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# Natural Variation in Yeast Stress Signaling Reveals Multiple Paths to Similar Phenotypes

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Natural Variation in Yeast Stress Signaling Reveals Multiple Paths to Similar Phenotypes

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**Abstract**

Natural environments are dynamic, and organisms must sense and respond to changing conditions. One common way organisms deal with stressful environments is through gene expression changes, allowing for stress acclimation and resistance. Variation in stress sensing and signaling can potentially play a large role in how individuals with different genetic backgrounds are more or less resilient to stress. However, the mechanisms underlying how gene expression variation affects organismal fitness is often obscure.

To understand connections between gene expression variation and stress defense phenotypes, we have been exploiting natural variation in *Saccharomyces cerevisiae* stress responses using a unique phenotype called acquired stress resistance, where cells that are pretreated with a sub-lethal dose of stress survive lethal high doses of stress. This response is observed in organisms ranging from bacteria to humans, though the specific mechanisms governing acquisition of higher stress resistance are poorly understood.

This dissertation explores the mechanistic underpinnings of natural variation in yeast stress responses and resistance, thus identifying strategies that I argue are likely conserved across diverse organisms. We first show that a commonly-used lab strain fails to acquire oxidative stress resistance when pretreated with ethanol, while a wild oak strain can. Using genetic mapping, we provided new evidence that Hap1p, heme-dependent transcription factor, was responsible for variation in this trait through the regulation of *CTT1*-encoding cytosolic catalase T— hydrogen peroxide scavenging enzyme. Interestingly, the lab strain can still acquire higher hydrogen peroxide resistance when pretreated with salt, and this cross protection requires *CTT1.* To determine whether *CTT1* was universally required for acquired hydrogen peroxide resistance, we tested over a dozen diverse yeast strains and found a wide range of catalase dependency suggesting that acquired hydrogen peroxide resistance arises through multiple anti-oxidant defense strategies. We used transcriptional profiling to identify potential

signaling pathways and transcription factors that regulate differentially-expressed modules of genes during salt or ethanol stress and potential compensatory oxidative stress proteins.

These experiments highlight the power of using yeast natural variation to uncover novel aspects of conserved signaling networks and stress defenses, providing a framework for understanding the mechanistic underpinnings of natural variation in other organisms.

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**Dedication**

Dedication In loving memory of Sandra Scholes and Vernon Scholes. I love you both so much. Grandma, thank you for all our phone conversations, your guidance, and pushing me to succeed. I, partially, owe my love of science to you and grandpa!

To Dad, thank you for never giving up on me and for your continuous love and support I wouldn’t be who I am today without your endless love.

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**List of Published Papers**

**Chapter 2:**

Stuecker TN, Scholes AN, Lewis JA. 2018. Linkage mapping of yeast cross protection connects gene expression variation to a higher-order organismal trait. *PLoS Genetics.* 14(4):e100733

**Submitted**

Supplemental 1:

Scholes, AN**,** Pollock, ED, and Lewis JA. A Wild Yeast Laboratory Activity: From Isolation to Brewing. Preprint available on Biorxiv at doi: https://www.biorxiv.org/content/10.1101/698589v1

Supplemental 2:

Scholes, AN and Lewis JA. Comparison of RNA Isolation Methods in Yeast on RNA-Seq: Implications for Differential Expression and Meta-Analyses. Preprint available on Biorxiv at doi: https://www.biorxiv.org/content/10.1101/728014v1

# Chapter 1 Introduction

## 1.1 Stress

Yeasts are essentially everywhere (1, 2). In fact, yeast have been shown to live on plants (1), oak trees (3), soil (1, 4), grapes (5), damaged fruits and berries (5), and insect guts (6-8). With natural environments being dynamic, organisms must be able to recognize and respond to various environmental conditions throughout their life. While multicellular organisms have specialized organs and tissues to assist with this, microorganisms, such as yeast, have evolved mechanisms for adapting to diverse environmental conditions. During stress, cells must be able to switch between cell growth, survival, and death. One way to survive stress is to alter gene expression and many studies have been conducted on how model organisms, like *Saccharomyces cerevisiae,* respond to different stress conditions (9-22). There are many similarities in the molecular responses to stress in yeast and mammalian systems (23-28).

### 1.1.1 Response to osmotic stress

Cells require sugars to survive, but when a cell is exposed to a plethora of sugars, like a freshly crushed grape, it is being exposed to osmotic stress. Osmotic stress occurs when cells are exposed to either increased or decreased concentrations of salt or sugars. Increased concentrations (hyperosmotic) outside the cells will immediately cause cells to lose intracellular water causing cell shrinkage. Decreased salt or sugar concentrations (hypoosmotic) outside the cell lead to water uptake causing cells to swell with the potential to burst, thus cells have evolved ways to combat changes in osmolarity to maintain cellular homeostasis (29). Osmotic stress has multiple effects on cells: morphological, transport, and metabolic adjustments (30). For cells to react to osmotic stress, first they must sense the stress. Highly conserved mitogen-activated protein kinase (MAPK) pathways can be activated by both intracellular and extracellular signals in response to osmotic stress. In humans, osmoregulation occurs via p38 and JNK kinase pathways (31), which have homologs in yeast (32).

In *S. cerevisiae*, the high osmolarity glycerol (HOG) MAPK pathway is used for osmoregulation. The HOG pathway consists of two branches (Figure 1.1). Sln1p branch is activated by hypoosmotic conditions, while the Sho1p branch is activated by hyperosmotic conditions. During osmotic stress Pbs2p (MAPKK) will phosphorylate Hog1p (33), triggering rapid translocation from the cytoplasm into the nucleus (34). Once in the nucleus, Hog1p interacts with transcription factors such as Msn2p, and paralog Msn4p, even though only a small fraction of genes are exclusively controlled by the HOG pathway (10, 35, 36). Msn2p and Msn4p are general stress transcription factors (11, 37, 38) activated by Hog1p during osmotic stress. Previous research has shown that osmo-regulated gene expression diminished in the *MSN2/4* double mutant were also strongly diminished in the *HOG1* mutant, while genes that were strongly diminished in the *HOG1* mutant were unaffected by the *MSN2/4* double mutation (35), suggesting the HOG pathway uses multiple transcription factors for osmotic stress response. Another transcriptional activator activated during osmotic stress is Skn7p. Skn7p has been shown to have genetic and physical interactions that link it to the HOG pathway (39, 40). Several observations indicate that Skn7p is responsible for cell swelling, responses to hypoosmotic signaling, and stress responses. As Skn7p is non-essential, it was intriguing that overexpression of Skn7p is lethal producing swollen cells (41). Skn7p is responsible for the activation of genes involved in cell wall assembly and seems to function opposite to the hyperosmotic branch of the HOG pathway.

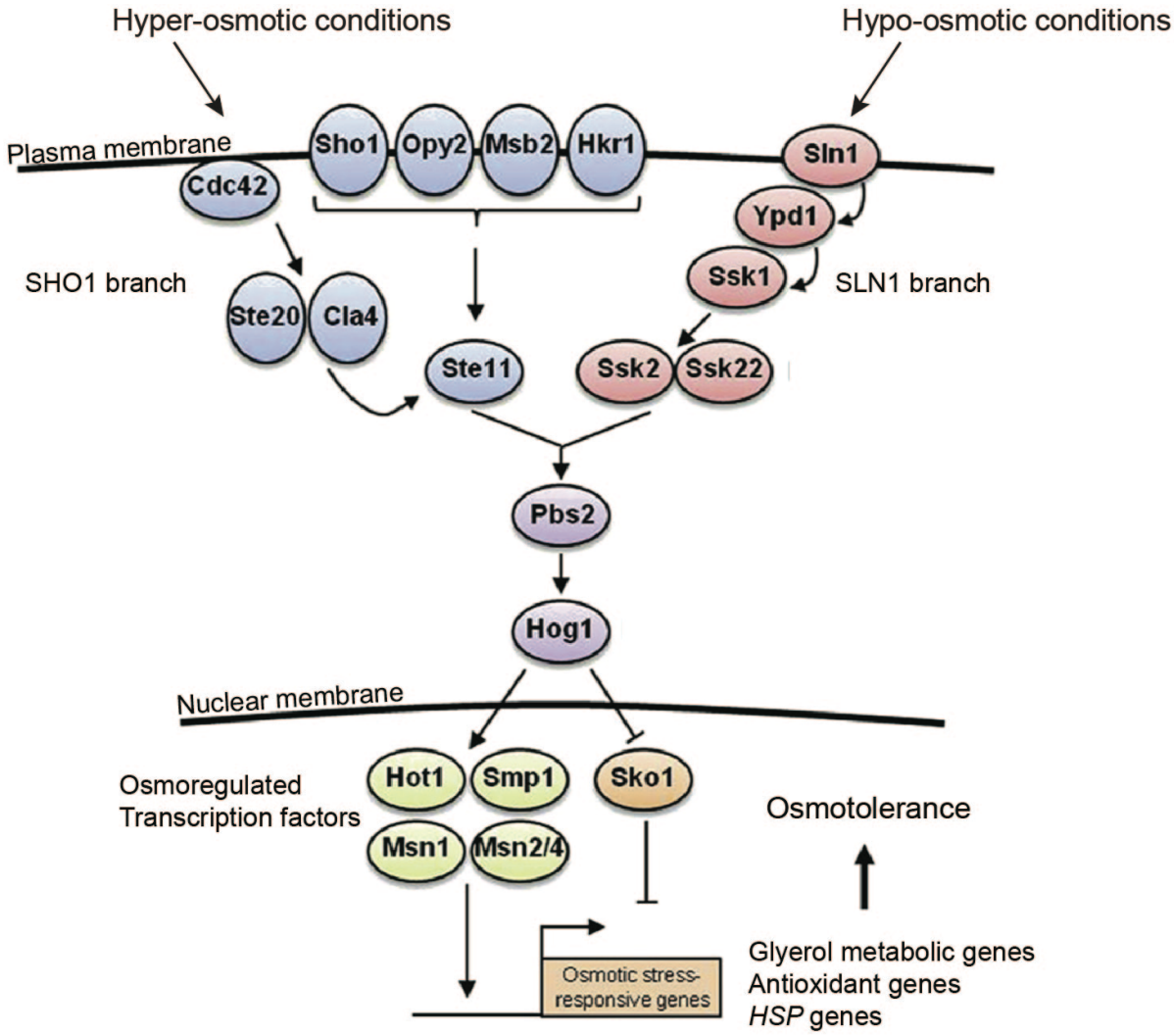


**Figure 1.1 *S. cerevisiae* HOG pathway.** HOG pathway consists of two branches. Modified from (42).

### 1.1.2 Response to salt stress

Another stress that can cause issues with homeostasis is salt stress. Increased external salt stress concentrations cause the cells to lose water, similarly to that which occurs during osmotic stress. When cells are exposed to salt it is both an osmotic and ionic stress. The gene expression changes induced by osmotic and salt stress are very similar (11, 35).

In yeast, while some ions are required for growth ($K^+$), there is no requirement for sodium (43). Interestingly, $K^+$ is the preferred intracellular cation, even though $Na^+$ is more prevalent in nature. *S. cerevisiae* can tolerate salt concentrations up to 2M NaCl (44). Growth in elevated NaCl concentrations changes the intracellular $Na^+/K^+$ ratio and high ratios are toxic. If ionic levels are too low this causes damage to the cell and inhibits growth (43). $Na^+$ efflux is mediated by the *ENA* locus, a plasma membrane $Na^+$ ATPase (45). Cells exposed to mild salt stress showed induction of *ENA1* via the HOG pathway (46). Hog1p is responsible for the proper induction of ~75% of the salt defense genes (36). Many of the genes induced during salt have the stress response element (STRE) in their promoter, therefore they are a target of the general stress transcription factors Msn2/4p (47).

### 1.1.3 Response to ethanol stress

There are two types of fermentation: lactic acid and ethanol. Mammalian cells can ferment sugars to lactate, in muscle and red blood cells, while yeast ferment sugars to ethanol. Yeast have been exploited for many years for their ability to produce ethanol (48). One of the most common fermentation stresses that yeast encounter is ethanol (17) and ethanol is toxic in high enough concentrations (49). Yeast had to develop appropriate mechanisms to counter the damages caused by ethanol. Ethanol can affect cells in multiple ways; increase membrane fluidity, denature proteins, and damage DNA (50, 51). One way cells counteract ethanol stress is by inducing genes involved in protein folding and membrane stabilization (17, 52). In fact, ethanol stress appears to induce expression changes similar to that of the response to heat

stress (53). Ethanol induces heat shock proteins (54) via Hsf1p and general stress transcription factors, Msn2/4p (55), HSPs aid in refolding proteins that may be denatured during ethanol stress.

### 1.1.4 Response to oxidative stress

Hydrogen peroxide is one of the most commonly occurring reactive oxygen species (ROS), either as a by-product of metabolism or from the environment (56). ROS can have multiple negative impacts depending on the molecule. Oxidative stress is thought to be involved with many human diseases, such as neurodegenerative disorders (57-59), cancer (60, 61), heart disease (62, 63), and ischemic stroke (64-66). ROS can cause protein aggregation or fragmentation (67), lipid peroxidation (68), DNA damage (69), and improper disulfide bond formation (70). During oxidative stress, there are multiple ways to cope with ROS, which is thought to help buffer the reducing environment (71-73). A common peroxide scavenging enzyme found is catalase. Catalase, a key enzyme responsible for decomposing hydrogen peroxide into water and oxygen, is one of the most efficient enzymes in the cell (74-76). Two of the other systems utilized are glutathione-, one of the most abundant thiols in the cell, and thioredoxin-dependent peroxidases (Figure 1.2) (77-79). ROS will oxidize glutathione and other thiol-specific antioxidants, followed by reduction by the thioredoxin and glutaredoxin systems to regenerate reducing activity. Catalase and glutathione are major peroxide scavengers (80) and are highly conserved (81, 82).

Ecologically, when sugar concentrations get low, yeast can switch to respiring the recently produced ethanol resulting in the generation of ROS (83). During oxidative stress in yeast, there are more than 900 genes induced (10, 11). Transcription factor Yeast AP1 (Yap1p) along with Skn7p are important for the majority of the hydrogen peroxide oxidative stress response (84), Figure 1.3. Yap1p is essential for its role in oxidative stress and in the absence of *YAP1* cells showed hypersensitivity to hydrogen peroxide (85). Yap1p is responsible for the

activation of glutathione and thioredoxin peroxidases (71, 86, 87). Skn7p has been shown to have genetic and physical interactions with Yap1p (87-89). Along with the HSPs and cell wall assembly proteins, Skn7p activates genes involved in oxidative stress (30, 87, 89). Oxidative stress can cause cell wall damage and as Skn7p plays a role in activating genes involved in cell wall assembly, this supports the idea that Skn7p assists Yap1p in oxidative stress response (30, 87). Msn2/4p and Hsf1p are also implicated in oxidative stress. In a double *msn2/4* mutant, cells showed hypersensitivity to hydrogen peroxide. Msn2/4p activates cytosolic catalase t (Ctt1p) and overexpression of Msn2p showed higher oxidative stress resistance (90). Hsf1p is co-regulated with Skn7p under hydrogen peroxide stress to aid in the expression of HSPs (91).

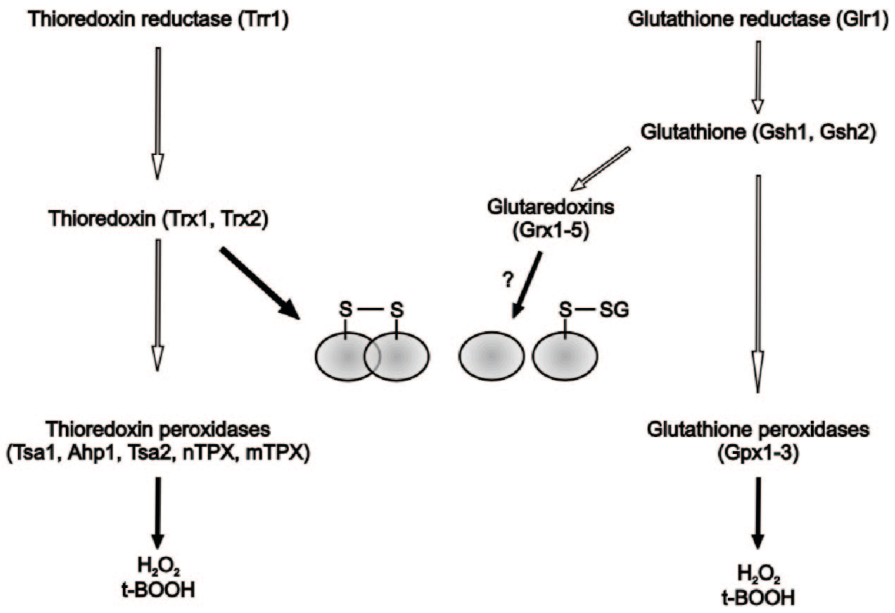


**Figure 1.2 Schematic of thioredoxin and glutathione peroxidases.** Modified from (84).

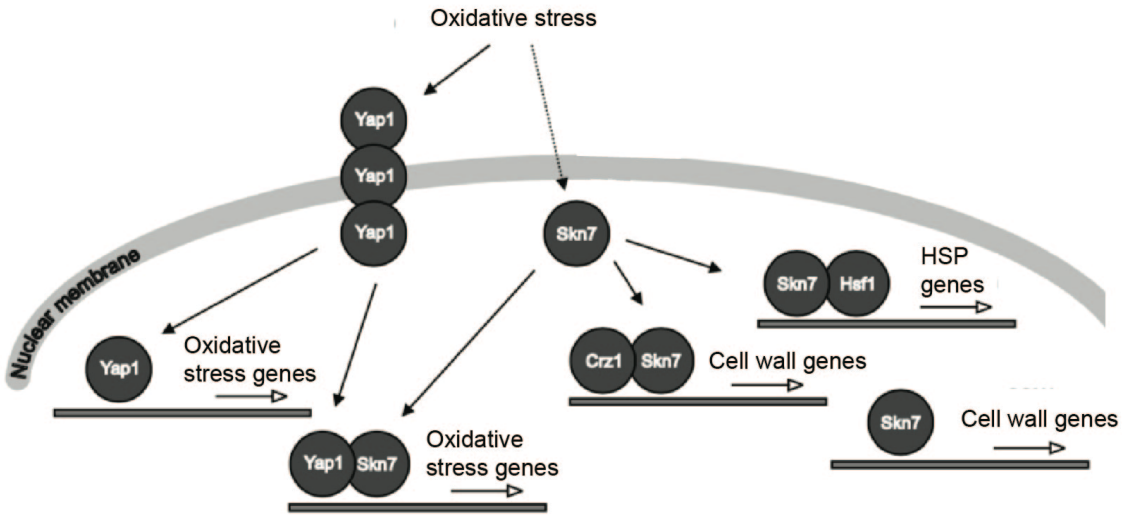


**Figure 1.3 Oxidative stress response via Skn7p and Yap1p schematic.** Modified from (84).

### 1.1.5 Environmental stress response

While yeast have stress-specific responses, there are a core set of genes being induced and repressed during multiple stress conditions, termed environmental stress response (10, 92). During stress conditions, genes involved in cell growth such as ribosome biogenesis and RNA metabolism (93), are down regulated (repressed ESR ~600 genes), while genes involved in carbohydrate metabolism, redox reactions, defense against ROS, DNA damage repair, cell wall modification, and protein folding are upregulated (induced ESR ~300 genes) (10, 47). The repressed growth and the associated factors (decreased transcription and translation) may help cells conserve energy while they adapt to the stressful environment and express stress defense genes (94). One of the original hypotheses was that this induced gene expression was required to survive the initial stress. Previous studies have found only a small fraction of the expressed genes are required to survive the initial stress condition (95-97).

Approximately 1/6$^{th}$ of the genes in the induced ESR have a STRE that general stress transcription factors, Msn2p and Msn4p, recognize (37, 98). Interestingly, ~60% of the induced ESR genes show a defect in the double mutant *msn2/4Δ* when exposed to heat or hydrogen peroxide (10). Studies have found that induction of gene expression does not equate to activated proteins (99, 100). In fact, some genes, like those involved in glycogen synthesis and degradation, are induced during stress but are post-translationally regulated (101). Although the gene expression responses are similar, they may be activated by different mechanisms. As mentioned previously, there are many similarities in the molecular responses to stress in yeast and higher eukaryotes. Under stress, many mammalian cell types have a similar response to that of the yeast ESR (23-28).

### 1.1.6 Acquired stress resistance

In natural environments, there are fluctuating concentrations of stressors that can occur in combination or sequentially. For example, yeast can be exposed to increased sugar

concentrations from fruit, resulting in osmotic stress, then the sun rises exposing the yeast to additional stress, heat. Accordingly, if cells could anticipate impending stress, that would be advantageous. We and others (Berry and Gasch) hypothesize since only a small fraction of genes induced are required to survive the initial stress, then the induction of gene expression (ESR) is to survive impending stress (92, 102-104). In fact, when cells are pretreated with mild stress, they are more likely to survive a subsequent more severe, otherwise lethal stress, in a phenomenon called acquired stress resistance. This acquired stress resistance (also known as adaptive response) is widespread across organisms ranging from bacteria to higher eukaryotes (105-117). Bacteria that are pretreated with mild starvation can survive higher doses of oxidative stress and radiation (115). In plants, a mild stress cross protects against increased drought and heat stress (106, 116). In humans, an ischemic episode can induce cardio protection (111, 117). Previous screens of yeast gene deletion libraries have found surprisingly little overlap between the genes necessary for surviving stress and genes that are induced by stress (13, 18, 21, 95, 97, 118-122), reiterating that organisms could be using acquired stress resistance to survive sequential stresses like those occurring in a natural setting. Instead, gene induction may be a better predictor of a gene's requirement for acquired stress resistance (96). Studies have shown that genes necessary for intrinsic (no pretreatment) and acquired resistance are largely non-overlapping (21, 96, 97), suggesting that mechanisms underlying intrinsic and acquired stress resistance are distinct. For acquired stress resistance, these stresses can be the same (same stress protection) or different conditions (cross protection). In yeast, acquired stress resistance is not universal, meaning not every stress protects the organism against every stress (92).

## 1.2 Natural variation

### 1.2.1 Natural variation

All around there is phenotypic variation. This variation can be intra- and interspecies variation (123). There are multiple causes for phenotypic variation, such as DNA mutations,

epigenetics, and gene expression variation. These DNA mutations can be single nucleotide polymorphisms (SNPs), insertions or deletions, or copy number variation (CNV) (124). In the human genome, there are at least 88 million variations, 84.7 million being SNPs (124). Several hundred thousand SNPs have been found across geographically different human populations (125). Genome variation is not just in coding sequences, studies have seen variation in non-coding sequences, as well (126-132). These genomic variations can cause differences in gene expression. In fact, gene expression variation has been linked to differences in physiology (133-137), morphology (138-144), metabolism (145-147), and behavior (148-151). Variation in gene expression is thought to play a majority role in the phenotypic variation between humans and chimpanzees (123, 152). Genetic variation is currently being used to better understand human diseases and why some individuals are more susceptible (153, 154).

### 1.2.2 Natural variation in yeast and stress resistance

One challenging matter with examining natural variation is not having an easily observable phenotype associated with it. For instance, some phenotypes may only be present under specific conditions. Studies are examining how individuals respond differently and found that it often depends on their individual genetic background. However, the understanding of the mechanisms underlying these so-called “gene-environment interactions” is lacking. Natural variation occurs in yeasts, both inter- and intraspecies (155). One example, commercial brewing yeast have been selected for many generations for high ethanol yields and specific ester production (156), while wild yeast have been selected to survive natural environments (157), making wild yeast rich with trait variation (1). There are over a thousand isolates of *S. cerevisiae*, and studies have been conducted to understand the strain variation between these wild isolates and the commonly-used laboratory strain. Some of the differences between wild and laboratory isolates are thought to be due to artificial selection in laboratory settings.

When comparing yeast genomes, the variation between these yeast isolates is <1% (1, 158, 159) with 1.65 million SNPs being found (0.13% of the genome), and nearly every open reading frame (ORF) had a CNV in at least one strain background (160). A comparative knock out study showed that 5% of essential genes in one strain background were not essential in the second strain background (161). Studies have been using commonly encountered stresses like heat (162), oxidative stress (163), alternate carbon sources (164, 165), and ethanol (102, 166), as well as other conditions (157, 167-170) to better understand the variation between yeast strains.

Recent studies have started using the acquired stress resistance phenotype to study natural variation (103, 104). As previously mentioned, acquired stress resistance is not universal. In the lab strain, a pretreatment of mild salt or hydrogen peroxide will protect against higher doses of salt and hydrogen peroxide, while heat stress will protect against ethanol, heat, salt and hydrogen peroxide (92). Mild ethanol does not provide cross protection against salt in the lab strain, while a wild vineyard strain does acquire salt resistance when pretreated with ethanol. Interestingly, both strains acquire salt resistance when they are pretreated with mild salt (103). Another study found extensive gene expression variation between the lab strain, a wild oak strain and wild vineyard strain (102, 166). Thus, acquired stress resistance is a great phenotyping tool for studying natural variation in yeast.

### 1.3 Synopsis

All organisms experience stress and must sense and respond accordingly. An individual's physiological response to different environmental conditions often depends on their individual genetic background. However, the mechanisms underlying these so-called "gene-environment interactions" are generally poorly understood. One challenge is that some phenotypes may only be present under specific conditions. We have been exploiting natural variation in *Saccharomyces cerevisiae* stress responses to understand the role of gene-

environment interactions in a phenotype called acquired stress resistance, a phenomenon where cells that are pretreated with a mild stress are more likely to survive a subsequent severe, otherwise lethal stress. This approach highlights the power of using natural variation to uncover novel aspects of conserved signaling networks, which may play a large role in gene-environment interactions.

### 1.4 Dissertation outline

This dissertation presents unique research focusing on using acquired stress resistance phenotype to understand natural variation in the model organism, *Saccharomyces cerevisiae*. Chapter 2 identified the genetic variation responsible for ethanol induced hydrogen peroxide cross protection between the lab and wild oak strain. Chapter 3 characterizes the variation of catalase dependency in acquired hydrogen peroxide resistance among wild yeast and reveals that some wild yeast have multiple means to provide hydrogen peroxide resistance.

## 1.5 References


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**Chapter 2 Linkage mapping of yeast cross protection connects gene expression variation to a higher-order organismal trait**

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## 2.1 Abstract

Gene expression variation is extensive in nature, and is hypothesized to play a major role in shaping phenotypic diversity. However, connecting differences in gene expression across individuals to higher-order organismal traits is not trivial. In many cases, gene expression variation may be evolutionarily neutral, and in other cases expression variation may only affect phenotype under specific conditions. To understand connections between gene expression variation and stress defense phenotypes, we have been leveraging extensive natural variation in the gene expression response to acute ethanol in laboratory and wild *Saccharomyces cerevisiae* strains. Previous work found that the genetic architecture underlying these expression differences included dozens of “hotspot” loci that affected many transcripts in trans. In the present study, we provide new evidence that one of these expression QTL hotspot loci affects natural variation in one particular stress defense phenotype—ethanol-induced cross protection against severe doses of H2O2. A major causative polymorphism is in the heme-activated transcription factor Hap1p, which we show directly impacts cross protection, but not the basal H2O2 resistance of unstressed cells. This provides further support that distinct cellular mechanisms underlie basal and acquired stress resistance. We also show that Hap1p-dependent cross protection relies on novel regulation of cytosolic catalase T (Ctt1p) during ethanol stress in a wild oak strain. Because ethanol accumulation precedes aerobic respiration and accompanying reactive oxygen species formation, wild strains with the ability to anticipate impending oxidative stress would likely be at an advantage. This study highlights how strategically chosen traits that better correlate with gene expression changes can improve our power to identify novel connections between gene expression variation and higher-order organismal phenotypes.

## 2.2 Author Summary

A major goal in genetics is to understand how individuals with different genetic makeups respond to their environment. Understanding these “gene-environment interactions” is important for the development of personalized medicine. For example, gene-environment interactions can explain why some people are more sensitive to certain drugs or are more likely to get certain cancers. While the underlying causes of gene-environment interactions are unclear, one possibility is that differences in gene expression across individuals are responsible. In this study, we examined that possibility using baker’s yeast as a model. We were interested in a phenomenon called acquired stress resistance, where cells exposed to a mild dose of one stress can become resistant to an otherwise lethal dose of severe stress. This response is observed in diverse organisms ranging from bacteria to humans, though the specific mechanisms governing acquisition of higher stress resistance are poorly understood. To understand the differences between yeast strains with and without the ability to acquire further stress resistance, we employed genetic mapping. We found that part of the variation in acquired stress resistance was due to sequence differences in a key regulatory protein, thus providing new insight into how different individuals respond to acute environmental change.

## 2.3 Introduction

A fundamental question in genetics is how individuals with extremely similar genetic makeups can have dramatically different characteristics. One hypothesis is that a small number of regulatory polymorphisms can have large effects on gene expression, leading to the extensive phenotypic variation we see across individuals. In fact, gene expression variation is hypothesized to underlie the extensive phenotypic differences we see between humans and chimpanzees despite >98% DNA sequence identity (1, 2). This hypothesis is supported by numerous examples of gene expression variation affecting higher-order organismal traits.

For example, human genome-wide association studies (GWAS) have found that a substantial fraction of disease-associated variants are concentrated in non-coding regulatory DNA regions (3-8). Further examples include gene expression variation being linked to differences in metabolism (9-11), physiology (12-16), morphology (17-23), and behavior (24-27).

While gene expression variation is pervasive, there is often a lack of obvious phenotypic change associated with differentially expressed genes. This can occur for a variety of reasons. First, a large fraction of expression variation has been postulated to be evolutionarily neutral with no effect on organismal fitness (28-30). Second, co-regulation of genes that share the same upstream signaling network and transcription factors can lead to genes whose expression differences correlate with phenotype but are not truly causative. Finally, some gene expression differences may truly affect phenotype, but only under specific conditions. For example, the predictive power of expression quantitative trait loci (eQTL) mapping studies on higher-order phenotypes can be poor unless multiple environments are considered (31). Similarly, tissue-restricted eQTLs are more likely to map to known disease-associated loci identified from GWAS than non-tissue-restricted eQTLs (32, 33).

