Negative Regulation of Haa1 by Casein Kinase I protein Hrr25 in Saccharomyces cerevisiae

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Negative Regulation of Haa1 by Casein Kinase I protein Hrr25 in Saccharomyces cerevisiae

A Thesis

Submitted by the Graduate Faculty of the
University of New Orleans
in partial fulfillment of the
requirements for the degree of

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In
Biological Sciences

By
Morgan Collins
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Dedication

To my parents, Carol Morgan and Jerry Collins, who always told me it was never too late to pursue my dreams, who have supported me through the good and the bad, and who have always encouraged me to be the best version of who I am. I would not have been able to do this without you. Thank you.
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The journey to get my Master’s degree has been long and convoluted. I feel very fortunate to be afforded the opportunity to continue my education and peruse my dream. This journey would not have been possible if it were not for the generous help and support of many people.

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Abstract

Haa1 is a transcription factor that adapts *Saccharomyces cerevisiae* cells to weak organic acid stresses by activating the expression of various genes. How Haa1 is activated by weak acids is not clear. This study proposes that Hrr25 is an important regulator of cellular adaptation to weak acid stress by inhibiting Haa1 through phosphorylation. *YRO2*, one of the targets of Haa1, shows increase in expression during stationary phase. This increase is due to basal activity of Haa1 and another, unknown, transcription factor. This study proposes that *Gsm1* is another transcription factor that regulates *YRO2* expression in the stationary phase. Finally, the mechanism of regulation of *YRO2* by Haa1 is largely unknown. This study identifies two possible Haa1-mediated cis-acting elements in the *YRO2* promoter.

Key Words: Casein kinase I protein; Hrr25; Haa1; Acetic acid stress response; Saccharomyces cerevisiae; Stationary phase
Introduction

Acetic Acid Stress Response Pathway

Cells employ signaling pathways to adapt to rapid changes in their environment. One of these pathways, the weak organic acid stress response pathway in *Saccharomyces cerevisiae*, is mediated by Haa1, a homolog of copper-regulated transcription factor Ace1 [1,2,3,4,5]. *HAA1* has a paralog called *CUP2* that resulted from a whole genome duplication [6]. Unlike Ace1 and Cup2, Haa1 activation is independent of copper statues of the cell [1]. Yeast has evolved to have different responses to different acids depending on the R group of the acid (R-COOH), and genome-wide transcriptional analyses have revealed many genes whose expression is affected by weak acid treatment [1,2,3,4,7,8,9]. Different organic acids affect the expression of different sets of genes involving a number of transcription factors, including Rim101, Msn2, Msn4, War1, and Haa1.

Weak acids, such as acetic acid and lactic acid, are able to diffuse though the membrane in the undissociated lipophilic form, but dissociates once in the almost neutral pH of the cytosol leading to an intracellular buildup of protons that lower the intracellular pH [4,10]. This decrease in pH, along with perturbations on the lipid organization and membrane permeability, and other changes in cells, is the likely cause of an extended lag phase in yeast cells in response to weak acid stress (reviewed in [11,12]). This lag phase can be further increased with the deletion of *HAA1* from a lag time of 17 hour in BY4741 parental strain in acetic acid, to 60 hours for *haa1Δ* strain under acetic acid conditions [1]. This indicates that Haa1 plays a major role in decreasing lag time by increasing transcription activation of acid stress response genes [1].
Indeed, Haa1 has been reported to be required for resistance in yeast to weak acids such as acetic, lactic, propionic and butyric acids [1,3]. Haa1 has also been reported to be required for the expression of 80% of the genes involved in the acetic acid stress response pathway [4].

Some target genes of Haa1 such as TPO2, TPO3 and YGP1 are known to play a direct role in the acetic acid stress response; other Haa1 gene targets such as YRO2, YER130c, YIR035c, YLR297w, and YPR157w are less well characterized but still show an increase in mRNA levels directly after exposure to weak acids [1,2,3,4,5,13]. TPO2 and TPO3 are two paralogs that also arose from whole genome duplication and encode for two polyamine transporters [6,14]. YGP1 encodes a highly glycosylated cell-wall related secreted protein [15]. TPO2, TPO3 and YGP1 all confer resistance to stresses caused by weak acids [1,2,4]. YRO2 is a gene that codes for a putative plasma membrane protein of unknown function, but is a paralog to protein Mrh1 [5,16,17]. Yro2 shows an increase in expression levels under acetic acid stress, and shows reduced expression in haa1Δ strains [5,16,17]. In addition, yro2Δ cells are hypersensitive to acetic acid indicating that Yro2 does plays a role in the acetic acid stress response pathway [16].

Though the mechanism for activation of each Haa1 targeted gene is still unknown, 55% of genes that show activation by Haa1 under acetic acid conditions have the binding site for Haa1 called the Haa1-responsive element (HRE), identified as (G/C)(A/C)GG(G/C)G, in their promoter regions [13]. Some genes, like YRO2, do not contain an HRE element in their promoters, and are believed to be regulated by an unknown protein that is regulated by Haa1.

Much has been done on identifying target genes of Haa1, but little work has been done on what regulates Haa1. Understanding the weak acid stress pathways is important for multiple reasons. Weak acid is used as a food preservative that inhibits the growth of microorganisms by greatly extending the lag period [1,4,12]. Many food spoilage microbes, including S. cerevisiae,
grow to be resistant to the weak acid used leading to loss of food products [4,12,13].
Understanding the weak acid response pathway better could help the food industry find better ways to preserve food. Weak acids are also used to make products such as plastics, cosmetics, and pharmaceuticals [12]. Some weak acids, such as artemisinic acid, artesunic acid and mycophenolic acid are used as drugs, whereas others, such as 2,4, dichlorophenoxyacetic acid, are used as pesticides [12]. S. cerevisiae is often used as a model to study cytotoxic effects and response to the weak acids used in pesticides and pharmaceuticals [12]. Understanding how genes work in these weak acid pathways can help engineer more efficient industrial production strains.

One of the most important applications for studies involving weak acid pathways is in the biofuel industry. Bioethanol made from lignocellulosic biomass, such as wood and agriculture waste, is a growing alternative fuel source to petroleum based fuels [18]. The ethanol is made using microbial fermentation, in which S. cerevisiae is a major player [18]. Xylose, the sugar found in wood and agricultural products, cannot be naturally fermented by yeast, so a genetically engineered version of S. cerevisiae has been made that is able to break down xylose [18]. However, the ethanol production from lignocellulosic hydrolysates by this yeast cell is limited by high concentrations of acetic acid and other weak acids that are given off as byproducts of the hydrolysate process [16,18,19,20,21]. Yeast strains with increased resistance to acetic acid could be of importance to biofuel industry for this reason. It has been shown that overexpression of Haa1 leads to increased acetic acid resistance, increased expression of Haa1-target genes, and increased yield of bioethanol [16,18,22,23]. Therefore, it is important to understand how Haa1 activity is regulated in response to acetic acid treatment in order to make more efficient biofuel production from lignocellulosic biomass.
Though it is known that Haa1 plays a major role in regulating the acetic acid stress response pathway, little is known of the mechanisms that regulate Haa1. Yak1, a serine/threonine protein kinase in the glucose-sensing system, has been reported to regulate acetic acid stress resistance via Haa1, but the underlying mechanism is still unknown [24]. A recent report suggested that Haa1 phosphorylation may play a role in Haa1 regulation [25]. Haa1 is found throughout the cell in a multiply phosphorylated state. However, under lactic acid stress, Haa1 translocates into the nucleus and displays a decrease in phosphorylation [26]. In the same study, it was also reported that Msn5, a karyopherin important for nuclear export and import of various cargo proteins, mediates nuclear export of Haa1. Thus, Haa1 phosphorylation seems to have a negative effect on its activity. Multiple phosphorylation sites of Haa1 have been revealed through high-throughput studies on protein phosphorylation [26,27,28]. Some of the phosphorylation sites are the consensus sites of protein kinase A and casein kinase I, indicating that these kinases might play a role in regulating Haa1.

