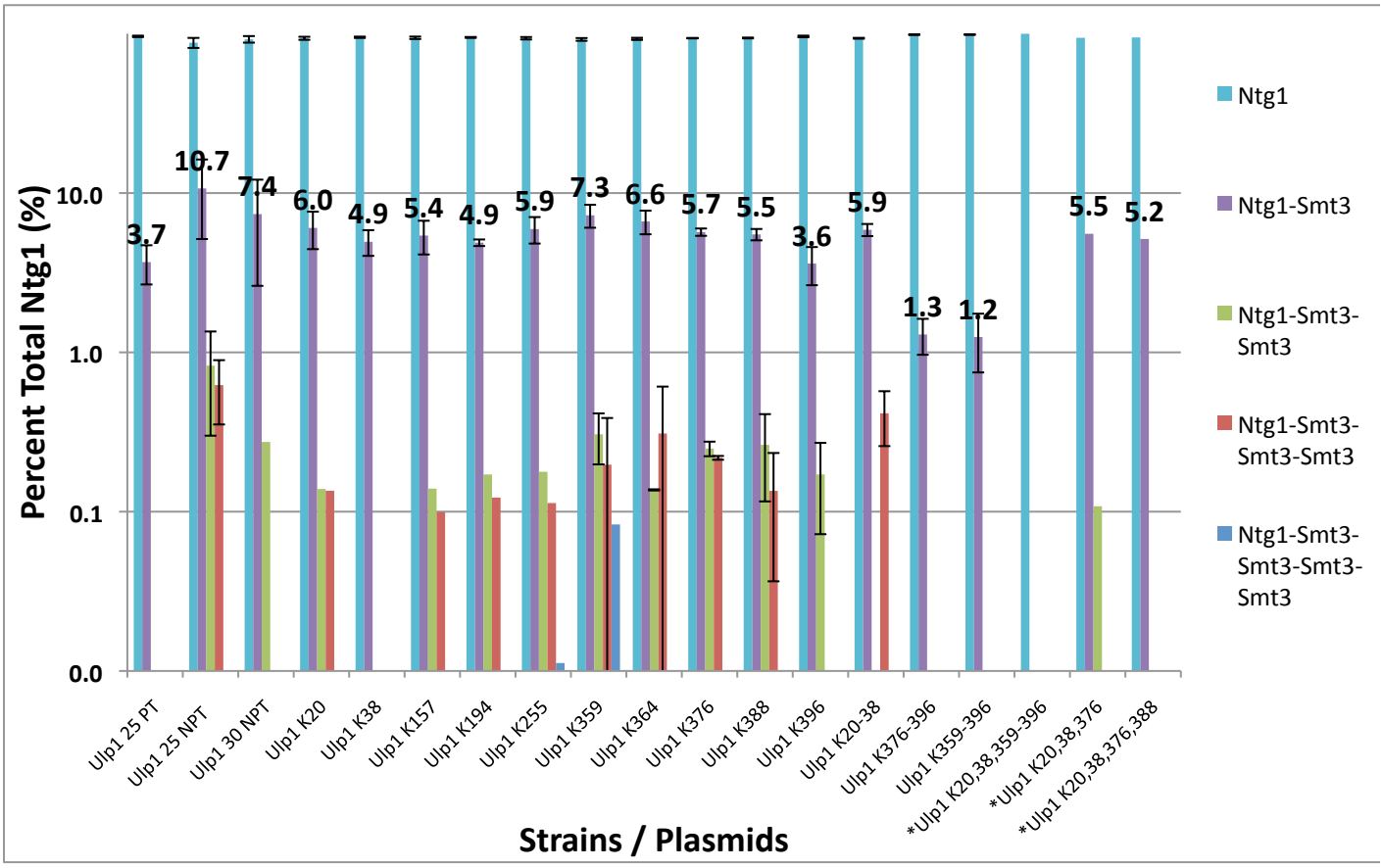

Percent Total Ntg1 (%)
10.0
1.0
0.1
0.0
3.7
10.7
7.4
6.0
4.9
5.4
4.9
5.9
7.3
6.6
5.7
5.5
3.6
5.9
1.3
1.2
5.5
5.2
Ulp1 25 PT
Ulp1 25 NPT
Ulp1 30 NPT
Ulp1 K20
Ulp1 K38
Ulp1 K157
Ulp1 K194
Ulp1 K255
Ulp1 K359
Ulp1 K364
Ulp1 K376
Ulp1 K388
Ulp1 K396
Ulp1 K20-38
Ulp1 K376-396
Ulp1 K359-396
*Ulp1 K20,38,359-396
*Ulp1 K20,38,376
*Ulp1 K20,38,376,388
Strains / Plasmids
Ntg1
Ntg1-Smt3
Ntg1-Smt3-Smt3
Ntg1-Smt3-Smt3-Smt3
Ntg1-Smt3-Smt3-Smt3-Smt3

