GENOME-WIDE MUTAGENESIS TO INVESTIGATE THE N-TERMINAL METHYLOME

The Protective Effects of Hsp31 and Other Methylated Proteins in Yeast

Abstract

The purpose of this study was to understand the role of methylation in regulating the cellular stress response of Hsp31 in *Saccharomyces cerevisiae* yeast cells. Hsp31 is known to be methylated by the N-terminal methyltransferase Tae1. Changing the methylation site can affect the methylation status of Hsp31, which may play a role in the protective activity of Hsp31 against cellular stress.

GLO1 is a gene in yeast involved in catalyzing the detoxification of methylglyoxal (MGO), which is a by-product of glycolysis. We established that *S. cerevisiae* in the *glo1Δ* and background is sensitive to cellular stress by MGO. Mutant strains in the *glo1Δ* background will simulate methylation levels, which can be used to determine if methylation increases or decreases the protective activity of Hsp31 under cellular stress. Hsp31 overexpression successfully rescues mutants in the *glo1Δ* background when treated with MGO. In a high throughput screen using CRISPR-based technology from Inscripta (Boulder, Colorado), a biotechnology company, we aim to replicate the results we observed in the *glo1Δ* background and potentially uncover new phenotypes in a series of experiments that will investigate the role of methylation in cellular processes in addition to oxidative stress, including heat stress and protein synthesis. The Inscripta library will allow us to collect data from approximately 1,000 mutants simultaneously rather than completing the experiment for each mutant individually. These results will provide much greater insight as to how methylation globally affects the cellular processes involved in the protective activity of Hsp31 and other substrates in *S. cerevisiae*.

Keywords

Saccharomyces cerevisiae, methylation, methyltransferase, cellular stress response, Hsp31, genome, methylome, mutation

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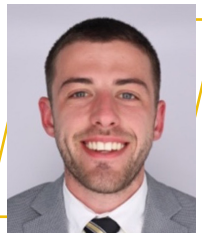

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INTRODUCTION

Saccharomyces cerevisiae yeast is considered a model organism for the human genome. In addition to being easy to manipulate, the yeast genome is also shockingly similar to the human genome, which is why several Nobel Prizes have been awarded to scientists for their work in yeast in recent history (Cervelli & Galli, 2021). Despite the similarities in genomes, though, humans are obviously much more complex organisms than simple baker's yeast. One key contributor to the complexity of humans lies in post-translational modifications, which are modifications that occur after a protein has been made that affect the functionality of the protein. Such modifications mediate the behavior of proteins, allowing one protein to have a variety of functions. One example of these modifications is methylation.

Methylation is the addition of one or more single, carbon-based chemical groups to the end of a protein. Proteins are methylated by enzymes called methyltransferases, which recognize specific amino acid sequences to methylate the protein. A subset of methyltransferase enzymes, N-terminal methyltransferases, are dedicated to methylating only the N-terminus of proteins in both humans (e.g., NTMT1) and yeast (e.g., Tae1) (Figure 1).

In yeast, the Tae1 enzyme recognizes the canonical X-P-K motif (X = A, P, or S) on the N-terminus of the protein.

When Tae1 recognizes this sequence, it methylates the N-terminus of the protein with one, two, or three methyl groups. The degree of protein methylation can impact the protein's function. Several proteins have demonstrated methylation in yeast (Figure 2), including up to 45 substrates. This study investigates how methylation affects the function of a protein called Hsp31, which contains an A-P-K motif. Mutations on this motif alter the methylation status of the protein. For example, substituting the alanine for a proline (A2P) results in increased methylation. Previous studies using mass spectrometry have confirmed that the in vivo level of methylation indicates that the A2P mutant is hypermethylated and the A2D mutant is hypomethylated (Chen, 2022).