In *S. cerevisiae*, casein kinase I has four isoforms, Yeast Casein Kinase 1(Yck1), Yck2, Yck3 and Hrr25, that are involved in regulation of DNA repair, morphogenesis, cell growth, glucose sensing, mitosis, amino acid sensing, and autophagy [29,30,31,32,33,34,35,36,37,38]. Casein Kinase I (CKI) proteins recognize serine/threonine residues in target proteins and are able to phosphorylate acidic substrates such as the milk protein, casein [29,31,39,40]. They are found throughout the cell in the nucleus, cytoplasm, and plasma membrane [39,40]. YCK1 and YCK2 are an essential gene pair needed for cell growth [31,41]; double mutants for *yck1 yck2* show abnormal bud development and impaired cytokinesis [41]. Yck1 and Yck2 also play an important role in transport vesicle formation in morphogenesis [41]. Yck3 has a similar molecular weight and isoelectric point as Yck1 and Yck2, but has a unique structural feature of a
catalytic domain that contains three insertions compared to Yck1 and Yck2 [41]. These insertions do not involve substrate affinity, so are believed to be involved with interactions with regulatory molecules [41]. Yck3 plays a role in negatively regulating vacuole fusion under hypertonic stress conditions [42]. Yck1, Yck2 and Yck3 are often associated with the membrane, whereas the Hrr25 kinase is mainly nuclear and so interacts differently with Yck targets [41]. Hrr25 has an N-terminal kinase domain made of 289 amino acids, and a 100 amino acid C-terminal region that is proline and glutamine rich (50% being proline or glutamine) [30,40]. The Hrr25 kinase domain has been reported to be required for Hrr25 function in vivo, but the function of the proline/glutamine rich region is largely unknown [30]. It is possible the proline/glutamine region plays a role as a structural feature needed for substrate interaction [30]. Hrr25 is known to play a role in the cell cycle, meiosis, and DNA repair [30,40,43]. Strains with hrr25Δ show delays in the cell cycle, slow growth, no sporulation, sensitivity to DNA damage, and large filamentous or extended cell bodies [30,40]. Because of the multiple roles that Hrr25 is known to play in the cell, it is believed to have many substrate targets in vivo [43].

This report identifies the casein kinase I protein Hrr25 as an important negative regulator of Haa1. In hrr25 mutant cells, the activity of Haa1 is increased, which correlates with partial dephosphorylation of Haa1. This study also determines that the C-terminal proline/glutamine rich region of Hrr25 is differentially needed for three different cellular processes. This study provides important insights into the regulation of Haa1 and the cellular responses of yeast to acetic acid.
Stationary Phase

Most experiments involving *S. cerevisiae*, such as those in this study done on with Haa1 and Hrr25, are done with rapidly dividing yeast grown in the logarithmic or exponential growth phases of yeast growth [44,45]. However, most eukaryotic organisms, including single-celled yeast and multi-celled organisms, spend most of their lives in a nonproliferation state referred to as the stationary phase, the quiescent state, or G₀ [44,45,46]. Due to this fact, another study was done to examine how Haa1 regulation worked when cells entered stationary phase.

In 2004, Gray et al. proposed a change to the nomenclature in yeast to term stationary phase as the state of cell saturation of the culture, whereas ‘quiescent’ would refer to the state of cell in the saturated culture [45,46]. In the stationary phase, cells can exist in both the quiescent and nonquiescent stages [47]. Change in cells causing the shift to stationary phase or quiescence occurs when the cells are deprived of an essential nutrient or nutrients such as nitrogen, phosphorous, sulfur, or, most commonly, carbon sources [44,45,46]. In a lab, stationary phase is normally accomplished by growing cells in rich media until the carbon source, such as glucose, is depleted [44,45,46]. During this time, cells go through three phases before reaching stationary phase [44]. The first phase is the exponential phase, where cells grow mostly by fermentation [44]. The next phase, the diauxic shift, occurs when glucose is depleted from the media, leading to the postdiauxic phase, where cells begin to get their energy from respiration [44]. During the postdiauxic phase, cells are extremely slow growing, distinguishing them from stationary phase where cells are mostly in a nonproliferating state [44,45,46].

Quiescent cells in the stationary phase undergo many morphological, physiological and biochemical changes in order to prepare the cell for a prolonged existence in a nutrient-depleted environment [44,45]. In fact, cells in stationary phase are extremely stable for long periods of
time and have been known to be viable for three months or more without added nutrients
to the media [44,45]. Quiescent cells in stationary phase differ from proliferating cell in that they
do not accumulate mass or volume, have a thicker and less porous cell wall, have more rounded
mitochondria, have more condensed chromosomes, and are unbudded, and refractile
[44,45,46,47]. Cells in stationary phase arrest before the Start step of G\textsubscript{1} in the cell cycle due to
the fact that G\textsubscript{1}-cyclins, which are posttranslationally regulated in a cell-cycle- dependent
manner and are needed for the cell to be able to perform Start, do not reaccumulate in nutrient-
starved cells [44]. Quiescent cells also undergo a decrease in transcription and translation; in
fact, the transcription rate for these cells can be 3-5 times lower than rapidly dividing cells
[45,46]. Cells also see a decrease in their mRNA degradation, and protein synthesis during
quiescence [45]. During stationary phase, cells have been reported to have half as much poly(A)
RNA of exponentially growing cells, and protein synthesis falls to less than 10% of that of
exponentially growing cells [44]. Though many genes are highly suppressed during stationary
phase, other genes, such are stress response genes, are highly induced. For example, stationary
phase cells show a high resistance to heat shock and high osmolarity, and genes associated with
these pathways are induced [45,48].

Many scientists believe the physiological and biochemical changes seen in cells in
stationary phase more closely resemble how yeast cells spend most of their lives in the natural
environment [45,46]. In addition, cells in most organisms are in a G\textsubscript{0} state at some point during
their life cycle [47]. This means studying quiescent cells can have applications in many different
fields such as infectious diseases, wound healing, sexual reproduction, stem-cell maintenance,
immunosuppressant therapies, cancer therapies, neurodegenerative disease, aging, etc [45,47].
Studying the mechanisms that cause cells to move into and out of quiescence as well as how
cells maintain the G\textsubscript{0} state can be helpful in many different areas of study.
**YRO2** has been reported to be induced in stationary phase as well as under acetic acid stress [16]. However, results from this study indicate that this induction is not completely Haa1-dependent. Indeed, other targets of Haa1 such as *TPO2* and *YGP1* show little increase or a decrease in expression in stationary phase suggesting that Haa1 is not induced in stationary phase. Therefore, it is possible that basal levels of Haa1 are responsible for *YRO2* induction in stationary phase, but full induction for *YRO2* is facilitated by another transcription factor that is active during stationary phase. This study identifies GSM1 as a possible transcription factor that is also responsible for regulation of YRO2 activity during stationary phase.
Material and Methods

Strains, plasmids, and growth media and growth conditions.
Yeast strains and plasmids used in this study are listed in Table 1 and 2, respectively. Yeast strains were grown at 30°C in YPD medium (1% Bacto-yeast extract, 2% Bacto-peptone, 2% Glucose), YNBcasD medium (0.67% yeast nitrogen base, 1% casamino acids, 2% dextrose), synthetic dextrose minimal media (SD) (0.67% yeast nitrogen base without amino acids, 2% glucose, 2% Bacto-agar), or MM4 medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 0.265% ammonium sulfate, 2% glucose, adjusted to pH 4) with or without 60 mM potassium acetate (adjusted to pH4). SD media was supplemented with leucine, histidine, methionine, lysine and uracil at standard concentrations to cover auxotrophic requirements if required [49].

