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Identification of Proteins that Contribute to Yeast Heat Stress by Lysine Acetylation

An Honors Thesis submitted in partial fulfillment of the requirements of Honors Studies

in the Department of Biological Sciences

By

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Biology

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Abstract

Evidence is emerging that protein lysine acetylation may be a novel type of post-translational modification (PTM) contributing to the mechanisms of yeast heat stress responses. Proteomics studies including ours have identified over 1,000 acetylated proteins in the yeast proteomes that are composed of about 6,000 proteins. Our lab recently identified 596 proteins that underwent acetylation changes during heat shock by mass spectrometry. However, the role of lysine acetylation on specific residues of specific proteins in yeast thermotolerance remains largely unknown. This study selected 43 proteins from our lab's previous work and examined their possible contributions to yeast heat stress responses. We found that knockout of 32 genes caused a growth defect in yeasts at 40 °C, suggesting these proteins are required for yeast innate thermotolerance. Among these 32 proteins, knockout of 5 genes including *rpl31a*, *sin3*, *aco1*, *adh1* and *pfk2* almost completed inhibited yeast growth at 40 °C, suggesting they are ideal candidates for further studies. Site-directed mutagenesis method was employed to replace the lysine K638 in Aco1p protein for the purpose of mimicking different states of acetylation; K638 was first replaced with alanine to examine whether this lysine residue was essential to yeast survival and growth, and then K638 was replaced by glutamine or arginine to mimic acetylated or un-acetylatable Aco1p, respectively. Similar work was performed for proteins Rpl31ap, Sin3p, and Hsp104p, whose function was confirmed in this study to be required for yeast innate and acquired thermotolerance. Work is in progress to examine if these manipulations will impact yeast thermotolerance. To conclude, this study identified that 32 protein with changing acetylation are required for yeast innate thermotolerance. In addition, this work generated mutant strains

harboring desired residues that are useful to examine the role of specific lysine residues whose acetylation may play a role in regulating the yeast heat stress responses.

Introduction

All living organisms must adapt to the constantly changing environment to maintain internal homeostasis. Cells have developed many regulatory mechanisms such as dynamic gene expression changes to combat stress; however, some of these methods are likely too slow to efficiently deal with very rapid environmental changes [1]. Complementary to the relatively slow responses, living organisms also utilize a process known as protein post translational modifications (PTMs) to cope with stress in a swift manner [2]. PTMs regulate protein functions in a very rapid manner by utilizing a minimal number of cellular resources; some proteins can be activated or deactivated immediately when PTM occurs. This makes PTMs well suited for the adaptation responses to environmental stresses [3]. PTMs are dynamically regulated, and dysregulations of PTMs are causatively associated with a broad spectrum of diseases.

PTMs refer to the rapid and reversible chemical modifications of existing proteins. It is estimated that humans have over 1 million proteins, and more than 200 types of PTMs have been discovered [3]. The most common types of PTMs include phosphorylation, glycosylation, ubiquitination, methylation, and acetylation, and different types of PTMs can interact with each other [3]. The yeast proteome has been well-established, and the total number of proteins in yeast is about 6, 000 [4, 5]. Each yeast cell has an average of about 80 million protein molecules [5]. Many PTMs have been discovered in yeast proteins. According to the Yeast Amino Acid Modification (YAAM)

database (http://yaam.ifc.unam.mx), at least 12 types of PTMs have been well characterized for a total of 121, 921 residues [6]. Interestingly, the highest number of yeast protein residues with PTMs are phosphorylation, ubiquitylation and acetylation, with 34,643, 8,851 and 6,971 residues for each type of PTM reported in the YAAM database [6], suggesting that acetylation is one of the three most abundant PTMs in yeast. Of note, the dynamic changes in protein phosphorylation during heat stress have been well described in previous studies, and ubiquitylation during heat stress in yeast have been described in our lab's recent publication [7, 8].

As an important form of PTMs, protein acetylation refers to the reversible addition of an acetyl group (CH_3CO) onto the amino groups of a protein. This reaction usually occurs on the lysine residues or the N-terminal of proteins [2]. The present study focuses on protein lysine acetylation. Lysine residues are normally positively charged due to their $–NH_2$ group. Acetylation of lysine neutralizes the positive charge, enhances the hydrophobicity, and increases the size of the side chain and therefore may affect protein confirmations, functions, stabilities, subcellular localizations, and interactions with other molecules [2, 9].

Protein lysine acetylation was first described in histones in the early 1960s [10]. More than two decades later, lysine acetylation was also discovered in non-histone proteins such as tubulin [11]. While early studies focused mainly on lysine acetylation in nucleus due to extensive histone acetylation, the rapid development in proteomics technologies enabled the identification of numerous non-histone proteins that are lysine acetylated, and these modified proteins are localized to nearly all the cellular compartments, suggesting that lysine acetylation may play regulatory roles in a wide

array of cellular processes. The role of lysine acetylation also expanded from controlling gene expressions, which was initially discovered with histone proteins, to directly affecting protein functions and regulating protein-protein interaction and even modulating the interactions with other forms of PTMs [9, 12, 13].

Like other forms of PTMs, the level of protein lysine acetylation is tightly regulated by both enzymatic and non-enzymatic processes [9]. The enzymatic control of protein acetylation mainly involves lysine acetyltransferases (KATs) and deacetylases (KDACs), which are responsible for the addition and removal of the acetyl group in the modified proteins, respectively [9]. Disruptions in the level of protein lysine acetylation have been implicated in many disorders, particularly cancers. Small molecules that can correct the dysfunctions in protein lysine acetylation are being actively explored in hoping to help treat diseases. A group of chemicals inhibiting KDACs and thus restoring the lysine acetylation homeostasis have been shown to be beneficial to many diseases. Indeed, the US Food and Drug Administration has approved four small molecule KDAC inhibitors for the treatment of various forms of blood cancers, such as peripheral T-cell lymphoma and multiple myeloma. These drugs include vorinostat, romidepsin, belinostat, and panobinostat, and they all exert their therapeutic effects via promotion of protein lysine acetylation by inhibiting KDACs [14]. These chemical inhibitors for the regulation of protein lysine acetylation have become valuable tools in elucidating the role of acetylation in many biological processes. In contrast, our current understandings on the non-enzymatic regulation of protein lysine acetylation are very limited.

A growing number of proteins have been found to undergo lysine acetylation under normal or stressed conditions. In human cells, 1750 proteins with 3600 lysine

acetylation sites have been reported [12]. In 48 phylogenetically diverse bacteria, an average of 190 proteins with an average of 508 lysine acetylation sites were identified in each organism [15]. In yeast, over 1,000 proteins were found to be susceptible to lysine acetylation [16]. Computational function-enrichment analysis showed that lysine acetylated proteins were enriched in many cellular processes such chromatin remodeling, cell cycle, nuclear transport in human cells[12], and metabolism related pathways, including glycolysis, pyruvate metabolism, and the tricarboxylic acid (TCA) cycle in bacteria [15]. In yeast, the acetylated proteins were enriched in the cytoplasm, nucleus, and mitochondria, and they were significantly overrepresented in amino acid metabolism, histone modification, protein synthesis and protein folding [16].

