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Regulation of the Vacuolar ATPase activity

Paulina Konarzewska

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Regulation of the Vacuolar ATPase activity

By

Paulina Konarzewska

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

2015
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

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In eukaryotes, V-ATPases play an essential role in cellular pH homeostasis as they transport hydrogen ions into the vacuoles. This assures an acidic vacuolar interior and normal physiological processes associated with this organelle. Studies show that mutations in any of the VMA genes encoding for subunits of the V-ATPase pump result in growth inhibition in the absence of inositol, suggesting that V-ATPases may play an important role in phospholipid biosynthesis or vice versa. It is not clear how VMA genes affect phospholipid biosynthesis and how the regulators of phospholipid biosynthetic genes may affect ATPase activity. Here, we employ biochemical and genetic analyses to understand the connection.

In the first part of the study, we evaluated how VMA3 an indispensable gene required for proper V-ATPase function affects phospholipid biosynthesis. Using growth analysis, we demonstrated that vma3Δ cells were sensitive to pH conditions in the absence of inositol. This result strongly suggests that VMA3 can influence phospholipid gene expression. Subsequently, we observed that HXK2 gene was down-regulated in vma3Δ cells using real-time PCR analysis. Acetic acid sensitivity assay further confirmed reduced expression of HXK2 gene in vma3Δ cells. As such, we have demonstrated that the VMA3 plays an important role in phospholipid biosynthesis through regulation of HXK2 gene expression.

Recent studies show that some phospholipid genes play important roles in vacuolar morphology and acidification. Among these genes are transcription factors, INO2 and OPI1. To determine their role on vacuolar function, we first performed CaCl₂ sensitivity test which screens for genes important in vacuolar ATPase activity. Our results showed that WT, opi1Δ and ino2Δ cells exhibited similar growth sensitivity at 10 mM CaCl₂ whereas at 60 mM CaCl₂ both opi1Δ and ino2Δ cells exhibited moderate increase in sensitivity compared to WT. As microscopic analyses did not show any significant differences in vacuolar morphology of opi1Δ or ino2Δ compared to WT, vacuolar pH analyses showed significant increases in vacuolar acidification of opi1Δ but not ino2Δ cells compared to WT. Moreover, we observed that some of the genes that encode for V-ATPases were down-regulated significantly in both opi1Δ and ino2Δ.
Furthermore, both *opi1Δ* and *ino2Δ* cells showed lower ATPase activity than WT cells, suggesting that the transcription factors affecting expression of phospholipid genes play an important role in V-ATPase pump activity and vacuolar homeostasis. It is possible that down-regulation of ATPase activity is to maintain normal H⁺ concentration in the vacuole and to compensate a pH imbalance caused by the lack of Opi1p or Ino2p. Together, our results have provided new insights into the connection between phospholipid biosynthesis and cellular pH homeostasis.
Acknowledgements

I would like to take this opportunity to thank my mentor, Dr. Chang-Hui Shen, for his supervision not only during my years in Ph.D program but also during my research years as an undergraduate student. His encouragement led me to join the Ph.D program and achieve not only years of research and teaching experience which already have a great impact on my professional life but also helped me build a stronger character. I would like to sincerely thank my examination committee, Dr. Alejandra Alonso, Dr. De. William L’Amoreaux, Dr. Gary Wen, and Dr. Christopher Corbo for their precious time to discuss my research with me and to encourage me to work harder in order to get everything done on time. I am extremely thankful and indebted to Dr. William L’Amoreaux who has been my “behind the scene person”. I am thankful for his helpful advice and remarks with regards to my thesis as well as my professional life. Finally, I would like to take this opportunity to express my gratefulness for Dr. Elena McCoy who has been on my committee and has always been interested in my research. Her support was especially appreciated during my doubtful times with regards of the tough program that I have chosen. She will forever be missed and never forgotten. I consider myself a very lucky person to be surrounded by such a wonderful people who throughout the years became like a family to me.

My affectionate gratitude goes to my dearest friends and laboratory partners for helping me in both personal and academic matters throughout many years. My special thanks go to graduate students Michelle Esposito and Goldie Lazarus for helping me accomplish my degree in many ways including help with calculations and suggestions in terms of experimental set ups. Equally, my deepest gratitude goes to undergraduate students Suzanne Ahmed, Jaclyn Trotta, and Brendon Ursomanno for devoting their time to help me with my experiments.

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Table of Contents

Chapter 1 .................................................................................................................................................. 1

1 Introduction ........................................................................................................................................... 1

1.1 Structure and composition of the V-ATPase .................................................................................. 1

1.2 Function and importance of V-ATPase in *Saccharomyces cerevisiae* yeast cell ....................... 5

1.3 Function and importance of V-ATPase in mammalian cell ......................................................... 7

1.4 Morphology of yeast's vacuole ....................................................................................................... 9

1.5 Factors affecting V-ATPase activity ............................................................................................... 12

1.6 Evolution of Vacuolar ATPases ..................................................................................................... 16

1.7 Significance ..................................................................................................................................... 19

Chapter 2 ............................................................................................................................................... 20

2 Materials and Methods ....................................................................................................................... 20

2.3.1 Total RNA preparation ............................................................................................................. 24

2.3.2 Determination of RNA concentrations and quantification .................................................... 28

2.3.3 ATPase Activity ....................................................................................................................... 29

2.3.4 Vacuolar pH determination ...................................................................................................... 34

2.3.5 Vacuolar morphology ............................................................................................................... 35

2.4 Cell Growth .................................................................................................................................... 36

2.4.1 Cell culture ............................................................................................................................. 36

2.4.2 Glycerol stock preparation ...................................................................................................... 36

Chapter 3 ............................................................................................................................................... 37

3 The role of *VMA3* gene in de novo inositol synthesis .................................................................. 37

3.1.1 Growth analysis ....................................................................................................................... 39

3.1.2 Total RNA preparation ........................................................................................................... 40

3.1.4 Analysis of RNA quantity ........................................................................................................ 42

3.2 Results ........................................................................................................................................... 43

3.2.1 Growth experiments to identify effects of pH and inositol availability on *vma3Δ* viability .... 43
3.2.2 mRNA analysis to identify which genes in phospholipid biosynthetic pathway are affected by VMA3 deletion……………………………………………………………………………………………..48

3.2.3 Growth experiments to confirm effects of vma3Δ upon HXK2 gene expression…………60

3.2.4 Discussion..................................................................................................................63

Chapter 4..........................................................................................................................64

4 The effect of the transcription factors from the phospholipid biosynthetic pathway upon vacuolar morphology and acidification.................................................................................64

4.1 Materials and methods........................................................................................................66

4.1.1 Growth analysis on YPD plates......................................................................................66

4.1.2 Growth analysis in liquid broth......................................................................................66

4.1.3 Vacuolar morphology..................................................................................................66

4.1.4 Vacuolar pH analysis..................................................................................................67

4.2 Results............................................................................................................................68

4.2.1 The effects of CaCl₂ upon OPI1 and INO2 deletion strain..............................................68

4.2.2 The effect of OPI1 and INO2 deletion strain upon vacuolar morphology and acidification….78

4.3 Discussion.......................................................................................................................84

Chapter 5..........................................................................................................................86

5 The effect of the transcription factors from the phospholipid biosynthetic pathway upon V-ATPase pump activity.........................................................................................86

5.1 Materials and methods........................................................................................................88

5.1.1 Total RNA preparation...............................................................................................88

5.1.5 Spheroplast formation and lysis..................................................................................90

5.1.6 Vacuolar flotation.......................................................................................................90

5.1.7 Protein determination................................................................................................90

5.1.2 Vacuolar H⁺ ATPase activity.......................................................................................93

5.2 Results............................................................................................................................96

5.2.1 RNA analysis to determine whether OPI1 and INO2 affect pump activity at molecular level .........................................................................................................................96

5.2.2 Vacuolar H⁺ ATPase activity.......................................................................................103

5.3 Discussion.......................................................................................................................106
List of Figures

Figure 1.1 Model of vacuolar proton translocation ATPase (V-ATPase) in yeast Saccharomyces cerevisiae.................................................................3

Figure 1.2 Transport mechanisms in yeast vacuole.....................................................................................................................6

Figure 1.3 Diagram illustrating vacuolar development in Saccharomyces cerevisiae.........................................................10

Figure 1.4 Phospholipid biosynthetic pathway.....................................................................................................................15

Figure 1.5 Evolutionary tree of proton ATPases..............................................................................................................18

Figure 2.1 Summary of gene expression analysis..............................................................................................................27

Figure 2.2 Coupled enzyme ATP regeneration assay system used to measure V-ATPase activity............................32

Figure 3.1 Growth pattern of WT cells at various conditions..........................................................................................45

Figure 3.2 Growth pattern of vma3Δ cells at various conditions....................................................................................46

Figure 3.3 qRT-PCR analysis of HXK2 gene expression...............................................................................................51

Figure 3.4 qRT-PCR analysis of INO1 gene expression...............................................................................................52

Figure 3.5 qRT-PCR analysis of INO2 gene expression...............................................................................................53

Figure 3.6 qRT-PCR analysis of OPI1 gene expression...............................................................................................54

Figure 3.7 qRT-PCR analysis of INM1 gene expression...............................................................................................55

Figure 3.8 qRT-PCR analysis of PIS1 gene expression...............................................................................................56

Figure 3.9 qRT-PCR analysis of VPS34 gene expression...............................................................................................57

Figure 3.10 qRT-PCR analysis of FAB1 gene expression...............................................................................................58

Figure 3.11 qRT-PCR analysis of IPK2 gene expression...............................................................................................59

Figure 3.12 Acetic acid growth sensitivity assay.......................................................................................................61

Figure 4.1 Growth sensitivity analysis in the presence of 60 mM CaCl2........................................................................69

Figure 4.2 Growth survival analysis of WT cells...............................................................................................................72

Figure 4.3 Growth survival analysis of vma3Δ cells...............................................................................................................73

Figure 4.4 Growth survival analysis of ino2Δ cells...............................................................................................................74

Figure 4.5 Growth survival analysis of opi1Δ cells...............................................................................................................75

Figure 4.6 Vacuolar morphology of cells in various conditions..................................................................................79

Figure 4.7 Vacuolar pH of WT in the presence and absence of inositol at PH 5.5 or pH 7.................................82
Figure 4.8 Vacuolar pH in the presence and absence of inositol at pH 5.5 or pH 7 of WT, opi1Δ, ino2Δ and vma3Δ .................................................................................................................................................. 83

Figure 5.1 qRT-PCR analysis for VMA genes expression................................................................................. 98

Figure 5.2 qRT-PCR analysis of VMA genes expression ino2Δ and opi1Δ cells................................................. 101

Figure 5.3 Vacuolar H+ ATPase analysis of WT in the presence and absence of inositol at pH 5.5 or pH 7.................................................................................................................................................. 104

Figure 5.4 Vacuolar H+ ATPase analysis of WT compared to vma3Δ, ino2Δ and opi1Δ in the presence and absence of inositol at pH 5.5 or pH 7.................................................................................................................................................. 105

Figure 5.5 Proposed OPI1 and INO2 function in the vacuolar pH homeostasis................................................. 108
List of Tables

Table 1.1 Genes encoding V-ATPase in yeast.................................................................4
Table 1.2 Phenotype characteristics of the morphological groups that define vps mutants..........11
Table 3.1 Yeast strains........................................................................................................39
Table 3.2 List of primers used in RNA analysis................................................................41
Table 3.3A Growth rate percentage of WT affected by pH change....................................47
Table 3.3B Growth rate percentage of WT affected by inositol availability..........................47
Table 3.4A Growth rate percentage of vma3Δ affected by pH change.................................47
Table 3.4B Growth rate percentage of vma3Δ affected by inositol availability....................47
Table 3.5 Growth rate percentage in vma3Δ compared to WT cells........................................47
Table 3.6 Function of the genes used in mRNA analyses.....................................................49
Table 3.7 Decrease in vma3Δ growth rate percentage at 24th hour of incubation in various concentrations of acetic acid in the presence and absence of inositol.................................................62
Table 4.1 Yeast strains........................................................................................................66
Table 4.2A Growth rate percentage of WT cells affected by pH of the media....................76
Table 4.2B Growth rate percentage of WT cells affected by inositol availability in the media.....76
Table 4.3A Growth rate percentage of vma3Δ affected by pH of the media..........................76
Table 4.3B Growth rate percentage of vma3Δ affected by inositol availability in the media.......76
Table 4.4A Growth rate percentage of ino2Δ affected by pH of the media............................77
Table 4.4B Growth rate percentage of ino2Δ affected by inositol availability in the media.........77
Table 4.5A Growth rate percentage of opi1Δ affected by pH of the media.............................77
Table 4.5B Growth rate percentage of opi1Δ affected by inositol availability in the media........77
Table 4.6 Vacuolar pH in the presence and absence of inositol at pH 5.5 or pH 7....................81
Table 5.1 Yeast strains........................................................................................................88
Table 5.2 List of primers used RNA analysis.......................................................................89
Table 5.3 Protein concentration of every experimental sample...........................................92
Table 5.4 Total V-ATPase inhibition in the presence of Concanamycin A.............................94
Table 5.5 Statistical analysis (p value) for VMA genes expression....................................102
<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTp’s</td>
<td>Deoxynucleotriphosphate</td>
</tr>
<tr>
<td>dTTP</td>
<td>Deoxythymidine triphosphate</td>
</tr>
<tr>
<td>dUTP</td>
<td>Deoxyuridine triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Real time quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROX dye</td>
<td>Carboxy-X-rhodamine dye</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SC</td>
<td>Synthetic Complete</td>
</tr>
<tr>
<td>SM</td>
<td>Spheroplast Medium</td>
</tr>
<tr>
<td>TE</td>
<td>Tris- ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>TES</td>
<td>Tris- ethylenediaminetetraacetic acid -sodium dodecyl sulphate buffer</td>
</tr>
<tr>
<td>TSA</td>
<td>Trichostatin A</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
</tbody>
</table>
YPD  Yeast extract peptone dextrose
Chapter 1

1 Introduction

1.1 Structure and composition of the V-ATPase

Vacuolar proton translocating ATPases (V-ATPases) are highly conserved among all eukaryotic organisms. They are large, multisubunit, membrane spanning complexes responsible for carrying out the active transport of protons across the membrane from the cytoplasm into intra-organellar compartments such as vacuoles (pH 5.5-6.2) and lysosomes (pH ~5) (Graham and Stevens, 1999, Ohira et al., 2006;). The V-ATPase pump is composed of two structurally distinct domains: the catalytic \( V_1 \) domain and the proton translocating \( V_0 \) domain. The \( V_1 \) domain (640 kDa) is associated with the ATP-binding sites and is localized on the cytosolic side of the membrane that surrounds the vacuole and lysosome. The \( V_0 \) domain (240kDa) is composed of peripheral membrane proteins as well as integral ones (Figure 1.1). Both domains are connected through a stalk-like structure that belongs to \( V_1 \) domain (Graham et al., 2003; Kane, 2005). The genes for each subunit of V-ATPase have been co-purified and identified. Most of the subunits are encoded by single copy vacuolar membrane ATPase (VMA) gene, and one subunit is encoded by \( VPH1 \) gene (Table 1.1) (Förster et al., 2000). The first eight subunits are found in the \( V_1 \) domain. All are peripheral proteins assigned A-H. Subunit A (69kDa) and subunit B (60kDa) form ATP catalytic nucleotide subunit responsible for ATP binding and hydrolysis. In addition, subunit A as well as subunit B regulate proton transport as well as dissociation of \( V_1 \) and \( V_0 \) domain (Shao et al., 2003; Wagner et al., 2004). Subunit C (42kDa) regulates \( V_1 \) and \( V_0 \) assembly and can reversibly dissociate from the enzyme in glucose deprivation, leading to dissociation of \( V_1 \) from \( V_0 \) domain (Curtis et al., 2002; Drory et al., 2004). Subunit D (32kDa) plays important role in \( V_1 \) and \( V_0 \) assembly and therefore ATPase activity and hydrogen transport (Xu and Forgac, 2000). Mutations in subunit D prevent association between \( V_1 \) and \( V_0 \) domains without affecting their assembly (Graham et al., 1995). Subunit E (27kDa) forms part of the peripheral stalk of V-ATPase and seems to regulate assembly and activity of the V-ATPase (Grüber et al., 2002; Lu et al., 2002). Subunit F (14kDa) is important in the assembly of the \( V_1 \) and \( V_0 \) sector (Nelson et al., 1994). Subunit G (16kDa) participates in stalk formation and maintains stable levels of
Subunit E (Charsky et al., 2000; Ohira et al., 2006). Subunit H (54kDa) is not required for the assembly of V₁ domain but it is needed for V-ATPase activation by closely interacting with subunit F from V₁ domain. Mutations in subunit H do not affect assembly of V₁ domain and increases ATPase activity (Ho et al., 2003; Jefferies et al., 2008).

The remaining six subunits are found in the V₀ domain. Most of them are integral proteins, designated as a, c, c’, c” and one a peripheral protein designated d. Subunit a (100kDa) is a specific vacuolar subunit, required for proper assembly of V-ATPase and proton transport (Leng et al., 1996; Kawasaki-Nishi et al., 2001). Subunit c (165kDa) is a hydrophobic polypeptide containing glutamic acid residue whose carboxyl side chain is predicted to be important for proton translocation (Umemoto et al., 1990; Owegi et al., 2006). Subunit c’ (17kDa) and c” (23kDa) are homologous to subunit c. Both contain glutamic acid residues important in proton translocation. The above proteolipid subunits have similar but not redundant functions, as mutations in each affects V-ATPase assembly and activity (Owegi et al., 2006). Subunit d (36kDa) is hydrophilic, non-integral membrane protein. This subunit plays important role in coupling ATP hydrolysis and proton transport (Bauerle et al. 1993).

