THE REGULATION OF YEAST HOMOTYPIC MEMBRANE FUSION BY CLASS C ABC TRANSPORTERS

BY

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DISSERTATION

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ABSTRACT

Maintenance of eukaryotic cellular homeostasis requires the fusion of vesicle membranes that is accomplished by a SNARE-mediated mechanism. Membrane fusion is the merger of two lipid bilayers into one continuous membrane. Multiprotein complexes that have been conserved in eukaryotes carry out the basic reactions of fusion. In *Saccharomyces cerevisiae*, homotypic vacuole fusion occurs in experimentally defined phases. Fusion priming does not involve contact between vacuoles but includes the disassembly of complexes of SNAREs on the same membrane (cis) by Sec18p (NSF) and its cochaperone Sec17p (α-SNAP). Tethering requires Ypt7p (a Rab GTPase) and the HOPS effector complex. SNARE complexes, including one R SNARE from a donor vacuole and three Q SNAREs from the acceptor vacuole, are formed in trans during docking of vacuoles. The membranes of the docked vacuoles are drawn together to form the “boundary domain” that resembles flat discs. The outer membranes are not in contact and come together at the boundary membrane to form the vertex ring. The vertex microdomain, enriched in fusogenic lipids and proteins, is the origin of fusion where the outer membranes are joined. Fusion culminates with the internalization of the luminal vesicle and mixing of luminal contents.

The core fusion machinery has been elucidated in liposomes but many regulatory factors are being discovered in the context of the yeast vacuole. Regulatory proteins and lipids as well as lipid modifiers have been described suggesting a complex system that regulates cellular traffic. The ABC transporter superfamily is present in all organisms and is responsible for actively transporting a wide range of substrates across the lipid bilayer. Several class C ABC transporters interact with factors that are
important in the fusion mechanism while others have been implicated in lipid translocation activity. The yeast vacuole contains five ABC transporters of the ABCC1 subfamily: Ycf1p, Bpt1p, Ybt1p, Vmr1 and Nft1. This project sought to identify the role of ABC transporters in membrane fusion as regulators of fusogenic proteins and lipids and their role in the mechanism of remodeling the membrane bilayer as lipid translocators.

Although Ybt1p was originally identified as a bile acid transporter, it has also been found to function in other capacities including the translocation of phosphatidylcholine to the vacuole lumen and the regulation of Ca\(^{2+}\) homeostasis. We found that deletion of YBT1 enhanced in vitro homotypic vacuole fusion by up to 50% relative to wild type vacuoles. The increased vacuole fusion was not due to aberrant protein sorting of SNAREs or recruitment of factors from the cytosol such as Ypt7p and the HOPS tethering complex. In addition, ybt1Δ vacuoles displayed no observable differences in the formation of SNARE complexes, interactions between SNAREs and HOPS, or formation of vertex microdomains. However, the absence of Ybt1p caused significant changes in Ca\(^{2+}\) transport during fusion. One difference was the prolonged Ca\(^{2+}\) influx exhibited by ybt1Δ vacuoles at the start of the fusion reaction. We also observed a striking delay in SNARE-dependent Ca\(^{2+}\) efflux. Evidence is presented that the delayed efflux in ybt1Δ vacuoles leads to the enhanced SNARE function.

Ycf1p, another ABCC family member that was originally characterized as a Cd\(^{2+}\) transporter, has also been found to physically interact with a wide array of proteins including factors that regulate vacuole homeostasis. Here we examined the role of Ycf1p and other ABCC transporters in the regulation of vacuole homotypic fusion. We found that deletion of YCF1 attenuated in vitro vacuole fusion by up to 40% relative to
wild type vacuoles. Plasmid-expressed wild type Ycf1p rescued the deletion phenotype; however, Ycf1p containing a mutation in the Walker A box of the first nucleotide binding domain (Ycf1p\textsuperscript{K669M}) was unable to complement the fusion defect of ycf1Δ vacuoles. This indicates that the ATPase activity of Ycf1p is required for its function in regulating fusion. In addition, we found that deleting YCF1 caused a striking decrease in vacuolar levels of the soluble SNARE Vam7p, whereas total cellular levels were not altered. The attenuated fusion of ycf1Δ vacuoles was rescued by the addition of recombinant Vam7p to \textit{in vitro} experiments. Thus, Ycf1p regulates fusion through the recruitment Vam7p to vacuolar membranes.

Membrane lipids are organized in an asymmetric fashion across the vesicle bilayer and this asymmetry must be maintained through lipid translocation. The translocation or flippase activity can be monitored using fluorescently labeled lipids. In this study, we describe a FRET-based assay to follow the translocation of the rhodamine-labeled phosphatidylethanolamine (RH-PE) in a real-time assay. We found that there is an ATP-dependant Rh-PE flippase on the vacuole and the ABC transporters Ycf1p, Bpt1p and Ypk9p are not responsible for the translocation activity. We also report that pH and osmolyte concentration do not affect the lipid translocation activity. However, modifying the bilayer fluidity with the addition of chlorpromazine (CPZ) abolished lipid translocation activity and propranolol inhibition of phosphatidic acid phosphatase activity increased the rate of lipid translocation.
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Membrane fusion is a fundamental process in the maintenance of eukaryotic cellular homeostasis. Membrane fusion is the merger of two lipid bilayers into one continuous membrane and the mechanisms have been conserved through eukaryotic organisms. In *Saccharomyces cerevisiae* vacuoles, homotypic vacuole fusion occurs in experimentally defined phases. While the core fusion machinery has been identified, a variety of fusion regulators continue to be identified as more is understood about the biochemical process. Because ATP-Binding Cassette (ABC) transporters have a wide range of substrates including ions, lipids, and peptides and their malfunction has been implicated in a variety of diseases, this dissertation examines their possible roles in regulating homotypic vacuole fusion. In this chapter I present an introduction to the stages and regulators of membrane fusion and an overview of ABC transporters with an emphasis on members from the Class C subfamily.