Despite decades of research, protein lysine acetylation remains incompletely understood. Though advancement in the proteomics technology has greatly facilitated the discovery of lysine acetylated proteins, deciphering the biological significance of lysine acetylation continues to be a labor-intensive endeavor with relatively slow progress. Numerous proteins have been reported to be modified by lysine acetylation under various conditions, but most findings have not been confirmed by independent groups and the functional impact of lysine acetylation on most proteins remain unclear. It is often unknown if perturbations in protein lysine acetylation are a cause or a result of certain biological processes. Limited evidence seems to suggest that acetylation of only very selective lysine residues causes a change in protein functions and thus leads to altered biological responses. Identification of the critical lysine residues whose acetylation regulates protein functions and even certain biological process is one of the urgent needs in this area.

Our lab recently found that protein lysine acetylation is required for the full acquisition of thermotolerance in yeast. Acquired thermotolerance refers to an organism's ability to better survive life-threatening high temperatures after being first exposed to mild, non-lethal heat stresses. Our lab found that yeast treated with KDAC inhibitors Trichostatin A and nicotinamide showed significant viability defects at 45°C after being subjected to a mild heat stress at 37°C, suggesting protein lysine acetylation plays an important role in acquired thermotolerance in yeast. To further elucidate the role of acetylation in heat stress response, the acetylome, that is, the entire set of lysine acetylated proteins, in yeast was profiled. Specifically, yeast were subjected to a 37°C heat shock for 10, 15, 30, 45, 60, 90, 120, and 240 minutes, and the acetylated proteins were enriched using an acetyllysine resin and analyzed using mass spectrometry. A total of 596 acetylated proteins were identified, among which 207 proteins showed significant fold changes ((Benjamini-Hochberg corrected p-value <0.05) in acetylation in at least one time point compared to the non-stressed groups. GO process enrichment (Princeton GO Term Finder) analysis found that the proteins with upregulated acetylation were enriched in protein folding and refolding, response to heat, nucleotide metabolic processes, small molecule metabolism, carbohydrate metabolism, while those with decreased acetylation were enriched in translation, nitrogen metabolism, gene expression, ribosome biogenesis and assembly and peptide metabolism, suggesting acetylation likely activates the cellular stress response (Rebecca E. Hardman, PhD Thesis: https://scholarworks.uark.edu/cgi/viewcontent.cgi?article=5039&context=etd).

To further test our hypothesis that protein lysine acetylation is a novel protective mechanism against heat stress in yeast, the present study aims to elucidate the role of

lysine acetylation of specific proteins on specific residues in mediating the heat stress responses in yeast. This study focused on the 596 acetylated proteins identified in our lab's previous study.

Materials and Methods

Strains and Growth Conditions

The wild-type yeast strain used in this study is BY4743 (S288c background; *MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 met15Δ0/MET15 ura3Δ0/ura3Δ0*). All protein knockout strains were homozygous diploid deletions in the BY4743 background purchased from Open Biosystems and are listed in Table 1. The deletions were generated by replacing each open reading frame with a KanMX cassette by homologous recombination. Yeast strains were cultured in yeast extract peptone dextrose (YPD) containing 1% yeast extract, 2% peptone, 3% dextrose under orbital shaking of 270 rpm.

Table 1. Yeast strains used in this study

Strain	Gene deletion	Genotype
JL 2	Wild Type	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 met15Δ0/MET15 ura3Δ0/ura3Δ0*
JL 327	*hsp104Δ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 met15Δ0/MET15 ura3Δ0/ura3Δ0 hsp104Δ/Δ*
JL 1165	*idp1Δ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 idp1::kanMX/idp1::kanMX*
JL 1166	*ssa4Δ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 ssa4::kanMX/ssa4::kanMX*
JL 1167	*eno1Δ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 eno1::kanMX/eno1::kanMX*
JL 1168	*tdh1Δ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 tdh1::kanMX/tdh1::kanMX*
JL 1170	*tdh3Δ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 tdh3::kanMX/tdh3::kanMX*
JL 1173	*tma19Δ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 tma19::kanMX/tma19::kanMX*

JL 1175	*ssa2Δ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 ssa2::kanMX/ssa2::kanMX*
JL 1176	*pdc1Δ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 pdc1::kanMX/pdc1::kanMX*
JL 1180	*aco1Δ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 aco1::kanMX/aco1::kanMX*
JL 1185	*snf2Δ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 snf2::kanMX/snf2::kanMX*
JL 1186	*sin3Δ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 sin3::kanMX/sin3::kanMX*
JL 1187	*rpp2aΔ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 rpp2a::kanMX/rpp2a::kanMX*
JL 1188	*adh1Δ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 adh1::kanMX/adh1::kanMX*
JL 1190	*lat1Δ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 lat1::kanMX/lat1::kanMX*
JL 1191	*tkl1Δ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 tkl1::kanMX/tkl1::kanMX*
JL 1236	*rpl20aΔ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 rpl20a::kanMX/rpl20a::kanMX*
JL 1238	*cpr6Δ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 cpr6::kanMX/cpr6::kanMX*
JL 1240	*hsp82Δ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 hsp82::kanMX/hsp82::kanMX*
JL 1243	*nsr1Δ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 nsr1::kanMX/nsr1::kanMX*
JL 1245	*rpl11bΔ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 rpl11b::kanMX/rpl11b::kanMX*
JL 1246	*rpl14aΔ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 rpl14a::kanMX/rpl14a::kanMX*
JL 1248	*rpl31aΔ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 rpl31a::kanMX/rpl31a::kanMX*
JL 1250	*rpl9aΔ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 rpl9a::kanMX/rpl9a::kanMX*
JL 1251	*rps1aΔ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 rps1a::kanMX/rps1a::kanMX*
JL 1252	*rps1bΔ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 rps1b::kanMX/rps1b::kanMX*
JL 1253	*rps7a/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 rps7a::kanMX/rps7a::kanMX*
JL 1254	*ssa1Δ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 ssa1::kanMX/ssa1::kanMX*
JL 1255	*sti1Δ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 sti1::kanMX/sti1::kanMX*
JL 1179	*adh3Δ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 adh3::kanMX/adh3::kanMX*
JL 1244	*pfk2Δ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 pfk2::kanMX/pfk2::kanMX*
JL 1242	*new1Δ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 new1::kanMX/new1::kanMX*