Interestingly, loss of any of the V₁ subunits has little or no effect on the stability of the rest of V₁ subunits. However, it does prevent interaction and assembly with V₀ domain to form functional V-ATPase pump. Also, loss of any of the V₁ subunits does not affect stability or assembly of V₀ subunits (Graham et al., 2000; Kane, 2005). Similarly, loss of any of the V₀ subunits has no effect on the stability of the rest of V₀ subunits but it does affect the assembly of V₀ domain without affecting assembly of V₁ domain (Kane, 2005; Graham et al., 2000). Additionally, mutations in any of the subunits belonging to V₁ or V₀ display similar conditional mutants: inability to grow in media buffered to neutral/basic pH or media containing high concentration of Ca^{2+} (Anraku et al., 1992).
Figure 1.1 Model of vacuolar proton translocation ATPase (V-ATPase) in yeast *Saccharomyces cerevisiae*. Catalytic domain (A-H) includes the ATP-binding sites and it is localized on the cytosolic side. Proton translocation domain (a, c, c’, c”, d) contains peripheral and integral proteins. (Graham et al., 2005).
<table>
<thead>
<tr>
<th>Gene</th>
<th>Subunit</th>
<th>Molecular mass (kDa)</th>
<th>Integral or peripheral</th>
<th>Proposed function</th>
</tr>
</thead>
<tbody>
<tr>
<td>V1</td>
<td>VMA1</td>
<td>A</td>
<td>peripheral</td>
<td>ATP binding subunit</td>
</tr>
<tr>
<td></td>
<td>VMA2</td>
<td>B</td>
<td>peripheral</td>
<td>ATP binding subunit</td>
</tr>
<tr>
<td></td>
<td>VMA4</td>
<td>E</td>
<td>peripheral</td>
<td>Assembly of V1 and V0</td>
</tr>
<tr>
<td></td>
<td>VMA5</td>
<td>C</td>
<td>peripheral</td>
<td>Activation of V-ATPase</td>
</tr>
<tr>
<td></td>
<td>VMA7</td>
<td>F</td>
<td>peripheral</td>
<td>Assembly of V1 and V0</td>
</tr>
<tr>
<td></td>
<td>VMA8</td>
<td>D</td>
<td>peripheral</td>
<td>Assembly of V1 and V0</td>
</tr>
<tr>
<td></td>
<td>VMA10</td>
<td>G</td>
<td>peripheral</td>
<td>Activation of V-ATPase</td>
</tr>
<tr>
<td></td>
<td>VMA13</td>
<td>H</td>
<td>peripheral</td>
<td>Activation of V-ATPase</td>
</tr>
<tr>
<td>V0</td>
<td>VPH1</td>
<td>a</td>
<td>integral</td>
<td>H+ transport</td>
</tr>
<tr>
<td></td>
<td>VMA3</td>
<td>c</td>
<td>integral</td>
<td>H+ transport</td>
</tr>
<tr>
<td></td>
<td>VMA6</td>
<td>d</td>
<td>peripheral</td>
<td>Assembly of V1 and V0</td>
</tr>
<tr>
<td></td>
<td>VMA11</td>
<td>c'</td>
<td>integral</td>
<td>H+ transport</td>
</tr>
<tr>
<td></td>
<td>VMA16</td>
<td>c&quot;</td>
<td>integral</td>
<td>H+ transport</td>
</tr>
</tbody>
</table>

Table 1.1 Genes encoding V-ATPase in yeast.
1.2 Function and importance of V-ATPase in *Saccharomyces cerevisiae*

The vacuolar hydrogen ATPases (V-ATPases) pump protons from the cytoplasm into the vacuole using energy generated from ATP hydrolysis. Mutations in vacuolar ATPase pumps result in conditional mutants that not only cannot acidify their vacuoles, but also fail to maintain optimum cytoplasmic pH (Graham and Stevens, 1999; Kane 2005). Therefore, V-ATPases play essential role in cellular pH homeostasis. As V-ATPases use energy to transfer protons into the vacuole, electrical potential differences build up across the membrane. This, in turn, is used to create movement of cations and solutes across the vacuolar membrane (Figure 1.2). Cytoplasmic calcium levels are maintained within a physiological range by transporting the cation into the vacuole via the Ca\(^{2+}/H^+\) antiporter, which is driven by vacuolar hydrogen ATPases. Mutants lacking functional V-ATPases accumulate 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). Heavy/toxic metal detoxification is also accommodated with help of vacuolar antiporters, which in turn relay on vacuolar hydrogen ATPases. Cadmium (Cd\(^{2+}\)), for example, is a heavy metal that causes oxidative stress in yeast cells, and its cytoplasmic levels are regulated by transporting it into the vacuole in exchange for hydrogen ions (Bryant *et al*., 1998; Sousa *et al*., 2014). Mutants defective in V-ATPase pumps exhibit hypersensitivity to all types of heavy/toxic metals present in the growth medium. Yet another function of vacuolar hydrogen ATPases is to promote hydrolytic activity of various enzymes and therefore promotes degradation, storage, and recycling of molecules such as nucleic acid, proteins, lipids, or sugars (Yamashiro *et al*., 1990; Forgac, 2007). As such, during nitrogen starvation, approximately 80% of total cellular protein content degradation occurs in the vacuole. Aside from molecules, organelles are also transported into the vacuole for degradation and turnover of their components. This way, amino acids, nucleic acids and lipids can be recycled in order to facilitate stress survival (Scott *et al*., 1996; Nakamura *et al*., 1997; Yorimitsu and Klionsky, 2005). Mutations that disrupt the V-ATPase pump interfere with vacuolar hydrolases maturation as well as cargo sorting into the vacuole (Yamashiro *et al*., 1990; Peters *et al*., 2001). Since the V-ATPase pump provides vacuolar acidification and electoral potential difference, alterations in the activity of the pump result in pleiotropic consequences that changes the physiology of the vacuole and ultimately the cell.
Figure 1.2 Transport mechanisms in yeast vacuole. (1) degradative vesicles bring cargo such as sugars, proteins and organelles into the vacuole for degradation or turnover with help of various hydrolytic enzymes. (2) V-ATPases maintain cellular pH homeostasis by transporting 2H⁺/1ATP from the cytoplasm into the vacuole. (3) and (4) cation antiports transport cations such as Ca²⁺, Na⁺, Zn²⁺, Cd²⁺ in exchange for H⁺.
1.3 Function and importance of V-ATPase in mammalian cell

Mammalian lysosomes seem to be analogous to yeast vacuoles and so they are also equipped with V-ATPases. Aside from lysosomes, V-ATPases are also found in Golgi-derived vesicles, clathrin-coated vesicles, secretory vesicles, endosomes and specialized cells (Wagner et al., 2004; Hinton et al., 2009). Therefore, acidification of different cellular compartments, mediated by V-ATPases provides optimal pH for diverse functions. For instance, acidic pH is required for the activation of specific proteases that are responsible for post translational cleavage and therefore activation of various precursors. Conversion of proinsulin to insulin occurs in such a manner and disruption of the intracellular proton gradient by ionophores abolishes such conversion (Mellman, 1992; Sun-Wadaa et al., 2014). V-ATPases’ activity also promotes transport and breakdown of macromolecules. For instance, dissociation of ligands such as low-density lipoproteins from their receptors relay on acidic environment since inhibition of V-ATPase using Bafilomycin A₁ prevents separation and release of ligand-receptor complexes (Mellman, 1992; Finbow and Harrison, 1997). Once dissociated, ligands can be degraded with help of hydrolases such as proteases, lipases, and nuclease whose function is also dependent upon acidic environment provided by V-ATPases (Beyenbach and Wieczorek, 2006). Aside from creating proton gradient, V-ATPases also generate membrane potential gradients to promote electrophoretic movement of ions and neurotransmitters. In synaptic areas of neurons, neurotransmitters serotonin or dopamine accumulate into synaptic vesicles through H⁺-dependent transport and inhibition of the V-ATPase by Bafilomycin A₁ diminishes the uptakes (Moriyama et al., 1992; Beyenbach and Wieczorek, 2006; Marshansky and Futai, 2008). Mammalian V-ATPase pumps are also found in the plasma membrane of specialized cells and their function is to transfer protons from the cytoplasm into the cell surroundings. Such a function of V-ATPase pumps facilitate bone restoration by osteoclasts, pH homeostasis in neutrophils and macrophages, acid secretion by intercalated cells of kidney, and angiogenesis by endothelial cells (Wagner et al., 2004). Defects in V-ATPases expressed in any of the specialized cells compromises their function and causes various genetic diseases such as osteopetrosis or distal renal tubular acidosis (Hinton et al., 2009). Plasma membrane V-ATPases are implicated in non-genetic diseases such as cancer. Invasive human breast tumor cells express abnormal levels of V-ATPases at their cell surface compared to poorly metastatic cells. These plasma membrane V-ATPases contribute to alkalization of the
tumor cell’s cytoplasm and at the same time acidification of the extracellular environment. These factors play important role in tumor survival, metastasis and drug resistance (Sennoune et al. 2004). It has been shown that cellular acidosis triggers apoptosis in mammalian cells. However, cancer cells are able to delay or even overcome programmed cell death with help of plasma membrane V-ATPases that remove excess of hydrogen ions into the surrounding of the cell and therefore maintain alkaline cytoplasmic pH (Sennoune et al., 2004). Reduced pH of the extracellular microenvironment may further stimulate secretion and activation of proteases, which in turn promote degradation and remodeling of the extracellular matrix. This in turn may contribute to cancer invasion and metastasis (Gocheva and Joyce et al., 2007). Acidic microenvironments also negatively affect chemotherapeutic drugs since most of them are neutralized due to acidic pH and therefore unable to enter the nucleus to perform its function (Fais et al., 2007). Inhibition of V-ATPases not only showed to induce apoptosis and drug accumulation but also to suppress cancer metastasis. V-ATPases are thus emerging as potential targets in the treatment of genetic and non-genetic diseases (Sennoune et al., 2004; Fais et al., 2007).
1.4 Morphology of yeast’s vacuole

In yeast cells, cytoplasmic organelles are inherited rather than synthesized. Therefore, partitioning of each organelle is required to guarantee that every daughter cell receives each type of organelle, including vacuoles (Conradt et al., 1992). Upon bud initiation (daughter cell formation), the large vacuole fragments into a cluster of smaller vacuoles (Figure 1.3). These vacuoles then form a tubule-vesicular structure that is then directed towards the emerging bud (Conradt et al., 1992). This allows for the transfer of inherited organelle into each daughter cell. As the bud increases in size, cluster vacuoles begin to fuse into one large compartment. As the bud increases in size, cluster vacuoles in both the daughter and mother cells begin to fuse into one large compartment. Finally, cells form septa, which initiate daughter cell separation from the mother cell. At this point, the large vacuole begins to fragment again so it can be transferred to yet another daughter cell (Wiemken et al., 1970).

Vacuolar morphology can be adversely affected due to alternations in lipid synthesis, mutations in vacuolar protein sorting (VPS) genes, vacuolar membrane ATPase (VMA) genes, and in genes regulating VMAs expression. Often, 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). Another example of defected vacuole is when mutant cells are defective for vacuolar segregation, and appear as one large vacuolar compartment that fails to extend into developing buds. In these mutants, V-ATPase fails to assemble (Raymond et al., 1992). Table 1.2 represents different vacuolar morphological groups found among different vacuolar protein sorting (VPS) mutants, but these morphological phenotypes are not limited to vps mutants.
Figure 1.3 Diagram illustrating vacuolar development in *Saccharomyces cerevisiae*.
<table>
<thead>
<tr>
<th>Class</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class A</td>
<td>Wild type vacuolar morphology where vacuoles frequently appear as 3-10 subcompartments that cluster to one region of the cytoplasm. V-ATPase is assembled normally.</td>
</tr>
<tr>
<td>Class B</td>
<td>Mutants have fragmented vacuoles exhibiting about 20 subcompartments. These compartments can appear clustered or distributed randomly throughout the cell. V-ATPase is assembled partially.</td>
</tr>
<tr>
<td>Class C</td>
<td>Mutants contain a mixture of vesicle like organelles and lipid droplets. V-ATPase is at least partially defective.</td>
</tr>
<tr>
<td>Class D</td>
<td>Mutants are defective for vacuolar segregation and therefore appear with one large vacuole that fails to fragment and extend into developing buds. V-ATPase fails to assemble properly.</td>
</tr>
<tr>
<td>Class E</td>
<td>Mutants contain vacuoles but the V-ATPase is present yet in another compartment.</td>
</tr>
<tr>
<td>Class F</td>
<td>Mutants contain large central vacuole surrounded by fragmented vacuolar subcompartments. V-ATPase is assembled normally.</td>
</tr>
</tbody>
</table>

Table 1.2 Phenotype characteristics of the morphological groups that define the vps mutants (Raymond et al., 1992).
1.5 Factors affecting V-ATPase activity

The levels of V-ATPase activity may be regulated at the post-assembly level with help from various factors such as glucose concentration, pH variation, lipid content and inositol availability. V-ATPase assembly is exquisitely sensitive to glucose availability; hence, glucose depravation leads toward rapid dissociation of fully assembled and active V-ATPase pumps into cytoplasmic V₁ domains and membrane bound V₀ domains (Parra and Kane, 1998; Kane, 2006). This effect is entirely reversible by addition of glucose to starved cells, resulting in reassembly of the V₁ and V₀ domains into functional V-ATPase (Parra and Kane, 1998). As glucose provides necessary energy for all biosynthetic processes in eukaryotic cells, glucose abundance is a major requirement for cells viability. Consequently, cells evolved elaborate signaling networks that control cellular response to changes in nutrient availability. ATP levels correlate with V-ATPase activity; glucose depravation leads to reduced ATP levels and inactivation of ATP hydrolysis by dissociation of V₁ domain into the cytosol, possibly to prevent unnecessary ATP turnover during starvation (Parra and Kane, 1998). Conversely, return of glucose restores ATP-driven proton transport due to increases in the intracellular pool of ATP (Li and Kane, 2009; Parra and Kane, 1998).

Recent evidence indicate that V-ATPase assembly is regulated by cytosolic pH. A drop in cytosolic pH causes V-ATPase disassembly into a cytoplasmic V₁ domain and a V₀ domain that remains embedded in the vacuole membrane. This can be entirely reversed when cytoplasmic pH becomes neutral or basic (Padilla-López and Pearce, 2006). At this point there is an increase in levels of V₁ assembly in the vacuoles suggesting that lower extracellular pH might downregulate activity of the pump and hydrogen transport (Padilla-López and Pearce, 2006; Diakov and Kane, 2010). Further, studies suggest that subunit a from the V-ATPase might mediate pH-dependent regulation of pump assembly and disassembly. This particular subunit is integrated in the vacuole membrane and its N-terminal-containing aspartic acid faces the outside of the vacuole. This amino acid can undergo reversible protonation and deprotonation due to pH changes. Studies using recombinantly expressed and purified N-terminal region of subunit a, showed that it becomes oligomerized in an acidic environment, suggesting that protonation of subunit a induces its oligomerization. Additional studies have shown that mutation of one of the
aspartic acids partially inhibited V-ATPase disassembly (Parra and Kane, 1998). When the combined effect of glucose and extracellular pH on the pump’s activity is considered, the neutral or basic extracellular pH suppresses the glucose depravation effect, preventing V-ATPase disassembly. Increasing V-ATPase activity in external alkaline/basic pH and suppression of its activity during periods of glucose starvation might be important to protect acidification of vacuole from the competing export of cytosolic protons across the membrane by P-ATPases (plasma membrane ATPases) and to conserve ATP when it is necessary (Diakov and Kane, 2010).

There is insufficient evidence that V-ATPase activity may be affected by inositol and genes involved in the phospholipid biosynthetic pathway (Figure 1.4). When inositol is present in growth media, it stimulates conversion of phosphatidic acid (PA) into phosphatidylinositol (PI). PI is further phosphorylated into phosphoinositide (3)P with help of phosphatidylinositol(PI) 3-kinase (Vps34p). Phosphoinositide (3)P can yet further be phosphorylated into phosphoinositide (3,5)P$_2$ with help of 1-phosphatidylinositol-3-phosphate 5-kinase (Fab1p) (Figure 1.4) (Odorizzi et al., 2000). Two genes in this phospholipid biosynthetic pathway are implicated in vacuolar acidification as well as vacuolar morphology. Studies have shown that $vps34$ mutants show almost no V-ATPase subunits in the membrane, which correlate with 30% reduction in V-ATPase activity as compared to wild type cells (Sambade et al., 2005). Hydrogen transport is also reduced as $vps34$ mutant cells demonstrate decreased levels of quinacrine localization in the vacuole (Wurmser et al., 1999; Sambade et al., 2005). Although $vps34$ mutants show normal morphology of the vacuole, development of new vacuole in daughter cell is delayed (Herman and Emr, 1990). Studies associated with $fab1\Delta$ mutants show kinetic delays in hydrolase maturation, which normally depends on acidic environment of the vacuole. This indicates that Fab1p may affect vacuolar acidification (Wurmser et al., 1999; Ho et al., 2012). Further, yeast cells overexpressing Fab1p show increased concentration of PI(3,5)P$_2$ and display shrunken vacuoles. Reduced levels of PI(3,5)P$_2$ lead to increase in the vacuolar size (Gary et al., 2002; Efe et al., 2005). This evidence suggests that Fab1p and PI(3,5)P$_2$ are necessary for organelle acidification as well as vacuolar morphology (Gary et al., 1998; Rudge et al., 2004). $OPI1$ is another important gene in the phospholipid biosynthetic pathway. Although there hasn’t been any research that associates its importance in vacuolar morphology or acidification, it has been shown that the product of this gene, Opi1p moves into the nucleus in pH and inositol dependent
manner (Loewen, 2012; Young et al., 2010). The Opi1 protein is localized on the endoplasmic reticulum where it is anchored to PA and Scs2p. PA having a polar head group can become protonated or deprotonated depending on the internal pH environment of the cell. In the acidic environment, it becomes protonated, leading to loss of affinity for Opi1p protein (Orij et al., 2011; Shin and Loewen, 2011). Free Opi1p translocates into the nucleus where it acts as a transcriptional suppressor upon more than 30 genes. In the neutral or basic environment, Opi1p has been shown to stay outside of the nucleus where it is anchored onto PA and Scs2p (Young et al., 2010). Inositol has a similar effect upon Opi1p. In the presence of inositol, PA becomes depleted upon its conversion into PI, and so Opi1p loses anchorage and is free to translocate into the nucleus where it regulates activity of other genes (Figure 1.4) (Loewen et al., 2004; Loewen, 2012). Studies have shown that vma mutants exhibit poor/no growth in the absence of external inositol. This indicates that vma mutants require inositol for growth. (Villa-García et al., 2011). One way to rescue the growth of vma mutants is by deleting OPI1 gene (Young et al., 2010). One might propose that Opi1p might also act upon VMA genes by repressing them in pH and/or inositol dependent manner.

