INVESTIGATING THE ROLE OF *HMX1* IN ROS TOLERANCE DURING MITOSIS AND MEIOSIS IN *SACCHAROMYCES CEREVISIAE*

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ABSTRACT

INVESTIGATING THE ROLE OF *HMX1* IN ROS TOLERANCE DURING MITOSIS AND MEIOSIS IN *SACCHAROMYCES CEREVISIAE*

by Kyle Kern

Organisms that have evolved to live in aerobic environment also need to be able to exist in the presence of toxic forms of oxygen, known as reactive oxygen species (ROS). ROS are highly reactive molecules that damage biomolecules and as a result inhibit cellular processes or causes cell death. During meiotic events ROS can cause damage to mitochondrial membranes and DNA that reduces the formation of viable daughter cells. In industry ROS accumulation in industrial organisms causes increased production time and decreased yields of target products. Cells are not defenseless however, and can utilize defense mechanisms, known as antioxidants, to combat cellular ROS accumulation. *HMX1* has recently been identified as a possible gene that regulates antioxidant reactions and has shown to be necessary in ROS tolerance in *Saccharomyces cerevisiae*. Tests were run using yeast strains engineered with *HMX1* deletions, *HMX1* overexpression, and mitochondrial GFP to study the effect of *HMX1* expression on cells mitotically dividing in the presence of hydrogen peroxide H_2O_2 and furfural. We also used these same strains to study the effect of *HMX1* expression during meiosis under normal conditions of cell starvation and in the presence of H_2O_2. We demonstrated that *HMX1* effects cell tolerance to H_2O_2 regardless of being a haploid or diploid. *HMX1* deletion shows damage to mitochondrial membranes when compared to wild-type and a loss of sporulation under exogenous ROS stress when grown in nutrient rich media. Our preliminary results show that overexpression of *HMX1* doesn't increase sporulation quantity but does seem to increase spore viability. Also indicated was that *HMX1* has a role in furfural tolerance in yeast undergoing mitotic division. Understanding these results and continued study of *HMX1* may lead to an increased

understanding of cellular response to ROS during mitosis and meiosis as well as engineering better yeast strains for industrial fermentation.

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CHAPTER I

INTRODUCTION

Saccharomyces cerevisiae is a valuable model organism

Saccharomyces cerevisiae is a species of budding yeast commonly used as a model organism for studying eukaryotic cell biology because it is small, easily grown in laboratory conditions, and there is centuries of genetic information available for study. *S. cerevisiae* is capable of fermenting mono- and -disaccharides under anaerobic or aerobic conditions and produce ethanol and carbon dioxide (Herskowitz, 1988). This metabolic process has been agriculturally important for thousands of years for the production of fermented food products, but has recently been in use for industrial purposes. Though there are many other organisms capable of fermenting, *S. cerevisiae's* ability to ferment aerobically as well as its survivability under alcoholic conditions makes it the preferred organism to produce fermentation products, such as ethanol and pharmaceuticals (Lin & Tanaka, 2006).

S. cerevisiae has two life stages, a haploid stage and a diploid stage. Haploid yeast cells can mitotically divide to create more cells, or they can mate with haploid yeast of an opposite mating type to create a diploid organism. Haploid cells have two mating types, "*MATa*" and "*MATα*" type, each with their own specific mating pheromone. A cell is sensitive to mating pheromones of the opposite mating type, and in a process called shmooing cells respond to the opposing mating type pheromone by forming projections towards the pheromone. Two opposing mating types will project towards each other, and when the two plasma membranes of the yeast of opposite mating types meet their cell walls will degrade and fuse together, their two nuclear envelopes fuse, and they will become a single diploid organism (Melloy, Shen, & Rose, 2007).

Diploid yeast can either divide by mitosis or enter into the specialized cell division of meiosis. Sporulation in *S. cerevisiae* occurs under conditions of starvation, and causes a cell to divide into 4 haploid cells. When sporulation is initiated meiosis 1 and 2 occurs creating 4 haploid nuclei surrounded by the nuclear envelope (Chu & Herskowitz, 1998). During the end of meiosis 2 the nuclear envelope begins to generate a prospore membrane, which engulfs one of the nuclei, the nuclear envelope surrounding the nuclei, and the surrounding cellular material to form a spore. These spores mature and push out towards the maternal cell wall, forming a structure known as an ascus. This ascus will soon deteriorate to produce 4 individual haploid cells which will eventually germinate to produce haploid cells (Neiman, 2005).

The spores are essential to yeast survival, not only because they are stress resistant but because they allow for genetic diversity. This genetic diversity can be achieved through genetic recombination and yeast mating (Ohta, Shibata, & Nicolas, 1994). In this fashion yeast spores are similar to gametes in more complex species, where haploids from one individual are formed with the purpose to fuse with haploids from another individual. Spores and gametes are formed in similar processes with each other, and are subject to some of the same stresses and constraints. With these similarities yeast sporulation can be used as a model for studying gametogenesis and cellular stress (Chu & Herskowitz, 1998).

Mitochondria are dynamic organelles during both mitotic and meiotic growth.

Mitochondria are organelles believed to originally be prokaryotic bacteria that where at engulfed by a eukaryotic cell and the two cells formed a symbiotic relationship. This theory is based on the presence of circular chromosomes within mitochondria that resemble bacterial chromosomes, and the unique formation of two mitochondrial membranes instead of the

traditional one (with the exception of chloroplasts, which are believed to have similar origins) (Gross & Debashish , 2011). The host cell and mitochondria are now intricately dependent and coordinated with each other. Mitochondria are essential in aiding in a variety of eukaryotic cell processes, including ATP generation, steroid synthesis, heme synthesis, and apoptosis regulation (Jouaville, *et al.*, 1999; Atamna & Boyle, 2005; Tanaka, et al., 2002; Duarte, *et al*., 2012). The mitochondria are dependent upon the cells as well, as up to 900 mitochondrial genes are transcribed from the nuclear chromosome and transported to the mitochondria. The mitochondrial genome and mitochondrial membrane structure are essential for mitochondrial function, and mitochondrial mutations or cellular membrane damage can lead to many diseases (Chan, 2006).

Mitochondrial morphology is maintained by regulating mitochondrial membrane fission and fusion events. *S. cerevisiae* has been important in identifying genes that regulate mitochondrial fission and fusion. These fission and fusion events are largely dependent upon the condition and stage of development of the cell. Mitochondrial fission and fusion play a role in maintaining and distributing mitochondrial DNA, regulating apoptosis, and optimizing spore production and viability (Gorsich & Shaw, 2004; Peraza-Reyes, Crider, & Pon, 2010). In addition mitochondrial morphology changes depending upon whether the cells are fermenting or respiring. In cells that are fermenting, mitochondria are few and less developed, but when they begin to respire mitochondria begin to enlarge and develop large cristae inlets. Certain types of stresses to a cell might cause the mitochondria to aggregate into clumps and reduce their function (Sauvaneta, *et al.*, 2010)

During meiosis mitochondria undergo a complex system of fusion and fission events that assure proper segregation of mitochondria to the four haploid spores. Once the cell commits to

meiosis mitochondria will fragment while maintaining an equal distribution of mitochondria. Upon entering meiosis mitochondria will fuse forming an elaborate network of membrane tubules. Once meiosis is complete and the haploid nuclei are packaged into spores, mitochondria will fragment and then shortly after will reform tubules (Miyakawa, *et al.*, 1984). During the entire process the mitochondria will be evenly distributed between the four new cells, which aids in overall spore fitness. However, if there is a disruption in the process of mitochondrial fission or fusion the mitochondrial segregation into spores becomes less efficient and some spores receive less or no mitochondria. Mitochondrial segregation issues can lead to either fewer viable spores or spores with reduced fitness (Gorsich & Shaw, 2004; Gross & Debashish , 2011)

Defining reactive oxygen species and their impact on organisms

Reactive oxygen species (ROS) are typically defined as chemically reactive molecules that contain oxygen. The most common biological forms of ROS are singlet oxygen ($^1O_2$), hydroxyl radicals ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), and superoxide anion ($O_2^{\bullet-}$) although many other types of molecules can be formed. ROS are highly reactive and will generally react with surrounding molecules in order to stabilize themselves, usually oxidizing or reducing those other molecules. These redox reactions can be especially destructive when ROS are generated in a biological system, as the available reactants are usually biomolecules essential in cellular processes. ROS can react with and damage carbohydrates, proteins, and lipids, which can have severe consequences, such as DNA damage or mutations, loss of protein function, and instability and loss of integrity of internal and exterior cellular membranes (Tamarit, *et al.*, 1998). ROS in a biological system can be generated by electrons from the electron transport chain "escaping" from either electron transport complexes or the carrier molecules and reducing cellular oxygen

(Liu, *et al.*, 2002). Other methods or ROS generation is from bombardment of radiation freeing electrons from molecular oxygen in the cellular environment or exposure to chemicals in the environment (Poljsak & Dahmane, 2012).

However, organisms are still capable of living in oxygenated environments and are not defenseless against the deleterious effects of ROS. Aerobic organisms have evolved to make use of antioxidants and cellular repair mechanisms in order to stave off ROS damage. Antioxidants are molecules that are capable of detoxifying ROS, rendering them harmless, and repair mechanisms are cellular processes set up to repair damage caused by ROS to cellular molecules. Antioxidants typically function in 2 major ways; the first is utilizing nutrients such as ascorbic acid and beta-carotene, which can react with ROS to form non-toxic products until they are either flushed from the body or reformed via other enzymatic processes. The second are enzymes like superoxide dismutase and catalases, which are capable of reducing or oxidizing ROS and are then reset via other molecules or enzymes (Herrero, *et al.*, 2008). ROS damage repair takes advantage of other damage repair mechanisms, such as base excision and nucleotide excision repair, to attempt to reverse the damage caused by ROS, though these mechanisms can also be mutagenic or otherwise problematic if used over extended periods of time (Croteau & Bohr, 1997).

