DISSECTING THE ROLE OF YEAST RIM8 IN THE INTERNALIZATION OF THE PLASMA MEMBRANE PROTON PUMP PMA1

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ABSTRACT

The vacuolar A-TPase (V-ATPase) is a proton pump that is found ubiquitiously throughout the cells. It uses the hydrolysis of ATP to transport protons across membranes, thereby maintaining homeostatic pH. pH control in the cells of an organism is vital, a disturbance in cellular pH may be lethal. The maintenance of homeostatic pH within the cell appears to be a result of the interplay between V-ATPases and proton exporters. In yeast and plants, the major proton exporter is the plasma membrane proton exporter, Pma1. Pma1 is the transporter that is primarily involved in the maintenance of cytosolic pH. In cells in which the function of V-ATPase has been compromised (*vma* mutants) Pma1 is partially mislocalized.

It is known that membrane transporters lacking the PY motif are endocytosed via the action of an Arrestin Related Trafficking (ART) protein, which translocates an E3 ligase into close proximity with the transporter, so as to allow for the ubiquitination of the transporter. Rim 8 is the ART protein (adaptor) that has been linked to the endocytosis of Pma1, along with E3 Ubiquitin ligase Rsp5. It is of interest to this project that Rim8 is well studied in its role as an adaptor in the alkaline ambient pH pathway.

We thus propose that there may be crosstalk between the ambient pH pathway and the pathway that leads to the internalization of Pma1. Therefore, in this body of work we seek to find other players that may be involved in the Pma1 pathway, as well as to elucidate the areas of interaction between Rim8 and Pma1. Our final goal is bringing a better understanding of the pathway that leads to the endocytosis of Pma1.

To answer the question posed in this work we monitored the growth phenotype and the localization of Pma1 in double mutants lacking both V-ATPase subunits and key players in the ambient pH pathway. In addition, we looked to see which cytosolically exposed terminal of Pma1 may be involved in the interaction with Rim8. In yet another experiment, we mutated Rim8 so as to find which areas of the adaptor were vital for Pma1 internalization. Our results showed that other players tested in the Rim pathway (*vma2*Δ*rim20*Δ and *vma2*Δ*vps23*Δ*)* were not required for Pma1 internalization. In addition we observed that mutations in Rim8 that compromise its function in the Rim pathway still allow Pma1 internalization, even though they show synthetic growth phenotypes with *vma2*Δ mutations. Two-hybrid assay could not detect the sites of interaction between Rim8 and Pma1 and new strategies will be employed to determine these sites. Changes in electrophoretic mobility of Rim8 suggested that Rim8 undergoes posttranslational modifications, and showed differences in *vma2*Δ mutants and WT mutants.

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Figures and Tables

General Introduction

The Function of the V-ATPase: Organelle acidification and V-ATPases

The vacuolar-type H^+-ATPase (V-ATPase) is a proton pump that is found ubiquitiously at the membranes of organelles, where it uses ATP hydrolysis to transport protons across the membrane thus maintaining homeostatic pH.[1] The proton pump mediates the intraorganelle acidification in an ATP-dependent manner and thus regulates vacuolar protein turnover, vesicular trafficking and vacuolar fusion.[1] It must be noted that in intracellular membrane compartments of eukaryotic cells V-ATPase is constitutive and is required for cell function.[2] The V-ATPases serve various functions in different organelle types, for example in endosomes, they generate an acidic pH so that the endocytosed ligand-receptor complexes can be uncoupled and therefore the receptor can be recycled to the cell surface.[1] The low pH generated by the V-ATPases is also required in organelles such as lysosomes that have digestive enzymes, as these enzymes need acidic environments in order to function.[1] Added to which, in the secretory vesicles the proton and the potential gradients that are created at the membranes, drive the uptake of small molecules.[1] In mammals and in midgut of insects[3], in specialized cells such as the renal intercalated cells, the V-ATPase is targeted to the plasma membrane, where it is seen to energize the membrane.[1] The energized plasma membranes adjust the pH of compartments as well as control the intake and loss of ions and fluids.[4] In osteoclasts and renal cells V-ATPase acidifies the extracellular medium, whereas in neutrophils and macrophages it controls cytoplasmic pH.[5] V-ATPases play a very important role in cells; in mammalian cells the loss of V-ATPase is lethal.[6] In yeast the loss of V-ATPase results in a more alkaline vacuolar pH and a slightly acidic cytosolic pH.

Consequences of loss of V-ATPase activity

Malfunction of the V-ATPase has been linked with renal acidosis, diabetes, osteopetrorosis and cancer.[7] In these illnesses a subunit isoform of the V-ATPase is mutated, but the pump itself is still present. For example in the case of osteopetrosis it is the TCIRG1 gene encoding the a3 subunit of V-ATPase that is mutated.[8] V-ATPase is essential and in mammals loss of V-ATPase causes embryotic lethality.[9] In yeast the *vma* mutants (lacking functional V-ATPase) are pH dependent conditionally lethal and fail to grow at a pH higher than pH 7. They are also seen to be sensitive to high extracellular calcium.[2] Mutants that result from the loss of V-ATPase, *vma* mutants, show a slow growth phenotype as well as sensitivity to many heavy metals, oxidants and drugs.[2] These mutants are unable to grow on non-fermentable carbon sources[10]as a result of their sensitivity to reactive oxidative stress. It must be noted, however, that the *vma* mutants have functioning mitochondria.[11] Due to the fact that the V-ATPase is not active, *vma* mutants show a loss of acidification in organelles that are otherwise acidified by V-ATPase.[10] Added to which they show severe defects in both sporulation and germination, however, this can be suppressed under low extracellular pH.[2]

Structure of the V-ATPase

The V-ATPase is a highly conserved multisubunit rotary proton pump that is found at the intracellular and, in some cases, plasma membranes of cells.[12] The structure of V-ATPase[13] is seen in (**Fig.1)** below as shown by *Oot et al*.

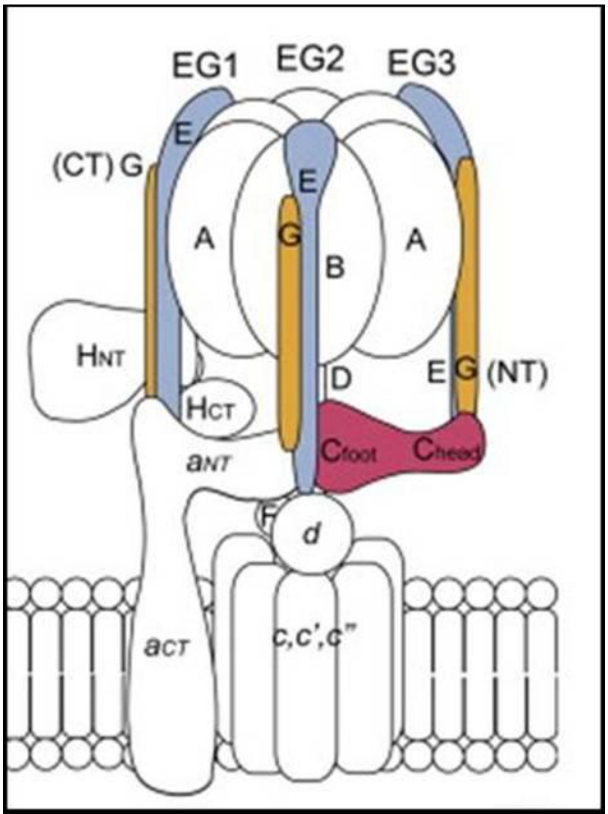


Figure1: The structure of V-ATPase showing the membranal V_0 and the peripheral V_1. *Oot et al.*

It is comprised of two complexes, the peripheral V_1 (650kDa) which contains the site for ATP hydrolysis and the membrane inserted V_0 (260kDa) which contains the proton pore.[1] The pump is comprised of 14 subunits which are each encoded by a single gene in yeast, except subunit a (the largest subunit).[10] V_1 sector has eight subunits- A, B, C, D, E, F, G and H, while the V_0 subunit has the subunits a, c, c', c", d and e.[2]

Letter Designation	MW/kDa	Gene Product
A	69	Vma1p
B	60	Vma2p
H	54	Vma13p
C	42	Vma5p
D	32	Vma8p
E	27	Vma4p
F	14	Vma7p
G	13	Vma10p
a	100	Vph1p
a	100	Stv1p
d	36	Vma6p
c’’	21	Vma16p
c’	17	Vma11p
c	17	Vma3p

*Table1: The Subunits of the V-ATPase and their gene product, edited from *Kane, P.M (2006).*[2]

Of interest is the largest subunit a, a 100kD subunit that has a buried Arg in its seventh transmembrane domian; this Arg appears to be essential for proton transport.[14] It is part of the stator, which is the stationary part of the V-ATPase complex.[1] It is the only subunit found in two different isoforms in yeast- Vph1 and Stv1. These subunits specify localization of the V-ATPase, specifically through their N-terminal cytosolic domain.[1] When Vph1 is present, the V-ATPase is predominantly found at the vacuole, while with Stv1 it is found at the Golgi apparatus/endosome, however it must be noted that during their biosynthesis, the two isoforms occupy the same compartments.[15] It is seen that the two isoforms have varying effects on vacuolar pH, when *STV1* is lost, there is little to no effect on the vacuolar pH, however, when *VPH1* is lost the vacuole become more alkaline.[15] It is observed that in *vph1*Δ mutants *STV1*can partially compensate for loss of *VPH1*, allowing for V-ATPase function, however, there are biochemical differences.[15]

These biochemical differences suggest that the two isoforms are not completely interchangeable. The a subunit must be present along with associated V_1 and V_0 for ATP to be hydrolyzed. ATP hydrolysis occurs at all three catalytic nucleotide binding sites, which are located at the interfaces of the A and B subunits.[1] It is seen that when the V_1 and V_0 subunits are held together this allows for proton translocation via the rotation of the central stalk (subunits D and F) and the c ring.[2] Thus it may be concluded that the hydrolysis of ATP in the A_3B_3 catalytic domains causes the rotation of the central rotor, this rotation leads to the movement of the c ring past the a subunit, allowing for a proton to be transported across the membrane.[16]

Regulation of V-ATPases

Yeast V-ATPase is known to undergo reversible disassembly, for example when yeast is deprived of glucose (its preferred carbon source) Vo and V_1 will disassemble, and when glucose is added the subunits will reassemble. It must be noted that the Vo and V_1 domains do not separate into individual subunits but remain as intact domains upon their dissociation.[2] Reversible disassembly was independently observed in the two very different systems of yeast and *Manduca sexta*, suggesting that it may be a general function of the V-ATPase.[2] In *Manduca sexta* it has been seen that the assembly of the Vo and V_1 is controlled by the C subunit, which seems to be phosphorylated and dissociates from both Vo and V_1, an event closely followed by a disassembly of these two domains.[10] In yeast, reversible dissociation is rapid, occurs posttranslationally[2] and does not use newly synthesized protein or many of the pathways that are activated by starvation.[1]

There are several triggers to dissociation of V-ATPase. In yeast, glucose depletion leads to disassembly and in insects it results from molting.[1] Dissociation and association seem to be independently controlled processes.[1] During V-ATPase dissociation, the V_0 and V_1 become separated and the pump loses both its ATPase and proton translocating activities.[17] Extracellular glucose concentrations regulate V-ATPase *in vivo* by regulating the association between the peripheral domain and the integral membrane domain. [18] It has been seen that in glucose-grown cells, when those cells are temporarily deprived of glucose (or the carbon source), there is a decrease in the number of assembled V-ATPase and thus lower levels of activity are observed in vacuoles.[18] There are several theories on why nutrition deprivation may cause disassembly. Parra and Kane (1998) theorize that during glucose deprivation there is an increase in V-ATPase disassembly in order to conserve cellular reserves of ATP and reassembly allows for the cells to be able to handle the acidification buildup.[18] It would appear that cytosolic pH may be second messenger for glucose in the protein kinase A (PKA) pathway- a pathway that is activated by glucose in yeast.[19] However, it must be noted that the exact molecular activation of the pathway is as yet unknown.[19] The rapid decrease in intracellular ATP may be the signal that triggers the V-ATPase disassembly.[18]

It is known that the glucose induced responses are not resulting from the known glucose pathways and thus the signaling may actually be a novel pathway.[18] Glucose activates the cAMP-dependent protein kinase A (PKA) pathway, a major glucose response pathway, however its connection with V-ATPase is complex because mechanisms of its activation remain unknown.[19] It is observed that a certain number of the assembled complexes do not disassemble during glucose deprivation. This may be because they are inactive pumps or possibly there must be a basal level of assembled pumps that must be maintained for cell survival.[18] There is a sharp drop in pH that takes places immediately after the addition of glucose to glucose starved cells. This drop in pH may be the signal that induces reassembly of the V-ATPase complex.[18] Since it is suggested that the vacuolar ATPase and plasma membrane proton pump Pma1 work in conjunction, it can be hypothesized that reassembled V-ATPase may be assisting Pma1 in raising the cytosolic pH after glucose addition.[18] It remains that though glucose may play a role in the regulation of V-ATPase, V-ATPase reversible dissociation remains pH dependent, with an optimal cytoplasmic pH of ~7.[18] There is increasing evidence that beyond mediating the organelle pH, V-ATPase appears to be needed for the correct transduction of signals and for the regulation of cell growth and survival.[19]

Pma1 is the major plasma membrane proton pump in yeast

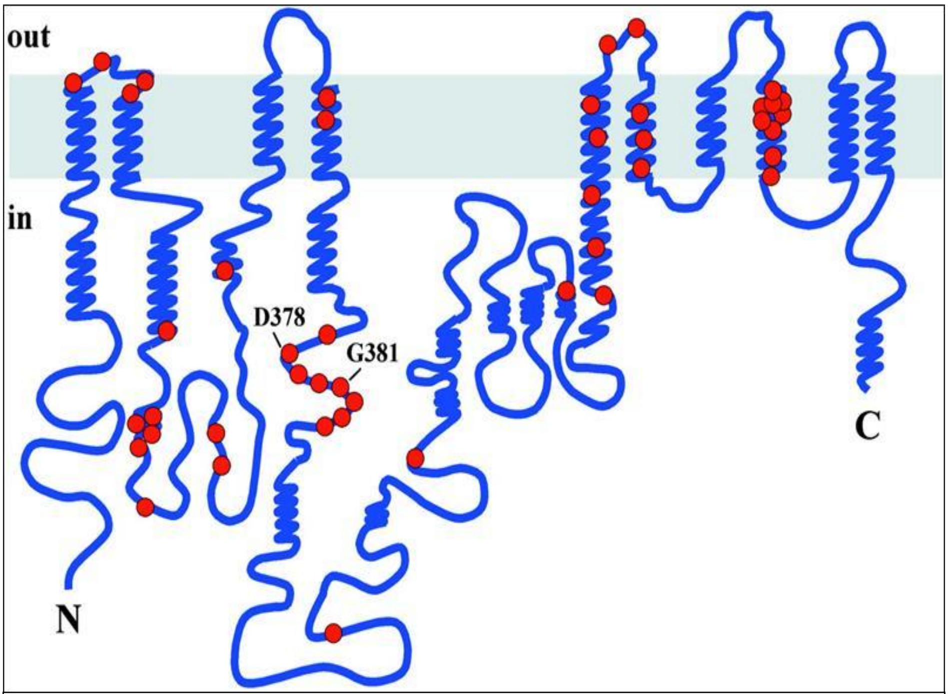


Figure 2: Predicted structure of plasma membrane proton pump Pma1. *Ferreira T. et al*.[48] Zig zag lines represent regions predicted to have α helical secondary structures, red circles mark positions at which mutations have been found to disrupt protein folding, block biogenesis and or lead to a dominant lethal phenotype. Of interest is D378, the catalytically important Asp residue which is phosphorylated by ATP during catalytic activity.

Pma1 is a polytopic P-type proton export pump that aids in the maintenance of cellular pH, along with V-ATPase in yeast. **(Fig.2)**[20] It is a 100 kDa ATPase with 10 transmembrane domains.[20] The protein is folded into 3 cytoplasmic domains (N, P, A) and these form its catalytic portion.[20] Pma1 is responsible for the export of protons in plants and fungi. In mammals this role is carried out by Na+/K+-ATPase which drives the activity of the Na+/H+ exchangers at the plasma membrane and thus protons are exported.[21] *Lecchi et al*. looked at the structure of fungi Pma1 ATPase and saw that the cytosolic C-terminus of the protein folds as a 13 amino acid alpha helix.[20] As in V-ATPases, glucose plays a role in Pma1 regulation, in both proteins glucose deprivation leads to posttranslational regulation.[18] Glucose causes a rapid and strong increase in

ATPase catalytic activity, secondly glucose metabolism results in a slow increase of PMA1 gene expression.[22] It has been seen that in carbon starved cells that are exposed to glucose there is phosphorylation of Ser/Thr at the C-terminal of Pma1, this may be the trigger for the ATPase activation.[20] Threonine appears to be constitutively phosphorylated, while Serine phosphorylation is activated soon after glucose addition.[20]

At the N-terminus the α-helix is more flexible and hydrophilic, whereas at the C-terminus it is more rigid and less hydrophilic and as such it is less likely to be exposed.[20] This C-terminus is inaccessible during carbon starvation, preventing its phosphorylation and thus activation of Pma1.[20] However, upon the addition of glucose, the C-terminal tail is released from its tight binding of regions of the N and or P domains, and it assumes an open, ATPase activated conformation.[20] In this conformation the 45 amino acids of the C-terminus protrude into the cytoplasm and mutagenesis experiments by *Lecchi et al.*, removing the last 18 amino acids of Pma1 resulted in the protein being constitutively activated in absence of glucose.[20] C-terminus plays a regulatory role in that when the cells are starved it binds tightly to N and/or P domains creating a low activity and low affinity form of Pma1.[20] **Fig.3** depicts the changes that Pma1 and its C-terminal undergo during glucose starvation. It was seen that phosphorylation of Pma1 was central because mutations that prevented phosphorylation locked Pma1 in a 'carbon-starved state', while mutations that mimicked phosphorylation locked the ATPase in a glucose-activated state.[20] It is theorized that the similar structure of the termini may also be observed in other eukaryotes as cytosolically exposed termini have been the site of interaction for other transmembranal proteins.