Hsp31 is a multifunctional heat shock protein, which means it helps the yeast respond to cellular damage and

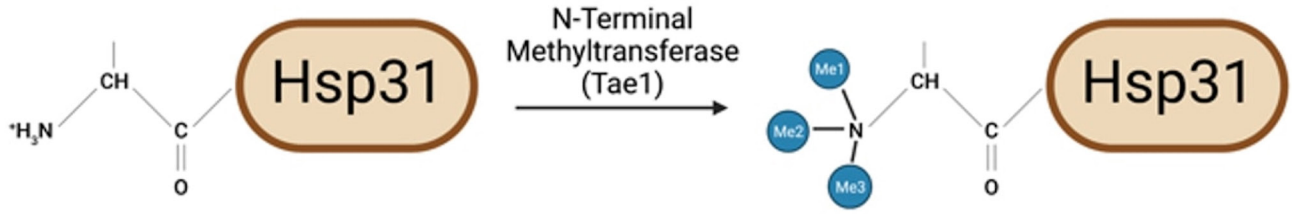

FIGURE 1. N-terminal methylation of Hsp31 by NTMT. Created with BioRender.com.

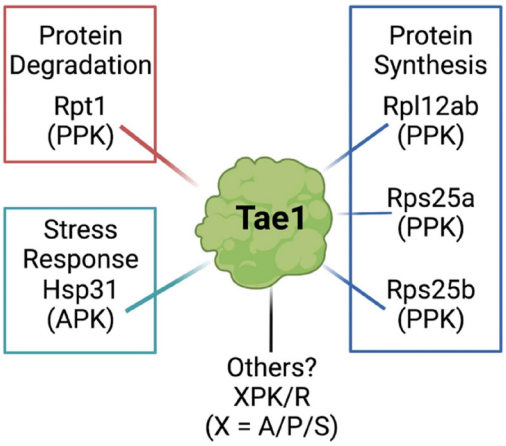

FIGURE 2. Substrates of Tae1. Created with BioRender.com.

stress, including heat, dicarbonyl metabolite, and oxidative stress. Hsp31 is also an ortholog, or parallel gene, of the DJ-1 gene in humans. The dysregulation of DJ-1 in humans is involved in cancer progression and Parkinson's disease (Jin, 2020; Repici & Giorgini, 2019). This study investigates the role of Hsp31 and methylation in response to induced cellular stress by hydrogen peroxide (H_2O_2) and methylglyoxal (MGO). The findings of this investigation will provide a greater understanding of the fundamental biological process involved in the progression of diseases related to cellular oxidative stress and are directly relevant to understanding the evolution of the methyltransferase enzymes and the role of methylation in humans.

METHODS

Strains and Reagents

The strains used in this study are listed in Table 1. Strains capable of artificially induced overexpression of Hsp31 were generated using a yeast BG1805 plasmid vector with a GAL1 promoter (Gelperin et al., 2005). Stress conditions were created using MGO from MP Biomedicals (155558) and H_2O_2 from Alfa Aesar (MFSD00011333).

Yeast Transformation

The *sod1* strain was obtained from the Dharmacon collection (Winzeler et al., 1999). For the transformation reaction, the LiAc/SS carrier DNA/PEG method was used (Gietz & Schiestl, 2007). The BG1805 plasmid vector was used with the WT BY4741 parental strain.

Galactose Induction

To overexpress Hsp31, the BG1805 plasmid was utilized. In this plasmid, the Hsp31 gene is under the control of the galactose promoter, so galactose induces the expression, or overexpression, of Hsp31. To artificially induce the overexpression of Hsp31, an adapted galactose induction protocol was used (Gelperin et al., 2005).

TABLE 1. *S. Cerevisiae* strains used in the study.

Strain	Genotype	Source
WT (BY4741)	MATa; *his3Δ1; leu2Δ0; met15Δ0; ura3Δ0 hsp31Δ::kanMX4* derivative of BY4741	Horizon Discovery YSC1048
glo1Δ	*glo1Δ::kanMX4* derivative of BY4741	This Study
sod1Δ	*sod1Δ::kanMX4* derivative of BY4741	This Study
tae1Δ	*tae1Δ::kanMX4* derivative of BY4741	This Study
yap1Δ	*yap1Δ::kanMX4* derivative of BY4741	This Study

Single colonies of transformants were inoculated in SC – URA supplemented with glucose using a sterile inoculating loop and grown overnight. The next day, the cultures were diluted to an optical density (OD) of 0.25 at 600 nm in 3 mL SC – URA supplemented with 2% raffinose so the OD would be approximately 1 the following day. On the third day, the ODs of each culture were measured, and then 1 mL of culture was added to 1 mL 3X YEP supplemented with 6% galactose and 1 mL sterile water. The cultures were induced for 3 h in a shaking incubator at 30°C and 200 rpm.