ROS as it relates to cellular and human health

Understanding the cellular effects ROS will help us to understand ROS-induced diseases, and possibly lead to better diagnoses and treatment of a wide variety of diseases. Under normal conditions there is a balance between the amount of ROS produced and the amount that can be remediated via antioxidant detoxification and damage repair mechanisms. However, cellular

stress that causes an increased production of ROS or a decreased production of antioxidants can cause ROS to accumulate, which will result in damage to the cell. Extreme ROS accumulation will generally cause a cell to induce apoptosis, or programmed cell death, in order to remove the damaged cell from the population (Yang, *et al.*, 1998). In a multicellular organism rapid ROS accumulation can also signal that a tissue has been injured and causes the release of anti-inflammatory molecules (Boldogh, et al., 2005). Prolonged ROS accumulation and subsequent cell damage have been attributed to many health problems, such as heart disease, liver disease, Alzheimer's, Parkinson's, and infertility (Moustafa, *et al.*, 2004; Lee *et al.*, 2008; Shimamoto, *et al.*, 2011; Buizza, *et al.*, 2012; Kim, *et al.*, 2013).

There are many factors that can produce ROS and its accumulation during gamete (sperm or egg formation) and lead to infertility. These problems include age, alcohol and drug use, heavy metals, industrial chemicals, radiation, and overall stress. One impact of ROS accumulation on fertility is damage to the overall health of sperm. Spermatagonia in testes produces male germ cells, and these male germ cells will undergo meiosis to produce haploid cells which will eventually become sperm cells. ROS accumulation has been shown to cause low sperm production, defective sperm morphology, and a decrease in sperm viability and fertilization capacities (Moustafa, *et al.*, 2004). Another associated defect is ROS damage to genetic material of sperm cells; ROS can cause single and double stranded breaks in DNA, deletions, and chromosomal beaks or loss of the complete choromosome. This damage can lead to an inability of proper sperm formation (Zini, *et al.*, 2009), while also being implicated in loss of epigenetic functions (Tunc & Tremellen, 2009).

Saccharomyces cerevisiae as a model organism for fertility studies

Saccharomyces cerevisiae is an excellent model organism for studying reproductive health as it relates to humans. Even though yeast do not produce eggs or sperm they do produce a haploid spore, which is analogous to human gametes. Yeast undergo meiosis and spore development under controlled conditions in vitro in an analogous manner as humans and other more derived organisms. In yeast we have identified that mitochondria function and morphology are vital to meiotic processes and to proper spore formation and viability. Mitochondrial fission and fusion mutants can cause severe defects in spore formation and result in inviable spore production due to misallocation of mitochondria to all the potential spores (Figure 1.) (Gorsich & Shaw, 2004). Mitochondrial damage and the resulting defects have been shown to be consistent in human gamete production (Okamoto & Shaw, 2005)

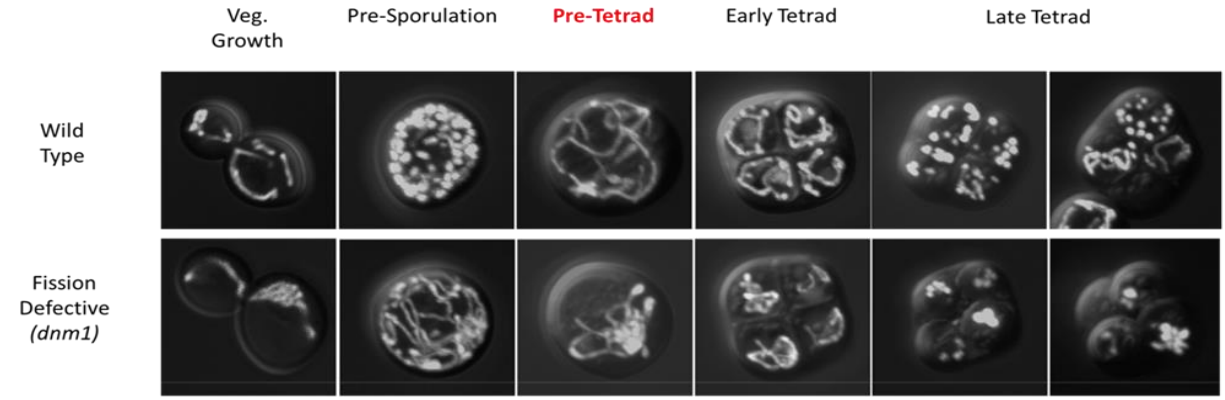

Figure 1. **Mitochondrial fission mutants show increased mitochondrial stress and reduced spore viability**
Wild type and *dnm1* fission mutants where transformed with mitochondrial GFP and sporulated, and cells were visualized at different stages of sporulation. It is clear in the mitochondrial fission mutants that the mitochondria are unable to function properly, and during the pre-tetrad stage aggregate to the polar regions of the cell. This negatively impacts tetrads and causes a mis-allotment of mitochondria and results in decreased spore viability. (Gorsich and Shaw, 2004)

Similar to the mitochondrial morphology mutants, defects in spore production can also be seen in yeast cells that undergo hydrogen peroxide induced ROS stress. Hydrogen peroxide damages mitochondrial cell membranes and causes a normally tubular structure to fragment and aggregate at the cellular poles during spore formation (Brown-Stokes and Gorsich, unpublished observations). Yeast strains engineered to overexpress *ZWF1* have increased tolerance to hydrogen peroxide and have been shown to increase meiotic events under exogenous hydrogen peroxide stress but also to increase the viability of all potential spores (Brown-Stokes and Gorsich, unpublished observations). Zwf1p is the rate limiting enzyme in the pentose phosphate pathway that generates NADPH, which serves as a co-factor for stress protective enzymes. Identifying other genes important in protecting yeast during spore production is vital in furthering our understanding of meiosis and gamete production during ROS stress. These findings will help us understand the impact of ROS stress on fertility.

Bioethanol methods and concerns

ROS impacts not only disease, but also yeast cell health as it relates to industrial fermentation of bioethanol. The current method of bioethanol production utilizes the fermentative preferences of *Saccharomyces cerevisiae* in order to ferment ethanol from sugar and hydrolyzed starch (unmodified starch is not capable of being fermented), most commonly found in agricultural products such as sugar cane and corn respectively. The most common sugar in these agricultural products is the monosaccharide glucose, which is efficiently fermented to ethanol and carbon dioxide (Lin & Tanaka, 2006). The problem is that though *S. cerevisiae* is more tolerant to ethanol compared to other fermenting organisms, it still can only survive in a concentration of about 15% ethanol by volume. This sensitivity to ethanol means

that distillation is still necessary and a large amount of substrate and labor is required to make a concentrated volume of ethanol. Further aggravation is that corn and sugar cane is a valuable food crop, and the competition for resources between food and bioethanol makes the process an extremely expensive and variable one (Tenenbaum, 2008).

A possible solution to this problem is to find alternative non-food substrates with which to ferment bioethanol. Lignocellulosic materials are possible alternatives for bioethanol production. These materials include grass, switch grass, wood chips, corn husks, and saw dust) (Gorsich, *et al.*, 2006). A current problem with the utilization of lignocellulosic material as a substrate for bioethanol production is that the hydrolysate created from lignocellulosic material contains pentoses as well hexoses of which yeast can only ferment hexoses. One of the common pentoses harvested from lignocellulosic lysate is xylose, a sugar that is not normally able to be utilized by *S. cerevisiae*. Though the problem of xylose utilization in *S. cerevisiae* is not solved, progress has been made by engineering *S. cerevisiae* with xylose metabolizing genes from *P. siptis*, a yeast species that is able to ferment xylose (Kötter & Michael, 1993). A second problem in using lignocellulosic material is that the fermentable hydrolysate produced from lignocellulose has over 100 toxic chemicals that inhibit cell growth and fermentation. These toxins include furfural and hydroxymethylfurfural, which are breakdown derivatives of xylose and glucose, respectively (Palmqvist & Hähn-Hägerdal, 2000). Furfural is a heterocyclic aldehyde that has been shown to cause an accumulation of reactive oxygen species and is an inhibitor of fermentation in *Saccharomyces cerevisiae* (Figure 2) (Allen, et al., 2010). *S. cerevisiae* can detoxify furfural by reducing it to 2-furanmethynol by using NADH-dependent alcohol dehydrogenase. In addition, the pentose phosphate pathway also protects yeast from furfural stress presumably by the NADPH this pathway produces, which is used as a cofactor for alcohol

reductases seen to detoxify furfural (Gorsich, *et al.*, 2006; Bowman *et al.*, 2010). Identifying furfural detoxifying genes and finding ways to engineer yeasts that are more tolerant to furfural and other inhibitors is vital if linocellulosic material is to become a viable resource for bioethanol.