Yeast Transformation and β-galactosidase assays.
For transformation, yeast cells were grown in YPD media and transformed using high the high efficiency method of Gietz et al [50]. YNBcasD or SD plates with appropriate amino acid supplements were used to select for yeast transformants. For β-galactosidase assays, yeast strains were grown in MM4 media with or without 60 mM acetic acid at 30°C for at least 6 generations to reach OD_{600} 0.5- 0.8 before collection for log phase growth. For comparison of stationary phase, cells were grown at 30°C until they reached an optical density at 600 nm(OD_{600}) of 0.4 (pre-log phase), 0.8 (mid-log phase), 1.6 (late log phase) and 2.4(stationary phase). Two to four independent cultures were grown and assays were carried out in duplicate for each sample. Cells were collected by centrifugation and β-galactosidase activity assays were conducted as described [49]. To summarize, cells were collected and centrifuged for approximately 5 minutes. Cells were then resuspended in breaking buffer [see Cold Spring
Harbor Protocol] and PMSF protease inhibitor was added to solution before cells were lysed using glass beads and vortexing. The cell extract was then removed. To perform the β-galactosidase assay, extract from each sample was added to Z-Buffer [see Cold Spring Harbor Protocol], and put in a 30°C water bath. ONPG substrate was then added to the vial and time monitored until color change occurred. The reaction was then stopped with a Na₂CO₃ solution, and the optical density measured at OD₄₂₀. Next the protein concentration was measured by adding protein extract to diluted Bradford reagent. Optical density was then measured at 595nm. Activity level was then calculated using results from the β-galactosidase assays and Bradford assay [49].

Total cellular proteins were prepared from yeast cells treated with 7.5% β-mercaptoethanol and 1.85N NaOH solution and precipitated with trichloroacetic acid as described [51]. Pellets from TCA precipitation were resuspended in 1x SDS-PAGE loading buffer with 100 mM dithiothreitol (DTT). Protein samples were boiled for 3 minutes before being separated by SDS-polyacrylamide gel electrophoresis. Proteins were then transferred to nitrocellulose membrane for immunoblotting. Haal1 with a C-terminal 3x HA epitope tag was probed with rat monoclonal high affinity anti-HA antibody (3F10, Roche) and followed by goat anti-rat horseradish peroxidase-conjugated polyclonal secondary antibody (Jackson ImmunoResearch Laboratories, Inc., PA, USA). Ilv5 was probed with rabbit anti-Ilv5 polyclonal antibodies. Chemiluminescence images of Western blots were captured using Bio-Rad Chemi- Doc photo documentation system and processed using the Bio-Rad Quantity One software.

Cell extract preparation, phosphatase treatment, and Western blotting. Total cellular proteins were prepared from yeast cells treated with 7.5% β-mercaptoethanol and 1.85N NaOH solution and precipitated with trichloroacetic acid as described [51]. Pellets from TCA precipitation were
resuspended in SDS-PAGE loading buffer with 100 mM dithiothreitol (DTT). Protein samples were boiled for 3 minutes before being separated by SDS-polyacrylamide gel electrophoresis. Proteins were then transferred to nitrocellulose membrane for immunoblotting. Haa1 with a C-terminal 3x HA epitope tag was probed with rat monoclonal high affinity anti-HA antibody (3F10, Roche) and followed by goat anti-rat horseradish peroxidase-conjugated polyclonal secondary antibody (Jackson ImmunoResearch Laboratories, Inc., PA, USA). Ilv5 was probed with rabbit anti-Ilv5 polyclonal antibodies [1]. Chemiluminescence images of Western blots were captured using Bio-Rad Chemi-Doc photo documentation system and processed using the Bio-Rad Quantity One software.

**Generation of an hrr25(E52D) mutant allele.**
A glutamate to aspartate mutation at residue 52 was introduced via site-directed mutagenesis by overlap extension using the polymerase chain reaction as described [43]. Briefly, a primer pair, 5’-gtcaTCTAGACCAGTGCTGAGTCATG-3’ and 5’-TTAAGTATCTGTAGACCGGAGTCATAGTCCAATTGAGGATGTCT-3’ (underline sequence represents restriction sites), were used to amplify an 841-bp HRR25 promoter and the coding sequence to Leu 59 including the E52D mutation using high-fidelity Pfu DNA polymerase. Another primer pair, 5’-
AGACATCCTCAATTGGACTATGACTCCCCGTCTCACAGATACTTTAA-3’ and 5’-gtcaGTCGACGTCTTCTCAGAGGCCCCCTCT-3’ were used to amplify the coding sequence from Arg 45 and a 332-bp sequence downstream of the open reading frame. These two PCR products were purified and subject to overlap extension in the presence of the primers 5’- gtcaTCTAGACCAGTGCTGAGTCATG-3’ and 5’-gtcaGTCGACGTCTTCTCAGAGGCCCCCTCT-3’. The final PCR product carrying an
E52D mutation was digested with XbaI and SalI restriction endonucleases and cloned into XbaI and XhoI sites of the vector pRS415.

**Differential interference contrast microscopy.**

A *hrr25Δ* mutant strain carrying the plasmids as indicated in Figure 6 was grown in SD medium to mid-log phase. For cells indicated in Figure 9, cells were grown in MM4 medium with addition of L-leucine, L-histidine, and L-methionine until they reached OD$_{600}$ of ~0.8 and ~2.4 (mid-log phase and stationary phase). Cells were concentrated by centrifugation and live cells were observed by differential interference contrast microscopy under a Nikon Plan Fluor 100x objective lens on a Nikon Eclipse E800 microscope. Digital images were acquired with a Photometrics Coolsnap fx CCD camera and Metamorph Imaging Software (Molecular Devices, Sunnyvale, CA) and processed using ImageJ software (National Institutes of Health).
Results

Haa1 is negatively regulated by Hrr25

Casein kinase I proteins Yck1, Yck2, and Yck3, do not play a significant role in Haa1-target gene expression.

Global phosphorylation analysis of yeast proteins has revealed that Haa1 is phosphorylated at 15 sites, including three at the casein kinase I motif [27,28]. It has been proposed that Haa1 dephosphorylation correlates with its activation [25]. To determine whether casein kinase I regulates the activity of Haa1, the expression of Haa1-target genes in wild-type and casein kinase I mutant cells were examined. To facilitate the analysis of Haa1-target gene expression, lacZ reporter genes fused downstream of the promoters of two well-established Haa1 targets, TPO2 and YRO2, on a low-copy centromeric plasmid were created. The resultant plasmids encoding TPO2-lacZ and YRO2-lacZ reporter genes were introduced into a BY4741 wild-type strain, and β-galactosidase activity assays on the transformants showed that they were induced by acetic acid treatment in an Haa1-dependent manner (Figure not shown).

To examine the role of each CKI, the expression of TPO2-lacZ and YRO2-lacZ reporter genes in individual casein kinase I mutants were examined. Saccharomyces cerevisiae has four casein kinase I isoforms, Yck1, Yck2, Yck3, and Hrr25, which are each involved in multiple cellular pathways, including DNA repair, ribosome biogenesis, cell morphogenesis, glucose sensing, amino acid sensing, vesicular trafficking, and autophagy [30,31,32,33,34,35,36,38]. Yck1 and Yck2 perform redundant functions and are together essential for cell growth. β-galactosidase activity assays require cells to grow several generations before being collected in the logarithmic phase, so a temperature-sensitive yck1Δ yck2Δ double mutant strain was used to determine whether Yck1 and Yck2 play a role in the regulation of Haa1. At the non-permissive
temperature 37 °C, *yck1Δ yck2*\textsuperscript{ts} double mutant fails to grow [31]. At the permissive temperature 26 °C, the double mutant has been shown to display defects in the phosphorylation of a known Yck1/2-substrate [37]. Thus, the double mutant cells carrying a *TPO2-lacZ* or *YRO2-lacZ* reporter gene were grown at 30 °C, the standard temperature to grow yeast cells. At this temperature in the minimal medium at pH 4 (MM4), wild-type cells divided approximately every 200 min while the isogenic *yck1Δ yck2*\textsuperscript{ts} mutant doubled approximately every 340 min (data not shown), indicating that the activity of the temperature-sensitive *yck2*\textsuperscript{ts} allele was indeed compromised. β-galactosidase activity assays show that *TPO2-lacZ* expression is slightly increased in *yck1Δ yck2*\textsuperscript{ts} mutant cells compared to the wild-type (Fig 1A). In contrast, *YRO2-lacZ* expression is increased by four-fold in the double mutant compared to the wild-type.

