The Importance of Vacuolar Ion Homeostasis and Trafficking in Hyphal Development and Virulence in *Candida albicans*

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The vacuole of *Candida albicans* plays a significant role in many processes including homeostasis control, cellular trafficking, dimorphic switching, and stress tolerance. Thus, understanding the factors affecting vacuole function is important for the identification of new drug targets needed in response to the world’s increasing levels of invasive infections and the growing issue of fungal drug resistance. Past studies have shown that vacuolar proton-translocating ATPases (V-ATPases) play a central role in pH homeostasis and filamentation. Vacuolar protein sorting components (VPS) regulate V-ATPases assembly and at the same time affect hyphal development. As well, vacuolar calcium exchange systems like Yvc1 and Pmc1 maintain cytosolic calcium levels while being affected by V-ATPases function. All these proteins play a role in the virulence and pathogenesis of *C. albicans*. This review highlights the relationships among V-ATPases, VPS, and vacuolar calcium exchange proteins while summarizing their importance in *C. albicans* infections.

Keywords: *Candida albicans*, vacuolar proton-translocating ATPases, vacuolar protein sorting components, vacuolar Ca²⁺ channel, virulence

INTRODUCTION

*Candida albicans* is an opportunistic fungal pathogen generating a high rate of mortality in systemic infections (Jenks et al., 2020). Due to *C. albicans*’ growing resistance to antifungal drugs, there is a great need to further study the pathways affecting its pathogenesis and virulence in order to discover new potential drug targets (Berman and Krysan, 2020). Vacuoles occupy 10–20% of the yeast cell’s volume and are involved in several cellular functions including ion homeostasis, stress response, cell differentiation, and adaptation to new environments (Armstrong, 2010). Thus, vacuolar function changes can have profound effects on the virulence of *C. albicans*, and targeting the vacuolar function of *C. albicans* may provide a new strategy for the development of antifungal drugs (Olsen, 2014).
C. albicans V-ATPases’ DISRUPTION IMPAIRS VACUOLAR ACIDIFICATION AND VIRULENCE

pH is a key consideration for pathogenic yeasts like C. albicans as it affects their virulence and dimorphic switching. pH homeostasis is not only required for sensing and responding to ambient pH, but also generating and transducing signals for secreting virulence factors (Patenaude et al., 2013; Du and Huang, 2016). The vacuolar pH is especially important for pathogenesis because vacuoles play a key role in cellular trafficking, and the defects in endosomal trafficking can affect the expression of adhesion and invasion membrane proteins (Kulkarny et al., 2014; Kim et al., 2019).

Maintaining vacuolar pH through acidification is the major role of the proton pumps called vacuolar proton-translocating ATPases (V-ATPases), which transport H+ from the cytoplasm into the vacuole (Parra et al., 2014). They contain a peripheral membrane subcomplex V₁ and an integral membrane subcomplex V₀ (Kane, 2007). The subcomplex V₁ consists of subunits A, B, C, D, E, F, G, H, which are encoded by the genes TFP1, VMA2, VMA5, VMA8, VMA4, VMA7, VMA10, and VMA13, respectively. The subcomplex V₀ includes subunits a, c, c', c″, d, e, which are encoded by genes VPH1/STV1, VMA3, VMA11, VMA16, VMA6, and C1_10750C_A (Table 1). For both the non-pathogenic model yeast S. cerevisiae and the pathogenic C. albicans, all subunits are encoded by single genes, except for the subunit a in V₀ which is encoded by the paralogs VPH1 and STV1 (Patenaude et al., 2013). The phenotypes of each subunit disruption mutant are summarized in Table 1.

Studies have shown that the structure of the V-ATPases in vacuoles plays an important role in pH balance and ion homeostasis (Veses et al., 2008). Factors affecting V-ATPase assembly and cellular trafficking also have strong influences on calcium ion homeostasis. All these functions are required for virulence and pathogenesis in C. albicans. The deletion of any one of the genes encoding subunits of the V-ATPases creates a Vma12 deficient (Vma12, vacuolar membrane ATPase activity) phenotype. Vma12 S. cerevisiae and C. albicans demonstrate similar functional patterns, showing increased sensitivity to high pH, heavy metal ions, and antifungal drugs (Kane, 2007). S. cerevisiae cells with the Vma12 phenotype also show slower growth compared to wild-type cells even at pH 5 and have defects in sporulation and germination (Kane, 2006).