V-ATPase pumps are also regulated at transcriptional level due to osmotic stress, heavy metal stress, temperature stress or acid stress. Interestingly, genes encoding different V-ATPase subunits are expressed in non-co-ordinated manner where some genes are upregulated while others are not affected depending on particular situation (Low et al., 1996). This might be due to different turnover rates of V-ATPase subunits, or due to different signaling pathways regulating the transcription of particular subunits (Diethz, 2001).
Figure 1.4 Phospholipid biosynthetic pathway.
1.6 Evolution of Vacuolar ATPases

The ATPase family are divided into three major groups: P-ATPases, F-ATPases and V-ATPases. The P-ATPases, also known as E₁E₂ type, are composed of a single catalytic subunit, approximately 100kDa, found in the plasma membrane of eukaryotic cells. This catalytic subunit operates with a phosphoenzyme intermediate, and is sensitive to low concentrations of vanadate. There are many different classes of P-ATPases, each responsible for transport of a specific type of ion such as Na⁺, K⁺, Ca²⁺ and H⁺ (Al-Awqati, 1986; Serrano, 1991). The F-ATPases also known as F₁F₀ type, are composed of a large, multisubunit enzyme, approximately 500kDa, found in plasma membranes of eubacteria as well as on the inner membrane of mitochondria and chloroplast. This multisubunit enzyme is divided into F₁ complex and F₀ complex. The F₁ catalytic complex is made of five subunits α to ε. The F₀ complex is composed of integral membrane proteins, as well as proteolipid that forms the bulk of the proton channel. F-ATPases function in ATP synthesis, but can also hydrolyze ATP as a result of proton transport (Al-Awqati, 1986; Serrano, 1991). Finally, the V-ATPases, also known as vacuolar type, are composed of a large multisubunit enzyme, approximately 500kDa, found in membranes of vacuoles, lysosomes, endosomes, Golgi, and coated vesicles of eukaryotic cells. The enzyme is comprised of a V₁ catalytic complex and V₀ integral membrane complex. V-ATPases function exclusively in proton gradient generation through ATP hydrolysis (Nelson et al., 1989; Serrano, 1991).

One could argue that P-ATPases and V-ATPases are closely related to each other due to common function and the fact that both are present in eukaryotic cells. Instead, biochemical properties of V-ATPases indicate that these enzymes are more closely related to F-ATPases, previously identified in bacteria, chloroplasts and mitochondria (Senior et al., 1983; Serrano, 1991). Both types of ATPases share similar structure as well as subunit composition. The mechanism of ATP binding, hydrolysis and H⁺ pumping activity is also similar in these two families (Zimniak et al., 1988; Nelson 1992). The amino acid sequence of both the A subunit and B subunit of V-ATPases are homologous with the β subunit and α subunit of F-ATPases respectively (Nelson 1992). Similarly, cDNA sequence of vacuolar proteolipid shows homology with proteolipid from F-ATPase. What distinguishes V-ATPase and F-ATPase from P-ATPase is the fact that both ATPases function without phosphoenzyme intermediates and are insensitive
to vanadate (Zimniak et al., 1988; Nelson et al., 1989). Furthermore, sequence analysis of the nucleotide binding site indicates that P-ATPases probably evolved independently from the other two ATPase families (Serrano, 1991).

Despite, the number of similarities between V-ATPases and F-ATPases, these two ATPases are adequately unique to fall into two individual families. Alignment between A subunit of V-ATPase with subunit B from mitochondria and subunit β from eubacteria revealed an insertion of about 90 amino acids in vacuolar ATPases. Also, proteolipids from vacuolar ATPases have been shown to appear twice as large in size due to gene duplication (Serrano, 1991; Nelson 1992). The size of the proteolipid may indicate transformation of eukaryotic V-ATPases to function exclusively in proton gradient generation through ATP hydrolysis (Nelson 1989; Nelson 1992). Both F-ATPases and V-ATPases containing short version of proteolipid are capable of ATP synthesis. On the other hand, removal of the catalytic sector of eukaryotic V-ATPases failed to generate proton gradients, but increased proton gradient formation in F-ATPases. This suggests that gene duplication in vacuolar proteolipid rendered V-ATPases into a proton pump that can no longer participate in ATP synthesis (Nelson 1989; Nelson 1992).

Since most prokaryotes do not contain E₁E₂ type of ATPases, it has been suggested that the original ATPase that first arose was of F₀F₁ type. Of all subunits of the F₁F₀ type ATPases, the proteolipid shares highly conserved sequence homology between bacteria, mitochondria, as well as chloroplast, and most likely it was the first subunit to evolve (Al-Awqati 1989). Based on a putative evolutionary tree from Nelson Hannah et al., 1989 (Figure 1.5), step 1 indicates evolution of the ATPase which function in both balancing the interior pH as well as ATP synthesis. Step 2 indicates evolution of the Vacuolar ATPase. Small changes which occurred in the proteolipid gene sequence, limited vacuolar ATPases activity to proton transport only. The remaining steps indicate evolution of ATPase in eubacteria as well as mitochondria and chloroplast (Al-Awqati, 1989; Nelson 1989). Based on this evolutionary tree, two subfamilies can be created; first subfamily composed of archaeabacterial ATPases together with vacuolar ATPases, and a second subfamily composed of eubacterial, mitochondrial and chloroplast ATPases (Nelson, 1989).
Figure 1.5 Evolutionary tree of proton ATPases. 1: evolution of ATPase present in current arachaebacteria. This enzyme has dual function; proton transport and ATP synthesis. 2: evolution of vacuolar ATPase present in eukaryotes. This enzyme is limited to function in proton transport only. 3: evolution of ATPase present in eubacteria. 4: evolution of ATPase present in bacterial cell membrane. This enzyme has dual function; proton transport and ATP synthesis. 5: evolution of ATPase present in mitochondria and chloroplast (Nelson, 1989).
1.7 Significance

V-ATPase pumps in *Saccharomyces cerevisiae* are very similar to those of higher eukaryotic cells both in the DNA sequence of subunit genes and overall structure (Curtis *et al.*, 2002). To date, 13 subunits have been identified and sequenced in the yeast V-ATPase and shown to be homologous with V-ATPase subunits of mammalian cells (Curtis *et al.*, 2002). Moreover, yeast has proven to be an excellent model organism to study V-ATPase pumps as it can survive and grow without the active enzyme unlike mammalian cells (Nelson, 2003).

In eukaryotic cells, acidification of different cellular compartments mediated by V-ATPases provides optimal pH for diverse functions. For instance, acidic pH is required for the activation of specific proteases that are responsible for post translational cleavage and therefore activation of various precursors, transport and breakdown of macromolecules, cell detoxification, ion transport and more (Hinton *et al.*, 2009). Abnormalities with regards to V-ATPase activity impairs the above processes and therefore disturbs cell’s homeostasis. For instance, abnormal levels of V-ATPases are implicated in cancer and although molecular silencing and pharmacologic inhibitors of the V-ATPases delay cancer growth, such approaches result in severe toxicity and other undesired problems (Hinton *et al.*, 2009). Dysfunctional V-ATPases are also implicated in neurodegenerative diseases such as Alzheimer’s disease and Parkinson’s disease (Wolfe *et al.*, 2013). Therefore understanding V-ATPase function and regulation may help us better understand the underlying causes for V-ATPase related diseases. It may also improve medical approaches towards V-ATPase associated disorders in a way to avoid or reduce side effects such as toxicity.
Chapter 2

2 Materials and Methods

2.1 Media and Agar

**SC + inositol (Synthetic complete media)** - 2% Dextrose (w/v) (Fisher Scientific), 0.67% YNB (yeast nitrogen base) with ammonium sulfate (w/v) (MP Biomedicals, Cat: 114027522), 0.079% CSM (complete supplement mixture) (w/v) (MP Biomedicals, Cat: 4500 022), 0.002 (W/V)% of *myo*-inositol.

**SC – inositol (Synthetic complete media)** - 2% Dextrose (w/v) (Fisher Scientific), 0.67% YNB without inositol (yeast nitrogen base) with ammonium sulfate (w/v) (MP Biomedicals, Cat: 4027 412), 0.079% CSM (complete supplement mixture) (w/v) (MP Biomedicals, Cat: 4500 022).

**SM (Spheroplast Medium)** – 2% Dextrose (w/v) (Fisher Scientific), 0.67% YNB (yeast nitrogen base) with ammonium sulfate (w/v) (MP Biomedicals, Cat: 114027522), 0.079% CSM (complete supplement mixture) (w/v) (MP Biomedicals, Cat: 4500 022), 18.22% D-sorbitol (w/v), 50mM Tris HCl (pH 8).

**YPD (yeast extract peptone dextrose) agar** – YPD agar was prepared by dissolving 10g of becto yeast extract (Difco, Cat: 212750), 20g of becto-peptone (Becton Dickinson, Cat: 211705), 20g of dextrose (Fisher Scientific), and 20g becto agar (Becton Dickinson, Cat: 214010) in 500mL water and autoclaved.
2.2 Reagents and Solutions

**ATPase solution:** 25mM Tris acetate pH 7 (Fisher Scientific), 25mM KCl (Fisher Scientific), 5mM MgCl$_2$ (Fisher Scientific), 2mM phosphoenolpyruvate (MP biomedicals, Cat: 153888), 2mM ATP (MP biomedicals, Cat: 100008), 0.5mM NADH (Research organics, Cat: 606 68 8), 30U L-lactate dehydrogenase (Roche, Cat: 10 127 876 001) and 30U pyruvate kinase (MP biomedicals, Cat: 151999), concanamycin A (Sigma, Cat: C9705).

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

**Buffer A** – 10mM 2-(N-morpholino)ethanesulfonic acid (MES)/Tris pH 6.9, 0.1mM MgCl$_2$, 12% Ficoll 400.

**Buffer B** – 10mM 2-(N-morpholino)ethanesulfonic acid (MES)/Tris pH 6.9, 0.5mM MgCl$_2$, 8% Ficoll 400.

**Buffer C** – 10mM 2-(N-morpholino)ethanesulfonic acid (MES)/Tris pH 6.9, 5mM MgCl$_2$, 25mM KCl.

**Calibration buffer** – solution of 50mM MES (BioWorld, Cat: 41320024 1), 50mM HEPES (Fisher Scientific, Cat: BP308), 50mM KCl (Fisher Scientific), 50mM NaCl (Fisher Scientific), 0.2M ammonium acetate (Fisher Scientific), 10mM sodium azide (Sigma, Cat: S8031), 10mM 2-deoxyglucose (Sigma, Cat: D8375) and 15µM nigericin was buffered to pH of 5, 5.5, 6 and 6.5 using hydrochloric acid or 1M NaOH.

**Chloroform** – 99.8% (Acros, Cat:61003-0040).

**DEPC treated water** – 0.1% of diethyl pyrocarbonate (Sigma) was added to distilled water and autoclaved.

**DNase 1** – RNase free DNase (Qiagen, Cat: 79254) 1500 units.

**EDTA** – 0.5M solution contains disodium EDTA 2H$_2$O 186.12 g/L (DNase RNase and proteases free (Quality Biological, Cat: 351 027 100)); pH 8.80 was adjusted using NaOH. Store at: 15-30°C.

**FM4-64** – 10mM of FM4-64 (Life technologies, Cat: T3166) was dissolved in DMSO.

**Glycerol** – 99.5% UltraPure™ Glycerol (Invitrogen, Cat: 15514-029).

**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]) – MES buffer was prepared by dissolving 20mM of MES (BioWorld, Cat: 41320024 1) in water, and buffering it using 1M NaOH (Fisher Scientific).

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

Pierce Modified Lowry Protein Assay Kit – ThermoScientific, Cat: 23240

Phenol saturated - pH 6.6 (Fisher Scientific, Cat: BP1750-400). Stored at 4°C.

Phenol buffer saturated - pH 4.3 (Shelton Scientific, Cat: IB05184). Stored at 4°C.

Proteinase K from tritirachium album – Stock solution was 20mg/ml (Sigma-Aldrich, Cat: P2038-100MG).

Proteinase inhibitor cocktail – 100X cocktail contains 104mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 80uM Aprotinin, 4mM Bestatin and 1.4 mM trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane(E64) in dimethylsulfoxide (DMSO) (Sigma- Aldrich, Cat: P8340-5ML).

RNA sample buffer – 2mM EDTA, 10mM Tris HCl (pH 8), 1% β-mercaptoethanol, 1% SDS and 10% glycerol.

Sodium acetate – 3M solution prepared by dissolving 408.1g sodium acetate in 1L distilled water. Adjusted the 5.3 pH using HCl. Stored at room temperature.

Sorbitol – 1M solution prepared by dissolving 9.10g of D-sorbitol (Acros organics, Cat: 132730010) in 50 ml distilled water. Sterilized by filtering or autoclaving.

SYBER® GreenER™ Two-Step qRT-PCR Kit Universal – This kit (Invitrogen, Cat No: 11765-100) consist of two kits,

- SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR kit (RT enzyme mix: RNaseOUT™ recombinant ribonuclease inhibitor and SuperScript™ III reverse transcriptase, 2X RT reaction mix: 2.5μM oligo (dT)20, 2.5ng/μl random hexamers, 10mM MgCl2 and dNTPs, and E.coli RNase H). Stored at -20°C.
• SYBR® GreenER™ qPCR SuperMix Universal kit (2X SYBR® GreenER™ qPCR SuperMix Universal: Taq DNA polymerase, SYBR® GreenER™ fluorescent dye, MgCl₂ dNTPs with dUTP instead of dTTP, UDG and ROX reference dye). Stored at 4°C.

**Taq Man Gene Expression Master Mix** – (Applied Biosystems, Cat: 4369016) Supplied at 2X concentration. The mix is optimized for real-time PCR quantitative analysis and contains AmpliTaq Gold® DNA Polymerase, UP (Ultra Pure), Uracil-DNA Glycosylase (UDG) and dTNPs with dUTP.

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

**TES (Tris-EDTA-SDS) buffer** – Solution was prepared by adding 10 mM Tris HCl pH 7.5, 10 mM EDTA and 0.5% SDS. Sterilized by autoclaving.

**Trichostatin A** – 10mM solution was prepared by dissolving 1mg trichostatin A in 330ul 100% ethanol (Wako, Cat: 203-17561)

**Tris HCl** – 1M solution was prepared by dissolving 78.82g Tris HCl in 500ml distilled autoclaved water. pH was adjusted using NaOH.

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

**10x Buffer** – Solution was prepared by combining 1M Tris HCl pH 8 and 1M MgCl₂ with DEPC water. Sterilized.
2.3 Experimental Protocols

2.3.1 Total RNA Preparation

2.3.1.1 Cell preparation

Cells were grown from a single colony until mid-logarithmic phase (optical density of 0.8) in SC media with 0.002% (w/v) of myo-inositol buffered to pH 5.5 or pH 7, followed by induction and repression for additional two hours in the absence and presence of 0.002% (w/v) of myo-inositol respectively at pH 5.5 or pH 7. Cell pellets were re-suspended in 400µl ice-cold autoclaved water and were spun down at 13000 rpm for 15 seconds at 4°C. The supernatant was discarded and the pellet was rinsed in 400µl TES buffer. The phenol chloroform extraction was then performed using an acid phenol (pH 4.3) followed by the ethanol precipitation.

2.3.1.2 Phenol (acid)-chloroform extraction

400µl of acid phenol (pH 4.3) was added into the dissolved pellet and was incubated at 65°C for an hour. The tube was then kept in ice for 5 minutes. It was subsequently spun down at 13000 rpm for 5 minutes at 4°C in order to separate the aqueous layer. 400µl of acid phenol (pH 4.3) was the added to the aqueous layer. The sample was then vortexed vigorously for 10 seconds and placed on ice for 5 minutes. It was then spun down at 13000 rpm for 5 minutes at 4°C to separate the aqueous layer, which then had 400µl of chloroform added to it. The tube was then vortexed vigorously and spun down at 13000 rpm for 5 minutes at 4°C. 360µl of the aqueous phase was transferred to a new tube, followed by the addition of 40µl of 3M sodium acetate (NaOAC) (pH 5.3) and 1ml of ice-cold 100% ethanol. The sample was then incubated in dry-ice for 20-25 minutes or at -80°C for an hour or at -20°C overnight. Next, it was spun down at 13000 rpm for 5 minutes at 4°C and the supernatant was removed. Then, it was washed with 1ml ice-cold 70% ethanol and the supernatant was removed. A speed-vacuum centrifuge was then used to remove all the ethanol. At the end of the ethanol precipitation the pellet was re-suspended in 200µl RNA sample buffer. Short term storage was conducted at -20°C and long term storage was conducted at -80°C.
2.3.1.3 DNase Treatment

According to the RNA quantifications, 10µg of RNA sample was added to 5µl of 10X Buffer and 3.5µl DNase-1 in a micro-centrifuge tube. Total volume was then increased to 50ul by adding DPEC-treated water. The sample was incubated at 37°C for an hour. Then the phenol chloroform extraction was performed, followed by ethanol precipitation. Phenol (pH 6.6): Chloroform ratio should be 3:1. At last, the pellet was re-suspended in 20µl DEPC treated water or TE made up of DPEC treated water.