Because the *yck1Δ yck2*\textsuperscript{ts} mutant effects on the two Haa1-target genes yielded different results, it was hypothesize that Yck1 and Yck2 play only a small role, if any, in the regulation of Haa1. Consistent with this notion, it was found that the expression of *YGP1*, another target gene of Haa1, was actually reduced by ~2-fold in *yck1Δ yck2*\textsuperscript{ts} double mutant cells compared to wild-type using β-galactosidase activity assays (data not shown).

Next, the expression of these two reporter genes in a *yck3Δ* mutant, which unlike the *yck1Δ yck2*\textsuperscript{ts} mutant, does not have a growth defect, was analyzed. Figure 1B shows that *yck3Δ* has no effect on the expression of *TPO2-lacZ* and *YRO2-lacZ* reporter genes, indicating that Yck3 does not play a significant a role in Haa1 regulation. Together, these data suggest that Yck1, Yck2, and Yck3 do not play important roles in Haa1-target gene expression.
Figure 1. β-galactosidase activity assays of TPO2-lacZ and YRO2-lacZ reporter genes.

β- galactosidase activity assays of TPO2-lacZ and YRO2-lacZ reporter genes in wild-type (LRB341) and isogenic yck1Δ yck2ts double mutant cells (LRB362) (panel A) and wild-type (BY4741) and yck3Δ mutant cells (ZLY4260) (panel B). Cells were grown in MM4 medium without and with 60 mM acetic acid and β-galactosidase activity assays were carried out as described in Materials and Methods.
**Hrr25 negatively regulates the expression of Haa1-target genes.**

Hrr25, the fourth isoform of casein kinase I in the budding yeast, has been reported to be an essential protein in the BY4741 background, and the essential function is linked to maturation of the pre-40S ribosomal subunit [36,52]. To examine a potential role of Hrr25 in the regulation of Haa1, a missense mutant allele of HRR25 was constructed in order to determine lacZ reporter gene expression. It was reported that an hrr25(E52D) mutation (glutamate 52 to aspartate) leads to viable mutant cells [53]. This mutant allele was generated via site-directed mutagenesis by overlap extension using the polymerase chain reaction (PCR). The recombinant plasmid encoding hrr25(E52D) was then introduced into a heterozygous HRR25/hrr25Δ diploid strain.

Transformants were sporulated and tetrad analysis was conducted to generate hrr25Δ mutant cells carrying the plasmid encoding the hrr25(E52D) mutated allele. After three days of growth on YPD medium, hrr25E52D mutant cells exhibited a mild growth defect (Fig. 2A). Surprisingly, after five days of growth, hrr25Δ mutant spores without inheriting plasmid encoding hrr25(E52D) grew into small colonies (Fig. 2B). In a different strain background, hrr25Δ leads to an extreme slow growth phenotype [30]. Our data indicate that Hrr25 is important but not essential for cell viability in the BY4741 background.

TPO2-lacZ and YRO2-lacZ reporter genes were then transformed into both the hrr25(E52D) and hrr25Δ mutants and β-galactosidase activity assays were performed on the transformants. Figure 2C shows that hrr25 mutations lead to increased expression of both reporter genes. Interestingly, hrr25Δ, which has a more severe growth defect than the hrr25(E52D) mutant allele, also leads to a stronger induction of the two lacZ reporter genes. A higher induction of Haa1-target gene expression by the stronger hrr25Δ mutant allele indicates that Hrr25 negatively regulates Haa1 function. The effect of hrr25Δ and hrr25(E52D) mutations on TPO2-lacZ and YRO2-lacZ reporter gene expression in cells grown in the presence of 60 mM
acetic acid, a condition which activates Haa1 were also examined. Figure 2D shows that, like cells grown in minimal medium without acetic acid, hrr25 mutations also increase TPO2-lacZ and YRO2-lacZ reporter gene expression in cells stimulated with acetic acid, with the more severe hrr25Δ mutant allele having a stronger effect. Together, these data indicate that Hrr25 negatively regulates Haa1 function under both basal and induced conditions.
Figure 2. Mutations in HRR25 increase the expression of TPO2-lacZ and YRO2-lacZ reporter genes.

(A) Generation of hrr25(E52D) missense mutant strains via tetrad analysis. BY4743 hrr25Δ::kanMX4/HRR25 diploid cells carrying a centromeric plasmid encoding an hrr25(E52D) mutant allele (pZD359) were sporulated and tetrads were dissected on YPD plate. The picture was taken after 3 days. M, hrr25(E52D) mutants. W, wild-type. (B) Generation of hrr25Δ mutant strains via tetrad analysis of BY4743 hrr25Δ::kanMX4/HRR25 cells. The picture was taken after 6 days. (C-D) The effect of hrr25(E52D) and hrr25Δ mutations on TPO2-lacZ and YRO2-lacZ reporter genes. Wild-type (BY4741) and isogenic hrr25(E52D) (ZLY4467) and hrr25Δ (ZLY4501) mutant cells were grown in MM4 medium without acetic acid (panel C) and with 60 mM acetic acid (panel D). β-galactosidase activity assays were carried out as described.
Haa1 is required for increased expression of TPO2-lacZ and YRO2-lacZ in hrr25(E52D) mutant cells.

Since Hrr25 is implicated in multiple cellular processes, it is conceivable that hrr25 mutations could activate Haa1-target gene expression in a pathway independent of Haa1. To test this possibility, an haa1Δ mutation was introduced into an hrr25(E52D) mutant to generate an hrr25(E52D) haa1Δ double mutant, which was then transformed with plasmids encoding TPO2-lacZ and YRO2-lacZ reporter genes. The resulting transformants, along with those of wild-type, an haa1Δ singe mutant, and an hrr25(E52D) single mutant, were grown in MM4 medium with and without the supplementation of 60 mM acetic acid and β-galactosidase activity assays were carried out. Figure 3 shows that increased expression of TPO2-lacZ and YRO2-lacZ in hrr25(E52D) single mutant cells is largely abolished by haa1Δ, indicating that Hrr25 negatively regulates Haa1 activity.
Figure 3. Increased expression of $TPO2$-$lacZ$ and $YRO2$-$lacZ$ reporter genes due to an $hrr25(E52D)$ mutation requires Haa1.

Wild-type (BY4741) and isogenic $haa1\Delta$(ZLY4043), $hrr25(E52D)$(ZLY4467) and $hrr25(E52D) haa1\Delta$ double mutant (ZLY4637) strains carrying centromeric plasmids encoding a $TPO2$-$lacZ$ or $YRO2$-$lacZ$ reporter gene were grown in MM4 medium without (panel A) and with 60 mM acetic acid (panel B) to mid-logarithmic phase. β- galactosidase activity assays were conducted as described.
Reduced Haa1 phosphorylation in hrr25(E52D) mutant cells.

Haa1 has been reported to be a phosphoprotein and its partial dephosphorylation correlates with its activation [25]. Our data on a negative regulatory role of Hrr25 on Haa1 prompted us to determine whether a mutation in HRR25 affects the phosphorylation state of Haa1. Accordingly, a plasmid encoding a 3xHA tag at the C terminus of Haa1 was generated to facilitate immunodetection of Haa1-HA using anti-HA antibody. It was confirmed that the Haa1-HA fusion under the control of the endogenous Haa1 promoter was functional based on its ability to rescue the acetic acid sensitivity phenotype of haa1Δ mutant cells (data not shown).