The case is similar for C. albicans; several studies have established the necessity of V-ATPases subunits in maintaining vacuolar pH and virulence. The vma4 and vma10 null mutants of C. albicans both show non-acidic compartments and attenuated virulence. Protease secretion is also defective in the null mutants, and this compromises their ability in host cell degradation and in immune evasion (Kim et al., 2019). When VMA2 expression is repressed, vacuolar acidification is inhibited causing abnormal vacuolar morphology, and autophagy is delayed as visualized by monitoring Ape1-GFP localization. The mutant shows the Vma12 growth phenotype and is avirulent in the C. elegans infection model (Rane et al., 2014a). The vma5 and vma7 null mutants of C. albicans are found to have the same defects in vacuolar acidification and are avirulent in a mouse model of systemic candidiasis (Poltermann et al., 2005; Zhang et al., 2017). C. albicans VMA3 is found to be functionally similar to S. cerevisiae VMA3 and, when its expression is disrupted, results in the loss of V-ATPase activity and vacuolar acidity. In addition, loss of VMA3 results in significantly attenuated macrophage killing (Rane et al., 2013). The deletion of Tfp1, the putative C. albicans homologue of S. cerevisiae Vma1, can cause a defect in vacuolar acidification and strongly reduces virulence (Jia et al., 2014). VPH2 encodes the homologue of Vma12, which is one of the V-ATPases assembly factors.

| Subcomplexes of V-ATPase | Subunits | Encoding genes | Phenotypes of null mutant |
|-------------------------|----------|----------------|---------------------------|
|                         |          |                | Vacular acidification | Hypal development | Virulence in systematic infection | References |
| V₁                     | A        | TFP1           | Decreased                | Locked in yeast   | Avirulent                       | Ja et al., 2014 |
|                         | B        | VMA2           | Decreased                | Locked in yeast   | Avirulent                       | Rane et al., 2014a |
|                         | C        | VMA5           | Decreased                | Locked in yeast   | Avirulent                       | Zhang et al., 2017 |
|                         | D        | VMA8           | –                        | –                | Avirulent                       | Kim et al., 2019 |
|                         | E        | VMA4           | Decreased                | Locked in yeast   | Avirulent                       | Poltermann et al., 2005 |
|                         | F        | VMA7           | Decreased                | Partial defect    | Avirulent                       |                  |
|                         | G        | VMA10          | Decreased                | Locked in yeast   | Avirulent                       | Kim et al., 2019 |
|                         | H        | VMA13          | –                        | –                | –                              |                  |
| V₂                     | a        | VPH1           | Decreased                | Partial defect    | Avirulent                       | Kane, 2006 |
|                         | c        | VMA3           | Uchanged                 | Partial defect    | Virulent                        | Kane, 2006 |
|                         | c'       | VMA11          | –                        | Locked in yeast   | –                              | Rane et al., 2014b |
|                         | c″       | VMA16          | –                        | –                | –                              |                  |
|                         | d        | VMA6           | Decreased                | Locked in yeast   | Avirulent                       | Jia et al., 2018b |
|                         | e        | C1_10750C_A    | Decreased                | –                | –                              |                  |

References:
Jia et al., 2018b
Kane, 2006
Kane, 2007
Kim et al., 2019
Kim et al., 2014
Parra et al., 2014
Patenaude et al., 2013
Rane et al., 2014a
Rane et al., 2014b
Rane et al., 2013
Poltermann et al., 2005
Zhang et al., 2017
Jia et al., 2014
Kulkarny et al., 2014
Du and Huang, 2016
Kane, 2006
and VMA6 encodes subunit d required for V1 domain assembly. Disruption of either of these two genes elevates vacuolar pH and weakens the virulence of *C. albicans* (Jia et al., 2018b).