Thus, a major challenge for connecting gene expression variation to downstream effects on higher-order traits is the choice of which conditions and traits to examine. To this end, we have been leveraging natural variation in the model eukaryote *Saccharomyces cerevisiae*, and

a phenotype called acquired stress resistance. Many studies have shown a poor correlation between genes that respond to stress and their importance for surviving stress (34-43). Thus, we and others have argued that the role of stress-activated gene expression is not to survive the initial insult, but instead protects cells from impending severe stress through a phenomenon called acquired stress resistance (44, 45). Acquired stress resistance (sometimes referred to as “induced tolerance” or the “adaptive response”) occurs when cells pretreated with a mild dose of stress gain the ability to survive an otherwise lethal dose of severe stress. Notably, acquired stress resistance can occur when the mild and severe stresses are the same (same-stress protection) or across pairs of different stresses (cross protection). This phenomenon has been observed in diverse organisms ranging from bacteria to higher eukaryotes including humans (44-50). The specific mechanisms governing acquisition of higher stress resistance are poorly understood, but there are wide reaching implications. In humans, ischemic preconditioning (transient ischemia followed by reperfusion—i.e. mild stress pretreatment followed by severe stress) may improve outcomes of cardiovascular surgery (51-54), while transient ischemic attacks (“mini-strokes”) may protect the brain during massive ischemic stroke (55-57). Thus, understanding the genetic basis of acquired stress resistance in model organisms holds promise for mitigating the effects of stress in humans.

A previous study found that a commonly used S288c lab strain is unable to acquire further ethanol resistance when pretreated with a mild dose of ethanol (44). We found this phenotype to be surprising, considering the unique role ethanol plays in the life history of *Saccharomyces* yeast, where the evolution of aerobic fermentation gave yeast an advantage over ethanol-sensitive competitors (58). Because ethanol is a self-imposed stress that induces a robust stress response (59-63), we expected that ethanol should provoke acquired stress resistance in wild yeast strains. Indeed, this turned out to be the case, with the majority of tested wild strains acquiring resistance to severe ethanol following a mild ethanol treatment (45). Furthermore, this phenotype correlated with extensive differences in the transcriptional

response to acute ethanol stress in the lab strain when compared to a wild vineyard (M22) and wild oak (YPS163) strain (>28% of S288c genes were differentially expressed at an FDR of 0.01) (45, 64). We performed linkage mapping of S288c crossed to a wild vineyard strain (M22) and wild oak strain (YPS163), and observed numerous “hotspots” where the same eQTL loci affect the expression of a large number of transcripts (anywhere from 10 – 500 transcripts per hotspot) (64).

In the present study, we provide new evidence that one of these eQTL hotspot loci affects natural variation in acquired stress resistance, namely the ability of ethanol to cross protect against oxidative stress in the form of hydrogen peroxide. The causative polymorphism is in the heme-activated transcription factor Hap1p, which we show directly impacts cross protection, but not the basal resistance of unstressed cells. Finally, we show that the Hap1p effect is mediated through novel regulation of cytosolic catalase T (Ctt1p) during ethanol stress in wild strains. This study highlights how strategically chosen traits that are better correlated with gene expression changes can improve our power to identify novel connections between gene expression variation and higher-order organismal phenotypes.

## 2.4 Materials and Methods

### 2.4.1 Strains and growth conditions

Strains and primers used in this study are listed in S2 and S3 Tables, respectively. The parental strains for QTL mapping were YPS163 (oak strain) and the S288c-derived DBY8268 (lab strain; referred to throughout the text as S288c). The construction of the S288c x YPS163 QTL mapping strain panel (44 $F_2$ progeny) is described in (65) (kindly provided by Justin Fay). Genotypes for the strain panel are listed in S4 Table. During the course of analyzing *HAP1* genotypes, we found one segregant (YS.15.2) to be a mixed population, so it was removed from subsequent analyses. Deletions in the BY4741 (S288c) background were obtained from Open Biosystems (now GE Dharmacon), with the exception of *hap1* (whose construction is described

in (45)). Deletions were moved into haploid *MATa* derivatives of DBY8268, M22, and YPS163 by homologous recombination with the deletion::KanMX cassette amplified from the appropriate yeast knockout strain (66). Homozygous *hap1*Δ strains of YPS1000 and Y10 were generated by moving the *hap1*Δ::KanMX allele from the BY4741 background into the strains, followed by sporulation and tetrad dissection. All deletions were verified by diagnostic PCR. DBY8268 containing a wild-type *HAP1* allele from YPS163 was constructed in two steps. First, the MX cassette from the *hap1*Δ::KanMX deletion was replaced with a URA3MX cassette, selecting for uracil prototrophy. Then, *URA3* was replaced with wild-type *HAP1* from YPS163 (amplified using primers 498-bp upstream and 1572-bp downstream of the *HAP1* ORF), while selecting for loss of *URA3* on 5-fluoroorotic acid (5-FOA) plates. Deletions and repair of *HAP1* were confirmed by diagnostic PCR (see S3 Table for primer sequences). YPS163 containing a $HAP1^{S288c}$ allele was constructed by first inserting a KanMX cassette into S288c 117-bp downstream of the Ty element to create JL1032. We then amplified and transformed the Ty element into YPS163 using primers that annealed 103-bp upstream of the Ty element and 177-bp downstream of the KanMX cassette, generating JL1069. Diploid strains for *HAP1* and *TOP3* reciprocal hemizygosity analysis were generated as follows. The hemizygote containing the wild-type S228c *HAP1* allele (JL580) was generated by mating JL140 (YPS163 *MATa ho*Δ::HygMX *hap1*Δ::KanMX) to JL506 (DBY8268 *MATα ho ura3 hap1*). The hemizygote containing the wild-type YPS163 allele (JL581) was generated by mating JL112 (YPS163 *MATα ho*Δ::HygMX *HAP1*) to JL533 (DBY8268 *MATa ho ura3 hap1*Δ::KanMX). The hemizygote containing the wild-type S288c *TOP3* allele (JL1107) was created by mating JL1066 (YPS163 *MATa ho*Δ::HygMX *top3*Δ*::KanMX*) to BY4742 (*MATα TOP3*). The hemizygote containing the wild-type YPS163 allele (JL1106) was created by mating JL1121 (BY4741 *MATa top3*Δ*::KanMX*) to JL112 (YPS163 *MATα ho*Δ::HygMX *TOP3*). All strains were grown in batch culture in YPD (1% yeast extract, 2% peptone, 2% dextrose) at 30°C with orbital shaking (270 rpm).

### 2.4.2 *HAP1* and *TOP3* Genotyping

To identify possible promoter polymorphisms, the *HAP1* promoters of the DBY8268 (JL505), YPS163 (JL111), and S288c $HAP1^{YPS163}$ (JL975) strains were amplified using primers that anneal 1091-bp upstream and 134-bp downstream of the *HAP1* start codon. PCR products were purified with a PureLink PCR cleanup kit (Invitrogen) and sequenced by Sanger Sequencing (Eurofins Genomics) using a primer that anneals 498-bp upstream of the *HAP1* start codon. Sequences were aligned to the S288c and YPS163 reference sequences using SnapGene v4.1 (GSL Biotech). This verified the presence of a 1-bp indel within a poly-A stretch that differs between S288c and YPS163. The S288c $HAP1^{YPS163}$ (JL975) strain contains the YPS163 *HAP1* promoter sequence. Additionally, the YPS163 strain containing the $HAP1^{S288c}$ was constructed to only contain the Ty element and not the S288c promoter polymorphism.

The *HAP1* allele of each segregant for the QTL mapping panel was genotyped by differential PCR analysis where the same forward primer (HAP1 int 3' F) was paired with two different reverse primers. One primer (Ty R) anneals specifically to the Ty element, yielding an 856-bp product when amplifying the S288c allele. The second primer (*HAP1* 3' end R) anneals 3' to the Ty element of $HAP1^{S288c}$, yielding a 570-bp product for $HAP1^{YPS163}$ and a 6.5-kb product for $HAP1^{S288c}$. Each segregant was genotyped using both sets of primer pairs, and only one segregant (YS.15.2) appeared to contain both *HAP1* alleles. Subsequent analysis of multiple colonies verified that YS.15.2 was a mixed population, and thus it was removed it from all subsequent analyses.

The *TOP3* alleles of S288c and YPS163 contain two non-synonymous SNPs at nucleotide positions 1,398 and 1,422. Segregant genotypes at *TOP3* were determined by analyzing restriction fragment length polymorphisms. *TOP3* was amplified using primers (*TOP3* up F and *TOP3* down R) that anneal ~500-bp upstream and downstream of the open reading frame, generating a 2.9-kb product. PCR products were digested with either 1) *Pst*I, which cuts at position 1,248 only within the $TOP3^{YPS163}$ ORF allele yielding 1.7- and 1.2-kb products, or (2)

*Kfl*I, which cuts at position 1,155 only within the *TOP3*$^{S288c}$ yielding 1.6- and 1.3-kb products. Genotypes for *HAP1* and *TOP3* are listed in S1 Table.

### 2.4.3 Cross protection assays

Cross-protection assays were performed as described in (44) with slight modifications. Briefly, 3-4 freshly streaked isolated colonies (<1 week old) were grown overnight to saturation, sub-cultured into 6 ml fresh media, and then grown for at least 8 generations (>12 h) to mid-exponential phase ($OD_{600}$ of 0.3 – 0.6) to reset any cellular memory of acquired stress resistance (67). Each culture was split into two cultures and pretreated with YPD media containing either a single mild "primary" dose or the same concentration of water as a mock-pretreatment control. Primary doses consisted of 5% v/v ethanol, 0.4 M NaCl, or 0.4 mM $H_2O_2$. Thereafter, mock and primary-treated cells were handled identically. Following 1-hour pretreatment at 30°C with orbital shaking (270 rpm), cells were collected by mild centrifugation at 1,500 x *g* for 3 min. Pelleted cells were resuspended in fresh medium to an $OD_{600}$ of 0.6, then diluted 3-fold into a microtiter plate containing a panel of severe "secondary" $H_2O_2$ doses ranging from 0.5 – 5.5 mM (0.5 mM increments; 150 µl total volume). Microtiter plates were sealed with air-permeable Rayon films (VWR), and cells were exposed to secondary stress for 2 hours at 30°C with 800 rpm shaking in a VWR symphony Incubating Microplate Shaker. Four µl of a 50-fold dilution was spotted onto YPD agar plates and grown 48 h at 30°C. Viability at each dose was scored using a 4-point semi-quantitative scale to score survival compared to a no-secondary stress (YPD only) control: 100% = 3 pts, 50-90% = 2 pts, 10-50% = 1 pt, or 0% (3 or less colonies) = 0 pts. An overall $H_2O_2$ tolerance score was calculated as the sum of scores over the 11 doses of secondary stress. Raw phenotypes for all acquired stress resistance assays can be found in S5 Table. A fully detailed acquired stress protocol has been deposited to protocols.io under doi dx.doi.org/10.17504/protocols.io.g7sbzne. Statistical analyses were performed using Prism 7 (GraphPad Software).

### 2.4.4 QTL mapping and Heritability Estimates

Phenotyping of the QTL mapping strain panel for basal and acquired $H_2O_2$ resistance was performed in biological duplicate. Because cross-protection assays on the entire strain panel could not all be performed at the same time, we sought to minimize day-to-day variability. We found that minor differences in temperature and shaking speed affected $H_2O_2$ resistance; as a result, we used a digital thermometer and tachometer to ensure standardization across experiments. Moreover, we found that differences in handling time were a critical determinant of experimental variability. To minimize this source of variability, all cell dilutions were performed quickly using multichannel pipettes, and no more than two microtiter plates were assayed during a single experiment. To ensure that replicates on a given day were reproducible, we always included the YPS163 wild-type parent as a reference.

Single mapping scans were performed using Haley-Knott regression (68) implemented through the R/QTL software package (69). Genotype probabilities were estimated at every cM across the genome using the calc.genoprob function. Significant LOD scores were determined by 100,000 permutations that randomly shuffled phenotype data (i.e. strain labels) relative to the genotype data. The maximum LOD scores for the permuted scans were sorted, and the 99$^{th}$ percentile was used to set the genome-wide FDR at 1%. This resulted in LOD cutoffs of 3.07 for QTL mapping of basal $H_2O_2$ resistance, and 4.24 for acquired $H_2O_2$ resistance.

Broad-sense heritability ($H^2$) was estimated from the segregant data as described in (70) using a random-effects ANOVA model implemented through the lmer function in the lme4 R package (71). $H^2$ was estimated using the equation $\frac{\sigma_G^2}{(\sigma_G^2+\sigma_E^2)}$, where $\sigma_G^2$ represents the genetic variance due to the effects of segregrant, and $\sigma_E^2$ represents the residual (error or environmental) variance. The proportion of variance explained by a QTL was estimated using the equation $1-10^{(-\frac{2}{n}*\mathrm{LOD})}$, where $n$ represents the number of segregants.

### 2.4.5 Quantitative PCR of *CTT1* expression and cellular peroxidase assays

Induction of *CTT1* by ethanol was assessed by real-time quantitative PCR (qPCR) using the Maxima SYBR q-PCR Master Mix (Thermo Fisher Scientific) and a Bio-Rad CFX96 Touch Real-Time PCR Detection System, according to the manufacturers' instructions. Cells were grown to mid-exponential phase ($OD_{600}$ of 0.3 – 0.6) as described for the cross-protection assays. Cells were collected by centrifugation at 1,500 x *g* for 3 minutes immediately prior to the addition of 5% v/v ethanol (unstressed sample) and 30 minutes post-ethanol treatment, which encompasses the peak of global expression changes to acute ethanol stress (45). Cell pellets were flash frozen in liquid nitrogen and stored at -80°C until processed. Total RNA was recovered by hot phenol extraction as previously described (72), and then purified with a Quick-RNA MiniPrep Plus Kit (Zymo Research) including on-column DNase I treatment. cDNA synthesis was performed as described (72), using 10 µg total RNA, 3 µg anchored oligo-dT (T20VN), and SuperScript III (Thermo Fisher Scientific). One ng cDNA was used as template for qPCR with the following parameters: initial denaturation at 95°C for 3 minutes followed by 40 cycles of 95°C for 15 seconds and 55°C annealing and elongation for 1 minute. Cq was determined using regression analysis, with baseline subtraction via curve fit. The presence of a single amplicon for each reaction was validated by melt curve analysis. The average of two technical replicates were used to determine relative *CTT1* mRNA abundance via the $\Delta\Delta$Cq method (73), by normalizing to an internal control gene (*ERV25*) whose expression is unaffected by ethanol stress and does not vary in expression between S288c and YPS163 (45). Primers for *CTT1* and *ERV25* were designed to span ~200 bp in the 3' region of each ORF (to decrease the likelihood of artifacts due to premature termination during cDNA synthesis), and for gene regions free of polymorphisms between S288c and YPS163 (see S3 Table for primer sequences). Three biological replicates were performed and statistical significance was assessed via a paired *t*-test using Prism 7 (GraphPad Software).

For peroxidase activity assays, mid-exponential phase cells were collected immediately prior to and 60 minutes post-ethanol treatment, to assess peroxidase activity levels during the induction of cross protection. Cells were collected by centrifugation at 1,500 x *g* for 3 minutes, washed twice in 50 mM potassium phosphate buffer, pH 7.0 ($KP_i$), flash frozen in liquid nitrogen, and then stored at -80°C until processed. For preparation of whole cell extracts, cells were thawed on ice, resuspended in 1 ml $KP_i$ buffer, and then transferred to 2-ml screw-cap tubes for bead beating. An equal volume (1 ml) of acid-washed glass beads (425 - 600 micron, Sigma-Aldrich) was added to each tube. Cells were lysed by four 30-second cycles of bead beating in a BioSpec Mini-Beadbeater-24 (3,500 oscillations/minute, 2 minutes on ice between cycles). Cellular debris was removed by centrifugation at 21,000 x *g* for 30 minutes at 4°C. The protein concentration of each lysate was measured by Bradford assay (Bio-Rad) using bovine serum albumin (BSA) as a standard (74). Peroxidase activity in cellular lysates was monitored as described (75), with slight modifications. Briefly, 50 µg of cell free extract was added to 1 ml of 15 mM $H_2O_2$ in $KP_i$ buffer. $H_2O_2$ decomposition was monitored continuously for 10 minutes in Quartz cuvettes (Starna Cells, Inc.) at 240 nm ($\varepsilon_{240}$ = 43.6 $M^{-1}$ $cm^{-1}$) using a SpectraMax Plus Spectrophotometer (Molecular Devices). One unit of catalase activity catalyzed the decomposition of 1 µmol of $H_2O_2$ per minute. For each sample, results represent the average of technical duplicates. To assess statistical significance, four biological replicates were performed and significance was assessed via a paired *t*-test using Prism 7 (GraphPad Software).

## 2.5 Results

### 2.5.1 The genetic basis of natural variation in yeast cross protection.

We previously found that an S288c-derived lab strain was unable to acquire further ethanol resistance when pretreated with a mild dose of ethanol, in contrast to the vast majority of ~50 diverse yeast strains (45). In addition to the S288c strain's acquired ethanol resistance defect, ethanol also failed to cross protect against other subsequent stresses (44, 76). In nature,

wild yeast cells ferment sugars to ethanol, and then shift to a respiratory metabolism that generates endogenous reactive oxygen species (77-79). Thus, we hypothesized that ethanol might cross protect against oxidative stress in wild yeast strains. We tested this hypothesis by assessing whether mild ethanol treatment would protect a wild oak strain (YPS163) from severe oxidative stress in the form of hydrogen peroxide ($H_2O_2$). Cross protection assays were performed by exposing cells to a mild, sublethal dose of ethanol (5% v/v) for 60 min, followed by exposure to a panel of 11 increasingly severe doses of $H_2O_2$ (see Materials and Methods). Confirming the observations of Berry and Gasch (44), ethanol failed to cross protect against $H_2O_2$ in S288c, and in fact slightly exacerbated $H_2O_2$ toxicity (Fig 1). In contrast, ethanol strongly cross protected against $H_2O_2$ in YPS163 (Fig 1).

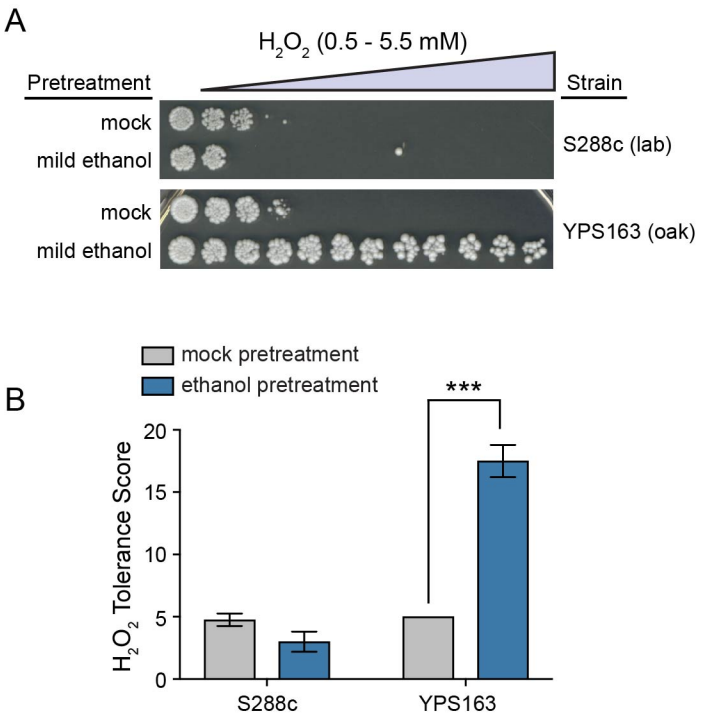



**Figure 2.1. Natural variation in ethanol-induced cross protection against $H_2O_2$.** (A) A representative acquired $H_2O_2$ resistance assay is shown. S288c (lab strain – DBY8268) and YPS163 (wild oak strain) were exposed to 5% ethanol or mock (5% water) pretreatment for 60 min, washed, exposed to 11 doses of severe $H_2O_2$ for 2 hr, and then plated to score viability. (B) A single survival score was calculated from the viability at all $H_2O_2$ doses (see Materials and Methods). Each plot shows the mean and standard deviation of 4 independent biological replicates. The replicates for mock-treated YPS163 all had the same tolerance score and thus zero standard deviation (see Table S1 for raw numerical data). Asterisks represent resistance that was significantly different from mock-treated cells (*** $P < 0.001$, *t*-test).

The inability of ethanol to induce acquired stress resistance in S288c correlates with thousands of differences in ethanol-dependent gene expression in comparison to wild strains that can acquire ethanol resistance (45, 64). In light of this observation, and the known dependency of cross protection on stress-activated gene expression changes (44), we hypothesized that differences in cross protection against $H_2O_2$ by ethanol may be linked to differential gene expression. To test this, we performed quantitative trait loci (QTL) mapping using the same mapping population as our original eQTL study that mapped the genetic architecture of ethanol-responsive gene expression (64). Specifically, we conducted QTL mapping of both basal and acquired $H_2O_2$ resistance in 43 $F_2$ progeny of S288c crossed with YPS163 (see Materials and Methods). While we found no significant QTLs for basal $H_2O_2$ resistance, we did find a significant QTL peak on chromosome XII that explained 38% of the variation in cross protection (Fig 2). It is unlikely that our failure to detect a chromosome XII QTL for basal $H_2O_2$ resistance was due to a lack of statistical power, because two independent basal $H_2O_2$ resistance QTL studies using millions of S288c x YPS163 $F_2$ segregants also found no significant associations at this locus (80, 81). Additionally, we estimated the heritability of phenotypic variation in basal resistance to be 0.79, which is slightly above the median value estimated by Bloom and colleagues for 46 yeast traits (70), and is only moderately lower than the heritability for cross protection (0.92). Lastly, the shape of the distribution of phenotypes in the $F_2$ were markedly different between basal and acquired $H_2O_2$ resistance, with basal resistance showing a transgressive segregation pattern and acquired resistance showing a continuous distribution (S1 Fig). Altogether, these results suggest that the genetic basis of natural variation in acquired stress resistance is distinct from the basal resistance of unstressed cells (see Discussion).

The significant QTL for cross protection was located near a known polymorphism in *HAP1*, a heme-dependent transcription factor that controls genes involved in aerobic respiration (82-84), sterol biosynthesis (85-87), and interestingly, oxidative stress (87, 88). S288c harbors a

known defect in *HAP1*, where a Ty1 transposon insertion in the 3' end of the gene's coding region has been shown to reduce its function (89). In fact, we previously hypothesized that the defective *HAP1* allele was responsible for the inability of S288c to acquire further resistance to ethanol. However, a YPS163 *hap1Δ* strain was still fully able to acquire ethanol resistance, despite notable differences in the gene expression response to ethanol in the mutant (45). Likewise, despite previous studies implicating Hap1p as a regulator of oxidative stress defense genes (87, 88), *HAP1* is apparently dispensable for same-stress acquired $H_2O_2$ resistance (47). These observations suggest that the molecular mechanisms underlying various acquired stress resistance phenotypes can differ, even when the identity of the secondary stress is the same.

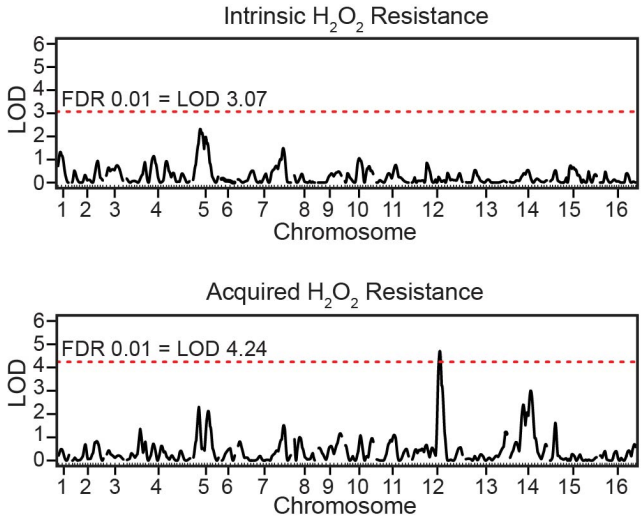
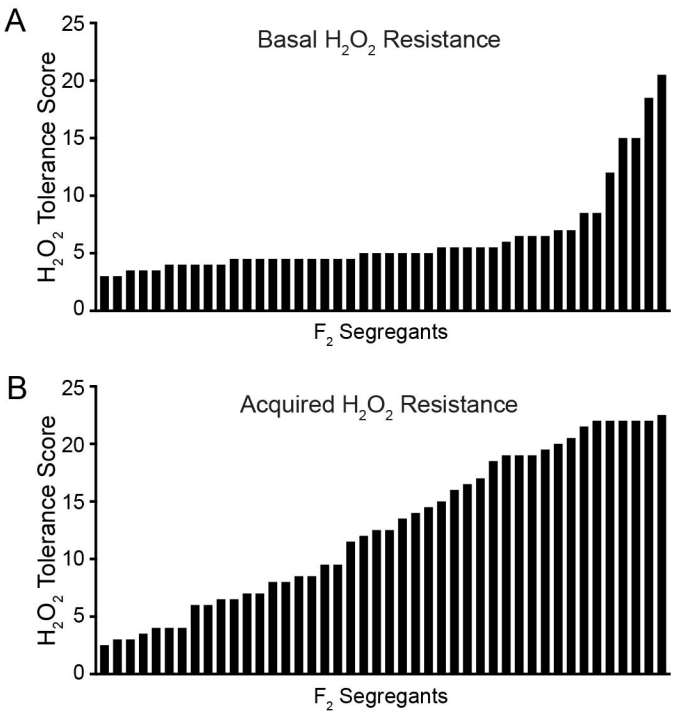



**Figure 2.2. The genetic basis of natural variation for basal and acquired stress resistance is distinct.** Linkage mapping of the S288c x YPS163 cross identified no significant QTLs for basal $H_2O_2$ resistance (top panel), but did identify a major QTL on chromosome XII for ethanol-induced cross protection against $H_2O_2$ (bottom panel). The red horizontal line denotes the LOD threshold for significance (1% FDR).




**Supp Figure 2.1. Distribution of phenotypes in the $F_2$ segregants.** Survival score plots indicating the mean of biological duplicates for (A) basal and (B) acquired $H_2O_2$ resistance.

### 2.5.2 A role for HAP1 in ethanol-induced cross protection against severe H2O2.

Because we previously implicated *HAP1* as a major ethanol-responsive eQTL hotspot affecting over 100 genes, we hypothesized that ethanol-induced cross protection against $H_2O_2$ may depend upon Hap1p-regulated genes. However, it was formally possible that *HAP1* was merely linked to the truly causal polymorphism. To distinguish between these possibilities, we generated deletion mutations in the YPS163 background for every non-essential gene within the 1.5-LOD support interval of the QTL peak (encompassing *IFH1* – *YCS4*). Of the 36 mutants tested, two showed significantly and highly diminished acquired $H_2O_2$ resistance (Fig 3 and S2 Fig), *hap1*Δ and *top3*Δ (encoding DNA topoisomerase III). To determine whether different alleles of *HAP1* and/or *TOP3* were responsible for natural variation in acquired $H_2O_2$ resistance, we applied an approach called reciprocal hemizygosity analysis (90), where the *TOP3* and *HAP1* alleles were analyzed in an otherwise isogenic S288c-YPS163 hybrid background (see Fig 4A for a schematic). In each of the two reciprocal strains, one allele of the candidate gene was deleted, producing a hybrid strain containing either the S288c or YPS163 allele in single copy (i.e. hemizygous for *TOP3* or *HAP1*). While we found only mild allelic effects for *TOP3*, the effects of different *HAP1* alleles were striking (Fig 4B and 4C). The hybrid strain containing the $HAP1^{YPS163}$ allele showed full cross protection, while the strain containing the $HAP1^{S288c}$ allele showed none. Thus, we examined the effects of *HAP1* on acquired $H_2O_2$ resistance further. Intriguingly, we found that the YPS163 *hap1*Δ mutant was unaffected for acquired $H_2O_2$ resistance when mild $H_2O_2$ or mild NaCl were used as mild stress pretreatments (Fig 5), suggesting that Hap1p plays a distinct role in ethanol-induced cross protection (see Discussion).

Finally, we performed allele swap experiments to examine the effects of the different *HAP1* alleles in the original parental backgrounds. We introduced only the Ty element from $HAP1^{S288c}$ into the YPS163 *HAP1* gene, and observed a loss of acquired $H_2O_2$ resistance similar to the YPS163 *hap1*Δ strain (Fig 6). We next tested whether repair of the defective *hap1* allele

in S288c could restore cross protection. Surprisingly, S288c repaired with *HAP1* $^{YPS163}$ was largely unable to acquire further $H_2O_2$ resistance (Fig 6). This additional layer of genetic.