The plasmid encoding HAA1-HA was then introduced into haa1Δ single and hrr25(E52D) haa1Δ double mutant cells, and transformants were grown in minimal medium with and without 60 mM acetic acid. Total cellular proteins were isolated, separated by SDS-polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane for Western blotting. Figure 4 shows that Haa1 migrates as diffuse mobility forms in otherwise wild-type cells, consistent with published results [25]. The diffuse migration pattern of Haa1 on Western blots have been attributed to phosphorylation. In hrr25(E52D) mutant cells in comparison to wild-type, the level of slower mobility forms of Haa1-HA is reduced, suggesting that Haa1 becomes partially dephosphorylated in hrr25(E52D) mutant cells. Lactic acid treatment has been reported to lead to Haa1 partial dephosphorylation [25]. However, we failed to detect Haa1 dephosphorylation in cells treated with acetic acid in both wild-type and hrr25(E52D) mutant cells (Fig. 4). Rather, a slight increase in the Haa1-HA protein level was detected due to acetic acid treatment. Since Hrr25 is a protein kinase, our data suggest that Hrr25 may directly regulate Haa1 activity through a physical interaction. To determine whether Hrr25 interacts with Haa1, an immunoprecipitation experiment was carried out. A plasmid was generated encoding a functional Hrr25 fusion
Figure 4. A mutation in *HRR25* leads to reduced phosphorylation of Haa1.

(A) An *hrr25(E52D)* mutation increases Haa1-HA mobility on SDS-PAGE. *haa1Δ* single (ZLY4043) and *hrr25(E52D) haa1Δ* double mutant (ZLY4637) strains carrying a centromeric plasmid encoding HA epitope-tagged *HAA1* were grown in MM4 medium without and with 60 mM acetic acid. Total cellular proteins were prepared, separated by SDS-PAGE, and Haa1-HA was detected by Western blotting. Ilv5, acetohydroxyacid reductoisomerase, was included as loading controls.
with a C-terminal myc tag under the control of its endogenous Hrr25 promoter and coexpressed Haa1-HA and Hrr25-myc in haa1Δhrr25Δ double mutant cells. Immunoprecipitation of Hrr25-myc with anti-myc antibodies failed to recover Haa1-HA in the immunoprecipitates despite multiple attempts. This result suggests that an interaction between these two proteins, if any, must be transient.

The C-terminal region of Hrr25 is differentially required for different cellular processes. Hrr25 has an N-terminal kinase domain (residues 1-295) and a C-terminal 200-residue region including a proline/glutamine-rich domain (residues 395-494) (Fig. 5A). Protein kinases often use regions outside the kinase domain for interaction with their substrates and/or cellular localization. Previously, it has been shown that the fission yeast S. pombe has two Hrr25 homologs: one has a C-terminal proline and glutamine rich domain and the other does not [54]. The former can restore normal cell growth to S. cerevisiae hrr25Δ mutant cells, which are extremely slow growing while the latter does not [54]. To determine whether the C-terminal region of Hrr25 is required for its function in the Haa1 pathway, a plasmid encoding myc-tagged Hrr25 kinase domain (Hrr25(KD), comprising residues 1 to 302) was generated to determine whether expression of HRR25(KD) under the control of the endogenous promoter in hrr25Δ mutant cells can lower TPO2-lacZ and YRO2-lacZ expression to wild-type levels. Accordingly, plasmids encoding myc-tagged full-length Hrr25 or Hrr25(KD) were transformed into hrr25Δ mutant cells carrying TPO2-lacZ or YRO2-lacZ reporter gene. The myc-tagged Hrr25 and Hrr25(KD) were confirmed to be expressed by using immunoblotting with antibody against the myc tag (Fig. 5B). The kinase domain of Hrr25 was expressed at a somewhat reduced level compared with full-length Hrr25. Figure 5C shows that, as expected, expression of full length Hrr25 from a centromeric plasmid in hrr25Δ mutant cells reduced the
Figure 5. The C-terminal region of Hrr25 is partially required for inhibiting Haa1-dependent gene expression.

(A) A schematic representation of Hrr25. (B) Western blot analysis of myc-tagged Hrr25 and Hrr25(KD). hrr25Δ mutant cells (ZLY4479) carrying control vector (pRS415) or centromeric plasmids encoding HRR25-myc (pZL3338) and HRR25(KD)-myc (pZL3361) were grown in MM4 medium and total cellular proteins were prepared. Hrr25-myc and Hrr25(KD)-myc were detected by Western blotting. Pgk1, phosphoglycerate kinase, was included as loading controls. (C) The Hrr25 kinase domain alone is partially functional in mediating Haa1-target gene expression. Strains described in panel B were transformed with centromeric plasmids encoding TPO2-lacZ or YRO2-lacZ. Transformants were grown in MM4 medium without and with 60 mM acetic acid as indicated and β-galactosidase activity assays were carried out as described.
expression of TPO2-lacZ and YRO2-lacZ reporter genes to wild-type levels. Interestingly, expression of Hrr25 kinase domain in hrr25Δ mutant cells significantly reduced the expression of both reporter genes, indicating that the kinase domain of Hrr25 alone is partially functional in inhibiting Haa1.

While growing cultures for β- galactosidase activity assays, we observed that the kinase domain of Hrr25 only marginally improved cell growth of the hrr25Δ mutant cells. A plate assay on the growth of hrr25Δ mutant cells carrying a plasmid encoding HRR25 or HRR25(KD), or an empty vector yielded similar result (Fig. 6A). It has been reported that cell morphology was abnormal in hrr25Δ cells [30]. Therefore, Hrr25’s C-terminal region was examined to see whether it is required for the role of Hrr25 in cell morphogenesis (Fig 6B). As reported, elongated and multi- budded cells were easily detected among hrr25Δ mutant cells. Expression of the kinase domain of Hrr25 in hrr25Δ mutant cells fully restored normal cell morphology, indicating that the C- terminal region of Hrr25 is dispensable for this process. Together, this indicates that the C- terminal region of Hrr25 is differentially required for three different cellular processes: dispensable for cell morphogenesis, important for cell growth, and partially required for Haa1 regulation.
Figure 6. Differential requirement of the C-terminal region of Hrr25 for cell growth and cell morphology.

(A) Hrr25 kinase domain alone is not sufficient to support cell growth. Strains described in Figure 5B were streaked on SD plate and the picture was taken after 3 days of growth at 30 °C.
(B) The Hrr25 kinase domain is fully functional in supporting normal cell morphology in hrr25Δ mutant cells. Strains described Figure 5B were grown in MM4 liquid medium to mid-logarithmic phase. Cells were imaged using differential interference contrast microscopy.
**YRO2 is induced during stationary phase**

**TPO2 and YRO2 show increased expression in stationary phase**

A 2014 study by Takabatake et al shows that YRO2 is induced both by acetic acid stress and by entering into the stationary phase. Haa1 plays a known role in the induction of YRO2 during acetic acid stress, but no link had been made between Haa1 and YRO2 induction during stationary phase. In order to see if Haa1 activity is induced by diauxic shift into stationary phase, the expression of three reporter genes - TPO2-lacZ, YGP1-lacZ, and YRO2-lacZ-of Haa1 were analyzed at four different growth points: early log phase (OD$_{600}$ ~0.4), mid-log phase (OD$_{600}$ ~0.8), late log phase (OD$_{600}$ ~1.6) and stationary phase (OD$_{600}$ ~2.4). Figure 7 shows that in wild-type cells, TPO2-lacZ showed an approximately three-fold increase in expression between mid-log phase and stationary phase. YGP1-lacZ expression showed a 6.5-fold decrease in expression between mid-log phase and stationary phase and YRO2-lacZ showed a ~19-fold increase in expression in the stationary phase compared to the mid-log phase. The increase in lacZ activity in for both TOP2 and YRO2 seen in stationary phase indicates these genes are being activated. However, the decrease in expression of YGP1-lacZ may indicate the induction of the reporter genes is not completely Haa1-dependent.