**Homotypic vacuole fusion and the core machinery**

Eukaryotic cellular homeostasis maintenance requires the trafficking of membrane-bound cargo through the endocytic and secretory pathways using membrane fusion mechanisms that are conserved in all eukaryotes (Jahn and Sudhof, 1999). Vacuoles (lysosomes) from *Saccharomyces cerevisiae* are used to examine the experimentally defined stages and regulators of membrane fusion (Figure 1.1). The first stage, priming, does not involve contact between vacuoles but includes the disassembly of complexes of SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein
receptors) on the same membrane (cis) by Sec18p (NSF) and its cochaperone Sec17p (α-SNAP) (Ungermann et al., 1998b). Next, tethering requires the Rab GTPase Ypt7p and the HOPS effector complex (Mayer and Wickner, 1997). SNARE complexes, including one R-SNARE from a donor vacuole and three Q-SNAREs from the acceptor vacuole, are formed in trans during docking of vacuoles. The membranes of the docked vacuoles are drawn together to form the boundary domain that resembles flattened discs. The outer membranes are not in contact and come together at the boundary membrane to form the vertex ring. The vertex microdomain, enriched in fusogenic lipids and proteins, is the origin of fusion where the outer membranes are joined. Fusion culminates with the internalization of the luminal vesicle and mixing of luminal contents (Wang et al., 2002).

The core machinery and regulators of fusion

The delivery of cargo in a membrane vesicle to its destination is finalized by the fusion of two membranes that is driven by a series of regulated stages. The basic reactions of fusion are carried out by protein complexes that have been conserved in eukaryotes (Jahn and Scheller, 2006). Yeast vacuole fusion requires numerous proteins and regulators (Table 1.1) including the Q-SNAREs Vam3p, Vti1p, Vam7p and the R-SNARE Nyv1p. In addition, vacuole fusion requires the Rab GTPase Ypt7p and its effector complex HOPS, actin, as well as the lipids ergosterol, diacylglycerol, phosphatidic acid, and multiple phosphoinositides (Wickner, 2010).

The final catalysts of fusion are SNARE proteins; however, at the start of the cascade, SNAREs are held in inactive cis-complexes on a single membrane. The
vacuole fusion pathway is initiated when cis-SNARE complexes are dissociated by the NSF homologue Sec18p and its co-chaperone α-SNAP/Sec17p in an ATP-dependent mechanism (Ungermann et al., 1998b). Membranes with activated SNAREs then tether and dock with partner membranes in a mechanism driven by the Rab GTPase Ypt7p and its effector complex HOPS (Mayer and Wickner, 1997; Seals et al., 2000). During the docking stage SNARE complexes form in trans between partner membranes where one membrane donates Nyv1p and the partner membrane donates Vam3p, Vti1p and Vam3p as a preformed 3Q-SNARE complex. Vacuoles are subsequently drawn together and become tightly apposed causing the deformation of vesicles and formation of a specialized membrane-raft microdomain called the vertex ring where the proteins and lipids that catalyze fusion laterally accumulate to create the site of fusion (Fratti et al., 2004; Wang et al., 2003; Wang et al., 2002).

Although the core fusion machinery has been identified, there is a growing collection of regulatory factors that modulate fusion by various means. These regulators include the protein kinase Yck3p (Cabrera et al., 2009), the lipid modifiers Pah1p (Sasser et al., 2012a) and Vps34p (Schu et al., 1993), as well as the Na⁺/H⁺ exchanger Nhx1p (Qiu and Fratti, 2010). Additional regulation may occur through regulating Ca²⁺ transport, phosphoinositide metabolism, or the translocation of lipids across the membrane bilayer.