JL 1247	*rpl16bΔ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 rpl16b::kanMX/rpl16b::kanMX*
JL 1249	*rpl7aΔ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 rpl7a::kanMX/rpl7a::kanMX*
JL 1174	*rps21aΔ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 rps21a::kanMX/rps21a::kanMX*
JL 1237	*yor051cΔ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 yor051c::kanMX/yor051c::kanMX*
JL 1178	*imd3Δ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 imd3::kanMX/imd3::kanMX*
JL 1182	*pdr16Δ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 pdr16::kanMX/pdr16::kanMX*
JL 1169	*hsp150Δ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 hsp150::kanMX/hsp150::kanMX*
JL 1189	*lsp1Δ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 lsp1::kanMX/lsp1::kanMX*
JL 1177	*stm1Δ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 stm1::kanMX/stm1::kanMX*
JL 1241	*htz1Δ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 htz1::kanMX/htz1::kanMX*
JL 1164	*hek2Δ/Δ*	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/METI5 ura3Δ0/ura3Δ0 hek2::kanMX/hek2::kanMX*

Growth curve assay

Yeast growth was measured to determine the effect of specific protein knockout on yeast heat sensitivity. Biological duplicates of protein knockout strains were cultured to saturation overnight in YPD at 25°C. A 1:500 dilution in 1 mL YPD was transferred into 24 well plates and sealed with a breathable sealing membrane. Growth curve experiments at both 40 °C stressed conditions and 30 °C unstressed conditions were performed using Biotek Eon Microplate spectrophotometer. The OD_{600} was measured every 15 min for 42 h. Results were compared to wild-type yeast and analyzed using GraphPad Prism Version 7.

Acquired thermotolerance assay

Yeast were cultured to saturation overnight in YPD at 25°C. To remove potential nutrient stress, yeast were sub-cultured into 6 ml fresh YPD. They were grown to

exponential phase as determined by OD_{600} of 0.3 to 0.6 measured by a Unico spectrophotometer.

Cells were then split into two groups. During primary stress, the control group received a 2-fold dilution using 25°C YPD and was incubated at 25°C for 1 h. The mild heat stress group received a 2-fold dilution using 55°C YDP to reach mild heat stress of 37°C and was incubated at 37°C for 1 h.

To remove primary stress after 1 h, both groups of yeast were collected by centrifugation at 1500xg for 3 min and resuspended in 25°C YPD to a final OD600 of 0.6. Then, 33 μL of cells were then pipetted into 1 row of a 96-well PCR plate that has been pre-warmed to 25°C. The plate was sealed with breathable sealing membrane. Yeast were incubated for 1 h in a gradient thermocycler with temperatures ranging from 42°C to 48°C. This serves as the secondary severe heat stress. Afterwards, 4 μl of a 20-fold dilution in YPD was spotted onto large agar plates and incubated at 30°C for 48 h. Each strain was performed in two biological duplicates.

Site directed mutagenesis

K638 in Aco1 was replaced by alanine to examine whether this lysine residue was necessary for protein function. It was also mutated to glutamine or arginine to mimic acetylated or un-acetylable lysine, respectively. A plasmid expressing the native *ACO1* gene for making mutants was first created. Aco1 gene was amplified by PCR reaction and inserted into pAG36 plasmid at the EcoRi-Spe1site via In-Fusion cloning. The PCR reaction consisted of 12.7 μL of polymerase free water, 5 μL of dNTPs, 0.2 μL of both forward and reverse Aco1 pAG36 primers, 3 μL of 25 mN MgSO4, 1μl of Lithium Acetate genomic DNA, 2.5 μL of KOD polymerase buffer, and 0.4 μL of KOD

polymerase. The reaction consisted of 35 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 45 sec, and elongation at 72°C for 3.5 min.

The three mutant yeast plasmids were generated using Stratagene Quickchange mutagenesis kit using protocols provided by the manufacturer. Mutations were verified with Sanger sequencing from Eurofins Genomics. Mutant plasmids were transformed into DH5α chemically competent *E. coli* cells using Ampicillin as selection marker. Transformation was verified by agarose gel electrophoresis of isolated mutant plasmid digested by *Xba*I and *Eco*RI restriction enzymes. All primers were designed using SnapGene online primer design tool and are listed in table 2.

Table 2. Primers used in this study

Primer Name	Sequence	Notes
ACO1 pAG36 F3	AAACGAGCTCG AATTCATGAAT GACGCCGGTCA CAC	Paired with "ACO1 pAG36 R3" to amplify 3,093 bp fragment of S288c Aco1 locus for cloning into pAG36. Product includes ACO1 gene as well as 462 bp upstream and 261 bp downstream flanking sequence.
ACO1 pAG36 R3	CGCATAGGCCA CTAGTACTGAG TACATAATCAG CAACAATATAA TATAAAAGTTTT GC	Paired with "ACO1 pAG36 F3" to amplify 3,093 bp fragment of S288c Aco1 locus for cloning into pAG36. Product includes ACO1 gene as well as 462 bp upstream and 261 bp downstream flanking sequence.
ACO1 K638A F	GTTAAAAATGT ATATACTGGTG AATACgcAGGTG TTCCAGACACT GCTAGAGATTA CAG	Paired with "ACO1 K638A R" for site directed mutagenesis of lysine 638 to alanine
ACO1 K638A R	CTGTAATCTCTA GCAGTGTCTGG AACACCTgcGTA TTCACCAGTAT ATACATTTTTAA C	Paired with "ACO1 K638A F" for site directed mutagenesis of lysine 638 to alanine
ACO1 K638Q F	GTTAAAAATGT ATATACTGGTG AATACcAAGGT GTTCCAGACAC	Paired with "ACO1 K638Q R" for site directed mutagenesis of lysine 638 to glutamine

	TGCTAGAGATTAC	
ACO1 K638Q R	GTAATCTCTAGCAGTGTCTGGAACACCTTgGTATTCACCAGTATATACATTTTTAAC	Paired with “ACO1 K638Q F” for site directed mutagenesis of lysine 638 to glutamine
ACO1 K638R F	GTTAAAAATGTATATACTGGTGAATACcgAGGTGTTCCAGACACTGCTAGAGATTAC	Paired with “ACO1 K638R R” for site directed mutagenesis of lysine 638 to arginine
ACO1 K638R R	GTAATCTCTAGCAGTGTCTGGAACACCTcgGTATTCACCAGTATATACATTTTTAAC	Paired with “ACO1 K638R F” for site directed mutagenesis of lysine 638 to arginine
ACO1 355 Seq F	CTGTCCACTGTGACCATTTGA	Anneals at the 355 nucleotides of S288c ACO1 gene to sequence the coding strand.
ACO1 639 Seq R	CCATAACATCAACGGCATCA	Anneals at 639 nucleotides of S288c ACO1 gene to sequence the non-coding strand.
ACO1 1350 Seq F	CAATGGGATCGTAGAGATATCAAG	Anneals at the 1350 nucleotide of S288c ACO1 gene to sequence the coding strand.
ACO1 1554 Seq F	TTCATGTTGAAACCACCACA	Anneals at the 1554 nucleotide of S288c ACO1 gene to sequence the coding strand
ACO1 1639 Seq R	TCTGCAGGTGGAGCTTGGTA	Anneals at the 1,639 nucleotides of S288c ACO1 gene to sequence non-coding strand.