Serial Dilution and Spotting

To study the effects of drug treatments on the growth of different yeast backgrounds, a serial dilution and spotting assay was performed. Following the 3 h induction period, cells were normalized to an OD of 1 using the ODs measured prior to induction. The normalized cells were spun down and resuspended in sterile water. Next, cells were serially diluted in sterile water in a 96-well plate. Stress conditions were introduced in two experimental protocols:

- Protocol A—Serially diluted cells were mixed with both sterile water and drug treatments (as denoted in each figure). Five µL of cell samples were then spotted on Greiner Bio-One square petri dishes with rich media (YPD) at various time points (as denoted in each figure) using the Avidien microPro 300 robot (300-0842).
- Protocol B—Immediately after serial dilution, 5 µL of serially diluted cells were spotted on agar plates, including a control YPD plate, and treatment plates with various stress conditions introduced by drug exposure (as denoted in each figure) using the Avidien robot.

Images of the plates were taken at various time points (as denoted in each figure) in a lighted black box to ensure clarity.

Protein Extraction

To extract Hsp31 protein for western blot analysis 200 µL Hsp31-expressing cell samples containing 10^8 cells were collected, and the optimized protein extraction for quantitative proteomics of yeast protocol was used (von der Haar, 2007). Following protein extraction, 15 µL of protein extract was loaded into an SDS-PAGE gel. The proteins were electrophoresed through the gel and transferred to blotting filter paper using the standard semi-dry blotting protocol and Bio-Rad Trans-Blot Turbo equipment.

Western Blot

To compare the relative levels of Hsp31 in each mutant strain, the standard western blotting protocol was used with a PVDF membrane (Mahmood & Yang, 2012). As a primary antibody to detect the Hsp31 protein, anti-HA rabbit antibody (Rockland [600-401-384]) was used at a 2,000-fold dilution. Another primary antibody was used to detect the control alpha-tubulin protein with 12G10 from the Developmental Studies Hybridoma Band (DSHB) at a 100-fold dilution. As a secondary antibody for Hsp31 detection, Goat anti-rabbit IgG DyLight 800 (Invitrogen; SA535571) was used. As a secondary antibody for alpha-tubulin detection, Goat anti-mouse IRDye 680RD (Li-Cor; 926-68070) was used. An Azure 600 imager from Azure Biosystems was used for multiplex fluorescent imaging of the blots.

N-Terminal Mutant Library Screen

A library of approximately 1,000 N-terminal mutants was generated by Inscripta (*MAD7 Nuclease | Inscripta* 2023) based on specifications pertaining to 45 substrates within the yeast genome that are likely to be methylated based on their N-terminal sequences. In addition to making the genomic edits, Inscripta also adds a DNA barcode sequence that allows the mutants to be identified. Samples from the frozen library were grown on YPD plates with hygromycin (100 mg/mL). Single colonies from the plates were transferred to individual wells in three 96-well plates and grown in liquid YPD with hygromycin (100 mg/mL) for 1–2 days. The Avidien robot was then used to spot 5 mL of the arrayed mutants onto plates with YPD or YPD and drug treatments (as denoted in each figure). The arrayed mutants were grown at 30°C for 1–2 days, and hits were identified based on sensitivity or resistance to the drug treatments. The

plasmids within identified strain hits were isolated using Zymoprep yeast plasmid miniprep II (D2004). Plasmids were submitted for whole plasmid sequencing by Eurofins genomics. The mutations responsible for each hit were then identified using the 12-base barcode in the amino acid sequence, which was cross referenced with the design library, which includes the barcode for each mutation. Identified hits were retested to confirm their phenotypes using the method described in Protocol B under "Serial Dilution and Spotting."