Proposed regulatory mechanism for Pma1 ATPase. *Lecchi S et al. (2007)*

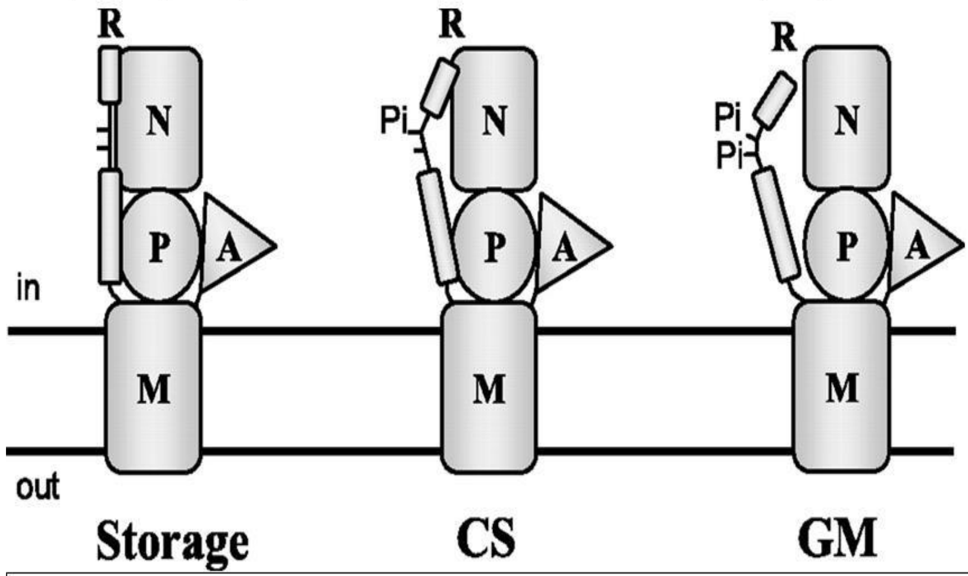


Figure3 : (above) shows proposed regulatory autoinhibition via the C-Terminal. (M)-membrane domain, (P) phosphorylation domain, (A) actuator domain, (N) nucleotide binding domain, (R) C-terminal tail/regulatory domain. It is seen here that during growth under carbon-starved conditions, phosphorylation at Thr912 release of the inhibition of the C-terminal, converting the ATPase to a low activity form (CS,centre). Upon glucose addition, there is additional phosphorylation at Ser911 and complete release from inhibition. The ATPase is now in high activity, high affinity form. (GM,right)

Found at the plasma membrane, Pma1 is not seen in continuous membrane distribution, but as a patchwork on the surface. This patchwork organization may result because it allows for the cell to perform a variety of highly regulated biological functions on the protein laden plasma membrane.[23] The plasma membrane is a specialized structure that mediates the import and export of molecules while simultaneously serving as a platform for various signalling complexes.[23] Pma1 is the most abundant protein on the plasma membrane. The electrochemical proton gradient that Pma1 generates is needed for nutrient uptake via secondary transporters.[22] The *PMA1* gene is essential in yeast and thus Pma1 is strongly regulated and never fully lost from the plasma membrane.

Pma1 mislocalization in *vma* mutants.

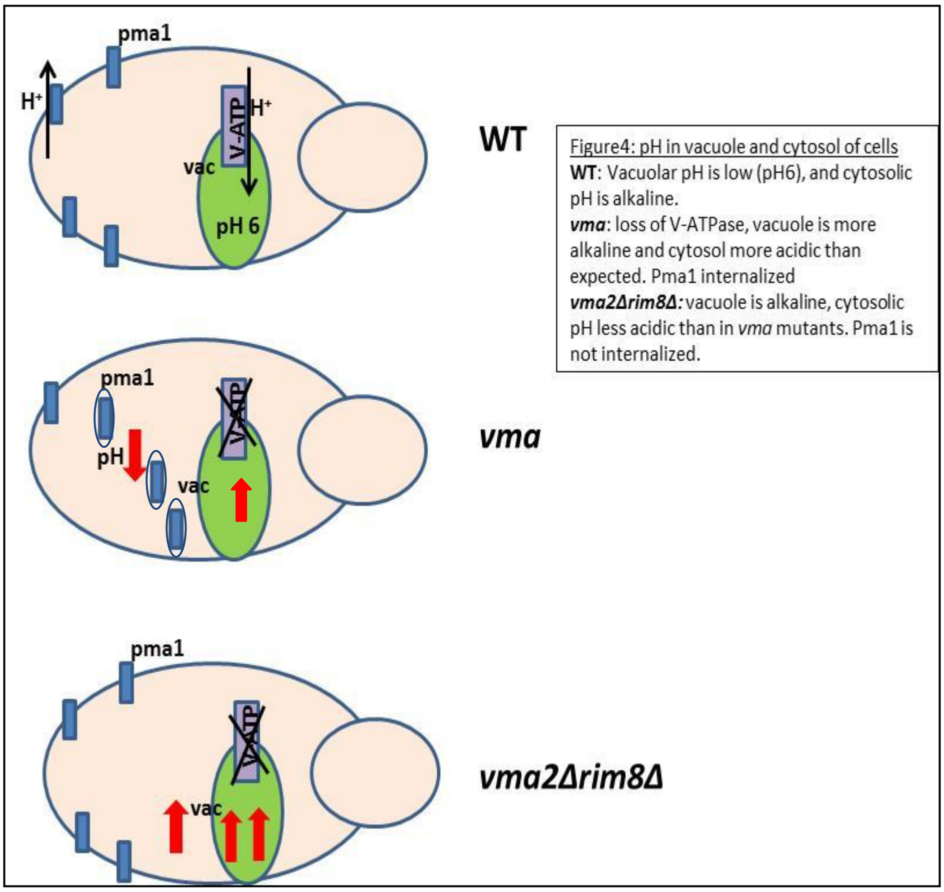


Figure4: pH in vacuole and cytosol of cells
WT: Vacuolar pH is low (pH6), and cytosolic pH is alkaline.
vma: loss of V-ATPase, vacuole is more alkaline and cytosol more acidic than expected. Pma1 internalized
vma2Δrim8Δ: vacuole is alkaline, cytosolic pH less acidic than in *vma* mutants. Pma1 is not internalized.

Surprisingly, it has been seen that in *vma* mutants there are higher levels of the Pma1 within the cell and decreased levels at the plasma membrane.[24] When V-ATPase is lost, the organelles become more alkaline and the cytosol more acidic than expected. It is thought that the acidic cytosol is largely due to the mislocalization of Pma1. The rate of proton export from the cell is reduced because the number of Pma1 pumps at the plasma membrane is reduced. **(Fig.4)**. Current work in the laboratory shows that in *vma* mutants, Pma1, like many plasma membrane permeases can be marked for endocytosis via ubiquitination by an E3 ligase.[21] When Pma1 is not internalized, as is seen in the *vph1*Δ mutant (lacks V-ATPase) and in *vma* double mutants that also lack either the adaptor protein or the ligase, it is seen that the cells have high levels of vacuolar alkalinization. The double mutant cells that cannot internalize Pma1 are seen to grow more poorly than cells with single mutant phenotypes that allow for Pma1 internalization. This suggests that Pma1 endocytosis is compensatory and aides in the survival of *vma* mutants and may play a role in the maintenance of the cells homeostatic pH.

It has been suggested that the pH in the vacuole is lowered by the passive uptake of weak acids from the extracellular fluid.[2] Such an uptake would be enhanced in intracellular environment that finds itself with more H^+ because the internalized Pma1 is not exporting the protons. Yeast thus becomes a good model to explore the above role of Pma1 because the disruption of the V-ATPase in higher eukaryotes is lethal, yet in yeast under specific conditions the cells can be studied.

It is known that plasma membrane proteins are shuttled to the vacuole in a constitutive manner for turnover, and also in a signal dependent manner when there is a need to downregulate the protein.[10] Endocytosis of the plasma membrane proteins works as a quality control and regulatory mechanism that allows for surface remodeling at the plasma membrane.[25] Thus one viable hypothesis is that the internalized Pma1 is being shuttled to the vacuole in the *vma* mutants, where it is degraded. In studied pathways is it seen that once the plasma membrane proteins have been marked via ubiquitination they are endocytosed and their recognition allows for the formation of multivesicular bodies (MVB) via the assembly of the ESCRT machinery.[26] When internalized into the MVBs the proteins are then sent to the vacuole for degradation.[26] The above hypothesis of vacuolar degradation of Pma1 is supported by *Matinez-Munoz and Kane* who showed that in *pep4* mutants (in which proteinase A, a vacuolar protease, is non-functional) in *vma* strains there is an accumulation of Pma1. Whereas, in *vma* strains with wild type proteinase A there were lower levels of Pma1 in the cytosol, suggesting that Pma1 was being cleared in the vacuole via action of proteinase A.[24]

<u>Ubiquitination and targeting of plasma membrane transporters</u>

Ubiquitin is a 76 amino acid peptide that is conjugated to other proteins.[27] Ubiquitination of proteins is possible because of the enzymatic reactions catalyzed by E1-E3 enzymes. The E3 ligase facilitates the addition of ubiquitin to substrates, and thus, it determines if the ubiquitination will be mono/poly ubiquitination. When the substrates of a plasma membrane transporter are in excess or if the cell is under stress, yeast plasma membrane transporters are endocytosed and sent to the vacuole for degradation.[28] Rsp5 is an E3 ubiquitin ligase[29], and its mechanism is well understood. The WW domain of the Rsp5 ligase recognizes the PY motif of the transporter, with the typical sequence of PPXY or LPXY.[28] Rsp5 is the yeast homolog of the mammalian ligase Nedd4.[29] There are several adaptor proteins (ARTS) that facilitate the ubiquitination of the transporter when the transporter lacks the PY motif, these adaptors bring the ligase in close proximity with the protein of interest (see below).[29] Rsp5 is characterized by its N-terminal phospholipid-binding C2 domain, its C-terminal HECT domain and its WW domains.[26] It is via the binding of the PY motif to one or more of the WW domains on the Rsp5 that the ligase recognizes the lysine (K) residues and ubiquitinates the protein of interest at these sites.[26] Rsp5 is responsible for ubiquitination of many membrane transporters as a response to high levels of ligands or of stress.[30]

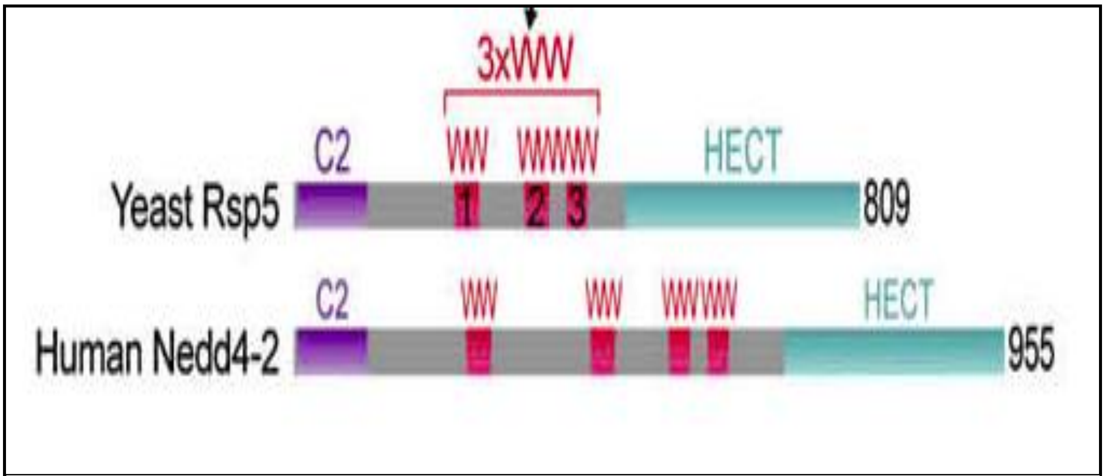


Figure5: The domains of Rsp5 and Nedd4 Ligases. *Charles H. Lin, Scott D. Emr et al (2008)*

<u>ART proteins that allow for endocytosis of the proteins at the Plasma Membrame</u>

ART proteins (arrestin related trafficking adaptor) are needed to bring the ubiquitinating E3 ligase Rsp5 to its substrate transporter and allow for ubiquitination.[31] Art proteins are named due to their similarities to arrestins. It must be noted that arrestins work in the manner that differs from that of Art proteins. In mammals, β-arrestins relay signals to the trafficking machinery by regulating the endocytosis of the G-protein-coupled receptors (GPCRs).[32] In yeast, however, α-arrestins, such as Aly2 (recruited to the aspartic acid/glutamic acid transporter Dip5) that control the intracellular sorting of the permeases.[32] Little is known about how signaling occurs in the cells. Arrestins are divided into α and β due to their primary sequence and structural features, the α-arrestins being ancestral and the β-arrestins more recently derived.[32] β-arrestins couple signaling with GPCRs (regulators), which are then stimulated and they transduce a whole range of kinases and transcription factors.[32] β-arrestins bind to the phosphorylated GPCRs and recruit a ubiquitin ligase, which then ubiquitinates both the arrestin and the receptor- leading to the endocytosis upon interaction with vesicle coat proteins.[32] Upon endocytosis the receptor may either be cycled back to the plasma membrane, degraded at the lysosome or form an endosomal signaling complex.[32] α-Arrestins are not as well studied but they are known to interact with the ubiquitin ligase Rsp5 via its HECT domain. [32] It is seen that α-arrestins mediate the endocytosis of plasma membrane proteins in response to environmental cues.[32] MacGurn *et al*. showed that TORC1 controls the uptake of nutrients in the cell by targeting the ART protein that ubiquitinates specific amino acid transporters at the plasma membrane.[33] In this pathway TORC1 inhibits the Npr1 kinase which in turn inhibits ART1 via N-terminal phosphorinhibition, thus preventing the ART from recruiting the E3 ligase Rsp5.[33] The N-terminal cluster of residues of Art1 are known to control the proteins translocation to the PM, thus phosphorylation prevents the ART from reaching the PM and allowing for transporter Can1 ubiquitination.[33] Proteomic analysis of yeast reveals that there is extensive phosphorylation of ART family proteins.[33] As with Art1 above, Art4 dephosphorylation promotes the glucose-induced endocytosis of its target, the lactate transporter Jen1.[34] The above endocytosis pathway appears to be substrate independent, rather the signaling is intracellular.[34] It must be noted that when Art4 is phosphorylated it is still able to interact with Rsp5 ligase, thus its phosphorylation seems to have little/no observable effect on this interaction.[34]

There are many structural similarities between arrestins and ART proteins, the most notable similarity being that both act as adaptors for ubiquitin ligase.[25] It can be seen that the interactions between β-arrestin 1 and AIP4 are very similar to the interactions that occur between the ART proteins and Rsp5. There is however one glaring difference, the arrestin proteins lack the PY motif.[25] This lack of the PY motif in arrestins suggest that the mechanism of interaction between the proteins and their ligases may be different. Additionally, where it has been seen that arrestins bind and recruit components of the endocytic machinery such as clathrin, the same has not been observed with ART proteins, in fact the residues that govern these interactions are not conserved in ARTs.[25] This supports the idea of mechanistic differences between the arrestins and the ART proteins

There are as yet ten identified ART proteins in yeast.[28] These ART proteins have been seen to share primary sequence homology as well as having predicted structural similarities.[25] There is a redundancy in how the ART proteins work, in that an ART may have more than one target, for example- Art 1 facilitates the ubiquitination of Mup1, Can1 and tat2.[28] Added to which a transporter can be targeted by more than one ART protein.[31] It may be that the Art protein recognizes related features in the transporters, such as a substrate-induced conformation or state, however further study must be done to fully understand this redundancy.[28]