Results

Selection of acetylated proteins identified by mass spectrometry for in-depth study

Our lab’s previous proteomics studies identified 596 acetylated proteins in yeast, and 207 of them showed significant changes in acetylation levels (Benjamini-Hochberg corrected p-value <0.05 as compared to the unstressed groups) at any point during a 4-h heat stress time course under 37 °C. It will be a daunting task to experimentally examine all the 596 acetylated yeast proteins in details. Therefore, the very first step of this project

was to narrow down the acetylated proteins to be further investigated. Based on literature reports of their possible involvement in heat stress, the number of acetylated lysine residues in each protein, and the availability of mutant strains in our lab, 43 proteins were selected for this project. The details of these 43 proteins were listed in Table 3. Among these proteins, 29 showed significant changes in acetylation levels during the 4-hour heat stress time course under 37°C, while the rest of 14 proteins did not show notable changes in acetylation during heat stress. It should be noted that proteins with no remarkable changes in acetylation during heat stress may still play a role in heat defense and therefore were included in this project.

These proteins were searched against the *Saccharomyces* Genome Database (SGD) to determine whether they have been reported to play a role in heat defense. A total of 20 proteins have been associated with yeast heat tolerance as reflected in the SGD, and the rest of the 9 proteins have not been reported (Table 3). Based on the lab's proteomic data, the specific lysine residues whose acetylation levels were changed during heat stress were also listed in Table 3.

Table 3. Forty-three proteins were selected for further investigations of their roles in yeast heat defense.

Category	**Protein Name**	**Lysine(s)**	**Reported heat sensitivity**
Protein folding	Cpr6p	K38	yes
	Hsp104p	K182, K218, K380, K470, K482, K559	yes
	Hsp82p	K178, K272, K387, K390, K426, K555	yes
	Ssa1p	K106, K316, K420, K69	yes
	Ssa2p	K243, K248, K455, K504, K86	no
	Ssa4p	K421, K505	no
	Sti1p	K337, K348, K442, K54	yes

Metabolic process	Aco1p	K638	yes
	Adh1p	K226, K315, K8	no
	Adh3p*	K108, K191, K342, K43	yes
	Eno1p	K132, K139, K241, K337, K338, K436, K56, K85	no
	Lat1p	K379	yes
	Pdc1p	K520	yes
	Pfk2p*	K275, K641, K66, K815	yes
	Tdh1p	K115, K184	no
	Tdh3p	K115, K137, K192, K261, K63	yes
	Tkl1p	K285, K303	yes
Transcription	Sin3p	K1417	yes
Translation	New1p*	K546	yes
	Rpl11bp	K166	yes
	Rpl14ap	K93	yes
	Rpl16bp*	K113, K133, K52	yes
	Rpl20ap	K141, K161	no
	Rpl31ap	K5	yes
	Rpl7ap*	K14, K155, K5	yes
	Rpl9ap	K87	yes
	Rpp2ap	K60	no
	Rps1ap	K45, K94	yes
	Rps1bp	K195	yes
	Rps21ap*	K5, K80	yes
	Rps7ap	K44	yes
	Yor051cp*	K3, K10	yes
Biosynthesis	Idp1p	K425	no
	Imd3p*	K261, K426, K519	no
	Pdr16p*	K98	no
Response to stress	Hsp150p*	K335	no
	Lsp1p*	K57	yes
	Stm1p*	K46	no
	Tma19p	K118, K81, K88	yes
DNA binding	Nsr1p	K6	yes
Chromatin remodeling	Htz1p*	K14	yes
	Snf2p	K1498	no
mRNA localization	Hek2p*	K82	no

Proteins were categorized by their major cellular functions and listed alphabetically in each category. The specific lysine residues that showed significant changes in acetylation

were also listed. Twenty-nine out of 43 proteins had statistically significant changes in acetylation as determined by log_2 changes compared to unstressed cells during 4-h heat stress. Of these 29 proteins, 21 have been reported to increase heat sensitivity and 8 proteins have not been reported as determined by the *Saccharomyces* Genome Database (SGD). The remaining 14 proteins did not have statistically significant changes in acetylation during the 4-h heat stress, and they are marked by * next to the protein name. Of these 14 proteins, 9 have been reported to affect heat sensitivity and 5 have not, as determined by the SGD.

Twenty-three proteins with significant changes in acetylation at 37 °C are required for yeast innate thermotolerance

We first aimed to examine if the 29 proteins with significant changes in acetylation during the 4-h heat stress time course at 37 °C are required for yeast innate thermotolerance, which refers to the yeast's ability to acclimate and grow under high temperatures. For this purpose, yeast knockout mutants were cultured at 40 °C for 42 h, and the cell number was determined by OD_{600} every 15 min. The time-dependent changes in OD_{600}, i.e., growth curves, were shown in Figure 1.

As compared to the wild-type yeast strain, yeast knockout mutants affected the growth curve in three different aspects. The first factor was their respective lag time, which refers to the initial periods when yeast strains were adapting before rapidly reproducing. This phase occurred at the beginning of the growth curve, where OD_{600} remained relatively low and constant. The second factor was the rate of exponential growth in which cells were rapidly reproducing. This was determined by the slope of the

growth curve during which OD_{600} was rapidly increasing. The last factor was the final density, which is the maximum population reached. This occurred near the end of the study, where OD_{600} was at its highest and remained constant. The growth curves of the knockout strains were compared to wild type strain based on these three factors and the overall patterns of changes were shown in Table 4.

Table 4. Patterns of changes in yeast growth curves under 40 °C induced by 29 protein knock out

	Lag time	Rate of exponential growth	Final density	Overall effects on innate thermotolerance	gene knockout
Figures 1A	+	+	++	Severe	*tdh1, rpl14a, rpl31a, sin3, aco1, adh1*
Figures 1B-C	+	+	+	Moderate	*idp1, rps7a, pdc1, rpl20a, hsp104, eno1, ssa1, cpr6, ssa4, lat1, tma19, tdh3*
Figures 1D	+	+	-	Mild	*tkl1, rpl11b, snf2, hsp82, nsr1*
Figures 1E	-	-	-	None	*rps1b, ssa2, rps1a, rpl9a, rpp2a, sti1*

+ indicates a significant change in the corresponding factor and – indicates no changes.

++ indicates that the final density determined by OD_{600} was below 0.6.

As shown in Figure 1A, a total of 6 mutants, i.e., *adh1*, *aco1*, *sin3*, *rpl31a*, *rpl14a* and *tdh1*, showed severe defects in innate thermotolerance, as demonstrated by the combination of longer lag time (i.e., OD_{600} reached exponential growth later than control), slower exponential growth rate (i.e., growth curve had a flatter slope than control), and a significantly lower final density (which we defined as an $OD_{600} < 0.6$) in their growth curves. Remarkably, deletion of *SIN3* (encoding a component of a large deacetylase complex) and *ADH1* (encoding alcohol dehydrogenase) completely inhibited yeast growth at 40 °C, i.e., their OD_{600} was unchanged over time (Figure 1A), and

deletion of the *ACO1* (encoding an essential enzyme in the TCA cycle) and *RPL31A* (encoding a component of the 60S ribosome subunit) led to minimal growth at 40 °C, i.e., their OD_{600} increased only slightly at 42 h. Knockout of these four proteins caused the strongest impairment in yeast innate thermotolerance in this study.