There is a different case for the VPH1 and STV1 genes, as they both encode for a subunit of V0. Both the VPH1 and STV1 genes need to be deleted to show a full Vma- phenotype (Kane, 2006). However, the vph1 null mutant is unable to acidify vacuolar compartments and is avirulent, while the stv1 null mutants can have their functions compensated by Vph1 and are shown to be virulent. This study shows that Vph1 plays a more important role in maintaining virulence for *C. albicans* than Stv1, although there is functional redundancy between the two isoforms that makes the effects of losing either one of them less significant than a regular Vma- phenotype (Patenaude et al., 2013).

As well as the genes directly coding for the components of V-ATPase, V-ATPase also require ergosterol to function properly. Erg mutants with disruptions in the last step of ergosterol synthesis also show a Vma- phenotype with an inability to grow in alkaline medium and failure to acidify the vacuole (Zhang and Rao, 2010). This suggests that ergosterol is necessary for V-ATPase activity. Other lipids may play a role in controlling V-ATPase activity as well. Sphingolipids with a C26 acyl group are critical for the activity of V-ATPase (Chung et al., 2003), and deletion of either Sur4 and Fen1, which are critical for sphingolipid biosynthesis, results in a milder version of the Vma- phenotype (Kane, 2006).

The deletion of genes coding vacuolar protein sorting components (VPS) like Vps28 and Vps32 also give rise to similar phenotypes to Vma- with enhanced sensitivity to alkaline pH and weakened virulence (Cornet et al., 2005). The null mutants of a subset of VPS genes like VPS34 or VPS15 abolish the uptake of quinacrine into the vacuole and lead to increased sensitivity to high pH with reduced V-ATPase activity due to a vacuolar acidification defect (Sambade et al., 2005). Certain VPS proteins like Vps34 are found to directly interact with Vma7 and may control the assembly of V-ATPase, so the vps34 null mutant has the same phenotypes as the vma7 null mutant in terms of vacuolar acidification and lower virulence (Poltermann et al., 2005).

Overall, the Vma- phenotype highlights the vacuole's role in maintaining ion homeostasis, and morphological transformation can also be impaired when V-ATPases or vacuolar trafficking pathways are defective.

**HYPHAL GROWTH DEFECTS AND CELL WALL CHANGES THROUGH V-ATPases INACTIVATION**

*C. albicans* are more capable of blocking phagosomal maturation and acidification when they have normal filamentation, and *C. albicans* invasion into oral and gastrointestinal tract epithelia involve hyphal form cells (Zhang and Rao, 2010), so filamentation could be used as a trait to assess virulence. The loss of hyphal growth can have several different vacuole-related genetic causes (Chen et al., 2020). Vma- mutants exhibit different degrees of defects in hyphal development. The vma3 and vma7 null mutants have essentially no filamentous growth in liquid Spider medium while filaments can be induced from wild-type cells. The deletion of TFPL, VPH2, or VMA6 also gives dramatic attenuation of *C. albicans* filamentous growth (Jia et al., 2014). In addition, the vph1 mutant shows deficiencies in hyphal formation while the stv1 null mutant has more normal filamentation. Interestingly, different hyphal development defects correspond with the inability to acidify vacuoles in the vph1 mutant but to lesser extent in the stv1 mutant. A link between vacuolar pH and hyphal formation is thus evident, and, as antifungal drugs that disrupt vacuolar pH also block hyphal growth, this suggests the V-ATPases may assist the signaling that induces hyphal formation (Patenaude et al., 2013).

In addition, the decreased activity of V-ATPase may influence cell wall synthesis through a reduction in the transport of secretory vesicles (Marshansky and Futai, 2008). For instance, vph2 or vma6 null mutants are hypersensitive to cell wall stresses and their cell wall composition changes significantly; the mutants contain more chitin and less β-1,3-glucan and phosphomannan (Jia et al., 2018b).