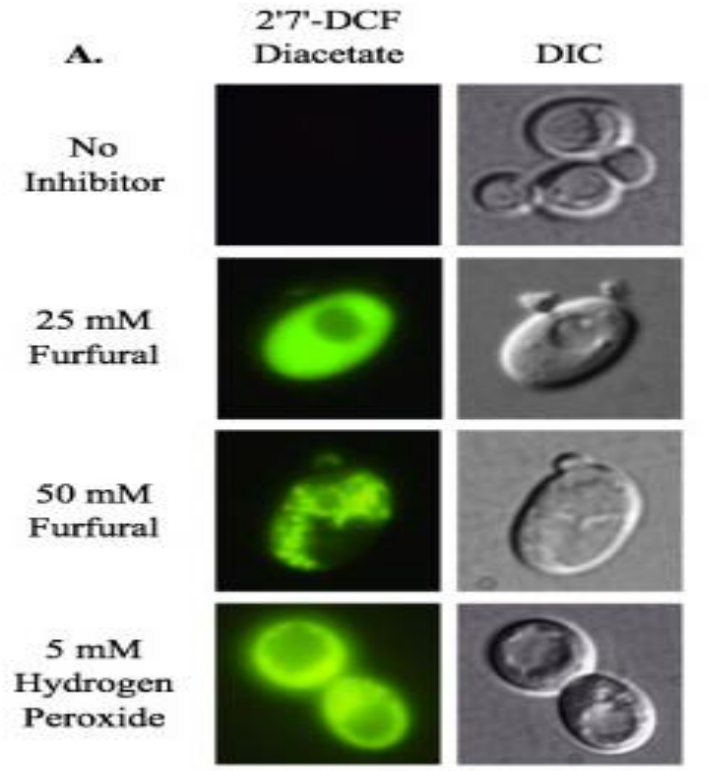

Figure 2. **Yeast cells undergoing exponential growth were incubated with furfural and hydrogen peroxide.**
A. (left) Cells incubated for 8 hours were stained with a ROS fluorescent indicator dye 2'7'-DCF Diacetate and (right) Differential Interference Contrast (DIC) shows cellular morphology and condition of represented yeast cells. (Allen, et al., 2010).

The yeast gene *HMX1* is important in stress tolerance.

HMX1 is a gene that might contribute both to the understanding of ROS stress on spore development and to furfural ROS tolerance. *HMX1* is found on the 12th chromosome and contains 951 base pairs. *HMX1* encodes heme oxygenase that is known to be involved with

heavy metal transport and has a recently identified role in tolerance to hydrogen peroxide (Collinson, *et al.*, 2011). In the presence of stress Hmx1p moves from the cytoplasm to the perinuclear regions for reason yet to be identified (Collinson, *et al.*, 2011). Studies have shown that *HMX1* mutants in haploid cells have reduced expression of known antioxidant genes such as *GPX1*, *GSH1*, and *CTT1*. Moreover, yeast that overexpress *HMX1* have an increased tolerance o H_2O_2 when compared to wild-type yeast. A possible explanation to these findings is that Hmx1p may indirectly or directly regulate the expression of these antioxidant genes (Collinson, *et al*., 2011).

Questions to be answered

A further understanding of the role of *HMX1* to yeast ROS tolerance response needs to be further investigated. This thesis will investigate two questions concerning *HMX1* and ROS: 1) *HMX1* role in spore development and 2) *HMX1* role in furfural tolerance.

Investigation 1: Is the tolerance to H_2O_2 provided by *HMX1* only found in haploid cells or does it have a similar function in diploid cells? In answering this question, wild-type, *hmx1::HIS* mutants, and overexpression mutants were observed using diploid yeast undergoing meiosis and mitosis in the presence of variable concentrations of hydrogen peroxide. Overall growth, spore production, spore viability, and mitochondrial morphology were assayed. It was shown that *hmx1* mutants have sensitivity to hydrogen peroxide during mitotic growth, during meiosis *HMX1* seems to have a roll in hydrogen peroxide tolerance as seen by stressed mitochondrial morphology and a reduction in spore production, though further investigations are needed.

Investigation 2: Does *HMX1* have a role in furfural tolerance as would be predicted by its demonstrated role in hydrogen peroxide tolerance? To answer this question I performed mitotic divisions using wild type and *hmx1* mutants and assessing their ability to recover from lag during conditions stressed by the presence of furfural. It was shown that wild-type cells could recover from lag earlier than the *hmx1* mutants during furfural incubation.

CHAPTER II

MATERIALS AND METHODS

S. cerevisiae general growth conditions

Yeast in this study was cultured using standard yeast techniques (Sherman, *et al.*, 1986; Guthrie & Fink, 1991). Strains utilized in this study were derived from a standard FY diploid strain *MATa/α ura3-52/ura3-52, his3Δ200/his3Δ200, leu2Δ1/leu2Δ1, trp1Δ63/trp1Δ63, lys2/lys2* or W303 strain *MATa/α ade2-1/ade2-1, leu2-3/leu2-3, his3-11,15/his3-11,15, trp1-1/trp1-1, ura3-1/ura3-1, can1-100/can1-100.* Using the yeast integrating vector, pRS403, a disruption cassette containing *HIS3* with *HMX1* flanking sequences was amplified and transformed into our yeast strains to disrupt *HMX1*. Transformations were performed via lithium acetate transformation in these strains to produce heterozygote *HMX1/hmx1::HIS3*. These diploid heterozygotes were then the sporulated to yield *MATa ura3-52, his3Δ200, leu2Δ1, trp1Δ63, lys2; MATα ura3-52, his3Δ200, leu2Δ1, trp1Δ63, lys2; MATa hmx1::HIS3 ura3-52, his3Δ200, leu2Δ1, trp1Δ63, lys2; MATα hmx1::HIS3 ura3-52, his3Δ200, leu2Δ1, trp1Δ63, lys2; MATa ade2-1, leu2-3, his3-11,15, trp1-1, ura3-1, can1-100; MATα ade2-1, leu2-3, his3-11,15, trp1-1, ura3-1, can1-100; MATa hmx1::HIS3 ade2-1, leu2-3, his3-11,15, trp1-1, ura3-1, can1-100; MATα hmx1::HIS3 ade2-1, leu2-3, his3-11,15, trp1-1, ura3-1, can1-100.* Sisters of the same genotype were then mated to produce diploids *MATa/α ura3-52/ura3-52, his3Δ200/his3Δ200, leu2Δ1/leu2Δ1, trp1Δ63/trp1Δ63, lys2/lys2; MATa/α hmx1::HIS3/hmx1::HIS3, ura3-52/ura3-52, his3Δ200/his3Δ200, leu2Δ1/leu2Δ1, trp1Δ63/trp1Δ63, lys2/lys2; MATa/α ade2-1/ade2-1, leu2-3/leu2-3, his3-11,15/his3-11,15, trp1-1/trp1-1, ura3-1/ura3-1, can1-100/can1-100; and MATa/α hmx1::HIS3/hmx1::HIS3, ade2-1/ade2-1, leu2-3/leu2-3, his3-11,15/his3-11,15, trp1-1/trp1-1,*

ura3-1/ura3-1, *can1-100/can1-100.* These cells were then transformed with *pRS-MET-425*, *HMX1/pRS-MET-425,* or *pVT100U-mtGFP* as indicated. Fitness issues arose with cells generated from W303 strains and studies with them were discontinued.

Sub-cloning *HMX1* into the yeast expression vector, *pRS425-HMX1*

HMX1 was amplified using the forward primer 5'-GGGGGGATTCATGGAGGACAGTAGCAATACAA-3' and reverse primer 5'-GGGGCTCGAGTTATACTATGCTAAGAAAACTC-3' (synthesized by Integrated DNA Technologies, Coralville IA) that contained *BamH1* and *Xho1* restriction enzyme sites, respectively. The amplified product contained *HMX1*'s open reading frame flanked by these two restriction sites. The amplified *HMX1* and the yeast expression vector, *pRS425-MET25*, was digested with *BamH1* and *Xho1* (New England Biolabs, Ipswitch MA) at 37°C for 18 hours, and then verified by gel electrophoresis. The digested products were then ligated to each other using T4 ligase, (New England BioLabs, Ipswitch MA) at 23°C for 18 hours. The ligated products were transformed by electroporation into competent *E. coli* cells (Invitrogen, Carlsbad CA) and incubated on LB-AMP solid plates at 37°C for 18-24 hours. Putative positive subclones were purified (Qiagen, Germantown MD) and verified to contain *HMX1* using restriction digest and PCR analyses. Verified subclones were sequenced by Michigan State University Research Technology Support Facility and transformed into yeast using a standard lithium acetate transformation protocol, and transformants selected on solid media lacking leucine (SD-LEU). The original *pRS425-MET25* plasmid was transformed into the other yeast subclones to show that no advantage is given from the plasmid itself, and to confer *LEU2* genes to all tested strains (Gorsich, Dien, Nichols, Slininger, Liu, & Skory, 2006).

Disruption of *HMX1* with *HIS3*

HMX1 was disrupted by replacing it with *HIS3*. Disruptions were made by amplifying *HIS3* from *pRS403* with the forward primer ATAACACAGCATATATACACACACACACATAAAATAACCGCAAAACTCTTGGCCTC CTCTAGT and reverse primer TTCATGTATATATTATGTTTGTATTTAGACTTTTTTTTTTATACTGACGCATCTGTGCG GTATT (synthesized by Integrated DNA Technologies, Coralville IA). The primers were engineered so the amplified product contained at the termini DNA sequences homologous to regions immediately up and down stream of *HMX1*'s open reading frame. This amplified *HIS3* product was then transformed into diploid yeast strains using a standard lithium acetate transformation protocol. Transformants were then selected on solid media lacking histidine (SD-HIS). Yeast growing on SD-HIS were verified by PCR to determine if *HMX1* was disrupted (Gutherie and Fink, 2002).