An ART Protein, Art9/Rim8, and its Association with Rsp5

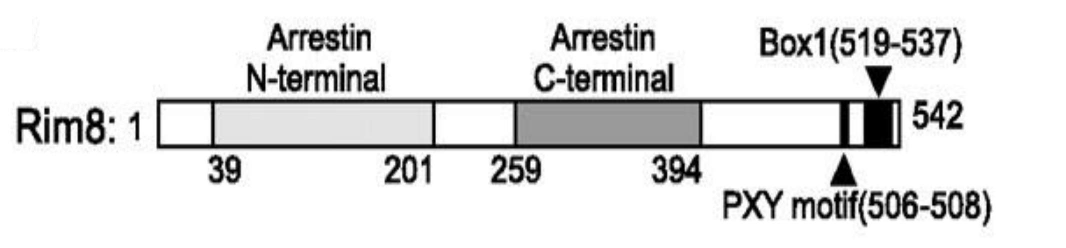


Figure6: The structure of Rim8, showing its various domains. *Herrador A. et al (2010)*

Art 9, also known as Rim8, is an adaptor that has been associated with the E3 ligase Rsp5 in the ubiquitination of the plasma membrane protein Rim21 **(Fig.6)**.[29] Rim8 in *S. cerevisiae* is ubiquitinated at K521 in the C-terminal region. It is at this amino acid that Rim8 is monoubiquitinated by the ligase, however it must be noted that Rim8 ubiquitination is constitutive.[35] It has been found that Rim8 is posttranslationally phosphorylated in response to neutral-alkaline pH and specific phosphorylation states are associated with its role in pathways.[36] Rim8 is found in the following three phosphorylation states: hypophosporylated state when Rim8 (82- to 87-kDa forms) is inactive at acidic pH, the intermediate phosphorylation state (87- to 95-kDa forms) and the hyperphosphorylated state (95- to 105-kDa forms).[36]

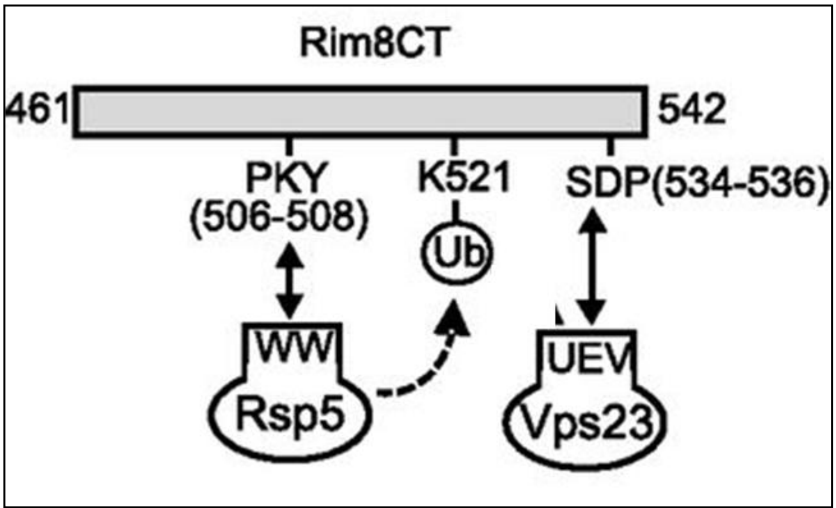
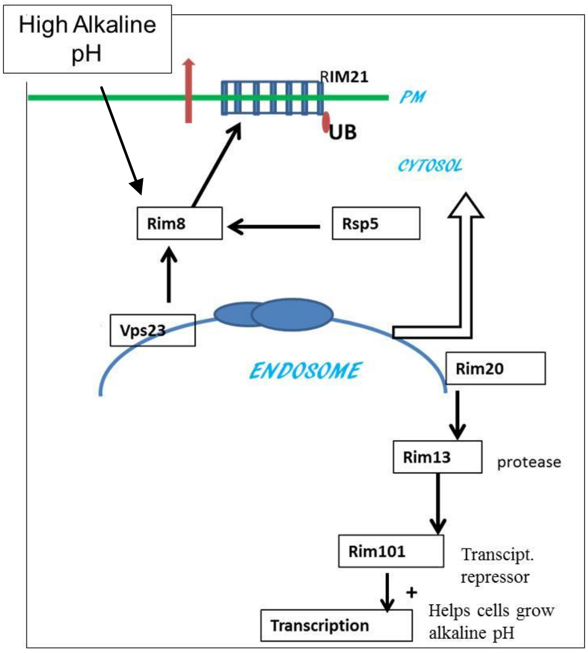


Figure7: showing the sites of interaction at the CT of Rim8. Adapted from *Herrador et al* (2013)

It is in the C-terminus that the PXY domain (506-508aa), which interacts with the WW domain of the ligase Rsp5, is located. Here we also find the SXP domain (533-536) which interacts with the ubiquitin E2 variant (UEV) domain of Vps23.[36] *Herrador et al.* showed that the C-terminus of Rim8 was not required for the binding of Rim8 to the 7TM protein Rim21, suggesting that Rim21 binds to the N-arrestin domain of the ART protein.[29]

The Role of Rim8 and Rsp5 in the Ambient pH Pathway

Rim8 is best known for its role in the ambient pH pathway, where it facilitates the ubiquitination of Rim21 and Dfg16, 7 transmembrane plasma membrane (PM) proteins.[35] In this context, it is associated with the activation of the extracellular pH sensing pathway, in which the transcription factor Rim101 is activated.[36] It appears that Rim8 bridges the signalling and the activation of this extracellular pH sensing pathway. Upon receiving a signal of high external pH, Rim8 is then believed to translocate Rsp5 to the plasma membrane where the 7TM Rim21 is ubiquitinated by the ligase.[29] The endocytosis of Rim21 leads to the activation of the scaffolding protein Rim20, which is known to interact with the C-terminal inhibitory domain of the Cys_2His_2 zinc-finger transcription factor Rim101.[35] Under acidic conditions the full length Rim101 is predominant and under alkaline conditions it is cleaved and is active as a transcription repressor.[35] Under acidic conditions Rim20 is cytosolic but when the extracellular pH becomes alkaline, Rim20 is found at the endosome, where its activation leads to the activation of the cysteine protease Rim13.[35] Rim20 binds to Rim101 where it acts as a protease substrate-recognizing adaptor. Upon binding of Rim20 to Rim101, Rim13 is recruited and it proteolytically cleaves the C-terminus of Rim101 which is thus activated.[35] The proteolytically processed Rim101 transcription factor translocates to the nucleus and promotes changes in gene expression which lead to the growth of cells and their survival in the elevated external pH.[36] Once Rim101 is activated, there is a decrease in the level of Rim8 as a result of its degradation.[36]



Current work in the lab: Pma1 and its possible association with the Ligase Rsp5 and Art9

It has been suggested that the alkalization of the organelles as a result of a loss of V-ATPase activity might mimic certain aspects of extracellular alkalization and thus share factors such as Rim8 with signaling from the V-ATPase to Pma1.[21] The lab therefore began to look at possible links between the ambient pH pathway and the internalization of Pma1, suggesting that there may be a possible crosstalk between the two pathways. Current work in the lab has shown that Pma1 ubiquitination seems to be dependent on Rim8 and Rsp5.[21] Mutations of *RIM8* and *RSP5* in *vma* mutants result in Pma1 being retained at the plasma membrane as well as synthetic growth defect. It is noted that both chronic and acute inhibition of V-ATPase activity result in the internalization of Pma1. To the knowledge of the lab, this is the first time that stimulus-dependent ubiquitination and endocytosis of wild-type Pma1 has been documented. Mutant forms of Pma1[37] have been shown to undergo ubiquitination and endocytosis, but this is beyond the scope of thesis work.

Thesis Goal

V-ATPase is a proton pump that is found at the membranes of acidic organelles, where it uses ATP hydrolysis to transport protons across the membrane and thus maintain pH. Loss of V-ATPase results in a more alkaline vacuolar pH and a slightly acidic cytosolic pH. Mutants that result from the loss of V-ATPase, *vma* mutants, show a slow growth phenotype. In such mutants Pma1, a P-type proton pump, is seen to be internalized. Pma1 is a plasma membrane proton pump that is responsible for the direct export of protons and aids V-ATPase in the maintenance of cytosolic pH. Without the adaptor Rim8 (ART9) and the E3 ligase Rsp5, Pma1 is not ubiquitinated and fails to internalize in *vma* mutants. This results in a synthetic growth phenotype in *vma2*Δ*rim8*Δ mutants. Rim8 is an important player in the ambient pH pathway.

We propose that there may be crosstalk between these two pathways.

We hypothesize that the internalization of Pma1 with the aid of Rim8, Rsp5 and other key players is necessary for homeostatic pH control which allows the cells with compromised V-ATPase to grow.

The goal of this thesis work is understand how Rim8 interacts with Pma1 and stimulates its ubiquitination, thus coming to a better understanding of the sites of interaction on Pma1 and Rim8. Secondly, this thesis aims to discover if other members of the ambient pH pathway are required for the endocytosis of Pma1. This thesis work will continue the laboratory's work on understanding the pathway of Pma1 internalization.

Materials and Methods

Construction of yeast strains and plasmids

Table 2: Oligonucleotides used in the experiments

Primers	Sequence
RIM8-2H5'	GGCCATGGAGATGTCGTTACTGAGACTGTGGAAC-3[1]
RIM8-2H3'	GCGGATCCATAGTCATCACAAGGGGGAGGATC-3[1]
Rim504Forw	TTAAGAGATTGGTTGGCCCCATTAAATGCATATGATATGACGATGTTCCACGGATCCCCGGGTTAATTAA-3[1]
Rim504Rev	TAGTTCCAACACGGCGCAAGCTGCGAGTATATGACTTGTTACCTGCACTTTGAATTCGAGCTCGTTTAAAC-3[1]
RIM8553	GAAGCAAGATGCGAACGTGAG-3[1]
Rim8-1000BamHI	GCGGATCCATACTCATATCACAGCCACTG-3[1]
Rim8-3HAup	TGCGTAATCAGGAACATCGTATGGATAAGCATAATCTGGAACATCATATGGGTACATGTTTGGTCAATGCTACCGATAG-3[1]
Rim8-3HAdown	TATCCATACGATGTTCCTGATTACGCATACCCATACGATGTTCCAGATTACGCTTCGTTACTGAGACTGTGGAAC-3[1]
Rim8+427Cla	GCATCGATGGTGCATTTTGAAACTAGGGAG-3[1]
Hind3Rim8-P2	GCAAGCTTCCACGTGAGGGAGCTCAAAGTTC-3[1]
Trunc504F	GCATATGATAGTGACGATGTTCCATAAAAAGTGCAGGTAACAAGTCAT-3[1]
Trunc504R	ATGACTTGTTACCTGCACTTTTTATGGAACATCGTCACTATCATATGC-3[1]
Pma1CT5'	GCGGATCCAGATGTCCACTTCTGAAGCCTTTG-3[1]
Pma1CT3'	GCCTCGAGTAGGTTTCCTTTTCGTGTTGAG-3[1]
Pma1NT5'	GCGGATCCTAATGACTGATACATCATCCTCTTC-3[1]
Pma1NT3'	GCCTCGAGATTCTTTTTCGTCAGCCATTTG-3[1]

*The oligonucleotides (oligos) that were used in this thesis were ordered from Eurofin MWG Operon.

Table 3: Yeast Strains

Strain Name	**Source**	**Genotype**
BY 4741 WT	Library strain	*MATa his3Δ1 leu2Δ0 ura3Δ0*
Y3656 *vma2Δ*	Library Strain	MATα his3Δ1 leu2Δ0 ura3Δ0 vma2Δ::Nat^r
BY 4741 *rim8Δ*	Library Strain	MATa his3Δ1 leu2Δ0 ura3Δ0 rim8Δ::kanMX
Y3656[38]*vma2Δrim8Δ*	Tetrad dissection: This study	MATα, ura3Δ0, leu2Δ0, his3Δ1, met15Δ0, lys2Δ0, can1ΔMFA1pr-HIS3-Mfα1pr-Nat^r
BY4741 RIM8-3HA	Lab strain-Anne Smardon	MATa his3Δ1 leu2Δ0 met15Δ0ura3Δ0 RIM8-3HA::KanMX6
MS1*vma2Δrim20Δ*	Tetrad dissection: This study	MATαhis3Δ1 leu2Δ0 ura3Δ0 rim20Δ::kanMX vma2Δ::Nat^r
MS2*vma2Δrim21Δ*	Tetrad dissection: This study	MATα his3Δ1 leu2Δ0 ura3Δ0 rim21Δ::kanMX vma2Δ::Nat^r
MS3*vma2Δvps23Δ*	Tetrad dissection: This study	MATα his3Δ1 leu2Δ0 ura3Δ0 vps23Δ::kanMX vma2Δ::Nat^r

*All Library Strains were from Euroscarf[39]All the double mutant strains made and used in this study were MATα.

The BY4741 yeast strain was used throughout this thesis work. The cells were grown on YEPD (1% yeast, 1% bactopeptone, 2% glucose) rich media or YEPD plates. The media was buffered at pH 5 or pH7.5 using the S&P buffer (50mM succinate and 50mM potassium phosphate). Some of the pH7.5 YEPD plates had Ca^{2+} added in order to create a more stringent growing environment and select for loss of growth of the *vma2Δ* yeast strains. In the mutagenesis steps, the yeast transformants were selected on uracil dropout plates, as the pRS316 plasmid has a Uracil gene and thus the transformants were grown on supplemented minimum media SD-ura media/plates (6.7g/liter yeast nitrogen base, 2%glucose, x10 amino acids-uracil).

Sporulation and Tetrad Dissection

To construct double deletion strains, the *vma2*Δ*::*NAT^r MAT α cells (Table2) were mixed with each of the Kan MX-marked haploid deletion strains in the BY4741 background. Mating was done in YEPD liquid media, incubated at 30˚C with shaking for 6 hours. The cells were then spun down and plated onto the selection plates (Nat^R+Kan plates), and the plates were incubated at 30˚C. The diploid colonies that came up were then used in sporulation. The colonies were grown in YEPD to 1OD/ml and then pelleted and washed once. They were then resuspended in sporulation media (0.25% yeast extract, 1.5% potassium acetate, 0.05% dextrose, 1X amino acid stock, H_2O), and incubated with shaking at 30˚C for 3 days. 5% Zymolyase 100T was added to the sporulated cells, and cells were incubated for 10mins at room temperature. The cells were then spread as a thin line on a YEPD plate and the dissection microscope was used to isolate single spores from a tetrad. The growth of these spores was monitored over a series of days. The spores were patched onto YEPD plates and then replica plated onto Kan, Nat and YEPD plates and growth was monitored. Double mutant spores were selected by their growth on all three of the plates (Kan, Nat and YEPD). Growth on both Kan and Nat showed that they had gained resistance from both the deletions and the *vma2*Δ respectively as a result of successful mating. Mating type was tested on the Mat a and the Matα plates which confirmed that the spores were haploid, as they would grow on one plate type and not the other. Some of the spores were Mat a and some were Mat α but for the purpose of consistency within my study I chose to use only the MAT α spores.

Growth Assays using Serial Dilutions

The yeast strains of interest were grown to 1OD/ml and then a 1:10 serial dilution was carried out. The dilutions were grown on various plate types: YEPD pH5, YEPD pH7.5, YEPD pH7.5+Ca^{2+}. The serial dilutions were allowed to grow over a series of days so as to monitor whether the deletions of the proteins in the ambient pH pathway would affect the growth of the *vma* mutants.

Preparation of Pma1 fragments of the CT and the NT for 2-Hybrid

The CT and the NT fragments of Pma1were produced using Pma1CT and Pma1NT pairs of oligos listed in **(Table2). Pma1CT5'** and **Pma1CT3' (Table2)** were used to synthesize the CT of Pma1 from aa875-aa918, and the oligos **Pma1NT5'** and **Pma1NT3' (Table2)** were used to synthesize the NT of Pma1 from aa1-aa111. These fragments were confirmed by agarose gel. The confirmed fragments were then ligated to the pGEM vector via overnight ligation at 4˚C. After these fragments were confirmed to be correct via sequencing, the CT and the NT fragments were both double digested with Bam HI and XhoI. The two-hybrid plasmid, pACT, was also cut with the same enzymes. The fragments were ligated and the resulting ligation product was transformed into DH5α cells. Transformants were digested with the restriction enzymes to confirm that the insert was present. The pACT-PMA1-CT and pACT-PMA1-NT plasmids were purified, and using the Gietz[40] yeast transformation protocol, transformed into PJ69-4α.[41] The PMA1-NT and -CT fragments were now ready for the 2-hybrid assay.