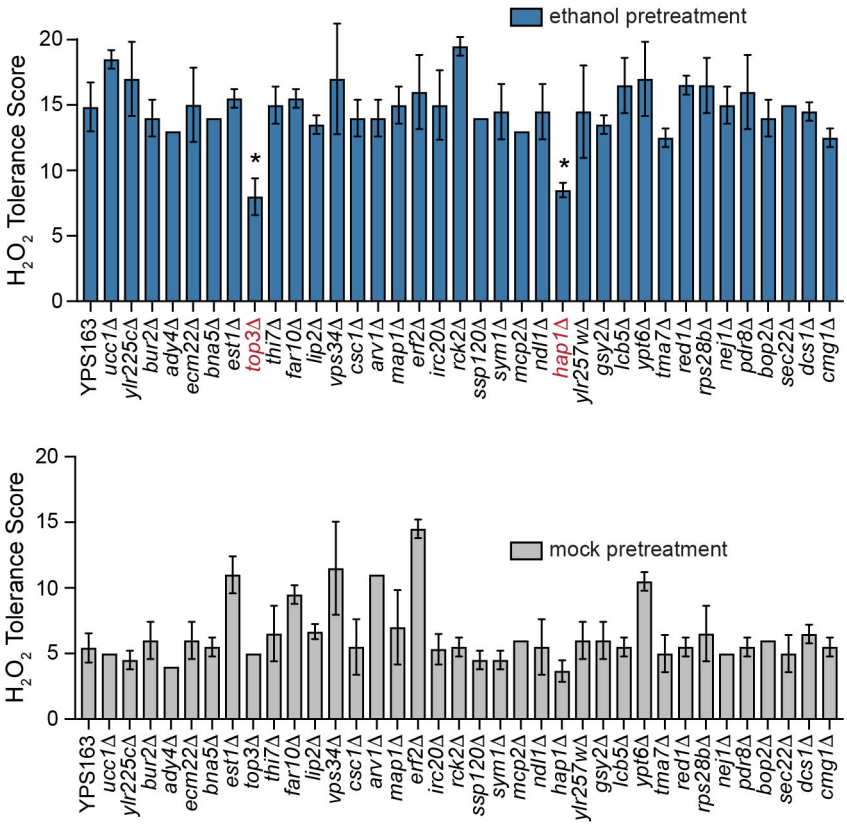
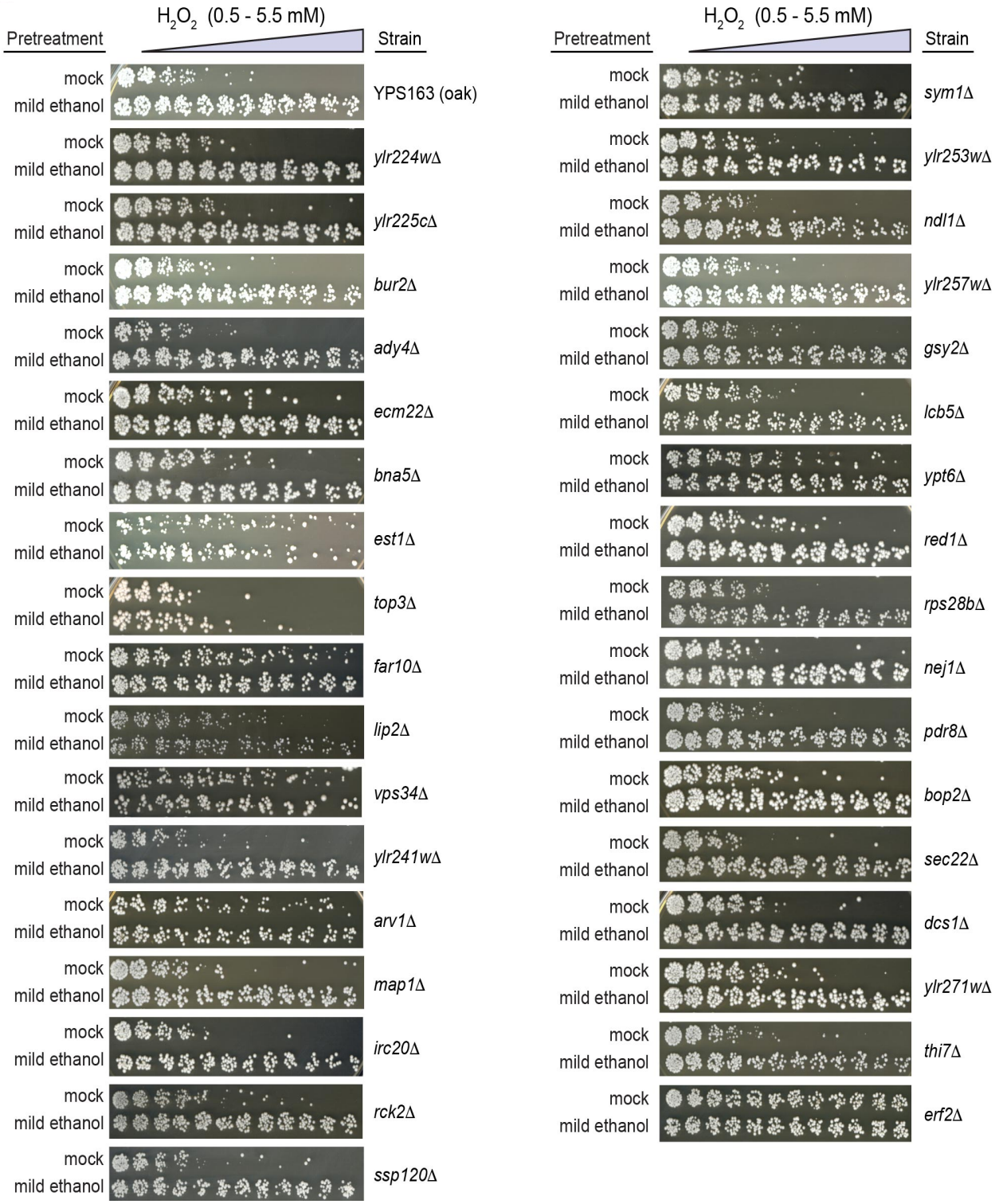



**Figure 2.3. Ethanol-induced cross protection against $H_2O_2$ in YPS163 requires *HAP1 and TOP3*.** Deletions of all non-essential genes within the 1.5-LOD support interval of the chromosome XII QTL peak were constructed in JL111 (YPS163 *MATa* haploid) background and tested for defects in acquired $H_2O_2$ resistance. Each plot shows the mean and standard deviation of 2 independent biological replicates, with the exception of the JL111 control (35 replicates). The replicates for several strains all had the same tolerance score and thus zero standard deviation (see Table S1 for raw numerical data). Asterisks represent acquired $H_2O_2$ resistance that was significantly lower than wild-type YPS163 (* $P < 0.001$, one-way ANOVA).




**Supp Figure 2.2. Representative acquired $H_2O_2$ resistance assays for candidate genes under the chromosome XII QTL peaks.** Representative acquired $H_2O_2$ resistance assays for wild-type YPS163 and each of 36 mutants generated for candidates falling within the 1.5-LOD support interval of the chromosome XII QTL peak.

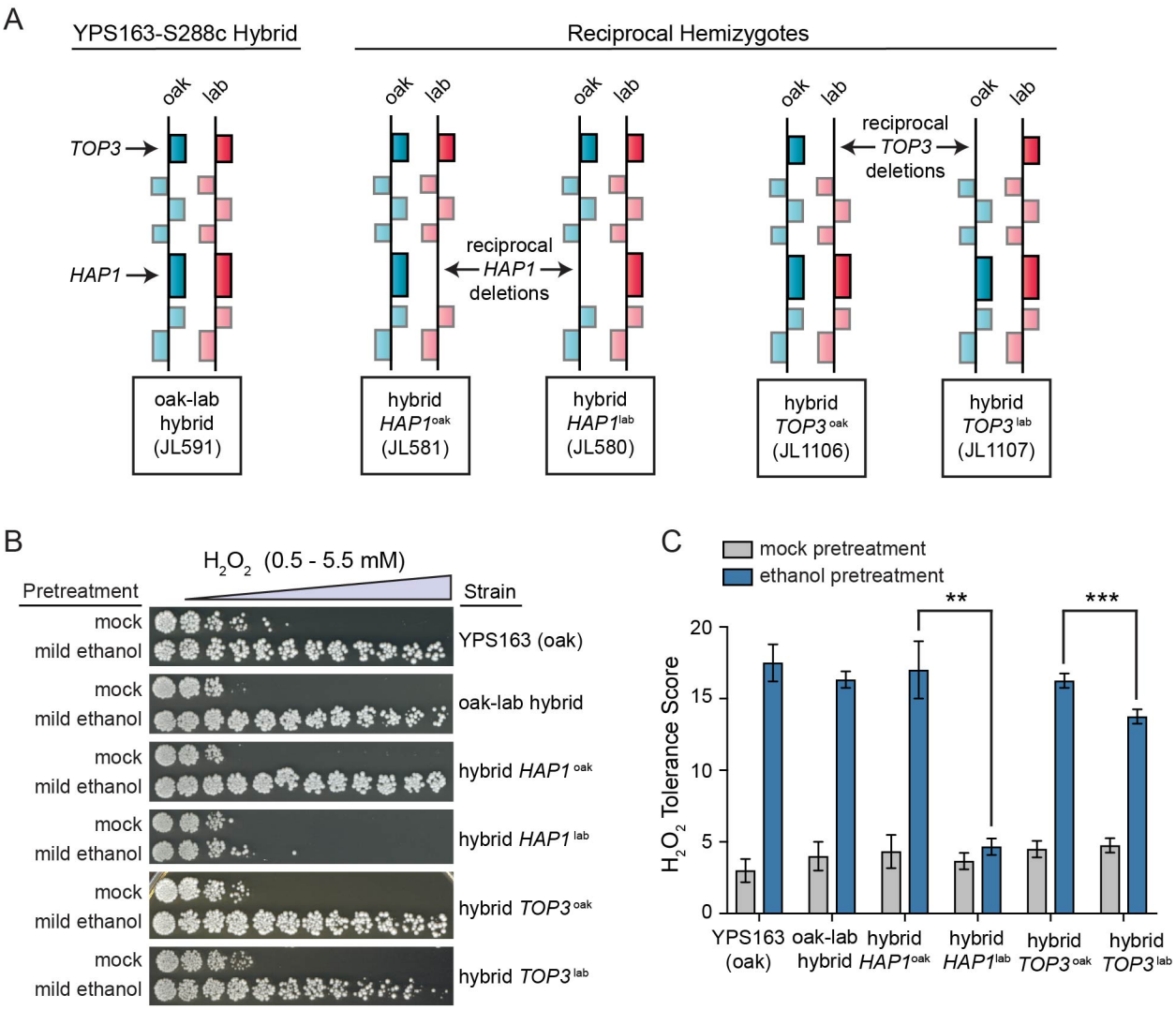



**Figure 2.4. Allelic variation in *HAP1* affects ethanol-induced cross protection against $H_2O_2$.** (A) Schematic of reciprocal hemizygosity analysis. Each block represents a gene, and each hybrid strain contains a single-copy deletion of *hap1* or *top3*, and a single copy of the respective S288c (lab) or YPS163 (oak) allele. (B) Representative acquired $H_2O_2$ resistance assays for wild-type YPS163, the YPS163-S288c hybrid, and the reciprocal hemizygotes. (C) Each survival score plot shows the mean and standard deviation of biological triplicates. Asterisks represent significant differences in acquired resistance between denoted strains (** $P < 0.01$, *** $P < 0.001$, ns = not significant ($P > 0.05$), *t*-test).

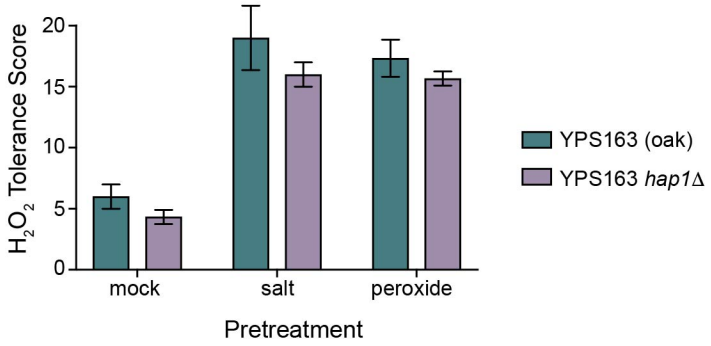



**Figure 2.5. *HAP1* is not required for acquired $H_2O_2$ resistance following mild $H_2O_2$ or mild NaCl pretreatments.** Cultures of wild-type YPS163 and the YPS163 *hap1*Δ mutant were split and exposed to either 0.4 mM $H_2O_2$, 0.4 M NaCl, or a mock (media only) treatment for 60 min, washed, exposed to 11 doses of severe $H_2O_2$ for 2 hr, and then plated to score viability. The survival scores across each of the 11 doses are plotted as the mean and standard deviation of biological triplicates.

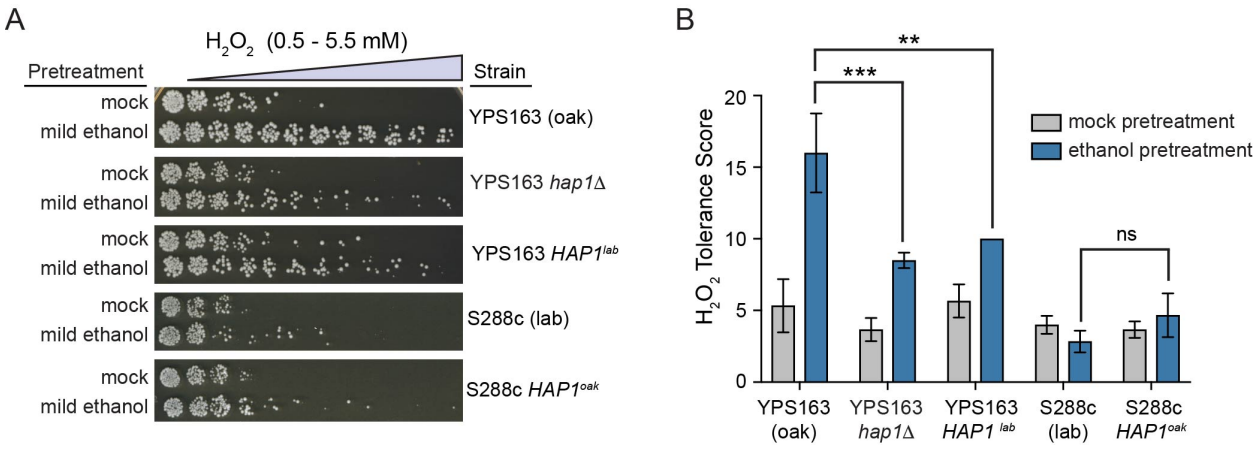
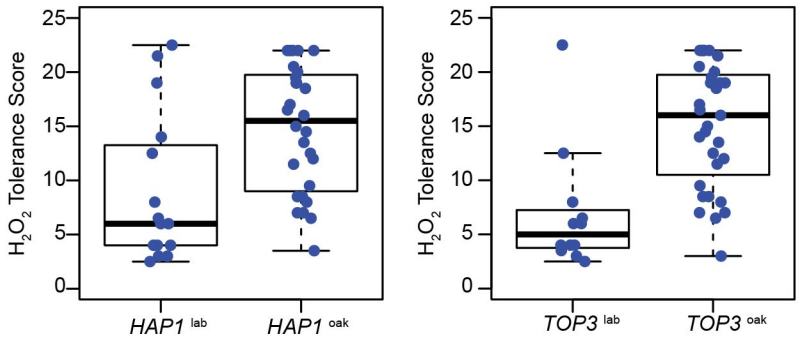
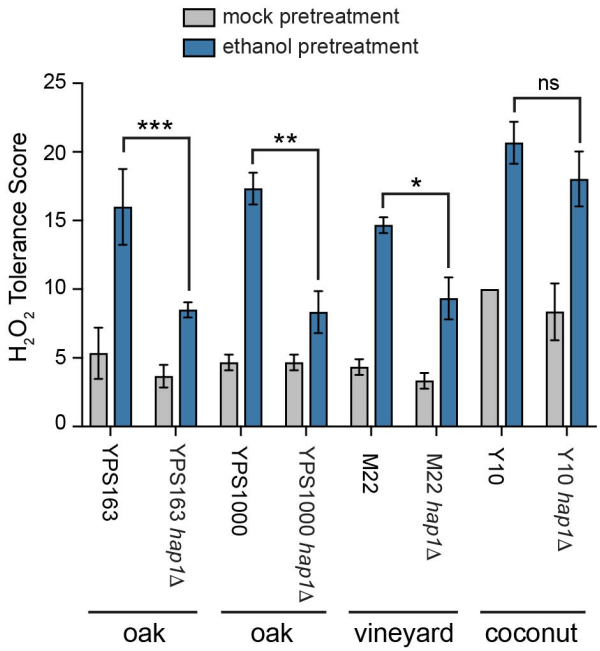



**Figure 2.6. Allele swaps suggest that *HAP1* is necessary for acquired $H_2O_2$ resistance in YPS163, but not sufficient to restore acquired $H_2O_2$ resistance in S288c.** (A) Representative acquired $H_2O_2$ resistance assays for wild-type YPS163 (oak), YPS163 *hap1*Δ mutant, YPS163 $HAP1^{S288c}$, and S288c $HAP1^{YPS163}$. (B) Each survival score plot shows the mean and standard deviation of at least biological triplicates. The replicates for YPS163 $HAP1^{S288c}$ all had the same tolerance score and thus zero standard deviation (see Table S1 for raw numerical data). Asterisks represent significant differences in acquired resistance between denoted strains (** $P < 0.01$, *** $P < 0.001$, ns = not significant ($P > 0.05$), *t*-test).

complexity suggests that S288c harbors additional polymorphisms that affect cross protection. To determine whether this was due to allelic variation in *TOP3*, the only other locus showing a difference in acquired $H_2O_2$ resistance, we genotyped each of the segregants at both the *HAP1* and *TOP3* loci. We identified two segregants with both the *HAP1*$^{YPS163}$ and *TOP3*$^{YPS163}$ alleles that were nonetheless unable to acquire further resistance (S3 Fig, S1 Table). These data, along with the continuous distribution of $F_2$ phenotypes (S1 Fig), is consistent with other loci outside of the chromosome XII QTL peak contributing to variation in acquired $H_2O_2$ resistance. Moreover, the causative alleles at these loci are apparently masked in YPS163-S288c hybrids that fully acquire $H_2O_2$ resistance, suggesting that they are recessive (see Discussion). We also noted during the genotyping that a small number of segregants contained the *HAP1*$^{S288c}$ (or *TOP3*$^{S288c}$) allele but were still able to acquire further $H_2O_2$ resistance (S3 Fig and S1 Table), suggesting that *HAP1* function is conditionally necessary in certain genetic backgrounds. To determine whether this was due to a unique genetic background for YPS163, we deleted *HAP1* in three additional wild strains. A wild oak (YPS1000) and wild vineyard (M22) strain showed defects in acquired $H_2O_2$ resistance similar to that of the YPS163 *hap1*$\Delta$ strain, while a wild coconut (Y10) strain showed a very slight defect (S4 Fig). Altogether, these results are consistent with *HAP1* being necessary for ethanol-induced cross protection against $H_2O_2$ in some genetic backgrounds, including those of several wild strains, but not others (see Discussion).




**Supp Figure 2.3. Effect plots for *HAP1* and *TOP3* alleles.** Boxplots and raw data points depict the distribution of segregant phenotypes depending on their alleles for either *HAP1* or *TOP3* (see methods for genotyping details).




**Supp Figure 2.4. *HAP1* is necessary for acquired $H_2O_2$ resistance in some wild strains.** Survival score plots indicating the mean and standard deviation of at least biological triplicates. The replicates for mock-treated Y10 all had the same tolerance score and thus zero standard deviation (see Table S1 for raw numerical data). Asterisks represent significant differences in acquired resistance between denoted strains (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns = not significant ($P > 0.05$), *t*-test).

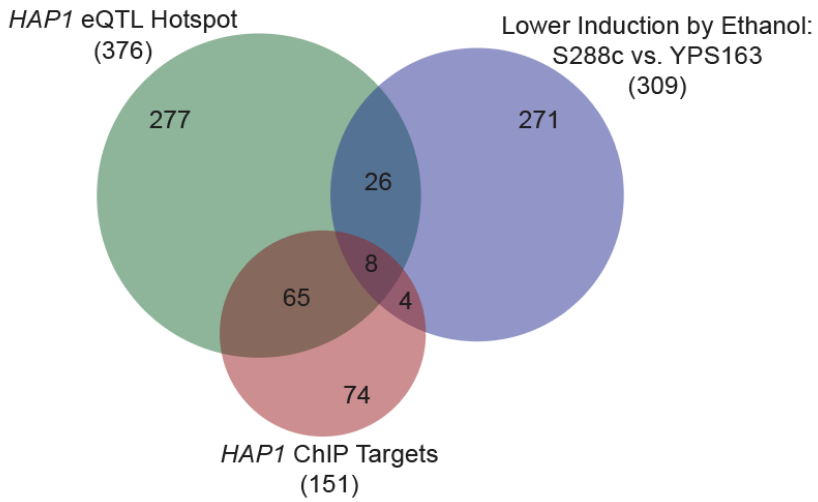
### 2.5.3 HAP1 affects catalase expression and peroxidase activity during ethanol stress.

Because Hap1p is a transcription factor, we hypothesized that acquired $H_2O_2$ resistance relied on Hap1p-dependent expression of a stress protectant protein. We reasoned that the putative stress protectant protein should have the following properties: i) a biological function consistent with $H_2O_2$ detoxification or damage repair, ii) reduced ethanol-responsive expression in S288c versus YPS163, iii) be a target gene of the *HAP1* eQTL hotspot, and iv) possess evidence of regulation by Hap1p.

We first looked for overlap between our previously identified *HAP1* eQTL hotspot (encompassing 376 genes) and genes with significantly reduced ethanol-responsive induction in S288c versus YPS163 (309 genes) (64). Thirty-four genes overlapped for both criteria, including several that directly defend against reactive oxygen species (*TSA2* encoding thioredoxin peroxidase, *SOD2* encoding mitochondrial manganese superoxide dismutase, *CTT1* encoding cytosolic catalase T, and *GSH1* encoding $\gamma$-glutamylcysteine synthetase (Fig 7A and S1 Table)). Of those 34 genes, 8 also had direct evidence of Hap1p binding to their promoters (91) (Fig 7B and S1 Table), including *CTT1* and *GSH1* (though both *TSA2* and *SOD2* have indirect evidence of regulation by Hap1p (92, 93)).

We first focused on *CTT1*, since it is both necessary for NaCl-induced cross protection against $H_2O_2$ in S288c (94), and sufficient to increase $H_2O_2$ resistance when exogenously overexpressed in S288c (67). We deleted *CTT1* in the YPS163 background, and found that ethanol-induced cross protection against $H_2O_2$ was completely eliminated (Fig 8). The complete lack of cross protection in the *ctt1*$\Delta$ mutant suggests that other peroxidases cannot compensate for the lack of catalase activity under this condition. Next, because *CTT1* was part of the *HAP1* eQTL hotspot (Fig 7C, plotted using the data described in (64)), we tested whether the S288c *HAP1* allele reduced *CTT1* expression during ethanol stress. To do this, we performed qPCR to measure *CTT1* mRNA induction following a 30-minute ethanol treatment (i.e. the peak ethanol response (45)). Consistent with our previous microarray data (45, 64), we saw lower induction of

A




B

| Gene | Description |
|---|---|
| *CTT1* | Cytosolic catalase T |
| *PIC2* | Mitochondrial copper and phosphate carrier |
| *YHB1* | Nitric oxide oxidoreductase |
| *GSH1* | γ-glutamylcysteine synthetase |
| *CYB2* | L-lactate cytochrome-c oxidoreductase |
| *FAA1* | Long chain fatty acyl-CoA synthetase |
| *PUT4* | Proline permease |
| *SUE1* | Degradation of unstable forms of cytochrome c |

C

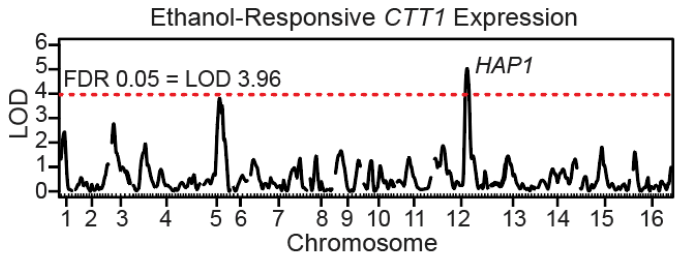



**Figure 2.7. Expression variation in Hap1p regulatory targets implicates oxidative stress defense genes as the direct effectors of ethanol-induced cross protection against $H_2O_2$.** (A) Overlap between genes that were *HAP1* eQTL hotspot targets from (64), genes with defective induction in S288c vs. YPS163 from (64), and direct targets of *HAP1* identified via ChIP experiments compiled from (91). (B) Descriptions of the eight genes that overlapped for all three criteria. (C) Previous eQTL mapping of the yeast ethanol response (newly plotted here using data described in (64)), implicated *HAP1* as causative for natural variation in *CTT1* induction levels during ethanol stress.

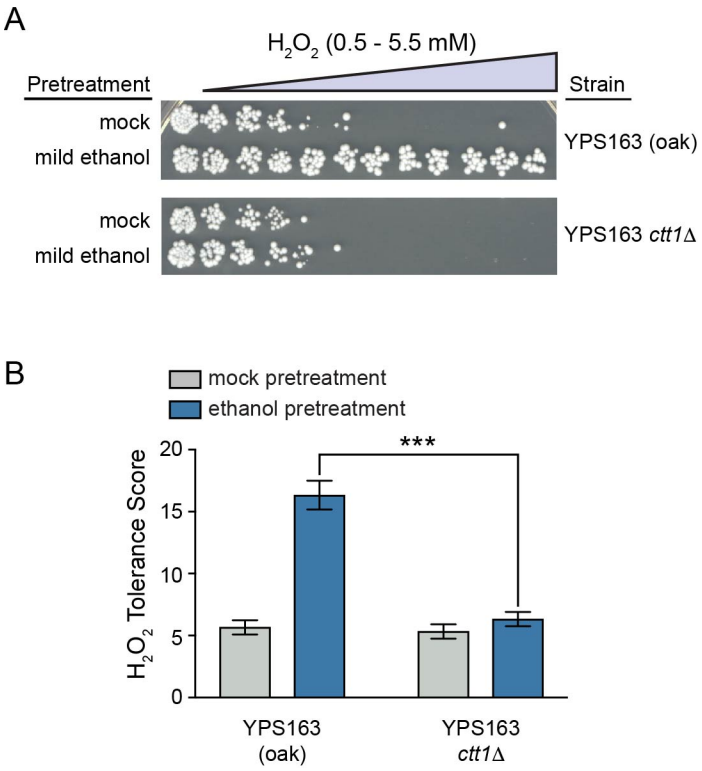



**Figure 2.8. *CTT1* function is necessary for ethanol-induced cross protection against $H_2O_2$.** (A) Representative acquired $H_2O_2$ resistance assays for wild-type YPS163 and the YPS163 *ctt1*Δ mutant. (B) Survival score plots indicating the mean and standard deviation of biological triplicates. Asterisks represent significant differences in acquired resistance between denoted strains (*** $P < 0.001$, t-test).

*CTT1* by ethanol in S288c relative to YPS163 (Fig 9a). Moreover, we saw dramatically reduced induction of *CTT1* in a YPS163 *hap1*Δ mutant compared to the wild-type YPS163 control (Fig 9a). Further support that *HAP1* is causative for reduced *CTT1* expression was provided by performing qPCR in the *HAP1* reciprocal hemizygotes, where we found that the $HAP1^{S288c}$ allele resulted in significantly reduced *CTT1* induction compared to the $HAP1^{YPS163}$ allele (Fig 9a).

To determine whether the differences in *CTT1* induction across strain backgrounds also manifested as differences in each strain's ability to detoxify $H_2O_2$, we measured *in vitro* peroxidase activity in cell-free extracts. We compared *in vitro* peroxidase activity in extracts from unstressed cells and cells exposed to ethanol stress for 60 minutes (i.e. the same pre-treatment time that induces acquired $H_2O_2$ resistance (see Materials and Methods)). For wild-type YPS163, ethanol strongly induced peroxidase activity, and this induction was completely dependent upon *CTT1* (Fig 9b). Mirroring *CTT1* gene expression patterns, the induction of peroxidase activity was reduced in a YPS163 *hap1*Δ mutant. Additionally, reciprocal hemizygosity analysis provided further support that lack of *HAP1* function results in decreased peroxidase activity, as the hybrid containing the $HAP1^{S288c}$ allele showed significantly reduced peroxidase activity following ethanol stress compared to the hybrid containing the $HAP1^{YPS163}$ allele (Fig 9b). Notably, the hybrid containing the $HAP1^{YPS163}$ allele had lower *CTT1* induction and *in vitro* peroxidase activity following ethanol shock than wild-type YPS163, despite equivalent levels of acquired $H_2O_2$ resistance in the strains. These results suggest that *HAP1* may play additional roles in acquired $H_2O_2$ resistance beyond $H_2O_2$ detoxification, depending upon the genetic background (see Discussion). Interestingly, S288c showed no induction of peroxidase activity upon ethanol treatment, despite modest induction of the *CTT1* transcript. This result is reminiscent of Ctt1p regulation during heat shock in the S288c background, where mRNA levels increase without a concomitant increase in protein levels (94). Thus, in addition to strain-specific differences in *CTT1* regulation at the RNA level, there are likely differences in regulation at the level of translation and/or protein stability.

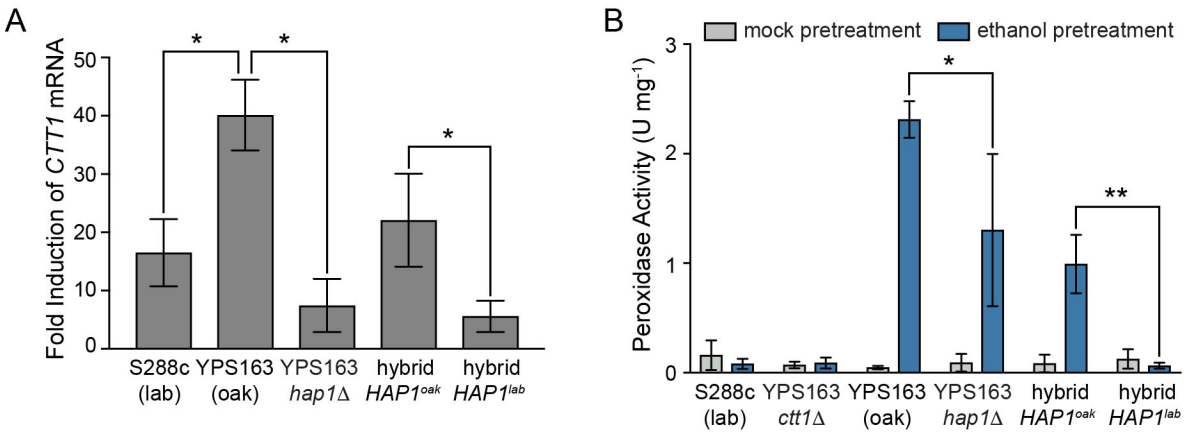



**Figure 2.9. *HAP1* is required for full induction of *CTT1* gene expression and cellular peroxidase activity during ethanol stress.** (A) Fold induction of *CTT1* mRNA in indicated strains following 30 min ethanol stress compared to unstressed cells, assessed by qPCR. (B) Peroxidase activity measured in cell-free extracts in either mock-treated or ethanol-stressed cells. The plots indicate the mean and standard deviation of biological triplicates (mRNA) or quadruplicates (peroxidase activity). Asterisks represent significant differences in *CTT1* mRNA induction or peroxidase activity between denoted strains (* $P < 0.05$, ** $P < 0.01$, paired *t*-test).