Yeast Mating

Wild-type yeast and yeast with *HMX1* disrupted (*hmx1::HIS3*) of mating type a and α were incubated at 30°C overnight (18-24hrs) at 225 RPM in 10mL of YPD media. These cultures were then diluted to 0.5OD_{600} U/mL and allowed to grow again to 1.0-1.5OD_{600} U/mL. Then 0.5OD_{600} U/mL of Wild-type or *hmx1::HIS3* mating type a and 0.5OD600 U/mL of the α mating type were combined to a final volume of 3mL and concentration of 1.0OD_{600} U/mL. These were then incubated at 25°C for 1 hour at 225RPM, then the RPM was reduced to 50RPM for 6-8 hours. Diploids were dissected and plated on YPD solid media. Yeast were confirmed as diploids by mating type affinity yeast assay.

Visualizing mitochondria

To view yeast mitochondrial membranes yeast strains were transformed via lithium acetate transformation with *pVT100U-mtGFP*. *pVT100U-mtGFP* encodes a mitochondrial targeted GFP that is constitutively expressed. These transformed yeast strains expressed this mitochondrial-targeted GFP that allows direct viewing of mitochondria using Nikon 80*i* eclipse fluorescent light microscope equipped with FITC HYQ filter (460-500 nm) (Westermann and Neupert, 2000). Mitochondria appear as tubular, fragmented, circular, aggregated, or net-like structures. In each experiment mitochondrial morphology and relative distribution was assayed.

DAPI staining

To visualize nuclear chromosomes and mitochondrial DNA yeast were stained with 4',6-Diamidino-2-Phenylindole (DAPI). Cells were fixed with 95% ethanol for 2 minutes and then immediately centrifuged and the cell pellet washed with sterile water. Next, DAPI was added at a concentration of 5mg/ml for 5 minutes. Cells were then centrifuged and washed with sterile water 5 times. Stained cells were then visualized using a Nikon 80*i* Eclipse fluorescent light microscope equipped with a UV filter.

Mitotic Growth Analysis

Wild-type yeast and yeast with *HMX1* disrupted (*hmx1::HIS3*) were incubated at 30°C overnight (18-24hrs) at 225 RPM in 10mL of Synthetic Dextrose (SD-Complete), Synthetic Dextrose without leucine (SD-LEU), or Synthetic Dextrose without uracil (SD-Ura). These cultures were then diluted to 0.5 OD_{600} U/ml in 10 ml of SD-Complete at 30°C at 225 RPM and grown for three hours. At this point the cultures are in exponential phase and back diluted again to 0.2 OD_{600} U/ml into 5ml of SD-Complete at 30°C 225 RPM.

At the second back dilution samples were divided between experimental and control groups, and the experimental groups received variable amounts of hydrogen peroxide (0, 2, 4, or 6mM) or furfural (0, 25, or 50mM). Cell density was determined at time points between 0-36 hours using a spectrometer set at 600nm (Thermo BioMate 3). (Gorsich, *et al*., 2006).

Yeast Sporulation

Diploid wild-type yeast and yeast with *HMX1* disrupted (hmx1::HIS3) were grown for 12-18 hours at 30°C 225 RPM in YPD liquid media, Synthetic Dextrose (SD-Complete), Synthetic Dextrose without leucine (SD-Leu), or Synthetic Dextrose without uracil (SD-Ura). These strains, yeast with overexpressed *HMX1* (*MET25-HMX1*), and yeast with *HMX1* disrupted (*hmx1*::*HIS3*) with overexpressed *HMX1* (*MET25-HMX1*) were grown for 12-18 hours at 30°C 225 RPM in SD-LEU liquid media. Cells were washed twice with Nanopure sterile water and incubated in sporulation media (1% potassium acetate supplemented with 10μg/ml adenine, arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, uracil, and valine) at a concentration of 1.3 OD_{600} U/ml with a total volume of 5mL in a 50mL glass shake flasks for 7 days. Every day for 7 days sporulations was assayed for the number of diads, triads, or tetrads present. Tetrads were further analyzed for their ability to successfully germinate all four spores by dissecting tetrads onto Yeast Dextrose Peptone (YPD) solid medium and incubating at $30^{\circ C}$ for 3-5 days (Gorsich & Shaw, 2004).

CHAPTER III

RESULTS

HMX1 mutation affects H_2O_2 tolerance in haploid cells during mitotic growth

Exponentially growing haploid wild-type and *HMX1* disruption (*hmx1::HIS3*) yeast were back diluted to $0.3OD_{600}$ U/mL in SD-Complete media and incubated with 0 and 2mM of hydrogen peroxide (H_2O_2). Cell densities (A_{600}) were measured every 3 hours to determine the effect of H_2O_2 on *hmx1::HIS3* mutants. Wild-type cells grown without hydrogen H_2O_2 grew better than *hmx1::HIS3* at all time points (Figure 3). Wild-type cells were able to recover by the 24 hour time point, but the *hmx1::HIS3* mutants were unable to recover at any time point measured (Figure 3). These results show that in our mutants we see ROS sensitivity, as was expected from previous literature (Collinson, et al., 2011).

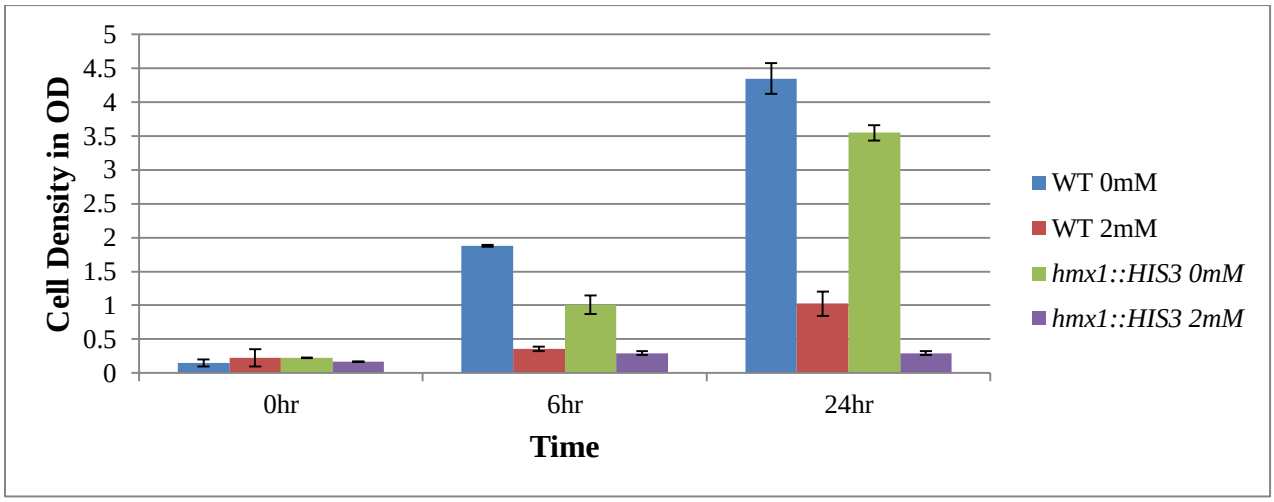

Figure 3. **Haploid *hmx1* mutants show increased ROS sensitivity compared to wild-type cells**
Exponentially growing haploid wild type and mutant cells were diluted and then incubated with either 0mM or 2mM hydrogen peroxide. At the 6 hour time point wild type achieved a higher concentration than mutants without the presence of inhibitor. After 24 hours the wild type cells incubated with 2mM H_2O_2 had started to resume logarithmic growth while the mutants in the same conditions showed no sign of exiting lag.

HMX1-MET25 shows no effect on haploid tolerance of hydrogen peroxide (H_2O_2)

Exponentially growing haploid yeast wild-type and HMX1 overexpressing yeast were diluted to 0.3OD_{600} U/mL in SD-LEU media, to select for the overexpression plasmid, and incubated with 0 and 2mM H_2O_2. Results were measured every 3 hours and set up observed for optical density. At 6 hours wild type without H_2O_2 grew better than *HMX1* overexpression, but by 24 hours they were both at similar concentrations (Figure 4). There was no difference seen between wild-type and *HMX1* overexpression grown in 2mM of H_2O_2 (Figure 4). These results show over expression of *HMX1* conferred no advantage when grown in the presence of H_2O_2.