2.3.1.4 Real-time quantitative RT-PCR (qRT-PCR)

The SYBER Green ER Two-Step qRT-PCR kit was used (Invitrogen, Cat No: 11765-100) to did the real time PCR. There were two steps (Figure 2.1).

1. Reverse transcriptase polymerase chain reaction (RT-PCR)

In the first step, c-DNA was made from mRNA using SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR kit. Each reaction contained approximately 1 ug of mRNA, 10μl 2X RT reaction mix and 2μl RT enzyme mix. A total volume of 20μl was achieved using DPEC treated water. Tubes were gently mixed and incubated at 25°C for 10 minutes followed by incubation at 50°C for 30 minutes. The reaction was terminated at 85°C for 5 minutes, and then chilled on ice. 1μl of E.coli RNase H was added and the tube was incubated at 37°C for 20 minutes. The reactions could be stored at -20°C until use.

2. Quantitative polymerase chain reaction (qPCR)

In the second step we did qPCR (SYBR Green method) to quantitate the amount of cDNA templates using SYBR® GreenER™ qPCR SuperMix Universal kit. To prepare each reaction, 7.8μl SYBR® GreenER™ qPCR SuperMix Universal, 0.4μl forward primer, 0.4μl reverse primer, 0.4μl ROX reference dye, and 2μl cDNA template (from the first step) was combined. The total volume was brought up to 25μl using autoclaved distilled water. The PCR plate was sealed and it was made sure that all of the components were at the bottom by mixing and brief centrifuging. The reactions were placed in a preheated thermal cycler programmed as described bellow. The real time instrument used was the 7500 real time PCR system (Applied Biosystems).
• 50 °C for 2 minutes hold
• 95 °C for 10 minutes hold
• 45 cycles of:
  • 95 °C for 15 seconds
  • 60 °C for 60 seconds
Figure 2.1 Summary of gene expression analysis.
2.3.2 Determination of RNA concentrations and quantification

DNA and RNA absorbed ultraviolet light at 260nm wavelength. It was possible to relate the amount of light absorbed to the concentration of the absorbed molecule using the Beer-Lambert Law. DNA concentration was achieved by spectroscopy at 260nm wavelength taking 1 optical density (OD) unit as equivalent to 50ug/ml of double-stranded DNA, 20 μg/ml of single-stranded oligonucleotide and 40ug/ml of single-stranded DNA or RNA (Sambrook et al., 1989). If the OD$_{260}$/OD$_{280}$ was significantly less than 1.8, then it was necessary to further purify the DNA by phenol chloroform extraction followed by ethanol precipitation.

Concentration of double stranded RNA = absorbance at 260nm * 40 * dilution factor

Target gene product quantities were estimated from the threshold amplification cycle number ($C_T$) using a Sequence Detection System Software. A Δ$C_T$ value was obtained by subtracting the respective gene $C_T$ from the $C_T$ value of the house keeping gene. Therefore, the relative mRNA levels were expressed as $2^{-\Delta C_T}$. 
2.3.3 ATPase Activity

2.3.3.1 Cells growth

Cells were grown from a single colony until mid-logarithmic phase (optical density of 0.8) in SC media with 0.002% (w/v) of myo-inositol buffered to pH 5.5 or pH 7, followed by induction and repression for additional two hours in the absence and presence of 0.002% (w/v) of myo-inositol respectively at pH 5.5 or pH 7.

2.3.3.2 Spheroplast formation

The yeast cell pellet was re-suspended in 25ml of spheroplast medium (SM). The growth medium was used to avoid starvation of the cells during the spheroplasting process. Sorbitol in the SM acted as an osmotic stabilizer. Cells were incubated at 30°C for 2-3 minutes and 20mg/ml of lyticase enzyme as well as 20mM of 2-mercaptoethanol was added. The incubation was continued at 30°C for about 40 minutes. To ensure complete digestion of the cell wall, optical density at 600nm was measured and compared between un-lysed cells vs. lysed cells. Once optical density doped to 5% of the staring value, digestion was complete.

Spheroplasts were then chilled on ice and spun down at 2200g for 5 minutes at 4°C in order to remove spheroplasting media. Spheroplasts were then washed two times in 25mL of 1M sorbitol to get rid of lytic enzyme and 2-mecaptoethanol.

2.3.3.3 Spheroplast lysis and vacuolar flotation

The specific density of isolated vacuoles from spheroplasts is generally not much higher than that of water. Therefore, method for isolating vacuoles was based on differential flotation. In this case, Ficoll was used in Buffer A, B, and C to increase density of the buffer for vacuoles to float on top.

Spheroplasts where lysed by resuspending the pellet in 25ml of Buffer A and homogenizing it using dounce homogenizer at 0°C. Lysate supernatant was centrifuged at 2200g for 10 minutes at 4°C to remove any unlysed spheroplasts.
Supernatant was transferred into a new polyallomer tube and overlaid with about half the volume of Buffer A. This was centrifuged at 60,000g for 30 minutes at 4°C. Next, white wafer floating on top was collected and further homogenized with 6ml of Buffer A. Everything was transferred into polyallomer tube and overlaid with the same volume of Buffer B. After centrifugation at 60,000g for 30 minutes at 4°C, white wafer floating on top was collected and resuspended in equal volume of 2x Buffer C. Suspension was further purified by passing the suspension up and down through a micropipette tip several times and diluted in equal volume of 1x Buffer C. Samples were then stored in -80°C until further use.

2.3.3.4 Protein determination

Before setting up and monitoring ATPase activity, it was important to determine protein concentration (µg/ml) using Modified Lowry Kit. Standard samples were prepared by diluting BSA (bovine serum albumin) in various concentrations of water. Then, 0.2mL of diluted standard samples and experimental samples were transferred into appropriately marked cuvettes and incubated with 1.0mL of Modified Lowry Reagent for 10min at room temperature. Next, 100µl of 1x Folin Reagent was added and everything was incubated for 30 minutes at room temperature. Absorbance for the standard samples and the experimental samples was collected in duplicates at 750nm using Lambda 35 spectrophotometer. Following, standard samples were plotted into graph: BSA absorbance vs. final BSA concentration in order to identify protein concentration in the experimental samples.
2.3.3.5 Vacuolar H$^+$ ATPase activity

The activity of the vacuolar ATPase was monitored using coupled enzyme ATP regeneration assay system as described by Lotscher et al., 1984. This assay is based on reaction in which decrease of NADH absorbance is proportional to the rate of ATP hydrolysis. In the assay, ATP hydrolysis allows for conversion of phosphoenol pyruvate into pyruvate. Then, as NADH is oxidized into NAD$^+$, pyruvate is further converted into lactate (Figure 2.2). All the reagents used in the assay are colorless, except for NADH which has high absorption at 340nm in comparison to NAD$^+$ which has low absorption at 340nm. Therefore, as ATP is being hydrolyzed, we should see reduction in NADH absorption.
Figure 2.2 Coupled enzyme ATP regeneration assay system used to measure V-ATPase activity.
5µg/ml of vacuolar vesicles were assayed in 1ml of solution containing 25mM Tris acetate pH 7, 25mM KCl, 5mM MgCl₂, 2mM phosphoenolpyruvate, 2mM ATP, 0.5mM NADH, 30U L-lactate dehydrogenase and 30U pyruvate kinase. The assay was performed by adding the vacuolar vesicles directly into a cuvette containing other components and immediately observing the change in absorbance at 340nm every minute for 10 minutes using Lambda 35 spectrophotometer. Control samples were prepared just like the experimental samples with addition of 1µM Concanamycin A that inhibited V-ATPase activity. Obtained values were plugged into below formula:

\[
\frac{\text{absorbance rate without inhibitor} - \text{absorbance rate with inhibitor}}{6.22} \times \frac{1000}{\text{volume assayed in microliters} \times \text{protein concentration of sample assayed in mg/ml}}
\]

*6.22 x 10⁻³ represents NADH excitation coefficient.
2.3.4 Vacuolar pH determination

Cells were grown from a single colony until mid-logarithmic phase (optical density of 0.8) in SC media with 0.002% (w/v) of myo-inositol buffered to pH 5.5 or pH 7, followed by induction and repression for additional two hours in the absence and presence of 0.002% (w/v) of myo-inositol respectively at pH 5.5 or pH 7. Next, cells were spun down for 3 minutes at 3400 rpm and resuspended in fresh media. 1mM of BCECF-AM dye was added and everything was incubated at 30°C for 30 minutes. Following, cells were spun down for 3 minutes at 3400 rpm and washed in appropriate SC media. At the end, cells were resuspended in appropriate SC media and placed on ice.

Calibration samples were prepared for every strain under every condition. Each calibration buffer containing 50mM MES, 50mM HEPES, 50mM KCl, 50mM NaCl, 0.2M ammonium acetate, 10mM sodium azide, 10mM 2-deoxyglucose and 15µM nigericin was buffered to pH of 5, 5.5, 6 and 6.5. 20µl of cells with BCECF-AM were added into 2ml of each calibration buffer and incubated for 30 min at 30°C. Experimental samples were prepared by resuspending 20µl of cells with BCECF-AM in 2ml of 1mM MES buffered to pH 5.5 or pH 7. Following, 200µl of experimental samples as well as calibration samples were transferred into 96 well plate and excitation wavelength at 450nm and 490nm with emission wavelength of 535nm was measured using SpectraMas M5 microplate reader. Calibration curve was generated by calculating the ratio of fluorescence at 490nm to 450nm for each calibration sample. The fluorescence ratio was then plotted vs. pH of every calibration buffer. Experimental samples were converted into fluorescence ratio as well and pH of vacuoles was determined using calibration curve.
2.3.5 Vacuolar Morphology

Cells were grown from a single colony until mid-logarithmic phase (optical density of 0.8) in SC media with 0.002% (w/v) of myo-inositol buffered to pH 5.5 or pH 7, followed by induction and repression for additional two hours in the absence and presence of 0.002% (w/v) of myo-inositol respectively at pH 5.5 or pH 7. Next, cells were spun down for 3 minutes at 3400 rpm and resuspended in fresh media. 16µM of FM4-64 dye was added and everything was incubated at 30°C for 30 minutes. Following, cells were spun down for 3 minutes at 3400 rpm and washed in appropriate SC media. At the end, cells were resuspended in appropriate SC media and transferred onto glass slides to be observed under 64x objectives of Leica microscope.
2.4 Cell Growth

2.4.1 Cell culture

Yeast strains were inoculated from a single colony into appropriate media. These cultures were grown at 30°C and 300rpm overnight. All the strains were subjected to inducing (absence of inositol) and repressing conditions (presence of inositol) at pH 5.5 or pH 7. The optical density was measured at 600 nm using Lambda 35 UV/VIS spectrometer (Perkin Elmer). The critical parameters in performing these experiments were inositol concentration, pH, temperature and the rotation speed.

2.4.2 Glycerol stock preparation

50% glycerol was prepared by mixing 100% glycerol and appropriate media in 1:1 ratio. Overnight culture of particular yeast strain was mixed with 50% glycerol in a 1: 1 ratio followed by immediate freezing in dry ice. Glycerol stocks were stored in -80°C.
3 The role of VMA3 gene in de novo inositol synthesis

Studies have shown that mutation in any of the VMA genes encoding for subunits of the V-ATPase pump, resulted in growth inhibition in the absence of inositol (ino- phenotype) (Young et al., 2010; Villa-Garcia et al., 2011). Whereas, inositol auxotrophy (ino- phenotype) in yeast cells is linked to dysregulation of the INO1 gene and/or other genes involved in de novo inositol synthesis and lipid metabolism (Donahue and Henry, 1981; Villa-Garcia et al., 2011). Although VMA genes have not been shown to regulate these genes (Figure 1.4), ino- phenotype in vma mutants suggests otherwise. Furthermore, it has been demonstrated that one way to rescue growth of vma mutants in the absence of inositol is through OPI1 deletion (Young, 2010). OPI1 is a transcriptional suppressor gene that plays an important role in de novo inositol synthesis. When inositol is present in the growth media, Opi1p translocates from the endoplasmic reticulum into the nucleus to inhibit de novo inositol synthesis by suppressing the Ino2p transcriptional activator protein. When inositol is absent in the growth media, Opi1p remains cytoplasmic, allowing the INO2 gene product to act upon INO1 gene, which stimulates de novo inositol synthesis (Figure 1.4). As such, the results from OPI1 deletion suggest a role of VMA gene(s) in de novo inositol synthesis and/or lipid metabolism through OPI1 regulation. Another study demonstrated that mutations in any of the VMA genes resulted in growth inhibition at pH 7 but not at pH 5.5 (Sambade et al., 2005). Cells with defective V-ATPase pumps are able to survive in acidic environment due to acidification of the vacuole through fluid phase endocytosis or yet another unknown pathway. However, when cells are grown in neutral or basic conditions, the vacuolar system can no longer be acidified and leads to lack of cell growth (Nelson and Nelson, 1990; Munn and Riezman, 1994). None of these studies, however, looked at the combined effect of inositol and pH to determine whether mutant cells possess conditional lethality due to lack of inositol production or imbalance in cytoplasmic and vacuolar pH. To elucidate the above question, we chose to study the effect of the VMA3 upon de novo inositol synthesis at various pHs. VMA3 encodes for hydrophobic polypeptide, a part of the proteolipid in V0 domain (Powell et al., 1986). Studies have shown that Vma3p is indispensable for proper proton transport across the vacuolar membrane. This is because deletion of VMA3 affects V-ATPase assembly as well as ATP hydrolysis and hydrogen transport.
Taken together, we proposed that VMA3p can affect *de novo* inositol synthesis. Thus, the deletion of *VMA3* should result in conditional lethality in the absence of inositol regardless of the medium pH. However, if *VMA3* knockout cells show growth inhibition in the absence of inositol at pH 7 but not at pH 5.5, then it will indicate that pH is the main factor driving mutant cells’ growth and not inositol. To test our hypothesis, we have performed growth analysis using growth media with and without inositol at pH 5.5 or pH 7. Further, RNA analysis was employed to identify which phospholipid genes are affected by Vma3p.
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 *vma3Δ* strains were grown in SC media (synthetic complete media) in the presence and absence of 0.002% (w/v) of *myo-inositol*. Each medium was buffered to pH 5.5 or pH 7.

For acetic acid hypersensitivity, *wild type* and *vma3Δ* strains were grown in SC media (synthetic complete media) in the presence and absence of 0.002% (w/v) of *myo-inositol* in the presence of 0mM, 40mM, 80mM, 120mM, 160mM or 200mM 1M acetic acid.

Growth experiments were performed in duplicates where the optical density (OD) of the cells and pH of the media were measured every two hours for 30 hours at 30°C.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genome type</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Wild type</em> (BY4741)</td>
<td>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</td>
</tr>
<tr>
<td><em>vma3Δ</em></td>
<td>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 deltaCUP5</td>
</tr>
</tbody>
</table>

Table 3.1 Yeast strains used in this study.
3.1.2 Total RNA preparation

Cells were grown from a single colony until mid-logarithmic phase (optical density of 0.8) in SC media with 0.002% (w/v) of myo-inositol buffered to pH 5.5 or pH 7, followed by induction and repression for additional two hours in the absence and presence of 0.002% (w/v) of myo-inositol respectively at pH 5.5 or pH 7. RNA was extracted from yeast cells through acid phenol (pH 4.3) chloroform extraction followed by ethanol precipitation. RNA concentration was quantified by reading absorbance at 260nm ($A_{260}$). DNase treatment was then employed to remove DNA. Subsequently, total RNA was stored at -20°C until further use. Each experiment was prepared in duplicates.

3.1.3 Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

mRNA was converted to cDNA using first strand synthesis kit. Subsequently, Syber Green® qPCR universal kit was used to quantify cDNA templates. Primers used in the qPCR reaction are shown in Table 3.2.
<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HXK2</strong> primers</td>
<td>5’ CCAATGGCCATCAACTGTGA ’3</td>
<td>5’ TGGCCTGGTCTTGGAGATTC ’3</td>
</tr>
<tr>
<td><strong>INO1</strong> primers</td>
<td>5’ CCATGGTTAGCCCAAACGA ’3</td>
<td>5’ GCCTTCAACCGTTGTGCA ’3</td>
</tr>
<tr>
<td><strong>INO2</strong> primers</td>
<td>5’ AGCGCTTTGTCTCAAGTTGT ’3</td>
<td>5’ TGGTCATTGCGCATAGGCTG ’3</td>
</tr>
<tr>
<td><strong>OPI1</strong> primers</td>
<td>5’ CTITGCACAAGCAAGATGGA ’3</td>
<td>5’ TTCCCGAAACCTGAGATCTTG ’3</td>
</tr>
<tr>
<td><strong>INM1</strong> primers</td>
<td>5’ GATCAAAACCCTGGCTCAAG ’3</td>
<td>5’ TGTGTTGTAGGGTCTCGTG ’3</td>
</tr>
<tr>
<td><strong>PIS1</strong> primers</td>
<td>5’ GGCAAACCCCTGGACAATGAC ’3</td>
<td>5’ GCTCATCTCATCTCATCT ’3</td>
</tr>
<tr>
<td><strong>VPS34</strong> primers</td>
<td>5’ CCAAGAAGGTCAGCATTGA ’3</td>
<td>5’ ACCCAGCCTTGAGCGGTAA ’3</td>
</tr>
<tr>
<td><strong>FAB1</strong> primers</td>
<td>5’ TCCTCAGTAGCAACAGCGAC ’3</td>
<td>5’ GTGCGCGCATAGGAATTCTG ’3</td>
</tr>
<tr>
<td><strong>IPK2</strong> primers</td>
<td>5’ TCCGCAAGGACTGGAATTCT ’3</td>
<td>5’ TGGGCATCCAAGACACAA ’3</td>
</tr>
<tr>
<td><strong>ACT1</strong> primers</td>
<td>5’ CCAAGCCGTTGGTTCTTGT ’3</td>
<td>5’ ACCGCGCAATCGATTCT ’3</td>
</tr>
</tbody>
</table>

**Table 3.2** List of primers used in RNA analysis.
3.1.4 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 house keeping gene. Therefore, the relative mRNA levels were expressed as $2^{\Delta C_t}$.
3.2 Results

3.2.1 Growth experiments to identify effects of pH and inositol availability on vma3Δ viability

In order to identify which factor plays the major role in conditional lethality of vma knockout cells, growth survival experiments were performed in the presence and absence of inositol at pH 5.5 or pH 7. The hypothesis states that if mutation in VMA3 gene affects de novo inositol synthesis, then knockout cells should only grow in the presence of inositol regardless of the medium pH. However, if VMA knockout cells show growth inhibition at pH 7 and not at pH 5.5 regardless of the inositol availability, then it will indicate that VMA mutants most likely do not affect de novo inositol synthesis or synthesis of inositol containing phospholipids. Instead, pH might be the factor inhibiting growth of knockout cells.