Preparation of Full Length Rim8 for 2-Hybrid

Full length Rim8 was produced using the **RIM8-2H5'** and the **RIM8-2H3'** oligos. Once the full length Rim8 was confirmed via agarose gel and sequencing, it was cut with the restriction enzymes Bam HI and NcoI, and the plasmid pAS2 was also cut with the same restriction enzymes. After the digestions had been purified, they were then ligated overnight at 16˚C using the Quickstick ligation method, producing a Rim8-pAS2 ligation product. These ligation products were then transformed into DH5α *Escherichia.coli* and DNA isolated from the transformants was digested to confirm the presence of the Rim8 insert. Once Rim8 had been confirmed the purified plasmids were used in the Gietz yeast transformation into the PJ69-4A strain. pAS2-RIM8 in PJ69-4A was used in the 2-hybrid assay. It must be noted that **RIM8553 (Table2)** is an internal oligo that was used as a primer in sequencing, so as to check the accuracy of the newly synthesised Rim8. Rim8 is too long (1626bp). [42]

To Test Which Termini of Pma1 (CT or NT) Interacts with the Full Length Rim8 2-hybrid Assay

The strains PJ69-4A/pAS-RIM8, PJ69-4α/pACT-Pma1CT, PJ69-4α/pACT-Pma1NT were made. A control strain was also made which contained the "empty" pACT vector (PJ69-4a/pACT). PJ69-4A/pAS-RIM8 was grown on SD$^-$trp plates as the pAS2 plasmid has a *TRP1* marker. Both PJ69-4α/pACT-Pma1CT and PJ69-4α/pACT-Pma1NT were grown on SD$^-$leu plates as the pACT plasmid is under the *LEU2* marker. The yeast strains used contained *ADE2* and *HIS3* under control of the *GAL1/10* promoter.[41] A standard 2-hybrid protocol (*Bartel, P.L et al*)[43] was followed and the resulting transformations were tested on the following plates: SD$^-$trp/leu/ade; SD$^-$trp/leu/ade/his; SD$^-$trp/leu/his; SD$^-$trp/leu/his+2mM AT. They were allowed to grow over a period of 6 days, at a temperature of 30°C.

Visualization of Rim8-HA by immunoblot

Preparing Whole Cell Lysates of Rim8 under various conditions

Both wild-type (Rim8-3HA) and *vma2*Δ-Rim8-3HA cells **(Table3)** were used in the experiments. Cells were grown in YEPD media to about 1OD/ml (log phase), and a total of 10 OD was harvested by centrifugation. The cells were then washed and resuspended in 10ml of fresh YEPD media. The wild type (WT) cells were exposed to various treatments. Some were challenged with 5mM and 20mM of acetic acid, and then they were incubated at 30˚C for a further 15 minutes. Some were put into YEP media (lacking glucose) and then incubated for a further 15 minutes. Of the cells that had been put in medium with no glucose (YEP), some then had 2% glucose added to them after 15minutes and were incubated for an additional 15 minutes.

The cells were then spun down and the supernatant removed. The pellets were frozen at -80˚C for 30 minutes or more. Lysis buffer, comprised of cracking buffer (50mM Tris-HCl ph6.8, 8M urea, 5%SDS, 1mM EDTA)+ 5% β-mercaptoethanol+ phosphatase inhibitors (40mM NaF and 1mM Na vanadate) and protease inhibitors (1mM PMSF, 1µg/ml leupeptin, 5µg/ml aproptinin and 2µg/ml pepstatin) was prepared.

To begin lysis, we added 100 ul heated lysis buffer to the pellets and this mixture was combined with 50µl of acid washed glass beads. Lysis was carried out via agitation of these contents. After lysis, 1% bromophenol blue was added and the proteins were heated at 65˚C before the beads were spun down again.

Analysis of Whole cell Lysates via Electrophoresis and Western Blotting

The contents of the whole cell lysates were run on an SDS PAGE gel under previously determined parameters (15 µl of lysate, equivalent to 1.5 OD of cells, was loaded), then transferred onto nitrocellulose paper for Western blotting. The blot was then probed with monoclonal antibody 16B12 against HA epitope (Covance), to probe for the 3HA tag on Rim8. Alkaline phosphatase-conjugated anti-mouse IgG secondary antibody (Promega) was used and the Rim8 was visualized after a brief incubation in carbonate buffer+5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetraolium (NBT).

Immunofluorescence microscopy of Pma1 using anti-Pma1 antibody

Immunofluorescence microscopy was performed as described previously.[24,44] The lab had first used immunofluorescence to localize Pma1 in a paper by *Martinez-Munoz and Kane* (2008).[24] Cells were grown to about 1OD/ml (log phase) and 1.2ml of 37% formaldehyde was added and the cells were incubated at 30˚C for an hour. The cells were then resuspended in 2 ml of fixative (4% paraformaldehyde) and were left shaking overnight at room temperature. Following this, the cells were lysed in 10mg/ml of zymolyase in SPM buffer (1.2M sorbitol, 50mM potassium phosphate pH 7.2, 1mM $MgCl_2$) for 1 hour at 30˚C. The cells were then washed in 1.2M sorbitol and applied to the 8-well multitest slide. The fixed cells were incubated with the primary mouse monoclonal antibody (Abcam ab4645) against Pma1 overnight and following this they were incubated with the secondary antibody (Alexa-Fluor 488-conjugated goat anti-mouse (Invitrogen)) for an hour. The slides were then washed stored at -20˚C. The cells and localization of Pma1 were visualized using the Zeiss Imager Z1 fluorescence microscope and Nomarski and FITC fluorescence optics.

<u>Mutagenesis of Rim8 at the CT, to Observe if CT is Required for Pma1 Internalization</u>

<u>Mutagenesis of Rim8 at the CT using in-genome deletion method</u>

The oligonucleotides **Rim504Forw** and **Rim504Rev (Table2)** were used in an attempt to use PCR based in-genome deletion [45] in Rim8 from aa505 to the end of the gene at aa542. The plasmid pFA6a-3HA-Kan-MX6 was used as the template so as to produce a mutated Rim8-3HA, here the full length RIM8 was C-terminally tagged. This C-terminally tagged RIM8 was made in the lab.* Rim8-3HA complements on growth assays but when we attempted to delete the C-terminal of Rim8 using the oligos stated above and with the parameters that were set for the in-genome methodology, however there is an essential gene that is too close to our deletion and it was disrupted during the deletion process. The resulting strain was temperature sensitive and had retarded growth at 30˚C. We then decided to pursue plasmid mutagenesis so as not to disrupt the genome.
*The RIM8-3HA was made by a lab member (Anne Smardon, PhD)

<u>Making of the Rim8-2HA-pRS316 plasmids for mutagenesis</u>

Rim8-1000BamHI and **Rim8-3HAup (Table2)** were used to add a Bam HI site 1000bp upstream of Rim8 and to add 2HA to the NT of Rim8 (HA2-Rim8).
Rim8+427Cla1 and **Rim8-3HAdown (Table2)** were used to add a ClaI site 427bp downstream of Rim8. The products of these two sets of oligos were fused via fusion PCR to form a Rim8 with 1000bp upstream, 427bp downstream and 2HA at the NT. The

piece that resulted was then used in site-directed (whole plasmid) mutagenesis; in which a deletion was made at the CT of Rim8. **Hind3Rim8-P2 (Table2)** was used in replacement of the **Rim8+427Cla1** in the amplification of Rim8 due to complications with the ClaI site. Once the BamHI-2HA-Rim8-HindIII fusion product had been confirmed via agarose gel and digestion at SalI sites, the fusion product was confirmed via sequencing. We then digested both the plasmid pRS316 and the Rim8-2HA product with Bam HI and HindIII and using Quickstick ligation (Stratagene) we ligated the cut pRS316 with the cut full length Rim8-2HA. The ligation product was then transformed into DH5α *E.coli* cells and grown on LB+Amp plates at 37˚C. The colonies had been confirmed to have the correct insert via a series of digestions to give the plasmid pRS316-HA-RIM8. This plasmid was then prepped for mutagenesis in which the CT of Rim8 was deleted as seen in *Herrador et al*.[29]

Quickchange mutagenesis via PCR

Mutagenesis was carried out using a lab developed protocol that was based off of the Quickchange XL Site-directed Mutagenesis kit (Stratagene). Oligonucleotides **Trunc504F and Trunc504R (Table2)** were ordered from Eurofin MWG. The **Trunc504F** and **Trunc504R** oligos were used in mutagenesis of the Rim8-2HA-pRS316 plasmid to make a truncation at the CT of Rim8, deleting from amino acid 505 to the end of the gene at 542.The template used in the PCR was full length HA-Rim8 in the pRS316 vector. The truncation primers were used in mutagenesis along with the Primestar ligase. The mutagenesis product was subjected to Dpn1 restriction digestion to remove any of the residual original templates and the mutagenesis product was then transformed into *E.coli*. Once the plasmid was isolated from *E.coli* and the deletion confirmed, it was introduced to the *rim8*Δ and the *vma2*Δ*rim8*Δ yeast strains via the Gietz yeast transformation. The yeast colonies that resulted from this transformation were used in western-blotting assay, growth assay and immunofluorescence assay, so as to compare the deletion mutant phenotype to that of the full length Rim8.

Results

Section A: How does loss of V-ATPase function lead to Pma1 internalization?

Fig.8A is an illustration of the known ambient pH pathway, as reported by *Maeda* (2012).[35] In the pathway, high external pH results in the recruitment of Rim8 and the ligase Rsp5 to the plasma membrane, where the 7TM plasma membrane receptor Rim21 is ubiquitinated and endocytosed. The internalization of Rim21 leads to Rim20 activating the protease Rim13. Rim13 then cleaves the C-terminus of the transcription factor Rim101[46]

Fig.8B is the pathway that my lab has hypothesized for Pma1 internalization. In this pathway it is seen that upon loss of V-ATPase, a signal is sent to Rim8 which allows for the adaptor to recruit the ligase Rsp5. Rim8 and Rsp5 then translocate to the plasma membrane where Pma1 is ubiquitinated and this leads to its endocytosis. The mislocalization of Pma1 allows for better pH control and the cells are able to survive. Better pH control is achieved because in acidic cytosolic environments, weak acids are able to enter organelles via permease action. They can thus acidify the organelles that had become alkaline as a result of a loss of V-ATPase at their membranes.

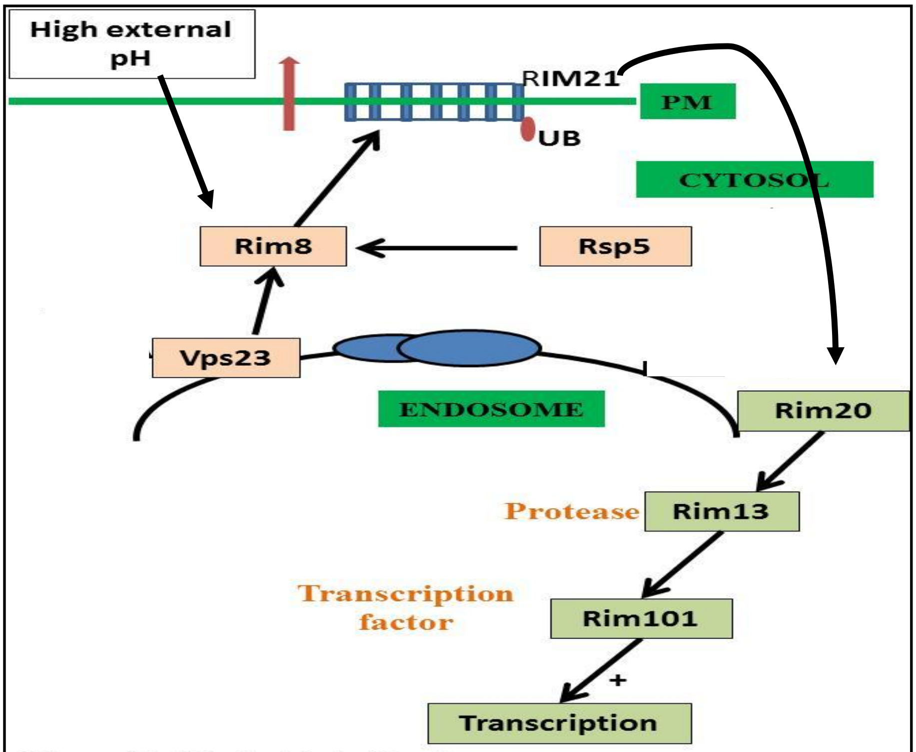


Figure 8A: The Ambient pH pathway

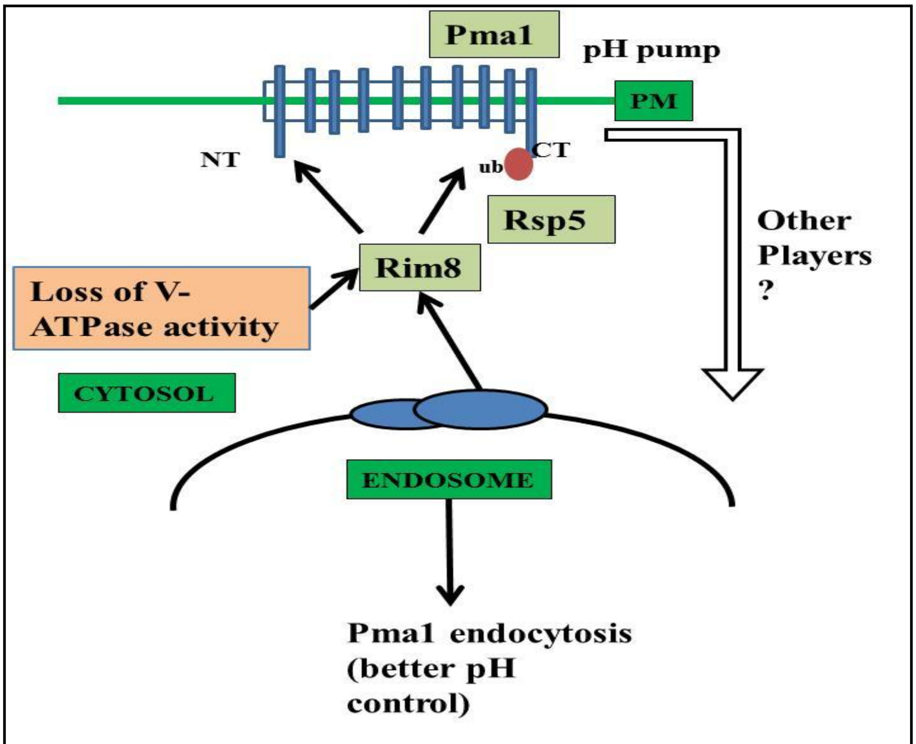


Figure 8B: The Proposed Pma1 Mislocalization Pathway

The following experiments were carried out in order to test our proposed hypothesis. Having seen from current work in the laboratory that ART/Rim8 and the ligase Rsp5 are required for the internalization of Pma1 and that these two proteins are part of the ambient pH pathway, we decided to test whether other players in the ambient pH pathway could be involved in Pma1 internalization. This would suggest crosstalk between the two pathways.

<u>Looking at the Growth of the Alkaline Ambient pH Pathway and V-ATPase *(vma)* double mutants</u>

Growth phenotypes may help to elucidate the effect of a particular mutation on the cell. Some mutations will show negative synthetic growth phenotypes. Such phenotypes would suggest that if the protein of interest is absent then the cell is negatively affected and thus the protein is needed for robust cell growth. Mutations of the V-ATPase show negative growth defects and the double mutants of the *vma* mutants and Rim8 or Rsp5 have been seen to show even more severe growth defects; these double mutants grow more poorly than the *vma* single mutant. This suggests that *vma* mutants and *rim8*Δ or *rsp5-1(*at the non-permissive temperature*)* have a negative synthetic interaction. Since Rim8 and Rsp5 are needed for Pma1 internalization, this suggests that Pma1 internalization helps the *vma* mutant cells to thrive.

In the growth assay below **(Fig.9)** we wanted to see if deletions of the other players in the ambient pH pathway showed the similar severe growth defects in combination with *vma2*Δ*:* that is growth that was poorer than that of the *vma2*Δ single mutant.

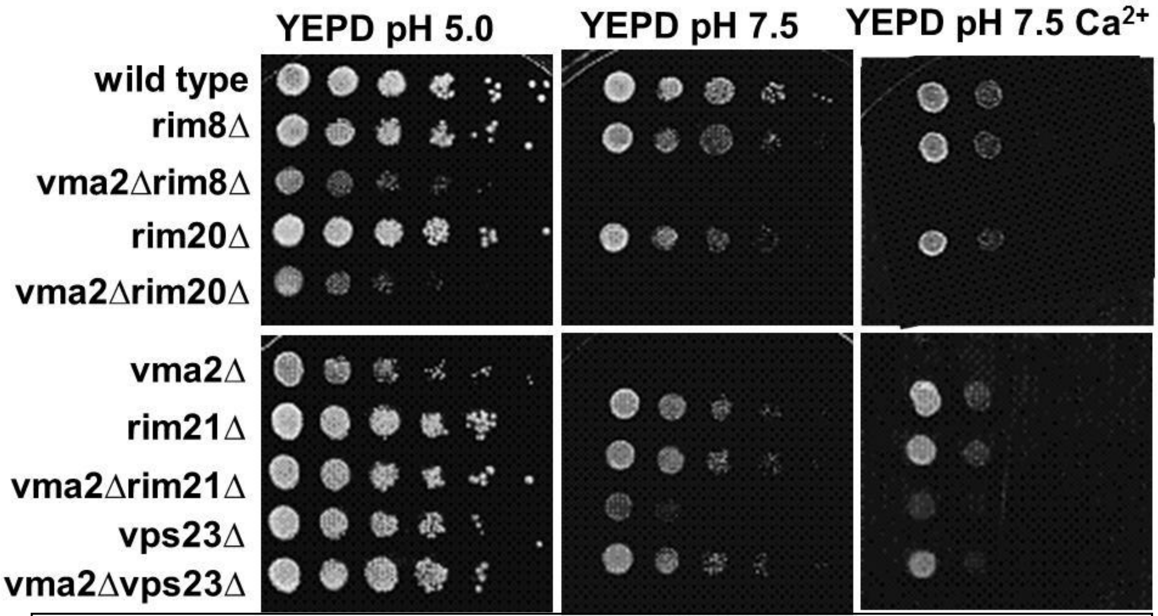


Figure 9: Growth of the alkaline ambient pH pathway and *vma* mutants
Each of the strains, WT*, rim8Δ,rim20Δ, rim21Δ, vps23Δ* and their *vma* double mutants were grown to 1OD and were diluted in 10-fold serial dilutions and then spotted onto the selected YEPD plates and incubated over 6 days at 30˚C.