**Ca²⁺ regulation of membrane fusion**

In exocytosis in specialized cells such as neurons, heterotypic membrane fusion and the subsequent release of the biologically active cargo is regulated by intracellular
Ca$^{2+}$ levels (Kendra et al., 2010). Ca$^{2+}$ is released from vacuoles during the process of homotypic vacuole fusion and was originally thought to bind to calmodulin to trigger the formation of protein complexes and fusion pores at the end of the process (Peters et al., 2001; Peters and Mayer, 1998). Although vacuole fusion is not triggered by a rise in Ca$^{2+}$ levels, there is evidence that Ca$^{2+}$ efflux plays a role by promoting bilayer mixing upon formation of trans-SNAREs (Merz and Wickner, 2004). Although the Q-SNARE Nyv1p acts as a negative regulator of Pmc1p, a vacuolar Ca$^{2+}$-ATPase that pumps calcium into the vacuole (Takita et al., 2001), later investigations found that Ca$^{2+}$ is not necessary for membrane fusion since vacuoles lacking both Pmc1p and Vcx1p, Ca$^{2+}$/H$^+$ exchanger, fuse as well as WT vacuoles (Ungermann et al., 1999); however, they did not measure Ca$^{2+}$ uptake or efflux. Yvc1p is a vacuolar Ca$^{2+}$-ATPase efflux pump, but its activity has not been implicated in the release of Ca$^{2+}$ due to trans-SNARE formation (Palmer et al., 2001). The class C ABC transporter Ycf1p has been shown to interact with Fab1p (Paumi et al., 2007), a PI(3)P 5-kinase that synthesizes phosphatidylinositol-3,5-bisphosphate and is essential for Yvc1 activation in response to hyperosmotic shock (Dong et al., 2010).

**Membrane asymmetry**

Membrane lipids are organized in an asymmetric fashion across the bilayer. The cytoplasmic surface of the eukaryotic bilayer contains an enrichment of phosphoinositides (PI), phosphatidic acid (PA), phosphatidylethanolamine (PE), and phosphatidylserine (PS) while the extracytoplasmic leaflet contains primarily phosphatidylcholine (PC) and sphingomyelin (Bretscher, 1972; Op den Kamp, 1979).
The thermodynamic barrier prevents passive transverse movement of the phospholipids from the inside to the outside (Kornberg and McConnell, 1971). Translocation of lipids is executed by integral membrane proteins that are referred to as lipid “flippases” and “floppases”, which establish lipid asymmetry between the inner and outer leaflets of a membrane (van Meer et al., 2008). ATP-dependent transporters that translocate lipids to the cytoplasmic face are called flippases while those that move lipids to the extracytoplasmic face are called floppases. Scramblase, an energy-independent transporter, eliminates lipid asymmetry important in apoptosis and the blood clotting cascade (Devaux et al., 2008). Translocation of lipids can cause changes in membrane shape that leads to fission or fusion (Papadopulos et al., 2007). Some of the putative flip/floppases belong to the ABC transporter superfamily (Gulshan and Moye-Rowley, 2011). Also, human multi-drug resistance associated protein (hMRP1) has been shown to transport PS analogues in human erythrocytes (Huang et al., 2004; Smriti et al., 2007) and fluorescently labeled PE accumulates in the plasma membrane of yeast deficient in the ABC transporter Yor1p (Decottignies et al., 1998). Together this indicates a potential role for yeast ABC transporters in maintaining lipid asymmetry.

**ABC transporters**

The ABC transporter superfamily is present in all organisms and is responsible for actively transporting a broad spectrum of substrates across the lipid bilayer. ABC transporters are formed from two homologous halves and each half consists of a conserved ABC signature, a nucleotide binding domain (NBD) for hydrolyzing nucleotides for active transport, and a membrane-spanning domain (MSD) consisting of
six transmembrane spans. A more detailed explanation of the structure and function of ABC transporters follows below. The ABCC (or multi-drug resistance associated protein, MRP) subfamily also contains an N-terminal extension composed of five transmembrane spans with an unclear function.

The yeast vacuole contains five ABC transporters of the ABCC subfamily: Ycf1p, Bpt1p, Ybt1p, Ymr1, and Nft1 (Paumi et al., 2009). Each plays a role in detoxifying yeast cells through the transport of glutathione conjugates: Ycf1p transports glutathione conjugated cadmium (Li et al., 1997), Bpt1p transports conjugated bilirubin and free glutathione (Petrovic et al., 2000), and Ybt1p transports bile acids (Sharma et al., 2003). Nft1 and Ymr1 have yet to be characterized for substrate specificity. In addition to transporting cadmium, Ycf1p has been reported to physically interact with various vacuolar proteins that are linked to membrane trafficking and fusion including the PI3P kinase Fab1p (Paumi et al., 2007). The goal of the research presented in this dissertation was to identify the role of ABC transporters in membrane fusion as regulators of fusogenic proteins and lipids and their role in the mechanism of remodeling the membrane bilayer as lipid translocators.

**Function and organization of ABC transporters**

ATP binding cassette (ABC) transporters comprise a large family of membrane proteins that catalyze the active transfer of a variety of solutes across biological membranes (Higgins, 1992). ABC transporters operate in all living cells from bacteria to human and have many functions within the cell. Most of them act as ATP-driven transporters, which use the energy of ATP hydrolysis to pump substrates against a
concentration gradient. However, ABC proteins can also function as ion channels, channel regulators, receptors, proteases and even as sensors of the environment (Higgins, 1995).

A variety of molecules are recognized as substrates ranging from ions, anticancer drugs, phospholipids, peptides, and antibiotics (Dean and Allikmets, 1995; Kuchler and Thorner, 1992). ABC transporters can function as importers and exporters. In bacteria, ABC pumps like LmrA or HlyB export toxins out of the bacterium conferring resistance (Blight et al., 1995; van Veen et al., 2001). ABC transporters are also employed to uptake nutrients like in the maltose importer MalFGK from *E. coli* or the histidine permease HisQMP2 from *S. typhimurium* (Ames and Lever, 1970; Bavoil et al., 1980).