TABLE 2. Methylation levels of N-terminal mutants.

N-Terminal Mutation	Methylation Status
WT	+
Vector	—
A2P	++
A2D	—
A2S	Unknown
A+	—
P3A	—
K4R	Unknown

RESULTS

Overexpression of the Hsp31 A2P Hypermethylation Mutant Protects Against MGO Stress

Although Hsp31 has methylglyoxalase activity (Bankapalli et al., 2015), its enzymatic activity has been reported to be relatively weak compared to other glyoxalase enzymes such as Glo1 and Glo2. By deleting the *GLO1* gene, the resulting strain (*glo1Δ*) is highly deficient in methylglyoxalase activity. The *glo1Δ* strain was utilized to evaluate survival of N-terminal mutants upon exposure to MGO following Hsp31 overexpression. The survival results are shown in Figure 3. Overexpression of WT Hsp31 increased survival of the *glo1Δ* mutants compared to the vector. Analysis of the Hsp31 N-terminal mutants identified differences in strain survival. Six N-terminal mutants were investigated, and their methylation status is summarized in Table 2. Using the method described in Protocol A, the A2P mutation demonstrated resistance upon exposure to 10 mM MGO for 28 h.

The wild-type and the A2S mutant also demonstrated resistance. Western blot analysis was performed on these strains to assess relative protein expression and protein stability. Protein levels were assessed immediately post induction and 18 h after the cells were suspended in sterile water and not actively growing. As shown in Figure 4, Hsp31 protein levels appear to be equivalent within a twofold range between different Hsp31 mutants. Hsp31 levels were also similar between strains immediately post induction (0 h) and 18 h after induction. Additional biological replicates are needed to quantitate and confirm these results.

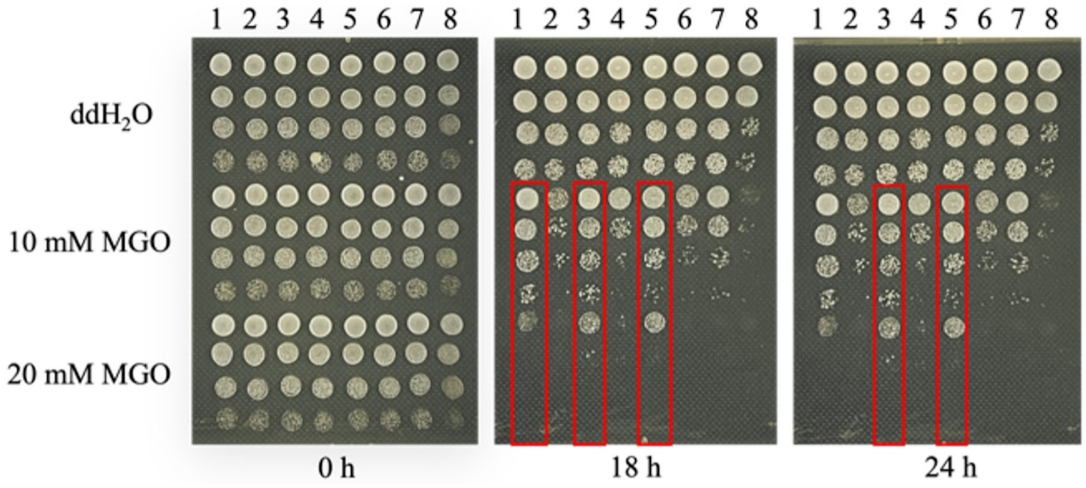

FIGURE 3. Survival results from MGO exposure in *glo1Δ* background using Protocol A. Strains include WT (1), Vector (2), A2P (3), A2D (4), A2S (5), A+ (6), P3A (7), K4R (8). Strains that have increased resistance are boxed in red.