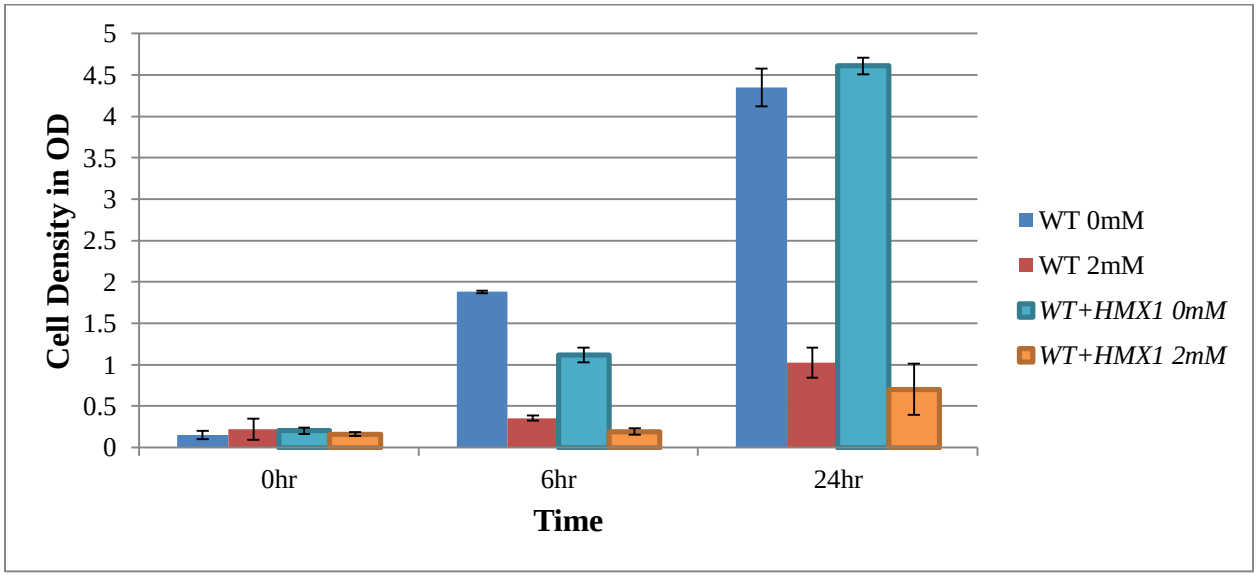

Figure 4. ***HMX1/MET25* shows no advantage to ROS tolerance compared to wild-type cells** Exponentially growing haploid wild type and *HMX1* overexpressing cells were diluted and incubated with either 0mM or 2mM hydrogen peroxide. At the 6 hour time point the uninhibited wild type cells had attained a higher density that the *HMX1* overexpressing cells. After 24 hours both the wild type and *HMX1* overexpressing cells incubated with 2mM H_2O_2 had exited lag and were at similar concentrations.

HMX1 affects H_2O_2 tolerance in diploid cells during mitotic growth

Wild-type and *hmx1::HIS3* mutant diploids were grown in SD-Complete media overnight and then diluted to $0.5OD_{600}$ U/mL and allowed to grow for 3 hours, then diluted to $0.3OD_{600}$ U/mL and incubated with 0, 2, 4, and 6mM of hydrogen peroxide. Optical densities were recorded at 0, 6, and 24 hours. There was no difference between wild-type and *hmx1::HIS3* mutant diploids when no H_2O_2 was present at 6 hours. When grown with 2 and 6mM H_2O_2 present there was a difference in cell growth at the 6 hour time point. At this time point, wild type had a higher cell density compared to the hmx1 mutant (Figure 5). The lower concentration of the mutants indicates that the *hmx1* mutants remained in lag phase longer than wild-type, and indicates that hmx1 mutants are more sensitive to hydrogen peroxide as diploids.

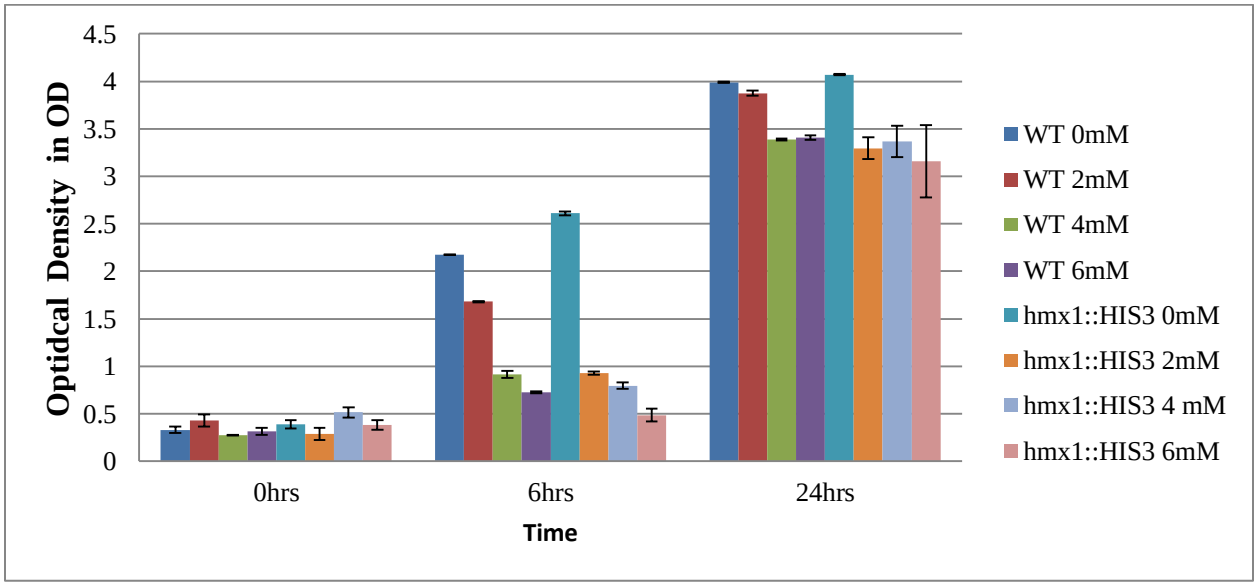

Figure 5. **Diploid *hmx1* mutants show ROS sensitivity when compared to wild-type** Exponentially growing diploid wild type and *HMX1* mutants were diluted and then incubated with 0, 2, 4 , and 6 mM hydrogen peroxide. At the 6 hour time point uninhibited wild type and mutants showed similar levels of cell density. However, the wild type cultures incubated with 2mM and 6mM H_2O_2 show a higher cell density than the *hmx1* mutants grown under the same conditions. After 24 hours all cell cultures had reached saturation

HMX1 affects sporulation of cells incubated with H_2O_2 when grown in YPD

Wild-type and *hmx1::HIS3* homozygous mutant diploids were grown in YPD media to saturation and then incubated in 1%KAc (potassium acetate) sporulation media for 10 days with either 0 mM or 2mM hydrogen peroxide. Every 24 hours the extent of sporulation was quantified by counting at least 100 cells using a compound light microscope. When no H_2O_2 was added there was no observed difference in sporulation observed between wild-type and mutant cells, where total sporulation events (asci containing 2-4 spores) averaged to approximately 13% of all cells (Figure 6 A). There were also no observed differences when the numbers of diads, triads, or tetrads were separated from the total sporulation events (Figure 6 C). This observation suggested that the loss of *HMX1* had no impact on spore formation under normal conditions. Next, wild-type and *hmx1* mutant diploids were sporulated with 2mM H_2O_2. There was a reduction in sporulation in the wild-type strain, from about 12% sporulation to about 7.5% sporulation.

However, in the *hmx1::HIS3* mutants a complete loss of sporulation was seen in the mutants in the presence of 2mM H_2O_2 (Figure 6 B, D). This reduction of sporulation seen in the wild-type cells is to be expected, as it has been known that H_2O_2 causes a reduction in the number of sporulation events (Tricia Brown-Stokes et al. Unpublished). The loss of the *HMX1* mutant's ability to sporulate in 2mM of H_2O_2 compared to the wild-type suggests that Hmx1p may play an essential role in ROS tolerance during sporulation. It is important to note that the mutant cells were not dead, and when incubated on fresh media are able to recover (result not shown).

Overexpression of *HMX1* has no effect on sporulation with cells incubated in the presence of H_2O_2

Wild-type yeast (with and without *HMX1* overexpressed) and *hmx1::HIS3* homozygous mutants (with and without *HMX1* overexpressed) were incubated in SD-LEU media overnight until they reached saturation, and were then incubated in 1% KAc sporulation media for 10 days with either 0mM or 2mM H_2O_2. Every 24 hours the extent of sporulation was quantified by counting at least 100 cells using a compound light microscope. There was no observed difference between total sporulation events (asci containing 2-4 spores) in any of the cultures without hydrogen peroxide (Figure 7 A). In addition, there was not any difference seen between types of sporulation events (diads, triads, or tetrads) without the presence of hydrogen peroxide (Figure 7 C). These results suggest that with no additional stress there is no conferred advantage to having *HMX1* overexpressed during sporulation. Surprisingly, there was no observed difference in the total sporulation events (asci containing 2-4 spores) in any of the strains tested with 2mM hydrogen peroxide (Figure 7 B), nor was there a difference in the type of sporulation (diads, triads, and tetrads) seen between genotypes with 2mM hydrogen peroxide (Figure 7 D). The absence of observed phenotype difference suggests that under H_2O_2 stress there is not a conferred advantage to having *HMX1* overexpressed during sporulation.

Next I investigated the conflicting observations that the hmx1 mutants were able to sporulate when sporulation experiments were initiated in minimal medium (SD-LEU; to select for the overexpression plasmid) compared to a loss of sporulation when sporulation experiments were initiated in rich medium (YPD). To assess whether it was the presence of *pRS425-MET25* or the minimal media used, sporulation experiments using wild-type and *hmx1::HIS3* homozygous mutants were performed in SD-Complete (synthetic minimal media containing all

essential nucleic acids and amino acids) and then sporulated with either 0mM or 2mM H_2O_2. These results were similar to results when grown in SD-LEU and not when grown in YPD. These data suggested that the difference in the ability of the hmx1 mutants to sporulate was linked to the media and not the presence of the *pRS425-MET25* (results not shown).