**HYPHAL DEVELOPMENT DEFECTS CAUSED BY DISRUPTION OF VACUOLAR TRAFFICKING GENES**

Hyphal formation in *C. albicans* is not only related to the vacuolar pH regulated by V-ATPases, but also related to vacuolar trafficking. Vacuolar trafficking involves the exchange of substances or vesicles between the vacuole and the endoplasmic reticulum, Golgi, mitochondria and other organelles, and is essential for maintaining the virulence of *C. albicans* (Bianchi et al., 2019). In *S. cerevisiae*, Vps21 was found to mediate vacuolar trafficking via an endosomal route, and a vps21 deletion in *C. albicans* causes a mild reduction in hyphal growth and virulence. Although the null mutant of aps3 alone does not produce an avirulent strain with a loss of filamentation, loss of function for both VPS21 and APS3 shows synthetic effects, generating pseudohyphae without vacuolated compartments and causing a significant decrease in virulence. This suggests that VPS21 and APS3 mediate vacuolar trafficking through distinct pathways and that the APS3 pathway is more significant when endosomal trafficking is disrupted (Palmer, 2010). The vps34 null mutant also has faulty vacuolar trafficking, with enlarged vacuoles and significantly less hyphal growth (Bruckmann et al., 2000). The vps11 null mutant has defects in filamentation and secreting proteases, and is completely unable to kill macrophages, resulting in a decrease in virulence (Palmer et al., 2003, 2005). Disruption of VPS1 by a regulatable tetracycline promoter produces defective filamentation and markedly reduced biofilm formation (Bernardo et al., 2008). It appears that disruption of vacuolar trafficking prevents vacuolation, compromises the regulation of turgor pressure that helps to provide a force for directional hyphal elongation, and prevents necessary factors like V-ATPase subunits from localizing in the vacuole. All these could be reasons why...
Vacuole Function on Virulence

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The vacuole is the site for calcium storage to maintain the optimum intracellular calcium level. The major vacuolar importer and exporters are the Ca\(^{2+}\) pump Pmc1 and the Ca\(^{2+}/H^+\) exchanger Vcx1, which are all vital to Ca\(^{2+}\) homeostasis (Cunningham, 2011).

Upon a hypotonic shock, vacuolar Yvc1 releases Ca\(^{2+}\) into the cytosol. A study on Yvc1’s importance for \textit{C. albicans} shows that the \textit{yvc1} null mutant has a much weaker calcium pulse under alkaline pH or hypertonic shock, and a second fluctuation, where Yvc1 releases vacuolar calcium in response to the stimuli to increase cytosolic calcium levels, is reduced. Yvc1 thus plays a part in mediating the increase of cytoplasmic calcium levels after external stimuli.

In addition, the \textit{yvc1} null mutant shows a reduction in hyphal development, producing mainly pseudohyphae, and has defects in biofilm development and hyphal polarized growth. Vcx1 has a role in activating expression of hypha-specific genes during hyphal growth, and the virulence of \textit{C. albicans} without Yvc1 is highly attenuated in a mouse model of systemic infection. Also, the damage ability of the \textit{yvc1} null mutant is significantly decreased compared to WT during invasion of human epithelial cells.

Yvc1 mediates stress resistance after stimulation by controlling cytoplasmic calcium levels and the subsequent activation of calcium signaling pathways, and it has a role in hyphal growth and re-orientation to host cells. These observations suggest why this putative vacuolar Ca\(^{2+}\) channel has an important part in maintaining the virulence of \textit{C. albicans} (Yu et al., 2014).

Pmc1 and Vcx1 sequester Ca\(^{2+}\) ions into the vacuole (Cunningham, 2011). The \textit{C. albicans} \textit{pmc1} null mutant was severely impaired when CaCl\(_2\) concentrations are high, while the \textit{vxc1} null mutant is unaffected. This suggests a significant role of Pmc1 in calcium homeostasis and stress tolerance. Also, the loss of \textit{PMC1} impairs the cell’s ability to form hyphae, and this negatively affects the \textit{pmc1} null mutant’s biofilm development, which is both related to the high calcium concentration caused by the loss of calcium detoxification performed by Pmc1. Furthermore, the \textit{pmc1} null mutant is avirulent in a mouse model of disseminated infection, while the \textit{vxc1} null mutant shows no difference compared to wild type in these aspects. Pmc1, with its calcium mediation function, has proved to be essential for the pathogenicity and virulence of \textit{C. albicans} (Luna-Tapia et al., 2019).