**Haa1 plays a role in induced expression of YRO2 and TPO2 in stationary phase**

To see if increased expression of TPO2-lacZ and YRO2-lacZ in stationary phase is Haa1-dependent, an haa1Δ strain carrying the plasmids encoding TPO2-lacZ, YRO2-lacZ and YGP1-lacZ reporter genes were grown in MM4 media and collected at the four previously mentioned growth points. β-galactosidase activity assays were then performed (Fig. 7). For cells carrying the plasmid encoding TPO2-lacZ, there was an approximately five-fold decrease in activity levels between wild-type cells and haa1Δ cells in cells grown to stationary phase (Fig. 7A).
Figure 7. β-galactosidase activity assay on TPO2-lacZ, YRO2-lacZ, and YGP1-lacZ reporter genes in wild-type and haa1Δ strains at four different time points.

Wild-type (BY4741) and isogenic haa1Δ(ZLY4043) strains carrying centromeric plasmids encoding a TPO2-lacZ (A), YGP1-lacZ (B), or YRO2-lacZ (C) reporter gene were grown in MM4 medium to early logarithmic (log) phase (OD 0.4), mid-log phase (OD 0.8), late log phase (OD 1.6) and stationary phase (OD 2.4). β-galactosidase activity assays were conducted as described in Material and Methods.
The decrease in activity levels in the *haa1Δ* strain suggests that TPO2 is dependent on Haa1 during stationary phase.

Like *TPO2*, *YRO2-lacZ* in shows a reduction activity level in the *haa1Δ* strain versus wild-type strain for cells grown to stationary phase (Fig. 7C). The almost 11-fold reduction *YRO2-lacZ* in activity seen between stationary wild-type cells and *haa1Δ* cells suggest that Haa1 also plays an important role in the increased expression of *YRO2-lacZ* seen during stationary phase.

Unlike *TPO2* and *YRO2*, *YGP1* does not show a decrease in expression between stationary phase wild-type versus *haa1Δ* mutant cells in stationary phase. In *haa1Δ* cells expressing *YGPl-lacZ*, Figure 7B shows that compared to wild-type cells there is no reduction in YGP1 expression. This suggests that expression of *YGP1* in stationary phase in not Haa1-dependent. The lack of change in expression level for *YGP1* between wild-type and *haa1Δ* cells, in combination with the decrease in activity levels seen between mid-log phase and stationary phase in both cell types suggest that not only is *YGP1* not Haa1 dependent during stationary phase, but also that Haa1 is not actually being activated in stationary phase. When Haa1 is induced during acetic acid stress response, an increase is activity is seen in *TPO2*, *YRO2*, and *YGP1*[1]. If Haa1 were being induced during stationary phase, then an increase in activity levels should be seen in all three reporter genes. The lack of increase seen in *YGP1* activity suggests that though Haa1 is present during stationary phase, it is not activated. The increased levels of activity seen in cells carrying plasmids encoding *TPO2-lacZ* and *YRO2-lacZ* is most likely due to basal level of expression of Haa1 found in the cell during stationary phase.
Induction of YRO2 in stationary phase is due to basal levels of Haa1 and an additional unknown transcription factor

Since YRO2-lacZ expression shows the highest level of induction during stationary phase conditions, YRO2 expression was further dissected. In order to confirm if down regulation of YRO2 reporter gene is due to haa1Δ and no other mutations in the haa1Δ strain, haa1Δ cells were transformed with a plasmid encoding HAA1-myc. Then, wild-type cells, haa1Δ cells, and haa1Δ with HAA1-myc plasmid were also transformed with the YRO2-lacZ reporter gene, and cells from all three strains were then grown in four different conditions: mid-log phase without acetic acid (−AA), mid-log phase with acetic acid (+AA), stationary phase −AA, and stationary phase +AA. The HAA1-myc plasmid in haa1Δ strain was expected to return the YRO2-lacZ activity levels to similar to wild-type levels if the activity change in the haa1Δ strain is based on haa1Δ mutation and no other mutations. The black bar in Figure 8 shows that the haa1Δ +HAA1-myc cells have YRO2-lacZ expression levels that are similar or greater than that of the wild-type YRO2-lacZ levels (white bar). This suggests that induction of YRO2-lacZ is Haa1 dependent under both under acetic acid conditions and stationary phase conditions, and that no other mutations are responsible.

The addition of the HAA1-myc plasmid to haa1Δ, cells recovered the phenotype of YRO2-lacZ activity at or above the levels found in wild-type cells. In fact, YRO2-lacZ activity was recovered to wild-type levels in +AA conditions, but above wild-type level in −AA conditions. Haa1 is known to be active under acetic acid conditions [1,3]. The full recovery of wild-type YRO2-lacZ expression levels seen in +AA is most likely due to the full activation of HAA1-myc under the acetic acid conditions. However, increase in activity of YRO2-lacZ over wild-type levels in haa1Δ +HAA1-myc cells suggests that Haa1 is not fully induced in wild-type cells. This make sense in mid-log phase −AA acid where Haa1 is not supposed to be induced; however, if Haa1 was being activated under during stationary phase, then YRO2-lacZ activity
levels should be similar for both wild-type and haa1Δ +HAA1-myc cells. Instead, the increase in YRO2 expression seen in haa1Δ +HAA1-myc cells over that seen in wild-type cells is possible because wild-type cells, with an extra copy of Haa1 plasmid, will give partial activation of Haa1 pathway, which is sensitive to Haa1 level even in the absence of induction. These results, taken in combination with those found in Figure 7, further suggest that Haa1 is not induced during stationary phase, but instead, increased levels Haa1- targeted genes is due to basal level of Haa1 expression seen in the cell.

In order to further investigate whether Haa1 is induced or not during stationary phase, WT cells with YRO2-lacZ reporter gene was compared under the four different conditions described above (Fig. 8). Since YRO2 is known to be induced by Haa1 under acetic acid stress conditions, then in wild type cells grown to log phase +AA, stationary phase –AA, and stationary phase +AA should show similar activity levels if stationary phase YRO2 induction is due to activation of Haa1. Instead, wild type cells (white bar) grown to stationary phase +AA show an additive phenotype of the YRO2-lacZ activity seen in log phase +AA, and stationary phase –AA. This additive phenotype seen when Haa1 is known to be induced by acetic acid, and induction of YRO2 target seen in stationary phase, suggest that high YRO2 induction seen in stationary phase is due the activation of another transcription factor in acting in addition to Haa1.

**HAA1-GFP does not show increased nuclear localization during stationary phase**

Nuclear translocation a requiring step for activation of Haa1[25]. Under weak acid conditions, where Haa1 is known to be induced, nuclear localization is seen in the majority of cells [25]. To further test the hypothesis that Haa1 is not induced during stationary phase conditions, haa1Δ mutant cells were transformed with a centromeric plasmid encoding
Figure 8. *Haa1-Amyc* plasmid restores wild-type *YRO2-lacZ* levels to *haa1Δ* cells.

*YRO2-lacZ* reporter gene expression for: wild-type (WT) cells grown with and without acetic acid; for *haa1Δ* cells grown with and without acetic acid; and for *haa1Δ* cells with pRS415-ADH1-HAA1-Amyc-2 plasmid grown with and without acetic acid.
GFP-tagged Haa1 or HAA1-HA. HAA1-GFP tag was used to determine the cellular localization of Haa1 and HAA1-HA was used as a control. Cells were grown in –AA conditions to mid-log phase and to stationary phase. Prior research in this lab has shown that the HAA1-GFP fusion construct is functional. Figure 9 shows that the GFP nuclear localization of HAA1-GFP was observed in ~20% of cells in log phase, but no increased nuclear localization of HAA1-GFP is seen in stationary phase cells. Nuclear localization of HAA1-GFP seen under non-induced condition is consistent with previous observations that Haa1 has a basal level of activity. These results further suggest that the activity of Haal is unlikely induced under stationary phase conditions.
Figure 9. Nuclear localization of HAA1-GFP in log phase and stationary phase.