The dysfunction of ABC transporters has been reported in various human diseases such as cystic fibrosis, adrenoleukodystrophy, familial hyperinsulinemic hypoglycemia of infancy, and Stargardt’s disease (Dean and Allikmets, 2001; Dean et al., 2001). A separate class of ABC transporters, P-glycoprotein (Pgp), is implicated in multidrug resistance (MDR). Overexpression of Pgp causes resistance to drugs used in chemotherapy (Gottesman and Pastan, 1993). Another important human ABC transporter is TAP (transporter associated with antigen processing), which translocates peptides from the cytosol to the ER and functions in the presentation of antigens at the cell surface (Lankat-Buttgereit and Tampe, 2002).

The ABC transport family shares a common topology consisting of two membrane-spanning domains (MSD) and two nucleotide-binding domains (NBD). The four domains can be arranged in any possible combination. The subunits may be
formed from separate polypeptides as is the case of several bacterial transporters. Most eukaryotic ABC transporters are expressed as single polypeptides. In half-size transporters each MSD is fused to an NBD. Several other arrangements are also possible but they are less frequently observed.

In addition to the core components, prokaryotic ABC transporters involved in solute uptake make use of a periplasmic binding protein to bind the substrate (van der Heide and Poolman, 2002). The MSDs span the membrane multiple times via putative \( \alpha \)-helices forming the substrate translocation channel through the membrane. The majority of ABC transporters contain twelve predicted \( \alpha \)-helices, but there are examples of ABC-transporters with a different number of \( \alpha \)-helices. Each MSD of the vitamin B12 transporter BtuCDE of \textit{E. coli} consists of 10 \( \alpha \)-helices and the MSDs of human TAP1 and TAP2 are predicted to possess 10 and 9 \( \alpha \)-helices, respectively (Lankat-Buttgereit and Tampe, 2002; Locher et al., 2002). The Class C transporters contain an N-terminal extension consisting of five \( \alpha \)-helices (Paumi et al., 2009). Some of the predicted membrane-spanning \( \alpha \)-helices may not be crucial for substrate translocation but may serve other functions such as membrane insertion or transporter regulation (Higgins, 2001).

The MSD, or the periplasmic binding protein in bacteria, determines the substrate specificity. As the types of substrates of ABC transporters is very broad, the MSDs generally share little homology (Holland and Blight, 1999). The NBDs drive the transport process by hydrolysis of ATP by acting as molecular motors. Amino acids of the NBDs share 25% identity suggesting that even transporters of unrelated function have conserved structure and function of NBDs (Schneider and Hunke, 1998).
The ability to mediate transport of substrates requires a balanced interaction between the MSDs and NBDs (and the periplasmic binding protein where applicable) (Higgins, 2001). The mechanism by which ATP hydrolysis changes NBD conformation and induces changes in MSD conformation for substrate translocation is not well understood. One of the recent focuses on ABC transporter research is to gain insights into the inter-domain communication (Oldham et al., 2008; Wen and Tajkhorshid, 2011).

The majority of the mechanism of how ABC domains work and communicate was gleaned from the characterization of isolated NBDs. Based on their primary structure the NBDs are characterized by five consensus motifs. The Walker A and Walker B motifs are found in all nucleotide binding proteins (Walker et al., 1982). The Walker A motif has the sequence ‘GxxGxGKS/T’, where x is any amino acid. The Walker B motif is less stringent with the sequence ‘Rx(6-8)φφφD’, where φ represents any hydrophobic residue. Some ATP-binding proteins contain an extended Walker B site with the consensus ‘Rx(6-8)φφφDEATSALD’ (Michaelis and Berkower, 1995). The distinguishing characteristic of the ABC family is the ‘C-loop’ with the consensus sequence ‘LSGGQ’, which is unique to ABC transporters and also known as the ABC signature motif (Bianchet et al., 1997; Higgins, 1992; Michaelis and Berkower, 1995). The C-loop is thought to be at the primary point of mutations that cause loss of function in ABC transporters (Hoof et al., 1994). An invariant histidine is placed downstream of the Walker B motif in the so-called switch II region, which is proposed to be involved in the hydrolysis of ATP (Nikaido and Ames, 1999). A conserved Q-loop is located between the Walker A motif and the C-loop. The Q-loop might undergo conformational changes during the catalytic cycle and function as a signal transducer between MSDs
and NBDs (Jones and George, 1999).

**ABC transporters in the yeast *Saccharomyces cerevisiae***

The yeast *S. cerevisiae* was the first eukaryotic organism whose complete genome was sequenced and revealed the presence of 30 ABC protein genes (Decottignies and Goffeau, 1997; Taglicht et al., 1998). Yeast ABC proteins are implicated in a variety of cellular functions ranging from drug resistance, pheromone secretion, stress response, and cellular detoxification. Here, a brief overview of the ABC subfamilies in *S. cerevisiae* will be provided (Paumi et al., 2009).

Based on phylogenetic analysis, yeast ABC proteins were classified into six subfamilies: MDR, PDR; MRP/CFTR, ALDp, YEF3, and RLI subfamily (Decottignies and Goffeau, 1997) and later reclassified as ABCA-ABCG to facilitate correlation of experimental findings between yeast and human ABC transporters (Paumi et al., 2009). The human ABCA subfamily is absent in yeast and there are two genes that do not fit within the current classification system.

