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The Interplay of Key Phospholipid Biosynthetic Enzymes and the Yeast V-ATPase Pump and their Role in Programmed Cell Death

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Abstract

Exposure of the yeast *Saccharomyces cerevisiae* to environmental stress can influence cell growth, physiology and differentiation, and thus result in a cell's adaptive response. During the course of an adaptive response, the yeast vacuoles play an important role in protecting cells from stress. Vacuoles are dynamic organelles that are similar to lysosomes in mammalian cells. The defect of a lysosome's function may cause various genetic and neurodegenerative diseases. The multi-subunit V-ATPase is the main regulator for vacuolar function and its activity plays a significant role in maintaining pH homeostasis. The V-ATPase is an ATP-driven proton pump which is required for vacuolar acidification. It has also been demonstrated that phospholipid biosynthetic genes might influence vacuolar morphology and function. However, the mechanistic link between phospholipid biosynthetic genes and vacuolar function has not been established. Recent studies have demonstrated that there is a regulatory role of Pah1p, a phospholipid biosynthetic gene, in V-ATPase disassembly and activity. Therefore, in this chapter we will use *Saccharomyces cerevisiae* as a model to discuss how Pah1p affects V-ATPase disassembly and activity and how Pah1p negatively affect vacuolar function. Furthermore, we propose a hypothesis to describe how Pah1p influences vacuolar function and programmed cell death through the regulation of V-ATPase.

Keywords: Phospholipid biosynthesis, Pah1p, programmed cell death, vacuolar activity, V-ATPase, pH homeostasis, Hxk2p

1. Introduction

The yeast vacuole is a crucial and dynamic organelle necessary for the survival of the cell. Adverse environmental factors, such as osmotic stress, toxic metal exposure, and alkaline pH conditions can wreak havoc on normal cellular processes and homeostasis. The vacuole helps play a critical role in protecting the cell via the induction of adaptive stress responses that are upregulated to shield the cell against many of these adverse environmental conditions. Of particular importance are the vacuolar proton translocating ATPase (V-ATPase) pumps. These energy requiring proton pumps are found in the membrane of the vacuole and can help defend the cell against many damaging environmental elements. These multi-subunit ATPase

complexes span the membrane and actively transport hydrogen ions into the lumen of the organelle. While V-ATPases have been highly characterized in vacuoles they are also present in a number of other organelles and cellular structures including lysosomes, Golgi complexes and the endosomes of eukaryotic cells [1–3]. Furthermore, in mammalian cells, these V-ATPase pumps have been found to be recruited to the cellular membranes of specialized cell types for the purpose of transporting protons across the membrane [1, 2].

Much of the research regarding V-ATPases has been conducted in the model system *Saccharomyces cerevisiae*. The main function of the V-ATPase pump is to acidify the organelle and maintain the internal acidic pH which is required for normal vacuolar function. Thus, the pump hydrolyzes ATP and drives the transport of protons across the membrane from the cytosol and into the lumen of the organelle [4]. The yeast vacuole is, in fact, one of the most highly acidic organelles documented in a cell, along with lysosomes in mammalian cells [4]. Vacuolar pH ranges in acidity from pH 5.0 to 6.5, depending on the specific environmental conditions [4–6]. In lysosomes, the internal pH is even lower, ranging from pH 4.5–5.0 [7]. Given the widespread localization of these pumps in various organelle membranes, V-ATPases are involved in a number of vital roles in cellular homeostasis including protein sorting and secretion, vesicular trafficking and zymogen activation [8]. Additionally, they have also been shown to be involved in endocytic and autophagic processes [1, 2, 9]. Since they play a vital position in maintaining cellular homeostasis, much research has been conducted to better understand just how important these pumps are. This review aims to specifically look at the significant role that V-ATPases play in maintaining pH homeostasis in yeast cells and how they are impacted by Pah1p, a key phosphatidate (PA) phosphatase in the lipid biosynthetic pathway that has been linked to apoptotic mechanisms via the regulation of this pump.

2. V-ATPase pump function

V-ATPase pumps have been shown to be crucial for proper cell function and survival. In fact, research experiments involving the deletion of genes that cause the ubiquitous loss of V-ATPase subunits show that it is lethal in almost all organisms, including *Drosophila* fruit flies and mice [10, 11]. Unlike most organisms however, yeast cells are still viable when V-ATPase subunit encoding genes are deleted. As a result, *Saccharomyces cerevisiae* has been an ideal model organism for studying V-ATPase function [12].

Deletion mutation studies in *S. cerevisiae* have shown that the vacuolar acidification process is impaired when genes that encode V-ATPase subunit are removed. Furthermore, growth phenotypes have indicated that cells with V-ATPase subunit deletions are sensitive to alkaline extracellular pH. While these mutants can grow in an extracellular environment of pH 5, they are unable to grow at pH 7.5. They are also sensitive to high levels of calcium in their growth medium, as well as when they are in the presence of heavy metals and oxidants. They also cannot grow on nonfermentable carbon sources [4, 12].