As shown in Figure 1B and 1C, twelve mutants showed moderate impairment of innate thermotolerance at 40 °C, as determined by a combination of longer lag time, slower exponential growth rate, and a moderate reduction in final density (arbitrarily determined by 0.6 < OD600 < 0.9). Of note, among these 12 knockout strains, *hsp104* knockout showed the lowest exponential growth rate, and rpl20a showed the lowest final density at 40 °C.

Figure 1D shows that 5 knockouts, including *tkl1, ssa1, snf2, hsp82*, and *nsr1*, caused mild defects in yeast innate thermotolerance at 40 °C, as determined by a combination of longer lag time, slower exponential growth rate and unchanged final density. In other words, their final OD_{600} reached similar levels as the control; however, they experienced both longer lag times and slower growth rate.

Lastly, knockout of six proteins did not affect yeast innate thermotolerance at 40 °C. These knockouts include ssa2, *rps1a*, *rps1b*, *rpl9a*, *rpp2a*, and *sti1*. As shown in figure 1E, the growth curves of these mutant strains almost overlapped with those of the wide-type yeast. They reached exponential growth phase at similar times as the control, did not experience slower growth rate, and their final OD_{600} reached similar densities as the wild-type yeast. Of note, the final OD_{600} densities of the *SSA2* (encoding an ATP binding protein) and *RPS1B* (encoding a protein in the 40S ribosome subunit) were even slightly higher than the wild-type yeast.

In addition to examining the yeast innate thermotolerance at 40°C, we also investigated if knockout of selective proteins impacts yeast growth at normal temperature, i.e., at 30 °C. The growth curves of select mutant strains at 30 °C, including knockouts of *tdh1*, *adh1*, *pdc1*, *spt5*, and *aco1*, were depicted in Figure 1F. These strains showed no significant growth defects at 30° C, indicating these proteins are not essential for the survival of yeast under normal condition.

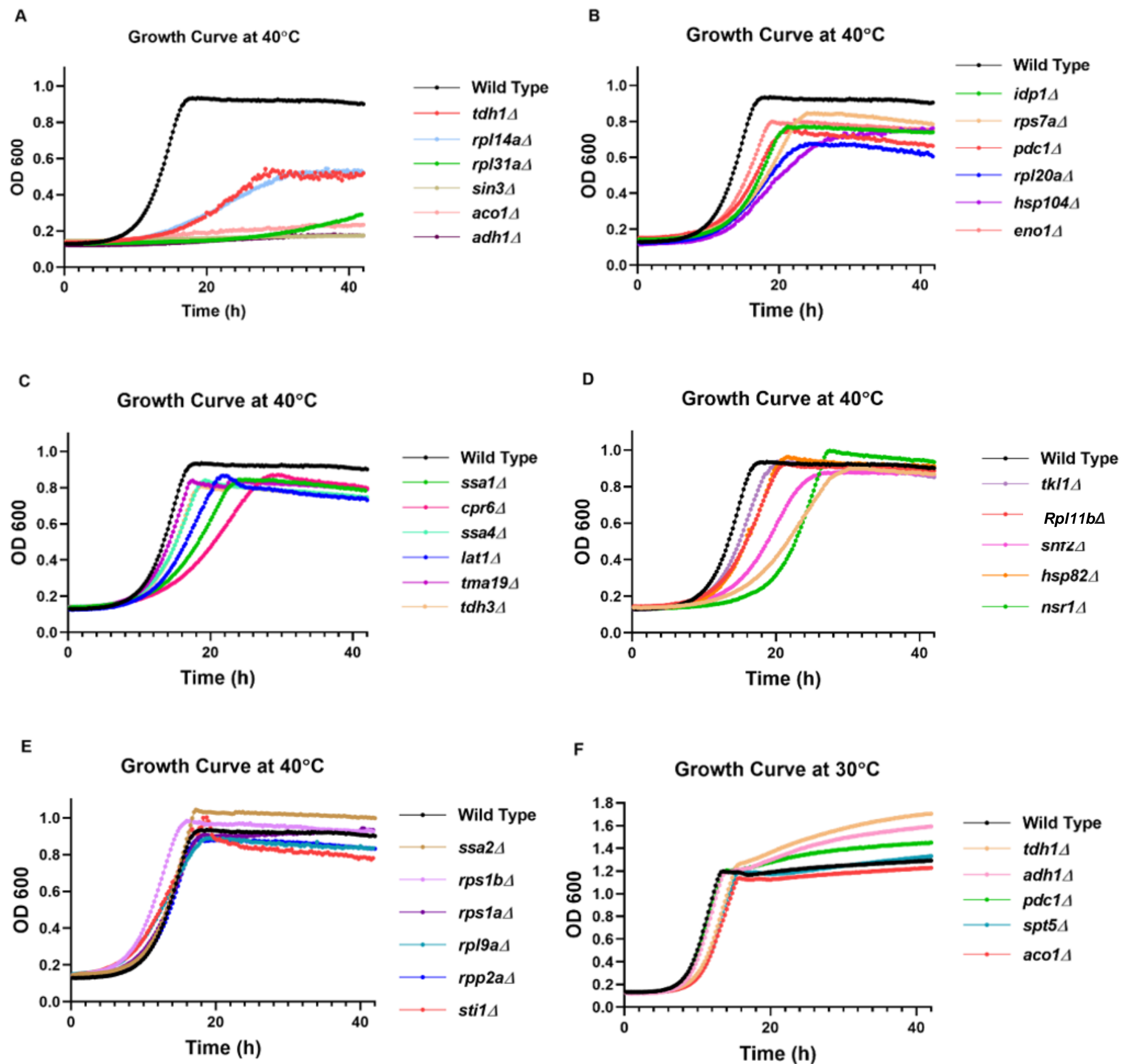

Figure 1. Effects of 29 protein knockout on innate thermotolerance in yeast at 40 °C or normal growth at 30 °C. Wild-type and 29 mutant strains were inoculated at either 40 °C (A-E) or 30°C (F) into an automated microplate spectrophotometer, and the OD_{600} was measured every 15 minutes over 42 h. At 40 °C heat stress, 6 mutant strains showed severe growth defects (A), 12 strains showed moderate growth defects (B and C), 5 strains showed mild growth defects (D), and 6 stains showed no significant changes (E). Five select strains with growth defects at 40 °C showed normal growth at the optimal temperature of 30 °C (F).