## 2.6 Discussion

In this study, we leveraged extensive natural variation in the yeast ethanol response to understand potential connections between gene expression variation and higher-order organismal traits. Previous screens of gene deletion libraries have found surprisingly little overlap between the genes necessary for surviving stress and genes that are induced by stress. (34-43). Instead, gene induction may be a better predictor of a gene's requirement for acquired stress resistance (94). Thus, we hypothesized that phenotypic variation in acquired stress resistance may be linked to natural variation in stress-activated gene expression. Our results provide a compelling case study in support of this notion—namely that a polymorphism in the *HAP1* transcription factor affects natural variation in acquired $H_2O_2$ resistance, but not the basal $H_2O_2$ resistance of unstressed cells. Forward genetic screens have shown that the genes necessary for basal and acquired resistance are largely non-overlapping (34, 36, 94), suggesting that mechanisms underlying basal and acquired stress resistance are distinct. We provide further genetic evidence to support this model. YPS163 *hap1*Δ mutants and the hybrid carrying the $HAP1^{S288c}$ allele had strong acquired $H_2O_2$ defects, but no differences in their basal $H_2O_2$ resistance (Figs 4 and 6). Moreover, the YPS163 *hap1*Δ mutant was affected only when ethanol was the mild pretreatment, and was able to fully acquire $H_2O_2$ resistance following mild $H_2O_2$ or mild NaCl (Fig 5). These results suggest that the mechanisms underlying acquired resistance differ depending upon the mild stress that provokes the response. Further dissection of the mechanisms underlying acquired stress resistance will provide a more integrated view of eukaryotic stress biology.

Our results reveal a new role for Hap1p in cross protection against $H_2O_2$ that has been lost in the S288c lab strain. We propose that a major mechanism underlying ethanol-induced cross protection against $H_2O_2$ is the induction of cytosolic catalase T (Ctt1p), and that in the YPS163 background, Hap1p is necessary for proper induction of *CTT1* during ethanol stress. We based this mechanism on the following observations. First, over-expression of *CTT1* in

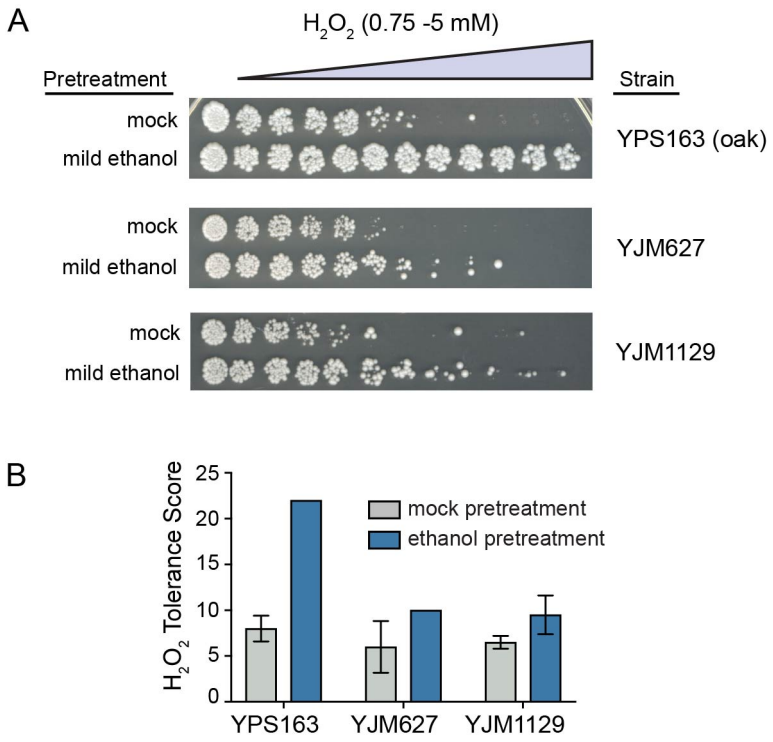
S288c is sufficient to induce high $H_2O_2$ resistance (67). Second, a YPS163 *ctt1*Δ mutant cannot acquire any further $H_2O_2$ resistance following ethanol pre-treatment (Fig. 8), suggesting that no other antioxidant defenses are able to compensate under this condition. Lastly, the defect in cross protection for the YPS163 *hap1*Δ mutant correlates with reduced *CTT1* expression and peroxidase activity during ethanol stress (compare Figs 6 and 9). How Hap1p is involved in the regulation of *CTT1* during ethanol stress remains an open question, but we offer some possibilities. Hap1p is activated by heme, thus promoting transcription of genes involved in respiration, ergosterol biosynthesis, and oxidative stress defense including *CTT1* (85, 86, 88, 92). Because heme biosynthesis requires oxygen, Hap1p is an indirect oxygen sensor and regulator of aerobically expressed genes (84, 85, 95). There is currently no evidence that heme levels are affected by ethanol stress, nor is there evidence that Hap1p is “super-activating” under certain conditions. Thus, we disfavor a mechanism of induction caused solely by Hap1p activation. Instead, we favor a mechanism where Hap1p interacts with other transcription factors at the *CTT1* promoter during ethanol stress, leading to full *CTT1* induction. One possibility that we favor is recruitment of the general stress transcription factor Msn2p, which plays a known role in acquired stress resistance (44, 45). We previously showed that a YPS163 *msn2*Δ mutant had no induction of *CTT1* mRNA during ethanol stress (45), suggesting that Msn2p was an essential activator for *CTT1* under this condition. The *CTT1* promoter region contains three Msn2p DNA-binding sites, two of which are ~100-bp away from the Hap1p binding site. Hap1p binding to the *CTT1* promoter could help recruit Msn2p during ethanol stress, possibly through chromatin remodeling that increases accessibility of the Msn2p binding sites as proposed by Elfving and colleagues (96).

What is the physiological role of Hap1p-dependent induction of *CTT1* during ethanol stress? One possibility is that regulation tied to the heme- and oxygen-sensing role of Hap1p ensures that *CTT1* induction only occurs under environmental conditions where reactive oxygen species (ROS) are most likely to be encountered—namely stressful conditions that are also

aerobic. In the context of ethanol stress, aerobic fermentation would lead to subsequent respiration of the produced ethanol and simultaneous ROS production. Under these conditions, *CTT1* induction leading to ethanol-mediated cross protection against ROS would likely confer a fitness advantage. On the other hand, during stressful yet anoxic conditions, Ctt1p and other ROS-scavenging proteins are likely unnecessary. Furthermore, because heme is not synthesized during anoxic conditions (84), Hap1p would fail to induce *CTT1* and other genes encoding non-essential heme-containing proteins. This may improve fitness by conserving energy used for biosynthesis and by redirecting limited heme to more essential heme-containing proteins.

The S288c lab strain has long been known to possess a defective *HAP1* allele (89). Apparently, the defective allele arose relatively recently, as only S288c contains a *HAP1* Ty1 insertion out of over 100 sequenced strains (97, 98). The lack of *HAP1* function in S288c could be due to relaxation of selective constraint, though others have argued in favor of positive selection for reduced ergosterol biosynthetic gene expression (99, 100). Regardless, the loss of ethanol-induced acquired $H_2O_2$ resistance is likely a secondary effect of the loss of Hap1p function. Intriguingly, we did find that two (non-S288c) domesticated yeast strains also lack ethanol-induced cross protection against $H_2O_2$ (S5 Fig), suggesting that phenotypic differences in acquired stress resistance may differentiate domesticated versus wild yeast. Because environmental stresses are likely encountered in combination or sequentially (101), acquired stress resistance is likely an important phenotype in certain natural ecological settings. Future studies directed at understanding differences in acquired stress resistance phenotypes in diverse wild yeast strains may provide unique insights into the ecology of yeast.

While our QTL mapping identified *HAP1* as the major effector of cross protection, we note that additional complexity remains unexplained. Notably, despite the strong cross protection defect in the YPS163 *hap1*Δ mutant, some residual cross protection persists that is absent in S288c (Fig 6). Intriguingly, the residual cross protection is also absent in the hybrid carrying the




**Supp Figure 2.5. Other non-S288c-derived yeast isolates lack ethanol-induced cross protection against $H_2O_2$.** (A) Representative acquired $H_2O_2$ resistance assays for wild-type YPS163, YJM627, and YJM1129. (B) Survival score plots indicating the mean and standard deviation of biological duplicates. The replicates for ethanol-treated YJM627 all had the same tolerance score and thus zero standard deviation (see Table S1 for raw numerical data).

*HAP1*$^{S288c}$ allele, suggesting the involvement of other genes depending upon the genetic background (Figs 4B and 4C). It is known that yeast strains with respiratory defects have increased ROS sensitivity (102, 103), potentially due to increased programmed cell death (104). It is possible that reduced respiratory activity and concomitant ROS sensitivity in strains lacking *HAP1* is exacerbated by genetic interactions with other alleles.

The lack of cross protection in S288c and the *HAP1*$^{S288c}$ hybrid correlates with the lack of inducible peroxidase activity following ethanol pretreatment in those strains. The lack of inducible peroxidase activity in S288c despite modest induction of *CTT1* mRNA could be due to translational regulation, which is supported by the observation that while mild heat shock induces *CTT1* mRNA, protein levels remain nearly undetectable (94). Strikingly, the hybrid carrying the *HAP1*$^{YPS163}$ allele still cross protects despite levels of *CTT1* mRNA induction and peroxidase activity that are lower than in the YPS163 *hap1*Δ strain that is unable to acquire further resistance (Fig 9). These data suggest that *HAP1* plays an additional role in ethanol-induced cross protection beyond $H_2O_2$ detoxification by Ctt1p. Moreover, the continuous distribution of the cross protection phenotype in the segregants (S1 Fig) and the results of allele swap experiments (Fig 6) strongly implicate other genes and processes in this complex trait. Specifically, the lack of complementation by the *HAP1*$^{YPS163}$ allele in the S288c background suggests that additional loci in S288c render *HAP1* necessary but not sufficient for cross protection in this background. Moreover, our genotyping of the segregants at *HAP1* revealed a small number that still possessed cross protection in the absence of functional *HAP1* (S3 Fig and S1 Table), suggesting that *HAP1* is dispensable in certain genetic backgrounds. We examined the effects of *hap1*Δ mutations in other wild strain backgrounds and found two additional strains with a strong *HAP1* requirement and a third strain with at most a mild *HAP1* effect (S4 Fig). This result, as well as those from other recent studies (105-107), suggests that these types of genetic background effects are likely the rule rather than the exception. Future

high resolution mapping experiments will be necessary to identify and characterize the source of these genetic background effects.

Gene expression variation is extensive in nature and is hypothesized to be a major driver of higher-order phenotypic variation. However, there are inherent challenges to connecting gene expression variation to higher-order organismal traits. Hundreds to thousands of genes are often differentially expressed across individuals, so identifying which particular transcripts exert effects on fitness is difficult. By studying acquired stress resistance—a phenotype better correlated with stress-activated gene expression changes—we were able to uncover a novel connection between gene expression variation and an organismal trait.

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### 2.7 Supplemental Information

All supplemental tables can be found here: https://journals.plos.org/plosgenetics/article?id=10.1371/journal.pgen.1007335#sec013

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# Chapter 3 Natural variation in stress signaling reveals multiple paths for acquiring higher stress resistance.

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Author contributions: Cader J. Locke constructed *GSH1* deletion strain and performed cross protection assays. I performed all other strain constructions. Dr. Tara N. Stuecker helped perform cross protection assays on transcription factor and *GSH1* mutants. I performed all other experiments and analyses in this study.

## 3.1 Abstract

An individual's physiological response to different environmental conditions often depends on their individual genetic background. However, the mechanism underlying these so-called "gene-environment interactions" are generally poorly understood. We have been exploiting natural variation in *Saccharomyces cerevisiae* stress responses to understand the role of gene-environment interactions in a unique phenotype called acquired stress resistance, where cells that are pretreated with a mild, sub-lethal dose of stress can then survive an otherwise lethal doses of severe stress. We have found that a commonly-used lab strain of yeast can acquire further oxidative stress resistance, but it depends on the pretreatment. For example, while mild salt pretreatment can induce hydrogen peroxide resistance, mild ethanol stress cannot. In contrast, most wild yeast strains can acquire peroxide resistance when pretreated with mild ethanol. Because salt-induced acquired peroxide resistance requires catalase activity in the lab strain, we tested whether catalase was necessary for acquired peroxide resistance in over a dozen diverse yeast strains. Surprisingly, we found a wide range of catalase dependency for acquired peroxide resistance, despite similar levels of acquired resistance in wild-type cells. We hypothesized that variation in catalase dependency was due to gene expression variation in oxidative stress defense genes. Transcriptional profiling revealed differential expression of these potentially compensatory oxidative stress genes, as well as the potential transcription factors regulating clusters of differentially expressed genes. Our approach highlights the power of using natural variation to reveal novel aspects of signaling networks, which may play a large role in shaping variation in gene-environment interactions across diverse organisms.

### 3.2 Introduction

How individuals respond to different environmental conditions often depends on their individual genetic background and can have profound effects on organismal fitness. While the mechanisms underlying these so-called “gene-environment interactions” is unclear, one possibility is that gene expression differences across individuals are responsible. Gene expression variation is a pervasive source of phenotypic variation in nature (1-4), and is even thought to underlie the extensive differences we see between humans and chimpanzees despite >98% DNA sequence identity between the two species (5, 6). One major challenge for connecting gene expression variation to higher-order organismal traits is the choice of trait to examine. In some cases, expression variation may be effectively neutral, while in other cases phenotypic effects may only be present under specific conditions (7, 8).

To understand the relationship between gene expression variation and gene-environment interactions, we have been leveraging extensive variation in *Saccharomyces cerevisiae* stress responses to understand their impact on a novel phenotype called acquired stress resistance, where cells pretreated with a mild sub-lethal stress gain the ability to survive an otherwise lethal severe dose of stress. These stresses can be the same (same stress protection) or different (cross stress protection).

We have argued that acquired stress resistance is a phenotype much better linked to gene expression than the intrinsic resistance of unstressed cells (9, 10) based on the following rationale. Many studies have shown a poor correlation between the genes that respond to stress and their importance for stress survival (11-13), thus suggesting that the purpose of stress-activated gene expression is not to survive the immediate insult. Additionally, yeast respond to diverse stresses by coordinating the expression of condition-specific genes with a large, common gene expression program called the environmental stress response (ESR) (14). Defective ESR expression correlates with diminished acquired stress resistance, suggesting that stress-activated gene expression changes may instead protect cells from future challenges

(9, 15). Beyond yeast, acquired stress resistance is widespread across organisms ranging from bacteria to higher eukaryotes (16-27). In both bacteria and mammalian cells, mild oxidative stress protects against hydrogen peroxide and radiation stresses (28, 29). Additionally, caloric restriction (i.e. starvation stress) and ischemic preconditioning (transient blocking of blood supply followed by reperfusion of oxygenated blood that causes mild oxidative stress) have been shown to induce cardio protection during heart attacks and heart surgery (30-32).Thus, understanding the mechanisms underlying acquired stress resistance has broad implications in fields ranging from food microbiology to human medicine.

We previously found that when a commonly-used lab strain was pretreated with mild ethanol, it failed to protect against higher levels of hydrogen peroxide, while a wild oak isolate could (10). This difference was genetically mapped to a mutation in the gene encoding the Hap1p transcription factor, which affected the expression of the gene encoding cytosolic catalase T (*CTT1*), a key hydrogen peroxide scavenging enzyme (10). Our previous results showed that lack of *CTT1* completely abolished ethanol-induced cross protection against hydrogen peroxide in the wild oak strain YPS163 (10). Likewise, an S288c (lab strain) *ctt1*$\Delta$ mutant completely lacks salt-induced cross protection against hydrogen peroxide (12), suggesting that there are perhaps no compensatory mechanisms of acquired hydrogen peroxide resistance.

In this study, we examined yeast strains from diverse genetic backgrounds and found surprising evidence for cytosolic catalase T, highly conserved protein hydrogen peroxide scavenging enzyme, and alternative mechanisms of cross protection against hydrogen peroxide. Depending upon strain background, these compensatory mechanisms can be activated by ethanol stress, salt stress, or both. Transcriptional profiling of the ethanol and salt responses, in diverse strains, further implicated possible regulators of these different protective mechanisms. Ultimately, this study highlights how superficially similar traits can have different

underlying molecular bases and provides a framework to examine natural variation in other organisms.

### 3.3 Materials and Methods

#### 3.3.1 Strain and growth conditions

All parental strains used in this study are listed in Table 1. All Homozygous strains were generated by sporulation and tetrad dissection before moving the *ctt1Δ*::KanMX allele from the YPS163 background (9), followed by another round sporulation and tetrad dissection to generate homozygous *ctt1Δ* strains. For construction of transcription factor and *GSH1* deletions, first, the MX cassette from *ctt1Δ*::KanMX deletion was replaced with a NatMX cassette (33), selecting for nourseothricin resistance followed by sporulation and tetrad dissection. Then the transcription factor or *GSH1* deletions were moved into homozygous YPS606 and YPS606 *ctt1Δ* (*ctt1Δ*::NatMX) by homologous recombination with the deletion::KanMX cassette amplified from the appropriate yeast knockout strain (34), followed by sporulation and tetrad dissection. To generate *msn2/msn4Δ* and *ctt1/msn2/msn4Δ*, the MX cassette from *msn4Δ*::KanMX deletion was replaced with a HygMX cassette, selecting for hygromycin resistance followed by sporulation and tetrad dissection before amplification and homologous recombination. For homozygous deletions *msn2/msn4Δ* and *ctt1/msn2/msn4Δ* strains were sporulated and dissected. All deletions were verified by diagnostic PCR.

#### 3.3.2 Cross protection assays

Cross protection assays were performed as described (15) with modifications. Briefly, overnight cultures were prepared using 3-4 isolated colonies from a freshly streaked plate (<1 week old) in YPD and grown to saturation at 30°C shaking at 270 rpm. Subcultures were grown for at least 8 generations overnight (35). Once cells reached mid-log ($OD_{600}$ 0.3-0.6), cultures

were split into a either a mock (YPD control) or mild experimental sample of either 5% (v/v) ethanol YPD or 0.4 M NaCl YPD. All samples incubated at 30°C shaking at 270 rpm for 1 hour (pretreatment). Cells were collected via centrifugation (1,500 x g for 3 minutes) and resuspend to $OD_{600}$ of 0.6 with fresh YPD. 50 μl of resuspended cells were transferred into a flat bottom 96-well plate containing 100 μl of increasing concentrations (0.75-5 mM) of hydrogen peroxide (secondary stress), sealed with a breathable membrane (VWR 60941-086), and incubated for 2 hours at 30°C with 800 rpm shaking in a VWR symphony Incubating Microplate Shaker. Cells were diluted (1:50) in fresh YPD in a flat bottom 96-well plate then 4 μl were spot plated onto YPD agar plates and grown for 48 hours at 30°C. Each colony was scored using a semi-quantitative scale comparing viability to the no secondary stress YPD control (100% viability = 3 pts, 50-90% viability =2 pts, 10-50% viability = 1 pt, and <10% or < 3 colonies = 0 pt) to determine hydrogen peroxide tolerance. A detailed protocol can be found on protocols.io (dx.doi.org/10.17504/protocols.io.g7sbzne). To determine WT percent max hydrogen peroxide acquisition:

$$WT\ percent\ max\ hydrogen\ peroxide = WT\ mild\ stress\ (pretreatment)\ tolerance\ score - WT\ intrinsic\ (no\ pretreatment) tolerance\ score$$

That value is now 100% as it is the wildtype phenotype. To calculate the percent max hydrogen peroxide of deletions:

$$Deleteion\ percent\ max\ hydrogen\ peroxide = ((deletion\ mild\ stress\ (pretreatment) tolerance\ score - deletion\ intrinsic\ (no\ pretreatment) tolerance\ score) / (WT\ mild\ stress\ (pretreatment) tolerance\ score - WT\ intrinsic\ (no\ pretreatment) tolerance\ score)) * 100$$

All statistical analyses on cross protection assays were performed using Prism 7 (GraphPad Software).

### 3.3.3 Cell collections, RNA extraction, library preparation and sequencing

Cells were grown to mid-log ($OD_{600}$ 0.3-0.6). A sample was taking before being exposed to ethanol (5% final concentration) or salt (0.4M NaCl). Cells were incubated for 30 minutes (9) for ethanol or 45 minutes for salt (15), before being collected via centrifugation and flash frozen in liquid nitrogen. Samples were stored at -80°C until ready for RNA extraction.

RNA was extracted using a hot phenol extraction as described in (36), followed by an off-column DNase I (Ambion AM2222) digestion. The total RNA was purified using Quick-RNA MiniPrep Plus Kit (Zymo Research R1057) along with an on-column DNase I digestion. Aglient TapeStation was used to determined RNA integrity. Total RNA concentrations were quantified by Qubit. A detailed protocol of the RNA isolation can be found here: (dx.doi.org/10.17504/protocols.io.inwcdfe). Libraries were prepared following KAPA mRNA HyperPrep Kit protocol (Roche 08098123702) using KAPA Single-Indexed Adapter Kit (Roche 08005699001) and an epMotion 5075 automatic pipetting machine. Briefly, 500ng total RNA was used as input. Libraries were amplified for 9 cycles and half reactions were used. A detailed protocol can be found on protocols.io (dx.doi.org/10.17504/protocols.io.uueewte). Libraries were sent to the University of Chicago Genomics Facility, where libraries were analyzed via Bioanalyzer high sensitivity tapes. Samples were pooled by replicates and a final AMPure bead cleanup was performed to remove any remaining adapters. To minimize against batch effects, all RNA-seq libraries replicates were constructed on the same day and each replicate was multiplexed and sequenced on a single lane of an Illumina HiSeq4000 instrument.

### 3.3.4 RNA Sequencing Analysis

All low-quality reads and adapters were trimmed using Trimmomatic (Version 0.38) (37) using the following command ILLUMINACLIP:Kapa_indices.fa:2:30:10 LEADING:3 TRAILING:3 MAXINFO:40:0.4 MINLEN:40. For generating each strain reference genome all reads were mapped using Bowtie2.0 (Version 2.3.4.1) (38) to S288c genome (Version Scer3). Variants were called using bcftools (Version 1.9) (39, 40) and new reference genomes were created using GATK3 GenomeAnalysisTK (Version 3.8-1-0) (41). Trimmed reads were then mapped to their new corresponding reference genome using STAR (Version 2.6.1) (42). RSEM (Version 1.3.1) was used to generate read counts (43). Differential expression was calculated using Bioconductor's edgeR (Version 3.26.4) using generalized linear model (44). For the generalized linear model, sample type (i.e. YPS606 Mock, YPS606 Ethanol, YPS606 Salt, M1 Mock…) biological replicate were used as factors. Two contrasts were made: strain specific response ($strain_1$ experimental - $strain_1$ control) and strain vs average response (($strain_1$ experimental - $strain_1$ control)/(average of all stress strain specific responses)). Only genes with at least 1 count per million (CPM) in at least one condition were included in analyses.

All hierarchical cluster were done using Cluster 3.0 (45) using Euclidean correlation, for RNA-Sequencing (Figure 3) and Centered Pearson correlation for tolerance scores (Figure 2), and centroid linkage with a weighted cutoff of 0.4. Java Treeview was used for heatmap cluster visualization (46). Functional enrichments of gene ontology (GO) categories were performed using Princeton's GO-TermFinder (https://go.princeton.edu/cgibin/GOTermFinder) (47), with Bonferroni-corrected P-values < 0.01 taken as significant. Possible regulators were determined by taking induced clusters and searching for all possible transcription factors using Yeastract (http://www.yeastract.com/formrankbytf.php) (48).

## 3.4 Results

### 3.4.1 Wild oak strain (YPS606) requires catalase (CTT1) for ethanol-induced cross protection, but not for salt-induced cross protection.

We previously reported that a commonly used lab strain of yeast fails to acquire higher hydrogen peroxide resistance when pretreated with ethanol but a wild oak strain (YPS163) can (10). Cells lacking *CTT1* encoding cytosolic catalase T, a key hydrogen peroxide scavenging enzyme, completely failed to acquire hydrogen peroxide resistance when pretreated with ethanol (10). To understand whether this catalase dependency was a general feature of acquired peroxide resistance in yeast, we tested whether *CTT1* was essential for acquired peroxide resistance in other diverse strain backgrounds. Cross protection assays were performed by exposing cells to a mild, sublethal dose of ethanol (5% v/v) or salt (0.4M NaCl) for 60 min, followed by exposure to a panel of 11 increasingly severe doses of hydrogen peroxide, which were used to calculate percent maximum hydrogen peroxide acquisition in the mutant relative to the wild-type control strain (see Material and Methods). For example, we saw a similar catalase dependent ethanol-induced hydrogen cross protection in another wild oak strain YPS606 (Figure 3.1A), implying that catalase was required for proper protection against hydrogen peroxide. Previous studies have been focused on variation in a strain's response to ethanol and ethanol-induced hydrogen peroxide acquisition (9, 10, 49) and because salt-induced acquired peroxide resistance required catalase activity in the S288c lab strain (12), we tested whether catalase was necessary for salt-induced acquired peroxide resistance in the YPS606 wild oak strain. Surprisingly, we found that there was still moderate residual cross protection in the absence of *CTT1* (Figure 3.1B).

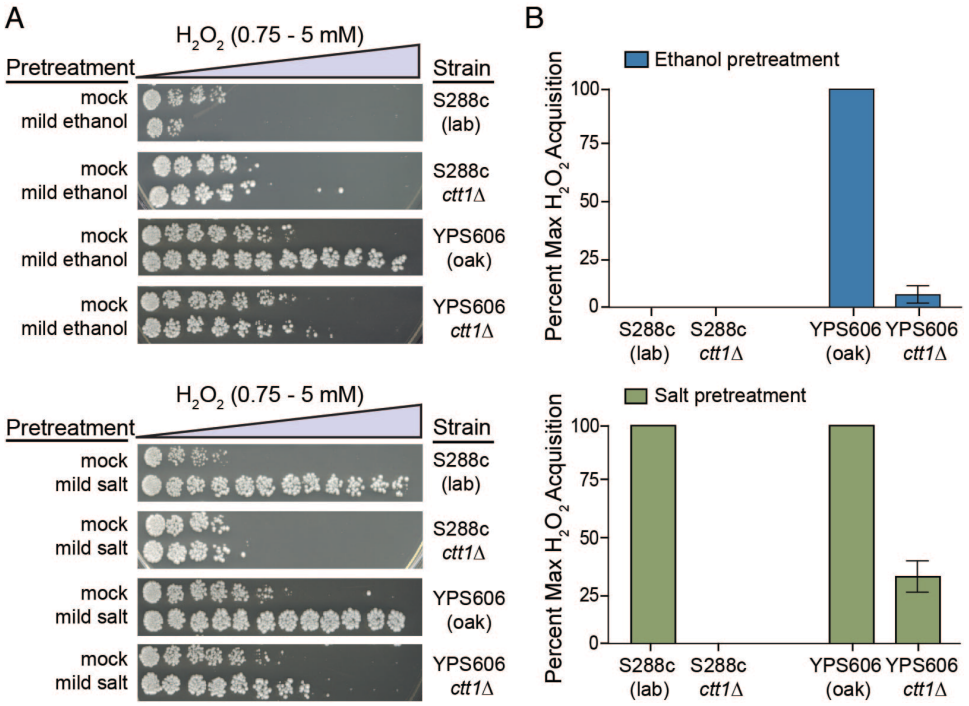



**Figure 3.1: Natural variation in ethanol- and salt-induced hydrogen peroxide cross protection.** (A) A representative acquired $H_2O_2$ resistance assay of wildtype and *ctt1Δ* of S288c (lab strain–DBY8268) and YPS606 (wild oak strain) is shown. S288c and YPS606 were exposed to either 5% ethanol, 0.4M NaCl, or mock (YPD control) pretreatment for 60 min, washed, exposed to 11 doses of severe $H_2O_2$ for 2 hr, and then plated to score viability. (B) Percent max $H_2O_2$ acquisition was calculated from the differences of viability of the pretreatment vs the mock control of the wildtype strain and set to 100%. Percent max $H_2O_2$ acquisition of the *ctt1Δ* was calculated the differences of viability of the pretreatment vs the mock control of the ctt1Δ divided by the percent max $H_2O_2$ acquisition value of the wildtype strain, multiplied by 100 to obtain the percent (see Materials and Methods). Each plot shows the mean and standard deviation of 3 independent biological replicates for S288c and 4 independent biological replicates for YPS606.

### 3.4.2 CTT1 is the key component, but not necessary for ethanol- or salt-induced hydrogen peroxide acquisition in some wild strains.