Double mutants *vma2Δrim21Δ, vma2Δ rim20Δ* and *vma2Δ vps23Δ* were made. These deletions were made on the premise that there was cross talk between the pathways and players in the ambient pH pathway may play a role in internalization of Pma1. *vma2Δrim21Δ* was made because Rim21 is found at the plasma membrane and is downstream of Rim8, thus we wanted to see the effect that upstream proteins may have on Rim8 pathway. *vma2Δ vps23Δ*was constructed because Vps23 is a subunit in ESCRT1 found at the endosome and it is known to bind to ubiquitinated cargo through a ubiquitin binding moiety. It interacts with Rim8 via its UEV domain and Rim8's SXP domain; Vps23 is also found upstream of Rim8.[35]*vma2Δ rim20Δ* was chosen because Rim20 is downstream of Rim8 and here we could observe downstream effects. Cells were grown to log phase and then 10-fold serial dilutions were carried out. Previous data shows that *vma* mutant cells grow robustly at pH5 but not at pH7.5, this suggests that at pH7.5 V-ATPase function is essential, added to which when the heavy metal Ca^{2+} is added there is no growth of *vma* mutants. The goal of this assay was to see whether some of the other players in the alkaline ambient pH pathway were needed in order for *vma* mutants to thrive. Using the YEPD, pH 5 plates, we looked for a double mutant that had poorer growth than the single mutants of *vma2*Δ and of the singly deleted proteins. Poorer growth would suggest that the deleted protein is required in a *vma* mutant.

We hypothesize that some of the key players in the ambient pH pathway may play a role in the internalization of Pma1. To test this, we mated *vma2*Δ mutants with mutants of single deletes of the players in the ambient pH pathway (Rim20, Rim21, Vps23), resulting in *vma* double delete mutants. We then isolated double mutants by tetrad dissection and genotyping of spores and carried out a serial dilution.

When the growth on the plates was studied, we saw as expected, that WT cells had the most robust growth on the plates. The single mutants also grew robustly; none of them exhibited the *vma* mutant phenotype- as they showed growth on pH5 as well as pH7.5 plates. The double mutant *vma2*Δ*rim8*Δ showed negative synthetic growth; its growth was poorer than that either the *vma2*Δ or the *rim8*Δ on the YEPD, pH 5 plate. The *vma2*Δ*rim20*Δ yielded a phenotype on the YEPD pH5 plates, where it showed poorer growth than both the *vma2*Δ and the single *rim20*Δ. This negative synthetic growth suggests that Rim20 may be required for *vma* mutants to thrive, which would be of interest in Pma1 internalization. The double mutants *vma2*Δ*rim21*Δ and *vma2*Δ*vps23*Δ showed no growth phenotype beyond the *vma2* growth phenotype.

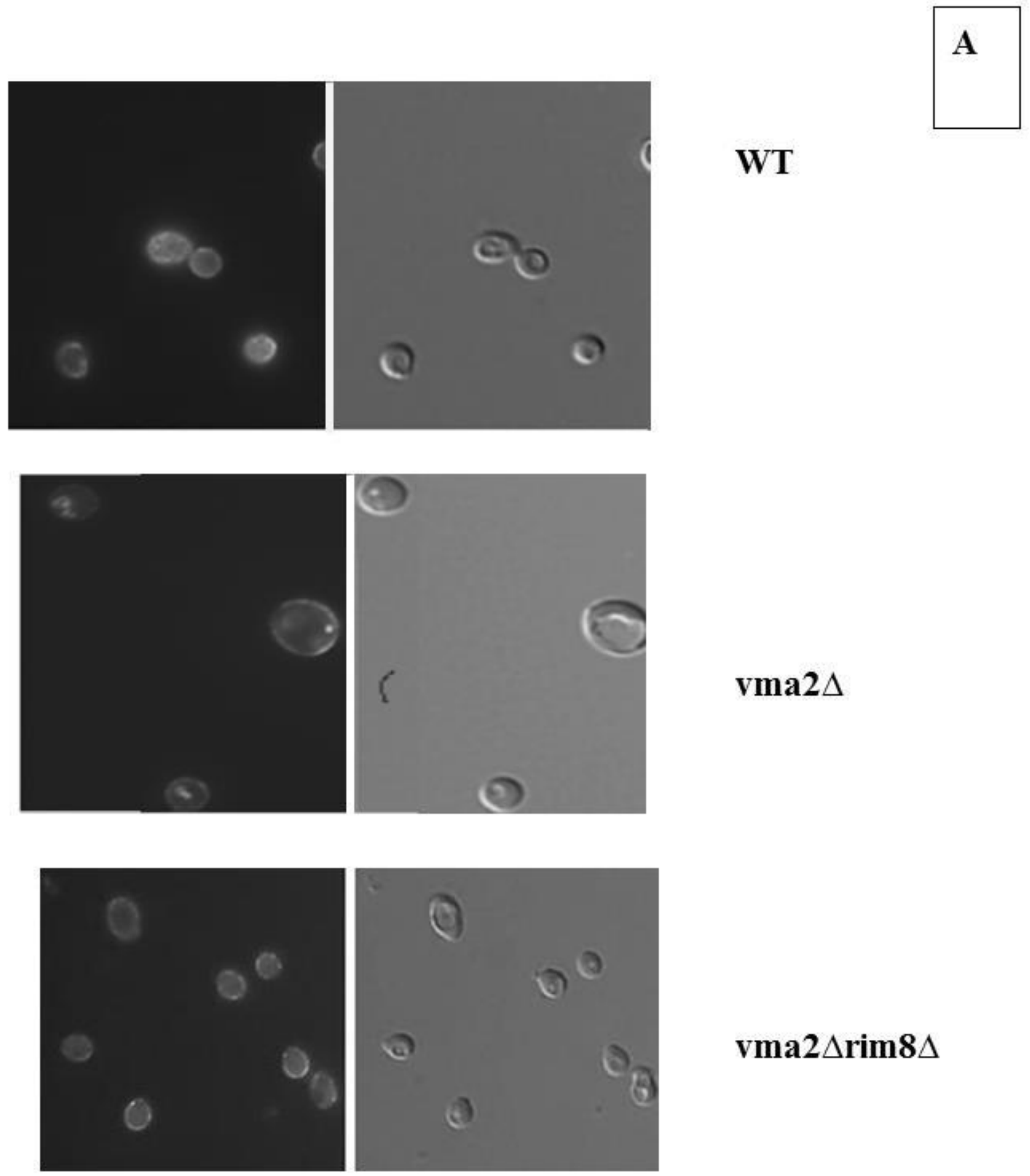
Mislocalization of Pma1 in *vma* mutants and proteins primarily associated with the alkaline ambient pH pathway

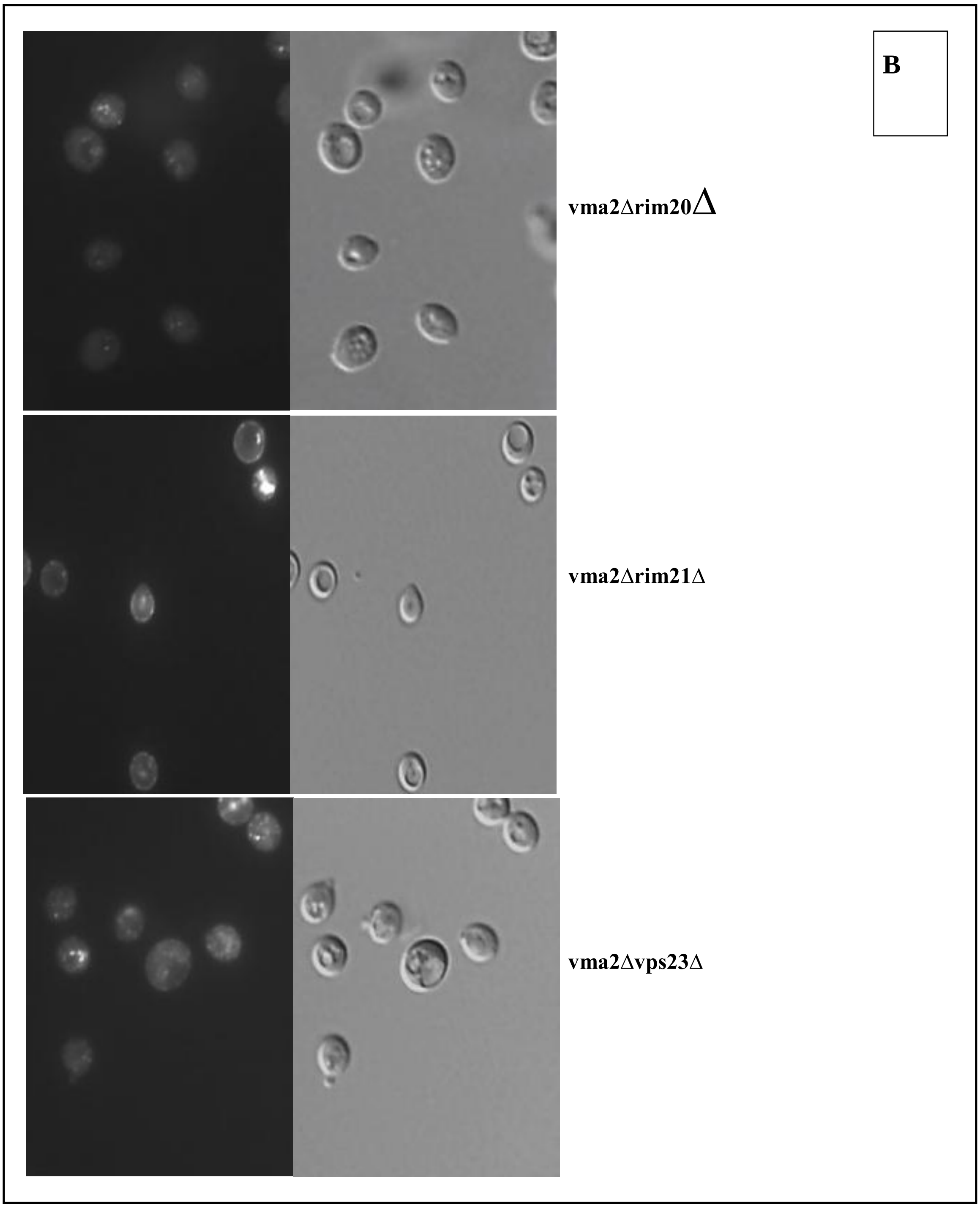
Pma1, being a plasma membrane proton pump is normally found at the plasma membrane in WT cell. Previous laboratory work showed that under conditions in which the V-ATPase is compromised, such as in *vma* mutants, Pma1 is internalized.[21] It was also shown via experimentation that Rim8 and Rsp5 were required for Pma1 internalization.[21] Rim8 brings the E3 ligase Rsp5 to the plasma membrane to ubiquitinate the Pma1 in preparation for internalization. Rim8 is known for its main role in the ambient pH pathway where it plays a role of an adaptor which facilitates the ubiquitination of plasma membrane transporter Rim21 by the ligase Rsp5.[35] If other elements of the Rim pathway are required, then we predict that when they have been deleted then the internalization of Pma1 in the *vma* mutant will be inhibited and the Pma1 will remain at the plasma membrane. The localization of Pma1 in *vma* double mutants was monitored using immunofluorescence and compared to that of the single mutants **(Fig. 10)**. The WT and the *vma2*Δ cells were used as controls. In the WT cells, where V-ATPase is fully functional, the Pma1 is not internalized and is found at the plasma membrane. In the *vma2*Δ cells it has been seen that Pma1 is endocytosed and found in vacuole of vacuolar protease-deficient cells[24] and appears to be in earlier endocytic compartments of protease replete cells.[24] We found that in the single mutants of the ambient pH players: *rim21*Δ*, rim20*Δ*, vps23*Δ *and rim8*Δ*,* Pma1 stayed at the plasma membrane (data not shown), this was expected because V-ATPase is fully functional in these mutants and thus Pma1 internalization is not expected.

The results showed the controls were as expected. In the wild type cells, Pma1 remained at the plasma membrane, the *vma2* Δ mutant showed that Pma1 is internalized.

The *vma2*Δ*rim8*Δ, did not show internalization of the Pma1, it stayed at the plasma membrane, this result supports previous unpublished work that was carried out in the laboratory, in that Rim8 is required for the internalization of Pma1 and in its absence (as seen in the double mutant) Pma1 fails to internalize. The *vma2*Δ*rim21*Δ, *vma2*Δ*rim20*Δ, *vma2*Δ*vps23*Δ double mutants all showed internalization of the Pma1, a phenotype that is similar to that seen with the *vma2*Δ mutant, this suggests that the proteins Rim20, Rim21 and Vps23 are not required in the internalization of Pma1. These proteins may not be playing a role in the Pma1 internalization pathway.

Figure10. Mislocalization of Pma1 in *vma* mutants and proteins primarily associated with the alkaline ambient pH pathway





<u>Figure 10</u><u>. Mislocalization of Pma1 in *vma* mutants and proteins primarily associated with the alkaline ambient pH pathway</u>
Pma1 was localized via immunofluorescence microscopy in wild type (WT) and vma mutants of the external pH pathway. Indirect immunofluorescence was used to produce the fluorescent images in the panels to the left. The cells had been (stained) with the primary monoclonal antibody against Pma1 and the secondary Alexa fluor 488-conjugated antibody. The panels to the right were imaged using Differential Interference Contrast (DIC). Both panels A and B were treated under the same conditions as detailed in the Materials and Methods.

The results of Rim21 and Vps23 in the growth assay **(Fig.9)** were consistent with those seen in the immunofluorescence **(Fig.10)** where the deletion of these two proteins does not affect the expected result of Pma1 localization. In this case the internalization of Pma1 in *vma* double mutants and its maintenance at the plasma membrane in single mutants of *vps23*Δ and *rim21*Δ was seen. *vma2*Δ*rim20*Δ shows a negative growth phenotype, this double mutant grows worse than the single mutants, this suggests that Rim20 is affecting the cell when V-ATPase is not functional. *vma2*Δ*rim20*Δ showed a negative synthetic growth phenotype **(Fig.9)**, like that observed with *vma2*Δ*rim8*Δ. Thus we expected that the protein Rim20 was needed for Pma1 internalization and thus its absence (in double mutant) would result in Pma1 being retained at the plasma membrane, as is observed with the *vma2*Δ*rim8*Δ mutant. However, it must be noted that this growth phenotype cannot be accounted for by failure to internalize Pma1, since the immunofluorescence data in **(Fig.10)** shows that deletion of Rim20 has no observable effect on Pma1 localization. In *vma2*Δ*rim20*Δ mutants the Pma1 was still internalized. This result suggests that Rim20 is needed for cell survival but is not required for the internalization of Pma1.

Section B: To understand how Rim8 interacts with the P-type plasma membrane proton pump Pma1- leading to the ubiquitination and endocytosis of Pma1

The internalization of plasma membrane proteins often requires their ubiquitination by a HECT-type ubiquitin ligase.[27] The HECT-type ligases interact with substrate proteins via WW domains in the ligase and PY domain in the substrate protein.[28] It is through this interaction that the ligase is able to recognize the lysine residues in the protein and ubiquitinate the protein at these sites.[26] In the event that the protein lacks the PY domain, an adaptor protein (also known as ARTs) may be used. These ARTs bring the ligase in close proximity with the protein that requires ubiquitination.[29] There are several ART proteins and Rim8 is one such ART protein.[29] In the endocytosis of Pma1 we hypothesize that Rim8 is translocating both the ligase Rsp5 and itself to the plasma membrane in order to ubiquitinate Pma1 and mark it for internalization. As seen in other plasma membrane proteins (such as Rim20)[35], there must be a sites of interaction between the plasma membrane protein and the adaptor. Here we aim to give further insight into the possible site of interaction of the Rim8 and further still- to find which cytosolically exposed terminus on Pma1 is the point of Rim8 interaction with the plasma membrane pump.

2-hybrid assay to look for interaction between the CT and the NT of Pma1 with full length Rim8

The 2 hybrid assay was used to detect a possible Pma1-Rim8 interaction, and to determine where Pma1 was interacting with Rim8. The 2-hybrid system was developed in order to identify a physical interaction between two genetically associated proteins.[41] We hypothesized that Rim8 might be binding to the N- or C-terminus of Pma1 because it had been observed that ubiquitination tends to take place at the cytosolically exposed termini of transporters, as is seen with Rim21 in the ambient pH pathway.[35] Pma1 is essential and thus Pma1 deletion mutants cannot be used to study its interaction with Rim8, thus we carry out a 2-hybrid with the termini of Pma1.