Ste6p, which belongs to the multidrug resistance (MDR/ABCB) subfamily, was the first known yeast ABC transporter and is one of the best characterised (Kuchler et al., 1989; McGrath and Varshavsky, 1989). While steady state localization of Ste6p appears to be the Golgi apparatus and endosome-like compartments, it functions at the plasma membrane and mediates ATP-dependent secretion of the yeast *a*-factor pheromone (Kuchler et al., 1993; Kuchler et al., 1989).

The other members of the ABCB subfamily are mitochondrial half-size ABC transporters. Atm1p is localized in the inner mitochondrial membrane with its NBD
facing the mitochondrial matrix (Leighton and Schatz, 1995) and functions as an exporter of Fe/S precursors (Kispal et al., 1999). Two other members have been found in the mitochondrial inner membrane, Mdl1p and Mdl2p. Mdl1p has been identified as an intracellular peptide exporter, while Mdl2p function has not been defined so far (Young et al., 2001).

The adrenoleukodystrophy family (ALD/ABCD) consists of two members, namely the PXA1/PAR2/SSH2/PAL1 (Shani et al., 1995) and PXA2/PAT1/YKL741 (Shani et al., 1996) genes. Both of them are half size transporters and putative orthologues of human ALDP and PMP70. They import long-chain fatty acids into the peroxisomes for subsequent β-oxidation (Hettema et al., 1996).

The pleiotropic drug resistance (PDR/ABCG) subfamily is the largest subfamily of ABC-proteins in S. cerevisiae. Pdr5p and Snq2p mediate PDR through an ATP-dependent drug efflux pumping hundreds of structurally and functionally unrelated compounds. They are functional homologues of mammalian P-gp (Balzi and Goffeau, 1995).

The best-characterized members of the multidrug resistance related protein (MRP/CFTR or ABCC) family are Yor1p and Ycf1p (Katzmann et al., 1999; Szczypka et al., 1994). Overexpression of Yor1p confers resistance to oligomycin, reveromycin A and organic anions (Cui et al., 1996; Li et al., 1996). Yor1p has also been implicated in the translocation of fluorescent phosphatidylethanolamine (Decottignies et al., 1998). Ycf1p plays a role in the detoxification of the cytosol by translocation of metal ions such as cadmium and arsenic as glutathione-S-conjugates and metal-containing peptides into the vacuole (Li et al., 1996; Szczypka et al., 1994). Bpt1p was classified as a
bilirubin translocator and also plays a similar role as Ycf1p (Petrovic et al., 2000). The ORF YLL048 encodes a full-length transporter (Ybt1p) that was reported to translocate bile acids into vacuoles (Ortiz et al., 1997). An integrated split-ubiquitin membrane yeast two-hybrid (iMYTH) analysis has shown that Ycf1p physically interacts with the PI3P 5-kinase Fab1p, which uses PI3P to produce PI(3,5)P₂, as well as the Rho1p nucleotide exchange factor, Tus1p (Paumi et al., 2007). Ycf1p has also been found to physically interact with Rho1p, a GTPase associated with actin dynamics (Lee et al., 2011). To obtain insight into the role of homotypic vacuole fusion regulation by class C ABC transporters, the yeast ABC transporters Ycf1p, Bpt1p and Ybt1p have been studied in detail in this PhD thesis.
## TABLES AND FIGURES

Table 1.1. Fusion factors.

| SNAREs soluble N-ethylmaleimide-sensitive factor attachment protein receptor | Nyv1 – R-SNARE  
Vam3 – Qa-SNARE  
Vti1 – Qb-SNARE  
Vam7 – Qc-SNARE |
|-------------------------|------------------|
| SNARE chaperones        | Sec18 (NSF) – AAA ATPase  
Sec17 (a-SNAP) – Sec18 co-chaperone  
HOPS – homotypic fusion and protein sorting complex. A heterohexameric complex composed of Vps11, 16, 18, 33, 39 & 41 |
| GTPases                 | Ypt7 – the vacuole Rab  
Rho1 – a Rho family GTPase |
| Lipids                  | Ergosterol - yeast cholesterol  
DAG - diacylglycerol  
PA - phosphatidic acid  
PC - phosphatidylcholine  
PE - phosphatidylethanolamine  
PS - phosphatidylserine  
PI - phosphatidylinositol that is phosphorylated to generate PI3P, PI4P, PI5P, PI(3,4)P2, PI(3,5)P2, PI(4,5)P2 & PI(3,4,5)P3 |

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Figure 1.1. The stages of fusion.


Cui, Z., D. Hirata, E. Tsuchiya, H. Osada, and T. Miyakawa. 1996. The multidrug resistance-associated protein (MRP) subfamily (Yrs1/Yor1) of Saccharomyces cerevisiae is important for the tolerance to a broad range of organic anions. J Biol Chem. 271:14712-6.