To investigate the role of *CTT1* in acquired stress resistance in wild strains, we performed ethanol and salt-induced cross protection assays in 13 strains from diverse environments (Table 3.1). When we tested the *CTT1* essentiality in wild oak strain YPS606, we saw a catalase dependent phenotype when pretreated with ethanol and an independent phenotype when pretreated with mild salt (Figure 3.1). Using our YPS606 data and previously reported YPS163 dependence on *CTT1* for ethanol-induced hydrogen peroxide acquisition (10), we hypothesized that similar wild strains would behave alike. Interestingly, the catalase requirement could differ if ethanol or salt was the mild stress pretreatment and only some strains required catalase for cross protection against hydrogen peroxide, Figure 3.2C. For instance, we saw four distinct groups of catalase dependency. In the absence of catalase, wild oak isolates (YPS606, YPS163 and YPS1000) acquired hydrogen peroxide resistance only when pretreated with salt and not ethanol. Strains M22 (vineyard isolate), YJM308 (clinical isolate), and Y12 (palm wine isolate), partially acquired hydrogen peroxide resistance when pretreated with either ethanol or salt, while other strain isolates: Y10 (natural isolate), Y2 (other fermentation isolate), M32 (vineyard isolate) and M1 (vineyard isolate) did not acquire hydrogen peroxide resistance when catalase was deleted. Lastly, we had strains that did not acquire hydrogen peroxide resistance when pretreated with ethanol (S288c, YJM1129, and YJM627). Intriguingly, all strains fully acquired hydrogen peroxide resistance when pretreated with salt when catalase was present (Supp Figure 3.1).

Table 3.1: Strains used to evaluate the natural variation of the environmental stress response.

| Strain ID | Strain Classification | Isolation Location |
|---|---|---|
| M22* | Vineyard | Vineyard, Tuscany, Italy |
| M32 | | Vineyard, Tuscany, Italy |
| M1* | | Vineyard, Italy |
| YPS163* | Oak | Oak soil sample, Pennsylvania |
| YPS1000 | | Oak exudate isolate, Mettlers Woods, NJ |
| YPS606* | | Bark of an oak tree, Lima, PA |
| Y10* | Natural | Coconut, Philippines |
| Y2 | Other Fermentation | Rum Fermentation, Trinidad |
| Y12 | | Palm wine, Ivory Coast |
| YJM1129* | | Distillery |
| YJM308* | Clinical | United States |
| DBY8268* | Lab | S288c derivative, California |
| YJM627 | | Y55 segregate, France |

* indicates strains were used for RNA-Sequencing.
Strains are color coated based on acquisition groups. Red denotes strains that are catalase independent. Blue denotes strains that are catalase dependent. Green denotes strains that are catalase dependent for ethanol and catalase independent for salt. Black denotes strains that fail to acquire hydrogen peroxide resistance when pretreated with ethanol.

As wine yeast showed increase resistance to sulfides (wine preservative) (50), we hypothesized catalase dependency might correlate with the environmental niche. Instead, we found the degree of *CTT1* dependency did not depend on the strain environment. We found that in strains that still acquire hydrogen peroxide resistance in the absence of catalase did not cluster based on environment (Figure 3.2), with the exception of the oak isolates. The wild oak strains all shared similar catalase dependencies was not altogether unexpected based on their relatively high levels of genetic similarity (51, 52).

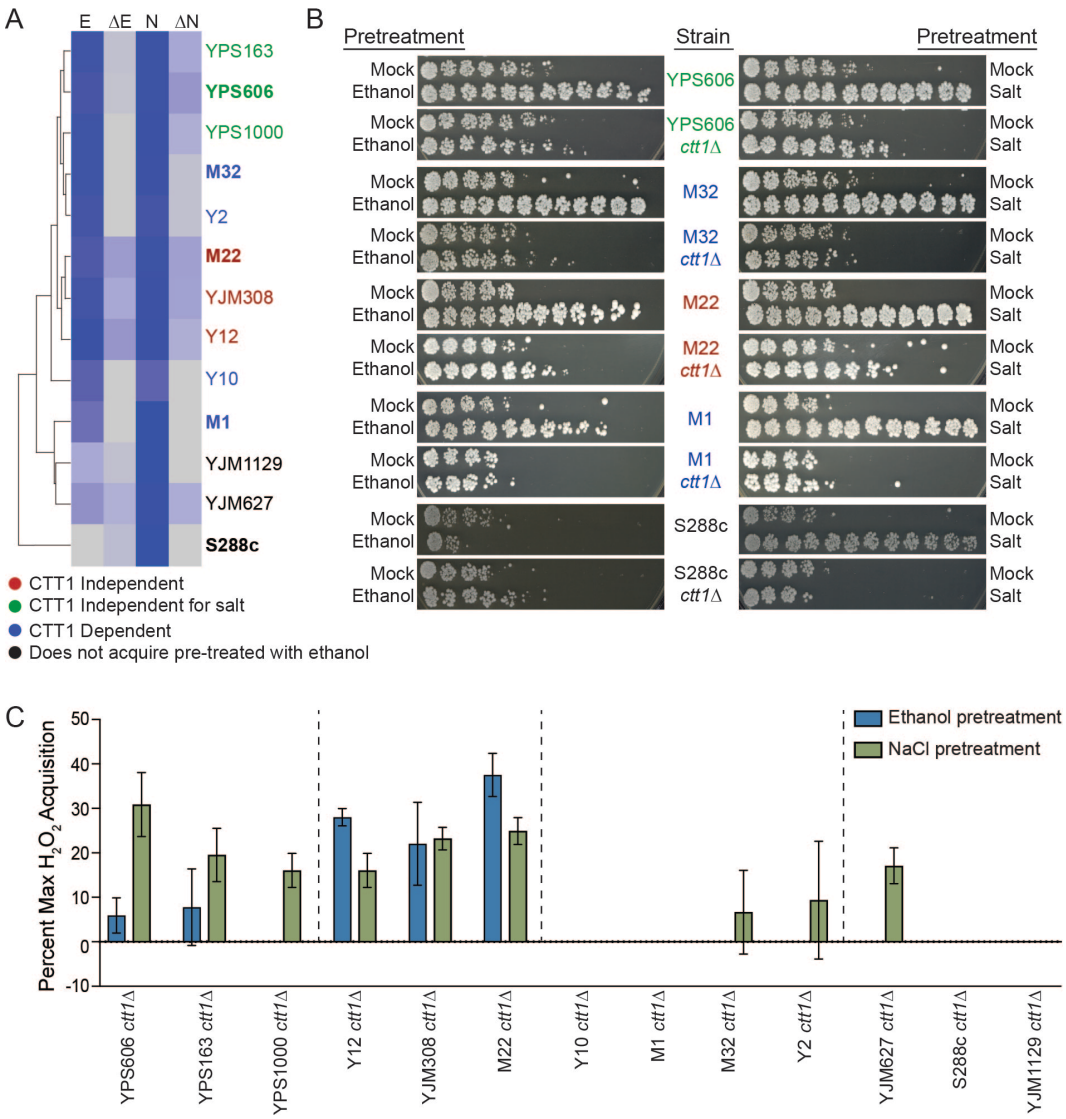
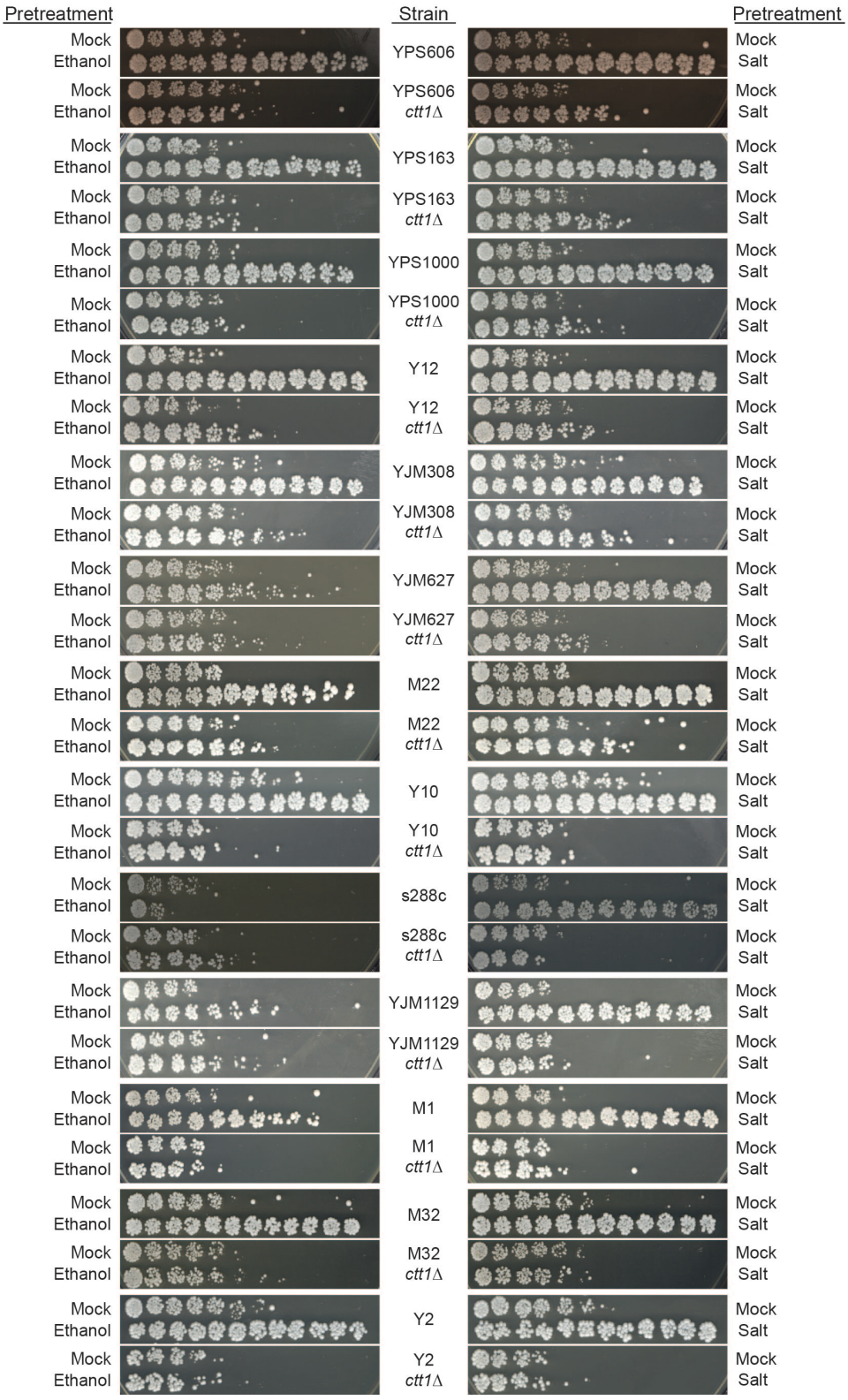



**Figure 3.2: Hierarchical clustering of hydrogen peroxide acquisition tolerance scores/phenotype.** (A) Strains were organized by hierarchical clustering using Cluster3.0 on average of tolerance scores replicates. Each row indicates a strain labeled on the right, and each column represents a different condition labeled on the top (E, ethanol; ΔE, *ctt1Δ* ethanol; N, NaCl; ΔN, *ctt1Δ* NaCl). Strains are color coated based on their acquisition groups. Dark blue colored boxes represent increased hydrogen peroxide acquisition and light gray indicates no hydrogen peroxide acquisition under the designated conditions.(B) Representative acquired $H_2O_2$ resistance assay of different clusters. (C) Four classes of hydrogen peroxide cross protection *CTT1* dependency indicating the mean and standard deviation of biological duplicates except for YPS163 (27 reps), YPS606 (4 reps), and S288c (3 reps).




**Supp Figure 3.1: Ethanol- and Salt-induced hydrogen peroxide acquisition varies based in wild strains.** Representatives of each cross protection assay shown. Biological duplicates were used except for YPS163 (27 reps), YPS606 (4 reps), and s288c (3 reps).

### 3.4.3 GSH1 is only partially responsible for the CTT1 independent ethanol and/or salt-induced hydrogen peroxide acquisition.

In the absence of *CTT1*, the wild oak strain YPS606 still acquired salt-induced hydrogen peroxide acquisition; therefore, another hydrogen peroxide scavenging enzyme is likely responsible. In addition to catalases, cells possess a number of ways to detoxify hydrogen peroxide with the thioredoxin-dependent or glutathione-dependent peroxidases being the most common (53-57). To hone in on the responsible alternative peroxidase, we first deleted the gene encoding the first step in glutathione biosynthesis (*GSH1*), both alone and in combination with *ctt1Δ* in wild oak (YPS606) strain, to determine whether a glutathione-dependent peroxidase was responsible for the partial *CTT1* independent salt-induced hydrogen peroxide acquisition. We used YPS606 as it showed one of the strongest *CTT1*-independent hydrogen peroxide acquisitions (Figure 3.2C). In the absence of *GSH1,* YPS606 fully acquired hydrogen peroxide acquisition when pretreated with salt, suggesting that *CTT1* is sufficient for maximal acquisition. When *GSH1* was deleted in combination with *CTT1*, we saw further reduction of hydrogen peroxide acquisition beyond the *ctt1*Δ alone (Figure 3.3), suggesting the alternative peroxidase, in YPS606, is partially glutathione dependent. This proposes that there are at least two routes for acquired hydrogen peroxide resistance. These findings reiterate that there are multiple ways for cells to combat hydrogen peroxide stress and it may vary based on strain background (see Discussion).

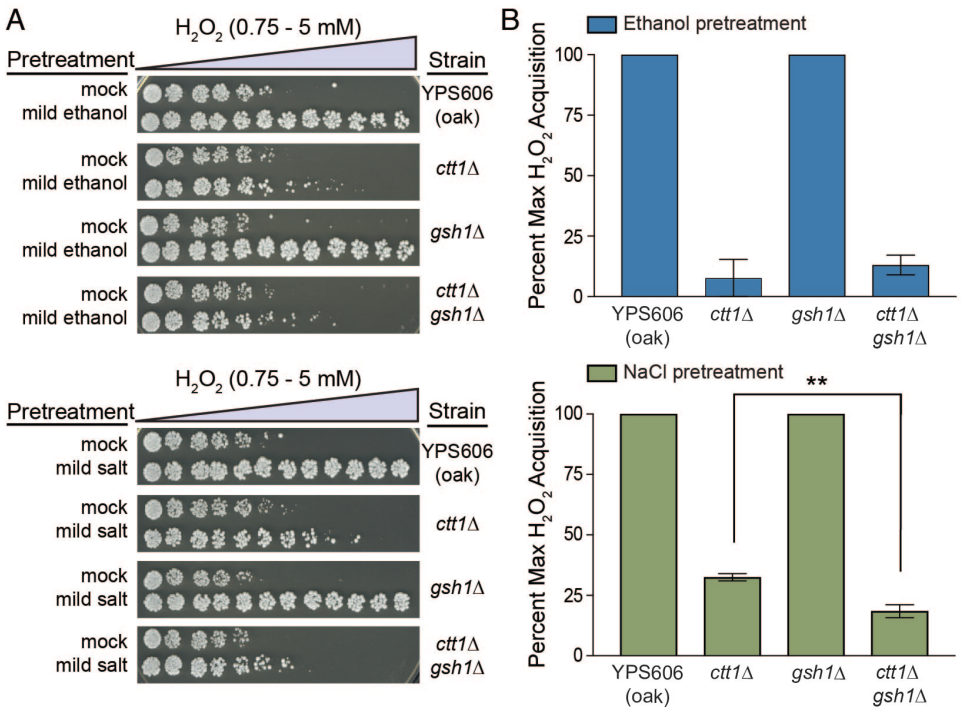



**Figure 3.3: *GSH1* is partially responsible for activating the alternative peroxidase under salt-induced hydrogen peroxide resistance in wild oak strain YPS606.** (A) A representative acquired $H_2O_2$ resistance assay of wildtype, *ctt1Δ, gsh1Δ, and ctt1Δ gsh1Δ* YPS606 is shown. Error bars indicate the mean and standard deviation of at least biological triplicates. Asterisks represent significant differences in percent max hydrogen peroxide acquisition between denoted strains (** P <0.01, *t-test*).

### 3.4.4 Extensive variation in the ethanol and salt responses between wild strains.

It has been previously shown that acquired stress resistance requires gene expression (9, 58) and variation in acquired resistance has been linked to variation in gene expression (59). Since we saw strain differences in hydrogen peroxide acquisition, we hypothesized that these phenotypic differences were due to gene expression variation. To address this hypothesis, we performed RNA-sequencing on strains that were exposed to 5% ethanol for 30 min (peak transcriptional response (9)) or 0.4M NaCl for 45 min (peak transcriptional response (15)) in biological triplicate. We chose two representatives from each catalase group, to gain a better understanding of the variation in response to ethanol and salt. For ethanol catalase independent for salt: YPS606 and YPS163; catalase independent for ethanol and salt: M22 and YJM308; catalase dependent for ethanol and salt: M1 and Y10; and did not acquire with ethanol: YJM1129 and S288c-derived strain DBY8268. We found a total of 3,918 genes with differential expression in ethanol responses (Figure 3.4A) and a total of 2,222 genes with differential expression in response to salt in any strain relative to the mean, Figure 3.4B (FDR < 0.01). While there was generous overlap between the differentially expressed genes (1,720 genes), each stress had genes that were stress specific (2,198 ethanol specific genes and 502 salt specific genes).

To identify patterns of co-regulated genes that differ across strains, we performed hierarchical clustering on all genes that were significantly differentially expressed in comparison to the mean expression value of all strains (FDR <0.01). We saw clusters enriched for the repressed ESR such as ribosome biogenesis, translation, and cell cycle. The induced ESR is also present with enrichments of detoxifying ROS, carbohydrate metabolism, and other catabolic processes. Even though we saw evidence of the ESR, we still noticed strain-specific differences. For example, S288c displayed a reduced response to ethanol when compared to the wild strains, showing lower repression of genes involved in the repressed ESR and lower induction of genes involved in the induced ESR. We have previously shown that S288c has

decreased *CTT1* expression compared to YPS163, and that ethanol fails to induce measurable peroxidase activity (10). Here, we saw the differences of catalase induction. *CTT1* independent strains M22 (3-fold increase) and YJM308 (8.5-fold increase) had higher *CTT1* expression compared to the mean of all strains, while S288c still induced *CTT1* but at a much lower degree (0.07-fold increase compared to the mean). Interestingly, YJM1129, the other strain that failed to acquire hydrogen peroxide acquisition when pretreated with ethanol, expressed *CTT1* equivalently to many strains that did acquire, which could suggest that *CTT1* may not be functional in that strain. However, when YJM1129 was pretreated with salt, we saw full acquisition in a catalase dependent manner, proving catalase is likely, at least, somewhat functional but perhaps has reduced expression or activity during ethanol stress due to translational or post-translational control.

Although all strains acquired hydrogen peroxide acquisition when pretreated with salt (Supp Figure 3.1), we still saw catalase dependent and independent phenotypes, suggesting different mechanisms for how to cope with hydrogen peroxide. Surprisingly, the vineyard strain, M1 had a much more muted response to salt compared to the other wild strains. M1 had lower expression of *CTT1* and alternative peroxidase candidates compared to the other wild strains, suggesting strain specific upstream regulation differences. Strains that acquired hydrogen peroxide acquisition in a catalase dependent manner (M1, YJM1129, and S288c) had lower induction of genes associated with carbohydrate metabolism and detoxifying ROS (Figure 3.4B) when exposed to mild salt. Interestingly Y10, a *CTT1* dependent strain for ethanol and salt-induced hydrogen peroxide acquisition, had similar gene expression changes as strains that are *CTT1* independent during salt stress. While wild oak strains, YPS606 and YPS163, still clustered close together we do see some strain specific gene expression variation in both ethanol and salt, suggesting natural variation in the wild isolates that are more genetically similar.

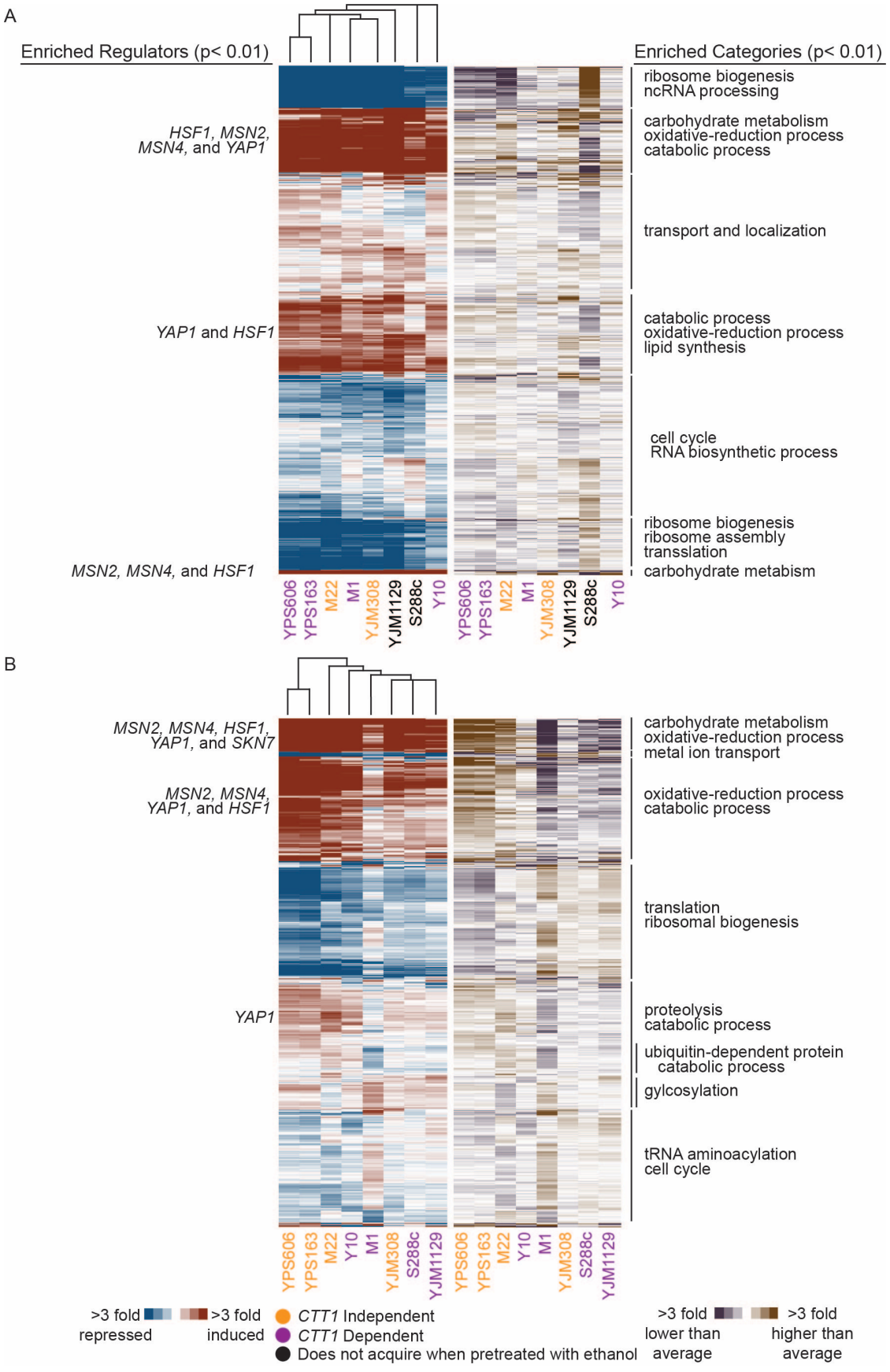

A
Enriched Regulators (p< 0.01)
Enriched Categories (p< 0.01)
HSF1, MSN2, MSN4, and YAP1
YAP1 and HSF1
MSN2, MSN4, and HSF1
ribosome biogenesis
ncRNA processing
carbohydrate metabolism
oxidative-reduction process
catabolic process
transport and localization
catabolic process
oxidative-reduction process
lipid synthesis
cell cycle
RNA biosynthetic process
ribosome biogenesis
ribosome assembly
transslation
carbohydrate metabism
YPS606
YPS163
M22
M1
YJM308
YJM1129
S288c
Y10
B
MSN2, MSN4, HSF1, YAP1, and SKN7
MSN2, MSN4, YAP1, and HSF1
YAP1
carbohydrate metabolism
oxidative-reduction process
metal ion transport
oxidative-reduction process
catabolic process
translation
ribosomal biogenesis
proteolysis
catabolic process
ubiquitin-dependent protein catabolic process
gylcosylation
tRNA aminoacylation
cell cycle
>3 fold repressed
>3 fold induced
CTT1 Independent
CTT1 Dependent
Does not acquire when pretreated with ethanol
>3 fold lower than average
>3 fold higher than average

**Figure 3.4: Variation in gene expression in response to ethanol and salt in wild strains**. $Log_2$ expression differences measured in denoted strains. Strains are color coated based on catalase dependency as described in Figure 2. A total of 3,918 genes with differential ethanol responses in any strain relative to the average and a total of 2,222 genes with differential salt responses in any strain relative to the average (FDR = 0.01) were organized by Euclidean clustering (see *Materials and Methods*). The left portion of the heat map displays expression changes in strain specific response to ethanol (panel A) or salt (panel B) across three biological replicates for catalase dependent for ethanol catalase independent for salt: YPS606 and YPS163, catalase independent for ethanol and salt: M22 and YJM308, catalase dependent for ethanol and salt: M1 and Y10, and did not acquire to ethanol: YJM1129 and S288c-derived strain DBY8268. Differences in ethanol and salt response for each wild strain vs. the mean of all strains are shown in the right portion of the figure. Each row represents a gene and each column represents a strain. Red indicates induced and blue indicates repressed expression in response to stress, while brown indicates higher expression compared to the average of all the strains and purple indicates lower expression to the average of all the strains, according to the key. Enriched functional groups (Bonferroni corrected $P < 0.01$) are annotated to the right.

### 3.4.5 MSN2/4 are responsible for ethanol-induced hydrogen peroxide acquisition, while SKN7 and YAP1 are partially responsible for the salt-induced hydrogen peroxide acquisition.

To identify potential regulators of the salt and ethanol responses that may be responsible for acquired hydrogen peroxide resistance, we looked for enrichment for DNA binding sites in the promoters of genes found within stress induced clusters. Our enrichments and previously known stress responsive transcription factors helped identify potential signaling pathways responsible for the expression divergence: *MSN2*, *MSN4*, *SKN7*, *HSF1*, and *YAP1*. To address their role in acquired hydrogen peroxide resistance, we deleted these transcription factors in the YPS606 strain background. Msn2p and Msn4p, general stress transcription factors (14, 58, 60), have been shown to play a role in osmotic shock (61) and are necessary for salt-induced hydrogen peroxide resistance in S288c. Surprisingly, we found that Msn2/4p are partially required for hydrogen peroxide acquisition in wild oak isolate YPS606 when pretreated with salt, but are necessary for ethanol-induced hydrogen peroxide cross protection, Figure 3.5A. When the *MSN2/4* deletion was combined with the *CTT1* deletion, we did not see a reduction in salt-induced hydrogen peroxide acquisition suggesting that the alternative peroxidase is not being regulated by Msn2/4p.

Another transcription factor involved in response to osmotic and oxidative stress (62-64), Skn7p was also enriched in our induced clusters. When *SKN7* was absent and cells were pretreated with ethanol, we saw a decrease in hydrogen peroxide resistance (Figure 3.5B), indicating Skn7p plays a role in response to ethanol stress in YPS606. *SKN7* deletion, by itself, did not yield in a reduction of salt-induced hydrogen peroxide acquisition; while in combination with *CTT1*, we saw significantly less hydrogen peroxide resistance indicating that *SKN7* is partially responsible for the alternative peroxidase activity. Heat shock factor 1, *HSF1*, responsible for activating gene expression in response to heat stress and ethanol (65-67), also showed up as a potential regulator. Since *HSF1* is an essential gene, we could only test a

heterozygous deletion strain. In this context, lack of Hsf1p did not affect salt-induced hydrogen peroxidase acquisition, suggesting it does not activate the alternative peroxidase, with the caveat that a clean deletion is not possible (Figure 3.5C). Interestingly, one cluster with high strain variation during salt stress was enriched for Yap1p binding sites. Yap1p is specifically activated during oxidative stress (56, 68, 69) and is responsible for inducing genes involved in detoxifying ROS, such as peroxidases (56, 70-72). When *YAP1* and *CTT1* were deleted we did not see a decrease in salt-induced hydrogen peroxide resistance. Yap1p has been shown to play a role in intrinsic acquisition (63). The decrease in the intrinsic hydrogen peroxide resistance results in an increase in percent maximum hydrogen peroxide acquisition well above 100%, as seen in Figure 3.5C. When looking at the hydrogen peroxide tolerance scores, we did see a decrease in salt-induced hydrogen peroxide acquisition, suggesting Yap1p plays a role in activating the compensating peroxidase, Supp Figure 3.2D.