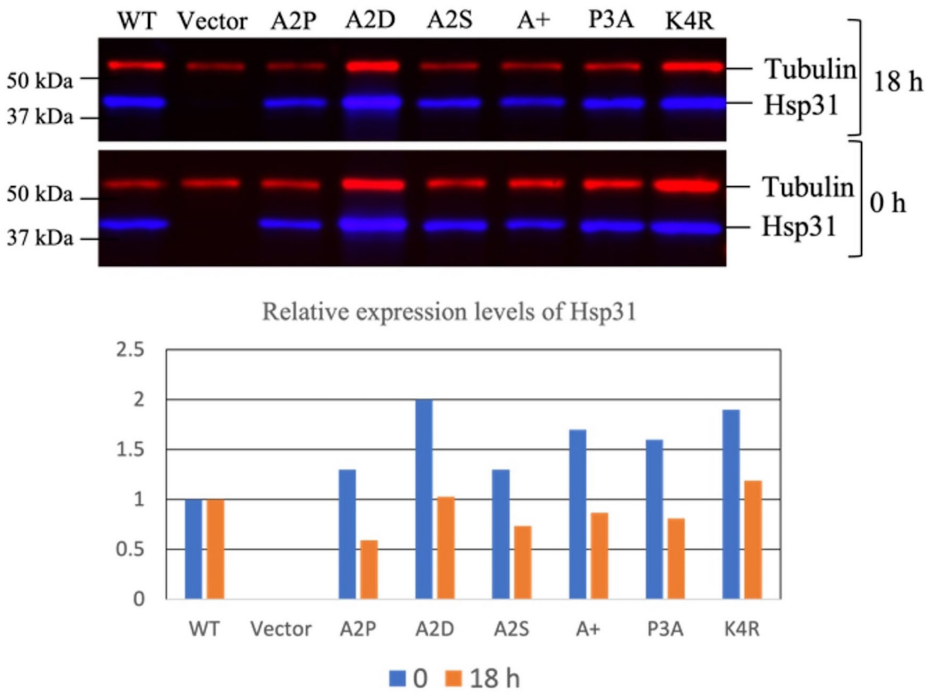

FIGURE 4. Western blot analysis in *glo1Δ* background at time points 0 h and 18 h. Strains include WT (1), Vector (2), A2P (3), A2D (4), A2S (5), A+ (6), P3A (7), K4R (8). The same cells used in Figure 3 were used to prepare lysates for this western blot. Blots were quantified using Image J and normalized to the a-tubulin in the same lane.

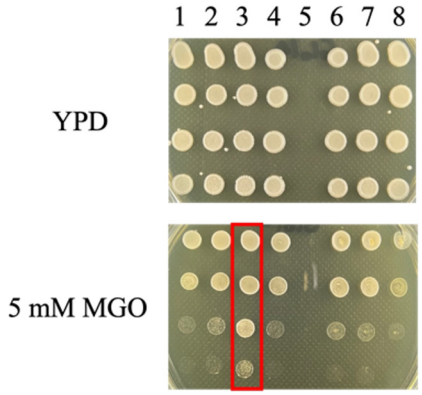

FIGURE 5. Survival results from MGO exposure in *glo1Δ* background using Protocol B. Strains include A2D (1), A2S (2), A2P (3) red outline, A+ (4), P3A (5), K4R (6), WT (7), and Vector (8). The P3A (5) strain was not tested in this experiment.

Using the method described in Protocol B, the A2P mutant also demonstrated resistance upon exposure to 5 mM MGO in the agar media (Figure 5).

Western blot analysis performed on these strains confirmed that Hsp31 expression at the spotting stage was similar in all strains tested (data not shown).

N-Terminal Mutant Library Screen to Examine Oxidative Stress Phenotypes

To assess whether N-terminal mutations of proteins affect function, the phenotypes of individual N-terminal mutant strains from a pooled strain library (Inscripta) were assessed. A small scrape of the frozen N-terminal mutant library was initially diluted and spread on plates to generate single colonies. Approximately 300 single colony mutants from the library were arrayed and grown on plates with different treatment conditions at varying concentrations of H_2O_2 and MGO. Six strain hits were identified that exhibited sensitivity to H_2O_2 or MGO (data not shown), and one strain demonstrated resistance to 15 mM H_2O_2 (Figure 6). Plasmid sequencing of the resistant strain indicated an A2P mutation in the histone H1 gene, *HHO1*, which is highly conserved between yeast and humans. The mutant also appeared to allow protection of the surrounding mutants in the array (Figure 6).