Figure 7

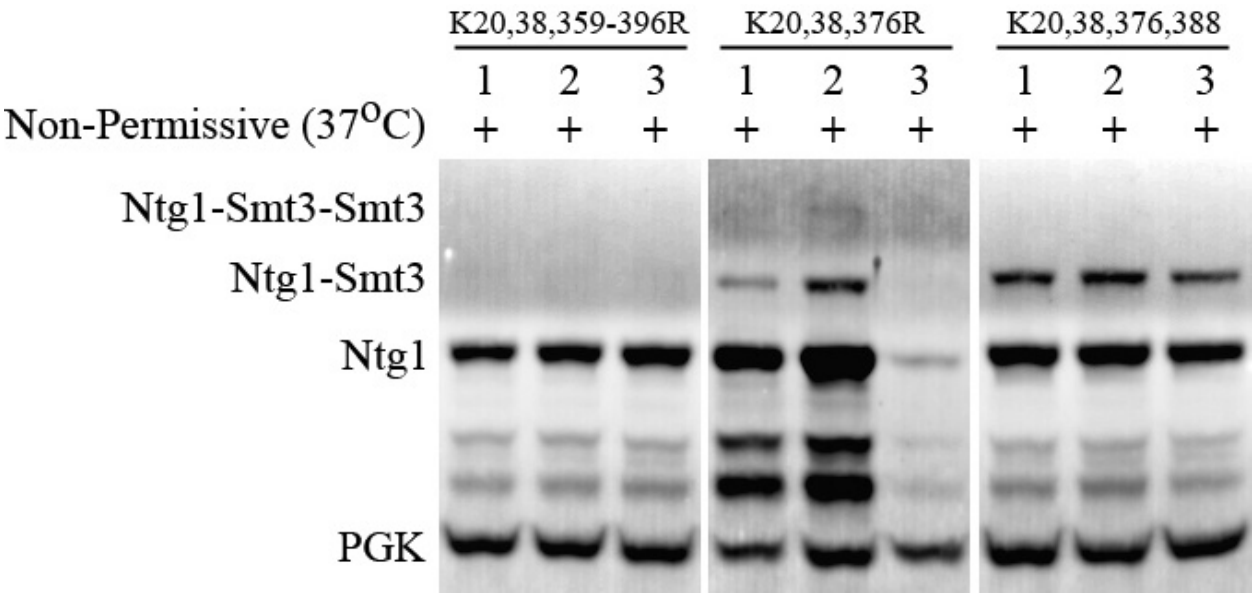

K20,38,359-396R
K20,38,376R
K20,38,376,388
1 2 3 1 2 3 1 2 3
Non-Permissive (37°C)
+ + + + + + + + +
Ntg1-Smt3-Smt3
Ntg1-Smt3
Ntg1
PGK