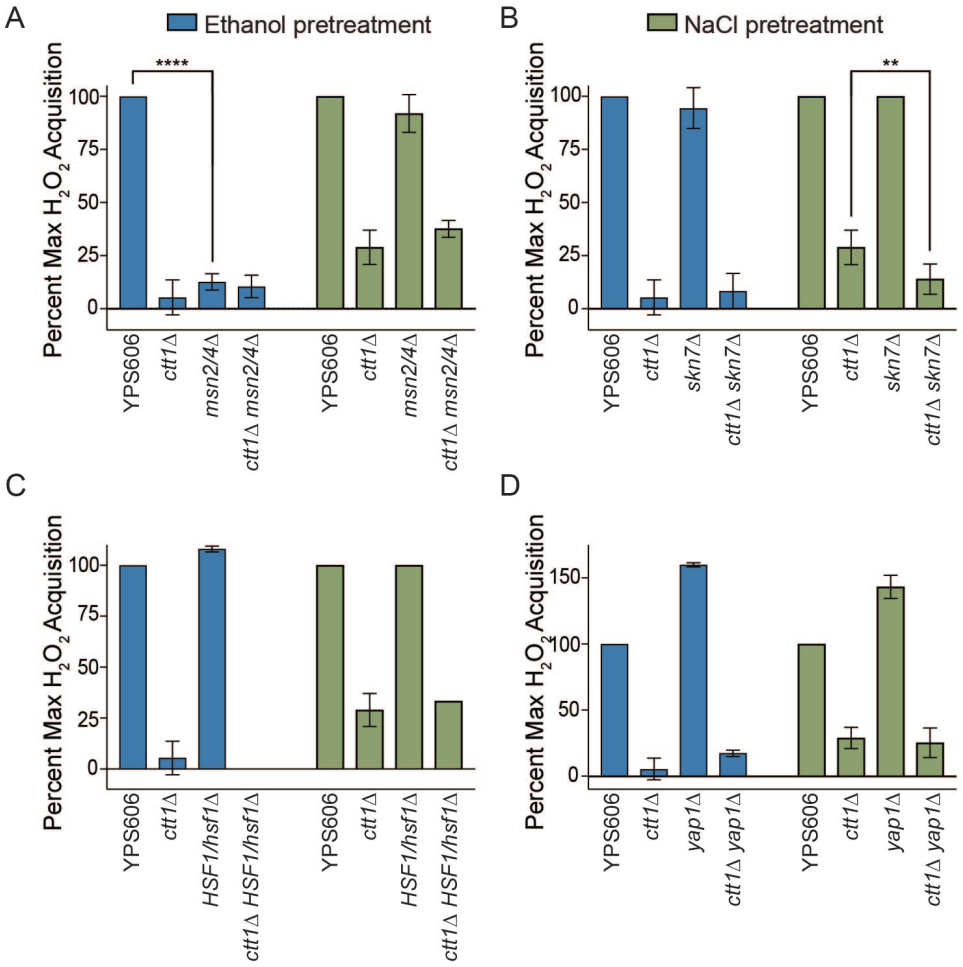
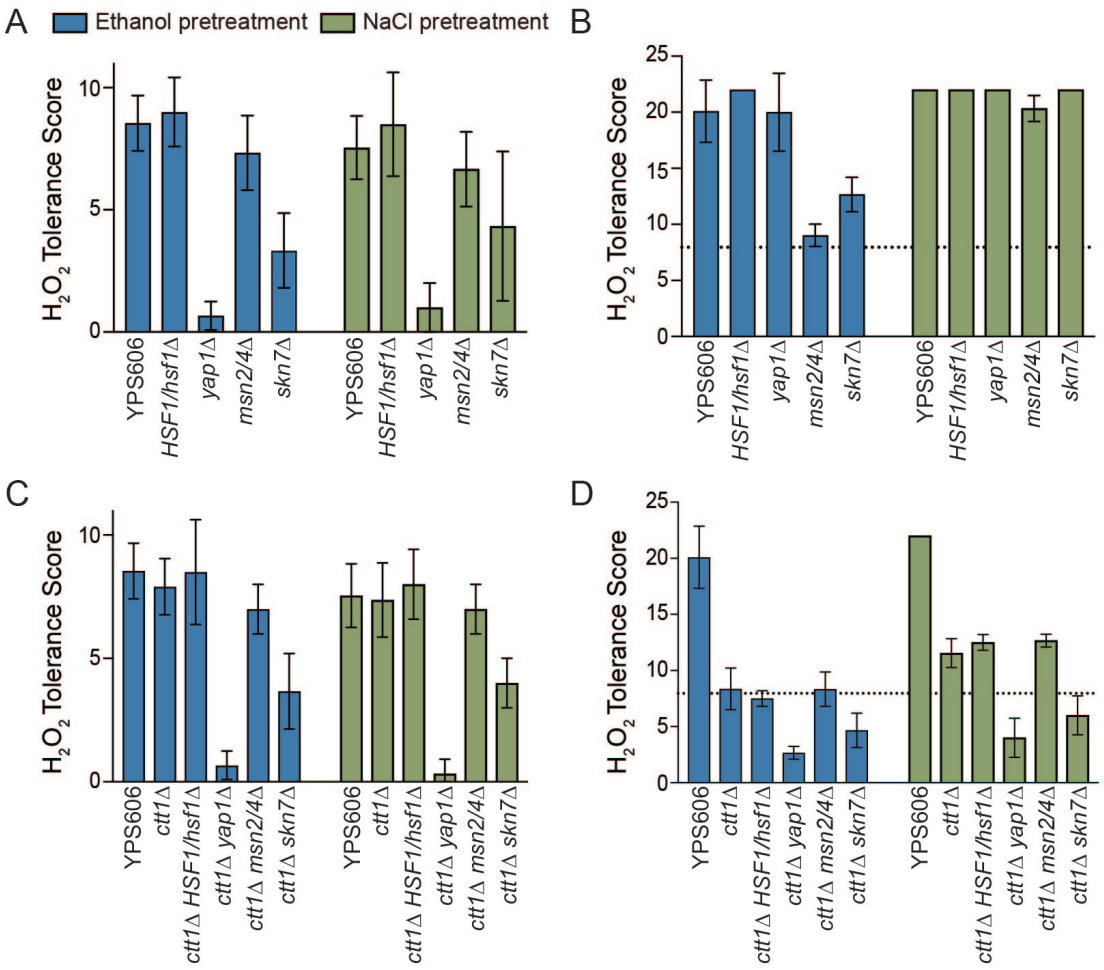



**Figure 3.5: *SKN7* is activating the alternative pathways are being activated during ethanol and salt stress while *MSN2/4* is required for ethanol-induced hydrogen peroxide cross protection.** Error bars indicate the mean and standard deviation of at least biological triplicates, except YPS606 *HSF1/hsf1Δ* (duplicate) and YPS606 (eleven reps). For YPS606 WT values are set to percent max $H_2O_2$ acquisition of 100%, thus zero standard deviation. Asterisks represent significant differences in percent max hydrogen peroxide acquisition between denoted strains (**P <0.01, ****P <0.0001, *t-test*).




**Supp Figure 3.2: Tolerance scores of transcription factor mutants.** Biological triplicate for all strains except YPS606 HSF1/hsf1Δ (duplicate) and YPS606 (eleven reps). The replicates for several strains all had the same tolerance score and thus zero standard deviation. Panel A and C show intrinsic levels of hydrogen peroxide resistance. Dotted line is the average of YPS606 intrinsic hydrogen peroxide tolerance score.

### 3.5 Discussion

In this study we leveraged acquired stress resistance to understand the different mechanisms underlying natural variation between gene expression and higher-ordered traits. It was previously shown that the S288c lab strain failed to acquire hydrogen peroxide acquisition when pretreated with ethanol (10) and S288c required catalase function for salt-induced cross protection against hydrogen peroxide (12). Additionally, we previously showed that catalase was required for ethanol-induced cross protection against hydrogen peroxide in the YPS163 oak strain (10). Here, we show that while this is true of some wild strains, this is not universal, and that some wild strains can acquire moderate levels of peroxide resistance in the absence of *CTT1*. While some strains required catalase only for ethanol-induced or salt-induced cross protection against hydrogen peroxide, we found that the catalase dependency also varied depending on the identity of mild stress. Previous studies have been focused on variation in a strain's response to ethanol and ethanol-induced hydrogen peroxide acquisition (9, 10, 49). Here, we looked at the variation among wild yeast in response to ethanol and salt stress. Surprisingly, when we tested this in the oak strain YPS606, we found that there was still moderate residual cross protection in the absence of *CTT1* only when salt was the pretreatment. Consistent with our previous results in wild oak strain YPS163 (10), wild oak strain YPS606 required catalase for proper hydrogen peroxide acquisition when pretreated with ethanol. Together with previous data, we hypothesized that strains of similar environment types would behave similar in response to ethanol and salt stress. Interestingly, when looking at ethanol or salt-induced hydrogen peroxide resistance, strains of similar environments did not cluster together or behave similarly with the exception of wild oak strains. We have contributed this to the close genetic relatedness of the oak strains (51, 52) while other strains, like vineyard strains, are more diverse (51, 73). Suggesting strains from similar niches have multiple ways of responding to stress. While catalase is one of the most efficient ways to break down hydrogen peroxide, there are multiple other enzymes that can detoxify ROS. Since catalase requires

heme to be active, perhaps these wild strains were exposed to iron limiting environments in their history and evolved other ways to combat ROS. Allowing them to be better buffered against hydrogen peroxide stress. We narrowed down that glutathione played a role in providing residual hydrogen peroxide acquisition in the absence of catalase. Glutathione and catalase have been shown to have overlapping roles in detoxifying cells of ROS (53). Future studies will determine the glutathione dependent peroxidase responsible for this catalase independent hydrogen peroxide acquisition.

We saw strain phenotypic differences in hydrogen peroxide acquisition, suggesting different wild yeast have different strategies for combating hydrogen peroxide and hypothesized that these are due to gene expression variation. To obtain a global view of stress defense physiology, we performed transcriptomics. Using this method, we could identify potential strategies that are used across all our strain backgrounds and which ones may be unique to particular strains. Although we saw conserved activation of the ESR, we did see strain-specific responses to both salt and ethanol. For example, we showed that S288c has decrease *CTT1* expression, seen previously (10), while YJM1129 had high *CTT1* expression yet still failed to acquire hydrogen peroxide resistance when pretreated with ethanol. We know *CTT1* is functional in YJM1129 because we see full hydrogen peroxide acquisition when pretreated with salt in a *CTT1* dependent manner. Thus, during ethanol there is likely translational or post-translational regulation occurring to reduce catalase levels and/or activity. Therefore, we argue that acquired hydrogen peroxide resistance occurs by different mechanisms depending on cellular experiences of the wild strains.

Further investigation of gene expression variation in ethanol and salt-induced hydrogen peroxide acquisition led to the discovery of potential regulators (*MSN2*, *MSN4*, *SKN7* and *YAP1*). We found that transcription factor Skn7p is partially responsible for salt-induced hydrogen peroxide acquisition. We saw that *skn7* deletion had less peroxide resistance with pretreated with ethanol. Skn7p has a role as a regulator that maintains cell wall integrity in yeast

(74, 75) and ethanol disrupts cell membranes (76). In the absence of Skn7p cells may be unable to control the genes associated with cell wall remodeling making them more susceptible. General stress transcription factors, Msn2/4p are responsible for fully induction of ethanol-induced cross protection against hydrogen peroxide. We previously reported this phenotype (9). One potential explanation is that Msn2p binding allows accessibility of *CTT1* promoter to other transcription factors (77), like Hap1p (10), so without *MSN2/4* we cannot obtain proper activation of *CTT1* leading to decreased acquisition. In the absence of Yap1p and Ctt1p, we did not see a see a decrease in percent maximum hydrogen peroxide acquisition. *YAP1* is known to play a critical role in intrinsic response (63); therefore, decreasing the intrinsic level of hydrogen peroxide acquisition. Since percent maximum hydrogen peroxide acquisition takes into account the intrinsic level of hydrogen peroxide acquisition, *YAP1* deletion displayed a much larger percent maximum hydrogen peroxide acquisition compared to wildtype cells (Figure 3.5). When we looked at the hydrogen peroxide raw tolerance scores (Supp Figure 3.2), we noted that Yap1p plays a role in salt-induced hydrogen peroxide acquisition. Yap1p has been shown to transcriptionally activate genes that encode alternative peroxidases such as those that are glutathione dependent (54, 70, 78) and thioredoxin dependent (56, 63, 68, 79). Thus, this is in agreement with our observation that the that the alternative peroxidase(s) is partially glutathione dependent.

In conclusion, we saw strain-specific variation in acquired stress resistance and in response to ethanol and salt. We observed different degrees of *CTT1* dependencies in hydrogen peroxide acquisition, suggesting that wild yeast have multiple methods to cope with hydrogen peroxide stress. Because these different anti-oxidant defense have different cofactor requirements, different strategies may be optimal under different environmental conditions (e.g. nutrient availability). This may have led to divergence in gene expression programs that favor one mechanism over another depending upon the activating stressor. Because stress responses share many conserved components (80, 81), this is likely a universal strategy.

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# Chapter 4 Conclusion

## 4.1 Summary of results

### 4.1.1 Hap1p responsible for ethanol-induced cross protection against hydrogen peroxide.

S288c, a lab strain, fails to acquire hydrogen peroxide resistance when pretreated with mild ethanol, whereas a wild oak strain, YPS163, can. We genetically mapped this to a transcription factor, Hap1p. We revealed a new role for Hap1p in cross protection against hydrogen peroxide that has been lost in the S288c lab strain, secondary to known ty1 insertion rendering it nonfunctional (1). To examine if Hap1p was solely responsible for the difference in acquisition between the two strain backgrounds, we constructed hemizygous hybrid strains. Wild oak strain YPS163 *hap1*Δ mutants and the hybrid carrying the $HAP1^{S288c}$ allele had strong hydrogen peroxide acquisition defects. YPS163 *hap1*Δ mutant was affected only when pretreated with mild ethanol but was able to fully acquire hydrogen peroxide resistance when pretreated with mild hydrogen peroxide or mild NaCl (Figure 2.5), suggesting this is a specific response to ethanol.

We found that Hap1p is necessary for proper induction of *CTT1*, cytosolic catalase T, during ethanol stress. We see a defect in cross protection for the YPS163 *hap1*Δ mutant and reduced *CTT1* expression and peroxidase activity during ethanol stress (Figure 2.6 and 2.9). YPS163 *ctt1*Δ mutant cannot acquire any further hydrogen peroxide resistance following ethanol pretreatment (Figure 2.8). The lack of cross protection in S288c and the $HAP1^{S288c}$ hybrid correlates with the lack of inducible peroxidase activity following ethanol pretreatment in those strains. Intriguingly, the hybrid carrying the $HAP1^{YPS163}$ allele still cross protects despite lower levels of *CTT1* mRNA induction and lower peroxidase activity than of YPS163 *hap1*Δ strain that is unable to acquire further resistance (Figure 2.9). These data suggest that *HAP1* plays an additional role in ethanol-induced cross protection beyond hydrogen peroxide detoxification by Ctt1p. Interestingly, the strain carrying the $HAP1^{YPS163}$ allele fails to fully complement the defect in the S288c background suggesting that additional loci in S288c render *HAP1* necessary but

not sufficient for cross protection. We surveyed other wild strains to determine the effects of *hap1*Δ mutations and found two additional strains with a strong *HAP1* requirement and (Supp Figure 2.4). This result, as well as those from other recent studies (2-4), suggests that these types of genetic background effects are likely the rule rather than the exception.

### 4.1.2 Natural variation in response to stress and catalase dependency in wild yeast.

It was previously shown that the S288c lab strain required catalase function for salt-induced cross protection against hydrogen peroxide (5). Additionally, we previously showed that catalase was required for ethanol-induced cross protection against hydrogen peroxide in the YPS163 oak strain (Chapter 2). Here we showed that while this is true of some wild strains, this is not universal and that some wild strains can acquire moderate levels of peroxide resistance in the absence of *CTT1*. When we pretreated wild oak strain YPS606 with ethanol, it acquired hydrogen peroxide resistance, in a *CTT1* dependent manner. Surprisingly, when we tested this in the oak strain YPS606, we found that there was still moderate residual cross protection in the absence of *CTT1* when pretreated with salt. When analyzed multiple wild strains, from diverse environments, we discovered four ways cells respond to hydrogen peroxide resistance: catalase independent for both ethanol and salt, catalase dependent for ethanol and independent for salt, completely dependent on catalase for ethanol and salt, does not acquire peroxide resistance when pretreated with ethanol. When looking at hydrogen peroxide tolerance, strains of similar environments did not cluster together, except for the wild oak strains, possibly due to the close genetic relatedness of the oak strains (6, 7). We proved that glutathione plays a role in providing residual hydrogen peroxide acquisition in the absence of catalase in the wild oak strain, YPS606.

We hypothesized that variation in gene expression variation may be responsible for the phenotypic differences in hydrogen peroxide acquisition. We showed that S288c has decreased *CTT1* expression which was also seen previously (Chapter 2) whereas distillery strain,

YJM1129, has high *CTT1* expression yet still fails to acquire hydrogen peroxide resistance when pretreated with ethanol. We know YJM1129 has a functional *CTT1* because we see full hydrogen peroxide acquisition when pretreated with salt in a *CTT1* dependent manner. Thus, we argue that acquired hydrogen peroxide resistance occurs by different mechanisms depending on cellular experiences of the wild strains.

We found potential regulators of the alternative peroxidase. First, we saw that *skn7Δ* mutation had less peroxide resistance with pretreated with ethanol and when performed in combination with *ctt1Δ* we saw a significant decrease in salt-induced hydrogen peroxide acquisition. Secondly, we witnessed that Msn2/4p is required for full induction of ethanol-induced cross protection against hydrogen peroxide. Finally, we saw that Yap1p, responsible for oxidation stress activation, plays a role in alternative peroxidase involved in salt-induced hydrogen peroxide acquisition.

**4.2 Future work**

Our study narrowed down the list of alternative peroxidases to those that are glutathione-dependent (Figure 3.3). Cross protection assays of the single mutations of the glutathione-dependent peroxidases will have to be performed to determine the peroxidase responsible for the *CTT1* independent salt-induced hydrogen peroxide acquisition phenotype. We have been investigating alternative peroxidases in the wild oak strain YPS606, but we see *CTT1* independent hydrogen peroxide acquisition in other wild strains, such as YJM308 (clinical strain), M22 (vineyard strain), and Y12 (palm wine strain). Additional single peroxidase mutations and acquired stress experiments will need to be performed to determine if these wild strains require the same peroxidase(s) to acquire hydrogen peroxide acquisition. This would give insight into just how many mechanisms wild yeasts use to combat ROS. Lastly, we saw

other yeast, YJM627, and YJM1129, who also fail to acquire when pretreated with ethanol (like the laboratory strain S288c), the reason as to why remains a mystery.

We have focused on determining the possible transcription factors regulating the acquired hydrogen peroxide stress acquisition, but there are multiple mechanisms of activation of these transcription factors. There are signaling cascades that are responsible for correct activation for these transcription factors. One highly conserved signaling cascade, HOG pathway, has been extensively studied (8-11). In *S. cerevisiae*, Hog1p is a mitogen-activated (MAP) kinase required for osmotic stress response (12). In other eukaryotes, such as mice, drosophila and humans, Hog1p homologs, are activated during multiple stresses (10, 13-16). We have shown that while Hog1p is necessary for salt-induced hydrogen peroxide, in some wild yeast *HOG1* mutants show stronger defects when salt is not the pretreatment (Figure 4.1). Further experiments need to be performed to understand how the HOG pathway is being activated and who Hog1p is activating during non-salt stresses.

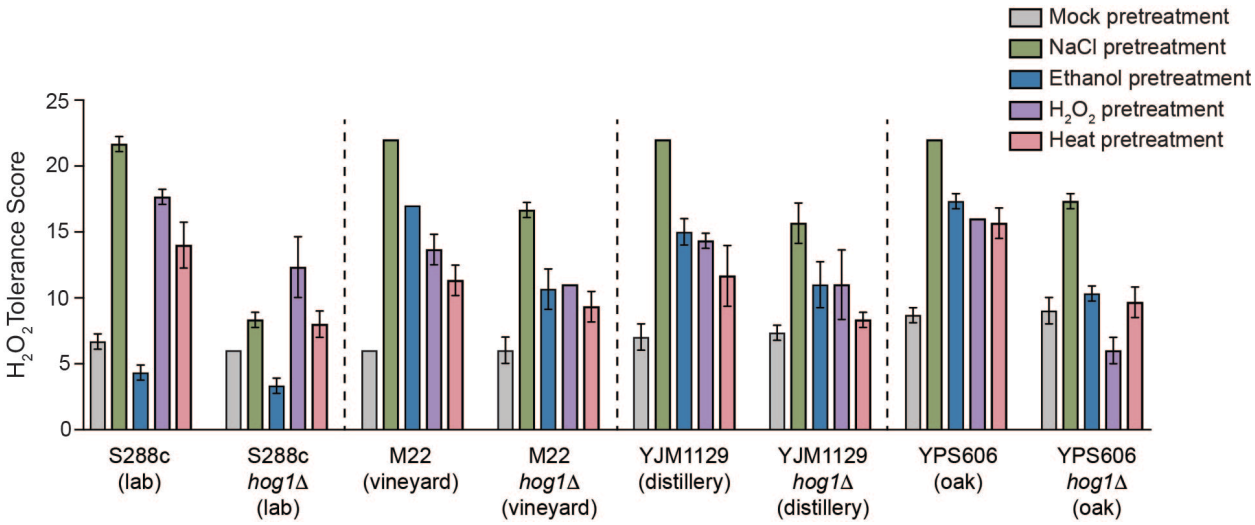


**Figure 4.1 Variation in *HOG1* dependency during multiple stress hydrogen peroxide acquisition.** Strains were exposed to either mock (YPD control), 0.4M NaCl, 5% ethanol, 0.4mM $H_2O_2$, or 37°C heat pretreatment for 60 min, washed, exposed to 11 doses of severe $H_2O_2$ for 2 hr, and then plated to score viability. Error bars indicate the mean and standard deviation of biological triplicates Replicates for several strains all had the same tolerance score and thus zero standard deviation.

### 4.3 Perspectives

The experiments discussed here demonstrate the benefits of using acquired stress resistance as a model for understanding natural variation of higher-order traits. The majority of studies in yeast have been conducted on the laboratory strain. Here, we have discovered differences between the laboratory strain and wild yeast and how they respond to stress. This study provides insight into multiple means of stress acquisition responses and insights into the ecology of yeast and reminds researchers that to fully understand how organisms respond to stress we need to look beyond the laboratory strains. Because of the conserved nature of the mechanisms to breakdown hydrogen peroxide this research could serve as a roadmap for studying natural variation in higher eukaryotes.

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## S1: A Wild Yeast Laboratory Activity: From Isolation to Brewing

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**S1.1 Abstract**

Microbial fermentation is a common form of metabolism that has been exploited by humans to great benefit. Industrial fermentation currently produces a myriad of products ranging from biofuels to pharmaceuticals. About one third of the world's food is fermented, and the brewing of fermented beverages in particular has an ancient and storied history. Because fermentation is so intertwined with our daily lives, the topic is easily relatable to students interested in real-world applications for microbiology. Here, we describe the curriculum for an inquiry-based laboratory course that combines yeast molecular ecology and brewing. The rationale for the course is to compare commercial *Saccharomyces cerevisiae* yeast strains, which have been domesticated through thousands of generations of selection, with wild yeast, where there is growing interest in their potentially unique brewing characteristics. Because wild yeast are so easy to isolate, identify, and characterize, this is a great opportunity to present key concepts in molecular ecology and genetics in a way that is relevant and accessible to students. We organized the course around three main modules: isolation and identification of wild yeast, phenotypic characterization of wild and commercial ale yeast strains, and scientific design of a brewing recipe and head-to-head comparison of the performance of a commercial and wild yeast strain in the brewing process. Pre and post assessment showed that students made significant gains in the learning objectives for the course, and students enjoyed connecting microbiology to a real-world application.

## S1.2 Introduction

Microbial fermentation is a ubiquitous form of metabolism that has been exploited by humans for thousands of years (1-4). About one third of the world's food is fermented (5), which of course has massive effects on global and local economies. Fermentation has a particularly rich history in the baking and brewing of alcoholic beverages, with the yeast *Saccharomyces cerevisiae* being among the oldest domesticated organisms (3, 6). While the first beer may have been brewed as long as 13,000 years ago (7), what we would now recognize as modern beer took shape in the Middle Ages, where malted barley was used as a source of fermentable sugars and hops were used as a bittering agent (8). During this time span, continuous selection of yeast in the brewing environment selected for a number of traits, including better utilization of wort carbon sources and increased fermentation efficiency. Modern brewing styles emerged from regional differences in brewing, and early brewers selected for yeast strains that complemented their brewing ingredients. For example, while the primary products of yeast fermentation are ethanol and carbon dioxide, a number of secondary products including esters and fusel alcohols are also produced that have unique flavor and aroma profiles (9). Certain beer styles (e.g. Belgian Lambic and German-style Hefeweizen) favor high levels of secondary fermentation products, while other styles favor little to none and consider these compounds to be "off flavors" (e.g. many Stouts and Amber Ales). The choice of yeast strain became a critical parameter for brewing design.

While brewers have most frequently used domesticated yeast strains, it's becoming increasingly clear that wild yeast strains are important reservoir for traits important to industrial fermentations including brewing (10). This can include novel metabolic capabilities, such as the ability to ferment complex carbohydrates in wort, or the ability to produce novel flavor compounds (11). Because wild yeast are so easy to isolate, phenotype, and genotype, this provides a unique opportunity for undergraduates in laboratory courses to engage in inquiry-based research. As such, we designed a course around the microbiology of brewing a

fermentation to provide a real-life application. We organized the course around three main modules: isolation and identification of wild yeast, phenotypic characterization of commercial and wild ale yeast strains, and scientific design of a brewing recipe and head-to-head comparison of the performance of a commercial and wild yeast strain in the brewing process.

### S1.2.1 Intended Audience and prerequisite student knowledge

This course was designed to provide our senior Biology majors with an upper-level Microbiology laboratory course. This course also provides an opportunity for students to write a research paper that can satisfy our university's writing requirement for graduation. Students should have some knowledge of molecular biology and biochemistry, particularly central metabolism and regulation of gene expression. As an upper-level course, students were required to have taken our sophomore-level Cell Biology and General Genetics courses and one of the associated introductory lab courses as prerequisites. While not required, we also suggested that our junior-level Prokaryote Biology course would be helpful.

### S1.2.2 Learning time

The laboratory was structured as a three credit-hour full-semester course (16 weeks). The class was scheduled to meet twice a week for three hours, and the approximate length of each lab can be found in the instructor's manual (Appendix 1). The majority of learning time and experiments took place in the laboratory. Some time outside of class was spent collecting wild yeast samples, reading relevant scientific literature, and completing assignments (laboratory notebooks, homework, oral presentation, and final research paper).

### S1.2.3 Leaving objectives

The overall goal of the course is to provide both conceptual learning and hands-on laboratory skills. Upon completion of the course, students should be able to:

1. Summarize and discuss primary research literature.
2. Predict where wild yeast can be isolated based on the natural ecology of yeast, and explain how one can enrich for yeast from environmental samples.
3. Explain why and how ITS sequencing is used to determine fungal species, and analyze ITS sequencing data to assign the species of an unknown isolate.
4. Describe the primary and secondary products of yeast fermentation, and how differences in fermentative metabolism across yeast strains impact brewing.
5. Analyze yeast phenotypic data for traits relevant to brewing, and then use those data to predict brewing outcomes.
6. Explain the role of each ingredient and step in the brewing process, and scientifically design and implement a brewing protocol.

**S1.3 Procedure**

While we provide detailed student and instructor instructions in the Appendices, here we will briefly describe the main modules of the course (Figure 1).

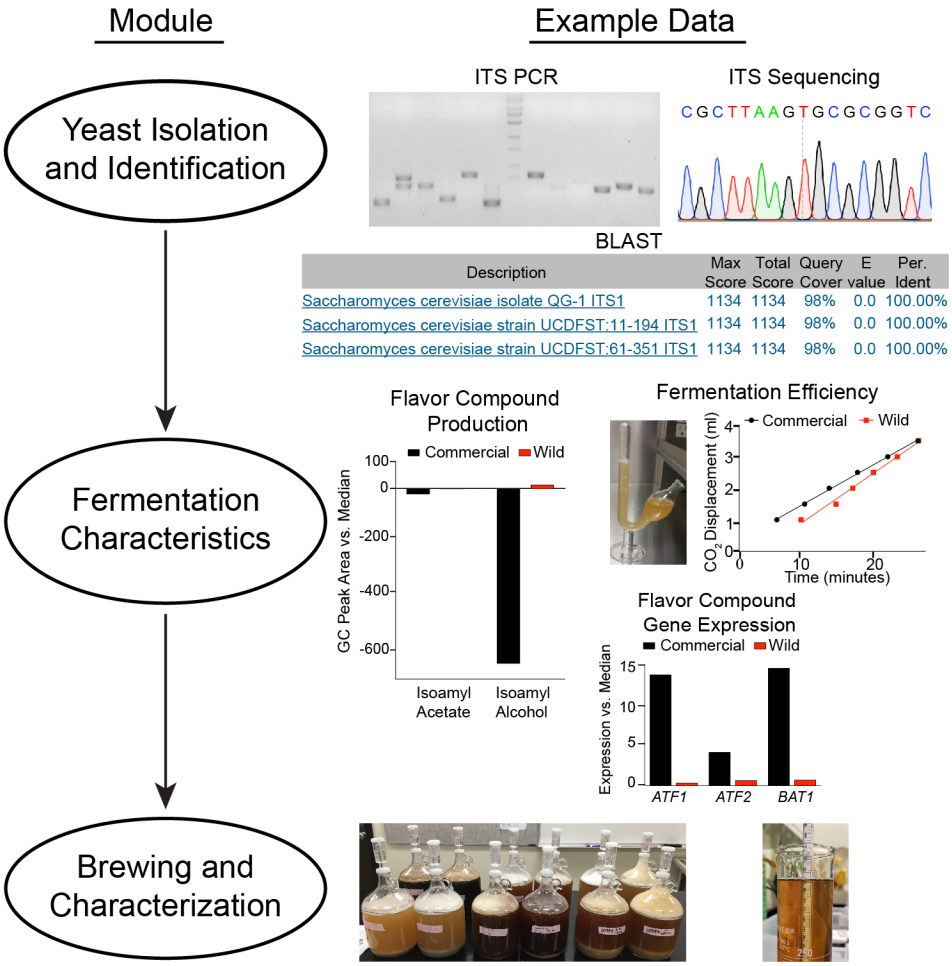



**Figure S1.1: Flowchart displaying the different course modules and example data.** ITS: internal transcribed spacer; PCR: polymerase chain reaction; BLAST: Basic Local Alignment Search Tool. qPCR: quantitative PCR; GC: gas chromatography.

### S1.3.1 Wild Yeast Isolation and Identification

Yeast are ubiquitous in the environment and can be found on a number of substrates ranging from rotting fruit to soil to tree bark (12). For the first part of this course, students are given materials to sample from nature to isolate wild yeast. Students then place the samples in liquid media that enriches for budding yeast, and samples showing evidence of fermentation (gas bubbles) are plated to identify colonies consistent with those of yeast, which can be confirmed for the presence of budding yeast via microscopy. Following successful yeast isolation, students then perform DNA extractions, PCR and sequence the ITS/5.8S ribosomal DNA locus that is frequently used to differentiate yeast species (13), and then perform BLAST analyses to determine the species of their isolated yeast.