A ΔPK mutant (deletion of the third and fourth amino acid) in the *ARB1* background and a Syn15 mutant (synonymous mutation at codon 15) in the *HHO1*

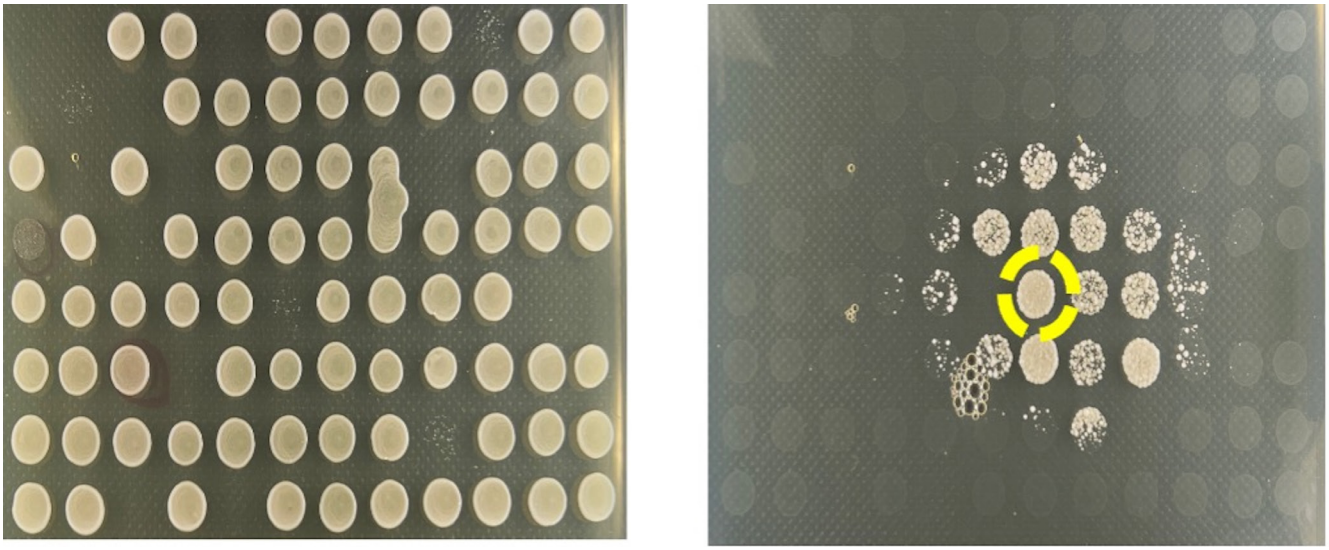

FIGURE 6. The plate on the left is plain YPD and the plate on the right contains 15mM H_2O_2 in the agar plate. The A2P mutant in the *HHO1* background (yellow circle) shows resistance and protective activity of the strains around it.