While V-ATPases are necessary for the vacuoles to maintain their internal acidic pH, they actually play a much more widespread role in the homeostasis of the cell by regulating cytoplasmic pH. Research has shown that cells with defective V-ATPase pumps are not only unable to maintain the acidity in their vacuoles but cannot uphold the necessary pH of the cell's cytoplasm [5, 13]. This is due to the fact that other pumps are dependent on V-ATPase activity which utilizes ATP to transport hydrogen ions into the vacuole. For instance, the $\text{Ca}^{+2}/\text{H}^{+}$ antiporter requires a

functional V-ATPase to transport calcium into the vacuole. If the V-ATPase is defective, calcium will enter the vacuole at a much slower rate and thus these mutants cannot grow well in presence of excess calcium as mentioned above [14–16].

Additionally, the vacuole plays a large role in detoxifying the cell of heavy metals via antiporters in the vacuole membrane. These pumps are also dependent on functional ATPases and rely on their activity to function properly. Cells with non-functional V-ATPases are unable to thrive in the presence of heavy metals, such as cadmium which causes oxidative stress in the cell [17, 18]. Furthermore, V-ATPases play an important part in promoting hydrolytic enzymes that degrade and recycle biomolecules [9, 19]. When a cell undergoes nitrogen starvation, nearly 4/5ths of the cell's protein degradation process takes place in the vacuole. Additionally, the vacuoles recycle organelles as well, which also helps to supply the cells with an abundance of amino acids and other biomolecule building blocks that can help the cell survive during times of stress [20–22]. V-ATPases therefore play a critical role in protecting the cell when exposed to various adverse environmental conditions and stress.

3. Structural composition of the vacuolar V-ATPase pump

Given the importance of V-ATPase pumps in maintaining intravacuolar acidity, the structural components of the pump have been well characterized in *Saccharomyces cerevisiae*. V-ATPase pumps are comprised of two distinctive domains that include the V1 domain and the V0 domain. The V1 domain is the catalytic component in the pump that is linked to the ATP binding sites and is located on the cytosolic part of the membrane. The V0 domain is the proton translocating component and consists of integral membrane proteins as well as peripheral ones. Both the V1 and V0 domains are linked together by a stalk-like structure within the V1 domain [13, 23]. These domains each consist of multiple subunits that come together to form the overall pump. V1 contains eight subunits, labeled A through H (A, B, C, D, E, F, G, and H) while the V0 domain contains 6 subunits labeled a, c, c', c'', d and e (**Figure 1**).

The 14 subunits of the V-ATPase pump are each encoded by their own gene, except for subunit a of the V0 domain which has two isoforms. 13 of these subunits are encoded by vacuolar membrane ATPase (VMA) genes [4]. These genes include *VMA1*, *VMA2*, *VMA3*, *VMA4*, *VMA5*, *VMA6*, *VMA7*, *VMA8*, *VMA9*, *VMA10*, *VMA11*, *VMA13*, *VMA16* (**Table 1**). The final subunit in the vacuolar V-ATPase is encoded by a gene named *VPH1* and encodes for the largest subunit in the pump, subunit a [15]. However, this subunit has two isoforms, with the second isoform being encoded by *STV1*. The *Stv1p* isoform is not located in the vacuolar membrane but is instead found in the V-ATPases located in the Golgi complex and endosome membranes. Research has shown that vacuolar V-ATPases that contain the *Vph1p* subunit are better at coupling the hydrolysis of ATP to the transport of hydrogen ions across the membrane. They also have increased assembly levels of the V-ATPase pump, and are more responsive to extracellular glucose levels compared to V-ATPases that contain *Stv1p* [4, 24]. For the purposes of this review, we will be focusing on vacuolar V-ATPases and will be looking at the *Vph1p* subunit.

Thus, eight of the V-ATPase subunits in the vacuole are found in the V1 domain and are encoded by *VMA1*, *VMA2*, *VMA4*, *VMA5*, *VMA7*, *VMA8*, *VMA10*, *VMA13*. The remaining six subunits are encoded by *VPH1*, *VMA3*, *VMA6*, *VMA9*, *VMA11*, and *VMA16* and are found in the V0 domain. A table outlining the various genes of the V-ATPase pump and the subunits encoded by them has been included (**Table 1**).

Figure 1. Model of the *Saccharomyces cerevisiae* vacuolar V-ATPase. The V₁ domain contains subunits A, B, C, D, E, F, G, and H. Subunits A and B each have three copies (not all depicted) and alternate in an A, B, A, B, A, B fashion in the pump. The V_o domain consists of subunits a, c, c', c'', d, and e.

Domain	Gene	Subunit
V ₁	VMA ₁	A
V ₁	VMA ₂	B
V ₁	VMA ₄	E
V ₁	VMA ₅	C
V ₁	VMA ₇	F
V ₁	VMA ₈	D
V ₁	VMA ₁₀	G

Domain	Gene	Subunit
V ₁	VMA ₁₃	H
V ₀	VPH ₁	a
V ₀	VMA ₃	c
V ₀	VMA ₆	d
V ₀	VMA ₉	e
V ₀	VMA ₁₁	c'
V ₀	VMA ₁₆	c''

Table 1.
The genes of the *Saccharomyces cerevisiae* vacuolar V-ATPase and their corresponding subunits.