(A) haa1Δ grown with plasmid encoding GFP-tagged Haa1 or HA-tagged Haa1 were grown in MM4 media to mid-log phase and stationary phase. Digital images were acquired with a Photometrics Coolsnap fx CCD camera and Metamorph Imaging Software and processed using ImageJ software. Approximately 20% of cells grown to log phase show nuclear localization of HAA1-GFP, whereas no increased nuclear localization of HAA1-GFP was seen in cells grown to stationary phase. (B) Example of increased nuclear localization seen under acetic acid stress conditions for cells grown to log phase compared to cells under non-stressed conditions for cells grown to log phase.
**GSM1 is a transcription factor that is induced during stationary phase**

To find the other transcription factor that induces \( YRO2-lacZ \) expression during stationary phase, seven different transcriptional activators were examined. During stationary phase, budding yeast shifts from using its preferred glucose carbon source to using a nonfermentable carbon source such as ethanol [55]. \( ADR1, CAT8, GSM1, MSN2, MSN4, RDS2 \) and \( SIP4 \) are all transcription factors for nonfermentable carbon utilization [55]. In order to find the unknown transcription factor that is inducing \( YRO2-lacZ \) during stationary phase, haploid deletion strains of each the seven transcription factors above were obtained from the yeast genome deletion project. Each mutant strain was then transformed with the plasmid encoding \( YRO2-lacZ \) reporter gene and transformants were grown to stationary phase. Figure 10 shows the results of the \( \beta \)-galactosidase assays preformed on these cells. As seen in the figure, \( gsm1\Delta \) shows the greatest reduction in \( YRO2-lacZ \) expression, suggesting Gsm1 might be the transcription factor working with Haa1 to activate the expression of YRO2 during stationary phase.

To see if \( GSM1 \) expression is induced during stationary phase, a \( GSM1-lacZ \) reporter gene was made by fusing the \( GSM1 \) promoter in front of \( lacZ \) in a centromeric plasmid construct. The resultant plasmid was then transformed into a wild-type strain and grown in MM4 media supplemented with or without acetic acid both log phase and stationary phase. Figure 11 shows that \( GSM1-lacZ \) is induced in stationary phase cells grown in the absence of acetic acid compared to the other three growth states. This result suggests that Gsm1 activity is likely to be increased through up-regulation of its expression in stationary phase.
Figure 10. *gsm1Δ* strain cells show decrease in *YRO2-lacZ* expression under stationary phase conditions.

Haploid deletion strains for transcription factors *ADR1, CAT8, GSM1, MSN2, MSN4, RDS2* and *SIP4* were transformed with a plasmid encoding *YRO2-lacZ* reporter gene. Cells were grown in MM4 media until stationary phase (OD$_{600}$ is ~ 2.4). A β- galactosidase activity assay was conducted as described.
**Figure 11.** *GSM1-lacZ* shows is induced stationary phase.

A wild-type strain carrying plasmid encoding the *GSM1-lacZ* reporter gene was grown under four conditions: log phase (OD$_{600}$~0.8) without acetic acid, log phase with acetic acid, stationary phase (OD$_{600}$~2.4) without acetic acid, and stationary phase with acetic acid treatment. β-galactosidase activity was determined as described in materials and methods.
To examine the possibility that Gsm1 works in conjunction with Haa1 in regulating YRO2 expression during stationary phase, a *gsm1Δ haa1Δ* mutant strain was made by introducing a *haa1Δ* mutation into the haploid *gsm1Δ* strain transformed a plasmid encoding the *YRO2-lacZ* reporter gene. Figure 12 shows *YRO2-lacZ* expression in a wild-type, *gsm1Δ, haa1Δ*, and *gsm1Δ haa1Δ* strains under four different conditions: log phase -AA treatment, log phase +AA treatment, stationary phase -AA treatment, stationary phase +AA treatment. Under all four treatments, the *gsm1Δ haa1Δ* double mutant shows an additive reduction in *YRO2-lacZ* expression. Under the log phase -AA treatment, *gsm1Δ* and *haa1Δ* result in 2-fold and 2.5-fold reduction in *YRO2* expression respectively, while the double mutation results in a 4.5-fold reduction in *YRO2* expression. Under stationary phase -AA, *gsm1Δ* shows a 1.4-fold reduction for *YRO2-lacZ*, while *haa1Δ* shows a 15-fold reduction, and the double mutant shows a 35-fold reduction in activity. Under treatment with acetic acid, during which the *HAA1* stress response pathway is active, the *gsm1Δ* single mutant shows only a 1 or 1.2-fold decrease for log and stationary phase compared to the *haa1Δ*, which shows ~6-fold and 13-fold reduction in *YRO2-lacZ* activity in the log and stationary phase respectively. A larger decrease in activity is seen with the *gsm1Δ haa1Δ* strain cells which show a 9-fold and 24-fold reduction compared to wild-type activity in the log and stationary phase cells +AA treatment. The greater reduction seen in the *gsm1Δ haa1Δ* strain compared to *haa1Δ* and *gsm1Δ* single mutation strains suggests that the Gsm1 and Haa1 transcription factors play an independent role in mediating *YRO2* expression.
Figure 12. Gsm1 and Haa1 play an additive role in mediating YRO2 expression.

gsm1Δ, haa1Δ, and gsm1Δhaa1Δ mutant strains, along with wild-type control, were transformed with plasmid encoding YRO2-lacZ reporter gene. Cells were grown with four different treatments: log phase (OD_{600} ~ 0.8, top row) without acetic acid treatment, log phase with acetic acid treatment, stationary phase (OD_{600} ~ 2.4, bottom row) without acetic acid treatment, stationary phase with acetic acid treatment. Note differences in scale due to extreme differences in β-galactosidase activity levels.
Discussion

Haa1 is important regulator that mediates adaptation to weak organic acids in yeast. How Haa1 is regulated is largely unknown. In this report, the casein kinase I protein Hrr25 was identified as a negative regulator of Haa1. The conclusion was based on the observation that the expression of Haa1-target genes is increased in hrr25 mutant cells, an effect that requires Haa1. There are four isoforms of casein kinase I proteins in yeast, and the data indicate that Hrr25 is the primary casein kinase I protein, if not the only one, that regulates the activity of Haa1.

Mutations in HRR25 lead to both activation of Haa1-target gene expression and increased dephosphorylation of Haa1, providing a potential mechanism for the regulation of Haa1. It has been reported that partial dephosphorylation of Haa1 due to lactic acid treatment correlates with increased activity of Haa1 [25]. The results in this report are consistent with these findings. However, there is an important difference between this study and the study by Sugiyama et al. In cells stimulated with acetic acid, which activates Haa1, no partial dephosphorylation of Haa1 was detected. Instead, a slight increase in the Haa1 protein level in cells treated with Haa1 was detected. This discrepancy could be due to the difference of stimulants used in these two studies, acetic acid in this study and lactic acid in the study by Sugiyama et al. [25]. It has been reported that different weak acids affect the expression of different set of genes (reviewed in [12]). Therefore, it is conceivable that yeast cells can differentiate between lactic acid stimulus and acetic acid stimulus, which may account for the difference in Haa1 phosphorylation between the studies. The significance of increased Haa1 protein level is still unclear. Overexpression of Haa1 leads to increased expression of its target genes [22]. When Haa1 is mildly overexpressed from a centromeric plasmid under the control of endogenous promoter in wild-type cells, the expression
of Haa1-target genes is only partially induced (our unpublished result). It is possible that both the Haa1 protein level and Haa1 dephosphorylation might both contribute to activation of the pathway.

It is unclear whether Hrr25 regulates Haa1 through direct phosphorylation since co-immunoprecipitation data did not show a direct physical interaction between these two proteins. However, considering that Hrr25 is a protein kinase, it is possible that an interaction between Hrr25 and Haa1 may be transient. Mutations were generated at the three casein kinase I consensus sites uncovered in global protein phosphorylation studies [27,28], but change in the activity of Haa1 was not seen (our unpublished results). It is possible that other sites may be targeted by Hrr25 or that combinations of phosphorylation sites are required for the regulation of Haa1.

