The Regulation of the Phosphatidate Phosphatase Gene \textit{PAH1} and Its Regulatory Role on Cell Homeostasis

Goldie Libby Sherr

\textit{The Graduate Center, City University of New York}

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THE REGULATION OF THE PHOSPHATIDATE PHOSPHATASE GENE PAH1 AND ITS REGULATORY ROLE ON CELL HOMEOSTASIS

by

GOLDIE LIBBY SHERR

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2016
The regulation of the phosphatidate phosphatase gene PAH1 and its regulatory role on cell homeostasis

By

Goldie Libby Sherr, M. Phil

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

The regulation of the phosphatidate phosphatase gene PAH1 and its regulatory role on cell homeostasis

By

Goldie Libby Sherr, M. Phil

Advisor: Dr. Chang Hui-Shen

The Saccharomyces cerevisiae gene, PAH1, encodes a phosphatidate (PA) phosphatase that plays a fundamental role in lipid metabolism. PA phosphatases are key enzymes that catalyze the PA dephosphorylation reaction to form diacylglycerides, the first step in the synthesis of triacylglycerols. Pah1p, one of the main PA phosphatases in yeast, has not only emerged as a key player in lipid biosynthetic pathways, but also acts as an important regulator of nuclear membrane biogenesis, the transcriptional regulation of many inositol-sensitive upstream activating sequence (UAS_INO) containing genes needed for phospholipid synthesis, vacuole homeostasis, and lipid droplet formation. Due to its crucial role in lipid and overall cell homeostasis, this thesis aimed to elucidate the role and regulation of the PAH1 gene. In the first part of the study, we tried to evaluate how PAH1 affects other genes in the lipid biosynthetic pathway as well as assess the impact that the crucial phospholipid precursors, inositol and choline, have on cellular growth. Results showed that while there was not a large difference between the growth rates of WT and pah1Δ strains when exposed to different concentrations of the precursors, the pah1Δ strain did tend to grow slightly better than WT in the presence of inositol. Furthermore, our RNA analysis showed that UAS_INO genes were upregulated in the pah1Δ strain when compared to WT cells, strongly suggesting the role of PAH1 as a negative regulator in the lipid biosynthesis pathway. Interestingly, the HXK2 gene was the only gene
tested that was downregulated in pah1Δ cells. Since HXK2 is involved in preventing apoptosis, this implied that Pah1p might be involved in cellular apoptosis. Additional growth experiments on pah1Δ were conducted in the presence of acetic acid and hydrogen peroxide. Results showed that pah1Δ cells fared significantly better than WT cells after exposure to these apoptotic reagents. RNA analysis confirmed these results, showing a significant upregulation of anti-apoptotic genes in pah1Δ. As such, this aim demonstrated that PAH1 plays an important regulatory role in phospholipid biosynthesis and suggested that it also plays a role in apoptosis by regulating anti-apoptotic genes as well.

For the second part of our study, we wanted to elucidate the conditions of PAH1 induction, as well as the ways in which it itself is regulated. Our experiments showed that PAH1 undergoes induction during the stationary phase of growth when inositol is present. These findings were subsequently used to help determine the chromatin remodelers involved in PAH1 gene expression. Chromatin remodelers play a significant role in modifying and restructuring the nucleosome and therefore play an essential role in the regulation of gene expression. Using growth curve analysis, qRT-PCR, and ChIP, we set out to determine which chromatin remodelers impact PAH1 gene expression. Our results showed that Snf2p plays a role in PAH1 gene induction and localizes at its promoter region. Interestingly, Snf2p is one of the chromatin remodelers important for INO1 regulation, one of the most crucial genes in inositol production, and a gene which is heavily regulated by PAH1. Taken together, these findings may indicate another mode by which PAH1 can affect INO1 expression; by possibly using the same remodeler to help keep gene expression in check. Overall, these findings give a better understanding of how PAH1 gene regulation is controlled at the chromatin level.
For the last part of our study, we decided to look into another organelle that *PAH1* impacts, the vacuole. Since *PAH1* has been previously documented to influence vacuole morphology by regulating the proteins involved in vacuole fusion, we wanted to determine if its presence also affects proper vacuole homeostasis, particularly the maintenance of acidification and the function of V-ATPase pumps. Using electron microscopy, we determined the vacuolar phenotype of *pah1Δ* cells, which consisted of fragmented vacuoles in both exponential and stationary phases of growth. This was followed by RNA analysis of V-ATPase genes. Our results demonstrated that all genes remained at similar or greater levels than WT in *pah1Δ* cells, suggesting that V-ATPase pump activity is not implicated despite the morphological defect. Growth experiments and vacuolar pH measurements confirmed this finding. In order to see if other important genes involved in vacuole fission and fusion were potential contributors to the mutant phenotype in *pah1Δ* cells, we performed qRT-PCR to measure gene expression. Results showed that the overexpression of genes like *FAB1* and *ATG18*, which are crucial for normal fragmentation, in *pah1Δ* cells can be a contributing factor to the fragmented vacuole phenotype.

Overall, our study has looked into three important areas in the cell with respect to *PAH1*. We have elucidated the ways in which it impacts the lipid biosynthetic pathway, the modes in which it itself is regulated, and the influence it has on vacuolar morphology and function. These findings can be used to further understand the functional role of *PAH1* and can provide insights into the ways in which *PAH1* impacts cell homeostasis.
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<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>$A_{260\text{nm}}$</td>
<td>Absorbance at 260 nanometer</td>
</tr>
<tr>
<td>$A_{600\text{nm}}$</td>
<td>Absorbance at 600 nanometer</td>
</tr>
<tr>
<td>ACT1</td>
<td>Actin-1, Constitutive housekeeping gene</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-triphosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<tr>
<td>ChIP</td>
<td>Chromatin Immunoprecipitation</td>
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<td>DAG</td>
<td>Diacylglyceride</td>
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<td>Messenger RNA</td>
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<td>-----------</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
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<td>Triacylglyceride</td>
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<td>TE</td>
<td>Tris- ethylenediaminetetraacetic acid</td>
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<td>TES</td>
<td>Tris- ethylenediaminetetraacetic acid -sodium dodecyl sulphate buffer</td>
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<tr>
<td>UAS\textsubscript{\textit{INO}}</td>
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<td>Zinc Responsive Element upstream activating sequence</td>
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<td>Yeast extract peptone dextrose</td>
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Goldie Libby (Lazarus) Sherr
Chapter 1

1. Introduction

1.1. Phospholipids

The plasma membrane is involved in many aspects of cellular development and growth. Its role as a permeability barrier for the cell is just one of its many functions. In addition to protecting the integrity of the internal cellular environment, it is also pivotal for supporting and maintaining cellular shape (Yeagle, 1989). Its roles are even further reaching due to the membrane’s several components. Phospholipids are the predominant molecules in cellular membranes and as such, function as one of the main structural components of the cell (Quinn et al., 1980). There, they form an amphipathic lipid bilayer, with their hydrophilic heads facing outward and their hydrophobic fatty acid tails facing inward (Van der Rest et al., 1995). This bilayer contains various proteins embedded throughout, along with cholesterol and carbohydrate groups, which attach to the lipids and proteins (Benga and Holmes, 1984). There are four major phospholipids found in the plasma membrane of the eukaryotic model organism Saccharomyces cerevisiae. These phospholipids include phosphatidylinositol, phosphatidylcholine, phosphatidylinositol, and phosphatidylerine and all are essential for cell survival (Figure 1.1; Iwanyshyn et al., 2004; Henry et al., 2012). In addition to acting as the structural basis of membranes, these phospholipids are involved in a number of other cellular processes that extend far beyond lipid metabolism. They provide the foundation for the assembly and performance of many catalytic reactions and assume roles as molecular chaperones, components in signaling pathways, and precursors to macromolecules and secondary messengers (Greenberg and Lopes; 1996; Dowhan, 1997; Henry and Patton-Vogt, 1998; Strahl and Thorner; 2007). Since these molecules are pivotal for cell function and survival, the regulation of these lipids is extensive.
The endoplasmic reticulum (ER) is the main site for lipid biosynthesis. There, phospholipids, as well as other fats, are synthesized via the lipid biosynthetic pathway (Carman and Henry, 1989; Van Meer et al., 2008; Henry et al. 2012). These pathways are highly regulated by various mechanisms, many of which target the transcription of lipid biosynthetic genes. This therefore helps ensure the proper production of lipids necessary for cell survival.
Figure 1.1: Main phospholipids found in the plasma membrane of *Saccharomyces cerevisiae*. (Henry *et al.*, 2012)
1.2. Transcriptional regulators of the lipid biosynthetic pathway

The regulation of phospholipid composition in membranes is essential for correct cellular growth and development (Cardozo-Gizzi and Caputo, 2013). *Saccharomyces cerevisiae* is a common model system used to better understand phospholipid synthesis and its regulation. While there are four main types of phospholipids found in *Saccharomyces cerevisiae*, their plasma membranes are mainly composed of the two phospholipids, phophatidylinositol and phosphatidylcholine (Carman and Henry, 1999; Santos-Rosa et al., 2005). The synthesis of these and other phospholipids, as well as membrane biogenesis in general, is mainly regulated by the crucial phospholipid precursors, inositol and choline. Both inositol and choline coordinately regulate phospholipid metabolism by the response to their availability (Kelley et al., 1998; Dowd et al., 2001; Boumann et al., 2006; Gaspar et al., 2006). When the intracellular concentration levels of inositol and choline are low, the transcriptional activator complex, Ino2p/Ino4p, activates the expression of many genes that encode phospholipid biosynthetic enzymes (Lopes and Henry, 1991; Carman and Henry, 1999; Graves and Henry, 2000). Alternatively, when the intracellular concentration of inositol is high, the negative regulator, Opi1p, a transcription factor localized in the endoplasmic reticulum, is translocated to the nucleus and represses the transcription of phospholipid biosynthetic genes (Carman and Henry, 1999; Carman and Han, 2011). Many of the genes necessary for phospholipid synthesis contain an inositol responsive *cis*-acting element in the promoter region called the UAS_{INO} element (Bachhawat et al., 1995; Carman and Henry, 1999). Thus, when inositol and choline are not present, the activator Ino2p/Ino4p specifically binds to the UAS_{INO} element and thus activates transcription (Carman and Henry, 1999; Carman and Han, 2011). However, when inositol and choline are abundant,
Opi1p, binds to the activator, Ino2p/Ino4p, and prevents transcription from occurring (Figure 1.2) (Carman and Henry, 1999; Carman and Han, 2011).
Figure 1.2: Induction and repression of $INO1$, a critical gene in the lipid biosynthetic pathway.

In the absence of inositol, the Ino2p/Ino4p activator complex binds to the promoter region at the $UAS_{INO}$ and allows for transcription of $INO1$. However in the presence of inositol, the repressor Opi1p, which is localized in the endoplasmic reticulum, is translocated into the nucleus and binds to the Ino2p/Ino4p activator complex. This represses transcription of $INO1$. 
While inositol and choline are two crucial phospholipid precursors that play a role in regulating and controlling lipid biosynthesis, there are many other factors that are involved in this regulation. Phosphatidate (PA) phosphatase enzymes have recently emerged as pivotal regulators of lipid metabolism. Their maintenance of PA and diacylglycerol (DAG) levels, two major components of the lipid biosynthetic pathway, helps regulate the synthesis of triacylglycerides and a variety of phospholipids (Pascaul and Carman, 2012). PA phosphatase (PAP) enzymes are responsible for catalyzing the reaction that dephosphorylates PA, which in turn produces DAG and a phosphate group (Figure 1.3) (Han et al, 2007). This reaction is crucial in lipid biosynthesis since DAG can be used to either generate triacylglycerol (TAG), which stores energy and fatty acids in lipid droplets or can be used to generate the phospholipids, phosphatidylethanolamine (PE), and phosphatidylcholine (PC) (Siniossoglou, 2009). Conversely, PA can be used to synthesize other phospholipids such as phosphatidylinositol (PI) and phosphatidylycerine (PS) if it is not first dephosphorylated by a PAP enzyme. Furthermore, it can also be used to synthesize phosphatidylethanolamine (PE), and phosphatidylcholine (PC) via the CDP-DAG pathway within the lipid biosynthetic pathway (Figure 1.3). Thus, the concentrations of PA and DAG are crucial in maintaining the production of phospholipid levels, as well as determining the types of phospholipids that are synthesized. Since PAP enzymes are responsible for regulating PA and DAG levels, they play a critical role in regulating the lipid biosynthetic pathway.
Figure 1.3: Lipid biosynthetic pathway of *Saccharomyces cerevisiae*.

(Wilmalathrathna *et al.* 2009)
1.3. Phosphatidate Phosphatase (PAP) Enzymes

As explained previously, one of the more recently discovered regulators of the lipid biosynthetic pathway are PAP enzymes. These enzymes catalyze the crucial step of PA dephosphorylation, which in turn produces DAG. In Saccharomyces cerevisiae, there have been multiple PAP enzymes identified that are encoded by genes found in the lipid biosynthetic pathway. These genes include PAH1 (formerly SMP2), DPP1, LPP1, and APP1 (Pascual and Carman, 2012). While all PAP enzyme-encoding genes share a common role in dephosphorylating PA, they each have their own distinct characteristics that make them unique. Their differences are quite vast and span a range of areas including physiological function, cellular localization, substrate specificity, and cofactor requirement (Figure 1.4).

One of the major distinguishing characteristics of PAP enzymes is the requirement of the Mg$^{2+}$ cofactor to function. Both the protein products of DPP1 and LPP1 are Mg$^{2+}$ independent, while those of PAH1 and APP1 require magnesium to work (Toke et al., 1998; Han et al., 2006; Chae et al., 2012). This is due to the different catalytic motifs present in each of these enzymes. Pah1p has the DXDX(T/V) motif that is common in the superfamily of Mg$^{2+}$ dependent phosphatase enzymes, which gives it its phosphatase activity (Han et al., 2007). Dpp1p and Lpp1p, however, contain a three domain lipid phosphatase motif that possesses their catalytic activity (Pascual and Carman; 2012). Lastly, the APP1 gene has only recently been discovered as a PAP encoding gene and as such, more research is still needed to help characterize its catalytic activity and other defining characteristics.

There are multiple other factors that distinguish these PAP enzymes from one another. Both Dpp1p and Lpp1p are small integral membrane proteins that have six transmembrane domains in their sequence (Toke et al., 1998; Pascual and Carman, 2012). Pah1p however is
considerably larger and does not contain any transmembrane domains (Han et al., 2006). These differences greatly affect their localization. Pah1p is primarily found in the cytosol, but is translocated to the membrane when needed to catalyze the reaction of converting PA, located on the ER membrane, into DAG (Karanasios et al., 2010). Pah1p has also been found to be present in the nucleus where it can act as a direct repressor of transcription for genes in the lipid biosynthetic pathway (Santos-Rosa et al., 2005). Dpp1p and Lpp1p however are located in the vacuole and Golgi apparatus membranes, respectively (Pascual and Carman, 2012). The distinct function of each PAP enzyme helps to play a role in its localization. Dpp1p and Lpp1p have been shown to be involved in signaling, while Pah1p is more directly involved with phospholipid production (Toke et al., 1998; Carman and Han, 2006). As a result, these enzymes’ physiological functions affect their localization.

Additionally, these PAP enzymes differ in their sensitivity to the thioreactive compound N-ethylmaleimide (NEM) and to the synthetic compound propranolol. While both the activity of Dpp1p and Pah1p are not affected by NEM, Lpp1p activity is essentially hindered by it (Wu et al., 1996; Furneisen and Carman, 2000; Han et al., 2006). The PAP activity of Pah1p however is impacted by propranolol and inhibits its ability to function properly. Propranolol affects Lpp1p activity as well, despite the fact that it is believed to inhibit the enzymes by interacting with the Mg$^{2+}$ binding location (Morlock et al., 1991; Furneisen and Carman, 2000; Pascual and Carman, 2012). Little is still known as to why these reagents affect these PAP enzymes differently. Due to this, both of these inhibiting compounds are rarely used as methods to distinguish between the enzymes, despite the fact that they impact these enzymes differently.

Lastly, the classes of PAP enzymes differ in respect to the specificity of their substrates. While Dpp1p and Lpp1p can catalyze reactions involving multiple lipid phosphate substrates
including PA, DGPP, and lysoPA, Pah1p is specific for the substrate PA (Kohei et al., 1984; Lin and Carman, 1989; Toke et al., 1998; Faulkner et al., 1999; Pascual and Carman, 2012). As mentioned earlier, the substrates and products that Dpp1p and Lpp1p act on have roles as signaling molecules. Thus, these enzymes are involved in lipid signaling more so than in de novo phospholipid and TAG synthesis (Toke et al., 1998; Carman and Han, 2006). Pah1p, however, has a pivotal role in the lipid biosynthetic pathway and is extremely important for de novo phospholipid synthesis and TAG production (Han et al., 2006; Sorger and Daum; 2003). Thus, while all these PAP enzymes contain PA phosphatase activities, their differences range in such a wide array of areas that their physiological functions are vastly different.
Phosphatidate Phosphatase Enzymes Found in *Saccharomyces cerevisiae*

<table>
<thead>
<tr>
<th></th>
<th><em>Dpp1p</em></th>
<th><em>Lpp1p</em></th>
<th><em>Pah1p</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg$^{2+}$ independent</td>
<td>Mg$^{2+}$ independent</td>
<td>Mg$^{2+}$ dependent</td>
<td></td>
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<tr>
<td>Integral membrane protein</td>
<td>Integral membrane protein</td>
<td>No transmembrane domains</td>
<td></td>
</tr>
<tr>
<td>Found in membrane (Vacuole)</td>
<td>Found in membrane (Golgi apparatus)</td>
<td>Found in cytosol; Translocated to nuclear/ER membrane</td>
<td></td>
</tr>
<tr>
<td>Multiple substrates</td>
<td>Multiple substrates</td>
<td>One substrate: PA</td>
<td></td>
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- The *APP1* PAP encoding enzyme was only recently discovered and more studies are being conducted to characterize it.

Figure 1.4: Chart depicting the differences of PAP enzymes found in *Saccharomyces cerevisiae*.

PAP enzymes are differentiated from one another based off a number of categories, including cofactor dependence, localization, catalytic motifs, physiological function, and substrate specificity.
1.4 PAH1: A crucial PAP-encoding gene

PAH1 is a yeast PAP gene which encodes the PA phosphatase, Pah1p. It is part of a subfamily of PA phosphatases that depend on the presence of Mg\(^{2+}\) for proper function (Han et al., 2006). Its protein product, Pah1p, is 863 amino acids long and has a molecular weight of about 95-kDa (Pascual and Carman, 2012). Since it is a PAP enzyme, Pah1p is necessary for catalyzing the key step of dephosphorylating PA to form DAG. Over the past few years, numerous studies have been conducted to characterize this protein and better understand the ways in which it acts. Pah1p contains a DXDX(T/V) catalytic motif within its haloacid dehalogenase (HAD)-like domain that gives it its catalytic function and contains an NLIP domain which currently has an unknown function (Han et al., 2007). The catalytic motif located in the HAD-like domain is necessary for its ability to dephosphorylate its substrate, as studies have shown that without it, Pah1p loses its phosphatase ability (Han et al., 2007). Additionally, Pah1p does not contain any transmembrane domains (Karanasios et al., 2010). As a result, in order to dephosphorylate PA, Pah1p must first be translocated onto the ER/nuclear membrane, where PA is situated, so that it can function and catalyze the reaction. Studies have shown that the translocation of Pah1p occurs when there are increased levels of PA in the cell. When PA levels rise, Pah1p is recruited onto the nuclear/ER membrane (Siniossoglou, 2009). This process is mediated by an amino terminal amphipathic helix that Pah1p contains in its amino terminal end (Karanasios et al., 2010). However, this recruitment only takes place when Pah1p is first dephosphorylated by a phosphatase complex of two proteins, the Nem1p-Spo7p complex (Karanasios et al., 201). Thus, Pah1p itself must first be dephosphorylated in order to obtain phosphatase ability and will remain inactive while in its phosphorylated state.
1.4.1 The activation and deactivation of Pah1p.

The Nem1p-Spo7p complex is responsible for dephosphorylating Pah1p. Both Nem1p and Spo7p come together to form a complex that is located in the endoplasmic reticulum membrane (O’Hara et al., 2006). There, they help recruit Pah1p to the membrane and dephosphorylate Pah1p, which allows Pah1p to bind to the membrane via its amphipathic helix (Figure 1.5). Specifically, the Nem7 subunit targets Pah1p to the membrane and once dephosphorylated by the Nem1p-Spo7p complex, the Pah1p helix becomes “primed” for binding to the membrane and can dephosphorylate PA to generate DAG for lipid synthesis (Karanasios et al., 2010).

Since dephosphorylation is the mechanism responsible for activating Pah1p, the deactivation of Pah1p was therefore hypothesized to be the result of its phosphorylation. Pah1p was discovered to be the target of many protein kinases, some of which include Pho85p-Pho80p, Cdc28p-cyclin B, protein kinase A, and protein kinase C (Su et al., 2012; Hsieh et al., 2015; Hsieh et al., 2016). Pah1p has been found to have multiple sites of phosphorylation (Figure 1.6), seven of which are in the Ser/Thr-Pro motif that are targeted by protein kinases regulated during the cell cycle (Choi et al., 2011, Hsieh et al., 2015). When these seven sites are phosphorylated, Pah1p function is negatively regulated and its phosphatase activity is inhibited. Research has shown that the protein kinase cyclin complex, Pho85p-Pho80p, phosphorylates Pah1p on all 7 sites (Choi et al., 2012; Su et al., 2014). When phosphorylated by Pho85-Pho80, Cdc28p-cyclin B, and protein kinase A, Pah1p is sequestered away from the membrane, where PA resides, and remains in the cytoplasm. There, it will remain inactive until it is recruited to the nuclear/ER membrane by the Nem1p-Spo7p complex and subsequently dephosphorylated. Thus, the activity
and localization of *PAHI* is heavily dependent on its phosphorylated and dephosphorylated states.

In addition, Pah1p is further regulated by proteasome protease activity and is targeted for degradation during the stationary phase of growth (Hsieh *et al*., 2015). It is specifically targeted by the 20S proteasome, and not the 26S proteasome, making it independent of an ubiquitin directed degradation. This mode of regulation is in turn administered by Pah1p’s phosphorylation state. When it is phosphorylated, and thus in its inactive state, degradation by the proteasome is greatly reduced. However, when dephosphorylated, it is highly subjected to degradation and therefore targeted in its active form (Hsieh *et al*., 2015). Thus, due to its importance in regulating the lipid biosynthetic pathway, Pah1p levels are heavily monitored in the cell.
Figure 1.5: Pah1p activation by the Nem1p-Spo7p complex.

(A) In its phosphorylated state, Pah1p is localized in the cytosol and inactive, (B) The Nem1p-Spo7p complex helps recruit Pah1p to the ER/nuclear membrane and dephosphorylates it, (C) Once dephosphorylated, Pah1p regains phosphatase activity and is able to catalyze the reaction which converts PA to DAG.
Figure 1.6: Phosphorylation sites of Pah1p.
1.4.2 *PAH1*: A Pivotal Player in Phospholipid Biosynthesis Regulation

While Pah1p is needed for catalyzing the reaction that produces DAG from PA, a crucial step in the eventual biosynthesis of triglycerides and other phospholipids, it also plays a major role in regulating the transcription of genes needed for phospholipid production. Many of the genes that encode enzymes necessary for phospholipids biogenesis contain a sequence in their promoters called the UAS$_{INO}$ (Santos-Rosa *et al.*, 2005). Out of 47 phospholipid genes, 22 have been identified to contain a UAS$_{INO}$ in their promoter region (Wimalarathna *et al.*, 2011). In *Saccharomyces cerevisiae*, the levels of PA play an important role in specifically controlling the transcription of biosynthetic genes that contain a UAS$_{INO}$. As explained earlier, the levels of PA heavily depend on Pah1p (Siniossoglou, 2009). Thus, when the levels of Pah1p are high, PA levels will be lower. Conversely, when levels of Pah1p are lower, levels of PA will be higher. This has major implications on the transcriptional regulation of genes in the lipid biosynthetic pathway, since the transcriptional repressor Opi1p is bound to PA on the ER/nuclear membrane (Loewen *et al.*, 2004). As a result, when PA levels are high, Opi1p will remain bound to PA on the ER membrane and will be unable to enter the nucleus and block transcription. This in turn will allow the transcriptional activator complex, Ino2p/Ino4p, to bind to the UAS$_{INO}$ and activate transcription (Siniossoglou, 2009). When the levels of PA are low however, nothing will prevent Opi1p from entering the nucleus, and it is thus able to repress transcription (Figure 1.7: Model 1) (Siniossoglou, 2009). Since Pah1p regulates PA levels, the presence of Pah1p plays a major role in the repression and derepression of phospholipid biosynthetic genes containing a UAS$_{INO}$ (Santos-Rosa *et al.*, 2005).

While the above model is one way to explain the role of Pah1p in mediating regulation in phospholipid gene transcription, a second model has also emerged to explain another way in
which Pah1p can act as a transcriptional regulator. A recent study using chromatin immunoprecipitation has shown that Pah1p can actually negatively regulate transcription by directly binding to the promoter regions of certain genes that contain a UAS<sub>INO</sub> (Santos-Rosa et al., 2005). Using three biosynthetic genes (INO1, INO2, and OPI3), it was found that when Pah1p becomes dephosphorylated by Nem1p-Spo7p, it will be recruited directly onto the promoter of these UAS<sub>INO</sub>-containing genes and can stop transcription (Santos-Rosa et al., 2005). These findings suggest a more direct role that Pah1p takes in preventing transcription from occurring; by physically entering the nucleus and impeding gene expression (Figure 1.7:Model 2). Thus, depending on the model of regulation used, the involvement of Pah1p in regulating gene transcription can be either direct or indirect.
Figure 1.7: Two models Pah1p acting as a negative regulator of lipid biosynthetic genes.

A) When the levels of PA are high, Opi1p, becomes bound to PA on the ER membrane and is unable to enter the nucleus. As a result, the transcriptional activator complex, Ino2p/Ino4p, binds to the UAS_{INO} and activates transcription. When PA levels are low however, nothing prevents Opi1p from entering the nucleus, and it is thus able to repress transcription. B) Additional model, which is identical as first model, except that Pah1p, also enters nucleus and binds promoter to block transcription.
1.4.3 Impact of \textit{PAH1} and Pah1p on cell physiology and homeostasis

Due to its importance in the lipid biosynthetic pathway, multiple studies have been conducted to help further characterize the important role of the \textit{PAH1} gene. Deletion experiments have been pivotal in gaining insight into the role of \textit{PAH}. Studies have shown that the \textit{pah1Δ} mutant has increased levels of PA in the cell and has an atypical increase in the expansion of the nuclear and ER membrane (Barbosa et al., 2015). The \textit{pah1Δ} mutant also shows an increase in expression of certain genes involved in phospholipid biosynthesis since the deletion of \textit{PAH1} leads to a derepression of enzymes in the CDP-diacylglycerol pathway and the Kennedy pathway, the two main pathways in the lipid biosynthetic pathway used for the synthesis of phospholipids (Soto-Cardalda et al., 2012). In addition, the levels of phospholipids, sterol esters and fatty acids rise in these mutants. This in turn leads to the cells having fatty acid toxicity (Fakas et al., 2011). Conversely, TAG synthesis in the \textit{pah1Δ} mutant is drastically lower. This is due to the lack of DAG, which Pah1p converts from PA, and is the precursor for TAG. Thus, without \textit{PAH1} present, DAG is unable to be produced and TAG cannot be synthesized. Moreover, studies have shown that cells lacking \textit{PAH1} also have a respiratory deficiency since they are unable to grow on non-fermentable carbon sources and exhibit temperature sensitivity (Hsieh et al., 2015).

\textit{PAH1} has also been shown to play important roles in the formation of lipid droplets (Adeyo et al., 2011). Cytosolic lipid droplets provide eukaryotic cells with a compact source of energy, but have also been shown to be used as storage units and are involved in organelle trafficking and interorganellar communication (Cermelli et al., 2006; Guo et al., 2009; Murphy et al., 2009; Zehmer et al., 2009; Salo et al., 2011). The production of lipid droplets depends on the presence of DAG, a product of the dephosphorylation of PA by Pah1p (Adeyo et al., 2011).
As such, studies have shown that the absence of \textit{PAH1} causes a marked decrease in lipid droplets in the cell, thereby confirming the importance of the presence of \textit{PAH1} in the formation of lipid droplets (Adeyo \textit{et al.}, 2011).

Adding to the list of areas in which this PAP enzyme affects, \textit{PAH1} has been shown to be crucial for vacuole morphology and homeostasis. The deletion of the \textit{PAH1} gene causes a morphological defect in the vacuole, in which the vacuole cannot remain intact and appears fragmented. A study has shown that this fragmentation is due to the implication of the vacuole fusion machinery when \textit{PAH1} is not present, leaving the vacuole fragments unable to fuse back together into a single vacuole (Sasser \textit{et al.}, 2012). This is due to the lack of phosphastase activity provided by Pah1p, preventing SNAREs from binding to Sec18p, the protein responsible for priming the SNARE complexes for fusion. In addition, when \textit{PAH1} is not present, a number of other crucial factors that make up the machinery for fusion are also absent, including Vps39p, which is a subunit of the homotypic fusion and vacuole protein sorting (HOPS) tethering complex, as well as phosphatidylinositol 3-phosphate, which is a lipid needed for SNARE activity and fusion (Sasser \textit{et al.}, 2012). Thus, \textit{PAH1} is critical for proper vacuole morphology and homeostasis.

Overall, the importance of \textit{PAH1} in proper cell function and development has been shown to be extensive. Its deletion has been shown to impact morphological structures, lipid production, and cellular function throughout the cell. Since its importance has only been recently discovered, more research is bound to highlight additional areas in the cell that it has an effect on.
1.4.4 Regulation of *PAH1*

While the regulation of Pah1p has been studied extensively at the protein level, the regulation of *PAH1* gene expression has only recently been emerging. The management of the *PAH1* gene is critical since it acts on the substrate PA. PA is the precursor molecule which is needed for multiple pathways in lipid synthesis and leads to the production of molecules such as phospholipids or TAGs, depending on the pathway taken. As mentioned earlier, it also tethers Opi1p to the ER membrane and thus helps control regulation of genes in the lipid biosynthetic pathway. During the exponential phase of growth, the level of PAP activity is generally low and phospholipids needed for the membrane are synthesized. Once cells reach the stationary phase, PA is mainly used to produce TAGs and as a result PA phosphatase activity is amplified (Pascual *et al.*, 2013).

Recently studies have shown that *PAH1* undergoes induction during times of zinc deficiency (Soto-Cardalda *et al.*, 2012). In *Saccharomyces cerevisiae*, the synthesis of membrane phospholipids is regulated in response to zinc homeostasis. During times of zinc depletion, zinc transporters are induced through Zap1p, while changes in the composition of phospholipid membranes occur as well (Eide, 2009). Certain phospholipid synthetic genes are induced by Zap1p while others are repressed by it. *PAH1* is one of the genes that become activated when zinc levels drop (Eide, 2009). This induction of Pah1p by zinc deficiency occurs via a transcriptional mechanism. Zap1p interacts with *PAH1* at one of the three upstream activating sequence zinc responsive elements (UAS$_{ZRE}$ sequences) on its promoter region (Soto-Cardalda *et al.*, 2012). As a result, *PAH1* plays a major role in phospholipid synthesis via zinc regulation.
In addition to regulation by zinc, recent studies have shown that induction of *PAH1* also occurs in response to palmitoleic acid (Fakas *et al.*, 2011). When growth medium is supplemented with palmitoleic acid, the level of Pah1p phosphatase activity increases, an observation that is not seen when palmitoleic acid is not introduced. This supplementation is then followed by an increase in the amount of TAG present in the cell. The induction of PA phosphatase activity by palmitoleic acid is specific for *PAH1*, and is not seen with any of the other PA phosphatase encoding genes. As of now, the mechanism for this means of regulation is still being studied and currently remains unclear as to how exactly *PAH1* is regulated in this manner (Fakas *et al.*, 2011).