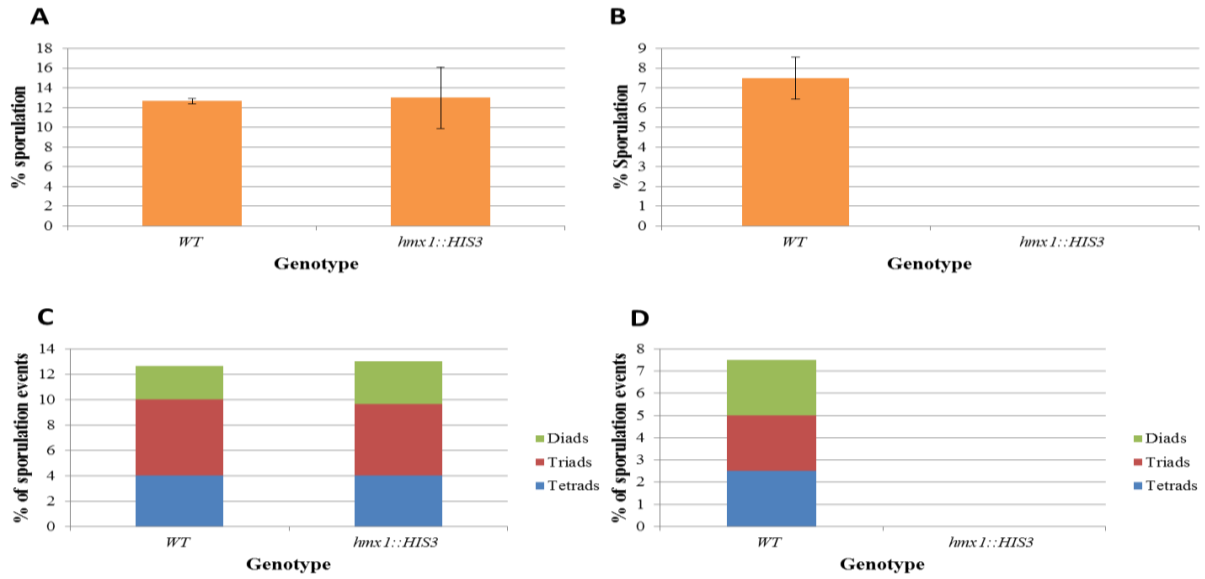

Figure 6. *hmx1* mutants show an inability to sporulate in the presence of 2mM hydrogen peroxide
Wild type and *hmx1* mutant diploid were sporulated for one week and the number and type of sporulation was counted. Percentages of total sporulation were assessed in cultures with no H_2O_2 (A.) or 2mM H_2O_2 (B.). There was no observed sporulation difference in cultures without inhibitor. In the presence of 2mM H_2O_2 wild type cultures were still able sporulate but no sporulation was seen in the mutant strains. Cultures sporulated in 0mM (C.) and 2mM (D.) H_2O_2 were assessed based on the number of spores formed during sporulation. There was no difference in the spore number between genotypes of cultures that sporulated.

HMX1 overexpression positively affects spore viability

Spore viability was tested using wild-type yeast (with and without *HMX1* overexpressed) and *hmx1::HIS3* homozygous mutants (with and without *HMX1* overexpressed) with either 0mM or 2mM H_2O_2 tested. Tetrads (asci with 4 spores) were digested with beta-glucuronidase to partially digest the asci. Following this the four spores were removed and allowed to grow on

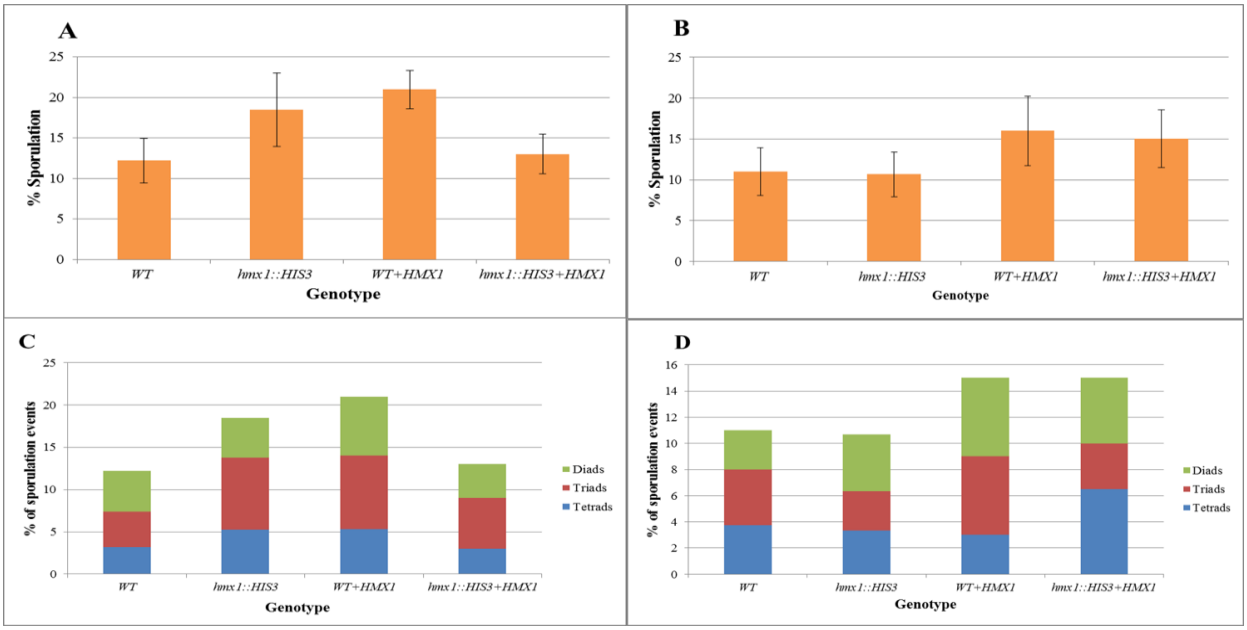

Figure 7. *HMX1* overexpression gives no ROS tolerance when compared to other genotypes Wild type, mutant, *HXM1* overexpressing, and mutants with *HMX1* overexpression were sporulated for 7 days and number and type of sporulation events were counted. Percentages of total sporulation were assessed for cultures sporulated without inhibitor (A.) and with 2mM H_2O_2 (B.). There was no difference in sporulation seen between genotypes in either uninhibited cultures or those grown in the presence of H_2O_2. Percentage of tetrads, triads, and diads were assessed after 7 days in cultures sporulated without inhibitor (C.) and with 2mM of H_2O_2 (D.). No noticeable difference was seen in the numbers of spores between genotypes regardless of presence of inhibitor.

YPD for approximately 3 days. Viable spores were able to form a colony, whereas non-viable spores were unable to form a colony. Results were then analyzed by taking the number of colonies formed by dissected spores/number of dissected spores plated.Spore viability of cultures without hydrogen peroxide was calculated at 90% or higher with no difference between wild type and *hmx1::HIS3* mutants (Figure 8). This result suggests that *HMX1* deletion has no effect on spore viability under non-stressed conditions. Preliminary results on spore viability with 2mM hydrogen peroxide show that while *HMX1* deletion did not affect spore viability, overexpression of *HMX1* aided spore viability (Figure 8). These are only preliminary results done with a very small number of trials, and thus more tests must be run to substantiate these

findings. In addition these data sets are only for those cultures grown in minimal medium in which *HMX1* has no significant impact on sporulation under any conditions. These cultures were used because cultures grown in YPD do not sporulate under conditions of H_2O_2 stress, therefor there are no spores available to dissect.

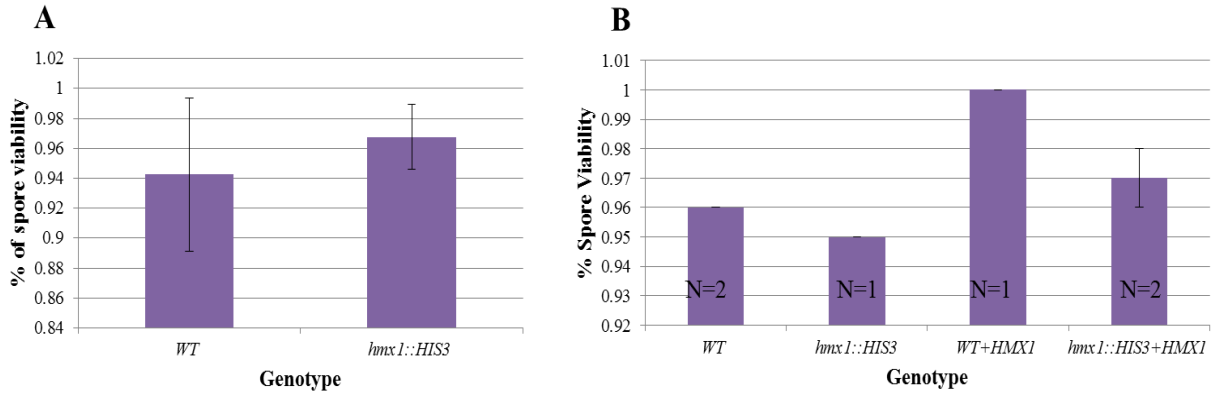

Figure 8. **Preliminary results show that *HMX1* overexpression increases spore viability under ROS stress**
Wild type and mutant cells sporulated with no H_2O_2 (A.) and wild type, mutant, *HMX1* overexpression, and mutant with *HMX1* overexpression sporulated under 2mM H_2O_2 stress (B.) were dissected and plated to assess spore viability. There was no difference seen in spore viability in cultures without H_2O_2 stress. Under 2mM H_2O_2 stress there was a noticeable increase in spore viability seen in cultures overexpressing *HMX1* when compared to the other genotypes.