4. V-ATPase subunit functions

The overall molecular mechanism of V-ATPase pump activity involves the hydrolysis of ATP by the V1 domain. This step provides the necessary energy needed to pump protons across the membrane and create a pH gradient that is needed for secondary transport systems [3, 25]. However, the specific functions of each of the subunits of the V1 and V0 domains are varied. The first two subunits of the V1 domain, subunit A and subunit B, of which there are three copies of each, are encoded by *VMA1* and *VMA2* respectively. They are the ATP binding subunits that hydrolyze ATP. Additionally, both aid in the regulation of proton transport and in the disassembly of the V1 and V0 domains [26, 27]. Subunit C, which is encoded by the *VMA5* gene, is needed to help activate the pump by regulating the assembly of the V1 domain to the V0 domain. Furthermore, it plays a key role in the disassembly of the pump since it dissociates from the enzyme during glucose starvation and separates the two domains, rendering the pump inactive [28, 29]. Subunit D is encoded by *VMA8* and is also crucial for the assembly of the two domains which is needed for pump activity and proton transport [30]. Subunit E, encoded by the *VMA4* gene, helps to form the peripheral structural stalk of the pump which is needed for proper assembly and function of the pump, while subunit F which is encoded by *VMA7* is a rotor subunit and needed for proper pump assembly [31–34]. Subunit G is encoded by *VMA10* and needed for proper stalk formation, while subunit H, which is encoded by *VMA13*, is required to inhibit ATP hydrolysis when V1 and V0 domains are dissociated and does so by interacting with subunit F [32, 35].

The subunits of the V0 domain are the remaining components of the V-ATPase pump that are primarily integral proteins, with the exception of subunit d which is a peripheral protein. Subunit a is encoded by the *VPH1* gene which are specific for V-ATPases localized in the vacuolar membrane. It is needed for the appropriate assembly of the pump as well as for the transport of hydrogen ions [24, 36]. Subunits c, c' and c'', which are encoded by *VMA3*, *VMA11*, and *VMA13* respectively, are suggested to be needed for proton translocation. All three subunits are crucial for V-ATPase function since a mutation in any of them will impair pump activity [37, 38]. Subunit d, which is encoded by *VMA6*, is the only peripheral V0 subunit and plays a key role in coupling ATP hydrolysis with proton transport [39]. Lastly, it is also important to note that the e subunit in the V0 domain, which is encoded by *VMA9*, was discovered much later than the rest of the subunits and as such has not been as well characterized [12]. Furthermore, *in vitro* studies have suggested that Vma9p is not needed for V-ATPase proton pumping activity since removal of Vma9p does not impact proton transport [40].

Importantly, the deletion of any one specific subunit in the V1 domain does not impact the stability of the remaining V1 subunits in the complex. However, it will impair the association and assembly of the entire V1 domain with the V0 domain, thus rendering the pump nonfunctional. Additionally, the deletion of any specific V1 subunit will not impact the stability of the V0 domain [13, 41]. Likewise, the loss of any one specific V0 subunit will not impact the stability of the remaining V0 subunits nor will it impact the assembly of the independent V1 domain. It will however impact the assembly of the V0 domain which is unable to produce a functional V-ATPase [13, 41]. It is important to note that if any of the thirteen genes that encode the V-ATPase pump are implicated (not including *VMA9*), the pump will not be able to work properly. Thus, if there is a mutation in any single subunit from either domain, the cell will be unable to grow in media whose pH is neutral or basic [42].

5. V-ATPase mechanism of disassembly and assembly

The disassembly of the V-ATPase is a crucial step required for regulating the activity of the pump. This mechanism involves the separation of the V1 and V0 domains and will ultimately inhibit the V-ATPase pump's function *in vivo*. When undergoing disassembly, the C subunit located in the V1 domain, which acts as a bridge between the V1 and V0 domains, will depart from the complex and cause the separation of the two domains [43, 44]. Subsequently, a conformational change in the H subunit inhibits ATP hydrolysis from occurring in the newly separated V1 complex. This occurs so that energy will not be unnecessarily used without the concurrent transportation of protons across the membrane [45–48]. Furthermore, once the domains have disassembled, the passive transport of hydrogen ions across the V0 complex is also prevented [49].

This mechanism is completely reversible however once the C subunit is brought back and re-bridges the V1 and V0 domains [43]. In *Saccharomyces cerevisiae*, re-assembling the V-ATPase pump utilizes a chaperone protein that is specific for V-ATPase pumps [50]. Specifically, this process requires RAVE, or the regulator of ATPases of Vacuoles and Endosome [3]. This is a chaperone complex that includes an adaptor protein, Skp1p and the functional subunits Rav1p and Rav2p [50, 51]. This RAVE chaperone complex helps to stabilize the V1 domain and facilitates in the process of reintroducing the C subunit to bridge V1 to V0 [52, 53]. Once completed, the V-ATPase pump will be fully functional once again.

6. Importance of V-ATPase activity in mammalian cells

The study of vacuolar function in *Saccharomyces cerevisiae*, and particularly the activity of the V-ATPase pump is important since yeast vacuoles are strikingly similar to the lysosomes in mammalian cells [52, 53]. The mammalian lysosome has an internal acidic pH that ranges from 4.2–5.3 and is primarily maintained by V-ATPase pumps that actively transport ions across the membrane to acidify the lysosome [54]. Similar to the acidic pH needed in the vacuole, the maintenance of lysosomal pH is required for the important hydrolytic activities and signaling roles of lysosomal homeostasis. Defects in V-ATPase machineries and functions have been associated with a number of neurodegenerative diseases, especially disorders associated with older age including some forms of Parkinson's disease and Alzheimer's disease [55–57]. In fact, the brain is one of the main organs that is the most significantly impacted by genetic diseases that interrupt normal lysosomal

function in cells. This underlies the importance of normal lysosome function in the central nervous system [54, 58]. Additionally, impairments to proper acidification in lysosomes have been involved with cell aging and longevity. Studies conducted in yeast models have shown that longer lifespans occurred when V-ATPase components were overexpressed [59, 60]. Additionally, there have been over fifty genetic disorders that have been linked to mutated genes that encode specific proteins of the lysosome, many of which are related to the acidification function [61–63].