Furthermore, *PAH1* expression is also regulated by the growth stage of the cell. Studies have shown that *PAH1* undergoes induction during the stationary phase of growth in yeast cells (Pascual *et al.*, 2013). This is consistent with the fact that *PAH1* is the main PAP encoding gene responsible for TAG formation. Since TAG production increases during stationary phase, when phospholipid synthesis decreases, it is logical that *PAH1* expression would increase during stationary phase to aid in the TAG production. Moreover, the addition of inositol aids in the induction of *PAH1* during stationary phase and increases its expression even more so. Interestingly, it was recently uncovered by Dr. Chang-Hui Shen’s lab that the *PAH1* gene also contains a UAS<sub>INO</sub> in its promoter region (Wimalarathna *et al.*, 2011). This is an important finding, since as mentioned earlier the UAS<sub>INO</sub> is the site where the Ino2p-Ino4p activator complex binds to initiate transcription and where the Opi1p repressor will dock when repressing transcription. Studies have also shown that Pah1p has a direct role in repressing transcription by binding to the UAS<sub>INO</sub> of genes (Santos-Rosa *et al.*, 2005). Thus, it would seem that inositol would play a role in helping to regulate its own expression.
1.5 Significance of Study

Pah1p is part of a family of proteins called lipins, which are types of PA phosphatases. There have been numerous PAP encoding genes found in higher eukaryotic organisms. Homologs of Pah1p have been discovered in plants, worms, flies, mice and humans (Golden et al., 2009; Nakamura et al., 2009; Han and Carman, 2010; Eastmond et al., 2011; Ugrankar et al., 2011; Fang et al., 2014). These genes have been shown to be crucial for the regulation of fat metabolism, adipogenesis, and the biogenesis of organelles (Reue and Zhang, 2008; Karanasios et al., 2010; Harris and Finck, 2011; Mietkiewska et al., 2011). These PAP enzymes are a part of the superfamily of haloacid dehalogenase (HAD)-like proteins, all of which possess the key DXDX(T/V) catalytic domain in the HAD-like domain. In addition, they also contain the NLIP domain, which as of yet has a mostly unknown function, but does contain a conserved glycine residue necessary for its fat regulating function, (Péterfy et al., 2001; Han et al., 2007).

Mammalian cells in particular have three lipins. These lipins include lipin-1, lipin-2, and lipin-3 (Reue and Dwyer, 2009). The Saccharomyces cerevisiae PAH1 gene is the yeast homologue of LPIN1, which encodes for the Lipin 1 protein (Han et al., 2006). LPIN1 contains the same greatly conserved domains found in PAH1. As explained earlier, it contains the N-LIP domain, as well as the HAD-like domain (in the C-LIP domain) which houses the DXDXT motif responsible for PA phosphatase activity. Lipin-1 is mainly found in muscle and adipose tissue and like its yeast homolog, is a PA phosphatase that plays a major role in phospholipid biosynthesis and TAG formation. Mammalian lipins are crucial for proper adipogenesis and are necessary for adipocyte differentiation to occur (Phan et al., 2005). Studies using mice have shown that knocking out lipin 1 inhibits normal development of adipose tissue (Péterfy et al., 2001). The lack of lipin 1 leads to lipodystrophy, a condition in which adipose tissue cannot develop
properly due to deficiency in adipogenesis. Conversely, the overexpression of lipin 1 has been shown to cause obesity (Agarwal and Garg, 2006; Han et al., 2007). As such, lipins play a major role in fat metabolism (Reue, 2009). Studying lipins and finding ways in which to target them for drug development could possibly be a means to treat obesity (Carman and Han, 2006). This could have profound implications, since obesity is a major health concern in the United States (Wang and Beydoun, 2007) and could help treat the obesity epidemic. Therefore, by using yeast as a model system and examining the functional role of Pah1p, we can get a better understanding of the role that lipins have in lipid metabolism and the ways in which they can be regulated.

Additionally, other metabolic disorders have also been associated with defects in lipins including lipodystrophy, insulin resistance, metabolic syndrome, type 2 diabetes, rhabdomyolysis and inflammation (Phan and Reue, 2005; Pascual and Carman, 2012). Mutations in the human LPIN1 gene have recently been discovered to be a newfound cause of the rare disease known as myoglobinuria, a condition in which rhabdomyolysis occurs and the skeletal muscle fibers deteriorate (Michot et al., 2010). Thus, by using yeast as a model organism to better understanding the role that the PAH1 gene plays both in the cell homoeostasis and in the lipid biosynthetic pathway, we can gain insight into the way it impacts fat metabolism and potential ways to cure these disorders.
2. Materials and Methods

2.1 Media and agar used in this study

**SC (Synthetic complete media)** – To make 1000mL, add 20g Dextrose (ThermoFisher Scientific), 6.7g Yeast Nitrogen Base with ammonium sulfate (MP Biomedicals, Cat: 114027522), 0.79g Complete Supplement Mixture (MP Biomedicals, Cat: 4500 022) and top to 1000mL with water. Autoclave.

**SC plus inositol** - To make 1000mL, add 20g Dextrose (ThermoFisher Scientific), 6.7g Yeast Nitrogen Base with ammonium sulfate (MP Biomedicals, Cat: 114027522), 0.79g Complete Supplement Mixture (MP Biomedicals, Cat: 4500 022), 10mg *myo*-inositol (Sigma, Cat: I5125) and top to 1000mL with water. Autoclave.

**SC minus inositol** - To make 1000mL, add 20g Dextrose (ThermoFisher Scientific), 6.7g YNB without inositol (yeast nitrogen base) with ammonium sulfate (MP Biomedicals, Cat: 4027 412), 0.79g Complete Supplement Mixture (MP Biomedicals, Cat: 4500 022) and top to 1000mL with water. Autoclave.

**SC minus zinc** - To make 1000mL, add 20g Dextrose (ThermoFisher Scientific), 6.7g Yeast Nitrogen Base minus copper minus zinc with ammonium sulfate (MP Biomedicals, Cat: 4027 412), 0.04mg/L copper sulfate, 0.79g Complete Supplement Mixture (MP Biomedicals, Cat: 4500 022) and top to 1000mL with water. Autoclave.

**SC plus geneticin** - To make 1000mL, add 20g Dextrose (ThermoFisher Scientific), 6.7g YNB without inositol (yeast nitrogen base) with ammonium sulfate (MP Biomedicals, Cat: 4027 412),
0.79g Complete Supplement Mixture (MP Biomedicals, Cat: 4500 022), 200μg/mL G418-Geneticin (ThermoFisher, Cat: 11811023) and top to 1000mL with water. Autoclave.

**SC buffered to pH 5.5/pH 7.0** - To make 1000mL, add 20g Dextrose (ThermoFisher Scientific), 6.7g Yeast Nitrogen Base with ammonium sulfate (MP Biomedicals, Cat: 114027522), 0.79g Complete Supplement Mixture (MP Biomedicals, Cat: 4500 022) Dissolved in 1:9 MES: H2O solution up to 1000mL. Buffer to desired pH using hydrochloric acid or NaOH before autoclaving.

**SC plus CaCl2 (Synthetic complete media)** – To make 1000mL, add 20g Dextrose (ThermoFisher Scientific), 6.7g Yeast Nitrogen Base with ammonium sulfate (MP Biomedicals, Cat: 114027522), 0.79g Complete Supplement Mixture (MP Biomedicals, Cat: 4500 022), 60mM calcium chloride, and top to 1000mL with water. Autoclave.

**SM (Spheroplast Medium)** – To make 1000mL, add 20g Dextrose (ThermoFisher Scientific), 6.7g Yeast Nitrogen Base with ammonium sulfate (MP Biomedicals, Cat: 114027522), 0.79g Complete Supplement Mixture (MP Biomedicals, Cat: 4500 022), 18.22% D-sorbitol (w/v), 50mM Tris HCl (pH 8), and top to 1000mL with water. Autoclave.

**YPD (yeast extract peptone dextrose)** – To make 1000mL, add 20g of bactoyeast extract (Difco, Cat: 212750), 40g of bacto-peptone (Becton Dickinson, Cat: 211705), 40g of dextrose (Fisher Scientific), and 20g bacto agar (Becton Dickinson, Cat: 214010) and top to 1000mL with water. Autoclave.

*To turn any of the above medium into plates add 1.5% Difco Agar (BD, Cat: 214530) to the solution as well before autoclaving.*
2.2 Reagents and Solutions

10x Buffer – To prepare, add: 1M Tris-HCl pH 8, 1M MgCl₂ to DEPC water. Filter sterilize.

Acid Phenol – pH 4.5 (Shelton Scientific, Cat: IB05184).

BCECF-AM – 1.6mM of BCECF-AM (Invitrogen, Cat: B3051) in DMSO.

Calibration buffer – To prepare add: 50mM HEPES (Fisher Scientific, Cat: BP308), 50mM KCl (Fisher Scientific), 50mM MES (BioWorld, Cat: 41320024 1), 50mM NaCl (Fisher Scientific), 0.2M ammonium acetate (Fisher Scientific), 10mM 2-deoxyglucose (Sigma, Cat: D8375), 10mM sodium azide (Sigma, Cat: S8031), and 15µM nigericin and buffer to desired pH using hydrochloric acid or sodium hydroxide.

Chloroform – (Acros, Cat: 61003-0040).

Deoxycholic Acid – (Sigma, Cat: D2510).

DEPC treated water – (Invitrogen, Cat: 46-2224)

DNase 1 – (Qiagen, Cat: 79254).

EDTA – 0.5M solution - DNase RNase and proteases free (Quality Biological, Cat: 351 027 100).

Formaldehyde: 37.5% (Sigma, Cat: 252549).

Glycerol – (Invitrogen, Cat: 15514-029).

Glycine – 2.5M (Fisher Scientific, Cat: G46-1).
**High Salt Buffer** – To prepare: Add 0.1% SDS, 2mM EDTA pH 8, 20mM Tris-HCl pH 8, 1% Triton X-100, 0.5M NaCl to water. Filter sterilize.

**LiCl Buffer** – To prepare: Add 250mM LiCl, 10mM Tris-HCl pH 8, 1mM EDTA pH 8, 1% Triton X-100, 0.1% deoxycholic acid to water. Filter sterilize.

**Low Salt Buffer** - To prepare: Add 0.1% SDS, 2mM EDTA pH 8, 20mM Tris-HCl pH 8, 1% Triton X-100, 0.15M NaCl to water. Filter sterilize.

**Lysis Buffer** – To prepare: 0.05M Tris HCl pH 7.5, 0.15 M NaCl, 1mM EDTA, 1% Triton X-100 to water. Filter sterilize.

**Lyticase enzyme** – 20mg/ml enzyme solution was prepared using powder lyticase from arthrobacter leuteus, crude (Sigma, Cat: L4025). Stored at -20°C.

**MES buffer** ([2-(N-morpholino) Ethanesulfonic acid]) – To prepare, add: 20mM MES (BioWorld, Cat: 41320024 1) to water, and buffer it using sodium hydroxide.

**Nigericin** –5mM of nigericin (Sigma, Cat: N7143) was dissolved in DMSO.

**Phenol saturated** - pH 6.6 (Fisher Scientific, Cat: BP1750-400).

**Proteinase K from tritirachium album** – (Sigma-Aldrich, Cat: P2038-100MG).

**Proteinase inhibitor cocktail** –100X cocktail (Sigma- Aldrich, Cat: P8340-5ML).

**RNA sample buffer** – To prepare, add: 2mM EDTA, 10mM Tris HCl (pH 8), 1% β-mercaptoethanol, 1% SDS and 10% glycerol and top with water.
**Sodium acetate** – Prepare by adding 204.05 g of sodium acetate in 500mL water to make a 3M solution. Adjust the pH using hydrochloric acid.

**Sorbitol** – Prepare by adding 9.10g of D-sorbitol (Acros organics, Cat: 132730010) in 50 ml distilled water to make 1M solution. Autoclave.

**SYBER® GreenER™ Two-Step qRT-PCR Kit Universal** (Invitrogen, Cat No: 11765-100), consists of two kits.

**Taq Man Gene Expression Master Mix** – (Applied Biosystems, Cat: 4369016).

**TE** – 50X solutions was prepared by adding 10mM Tris and 0.1mM EDTA. Sterilized by filtering.

**TES (Tris-EDTA-SDS) buffer** – To prepare, add: 10 mM Tris HCl pH 7.5, 10 mM EDTA and 0.5% SDS to deionized water. Filter sterilize.

**Trichostatin A** - 10mM solution was prepared by adding 1mg trichostatin A to 330ul 100% ethanol (Wako, Cat: 203-17561) and allowing to dissolve.

**Tris HCl** – 1M solution was prepared by adding 39.41g Tris HCl in 250ml distilled autoclaved water. Sodium hydroxide was used to buffer the pH.

**Triton X-100** – (Sigma, Cat: T8532-500ML).
2.3 Yeast Strains

Table 2.1: Genotypes of yeast strains used in this study.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genome type</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (BY4741)</td>
<td>( \text{MATa \ his3}^\Delta 200 \ \text{leu2}^\Delta 0 \ \text{met15}^\Delta 0 \ \text{ura3}^\Delta 0 )</td>
</tr>
<tr>
<td>WT (BY4733)</td>
<td>( \text{MATa \ his3}^\Delta 200 \ \text{leu2}^\Delta 0 \ \text{met15}^\Delta 0 \ \text{trp1}^\Delta 63 \ \text{ura3}^\Delta 0 )</td>
</tr>
<tr>
<td>( \text{pah1}^\Delta )</td>
<td>( \text{MATa \ his3}^\Delta 200 \ \text{leu2}^\Delta 0 \ \text{met15}^\Delta 0 \ \text{ura3}^\Delta 0 \ \text{pah1}^\Delta )</td>
</tr>
<tr>
<td>( \text{snf2}^\Delta )</td>
<td>( \text{MATa \ his3}^\Delta 200 \ \text{leu2}^\Delta 0 \ \text{met15}^\Delta 0 \ \text{ura3}^\Delta 0 \ \text{snf2}^\Delta )</td>
</tr>
<tr>
<td>( \text{ino80}^\Delta )</td>
<td>( \text{MATa \ ino80}^\Delta::\text{trp1} \ \text{his3}^\Delta 200 \ \text{leu2}^\Delta 0 \ \text{met15}^\Delta 0 \ \text{trp1}^\Delta 63 \ \text{ura3}^\Delta 0 )</td>
</tr>
</tbody>
</table>
2.4 Experimental Procedures: Cell Growth

2.4.1 Cell culture

Yeast strains were inoculated from a single colony into appropriate media. Cultures were grown at 30°C at 300 rpm overnight. The optical density was measured at $A_{600\text{nm}}$ using Lambda 35 UV/VIS spectrometer (Perkin Elmer).

2.4.2 Glycerol stock preparation

Glycerol stocks were prepared by mixing 100% glycerol and appropriate media in 1:1 ratio. Overnight culture of particular yeast strain was mixed with glycerol followed by immediate freezing in dry ice. Glycerol stocks were stored in -80°C.

2.5 Growth Analysis

Wild type and $pah1\Delta$ strains were grown in SC media (synthetic complete media) in the presence and absence of 100μM of myo-inositol, the presence and absence of 100μM choline, as well as 10μM of inositol and 10μM of choline. They were all started at an optical density ($A_{600}$) of around 0.2. Growth experiments were performed in duplicates, where the optical density of the cells were measured every two hours for the first eight hours and then again at the 24th hour until stationary phase was reached. Cells were grown at 30°C.

The average of the 24th hour OD readings for each strain and condition was used in the calculation to derive the average number of cell generations for that specific cell type:
Average # of generations = [(log# of cells at the end of 24 hours – log# of cells at the start of 24 hours)/log(2)]

This was then followed by determining the generation time in minutes/generation using the average # of generations:

Generation time (min/gen) = [(60min/hr * 24)/number of generations in 24 hours]

For the acetic acid hypersensitivity growth curve the same calculations were performed. The strains used for this growth curve, wild type and pah1Δ strains, were the same listed in Table 3.1 and were grown in SC media (synthetic complete media) in the presence of 100μM myo-inositol as well as in the presence of 0mM, 5mM, 10mM, 25mM, 50mM, and 100mM acetic acid.

2.6 Total mRNA Preparation

2.6.1. Cell preparation and RNA isolation

WT and pah1Δ cells (Table 3.1) were grown from a single colony until mid-logarithmic phase (0.8 A600) in SC media with 10μM myo-inositol, followed by an additional two hours in the presence of 100μM myo-inositol. RNA was extracted from yeast cells through acid phenol (pH 4.3) chloroform extraction in which the cells were resuspended in TES (10mM EDTA; 10mM Tris-HCl pH 7.5; 0.5% SDS), and then incubated at 65°C with equal parts acid phenol for 60 minutes. This was followed by a five-minute incubation on ice before centrifugation at 4°C. The top layer of the sample was transferred to a new test tube and mixed with equal parts acid phenol for a second time. Samples were incubated on ice for 5 minutes before centrifugation at 4°C and then separated by transferring the top layer of the sample to a fresh test tube and mixing
with equal parts chloroform. Samples were centrifuged, then separated again from their bottom layer and transferred to a new test tube. Ethanol precipitation was then performed by adding 1 mL of 100% ethanol and 40μL of 3M NaOAc to the sample. The samples were then stored in dry ice for 20 minutes, followed by a fifteen-minute centrifugation. The supernatant was discarded and pellets were washed in ethanol (70%) and centrifuged again for three minutes. The supernatant was removed and the pellets were left to dry for 10 minutes. This was followed by resuspending the pellets in RNA sample buffer. RNA concentration was quantified by reading absorbance at 260nm ($A_{260}$). Subsequently, total RNA was stored at -80°C until further use.

### 2.6.2 DNase Treatment of RNA samples

DNase treatment was performed to remove any DNA from the isolated RNA samples. RNA was mixed with 10X buffer (0.1 M Tris-HCl pH 8, 5mM CaCl$_2$ and 0.025M MgCl$_2$), DNase, and DEPC water. Samples were placed in 37°C water bath for 60 minutes, followed by phenol:chloroform extraction. A 3:1 ratio of phenol:chloroform was added to the samples, mixed and then placed on ice for a five minute incubation. Samples were then centrifuged for 10 minutes and then the top layer was transferred to a new test tube to be used for ethanol precipitation. Ethanol (100%) and 20μL of 3M NaOAc were added to supernatant, mixed and then placed on dry ice for 20 minutes. Samples were centrifuged for 13 minutes, followed by removal of supernatant, and a washing of the pellet in 70% ethanol. This was followed by centrifugation and the removal of the supernatant. Pellets were placed in a speed vacuum to dry and then resuspended in TE buffer that was prepared using DEPC water. RNA concentration was again quantified by reading absorbance at 260nm.
2.6.3 Preparation of cDNA: First Strand Synthesis

In order to prepare the cDNA needed for qRT-PCR, the SuperScript III First Strand Synthesis SuperMix kit from Invitrogen (Catalog: 11752-050) was used. The reaction was performed using a polymerase chain reaction (PCR) thermocycler. Each DNase free mRNA sample (1 μg) was mixed with 2x RT Reaction mix, RT enzyme mix, and DEPC water. Samples were placed in the PCR thermocycler and underwent the reaction using the following thermocycler program: 10 minute incubation at 25°C, 30 minute incubation at 50°C, and 5 minute incubation at 85°C. Samples were then cooled on ice for a few minutes before adding *E. coli* RNase H and then placed in a 37°C waterbath for 20-30 minutes. First strand synthesis samples were stored at -20°C until further use.

2.6.4 Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

The cDNA prepared from first strand synthesis was amplified and quantified using qRT-PCR. The SYBR GreenER® qPCR Supermix universal kit from Invitrogen (catalog number: 11762-500) was used to quantify cDNA templates. Forward and reverse primers were used that targeted the open reading frame (ORF) in the qPCR reaction. The reaction setup for each sample contained the following: 10μL of SYBR GreenER® qPCR Supermix, 0.4 μL each of forward and reverse primers, 0.4 μL of ROX reference dye, 1 μL of the cDNA template, and RNA and DNA free water to a volume of 20μL. Samples were added to a 96 well plate and then sealed and placed in the Applied Biosystems 7500 real time PCR machine. The program used had a 2 minute incubation at 50°C, followed by 10 minutes at 95°C, and 40 cycles of 15 seconds at 90°C and 1 minute at 60°C. PCR reactions were performed in triplicates.
2.6.5 Analysis of RNA quantity

A ΔC\(_T\) value was obtained by subtracting the respective gene C\(_T\) from the C\(_T\) value of the constitutive housekeeping gene (ACT1). Therefore, the relative mRNA levels were expressed as \(2^{\Delta C_T}\). Data results were graphed as a mean of the repeats ± standard deviation.

2.7 Cell Survival and Serial Dilutions for Acetic Acid Sensitivity Assay

Yeast strains were grown in synthetic complete media with 100 μM of inositol over night at 30°C. For acetic acid cell survival and sensitivity, cells were spun down and diluted to an \(A_{600\text{nm}}\) of 1.0 and placed in pH 3 SC media containing either 0mM acetic acid, 80mM acetic acid, and 120mM acetic acid (methods followed from Agimoni; 2013) and grown at 30°C, 300 RPM, for 200 minutes. Cells were collected at the following time points: 0 minutes, 90 minutes, 200 minutes. At each time point, all cells were diluted to \(A_{600\text{nm}}\) of 1.0 and then serially diluted by performing three 100-fold serial dilutions (\(10^{-6}\)). 15μL of cells were then plated on YPD plates for each strain and each time point from the \(10^{-6}\) dilution. After 200 minutes, instead of three 10-fold serial dilutions, five 10-fold serial dilutions were performed. 15μL of cells were still plated on YPD plates from the \(10^{-6}\) dilution, but on a separate YPD plate, a 5μL droplet of each dilution (starting from \(10^{-2}\) to \(10^{-10}\)) was plated as well for each condition. Plates were placed in a 30°C incubator for three days. Similar to calculating colony forming units (CFUs), cells were counted on each plate to determine cell survival and viability. Three to four repeats were conducted per condition and then averaged ± standard deviation. Mean data was converted into percentages and plotted as cell survival against time. Serial dilution plates were photographically captured.
2.8 Cell Survival and Serial Dilutions for Hydrogen Peroxide Sensitivity Assay

Yeast strains from Table 3.1 were grown in synthetic complete media with 100 μM of inositol over night at 30°C. For hydrogen peroxide exposed cell survival and sensitivity, cells were spun down and diluted to an $A_{600nm}$ of 1.0 and placed in synthetic complete media with 100 μM of inositol that contained either 0mM H$_2$O$_2$ or 1mM H$_2$O$_2$ (Agimoni; 2016) and grown at 30°C, 300 RPM, for 4 hours. Cells were collected at the following time points: Hour 0, Hour 2, and Hour 4. At each time point, all cells were diluted to $A_{600nm}$ of 1.0 and then serially diluted by performing three 10-fold serial dilutions ($10^{-6}$). 15μL of cells were then plated on YPD plates for each strain and each time point from the $10^{-6}$ dilution. After 4 hours, instead of three 100-fold serial dilutions, five 100-fold serial dilutions were performed. 15μL of cells were still plated on YPD plates from the $10^{-6}$ dilution, but on a separate YPD plate, a 5μL droplet of each dilution (starting from $10^{-2}$ to $10^{10}$) was plated as well for each condition. Plates were placed in a 30°C incubator for three days. Similar to calculating colony forming units (CFUs), cells were counted on each plate to determine cell survival and viability. Three to four repeats were conducted per condition and then averaged ± standard deviation. Mean data was converted into percentages and plotted as cell survival against time. Serial dilution plates were photographically captured.
2.8 Chromatin Immunoprecipitation (ChIP)

2.8.1. Cell preparation and crosslinking

ChIP was performed on WT strain (Table 4.1). WT was streaked from a single colony and grown in 6mL SC media with 10µM inositol until saturation. Cells were transferred to 250mL of SC media with 100µM inositol and grown to stationary phase so that cells would be harvested in a stage where PAHL would be induced. Prior to harvesting, cells were cross-linked with formaldehyde for 25 minutes and then quenched with glycine for an additional 5 minutes. Cells were then pelleted, collected and then treated with cell wash buffer (20mM Tris-HCl pH8; 200mM NaCl) to be washed. Cells were then stored at -80°C until they were ready to be lysed.

2.8.2. Lysate Preparation

In order to lyse cells, the pellets were mixed with 400µl of lysis buffer that contained PMSF (50mM Tris-HCl pH 7.5, 0.15 M NaCl, 1mM EDTA pH 8, 1% Triton X-100, 1.5µM trichostatin A, 0.2mM PMSF) as well as 0.4µl Protease inhibitor cocktail set III (CalBioChem Catalog number: 539134). The cells with the lysis buffer mixture were then carefully added to acid washed glass beads in an epindorf tube and subsequently vortexed at 4°C for 20-25minutes. Cells were then separated from beads by centrifugation at 2K for 10 seconds. The supernatant was collected and placed in a new test tube so that it could undergo centrifugation at 13K for 15 minutes at 4°C. Supernatant was discarded and the remaining pellet was mixed with chilled lysis buffer that did not contain PMSF. Samples were sonicated and then centrifuged at 2K for 60 seconds at 4°C. Supernatant was then placed in a fresh test tube and stored in -80°C.
2.8.3. Input sample preparation

Elution buffer (1% SDS + 0.1 M NaHCO₃) and 5M NaCl were added to the 10μl of lysate. Tubes were incubated at 65°C for 4 hrs. Then, 8μl 0.5M EDTA, 16μl 1M Tris HCl pH 6.5 and 1μl Protinase K were added to the tubes and incubated again at 45°C for 1 hour. After this, phenol chloroform extraction was performed, followed by the ethanol precipitation. DNA quantification was done using spectrophotometer.

2.8.4. Immunoprecipitation

Sonicated lysates that contained 11μg total DNA were used for immunoprecipitation (IP). They were diluted 10-fold in ChIP dilution buffer and incubated with 1μL TSA and 1μL protease inhibitor cocktail, along with 35 μL protein A agarose slurry. Samples were placed at 4°C for an hour on a rotation rack to preclear and reduce nonspecific background. They were then briefly centrifuged at 2K for 15 seconds and the supernatant was transferred to a new epindorf tube. Antibodies against Snf2p were added and allowed to incubate overnight. The negative control samples (mock samples) were not treated with antibodies.

After overnight IP, 60 μL protein A agarose slurry was added to samples and incubated for one hour. Samples were briefly centrifuged were washed once with low salt buffer, high salt buffer, and LiCl solution, respectively. Subsequently, the samples were washed with 1X TE and then washed again with 1X TE. The protein-DNA complex was eluted from the resin by adding 250μl of freshly prepared elution buffer and rotating for two fifteen-minute time intervals. The supernatant was collected after centrifugation and 5M NaCl was added to each test tube to
reverse cross-link the samples by heating them at 65°C for 5-6 hours. Then, 8ul of 0.5 M EDTA, 16ul of 1M Tris-HCl, pH6.5 and 2 µl of Proteinase K was added and incubated for 2 hours at 45°C. The DNA was then recovered by phenol/chloroform extraction and ethanol precipitation. Finally, the pellet was re-suspended in 20 µl of TE.

2.8.5. Quantitative PCR (qPCR)

qPCR was used to analyze and determine the abundance of DNA fragments that were bound by the protein of interest. Mock and input samples were also tested. Input samples were prepared as all the DNA sequences in the genome from the lysate of the cell without any selection or IP. Mock samples were prepared exactly as IP samples, just without the addition of an antibody to be used to subtract out any background signal. qPCR was performed using the Applied Biosystems, 7500 real-time PCR system). IP samples were calculated as follows:

\[(2^{\Delta \text{Ct}_\text{IP} - \Delta \text{Ct}_\text{mock})} - (\text{Input Ct} \# - \text{mock Ct} \#) \times (A_{260} \times 50 \mu g/mL \times 50 \text{ dilution factor})\]

The IP samples were then graphed as an average of repeats ± standard deviation that was then normalized to mock and input samples.

2.9 Vacuolar Morphology: Electron Microscopy

WT and pah1∆ were grown from a single colony until mid-logarithmic phase (exponential phase) and stationary phase in SC media with 0.002% (w/v) of myo-inositol. Cells were fixed with glutaraldehyde, calcium chloride, and sodium cacodylate buffer for one hour on
ice. The fixative was removed and cells were washed twice with the sodium cacodylate buffer. Cells were then treated with lyticase for about 30 minutes at room temperature to break open the cell wall. Following, cells were washed with buffer and then treated with osmium tetroxide for two hours on ice. They were then washed with the buffer and left overnight in 4°C. The next day, cells were dehydrated with 50%, 60%, and 70% ethanol, followed by an overnight incubation with 70% ethanol and 3% uranyl acetate reagent. Cells were washed with 80%, 90%, and 100% ethanol, followed by two incubations/washes with propylene oxide. A 1:1 ratio of propylene oxide and resin was added, followed by fresh resin to embed and placed in the oven to set. Samples were cut into ultra-thin sections and prepared for imaging using the Leica UCT Ultramicrotome. EM microscopy was performed using the Fei Technai Spirit Transmission Electron Microscope.

### 2.10 Growth analysis on SC and YPD plates (containing either CaCl$_2$ or buffered pH)

Individual colonies were incubated overnight in SC + 100mM inositol media (dextrose-nitrogen base-amino acids). On the following day, each strain's OD was measured and then either diluted or concentrated so that all strains contained the same OD for their starting point. The strains were then diluted 100 fold in SC medium and 5ul of each diluted strain were plated on SC+ inositol plates that contained 60mM of CaCl$_2$ for the calcium chloride sensitivity assay and on YPD plates buffered to pH 5.5 and pH 7.0 for the pH sensitivity assay. Plates were incubated at 30°C for two days before analyzed.
2.11 Growth analysis in liquid broth buffered to appropriate pH

Cells were grown in SC media (synthetic complete media) containing 0.002% (w/v) of myo-inositol. Media was buffered to pH 5.5 and pH 7. Growth experiments were performed in duplicates where the optical density (OD) of the cells and pH of the growth medium was measured every two hours for 8 hours and then resumed at 24 hours to 30 hours at 30°C.

2.12 Vacuolar pH analysis

WT, pah1Δ and vma3Δ cells were grown until stationary phase in SC media with 100μM of inositol buffered to pH 7.0. Cells were then incubated with 1mM of BCECF-AM dye for 30 to 40 minutes at 30°C in the dark. Cells were then spun down, washed twice with media, and resuspended in fresh media. Cells were then stored on ice. Next, calibration samples were made for all three strains. Cells with the BCECF dye were incubated with 15μM of nigericin and the calibration buffers (50mM HEPES, 50mM MES, 10mM 2-deoxyglucose, 50mM potassium chloride, 50mM sodium chloride, 0.2M ammonium acetate, 10mM 2-deoxyglucose) that ranged from pH 5.0 to pH 7.0 for thirty minutes at 30°C. The unknown samples were prepared by resuspending cells with BCECF with 1mM MES buffer at pH 7. All samples were plated in a 96 well plate. The SpectraMas M5 microplate reader was used to measure the excitation wavelength (450nm and 490nm) with emission wavelength of 535nm. The calibration curves were made by taking the ratio of fluorescence at 490nm to 450nm. The fluorescence ratio was then plotted against pH (Figure 5.3A and 5.3B). Unknown samples were then plotted on standard curve to determine their pH.
Chapter 3

Characterization of the PAH1 gene in the lipid biosynthetic pathway

PAH1 encodes the PAP enzyme, Pah1p, which plays a crucial role in regulating the lipid biosynthetic pathway. The negative regulator Opi1p, which is bound to PA on the nuclear-ER membrane during gene activation, enters the nucleus when gene repression is required. It binds to the promoter region of UAS\textsubscript{INO} genes and blocks transcription. Conversely, when these genes are in need of activation, the Ino2p-Ino4p activator complex binds to the UAS\textsubscript{INO} of these genes and Opi1p will remain bound to PA outside of the nucleus. This process however has been shown to be heavily dependent on Pah1p, which mediates the levels of PA by catalyzing the step of PA dephosphorylation and turning it into DAG. It thus diminishes PA levels, thereby allowing Opi1p to enter the nucleus. As such, Pah1p negatively regulates these genes. However the effect it has on specific genes has only been minimally researched.