First we isolated the termini of Pma1 via PCR, producing a NT (1-111) fragment and a CT (875-918) fragment. With these two pieces we would be able to observe which termini full length Rim8 interacted with. We inserted the Pma1 fragments into the plasmid pACT vector, resulting in two pieces: pACT -Pma1NT and pACT- Pma1CT. Full length Rim8 was inserted into the pAS plasmid to produce the piece pAS- Rim8. We transformed pAS+ Rim8 into the MAT a yeast two hybrid strain (PJ69-4a) and pACT-Pma1NT and pACT-Pma1CT each were individually transformed into pj69-4α. **(Fig.11)**. A control cross was carried out, in which 'empty' pACT (with no Pma1 fragment) was crossed with pAS-Rim8, this cross should show no growth on the test plates because there is no protein for Rim8 to interact with in this plasmid. All the crosses were expected to grow on the SD plates that lacked both tryptophan and leucine (SD-Trp-Leu plates) because the plasmid pACT has the selectable marker *LEU2* and the plasmid pAS has the selectable marker *TRP1* thus successful crosses of the two plasmids would grow on plates lacking both tryptophan and leucine.

HIS3 and *ADE2* genes are part of the histidine and the adenine biosynthetic pathways. The *HIS3* reporter gene is expressed under control of the *GAL1/10* promoter in the pj69-4 yeast strains but is very 'leaky', thus the drug 3-aminotriazole (2mM AT) is added to reduce this 'leakiness'.[41] The pj69-4A strain also contains a second reporter, *GAL2-ADE2*, that is very sensitive and has low background.[41] In the presence of a two-hybrid interaction, the strain can grow on plates lacking adenine, hence we tested our crosses on SD-Trp-Leu-Ade, and SD-Trp-Leu-His-Ade as well. When the pj69-4 yeast strains do not have enough adenine (needed for the AMP pathway) they will turn pink in color, this is seen in our results in the SD plates that lack Adenine.. The expression of *ADE2* as a result of two-hybrid interaction causes the cells to become pink or white.

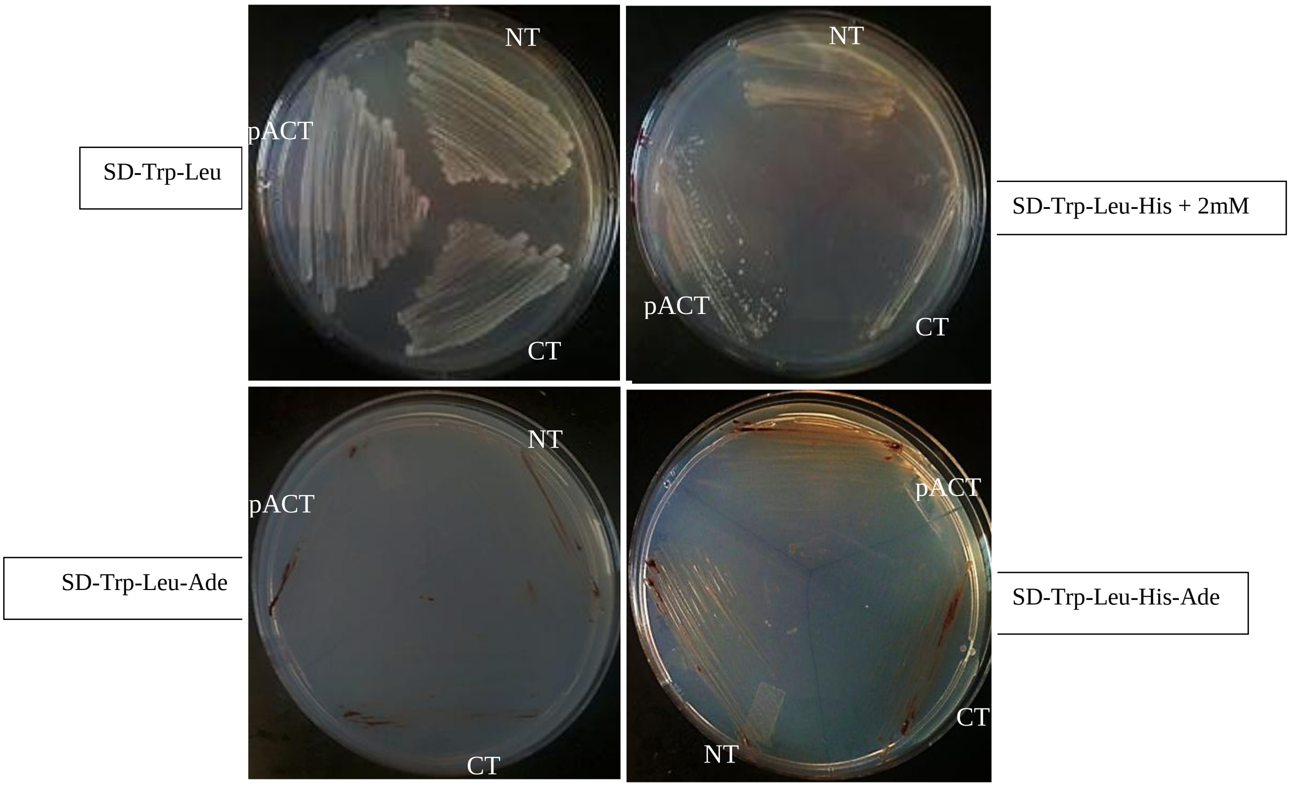


Figure 11: 2-hybrid assay to look for interaction between the CT and the NT of Pma1 with full length Rim8
pAS-RIM8 in PJ69-4a was crossed with either pACT-PMA1NT or pACT-PMA1CT in PJ69-4α. These crosses were grown on SD-Trp-Leu plates and then restreaked onto the plates of interest: SD-Trp-Leu-Ade, SD-Trp-Leu-His+2mM AT, SD-Trp-Leu-His-Ade. The plates were allowed to grow over 6 days and their growth was recorded. pACT only was transformed into plasmid and used as a control.

In the above figure **(Fig.11)** we see that the SD-Trp-Leu plates showed growth on all of the crosses as expected, this is an indication that the crosses were successful. We expected that either the CT or the NT might interact with the full length Rim8 and showing more significant growth on the test plates. With such a result we would have been able to conclude which terminus was the site for Rim8 interaction. However, our results show that both CT and NT show somewhat similar interaction with Rim8 to the empty pACT plasmid. NT may show a bit more growth but it is not significant enough for us to conclude that this is the terminus with which Rim8 interacts.

Western blot analysis of the vma2Δ-rim8-3HA and WT Rim8-3HA under various conditions

As described above, we know that Rim8 is the ART protein that is needed for Pma1 ubiquitination and eventual endocytosis. We hypothesize that it acts as an adaptor protein which brings the ligase Rsp5 to the plasma membrane.[29] Phosphorylation appears to be a method of regulating ARTs as seen with Art1 adaptor (note that ART1 is an adaptor for multiple permeases). Art1 is phosphorylated by the kinase nitrogen permease reactivator 1 (Npr1), a negative regulator of endocytosis, and thus the ART is inhibited.[33] In the case of Art1 it was known that there was more than one form of the ART because it exhibited itself as a double band on an SDS-PAGE gel, when this ART was subjected to phosphatase treatment the doublet collapsed to a single band. This indicated that the Art1 had a phosphorylated form.[33]

Phosphorylation may cause a band to run lower because the negative charges of the phosphate groups that allow the protein to migrate faster through the SDS-PAGE gel. In Art1, when the adaptor was treated to a variety of media conditions and cellular stresses (starvation in minimal media), the ART became hyperphosphorylated, while in nutrient rich media it was dephosphorylated.[33] It was shown that dephosphorylation was a result of TORC1 signaling, which then allowed for the endocytosis of Can1.[33] Thus phosphorylation regulation of the Art1 seems to be under the control of the TORC1-Npr1 negative kinase signaling cascade.[33]

Below **(Fig.12)** is the model of how TORC1-Npr1 may be regulating Art1, and a model for possible phosphorylated regulation of other ART proteins in response to kinases and phosphatases.

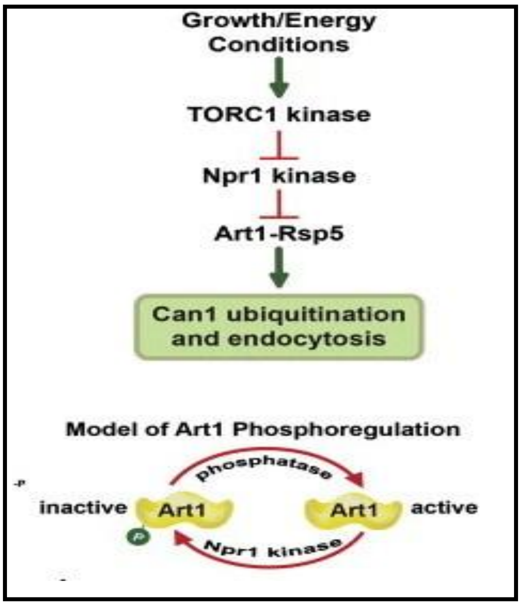


Figure 12: Model of TORC1-Npr1 regulation of Art1 via phosphorylation. In its phosphorylated form Art1 is inactive and endocytosis is inhibited.

In this thesis, western blotting is used to visualize the mobility of the HA-tagged Rim8, (Rim8-3HA) with the hypothesis that as with Art1 a shift might be seen in electrophoretic mobility and such a shift might provide insights into how Rim8 was sensing loss of V-ATPase activity.

The cells were subjected to several conditions and then the Rim8-3HA probed to see if there was a shift in mobility. The WT and the *vma2Δ* cells were used as controls in order to compare them with the treated cells. Both the WT and the *vma2Δ* cells showed multiple banding. Each had three bands, with the lower band being much darker than the upper two bands, and the middle band the faintest of the three bands.

Figure 13: Western blot analysis of the vma2Δ-rim8-3HA and WT Rim8-3HA under various conditions

Cells were grown at 30˚C in YEPD pH5 media and were treated with acetic acid and glucose deprivation. An equal amount of the whole cell lysates was then loaded onto the SDS PAGE gels and the transferred onto nitrocellulose blots. The blots were probed with anti-HA antibodies and then visualized.

*visualization of the samples probed with anti-HA was done via western blotting.

All the cells in the experiment were grown in the YEPD media to 1OD and divided into separate cultures. The WT cells were then treated with acetic acid or were deprived of glucose. To one set of the cells that had been briefly glucose deprived, 2% glucose was later added back. After the cells had been subjected to the various conditions- whole cell lysates were prepared. We wanted to observe whether the low cytosolic pH present in the *vma* mutant[24] (mimicked using the permeable acetic acid) or glucose deprivation (generating acute loss of V-ATPase activity)[19] had an effect on the level or mobility of

Rim8 in the cells. In cells in which V-ATPase is non-functional there is an increase in the cytosolic H^+ concentration, since the pump in not pumping protons into the organelles. In addition, Pma1 is internalized, which means that protons are not being pumped out of the cytosol as efficiently, this would give rise to the acidic pH that is mimicked by the acetic acid. Literature has shown that external pH mobilizes Rim8 in the ambient pH pathway leading to internalization of the plasma membrane transporter. Glucose deprivation was used as a second method of generating loss of V-ATPase activity. It has also been shown that glucose deprivation leads to the disassembly of the V-ATPase and readdition of glucose to these glucose deprived conditions will result in the reassembly of the V-ATPase and restoration of activity.

We would expect that the cells that have been treated with acetic acid and those that have been deprived of glucose may show a more *vma* mutant phenotype. While the cells in which glucose was added back, thus allowing reassembly may show a more WT phenotype. After the whole cell lysate was carried out, equal amounts of the lysates were loaded onto an SDS PAGE gel. Nitrocellulose blot was visualized in order to see the distribution of bands for the HA-tagged Rim8.

In the treated cells the following observations were made. There were some variations in the intensity of the banding patterns in the samples; however, these variations were not significant enough to make conclusive observations. The 5mM acetic acid banding shows that the bands are barely discernible, however previous repetitions of this experiment have shown that the 5mM and 20mM show a similar band pattern.   (Note that the lighter 5mM lane is because of a bubble during transfer.) The signal of the 5mM and 20mM acetic acid is not as strong as that observed in WT, they show a band pattern similar to that of *vma2*Δ. The cells that were deprived of glucose show banding that is comparable to that of *vma2*Δ cells. When glucose was readded, as seen in the following lane, the bands were similar to those of WT, differing from those merely deprived of glucose because their upper band is darker. The dark upper band is also observed in the WT cells. The results of the western blotting experiment were not conclusive and further study must be carried out.

The multiple banding at the size of the Rim8-3HA protein is of interest. Three distinct bands were seen, with the lowest of the bands showing a higher intensity that the other two bands. This banding pattern was seen on all the samples loaded. The bands may signal a possible posttranslational modification, such as ubiquitination or phosphorylation. The size of the bands suggests that they may be too small to indicate ubiquitination of the protein. The samples will be treated with phosphatases to conclude whether the bands are due to phosphorylation or some other signal. If the bands are a result of phosphorylation, then treatment will result in the collapse of the bands

Mutational analysis of *RIM8*

Rim8 is made up of an N-terminus arrestin domain, which is its site for association with the target protein. In addition Rim8 has a C-terminus domain which has a PXY motif , which is the site for association with the ligase Rsp5; the lysine at 521aa, which is the site for Rim8 ubiquitination and the SXP domain which interacts with Vps23.[29]We have shown that both Rsp5 and Rim8 are required for the endocytosis of Pma1.[21] and thus we predicted that the C-terminus of the adaptor is necessary for its role in the endocytosis of Pma1. In our construction of the HA-tagged Rim8 we initially had a genomic C-terminally tagged Rim-3HA. Rim8-3HA was functional however, upon deletion of its C-terminal amino acid 505-542, mutated Rim8-3HA was temperature sensitive and showed poor growth at 30˚C. It was found that mutation of Rim8-3HA results in a disruption of an essential gene that was close to the C-terminus of Rim8. Thus we constructed an in-plasmid Rim8- HA2-Rim8. The N-terminally tagged HA2-Rim8 was used in-plasmid due to complications with the in-genome mutagenesis: there is an essential gene near the N-terminus of Rim8 that was disrupted when we added the tag to Rim8 and thus the product that resulted was incorrect. The in-plasmid mutagenesis gave us the correct product- HA2-Rim8. After adding the HA-tag to the N-terminus of Rim8 we then inserted Rim8-2HA into the plasmid pRS316. Then we used PCR mutagenesis (see Materials and Methods) to remove amino acids 505-542(C-terminal end of Rim8). This truncated Rim8 (referred to as *rim8*-mut) was then used in the following experiments. We added our wild-type and mutant RIM8 plasmid to *rim8*Δ and *rim8*Δ*vma2*Δ via transformation.

The truncation of Rim8 was confirmed via sequencing. **Fig. 14** shows the truncation that was made in Rim8.

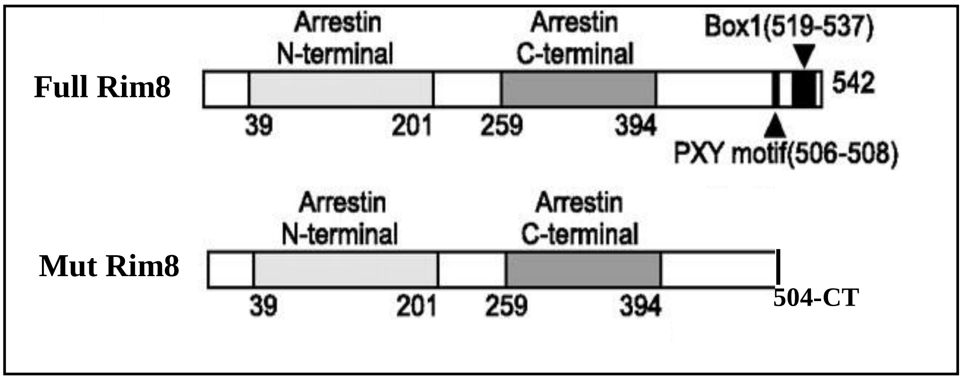


Figure 14: Full Rim8 is shown and below it is seen the mutated Rim8, in which a truncation has been made at the CT from, 505-542aa.

Part 1:Effect of Mutant Rim8 on Pma1 Internalization

Once this truncation was confirmed we then looked to see the effect of the truncated Rim8 in a single mutant- *rim8*Δ and in a double mutant *vma2*Δ*rim8*Δ. We looked at internalization of Pma1 using immunofluorescence (as shown above and described in Materials and Method). **(Fig.15)** WT (Rim8-3HA) as well as *vma2*Δ were used as controls. With the WT cells we know that the V-ATPase is active and thus there is no Pma1 internalization, Pma1 is seen at the plasma membrane. The *vma2*Δ cells in which V-ATPase is non-functional have been seen to show endocytosis of Pma1, we expect to see lower concentrations of Pma1 at the plasma membrane as well as internalized Pma1.

Figure 15: Pma1 was localized via immunofluorescence microscopy.