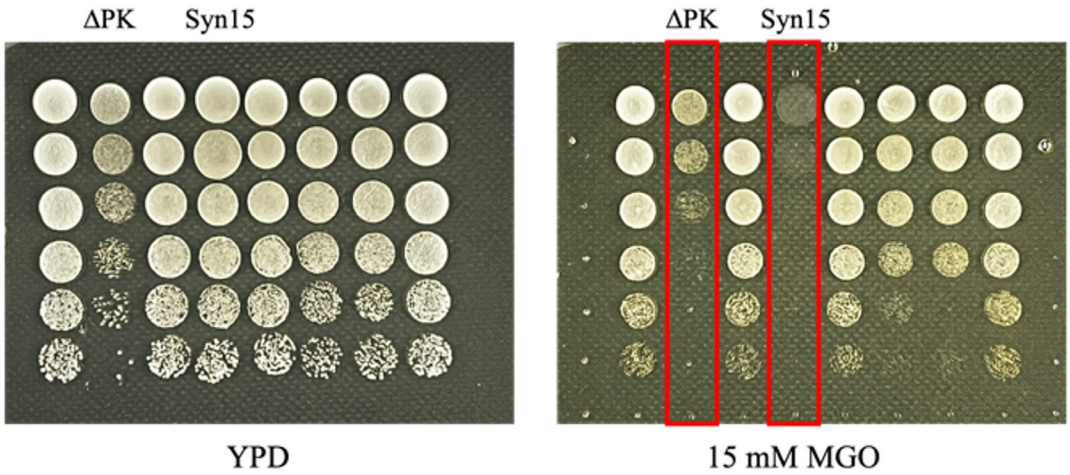

FIGURE 7. Results from retesting the high throughput screen hits for sensitivity to MGO. The *arb1* ΔPK and *hho1* syn15 mutants are outlined in red.

background both demonstrated sensitivity to 15 mM and 25 mM MGO, and these phenotypes were confirmed in the retest on 15 mM MGO (Figure 7).

Additionally, two other mutants displayed slight sensitivities in the screen, but more investigation into their phenotypes is needed.

DISCUSSION

Previous studies have documented a link between elevated N-terminal methylation and response to stress (Dai et al., 2013; Villar-Garea et al., 2012). Hsp31 is a small heat shock protein that plays a crucial role in protecting cells from oxidative stress, regulating protein homeostasis, and preventing the accumulation of damaged proteins in cells. The N-terminal region of Hsp31 contains a methylation motif sequence, which suggests that this protein may be subject to N-terminal methylation.

Given the role of Hsp31 in stress response and the presence of the N-terminal methylation motif sequence, we hypothesize that N-terminal methylation of Hsp31 could modulate its function.

The Role of Methylation in Regulating the Stress Response of Hsp31

This hypothesis was tested by serially diluting normalized mutant strains and exposing them to stress conditions, including H_2O_2 and MGO, either in liquid suspensions or in agar media. In both conditions, the A2P mutant, which increases methylation levels, showed greater resistance to MGO stress in the *glo1Δ* background compared to WT. Primitive data suggests a trend that methylation deficient strains are more sensitive to MGO stress, but further investigation is needed.

Because we did not detect methylation directly, it could be the case that the amino acid substitutions themselves are responsible for the altered function of Hsp31 in each mutant. Based on western blot analysis, though, it does not seem likely that the stability of Hsp31 is diminished over the time tested in this study, which is consistent with the long half-life of 31 h for Hsp31 and 20 h for alpha-tubulin (Christiano et al., 2014). This finding also supports the idea that methylation is responsible for the change in protein function rather than the amino acid substitutions themselves.

N-Terminal Mutant Library Screen

The purpose of the N-terminal mutant library screen was to determine whether the high throughput screening method could be used to identify hits and correlate mutations with stress conditions. In the *HHO1* background, two mutants were identified as hits. The A2P mutant, which increases methylation levels, demonstrated resistance to H_2O_2 stress. The Syn15 mutant is a synonymous mutation at codon 15, so it should not affect protein function, yet the Syn15 mutant demonstrated sensitivity to MGO stress. Although Syn15 is a synonymous mutation and was not expected to produce a phenotype, a recent study suggests synonymous mutations could affect gene expression (Shen et al., 2022), although another recent preprint disputes this idea (Kruglyak et al., 2023). The *hho1Δ* strain demonstrated sensitivity to oxidative stress (Brown et al., 2006). A third hit was identified in the *ARB1* background in the ΔPK mutant, which decreases methylation levels, and demonstrated sensitivity to MGO stress. It should be noted that the ΔPK mutant also displayed reduced fitness even on YPD with no stress. *ARB1* is an essential gene (Dong et al., 2005; Giaever et al., 2002), so it is more likely to show a growth phenotype under nonstress conditions.

This initial pilot screen demonstrated that the high throughput screening method can be used to identify hits and correlate mutations with stress conditions. The results of this pilot screen need to be validated by transforming the isolated plasmids into a WT strain to verify that the mutation was responsible for the observed phenotype. The use of high throughput screening creates a foundation for future experiments by gaining a more global perspective of the role of N-terminal methylation.

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