Mutants show increased aberrant mitochondrial morphology under normal sporulation conditions

Wild type and *hmx1::HIS3* homozygous mutant diploids with mitochondrial GFP (*pVT100U-mtGFP*) (Westermann & Neupert, 2000) were grown overnight in SD-URA to saturation, and then sporulated in 1%KAc sporulation media and incubated for 6 days. Mitochondrial morphology was measured using fluorescence microscopy at the start of sporulation and every 24 hours afterwards. Normal mitochondrial morphology during sporulation is dynamic and goes through several morphology changes. Specifically, during

meiosis these fragments form an evenly distributed tubular mitochondrial network. During meiosis stressed mitochondria appeared primarily as one of the following categories: 1.) aggregated to one part of the cell, or 2.) undefined, which appear as the cell being fully fluorescent (Figure 9 A). *hmx1::HIS3* strains at one day of sporulation had 14% stressed mitochondria when compared to wild-type which had about 5%, and this difference is seen until around day 6 when both wild-type and mutants showed uniformed tubular mitochondrial morphologies (Figure 9 A). This mitochondrial stress does not seem to impact the abilities of cells to sporulate, but could possibly explain the difference in spore viability.

Mitochondrial morphology shows less difference under 6mM H_2O_2 stress

Wild-type and *hmx1::HIS3* mutant diploids with mitochondrial GFP (Westermann & Neupert, 2000) were grown overnight in SD-URA to saturation and then sporulated in 1%KAc sporulation media and incubated for 6 days with 6mM H_2O_2. Mitochondrial morphology was viewed via fluorescence microscopy at the start of sporulation and every 24 hours afterwards. Both wild-type and *hmx1::HIS3* showed similar levels of mitochondrial stress at day one (50-60%), and these levels persisted throughout the 6 day period (Figure 9 B). It was also seen that the majority of stressed mitochondrial morphology was aggregated at day 1, but progressively ambiguous mitochondria became more present, being over half of the stressed morphology by day 6 (Figure 9 B).

Preliminary mitochondrial morphology results during sporulation with 2mM hydrogen peroxide suggest that there is an increased level of aggregation in the first two days of sporulation. In addition, a new mitochondrial morphology phenotype appeared which were constricted tubular mitochondria, mitochondria which were evenly distributed and tubular but

constricted at points along the mitochondrial network. During these first two days there are also signs of cell rupturing, possibly due to programed cell death (Fiers et al.,1999). After 3 days the amount of aberrant mitochondria reduces and the cells begin to sporulate.

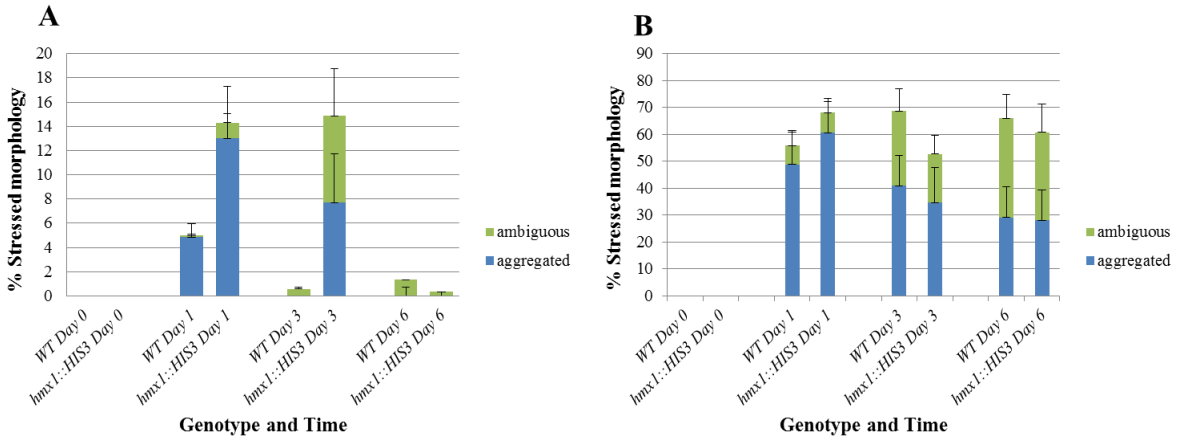

Figure 9. *hmx1* mutants showed increased signs of mitochondrial stress when sporulated: Wild type and mutant cultures were transformed with mitochondrial GFP and sporulated for 7 days with either 0mM H_2O_2 (A.) or 6mM H_2O_2 (B.). Cultures were assessed for mitochondrial morphology at the start of sporulation and every day after, shown are major time points at day 0, day 1, day 3, and day 6. Mutant cultures grown without inhibitor show a noticeable increase in mitochondrial stress during days 1 and 3 when compared to the wild type cells. However, this difference in morphology is not seen in cells stressed with 6mM of H_2O_2, where both cultures experience similar levels of mitochondrial stress.

No differences observed in overall chromosomal appearance during sporulation

To assay overall nuclear and mitochondrial (mtDNA) chromosomal morphology, I stained the cellular DNA with the DNA-specific dye, DAPI, during sporulation of wild type and *hmx1::HIS3* homozygous mutants with either 0 or 2mM hydrogen peroxide. Normal nuclear chromosomes stain as constricted spheres and mtDNA as several cytoplasmic small spheres. When stressed with ROS nuclear chromosomes should appear as disorganized structures in the nucleus and mtDNA often are difficult to observe. There was no significant difference in the DAPI defects seen in wild type or mutants under any conditions (data not shown)

HMX1 affects furfural tolerance during mitotic growth

Exponentially growing wild-type and *hmx1::HIS3* homozygous mutants were diluted to 0.3OD_{600} U/mL and incubated with 0, 25, and 50mM amounts of furfural. Optical densities were recorded at the start of the test, and then every three hours after until saturation was seen. In the cultures incubated with 25mM furfural, wild type resumed logarithmic growth at about 15 hours, while *hmx1* mutants took 18 to 21 hours to reach logarithmic growth (Figure 10). Cultures grown in 50mM furfural never resumed growth, even after 72 hours (results not shown). The longer time it took for mutants to resume logarithmic growth suggests that *HMX1* has a role in furfural tolerance.

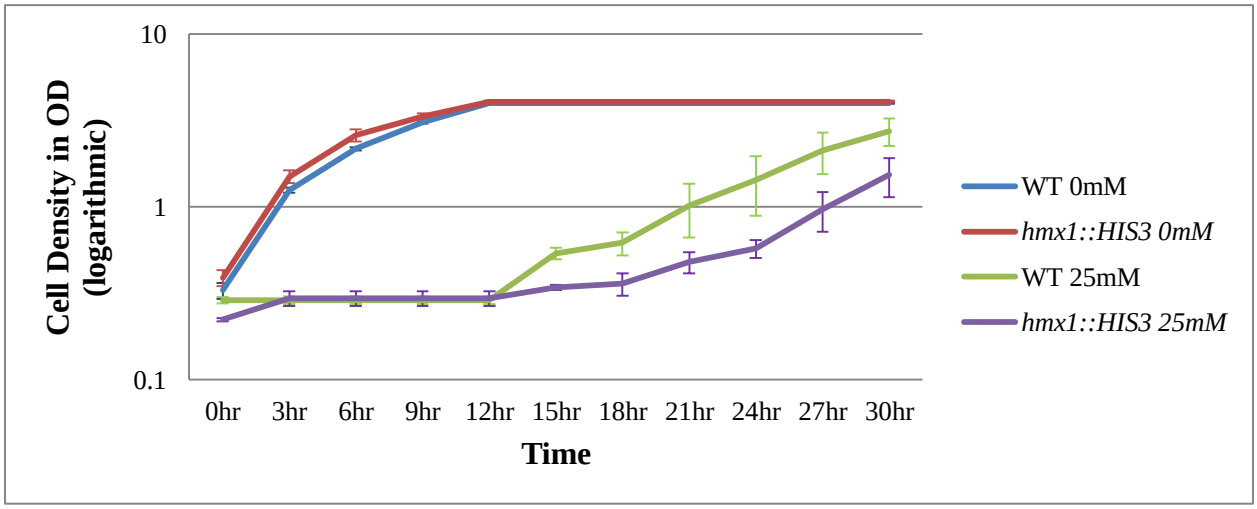

Figure 10. ***hmx1* mutants show increased sensitivity to furfural toxicity compared to wild-type**
Exponentially growing diploid wild type and mutants were diluted and then incubated with 25mM of furfural and assessed of cell density. Wild-type cells recovered from lag at about 15 hours, an improvement on mutants which seemed to take 18-21 hours to exit lag.

CHAPTER IV

DISCUSSION

The haploid growth assay of wild-type and *hmx1::HIS3* mutants under H_2O_2 stress indicated the expected result that *HMX1* deletion induces loss of ROS tolerance. It was exciting to see that the mitochondrial morphology observations taken during these growth assays seemed to correspond with the results seen from the assays and the mitochondrial morphology appeared to be indicative of the observations seen in recovery from lag. Overexpression of *HMX1* did not seem to grant any sort of benefit to lag recovery during H_2O_2 stress in haploid cells, which goes against the published understanding of *HMX1* (Collinson, *et al*., 2011). Unfortunately mutants with both *HMX1* overexpression and mitochondrial GFP were not engineered and used, for it would be interesting to see if *HMX1* overexpression confers an advantage to mitochondrial stability, a phenomenon that may not be directly seen in recovery from lag but may indicate an overall health advantage and tolerance to ROS.