While studies have shown Pah1p negatively regulates the lipid biosynthetic pathway, its effect has only been tested on three biosynthetic genes, \textit{INO1}, \textit{INO2}, and \textit{OPI3}. For instance, while \textit{INO1} is a good model gene to use, these studies have not shown how Pah1p affects other genes important in inositol production. Thus, the functional role of Pah1p in regulating other genes in the pathway of inositol production has yet to be investigated. Furthermore, the impact of PAH1 on the transcription of the other genes nearby in the lipid biosynthetic pathway has not been directly studied as well. The lipid biosynthetic pathway consists of over 50 genes and a more thorough analysis of the effect of Pah1p on these genes, both those containing a UAS\textsubscript{INO} and those without, is needed. Therefore, we aim to investigate PAH1’s effect on a number of sub-pathways within the lipid biosynthetic pathway.
In addition, further studies have shown that the synthesis of phospholipids, as well as membrane biogenesis in general, is mainly regulated by the crucial phospholipid precursors, inositol and choline. However, the relationship of these precursors in conjunction with the regulatory role of PAH1 has not been fully studied. In order to elucidate the above relationship, we chose to study the effect of PAH1 upon the entire process of de novo inositol synthesis as well as look at its impact on other crucial genes in the lipid biosynthetic pathway in the presence of inositol.

To test our hypothesis, and examine the implications of PAH1 on lipid biosynthetic gene regulation, especially in relation with the two crucial phospholipid precursors inositol and choline, growth analysis followed by RNA analysis, was performed. Additionally, we used a sensitivity assay to confirm the results of HXK2 gene regulation, a gene which was downregulated by pah1Δ, a result which we had not previously encountered before. Since PAH1 negatively regulates certain genes in the lipid biosynthetic pathway, its deletion should cause an upregulation of these genes or at least remain at similar levels to wild type for the genes that is does not affect. Finding a gene in which the reverse happens, suggests that PAH1 affects pathways other than the lipid biosynthetic pathway and may be involved in other areas of cell homeostasis.
3.1 Materials and methods

3.1.1 Growth analysis

Yeast strains used in this experiment are shown in Table 3.1. *Wild type* and *pah1Δ* strains were grown in SC media (synthetic complete media) in the presence and absence of 100μM of *myo-inositol*, the presence and absence of 100μM choline, as well as 10μM of inositol and 10μM of choline. They were all started at an optical density (A600) of around 0.2. Growth experiments were performed in duplicates, where the optical density of the cells were measured every two hours for the first eight hours and then again at the 24th hour until stationary phase was reached. Cells were grown at 30°C.

Table 3.1: Yeast strain genotypes used in Chapter 3

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genome type</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Wild type</em> (BY4741)</td>
<td><em>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</em></td>
</tr>
<tr>
<td><em>pah1Δ</em></td>
<td><em>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</em></td>
</tr>
</tbody>
</table>

The average of the 24th hour OD readings for each strain and condition was used in the calculation to derive the average number of cell generations for that specific cell type:

Average # of generations = [(log# of cells at the end of 24 hours – log# of cells at the start of 24 hours)/log(2)]

This was then followed by determining the generation time in minutes/generation using the average # of generations:

Generation time (min/gen) = [(60min/hr * 24)/number of generations in 24 hours]
For the acetic acid hypersensitivity growth curve the same calculations were performed. The strains used for this growth curve, wild type and pah1Δ strains, were the same listed in Table 3.1 and were grown in SC media (synthetic complete media) in the presence of 100μM myo-inositol as well as in the presence of 0mM, 5mM, 10mM, 25mM, 50mM, and 100mM acetic acid.

3.1.2 RNA analysis: Total mRNA preparation and quantification

WT and pah1Δ cells (Table 3.1) were grown from a single colony until mid-logarithmic phase (0.8 A₆₀₀) in SC media with 10μM myo-inositol, followed by an additional two hours in the presence of 100μM myo-inositol. RNA was extracted from yeast cells through acid phenol (pH 4.3) chloroform extraction in which the cells were resuspended in TES (10mM EDTA; 10mM Tris-HCl pH 7.5; 0.5% SDS), and then incubated at 65°C with equal parts acid phenol for 60 minutes. This was followed by a five-minute incubation on ice before centrifugation at 4°C. The top layer of the sample was transferred to a new test tube and mixed with equal parts acid phenol for a second time. Samples were incubated on ice for 5 minutes before centrifugation at 4°C and then separated by transferring the top layer of the sample to a fresh test tube and mixing with equal parts chloroform. Samples were centrifuged, then separated again from their bottom layer and transferred to a new test tube. Ethanol precipitation was then performed by adding 1 mL of 100% ethanol and 40μL of 3M NaOAc to the sample. The samples were then stored in dry ice for 20 minutes, followed by a fifteen-minute centrifugation. The supernatant was discarded and pellets were washed in ethanol (70%) and centrifuged again for three minutes. The supernatant was removed and the pellets were left to dry for 10 minutes. This was followed by resuspending the pellets in RNA sample buffer. RNA concentration was quantified by reading absorbance at 260nm (A₂₆₀). Subsequently, total RNA was stored at -80°C until further use.
3.1.3 DNase Treatment of RNA samples

DNase treatment was performed to remove any DNA from the isolated RNA samples. RNA was mixed with 10X buffer (0.1 M Tris-HCl pH 8, 5mM CaCl$_2$ and 0.025M MgCl$_2$), DNase, and DEPC water. Samples were placed in 37°C water bath for 60 minutes, followed by phenol:chloroform extraction. A 3:1 ratio of phenol:chloroform was added to the samples, mixed and then placed on ice for a five minute incubation. Samples were then centrifuged for 10 minutes and then the top layer was transferred to a new test tube to be used for ethanol precipitation. Ethanol (100%) and 20μL of 3M NaOAc were added to supernatant, mixed and then placed on dry ice for 20 minutes. Samples were centrifuged for 13 minutes, followed by removal of supernatant, and a washing of the pellet in 70% ethanol. This was followed by centrifugation and the removal of the supernatant. Pellets were placed in a speed vacuum to dry and then resuspended in TE buffer that was prepared using DEPC water. RNA concentration was again quantified by reading absorbance at 260nm.

3.1.4 Preparation of cDNA: First Strand Synthesis

In order to prepare the cDNA needed for qRT-PCR, the SuperScript III First Strand Synthesis SuperMix kit from Invitrogen (Catalog: 11752-050) was used. The reaction was performed using a polymerase chain reaction (PCR) thermocycler. Each DNase free mRNA sample (1 μg) was mixed with 2x RT Reaction mix, RT enzyme mix, and DEPC water. Samples were placed in the PCR thermocycler and underwent the reaction using the following thermocycler program: 10 minute incubation at 25°C, 30 minute incubation at 50°C, and 5 minute incubation at 85°C. Samples were then cooled on ice for a few minutes before adding E.
coli RNase H and then placed in a 37°C waterbath for 20-30 minutes. First strand synthesis samples were stored at -20°C until further use.

3.1.5 Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

The cDNA prepared from first strand synthesis was amplified and quantified using qRT-PCR. The SYBR GreenER® qPCR Supermix universal kit from Invitrogen (catalog number: 11762-500) was used to quantify cDNA templates. Forward and reverse primers were used that targeted the open reading frame (ORF) in the qPCR reaction. Primers used in this study are shown in Table 3.2.
Table 3.2: List of primers used for qRT-PCR for RNA analysis

<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>HXK2</td>
<td>5’ CCAATGGCCATCAACTGTGA ‘3</td>
<td>5’ TGGCCTGGTCTTTGGAGATTC ‘3</td>
</tr>
<tr>
<td>INO1</td>
<td>5’ CCATGGTTAGCCCAACGA ‘3</td>
<td>5’ GCCTTCAAGCGTTGTTGCA ‘3</td>
</tr>
<tr>
<td>DPP1</td>
<td>5’ AAGGCTTGCCATTGGACACT ‘3</td>
<td>5’ CCCAGTCTCTGCAAAGCTTTTC ‘3</td>
</tr>
<tr>
<td>LPP1</td>
<td>5’ AAAGCACTCAAAGCGGACAT ‘3</td>
<td>5’ GGGCACAAATGCAACTTCT ‘3</td>
</tr>
<tr>
<td>INM1</td>
<td>5’ GGGATGGTGGTTGCTATTCG ‘3</td>
<td>5’ TGTTCCCCTCACAGCCAAAT ‘3</td>
</tr>
<tr>
<td>PIS1</td>
<td>5’ GATGCAGCTAGACGGAAACCAT ‘3</td>
<td>5’ TCAAGCCAGCGGTACCTGGAT ‘3</td>
</tr>
<tr>
<td>APP1</td>
<td>5’ CCAAGAAGGTGCCCCATTGA ‘3</td>
<td>5’ ACCCAGCCTTGTAGCGGTAA ‘3</td>
</tr>
<tr>
<td>TGL3</td>
<td>5’ TCCTCGATAGCAACAGCGAC ‘3</td>
<td>5’ GTGCAGCGCATAGGAATCTCT ‘3</td>
</tr>
<tr>
<td>TGL4</td>
<td>5’ TCCGCAGGAACTGGAATTCT ‘3</td>
<td>5’ TGGGCATCCAAGAGCACAA ‘3</td>
</tr>
<tr>
<td>ACT1</td>
<td>5’ CCAAGCCGTTTGTCTCTTGT ‘3</td>
<td>5’ ACCGGCCAAATCGATTCTC ‘3</td>
</tr>
<tr>
<td>DGA1</td>
<td>5’ GCGACCCCTTTTGAAAGGA ‘3</td>
<td>5’ TGCTGGTGCAGAGGATTC ‘3</td>
</tr>
<tr>
<td><strong>TGL5 primers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward: 5’ ACGCCGCTATTCGAGAAAGA 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reverse: 5’ GACGAGAAATGGGCGATGTCA 3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>CDS1 primers</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward: 5’ CAAGGCAGCTCCATCACATA 3</td>
</tr>
<tr>
<td>Reverse: 5’ TGCAGGACTCATGCCATTTA 3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>BIR1 primers</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward: 5’ GGGGGTTTCGGACTGGAAAA 3</td>
</tr>
<tr>
<td>Reverse: 5’ GCTGGAGTCGTATCGCATGA 3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>TRR1 primers</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward: 5’ GGGGGTTTCGGACTGGAAAA 3</td>
</tr>
<tr>
<td>Reverse: 5’ GCTGGAGTCGTATCGCATGA 3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>TRX1 primers</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward: 5’ ACTTGGTGCGGTCCATGTAA 3</td>
</tr>
<tr>
<td>Reverse: 5’ TTGGCACCAACAACCTTTGC 3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>TRX2 primers</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward: 5’ AGTGCTTTAGCATCTGGCGA 3</td>
</tr>
<tr>
<td>Reverse: 5’ CTGGTAACCTCCTTACCGCC 3</td>
</tr>
</tbody>
</table>

The reaction setup for each sample contained the following: 10μL of SYBR GreenER® qPCR Supermix, 0.4 μL each of forward and reverse primers, 0.4 μL of ROX reference dye, 1 μL of the cDNA template, and RNA and DNA free water to a volume of 20μL. Samples were added to a 96 well plate and then sealed and placed in the Applied Biosystems 7500 real time PCR machine. The program used had a 2 minute incubation at 50°C, followed by 10 minutes at 95°C, and 40 cycles of 15 seconds at 90°C and 1 minute at 60°C. PCR reactions were performed in triplicates.
3.1.6 Analysis of RNA quantity

A $\Delta C_T$ value was obtained by subtracting the respective gene $C_T$ from the $C_T$ value of the constitutive housekeeping gene ($ACT1$). Therefore, the relative mRNA levels were expressed as $2^{\Delta C_T}$. Data results were graphed as a mean of the repeats ±standard deviation.

3.1.7 Acetic Acid Sensitivity Growth Analysis

Using the yeast strains listed in Table 3.1, growth analysis was performed under varying concentrations of acetic acid. Yeast strains were grown in synthetic complete media with 100 μM of inositol over night at 30°C and subsequently washed and diluted to $A_{600nm}$ 0.2. Cells from both strains were placed in medium containing either 0mM, 5 mM, 10mM, 25mM, 50mM and 100mM acetic acid and grown at 30°C and 300 RPM. Growth of the cells was measured spectrophotometrically using $A_{600nm}$ and were measured every two hours for the first eight hours and then again at the 24th hour until stationary phase was reached. Calculations to analyze data were the same as 3.1.1 growth analysis.

3.1.8 Cell Survival and Serial Dilutions for Acetic Acid Sensitivity Assay

Yeast strains from Table 3.1 were grown in synthetic complete media with 100 μM of inositol over night at 30°C. For acetic acid cell survival and sensitivity, cells were spun down and diluted to an $A_{600nm}$ of 1.0 and placed in pH 3 SC media containing either 0mM acetic acid, 80mM acetic acid, and 120mM acetic acid (methods followed from Agimon; 2013) and grown at 30°C, 300 RPM, for 200 minutes. Cells were collected at the following time points: 0 minutes, 90 minutes, 200 minutes. At each time point, all cells were diluted to $A_{600nm}$ of 1.0 and then
serially diluted by performing three 100-fold serial dilutions \((10^6)\). 15μL of cells were then plated on YPD plates for each strain and each time point from the \(10^6\) dilution. After 200 minutes, instead of three 10-fold serial dilutions, five 10-fold serial dilutions were performed. 15μL of cells were still plated on YPD plates from the \(10^6\) dilution, but on a separate YPD plate, a 5μL droplet of each dilution (starting from \(10^{-2}\) to \(10^{-10}\)) was plated as well for each condition. Plates were placed in a 30°C incubator for three days. Similar to calculating colony forming units (CFUs), cells were counted on each plate to determine cell survival and viability. Three to four repeats were conducted per condition and then averaged \(\pm\) standard deviation. Mean data was converted into percentages and plotted as cell survival against time. Serial dilution plates were photographically captured.

### 3.1.9 Cell Survival and Serial Dilutions for Hydrogen Peroxide Sensitivity Assay

Yeast strains from Table 3.1 were grown in synthetic complete media with 100 μM of inositol over night at 30°C. For hydrogen peroxide exposed cell survival and sensitivity, cells were spun down and diluted to an \(A_{600nm}\) of 1.0 and placed in synthetic complete media with 100 μM of inositol that contained either 0mM \(H_2O_2\) or 1mM \(H_2O_2\) (Agimoni et al., 2016) and grown at 30°C, 300 RPM, for 4 hours. Cells were collected at the following time points: Hour 0, Hour 2, and Hour 4. At each time point, all cells were diluted to \(A_{600nm}\) of 1.0 and then serially diluted by performing three 10-fold serial dilutions \((10^6)\). 15μL of cells were then plated on YPD plates for each strain and each time point from the \(10^6\) dilution. After 4 hours, instead of three 100-fold serial dilutions, five 100-fold serial dilutions were performed. 15μL of cells were still plated on YPD plates from the \(10^6\) dilution, but on a separate YPD plate, a 5μL droplet of each dilution
(starting from $10^2$ to $10^{10}$) was plated as well for each condition. Plates were placed in a 30°C incubator for three days. Similar to calculating colony forming units (CFUs), cells were counted on each plate to determine cell survival and viability. Three to four repeats were conducted per condition and then averaged ± standard deviation. Mean data was converted into percentages and plotted as cell survival against time. Serial dilution plates were photographically captured.
3.2 Results

3.2.1 The \textit{pah1Δ} mutant exhibits a greater growth pattern than WT cells, with the greatest difference seen under conditions of high concentrations of inositol and choline present.

Results showed that in media containing neither inositol nor choline, both WT and \textit{pah1Δ} grew well, with the WT strain reaching an average optical density (OD) $A_{600nm}$ at hour 24 of approximately 5.3 ± 0.28. The \textit{pah1Δ} mutant however had a slightly higher growth than WT at hour 24 with an average optical density of 5.8 ± 0.14, and grew 5.40% ± 3.56 better than WT (Figure 3.1A; Table 3.3; Table 3.4). This percent difference in growth however was not significant ($p=0.15$). For cells grown in media that contained 10µM of inositol and 0µM of choline the growth difference was even greater between WT and the \textit{pah1Δ} mutant, with a percent growth difference of 7.75% ± 0.71 (Figure 3.1E, Table 3.4), though again the difference in growth was not significant ($p=0.185$). The biggest change in growth however occurred between the two strains when there was at least 100µM of inositol or 100µM of choline in the media. For WT cells grown in media containing 100µM of inositol and 100µM of choline, the average OD was about 5.4 ± 0.14, while the average OD of the \textit{pah1Δ} mutant cells was 6.5 ± 0.35, causing \textit{pah1Δ} mutant cells to have a significant difference in growth that was 10.8% ± 1.66 than WT ($p=0.05$; Figure 3.1B; Table 3.3; Table 3.4). Similar differences in growth were observed for cells grown in media that contained just one of crucial phospholipid precursors at the 100µM concentration level. The \textit{pah1Δ} strain grew 11.7% ± 1.17 better than WT when grown in media containing 100µM choline and 0µM inositol, and 11.6% ± 3.61 better when grown in media containing 100µM choline and 10µM inositol (Figures 3.1D, 3.1F; Table 3.4). Interestingly though, only the latter was significant, signifying the importance of inositol in the media. The biggest percent growth difference that was significant occurred when cells were
grown in media containing 100µM inositol and 0µM choline. The average OD of WT cells at hour 24 was 5.3 ± 0.42, while pah1Δ mutant cells had an average OD of 6.7 ± 0.07, which led to an increase of 12.3% ± 0.46 in growth and a p value of p=0.047 (Figure 3.1C; Table 3.1; Table 3.4).

Consistent with our results, when the number of generations per 24 hour period was calculated, along with the generation times, pah1Δ strain had a higher number of generations and an overall faster generation time than WT. For instance, for cells grown in media that had neither inositol nor choline present, WT had 4.27 generations in 24 hours and a generation time of 337.32 minutes per generation compared to the 4.48 generations of the pah1Δ strain and a generation time of 321.43 minutes/generation (Table 3.3). For cells grown in media containing 100µM inositol and 0µM choline the number of generations for WT was 4.35 generations/24 hours and for pah1Δ was 4.95 generations/24 hours. The generation time for WT in this condition was 331.05 minutes/generation while for pah1Δ was 290.82 minutes per generation. Taken together, these results demonstrated that the pah1Δ strain exhibited a higher growth pattern than WT in all conditions tested, but showed an even greater difference when higher concentrations of inositol and choline were present.
Figure 3.1: The *pah1Δ* mutant exhibits higher growth patterns compared to WT in all conditions tested, with significant differences for conditions that contain 100 μM inositol or 100 μM choline with 10 μM inositol.

WT and *pah1Δ* cells were grown in 10μM inositol SC media and were then washed and diluted to an Absorbance$_{600nm}$ of 0.2 in SC media containing various concentrations of inositol and choline. Optical density ($A_{600nm}$) was read for first 8 hours of exponential phase and then resumed at 21 hours and taken until stationary phase was reached.
WT and \textit{pah1Δ} mutant exhibit no significant difference of growth between growth conditions, except for the \textit{pah1Δ} mutant between the 0 μM inositol and 100 μM inositol conditions. WT and \textit{pah1Δ} cells were grown in 10μM inositol SC media and were then washed and diluted to an Absorbance of 0.2 in SC media containing various concentrations of inositol and choline. Optical density (A$_{600nm}$) was read for first 8 hours of exponential phase and then resumed at 21 hours and taken until stationary phase was reached.
<table>
<thead>
<tr>
<th>Condition</th>
<th>Average O.D WT (24th Hour)</th>
<th>Average O.D pah1Δ (24th Hour)</th>
<th># of generations in 24 hours for WT</th>
<th># of generations in 24 hours for pah1Δ</th>
<th>Generation Time (min/generation) for WT</th>
<th>Generation Time (min/generation) for pah1Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0µM Inositol, 0µM Choline</td>
<td>5.3 ± 0.28</td>
<td>5.8 ± 0.14</td>
<td>4.27</td>
<td>4.48</td>
<td>337.32</td>
<td>321.43</td>
</tr>
<tr>
<td>100µM Inositol, 100µM Choline</td>
<td>5.4 ± 0.14</td>
<td>6.5 ± 0.35</td>
<td>4.38</td>
<td>4.91</td>
<td>329.01</td>
<td>293.44</td>
</tr>
<tr>
<td>100µM Inositol, 0µM Choline</td>
<td>5.3 ± 0.42</td>
<td>6.7 ± 0.07</td>
<td>4.35</td>
<td>4.95</td>
<td>331.05</td>
<td>290.82</td>
</tr>
<tr>
<td>0µM Inositol, 100µM Choline</td>
<td>4.5 ± 1.8</td>
<td>5.9 ± 1.6</td>
<td>4.55</td>
<td>4.68</td>
<td>316.54</td>
<td>307.6</td>
</tr>
<tr>
<td>10µM Inositol, 0µM Choline</td>
<td>4.6 ± 0.35</td>
<td>5.8 ± 0.78</td>
<td>4.08</td>
<td>4.61</td>
<td>353.35</td>
<td>312.14</td>
</tr>
<tr>
<td>10µM Inositol, 100µM Choline</td>
<td>4.8 ± 0.28</td>
<td>6.4 ± 0.21</td>
<td>4.18</td>
<td>4.53</td>
<td>344.55</td>
<td>317.9</td>
</tr>
</tbody>
</table>

Table 3.4: *pah1Δ* vs WT Percent Difference in Growth

<table>
<thead>
<tr>
<th>Condition</th>
<th>Percent Growth Difference between <em>pah1Δ</em> and WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>0µM Inositol, 0µM Choline</td>
<td>5.40% ± 3.56</td>
</tr>
<tr>
<td>100µM Inositol, 100µM Choline</td>
<td>10.8% ± 1.66  (<em>p</em>=0.05)</td>
</tr>
<tr>
<td>100µM Inositol, 0µM Choline</td>
<td>12.3% ± 0.46  (<em>p</em>=0.047)</td>
</tr>
<tr>
<td>0µM Inositol, 100µM Choline</td>
<td>11.7% ± 1.17</td>
</tr>
<tr>
<td>10µM Inositol, 0µM Choline</td>
<td>7.75% ± 0.71</td>
</tr>
<tr>
<td>10µM Inositol, 100µM Choline</td>
<td>11.6% ± 3.61  (<em>p</em>=0.025)</td>
</tr>
</tbody>
</table>
3.2.2 The *pah1Δ* strain has higher gene expression for UAS<sub>INO</sub> genes and some non UAS<sub>INO</sub> genes, thus exhibiting the importance of Pah1p as a negative regulator, while having the reverse effect for the *HXK2* gene.

Growth analysis suggested that *pah1Δ* exhibits greater differences from WT when grown in the presence of 100μM of inositol. In order to better elucidate the role of *PAH1* on gene regulation, we decided to examine its impact on closely related genes in the lipid biosynthetic pathway. The effect of *PAH1* on other genes has only been minimally tested and most importantly not on all genes involved in inositol synthesis. While *INO1* has been studied previously to determine the impact that *PAH1* has on it, other genes in the same pathway have not (Santos-Rosa *et al*., 2005). Furthermore, *INO1* expression has yet to be examined in a study, which tests both the importance of *PAH1* as well as the crucial phospholipid precursor, inositol, together on its gene expression. In order to test this, we decided to perform a time course RNA analysis study. Since the presence of inositol led to the greatest difference in growth analysis, our RNA analysis was carried out by introducing inositol to the cells and collecting those cells throughout their growth process at designated time points (0, 60 and 120 minutes). The mRNA from the WT and *pah1Δ* strains was then used as a template to be converted into cDNA and subsequently quantified using qRT-PCR, with primers targeting the open reading frame of the genes being tested. Genes chosen for this analysis included those in the lipid biosynthetic pathway that were nearby to *PAH1*. These genes included *PIS1, CDS1, DPP1, LPP1, APP1, TGL3, TGL4, TGL5,* and *DGA1*. Genes that are involved in *de novo* inositol synthesis were also tested. These included *HXK2, INO1,* and *INM1*. Lastly, our results were normalized using the constitutive housekeeping gene, *ACT1*, which acted as our internal control. However, we used an additional control, *POL1*, another constitutive housekeeping gene, to serve as an external control. Thus, *POL1* gene expression was tested as well.
Our results showed and confirmed that PAH1 does in fact negatively regulate UAS\textsubscript{INO} genes, including all the novel UAS\textsubscript{INO} genes tested (Figure 3.3). Perhaps one of the most regulated UAS\textsubscript{INO} genes by PAH1 is INO1 (Figure 3.3A). As can be seen, INO1 expression is significantly higher in pah1Δ cells than in WT for all time points collected, heavily suggesting that Pah1p acts as a negative regulator when present. Starting from time point 0 minutes, INO1 expression in pah1Δ is about double that of WT (0.271 ± 0.02 and 0.546 ± 0.016; \( p < 0.01 \)). Under normal circumstances, the addition of inositol should repress the transcription of INO1, since the cell no longer requires the production of inositol as it is now available in the environment. This is clearly seen in WT, with INO1 expression plummeting drastically (0.007±0.0003) after 60 minutes of exposure to inositol (Figure 3.3A). While there is a decrease in INO1 expression in pah1Δ at 60 minutes compared to 0 minutes, there is still a relatively high level of INO1 expression, which is comparable to levels in WT at time point 0 minutes (0.20±0.04). This suggests that while other regulatory machinery is still employed to help suppress INO1 expression, the deletion of PAH1 omits a key component that is needed for INO1 repression. Thus, it still exhibits INO1 mRNA after exposure to inositol that is comparable to levels found in WT without the addition of inositol. The difference of expressions levels between both strains is significant at 60 minutes \( (p=0.002) \). The same effect is still seen after 120 minutes with INO1 expression levels in WT being significantly lower than those of pah1Δ (0.009±0.002 and 0.21±0.016; \( p < 0.01 \)) as well (Figure 3.3A).

While the repressing regulatory role of PAH1 is most clearly seen with INO1, the effect is similar with other UAS\textsubscript{INO} genes tested as well. Genes such as PIS1, CDS1, and INM1, all of which contain a UAS\textsubscript{INO} have significantly higher levels of expression, at all tested time points, in pah1Δ cells than in WT cells (Figure 3.3B, 3.3C, and 3.3D). The TGL4 gene, which is one of
the genes responsible for converting triacylglycerols back into diacylglycerols, also has significantly higher expression levels in \( pah1\Delta \) cells than in WT (Figure 3.3E), strongly suggesting that \( PAH1 \) does not only regulate \( \text{UAS}_{\text{INO}} \) genes, but those without \( \text{UAS}_{\text{INO}} \) as well. However, it should be noted that most non-\( \text{UAS}_{\text{INO}} \) genes tested in this study did not have a significant difference between gene expression in WT and \( pah1\Delta \). These genes included \( LPP1, DPP1, APP1, TGL3, TGL5, \) and \( DGA1 \) (Figures 3.4). Therefore while \( PAH1 \) can affect gene expression of other genes, its main impact is on genes that contain a \( \text{UAS}_{\text{INO}} \) since all genes with a \( \text{UAS}_{\text{INO}} \) that were tested showed significant upregulation compared to WT. Additionally we also did RNA analysis on \( POL1 \), which acted as our external control. \( POL1 \) is a constitutive housekeeping gene and thus should not exhibit any changes between WT or \( pah1\Delta \), nor should it change in response to inositol. As can be seen, \( POL1 \) expression remained the same for all strains and time points collected after introduction of inositol (Figure 3.4G), thus acting as the control gene for this experiment.

Another interesting observation that was noted was that the \( pah1\Delta \) strain did not always respond to the addition of inositol the way that WT did. Most genes in the lipid biosynthetic pathway will either upregulate or downregulate in response to the addition of inositol. For instance, as was noted earlier, the \( INO1 \) gene will greatly downregulate in response to inositol, which was an effect we saw in both WT and \( pah1\Delta \), to varying degrees. The expression level pattern in response to inositol of other genes that we tested were also consistent with previous findings (Wimalarathna et al., 2011), including \( INM1 \) (upregulated), \( CDS1 \) (downregulated), and \( TGL3 \) (downregulated). For other genes, however, \( pah1\Delta \) did not necessarily respond to inositol the same way that WT did. For example, \( PIS1 \) should become upregulated in the presence of inositol (Wimalarathna et al., 2011). The results that we collected for WT was in accordance
with that trend and upregulated, as the *PIS1* mRNA levels increased significantly after 60 and 120 minutes \( (p=0.013; \ p=0.017) \). However, the mRNA levels of *pah1Δ* did not significantly increase in response to inositol (Figure 3.3B). While this phenomenon was not seen in most genes tested, it was seen in a few (i.e., *PIS1*, Figure 3.3B and *TGL4*, Figure 3.3E). This suggests that the effect that the inositol would usually play in gene expression is canceled out by the constant upregulation that the *pah1Δ* causes. Thus, in genes like *INO1*, which is involved in the rate limiting step of de novo inositol synthesis (Greenberg and Lopes, 1996) there would be multiple modes of regulation, with *PAH1* being just one method of repression. However there may be other genes that do not possess as many modes of regulation and whose main regulatory system relies heavily upon the repressive role of *PAH1*, thus being dependent on its function. Therefore, for the genes that lack the Pah1p repressor, their constant upregulation would be indifferent to the effects of inositol or other precursors.