V-ATPases have been found in the cellular membranes of certain specialized cells in mammals. Impairments in these V-ATPases cause an array of genetic disorders. For example, V-ATPases that are found in intercalated cells of the distal tubule and the collecting ducts of the kidney play a crucial role in maintaining acid–base homeostasis. In humans, defects in the specific genes that encode V-ATPase subunits, such as a mutation in the renal isoform of subunit B or in subunit a, leads to the inherited disease of renal tubule acidosis [64, 65]. Furthermore, V-ATPase pumps have also been found to be located in the cellular membrane of osteoclast cells and are required for the process of bone resorption. As a result, genetic mutations in V-ATPase encoding genes, such as in subunit a, has been associated with osteopetrosis, which causes skeletal abnormalities caused by lack of bone degradation [64, 65]. Furthermore, V-ATPase activity has been linked to nongenetic diseases, such as cancer. The key role that these pumps play in tumor and cancer cell lines will be discussed further in a later section. Given the importance of V-ATPases with regard to diseases, research in yeast vacuoles has been crucial in helping better understand the role that these pumps play in cellular homeostasis.

7. Regulation of the lipid biosynthetic pathway

The relationship between V-ATPases and the lipid biosynthetic pathway is only recently becoming better understood. The lipid biosynthetic pathway is a highly controlled pathway that synthesizes the crucial membrane phospholipids, in addition to other various fats and lipids that are necessary for cell survival [66–68]. The transcription of genes in this pathway is tightly governed by a number of key regulators. One of these primary regulators is inositol, a crucial phospholipid precursor that plays a key role in regulating phospholipid metabolism based on its availability [69–72]. When present in the growth medium, inositol is not required to be synthesized by the cell and thus the genes involved in its production are turned off. If inositol is absent in the growth medium, then the cell will need to produce the crucial phospholipid precursor. Multiple genes in the phospholipid biosynthetic pathway have a UAS_{INO} , or an inositol responsive cis-acting element, that is located in their promoter regions [73, 74]. This region is the binding site for the Ino2p/Ino4p activator which activates the transcription of genes needed to produce inositol. Conversely, when inositol is richly present in the media, the Opi1p repressor will bind to the Ino2p/Ino4p activator and thus repress transcription from occurring [74, 75].

Another crucial regulator of the lipid biosynthetic pathway is the phosphatidate (PA) phosphatase enzyme, Pah1p. Pah1p, which is encoded by the *PAH1* gene, catalyzes the crucial reaction that dephosphorylates PA and leads to the production of diacylglycerol (DAG) and a phosphate group in the lipid biosynthetic pathway [76]. Both PA and DAG are two central players in this pathway and their levels are highly regulated, which in turn regulates the synthesis of triacylglycerides and membrane phospholipids [77]. Depending on the pathway taken, DAG can either be the precursor of the phospholipids phosphatidylcholine and phosphatidylethanolamine or can be converted into triacylglycerol (TAG). PA on the other hand can generate all

four major phospholipids that are found in the membrane of *Saccharomyces cerevisiae*. If it is not dephosphorylated and converted into DAG, then PA can be used to manufacture phosphatidylinositol and phosphatidylserine and can also be used to produce phosphatidylcholine and phosphatidylethanolamine [77].

Since PA and DAG play such critical roles in the lipid biosynthetic pathway, they are both highly regulated by the key PA phosphatase enzyme, Pah1p. While there are other PA phosphatase enzymes, Pah1p is considered to participate more directly in the synthesis of phospholipids and plays a pivotal regulatory role in the pathway [78, 79] and is essential for the *de novo* synthesis of membrane phospholipids and TAG [80, 81]. Pah1p is mainly located in the cytosol where it can be easily translocated onto the endoplasmic reticulum to catalyze the conversion of PA into DAG [82]. However, Pah1p's regulatory role in the lipid biosynthetic pathway extends even further due to the fact that it exerts transcriptional regulation over other genes involved in the manufacturing of phospholipids. As explained earlier, numerous genes in the lipid biosynthetic pathway contain a UAS_{INO} element in their promoter regions which acts as a binding site for the transcriptional activator, Ino2p/Ino4p [83, 84]. The levels of PA actually help control the transcription of UAS_{INO} containing genes, since Opi1p, which is a negative regulator of transcription, is physically connected to PA on the endoplasmic reticulum and nuclear membrane [85]. Thus, when there are greater levels of PA, the Opi1p repressor will continue to remain tethered to PA on the ER/nuclear membrane which will prevent it from being able to cross into the nucleus and prevent the transcription of UAS_{INO} containing genes [86]. Conversely, when PA levels are decreased, Opi1p is free to cross the nuclear membrane and bind to the Ino2p/Ino4p activator complex on the UAS_{INO} and block transcription. Since Pah1p regulates PA levels by catalyzing the reaction to turn PA into DAG, it indirectly impacts the transcription of other lipid biosynthetic genes. Therefore, higher concentrations of Pah1p will lead to less PA which represses transcription of genes with a UAS_{INO}, while lower concentrations of Pah1p leads to increased amounts of PA and activates transcription of genes with a UAS_{INO} [86]. However, additional research has uncovered that Pah1p actually plays a more direct role in the regulation of lipid biosynthetic genes as well. Studies have found that Pah1p is located in the nucleus as well so that it can directly act as a repressor for UAS_{INO} genes in the pathway [83]. In fact, it can directly bind to the promoter of UAS_{INO} containing genes and physically block gene expression. It is therefore not surprising that studies looking into the impact of deleting the *PAH1* gene show that there is an upregulation of gene expression of UAS_{INO} containing genes [87].