Our results showed that the WT cells grew well in the presence of inositol at pH 5.5. In the absence of inositol, WT cells grew slower than in the presence of inositol (Figure 3.1A). The growth rate decreased about 17.24% (Table 3.3B). It is possible that WT cells need to activate phospholipid biosynthetic gene expression to adopt themselves to the given condition. At pH 7, we observed that the growth patterns were very similar to those at pH 5.5, both in the presence and absence of inositol respectively. However, we still noted some differences. First, there was a 4.80% decrease in growth rate in cells grown at pH 7 in the presence of inositol as compared to cells growth at pH 5.5 in the presence of inositol. Secondly, there was a 17.99% decrease in growth rate in cells grown at pH 7 in the absence of inositol as compared to the cells growth at pH 5.5 in the absence of inositol (Table 3.3A). Although the drop in cells growth was negligible, it seems that cells preferred growth in an acidic environment and in the presence of inositol.

With cells growth, pH of the medium dropped accordingly. For the first 6 hours there was a minimal drop in pH, due to absence or minimal cell division. For the next 18 hours, the media pH began to drop as cells entered log phase during which rapid cell division occurred. After that, the media pH stabilized and remained the same over the next 6 hours where the culture reached plateau (Figure 3.1B).

We observed that vma3Δ cells also grew well at pH 5.5 in the presence of inositol (Figure 3.2A). In the absence of inositol in the external media, vma3Δ cells showed reduced growth rate compared to cells growth in the presence of inositol by decreasing 42.31% (Table 3.4B). This reduction in growth was much
higher than in WT cells which was only 17.24%. The severe growth reduction may indicate the effect of Vma3p on phospholipid biosynthetic pathway gene expression. We also observed that vma3Δ cells were nonviable in media buffered to pH 7 (Figure 3.2A). Furthermore, we observed that pH value of the media remained unchanged as cells were nonviable (Figure 3.2B).
Figure 3.1 Growth pattern of WT cells at various conditions. (A) WT cells grew well in the presence and absence of inositol at pH 5.5 and pH 7. (B) The pH of growth media dropped over time as cells began to produce waste material.
Figure 3.2 Growth pattern of \textit{vma3Δ} cells at various conditions. (A) \textit{vma3Δ} cells were nonviable at pH 7 regardless of inositol availability. At pH 5.5 cells grew well in the presence of inositol, whereas in the absence of inositol cells growth was reduced. (B) The pH of growth media dropped over time as cells were grown at pH 5.5 due to production of waste materials. The pH of growth media at pH 7 remained unchanged as cells were nonviable.
### Table 3.3A Growth rate percentage of WT affected by pH change. Although WT cells grew well in acidic and neutral pH, the growth was optimal at pH 5.5.

<table>
<thead>
<tr>
<th></th>
<th>OD at 24h</th>
<th>Change in cell doubling time (Folds)</th>
<th>Decrease in growth rate %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>plus 5.5</td>
<td>5.105</td>
<td>4.86</td>
<td>1.050411523</td>
</tr>
<tr>
<td>minus 5.5</td>
<td>4.225</td>
<td>3.465</td>
<td>1.219336219</td>
</tr>
</tbody>
</table>

### Table 3.3B Growth rate percentage of WT affected by inositol availability. Although WT cells grew well in the presence and absence of inositol, the growth was optimal in the presence of inositol in the growth media.

<table>
<thead>
<tr>
<th></th>
<th>OD at 24h</th>
<th>Change in cell doubling time (Folds)</th>
<th>Decrease in growth rate %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>plus 5.5</td>
<td>5.105</td>
<td>4.86</td>
<td>1.050411523</td>
</tr>
<tr>
<td>minus 5.5</td>
<td>4.225</td>
<td>3.465</td>
<td>1.219336219</td>
</tr>
</tbody>
</table>

### Table 3.4A Growth rate percentage of vma3Δ affected by pH change. The vma3Δ cells grew at pH 5.5 but not at pH 7.

<table>
<thead>
<tr>
<th></th>
<th>OD at 24h</th>
<th>Change in cell doubling time (Folds)</th>
<th>Decrease in growth rate %</th>
</tr>
</thead>
<tbody>
<tr>
<td>vma3Δ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>plus 5.5</td>
<td>5.07</td>
<td>0.165</td>
<td>30.72727273</td>
</tr>
<tr>
<td>minus 5.5</td>
<td>2.925</td>
<td>0.155</td>
<td>18.87096774</td>
</tr>
</tbody>
</table>

### Table 3.4B Growth rate percentage of vma3Δ affected by inositol availability. Although vma3Δ cells grew at pH 5.5 in the presence and absence of inositol, the growth was optimal as inositol was present in the growth media.

<table>
<thead>
<tr>
<th></th>
<th>OD at 24h</th>
<th>Change in cell doubling time (Folds)</th>
<th>Decrease in growth rate %</th>
</tr>
</thead>
<tbody>
<tr>
<td>vma3Δ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>plus 5.5</td>
<td>5.07</td>
<td>2.925</td>
<td>1.7333333333</td>
</tr>
<tr>
<td>minus 5.5</td>
<td>0.165</td>
<td>0.155</td>
<td>1.064516129</td>
</tr>
</tbody>
</table>

### Table 3.5 Growth rate percentage in vma3Δ compared to WT cells.

<table>
<thead>
<tr>
<th>OD at 24h</th>
<th>WT</th>
<th>vma3Δ</th>
<th>Change in cell doubling time (Folds)</th>
<th>Decrease in growth rate %</th>
</tr>
</thead>
<tbody>
<tr>
<td>plus pH 5.5</td>
<td>5.105</td>
<td>5.07</td>
<td>1.006903353</td>
<td>0.69%</td>
</tr>
<tr>
<td>minus pH 5.5</td>
<td>4.225</td>
<td>2.925</td>
<td>1.444444444</td>
<td>30.77%</td>
</tr>
<tr>
<td>plus pH 7</td>
<td>4.86</td>
<td>0.165</td>
<td>2.945454545</td>
<td>96.60%</td>
</tr>
<tr>
<td>minus pH 7</td>
<td>3.465</td>
<td>0.155</td>
<td>22.35483871</td>
<td>95.53%</td>
</tr>
</tbody>
</table>
3.2.2 mRNA analysis to identify which genes in phospholipid biosynthetic pathway are affected by VMA3 deletion

The growth survival experiment demonstrated growth reduction of vma3Δ cells in the absence of inositol at pH 5.5. This suggested that VMA3 might affect expression genes involved in de novo inositol synthesis, or synthesis of inositol containing phospholipids. To confirm this, we performed mRNA analysis focusing on genes involved in de novo inositol synthesis (HXK2, INO1, INO2, OPI1, and INM1) as well as genes involved in phospholipid metabolism (PIS1, VPS34, FAB1, and IPK2). The function of each gene is listed in Table 3.6. Our hypothesis argued that if vma knockout affects these gene(s), then there should be a reduced mRNA expression of affected genes. However, if vma3Δ does not affect de novo inositol synthesis and/or lipid metabolism then the expression of these genes should be similar WT cells. The qRT-PCR was employed to examine gene expression levels under various growth conditions. These conditions included: plus inositol at pH 5.5, minus inositol at pH 5.5, plus inositol at pH 7, and minus inositol at pH 7.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>HXK2</em></td>
<td>Catalyzes phosphorylation of glucose into glucose-6-phosphate.</td>
</tr>
<tr>
<td><em>INO1</em></td>
<td>Involved in synthesis of inositol phosphates and inositol containing phospholipids.</td>
</tr>
<tr>
<td><em>INO2</em></td>
<td>Transcription activator that binds inositol/choline-responsive elements (ICREs), required for derepression of phospholipid biosynthetic genes in response to inositol deprivation.</td>
</tr>
<tr>
<td><em>OPI1</em></td>
<td>Negatively regulates phospholipid biosynthetic genes in the presence of inositol by interacting with Ino2p.</td>
</tr>
<tr>
<td><em>INM1</em></td>
<td>Involved in biosynthesis of inositol.</td>
</tr>
<tr>
<td><em>PIS1</em></td>
<td>Required for biosynthesis of phosphatidylinositol.</td>
</tr>
<tr>
<td><em>VPS34</em></td>
<td>Converts phosphatidylinositol into phosphatidylinositol-3-phosphate.</td>
</tr>
<tr>
<td><em>FAB1</em></td>
<td>Converts phosphatidylinositol-3-phosphate into phosphatidylinositol-3-5-phosphate.</td>
</tr>
<tr>
<td><em>IPK2</em></td>
<td>Converts phosphatidylinositol-3-5-phosphate into phosphatidylinositol-3-5-6-phosphate.</td>
</tr>
</tbody>
</table>

**Table 3.6** Function of the genes used in mRNA analyses.
Our mRNA analysis showed that both WT cells and vma3Δ cells have similar expression patterns of most of the phospholipid gene expression (Figure 3.4A, 3.4B; 3.5A, 3.5B; 3.6A, 3.6B; 3.7A, 3.7B; 3.8A, 3.8B; 3.9A, 3.9B; 3.10A, 3.10B; 3.11A, 3.11B). These results suggest that VMA3 deletion does not affect expression of these genes. However, we did observe that HXK2 expression was significantly reduced in vma3Δ indicating that VMA3 might be involved in de novo inositol synthesis (Figure 3.3A and 3.3B).
Figure 3.3 qRT-PCR analysis of *HXK2* gene expression. The *vma3Δ* showed significantly reduced *HXK2* gene expression in the presence and absence of inositol (A) at pH 5.5 and (B) at pH 7 compared to WT.
Figure 3.4 qRT-PCR analysis of INO1 gene expression. The vma3Δ showed similar INO1 gene expression as WT in the presence and absence of inositol (A) at pH 5.5 and (B) at pH 7.
Figure 3.5 qRT-PCR analysis of INO2 gene expression. The vma3Δ showed similar INO2 gene expression as WT in the presence and absence of inositol (A) at pH 5.5 and (B) at pH 7.
Figure 3.6 qRT-PCR analysis of OPI1 gene expression. The vma3Δ showed similar OPI1 gene expression as WT in the presence and absence of inositol (A) at pH 5.5 and (B) at pH 7.
Figure 3.7 qRT-PCR analysis of INM1 gene expression. The vma3Δ showed similar INM1 gene expression as WT in the presence and absence of inositol (A) at pH 5.5 and (B) at pH 7.
Figure 3.8 qRT-PCR analysis of PIS1 gene expression. The vma3Δ showed similar PIS1 gene expression as WT in the presence and absence of inositol (A) at pH 5.5 and (B) at pH 7.
Figure 3.9 qRT-PCR analysis of VPS34 gene expression. The vma3Δ showed similar VPS34 gene expression as WT in the presence and absence of inositol (A) at pH 5.5 and (B) at pH 7.
Figure 3.10 qRT-PCR analysis of FAB1 gene expression. The vma3Δ showed similar FAB1 gene expression as WT in the presence and absence of inositol (A) at pH 5.5 and (B) at pH 7.
Figure 3.11 qRT-PCR analysis of IPK2 gene expression. The vma3Δ showed similar IPK2 gene expression as WT in the presence and absence of inositol (A) at pH 5.5 and (B) at pH 7.
3.2.3 Growth experiment to confirm effects of \textit{vma3Δ} upon \textit{HXK2} gene expression

Our mRNA analysis showed that \textit{HXK2} expression was significantly reduced in \textit{vma3Δ} cells. This gene encodes for hexokinase-2-protein during growth on glucose (Moreno and Herrero, 2002). Hxk2p plays an important role in glucose metabolism as it phosphorylates glucose at C6 which can be further phosphorylated in glycolytic pathway, or converted into inositol-3-phosphate in phospholipid biosynthetic pathway.

Further, studies have shown that \textit{hxk2Δ} cells demonstrate acetic acid hypersensitivity as \textit{HXK2} protects yeast cells against acetic-acid-induced apoptosis (Amigoni, 2013). Therefore, to confirm that the absence of \textit{VMA3} downregulates the expression of \textit{HXK2}, we performed acetic acid sensitivity analysis.

Our results showed that WT cells grew well in the presence of inositol. As we began to introduce different concentrations of acetic acid into the growth media WT cells survival began to change. At 40 mM, 80 mM, 120 mM and 160 mM acetic acid there was slight but not significant growth reduction (Figure 3.12A). In the presence of 200 mM acetic acid, however, the growth of WT cells dropped as much as 28% compared to 0 mM acetic acid, demonstrating toxic levels of acetic acid upon WT cells growth (Table 3.7). Likewise, the \textit{vma3Δ} grew well in 0mM, 40 mM, 80 mM, 120 mM and 160 mM acetic acid, whereas at 200 mM cells growth was reduced by 39% (Figure 3.12B).

In the absence of inositol, both WT and \textit{vma3Δ} cells grew well at 0 mM, 40 mM and 80 mM acetic acid. WT cells began to show sensitivity to acetic acid at 120 mM, 160 mM and 200 mM acetic acid, and its growth dropped 20%, 37%, and 57% compared to 0 mM, respectively. The \textit{vma3Δ} cells showed to be even more sensitive to acetic acid as their growth was reduced by 32%, 56% and 68% compared to 0 mM. As such, both WT and \textit{vma3Δ} cells showed sensitivity to acetic acid especially in the absence of inositol, although growth reduction of \textit{vma3Δ} cells was shown to be more severe compared to WT. These results confirmed downregulation of \textit{HXK2} gene in \textit{vma3Δ} cells as their growth in acetic acid was more affected compared to WT.
Figure 3.12 Acetic acid growth sensitivity assay (A) WT cells, (B) vma3Δ cells. Although WT and vma3Δ cells showed reduced cell growth as concentration of acetic acid increased gradually, vma3Δ displayed increased sensitivity compared to WT at 200mM in the presence of inositol, and in the absence of inositol at 120mM, 160mM and 200mM.
<table>
<thead>
<tr>
<th>24h</th>
<th>WT</th>
<th>Change in cell doubling time (Folds)</th>
<th>Decrease in growth rate %</th>
<th>vma3Δ</th>
<th>Change in cell doubling time (Folds)</th>
<th>Decrease in growth rate %</th>
</tr>
</thead>
<tbody>
<tr>
<td>plus 0mM</td>
<td>5.4</td>
<td></td>
<td></td>
<td></td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>plus 40mM</td>
<td>5.4</td>
<td>1</td>
<td>100.00%</td>
<td>6.2</td>
<td>1.0333333333333333</td>
<td>103.33%</td>
</tr>
<tr>
<td>plus 80mM</td>
<td>4.7</td>
<td>1.14893617</td>
<td>87.04%</td>
<td>5.5</td>
<td>1.09090909091</td>
<td>91.67%</td>
</tr>
<tr>
<td>plus 120mM</td>
<td>5</td>
<td>1.08</td>
<td>92.59%</td>
<td>5.4</td>
<td>0.9</td>
<td>90.00%</td>
</tr>
<tr>
<td>plus 160mM</td>
<td>4.6</td>
<td>1.173913043</td>
<td>85.19%</td>
<td>5.5</td>
<td>1.09090909091</td>
<td>91.67%</td>
</tr>
<tr>
<td>plus 200mM</td>
<td>3.9</td>
<td>1.384615385</td>
<td>72.22%</td>
<td>3.7</td>
<td>1.621621622</td>
<td>61.67%</td>
</tr>
<tr>
<td>minus 0mM</td>
<td>4</td>
<td>1.35</td>
<td>100%</td>
<td>3.4</td>
<td>1.764705882</td>
<td>100%</td>
</tr>
<tr>
<td>minus 40mM</td>
<td>4.6</td>
<td>1.15</td>
<td>115%</td>
<td>5.9</td>
<td>1.735294118</td>
<td>174%</td>
</tr>
<tr>
<td>minus 80mM</td>
<td>4</td>
<td>1</td>
<td>100%</td>
<td>4.9</td>
<td>1.441176471</td>
<td>144%</td>
</tr>
<tr>
<td>minus 120mM</td>
<td>3.2</td>
<td>1.25</td>
<td>80%</td>
<td>2.3</td>
<td>1.47826087</td>
<td>68%</td>
</tr>
<tr>
<td>minus 160mM</td>
<td>2.5</td>
<td>1.6</td>
<td>63%</td>
<td>1.5</td>
<td>2.266666667</td>
<td>44%</td>
</tr>
<tr>
<td>minus 200mM</td>
<td>1.7</td>
<td>2.352941176</td>
<td>43%</td>
<td>1.1</td>
<td>3.090909091</td>
<td>32%</td>
</tr>
</tbody>
</table>

**Table 3.7** Decrease in WT and vma3Δ growth rate percentage at the 24th hour of incubation in various concentrations of acetic acid in the presence and absence of inositol.
3.2.4 Discussion

In an attempt to identify how VMA3 affects phospholipid biosynthesis, we performed growth analyses under four conditions: plus inositol at pH 5.5, minus inositol at pH 5.5, plus inositol at pH 7, and minus inositol at pH 7. We then conducted mRNA analyses and acetic acid sensitivity test to further confirm our findings. Yeast strains used in the above experiments included wild type and vma3Δ.