Iron homeostasis has been found to be critical for the regulation of commensalism and pathogenicity of \textit{C. albicans} (Noble et al., 2017; Tripathi et al., 2020). Because they maintain the major iron pools in fungi, mitochondria and vacuoles play central roles in modulating intracellular iron homeostasis. The major iron importer Ccc1 and exporter Smf3 are confirmed to regulate both cellular iron levels and hyphal development in \textit{C. albicans}. However, the hyphal development and virulence deficiencies caused by \textit{CCC1} and \textit{SMF3} knockouts are not as significant as those caused by the disruption of the mitochondrial iron transporter \textit{MRS4}. In addition, \textit{CCC1} disruption could rescue the filamentous development and virulence in the \textit{mrs4\Delta/\Delta} mutant, which suggests an opposing influence of \textit{MRS4} and \textit{Ccc1} on iron homeostasis (Xu et al., 2014).

![V-ATPases SUBUNITS AND ASSEMBLY FACTORS MAINTAIN VACUOLAR CALCIUM HOMEOSTASIS](image-url)

V-ATPases SUBUNITS AND ASSEMBLY FACTORS MAINTAIN VACUOLAR CALCIUM HOMEOSTASIS

Vacuolar calcium channels have been found to be affected by other regulators, especially those related to V-ATPases. For example, the absence of the assembly factor Vph2 of the V-ATPase and the loss of Tfp1, the subunit a of the V, domain, causes abnormal localization of Yvc1 and leads to the disruption of...
calcium transport from the vacuoles to the cytosol (Peng et al., 2020). The vph2 null mutant has attenuated pathogenicity (Jia et al., 2018b), and the tfp1 null mutant has significantly increased cytosolic calcium levels, indicating its importance in ion homeostasis (Jia et al., 2015). The tfp1 pmc1 double mutant has increased disruption in calcium homeostasis compared to the pmc1 null mutant alone. The vph2 or vma6 null mutants give rise to abnormal localization of Tfp1, which consequently affects the vacuolar calcium channel Yvc1 (Jia et al., 2018a). Overall, the proteins involved in vacuolar protein or ion transport mentioned above are critical for maintaining the pathogenicity of C. albicans.

CONCLUSION AND PERSPECTIVES

In summary, studies have found C. albicans virulence is affected by several aspects of vacuolar function including vacuolar pH, vacuolar trafficking, calcium homeostasis, and iron homeostasis. The master pump V-ATPase maintains vacuolar pH and is crucial for pathogenesis and virulence, and its loss of activity also affects hyphal growth and calcium channel function. As well, the calcium and iron channels are necessary for filamentation and biofilm development in C. albicans. Vacular trafficking also controls vacuolar morphology, V-ATPase activity, autophagy, and hyphal growth, elaborating the role of VPS genes in the pathogenicity of C. albicans. This vacuolar trafficking process also involves the interaction of multiple protein families, such as Rho/Rab GTPases, guanylate exchange factors, the HOPS (homotypic fusion and vacuole protein sorting) complex, and the SNARE (soluble NSF attachment protein receptor) complex; this extensive system has not been detailed in this focused review (Bröcker et al., 2010). Moreover, vacuolar fusion can influence hyphal compartments; the highly fragmented vacuoles in C. albicans enable hyphal extensions and septation with reduced branching frequencies. These interconnected pathways may have further potential as targets for future antifungal drug discovery. Thus, further research is still needed to fully understand both morphogenesis and the role of vacuoles in the mechanisms behind pathogenesis and virulence in C. albicans.

AUTHOR CONTRIBUTIONS

LY conceived and wrote the review. QL and YJ conceived and searched the references. All authors contributed to the article and approved the submitted version.

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