Perhaps one of the most surprising results from the RNA analysis we discovered was regarding the *HXK2* gene, a gene that codes for hexokinase-2 protein during growth on glucose, and which is necessary for the production of inositol (Moreno and Herrero, 2002). As was mentioned earlier, aside for the *INO1* gene, the other genes necessary for the production of inositol has yet to be looked into in regard to their expression levels in *pah1Δ* cells. We therefore looked into expression of *INM1* (as explained above) and *HXK2* in the *pah1Δ* mutant strain since both are crucial genes needed for *de novo* inositol synthesis (Figure 1.3). Being a UAS*INO* gene, *INM1* followed the pattern of *INO1*, in that its expression level was higher in *pah1Δ* than in WT. While *HXK2* does not contain a UAS*INO*, we were curious to see if its expression would still be higher in *pah1Δ* than in WT since it plays such a crucial role in inositol production. While we hypothesized that it would be probable to see an increased expression level of *HXK2* in *pah1Δ*
compared to WT, it would not be surprising if levels remained comparable to each other since the majority of non-\(\text{UAS}_{\text{INO}}\) genes did not show a significant difference between the strains. Results showed that at 0 minutes, \(\text{HXK2}\) levels were in fact comparable between WT cells and \(\text{pah1}\Delta\) cells and were not significantly different (0.227±0.041 and 0.185±0.038; \(p=0.398\)). However, with the addition of inositol, the expression of \(\text{HXK2}\) increased with time in the WT strain after 60 minutes and 120 minutes (Figure 3.5). This was expected since \(\text{HXK2}\) had previously been determined to upregulate in response to inositol (Wimalarathna et al., 2011). \(\text{HXK2}\) levels however did not change in the \(\text{pah1}\Delta\) cells as they did in WT. While this phenomenon of \(\text{pah1}\Delta\) cells not following the same response to inositol as WT was seen with other genes (i.e. \(\text{PIS1, TGL4}\)), this situation was unique in that WT had higher gene expression compared to \(\text{pah1}\Delta\). Stated differently, in all the other genes we tested there was either no significant difference between WT and \(\text{pah1}\Delta\) or, \(\text{pah1}\Delta\) cells always exhibited higher gene expression levels. In this case, however, \(\text{HXK2}\) gene expression was vastly greater in WT. At 60 minutes, \(\text{HXK2}\) expression levels in WT was more than quadruple that in \(\text{pah1}\Delta\) (0.673±0.037 and 0.161±0.003; \(p=0.003\)) and at 120 minutes, it was almost triple the amount (0.416±0.041 and 0.169±0.04; \(p=0.026\)). Thus, \(\text{HXK2}\) is the sole gene in this study that actually had expression levels lower in \(\text{pah1}\Delta\) than in WT.
**INO1 Gene Expression**

- **Normalized mRNA level (INO1/ACT1)**
  - **WT**
  - **pah1Δ**

<table>
<thead>
<tr>
<th>Time</th>
<th>WT</th>
<th>pah1Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Min</td>
<td>0.5</td>
<td>0.2</td>
</tr>
<tr>
<td>60 Min</td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>120 Min</td>
<td>0.2</td>
<td>0.2</td>
</tr>
</tbody>
</table>

- *p* values:
  - 0.002
  - 0.004
  - 0.005
  - 0.002
  - 0.02
  - 0.003
**PIS1 Gene Expression**

- **Normalized mRNA Levels (PIS1/ACT1)**
- **p** = 0.03
- **p** = 0.013
- **p** = 0.044
- **p** = 0.031
- **p** = 0.017
CDS1 Gene Expression

Normalized mRNA Level (CDS1/ACT1)

WT  pah1Δ

0 Min  60 Min  120 Min

p=0.02  p=0.05  p=0.046

p=0.006  p=0.038
**INM1 Gene Expression**

Normalized mRNA Level (INM1/ACT1)

- **WT** and **pah1Δ**
  - 0 Min: $p = 0.036$
  - 60 Min: $p = 0.01$
  - 120 Min: $p = 0.044$

$\bar{p}$ values indicate statistical significance in gene expression levels between wild type (WT) and pah1Δ strains at different time points.
Figure 3.3: The \textit{pah1\Delta} mutant exhibits an upregulation in gene expression of UAS\textsubscript{INO} genes and some non-UAS\textsubscript{INO} genes compared to WT.

WT cells were grown to mid logarithmic phase (1.0±0.2 \textit{A}\textsubscript{600nm}) in 10\textmu M inositol SC media and were then washed and grown in SC media that contained 100\textmu M inositol. mRNA was then extracted from cells using acid phenol chloroform and purified further with treatment of DNase. RNA was converted to cDNA and was amplified/quantified using qPCR with primers targeting the open reading frame sequence of tested gene. Expression levels of mRNA were normalized to the housekeeping gene \textit{ACT1}. All experiments were done with at least two repeat colonies and all qPCR reactions were done in triplicates. Data was graphed as a mean ± standard deviation.
A)

**LPP1 Gene Expression**

<table>
<thead>
<tr>
<th>Time</th>
<th>WT</th>
<th>pah1Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 Min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>120 Min</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Normalized mRNA Level (LPP1/ACT1)
### APP1 Gene Expression

<table>
<thead>
<tr>
<th>Time</th>
<th>WT</th>
<th>pah1Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 Min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>120 Min</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Normalized mRNA Levels (APP1/ACT1)

- Graph showing expression levels for WT and pah1Δ strains at 0, 60, and 120 minutes.
D)

**TGL3 Gene Expression**

<table>
<thead>
<tr>
<th>Time</th>
<th>WT</th>
<th>pah1Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Min</td>
<td>0.006</td>
<td>0.005</td>
</tr>
<tr>
<td>60 Min</td>
<td>0.004</td>
<td>0.003</td>
</tr>
<tr>
<td>120 Min</td>
<td>0.006</td>
<td>0.005</td>
</tr>
</tbody>
</table>

*p* = 0.022, *p* = 0.029, *p* = 0.035
E)

**TGL5 Gene Expression**

![Bar chart showing normalized mRNA levels (TGL5/ACT1) for WT and pah1Δ strains at 0 Min, 60 Min, and 120 Min.](chart)

- **0 Min**
  - WT: ~0.004
  - pah1Δ: ~0.004

- **60 Min**
  - WT: ~0.008
  - pah1Δ: ~0.008

- **120 Min**
  - WT: ~0.006
  - pah1Δ: ~0.006

Normalized mRNA Levels (TGL5/ACT1)
**DGA1 Gene Expression**

![Graph showing DGA1 Gene Expression](image)

- **Normalized mRNA Levels (DGA1/ACT1)**

- **Conditions**:
  - WT
  - pah1Δ

- **Time Points**:
  - 0 Min
  - 60 Min
  - 120 Min
Figure 3.4: The pah1Δ mutant exhibits similar gene expression levels to WT for most non-UASINO genes.

WT cells were grown to mid logarithmic phase (1.0±0.2 A₆₀₀nm) in 10μM inositol SC media and were subsequently washed and grown in SC media that contained 100μM inositol. mRNA was then extracted from cells using acid phenol chloroform and purified further with treatment of DNase. RNA was converted to cDNA and was amplified/quantified using qPCR with primers targeting the open reading frame sequence of tested gene. Expression levels of mRNA were normalized to the housekeeping gene ACT1. All experiments were done with at least two repeat colonies and all qPCR reactions were done in triplicates. Data was graphed as a mean ± standard deviation.
**Figure 3.5**: *HXK2* gene expression remains constant in the *pah1Δ* mutant strain and does not up-regulate in response to inositol as does WT.

WT cells were grown to mid logarithmic phase (1.0±0.2 A$_{600}$nm) in 10µM inositol SC media and were subsequently washed and grown in SC media that contained 100µM inositol. mRNA was then extracted from cells using acid phenol chloroform and purified further with treatment of DNase. RNA was converted to cDNA and was amplified/quantified using qPCR with primers targeting the open reading frame sequence of tested gene. Expression levels of mRNA were normalized to the housekeeping gene *ACT1*. All experiments were done with at least two repeat colonies and all qPCR reactions were done in triplicates. Data was graphed as a mean ± standard deviation.
3.2.3 The \textit{pah1}\Delta mutant exhibits higher tolerance to acetic acid induced apoptosis than WT.

While nearly all genes tested during RNA analysis were either upregulated or remained at similar levels to wildtype, there was one gene that did not upregulate or remain at similar levels to WT with the introduction of inositol. Our mRNA analysis showed that \textit{HXXK2} expression was significantly reduced in \textit{pah1}\Delta cells compared to WT when inositol was introduced. While \textit{HXXK2} levels increased in wildtype after induction with inositol, the levels did not in \textit{pah1}\Delta cells. \textit{HXXK2} encodes for the hexokinase-2-protein during growth on glucose (Moreno and Herrero, 2002). It plays a crucial role in glucose metabolism since it’s responsible for phosphorylating glucose at C6 which can be further phosphorylated in the glycolytic pathway, or can be converted into inositol-3-phosphate in the phospholipid biosynthetic pathway.

Furthermore, other research has shown that \textit{HXXK2} may play a role in protecting cells against apoptosis since previous studies have shown that \textit{hxk2}\Delta cells demonstrated acetic acid hypersensitivity (Amigoni; 2013). Therefore, we hypothesized that since \textit{HXXK2} levels remained significantly lower in \textit{pah1}\Delta cells compared to wildtype it would be more susceptible to acetic acid induced apoptosis than wildtype.

Our results showed that WT cells grew well under normal conditions in SC media. As we began to introduce different concentrations of acetic acid into the growth media WT cells survival began to change (Figure 3.5). At 5mM, 10mM, and 25mM concentrations of acetic acid, growth rate decreased to 3.58\% ± 4.07, 12.05\% ± 0.07, and 21.08\% ± 3.35, respectively (Table 3.5). WT cells were unable to grow anymore at a 50mM concentration of acetic acid. For \textit{pah1}\Delta cells the trend of cell growth was similar to WT when higher concentrations of acetic acid were introduced (Figure 3.7). At 5mM, 10mM, and 25mM concentrations of acetic acid, the growth
rate decreased to 2.18% ± 1.82, 9.86% ± 1.76, and 27.59% ± 7.95, respectively (Table 3.6).

There was no significant difference between the 5mM, 10mM, and 25mM growth drop between WT and pah1Δ. However unlike WT cells, at the 50mM acetic acid concentration pah1Δ cells were able to grow to a small degree. Their growth rate dropped to 94.69% ± 0.26 compared to 0mM, but this was statistically significant (p<0.01) compared to the 100% decrease in growth of WT.

These results were therefore surprising, since pah1Δ cells seemed to grow even better than wildtype despite the fact that HXK2 levels were diminished in pah1Δ. In order to confirm this finding further, we decided to approach this question differently and characterize the relationship of pah1Δ cells to apoptosis with a more sensitive assay. Instead of measuring cell growth in the presence of acetic acid, we decided to assess cell sensitivity by performing a “death curve” and looking at cell survival (Amigoni; 2013). This would more accurately portray the apoptotic effect of acetic acid. In addition, cells would only be exposed for 90 minutes and 200 minutes at higher concentrations of acetic acid (80mM and 120mM) and then be grown on regular YNB plates to assess their recovery.

Our results confirmed our initial observation, but to an even greater extent. Upon just an initial glance at the growth plates containing acetic acid exposed cells, there were an overwhelmingly greater number of pah1Δ colonies than WT (Figure 3.9). This was seen for both cells exposed to 80mM acetic acid and 120mM acetic acid. Though they were smaller colonies (which is consistent with growth rate of pah1Δ on plates), the number of colonies for pah1Δ could be seen to far outnumber WT even before quantifying the results. Once quantified, the data showed that pah1Δ cells survived significantly better than WT. At 0mM concentration of acetic acid, both WT and pah1Δ exhibited a similar trend in cell survival as was to be expected. WT
cell number increased from 100% to 143.2%, while \textit{pah1Δ} increased from 100% to 146.1% after 90 minutes. Both cells declined slightly after 200 minutes, but at a comparable rate with cells being at 86.6% for WT and 78.3% for \textit{pah1Δ}, respectively (Figure 3.10C). At an exposure of 80mM acetic acid, cell survival differed drastically between both strains. After only 90 minutes, WT cell survival plummeted from 100% down to only 26.03% and after 200 minutes, it decreased to 2.05% (Figure 3.10A). Exposure to 120mM acetic acid yielded even stronger declines in cell survival for WT. At 90 minutes, WT cell survival went down 6.85% and after 200 minutes, it decreased to 0.68% (Figure 3.10A). The \textit{pah1Δ} cells however fared much better when exposed to acetic acid. At 80mM acetic acid, cells actually grew after 90 minutes of exposure going from 100% to 122.9% and then dropping in number after 200 minutes of exposure down to 109.6% (Figure 3.10B). At 120mM, the exposure to acetic acid had a more drastic effect on \textit{pah1Δ} but was still significantly better than WT, with cells dropping down to 65.2% after 90 minutes and 23.7% after 200 minutes (Figure 3.10B). Thus \textit{pah1Δ} cells seem much more likely to withstand acetic acid induced apoptosis than WT.

These results were further confirmed with our serial dilution sensitivity assay. This assay was performed at the end of our experiment, in which the cells that had been growing for 200 minutes in various concentrations of acetic acid were diluted to the same OD and then plated in five 100-fold dilutions. At 0mM acetic acid, both WT and \textit{pah1Δ} grew at comparable rates and both had nice cell growth visible at $10^{-8}$ dilution (Figure 3.11A). At 80mM acetic acid, \textit{pah1Δ} still grew well, with the last visible growth still seen at the $10^{-8}$ dilution but on a much smaller scale than had been at 0mM. The last visible growth for WT cells at this concentration was at the $10^{-6}$ dilution and again, cell growth was minimal (Figure 3.11B). Lastly at 120mM acetic acid, \textit{pah1Δ} cells grew nicely all the way to the $10^{-6}$ dilution. The last visible growth for WT was only
at the $10^2$ dilution (Figure 3.11C). Taken together, these experiments show that the \textit{pah1Δ} mutant can survive at much higher concentrations of acetic acid than can WT. These finding are extremely interesting since \textit{pah1Δ} has lower expression levels of \textit{HXK2}, a gene that has been shown to be involved in protecting against apoptosis. Despite lower \textit{HXK2} expression levels than WT cells grown in SC media with inositol, \textit{pah1Δ} is greater at withstanding apoptosis than WT.
Figure 3.6: WT growth diminishes with increasing concentrations of acetic acid.

WT and *pah1A* cells were grown in SC+ media and were then washed and diluted to an Absorbance <sub>600nm</sub> of 0.2 in SC media containing various concentrations of acetic acid. Optical density (A<sub>600nm</sub>) was read for the first 8 hours of exponential phase and then was resumed at 24 hours and read until stationary phase was reached.

Table 3.5: WT Percent Difference in Growth at 24<sup>th</sup> Hour

<table>
<thead>
<tr>
<th>WT: Acetic Acid Concentration Comparison</th>
<th>Decrease in growth rate %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0mM to 5mM</td>
<td>3.58% ± 4.07</td>
</tr>
<tr>
<td>0mM to 10mM</td>
<td>12.05% ± 0.07</td>
</tr>
<tr>
<td>0mM to 25mM</td>
<td>21.08% ± 3.35</td>
</tr>
<tr>
<td>0mM to 50mM</td>
<td>100% ± 0.00</td>
</tr>
<tr>
<td>0mM to 100mM</td>
<td>100% ± 0.00</td>
</tr>
</tbody>
</table>
Figure 3.7: *pah1Δ* growth diminishes with increasing concentrations of acetic acid.

*WT* and *pah1Δ* cells were grown in SC+ media and were then washed and diluted to an Absorbance_{600nm} of 0.2 in SC media containing various concentrations of acetic acid. Optical density (A_{600nm}) was read for the first 8 hours of exponential phase and then was resumed at 24 hours and read until stationary phase was reached.

Table 3.6: *pah1Δ* Percent Difference in Growth at 24th Hour

<table>
<thead>
<tr>
<th><em>pah1Δ</em>: Acetic Acid Concentration Comparison</th>
<th>Decrease in growth rate %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM</td>
<td>2.18% ± 1.82</td>
</tr>
<tr>
<td>0 mM</td>
<td>9.86% ± 1.76</td>
</tr>
<tr>
<td>0 mM</td>
<td>27.59% ± 7.95</td>
</tr>
<tr>
<td>0 mM</td>
<td>94.69% ± 0.26</td>
</tr>
<tr>
<td>0 mM</td>
<td>100% ± 0.00</td>
</tr>
</tbody>
</table>
Figure 3.8: WT and pah1Δ cell growth diminishes with increasing concentrations of acetic acid.

WT and pah1Δ cells were grown in SC+ media and were then washed and diluted to an Absorbance$_{600\text{nm}}$ of 0.2 in SC media containing various concentrations of acetic acid. Optical density (A$_{600\text{nm}}$) was read for the first 8 hours of exponential phase and then was resumed at 24 hours and read until stationary phase was reached.

Table 3.7: WT and pah1Δ Growth Experiment 24th Hour Analyses

<table>
<thead>
<tr>
<th>Condition</th>
<th>Average O.D WT (24th Hour)</th>
<th>Average O.D pah1Δ (24th Hour)</th>
<th># of generations in 24 hours for WT</th>
<th># of generations in 24 hours for pah1Δ</th>
<th>Generation Time (min/generation) for WT</th>
<th>Generation Time (min/generation) for pah1Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0mM Acetic Acid</td>
<td>5.2 ± 0.0</td>
<td>6.6 ± 0.0</td>
<td>4.700908133</td>
<td>4.940549753</td>
<td>306.3237909</td>
<td>291.4655397</td>
</tr>
<tr>
<td>5mM Acetic Acid</td>
<td>4.3 ± 0.28</td>
<td>6.0 ± 0.57</td>
<td>4.533883972</td>
<td>4.836983241</td>
<td>317.6084807</td>
<td>297.7062206</td>
</tr>
<tr>
<td>10mM Acetic Acid</td>
<td>3.3 ± 0.28</td>
<td>4.6 ± 0.14</td>
<td>4.157283095</td>
<td>4.45361402</td>
<td>346.3800678</td>
<td>323.332413</td>
</tr>
<tr>
<td>25mM Acetic Acid</td>
<td>2.45 ± 0.49</td>
<td>2.45 ± 0.35</td>
<td>3.727556</td>
<td>3.579442602</td>
<td>386.3121037</td>
<td>402.2972736</td>
</tr>
<tr>
<td>50mM Acetic Acid</td>
<td>0.21 ± 0.01</td>
<td>0.27 ± 0.03</td>
<td>0.072156976</td>
<td>0.263060618</td>
<td>19956.49049</td>
<td>5474.023479</td>
</tr>
<tr>
<td>100mM Acetic Acid</td>
<td>0.2 ± 0.0</td>
<td>0.18 ± 0.01</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 3.9: The pah1Δ mutant has greater cell survival to acetic acid induced apoptosis than WT.

WT and pah1Δ cellular growth on YPD plates after 0, 90 and 200 minute exposures to 80mM acetic acid. All cells were initially grown in synthetic complete medium with 100 μM inositol overnight and then diluted to an A600nm of 1.0 and grown in pH 3 SC media with appropriate concentration of acetic acid. Cells aliquots were collected at predetermined time points and diluted back again to a starting optical density (A600nm) of 1.0 (to ensure same cell number for each strain). Three serial dilutions were performed and plated from the 10⁶ dilution for each condition of each strain. YPD plates were grown for 3 days at 30°C. Experiments were repeated in triplicates.
A)

WT Acetic Acid Sensitivity

B)

pah1Δ Acetic Acid Sensitivity
Figure 3.10: The *pah1Δ* mutant has greater cell survival to acetic acid induced apoptosis than WT.

WT and *pah1Δ* cellular growth on YPD plates after 0, 90 and 200 minute exposures to 80mM acetic acid. All cells were initially grown in synthetic complete medium with 100 μM inositol overnight and then diluted to an $A_{600nm}$ of 1.0 and grown in pH 3 SC media with appropriate concentration of acetic acid. Cells aliquots were collected at predetermined time points and diluted back again to a starting optical density ($A_{600nm}$) of 1.0 (to ensure same cell number for each strain). Three serial dilutions were performed and plated from the $10^{-6}$ dilution for each condition of each strain. YPD plates were grown for 3 days at 30°C. Experiments were repeated in triplicates.
A) 0mM Acetic Acid

WT

pah1Δ

B) 80mM Acetic Acid

WT

pah1Δ
Figure 3.11: The pah1Δ mutant has less sensitivity to acetic acid and can withstand exposure to higher concentrations of acetic acid compared to WT.

WT and pah1Δ cellular growth on YPD plates after 200 minute exposure to (A) 0mM acetic acid, (B) 80mM acetic acid, (C) 120mM acetic acid. All cells were initially grown in synthetic complete medium with 100 μM inositol overnight and then diluted to an A600nm of 1.0 and grown in pH 3 SC media with 0, 80, or 120mM acetic acid. Cells were collected after 200 minutes and diluted back again to a starting optical density (A600nm) of 1.0 (to ensure same cell number for each strain). Five serial dilutions were performed and plated for each condition of each strain. YPD plates were grown for 3 days at 30°C. Plates were repeated in triplicates.
3.2.4 The \textit{pah1Δ} mutant exhibit higher tolerance to hydrogen peroxide induced apoptosis than WT.

Due to the surprising results that \textit{pah1Δ} cells were better at withstanding acetic acid induced apoptosis than WT despite having lower \textit{HXK2} levels in SC with 100 μM inositol media, we wanted to confirm that \textit{pah1Δ} cells were in fact able to resist apoptosis that was caused by other reagents as well. This would be done conducting the same experiment that was performed with acetic acid but with another apoptotic factor. We therefore decided to expose the cells to a well-known apoptotic reagent, hydrogen peroxide. Both WT and \textit{pah1Δ} were grown under normal conditions and then employed the death curve assay using hydrogen peroxide.

Results yielded similar outcomes in terms of the anti-apoptotic property of \textit{pah1Δ} cells compared to WT. Cell survival dropped drastically in WT cells when they were exposed to 1mM of hydrogen peroxide. This could be seen after just looking at the colony growth of both strains on the YPD plates (Figure 3.12). This was confirmed after quantification. After 2 hours of exposure to the reagent, WT cell survival went from 100% down to just 5.98%. After 4 hours of exposure, cells continued to die yielding only a 0.71% of survival. The \textit{pah1Δ} cells however fared much better. While exposure to hydrogen peroxide caused cell death, it was not as severe as WT. After 2 hours of exposure to the reagent, \textit{pah1Δ} cells went from 100% cell survival down to 21.4% and after 4 hours of exposure even recovered slightly and maintained a 34.5% survival (Figure 3.13A). Thus, after observing both strains after 4 hours of exposure to hydrogen peroxide, \textit{pah1Δ} cells significantly survived better (p=0.001) than WT (Figure 3.13B). These results were confirmed further with our serial dilution sensitivity assay. Both WT and \textit{pah1Δ} cells grew well and were both visible at the $10^{-10}$ dilution. However once these strains were exposed to 1mM hydrogen peroxide, WT was only seen at the $10^{-2}$ dilution, while \textit{pah1Δ} still
grew to the $10^{-10}$ dilution (Figure 3.14). Taken together, these results confirm that $pah1\Delta$ withstands hydrogen peroxide better than WT and is able to resist apoptosis better as well.
Figure 3.12: The \textit{pah1\Delta} mutant has greater cell survival to H$_2$O$_2$-induced apoptosis than WT after just 2 hours of exposure and even greater after 4 hours.

WT and \textit{pah1\Delta} cellular growth on YPD plates after 0, 2 or 4 hours of exposure to 1mM hydrogen peroxide. All cells were initially grown in synthetic complete medium with 100 μM inositol overnight and then diluted to an A$_{600\text{nm}}$ of 1.0 and grown in SC media with 100 μM inositol and with or without the addition of hydrogen peroxide. Cell aliquots were collected at predetermined time points and diluted back again to a starting optical density (A$_{600\text{nm}}$) of 1.0. Three serial dilutions were performed and plated from the $10^{-6}$ dilution for each condition of each strain. YPD plates were grown for 3 days at 30°C. Plates were repeated in triplicates.
A)

Cell Survival to Exposure of Hydrogen Peroxide

- WT 0mM
- pah1Δ 0mM
- WT 1mM
- pah1Δ 1mM

Cell Survival (%)

Time (Hour)
Figure 3.13: The \textit{pah1\Delta} mutant has less sensitivity to hydrogen peroxide and can withstand longer exposure compared to WT.

WT and \textit{pah1\Delta} cellular growth on YPD plates after 0, 2 and 4 hours exposure to 0mM and 1mM acetic acid. All cells were initially grown in synthetic complete medium with 100 μM inositol overnight and then diluted to an $A_{600nm}$ of 1.0 and grown in SC+ media with appropriate concentration of acetic acid. Cells aliquots were collected at predetermined time points and diluted back again to a starting optical density ($A_{600nm}$) of 1.0 (to ensure same cell number for each strain). Three serial dilutions were performed and plated from the $10^6$ dilution for each condition of each strain. YPD plates were grown for 3 days at 30°C. Experiments were repeated in at least triplicates.
Figure 3.14: The pah1Δ mutant has less sensitivity to hydrogen peroxide and can withstand longer exposure compared to WT.

WT and pah1Δ cellular growth on YPD plates after 4 hours of exposure to (A) 0mM hydrogen peroxide, (B) 1mM hydrogen peroxide. All cells were initially grown in synthetic complete medium with 100 μM inositol overnight and then diluted to an A_{600nm} of 1.0 and grown in SC media with 100 μM inositol and with or without the addition of hydrogen peroxide. Cells were collected after 4 hours and diluted back again to a starting optical density (A_{600nm}) of 1.0 (to ensure same cell number for each strain). Five serial dilutions were performed and plated for each condition of each strain. YPD plates were grown for 3 days at 30°C. Plates were repeated in triplicates.
3.2.5 Anti-apoptotic genes are upregulated in \textit{pah1}\textsuperscript{Δ} cells compared to WT when subjected to hydrogen peroxide exposure.

Due to the fact that our previous experiments showed that \textit{pah1}\textsuperscript{Δ} cells are better able to withstand exposure to apoptotic inducing agents than WT, we wanted to confirm this finding further. Since \textit{pah1}\textsuperscript{Δ} cells seem to possess a somewhat anti-apoptotic property compared to WT, we hypothesized that \textit{pah1}\textsuperscript{Δ} cells would have higher expression of anti-apoptotic genes than WT. In order to test this hypothesis, we performed mRNA analysis using genes that have been previously documented to prevent apoptosis. Cells were grown in SC medium with 100μM inositol and upon reaching exponential phase about half were collected. The other half of the cells was then exposed to 1μM hydrogen peroxide for four hours and then collected. Both the cells that were treated with hydrogen peroxide and those that were collected before treatment were processed for their RNA and then tested to assess the levels of anti-apoptotic genes.

The first gene that we tested was \textit{BIR1}, which has an important role in counteracting apoptosis. It is the only gene in \textit{Saccharomyces cerevisiae} that belongs to the inhibitor of apoptosis protein (IAPs) family. Genes in this family are able to modulate caspases which become activated during apoptosis (Owsianowski \textit{et al.}, 2007). Since it plays a major role in responding to apoptotic factors, we decided to test it. Results showed that before treatment, both WT cells and \textit{pah1}\textsuperscript{Δ} cells exhibited similar expression levels of \textit{BIR1} (0.09 ± 0.022 and 0.11 ± 0.023; Figure 3.15). However after exposure to hydrogen peroxide, WT cells had a drastic decrease in \textit{BIR1} expression compared to unexposed levels (0.01±0.001 and 0.09 ± 0.022; \(p=0.045\)). The \textit{pah1}\textsuperscript{Δ} mutant cells however did not have a decrease in expression. In fact, expression levels rose when compared to the cells before treatment (0.19 ± 0.051 and 0.11 ± 0.023), however the change was not significant enough to be considered substantial. Even so,
these findings suggest that \( \textit{pah1} \Delta \) mutant cells have significantly greater \( \textit{BIR1} \) expression after hydrogen peroxide exposure than WT (0.19 ± 0.051 and 0.01±0.001; \( p=0.037 \)), which would give them greater protection against apoptosis.

In order to strengthen this finding further we decided to examine the genes of the thioredoxin system. The thioredoxin system helps maintain a reduced cellular environment and consists of thioredoxins that are heat stable proteins which reduce various other proteins and thus protect against oxidative damage (Grant, 2001; Holmgren, 1989). The cytoplasmic thioredoxin system of \( \textit{Saccharomyces cerevisiae} \) consists of the thioredoxin encoding genes \( \textit{TRX1} \) and \( \textit{TRX2} \), in addition to \( \textit{TRR1} \) which encodes for a thioredoxin reductase, an important regulatory enzyme of the system. Together, these genes protect the cell against oxidative stress by regulating the redox state of the thioredoxin system. Previous research has shown that deletion of the \( \textit{TRX2} \) gene leads to extreme sensitivity to hydrogen peroxide (Kuge and Jones; 1994), suggesting its role in protecting the cells against the reagent. We therefore decided to test these genes and examine their levels in both WT and \( \textit{pah1} \Delta \) mutant cells.

Our results showed that before treatment with hydrogen peroxide, both WT and \( \textit{pah1} \Delta \) cells exhibited similar expression levels for \( \textit{TRX2} \) (0.84±0.23 and 1.04±0.01) and \( \textit{TRR1} \) (1.04±0.15 and 1.3±0.32). However after exposure to the reagent for four hours, both \( \textit{TRX2} \) and \( \textit{TRR1} \) expression was significantly higher in \( \textit{pah1} \Delta \) cells than in WT cells (\( \textit{TRX2}: 0.34±0.12 \) and \( 4.13 \pm 1.29, \ p=0.05; \textit{TRR1}: 0.6 \pm 0.02 \) and \( 3.9±1.1, \ p=0.05 \)). The third gene, \( \textit{TRX1} \), actually had higher gene expression levels in \( \textit{pah1} \Delta \) cells both before and after treatment of hydrogen peroxide compared to WT (Figure 3.16A). Thus, our data suggests that the reason \( \textit{pah1} \Delta \) cells
have greater protection from hydrogen peroxide might be a result of the increase in expression of the anti-apoptotic gene \textit{BIRI} (Figure 3.15) as well as \textit{TRX1}, \textit{TRX2} and \textit{TRR1} (Figure 3.16).
Figure 3.15: WT cells have a decrease in BIR1 gene expression, a gene that is involved in inhibiting apoptosis, after exposure to hydrogen peroxide, while the pah1Δ mutant maintains levels.

WT cells were grown to mid logarithmic phase (1.0±0.2 A_{600nm}) in 10µM inositol SC media and were subsequently washed and grown in SC media that contained 100µM inositol and 1µM hydrogen peroxide for 4 hours. mRNA was then extracted from cells using acid phenol chloroform and purified further with treatment of DNase. RNA was converted to cDNA and was amplified/quantified using qPCR with primers targeting the open reading frame sequence of tested gene. Expression levels of mRNA were normalized to the housekeeping gene ACT1. All experiments were done with at least two repeat colonies. Data was graphed as a mean ± standard deviation.
A) 

TRX1 Gene Expression

- No Treatment
- After 1μM H2O2 Treatment

B) 

TRX2 Gene Expression

- Before Treatment
- After 1μM H2O2 Treatment

p-values: p=0.03, p=0.04, p=0.05
Figure 3.16: The \textit{pah1Δ} mutant exhibits higher expression levels of genes within the thioredoxin system compared to WT after hydrogen peroxide exposure.

WT cells were grown to mid logarithmic phase (1.0±0.2 \textit{A}_{600nm}) in 10\mu M inositol SC media and were subsequently washed and grown in SC media that contained 100\mu M inositol and 1\mu M hydrogen peroxide for 4 hours. mRNA was then extracted from cells using acid phenol chloroform and purified further with treatment of DNase. RNA was converted to cDNA and was amplified/quantified using qPCR with primers targeting the open reading frame sequence of tested gene. Expression levels of mRNA were normalized to the housekeeping gene \textit{ACT1}. All experiments were done with at least two repeat colonies. Data was graphed as a mean ± standard deviation.
3.3 Discussion

In the yeast *Saccharomyces cerevisiae*, PAH1 has been shown to be an important gene in the lipid biosynthetic pathway. In an attempt to better characterize its role as a regulator as well as determine the effect of the two crucial phospholipid precursor molecules, inositol and choline, on its function we employed a number of experiments including growth curve analyses and time course RNA analysis via qRT-PCR for both the wildtype strain and the pah1Δ mutant strain.

Growth curve experiments showed that while both strains grew well in media containing various concentrations of inositol and choline, the pah1Δ mutant always seemed to grow a little bit better. This effect was specifically significant however when cells were grown in the presence of either 100μM inositol or 100μM choline with some inositol present. This indicated the importance of these molecules in the increased growth of the pah1Δ strain. In addition, the greater growth pattern of pah1Δ is most probably due to the fact that many of the genes involved in phospholipid biosynthesis are unregulated when PAH1 is deleted and thus leads to greater growth.