Growth experiments showed that wild type strain grew well in all experimental conditions. However, vma3Δ cells grew only at pH 5.5 regardless of the presence or absence of inositol, although growth in the absence of inositol was reduced. This indicated involvement vma3Δ in de novo inositol synthesis in the phospholipid biosynthetic pathway.

RNA analyses was performed to elucidate the above assumption by investigating following genes expression; HXK2, INO1, INO2, OPI1, INM1, PIS1, VPS34, FAB1, and IPK2. Results demonstrated that only HXK2 gene showed significantly lower expression in the vma3Δ compared to WT cells. The product of HXK2 gene expression is important to phosphorylate glucose to glucose-6-phosphate, which then can be converted into inositol or pyruvate depending upon the biosynthesis pathway that dominates other environmental conditions. HXK2 plays crucial role when cells are grown in the media lacking inositol, as it is involved in early steps of de novo inositol synthesis in phospholipid biosynthetic pathway (Figure 1.4) thus, downregulation of HXK2 in vma3Δ cells explains reduced growth in the absence of inositol.

It has been demonstrated that addition of acetic acid, a well-known apoptotic stimulus in yeast cell, demonstrated relocalization of active Ras into the mitochondria. Furthermore, addition of acetic acid to hxx2Δ cells showed increased localization of active Ras in mitochondria and cell death compared with WT. Since HXK2 is downregulated in vma3Δ cells (Figure 3.3A, Figure 3.3B), it was reasonable to observe that vma3Δ cells were more sensitive to acetic acid than WT cells. This highlights a new possible role of VMA3 as an anti-apoptotic factor for yeast cells. Therefore, in the future we would like to examine whether vma3Δ causes increase in apoptotic cells with regards to biochemical and morphological changes that are typical of apoptosis such as radical oxygen species production and DNA fragmentation.
It has been shown that morphology of the vacuole changes throughout the cell cycle of yeast cells. First, during daughter cell/bud formation vacuole appears fragmented producing vesicular structures (Wiemken et al., 1970). These vesicular structures are being transported into the bud. With increasing bud size, the vacuolar size increases as well, indicating that vesicular structures fuse together in the daughter cell as well as the mother (Weisman L. S et al., 1987). Once daughter cell buds off, the vacuole begins to fragment again so it can be further transferred into a new daughter cell. It has been shown that lipid composition of the vacuole plays important role in vacuolar inheritance, vacuolar morphology and vacuolar acidification. Unlike plasma membrane, vacuolar membrane does not contain sphingolipid-rich lipid rafts. Furthermore, the vacuole is composed of regulatory lipids such as ergosterol, diacylglycerol, phosphatidylinositol 3-phosphate (PI(3)P) and phosphatidylinositol 4-phosphate (PI(4)P) which play important role in vacuolar morphology (Li and Kane, 2009). The major phosphoinositide species present in the membrane of the vacuoles include phosphatidylinositol 3,5-bisphosphate (PI(3,5)P2) that is generated from PI(3)P with help of Fab1p (Figure 1.4). Studies have shown that PI(3,5)P2 plays important role in vacuolar homeostasis as deletion or overexpression of FAB1 gene, resulted in enlarged or fragmented vacuoles, with neutral vacuolar pH due to reduced vacuolar H+–ATPase activity. These mutants also exhibited poor growth at high extracellular pH and in the presence of CaCl2 (Yamamoto et al., 1995; Li et al., 2014). VPS34 is yet another gene in the phospholipid biosynthetic pathway affecting vacuolar homeostasis. Its product, Vps34p phosphorylates phosphatidylinositol (PI) to form PI(3)P which can be further phosphorylated into PI(3,5)P2 (Figure 1.4). Although mutations in VPS34 gene resulted in normal vacuolar morphology, the pH of the vacuole appeared neutral due to reduced vacuolar H+–ATPase activity (Herman and Scott, 1990; Sambade, 2005). These mutants also exhibited minimal growth at high extracellular pH and the presence of CaCl2 (Sambade et al., 2005).

OPI1 and INO2 are two major transcription factors that regulate expression of genes required for phospholipid biosynthesis (Figure 1.4). Opi1p is situated outside of the nucleus and moves into the
nucleus in pH and inositol dependent manner. In acidic pH or in the presence of inositol, Opi1p moves into the nucleus where it acts as a negative regulator upon genes required for phospholipid biosynthesis. Whereas in the neutral/basic pH or in the absence of inositol, it stays outside of the nucleus (Young et al., 2010; Loewen, 2012). Ino2p is situated inside of the nucleus and it acts as transcriptional activator for de novo inositol synthesis as deletion of INO2 prevents cells from growing in the absence of inositol (Wagner Christian et al., 1999). Since both Opi1p and Ino2p work together to regulate de novo inositol synthesis that can further be phosphorylated into various phospholipids, we wanted to examine their importance in vacuolar homeostasis.

To test the hypothesis that OPI1 and INO2 may regulate vacuolar morphology and acidification, we conducted growth survival analysis in the presence of CaCl$_2$ at various pH ranges and in the presence or absence of inositol, followed by microscopic analysis and vacuolar acidification assay. Yeast strains used in these experiments included wild type, vma3Δ, ino2Δ and opi1Δ. Wild type was used as the positive control, where the vacuoles have been shown to occupy a small fraction of the total cell volume with vacuolar pH 5.5-6.2 (Preston et al., 1989; Salcedo et al., 2012). The vma3Δ was used as yet another control that has been shown to exhibit normal vacuolar morphology, with neutral pH of the vacuole as a result of defective vacuolar H$^+$-ATPase activity (Tanida et al., 1994; Martinez-Muñoz et al., 2008). This mutant also showed inability to growth at high extracellular pH and the presence of CaCl$_2$. 

65
4.1 Materials and methods

4.1.1 Growth analysis on YPD plates

Yeast strains used in this experiment are shown in Table 4.1. Individual colony of wild type, vma3Δ, opi1Δ and ino2Δ was incubated overnight in 6ml YPD media (yeast-extract-peptone-dextrose). Following day, each strain was diluted 10 folds in YPD medium and 5ul of each diluted strain was plated on YPD plate. YPD medium and plates contained 60mM of CaCl\textsubscript{2} and were buffered to pH 5.5 or pH 7. Plates were incubated at 30°C overnight before analyzing the results.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genome type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (BY4741)</td>
<td>MAT\text{a} his3\Delta1 leu2\Delta0 met15\Delta0 ura3\Delta0</td>
</tr>
<tr>
<td>vma3\Delta</td>
<td>MAT\text{a} his3\Delta1 leu2\Delta0 met15\Delta0 ura3\Delta0 ΔCUP5</td>
</tr>
<tr>
<td>opi1\Delta</td>
<td>MAT\text{a} his3\Delta1 leu2\Delta0 met15\Delta0 ura3\Delta0 ΔOPI1</td>
</tr>
<tr>
<td>ino2\Delta</td>
<td>MAT\text{a} his3\Delta1 leu2\Delta0 met15\Delta0 ura3\Delta0 ΔINO2</td>
</tr>
</tbody>
</table>

Table 4.1 Yeast strains used in this study.

4.1.2 Growth analysis in liquid broth

Wild type, opi1\Delta, ino2\Delta and vma3\Delta cells were grown in SC media (synthetic complete media) containing 10mM CaCl\textsubscript{2}. Media was buffered to pH 5.5 or pH 7 with or without 0.002% (w/v) of myo-inositol.

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 30 hours at 30°C.

4.1.3 Vacuolar Morphology

Wild type, opi1\Delta, ino2\Delta and vma3\Delta cells were grown from a single colony until mid-logarithmic phase (optical density of 0.8) in SC media with 0.002% (w/v) of myo-inositol buffered to pH 5.5 or pH 7, followed by induction and repression for additional two hours in the absence and presence of 0.002% (w/v) of myo-inositol respectively at pH 5.5 or pH 7. Cells were then incubated with 16µM of FM4-64 dye for 30 minutes at 30°C. Following, cells were washed twice, resuspended in fresh media and transferred onto glass slides to be observed under 64x objectives of Leica microscope.
4.1.4 Vacuolar pH analysis

Wild type, opi1Δ, ino2Δ and vma3Δ cells were grown until mid-logarithmic phase (optical density of 0.8) in SC media with 0.002 (W/V)% of myo-inositol buffered to pH 5.5 or pH 7, followed by induction and repression for additional two hours in the absence and presence of 0.002% (w/v) of myo-inositol respectively at pH 5.5 or pH 7. Cells were then incubated with 1mM of BCECF-AM dye for 30 minutes at 30°C. Following, cells were washed twice, resuspended in fresh media and placed on ice.

Calibration samples were prepared for every strain under every condition. Cells with BCECF were incubated with calibration buffers at various pHs for 30 minutes at 30°C. Experimental samples were prepared by resuspending cells with BCECF with 1mM MES buffer at pH 5.5 or pH 7. Experimental sample as well as calibration samples were transferred into 96 well plate and excitation wavelength at 450nm and 490nm with emission wavelength of 535nm was measured using SpectraMas M5 microplate reader. Calibration curve was generated by calculating the ratio of fluorescence at 490nm to 450nm for each calibration sample. The fluorescence ratio was then plotted vs pH to obtain calibration. Experimental samples were converted into fluorescence ratio as well and pH of vacuole was determined using standard curve.
4.2 Results

4.2.1 The effects of CaCl\(_2\) upon \textit{OPI1} and \textit{INO2} deletion strain

Since previous studies showed that \textit{FAB1}, \textit{VPS34} and \textit{VMA3} cells were sensitive to CaCl\(_2\) and neutral pH, we wanted to examine whether \textit{opi1Δ} and \textit{ino2Δ} follow the same conditional phenotype.

For WT cells, we observed that cells grew well on YPD plate at pH 5.5 containing 60 mM CaCl\(_2\) and the last visible growth was seen at 10\(^{10}\) fold dilution (Figure 4.1A). Whereas, growth of WT cells on YPD plate at pH 7 containing 60 mM CaCl\(_2\) was reduced and the last visible growth was seen at 10\(^6\) fold dilution (Figure 4.1B). Moreover, the last visible growth of \textit{vma3Δ} cells on YPD plate at pH 5.5 containing 60 mM CaCl\(_2\) was seen at 10\(^2\) fold dilution (Figure 4.1A), whereas growth on YPD plate at pH 7 containing 60 mM CaCl\(_2\) was completely absent (Figure 4.1B). As such, this result demonstrated that \textit{vma3Δ} can serve as a good negative control.

We then turned our attention into sensitivity experiments with respect to \textit{ino2Δ} and \textit{opi1Δ}. The \textit{INO2} deletion strain grew well on YPD plate at pH 5.5 containing 60 mM CaCl\(_2\) and the last visible growth was seen at 10\(^8\) fold dilution (Figure 4.1A). Whereas, growth on YPD plate at pH 7 containing 60 mM CaCl\(_2\) was reduced and the last visible growth was seen at 10\(^4\) fold dilution (Figure 4.1B).

For \textit{OPI1} deletion cells, growth sensitivity experiment indicated that this strain grew well on YPD plate at pH 5.5 containing 60mM CaCl\(_2\) and the last visible growth was seen at 10\(^{10}\) fold dilution (Figure 4.1A). Whereas, growth of \textit{OPI1} deletion strain on YPD plate at pH 7 containing 60mM CaCl\(_2\) was reduced and the last visible growth was seen at 10\(^4\) fold dilution (Figure 4.1B). As such, sensitivity experiments showed that growth of \textit{ino2Δ}, \textit{opi1Δ} and WT cells was sensitive to CaCl\(_2\) at pH 7, although \textit{opi1Δ} and \textit{ino2Δ} showed moderate increase in sensitivity compared to WT.
Figure 4.1 Growth sensitivity analysis in the presence of 60 mM CaCl₂. (A) There is a visible growth of all strains at pH 5.5. (B) Growth of WT, ino2Δ and opi1Δ was reduced at pH 7, whereas vma3Δ cells were nonviable.
We next examined growth survival with 10 mM of CaCl$_2$ in the presence or absence of inositol to get better understanding of the $OPI1$ and $INO2$ deletion upon cells survival under this unfavorable condition.

For the WT cells, we observed that cells grew well at pH 5.5, although there was a 22.27% decrease in growth in the absence of inositol as compared to the growth in the presence of inositol (Table 4.2B). There was even greater growth reduction (65.28%) in the presence of inositol at pH 7 compared to cells growth in the presence of inositol at pH 5.5. In the absence of inositol at pH 7 there was a 75% decrease in growth compared to cells growth in the absence of inositol at pH 5.5 (Figure 4.2A). We also examined pH of the growth medium and results indicated that as cells grew, pH of the media dropped accordingly (Figure 4.2B). As such, growth of WT cells in the presence of CaCl$_2$ was more affected by media pH than inositol availability.

For $vma3\Delta$, growth survival experiment showed that these mutants grew well in low concentrations of CaCl$_2$ as long as the medium pH remained acidic (Figure 4.3A). However, cells were nonviable when grown in media buffered to pH 7 regardless of inositol availability. Change in pH of the growth media corresponded with the growth of cells. As cells were viable at pH 5.5, pH of the media dropped accordingly. Whereas, pH of media buffered to pH 7 did not change over time as cells were nonviable in neutral environment (Figure 4.3B). This indicated that $vma3\Delta$ cells survival in the presence of CaCl$_2$ was dependent upon acidic environment of the medium.

The $ino2\Delta$ cells grew well in the presence of inositol at pH 5.5 (Figure 4.4A). However, there was a 59.66% decrease in growth in the presence of inositol at pH 7 compared to the growth in the presence of inositol at pH 5.5. The $ino2\Delta$ cells were nonviable in the absence of inositol at pH 5.5 and pH 7 (Table 4.4A). We also observed drop in pH over time in media containing inositol at pH 5.5 and pH 7. However, because cells were nonviable in the absence of inositol at pH 5.5 or pH 7, pH of the media remained constant (Figure 4.4B). Here, $ino2\Delta$ cells demonstrated pH and inositol sensitivity.

The $opi1\Delta$ cells grew well at pH 5.5 in the presence and absence of inositol (Figure 4.5A). However, there was a 59.46% decrease in cells growth in the presence of inositol at pH 7 compared to cells growth in the presence of inositol at pH 5.5. Similarly, there was a 61.37% decrease in cells growth in the absence of
inositol at pH 7 compared to cells growth in the absence of inositol at pH 5.5 (Table 4.5A). As such, growth of \textit{opi1}\Delta cells was mainly affected by pH of the medium.

Taken together, we observed that \textit{ino2}\Delta and \textit{opi1}\Delta were sensitive to 10 mM CaCl\textsubscript{2} at pH 7 in similar way to WT cells, whereas at 60 mM mutants showed moderately increased sensitivity compared to WT cells. On the other hand, \textit{vma3}\Delta was completely nonviable at pH 7.
Figure 4.2 Growth survival analysis of WT cells. (A) Cells grew in the presence and absence of inositol at pH 5.5, whereas at pH 7 cells growth was reduced. (B) The pH of growth media dropped as cells began to produce waste material.
Figure 4.3 Growth survival analysis of vma3Δ cells. (A) Cells grew well at pH 5.5, whereas at pH 7 cells were nonviable. (B) The pH of growth media dropped over time due to waste material production at pH 5.5, whereas at pH 7 pH of growth media did not change much as cells were nonviable.
Figure 4.4 Growth survival analysis of *ino2Δ* cells. (A) Cells grew well in the presence of inositol regardless of media pH, whereas in the absence of inositol, cells were nonviable. (B) The pH of growth media dropped over time in the presence of inositol, whereas in the absence of inositol, pH of growth media remained unchanged.
Figure 4.5 Growth survival analysis of opi1Δ cells. (A) Cells grew well in the presence and absence of inositol at pH 5.5, whereas at pH 7 cells growth was reduced. (B) The pH of growth media dropped over time as cells began to produce waste material.
<table>
<thead>
<tr>
<th>Wild type</th>
<th>OD at 24h</th>
<th>Change in cell doubling time (Folds)</th>
<th>Decrease in growth rate %</th>
</tr>
</thead>
<tbody>
<tr>
<td>plus 5.5</td>
<td>plus 7</td>
<td>4.94 1.715</td>
<td>65.28%</td>
</tr>
<tr>
<td>minus 5.5</td>
<td>minus 7</td>
<td>3.84 0.96</td>
<td>75.00%</td>
</tr>
</tbody>
</table>

**Table 4.2A** Growth rate percentage of WT cells affected by pH of the media.

<table>
<thead>
<tr>
<th>Wild type</th>
<th>OD at 24h</th>
<th>Change in cell doubling time (Folds)</th>
<th>Decrease in growth rate %</th>
</tr>
</thead>
<tbody>
<tr>
<td>plus 5.5</td>
<td>minus 5.5</td>
<td>4.94 3.84</td>
<td>22.27%</td>
</tr>
<tr>
<td>plus 7</td>
<td>minus 7</td>
<td>1.715 0.96</td>
<td>44.02%</td>
</tr>
</tbody>
</table>

**Table 4.2B** Growth rate percentage of WT cells affected by inositol availability in the media.

<table>
<thead>
<tr>
<th>vma3Δ</th>
<th>OD at 24h</th>
<th>Change in cell doubling time (Folds)</th>
<th>Decrease in growth rate %</th>
</tr>
</thead>
<tbody>
<tr>
<td>plus 5.5</td>
<td>plus 7</td>
<td>5.7 0.54</td>
<td>90.35%</td>
</tr>
<tr>
<td>minus 5.5</td>
<td>minus 7</td>
<td>5.69 0.385</td>
<td>93.15%</td>
</tr>
</tbody>
</table>

**Table 4.3A** Growth rate percentage of vma3Δ affected by pH of the media.

<table>
<thead>
<tr>
<th>vma3Δ</th>
<th>OD at 24h</th>
<th>Change in cell doubling time (Folds)</th>
<th>Decrease in growth rate %</th>
</tr>
</thead>
<tbody>
<tr>
<td>plus 5.5</td>
<td>minus 5.5</td>
<td>5.7 5.69</td>
<td>0.18%</td>
</tr>
<tr>
<td>plus 7</td>
<td>minus 7</td>
<td>0.54 0.385</td>
<td>28.70%</td>
</tr>
</tbody>
</table>

**Table 4.3B** Growth rate percentage of vma3Δ affected by inositol availability in the media.
### Table 4.4A Growth rate percentage of \(ino2\Delta\) affected by pH of the media.