### S1.3.2 Wild and Commercial Yeast Phenotypic Characterization

Students are then paired, and half of the class is charged with phenotypically characterizing different wild *S. cerevisiae* strain, and the other half of the class will characterize different commercial brewing strains. First the entire group learns how to “mash” malted grains together (which they will need to understand for the following module). The resulting wort from each group is then pooled and autoclaved to generate a standardized “beer media” to characterize all of the strains. Phenotypes for characterization include fermentation rate, quantitative PCR of mRNA levels for genes known to be responsible for ester and fusel alcohol production, and gas chromatography-mass spectrometry analysis of fermented beer media to directly quantify secondary metabolite levels.

### S1.3.3 Wild and Commercial Yeast Brewing and Beer Characterization

The final module has student pairs join to form a larger group to design a brewing recipe where they will compete a wild and commercial *S. cerevisiae* strain head-to-head. Student pairs share their data with each other and then design a brewing recipe that fits the characteristics of

one or both of their yeast strains. Here students gain hands-on experience for all of the major steps of brewing: mashing, boiling, and fermentation. During mashing, the grains are mixed with water and heated to a temperature that activates the alpha and beta amylases that naturally occur in malted grains. This leads to conversion of the grain starches into sugars that can be fermented by yeast (with the added wrinkle that alpha and beta amylases are most active at different temperatures, leading to different sugar profiles in the final wort depending on mash temperature). “Roasting” of the malted grains at different temperatures and times leads to lighter or darker malts (with darker malts having fewer active amylases and more Maillard products that are not fermentable). Following mashing, hops are generally added to the resulting sweet wort, which is then boiled. Boiling partially sterilizes the wort, and isomerizes hop alpha-acids leading to characteristic bitterness (with different varieties of hops containing differing amounts of alpha acids and other flavor compounds). Hop iso-alpha-acids also are bacteriostatic against many Gram-positive bacteria (14, 15). Finally, the wort is chilled, the yeast are “pitched”, and fermentation converts the wort sugars to mainly ethanol and $CO_2$ along with secondary esters and alcohols.

For this course, we used the “brew in a bag” method, where the grains are placed in a bag that is submerged during the mashing process. Following mashing, the bag is simply removed and squeezed to drain the residual sweet wort. Then the sweet wort is brought to a boil for sterilization and hop additions, cooled to allow for yeast pitching, fermented for 3 weeks (typical for many ales), and finally bottle conditioned for 2 weeks. Following brewing, students measured their beers’ final gravities (to determine percent attenuation and alcohol percentage), color, bitterness, and secondary flavor compounds. Students also had the option of participating in a voluntary taste test of the final beers. Below is an example of a student-designed recipe built around a low-ester producing and highly fermentative yeast strain:

### S1.3.4 Recipe: Blood Orange Ginger American Ale

Ingredients

**Grain (target original gravity 1.068)**

4.96 lb 2-row U.S. Pale Malt

1.3 lb Briess Aromatic Munich Malt

0.43 lb Flaked Wheat

**Hops (target IBU 86)**

0.58 oz Citra – boiled for 60 min

**Additives**

1 Whirfloc Tablet (Irish moss; clarifying agent)– boiled for last 5 min

0.5 oz Blood Orange Extract – boiled for last 5 min

0.4 oz Sliced Ginger Root – boiled for last 10 min

**Mashing**

1. Heat 3.5 gallons of ultra-pure water in stockpot to 67°C.
2. Add all grain to the “brew bag” within the stockpot and mash at 67°C for 60 minutes.
3. Pull out brew bag and squeeze to drain excess wort. Discard spent grain

***Boiling***

4. Raise mash to a rolling boil.
5. Add Citra hops to a hop bag and add to boiling wort.
6. Incubate for 60 minutes.
7. With 10 minutes left in the boil, add 0.4 oz sliced ginger root.
8. With 5 minutes left, add 0.5 oz blood orange extract and 1 Whirfloc tablet.

***Fermentation***

9. Cool wort to near room temperature using a wort chiller.
10. Add cooled wort and 65.5 billion yeast cells to a 1-gallon fermentation growler.
11. Add airlock and fill with water diluted Star San sanitizer.
12. Transfer 250mL of the remaining wort to graduated cylinder and measure initial gravity with a hydrometer
13. Place fermentation growlers in a dark area at room temperature for 3 weeks.

***Bottle Conditioning***

14. Add 6.6 ml 50% glucose (priming sugar for carbonation) to sterilized 16 oz amber swing-neck bottle.
15. Auto-siphon the beer into a sterile 16oz amber bottle.
16. Transfer 250 ml of the remaining beer to graduated cylinder and measure final gravity with a hydrometer.
17. Incubate at room temperature for 2 weeks in the dark to carbonate the beer.

**S1.3.5 Materials**

Materials are listed for a class of 24 students working individually for the initial yeast isolation, and then in pairs for the subsequent experiments. Materials (including media recipes) and equipment are listed in Appendix 1.

**S1.3.6 Student instructions**

Student handouts or manual can be made from adapting the instructor's manual (Appendix 1). Adapting instructions can be found on page 2 of the instructor manual. Students were required to maintain a lab notebook with detailed rationale, methods, results, and discussion sections. An example lab notebook entry can be given to students to serve as a

guide (Appendix 3). The notebook was collected three times during the 16-week course. Students were also responsible for preparing a ten-minute fermentation-related oral presentation, along with a final term paper describing their scientifically-designed brewing recipe in journal article format.

### S1.3.7 Faculty instructions

Detailed faculty instructions for lab activities can be found in the instructor manual (Appendix 1). Lab lectures and active learning activities (clicker questions and group discussions) can be found in Appendix 2. Instructor materials for all graded assignments including associated rubrics can be found in Appendix 4.

### S1.3.8 Outcomes and issues for discussion with students

Because this is a research-based course, anticipated outcomes are not guaranteed. Not all students are guaranteed to isolate yeast for molecular characterization. Those students should be provided with a wild yeast isolate, either from another classmate who isolated more than one unique strain, or from the instructor. Likewise, there is no guarantee that the class will isolate enough wild *S. cerevisiae* strains for subsequent experiments, so the instructors should be prepared to supply wild *S. cerevisiae* strains as a backup. Wild yeast strains can be ordered from the ARS Culture Collection (https://nrrl.ncaur.usda.gov), but the corresponding author (Dr. Jeff Lewis) is happy to send wild *S. cerevisiae* strains upon request. It is helpful to cryopreserve all positively screened wild *S. cerevisiae* strains so that they can be used in future classes if necessary.

### S1.3.9 Suggestions for determining student learning

A pre- and post-laboratory exam and survey (Appendix 4) were administered to students. The 15-question exam consisted of an equal number of multiple choice, true-false,

and short answer questions. The 5-question survey measured student perceptions of proficiency using a Likert-like scale. We did not use quizzes, midterms, or a final exam to assess student learning, though those could certainly be implemented. The ability to summarize and discuss the primary literature was assessed via homework assignments and a short (10-12 min) oral presentation. For each module, laboratory notebooks were graded to assess student learning. A final paper in the form of a primary research article was used as an additional summative assessment of student learning.

**S1.3.10 Safety issues**

Students must demonstrate competency with BSL1 safety procedures before working with unknown samples that require BSL2 precautions. Because this in an upper-level course that requires prerequisite BSL1 level lab activities, students were mostly familiar with BSL1 precautions. Nonetheless, students received important safety training on proper BSL1 and BSL2 procedures, and were required to demonstrate proficiency with BSL1 procedures before performing BSL2 procedures including safe handling of potentially pathogenic unknown organisms (16). Students were required to wear personal protective equipment (gloves, lab coat, eye protection) at all times, and received instructions for how to minimize aerosolizing cultures. All bench surfaces and objects on the laboratory bench were disinfected after each class with 70% ethanol. The instructors were responsible for autoclaving all plates and contaminated materials after every class according to the minimal standards set by the ASM Biosafety Guidelines (16). All chemicals in this course are low risk biohazardous agents except for methylene blue, hydrochloric acid, iodine, and iso-octane, which were discarded according to the institutional biohazard waste disposal guidelines.

All ingredients used for brewing were food grade, and brewing was conducted in a space safe for food handling. While many different types of wild yeast can be used for brewing, we were cautious to only use wild *Saccharomyces cerevisiae*. This activity and the associated

research was submitted to the University of Arkansas IRB Committee (Protocol No. 1807133914) and determined to be exempt. The course also included optional tours of a local craft brewery (Core Brewing in Springdale, AR) and a local homebrew store (Steve's Brew Shop in Fayetteville, AR), as well as an optional taste test of the final beers. We recognized that tasting of alcohol beverages is a potentially sensitive subject, so we worked closely with the university administration to ensure that we complied with all university regulations and guidelines. We came up with the following guidelines for beer tasting: 1) Tasting is entirely optional. Any students who do not wish to participate do not have to, and the tasting will have no impact on student grades. 2) Only students 21 years of age or older may participate in tasting. A valid photo ID with birth date will be required. IDs will be checked by the trained staff at Core Brewing. 3) Tasting will only occur at Core Brewing. There will be no tasting of alcoholic beverages on campus. 4) Tasting will be through the sip and spit method only. There will be no drinking of the beer. 5) Students must sign a waiver that includes the above information, as well as a statement that they will act responsibly.

### S1.4 Discussion

#### S1.4.1 Field testing

This class was developed, and field tested through two years as an upper-level research-based undergraduate course at the University of Arkansas (23 students in 2017, and 24 students in 2018). Students worked independently for yeast isolation, in pairs for yeast sequencing and characterization, and in groups of four (two pairs) for brewing. Discussions within and between groups were encouraged. For yeast isolation, 37 / 47 students successfully isolated wild budding yeast. Based on ITS sequencing, 6 / 37 isolates were *S. cerevisiae*. Several other species were identified including *S. paradoxus*, *S. cariocanus*, *Pichia* species (*P. kudriavzevii, P. kluyveri, P. fermentans*, *P. terricola*), *Meyerozyma caribbica*, *Lachancea fermentati*, *Wickerhamomyces anomalus, Kodamaea ohmeri*, and *Debaryomyces sp*. Further

characterization only proceeded with wild *S. cerevisiae* strains. While all-grain brewing may seem intimidating for novices, the “brew in a bag” method dramatically simplifies the process, and works extremely well for the small volumes being brewed in the course. Neither the instructors nor most of the students had any experience brewing, but every group in both student cohorts was able to successfully brew beer.

In the second offering of the course, we changed the focus of the brewing module to be more “yeast centric.” We did this by having pairs of lab partners—each working with either a commercial brewing strain or a wild *S. cerevisiae* strain—scientifically design a single brewing recipe to compete the yeast strains. This allowed each group of students to predict how the final beer would change depending on the properties of the yeast, and then test these predictions in the final characterization of the beer.

Overall, student feedback on the course was highly positive. Anonymous online evaluations rated the course very highly on a 1 (very poor) through 5 (excellent) Likert-like scale, with a 2017 rating of 4.80 / 5 (compared to a departmental mean of 3.96) and a 2018 rating of 4.79 / 5 (compared to a departmental mean of 3.83). Student comments pointed to a particular appreciation of connecting molecular biology to real-world applications.

Examples include:

- *I was able to learn about genetics through real life situations, and to apply what I learned, in a way that made much more sense than my general genetics course ever did.*
- *This class is a great example of helping students to understand complex concepts by utilizing an interesting life-application.*
- *Great reminder of some biology concepts that did not seem applicable to real life when taught in another course.*

### S1.4.2 Evidence of student learning

Student learning was assessed using a variety of methods (Table 1). Take-home problem sets (Appendix 3) were used to assess understanding of the assigned readings. Lab notebook entries were used to assess students' abilities to understand the rationale for their experiments as well as their design, analyses, and interpretations. Students were evaluated on their ability to present short (10-15 min) mini-lectures on their choice of topics related to microbial fermentation. Last final written report in the format of a primary research article was used to assess students' abilities to synthesize what they learned. Rubrics can be found in Appendix 3**.**

**Table SA1.1: Learning objectives and their corresponding methods of assessment.**

| Learning Objective | Assessment |
|---|---|
| Summarize and discuss primary research literature. | Homework, presentation, final paper, pre/post survey |
| Predict where wild yeast can be isolated based on the natural ecology of yeast, and explain how one can enrich for yeast from environmental samples. | Homework, lab notebook, pre/post exam, pre/post survey |
| Explain why and how ITS sequencing is used to determine fungal species, and analyze ITS sequencing data to assign the species of an unknown isolate. | Homework, lab notebook, pre/post exam, pre/post survey |

**Table SA1.1 (Cont.)**

| Learning Objective | Assessment |
|---|---|
| Describe the primary and secondary products of yeast fermentation, and how differences in fermentative metabolism across yeast strain impact brewing. | Lab notebook, final paper, pre/post exam, pre/post survey |
| Analyze yeast phenotypic data for traits relevant to brewing, and then use those data to predict brewing outcomes. | Lab notebook, final paper, pre/post exam, pre/post survey |
| Explain the role of each ingredient and step in the brewing process, and scientifically design and implement a brewing protocol. | Lab notebook, final paper, pre/post exam, pre/post survey |

We measured changes in student learning with pre- and post-tests, and we assessed changes in student perceptions of their skills and knowledge with pre- and post-surveys (see Appendix 4 for exam and survey questions). The average pre-test score was 26% correct, which rose to 67% following participation in the course (Figure 2). This was statistically significant ($p = 6 \times 10^{-14}$, two-tailed unpaired Mann-Whitney U test), and of large effect (Cliff's delta = 1). Additionally, students showed significant increases in learning for the majority of the questions (Figure 3). We should note that formal assessment in the course did not include any exams, so these gains are more likely to reflect long-term understanding instead of short-term memorization. Students also self-reported their perceptions of competency on pre- and post-surveys. Following the course, students showed significantly higher confidence in their abilities to isolate wild yeast from nature, use molecular biology and phylogenetics to identify yeast species, describe the major steps in brewing, and brew beer on their own (Figure 4). Coming into the class, students felt confident with reading scientific articles, though they may have still showed a small gain in confidence following the course ($p = 0.08$, two-way ANOVA, Fisher's LSD).

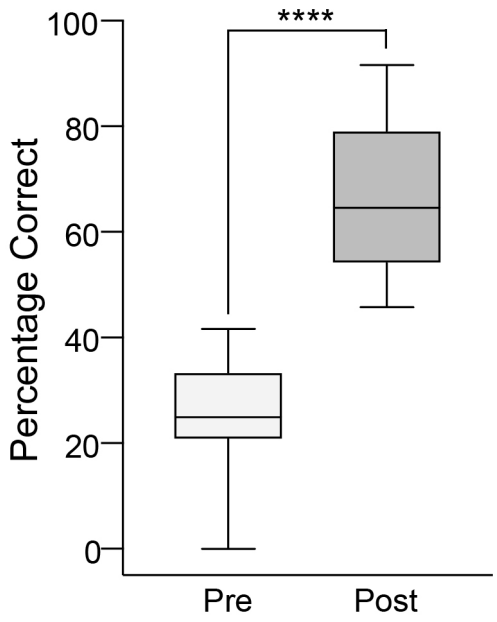



**Figure SA1.2: Pre- and post-exams show significant gains in student learning.** The boxplot depicts the median and interquartile range, and the whiskers depict the range. **** $P = 6 \times 10^{-14}$, two-tailed unpaired Mann-Whitney U test.

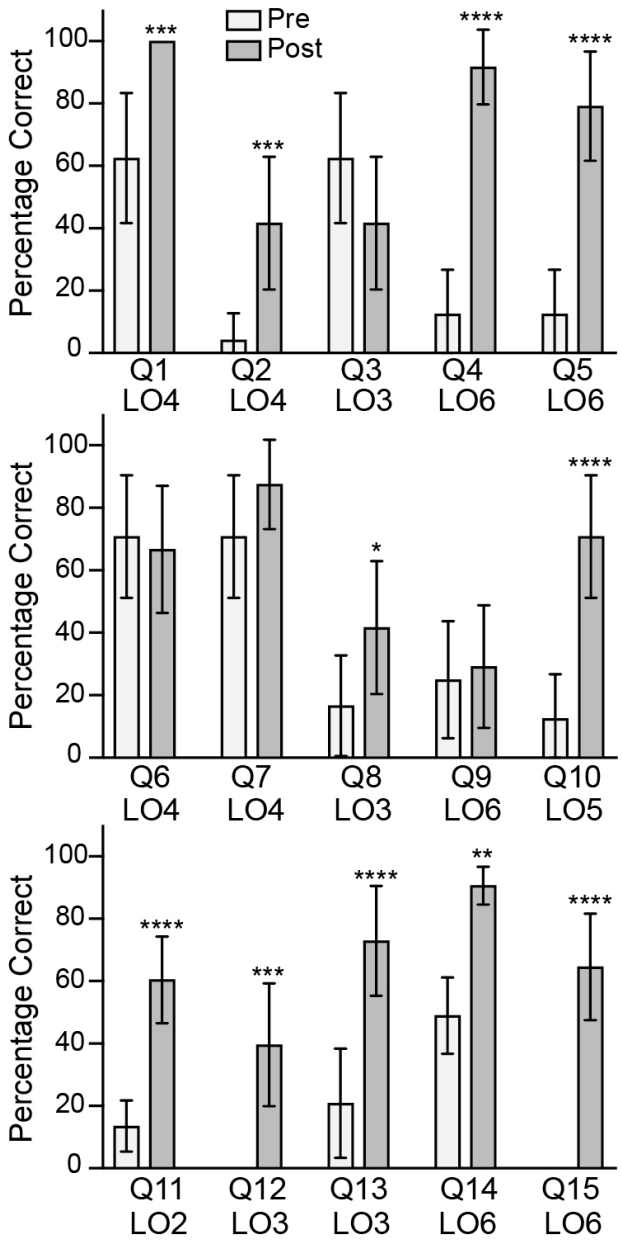
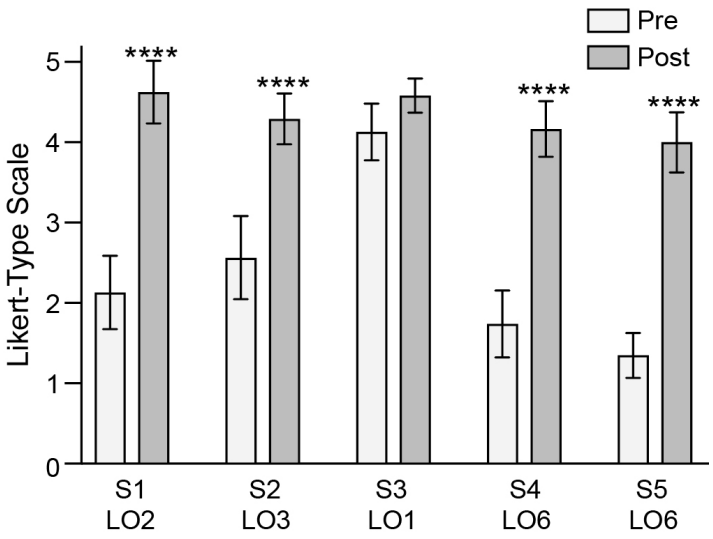



**Figure SA1.3: Individual item responses for pre- and post-exam scores.** Exam questions (Q1 – Q15) can be found in Appendix 5. LO denotes the learning objectives. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$, two-way ANOVA, Fisher's LSD test.




S1: Can find and isolate wild yeast from nature

S2: Can molecularly identify an unknown yeast species

S3: Comfortable reading and discussing papers

S4: Can describe all of the major steps in brewing

S5: Able to brew beer on own

**Figure SA1.4: Pre- and post-survey shows increase in self-reported perceptions in student ability.** Survey questions (S1 – S5) can be found in Appendix 5. LO denotes the learning objectives. **** $P < 0.0001$, two-way ANOVA, Fisher's LSD test.

### S1.4.2 Possible modifications

This course was designed and offered twice as an upper-level course that met twice a week for one semester. There are several modifications that could be included for a shorter course. For example, the yeast isolation can be shortened by the instructor plate or streak colonies from fermentation-positive cultures. Additionally, the brewing module can be shortened by using commercial malt extracts instead of mashing whole grains. Optional activities that could be omitted include a guest lecture from a local craft brewer, and tours of both a local craft brewery and homebrew store.

One of the optional modules we included was strain characterization of flavor compound formation (e.g. volatile esters and fusel alcohols), which we did both at the gene expression level via quantitative real-time PCR (qPCR) and directly via gas chromatography-mass spectrometry (GC-MS). We understand that some instructors may not have access or funds to include these modules. One cheaper alternative to qPCR would be semi-quantitative PCR (17). An alternative to GC-MS is sensory analysis, where students can be trained to identify esters and fusel alcohols by taste (individual flavor standards may also be purchased from FlavorActiV to facilitate compound identification).

### S1.5 Acknowledgements

Funding for this project was supported in part by National Science Foundation grant no. IOS-1656602. We are thankful to Ron Schmidt of Core Brewing and Distilling, LLC. for providing a guest lecture and a guided brewery tour. We also thank Steve Wilkes for giving a guided tour of Steve's Brew Shop. The authors declare that there are no conflicts of interest.

### S1.6 Supplemental Information

Supplemental information can be found here:
https://www.biorxiv.org/content/10.1101/698589v1

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## S2: Comparison of RNA Isolation Methods on RNA-Seq: Implications for Differential Expression and Meta-Analyses.

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Author contributions: Jeffrey A. Lewis. conceived and designed the project and performed experiments and analyses. I performed experiments and analysis.

This chapter has been submitted for publication.

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## S2.1 Abstract

Technical variation across different batches of RNA-seq experiments can clearly produce spurious signals of differential expression and reduce our power to detect true differences. Thus, it is important to identify major sources of these so-called “batch effects” to eliminate them from study design. Based on the different chemistries of “classic” phenol extraction of RNA compared to common commercial RNA isolation kits, we hypothesized that specific mRNAs may be preferentially extracted depending upon method, which could masquerade as differential expression in downstream RNA-seq analyses. We tested this hypothesis and found that phenol extraction preferentially isolated membrane-associated mRNAs, thus resulting in spurious signals of differential expression. Within a self-contained experimental batch (e.g. control versus treatment), the method of RNA isolation had little effect on the ability to identify differentially expressed transcripts. However, we suggest that researchers performing meta-analyses across different experimental batches strongly consider the RNA isolation methods for each experiment.

## S2.2 Background

The decreasing cost of massively parallel sequencing had led to an explosion of transcriptomic datasets. This large number of datasets has allowed for meta-analyses, which can be valuable due to their increase in statistical power. However, researchers performing meta-analyses on transcriptomic datasets need to be cautious in their use and be aware of so-called “batch effects,” where technical differences between experimental batches can clearly produce spurious signals of differential expression and reduce our power to detect true differences.

In some cases the sources of batch effects are known and can be avoided. Some well-known batch effects include sequencing lane effects, library construction protocol, and RNA quality (1-3). Other sources of batch effects clearly exist but remain unknown. While batch effects can sometimes be accounted for this comes with some major caveats. If the batch effect completely confounds the experimental design, for example with different sequencing lanes being used for controls and treatments, statistically accounting for the batch effect will remove any “real” signal (4). Even in the case where the batch effect is not a complete confounder, accounting for batch can reduce our power to detect true biological signal (5). Thus, a better understanding of the sources of batch effects can help us to avoid them.

In this study, we examined the effects of RNA isolation method as a possible source of batch effects in RNA-seq design. It is well known that the RNA distribution within cells is not uniform. Newly synthesized pre-mRNAs are processed in the nucleus before being exported. Once exported, mRNAs are frequently trafficked to specific subcellular sites as a mechanism for spatially controlling protein synthesis. Indeed, perhaps the most widespread example of mRNA localization is that used for spatial control of protein synthesis, where mRNAs encoding secreted and membrane proteins are translated at the ER membrane allowing for proper protein localization and folding (6).

Despite the widespread acknowledgement that mRNAs are differentially localized within the cell, there has been a paucity of studies examining whether “common” RNA extraction methods are equivalent in their abilities to extract differentially localized RNA species, and whether the method of RNA isolation affects our ability to detect differentially expressed transcripts. Sultan and colleagues compared two RNA isolation methods (Qiagen RNeasy kit and guanidinium-phenol (TRIzol) extraction) and two library selection schemes (poly-A enrichment and rRNA depletion) on downstream transcript abundance estimates, and found that rRNA depletion was particularly sensitive to the RNA extraction method (2). However, their comparisons were done using only two biological replicates, and they only examined transcript abundance across technical replicates and not whether the method of extraction affects the ability to detect differential expression in the types of sample comparisons that biologists frequently care about (e.g. wild-type versus mutant or treatment versus control).

Thus, we sought to systematically examine whether three common RNA isolation methods led to differences in transcript abundance and/or our ability to detect differential expression between two experimental conditions in the form of the *Saccharomyces cerevisiae* heat shock response. The different RNA isolation methods were the classic “hot acid phenol” method, and the two most commonly-used types of kits (7)—a silica-based column kit (Qiagen RNeasy Kit) and a guanidinium-phenol (TRIzol)-based kit (Zymo Research Direct-zol), hereafter referred to as the Phenol, RNeasy, and Direct-zol methods. Based on the combined chemistries of sodium dodecyl sulfate (SDS) and phenol on cellular membranes (8, 9), we hypothesized that the Phenol method would better solubilize membrane-associated mRNAs. To test this hypothesis, and whether the choice of RNA isolation method had downstream effects on our ability to detect differentially expression transcripts, we collected four biological replicates of the model yeast *Saccharomyces cerevisiae* before and after a 20-minute heat shock. Importantly, each biological sample was split into three identical technical replicates that differed only in their mode of RNA isolation. This allowed us to systematically test whether the RNA isolation method

affects relative transcript abundance between technical replicates, and whether that matters for differential expression analysis.

Our analysis found a striking number of transcripts (nearly 1/3 of the genome) that appeared “differentially” expressed when comparing the Phenol method to either Kit method, and a small number of differences when comparing the Kit methods to each other. Transcripts over-represented by Phenol extraction compared to either Kit were enriched for membrane proteins, suggesting that indeed the SDS plus phenol better extracts those species of mRNA. Importantly, there were virtually no differences when comparing differential expression for the heat shock response within samples where RNA was isolated via same method. Based on these results, we strongly recommend that meta-analyses be performed on groups of experiments with common RNA isolation methods.

**S2.3 Methods**

**S2.3.1 Yeast Growth and Sampling Procedures**

All experiments were performed using yeast strain BY4741 (S288c background; MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), obtained from Open Biosystems. To compare RNA isolation methods, we collected three identical 10-ml ‘technical’ replicates for each biological replicate (4 biological replicates in total). Cells were grown >8 generations in 100-ml synthetic complete medium (SC) (16) at 30°C with orbital shaking (270 rpm) shaking to mid-exponential phase ($OD_{600}$ of 0.3 – 0.6), and 10-ml samples were removed representing the unstressed control. For heat shock treatment, one volume of 55°C medium was added to the remaining culture, immediately bringing the final temperature to 37°C, and the culture was incubated at 37°C for another 20 minutes before removing 10-ml samples. Both unstressed and heat shocked cells were collected by centrifugation at 1,500 x *g* for 3 minutes, and cell pellets were flash frozen in liquid nitrogen and stored at -80°C until processing.

### S2.3.2 RNA Isolation Methods

#### S2.3.2.1 Hot Phenol Isolation

Cells were lysed and RNA was isolated using a standard hot phenol method as described (17), and a detailed protocol can be found on the protocols.io repository under DOI dx.doi.org/10.17504/protocols.io.inwcdfe. Briefly, 1 volume of acid saturated phenol and 1 volume of lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM EDTA, 0.5% SDS) were added to frozen cell pellets, vortexed, and then placed in a 65°C preheated Multi-Therm incubated vortexer (Benchmark Scientific) at 1500 rpm for 45 minutes. Samples were centrifuged for 10 min at 4°C at maximum speed in a microcentrifuge, extracted once more with phenol, once with chloroform, and then precipitated overnight at -20°C with 0.1 volumes of sodium acetate (pH 5.2) and 2.5 volumes of 100% ethanol. Precipitated RNA was washed once with 70% ethanol and then resuspended in TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA). The phenol extracted RNA was then 'cleaned' using an RNeasy Miniprep Kit with optional on-column DNase treatment according to the manufacturer's instructions.

#### S2.3.2.2 RNA Isolation with Two Different Miniprep Kits

RNA was extracted using two different kits: the Qiagen RNeasy Mini Kit (Cat. 74104) and the Zymo Research Direct-zol RNA Miniprep Kit (Cat. R2050). Cell concentrations were all below the maximum recommendation of 5 x $10^7$ cells from both manufacturers (ranging from 2.5 x $10^7$ – 4.5 x $10^7$ cells). For both kits, we mechanically lysed cells with a Beadbeater-24 (3,500 oscillations/minute, 45 seconds on ice between cycles). Mechanical lysis was performed in 2-ml screw-capped tubes containing an equal volume (600 µl) of lysis buffer (RLT for RNeasy or TRI reagent for Direct-zol) and acid-washed glass beads (425-600 micron, Sigma-Aldrich).

RNA was then purified according to each manufacturer's protocol for yeast, including the optional on-column DNase digestion. For all samples, RNA was quantitated using a Qubit RNA

HS Assay kit and Qubit fluorometer according to the manufacturer's instructions. The RNA integrity number (RIN) for each sample was measured using an Agilent 2200 TapeStation. RNA concentrations and RIN values for each sample can be found in Supplementary Table 1.

### S2.3.2 RNA Sequencing and Analysis

RNA-seq libraries were prepared from polyA-enriched RNA using the KAPA Biosystems mRNA HyperPrep Kit (KK8581) and KAPA Single-Indexed Adapter Set A+B (KK8700), according to manufacturer's instructions. We started with 500 ng total RNA, fragmentation time (6 min) was optimized to generate 200-300-nt RNA fragments, and the libraries were amplified with 9 cycles of PCR. All libraries were constructed in a single batch through an automated Eppendorf epMotion 5075 liquid handling robot, and detailed a protocol can be found on protocols.io under DOI dx.doi.org/10.17504/protocols.io.uueewte. cDNA libraries were sequenced on a HiSeq4000 at the University of Chicago Genomics Facility, generating single-end 50-bp reads.