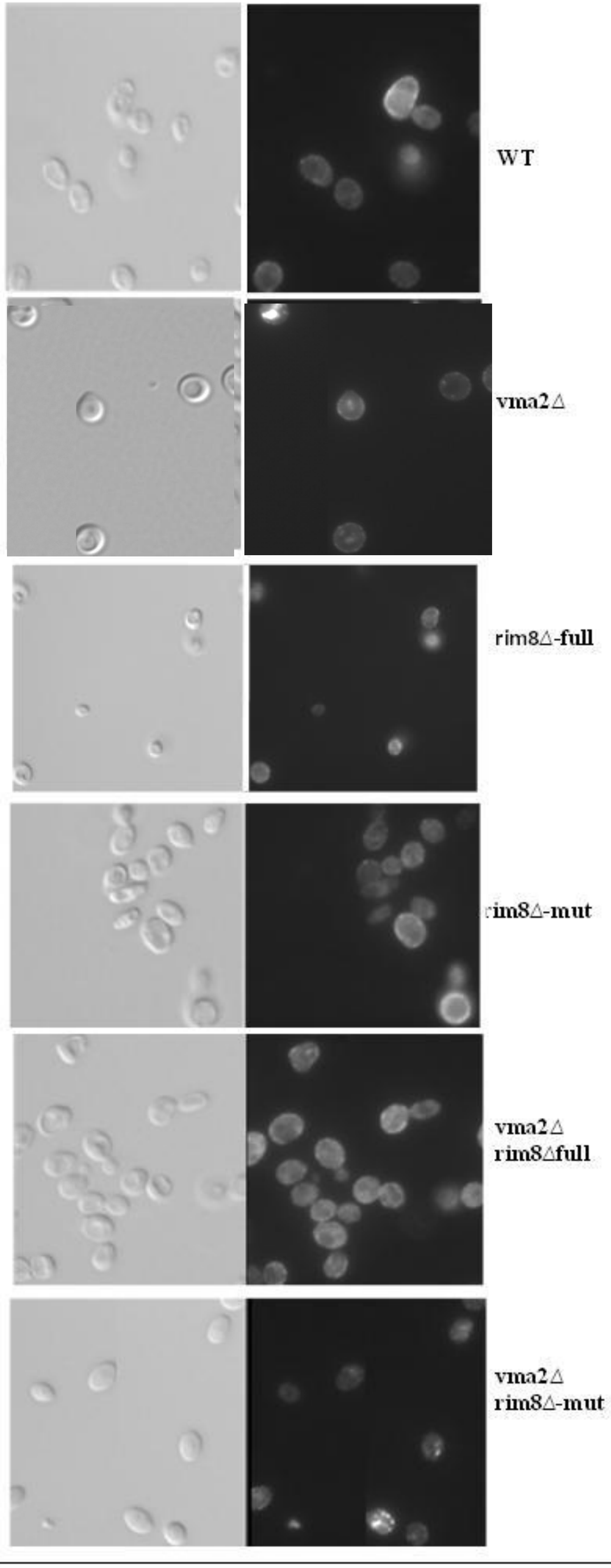
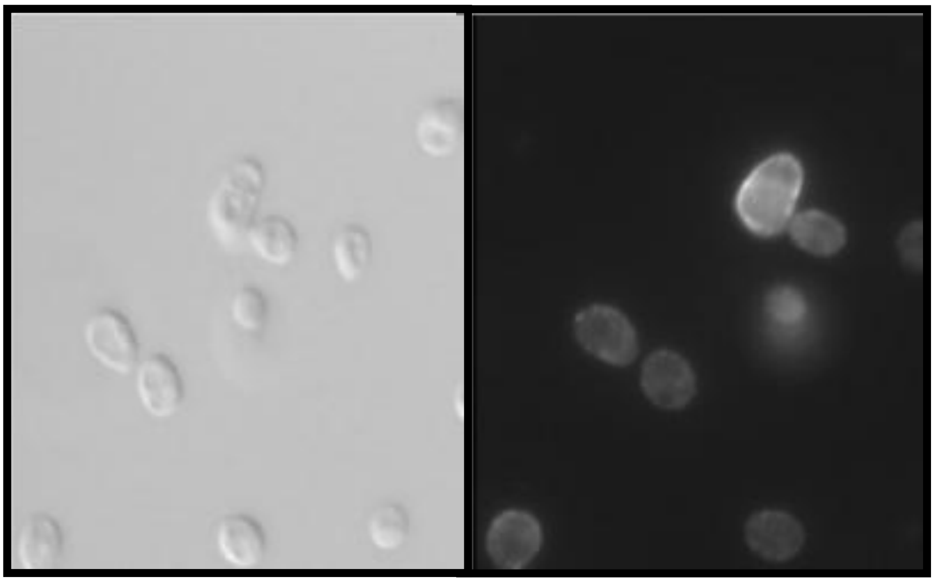
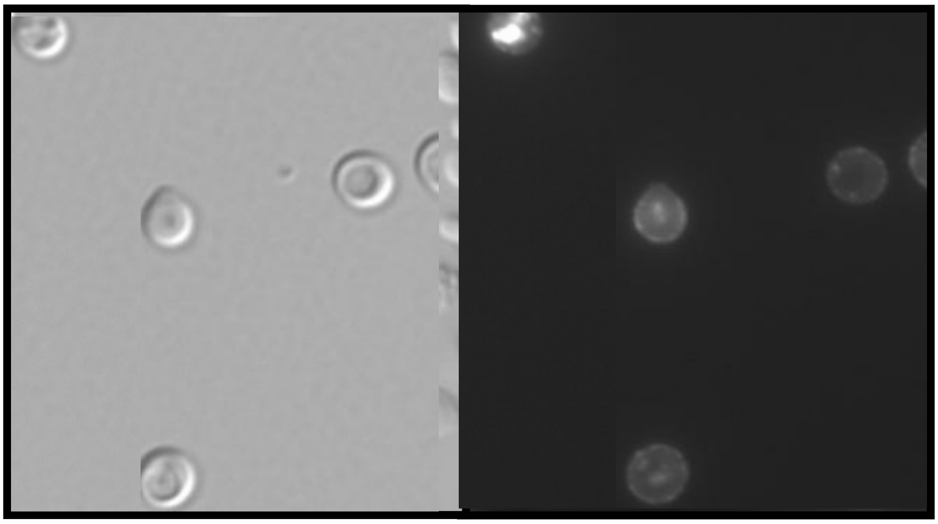


Figure 15: Pma1 was localized via immunofluorescence microscopy. Seen above. Indirect immunofluorescence was used to produce the fluorescent images in the upper panels. The cells had been (stained) with the 1˚ monoclonal antibody against Pma1 and the 2˚ Alexa fluor 488-conjugated antibody. The lower panels were imaged using Differential Interference Contrast (DIC).

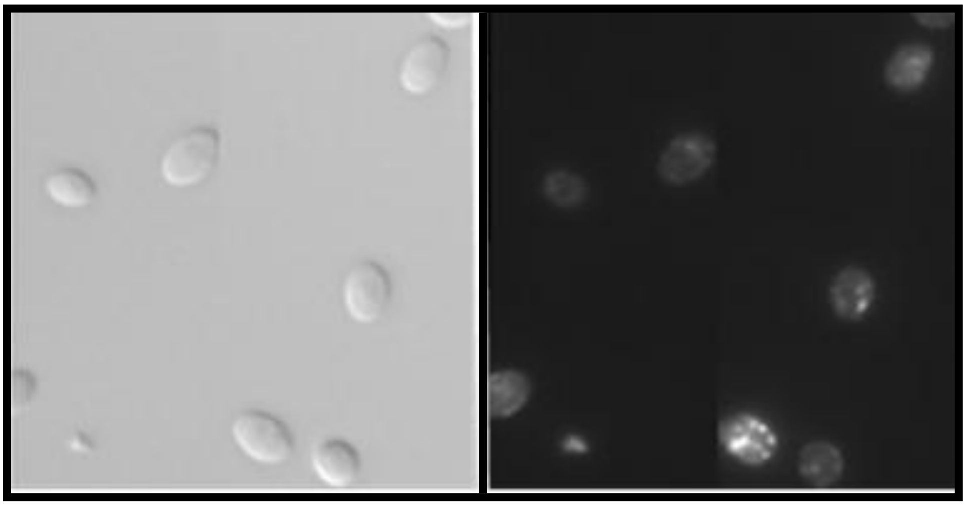
Figure 16. Seen below is an enlargement of WT and vma2Δ and vma2Δrim8Δ-mut*RIM*8 cells from Fig.15



WT



vma2Δ



vma2Δrim8Δ-mut *RIM8*

We expected that the full Rim8 would complement the *rim8*Δ mutation and result in a mutant with a *vma2*Δ phenotype and Pma1 internalization. In the mutant with the truncated Rim8, we expected that if the Rim8 were non-functional, then Pma1 would be retained at the plasma membrane. However, if the Pma1 was seen to be internalized this would have several implications. The results yielded by the immunofluorescence assay are seen above in **(Fig.15).**

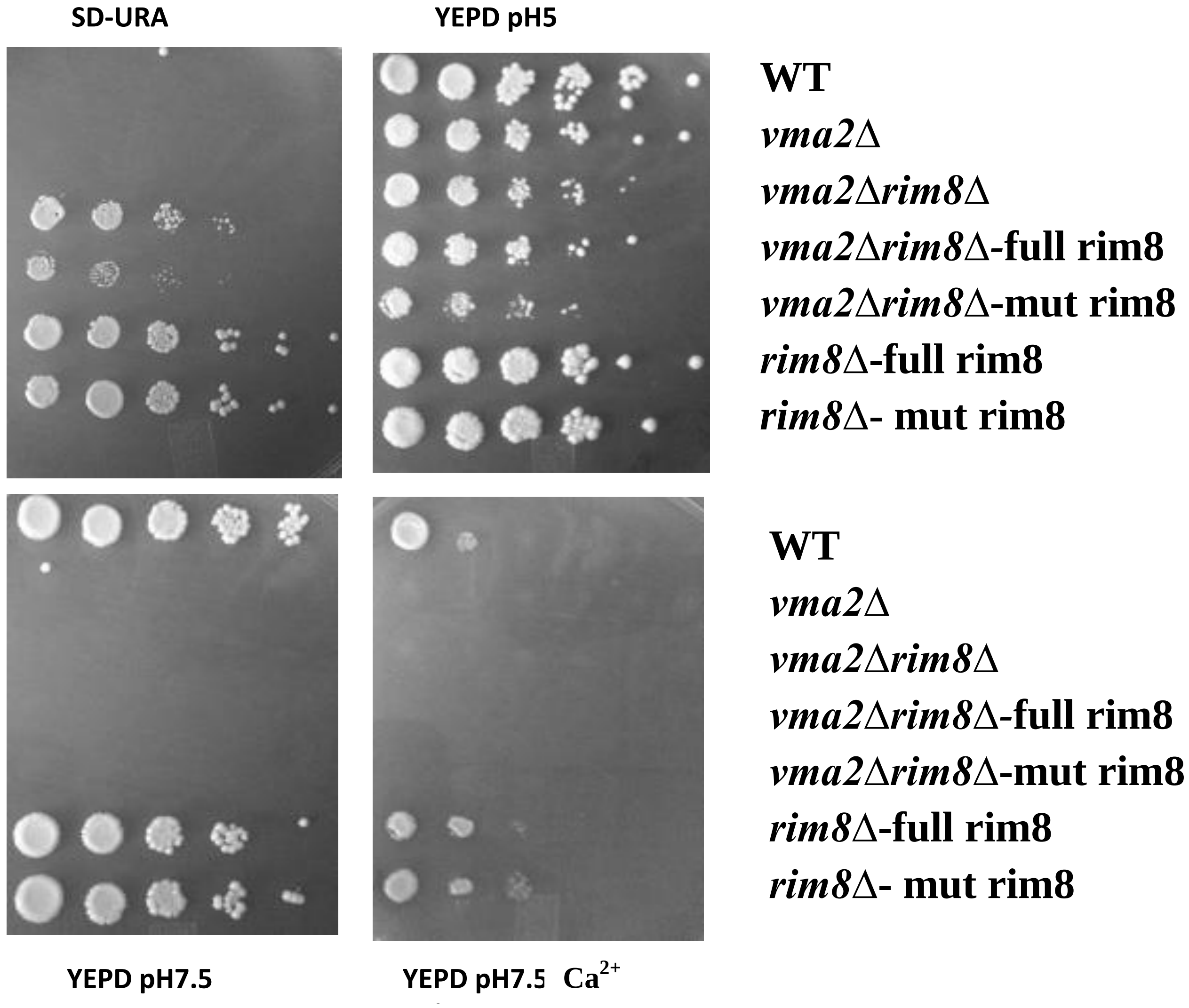
The controls yielded the expected results. WT cells showed Pma1 localized at the plasma membrane as V-ATPase is functional, the *vma2*Δ cells showed that Pma1 was internalized, as has been shown when V-ATPase is non-functional. *rim8*Δ*-full* and *rim8*Δ*-mut* (truncated Rim8) both showed Pma1 at the plasma membrane and this was expected because V-ATPase is not disrupted in these mutants and is fully functional, thus there is no endocytosis of Pma1, rather these cells show a phenotype that is more similar to that of WT cells than that of *vma2*Δ cells. The *vma2*Δ*rim8*Δ*-full* showed that Pma1 was internalized, this is consistent with the results of a *vma2*Δ mutant that has a functioning Rim8, thus we suggest that the HA-tagged RIM8 has functionally replaced the deleted Rim8 in this *vma2*Δ*rim8*Δ mutant. The *vma2*Δ*rim8*Δ*-mut* (truncated Rim8) also shows that Pma1 is internalized. Further study must be carried out to test the mechanism behind the internalization of Pma1 in *vma2*Δ*rim8*Δ*-mut*.

Part2: Looking at the Effect of Mutant Rim8 on Growth

We also checked the growth phenotype that resulted from the truncated Rim8. As shown above **(Fig.9)**, *vma2*Δ*rim8*Δ grows worse than either of the single mutants (*vma2*Δ or *rim8*Δ). Using the dilution assay described above we will be able to determine whether *vma2*Δ*rim8*Δ-mutated rim8 yields a growth phenotype that grows poorer than its single mutants or if the presence of truncated Rim8 complements or partially complements the *rim8*Δ mutant.

We used SD-URA plates as a control because only the cells containing the plasmids should grow on these plates. YEPD plates were used as the test plates, our results were taken from the growth on YEPD pH5 plates, as this is the optimum pH for *vma* mutants. YEPD pH7.5 is another control plate on which none of the *vma* should grow, as it has been previously shown that the mutants fail to thrive at this pH. *rim8*Δ transformed with full RIM8 was expected to have better growth than *vma2*Δ*rim8*Δ,as the single mutant had shown better growth than than the double mutant **(Fig 9 above)**. *rim8*Δ has no growth defect because V-ATPase is fully functional and thus it would show better growth than those strains that exhibit a negative synthetic growth phenotype. We would observe how the *vma2*Δ*rim8*Δ-full *RIM8* and the *vma2*Δ*rim8*Δ- mutated *RIM8* grew.

(Fig.17) below shows the results of the growth assay, it must be noted that the assay was recorded after 3 days, because these cells would have been overgrown if left at 30˚C for longer.



<u>**Figure 17:** Looking at the Effect of Mutant Rim8 on Growth</u>
Each of the strains, WT, *vma2*Δ, *vma2*Δ*rim8*Δ, *vma2*Δ*rim8*Δ-full rim8, *vma2*Δ*rim8*Δ- mutated rim8, *rim8*Δ-full rim8 and *rim8*Δ-mutated rim8, were grown to 1OD and were diluted in 10-fold serial dilutions and then spotted onto the selected YEPD plates as well as SD-URA and incubated over 3 days at 30˚C.

The results show that WT grew the most robustly, as was expected, as can be seen on the YEPD pH5 plate. *vma2*Δ grew more poorly than the WT and the double mutants *vma2*Δ*rim8*Δ showed growth that was poorer than both WT and *vma2*Δ*.* As expected *rim8*Δ-full rim8 grew with no discernible phenotype and *rim8*Δ-mut rim8 seemed to grow slightly less than *rim8*Δ-full rim8, but overall had robust growth. The *vma2*Δ*rim8*Δ showed the slowest growing phenotype, the *vma2*Δ*rim8*Δ alone showed poorer growth than *vma2*Δ*rim8*Δ-full *RIM8* but better growth than *vma2*Δ*rim8*Δ-mut *RIM8. vma2*Δ*rim8*Δ-mut rim8 showed the poorest growth, suggesting that the truncated Rim8 does not fully compensate for full Rim8, in fact since is shows poorer growth than *vma2*Δ*rim8*Δ one may suggest that it may have an adverse effect on the cells. This is discussed in future sections. The control plates were as expected- on the SD-Ura plates, only the cells containing the plasmid grew, and none of the *vma* mutants grew on the plates that had pH7.5 and those that had pH7.5 and calcium.