<table>
<thead>
<tr>
<th></th>
<th>OD at 24h</th>
<th>Change in cell doubling time (Folds)</th>
<th>Decrease in growth rate %</th>
</tr>
</thead>
<tbody>
<tr>
<td>plus 5.5</td>
<td>plus 7</td>
<td>6.445</td>
<td>2.6</td>
</tr>
<tr>
<td>minus 5.5</td>
<td>minus 7</td>
<td>0.55</td>
<td>0.44</td>
</tr>
</tbody>
</table>

### Table 4.4B Growth rate percentage of \(ino2\Delta\) affected by inositol availability in the media.

<table>
<thead>
<tr>
<th></th>
<th>OD at 24h</th>
<th>Change in cell doubling time (Folds)</th>
<th>Decrease in growth rate %</th>
</tr>
</thead>
<tbody>
<tr>
<td>plus 5.5</td>
<td>minus 5.5</td>
<td>6.445</td>
<td>0.55</td>
</tr>
<tr>
<td>plus 7</td>
<td>minus 7</td>
<td>2.6</td>
<td>0.44</td>
</tr>
</tbody>
</table>

### Table 4.5A Growth rate percentage of \(opi1\Delta\) affected by pH of the media.

<table>
<thead>
<tr>
<th></th>
<th>OD at 24h</th>
<th>Change in cell doubling time (Folds)</th>
<th>Decrease in growth rate %</th>
</tr>
</thead>
<tbody>
<tr>
<td>plus 5.5</td>
<td>plus 7</td>
<td>5.945</td>
<td>2.41</td>
</tr>
<tr>
<td>minus 5.5</td>
<td>minus 7</td>
<td>6.245</td>
<td>2.4</td>
</tr>
</tbody>
</table>

### Table 4.5B Growth rate percentage of \(opi1\Delta\) affected by inositol availability in the media.

<table>
<thead>
<tr>
<th></th>
<th>OD at 24h</th>
<th>Change in cell doubling time (Folds)</th>
<th>Decrease in growth rate %</th>
</tr>
</thead>
<tbody>
<tr>
<td>plus 5.5</td>
<td>minus 5.5</td>
<td>5.945</td>
<td>6.245</td>
</tr>
<tr>
<td>plus 7</td>
<td>minus 7</td>
<td>2.41</td>
<td>2.41</td>
</tr>
</tbody>
</table>
4.2.2 The effect of *OPI1* and *INO2* deletion strain upon vacuolar morphology and acidification

Growth analysis demonstrated that *opi1Δ* and *ino2Δ* cells were sensitive to 60 mM CaCl$_2$. To further determine their involvement with regards to vacuolar function, we conducted microscope analysis and vacuolar pH assay.

Our results showed that at pH 5.5 regardless of inositol availability, vacuoles in WT cells occupied small area of the total cell’s volume. At pH 7, WT cells exhibited enlarged vacuoles that occupied most cell’s surfaces (Figure 4.6). The other three mutants showed similar morphology as WT at pH 5.5 and pH 7, except that *ino2Δ* exhibited fragmented vacuoles at pH 5.5. As such, morphology of the vacuole does not always reflect upon its acidification.
Chapter 5

The effect of the transcription factors from the phospholipid biosynthetic pathway upon VMAs gene expression

In yeast, the protein product of the OPI1 gene (the transcriptional repressor) is located on the endoplasmic reticulum (ER) through interaction with phosphatidic acid (PA) and vesicle-associated membrane protein-associated Scs2p. Inositol is a potent regulator of Opi1p. Addition of myo-inositol to the growth medium results in depletion of phosphatidic acid at the endoplasmic reticulum though its conversion into phosphatidylinositol (PI) (Loewen C.J 2012). Depletion of phosphatidic acid reduces its interaction with Opi1p. Therefore, Opi1p is free to translocate into the nucleus where it acts as a negative regulator on more than 30 genes. A well-known example target gene of Opi1p is INO1. This gene plays an important role in de novo inositol synthesis. Therefore, when inositol is present in the growth media, ...

Figure 4.6 Vacuolar morphology of cells in various conditions. Right side represents DIC images of the yeast cells with vacuoles. Left side represents images of yeast vacuoles using Fm4-64 dye (red). At pH 5.5, vacuoles occupied small area of total cells volume in all the above strains. At pH 7, vacuoles appeared enlarged in all the above strains.
Next, vacuolar acidification assay was employed. WT cells grown in media buffered to pH 5.5 maintained acidic pH of their vacuoles regardless of inositol availability (Figure 4.7). Although vacuolar pH became significantly less acidic as cells were grown in media buffered to pH 7, the pH was still within normal vacuolar pH range of 5.5-6.2 (Table 4.6). This suggested that luminal acidification is well maintained in acidic or basic extracellular environment.

The vma3Δ cells displayed significantly less acidic vacuoles compared to WT when grown in media buffered to pH 5.5, but the pH was still within the normal range (Table 4.6). As vma3Δ were switched to media buffered to pH 7, their vacuoles were no longer within the normal vacuolar pH range (Figure 4.8). As for vma3Δ alone, vacuolar pH was significantly increased at pH 7 compared to pH 5.5. This suggested defective acidification of vacuoles at pH 7 (p = 0.0002).

The ino2Δ and opi1Δ cells, maintained acidic vacuoles when grown in media buffered to pH 5.5 and pH 7 (Table 4.6). Although, vacuoles in opi1Δ cells were significantly more acidic compared to WT cells (Figure 4.8). When vacuolar pH was compared between media pH 5.5 and pH 7 within ino2Δ and opi1Δ, vacuoles were significantly less acidic at pH 7 compared to pH 5.5 (opi1Δ plus inositol pH 5.5 vs. pH 7 p = 0.0005, minus inositol pH 5.5 vs. pH 7 p = 0.0002; ino2Δ plus inositol pH 5.5 vs. pH 7 p = 0.02, minus inositol pH 5.5 vs. pH 7 p = 0.001).
Table 4.6 Vacuolar pH in the presence and absence of inositol at pH 5.5 or pH 7. WT and ino2Δ cells maintained acidic pH of the vacuole at pH 5.5 and pH 7. Vacuolar pH of opi1Δ cells was significantly lower compared to WT. Vacuolar pH of vma3Δ remained acidic at pH 5.5 only.

<table>
<thead>
<tr>
<th></th>
<th>Wild type</th>
<th>vma3Δ</th>
<th>ino2Δ</th>
<th>opi1Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>plus inositol pH 5.5</td>
<td>5.583037</td>
<td>6.122117</td>
<td>5.645327</td>
<td>5.374503</td>
</tr>
<tr>
<td>minus inositol pH 5.5</td>
<td>5.474114</td>
<td>6.09836</td>
<td>5.55215</td>
<td>5.211802</td>
</tr>
<tr>
<td>plus inositol pH 7</td>
<td>5.970524</td>
<td>6.903896</td>
<td>5.792556</td>
<td>5.694734</td>
</tr>
<tr>
<td>minus inositol pH 7</td>
<td>5.859394</td>
<td>6.876762</td>
<td>5.779245</td>
<td>5.635958</td>
</tr>
</tbody>
</table>
Figure 4.7 Vacuolar pH of WT in the presence and absence of inositol at pH 5.5 and pH 7. Cells maintained acidic pH of the vacuole as cells were grown in media buffered to pH 5.5 and pH 7.
Figure 4.8 Vacuolar pH in the presence and absence of inositol at pH 5.5 or pH 7 of WT, *opi1Δ, ino2Δ* and *vma3Δ*. WT and *ino2Δ* cells maintained acidic pH of the vacuole at pH 5.5 and pH 7. Vacuolar pH of *opi1Δ* cells was significantly lower compared to WT. Vacuolar pH of *vma3Δ* remained acidic at pH 5.5 only.
4.3 Discussion

The products of \textit{VPS34} and \textit{FAB1} are very important in phospholipid biosynthesis as they catalyze conversion of PI to PI(3,5)P2. The fact that both \textit{VPS34} and \textit{FAB1} are important in vacuolar homeostasis has prompted us to propose that the transcriptional regulators of phospholipid biosynthesis may also play important role in vacuole function and activity.

\textit{OPI1} and \textit{INO2} are two major transcription factors that regulate expression of genes required for phospholipid biosynthesis (Figure 1.4). Both, Opi1p and Ino2p work together to regulate \textit{de novo} inositol synthesis that can further be phosphorylated into various phospholipids. One of the most important products of the phospholipid biosynthesis is the PI which is the substrate for the products catalyzed by Vps34p and Fab1p. As such, it is possible that \textit{OPI1} and \textit{INO2} may affect vacuolar homeostasis through the regulation of phospholipid biosynthesis.

Growth sensitivity experiment showed that WT, \textit{vma3Δ}, \textit{ino2Δ} and \textit{opi1Δ} grew well in growth medium buffered to pH 5.5 containing 10 mM CaCl$_2$. As cells were switched to growth medium buffered to pH 7 containing CaCl$_2$ WT, \textit{ino2Δ} and \textit{opi1Δ} showed growth reduction, whereas \textit{vma3Δ} was completely nonviable. As concentration of CaCl$_2$ was increased to 60 mM, \textit{opi1Δ} and \textit{ino2Δ} showed moderate growth reduction compared to WT.

Microscopic analysis and vacuolar acidification were employed to further elucidate the importance of \textit{OPI1} and \textit{INO2} in vacuolar homeostasis. Analysis of vacuolar morphology indicated that WT, \textit{vma3Δ} and \textit{opi1Δ} displayed vacuoles that occupied small area of the cell when grown in media buffered to pH 5.5. Whereas, \textit{ino2Δ} displayed fragmented vacuoles. On the other hand, all strains grown in media buffered to pH 7, exhibited enlarged vacuoles that occupied most of cell’s surface. This indicated that the presence of \textit{INO2} may play important in vacuolar morphology when cells are grown in media buffered to pH 5.5.

Lastly, vacuolar acidification assay showed that only \textit{vma3Δ} cells displayed notably less acidic vacuoles as cells were grown in media buffered to pH 5.5 and pH 7. Whereas, \textit{opi1Δ} displayed notably more acidic pH compared to WT.
Taken together, the above results demonstrated sensitivity of \textit{opi1\textDelta} and \textit{ino2\textDelta} cells in high concentration of CaCl$_2$, although \textit{opi1\textDelta} cells showed significantly increased vacuolar acidification as compared to \textit{ino2\textDelta} and WT cells. Therefore, we decided to characterize the function of both transcription factors upon V-ATPase pump activity at molecular and physiological level.
5 The effect of the transcription factors from the phospholipid biosynthetic pathway upon V-ATPase pump activity

In yeast, the protein product of the \textit{OPI1} gene (the transcriptional repressor) is located on the endoplasmic reticulum (ER) through interaction with phosphatidic acid (PA) and integral membrane protein Scs2p. Inositol is a potent regulator of Opi1p. Addition of \textit{myo-inositol} to the growth medium results in depletion of phosphatidic acid at the endoplasmic reticulum though its conversion into phosphatidylinositol (PI) (Loewen 2012). Depletion of phosphatidic acid reduces its interaction with Opi1p. Therefore, Opi1p is free to translocate into the nucleus where it acts as a negative regulator on more than 30 genes. A well-known example target gene of Opi1p is \textit{INO1}. This gene plays important role in \textit{de novo} inositol synthesis (Figure 1.4). Therefore, when inositol is present in the growth media, Opi1p is free to translocate into the nucleus from endoplasmic reticulum where it indirectly affects binding of Ino2p transcriptional activator with \textit{INO1} gene and therefore reduces its transcription. When inositol is absent in the growth media, Opi1p stays outside of the nucleus allowing for \textit{INO1} gene induction (Brickner J. H \textit{et al}., 2004).

Further, the pH is another potent regulator of Opi1p. Phosphatidic acid is an anionic type of lipid with negatively changed phosphomonoester head-group and hydrophobic diacylglycerol backbone. Therefore, the negatively charged phosphomonoester head-group allows for nonspecific electrostatic interactions with clusters of positively changed amino acids of effector proteins such as Opi1p (Shin J. H \textit{et al}., 2011). However, the interaction between phosphatidic acid and Opi1p fluctuates depending upon the pH of cytosol. It has been shown that due to drop in pH, negatively charged phosphomonoester head-group becomes protonated and binding of Opi1p is decreased. This allows Opi1p to translocate into the nucleus where it can repress transcription of various genes. Whereas, neutral or basic pH allows Opi1p to stay outside of the nucleus due to greater affinity for deprotonated phosphatidic acid (Young B. P \textit{et al}., 2010).

Our results showed that deletion of \textit{OPI1} but not \textit{INO2} significantly lowered vacuolar pH compared to WT. Therefore, it was of our interest to investigate the role of transcription factors with regards to vacuolar H+-
ATPases activity at molecular and physiological levels. We hypothesized that deletion of \textit{OP1} but not \textit{INO2} should increase VMAs gene expression and therefore V-ATPase activity as a result of increased vacuolar acidification.
5.1 Materials and methods

5.1.1 Total RNA preparation

Yeast strains used in this experiment are shown in Table 5.1. WT, opi1Δ, ino2Δ and vma3Δ were grown from a single colony until mid-logarithmic phase (optical density of 0.8) in SC media with 0.002% (w/v) of myo-inositol buffered to pH 5.5 or pH 7, followed by induction and repression for additional two hours in the absence and presence of 0.002% (w/v) of myo-inositol respectively at pH 5.5 or pH 7. RNA was extracted from yeast cells through acid phenol (pH 4.3) chloroform extraction followed by ethanol precipitation. RNA concentration was quantified by reading absorbance at 260nm (A260). DNase treatment was employed to remove DNA. Subsequently, total RNA was stored at -20°C until further use. Each experiment was prepared in duplicates.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genome type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (BY4741)</td>
<td>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</td>
</tr>
<tr>
<td>opi1Δ</td>
<td>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ΔOPI1</td>
</tr>
<tr>
<td>ino2Δ</td>
<td>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ΔINO2</td>
</tr>
<tr>
<td>vma3Δ</td>
<td>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ΔCUP5</td>
</tr>
</tbody>
</table>

Table 5.1 Yeast strains used in this study.

5.1.2 Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

mRNA was converted to cDNA using first strand synthesis kit. Subsequently, Syber Green® qPCR universal kit was used to quantify cDNA templates. Primers used in the qPCR reaction are shown in Table 5.2.
<table>
<thead>
<tr>
<th>Prime</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>VMA1</td>
<td>5’ GCCGTTTGTCTCGTACCATT 3’</td>
<td>5’ AACATGGGAAACCCAACAGAG 3’</td>
</tr>
<tr>
<td>VMA2</td>
<td>5’ AACGGAACGTGATGTTGGA 3’</td>
<td>5’ AGTGATGGACCCGGTACGAC 3’</td>
</tr>
<tr>
<td>VMA4</td>
<td>5’ CATTGACGGCAACTTCAAGA 3’</td>
<td>5’ CTTGACTCCTGCCCCGTGT 3’</td>
</tr>
<tr>
<td>VMA5</td>
<td>5’ TAACTTGGCTGTGCTGAGA 3’</td>
<td>5’ TCAGCATCCTGTGCAATAC 3’</td>
</tr>
<tr>
<td>VMA7</td>
<td>5’ TTATTGTAGCCGGGATTGG 3’</td>
<td>5’ GGTITCCCGCAGATGTTGT 3’</td>
</tr>
<tr>
<td>VMA8</td>
<td>5’ TGGACTGGTATCCTGACAGA 3’</td>
<td>5’ TCGTCTTGCGCTTCAAA 3’</td>
</tr>
<tr>
<td>VMA9</td>
<td>5’ AACTGGCCGTGATACCAGAG 3’</td>
<td>5’ GGTTAAAGGTCAGCAACCA 3’</td>
</tr>
<tr>
<td>VMA10</td>
<td>5’ TGGGTTAGCCGGGATTGG 3’</td>
<td>5’ GTGATGTTGCTTCAAA 3’</td>
</tr>
<tr>
<td>VMA13</td>
<td>5’ TTTCTTTGGTGCCATTGGTT 3’</td>
<td>5’ CAGGTGCCGAATTTCTTCA 3’</td>
</tr>
<tr>
<td>VMA11</td>
<td>5’ GCCATTTATGGGCTTGTTGT 3’</td>
<td>5’ ACAGTCACCGACCATACCA 3’</td>
</tr>
<tr>
<td>VMA12</td>
<td>5’ GCCATTTATGGGCTTGTTGT 3’</td>
<td>5’ ACAGTCACCGACCATACCA 3’</td>
</tr>
</tbody>
</table>

**Table 5.2** List of primers used in RNA analysis.
5.1.3 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 house keeping gene. Therefore, the relative mRNA levels were expressed as \( 2^{\Delta C_T} \).