CHAPTER 2: THE YEAST VACUOLAR ABC TRANSPORTER Ybt1p REGULATES MEMBRANE FUSION THROUGH Ca\textsuperscript{2+} TRANSPORT MODULATION\textsuperscript{1}

ABSTRACT

Ybt1p is a class C ABC transporter that is localized to the vacuole of *Saccharomyces cerevisiae*. Although Ybt1p was originally identified as a bile acid transporter, it has also been found to function in other capacities including the translocation of phosphatidylcholine to the vacuole lumen, and the regulation of Ca\textsuperscript{2+} homeostasis. In this study, we found that deletion of *YBT1* enhanced in vitro homotypic vacuole fusion by up to 50\% relative to wild type vacuoles. The increased vacuole fusion was not due to aberrant protein sorting of SNAREs or recruitment of factors from the cytosol such as Ypt7p and the HOPS tethering complex. In addition, *ybt1Δ* vacuoles displayed no observable differences in the formation of SNARE complexes, interactions between SNAREs and HOPS, or formation of vertex microdomains. However, the absence of Ybt1p caused significant changes in Ca\textsuperscript{2+} transport during fusion. One difference was the prolonged Ca\textsuperscript{2+} influx exhibited by *ybt1Δ* vacuoles at the start of the fusion reaction. We also observed a striking delay in SNARE-dependent Ca\textsuperscript{2+} efflux. Because vacuole fusion can be inhibited by high Ca\textsuperscript{2+} concentrations, we posit that the delayed efflux in *ybt1Δ* vacuoles leads to the enhanced SNARE function.

\textsuperscript{1} This chapter appeared in its entirety in the *Biochemical Journal* and is referred to later in this dissertation as “Sasser et al 2012b”. Sasser, T.L., M. Padolina, and R.A. Fratti. 2012. The Yeast Vacuolar ABC Transporter Ybt1p Regulates Membrane Fusion Through Ca\textsuperscript{2+} Transport Modulation. *Biochem J.* 448(3):365-72

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INTRODUCTION

Maintaining eukaryotic cellular homeostasis requires the trafficking of membrane-bound cargo through the endocytic and secretory pathways using mechanisms that are conserved in all eukaryotes (Jahn and Sudhof, 1999). The delivery of cargo to its destination is finalized by the fusion of two membranes that is driven through a series of regulated stages. Here, we used vacuoles (lysosomes) from Saccharomyces cerevisiae to examine the regulation of membrane fusion. Yeast vacuole fusion requires numerous proteins including the Q-SNAREs Vam3p, Vti1p, Vam7p and the R-SNARE Nyv1p. In addition, vacuole fusion requires the Rab GTPase Ypt7p and its effector complex HOPS, actin, as well as the lipids ergosterol, diacylglycerol, phosphatidic acid, and phosphoinositides (Wickner, 2010).

The final catalysts of fusion are SNARE proteins, however, at the start of the cascade, SNAREs are held in inactive cis-complexes on a single membrane. The vacuole fusion pathway is initiated when cis-SNARE complexes are dissociated by the NSF homologue Sec18p and its co-chaperone α-SNAP/Sec17p in an ATP-dependent mechanism (Ungermann et al., 1998b). Membranes with activated SNAREs then tether and dock with partner membranes in a mechanism driven by Ypt7p and its effector complex HOPS (Mayer and Wickner, 1997; Seals et al., 2000). During the docking stage SNARE complexes form in trans between partner membranes where one membrane donates Nyv1p and the partner membrane donates Vam3p, Vti1p and Vam7p as a preformed 3Q-SNARE complex. In conjunction with these events, vacuoles are drawn together and become tightly apposed causing the deformation of vesicles.
and formation of a specialized membrane-raft microdomain called the vertex ring where the proteins and lipids that catalyze fusion laterally accumulate to create the site of fusion (Fratti et al., 2004; Wang et al., 2003; Wang et al., 2002).

Although the core fusion machinery has been identified, there is a growing collection of regulatory factors that modulate fusion by various means. These regulators include the protein kinase Yck3p (Cabrera et al., 2009), the lipid modifiers Pah1p (Sasser et al., 2012a) and Vps34p (Schu et al., 1993), as well as the Na\(^+\)/H\(^+\) exchanger Nhx1p (Qiu and Fratti, 2010). Additional regulation may occur through controlling Ca\(^{2+}\) transport or the translocation of lipids across the membrane bilayer. The latter function is executed by polytopic membrane proteins that are referred to as lipid “flippases” and “floppases” that establish lipid asymmetry between the inner and outer leaflets of a membrane (van Meer et al., 2008). Some of the putative flip/floppases belong to the ABC transporter superfamily (Gulshan and Moye-Rowley, 2011).