Figure 8

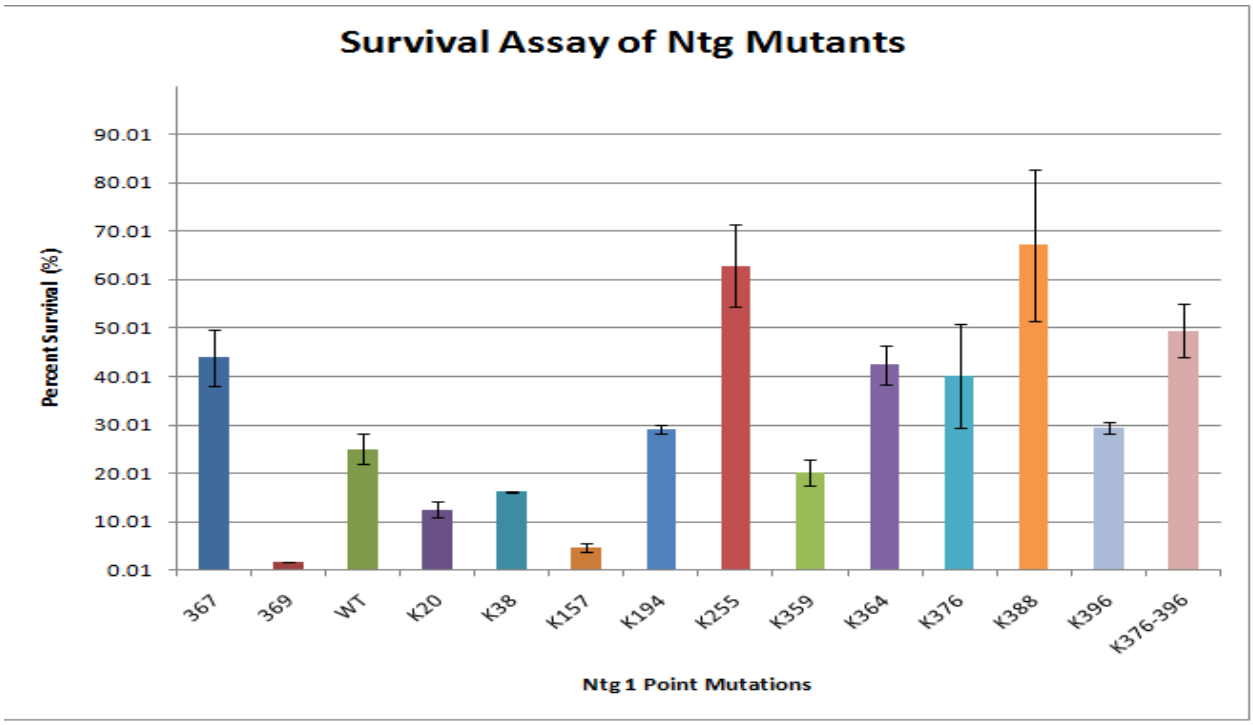
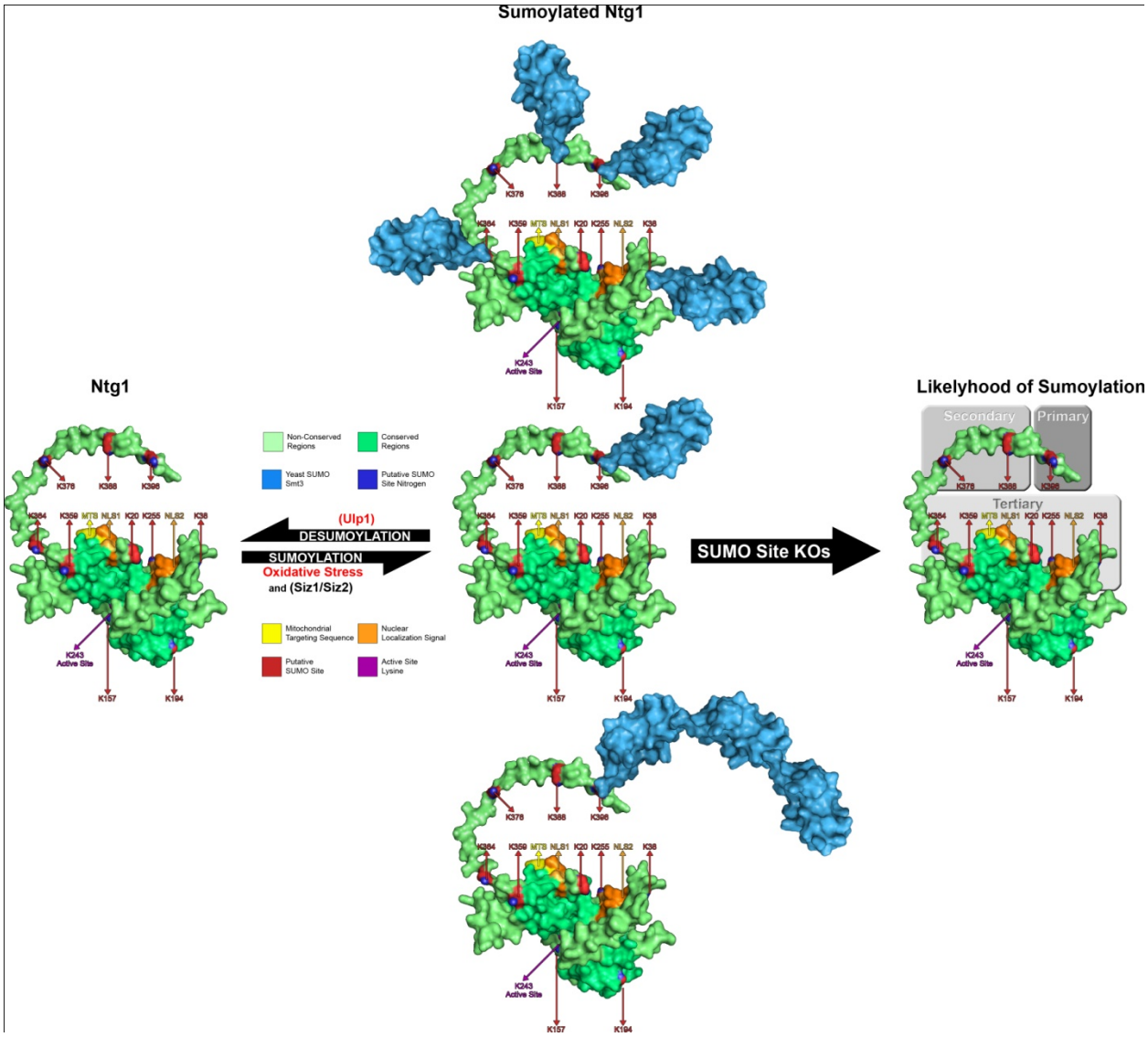