5.1.4 Cells growth for ATPase assay

Cells were grown from a single colony until mid-logarithmic phase (optical density of 0.8) in SC media with 0.002% (w/v) of myo-inositol buffered to pH 5.5 or pH 7, followed by induction and repression for additional two hours in the absence and presence of 0.002% (w/v) of myo-inositol respectively at pH 5.5 or pH 7.

5.1.5 Spheroplast formation and lysis

Cell wall was lysed using spheroplasting medium with 2-mercaptoethanol and lytic enzyme at 30°C. After cell lysis was complete, spheroplasts were washed in 1M Sorbitol and homogenized in Buffer A at 0°C. Lysate supernatant was then centrifuged to remove any unlysed spheroplasts.

5.1.6 Vacuolar flotation

To isolate vacuolar vesicles, supernatant was transferred into polyallomer tube and overlaid with half the volume of Buffer A. Followed by centrifugation, white wafer floating on top was collected and further homogenized with Buffer A. Everything was transferred into polyallomer tube and overlaid with the same volume of Buffer B. Once again, white wafer floating on top was collected and resuspended in equal volume of 2x Buffer C. Suspension was homogenized using small pipette and diluted in equal volume of 1x Buffer C.

5.1.7 Protein determination

Concentration of proteins (µg/ml) was obtained using Modified Lowry Kit. Control values were prepared by diluting BSA in various concentrations of water. Control samples and experimental samples were resuspended with Modified Lowry Reagent as well as 1x Folin Reagent, and the absorbance was
collected at 750nm. Control samples were used to generate calibration curves which in turn were used to determine protein concentration of experimental samples (Table 5.3).
<table>
<thead>
<tr>
<th>µg/ml</th>
<th>WT</th>
<th>ino2Δ</th>
<th>opi1Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>plus inositol pH 5.5 (repeat 1)</td>
<td>229.964</td>
<td>17.35648</td>
<td>23.82168</td>
</tr>
<tr>
<td>plus inositol pH 5.5 (repeat 2)</td>
<td>281.5</td>
<td>17.24824</td>
<td>27.59164</td>
</tr>
<tr>
<td>minus inositol pH 5.5 (repeat 1)</td>
<td>312.429</td>
<td>20.32203</td>
<td>28.34582</td>
</tr>
<tr>
<td>minus inositol pH 5.5 (repeat 2)</td>
<td>209.321</td>
<td>13.02866</td>
<td>23.15198</td>
</tr>
<tr>
<td>plus inositol pH 7 (repeat 1)</td>
<td>46.2469</td>
<td>23.98903</td>
<td>18.34603</td>
</tr>
<tr>
<td>plus inositol pH 7 (repeat 2)</td>
<td>40.8548</td>
<td>18.32221</td>
<td>14.59526</td>
</tr>
<tr>
<td>minus inositol pH 7 (repeat 1)</td>
<td>38.7481</td>
<td>19.321</td>
<td>14.06013</td>
</tr>
<tr>
<td>minus inositol pH 7 (repeat 2)</td>
<td>39.8406</td>
<td>20.22245</td>
<td>16.46195</td>
</tr>
</tbody>
</table>

**Table 5.3** Protein concentration of every experimental sample.
5.2.1 Vacuolar H\textsuperscript{+} ATPase activity

Yeast strains used in this experiment are shown in Table 5.1. The assay was performed by adding vacuolar vesicles directly into a cuvette containing reaction mixture (25mM Tris acetate pH 7, 25mM KCl, 5mM MgCl\textsubscript{2}, 2mM phosphoenolpyruvate, 2mM ATP, 0.5mM NADH, 30U L-lactate dehydrogenase and 30U pyruvate kinase) and immediately observing change in absorbance at 340nm using time drive mode Lambda 35 spectrophotometer. Control samples were prepared the same way with addition of Concanamycin A (V-ATPase pump inhibitor) (Table 5.4).
<table>
<thead>
<tr>
<th>Strain</th>
<th>ino2Δ</th>
<th>Average inhibition by concanamycin A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmol/min/mg</td>
<td>%</td>
</tr>
<tr>
<td>w/ inositol pH 5.5</td>
<td>0.005088463</td>
<td>86.87448728</td>
</tr>
<tr>
<td>w/o inositol pH 5.5</td>
<td>0.007493219</td>
<td>87.2433377</td>
</tr>
<tr>
<td>w/ inositol pH 7</td>
<td>0.006662254</td>
<td>87.8920496</td>
</tr>
<tr>
<td>w/o inositol pH 7</td>
<td>0.006901646</td>
<td>88.78338279</td>
</tr>
<tr>
<td>Strain</td>
<td>opi1Δ</td>
<td>Average inhibition by concanamycin A</td>
</tr>
<tr>
<td></td>
<td>µmol/min/mg</td>
<td>%</td>
</tr>
<tr>
<td>w/ inositol pH 5.5</td>
<td>0.009878605</td>
<td>88.81469115</td>
</tr>
<tr>
<td>w/o inositol pH 5.5</td>
<td>0.012958849</td>
<td>82.11091234</td>
</tr>
<tr>
<td>w/ inositol pH 7</td>
<td>0.007954059</td>
<td>86.5060241</td>
</tr>
<tr>
<td>w/o inositol pH 7</td>
<td>0.006459627</td>
<td>86.90095847</td>
</tr>
<tr>
<td>Strain</td>
<td>vma3Δ</td>
<td>Average inhibition by concanamycin A</td>
</tr>
<tr>
<td></td>
<td>µmol/min/mg</td>
<td>%</td>
</tr>
<tr>
<td>w/ inositol pH 5.5</td>
<td>0.007568043</td>
<td>87.85446763</td>
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<tr>
<td>w/o inositol pH 5.5</td>
<td>0.004519296</td>
<td>89.48805461</td>
</tr>
<tr>
<td>w/ inositol pH 7</td>
<td>0.006793589</td>
<td>88.1332408</td>
</tr>
<tr>
<td>w/o inositol pH 7</td>
<td>0.007345579</td>
<td>87.38095238</td>
</tr>
<tr>
<td>Strain</td>
<td>wild type</td>
<td>Average inhibition by concanamycin A</td>
</tr>
<tr>
<td></td>
<td>µmol/min/mg</td>
<td>%</td>
</tr>
<tr>
<td>w/ inositol pH 5.5</td>
<td>0.00588725</td>
<td>89.22426113</td>
</tr>
<tr>
<td>w/o inositol pH 5.5</td>
<td>0.009081195</td>
<td>83.0126132</td>
</tr>
<tr>
<td>w/ inositol pH 7</td>
<td>0.018498339</td>
<td>86.77859391</td>
</tr>
<tr>
<td>w/o inositol pH 7</td>
<td>0.018225386</td>
<td>85.53553554</td>
</tr>
</tbody>
</table>

**Table 5.4** Total V-ATPase inhibition in the presence of Concanamycin A.
Obtained values were plugged into below formula:

\[
\frac{(\text{absorbance rate without inhibitor} - \text{absorbance rate with inhibitor}) \times 1000}{\text{volume assayed in microliters}} \times \frac{\text{protein concentration of sample assayed in mg/ml}}{6.22}
\]

* 6.22 is the coefficient of NADH at 340 nm.
5.2 Results

5.2.1 RNA analysis to determine whether \textit{OPI1} and \textit{INO2} affect pump activity at molecular level

To examine whether transcription factors; \textit{OPI1} and \textit{INO2} regulate V-ATPase pump at molecular level, we performed mRNA analyses focusing on the 13 \textit{VMA} genes (\textit{VMA1, VMA2, VMA4, VMA5, VMA7, VMA8, VMA10, VMA13, VPH1, VMA3, VMA6, VMA11, and VMA16}) whose products constitute the components of the V-ATPase pump. We proposed that deletion of \textit{OPI1} but not \textit{INO2} should upregulate \textit{VMAs} gene expression as a result of increased vacuolar acidification.

mRNA analysis indicated that \textit{VMA} genes can be classified into three different types of expression patterns upon the change of pH value and inositol availability. Type 1 expression pattern showed increased expression levels when media component changed from with inositol to without inositol at pH 5.5. At pH 7 regardless of inositol availability, expression levels of \textit{VMA} genes maintained constant. Here, \textit{VMA1, VMA2, VMA6, VMA8, and VMA11} have been classified into Type 1 (Figure 5.1A). For the Type 2, expression pattern showed no changes of expression levels when the media’s component changed from with inositol to without inositol at pH 5.5 or pH 7. Genes in Type 2 included \textit{VMA3, VMA4, VMA7, VMA16} and \textit{VPH1} (Figure 5.1B). For the Type 3, there was an increase in genes expression when the media’s changed from with inositol to without inositol at pH 5.5 and pH 7. \textit{VMA5, VMA10} and \textit{VMA13} have been classified into Type 3 (Figure 5.1C).

The above results showed that \textit{VMA} genes are not uniformly affected by inositol availability or pH value of the media. Similar results where different \textit{VMA} genes have been expressed in non-co-ordinated way has been demonstrated in plants, and suggested explanation was that non-co-ordinated regulation of expression might be due to different stress conditions, different turnover rates of the subunits, due to different signaling pathways regulating the transcription of individual subunits, or simply because certain \textit{VMA} genes might not be limited to V-ATPase function only (Dietz \textit{et al.}, 2001). Therefore, these genes have been classified into three Types based on their expression pattern. The products of \textit{VMA} genes in Type 1 have shown to stimulate ATP hydrolysis, H+ transport and assembly of \textit{V}1 complex with \textit{V}0 complex. Products of \textit{VMA} genes in Type 2 function in V-ATPase assembly and H+ transport. Whereas,
products of VMA genes in Type 3 govern activation of the pump. Moreover, only expression pattern of genes in Type 3 showed to occur in inositol dependent manner.
Figure 5.1 qRT-PCR analysis for VMA genes expression. (A) In Type 1, genes are downregulated in the presence of inositol at pH 5.5 only. (B) In Type 2, genes are unaffected by the pH or inositol availability. (C) In Type 3, genes are downregulated in the presence of inositol at pH 5.5 and pH 7.
We next compared WT gene expression of Type 1, Type 2 and Type 3 with VMAs from \textit{opi1}\Delta and \textit{ino2}\Delta to determine its effect upon V-ATPase pump activity at molecular level.

For \textit{ino2}\Delta, gene expression in Type 1 and Type 2 did not follow the same pattern as in WT (Figure 5.2A, Figure 5.2B). Instead different \textit{VMA} genes were either upregulated, downregulated or unaffected compared to WT (Table 5.5A). Similar results have been demonstrated for \textit{opi1}\Delta (Figure 52A, Figure 5.2B).

Whereas, Type 3 mRNA analysis in \textit{opi1}\Delta and \textit{ino2}\Delta showed significantly lower gene expression in the absence of inositol at pH 5.5 and pH 7 compared to WT. Taken together, deletion of \textit{OPI1} or \textit{INO2} dysregulated expression of \textit{VMA} genes which could in fact affect pump activity.
A

B
Figure 5.2 qRT-PCR analysis of VMA genes expression in ino2Δ and opi1Δ cells. (A) Type 1 and (B) Type 2, ino2Δ and opi1Δ displayed downregulated and upregulated VMA genes as compared to WT. (C) In Type 3, ino2Δ and opi1Δ displayed downregulation of VMA genes in the absence of inositol at pH 5.5 and pH 7 as compared to WT.
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**Table 5.5** Statistical analysis (p value) for VMA genes expression. (A) Type 1. (B) Type 2. (C) Type 3.
5.2.2 Vacuolar H\(^{+}\) - ATPase activity

Since opi1Δ and ino2Δ showed different mRNA expression of VMA genes compared to WT, we wanted to determine its effect upon V-ATPase pump activity at physiological level.

For the wild type, V-ATPase activity was significantly higher as cells were grown in media buffered to pH 7 compared to cells grown in media buffered to pH 5.5 (Figure 5.3). Pump activity was also significantly higher in the absence of inositol at pH 5.5 compared to pump activity in the presence of inositol at pH 5.5. This suggested that V-ATPase pump activity is sensitive to neutral pH of the media and inositol availability. For the opi1Δ, ino2Δ and vma3Δ, V-ATPase activity was significantly reduced at pH 7 in the presence or absence of inositol compared to WT (Figure 5.4A, Figure 5.4B, Figure 5.4C).

Taken together, OPI1 and INO2 deletion reduced V-ATPase pump activity, and this could have been a result of dysregulated VMA genes expression in the above mutant cells.
Figure 5.3 Vacuolar H\textsuperscript{+}-ATPase analysis of WT in the presence and absence of inositol at pH 5.5 and pH 7. V-ATPase activity was significantly higher at pH 7 compared to pH 5.5.
Figure 5.4 Vacuolar H⁺-ATPase analysis of WT compared to vma3Δ, ino2Δ and opi1Δ in the presence and absence of inositol at pH 5.5 or pH 7. V-ATPase activity at pH 7 was significantly lower in vma3Δ, ino2Δ and opi1Δ compared to WT.
5.3 Discussion

Our results showed that \textit{vma3\Delta} cells were restricted to growth in acidic environment, as their vacuoles remained acidic only when cells were grown in media buffered to pH 5.5. Furthermore, we showed that pump activity in \textit{vma3\Delta} cells was not functional due to lack of \textit{VMA3} gene. Therefore, \textit{vma3\Delta} could only relay on alternative vacuolar acidification from acidic media to accompany all physiological processes necessary for cells survival. We also showed that WT cells were able to maintain acidic environment of the vacuole regardless of the external media pH, although pH of the vacuoles was significantly higher as cells were grown in media buffered to pH 7. On top of that, we showed that pump activity in WT cells increased significantly at pH 7. Therefore, a key to vacuolar homeostasis at neutral pH is the functional V-ATPase pump.

We do not show direct evidence that Opi1 and Ino2p are involved in the regulation of V-ATPase activity. However, we propose that Opi1p and Ino2p may have a role in balancing or regulating vacuolar pH. Here, we showed that \textit{opi1\Delta} cells displayed low vacuolar pH, although the V-ATPase activity was reduced. Therefore we propose that Opi1p could control H\textsuperscript{+} efflux from the vacuoles as well as influx to maintain an optimal vacuolar pH for the different vacuolar processes. During logarithmic phase of cells growth, high glucose level from the medium leads to an increase in ATP synthesis through the glycolytic pathway. Several studies have demonstrated a direct interaction between various glycolytic enzymes such as aldolase or phosphofructokinase-1 with some of the subunits that make up the V-ATPase pump. Moreover, it has been proposed that the V-ATPase and glycolytic enzymes form a complex in the vacuolar membrane to maximize the efficiency of the energy provision to acidify the vacuoles. These studies imply that the energy source for V-ATPase proton pumping is through glycolysis. It could therefore be hypothesized that \textit{in vivo} proton pumping into the vacuole would occur primarily during logarithmic growth when there is accessible ATP production from glycolysis. Under these conditions, Opi1p may be involved in the uncoupling of ATP hydrolysis and H\textsuperscript{+} transport of the V-ATPase complex and promoting H\textsuperscript{+} leakage from the vacuolar lumen into the cytosol. This could potentially regulate an excess of protons in the vacuole and prevent vacuolar acidosis.
In the absence of Opi1p, protons accumulate into the vacuole and the V-ATPase activity drops significantly. This action is to prevent further acidification of the vacuole especially when cells are grown in the acidic environment. Therefore, Opi1p could be a protein involved in H\(^{+}\) transport from the vacuole to the cytosol when the hydrogen concentration in the vacuolar lumen reaches high values in much similar way as uncoupling proteins do in mitochondria of mammalian cells. These uncoupling proteins transport protons across the mitochondrial inner membrane and divert energy from ATP synthesis to thermogenesis in the mitochondria of brown adipose tissue and are involved in decreasing the concentration of reactive oxygen species inside the mitochondria. Although the role of Opi1p in the vacuole would be somewhat different from the uncoupling proteins in the mitochondria, it is possible that these protons could share a similar mechanism to transport protons across the membrane.

On the other hand, Ino2p might have a slightly different role in vacuolar acidification. Ino2p may control influx through V-ATPase pump but not the hydrogen efflux from the vacuoles to maintain an optimal vacuolar pH for the different vacuolar processes. This is because the vacuolar pH is similar to WT but the V-ATPase activity is low in the absence of Ino2p. Furthermore, we observed significant downregulation of VMA5, VMA10 and VMA13 genes in the absence of Ino2p. These three gene produces are responsible for V-ATPase activation. Therefore, it is possible that Ino2p regulate the V-ATPase activity but not the efflux so that the excess of proton can be transported into the cytosol.

Here we have proposed a hypothesis to explain how Opi1p and Ino2p are involved in the regulation of protons in the vacuole. We explain how they control H\(^{+}\) efflux from the vacuoles as well as proton influx through V-ATPase pump to maintain an optimal vacuolar pH at various conditions. However, further biochemical experimentation is required to confirm this hypothesis such as experiments to show the association of V0 and V1 subunits of the vacuolar ATPase in different yeast strains.
Figure 5.5 Proposed *OPI1* and *INO2* function in the vacuolar pH homeostasis. In the wild type cell vacuolar pH is maintained normal due to V-ATPase pump and other channels and transporters. The *OPI1* deletion prevents hydrogen escape from the vacuole, leading to increase in vacuolar acidification. Moreover, it also reduces V-ATPase pump to prevent cellular acidosis. The *INO2* deletion regulates vacuolar homeostasis in yet another way. It reduces V-ATPase pump activity yet maintains normal vacuolar pH due to unidentified upregulated/activated acidification mechanism.
References


44. Loewen, Christopher JR. "Lipids as conductors in the orchestra of life." *F1000 biology reports* 4 (2012).