Nine proteins with no significant changes in acetylation at 37 °C are required for yeast innate thermotolerance

Fourteen proteins without significant changes in acetylation during 4-h heat stress time course at 37 °C were selected because a large portion of these proteins (9 out of 14) affected yeast heat sensitivity according to the SGD; therefore, it is suspected that their basal acetylation may still play a role in heat defense even if acetylation levels were not changed in statistically significant manners during the 4-h heat stress at 30 °C. To examine if these 14 proteins are required for yeast innate thermotolerance, their knockout mutants were heat stressed at 40 °C for 42 h, and the OD_{600} measured every 15 min. The time-dependent changes in OD_{600} were shown in Figure 2.

Similar to findings in the previous section, fourteen of these yeast gene knockouts affected the growth curve under 40 °C in three aspects. The first was lag time, which is the initial periods when yeast strains were adapting to new environment. The second was the rate of exponential growth, in which yeast are rapidly reproducing. The third is the final density, or maximum population reached near the end of the growth curve. The growth curves of the knockout strains were compared to wild-type strain based on these three factors and the overall patterns of changes were shown in Table 5.

Table 5. Patterns of changes in yeast growth curved under 40 °C induced by 14 protein knock out

	Lag time	Rate of exponential growth	Final density	Overall effects on innate thermotolerance	Gene knockout
Figures 2A	+	+	++	Severe	*htz1, pfk2*
Figures 2B-C	+	+	+	Moderate	*rpl16b, new1, pdr16, yor051c, isp1, adh3, hsp150*

Figures 2D	-	-	-	None	*rpl7a, stm1, imd3, rps21a, h1kr*

+ indicates a significant change in the corresponding factor and – indicates no changes.

++ indicates that the final density determined by OD_{600} was below 0.6.

As shown in Figure 2A, two yeast knockouts caused severe impairment in yeast innate thermotolerance at 40 °C. The first is the deletion of *PFK2* (encoding rate regulating enzyme of the glycolysis and gluconeogenesis cycle), and its mutation completely inhibited yeast growth at 40 °C. The second is the deletion of *HTZ1* (encodes a member of H2A histone), whose mutation drastically increased the lag time, reduced the rate of exponential growth, and remarkably decreased the final density ($OD_{600} < 0.4$) as compared to the wild-type yeast strain.

As shown in figure 2B and 2C, seven knockout mutants including *rpl16b, new1, pdr16, yor051c, isp1, adh3,* and *hsp150* caused moderate defect in yeast innate thermotolerance at 40 °C, as determined by a defect in all three factors including the lag time, rate of exponential growth, and final density ($0.6 < OD_{600} < 0.9$).

Figure 2D shows that five gene knockouts including *rpl7a, stm1, imd3, rps21a*, and *hek2* did not affect the innate thermotolerance in yeast at 40 °C, as these mutant strains did not show significant defects in lag time, rate of exponential growth, or final OD600 density compared to the control yeast. Notably, knockout of *RPL7A* (encodes a ribosomal protein of the 60S subunit) slightly reduced lag time and allowed cells to reach higher final OD_{600} as compared to control.

The growth rate of five select mutant strains were also tested at normal temperature, i.e., 30 °C, and the results were depicted in Figure 2D. While *pdr16* knockout did not affect the growth curve at 30 °C, the other four mutant strains, including

deletion of *pfk2, htz1, spt5* and *yor051c*, showed mild defects in growth curve at 30 °C, suggesting these four proteins are required for survival of yeast under normal conditions.

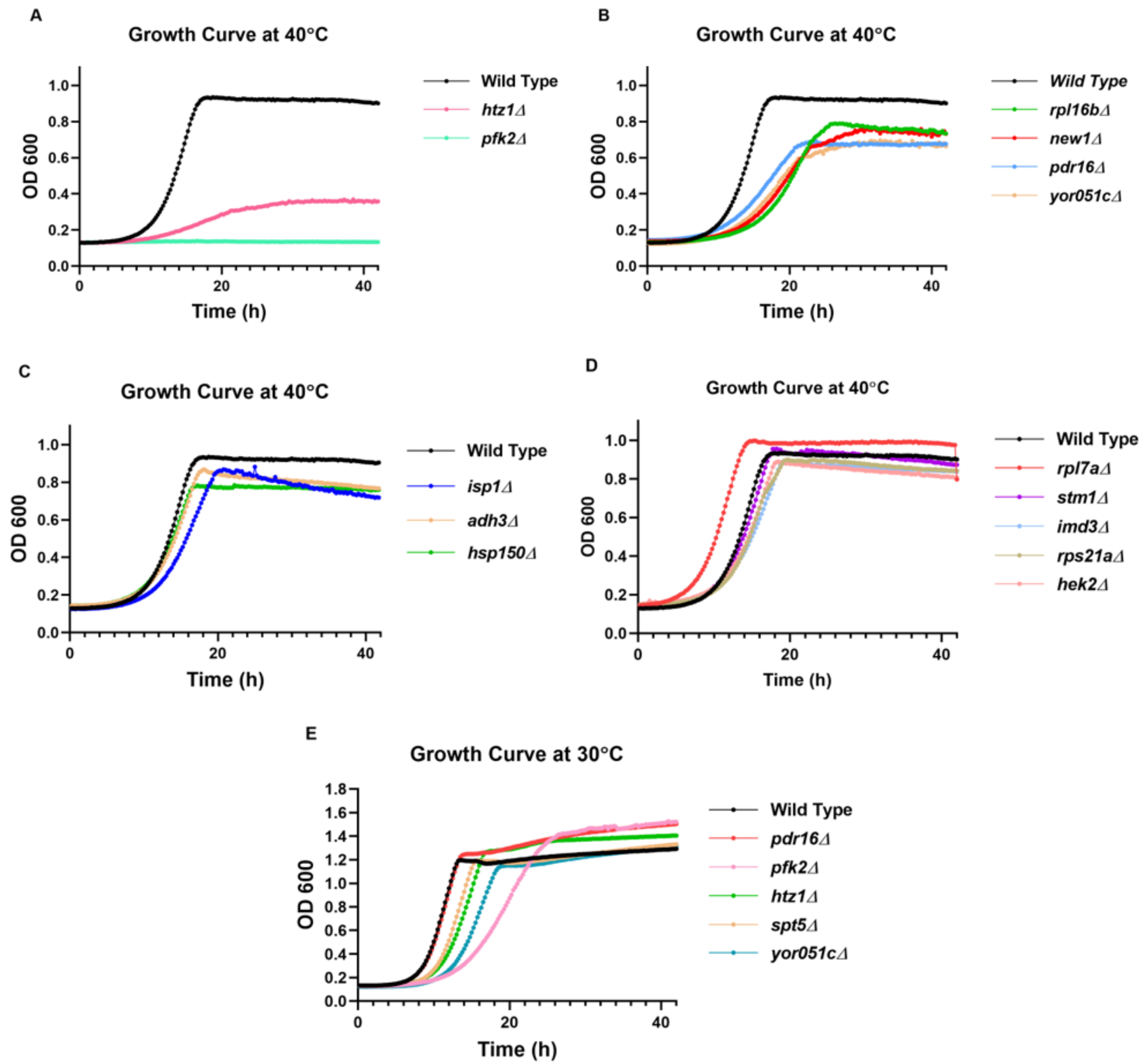

Figure 2. Effects of 14 protein knockout on innate thermotolerance in yeast at 40 °C or normal growth at 30 °C. Wild-type and 14 mutant strains were inoculated at either 40 °C (A-D) or 30°C (E) into an automated microplate spectrophotometer, and the OD_{600} was measured every 15 minutes over 42 h. At 40 °C heat stress, 2 mutant strains showed severe growth defects (A), 7 strains showed moderate growth defects (B and C), and 5

stains showed no significant changes (D). Five select strains with growth defects at 40 °C showed normal or mildly impaired growth at the optimal temperature of 30 °C (E).