Survival Assay of Ntg Mutants
Percent Survival (%)
90.01
80.01
70.01
60.01
50.01
40.01
30.01
20.01
10.01
0.01
367
369
WT
K20
K38
K157
K194
K255
K359
K364
K376
K388
K396
K376-396
Ntg 1 Point Mutations

Figure 9


Sumoylated Ntg1
Ntg1
Likelyhood of Sumoylation
Secondary
Primary
Tertiary
Non-Conserved Regions
Conserved Regions
Yeast SUMO Smt3
Putative SUMO Site Nitrogen
(Ulp1)
DESUMOYLATION
SUMOYLATION
Oxidative Stress
and (Siz1/Siz2)
SUMO Site KOs
Mitochondrial Targeting Sequence
Nuclear Localization Signal
Putative SUMO Site
Active Site Lysine
K376
K388
K396
K364
K359
MTS
NLS1
K20
K255
NLS2
K36
K243
Active Site
K157
K194

Tables

Table 1. Strain Genotypes

Strains	Relevant Genotype	Source
(DSC 470)	MATa ntg1::hphMX4, his7-1, lys2Δ5'::LEU-lys2Δ3', ade5-1, trp1-289, ura3-52	This study
(DSC 369)	MATa ntg1::hphMX4, ntg2::BSD, apn1::TRP1, rad1::kanMX, his7-1, lys2Δ3'::LEU-lys2Δ5', ade5-1 ura3-52	(*19*)
(Ulp1*ts*)	MATa ulp1Δ::HIS3 LEU2::ulp1- 333	(*52*)

Table 2. Putative Sumoylation Site Priority List

Predicted SUMO site	Score	Consensus Sequence	Location on Ntg1
K20	9.0	Yes	N-terminus near NLS
K38	9.0	Yes	N-terminus near NLS
K376	8.5	Yes	C-terminus
K388	8.5	Yes	C-terminus
K396	8.0	Yes	C-terminus
K364	5.0	No	C-terminus
K157	4.0	No	Middle
K255	5.0	No	Middle
K194	2.0	No	Middle
K359	2.0	No	C-terminus

Table 3. Ntg1 Mutants Created

	Ntg1 with Putative SUMO site knockouts										Effect on Sumoylation
	K20	K38	K157	K194	K255	K359	K364	K376	K388	K396	No
K20R											No
K38R											No
K157R											No
K194R											No
K255R											No
K359R											No
K364R											No
K376R											No
K388R											No
K396R											Yes,
K20,38R											Missing 2nd higher molecular weight band
K20,38,376R											No
K20,38,376,388R											No
K376-396R											Drastic Reduction
K359-396R											Drastic Reduction
K20,38,359-396											Yes, no sumoylation

Table 4. Mutation Rates of Ntg1 SUMO KO Mutants on CAN Plates

Strain	Mutation Rate	Plasmid Retention
367	1.97E-06	-
369	4.91E-04	-
WT	3.89E-05	85.44%
K20	-	99.19%
K38	2.23E-05	76.96%
K157	2.65E-05	71.86%
K194	1.06E-04	84.55%
K255		
K359		
K364	4.02E-05	73.36%
K376	3.29E-06	82.90%
K388	-	82.65%
K396	4.92E-05	80.57%
K376-396	2.37E-05	81.25%

Figures and Table Legends

Figure 1. **Homology of Ntg1 with hNth1 and endonuclease III**. Comparison of the primary sequence of Ntg1, human homologue hNth1, and E. coli homologue endonuclease III.

Figure 2. **Location of putative SUMO modification sites on Ntg1**. The 10 putative SUMO modification sites predicted from the SUMO site prediction programs are shown in red on the primary sequence of Ntg1.