In addition to its regulatory role, deletion experiments have shown just how pivotal Pah1p is to overall cell homeostasis. Mutants that lack the *PAH1* gene have much higher levels of PA present in the cell. Additionally, these cells contain much lower levels of TAG due to the loss of the Pah1p phosphatase activity and the conversion of PA into DAG, which is the precursor of TAG. Furthermore, there is an abnormal expansion of the nuclear and endoplasmic reticulum membrane as well as increased levels of phospholipids, sterol esters and fatty acids in cell lines lacking *PAH1* [88, 89]. This is because, the gene expression of UAS_{INO} genes in the lipid biosynthetic pathway is upregulated. This is due to the lack of Pah1p which causes a derepression of genes that are typically repressed in the presence of Pah1p in the CDP-DAG pathway and Kennedy pathways, both of which lead to the synthesis of membrane phospholipids [87]. These mutant strains also experience fatty acid toxicity due to the higher levels of lipids present [89]. *PAH1* has also been shown to be needed for lipid droplet formation, which is dependent on the presence of DAG. Thus, in the absence of *PAH1* and the resulting lower concentrations of DAG, there is a decrease in the concentration of lipid droplets in these cell [90]. Furthermore,

cells that are missing *PAH1* are not able to grow in the presence of non-fermentable carbon and are also temperature sensitive [91]. Most notably for this review article, *PAH1* has been shown to be important for vacuole morphology and function. Given the importance of V-ATPase activity in overall cell homeostasis, the link between Pah1p and vacuole function is important to understand and will now be explored in the upcoming sections.

8. The role of *PAH1* in vacuolar morphology and function

Interestingly, studies have revealed the importance of the *PAH1* gene in vacuolar homeostasis and morphology. Research has shown that cells lacking *PAH1* have morphologically defective vacuoles that remain interminably fragmented [92, 93]. While vacuoles typically undergo fragmentation in *Saccharomyces cerevisiae* during the budding process so that the new daughter cell can obtain and inherit these organelles, the vacuoles do not remain fragmented indefinitely [94]. This normal mechanism of fragmentation begins when the budding process is initiated and the vacuole in the mother cell fragments into a series of mini vacuoles. This is followed by the development of an elongated-vesicular structure by the mini vacuoles, which guides the structures to the newly budding yeast cell [94]. Once these mini fragmented vacuoles are inherited and the newly budding cell grows, the collection of vacuoles fuse together to recreate a single large vacuole in both the mother cell and daughter cell. The two cells are separated once septa are formed and this cycle can be repeated as more daughter cells are made [95]. However, cells that lack *PAH1* are unable to keep their vacuoles unfragmented and whole [92, 93]. Research studies have found that this is due to the fusion machinery having been implicated when *PAH1* is deleted and thus prevents the vacuoles from fusing back together. Without the enzymatic phosphatase activity that exists when *PAH1* is present, the SNARES are unable to bind to Sec18p, which is the protein required to prime the SNARE complex for the fusion mechanism. Furthermore, the deletion of *PAH1* also causes a number of other key fusion machinery components to be absent. This includes Vps39p, which is a component of the homotypic fusion and vacuole protein sorting (HOPS) tethering complex. Additionally, phosphatidylinositol 3-phosphate, a lipid that is needed for SNARE function and fusion, is also absent [92]. Moreover, Pah1p is also needed to recruit, Vps34p, which is the phosphatidylinositol 3-kinase needed for vacuolar fusion [92]. As such, vacuoles in cells that lack *PAH1* remain fragmented without ever fusing into a single vacuole.

Since irregularities in vacuole morphology have been linked to implications in V-ATPase pump activity, there is reason to suspect a relationship between Pah1p and V-ATPase activity given the important role that Pah1p plays in vacuolar morphology [93, 94]. On the other hand, reports have found that vacuoles with V-ATPase pumps that have a decrease in their level of acidifying the vacuole can actually lead to increased fusion of the vacuoles. One particular study found that vacuoles that experience deacidification often have an increased level of fusion whereas mutant vacuoles with internal pHs that have maintained its acidity may have fusion impeded [96]. Thus, the uncertainty as to whether the role of Pah1p and its impact on the morphological structure of vacuoles was related to proper V-ATPase pump function remained.