Hsp104p is necessary for full innate thermotolerance and fully acquired thermotolerance in yeast

Given the well-established role of Hsp104p in heat stress, it was chosen as a positive control for establishing various assays for exploring the contribution of lysine acetylation in heat defense. Our recent proteomics study found that four lysine residues of Hsp104p (K182, K380, K384 and K205) were acetylated in *S. cerevisiae* during heat stress. Notably, acetylation during heat shock increased for residues K348 and K205. These findings warrant further investigations on the causative relation between Hsp104p acetylation and yeast heat stress. We first examined the role of Hsp104p in yeast innate thermotolerance and acquired thermotolerance using the lab *S. cerevisiae* strain and its *hsp104* mutants. Each strain was first grown to mid-log phase in YPD and split into two groups. One group received a pretreatment of 25 °C mock heat shock for 1 h, and another group received a pre-treatment of 37 °C mild heat shock for 1 h. Both groups were then subjected to twelve severe heat shock temperatures ranging from 42°C to 48°C for 1 h, and cells were spotted onto agar plates to evaluate their growth.

In the mock stress pre-treated groups, *hsp104* mutants did not grow well as compared to the wild type under severe heat stresses at 42°C to 48°C, suggesting that Hsp104p is necessary to achieve full innate thermotolerance. Additionally, when the wild type was subjected to mild heat stress pre-treatment, the yeast became more resistant to subsequent severe heat stresses as compared to the groups that were mock stressed. In

contrast, the *hsp104* mutants were only partially protected by the prior mild heat stress (37°C for 1 h) when exposed to severe heat stresses at 42°C to 48°C. These results indicate that Hsp104p is also required for full acquired thermotolerance.

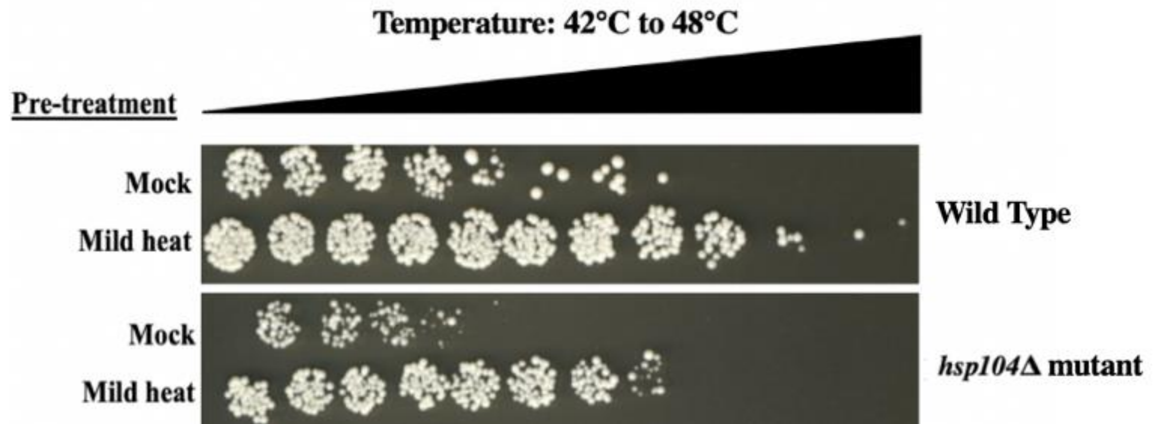

Figure 1. Role of Hsp104p in innate thermotolerance and acquired thermotolerance in yeast. Wild-type and the *hsp104* mutant were subjected to a 1-h mild heat stress (37 °C) or mock stress (25 °C) before being exposed to severe heat stresses at 42 °C to 48 °C for 1 h. Then the yeast were diluted and spotted on agar plates and incubated at 30 °C for 48 h. The figure is representative of at least three independent experiments.

Role of K638 acetylation in aconitase 1 protein (Aco1p) during yeast heat stress

Aco1p has been implicated in yeast heat sensitivity in a previous report [17]. We confirmed that *aco1* knockout decreased the yeast's ability to survive under heat stress at 40 °C (Figure 1A) and this protein underwent significant acetylation changes at only 1 lysine residue (K638) during heat stress (Table 3), making it a good candidate to conduct further study on the role of acetylation in Aco1p during yeast heat stress. We used site-directed mutagenesis to switch K638 to different amino acids to mimic different states of

acetylation. Specifically, K638 was first replaced with alanine to examine whether this lysine residue was essential to yeast survival and growth. Then, K638 was replaced by glutamine or arginine to mimic acetylated or un-acetylatable aco1p, respectively. Three *E. coli* plasmids containing the *ACO1* gene have been prepared and modified for the creation of K638A, K638Q and K638R mutants. DNA sequencing was performed, and the results showed that the desired changes at K638 have been successfully introduced. These plasmids will be transformed into yeast genome in the future to examine the effect of different states of acetylation at K638 on yeast heat tolerance.

Discussion

Living organisms have developed a variety of mechanisms in response to the ever-changing environments such as increased temperatures. Many of these defense mechanisms are conserved across species and yeast has been widely used as a model organism for studying heat stress responses. To cope with heat stress, the yeast can selectively alter the gene and protein expressions and change the metabolism pathways [18]. One of the best characterized changes are the upregulation of molecular chaperones such as the heat shock protein (Hsp) family [19]. However, the mechanism for yeast heat stress responses remains incompletely understood. The purpose of this study is to identify the proteins whose acetylation may serve as a novel mechanism for yeast to deal with heat stress.

Recent proteomics studies have consistently identified many types of PTMs, including lysine acetylation, in the Hsp family, notably the Hsp70 proteins [20], suggesting that PTMs may play a role in yeast heat defense. Our proteomics data showed

that the ubiquitination, phosphorylation [7], and acetylation levels of many proteins were altered when yeast were subjected to heat stress implying that lysine acetylation may be a novel mechanism for yeast to adapt to heat stress. Literature reports on the relation between protein lysine acetylation and heat stress, particularly in yeast, are extremely limited. Though a recent study showed that heat stress caused an increase of histone acetylation in C. elegans, the changes of acetylation in non-histone proteins have not been reported [21]. Our proteomics data provided a high-level overview of the changes of protein lysine acetylation under physiological and heat stressed conditions in yeast, but further studies are needed to understand the role of specific proteins and even specific lysine residues. To achieve this goal, a battery of experiments will be needed, and the results in this study lay an important foundation for future endeavors.