RNA analysis confirmed the role of the PAH1 gene as a negative regulator in the lipid biosynthetic pathway. The following genes were examined in the lipid biosynthetic pathway to see the effect that the deletion of PAH1 caused on their expression levels: *DPP1, LPP1, APP1, TGL3, TGL4, TGL5, DGA1, CDS1, PIS1, INO1, INM1, HXK2,* and *POL1*. Most of these genes were chosen due to their close proximity to PAH1 in the lipid biosynthetic pathway. *POL1*, while not in the lipid biosynthetic pathway, was chosen to act as the external control since it is a constitutive housekeeping gene and should not be affected by PAH1. The other genes were chosen due to their involvement in inositol production, and while *INO1* expression had been looked into previously in respect to the affect PAH1 has on it, the other genes had not been.
Results confirmed that UAS\textsubscript{INO} genes upregulate in the \textit{pah1\textDelta} strain compared to WT, thus confirming that \textit{PAH1} negatively regulates UAS\textsubscript{INO} genes. This was seen for all UAS\textsubscript{INO} genes tested in this study. However, our results showed that \textit{PAH1} can regulate non-UAS\textsubscript{INO} genes as well. While most non-UAS\textsubscript{INO} genes showed no significant difference between WT and \textit{pah1\textDelta}, some did, including \textit{TGL4}, thus suggesting that \textit{PAH1} can regulate non-UAS\textsubscript{INO} genes.

In addition, the \textit{pah1\textDelta} strain did not always respond to the addition of inositol the same way that WT did. Most genes in the lipid biosynthetic pathway will either upregulate or downregulate in response to the addition of inositol. While this phenomenon was not seen in most genes tested, it was seen in a few (\textit{PIS1}, Figure 3.4B and \textit{TGL4}, Figure 3.2E). This suggested that the effect that inositol would usually play in gene expression is canceled out by the constant upregulation that the \textit{pah1\textDelta} mutant causes. Thus, in genes like \textit{INO1}, which are involved in the rate limiting step of \textit{de novo} inositol synthesis (Ford \textit{et al.}, 2007, 2008; Esposito \textit{et al.}, 2009; Konarzewska \textit{et al.}, 2012) there would be multiple modes of regulation, with \textit{PAH1} being just one mode of repression. However there may be other genes that do not possess as many methods of regulation and whose main regulatory system would rely heavily upon the repressive role of \textit{PAH1}, thus being dependent on its function. Therefore, for these genes that lack the Pah1p repressor, their constant upregulation would be indifferent to the effects of inositol or other precursors.

Another interesting finding of our RNA analysis showed that one of the genes involved in inositol synthesis, \textit{HXK2}, did not have a significant difference in expression in \textit{pah1\textDelta}, compared to WT. However once inositol was introduced, only \textit{HXK2} levels in WT were upregulated while those in \textit{pah1\textDelta}, were not. This led to a significant difference in \textit{HXK2} expression level, with levels in \textit{pah1\textDelta} being significantly lower than WT. This was the only gene in our study which
expressed lower levels in \( pah1\Delta \) than WT, with all other genes either being upregulated or remaining comparable to WT.

The lower levels of \( HXK2 \) in \( pah1\Delta \) cells grown with the addition of inositol led us to believe that these cells would be more susceptible to apoptosis, since \( HXK2 \) has been shown to be involved in preventing apoptosis (Agimoni, 2013). It was therefore quite surprising when testing this hypothesis, that \( pah1\Delta \) fared far better than WT at resisting apoptosis. Cell survival was markedly increased in \( pah1\Delta \) compared to WT when exposed to high levels of acetic acid. This result was confirmed again using another apoptotic reagent, hydrogen peroxide. Again, \( pah1\Delta \) cell survival was significantly higher than WT when exposed to this apoptotic reagent.

Taken together, these results greatly suggest that \( pah1\Delta \) cells can withstand apoptosis much better than WT cells, despite the fact that there are lower levels of \( HXK2 \). This finding was confirmed further when RNA analysis was performed on cells treated with hydrogen peroxide and then qPCR was conducted to measure mRNA levels of anti-apoptotic genes in WT and \( pah1\Delta \) cells. As our hypothesis surmised, RNA levels for anti-apoptotic genes such as \( BIR1 \) was upregulated in \( pah1\Delta \) cells compared to WT cells after exposure to hydrogen peroxide. Genes in the thioredoxin system, which protect the cell against oxidative damage, also had higher expression levels in \( pah1\Delta \) cells compared to WT. This is an interesting and novel finding, since this is the first reporting of \( pah1\Delta \) displaying anti-apoptotic properties. This suggests a whole new area of study in which \( PAH1 \) may be involved in regulating genes involved in the apoptotic pathway. Further studies would be necessary to directly confirm this relationship and to identify other genes that would be affected \( PAH1 \).
Chapter 4

Regulation of the PAH1 gene

Studies have shown that the PAH1 gene plays a crucial role in lipid homeostasis. PAH1 encodes for the phosphatidate phosphatase that catalyzes the pivotal step in the synthesis of triacylglycerol. It dephosphorylates PA, which in turn is converted into diacylglycerol, a molecule which can be used to form triacylglycerides as well as be used in the formation of lipid droplets. In addition, its impact on PA and the way in which it regulates is abundance, helps maintain nuclear/endoplasmic reticulum growth. PAH1 has also been shown to be involved in negatively regulating the lipid biosynthetic pathway as well as maintaining proper protein trafficking needed for vacuole homeostasis. It therefore plays a pivotal role in not just lipid metabolism, but on overall cell homeostasis.

While a large extent of research has gone into characterizing the impact of PAH1 on cell homeostasis, the regulation of the PAH1 gene still needs to be elucidated. The regulation of Pah1p, the protein product of PAH1, however has greatly been characterized. Studies have shown the significance of phosphorylation and dephosphorylation deactivating and activating Pah1p. In order to attain phosphatase activity, Pah1p must be dephosphorylated and in order to be deactivated it must be phosphorylated (Santos-Rosa et al., 2005). Thus, phosphorylation and dephosphorylation play a major role in the regulation of Pah1p.

Few studies however have looked into the factors that impact PAH1 gene regulation, though recent developments have found a few important conditions in which PAH1 expression is impacted. Research has shown that the PAP activity encoded by the PAH1 gene is affected by intracellular levels of zinc. Zinc is an essential nutrient in yeast and higher eukaryotes and tight
regulation of its intracellular levels is wielded through the use of zinc transporters. Thus, if there is a deficiency in zinc, there is an induction of activity of these transporters as well as a change in the composition of membrane phospholipids (Soto-Cardalda, 2012). PAHI contains zinc-responsive upstream activating sequences in its promoter region, which becomes the binding site of Zap1p, a protein which binds there when zinc levels deplete, thus inducing transcription of the PAHI gene. As a result, PAHI is induced when zinc levels decrease.

Additionally, a study by Pascual et al. has found that the crucial phospholipid precursor, inositol, can cause the upregulation of the PAHI gene expression as well, but only in the stationary phase of growth (Pascual et al., 2013). The reason why this is so interesting is because many genes usually become impacted by this crucial precursor within a few hours of being introduced to it in the growth medium. For instance, as was shown in chapter 3, INO1 gene expression responds to inositol within just 60 minutes of exposure. We therefore wanted to elucidate the role of inositol in upregulating the PAHI gene. RNA analysis is a very effective method in determining transcription rates, and while other studies have shown the Pah1p protein levels to increase in the stationary phase, PAHI transcriptional activity has yet to be looked at. We therefore wanted to further clarify the extent to which PAHI was being induced by inositol based on the stage of growth. In addition, no one has yet compared the multiple inducing factors and determined which had the strongest effect on the upregulation of PAHI. We therefore want to find the best condition under which PAHI would be upregulated.

Furthermore, the importance of PAHI on fat regulation has been proven to be crucial for cell homeostasis (Han et al., 2007, Adeyo et al., 2011; Sasser et al., 2012; Soto-Cardalda et al., 2012). Therefore, insight into the different ways in which PAHI is regulated is crucial for a better understanding of how this gene functions. One area of study that has yet to be looked at in
terms of \textit{PAH1} gene regulation is the chromatin remodeler activity at the \textit{PAH1} promoter. Chromatin remodelers perform one of the most significant roles in modulating gene expression. They play a crucial role in modifying and restructuring the nucleosome, one of the most basic levels of DNA packaging that consists of DNA wrapped around eight histone proteins (Clapier and Cairns, 2009). As such chromatin remodelers are responsible for making the highly packaged DNA accessible so that transcription can occur. They therefore play an essential role in the regulation of gene expression. Here, we intended to identify which chromatin remodelers play a role in \textit{PAH1} gene expression. In order to address this question, growth curves of chromatin remodeler deletion strains were conducted to show the importance of these remodelers on cell growth. Next, we performed real time PCR on both WT and chromatin remodeler deletion strains using the \textit{PAH1} primer to assess \textit{PAH1} expression levels. This was followed by chromatin immunoprecipitation to determine the presence of the remodeler at the \textit{PAH1} promoter region.
4.1 Materials and Methods

4.1.1 RNA analysis: Total mRNA preparation and quantification

WT and \textit{pah1Δ} cells were grown from a single colony until mid-logarithmic phase (0.8 \( A_{600} \)) in SC media with 10\( \mu \)M \textit{myo-inositol}, followed by an additional two hours in the presence of 100\( \mu \)M \textit{myo-inositol}. RNA was extracted from yeast cells through acid phenol (pH 4.3) chloroform extraction in which the cells were resuspended in TES (10mM EDTA; 10mM Tris-HCl pH 7.5; 0.5% SDS), and then incubated at 65°C with equal parts acid phenol for 60 minutes. This was followed by a five-minute incubation on ice before centrifugation at 4°C. The top layer of the sample was transferred to a new test tube and mixed with equal parts acid phenol for a second time. Samples were incubated on ice for 5 minutes before centrifugation at 4°C and then separated by transferring the top layer of the sample to a fresh test tube and mixing with equal parts chloroform. Samples were centrifuged, then separated again from their bottom layer and transferred to a new test tube. Ethanol precipitation was then performed by adding 1 mL of 100% ethanol and 40\( \mu \)L of 3M NaOAc to the sample. The samples were then stored in dry ice for 20 minutes, followed by a fifteen-minute centrifugation. The supernatant was discarded and pellets were washed in ethanol (70%) and centrifuged again for three minutes. The supernatant was removed and the pellets were left to dry for 10 minutes. This was followed by resuspending the pellets in RNA sample buffer. RNA concentration was quantified by reading absorbance at 260nm (\( A_{260} \)). Subsequently, total RNA was stored at -80°C until further use.
4.1.2 DNase Treatment of RNA samples

DNase treatment was performed to remove any DNA from the isolated RNA samples. RNA was mixed with 10X buffer (0.1 M Tris-HCl pH 8, 5mM CaCl₂ and 0.025M MgCl₂), DNase, and DEPC water. Samples were placed in 37°C water bath for 60 minutes, followed by phenol:chloroform extraction. A 3:1 ratio of phenol:chloroform was added to the samples, mixed and then placed on ice for a five minute incubation. Samples were then centrifuged for 10 minutes and then the top layer was transferred to a new test tube to be used for ethanol precipitation. Ethanol (100%) and 20μL of 3M NaOAc were added to supernatant, mixed and then placed on dry ice for 20 minutes. Samples were centrifuged for 13 minutes, followed by removal of supernatant, and a washing of the pellet in 70% ethanol. This was followed by centrifugation and the removal of the supernatant. Pellets were placed in a speed vacuum to dry and then resuspended in TE buffer that was prepared using DEPC water. RNA concentration was again quantified by reading absorbance at 260nm.

4.1.3 Preparation of cDNA: First Strand Synthesis

In order to prepare the cDNA needed for qRT-PCR, the SuperScript III First Strand Synthesis SuperMix kit from Invitrogen (Catalog: 11752-050) was used. The reaction was performed using a polymerase chain reaction (PCR) thermocycler. Each DNase free mRNA sample (1 μg) was mixed with 2x RT Reaction mix, RT enzyme mix, and DEPC water. Samples were placed in the PCR thermocycler and underwent the reaction using the following thermocycler program: ten-minute incubation at 25°C, 30 minute incubation at 50°C, and 5 minute incubation at 85°C. Samples were then cooled on ice for a few minutes before adding E.
coli RNase H and then placed in a 37°C waterbath for 20-30 minutes. First strand synthesis samples were stored at -20°C until further use.

4.1.4 Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

The cDNA prepared from first strand synthesis was amplified and quantified using qRT-PCR. The SYBR GreenER® qPCR Supermix universal kit from Invitrogen (catalog number: 11762-500) was used to quantify cDNA templates. Forward and reverse primers were used that targeted the open reading frame (ORF) in the qPCR reaction. Primers used in this study are shown in Figure 4.1 and Table 4.1.
Table 4. 1: List of primers used for qRT-PCR for RNA analysis

<table>
<thead>
<tr>
<th>PAH1 primers</th>
<th>ACT1 primers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Forward:</strong></td>
<td><strong>Forward:</strong></td>
</tr>
<tr>
<td>5’CCACTTTGCATCGCCAAAACCT ‘3</td>
<td>5’ CCAAGCCGTTTTTGTCTCTGT ‘3</td>
</tr>
<tr>
<td><strong>Reverse:</strong></td>
<td><strong>Reverse:</strong></td>
</tr>
<tr>
<td>5’ TAGTCGCGGCTGCAGAAGAT’3</td>
<td>5’ ACCGGCCAAATCGATTCTC ‘3</td>
</tr>
</tbody>
</table>

Figure 4.1: Primers used in qPCR experiment.

Forwards and revers primers used in mRNA experiments to target the ORF of PAH1 and ACT1 genes.
4.1.6 Growth experiment for the chromatin remodeler deletion strains.

WT, *snf2Δ*, and *ino80Δ* yeast strains (Table 4.2) were grown in SC media (synthetic complete media) in the presence and absence of 100μM of *myo-inositol*. Strains were all started at an optical density (*A*$_{600}$) of around 0.2. Growth experiments were performed in duplicates, where the optical density of the cells was measured every two hours for the first eight hours and then again at the 24th hour until stationary phase was reached. Cells were grown at 30°C.

Calculations:

The average of the 24th hour OD readings for each strain and condition was used in the calculation to derive the average number of cell generations for that specific cell type:

Average # of generations = \([(\log# \text{ of cells at the end of 24 hours} – \log# \text{ of cells at the start of 24 hours})/\log(2)]\)

This was followed by determining the generation time in minutes/generation using the average # of generations:

Generation time (min/gen) = \([(60\text{min/hr} \times 24)/\text{number of generations in 24 hours}]\)
Table 4. 2: Yeast strain genotypes used in Chapter 4

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genome type</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (BY4741)</td>
<td>MATa his3Δ1 leu2Δ0 met15Δ0  ura3Δ0</td>
</tr>
<tr>
<td>pah1Δ</td>
<td>MATa his3Δ1 leu2Δ0 met15Δ0  ura3Δ0 pah1Δ</td>
</tr>
<tr>
<td>snf2Δ</td>
<td>MATa his3Δ1 leu2Δ0 met15Δ0  ura3Δ0 snf2Δ</td>
</tr>
<tr>
<td>ino80Δ</td>
<td>MATa ino80Δ::trp1 his3Δ200 leu2Δ0 met15Δ0 trp1Δ63 ura3Δ0</td>
</tr>
</tbody>
</table>
4.1.5 Chromatin immunoprecipitation (ChIP)

4.1.5.1 Cell Preparation and Crosslinking

ChIP was performed on WT strain (Table 4.2). WT was streaked from a single colony and grown in 6mL SC media with 10μM myo-inositol until saturation. Cells were transferred to 250mL of SC media with 100μM myo-inositol and grown to stationary phase so that cells would be harvested in a stage where PAHI would be induced. Prior to harvesting, cells were crosslinked with formaldehyde for 25 minutes and then quenched with glycine for an additional 5 minutes. Cells were then pelleted, collected and then treated with cell wash buffer (20mM Tris-HCl pH8; 200mM NaCl) to be washed. Cells were then stored at -80°C until they were ready to be lysed.

4.1.5.2 Lysate Preparation

In order to lyse cells, the pellets were mixed with 400μl of lysis buffer that contained PMSF (50mM Tris-HCl pH 7.5, 0.15 M NaCl, 1mM EDTA pH 8, 1% Triton X-100, 1.5μM trichostatin A, 0.2mM PMSF) as well as 0.4μl Protease inhibitor cocktail set III (CalBioChem Catalog number: 539134). The cells with the lysis buffer mixture were then carefully added to acid washed glass beads in an Epindorf tube and subsequently vortexed at 4°C for 20-25minutes. Cells were then separated from beads by centrifugation at 2K for 10 seconds. The supernatant was collected and placed in a new test tube so that it could undergo centrifugation at 13K for 15 minutes at 4°C. Supernatant was discarded and the remaining pellet was mixed with chilled lysis buffer that did not contain PMSF. Samples were sonicated and then centrifuged at 2K for 60 seconds at 4°C. Supernatant was then placed in a fresh test tube and stored in -80°C.
4.1.5.3 Input Sample Preparation

Elution buffer (1% SDS + 0.1 M NaHCO$_3$) and 5M NaCl were added to the 10μl of lysate. Tubes were incubated at 65°C for 4 hrs. Then, 8μl 0.5M EDTA, 16μl 1M Tris HCl pH 6.5 and 1μl Protease K were added to the tubes and incubated again at 45°C for 1 hour. After this, phenol chloroform extraction was performed, followed by the ethanol precipitation. DNA quantification was done using spectrophotometer.

4.1.5.4 Immunoprecipitation

Sonicated lysates that contained 11μg of total DNA were used for immunoprecipitation (IP). They were diluted 10-fold in ChIP dilution buffer and incubated with 1μL TSA and 1μL protease inhibitor cocktail, along with 35 μL protein A agarose slurry. Samples were placed at 4°C for an hour on a rotation rack to preclear and reduce nonspecific background. They were then briefly centrifuged at 2K for 15 seconds and the supernatant was transferred to a new Epindorf tube. Antibodies against Snf2p were added and allowed to incubate overnight. The negative control samples (mock samples) were not treated with antibodies.

After overnight IP, 60 μL protein A agarose slurry was added to samples and incubated for one hour. Samples were briefly centrifuged and washed once each with low salt buffer, high salt buffer, and LiCl solution, respectively. Subsequently, the samples were washed with 1X TE twice. The protein-DNA complex was eluted from the resin by adding 250μl of freshly prepared elution buffer and rotated for two fifteen-minute time intervals. The supernatant was collected after centrifugation and 5M NaCl was added to each test tube to reverse cross-link the samples by heating them at 65°C for 5-6 hours. Then, 8ul of 0.5 M EDTA, 16ul of 1M Tris-HCl, pH6.5 and 2 μl of Proteinase K was added and incubated for 2 hours at 45°C. The DNA was then
recovered by phenol/chloroform extraction and ethanol precipitation. Finally, the pellet was re-suspended in 20 μl of TE.

4.1.5.5 Quantitative PCR (qPCR)

qPCR was used to quantify the abundance of DNA fragments that were bound by the protein of interest. Mock and input samples were also tested. Input samples were prepared as all the DNA sequences in the genome from the lysate of the cell without any selection or IP. Mock samples were prepared exactly as IP samples, just without the addition of an antibody to be used to subtract out any background signal. qPCR was performed using the Applied Biosystems, 7500 real-time PCR system). Forward and reverse primers were used that target different areas of the URS and ORF in the qPCR reaction. Primers used in this study are shown in Figure 4.2 and Table 4.3.

IP samples were calculated as follows:

\[
(2^{\Delta \text{Ct} \# (\text{IP} \# - \text{mock} \#) - (\text{Input} \# - \text{mock} \#)}) \times (A_{260} \times 50 \mu g/mL \times 50 \text{ dilution factor})
\]

The IP samples were then graphed as an average of repeats ± standard deviation that was then normalized to mock and input samples.
Table 4.3: List of Primers Used for qPCR (On ChIP samples)

<table>
<thead>
<tr>
<th>ChIP Primers</th>
<th>Forward Primers C</th>
<th>Forward Primers B</th>
<th>Forward Primers A</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PAH1(-600 to -400 base pairs upstream of ORF) primers:</strong></td>
<td>5' CAGTTGGGTCTTTGTACTTATC 3'</td>
<td>5' AGTTTGGACCCCTGTTCAAG 3'</td>
<td></td>
</tr>
<tr>
<td>Forward:</td>
<td>5' AGGGTCCAAACTCATTTGTATATC 3'</td>
<td>5' GTCCCTTGCTTTTCTTCTACC 3'</td>
<td></td>
</tr>
<tr>
<td>Reverse:</td>
<td>5' AATCGACGATGTTGTCTATC 3'</td>
<td>5' GCTTCTCCAGAATCCTCAG 3'</td>
<td></td>
</tr>
<tr>
<td><strong>PAH1 ORF primers:</strong></td>
<td>5' ATGCAGTACGAGGCAAGAG 3'</td>
<td>5' GCCTTCTCCAGAATCCTCAG 3'</td>
<td></td>
</tr>
<tr>
<td>Forward:</td>
<td>5' ATGCAGTACGAGGCAAGAG 3'</td>
<td>5' GCCTTCTCCAGAATCCTCAG 3'</td>
<td></td>
</tr>
<tr>
<td>Reverse:</td>
<td>5' ATGCAGTACGAGGCAAGAG 3'</td>
<td>5' GCCTTCTCCAGAATCCTCAG 3'</td>
<td></td>
</tr>
</tbody>
</table>

*Used for Input, IP, and Mock samples

Figure 4. 2: PAH1 URS Map and Primers used for qPCR on ChIP samples.
4.2 Results

4.2.1 *PAH1* is induced under various conditions, with greatest induction occurring during stationary phase with 100µM inositol added to media.

In order to find the conditions in which *PAH1* gene expression is induced we started off by performing RNA analysis. Since the impact of a gene’s effect on the cell is greatest when the gene is at its highest expression level, it was important to find the conditions at which the gene is induced before we looked into other regulatory factors. Previous studies have shown that the *PAH1* promoter region includes both a UAS\textsubscript{ZRE} (Soto-Cardalda, 2012) as well as a UAS\textsubscript{INO} (Wimalarathna, 2011), indicating that both zinc and inositol impact the induction of *PAH1*. In fact, studies have demonstrated that the removal of zinc from growth media as well as addition of inositol and entry into stationary phase cause *PAH1* to become upregulated. However, there has been no study comparing these conditions to determine which of these factors cause the highest upregulation of *PAH1*. This information is crucial, since all other experiments will rely on this information so that *PAH1* can be expressed at its maximum expression level. To confirm the best conditions of *PAH1* induction, we conducted RNA analysis to measure *PAH1* transcription levels in WT cells grown in various environments. These environments included cells grown in regular SC media and were compared to those grown in SC media without zinc. Cells were also grown in SC media containing various concentration of inositol during exponential phase and were compared to cells grown in SC media with various concentrations of inositol during stationary phase. The RNA of these cell samples were isolated, subjected to DNase treatment to remove any DNA, and subsequently used to perform quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) by turning the mRNA into cDNA. For
repressing and inducing conditions, the results were examined at the open reading frame (ORF) of \textit{PAH1} and were subsequently normalized to the housekeeping gene \textit{ACT1}.

The first factor that we tested was the impact of zinc on \textit{PAH1} gene expression. The RNA levels demonstrated a significant difference ($p=0.04$) between cells grown with zinc versus those grown without, with the cells grown in media lacking zinc to have higher \textit{PAH1} expression levels (Figure 4.3). Our results clearly showed that zinc is a negative regulator to \textit{PAH1} expression. These findings are in agreement with previous results (Soto-Cardalda et al., 2012).

Next, we compared the levels of \textit{PAH1} in cells grown with normal zinc levels, but with 100µM inositol added. Cells were collected in both the exponential phase and stationary phase of cell growth. Again, \textit{PAH1} RNA levels demonstrated a significant difference ($p=0.001$) between cells collected in exponential phase and those in stationary phase. \textit{PAH1} was substantially upregulated in stationary phase compared to levels in exponential phase (Figure 4.4). This too was in agreement with previous findings (Pascual et al., 2013). However, the true test was to determine which of the above conditions (Figure 4.3, Figure 4.4) had the greatest impact on \textit{PAH1} levels. When compared, it can be seen that cells grown with 100µM inositol and collected in stationary phase were significantly higher than cells grown in media without zinc ($0.017 \pm 0.0004$ and $0.0056 \pm 0.00061; p=0.023$) (Figure 4.5). This novel finding demonstrated that while \textit{PAH1} can be upregulated in various environments, it is most highly induced when cells enter stationary phase and have excess inositol in their media.

In order to further clarify our finding we wanted to determine whether it was the inositol or the growth stage that most greatly impacted \textit{PAH1} expression. We therefore performed additional RNA analysis of \textit{PAH1} gene expression in cells grown in various concentrations of
inositol in both exponential and stationary phases. The results showed that while \textit{PAH1} was induced during the stationary phase in all cells that have inositol, they are significantly induced during the stationary phase when in the presence of 100\(\mu\)M inositol, a condition that contains excess amounts of inositol (Figure 4.6).

While differences in \textit{PAH1} expression are seen in the stationary phase based off of the amount of inositol present, the same could not be said for the exponential phase of growth. The various concentrations of inositol had no impact on \textit{PAH1} levels in exponential phase, thus demonstrating that it is the growth phase that plays a more significant role in \textit{PAH1} gene induction than the inositol. However, it seems that in order for induction to occur, at least some inositol must be present in the media. Cells that were grown without inositol did not show a significant change in \textit{PAH1} levels in exponential versus stationary phase (Figure 4.6). These data therefore demonstrate that the highest level of \textit{PAH1} expression is found in cells that have entered the stationary phase of growth and are grown with excess inositol.
Figure 4.3: *PAH1* is induced in growth environments that do not contain zinc.

WT cells were grown to mid logarithmic phase (1.0±0.2 A$_{600nm}$) in 10µM inositol SC media and were subsequently washed and grown in either regular SC media (which contains zinc) or SC media lacking zinc. mRNA was then extracted from cells using acid phenol chloroform and purified further with treatment of DNase. RNA was converted to cDNA and was amplified/quantified using qPCR with primers targeting the open reading frame sequence of PAH1. Expression levels of mRNA were normalized to the housekeeping gene *ACT1*. All experiments were done with at least two repeat colonies and all qPCR reactions were done in triplicates. Data was graphed as a mean ± standard deviation.
Figure 4.4: \textit{PAH1} gene expression is significantly induced in cells grown to stationary phase with 100\(\mu\)M inositol and normal levels of zinc.

WT cells were grown to mid logarithmic/exponential phase and stationary phase in 100\(\mu\)M inositol SC media and collected. mRNA was then extracted from cells using acid phenol chloroform and purified further with treatment of DNase. RNA was converted to cDNA and was amplified/quantified using qPCR with primers targeting the open reading frame sequence of \textit{PAH1}. Expression levels of mRNA were normalized to the housekeeping gene \textit{ACT1}. All experiments were done with at least two repeat colonies and all qPCR reactions were done in triplicates. Data was graphed as a mean ± standard deviation.
Figure 4.5: *PAH1* gene induction is significantly higher in cells grown to stationary phase with 100μM inositol compared to cells grown without zinc.

WT cells were grown in 10μM inositol SC media and were subsequently washed and grown in either SC media lacking zinc grown to mid logarithmic phase or SC media containing 100μM inositol grown to stationary phase. mRNA was then extracted from cells using acid phenol chloroform and purified further with treatment of DNase. RNA was converted to cDNA and was amplified/quantified using qPCR with primers targeting the open reading frame sequence of PAH1. Expression levels of mRNA were normalized to the housekeeping gene *ACT1*. All experiments were done with at least two repeat colonies and all qPCR reactions were done in triplicates. Data was graphed as a mean ± standard deviation.
Figure 4.6: \textit{PAH1} gene induction is significantly higher in cells grown to stationary phase with 100\(\mu\)M inositol compared to cells grown with less inositol in stationary phase.

WT cells were grown in 10\(\mu\)M inositol SC media and were subsequently washed and grown in various concentrations of inositol (0, 10, 100 \(\mu\)M) and collected in both exponential and stationary phases. mRNA was then extracted from cells using acid phenol chloroform and purified further with treatment of DNase. RNA was converted to cDNA and was amplified/quantified using qPCR with primers targeting the open reading frame sequence of \textit{PAH1}. Expression levels of mRNA were normalized to the housekeeping gene \textit{ACT1}. All experiments were done with at least two repeat colonies and all qPCR reactions were done in triplicates. Data was graphed as a mean ± standard deviation.
4.2.2 Snf2p and Ino80p are crucial chromatin remodelers for INO1 expression, a gene regulated by PAH1.

Since we found that the best inducing condition to upregulate the PAH1 gene was under stationary phase with excess inositol, we wanted to use this information to find possible chromatin remodelers that impact PAH1 gene expression. Chromatin remodelers play a crucial role in modifying and restructuring the nucleosome, one of the most basic levels of DNA packaging that consists of DNA wrapped around eight histone proteins. As such chromatin remodelers are responsible for making the highly packaged DNA accessible so that transcription can occur. We therefore wanted to look into possible chromatin remodelers that may affect PAH1 gene expression. Since inositol plays a role in PAH1 upregulation, we decided to look at the two chromatin remodelers which are important for INO1 expression, Snf2p and Ino80p.

INO1 is the gene responsible for the rate limiting step of de novo inositol synthesis. Thus, since PAH1 is impacted by inositol, it is likely that it would have the same remodelers as INO1. Additionally, since INO1 is highly regulated by PAH1, as was shown in our first aim (Figure 3.3A), we hypothesize that that they would use the same remodelers to help keep gene expression in check.

We therefore decided to first demonstrate the importance of SNF2 and INO80 in inositol production by doing growth curve analysis. WT, snf2Δ and ino80Δ strains were grown in either SC media that was depleted of inositol or contained 100μM inositol and observed until stationary phase was reached. Results showed that all strains grew well in conditions that contained excess inositol. WT, snf2Δ and ino80Δ strains all exhibited optical densities that were higher than 5.5 at hour 24 (A600 of 5.8±0.28, 6.3±0.00, and 5.65±0.08, respectively; Figure 4.7A) and all had similar numbers of generations and generation times (Table 4.4). However, cell growth in media
depleted of inositol showed vastly different results for the mutants. WT cells grew slightly poorer in 0μM inositol conditions than in 100μM inositol, with an A$_{600}$ of 4.65±0.35 (Figure 4.6B). However, $snf2\Delta$ and $ino80\Delta$ strains were unable to properly grow in conditions that lacked inositol and had extremely low optical densities (A$_{600}$ of 0.37±.07 and 0.30±.08, respectively) as well as incredibly long generation times (Table 4.4). The difference between $snf2\Delta$ cells grown in excess inositol and those grown without inositol was a staggering 85.4% difference in growth rate and the difference for $ino80\Delta$ was even higher at 90% (Table 4.5). The difference between WT cells grown without inositol and $snf2\Delta$ cells grown without inositol was 82.7% and the difference between WT and $ino80\Delta$ under inositol depletion was 87.65% (Table 4.6). Thus, these results demonstrate that the Snf2p and Ino80p chromatin remodelers play a critical role in inositol production, since without their presence inositol is unable to be synthesized and cells cannot grow properly. As such, they may be involved in PAH1 regulation which is induced by inositol.
Figure 4.7: snf2Δ and ino80Δ have moderate to severe reductions in growth when grown without inositol, indicating the important role of the Snf2p and Ino80p chromatin remodelers in INO1 activation.