Given the fact that Pah1p regulates genes in the lipid biosynthetic pathway, as well as helps play a critical role in the maintenance of proper vacuole morphology and function, additional studies were aimed to look at whether Pah1p plays a role in regulating the genes that encode for the V-ATPase pump and its impact on pump activity. Growth experiments have shown that cells lines lacking *PAH1* actually grew

better in neutral environments compared to wildtype. Since neutral environments can impact cytoplasmic and vacuolar pH, a properly functioning V-ATPase pump is required to ensure that pH homeostasis is maintained in the vacuole and the entire cell, indicating that *pah1Δ* cells did not negatively impact V-ATPase activity even though the morphological structure is affected. This was proven with subsequent experimentation that measured vacuolar pH and actually showed that *pah1Δ* cells were even better than wildtype cells at acidifying their vacuoles, with an average internal vacuolar pH of 5.89 in their vacuoles compared to pH 6.0 in wildtype. Therefore, while Pah1p does cause morphological disturbances to the vacuole it does not adversely impact V-ATPase pump activity. This however is not a contradiction since cell lines that have mutations that cause abnormalities in vacuole morphology and fragmentation can either have fully functioning V-ATPase pumps or conversely pumps that have defects in their assembly [97, 98].

Interestingly, other research studies have shown that V-ATPase function can adversely impact the fusion of vacuoles *in vivo* [96]. Therefore, it is likely that the increased acidity in the *pah1Δ* cells contribute to the fragmentation of the vacuole seen in these cell lines. This led to the discovery that perhaps Pah1p plays a regulatory role over V-ATPase genes as well, since pump activity was upregulated in the *pah1Δ* strain. RNA analysis experimentation showed that 11 of the 13 vacuolar membrane ATPase genes were upregulated in the V-ATPase pump, including *VMA3*, *VMA6* and *VMA16* which are all involved in the transport of hydrogen ions into the vacuole [93]. This indicates a potential role of Pah1p acting as a repressor for these genes since they are seemingly negatively regulated in the presence of Pah1p. Many of the 11 genes that were impacted by the deletion of *PAH1* contain a *UAS_{INO}*, which as mentioned earlier are types of genes that Pah1p negatively regulates in the lipid biosynthetic pathway. The *VMA* genes that possess a *UAS_{INO}* in their promoters include *VMA1*, *VMA5*, *VMA8*, *VMA13* and *VMA16* [93]. There is therefore a molecular relationship between *PAH1* and the genes that encode V-ATPases since it appears that Pah1p can directly negatively impact these genes by binding to their promoters.

9. V-ATPases and glucose metabolism

One important process that V-ATPase pumps have been found to be associated with is glucose metabolism. In fact, research has shown that V-ATPase pump activity is actually regulated by glucose. In both yeast and mammalian cells, a key inducer of the V-ATPase pump disassembly is glucose depletion [20, 43, 99]. When glucose is scarce, the V1 and V0 domains will disassemble and pump activity is unable to occur. Conversely, an increase in glucose levels, which triggers the activation of glycolysis, will lead to the reassembly of the V-ATPase pump and lead to an increase in pump activity. This has been found to occur in both yeast cells [8, 43] and mammalian cells [99, 100]. This process is extremely significant since during times of glucose depletion the cell aims to preserve energy. By disassembling the V-ATPase pump, it prevents unnecessary usage of ATP. Furthermore, in times of glucose abundance, the reassembling of the pump allows for a functioning V-ATPase that can lower the additional acidification of the cytosol that occurs during an uptick in glycolysis [1, 2, 25]. Research has shown that this cycle of the V-ATPase pump being disassembled and reassembled is proportional to the concentration of glucose available in the cell. This is important because it indicates that V-ATPase pumps are highly attuned to glucose metabolism and energy levels [8, 43, 101, 102].

In yeast, the highly characterized glucose sensing signaling mechanism which regulates the assembly of the V-ATPase pump is the Ras/cAMP/Protein Kinase A

(PKA) pathway [3, 103]. The GTP coupled protein, Ras, is inhibited by two GTPase activating proteins named Ira1p and Ira2p. When glucose is present, Ira1p and Ira2p are inhibited and Ras can stimulate the production of cAMP via adenylate cyclase. Once cAMP levels are high enough, the PKA regulatory subunit will dissociate and be free to trigger PKA's kinase activity. Additionally, studies have indicated that the assembly of the V-ATPase takes place as a result of acidification of the cytosol due to high levels of glycolysis and that this leads to changes in PKA [104, 105]. The presence of glucose, after a period of depletion, will activate PKA and thereby stimulate the assembly of the V-ATPase pump. This mechanism seemingly creates a positive feedback loop, since the heightened assembly of V-ATPase pumps aids in the maintenance of the pH in the cytosol and can stimulate PKA signaling. This in turn will cause an upregulation in glycolysis which further boosts the assembly of V-ATPase pumps and helps facilitate the switch from respiratory to fermentative growth [3, 25].