The ABC transporter superfamily is present in all organisms and is responsible for actively transporting a wide range of substrates across lipid bilayers. Loss of function mutations in ABC transporters result in a number of inherited human diseases including the lung and digestive disorder cystic fibrosis, the cholesterol transport disorder Tangier’s disease, and the elastic tissue disorder pseudoxanthoma elasticum (Paumi et al., 2009). ABC transporters are formed from two homologous halves that contain a nucleotide-binding domain (NBD) and a membrane-spanning domain (MSD) consisting of six transmembrane spans. Human ABC transporters are divided into seven subfamilies (ABCA to ABCG). Many of the best-characterized ABC transporters belong to the Class C subfamily (ABCC). With the exception of CFTR (cystic fibrosis
transmembrane conductance regulator), ABCC family members, also known as Multidrug Resistance-associated Proteins (MRP), contain an N-terminal extension composed of five transmembrane motifs and a short cytosolic loop. The yeast vacuole harbors five ABCC transporters: the yeast cadmium factor, Ycf1p (Szczypka et al., 1994; Wemmie and Moye-Rowley, 1997); two bile acid and pigment transporters, Bpt1p and Ybt1p (Ortiz et al., 1997; Petrovic et al., 2000); and two less well-characterized transporters, Vmr1p and Nft1p (Paumi et al., 2009). Ybt1p was first described as an ATP-dependent bile acid transporter (Ortiz et al., 1997) and is similar in structure to the human MRP transporter. Ybt1p is highly expressed on the yeast vacuole (Ghaemmaghami et al., 2003) and also plays a part in ade2 pigment transport (Sharma et al., 2003). Recently, a novel function was described for Ybt1p showing that it translocates phosphatidylcholine from the outer leaflet of the vacuole to the inner leaflet for degradation and choline recycling (Gulshan and Moye-Rowley, 2011).
MATERIALS AND METHODS

Reagents

Reagents were dissolved in PS buffer (20 mM PIPES-KOH, pH 6.8, 200 mM sorbitol). Antibodies against Vam3p (Nichols et al., 1997), Sec17p (Haas and Wickner, 1996), Nyv1p (Ungermann et al., 1998a), Sec18p (Mayer et al., 1996), Ypt7p (Mayer and Wickner, 1997) were described previously. The recombinant proteins His6-Gyp1-56 (Wang et al., 2003), GDI (Starai et al., 2007), GST-FYVE (Gillooly et al., 2000), MED (Fratti et al., 2004), His6-MTM1 (Taylor et al., 2000) and GST-Vam7p (Fratti et al., 2007; Fratti and Wickner, 2007) were prepared as described and stored in PS buffer with 125 mM KCl.

Strains

BJ3505 (MATα pep4::HIS3 prb1-Δ1.6R his3-200 lys2-801 trp1Δ101 (gal3) ura3-52 gal2 can1) and DKY6281 (MATα leu2-3 pho8::TRP1 leu 2-112 ura3-52 his3-D200 trp1-D901 lys2-801) were used for fusion assays (Haas et al., 1995). BJ3505 CBP-Vam3 nyv1Δ was used for trans-SNARE complex isolation (Collins and Wickner, 2007) (Table 1). YBT1 was deleted from BJ3505, DKY6281, and BJ3505 CBP-Vam3 nyv1Δ by homologous recombination. YBT1 was deleted from BJ3505 and DKY6281 with the kanMX6 cassette using PCR products amplified from pFA6a-kanMx6 (Longtine et al., 1998) with homology flanking the YBT1 coding sequence with forward primer 5’- GTG TGC GCA TCT GCA AAG GTA CGT TGT GAC TAA TGA ACG GAT CCC CGG GTT AAT TAA -3’ and reverse primer 5’- TCA GTA AAA GTT CAT TGG ATC AGA TTT
CCT TCA AAG ACG CGA ATT CGA GCT TTA AAC -3’. The PCR product was transformed into BJ3505 and DKY6281 by standard lithium acetate methods and plated on YPD media containing G418 (250 µg/L) to generate BJ3505 \( ybt1\Delta::kanMX6 \) (RFY28) and DKY6281 \( ybt1\Delta::kanMX6 \) (RFY29). \( YBT1 \) was deleted from BJ3505 CBP-Vam3 \( nyv1\Delta \) with the \( hghMX4 \) cassette using PCR product amplified from pAG32 (Goldstein and McCusker, 1999) with homology flanking the \( YBT1 \) coding sequence with forward primer 5' - GTG TGC GCA TCT GCA AAG AAC GTA CGT TGT GAC TAA TGA AAT AGG CCA CTA GTG GAT CTG -3' and reverse primer 5' - TCA GTA AAA GTT CAT TGG ATC AGA TTT CCT TCA AAG ACG CTC AGC TGA AGC TTC GTA CGC -3'. The PCR product was transformed into BJ3505 CBP-Vam3 \( nyv1\Delta \) by standard lithium acetate methods and plated on YPD media containing hygromycin (250 µg/mL) to generate BJ3505 CBP-Vam3 \( nyv1\Delta \ ybt1::hghMX4 \) (RFY30). For vacuole localization studies

**Vacuole isolation and in vitro vacuole fusion**

Vacuoles were isolated by floatation as described (Haas et al., 1995). Standard in vitro fusion reactions (30 µL) contained 3 µg each of vacuoles from BJ3505 and DKY6281 backgrounds, fusion reaction buffer (20 mM PIPES-KOH pH 6.8, 200 mM sorbitol, 125 mM KCl, 5 mM MgCl2), ATP regenerating system (1 mM ATP, 0.1 mg/mL creatine kinase, 29 mM creatine phosphate), 10 µM CoA, and 283 nM IB2. Reactions were incubated at 27°C and Pho8p activity was assayed in 250 mM Tris-Cl, pH 8.5, 0.4% Triton X-100, 10 mM MgCl2, 1 mM \( p \)-nitrophenyl phosphate. Fusion units were measured by determining the \( p \)-nitrophenolate produced min\(^{-1}\)·µg\(^{-1}\) \( pep4\Delta \) vacuole and
absorbance was detected at 400 nm.