Reads were trimmed of low-quality reads and adapter sequence (KAPA v1 indices) using Trimmomatic (version 0.32) (18), with the following commands: ILLUMINACLIP:Kapa_indices.fa:2:30:10 LEADING:3 TRAILING:3 MAXINFO:40:0.4 MINLEN:40 . Reads were mapped to the S288c genome (version Scer3), using STAR (version 020201) (19). Mapping statistics can be found in Supplementary Table 2. Transcripts per million (TPM) and expected counts for each gene were calculated using RSEM (version 1.3.1) (20). The RSEM output can be found in Supplementary File 1.

Differential expression analysis was conducted using the Bioconductor package edgeR (version 3.22.3) using the quasi-likelihood (QL) framework. For the QL model, sample type (i.e. Phenol unstressed, Phenol heat shock, RNeasy unstressed…) and biological replicate were used as factors. To account for differences in RIN across samples, we also performed a separate analysis that included sample type, replicate, and RIN as factors in the model. To

control for differences in sequencing depth across samples, the edgeR function thincounts was used to randomly subsample counts across all samples to be equal to the sample with the lowest number of total counts (8,678,188). Only genes with at least 1 count per million (CPM) in at least one condition were included for TMM normalization and differential expression analysis. All RNA-seq data are available through the National Institutes of Health Gene Expression Omnibus (GEO) database under accession no. GSE135430, and the edgeR outputs can be found in Supplementary File 2.

Principle component analysis (PCA) was performed using ClustVis (21) on ln-transformed TPM values for all transcripts included in the differential expression analysis, using unit variance scaling and singular value decomposition. Hierarchical clustering was performed with Cluster 3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm) using uncentered Pearson correlation and centroid linkage as the metric (22). RNA-seq samples were weighted using a cutoff value of 0.4 and an exponent value of 1. Functional enrichments of gene ontology (GO) categories were performed using GO-TermFinder (https://go.princeton.edu/cgi-bin/GOTermFinder) (23), with Bonferroni-corrected *P*-values < 0.01 taken as significant. Complete lists of enriched categories can be found in Supplementary File 3.

**S2.3 Data availability**

All RNA-seq data are available through the National Institutes of Health Gene Expression Omnibus (GEO) database under accession no. GSE135430. The analyses generated during this study are included in the supplementary information files.

**S2.4 Results**

**S.2.4.1 Experimental setup**

To test whether RNA extraction methods impact between-sample comparisons and the power to identify differentially expressed genes, we used the well-characterized yeast heat

shock response as an environmental perturbation. We collected four biological replicates for comparison. For each biological replicate, three “technical replicate” samples were collected to understand the impact of RNA extraction method. The only difference between was that each technical replicate had their RNA extracted by one of three methods: classic hot acid phenol (Phenol method), a silica-based column kit (RNeasy Method) and a guanidinium-phenol (TRIzol)-based kit (Direct-zol Method) (Fig. 1). RNA isolated via the Phenol method was subsequently “cleaned” with a Qiagen RNeasy Kit using the optional on-column DNase treatment, thus controlling for both DNase treatment and potential differential binding of different RNA species to the column. To minimize against batch effects other than RNA extraction method, all RNA-seq libraries were constructed on the same day using an automated robotic platform, and all libraries were multiplexed and sequenced on a single lane of an Illumina HiSeq4000 instrument.

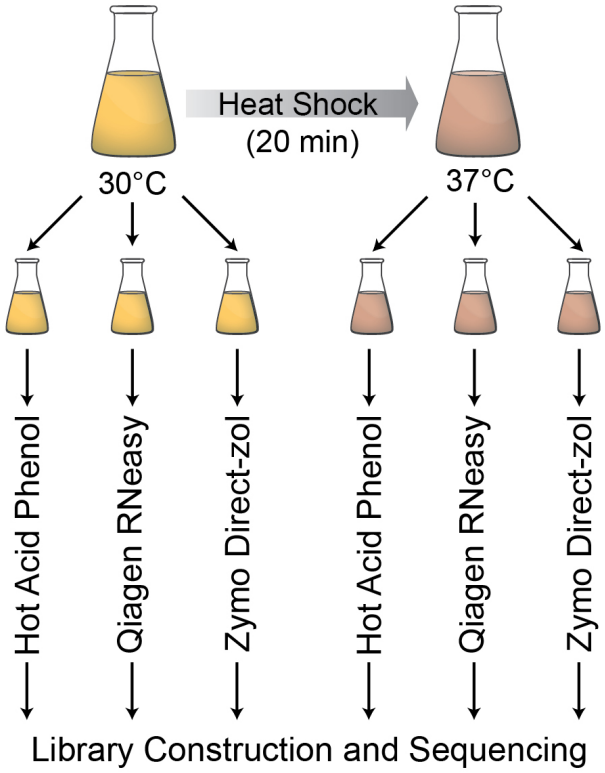



**Figure. SA2.1. Schematic of the experimental design.** Yeast cells were grown to mid-exponential phase at 30°C, unstressed control samples were collected, and then cells were shifted to a 37°C heat shock with samples collected after 20 minutes. For both unstressed and stressed cells, we collected three identical samples (technical replicates), and RNA was isolated using either hot acid phenol extraction, a Qiagen RNeasy Kit, or a Zymo Research Direct-zol RNA Kit. Libraries were constructed in a single batch using a liquid handling robot, and then were pooled and sequenced on a single Illumina HiSeq4000 lane.

**S2.4.2 Differences in relative transcript abundance between phenol-extracted RNA and kit-extracted RNA.**

All of the RNA isolation methods yielded generally high quality RNA, as defined by a RIN of 9.0 or above, though the phenol extracted RNA averaged significantly higher RIN values than those isolated from the Direct-zol kit (9.96 vs. 9.33; $p = 2 \times 10^{-6}$, *t*-test) or the RNeasy kit (9.96 vs. 9.79; $p = 0.01$, *t*-test). (Supplementary Table S1). The percentage of total mapped reads was similar across samples, with slight (though significant) differences (Supplementary Table 2). There were larger differences in the percentage of uniquely mapped reads across RNA isolation methods (Supplementary Table 2). These differences did not correlate with RNA integrity, as the Direct-zol samples had the lowest RIN values and highest uniquely and total mapped reads. Overall, we feel that the both the RNA quality and read mapping would not raise any red flags in laboratories performing RNA-seq on either their own samples, or conducting a meta-analysis, though those values can be used a factor to be controlled for in differential expression analysis (3).

We were particular interested in whether differences in the RNA isolation method could masquerade as “differential” expression due to differences in transcript quantification. We first performed principal component analysis (PCA) (Fig. 2). Not surprisingly, a substantial proportion of the variance (50.5%) was explained by treatment (unstressed versus heat shock). The second principal component corresponded to RNA isolation method and explained 26.9% of the variation. Samples with RNA isolated by the two different kit methods clustered together, with the Phenol-isolated samples forming a separate cluster. It could seem counterintuitive that Direct-zol and Phenol methods would be so dissimilar, considering that both methods use phenol. However, the Direct-zol method uses a milder detergent than SDS (sarkosyl), is performed at room temperatures instead of 65°C, and samples are exposed to phenol for 10 minutes instead of 45 minutes. We speculate these differences with the Phenol method result in both silica-column-based kits behaving similarly (see Discussion).

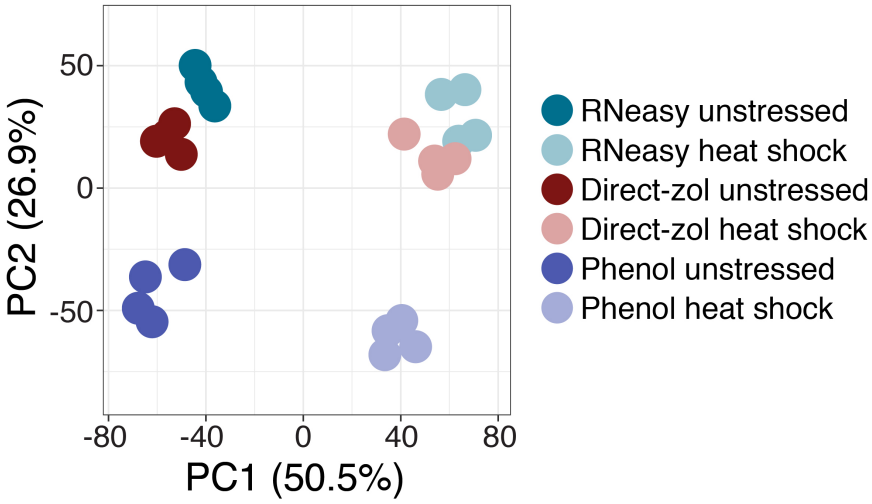



**Figure. SA2.2. Principal component analysis (PCA) strongly implicates RNA isolation method as a batch effect**. PCA on TPMs for each sample (see methods) shows clear separation on both treatment (PC1) and RNA isolation method (PC2). Kit samples were more similar to each other than they were to the Phenol sample.

To visualize differences in transcript abundance across RNA isolation methods, we performed hierarchical clustering on the TPMs of the unstressed samples (Fig. 3a). Hierarchical clustering of the samples largely recapitulated the patterns of PCA—again, the Phenol-isolated samples formed a discreet cluster distinct from the two kits. The RNeasy- and Direct-zol-isolated samples also had far fewer visible differences. To quantify these differences, we used edgeR to identify transcripts with significantly differential abundance in pairwise comparisons of each RNA isolation method (FDR < 0.01, see Methods). Pairwise comparisons of the Phenol method with each Kit method identified a large number of transcripts with differential abundance: 2,430 transcripts (Phenol vs. RNeasy) and 2,512 transcripts (Phenol vs. Direct-zol) comparison. Of those transcripts with differential abundance in both comparisons, 1,917 overlapped, which was highly significant ($P = 1 \times 10^{-520}$, Fisher's exact test) (Fig. 3c). In contrast, only 230 transcripts had differential abundance when comparing the kits to each other, suggesting only slight differences.

To better visualize these differences, we performed hierarchical clustering on all 3,127 transcripts with significantly differential abundance (FDR < 0.01) in any pairwise comparison of RNA isolation method (Fig. 3b). We found striking functional gene ontology (GO) enrichments for transcripts with higher or lower abundance in the phenol-extracted samples compared to both kits. Transcripts with higher abundance in phenol-extracted RNA in comparison to both kits were strongly enriched for transmembrane transport ($P < 4 \times 10^{-68}$), establishment of localization ($P < 9 \times 10^{-54}$), lipid metabolism ($P < 1\text{x } 10^{-27}$), and cell wall organization ($P < 1 \times 10^{-18}$). Looking more closely at the cellular component GO enrichments, transcripts with higher abundance in the phenol samples were strongly enriched for those encoding intrinsic membrane proteins ($P < 4 \times 10^{-191}$), as well as proteins localized to the endoplasmic reticulum ($P < 6 \times 10^{-84}$), cell periphery ($P < 3 \times 10^{-80}$), and the vacuole ($P < 3 \times 10^{-53}$). In contrast, mRNAs with lower relative abundance in the phenol samples were enriched for nuclear in localization ($P < 3 \times 10^{-60}$), and included those encoding functions related to nucleic acid metabolism ($P < 1 \times 10^{-38}$), RNA

metabolism ($P < 6 \times 10^{-28}$), chromosome organization ($P < 4 \times 10^{-17}$), and gene expression ($P < 8 \times 10^{-17}$).

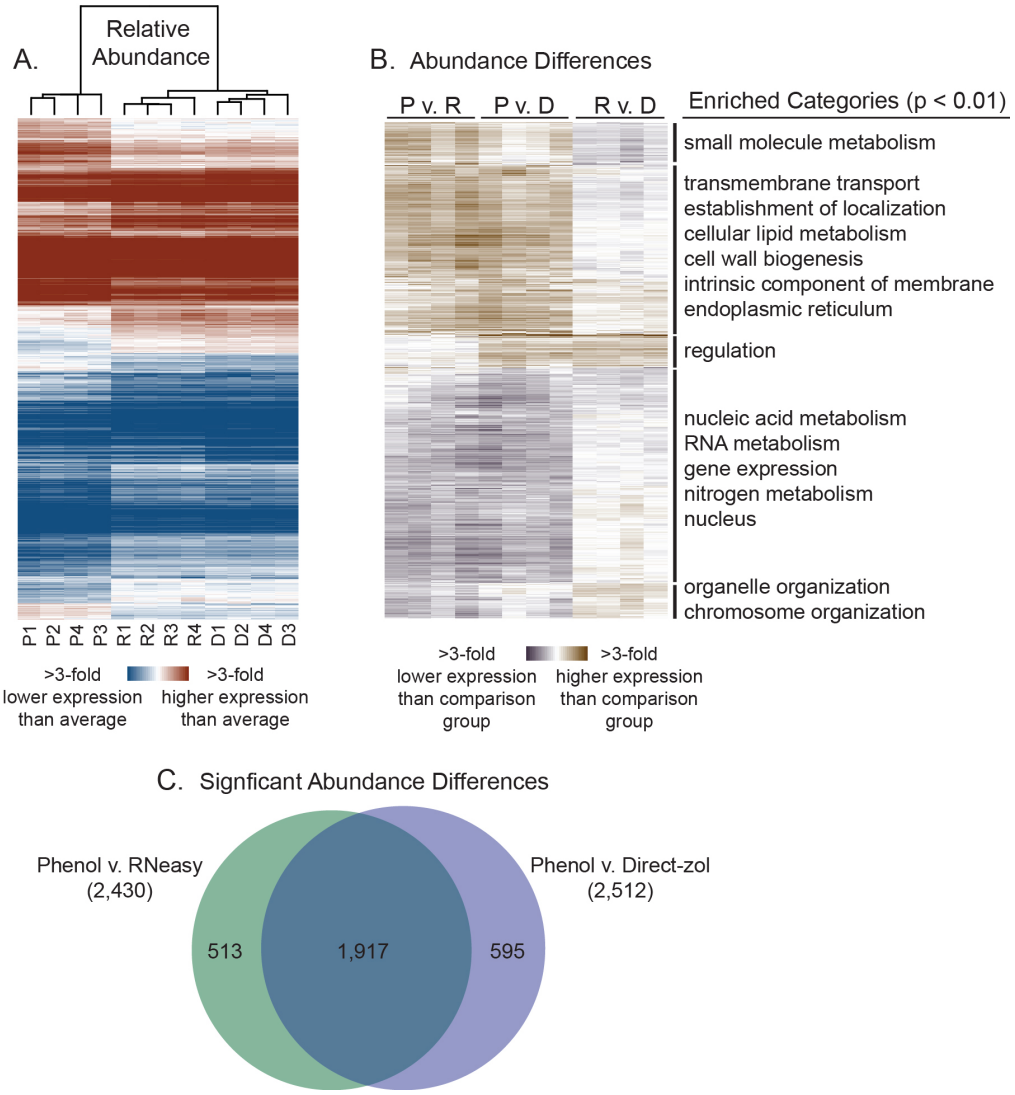
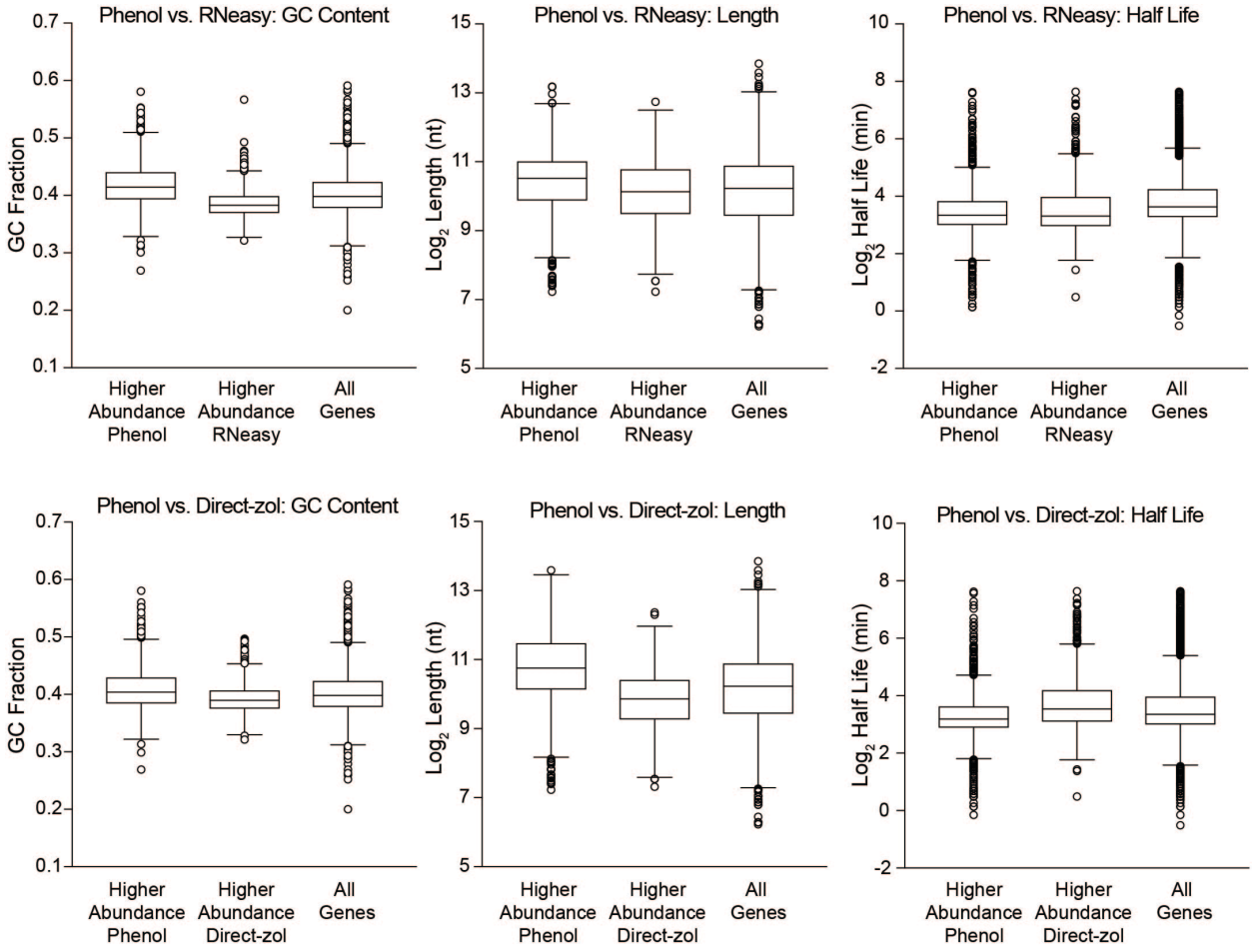



**Figure. SA2.3. Phenol preferentially extracts mRNAs that encode for membrane proteins.** **a** Hierarchical clustering of unstressed samples (P = Phenol, R = RNeasy, D = Direct-zol). Clustering on relative transcript abundance (TPMs) reveals differences depending upon RNA isolation method, while clustering on sample identity shows that the Phenol method diverges from both Kits. Red indicates higher than average transcript abundance within a sample, and blue indicates lower than average transcript abundance. **b** Hierarchical clustering of 3,127 transcripts with significantly differential abundance (FDR < 0.01) in any pairwise comparisons between each RNA isolation method. Brown indicates higher expression than the comparison group (e.g. Phenol in the P v. R column) and violet indicates lower expression than the comparison group (e.g. RNeasy in the P v. R column). Enriched Gene Ontology (GO) categories (Bonferroni-corrected *P* < 0.01) are shown on the right. Complete GO enrichments for each cluster can be found in Supplementary File 3. **c** Overlap between transcripts with significantly differential abundance (FDR < 0.01) in the Phenol v. RNeasy and Phenol v. Direct-zol comparisons.

**S2.4.3 Properties of transcripts with spurious differential expression.**

That Phenol-isolated samples have higher transcript abundance for mRNAs encoding membrane proteins fits with the hypothesis that the Phenol method better solubilizes that species of mRNA. Another possibility is that differences in transcript degradation rates are responsible for the spurious patterns of differential expression. Because GC content and transcript length correlate with in vivo mRNA degradation rates (3), we examined those relationships in our data. Transcripts with significantly higher or lower abundance in Phenol-extracted samples compared to each Kit method had significantly higher GC content and gene length (Supplementary Fig. 1). We also examined the relationship between differential abundance and direct estimates of *in vivo* transcript stability (half-lives) from Neymotin and colleagues (10). We did find a significant difference in the Phenol vs. Direct-zol comparison, but not for the Phenol vs. RNeasy comparison. To determine how much of the variation was explained by GC content, gene length, and transcript half-life, we performed linear regression of those parameters on the average fold changes for phenol-extracted samples vs. the kits. Both GC content and transcript length showed weak to moderate correlation ($r$ = 0.06 – 0.32) with $\log_2$ fold changes, depending upon the comparison group, while estimated *in vivo* half-life weakly correlated with $\log_2$ fold changes in either comparison (Supplementary Table 3). Because differences in GC content and length are associated with differences in transcript degradation rates *in vitro* (3), we repeated the edgeR analysis using RIN as a factor. We expected that because the RIN values for the Direct-zol samples were all lower than the others, using RIN as a covariate would eliminate most of the signal for differential expression. This turned out to be correct—we identified 788 “differentially” expressed genes in the Phenol vs. Direct-zol comparison compared to 2,513 when RIN was not included as a factor. The surviving differentially expressed transcripts with higher expression in the Phenol-isolated samples relative to the Direct-zol isolated samples were still strongly enriched for those encoding intrinsic membrane proteins ($P < 3 \times 10^{-100}$). Because the RNeasy-isolated samples had relatively high




**Supp Figure. SA1.1. Properties of transcripts with differential abundance depending upon RNA isolation method.**

RIN values relative to the Direct-zol-isolated samples, the vast majority of transcripts with differential expression were retained as significant when accounting for RIN in the edgeR QL model (2,362 / 2,430). Because of the substantial overlap between genes called as differentially expressed in the Phenol vs. RNeasy and Phenol vs. Direct-zol comparisons, we hypothesize that the that differing chemistries in the extraction are responsible for the batch effect, and not RNA degradation (see Discussion).

### S2.4.4 Differences in RNA isolation method have little effect on the ability to detect differential expression with a batch.

The striking differences in transcript abundance depending on RNA isolation could conceivably affect the ability to detect differential expression. To test this, we examined our ability to detect differential expression in cells shifted from 30°C to 37°C for 20 minutes—the classic yeast heat shock response. We identified ~3,800 differentially expressed transcripts for all three RNA isolation methods, with substantial overlap for all three (Fig. 4). Hierarchical clustering yielded no clear pattern among differentially expressed transcripts that were missed in sample set over another (Fig. 4). We also detected zero transcripts that had significant fold change differences in their heat shock response in any pairwise comparison between RNA isolation methods (Supplementary File 2). We hypothesize that at sufficient sequencing depth, the ability to detect differential expression is robust to the modest differences in transcript counts caused by differences in RNA isolation method.

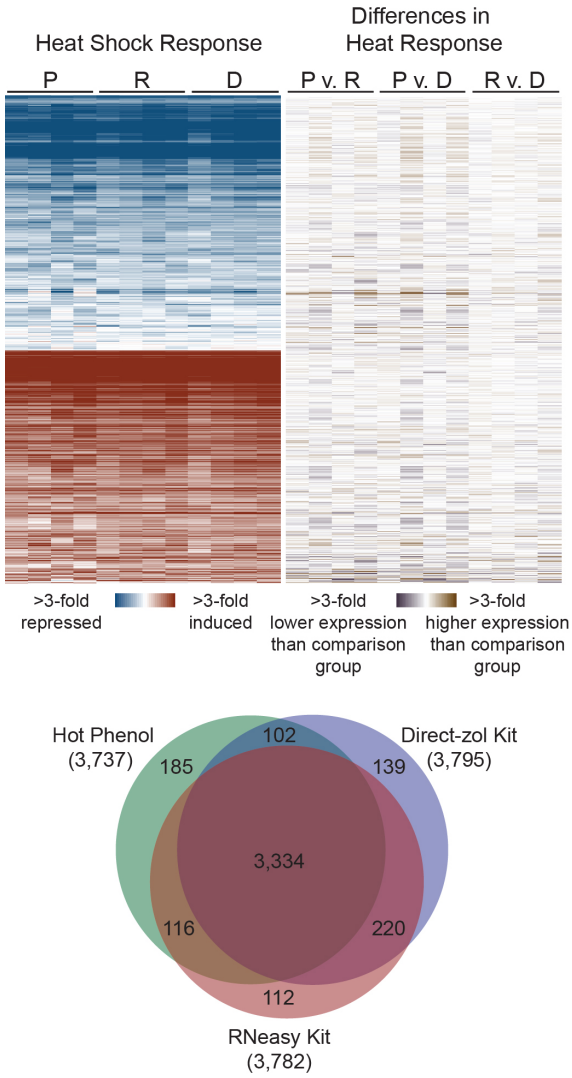



**Figure. SA2.4. The method of RNA extraction has little effect on differential expression analysis.** Hierarchical clustering of median-centered $\log_2$-fold TPM changes for 4,232 transcripts that were differentially expressed in response to heat (FDR < 0.01) in at least one set of samples (P = phenol, R = RNeasy, D = Direct-zol). The left portion of the heat map displays gene expression changes during heat shock across the four biological replicates, with red indicating genes induced by heat shock, and blue indicating genes repressed by heat shock. The right portion shows differences in abundance in pairwise comparisons between each RNA isolation method, with brown indicating higher expression than the comparison group, and violet indicating lower expression than the comparison group. The Venn Diagram depicts overlap between differentially expressed genes in the Phenol, RNeasy, and Direct-zol isolated samples.

**S2.5 Discussion**

In this study, we tested whether differences in RNA isolation method affect relative transcript abundance between samples, and whether the RNA isolation method impacts our ability to detect differential expression. Our results suggest that differences in RNA isolation method can substantially affect relative transcript abundance, and we see thousands of differences in transcript abundance when comparing hot acid phenol extraction with an RNeasy or Direct-zol kit. It is well established that mRNAs encoding membrane and secreted proteins are anchored to the membrane during translation (11). That transcripts with higher abundance in the Phenol-isolated samples are strongly enriched for encoding membrane proteins suggests the Phenol method better solubilizes those mRNAs. Because relatively more membrane-associated mRNAs are being extracted, there must be relatively less abundance of other mRNAs. Thus, we see decreased abundance of certain nuclear transcripts, which were already more lowly expressed, and thus likely more sensitive to appearing “repressed.”

We disfavor the alternative hypothesis that we are capturing differences in transcript degradation rates for a number of reasons. First, while we do see differences in RIN values across the different RNA isolation methods, the differences are relatively small, and our RIN values are all much higher than the points where other studies identified them as confounding RNA-seq analysis (3, 12). Second, it is likely that any degradation that is occurring in our samples is happening *in vitro* during RNA isolation, and Opitz and colleagues have found that *in vitro* RNA degradation rates are likely relatively equal across transcripts and thus have little effect on differential expression analysis (13). And while RNA degradation rates *in vivo* are strongly biased and can lead to spurious functional enrichments in downstream analysis, we found little relationship between estimated mRNA half-lives from (10) and fold-changes in comparisons between kits. Only one of the Phenol vs. Kit comparisons showed a significant difference in half-lives, but the correlation was still rather poor ($r^2$ = 0.02). And while transcripts with higher relative abundance in the phenol-extracted samples versus the kits had higher GC

content and gene length, which both correlate with higher *in vivo* degradation rates (3), the correlation between those parameters and fold-change differences was not strong (Supplementary Table 3). Notably, GC content and gene length are not random, and membrane proteins tend to be longer and have higher GC content than average (14, 15). Finally, if RNA degradation is responsible, it is somewhat hard to reconcile that we see similar patterns of "differential" expression when comparing the Phenol vs. Direct-zol or RNeasy kits, even though the RNeasy kits have quite a bit higher RIN values.

Regardless of the cause of these differences between hot-phenol extracted samples and kits, it clear that this can represent a large source of batch-effect variation between samples whose RNA has been isolated via different methods. Within an individual lab, we are largely agnostic. The method of RNA isolation had little effect on the ability to identify differentially expressed transcripts in our heat shock test case. Thus, experiments within a single lab are unlikely to be affected by the choice of RNA isolation method as long as the same method is used throughout an experiment. For meta-analyses however, we recommend that researchers avoid comparing experiments where the RNA isolation methods differ.

**S2.6 Acknowledgements**

We thank Zymo Research for providing a free sample of the Direct-zol RNA Kit, Dr. Elizabeth Ruck for running samples on the TapeStation, and Dr. Andrew Alverson for the use of equipment.

**S.2.7 Supplemental Information**

Supplemental Information can be found:
https://www.biorxiv.org/content/10.1101/728014v1

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# Chapter 6:Appendix

UNIVERSITY OF ARKANSAS

*Office of Research Compliance*

July 24, 2019

MEMORANDUM

| | |
|---|---|
| TO: | Dr. Jeffrey Lewis |
| FROM: | Ines Pinto, Biosafety Committee Chair |
| RE: | Protocol Renewal |
| PROTOCOL #: | 14007 |
| PROTOCOL TITLE: | Genomics and Physiology of Microbial Stress Responses |
| APPROVED PROJECT PERIOD: | **Start Date** July 11, 2013 **Expiration Date** July 10, 2022 |

The Institutional Biosafety Committee (IBC) has approved your request, dated July 8, 2019, to renew IBC # 14007, "Genomics and Physiology of Microbial Stress Responses".

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.

1424 W. Martin Luther King, Jr. • Fayetteville, AR 72701
Voice (479) 575-4572 • Fax (479) 575-6527

UNIVERSITY OF ARKANSAS

J. William Fulbright College of Arts and Sciences
*Department of Biological Sciences*

Date: December 5, 2019

To: Whom it may concern

From: Dr. Jeffrey A. Lewis
Research Advisor

Subject: Amanda Scholes Dissertation

The majority of the work presented in this dissertation was performed by Amanda Scholes as described in chapter author contributions. All biosafety training was completed and maintained in accordance with University of Arkansas and IBC policies regarding IBC protocol #14007.

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Assistant Professor of Biological Sciences

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