10. V-ATPase and cancer

As mentioned earlier, V-ATPase pump activity and function has been associated with various diseases. However, one such non genetic disease that these pumps have been linked to is cancer [106]. Normal V-ATPase pump functioning is crucial for various signaling pathways in the cell. In fact, many of the pathways that lose their homeostatic control during cancer require V-ATPase pumps for proper functioning, such as the Notch signaling pathway and the Wnt/ β -catenin signaling pathway [106–108]. Moreover, proper pump activity is required for the activation of the mechanistic target of rapamycin complex, or mTORC1, which is a common pathway implicated in cancer [109–113]. However, studies have shown more direct roles of V-ATPase pumps in cancer. For example, when V-ATPase pumps are inhibited in human tumor cells, results showed programmed cell death is induced [114–119]. These findings indicate that cancer cells rely on V-ATPase activity much more than noncancerous cells do in order to remain viable [115, 116, 119]. This is because cancerous cells have been shown to generate increased levels of acidity [120]. Since V-ATPase pumps play the critical role of removing acids out of the cell and help to preserve a neutral pH in the cytosol, cancer cells heavily rely upon V-ATPases to promote the alkalinization of their cytosol and to pump protons into the extracellular space [1, 2, 121]. It has been hypothesized that expression of these pumps may upregulate or may be specifically localized to the cellular membrane by expressing certain isoforms that target the V-ATPases to the plasma membrane [64, 65, 122, 123]. In this way, tumors are thus able to dodge programmed cell death and can proliferate [106]. In fact, V-ATPase pumps have been found in the plasma membrane of numerous invasive cancer cell lines while not in non invasive ones [124]. For example, an invasive line of breast tumor cells revealed higher levels of the V-ATPase pump on the cell surface compared to noninvasive breast cancer cells [124]. As explained above, these pumps aid in the maintenance of cytosolic pH. The impact of V-ATPases has also been observed in various other invasive cancerous cell lines, including liver cells, esophageal cells, ovarian cells, lung cells, prostate cells and pancreas cells amongst others [106, 110, 111, 125–129]. Research has shown that inhibiting V-ATPase pumps disrupts the pH balance and causes a more acidic pH in the cell which leads to higher levels of apoptosis [130].

Furthermore, V-ATPase pumps are not only important for regulating the pH of the cytosol, but for regulating the pH of other organelles as well [106]. Implications of protein production and shortages in nutrients can lead to ER stress in cancer cells. As a result, lysosomes are activated by the Bax inhibitor-1 in response to the

ER stress in order to raise protein turnover [131]. Studies have shown that blocking ATPase pump activity barred this from occurring and ultimately led to cell death [131]. Moreover, V-ATPases in the cell membrane have also been proposed to play a crucial role in the migration and invasion of cancer cells. Studies have shown that when V-ATPase pumps were inhibited in invasive breast cancer cells, the migration and invasiveness of these cells was diminished [1, 2, 124]. This was further proven in other cancer lines as well, such as cancerous pancreas cells [125]. Thus, the relationship between V-ATPases and cancer, and subsequently its link to apoptosis, has been an active area of research. However, the role of V-ATPases in yeast and its connection to apoptosis has not been as well characterized compared to that of human cell lines. This review will now look at a possible mechanism by which V-ATPase activity can be linked to apoptosis via its regulation by key players of the lipid biosynthetic pathway.

11. V-ATPase genes and the lipid biosynthetic pathway

As explained earlier, inositol is one of the key phospholipid precursors whose presence is essential in regulating phospholipid metabolism [69–72]. When present in the growth medium, inositol production is repressed. If inositol is absent in the growth medium, then the genes involved in its production are upregulated. Research has shown that cells that lack one or more of the vacuolar membrane ATPase genes exhibited defects in growth when cultured in media without inositol [132, 133]. However, these defects were able to be reversed if *OPI1*, the gene that encodes the Opi1p repressor, was deleted [133]. Additionally, cells that lack one or more V-ATPase genes have higher buildups of oxidant molecules which may indicate their relationship to protecting the cell and their role in preventing cell death.

Recent studies have shown that one of the key vacuolar membrane ATPase genes, *VMA3*, plays a significant role in regulating the synthesis of phospholipids. Growth experiments that were performed with cell lines lacking the *VMA3* gene exhibited a growth defect that had cells growing much slower when cultured without inositol in their media compared to wildtype cells [134]. *VMA3*'s role in *de novo* phospholipid production was further clarified when mRNA studies showed *HXK2*, another important gene in the phospholipid biosynthetic pathway required for inositol production, had significantly lower mRNA levels in the cells lacking *VMA3* compared to wildtype. Thus, *VMA3* was revealed to impact the phospholipid biosynthetic pathway by specifically regulating the transcription of the *HXK2* gene [134]. This finding was particularly interesting since other studies have shown that cells lacking *HXK2* exhibit a growth sensitivity to acetic acid [135], which is a growth condition that has long been used to screen for apoptosis. Earlier studies have hypothesized that *HXK2* plays a role in shielding cells from programmed cell death since cells that have had the *HXK2* gene deleted had an accrual of activated Ras by the mitochondria [135]. Thus, further experimentation from this study showed that *vma3Δ* cells grew significantly slower in the presence of acetic acid compared to wildtype and were even more sensitive to this apoptotic inducing agent when grown without inositol present. Taken together, these findings indicate that the deletion of *VMA3* leads to decreased transcription of the *HXK2* gene, which ultimately leads to cells being more sensitive to acetic acid. This therefore demonstrates that *VMA3* plays an important regulatory role in apoptosis [134].