A) WT, ino80Δ, and snf2Δ cells were grown in 10μM inositol SC media and were then washed and diluted to an Absorbance_{600nm} of 0.2 in SC media that represented either the inducing or repressing conditions. Cells were grown in both inositol depleted media (inducing condition for the INO1 gene) or inositol rich media that contained 100μM inositol (repressing condition). The optical density (A_{600nm}) was read for first 8 hours of exponential phase and then resumed at 24 hours and taken until stationary phase was reached. B) Graph depicting only the 0μM inositol conditions.
Table 4.4: 24\textsuperscript{th} Hour Growth Analysis

<table>
<thead>
<tr>
<th>Condition</th>
<th>Average O.D 0μM Inositol (24th Hour)</th>
<th>Average O.D 100μM Inositol (24th Hour)</th>
<th># of generations in 24 hours for 0μM Inositol</th>
<th># of generations in 24 hours for 100μM Inositol</th>
<th>Generation Time (min/generation) for 0μM Inositol</th>
<th>Generation Time (min/generation) for 100μM Inositol</th>
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</thead>
<tbody>
<tr>
<td>WT</td>
<td>4.65±0.35</td>
<td>5.8±0.28</td>
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<td>4.372989908</td>
<td>355.1928246</td>
<td>329.2941512</td>
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<tr>
<td>snf2Δ</td>
<td>0.37±0.07</td>
<td>6.3±0.00</td>
<td>0.717671781</td>
<td>4.873428869</td>
<td>2006.488257</td>
<td>295.4798436</td>
</tr>
<tr>
<td>ino80Δ</td>
<td>0.30±0.08</td>
<td>5.65±0.08</td>
<td>0.525143372</td>
<td>5.098220942</td>
<td>2742.108303</td>
<td>282.4514701</td>
</tr>
</tbody>
</table>

Table 4.5: Comparison of growth differences between 0μM Inositol to 100μM Inositol

<table>
<thead>
<tr>
<th>Strain Comparison</th>
<th>Change in cell doubling time (Folds)</th>
<th>Difference in growth rate %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type 0μM Inositol</td>
<td>Wild Type 100μM Inositol</td>
<td>1.079154012</td>
</tr>
<tr>
<td>snf2Δ 0μM Inositol</td>
<td>snf2Δ 100μM Inositol</td>
<td>7.38541158</td>
</tr>
<tr>
<td>ino80Δ 0μM Inositol</td>
<td>ino80Δ 100μM Inositol</td>
<td>11.55788708</td>
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</table>

Table 4.6: Comparison of growth differences between strains in 0μM Inositol

<table>
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<th>Strain Comparison</th>
<th>Change in cell doubling time (Folds)</th>
<th>Difference in growth rate %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type 0μM Inositol</td>
<td>snf2Δ 0μM Inositol</td>
<td>6.05231592</td>
</tr>
<tr>
<td>Wild Type 0μM Inositol</td>
<td>ino80Δ 0μM Inositol</td>
<td>8.977775579</td>
</tr>
<tr>
<td>snf2Δ 0μM Inositol</td>
<td>ino80Δ 0μM Inositol</td>
<td>1.450415783</td>
</tr>
</tbody>
</table>

Table 4.7: Comparison of growth differences between strains in 100μM Inositol

<table>
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<th>Strain Comparison</th>
<th>Change in cell doubling time (Folds)</th>
<th>Difference in growth rate %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type 100μM Inositol</td>
<td>snf2Δ 100μM Inositol</td>
<td>1.117487271</td>
</tr>
<tr>
<td>Wild Type 100μM Inositol</td>
<td>ino80Δ 100μM Inositol</td>
<td>1.170583464</td>
</tr>
<tr>
<td>ino80Δ 100μM Inositol</td>
<td>snf2Δ 100μM Inositol</td>
<td>1.047222226</td>
</tr>
</tbody>
</table>
4.2.3 The snf2Δ mutant, but not the ino80Δ mutant, exhibits lower PAH1 expressions levels than WT during stationary phase, suggesting a possible role of Snf2p as an activator of PAH1 expression.

Once we confirmed the importance of Snf2p and Ino80p in inositol production we set out to determine whether these chromatin remodelers actually impact PAH1 gene expression. We accomplished this by employing RNA analysis and looked at PAH1 expression in WT, ino80Δ, and snf2Δ. Cells were grown in 100μM inositol and were subsequently collected in both exponential phase and stationary phase. Cells were collected in both phases so that we could examine PAH1 expression in PAH1’s induced state.

Results showed that PAH1 expression levels were comparable between WT and the ino80Δ mutant. While there is a significant difference within each strain between exponential phase and stationary phase for both WT (0.0028±0.0002 and 0.0085±0.0019; p=0.05) and ino80Δ (0.0045 ±0.0012 and 0.0141±0.0009; p=0.01), there was no significant difference in PAH1 levels between the two strains in either exponential phase or more importantly in stationary phase (Figure 4.8). Thus, these findings suggest that Ino80p does not play a role in PAH1 activation.

Yet while Ino80p did not seem to play a role in PAH1 expression, the other chromatin remodeler that we focused on, Snf2p, does seem to play a role. Our RNA results for PAH1 gene expression in WT and the snf2Δ mutant show that PAH1 levels in the snf2Δ mutant is significantly lower than that in WT (0.0075±0.001 and 0.004±0.0007; p=0.022) during stationary phase (Figure 4.9). Since PAH1 has the highest expression level in stationary phase when inositol is present, this suggests that Snf2p is involved in PAH1 activation, since PAH1 levels are significantly lower in the snf2Δ mutant, where Snf2p is not present.
Figure 4.8: The *ino80Δ* mutant exhibits similar *PAH1* gene expression levels to WT for both stationary and exponential phases.

WT and *ino80Δ* mutant cells were grown to mid logarithmic phase as well as stationary phase in 100µM inositol SC media. mRNA was then extracted from cells using acid phenol chloroform and purified further with treatment of DNase. RNA was converted to cDNA and was amplified/quantified using qPCR with primers targeting the open reading frame sequence of tested gene. Expression levels of mRNA were normalized to the housekeeping gene *ACT1*. All experiments were done with at least two repeat colonies and all qPCR reactions were done in triplicates. Data was graphed as a mean ± standard deviation.
PAH1 gene regulation

![Bar graph showing gene expression levels for WT and snf2Δ in exponential and stationary phases.]

$p = 0.04$  
$p = 0.022$  
$p = 0.014$

Figure 4.9: The snf2Δ mutant exhibits lower PAH1 gene expression levels than WT at the stationary phase.

WT and snf2Δ mutant cells were grown to mid logarithmic phase as well as stationary phase in 100µM inositol SC media. mRNA was then extracted from cells using acid phenol chloroform and purified further with treatment of DNase. RNA was converted to cDNA and was amplified/quantified using qPCR with primers targeting the open reading frame sequence of tested gene. Expression levels of mRNA were normalized to the housekeeping gene ACT1. All experiments were done with at least two repeat colonies and all qPCR reactions were done in triplicates. Data was graphed as a mean ± standard deviation.
4.2.4 Snf2p is present at the *PAH1* promoter.

Since our RNA results suggested that the chromatin remodeler Snf2p plays a role in *PAH1* activation, we wanted to confirm this finding further by detecting Snf2p at the promoter region of *PAH1*. This was done by performing chromatin immunoprecipitation (ChIP), a process by which proteins associated with specific regions of DNA are pulled down and targeted with an antibody. In order to do this, we used a Snf2p antibody to pull down the Snf2p that was bound to DNA in WT cells and employed quantitative real time PCR to examine the presence of Snf2p at the *PAH1* promoter region under inducing conditions.

Primers targeting multiple locations of the upstream regulatory sequence (*URS*) and the open reading frame (*ORF*) of *PAH1* were used and IP signals from qPCR were normalized to the *PAH1* input and mock samples. Since Snf2p should be mainly found at the promoter region, the *ORF* primers served as our external control. Our results showed minimal IP signals at the *ORF* region. However, there was a significant increase in IP signals in the promoter region that covered the region of -1 to -200 base pairs upstream of the *ORF* in the *PAH1* gene (*p*<0.01) suggesting that Snf2p is present in that region (Figure 4.9). Furthermore, IP signals increased even more drastically from the -200 to -400 base pair region compared to the *ORF* (*p*=0.027), suggesting that Snf2p accumulates mainly in this region of the *PAH1* promoter. Lastly, there was an increase of IP signal found at the -400 to -600 base pair region upstream that was significantly greater than signals found at the ORF (*p*=0.02), however the increase in IP signal was not nearly as high as those found at -200 to -400 base pairs upstream. This therefore implies that Snf2p is present at the -400 to -600 base pair region of the *PAH1* promoter, but not to the same extent as is found at -200 to -400 region (Figure 4.10). Taken together, these findings suggest that Snf2p is...
found throughout the *PAHI* promoter, but is most heavily concentrated at the region of -200 to -400 base pairs upstream of the *ORF* and is therefore involved in *PAHI* gene induction.
Figure 4.10: Snf2p is present at the PAH1 promoter region.

Quantitative real time PCR (qPCR) analysis of DNA from WT cells that was immunoprecipitated via the process of chromatin immunoprecipitation (ChIP). WT cells were grown to stationary phase in 100µM inositol SC media, cross-linked, lysed, and quantified. This was followed by immunoprecipitation with an antibody against Snf2p. The IP for the PAH1 promoter was graphed as the average of the repeat samples ± standard deviation. It was normalized via the input samples (all of the genomic DNA sequences from the collected lysate without any selection or immunoprecipitation) and the mock samples (samples that represent the signal background, since all the ChIP steps were done on these lysate samples but no antibody was added, thus theoretically having no immunoprecipitated DNA).
4.3 Discussion

Due to the fact that the *PAH1* gene plays a pivotal role in the lipid biosynthetic pathway and acts as a key regulator of many of these genes, it was important to get a better understanding of how it itself is regulated. While there had previously been some research done to help clarify this, not enough had been done to help define the parameters in which *PAH1* was best induced. Since this information is crucial for studying a gene, especially when experiments need to be performed at peak gene expression, it was critical to determine the best inducing condition for *PAH1*. In addition, no one had yet looked into the chromatin remodelers that affect *PAH1* gene expression. Since chromatin remodelers play a crucial role in modifying and restructuring the nucleosome they therefore play an essential role in the regulation of gene expression. It was therefore an important aspect of gene regulation that we wanted to examine in *PAH1*.

Through RNA analysis and qPCR, we were able to define the best condition for *PAH1* gene induction. While *PAH1* expression is significantly upregulated in response to the depletion of the essential mineral zinc (Figure 4.3), it paled in comparison to the effects of high concentrations of inositol and the stationary phase on *PAH1* gene expression (Figures 4.4 and 4.5). While the stationary phase of growth had a greater impact on *PAH1* induction than inositol alone, since cells grown with excess inositol and collected in exponential phase did not have an expression of *PAH1* nearly as high, the two conditions combined gave the highest levels of *PAH1* transcription (Figure 4.6). Taken together, these results showed that while there may be various conditions under which *PAH1* is induced, it is most greatly induced when cells are grown in excess inositol and have entered stationary phase.

Both the depletion of zinc and the addition of excess inositol in stationary phase were not unlikely factors to induce the *PAH1* gene. This is because the *PAH1* promoter region contains
both a UAS\textsubscript{INO} (Wimalarathna, 2011) as well as three upstream activating sequence zinc-responsive elements (UAS\textsubscript{ZRE}) (Soto-Cardalda, 2012). The UAS\textsubscript{INO} is the inositol sensitive upstream activating sequence located in the promoter region of many genes in the lipid biosynthetic pathway and is the binding site for activators such as Ino2p and Ino4p in response to a need for inositol, while the three UAS\textsubscript{ZRE} are the binding sites for zinc responsive transcription factors that localize under conditions of zinc deficiency (Soto-Cardalda, 2012). Thus, the depletion of zinc or addition of inositol is recognized by these sites upstream of the \textit{PAH1} gene and therefore upregulate in response to this condition.

Once we verified the inducing conditions of the \textit{PAH1} gene, we wanted to look into which chromatin remodelers played a significant role in regulating \textit{PAH1} gene expression. Chromatin remodeling plays one of the most important roles in gene regulation. Remodelers are responsible for modifying and restructuring the nucleosome, one of the most basic levels of DNA packaging that consists of DNA wrapped around eight histone proteins (Clapier and Cairns, 2009). As such chromatin remodelers are responsible for making the highly packaged DNA accessible so that transcription can occur. They therefore play an essential role in the regulation of gene expression. Being that this is an area that has yet to be looked into in regard to \textit{PAH1} expression, we therefore wanted to look into possible chromatin remodelers that may affect \textit{PAH1} gene expression.

Since inositol plays such an important role in \textit{PAH1} upregulation in stationary phase cells (Figure 4.6), we decided to look at the two chromatin remodelers that impact \textit{INO1} expression, which is the gene responsible for the rate limiting step of \textit{de novo} inositol synthesis, to see if they might impact the \textit{PAH1} gene as well. These chromatin remodelers are Snf2p and Ino80p. Another reason why we chose to look into these two chromatin remodelers was because \textit{PAH1}...
has been shown to regulate the \textit{INO1} gene (Figure 3.2A). It therefore may be possible that they would use the same remodelers as another means to help keep gene expression in check. We therefore decided to first demonstrate the importance of Snf2p and Ino80p in inositol production by doing growth curve analysis using WT, \textit{snf2}Δ, and \textit{ino80}Δ strains grown in both inducing and repressing conditions. Growth analysis showed that while all strains grew well in media that contained excess inositol (100μM), only the \textit{wild type} strain grew well when no inositol was present. Both \textit{snf2}Δ and \textit{ino80}Δ strains were unable to grow under inositol depleted conditions (Figure 4.7B). This was due to the fact that in order to activate the \textit{INO1} gene, both Snf2p and Ino80p chromatin remodelers had to be functional and present in order to activate \textit{INO1} and allow transcription to begin. Thus when inositol was present and \textit{INO1} did not need to operate, all strains grew fine. Yet when no inositol was present, the \textit{snf2}Δ and \textit{ino80}Δ strains were unable to grow since \textit{INO1} was unable to activate.

Once we confirmed the crucial role of Snf2p and Ino80p chromatin remodelers in \textit{INO1} gene activation, we wanted to see if they played a role in regulating \textit{PAH1} as well. In order to do this, we employed RNA analysis and studied \textit{PAH1} gene expression in the \textit{snf2}Δ and \textit{ino80}Δ strains and compared them to those in WT. Results showed that \textit{PAH1} expression levels in the \textit{ino80}Δ strain were similar to that in WT, specifically levels in the stationary phase, the phase in which \textit{PAH1} gene expression is induced under excess inositol conditions (100μM inositol). Thus, our findings suggested that Ino80p does not play a role in \textit{PAH1} gene regulation. However, results from the \textit{snf2}Δ mutant RNA analysis exhibited a significant change in expression levels between the mutant strain and WT. The expression levels of \textit{PAH1} were significantly reduced in the stationary phase of \textit{snf2}Δ compared to that in WT suggesting that
Snf2p plays a role in activating the *PAH1* gene. This is a novel finding since there has been little to no research conducted on *PAH1* regulation in regard to factors that affect chromatin structure.

In order to confirm this finding further, we performed ChIP analysis to detect the presence of Snf2p at the promoter region of *PAH1* and to locate where on the promoter region it specifically localizes. Our data showed that IP levels were significantly higher in the *PAH1* promoter region than at the ORF, suggesting that Snf2p is present throughout the promoter region but is most concentrated between the region of -200 to -400 base pairs. Interestingly, *PAH1* contains an upstream activating sequence zinc-responsive element (UAS\(_{ZRE}\)) at the -200 to -210 base pair region. This falls in the region in which we saw the highest IP values for the presence of Snf2p at the *PAH1* promoter. As mentioned earlier, the UAS\(_{ZRE}\) is the binding sites for zinc responsive transcription factors that localize under conditions of zinc deficiency (Soto-Cardalda *et al.*, 2012). Since the depletion of zinc is a factor that induces *PAH1* gene expression, it would seem likely for Snf2p to localize in this region in order to aid in the activation process.

Taken together, our data suggests that Snf2p is present at this crucial region of the *PAH1* promoter (-200 to -400 base pairs) and we hypothesize that the reason it accumulates there is because when activation is necessary it makes the chromatin accessible where the UAS\(_{ZRE}\) is located and allows for further activation. Moreover, the second region with the highest IP values (-400 to -600 base pairs) contains a UAS\(_{INO}\) at -585 to -593 of the *PAH1* promoter region (Wimalaratna *et al.*, 2011). The UAS\(_{INO}\) is the inositol sensitive upstream activating sequence located in the promoter region of many genes in the lipid biosynthetic pathway and is the binding site for activators such as Ino2p and Ino4p in response to inositol. Since the addition of inositol in the stationary phase is another factor that induces *PAH1* gene expression, the presence of
Snf2p at this location seems likely as well. Thus, our data suggests that the presence of the Snf2p chromatin remodeler at the PAH1 promoter seem to be in regions where activation is necessary.

Interestingly, Snf2p is one of the chromatin remodelers important for INO1 regulation. INO1 is one of the most crucial genes in inositol production and happens to be heavily regulated by PAH1. PAH1 acts by repressing the INO1 gene. As was determined in Figure 3.3A, the pah1Δ strain has upregulated levels of INO1, thus confirming that when Pah1p is present it acts as a repressor of INO1. Taken together, these findings suggest another mode by which PAH1 affects INO1 expression, by possibly using the same remodeler to help keep gene expression in check. We therefore hypothesize another model in which PAH1 control INO1 gene expression. If INO1 needs to be activated, the Snf2p chromatin remodeler will collect at the INO1 promoter, leaving less of the remodeler available for PAH1 activation, which will then lessen the repressive role of Pah1p on INO1. Conversely, if PAH1 is activated, more Snf2p will be localized at the PAH1 promoter region, forcing more away from INO1 and thus decreasing the activity of the INO1 gene. This model therefore suggests an additional way in which PAH1 can control INO1 expression.
Chapter 5

The effect of PAH1 upon vacuolar morphology and V-ATPase pump activity.

The role of the PAH1 gene in maintaining cell homeostasis has been shown to be quite extensive. In addition to regulating the phospholipid biosynthetic pathway, its effect on various organelles has been shown to be crucial. For instance, PAH1 plays a role in maintaining normal nuclear and endoplasmic reticulum membrane morphology. When PAH1 is deleted, abnormal expansion of the nuclear endoplasmic reticulum membrane occurs in the cell (Santos-Rosa et al., 2005). This is due to the fact that many genes in the lipid biosynthetic pathway upregulate when PAH1 is unavailable to repress them, and as such, cause an increase in membrane expansion. Yet another organelle that is greatly impacted by the PAH1 gene is the vacuole. Morphological studies have shown that pah1Δ cells exhibit a change in vacuolar morphology, with vacuoles appearing fragmented as compared to WT (Sasser et al., 2012).

While vacuole fragmentation is a normal part of cell homeostasis and occurs in WT cells prior to cell division, abnormal fragmentation is caused by defects in lipid synthesis. Under normal conditions, vacuoles fragment and partition in yeast cells so that during daughter cell formation, the newly budded cell can receive the organelle (Conradt et al., 1992). This is due to the fact that in Saccharomyces cerevisiae, cytoplasmic organelles are not synthesized by the daughter cell, but inherited from the mother. Thus, upon bud initiation, the main vacuole in the mother cell will fragment into a collection of smaller vacuoles (Figure 5.1). A tubule-vesicular structure develops from these smaller vacuoles and directs itself toward the newly budding daughter cell (Conradt et al., 1992). In this way, the organelle is transferred and inherited by the new cell. As the budding cell increases in its size, the small group of vacuoles starts to fuse back into one large vacuole, both in the mother and daughter cells. Lastly, the cells form septa, which
separate the two cells from each other. This cycle of vacuole fragmentation can then begin again to be given to new daughter cells (Wiemken et al., 1970).

This process however is heavily impacted by the lipid biosynthetic pathway. Defects in lipid metabolism are associated with abnormal vacuole fragmentation and disruptions in vacuolar function. \textit{PAH1} has recently emerged as a key gene that influences proper vacuole morphology. Its deletion has been associated with abnormal vacuole fragmentation, with vacuoles unable to fuse back together. One study has shown that the lack of Pah1p phosphatase activity prevents the binding of SNARES and is devoid of components of the HOPS tethering complex needed for vacuolar fusion (Sasser et al.; 2012). Thus, vacuolar morphology is implicated in \textit{pah1∆} mutant. On the other hand, irregular vacuole morphology has also been related to atypical vacuolar acidification as a result of dysfunctional V-ATPase pumps. Studies have shown that some phenotypes of mutant cells with abnormal vacuoles include vacuole fragments that collect to one area or disperse all over the cell. These cells can have working V-ATPase pumps or have a defect in the assemblage of the V-ATPase pump (Weisman et al., 1987; Raymond et al., 1992). Therefore, it is possible that \textit{PAH1} may affect the vacuole or may implicate the V-ATPase pump and thus alter its morphology through the regulation of lipid biosynthesis.

In eukaryotic cells, vacuolar proton translocating ATPases (V-ATPases) play an important role in acidifying the vacuole. They span the membrane of the vacuole and are quite large, consisting of two main subunits, an integral one and a peripheral one (Figure 5.2). Their role in vacuole homeostasis is essential as they regulate the pH by actively transporting hydrogens from the cytoplasm into the organelle and thus maintain their acidic pH (pH ~6.0) which allows it to carry out its normal physiological activities. There are 13 genes needed to properly assemble the V-ATPase (Table 5.1). If any of them are implicated, the pump will be
unable to function properly. Interestingly, irregular vacuolar morphology has also been related to atypical vacuolar acidification as a result of V-ATPase pumps that are not functional. Some of the phenotypic characteristics include vacuoles that are fragmented. Since fragmented vacuoles is a phenotype that is also seen in pah1Δ cells, and is this phenotype is often associated with dysfunctional V-ATPase pumps there is a possibility that PAH1 can negatively impact one or more genes of the V-ATPase pump.

Although it has been shown that PAH1 plays an important role in lipid synthesis and may affect the vacuole through V-ATPase pump activity, to date, little research has been done on the effect of PAH1 on V-ATPases. We therefore wanted to better understand how Pah1p affects vacuolar morphology and its functional activity. In order to test this, electron microscopy was performed to visualize the morphological changes of the vacuole in the absence of Pah1p. While there are many types of microscopy that can detect differences in cellular structure, electron microscopy is one of the best types to visualize morphological differences. To date, it has yet to be used to look at vacuolar morphology in pah1Δ cells. Subsequently, RNA analysis of V-ATPase pump genes were examined followed by calcium chloride and pH sensitivity assays. Lastly, we concluded our experiments by measuring vacuolar pH, in order to gauge the ability of the V-ATPase pump to acidify the vacuole. This was followed by RNA analysis of other fusion genes in the lipid biosynthetic pathway that could affect the vacuole.
Figure 5.1: Vacuole fragmentation in *Saccharomyces cerevisiae*

A) Diagram illustrating vacuolar development in *Saccharomyces cerevisiae* (Konarzewska, 2015) B) EM image of budding yeast cell with fragmented vacuole.
Figure 5.2: Vacuolar proton translocation ATPase diagram.

Table 5.1: V-ATPase encoding genes (Konarzewska, 2015).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Subunit</th>
<th>Molecular mass (kDa)</th>
<th>Integral or peripheral</th>
<th>Proposed function</th>
</tr>
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<tbody>
<tr>
<td>V₁</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VMA1</td>
<td>A</td>
<td>69</td>
<td>peripheral</td>
<td>ATP binding subunit</td>
</tr>
<tr>
<td>VMA2</td>
<td>B</td>
<td>60</td>
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<td>ATP binding subunit</td>
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<td>VMA5</td>
<td>C</td>
<td>42</td>
<td>peripheral</td>
<td>Activation of V-ATPase</td>
</tr>
<tr>
<td>VMA7</td>
<td>F</td>
<td>14</td>
<td>peripheral</td>
<td>Assembly of V₁ and V₀</td>
</tr>
<tr>
<td>VMA8</td>
<td>D</td>
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<td>Assembly of V₁ and V₀</td>
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<tr>
<td>VMA10</td>
<td>G</td>
<td>16</td>
<td>peripheral</td>
<td>Activation of V-ATPase</td>
</tr>
<tr>
<td>VMA13</td>
<td>H</td>
<td>54</td>
<td>peripheral</td>
<td>Activation of V-ATPase</td>
</tr>
<tr>
<td>V₀</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VPH1</td>
<td>a</td>
<td>100</td>
<td>integral</td>
<td>H⁺ transport</td>
</tr>
<tr>
<td>VMA3</td>
<td>c</td>
<td>165</td>
<td>integral</td>
<td>H⁺ transport</td>
</tr>
<tr>
<td>VMA6</td>
<td>d</td>
<td>36</td>
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<td>Assembly of V₁ and V₀</td>
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<tr>
<td>VMA11</td>
<td>c'</td>
<td>17</td>
<td>integral</td>
<td>H⁺ transport</td>
</tr>
<tr>
<td>VMA16</td>
<td>c&quot;</td>
<td>23</td>
<td>integral</td>
<td>H⁺ transport</td>
</tr>
</tbody>
</table>
5.1 Materials and methods

5.1.1 Vacuolar Morphology: Electron Microscopy

WT and \textit{pah1Δ} were grown from a single colony until mid-logarithmic phase (exponential phase) and stationary phase in SC media with 0.002\% (w/v) of \textit{myo-inositol}. Cells were fixed with glutaraldehyde, calcium chloride, and sodium cacodylate buffer for one hour on ice. The fixative was removed and cells were washed twice with the sodium cacodylate buffer. Cells were then treated with lyticase for about 30 minutes at room temperature to break open the cell wall. Following, cells were washed with buffer and then treated with osmium tetroxide for two hours on ice. They were then washed with the buffer and left overnight in 4°C. The next day, cells were dehydrated with 50\%, 60\%, and 70\% ethanol, followed by an overnight incubation with 70\% ethanol and 3\% uranyl acetate reagent. Cells were washed with 80\%, 90\%, and 100\% ethanol, followed by two incubations/washes with propylene oxide. A 1:1 ratio of propylene oxide and resin was added, followed by fresh resin to embed and placed in the oven to set. Samples were cut into ultra-thin sections and prepared for imaging using the Leica UCT Ultramicrotome. EM microscopy was performed using the Fei Technai Spirit Transmission Electron Microscope.

5.1.2 RNA analysis: Total mRNA preparation and quantification

WT and \textit{pah1Δ} cells (Table 3.1) were grown from a single colony until mid-logarithmic phase (0.8 A_{600}) in SC media with 10\muM \textit{myo-inositol}, followed by an additional two hours in the presence of 100\muM \textit{myo-inositol}. RNA was extracted from yeast cells through acid phenol (pH 4.3) chloroform extraction in which the cells were resuspended in TES (10mM EDTA; 10mM Tris-HCl pH 7.5; 0.5\% SDS), and then incubated at 65°C with equal parts acid phenol for
60 minutes. This was followed by a 5 minute incubation on ice before centrifugation at 4°C. The top layer of the sample was transferred to a new test tube and mixed with equal parts acid phenol for a second time. Samples were incubated on ice for 5 minutes before centrifugation at 4°C and then separated by transferring the top layer of the sample to a fresh test tube and mixing with equal parts chloroform. Samples were centrifuged, then separated again from their bottom layer and transferred to a new test tube. Ethanol precipitation was then performed by adding 1 mL of 100% ethanol and 40μL of 3M NaOAc to the sample. The samples were then stored in dry ice for 20 minutes, followed by a 15 minute centrifugation. The supernatant was discarded and pellets were washed in ethanol (70%) and centrifuged again for three minutes. The supernatant was removed and the pellets were left to dry for 10 minutes. This was followed by resuspending the pellets in RNA sample buffer. RNA concentration was quantified by reading absorbance at 260nm (A_{260}). Subsequently, total RNA was stored at -80°C until further use.

### 5.1.3 DNase Treatment of RNA samples

DNase treatment was performed to remove any DNA from the isolated RNA samples. RNA was mixed with 10X buffer (0.1 M Tris-HCl pH 8, 5mM CaCl\_2 and 0.025M MgCl\_2), DNase, and DEPC water. Samples were placed in 37°C water bath for 60 minutes, followed by phenol:chloroform extraction. A 3:1 ratio of phenol:chloroform was added to the samples, mixed and then placed on ice for a five minute incubation. Samples were then centrifuged for 10 minutes and then the top layer was transferred to a new test tube to be used for ethanol precipitation. Ethanol (100%) and 20μL of 3M NaOAc were added to supernatant, mixed and then placed on dry ice for 20 minutes. Samples were centrifuged for 13 minutes, followed by removal of supernatant, and a washing of the pellet in 70% ethanol. This was followed by
centrifugation and the removal of the supernatant. Pellets were placed in a speed vacuum to dry and then resuspended in TE buffer that was prepared using DEPC water. RNA concentration was again quantified by reading absorbance at 260nm.

5.1.4 Preparation of cDNA: First Strand Synthesis

In order to prepare the cDNA needed for qRT-PCR, the SuperScript III First Strand Synthesis SuperMix kit from Invitrogen (Catalog: 11752-050) was used. The reaction was performed using a polymerase chain reaction (PCR) thermocycler. Each DNase free mRNA sample (1 μg) was mixed with 2x RT Reaction mix, RT enzyme mix, and DEPC water. Samples were placed in the PCR thermocycler and underwent the reaction using the following thermocycler program: 10 minute incubation at 25°C, 30 minute incubation at 50°C, and 5 minute incubation at 85°C. Samples were then cooled on ice for a few minutes before adding E. coli RNase H and then placed in a 37°C waterbath for 20-30 minutes. First strand synthesis samples were stored at -20°C until further use.