The results observed when diploids were analyzed for mitotic growth when stressed with hydrogen peroxide show that even with the extra robustness granted from being a diploid *HMX1* deletion still increases sensitivity to hydrogen peroxide. It should be noted however that the sensitivity is greatly reduced when compared to haploids, the effect in diploids is only present for a generation cycle, after that the mutants are seen recovering from lag and grow normally. It should also be noted that these observations were performed with synthetic minimal culture media, in a more complete media there may be even less impact on the loss of *HMX1* if more nutrients were available. Then again, there may be a starker contrast in YPD media as meiotic

studies show a greater difference than in SD medias, although as the cells are never starved in mitotic studies this may not be a major factor.

A similar effect of *HMX1* was seen on furfural tolerance, as the wild-type cells were able to exit lag quicker than the mutant cells. This result is encouraging, as it may open up new insights into furfural tolerance, since it is believed that *HMX1* upregulates other ROS detoxifying enzyme it may be that *HMX1* also regulates factors that regulate furfural detoxification. One unexpected issue was the varying times at which cells come out of lag during furfural incubation compared to hydrogen peroxide. The same issues that were seen in the studies with hydrogen peroxide was also seen here, where there is only a generation or two in the recovery time between mutant and wild-type, and the cultures were grown in SD media and we may see a difference in sensitivity if grown in a complete media.

Sporulation of these diploids however seems to be a bit more of a mystery as the results done in one media seem to refute the results done in another. It was relatively consistent that sporulation without hydrogen peroxide was not visibly effected by disruption of *HMX1*. This consistency is not entirely surprising, as mitotic growth is not impacted by *HMX1* under normal conditions. However when mitochondrial morphology was analyzed *hmx1::HIS3* mutants show 10% or more mitochondrial defects during the first few days of sporulation than the WT cells. This result indicates that even though there is no external or sporulation phenotypes occurring during uninhibited sporulation the mutant still experience greater mitochondrial stress than the wild type, and could indicate that *HMX1* has an impact on ROS tolerance during meiosis. It should be noted that the mitochondrial morphology studies were done in SD-URA, a synthetic media that as we will see may have an inherent effect on ROS tolerance in sporulating cells.

A major difference comes in when we look at the effect of what happens when we add hydrogen peroxide to the cultures during sporulation. When *HMX1* deleted cells were grown in YPD and then incubated in the starvation media with hydrogen peroxide there is no sporulation occurring at all, a complete contrast to the sporulation we see happening in the WT cells. This contrast would argue on the basis that *HMX1* is crucial to the ability of cells to sporulate under stressed conditions. However when grown in SD-Complete and SD-LEU medias this difference is not seen, and in fact sporulation is not only present in *HMX1* disrupted cells in the presence of hydrogen peroxide, but it is equal to the sporulation seen in wild-type cells.

One possible explanation is the condition of the cells prior to entering the sporulation media. Cells grown in YPD complete media have more nutrients available, and there is a greater possibility of generating intracellular ROS or other stress factors prior to incubation with hydrogen peroxide. This stress may not have an impact on the ability of cells to sporulate normally, but when an added stressor (extracellular hydrogen peroxide) is present the cell shuts down at a checkpoint during meiosis and sporulation never occurs. In the complete media these extra stressors are not present and the cell does not initiate checkpoint stops. A similar factor may be the concentration of cells prior to incubation in the starvation media. Cells were grown overnight, but their initial optical density was not carefully regulated, but in general cells grown in complete media are capable of obtaining a higher concentration than those grown in SD medias (unpublished observations). Perhaps the cells that have reached higher concentrations have accumulated more oxidative damage during growth and are thus weakened in comparison to cells grown in SD medias.

There may also be the issue of enzyme availability to mutants during the time of starvation and the necessity to devote energy to both cellular defense and sporulation may limit

the mutants grown in YPD. It is believed that *HMX1* is involved with regulating stress tolerance genes; perhaps in cells without *HMX1* the basal amount of enzymes produced is able to overcome most intracellular generated ROS. When these cells starved under normal conditions the basal enzyme amount is able to detoxify the necessary ROS and still have enough energy to undergo sporulation. However, when extracellular hydrogen peroxide is added perhaps the basal level of detoxifying enzymes require more energy to detoxify the chemical and therefore the energy required for sporulation is not available. In wild-type cells, *HMX1* may up-regulate the amount of ROS detoxifying enzyme during stationary phase, and when these cells are starved and extracellular hydrogen peroxide is added the increased amount of detoxifying enzymes are capable of detoxifying the hydrogen peroxide and still have enough energy to sporulate. In SD media, perhaps the reduced amount of cellular stress involved means that in the mutants there is still enough detoxifying capability available to detoxify hydrogen peroxide and still undergo sporulation. These are of course only speculation and will require further testing to be able to ascertain if any of them are the cause of the discrepancy seen between media types.

From the spore viability data that were collected we assume that there are no major sporulation defects occurring in *hmx1* mutants whether sporulated under normal conditions or incubated with hydrogen peroxide when grown in SD-Leu. It seems if the tetrads are able to be formed a majority of the spores are viable. An exciting preliminary finding is that it appears from initial results that *HMX1* overexpression may increase spore viability even in the presence of H_2O_2. We were unable to test for spore defects in YPD under hydrogen peroxide stress as there are no tetrads formed.

Unfortunately there is some question as to the effectiveness of the *HMX1::MET25* plasmid that was engineered. Although sequencing proved that it was indeed *HMX1* on the

plasmid, that the plasmid was functional in granting leucine production, there was no apparent conferred benefit to either wild-type or rescue in the *HMX1* mutants during mitotic growth. The lack of conferred benefit is puzzling as previous publications have shown that overexpression of *HMX1* increases ROS tolerance, and that overexpression of *HMX1* in mutants should rescue the genotype (Collinson, et al., 2011). There was also no conferred benefit to *HMX1::MET25* expression in sporulation numbers either normally or incubated with hydrogen peroxide. Previous studies were done using a galactose-inducible promoter (Collinson, *et al.*, 2011), so perhaps an issue with the *MET25* promoter is responsible for this result. There may also be an issue with the requirement to be grown in leucine poor media, and that somehow the use of this media negatively impacted the function of the *HMX1* plasmid.

The experiments that were performed were different that those that were performed by other labs. The tests performed by Collinson (Collinson, *et al*., 2011) tested for surviving cells after 6 hours of H_2O_2 stress, while our tests look at recovery from lag and number of sporulation events. Perhaps there are more cells that survive at 6 hours when *HMX1* is overexpressed, but the cells still take the same amount of time to recuperate during lag. The same may be true during sporulation, the cells may be more resilient due to the overexpression of *HMX1* and spore viability may increase, but the advantage may not be seen in increasing sporulation. The lack of lag recovery may be resultant in the fact that though there is more antioxidant enzyme production induced by overexpression of *HMX1*, the lack of other necessary factors such as co-factors and available energy does not allow these enzymes to recover from lag any faster or increase sporulation compared to wild type. This would indicate that indeed our plasmid is still functional, the test performed were not suited to accessing its functionality.

One major goal is to look into the impact of using complete media over synthetic media and the effect this has on mutant sensitivity to ROS. Mitotic growth assays should be run in both complete and synthetic media to assess wither there is a different sensitivity in mutants over wild-type while grown in these differing medias. ROS staining previous to sporulation and during sporulation should be performed in varying media in order to assess the overall state of ROS accumulation during these times. It would also be pertinent to attempt to standardize the concentration of cells prior to sporulation, and to investigate if differing concentrations of cells in stationary phase can cause a difference in sporulating ability.

Another major step we would like to accomplish is to induce a chromosomal expression of GFP so that it could be observed in mitochondria during sporulation in complete media. We know in complex media that sporulation is loss in *HMX1* mutants exposed to hydrogen peroxide, is this shown in a mitochondrial morphology? It would also be interesting to see if mitochondrial morphology is different in stressed mutants grown in SD media compared to complete media. The results obtained with respect to mitochondrial morphology data may have suffered from the same results as the other sporulation trials did, where SD-media showed a different phenotype than what would be seen in using complete media.

Further analysis should be performed in order to attempt to understand if the *HMX1/MET25* plasmid is properly functioning in overexpressing *HMX1*. If the overexpression plasmid is functioning properly then these experiments should be run again using both the *HMX1/MET25* plasmid and a galactose inducible plasmid to see if the results vary. Different medias appear to yield different results in regards to some of the data collected with *HMX1*, perhaps this too is a case where galactose yields a different variable not originally accounted for. If *HMX1/MET25* is not functioning properly, then there will be continued attempts to engineer a

HMX1 overexpressing plasmid. If a reliable plasmid is able to be created we would like to perform these experiments again using that plasmid to see if the result remains consistent, especially during sporulation. It would also be advantageous to induce a chromosomal overexpression of *HMX1* so that these strains could be grown in complete media, and therefore it could be seen if a similar situation as seen in the *hmx1::HIS3* mutants and the differences seen in growth media could be avoided.

We would also like to produce a cell line that both overexpresses *HMX1* and other cellular ROS tolerance genes, such as *ZWF1*. *ZWF1* codes for glucose-6-phosphate dehydrogenase and is used to generate NADPH, which goes to reduce many important co-factors involved in ROS detoxification, and its overexpression has been shown to increase ROS tolerance in both sporulation and mitotic growth. We would like to see if a synergistic effect could be created with these mutants during mitotic growth and meiosis, where the increased presence of detoxifying enzymes and reducing co-factor could create a cell with superior ROS tolerance when compared to overexpression of one gene alone. A genetic screen should also be performed to identify what genes are being regulated by *HMX1* during furfural stress. Any novel discovery made from analysis of genes regulated by *HMX1* could lead the way to future research in understanding furfural detoxification as well as advancing the goal of creating a furfural tolerance yeast cell.

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