To elucidate the role of lysine acetylation in yeast heat defense, an essential first step is to identify the acetylated proteins whose functions are required for maintaining yeast heat tolerance. If a protein is not required for yeast heat defense, its lysine acetylation will unlikely be an important regulatory mechanism contributing to yeast thermotolerance. This study showed that individual knockout of 11 proteins whose lysine acetylation levels were regulated during 37 °C heat stress did not affect the yeast growth curves under the temperature of 40 °C (Figure 1 and 2), suggesting that lysine acetylation is unlikely an important mechanism for these 11 proteins to help yeast cope with heat stress. In contrast, individual knockout of 32 proteins indeed caused a defect in yeast innate heat tolerance at 40 °C (Figure 1 and 2), suggesting these proteins are good candidates for further studies to examine the role of lysine acetylation in heat stress. Of the 43 proteins selected, over 74% of them showed expected results, i.e., their deletions

caused a defect in the innate heat thermotolerance. Data in this study provide important insights into how to narrow down the list of proteins to be explored in more details regarding lysine acetylation regulating yeast heat stress responses.

Under our study conditions, 5 gene knockouts including *rpl31a*, *sin3*, *aco1*, *adh1* (Figure 1A) and *pfk2* (Figure 2A) almost completely abolished the yeast innate thermotolerance at 40 °C. While these proteins have been reported to be associated with heat sensitivity in yeast in the SGD database, here we provide confirmatory data supporting the role of these proteins in heat defense. Given the strong effects observed here, these 5 proteins may serve as the best candidates for further studies. Additional advantage of these proteins is that they have a limited number of lysine residues, i.e., 1-4, that were acetylated (Table 3). This makes them the ideal candidates for manipulating the lysine residues for the purpose of mimicking the acetylation status. Based on these rationales, we chose Aco1p, Sin3p and
Rpl31ap as our top three choices, as they all have a single lysine residue that was acetylated, and their acetylation levels were significantly changed in our previous proteomics study (Table 3). The other two proteins, Adh1p and Pfk2p, have 3 and 4 lysine residues that were acetylated, respectively, and their acetylation levels were not remarkably regulated during heat stress at 37°C (Table 3), making them the less ideal candidates for further investigations.

Aco1p is a multifunctional protein that is involved both in the tricarboxylic acid cycle and in maintain mitochondrial DNA stability in yeast [22]. Aco2p is another isozyme of aconitase in yeast, but its function remains unclear. In humans and rodents, lysine acetylation at multiple residues of Aco2p activates its enzyme activities [23].

However, lysine acetylation on Aco1p, particularly in yeast, has not been investigated. Our finding that Aco1p knockout almost completely abolished yeast ability to survive under 40 °C are consistent with previous report [17]. Our proteomic data that lysine acetylation at K638 of Aco1p was perturbed during heat stress strongly suggest Aco1p is good protein candidate for exploring lysine acetylation in regulating heat stress. The plasmids for K638A, K638Q and K638R mutation on Aco1p have been successfully prepared in this study, and they will provide important tools to clarify the role of acetylation on K638 in yeast thermotolerance. The work on the other two top candidate proteins identified by this study, Sin3p and Rpl31ap, are in progress.

Our finding that knockout of some proteins affected the yeast's ability to cope with heat stress are consistent with literature reports. For example, our phosphofructokinase-2 (*PFK2*) knockout mutant showing the greatest defect in yeast heat tolerance is consistent with a previous publication, in which a high-throughput genetic screen system was used to identify essential genes for yeast survival under heat stress [17]. Many findings based on high-throughput screening systems have not been confirmed by independent groups, and our data provide the confirmatory evidence supporting the notion that PFK2p is required for yeast to cope with heat stress. Interestingly, lysine acetylation at K3 has been reported to regulate PFK2p activities under hypo-osmotic stress [24]. We speculate that lysine acetylation on PFK2p may play a role in regulating yeast heat thermotolerance and studies are in progress to test this hypothesis.

In addition to confirming reported findings, this study also clarified the role of 14 proteins in yeast innate thermotolerance. As shown in Table 3, a total of 14 proteins

tested in this study has not been reported to increase yeast heat sensitivity in the SGD database. Here we found 14 yeast mutant knockouts, *ssa2, rpp2a, imd3, stm1* and *hek2*, did not affect yeast's ability to survive and grow under 40 °C, indicating they are unlikely to be required for innate thermotolerance. On the other hand, knockout of the rest of 10 genes, particularly *tdh1*, caused notable defects in yeast growth curve at 40 °C, suggesting they are required for the innate thermotolerance. Further investigations are warranted to explore the role of these 10 proteins in regulating yeast heat stress responses.

The findings that Hsp104p knockout compromised both the innate thermotolerance and acquired thermotolerance in yeast (Figure 2) are consistent with previous report [25].We further created the K205A, K358A, K358Q, and K358R, which mimics either acetylated or un-acetylable lysine, mutant strains to examine whether lysine acetylation affects yeast heat stress responses and this work is in progress. While we were working on the creation of these lysine mutant strain, a new publication became available and provided some evidence supporting our original hypothesis [26]. It was found that mutating two or more, but not any single, lysine residues to glutamine to mimic acetylation of Hsp70p not only rendered yeast more sensitive to heat stress but also decreased the yeast's capability to survive after an acute heat stress at 47 °C [26], suggesting that deacetylation of Hsp70p at multiple lysine residues is likely a rapidly mobilized protective mechanism against heat stress in yeast. As the most important protein for yeast to cope with heat stress, Hsp104p interacts with Hsp70p and other proteins to help rescue the damaged proteins during stress [27]. The functional changes of lysine acetylation on Hsp104p and yeast heat stress, to be investigated using our lysine

mutant strains, may provide deeper understandings into the role of acetylation in regulating heat stress.

To summarize, this study identified 23 proteins whose lysine acetylation was perturbed during the 37 °C heat stress are required for yeast to maintain innate thermotolerance at 40 °C. In addition, 9 proteins whose lysine acetylation was unchanged during the 37 °C heat stress are found to be required for yeast to maintain innate thermotolerance at 40 °C. Furthermore, 4 proteins were found to be required for yeast normal growth at 30 °C. Among these proteins, Hsp104p was found to be required for yeast full acquired thermotolerance, and the lysine residues of Hsp104p and Aco1p were successfully switched to the desired acetylation mimic and unacetylable residues using site directed mutagenesis technology. The results in this study will provide useful information and necessary tools for further studies to elucidate the role the protein lysine acetylation in mediating the heat stress responses in yeast.

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