5.1.5 Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

The cDNA prepared from first strand synthesis was amplified and quantified using qRT-PCR. The SYBR GreenER® qPCR Supermix universal kit from Invitrogen (catalog number: 11762-500) was used to quantify cDNA templates. Forward and reverse primers were used that targeted the open reading frame (ORF) in the qPCR reaction. Primers used in this study are shown in Table 5.1.
Table 5.2: List of primers used for qRT-PCR for RNA analysis

<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>VMA1 primers:</td>
<td>5’ GCCGTTTGTCTCGTACCATT 3’</td>
<td>5’ AACATGGGAACCCAAACAGAG 3’</td>
</tr>
<tr>
<td>VMA2 primers:</td>
<td>5’ AACGGAACGTATGTGTTGA 3’</td>
<td>5’ AGTGATGGACCCGTTACGAC 3’</td>
</tr>
<tr>
<td>VMA4 primers:</td>
<td>5’ CATTGACGGCAACTTCGAAGA 3’</td>
<td>5’ CTTGACTCGTCCCCGTGT 3’</td>
</tr>
<tr>
<td>VMA5 primers:</td>
<td>5’ TAACCTGGCTGCTGCTGAGA 3’</td>
<td>5’ TCAGCATTTCCTGCAACTAC 3’</td>
</tr>
<tr>
<td>VMA7 primers:</td>
<td>5’ TTATTTGTTAGCCGGAGATTGA 3’</td>
<td>5’ AGATTTCTGGCTGCTTGA 3’</td>
</tr>
<tr>
<td>VMA8 primers:</td>
<td>5’ CTTGGCCGAAGTTTCTATG 3’</td>
<td>5’ AGATTTCCTTGGCTCGTTGA 3’</td>
</tr>
<tr>
<td>VMA10 primers:</td>
<td>5’ TGGACTGGTACCTCGTGACA 3’</td>
<td>5’ TCAGTCTCTGGGTCTGTTCA 3’</td>
</tr>
<tr>
<td>VMA13 primers:</td>
<td>5’ AACTGGCCGTGATACCAGAG 3’</td>
<td>5’ GGGTTAAAGGTCAGCAACCA 3’</td>
</tr>
<tr>
<td>VPH1 primers:</td>
<td>5’ TTGGTGAAATGATCGCAAGA 3’</td>
<td>5’ TGTGGGTAAACCAGCATTGA 3’</td>
</tr>
<tr>
<td>VMA3 primers:</td>
<td>5’ TTTCTTGTGCTGCCATTGGTT 3’</td>
<td>5’ GGCAATTAACCCGCTCATA 3’</td>
</tr>
<tr>
<td>VMA6 primers:</td>
<td>5’ TTGCCTACGTTGAGTGTTGC 3’</td>
<td>5’ CAGGTTCCCGAATTTCTTCA 3’</td>
</tr>
<tr>
<td>VMA11 primers:</td>
<td>5’ GCCATTTATGGGCTTGTTGT 3’</td>
<td>5’ ACGTCACCAGCATACCAAT 3’</td>
</tr>
<tr>
<td>VMA16 primers:</td>
<td>5’ GTGGTCTACCTTGACGCATT 3’</td>
<td>5’ ACGTACCCAGCATACCAAT 3’</td>
</tr>
<tr>
<td>ACT1 primers:</td>
<td>5’ CCAAGCCTTTGTGTCCTTGT 3’</td>
<td>5’ ACGGCCAAACATCGATTTC 3’</td>
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</tbody>
</table>
**FAB1 primers:**
Forward: 5’ TCCTCGATAGCAACAGCGAC 3’
Reverse: 5’ GTGCGCGCATAGGAATTCTG 3’

**VPS34 primers:**
Forward: 5’ CCAAGAAGGGTGCCATTGA 3’
Reverse: ACCCAGCCTTGTAGCGGTAA 3’

**VPS1 primers:**
Forward: 5’ TC ACTGTGTTGTCGGTTCCCAG3’
Reverse: 5’ TCCCTCGTTCTGGTGCTTTTC 3’

**ATG18 primers:**
Forward: 5’ GTGGCAACACAGAGACCAGT 3’
Reverse: 5’ TGTTACC CGCGTTACTGCTT 3’

The reaction setup for each sample contained the following: 10μL of SYBR GreenER® qPCR Supermix, 0.4 μL each of forward and reverse primers, 0.4 μL of ROX reference dye, 1 μL of the cDNA template, and RNA and DNA free water to a volume of 20μL. Samples were added to a 96 well plate and then sealed and placed in the Applied Biosystems 7500 real time PCR machine. The program used had a 2 minute incubation at 50°C, followed by 10 minutes at 95°C, and 40 cycles of 15 seconds at 90°C and 1 minute at 60°C. PCR reactions were performed in triplicates.

**5.1.6 Analysis of RNA quantity**

A ΔC_T value was obtained by subtracting the respective gene C_T from the C_T value of the constitutive housekeeping gene (*ACT1*). Therefore, the relative mRNA levels were expressed as 2^{ΔC_T}. Data results were graphed as a mean of the repeats ± standard deviation.
5.1.7 Growth analysis on SC and YPD plates (CaCl$_2$ and pH)

The yeast strains that were used in this experiment are shown in Table 5.3. Individual colonies of WT, vma3Δ, and pah1Δ were incubated overnight in SC + 100mM inositol media (dextrose-nitrogen base-amino acids). On the following day, each strain's OD was measured and then either diluted or concentrated so that all strains contained the same OD for their starting point. The strains were then diluted 100 fold in SC medium and 5ul of each diluted strain were plated on SC+ inositol plates that contained 60mM of CaCl$_2$ for the calcium chloride sensitivity assay and on YPD plates buffered to pH 5.5 and pH 7.0 for the pH sensitivity assay. Plates were incubated at 30°C for two days before analyzed.

Table 5.3: List of strains used in Chapter 5

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genome type</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (BY4741)</td>
<td>$MATa$ his3Δ1 leu2Δ0 met15Δ0 ura3Δ</td>
</tr>
<tr>
<td>vma3Δ</td>
<td>$MATa$ his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Δvma3</td>
</tr>
<tr>
<td>pah1Δ</td>
<td>$MATa$ his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Δpah1</td>
</tr>
</tbody>
</table>

5.1.8 Growth analysis in liquid broth

WT, pah1Δ, and vma3Δ cells were grown in SC media containing 100μM of inositol. Media was buffered to pH 7. Growth experiments were performed in duplicates where the optical density (OD) of the cells and pH of the growth medium was measured every two hours for 8 hours and then resumed at 24 hours to 30 hours at 30°C.
5.1.9 Vacuolar pH analysis

WT, pah1Δ and vma3Δ cells were grown until stationary phase in SC media with 100μM of inositol buffered to pH 7.0. Cells were then incubated with 1mM of BCECF-AM dye for 30 to 40 minutes at 30°C in the dark. Cells were then spun down, washed twice with media, and resuspended in fresh media. Cells were then stored on ice. Next, calibration samples were made for all three strains. Cells with the BCECF dye were incubated with 15μM of nigericin and the calibration buffers (50mM HEPES, 50mM MES, 10mM 2-deoxyglucose, 50mM potassium chloride, 50mM sodium chloride, 0.2M ammonium acetate, 10mM 2-deoxyglucose) that ranged from pH 5.0 to pH 7.0 for thirty minutes at 30°C. The unknown samples were prepared by resuspending cells with BCECF with 1mM MES buffer at pH 7. All samples were plated in a 96 well plate. The SpectraMas M5 microplate reader was used to measure the excitation wavelength (450nm and 490nm) with emission wavelength of 535nm. The calibration curves were made by taking the ratio of fluorescence at 490nm to 450nm. The fluorescence ratio was then plotted against pH (Figure 5.3A and 5.3B). Unknown samples were then plotted on standard curve to determine their pH.

A)
Figure 5.3: Calibration curve for vacuolar pH determination of cells (WT, pah1Δ, vma3Δ) grown in pH 7.0 medium. A) Repeat 1 B) Repeat 2.
5.2 Results

5.2.1 Vacuolar morphology in the pah1Δ strain is distorted in both exponential and stationary phase cells, resulting in fragmented vacuoles.

A previous study has shown that pah1Δ cells exhibit fragmented vacuoles (Sasser et al. 2012). Unlike WT cells however, pah1Δ cells are unable to bind SNARES and are devoid of components of the HOPS tethering complex needed for vacuolar fusion. This morphological defect in the vacuole was exhibited for pah1Δ cells in the exponential phase and was captured using a Zeiss Axio Observer Z1 inverted microscope. We however confirmed in chapter 4 that the PAH1 gene is upregulated in the stationary phase of growth when grown in the presence of 100μM inositol. As a result, we wanted to look at the vacuolar morphology of pah1Δ in the exponential phase of growth as well as the stationary phase and confirm the morphology when PAH1 is at inducing conditions. In addition, electron microscope (EM) is known for its high magnification and high resolution properties making it ideal to examine morphological structures in cells. We therefore decided to use this type of microscopy since it had not been used to visualize the vacuolar defect in the pah1Δ cells prior to this.

Results showed that while WT cells had many fragmented vacuoles, due to the normal fragmentation process that occurs in vacuoles during cell division, there was also a good percentage that contained large singular vacuoles. This was greatly contrasted to the pah1Δ cells, which mainly exhibited fragmented vacuoles (Figure 5.4A). Both the exponential phase and the stationary phase displayed vacuoles that were unable to fuse their fragments back together, demonstrating that the necessary fusion machinery is unavailable in pah1Δ cells no matter at what stage of growth they are in. This is likely due to the fact that the phosphatase activity encoded by PAH1 is unavailable in the mutant and as such impacts the lipid biosynthetic
pathway which produces lipids, such as phosphatidylinositol 3-phosphate, that are required for
SNARE activity and vacuole fusion. However, upon quantification, it was seen that the number
of fragmented vacuoles between WT and pah1Δ cells greatly differed in exponential phase and
stationary phase. In exponential phase, about 74.1% of wild type cells contained fragmented
vacuoles compared to the 96.4% of pah1Δ cells that had fragmented vacuoles (Figure 4.5B). In
stationary phase however, only 49.89% of wild type cells contained fragmented vacuoles, a
significant decrease from exponential phase, while pah1Δ cells contained 97% fragmented
vacuole, a value similar to that in exponential phase (Figure 5.4B). Thus, stationary phase cells
showed a much more significant difference in the number of fragmented vacuoles. This is due to
the fact that in exponential phase, cells are at their peak stage of growth and are constantly
dividing. Thus, our results showed that a large percentage of WT cells had fragmented vacuole in
this stage, due to the normal process of cell division. However, once they reached stationary
phase their growth rate steadied and less cells were dividing, leaving only a smaller number of
cells with fragmented vacuoles. For pah1Δ cells however, their lack of fusion machinery resulted
in fragmented vacuoles in almost all cells regardless of their stage in growth.

Lastly, it should be noted that in accordance with previous studies, a portion of the pah1Δ
cells examined in stationary phase had undergone apoptosis, a phenotype that is seen in pah1Δ
stationary cells as a result of the excess fatty acids that accumulate due to the blockage of TAG
synthesis (Fakas et al.; 2011). We had therefore collected cells just prior to entering stationary
phase, when PAH1 induction has been shown to occur, but there were still cell that already
exhibited the hallmarks of apoptosis.
Figure 5.4: The pah1Δ mutant strain has fragmented vacuoles in both exponential and stationary phases of growth, but has a more significant difference in stationary phase when compared to WT.

A) Electron microscopy images of vacuoles in WT and pah1Δ cells in both exponential and stationary phase. B) Quantification of fragmented vacuoles in all cell lines tested.

WT and pah1Δ were grown in SC media and subsequently washed and collected at exponential phase of growth and stationary phase. Cells were fixed, treated with lyticase to break open the cell wall, and treated with additional fixative. They were then dehydrated with 50%, 60%, and 70% ethanol, followed by an overnight incubation with 70% ethanol and 3% uranyl acetate reagent. Cells were washed with 80%, 90%, and 100% ethanol, followed by two washes of propylene oxide. A 1:1 ratio of propylene oxide and resin was added, followed by fresh resin, which was placed in oven to set. Samples were cut and prepared for imaging.
5.2.2 The effect of PAHI deletion on genes encoding V-ATPases

Since the morphology of vacuoles in pah1Δ is distorted (Figure 5.4A), we wanted to see if other functions of the vacuole were implicated as well. One important aspect of vacuolar homeostasis is the acidification of the vacuole via the V-ATPase pump. In order to maintain an acidic pH, the V-ATPase pump must be functional. V-ATPases are responsible for transporting hydrogen ions into the vacuole. This assures that there is an acidic vacuolar interior and that normal physiological processes that are associated with this organelle can occur. Interestingly enough, inhibition of V-ATPase function is directly related to a distortion in vacuole morphology. This distortion occurs as a result of the inability of the V-ATPase pump to function. V-ATPases thus play a crucial role in the morphology of the vacuole. As such, since pah1Δ cells have the fragmented vacuole phenotype, we were interested in looking at whether the V-ATPase pump was implicated. We therefore performed RNA analysis on the genes that encode the proteins that comprise the V-ATPase pump. Cells were collected right before stationary phase and their RNA was isolated. We then treated them with DNase and converted the RNA to cDNA before performing qPCR. Results showed that none of the V-ATPase pump genes were downregulated or negatively implicated, which would render them transcriptionally inactive (Figure 5.5). In fact, multiple of these V-ATPase encoding genes actually had an upregulation of gene expression compared to WT. This is unsurprising given the regulatory role of PAHI and the fact that its deletion has been shown to cause an increase in gene expression (Santos-Rosa et al., 2005). Based off of these results it is likely that the V-ATPase is still able to function properly since the genes of the pump transcribe just as well or even better compared to WT.
Figure 5.5: The *pah1Δ* cells exhibit higher or similar expression levels of V-ATPase genes to WT, with no genes being downregulated.

WT and *pah1Δ* cells were grown to stationary phase in SC media that contained 100μM inositol. mRNA was extracted from cells using acid phenol chloroform and purified further with treatment of DNase. RNA was converted to cDNA and was amplified/quantified using qPCR with primers targeting the open reading frame sequence of tested gene. Expression levels of mRNA were normalized to the housekeeping gene *ACT1*. All experiments were done with at least two repeat colonies and all qPCR reactions were done in triplicates. Data was graphed as a mean ± standard deviation.
5.2.3 The effects of CaCl$_2$ and pH upon the PAH1 deletion strain

Based off of our RNA findings, it seemed that the V-ATPase pump was not implicated in $pah1\Delta$ cells. In order to confirm this finding further we decided to perform growth analysis experiments to determine if $pah1\Delta$ is able to withstand conditions that cells without functional V-ATPases cannot. We used $vma3\Delta$ as our negative control, since $VMA3$ is one of the genes that encodes a portion of the V-ATPase pump and without which will render the pump inactive. Previous studies have shown that $vma3\Delta$ cells were sensitive to CaCl$_2$. This is due to the fact that cytoplasmic calcium levels are maintained within a physiological range by transporting the calcium cation into the vacuole via the Ca$^{2+}$/H$^+$ antiporter. This process is driven by vacuolar hydrogen ATPases. Mutants that lack functional V-ATPases amass calcium into the vacuole at slower rates and therefore exhibit minimal growth in the presence of elevated calcium levels as compared to wild type (Ohya et al., 1991; Clemens et al., 1999; Förster et al., 2000). In addition, studies have shown that cells that do not have functioning V-ATPases cannot acidify the vacuole and as such are unable to survive well in high pH conditions. We therefore wanted to examine whether $pah1\Delta$ would follow the same conditional phenotype or would be able to withstand these conditions rendering their V-ATPases functional. We thus performed a growth sensitivity assay by conducting a serial dilution experiment using CaCl$_2$ plates as well as a serial dilution experiment using different pHs as well as a pH growth curve.

For WT cells, we observed that cells grew well on SC plus 100uM inositol plates that contained 60 mM CaCl$_2$ and that the last visible growth was seen at $10^8$ fold dilution (Figure 5.6A). For the $vma3\Delta$ cells, which are sensitive to CaCl$_2$, the last visible growth was at $10^4$ fold dilution (Figure 5.6A). For the $pah1\Delta$ strain, the last visible growth was seen at $10^6$ fold dilution. The $pah1\Delta$ strain was therefore able to grow better than the $vma3\Delta$ cells in the presence of
CaCl₂. We then followed this experiment by performing a similar type of sensitivity assay, except this time used plates that were buffered to a specific pH (pH 5.5 and pH 7.0). Cells that do not have properly functioning V-ATPases are unable to grow well in non-acidic medium. As such, we decided to perform growth curve analysis of cells grown in both acidic and neutral conditions to see whether pah1Δ could handle a more neutral growth environment. Results showed that all three strains grew well in the more acidic pH, with all reaching the 10⁻⁶ dilution, and WT extending a little further and reaching the 10⁻⁸ dilution (Figure 5.5B). Results differed vastly though for the pH 7.0 plates. While WT still grew relatively the same and reached growth at the 10⁻⁸ dilution, the vma3Δ mutant was unable to grow at all and exhibited no growth at the lowest dilution of 10⁻². However, pah1Δ cells grew well and had comparable growth to WT when it grew on more acidic medium, reaching the 10⁻⁶ dilution (Figure 5.6C). Thus, these results suggest that pah1Δ cells are more similar to WT in regard to a functional V-ATPase.

In order to quantify the above results, we decided to perform a growth curve to measure optical density of cells grown in different pHs since this condition most clearly exhibited the difference between pah1Δ cells and vma3Δ cells. Results showed that similar to the serial dilution plates, all cells grew well in the acidic media that was buffered to pH 5.5 and all had similar number of generations and generation times (Table 5.6). By the 24th hour of growth analysis in pH 5.5, WT had 4.2 generations of cells and a generation time of 343.8 minutes/generation. The pah1Δ mutant had 4.6 generations and a generation time of 312.5 minutes/generation and the vma3Δ mutant had 4.7 generations and a generation time of 306.3 minutes/generation (Figure 5.7, Table 5.6). At pH 7 however the V-ATPase pump had to work harder and thus cells that did not have properly functioning pumps were unable to survive well. Results showed that vma3Δ cells that grew in media buffered to pH 7.0 were not able to grow
much since they did not have properly functioning V-ATPase pumps. At 24 hours, they only reached an O.D of 1.2 ± 0.14 and had about 2.4 generations with a generation time of 612.1 minutes/generation. WT cells however grew fine in the more neutral media. At the 24th hour analysis, they reached an O.D of 5.67 ± 1.46 and had about 4.5 generations with a generation time of 321.9 minutes/generation (Figure 5.8, Table 5.6). In terms of their growth, they grew 47.4% better than vma3Δ (Table 5.5). Thus, both our negative control and positive control exhibited expected results. Results for our experimental strain showed that pah1Δ grew fine under neutral conditions in accordance with our serial dilution experiment. At hour 24, it had an O.D of 5.03 ± 0.21 and had about 4.98 generations with a generation time of 289.3 minutes/generation. They grew 53.7% better than the vma3Δ mutant did. Thus, pah1Δ exhibited results that were significantly more similar to WT than to vma3Δ (Figure 5.9). Taken together with the RNA analysis, these findings suggest that V-ATPase pump activity functions properly in the pah1Δ mutant despite its morphological abnormalities.
Figure 5.6: The \textit{pah1}\textsuperscript{Δ} cells exhibit cellular growth more similar to WT than to \textit{vma3}\textsuperscript{Δ} when grown in the presence of CaCl\textsubscript{2} and neutral pH.

WT, \textit{pah1}\textsuperscript{Δ}, and \textit{vma3}\textsuperscript{Δ} cellular growth on A) SC+inositol plates containing 60mM CaCl\textsubscript{2} B) YPD plates buffered to pH 5.0 and C) YPD plates buffered to pH 7.0. Cells were diluted to same O.D and dive serial dilutions were performed and plated for each strain. YPD plates were grown for 3 days at 30°C.
Figure 5.7: The *pah1Δ* mutant strain grows well in pH 5.5 media, similar to WT and *vma3Δ* cells.

WT, *pah1Δ*, and *vma3Δ* were grown in SC media and subsequently washed and diluted to an optical density (OD) of 0.2 in SC media with 100 µM inositol buffered to pH 5.5. The OD was then taken every 2 hours for the first 8 hours and then grown overnight and resumed at the 24th hour and continued until stationary phase.

Table 5.4: Growth rate percentage and fold change for cells grown in pH 5.5 media

<table>
<thead>
<tr>
<th>Strain Comparison</th>
<th>Difference in cell doubling time (Folds)</th>
<th>Difference in growth rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td><em>pah1Δ</em></td>
<td>1.10018142</td>
</tr>
<tr>
<td>Wild Type</td>
<td><em>vma3Δ</em></td>
<td>1.122414954</td>
</tr>
<tr>
<td><em>pah1Δ</em></td>
<td><em>vma3Δ</em></td>
<td>1.020208971</td>
</tr>
</tbody>
</table>
Figure 5.8: The \textit{pah1Δ} mutant strain grows well in pH 7.0 media and exhibits similar growth pattern to WT, unlike \textit{vma3Δ} which does not grow well.

WT, \textit{pah1Δ}, and \textit{vma3Δ} were grown in SC media and subsequently washed and diluted to an optical density (OD) of 0.2 in SC media with 100 \textmu M inositol buffered to pH 7.0. The OD was then taken every 2 hours for the first 8 hours and then grown overnight and resumed at the 24\textsuperscript{th} hour and continued until stationary phase.

Table 5.5: Growth rate percentage and fold change affected by pH change to pH 7.0

<table>
<thead>
<tr>
<th>Strain Comparison</th>
<th>Difference in cell doubling time (Folds)</th>
<th>Difference in growth rate %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>\textit{pah1Δ}</td>
<td>1.112581304</td>
</tr>
<tr>
<td>Wild Type</td>
<td>\textit{vma3Δ}</td>
<td>1.901486493</td>
</tr>
<tr>
<td>\textit{pah1Δ}</td>
<td>\textit{vma3Δ}</td>
<td>2.115558321</td>
</tr>
</tbody>
</table>
Figure 5.9: The *pah1Δ* cells grow fine in pH 7.0 media, heavily suggesting that they do not implicate the v-ATPase pump.

*WT, pah1Δ,* and *vma3Δ* were grown in SC media and subsequently washed and diluted to an optical density (OD) of 0.2 in SC media with 100 µM inositol buffered to pH 7.0. The OD was then taken every 2 hours for the first 8 hours and then grown overnight and resumed at the 24th hour and continued until stationary phase.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Average O.D pH 5.5 (24th Hour)</th>
<th>Average O.D pH 7.0 (24th Hour)</th>
<th># of generations in 24 hours for pH 5.5</th>
<th># of generations in 24 hours for pH 7.0</th>
<th>Generation Time (min/generation) for pH 5.5</th>
<th>Generation Time (min/generation) for pH 7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>4.875±0.275</td>
<td>5.67±1.46</td>
<td>4.18820875</td>
<td>4.47331573</td>
<td>343.8224038</td>
<td>321.9088674</td>
</tr>
<tr>
<td><em>pah1Δ</em></td>
<td>6.5±0.173</td>
<td>5.03±0.208</td>
<td>4.60778945</td>
<td>4.976927447</td>
<td>312.5142795</td>
<td>289.3351401</td>
</tr>
<tr>
<td><em>vma3Δ</em></td>
<td>6.067±0.153</td>
<td>1.2±0.141</td>
<td>4.700908133</td>
<td>2.352536159</td>
<td>306.3237909</td>
<td>612.1053633</td>
</tr>
</tbody>
</table>
5.2.4 The pah1Δ strain exhibits a vacuolar pH that is significantly similar to WT and not vma3Δ cells.

The CaCl$_2$ and pH sensitivity assays and growth analyses suggested that the vma3Δ V-ATPase pump was not negatively implicated by the deletion of the PAH1 gene, despite the fact that morphology is altered. In order to further confirm these results and characterize the vacuole conditions in pah1Δ better, the vacuolar acidification assay was employed. This assay was used to measure vacuolar pH to fully confirm the functionality of the pump. The experiment was performed in media that was neutral in pH (pH 7.0) since at these conditions the pump would have to continuously work to acidify the vacuole. The assay was performed on cells entering the stationary phase of growth (with 100µM inositol) since this is when PAH1 induction is at its peak.

Results showed that WT cells that were grown in media buffered to pH 7.0 maintained the acidic pH of their vacuoles and remained within the normal physiological pH range of 5.5-6.2. The vacuolar pH measured for the WT vacuoles was 6.0±0.07. The pH measured for the vacuoles of vma3Δ, our negative control, however was not within the normal range. They had an average vacuolar pH of 6.6 ± 0.11, confirming that these cells were unable to maintain the acidity required and were defective in vacuolar acidification at pH 7. The vacuolar pH measured in pah1Δ cells however was 5.9 ± 0.03 and fell within the normal range of acidic pH values for the vacuole (Figure 5.10). These results suggest that acidification is well maintained in pah1Δ cells when grown in an extracellular environment that is neutral. They are thus similar to WT in this respect and did not show any significant difference in pH value (p=0.11). The pah1Δ cells however did show a significant difference to vma3Δ cells (p=0.0066), whose vacuoles were unable to properly acidify in a more neutral to basic growth environment. These finding
therefore suggest that V-ATPase pump functioning is not affected in *pah1A* cells despite the fact that vacuolar morphology is distorted.
Figure 5.10: The \textit{pah1Δ} cells exhibit a vacuolar pH similar to WT, indicating that v-ATPase pump activity is not affected by deletion of \textit{PAH1}.

\textit{WT, pah1Δ, and vma3Δ} were grown in SC media with 100\,µM inositol and subsequently washed and diluted and grown overnight in SC media with 100 \,µM inositol buffered to pH 7.0. Cells were grown to stationary phase, spun down, diluted to same cell number and treated with BCECF dye. Cells were grown for 40 minutes at 30ºC and then collected and washed. Calibration samples in a range of pHs were prepared and incubated for 40 minutes with nigericin. Experimental and calibration samples were plated on 96 well plate. The excitation wavelength at 450nm and 490nm with emission wavelength of 535nm was measured using the SpectraMas M5 microplate reader. Calibration curve was generated by calculating the ratio of fluorescence at 490nm to 450nm for each calibration sample.
5.2.5 Upregulation of genes involved in vacuolar fusion and fission may play a role in the fragmented vacuole phenotype in \textit{pah1\Delta}.

Since our previous findings indicated that vacuolar acidification is unaffected in the \textit{pah1\Delta} mutant, we decided to look into other genes involved in vacuolar fusion that may be affected. There are a number of different genes that are employed and necessary for proper vacuolar fission and fusion to occur. Some of the key genes involved in this process include \textit{VPS1}, a gene which encodes a dyamin like GTPase that generates force which supports the progression of fission (Rooji \textit{et al.}, 2010), and \textit{VPS34}, a phosphatidylinositol (PI) 3-kinase encoding gene that synthesizes PI-3-phosphate, a lipid necessary for vacuolar fission and fusion (Herman and Emr, 1990). In addition, \textit{FAB1} encodes for the vacuolar membrane kinase which produces phosphatidylinositol (3,5)P$_2$, a step which is crucial for vacuole vesiculation, while \textit{ATG18} helps regulate Fab1p activity (Cooke \textit{et al.}, 1998; Zeiger and Mayer; 2012). Since these genes play a crucial role in vacuolar homeostasis, we decided to examine their expression levels in \textit{pah1\Delta} cells to see if \textit{PAH1} affects their expression levels and could thus be a contributing factor to the mutant vacuole phenotype.

Results showed that for all four genes tested, expression was upregulated in \textit{pah1\Delta} cells compared to WT in stationary phase (Figure 5.11). The upregulation of these genes in \textit{pah1\Delta} cells was not surprising given our previous findings that \textit{PAH1} negatively regulates many genes in the biosynthetic pathway. However what was surprising was to find that genes such as \textit{VPS34} was overexpressed, despite the fact that previous research has found the vacuole to be devoid of multiple components of the fusion machinery, including Vps34p (Sasser \textit{et al.}, 2012). These findings therefore suggest that the inability of vacuolar fusion does not arise from a defect...
in v-ATPase gene expression or the downregulation of these genes, but is rather a recruitment issue of proteins that are unable to be brought to the vacuole.

In addition, our results also yielded another possible explanation as to why there is an increased amount of vacuole fragmentation in pah1Δ. Interestingly, the deletion of FAB1 in previous studies has been shown to cause an inability of the vacuole to fragment, denoting its important role in the fission process (Weisman, 2003; Zieger and Mayer, 2012). Thus, cells with this defect only contain single enlarged vacuoles. This phenotype is also seen with the deletion of VPS34 and ATG18 (Zieger and Mayer, 2012). It is therefore possible that the upregulation of these genes as compared to WT can contribute to the fragmented phenotype since their overexpression would lead to increased fission.
Figure 5.11: Upregulation of vacuolar fission genes in the pah1Δ strain.

WT and pah1Δ cells were grown to stationary phase in SC media that contained 100μM inositol. mRNA was extracted from cells using acid phenol chloroform and purified further with treatment of DNase. RNA was converted to cDNA and was amplified/quantified using qPCR with primers targeting the open reading frame sequence of tested gene. Expression levels of mRNA were normalized to the housekeeping gene ACT1. All experiments were done with at least two repeat colonies and all qPCR reactions were done in triplicates. Data was graphed as a mean ± standard deviation.
5.3 Discussion

The effect of *PAH1* and the phosphatase enzyme it encodes is not merely limited to the lipid biosynthetic pathway. Research has shown that its influence is actually widespread throughout the cell and impacts multiple organelles. One such organelle is the vacuole. While it has been documented that vacuolar morphology is implicated in the *pah1Δ* cell and exhibits a fragmented vacuole phenotype (Sasser et al.; 2012), limited information is available on how else *PAH1* affects vacuole homeostasis. Since normal vacuolar morphology is compromised in the *pah1Δ* mutant we therefore wanted to test if other factors involved in vacuolar homeostasis are affected. Many times, atypical vacuole morphology is connected to irregular vacuolar acidification as a result of dysfunctional V-ATPase pumps. Studies have shown that some phenotypes of mutant cells with abnormal vacuoles include vacuole fragments that collect to one area or disperse all over the cell. These cells can have working V-ATPase pumps or have a defect in the assemblage of the V-ATPase pump (Weisman *et al*., 1987; Raymond *et al*., 1992). Since *PAH1* plays such an important role in lipid synthesis, it could affect the V-ATPase pump and thus alter its morphology. In additional, overall vacuole homeostasis could be impacted. We therefore wanted to look into these other factors that implicate vacuolar morphology and see if *PAH1* affects them.

In order to test this, we started off by performing electron microscopy on the *pah1Δ* mutant to characterize the vacuole phenotype. This was important for two reasons. First, previously published studies have only imaged cells in the exponential phase of growth. However, as was determined by earlier experiments (Chapter 4), *PAH1* is induced in stationary phase of growth when grown with excess inositol. We therefore wanted to characterize the phenotype not only in exponential phase, but also during *PAH1*’s highest induction stage, which
is during stationary phase. Secondly, there has yet to be any published studies imaging vacuolar morphology for \( pah1\Delta \) cells using electron microscopy. Since this form of microscopy is renowned for its high resolution and magnification and can clearly capture detail of cell structure, we decided to employ it.

Our results showed that while WT cells had normal fragmentation of the vacuole due to the process of cell division, many cells also contained large singular vacuoles in both exponential and stationary phases of growth. This greatly differed from the vacuoles seen in \( pah1\Delta \) in which vacuoles were fragmented. This phenotype was found in both exponential and stationary phases of growth for \( pah1\Delta \), demonstrating that the lack of \( PAH1 \) phosphatase activity directly impacts vacuole fusion regardless of an increased induction of \( PAH1 \) in the stationary phase. This is unsurprising since even in exponential phase, \( PAH1 \) transcription is still active, though not to the extent it is at during stationary phase. Still, the lack of \( PAH1 \) means that the phosphatase activity encoded by it is unavailable and as such impacts the lipid biosynthetic pathway which produces lipids, such as phosphatidylinositol 3-phosphate, that are required for SNARE activity and vacuole fusion. Thus, both exponential and stationary phase cells in \( pah1\Delta \) exhibit the fragmented vacuole phenotype. It should also be noted that another difference between cells in both phases of growth was that there were a number of \( pah1\Delta \) cells that had undergone apoptosis in stationary phase. This is due to the toxic buildup of fatty acids that occurs in the mutant since \( PAH1 \) is unavailable to convert PA into DAG which eventually is converted into TAG. Therefore, even though we collected cells slightly prior to stationary phase, some cells underwent apoptosis.

Once we confirmed the morphological distortion of the vacuoles of \( pah1\Delta \) cells, we set out to determine if the V-ATPase pump is implicated. In order to do this, we performed RNA
analysis on the genes which encode the necessary proteins that make up the pump. Downregulation or unavailability of just one gene will essentially make the pump inactive so it was important to test all genes in the study. Results showed that not only were RNA levels comparable to that of WT, some genes were even upregulated (Figure 5.5). This result was not surprising since our previous findings have shown that the deletion of PAH1 causes the upregulation of many genes in the lipid biosynthetic pathway. However, it suggested that V-ATPase pumps were functional, based off of the RNA analysis.

In order to confirm the above finding, we performed sensitive assays and growth curve analysis using conditions that cells without functional V-ATPases would not be able to withstand. These conditions include higher concentrations of CaCl₂ in growth medium as well as a more neutral to basic pH. We used the vma3Δ as our negative control, since VMA3 is one of the genes that encodes for a protein of the V-ATPase pump. Results showed that while WT grew fine in 60mM CaCl₂ and vma3Δ did not, the pah1Δ mutant grew fairly well. Results were even stronger when cells were grown in varying pHs. While all cells grew well in the acidic pH 5.5 media, only vma3Δ did not grow well in pH 7.0. The pah1Δ mutant grew just as well in pH 7.0 as it had in pH 5.5, similar to WT. This was yet again confirmed when we performed growth curve analysis and actually quantified the serial dilution using optical density. Therefore, our results heavily suggested that V-ATPases are functional in the pah1Δ mutant despite the morphological distortion of the vacuole.