Msn5 has been reported to be required for Haa1 nuclear export. For many nuclear cargo proteins, including Pho4, Aft1, HO endonuclease, Mig1, and Crz1 [26,56,57,58,59,60,61], their phosphorylation is required for both an interaction with Msn5 and nuclear export. Therefore, Haa1 phosphorylation may play a similar role in its nuclear export. Future work will be directed towards the identification of Haa1 phosphorylation sites that may be required for nuclear export and interaction with Msn5.

Hrr25 has a 200-residue C-terminal region including a proline and glutamine rich domain. Data suggest that the Hrr25 sequence outside of the kinase domain is differentially required for three different cellular processes. Despite high similarity in their kinase domains, the four casein kinase I proteins have no significant sequence homology in their C-terminal regions. Both Yck1 and Yck2 have a glutamine rich domain at their C-terminal regions while the C-terminal region of Yck3 is enriched in serine and asparagine residues. Yck1, Yck2 and Yck3 all have a cys-cys dipeptide at their C-termini, which is required for palmitoylation and their
subsequent membrane targeting [62,63,64,65,66]. The involvement of Hrr25 in multiple cellular processes is also reflected in its cellular localization. It has been reported that it localizes in the nucleus, spindle pole bodies, the bud neck, the septin ring, the Golgi, and the P-bodies [67,68,69]. Different sequence elements of the C-terminal region of Hrr25 are likely to be required for interaction with different partners in various cellular processes or different cellular localization. This study found that the C-terminal region of Hrr25 is critical for interaction with target protein(s) that is important for cell growth. However, the C-terminal region is completely dispensable for cell morphogenesis, which could be related to its function and localization to bud neck and/or septin rings. The role of the C-terminal region in the regulation of Haa1 is complicated by the fact that the kinase domain alone is expressed at a lower level compared to the full-length protein (Fig. 5B). The kinase domain itself, even at a reduced level, can partially complement hrr25Δ in mediating the expression of Haa1-target genes, suggesting that the C-terminal region of Hrr25 is only partially required, if not dispensable, for Haa1 regulation. The mechanism behind the reduced expression level of the kinase domain itself is still unclear. Construction and characterization of fine, nested truncation alleles in the C-terminal region of Hrr25 will provide valuable insights into the role of its C-terminal domain in multiple cellular processes.

Most eukaryotic cells spend their lives in a state nonproliferation of stage of G0 [44,45,46]. In yeast cells, this phase occurs when the yeast is deprived of an essential nutrient, such as a carbon source[44,45]. The depletion of a carbon source causes the yeast cells to go through a diauxic shift resulting in a saturation of the culture known as stationary phase[45]. Once in stationary phase, many cells survive in the quiescent or G0 state for long periods of time until the essential nutrient source is reintroduced and the cell can continue with the cell cycle [44,45,47]. During stationary phase, transcription and protein synthesis are severely reduced in
the cell [44,45]. However, some genes, such as those involved in stress response pathways, show an increase in activity [16,45]. Results in this study show that YRO2, a Haa1 gene target, is induced during stationary phase, but that Haa1 itself is not induced. This model is based on the marginal induction of TPO2, and decrease in expression of YGP1 seen during stationary phase. TPO2 and YGP1 are also Haa1 gene targets, and both show an increase in expression when Haa1 is induced under acetic acid stress response. The lack of significant increase seen in TPO2 and the decrease in expression seen in YGP1 suggests that Haa1 is not induced during stationary phase. In further support this idea, similar levels of expression for YGP1-lacZ are seen in both wild-type cells and haa1∆ mutant cells during stationary phase (Fig. 8), suggesting that Haa1 is not being induced. GFP localization data suggests that Haa1 is not localized to the nucleus during stationary phase, but is instead found throughout the cell. Since translocation is a required step for activation of Haa1, the lack of nuclear localization seen in stationary phase also suggests that Haa1 is not fully activated in this state, but instead has only a basal level of expression. Therefore, increased activity of YRO2 seen during stationary phase is most likely due to basal levels of Haa1 expression.

Since it is likely Haa1 is not being induced under stationary phase, it is possible that another transcription factor is being induced under stationary phase conditions and is working in conjunction with Haa1 to induce YRO2 expression. Indeed, Figure 8 shows that YRO2-lacZ expression shows an additive increase in cells grown both with acetic acid and in stationary phase. This additive increase in expression suggests that another transcription factor, in addition to Haa1, is working to regulate YRO2 activity during stationary phase. This study found that GSM1, Glucose starvation modulator 1, a transcription factor involved with transcriptional regulation of the respiratory pathway and energy metabolism, is induced during stationary phase.
Data from Figure 12 further suggests that Gsm1 is a transcription factor that is working in conjunction with Haa1 to induce YRO2 during stationary phase. The gsm1Δhaa1Δ in yeast shows a 35-fold decrease in YRO2-lacZ expression during stationary phase without acetic acid. Still, YRO2-lacZ activity is not completely abolished. It is possible that YRO2 in stationary conditions is also being regulated by yet another transcription factor in combination with Gsm1 and Haa1. More work is needed to further investigate YRO2 regulation.

The function of Yro2 is largely unknown, but the protein has shown to be predominantly located to the plasma membrane [16]. It is a homolog of the heat shock protein HSP30 and has been shown to be induced under heat shock [16,73]. Since Yro2 does not appear to play a role in metabolism during the acetic acid stress response but instead localizes to the plasma membrane, it is possible Yro2 plays a role in changing the composition of the plasma membrane to make it more resistant to environmental stresses [16]. Indeed, YRO2 shows an increases in expression during both heat shock, and stationary phase where membrane composition is changed [48]. Cells in stationary phase are known to be more heat shock-resistant than cells going through the rapid growth found during log phase [48]. Since neither Gsm1 nor Haa1 are associated with heat shock resistance in yeast, it possible any additional regulatory factor(s) for YRO2 might be involved in regulation of the heat shock pathways. Further research would need to be done to investigate this theory.

Findings from this study identify a previously unknown regulator of Haa1. Though Haa1 targets have been widely studied, this is the first study to report a specific negative regulator of Haa1. This finding has application in the biofuel industry and could help engineer more efficient acetic acid-resistant strains that would help in biofuel production. This study also identified a possible regulator of YRO2 during stationary phase conditions. YRO2 plays an unknown role in the cell; understanding more about its regulation and targets could help to identify its function in the
future. Cell studies on stationary phase yeast cells are also important because stationary phase cells more closely resemble cells found in the human body than exponentially growing cells.

Understanding of how regulation in stationary phase yeast cell works could help with understanding of how regulation works in other eukaryotic cells, which in turn has applications in many biomedical fields.
References


### Appendix

#### Table 1: *S. cerevisiae* strains used in this study

<table>
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<th>Strain</th>
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<td>[74]</td>
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<td>LRB362</td>
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<td>Table 2: Plasmids used in this study</td>
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### Table 3: Primers used in this study

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</table>
Vita

Morgan Elizabeth Collins was born in Mount Pleasant, South Carolina to parents Carol Morgan and Jerry Collins. She attended high school at St. Vincent’s Academy in Savannah, Georgia and graduated in 2004. In 2008, she received her Bachelors of Science from Shorter College in Rome, Georgia with a major in Biology and a minor in Chemistry. In 2012, she received her post-baccalaureate teaching certification from Armstrong Atlantic State University in Savannah, GA. She taught Biology for three years at Robert W. Groves High School in Garden City, Georgia, before moving to New Orleans in the Fall of 2014. She was admitted into Biological Sciences Graduate Department in the Spring of 2015, and joined the research group of Dr. Zhengchang Liu in order to pursue her Masters of Science degree. Her goal is to continue her education and pursue a career in academia.