Discussion

Preliminary data from the laboratory showed that our pathway of interest- that of Pma1 internalization, requires the both the ART protein Rim8 and the E3 Ubiquitin ligase Rsp5, proteins also required in the ambient pH pathway.[21] We thus propose that there may be crosstalk between these two pathways. Thus we hypothesize that the internalization of Pma1 (in *vma* mutants) with the aid of Rim8, Rsp5 and other key players is necessary for homeostatic pH control which allows the cells with compromised V-ATPase to grow. The interdependence between Pma1 and V-ATPase had been shown previously in *Martinez-Munoz and Kane*. [24]

Current work in the laboratory has shown that in *vma* mutants without the adaptor Rim8 and the E3 ligase Rsp5, Pma1 is not be ubiquitinated and is retained at the plasma membrane. In the mutants in which Pma1 fails to internalize, the cells show poor growth.

<u>Do key players of the Alkaline Ambient pH pathway show a growth phenotype in *vma2*Δ mutants?</u>

Here the growth assay was used to see if the deletion of the proteins that were seen to be key players in the ambient pathway resulted in a growth defect in *vma* mutants. These deletions were done in *vma* mutants, resulting in double mutant strains, because Pma1 internalization is observed in *vma* mutants.[24] Added to which, it had been observed that the failure to internalize Pma1 in *vma* mutants resulted in a negative growth phenotype.[21]

In **Fig.9** we see that *vma2*Δ*rim21*Δ and *vma2*Δ *vps23*Δ show no growth defect showed a synthetic negative growth. The above results suggest that the 7TM protein

Rim21, found downstream of Rim8[35], is not involved in the internalization of Pma1. Vps23 did not show a phenotype as well, suggesting that it is not participating in the pathway that results in mislocalization of Pma1. It is known that Vps23, an ESCRT 1 subunit[35] found at the late endosome interacts directly with Rim8 via the Rim8 SXP motif and the ubiquitin E2 variant (UEV) domain.[35] It must be noted that Vps23, is found downstream of Rim8 in the pathway. This data suggests that upstream proteins in the ambient pH pathway are neither trigger nor response to a signal coming from loss of V-ATPase activity that results in activation of Rim8 and the eventual internalization of Pma1. Rim20 yielded an interesting result in that its negative growth phenotype suggested that Rim20 might be required for Pma1internalization. Rim20, found downstream of Rim8, is different from the other two proteins that were tested in that it is found downstream of Rim8 in the ambient pH pathway where it functions as a protease substrate-recognizing adaptor.[35] The negative growth observed in *vma2*Δ *rim20*Δ is also seen with *vma2*Δ *rim8*Δ and *vma2*Δ *rps5-1* which have shown that Rim8 and Rsp5 are essential in Pma1 internalization in published lab data.[21] In them, Pma1 is retained at the plasma membrane.[15]

Using the same double deletion strains, we carried out immunofluorescence experiments so as to detect the localization of Pma1 in these mutants. Results show that *vma2*Δ*rim21*Δ and *vma2*Δ *vps23*Δ allowed for the internalization of Pma1 **(Fig10)**. They showed the mislocalization of Pma1 as seen in *vma* mutants. The *vma2*Δ*rim20*Δ mutant showed endocytosis of Pma1 similar to that seen in *vma* mutants, a result that is not expected from the growth assay **(Fig.9)**. Growth assay had shown that the double mutant showed a negative growth phenotype that was similar to that seen with *vma2*Δ*rim8*Δ, suggesting that the protein is essential for the internalization of Pma1. This lack of Pma1 retention at plasma membrane raises questions about the role of Rim20 in the mislocalization of Pma1. In the ambient pH pathway Rim20 is found downstream of internalization of Rim21, where it signals to Rim13 for the cleavage of Rim101. It is possible that in the Pma1 internalization pathway that Rim20 plays a similar role, that is leading to the activation of conditions that allow the cells to survive at the high cytosolic pH created by the loss of V-ATPase and internalization of Pma1. In such a role, Pma1 internalization might occur independently of Rim20 and if Rim20 is absent the mutant will not develop the correct conditions for its robust survival, hence the negative growth phenotype seen with the *vma2*Δ*rim20*Δ mutant. Given that Rim20 binds to the ESCRTIII complex,[46] it may be suggested that ESCRTIII function is required for *vma* mutants to survive at high pH but not for Pma1 mislocalization.

Martinez-Munoz and Kane showed that upon loss of V-ATPase, cytosolic pH is much lower than expected.[24] V-ATPase acidifies vacuoles by pumping protons from the cytosol into the vacuole. Loss of V-ATPase makes the cytosol more acidic and Pma1 internalization increases this acidity because there is a decrease of protons pumped out of the cell due to loss of Pma1 at the plasma membrane. The cytosolic pH in *vma* mutants is lower than expected and vacuolar pH is higher than in WT cells, yet the cells are able to survive. It is plausible that the internalization of Pma1 activates Rim20 and it brings about a cascade of events (involving yet unknown players) that aid in cell survival. Consistent with such a mechanism, *vma2*Δ*rim20*Δ does not result in lethality, it results in conditions that cause poor cell growth.

Overall these results suggested that not all members of the ambient pH pathway are required for the endocytosis of Pma1- the idea that there is crosstalk between the two pathways seems unlikely. However Rim8 is now seen to have two independent roles on Rim21 and Pma1, this is supported by *Nikko et al.*[28] in which it is seen that Art1 along with other ARTs have more than one target transporter and in addition they show a redundancy in that two ARTs (e.g. Art1 and Art2 with transporter Tat2) act on the same transporter. Our results support that it is highly likely that Rim8 is an adaptor that is found in at least two pathways.

What is the signal that results in the activation of Rim8?

It has been observed that Art1 is phosphorylated. Phosphorylated Art1 is inactive and not able to bring about the ubiquitination of its target plasma membrane transporters.[33] Thus Art1 dephosphorylation is the signal that allows it to recruit Rsp5 and result in the endocytosis of transporters such as Can1.[33] *MacGurn et al.* (2011) were able to show that Art1 is indeed modified by phosphorylation by looking at the adaptor's electrophoresis mobility shifts and then treating the cells with phosphatase. Here we used western blotting **(Fig. 13)** to visualize the mobility shift of HA-tagged Rim8 in cells that had been treated with various conditions. Three distinct bands were seen with Rim8-3HA, with the lowest of the bands showing a higher intensity that the other two bands. This banding pattern was seen on all the samples loaded. It must be noted that Art1 also ran as multiple bands (doublet) on the western blot. Though all the Rim8-3HA samples showed three bands, independent of the conditions they had been treated with, the intensity of the bands varied. WT cells and the cells that had been initially deprived of glucose and then had glucose readded to them both showed darker lower and upper bands than *vma* mutants. Glucose deprivation results in the disassembly of the V-ATPase (acute loss of function), creating a *vma* like mutant, however V-ATPase is able to reversibly reassemble upon readdition of glucose.[47] Thus these cells that had glucose readded had functional V-ATPase and their banding similarity to WT cells is expected. The cells that were deprived of glucose (V-ATPase disassembled) showed a banding pattern that was similar to that of *vma* mutants, as expected. Some of the cells were treated with acetic acid, at 5mM and at 20mM concentrations. Acetic acid creates a low cytosolic pH (mimicking the cytosolic pH of *vma* mutants), thus it was not surprising that these cells showed a banding pattern that was similar to that seen in the *vma* mutants. It must be noted that there was no discernible difference on the banding patterns of the two concentrations of acetic acid used. Deprivation of glucose and addition of acetic acid create a cytosolic environment that mimics that of *vma* mutants. It has not yet been determined whether Pma1 is activated under these conditions; with future immunofluorescence experiments we may determine the location of Pma1 in these conditions. The multiple banding pattern suggests that there is a modification on Rim8. We suggest that the modification may be either ubiquitination or phosphorylation, as these modifications have been seen on other ART proteins[28]and on Rim8.[29] However, ubiquitination is not likely because the shift seen in Rim8 is not large enough to account for ubiquitination.

If phosphorylation of Rim8 is represented by the bands and phosphorylation is an inhibitor of Rim8 then the data in (**Fig.12)** suggests that depending on the extracellular conditions, the phosphorylation state of Rim8 may vary, and the signal it transmits to Pma1 may vary. Further study must be conducted to find the identity of this Rim8 modification. If the bands are a result of phosphorylation, then treatment with phosphatase will result in the collapse of the bands and confirmation of Rim8 phosphorylation.

At which point on the proteins are Pma1 (CT or NT) and Rim8 associating with each other

As stated above, previous data obtained in the laboratory showed that both Rsp5 and Rim8 were required for the ubiquitination of Pma1.[21] We wanted to find where Pma1 associated with Rim8, as well as which part of Rim8 is required in its association. We began by testing if either the C (875-918aa) or the N (1-111aa) termini of Pma1 were the sites of Rim8 interaction because predicted structural analysis of Pma1 has shown that the termini of the plasma membrane protein are cytosolically exposed. These ends were chosen because it has been observed in other transporters (permeases such as Rim21 and Can1) that the exposed N-termini are the site of ubiquitination by Rsp5. The 2-hyrbid assay was used to determine if there was a strong physical interaction between full length Rim8 and either of the termini. Such an interaction (seen by growth on selection plates) would suggest that that particular terminal was the site for Pma1 association with Rim8.

Fig.11 shows that the contructs Pma1NT+Rim8 and Pma1CT+Rim8 both showed growth, with Pma1NT+Rim8 showing slightly more growth. However, Rim8+the empty pACT plasmid, the negative control, also showed growth. Therefore we cannot conclusively say that there was interaction between Rim8 and either termini of Pma1.

These results were not able to definitively show which terminal of Pma1 associated with Rim8. It must be noted that the 2-hybrid assay is by no means an exhaustive experiment. Other more precise experiments may be carried out to look for association between the proteins. Such an experiment is discussed further in the following section (Future directions). In addition, the 2-hybrid assay only shows physical interaction between two proteins, in light of the results one must ask whether there is a physical interaction between Rim8 and Pma1. Could there be a linker protein that allows for the two proteins to be in close proximity?

There is evidence to support the association of Pma1 and Rim8 in as yet unpublished work from the lab. A Co-IP was done with comparable levels of Rim8-HA in both wild type and *vma2*Δ mutants.[21] Rim8-HA was immunoprecipitated from both the WT and *vma2*Δ mutants, and coprecipitation of Pma1 was determined by immunoblotting. Both proteins were present, suggesting that Rim8 is bound to Pma1. However, because of the presence of Rim8 in both WT and *vma2*Δ mutants, it appears that the recruitment of Rim8 to Pma1 is not a specific response to V-ATPase loss.[21] It is possible that Rim8 is located near the plasma membrane and a signal is sent to it that modifies the adaptor, making it active and allowing for it to carry out its function. In such a situation, Rim8 in WT cells would be found at the plasma membrane but would be inactive, whereas in *vma* mutants it would be activated. Further study must be carried out.

What is the phenotypic effect of C-terminally truncated Rim8 on growth and Pma1 localization

In the ambient pH pathway it was seen that the C-terminal domain of Rim8 showed the most activity and sites of protein modifications.[29] It is at the C-terminal of Rim8 that ligase Rsp5 binds to Rim8 to be translocated to the plasma membrane in the Rim pathways. We truncated Rim8 removing the amino acids 504-542. We wanted to observe whether the truncated Rim8 was still able to carry out Rim8 function, which would be seen via cell growth assays. After transformation into a *vma2*Δ *rim8*Δ mutant, a negative growth phenotype would suggest that the truncated Rim8 is not able to complement the *rim8*Δ mutation. We also looked at the localization of Pma1 via immunofluorescence. Failure to mislocalize Pma1 in the presence of truncated Rim8 would also suggest that truncated Rim8 has failed to carry out Rim8 function and thus the C-terminal would be deemed necessary for Rim8 function. In **Fig.16** the results of the growth assay are seen. *vma2*Δ*rim8*Δ-full RIM8, in which we had added N-terminally tagged Rim8 to the double delete, did not show a negative phenotype. This is expected because the full length Rim8 on the plasmid should compensate for the *rim8*Δ mutation. We expected that in these strains Pma1 would be endocytosed. **Fig.15** showed that in *vma2*Δ*rim8*Δ-full RIM8, Pma1 is internalized and thus the Rim8 is functional. *vma2*Δ*rim8*Δ-mut RIM8 showed a negative growth phenotype, with poorer growth than the single mutants, this suggests that the mutated Rim8 fails to carry out Rim8 function. We thus expected that Pma1 would be retained at the plasma membrane in these mutants. Unexpectedly, *vma2*Δ*rim8*Δ-mut RIM8 showed internalization of Pma1. This result is unexpected. Both previous results with RIM8 mutations,[29] and the growth assay suggests that mutated Rim8 should be non-functional; however immunofluorescence shows that Pma1 is internalized in presence of mutated Rim8.

In *vma2*Δ*rim8*Δ there is no Rim8 protein present in the cell, differing from *vma2*Δ*rim8*Δ-mut RIM8 in which Rim8 is present but in a mutated form. It is possible that another ART may be working with ART9/Rim8 and would be able to functionally substitute for the mutated Rim8 or work in combination with it. *Nikko et al*. would support this hypothesis as they have shown that there is a functional redundancy amongst adaptors.[28] They showed that several adaptors appear to work on the same transporter, for example, both Art2 and Art8 are adaptors for the transporter Smf1.[28] They demonstrated that these transporters showed sequence homology. Their data suggests that there is homology between Rim8 and Art5. Thus one must question whether in the presence of mutated Rim8, if Art5 becomes the primary adaptor for Pma1 leading to the transporter's mislocalization. However, it would still be puzzling that Art5 does not substitute in a *vma2*Δ*rim8*Δ mutant.

Secondly, it is possible that Rim8 might bind to Rsp5 at a site that is different from the PXY motif and this would allow for it to still translocate to the plasma membrane and bring about endocytosis of Pma1. *Herrador et al*. showed the PXY as the site of Rsp5 binding but the possibility that there may be multiple sites of binding must be explored further. For example, *Herrador* demonstrated two modes of interaction between Rim8 and Vps23. Rim8 may bind Vps23 via the SXP motif or the P(S/T)XP motif, since it appears that these two motifs have the same function- interaction with the UEV domain of Vps23.[29]

This body of work has shown that the other players of the alkaline ambient pH pathway (Rim21, Vps23) do not play a role in the pathway leading to Pma1 internalization. Rim20 seems as though it may play a role that is as yet undetermined. On these findings, it can be surmised that the two pathways do not seem to be cross-linked. We are closer to finding the sites of association between Rim8 and Pma1. The work has shown that there is a posttranslational modification on Rim8 and further study will be able to identify this modification. The thesis adds on to the work of the laboratory that aims to find the pathway of Pma1 mislocalization in response to loss of V-ATPase activity.

Future directions

The anomalous result that we obtained with the double mutant *vma2Δrim20Δ* must be further explored. We will first find proteins that are associated with Rim20 (and proteins of ESCRT III) and begin to delete these proteins in *vma* mutants. We will then look at the growth of the double mutants and the localization of Pma1 in these mutants. This will help us to elucidate the role of ESCRT III and hence the associated Rim20 in Pma1 mislocalization and in *vma* mutants. It may be of interest to measure the changes in pH in the cytosol with the deletion of Rim20 and other associated proteins in *vma* mutants, here we will use the yeast pHluorin pH-sensing system.[24]

Our analysis of the western blot of Rim8-3HA **(Fig.13)** yielded some interesting possibilities for the modification of Rim8. To test whether the modification seen in Rim8 is phosphorylation, we will treat the cells with phosphatase and observe whether the bands collapse. We may also tag ubiquitin with HA and in cells containing Rim8. We would then pull down Rim8 and then probe with HA, we can then compare the banding pattern that we get from this experiment with that of **Fig.13** and determine whether the modification is a result of ubiquitination..On establishing whether ubiquitination is occurring we could then test the conditions used in **Fig.13.** In addition to this we would carry out immunofluorescence microscopy so as to visualize Pma1 localization in these cells.. This will allow us to further build on the results that we have thus far collected in this experiment.

Because the results of the 2-hybrid were inconclusive we will employ an alternative method of determining which terminal of Pma1 is associating with Rim8. Using an experiment conducted by *Lin et al*.[25] as a basis, we will construct a chimera that consists of a non-essential permease such as Mup1, with either the NT or the CT cytosolic tail of Pma1 replacing one of the terminals in the transporter. A GFP tag will be added to the terminal that does not have the Pma1 terminal attached. We will then analyze the localization of this protein in various mutants using fluorescence microscopy. In this experiment, we would expect that if one of the termini is the one that is required for Pma1 interaction with Rim8, then when that terminal is part of the chimera, the transporter will be internalized in *vma* mutants. Alternatively, it is possible that both termini will result in the endocytosis of the permease, suggesting that Rim8 can interact with either terminus.

We must also explore the sites of interaction between Rim8 and the ligase Rsp5. It has been shown that the adaptor brings the ligase and the transporter in close contact because it has sites of interaction with both proteins.[29] Seeing the anomalous results obtained with the mutated Rim8, it may be of interest to mutate specific sites at the C-terminus of Rim8, rather than the full deletion that we carried out in the above work. Initially we would like to mutate the PXY motif of Rim8, as this is the site shown to interact with Rsp5 in activation of the Rim pathway. This will help us in determining whether there are multiple sites of Rsp5 association on Rim8, maybe even nearer to the N-terminus of the adaptor. If it is possible that there are multiple sites of interaction then new strategies will have to be employed so as to determine the site of Pma1 association on Rim8.

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