In order to verify our findings and characterize the vacuolar condition of pah1Δ we performed one last experiment to show that the V-ATPase pump in pah1Δ mutant is not negatively implicated and can function properly. We employed a vacuolar pH assay in which we measured the internal pH of the vacuole to determine whether or not the pump was able to
perform acidification. Results showed as we expected. While vma3Δ vacuoles did not fall into the normal acidic vacuolar pH range of 5.5-6.2 when cells were grown in pH 7.0 media, both WT and pah1Δ did. This therefore confirmed our previous findings and validated our conclusion that the V-ATPase pumps in pah1Δ function as they should and are not implicated as a result of the fragmented phenotype. Thus, individual fragments of the vacuole each have properly functioning V-ATPases.

Since our findings indicated that vacuolar acidification is unaffected in the pah1Δ mutant and that overall vacuole function is seemingly not impacted, we decided to look into other genes involved in vacuolar fusion and fission that may be implicated and could cause the fragmented phenotype. Results showed that for all four genes tested, expression was upregulated in pah1Δ cells compared to WT in stationary phase (Figure 5.11). The upregulation of these genes in pah1Δ cells was not surprising given our previous findings that PAH1 negatively regulates many genes in the biosynthetic pathway. These findings therefore suggest that the inability of vacuole fusion does not arise from a defect in gene expression or the downregulation of these genes needed, but is rather a recruitment issue of proteins that are unable to be brought into the vacuole. In addition, our results also yielded another possible explanation for the increased amount of vacuole fragmentation in pah1Δ. Interestingly, the deletion of FAB1 in previous studies has been shown to cause an inability to fragment, which denotes its important role in the fission process (Weisman, 2003; Zieger and Mayer, 2012). Thus, cells with this defect only contain single enlarged vacuoles. This phenotype is also seen with the deletion of VPS34 and ATG18. It is therefore possible that the upregulation and the overexpression of these genes as compared to WT, can contribute to the fragmented phenotype, regardless of their absence in the vacuole. Future studies therefore can be performed to confirm this hypothesis and connect the
overexpression of these genes to vacuolar fragmentation in the *pah1Δ* mutant as well as other impacts it may have on the cell.
Chapter 6

Concluding Remarks

Our study has looked into three important areas with respect to *PAH1*. We have elucidated the ways in which it impacts the lipid biosynthetic pathway, the modes in which it itself is regulated, and the influence it has on vacuolar morphology and function. Future areas of study include further elucidating the role that *PAH1* plays in regulating apoptosis and as well as identifying additional mechanisms of control over *PAH1*, such as the histone modifying enzymes involved. Overall, the findings of this thesis can be used to further understand the functional role of *PAH1* and can provide insights into the ways in which *PAH1* affects cell homeostasis.
Appendix

**Title:** The absence of Pah1p leads to slightly increased vacuolar acidification

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Abstract

In yeast, PAH1 plays an important role in cell homeostasis and lipid biosynthesis. Here, we showed that PAH1 is most greatly induced at the stationary stage compared to other induction conditions. Subsequently, we observed that the absence of Pah1p leads to unusual upregulated expression of certain V-ATPase genes and a more acidic vacuole internal environment, suggesting an important role for Pah1p in maintaining the V-ATPase pump. These observations provide evidence for PAH1 induction and its role in vacuolar acidification. As such, we provide evidence which describes novel insight into the role of PAH1 in vacuole homeostasis.

Key words: PAH1; vacuole; V-ATPase; fragmentation; pump activity; acidic pH.
Introduction

The regulation of phospholipid composition in membranes is essential for correct cellular growth and development (Cardozo-Gizzi and Caputo, 2013). *Saccharomyces cerevisiae* is a common model system used to better understand phospholipid synthesis and its regulation. There are four main phospholipids found in *Saccharomyces cerevisiae*, including phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine, and phosphatidylcholine (Carman and Henry, 1999; Santos-Rosa et al., 2005). The synthesis of these and other phospholipids, as well as membrane biogenesis in general, is mainly regulated by the crucial phospholipid precursors, inositol and choline (Kelley *et al*., 1988; Dowd *et al*., 2001; Boumann *et al*., 2006; Gaspar *et al*., 2006). PA phosphatase (PAP) enzymes are responsible for catalyzing the reaction that dephosphorylates PA, which in turn produces DAG and a phosphate group (Figure 1A) (Han *et al*., 2007). This reaction is crucial in lipid biosynthesis, since DAG can be used to either generate triacylglycerol (TAG), which stores energy and fatty acids in lipid droplets or can be used to generate the phospholipids, phosphatidylethanolamine (PE), and phosphotidylcholine (PC) via the Kennedy pathway (Siniossoglou, 2009). The concentrations of PA and DAG are crucial in maintaining the production of phospholipid levels, as well as determining the types of phospholipids that are synthesized (Pascual and Carman, 2012). Since PAP enzymes are responsible for regulating PA and DAG levels, they play a critical role in regulating the lipid biosynthetic pathway.

While *Saccharomyces cerevisiae* contain four PAP enzymes, *PAH1* encodes for the PA phosphatase, Pah1p, which is the only PAP enzyme responsible for *de novo* TAG and phospholipid synthesis (Han *et al*., 2006; O’Hara *et al*., 2006). It is part of a subfamily of PA phosphatases that depend on the presence of Mg$^{2+}$ for proper function (Han *et al*., 2006) and are
found in higher organisms as well including flies, worms, plants, mice and humans (Golden et al., 2009; Nakamura et al., 2009; Han and Carman, 2010; Eastmond et al., 2011; Ugrankar et al., 2011; Fang et al., 2014). Since it is a PAP enzyme, Pah1p is necessary for catalyzing the key step of dephosphorylating PA to form DAG.

The role of the PAH1 in maintaining cell homeostasis has been shown to be quite extensive. Yet one organelle that is greatly impacted by the PAH1 gene is the vacuole. Morphological studies have shown that pah1Δ cells exhibit a change in vacuolar morphology, with vacuoles appearing fragmented as compared to WT (Sasser et al., 2012). While vacuole fragmentation is a normal part of cell homeostasis and occurs in WT cells prior to cell division, abnormal fragmentation is caused by defects in lipid synthesis. Under normal conditions, vacuoles fragment and partition in yeast cells so that during daughter cell formation, the newly budded cell can receive the organelle (Conradt et al., 1992). This is due to the fact that in Saccharomyces cerevisiae, cytoplasmic organelles are not synthesized by the daughter cell, but rather, are inherited from the mother. This process however is heavily impacted by the lipid biosynthetic pathway. Defects in lipid metabolism are associated with abnormal vacuole fragmentation and disruptions in vacuolar function. PAH1 has recently emerged as a key gene that influences proper vacuole morphology. Its deletion has been associated with abnormal vacuole fragmentation, with vacuoles being unable to fuse back together. One study has shown that the lack of Pah1p phosphatase activity prevents the binding of SNARES and is devoid of components of the HOPS tethering complex needed for vacuolar fusion (Sasser et al.; 2012). Thus, vacuolar morphology is implicated in pah1Δ mutant. On the other hand, irregular vacuole morphology has also been related to atypical vacuolar acidification as a result of dysfunctional V-ATPase pumps. Studies have shown that some phenotypes of mutant cells with abnormal
vacuoles include vacuole fragments that collect to one area or disperse all over the cell. These cells can have working V-ATPase pumps or have a defect in the assemblage of the V-ATPase pump (Weisman et al., 1987; Raymond et al., 1992). Therefore, it is possible that PAH1 may affect the vacuole or may implicate the V-ATPase pump and thus alter its morphology through the regulation of lipid biosynthesis.

Although it has been shown that PAH1 plays an important role in lipid synthesis and may affect the vacuole through V-ATPase pump activity, to date, little research has been done on the effect of PAH1 on V-ATPases. We therefore wanted to better understand how Pah1p affects vacuolar morphology and its functional activity. In order to test this, RNA analysis was used to determine the best conditions for PAH1 induction, a condition to be used in subsequent experiments. Once determined, a growth curve sensitivity assay and the measurement of vacuolar pH were employed. Lastly, we examined how Pah1p affects the V-ATPase pump by performing RNA analysis of all V-ATPase genes.
Materials and methods

Yeast strains:

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genome type</th>
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<tr>
<td>WT (BY4741)</td>
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</tr>
<tr>
<td>vma3Δ</td>
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</tr>
<tr>
<td>pah1Δ</td>
<td>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Δpah1</td>
</tr>
</tbody>
</table>

Growth curve sensitivity assay:

Individual colonies of wild type (WT), vma3Δ, and pah1Δ were incubated overnight in SC media (dextrose-nitrogen base-amino acids) with 100mM inositol. Cells were diluted to an optical density of (A$_{600}$) of around 0.2-0.3 and grown in SC media with 100μM inositol buffered to pH 7.0. The optical density was subsequently recorded for every two hours over an eight hour period and then resumed at 24 hours until stationary phase was reached. For sensitivity analysis on plates, individual colonies of WT, vma3Δ, and pah1Δ were incubated overnight in SC media (dextrose-nitrogen base-amino acids) with 100mM inositol. On the following day, each strain's OD was measured and then either diluted or concentrated so that all strains contained the same OD for their starting point. The strains were then diluted 100 fold in SC medium and 5ul of each diluted strain were plated on YPD plates buffered to pH 5.5 and pH 7.0 for the pH sensitivity assay. Plates were incubated at 30°C for three days before analyzed.
Vacuolar pH analysis:

WT, pah1Δ and vma3Δ cells were grown until stationary phase in SC media with 100μM of inositol buffered to pH 7.0. Cells were then incubated with 1mM of BCECF-AM dye for 30 to 40 minutes at 30°C in the dark. Cells were then spun down, washed twice with media, and resuspended in fresh media. Cells were then stored on ice. Next, calibration samples were made for all three strains. Cells with the BCECF dye were incubated with 15μM of nigericin and the calibration buffers (50mM HEPES, 50mM MES, 10mM 2-deoxyglucose, 50mM potassium chloride, 50mM sodium chloride, 0.2M ammonium acetate, 10mM 2-deoxyglucose) that ranged from pH 5.0 to pH 7.0 for thirty minutes at 30°C. The unknown samples were prepared by resuspending cells with BCECF with 1mM MES buffer at pH 7. All samples were plated in a 96 well plate. The SpectraMas M5 microplate reader was used to measure the excitation wavelength (450nm and 490nm) with emission wavelength of 535nm. The calibration curves were made by taking the ratio of fluorescence at 490nm to 450nm. The fluorescence ratio was then plotted against pH (Figure 5.3A and 5.3B). Unknown samples were then plotted on standard curve to determine their pH.

Total mRNA preparation and quantification

Cells were grown from a single colony until desired optical density in SC media and collected. RNA was extracted from yeast cells through acid phenol (pH 4.3) chloroform extraction in which the cells were resuspended in TES (10mM EDTA; 10mM Tris-HCl pH 7.5; 0.5% SDS), and then incubated at 65°C with equal parts acid phenol for 60 minutes. This was followed by phenol-chloroform extraction and ethanol precipitation. Remaining pellets were resuspended in RNA sample buffer (10mM Tris HCl pH 8, 2mM EDTA, 1% SDS, 10% glycerol, and 1%
β-mercaptoethanol). RNA concentration was quantified by reading absorbance at 260nm. Subsequently, total RNA was stored at -80°C until further use.

**DNase Treatment of RNA samples**

DNase treatment was performed to remove any DNA from the isolated RNA samples. RNA was mixed with 10X buffer (0.1 M Tris-HCl pH 8, 5mM CaCl2 and 0.025M MgCl2), DNase, and DEPC water. Samples were placed in 37°C water bath for 60 minutes, followed by phenol:chloroform extraction and ethanol precipitation. Pellets were placed in a speed vacuum to dry and then resuspended in TE buffer that was prepared using DEPC water. RNA concentration was again quantified by reading absorbance at 260nm.

**Preparation of cDNA: First Strand Synthesis**

In order to prepare the cDNA needed for qRT-PCR, the SuperScript III First Strand Synthesis SuperMix kit from Invitrogen (Catalog: 11752-050) was used. The reaction was performed using a polymerase chain reaction (PCR) thermocycler. First strand synthesis samples were stored at -20°C until further use.

**Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) and analysis:**

The cDNA prepared from first strand synthesis was amplified and quantified using qRT-PCR. The SYBR GreenER® qPCR Supermix universal kit from Invitrogen (catalog number: 11762-500) was used to quantify cDNA templates. Forward and reverse primers were used that targeted the open reading frame (ORF) in the qPCR reaction. Samples were added to a 96 well plate and then sealed and placed in the Applied Biosystems 7500 real time PCR machine. PCR reactions were performed in triplicates. A ΔC_T value was obtained by subtracting the respective gene C_T
from the $C_T$ value of the constitutive housekeeping gene (ACT1). Therefore, the relative mRNA levels were expressed as $2^{\Delta C_T}$. Data results were graphed as a mean of the repeats ± standard deviation.
Results

1. Pah1p is most greatly expressed at nearing stationary phase.

Since the impact of a gene’s effect on the cell is greatest when the gene is at its highest expression level, it was important to find the best conditions at which the gene is induced for further experimentation. Previous studies have shown that the PAH1 promoter region includes both UASZRE sequences (Soto-Cardalda, 2012) as well as a UASINO (Wimalarathna, 2011), indicating that both zinc and inositol impact the induction of PAH1 (Soto-Cardalda et al., 2012; Pascual et al., 2013). However, it is still unknown in regard to which of these factors can upregulate PAH1 expression at its maximum levels as a comparison study has yet to be performed. In order to test the effect of Pah1p in vacuolar conditions, we first wanted to determine the best inducing conditions of PAH1.

RNA analysis was performed to determine the conditions for maximum Pah1p upregulation. In the absence of zinc, PAH1 is significantly upregulated in WT cells compared to the conditions with zinc. Next, we compared the levels of Pah1p in cells grown with normal zinc levels, but with 100μM inositol added (Figure 1B). Cells were collected in both the exponential phase and stationary phase of cell growth. Again, PAH1 mRNA levels demonstrated a significant difference between cells collected in exponential phase and those in stationary phase. PAH1 was substantially upregulated in stationary phase compared to levels in exponential phase (Figure 1C). When compared, it can be seen that cells grown with 100μM inositol and collected in stationary phase were significantly higher than cells grown in media without zinc (0.017 ± 0.0004 and 0.0056 ± 0.00061; p=0.023) (Figure 1C). This novel finding demonstrated that while PAH1 can be upregulated in various environments, it is most highly induced when cells enter stationary phase and have excess inositol in their media.
2. The absence of Pah1p can lead to vacuolar fragmentation at stationary phase.

Since we now determined the best inducing condition of PAH1, we continued to examine its effect on vacuole morphology and homeostasis. A previous study has shown that pah1Δ cells exhibit fragmented vacuoles in the exponential phase (Sasser et al. 2012). Here, we wanted to look at the vacuolar morphology of pah1Δ in the exponential phase of growth as well as the stationary phase and confirm the morphology when PAH1 is at inducing conditions.

Results showed that while WT cells had many fragmented vacuoles at the exponential stage, the morphology could be attributed to the normal fragmentation process that occurs in vacuoles during cell division. In the stationary phase, most cells contained large singular vacuoles. This was greatly contrasted to the pah1Δ cells, which mainly exhibited fragmented vacuoles. Both the exponential phase and the stationary phase displayed vacuoles that were unable to fuse their fragments back together, demonstrating that the necessary fusion machinery is unavailable in pah1Δ cells no matter at what stage of growth they are in (Figure 2A). It was seen that the number of fragmented vacuoles between WT and pah1Δ cells greatly differed in exponential phase and stationary phase. In exponential phase, about 74.1% of wild type cells contained fragmented vacuoles compared to the 96.4% of pah1Δ cells that had fragmented vacuoles (Figure 2B). In stationary phase however, only 49.89% of wild type cells contained fragmented vacuoles, a significant decrease from exponential phase, while pah1Δ cells contained 97% fragmented vacuole, a value similar to that in exponential phase (Figure 2B). Thus, stationary phase cells showed a much more significant difference in the number of fragmented vacuoles. This is due to the fact that in exponential phase, cells are at their peak stage of growth and are constantly dividing. Thus, our results showed that a large percentage of WT cells had fragmented vacuole in this stage, due to the normal process of cell division. However, once they
reached stationary phase their growth rate steadied and less cells were dividing, leaving only a smaller number of cells with fragmented vacuoles. For \textit{pah1}\textDelta\ cells however, their lack of fusion machinery resulted in fragmented vacuoles in almost all cells regardless of their stage in growth.

3. The absence of Pah1p can sustain growth in neutral environments suggesting maintenance of V-ATPase pump activity

Since the morphology of vacuoles in \textit{pah1}\textDelta\ is distorted, we wanted to see if other functions of the vacuole were implicated as well. One important aspect of vacuolar homeostasis is the acidification of the vacuole via the V-ATPase pump. In order to maintain an internal acidic pH in the vacuole, the V-ATPase pump must be functional. V-ATPases are responsible for transporting hydrogen ions into the vacuole. This ensures that there is an acidic vacuolar interior and that normal vacuolar processes can occur. Interestingly enough, inhibition of V-ATPase function is directly related to a distortion in vacuole morphology. This distortion occurs as a result of the inability of the V-ATPase pump to function. V-ATPases thus play a crucial role in the morphology of the vacuole. However, not all defects in vacuole morphology impact V-ATPase pump activity and thus must be determined. As such, since \textit{pah1}\textDelta\ cells have the fragmented vacuole phenotype, we wanted to look at whether the V-ATPase pump are implicated or not. One of the most basic ways to determine functional V-ATPase pump activity is via a pH sensitivity growth assay. Strains that do not contain a functional V-ATPase are unable to grow well in an environment with a neutral and basic pH (Kane, 2006). We therefore decided to perform growth sensitivity analysis experiments to determine if \textit{pah1}\textDelta\ is able to withstand conditions that cells without functional V-ATPases cannot. The \textit{vma3}\textDelta\ strain was used
as the negative control, since it does not contain a functional V-ATPase pump and should not grow well in a neutral pH.

Results showed that WT cells, \textit{vma3Δ} cells and \textit{pah1Δ} cells grew well in the more acidic pH, with all reaching the $10^{-6}$ dilution, and WT extending a little further and reaching the $10^{-8}$ dilution (Figure 3A). Results differed vastly though for the pH 7.0 plates. While WT still grew relatively the same and reached growth at the $10^{-8}$ dilution, the \textit{vma3Δ} mutant was unable to grow at all and exhibited no growth at the lowest dilution of $10^{-2}$. However, \textit{pah1Δ} cells grew well and had comparable growth to WT when it grew on more acidic medium, reaching the $10^{-6}$ dilution (Figure 3A). We then performed a growth curve experiment to further quantify the above findings. Results showed that while all strains grew well in the acidic medium, both WT and \textit{pah1Δ} cells also grew well in pH 7.0 liquid medium, while \textit{vma3Δ} cells did not. WT grew 1.9 folds better than \textit{vma3Δ}, while \textit{pah1Δ} cells grew 2.1 fold better than \textit{vma3Δ} in the neutral environment (Figure 3B). Thus, these results suggest that \textit{pah1Δ} cells are more similar to WT with regard to growth in neutral environments and likely V-ATPase activity.

4. The absence of Pah1p leads to a more acidic vacuole and an upregulation of V-ATPase genes in stationary phase.

The pH sensitivity growth assays suggested that the \textit{pah1Δ} V-ATPase pump was not negatively implicated by the deletion of the \textit{PAH1} gene, despite the fact that morphology is altered (Figure 2). In order to further confirm these results and characterize the vacuole conditions in \textit{pah1Δ} better, we employed the vacuolar acidification assay. This assay was used to measure vacuolar pH to fully confirm the functionality of the pump. The experiment was
conducted with media that was neutral (pH 7.0), since at these conditions the pump would have to continuously work to acidify the vacuole. The assay was performed on cells entering the stationary phase of growth (with 100µM inositol) since this is when \textit{PAH1} induction is at its peak. Results showed that WT cells that were grown in media buffered to pH 7.0 maintained the acidic pH of their vacuoles and remained within the normal physiological pH range of ~6.0 (Graham and Stevens, 1999). The vacuolar pH measured for the WT vacuoles was 6.00± 0.04 (Figure 3A). The pH measured for the vacuoles of \textit{vma3Δ}, our negative control, however was not within the normal range. They had an average vacuolar pH of 6.72 ± 0.11, confirming that these cells were unable to maintain the acidity required, and thus they were defective in vacuolar acidification at pH 7. The vacuolar pH measured in \textit{pah1Δ} cells however was 5.89 ± 0.001 (Figure 3A). These results suggest that acidification is well maintained in \textit{pah1Δ} cells when grown in a neutral extracellular environment and remains within the normal physiological range. Interestingly, the difference in pH between WT and \textit{pah1Δ} cells was significant ($p$=0.047), suggesting that \textit{pah1Δ} cells are better at acidifying the cell than WT. These findings therefore suggest that V-ATPase pump functioning is not downregulated in \textit{pah1Δ} cells despite the fact that vacuolar morphology is distorted and is actually better than WT.

Since the acidification assay experiment in which vacuolar pH was measured suggested that \textit{pah1Δ} cells can actually acidify their vacuoles better than WT. We therefore decided to perform RNA analysis on the genes that encode for the components that comprise the V-ATPase pump. There are 13 genes that are responsible for making up the two main domains of the V-ATPase pump. All genes must be functional in order for pump activity to work properly (Graham \textit{et al.}, 2000; Kane, 2005). Therefore, if even one gene is implicated, pump function is altered. Conversely, it may be that if one or more genes is upregulated, pH levels regulated by V-
ATPase pump activity can be affected. Cells were collected right before stationary phase and their RNA was isolated. We then treated them with DNase and converted the RNA to cDNA before performing qPCR. Results showed that none of the V-ATPase pump genes were downregulated or negatively implicated, which would render them transcriptionally inactive. In fact, multiple of these V-ATPase encoding genes actually had an upregulation of gene expression compared to WT (Figure 4B). This is unsurprising given the regulatory role of PAH1 and the fact that its deletion has been shown to cause an increase in gene expression (Santos-Rosa et al., 2005), however its effect has mainly been proven on genes that contain a UASINO. These results lead us to believe that perhaps the pump functions better as a result of this upregulation. Based off of these results it is likely that the V-ATPase is still able to function properly and possibly better since the genes of the pump transcribe just as well or even greater compared to WT.
Discussion

The effect of PAH1 and the phosphatase enzyme it encodes is not merely limited to the lipid biosynthetic pathway. It has shown that its influence is actually widespread throughout the cell and impacts multiple organelles. One such organelle is the vacuole. While it has been documented that vacuolar morphology is implicated in the pah1Δ cell and exhibits a fragmented vacuole phenotype (Sasser et al.; 2012), limited information is available on how else PAH1 affects vacuole homeostasis. Since normal vacuolar morphology is compromised in the pah1Δ mutant we therefore wanted to test if other factors involved in vacuolar homeostasis are affected. Many times, abnormal vacuolar morphology is also associated with abnormal vacuolar acidification due to dysfunctional V-ATPase pumps. Some of the phenotypic characteristics of abnormal vacuoles include fragmented vacuoles that cluster to one region or are distributed randomly throughout the cell. These mutants seem to either have functional V-ATPase or are partially defective in the assembly of V-ATPase (Weisman et al., 1987; Raymond et al., 1992). Since PAH1 plays such an important role in lipid synthesis, it could affect the V-ATPase pump and thus alter its morphology. In additional, overall vacuole homeostasis could be impacted. We therefore wanted to look into these other factors that implicate vacuolar morphology and see if PAH1 affects them.

In order to test this, we started off by performing electron microscopy on the pah1Δ mutant to characterize the vacuole phenotype. This was important because we wanted to characterize the phenotype not only in exponential phase, but also during PAH1’s highest induction stage, which is during stationary phase. Additionally, electron microscopy is renowned for its high resolution and magnification that can clearly capture detail of cell structure. We thus decided to employ it.
Our results showed that while WT cells had normal fragmentation of the vacuole due to the process of cell division, many cells also contained large singular vacuoles in both exponential and stationary phases of growth. This greatly differed from the vacuoles seen in pah1Δ in which vacuoles were fragmented. This phenotype was found in both exponential and stationary phases of growth for pah1Δ, demonstrating that the lack of PAH1 phosphatase activity directly impacts vacuole fusion regardless of an increased induction of PAH1 in the stationary phase. This is unsurprising since even in exponential phase, PAH1 transcription is still active, though not to the extent it is at during stationary phase. Still, the lack of PAH1 means that the phosphatase activity encoded by it is unavailable and as such impacts the lipid biosynthetic pathway which produces lipids, such as phosphatidylinositol 3-phosphate, that are required for SNARE activity and vacuole fusion. Thus, both exponential and stationary phase cells in pah1Δ exhibit the fragmented vacuole phenotype.

In order to determine if the fragmented vacuole phenotype impacts V-ATPase pump activity, we started off by performing growth sensitivity assays. This was important because strains defective in V-ATPase pump activity have been shown to be unable to grow in neutral pHs (Kane, 2006). In the sensitive assays, we observed that all cells grew well in the acidic pH 5.5 media, only vma3Δ did not grow well in pH 7.0. The pah1Δ mutant grew just as well in pH 7.0 as it had in pH 5.5, similar to WT. Further growth analysis in liquid broth, showed that while there was no significant difference in growth between WT and the pah1Δ mutant, both WT and pah1Δ cells each exhibited around a 2 fold greater difference in growth when compared to vma3Δ. Furthermore, the V-ATPase pump in pah1Δ mutant is not negatively implicated and can function properly as was determined by the acidification determination assay in which vacuolar pH was measured. While vma3Δ vacuoles did not fall into the normal acidic vacuolar pH range
of around 6.0 when cells were grown in pH 7.0 media, both WT and \textit{pah1}\textDelta did. This therefore confirmed that the V-ATPase pumps in \textit{pah1}\textDelta function as they should and are not implicated as a result of the fragmented phenotype. Interestingly, however, was that \textit{pah1}\textDelta cells actually exhibited a slightly more acidic vacuolar pH than WT that was significantly different. Therefore, our results suggested that while V-ATPases are functional in the \textit{pah1}\textDelta mutant despite the morphological distortion of the vacuole, they may actually work better at acidification.

In order to determine the cause of an increased vacuolar acidification in the absence of Pah1p, we decided to look into genes involved in the structure of the V-ATPase pump. Previous findings have demonstrated that defects in any one of the 13 genes can render the pump inactive, so it is likely that an overexpression could have the reverse effect. RNA analysis results showed that there are multiple genes of the V-ATPase pump upregulated in the \textit{pah1}\textDelta mutant compared to WT during stationary phase. Interestingly, of the genes that are upregulated, two of them, \textit{VMA1} and \textit{VMA2}, encode for ATP binding subunits that help regulate ATPase activity and proton transport (Shao \textit{et al.}, 2003), while another, \textit{VMA16}, encodes for a proteolipid subunit that is responsible for hydrogen ion translocation (Hirata \textit{et al.}, 1997). Thus, they are extremely important for pump activity and acidification and their overexpression can likely be responsible for the slightly more acidic pH found in the \textit{pah1}\textDelta mutant. The reason for the upregulation of these specific genes and why Pah1p impacts them is still unclear. More analysis will need to be done to determine the explanation for this.

In addition, our results also yielded another possible explanation for the increased amount of vacuole fragmentation in \textit{pah1}\textDelta that was determined by Sasser \textit{et al.}, and which we thought might impact the V-ATPase pump. Recent studies have shown that de-acidification of the vacuole actually induces vacuolar fusion and that mutated vacuoles that retained their internal
acidic pH had a blockage of this fusion (Desfougères et al., 2016). Since pah1Δ cells contain a slightly more acidic vacuolar pH than WT, it may be possible that their acidity could be a contributing factor to the fragmented vacuole phenotype as well if they are unable to deacidify properly. Whether the slightly more acidic pH in pah1Δ cells could play such a role in the morphological defect remains unanswered, especially since the vacuolar pH of pah1Δ cells still remains within the normal physiological range. Future studies would need to be performed to confirm this hypothesis and connect the increased vacuolar acidity to vacuolar fragmentation in the pah1Δ mutant as well as other impacts it may have on the cell. In addition,
Figure 1: PAH1 undergoes maximum induction during stationary phase of growth with excess inositol in medium. A) PAH1 catalyzes the reaction that dephosphorylates phosphatidic acid (PA) and converts it into diacylglycerol (DAG), which can be used to produce triacylglycerol (TAG). B) RNA analysis of the effect of different inducing conditions on PAH1 gene expression. WT cells were grown to mid logarithmic phase (1.0±0.2 A600nm) in 10µM inositol SC media and were subsequently washed and grown in respective media. mRNA was then extracted from cells using acid phenol chloroform and purified further with treatment of DNase. RNA was converted to cDNA and was amplified/quantified using qPCR with primers targeting the open reading frame sequence of PAH1. Expression levels of mRNA were normalized to the housekeeping gene ACT1. All experiments were done with at least two repeat colonies and all qPCR reactions were done in triplicates. Data was graphed as a mean ± standard deviation.
Figure 2: The absence of Pah1p can lead to vacuolar fragmentation at exponential and stationary phases of growth. A) Electron microscopy images of WT and pah1Δ cells in exponential and stationary phases of growth. B) Quantification of cells with fragmented vacuoles in WT and pah1Δ cells in both exponential and stationary phases of growth. WT and pah1Δ were grown in SC media and subsequently washed and collected at exponential phase of growth and stationary phase. Cells were fixed with glutaraldehyde, treated with lyticase, and subsequently fixed again with osmium tetroxide. They were dehydrated with 50%, 60%, and 70% ethanol, followed by an overnight incubation with 70% ethanol and 3% uranyl acetate reagent. Cells were washed with 80%, 90%, and 100% ethanol, followed by two washes of propylene oxide. A 1:1 ratio of propylene oxide and resin was added, followed by fresh resin, which was placed in oven to set. Samples were cut into ultra-thin sections and prepared for imaging using the Leica UCT Ultramicrotome. EM microscopy was performed using the Fei Technai Spirit Transmission Electron Microscope.
Figure 3: *pah1Δ* cells can grow in neutral pH suggesting that the absence of Pah1p does not affect vacuolar pump activity. A) pH sensitivity assay and serial dilution plates depicting the ability of *pah1Δ* cells to grow well on both acidic (pH 5.5) and neutral (pH 7.0) agar plates unlike the V-ATPase deficient *vma3Δ* mutant. B) Growth Curve analysis and quantification of WT, *pah1Δ* and *vma3Δ* cells in pH 5.5 and pH 7.0 liquid media. WT: (●), *pah1Δ* (■), *vma3Δ* (▲).
Figure 4: The \textit{pah1Δ} mutant has an internal vacuole pH within the normal physiological range, yet is more acidic than WT, and genes involved in V-ATPase pump structure and activity are upregulated in \textit{pah1Δ} cells during stationary phase. A) Vacuolar acidification assay showing that \textit{pah1Δ} cells have an internal vacuole pH comparable to WT and that which is within the normal physiological range, indicating that vacuolar pump activity and the acidification process in unaffected \textit{pah1Δ} cells. B) RNA analysis showing the upregulation of multiple genes of the V-ATPase pump. Expression levels of mRNA were normalized to the housekeeping gene \textit{ACT1}. Data was graphed as a mean ± standard deviation. WT: ( ░ ) and \textit{pah1Δ}: ( █ ).
Appendix References


References:


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