Figure 3. **Sumoylation of Ntg1 with single amino acid substitutions.** Immunoblot analysis of wildtype Ntg1, K20R, K38R, K157R, K194R, K255R, K359R, K364R, K376R, K388R, and K396R in Δ*ntg1* cells shows the steady-state levels of Ntg1 and sumoylated Ntg1. All of the cells, except as indicated, were treated in 20 mM H_2O_2 for 1 hour. The immunoblot was performed as described in Material and Methods.

Figure 4. **Detection of sumoylation induced from hydrogen peroxide treatment.** Immunoblot analysis of wildtype Ntg1-TAP, K20R, K38R, and K157R in Δ*ntg1* cells shows the steady-state levels of Ntg1 and sumoylated Ntg1. PGK was used as the loading control. All the cells, except as indicated, were treated in 20 mM H_2O_2 for 1 hour. The immunoblot was performed as described in Material and Methods and scanned using the Typhoon imager (GE Life Sciences).

Figure 5. **Sumoylation of Ntg1 with single sumoylation site mutations, and combination mutants.** Immunoblot analysis of wildtype Ntg1-TAP, K20R, K38R, K157R, K194R, K255R, K359R, K364R, K376R, K388R, K396R, K20,38R, K376-396R, and K359-396R in Ulp1*ts*

cells shows the steady-state levels of Ntg1 and sumoylated Ntg1. PGK was used as the loading control. All the cells, except as indicated, were incubated at the non-permissive temperature, 37^{o}C. The immunoblot was performed as described in Material and Methods and scanned using the Typhoon imager (GE Life Sciences).

Figure 8. **Quantification of typhoon image of Ntg1 mutants.** Quantification of the steady-state levels of Ntg1 and sumoylated Ntg1 from typhoon scans using Imagequant TL. The data points represent the percentage of total of steady-state level of Ntg1 that is sumoylated. The data comes from an average of three experiments. *Data for K20,38,359-396R, K20,38,376R, and K20,38,376,388R are from separate experiments.

Figure 9. **Sumoylation of K20,38,376R, K20,38,359-396R, and K20,38,376,388R mutants.** Immunoblot analysis of Ntg1-TAP with K20,38,376R, K20,38,359-396R , and 38,376,388R mutations a in Ulp1*ts* cells shows the steady-state levels of Ntg1 and sumoylated Ntg1. PGK was used as the loading control. All the cells were incubated at the non-permissive temperature, 37^{o}C. The immunoblot was performed as described in Material and Methods and scanned using the Typhoon imager (GE Life Sciences).

Figure 10. **Hydrogen Peroxide Cytotoxicity Experiment.** The H_2O_2 sensitivity of the control strain with wildtype DNA repair capacity (DSC367), BER/NER deficient cells (DSC 369), wildtype Ntg1 and Ntg1 mutants in BER/NER deficient cells was assessed. Cells were treated in 4 mM H_2O_2 for 1 hour as described in Material and Methods. Percent survival was determined by counting colonies and comparing colony formation to non-exposed cells. Errors bars represent standard deviation between duplicates. This was taken from one experiment.

Figure 11. **Model of Ntg1 Sumoylation.** Sumoylation of Ntg1 occurs primarily at K396, and then secondarily at K376 and K388 on the C-terminus. If the C-terminus sites are unavailable, then sumoylation can occur at K20 or K38. Sumoylation can either occur at multiple sites or as a polySUMO chain. Sumoylation of Ntg1 occurs mainly through the activity of Siz1 and desumoylation occurs mainly through the activity of Ulp1. The model of Ntg1 is derived from an overlay with endonuclease III, and the unconserved c-terminus was added on to show sumoylation sites. Blue structures represent Smt-3. Putative SUMO modification sites are labeled.

Table 1. **Strain Genotypes.** Genotypes of yeast strains used in this project.

Table 2. **Putative sumoylation sites priority list.** The most likely sites of sumoylation of Ntg1 based on the average score from 5 SUMO site prediction software SUMOSp 1.0, SUMOSp 2.0, PCL SUMO, SUMO Plot, and SUMOPre.

Table 3. **List of successfully created Ntg1 mutants**. All of the Ntg1 with sumoylation site mutations that were made using site-direct mutagenesis. Red indicates that the mutant was successfully made, whereas black indicates that the mutation had an effect on sumoylation.

Table 4. **Results of mutation rates assay**. The mutation rate and plasmid retention was calculated as described in the Material and Methods.

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