HXK2 plays an important role in glucose metabolism and encodes the hexokinase-2 enzyme that catalyzes the reaction which converts glucose into glucose-6-phosphate. Thus, *HXK2* is regulated by the presence of glucose. Studies have shown that *HXK2* is regulated by two important transcription factors, Rgt1 and

Med8, that cause the deregulation of *HXK2* when glucose is not present [136]. The findings by Konarzewska *et al.* have now also shown that *HXK2* is also regulated by Vma3p. Thus, there is a clear relationship between V-ATPases and the phospholipid biosynthetic pathway since Vma3p has been shown to upregulate the *HXK2* gene. Furthermore, it has been shown that by regulating the *HXK2* gene, *VMA3* has an important protective role when it comes to acetic acid induced apoptosis. This is therefore an important link between how V-ATPases relate to the lipid biosynthetic pathway.

12. A working model

As first mentioned in a 2017 article by Konarzewska *et al.*, a new model is now being hypothesized as to how Pah1p impacts apoptosis by regulating the V-ATPase *VMA3* gene, which in turn has a regulatory role over *HXK2* (**Figure 2**). This model starts with the availability of glucose that leads to a cytosolic pH, which creates a more alkaline internal environment, and will also activate protein kinase A [25]. This in turn causes the assembly of V-ATPase machinery and the formation of a functional V-ATPase pump. During this time, PA levels remain high while Pah1p's concentration is low. When PA is abundant due to lower Pah1p levels, it will follow the CDP-DAG pathway and create membrane phospholipids. Since V-ATPase assembly is being promoted, the *VMA* genes that encode these subunits are upregulated. Importantly, one of *VMA* genes yields Vma3p which is a key player in this model. Additionally, the lipid Phosphatidylinositol 3,5-bisphosphate can also help in the activation of the V-ATPase pump and as more V-ATPase pumps assemble and accumulate the alkaline cytosolic pH will be upheld [137]. Protein kinase A signaling can be stimulated by the newly assembled V-ATPase pumps, which leads to a positive feedback loop and increases the numbers of V-ATPase pumps being assembled. Additionally, there will be an upregulation of glycolysis which will lead to an abrupt changeover to fermentative growth. Regardless of whether the Ras/cAMP/PKA pathway is upstream or downstream of the V-ATPase pump assembly pathway [104, 134], Ras will be largely found by the cell membrane and inside the nucleus. Furthermore, the *HXK2* gene will exhibit maximal expression due to the high levels of glucose and the gene product Hxk2p will catalyze the reaction to convert glucose into Glucose-6-phosphate. Given the role of Hxk2p in apoptosis, the cell will have a greater resistance against acetic-acid induced apoptosis.

Conversely, when glucose is not abundantly present, the machinery for the V-ATPase pumps will undergo disassembly due to the less alkaline cytosolic environment. The *PAH1* gene will be upregulated and thus result in increased production of the active Pah1p which can execute its phosphatase activity on PA. This in turn will cause PA to be led along the pathway that converts it to DAG and ultimately TAG, thus minimizing the production of membrane phospholipids from PA. Furthermore, the increased amounts of Pah1p will repress most vacuolar membrane ATPase gene expression which also prevents further V-ATPase assembly and enhances its disassembly. Being that one of the *VMA* genes that Pah1p represses is *VMA3*, there will be less Vma3p available. Vma3p's regulation over *HXK2* leads to a decrease in Hxk2p. During this time, Ras will build up in the internal membranes and mitochondria which can ultimately cause dysfunction of the mitochondria and amplified ROS production [138]. This will therefore make cells more susceptible to acetic acid induced programmed cell death. Thus, Pah1p likely plays a major role in this apoptotic pathway given its regulatory role over Vma3p.

Figure 2. Proposed model first hypothesized in [134] for Pah1p as an apoptotic regulator via the regulation of Vma3p, which subsequently regulates Hxk2p. When glucose is available, V-ATPase pump assembly occurs due to the activation of PKA and cytosolic alkalinization as a result of glycolysis. Concurrently, Pah1p levels are decreased, which promotes the CDP-DAG pathway and PA being converted to membrane phospholipids. Vma3p, amongst other VMA genes, is upregulated due to lack of Pah1p and the increased levels of pump assembly uphold the cytosolic alkalinization. Furthermore, this leads to increased Hxk2p expression which results in the resistance of acetic acid induced apoptosis. Conversely, if glucose is not available then V-ATPase pumps undergo disassembly. Pah1p levels are high, which catalyzes the reaction of PA conversion to DAG which ultimately increases TAG levels. Furthermore, there is downregulation of VMA3, along with other VMA genes. This leads to decreased pump assembly, low levels of Hxk2p and an increase of Ras in the mitochondria and membranes. Ultimately, acetic acid induced apoptosis is promoted under these conditions [134].

13. Conclusion

Overall, this review shows the connection between the V-ATPase pump and the lipid biosynthetic pathway PA phosphatase regulator, Pah1p. Based on the regulatory role that Pah1p has over the VMA genes and specifically the VMA3 gene, a new model has emerged regarding how it can possibly influence acetic acid induced apoptosis through the regulation of V-ATPase genes. While more research studies will be needed to confirm this model, these studies have indicated how Vma3p can act as potential anti apoptotic factor in *Saccharomyces cerevisiae*. These findings thus

highlight the importance Pah1p on vacuolar function and on cell induced apoptosis via Vma3p.

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