**GST-Vam7p SNARE complex isolation and bypass fusion**

SNARE complex isolation was performed as described previously using GST-Vam7p (Fratti et al., 2007; Fratti and Wickner, 2007). Briefly, large-scale 6X fusion reactions (180 µL) were incubated with 85 µg/mL anti-Sec17p IgG to block priming. After 15 min, 43 µg/ml anti-Vam3p IgG was added to selected reactions and incubated for an additional 5 min before adding 400 nM GST-Vam7p. After a total of 90 min, reactions were placed on ice for 5 min and 30 µL aliquots were removed to measure Pho8p activity. The remaining 150 µL reactions were sedimented (11,000 g, 10 min, 4°C), and the supernatants removed before extracting vacuoles with solubilization buffer (SB: 20 mM HEPES-KOH, pH 7.4, 100 mM NaCl, 2 mM EDTA, 20% glycerol, 0.5% Triton X-100, 1 mM DTT) with protease inhibitors (1 mM PMSF, 10 µM Pefabloc-SC, 5 µM pepstatin A, and 1 µM leupeptin). Vacuole pellets were overlaid with 100 µL SB and resuspended gently. An additional 100 µL SB was added, gently mixed, and incubated on ice for 20 minutes. Insoluble debris was sedimented (16,000 g, 10 min, 4°C) and 176 µL of supernatants were removed and placed in chilled tubes. Next, 16 µL was removed from each reaction as 10% total samples, mixed with 8 µL of 3X SDS loading buffer and heated (95°C, 5 min). Equilibrated glutathione Sepharose 4B beads (30 µL) were incubated with the remaining extracts (15 hours, 4°C, nutation). Beads were sedimented and washed 5X with 1 mL SB (3,000 rpm, 2 minutes, 4°C), and bound material was eluted with 40 µL 1X SDS loading buffer. Protein complexes were examined by immunoblotting. Secondary antibodies conjugated to alkaline phosphatase were used
with ECF reagent (GE Healthcare).

**Trans-SNARE complex assay**

Analysis of *trans*-SNARE complex formation was conducted as described with some modifications (Collins and Wickner, 2007; Jun and Wickner, 2007). Complex formation was compared between reactions containing vacuoles from RFY29 and RFY30 relative to those with BJ3505-CBP-Vam3p *nyv1Δ* and DKY6281 vacuoles. The *trans*-SNARE assays were performed using 16X large-scale reactions (480 µL) containing 48 µg of vacuoles each from BJ3505 CBP-Vam3 *nyv1Δ* and DKY6281 backgrounds and incubated at 27°C for 60 minutes. After incubation, reactions were placed on ice for 5 minutes and 30 µL was withdrawn from each sample to assay Pho8p activity. The remaining 450 µL samples were centrifuged (13,000 g, 15 minutes, 4°C) and the supernatants decanted. Vacuole pellets were overlaid with 200 µL ice-cold solubilization buffer (20 mM Tris-Cl pH 7.5, 150 mM NaCl, 1 mM MgCl₂ 0.5% Nonidet P-40 alternative, 10% glycerol) with protease inhibitors (0.46 µg/mL leupeptin, 3.5 µg/mL pepstatin, 2.4 µg/mL pefabloc-SC, 1 mM PMSF), and gently resuspended. Solubilization buffer was added to a final volume of 600 µL and extracts were mixed (20 minutes, 4°C, nutation). Detergent-insoluble debris was removed by centrifugation (16,000 g, 20 minutes, 4°C). Supernatants were transferred to fresh tubes and 10% of the extract was removed for input samples. The remaining extracts were brought to 2 mM CaCl₂. CBP-Vam3p complexes were incubated with 50 µL equilibrated calmodulin Sepharose 4B (GE Healthcare) (4°C, 12 hours, nutation). Beads were collected by centrifugation (4,000 g, 2 minutes, 4°C) and suspended four times with the solubilization buffer
followed by bead sedimentation. Bound proteins were eluted with SDS sample buffer containing 5 mM EGTA and heated at 95°C for 5 minutes. The samples were used for SDS-PAGE analysis and immunoblotting.

**Lipid mixing**

Lipid mixing assays were conducted using Lissamine rhodamine B (Rh-PE; Invitrogen) as described with minor modifications (Sasser et al., 2012a). Briefly, BJ3505 background vacuoles (300 µg) were incubated in 400 µL of PS buffer containing 150 µM Rh-PE (10 minutes, 4°C, nutating). Samples were mixed with 15% Ficoll in PS buffer (wt/vol) and transferred to a polyallomer tube (11 x 60 mm). Samples were overlaid with 1.0 mL each of 8%, 4%, and 0% Ficoll. Labeled vacuoles were re-isolated by centrifugation using a SW60 Ti rotor (105,200 g, 25 minutes, 4°C) and recovered from the 0 - 4%-Ficoll interface. Lipid mixing assays (90 µL) contained 2 µg of Rh-PE-labeled vacuoles and 16 µg of unlabeled vacuoles in fusion buffer. Reaction mixtures were transferred to a black, half-volume 96-well flat-bottom microtiter plate with nonbinding surface (Corning). Rhodamine fluorescence ($\lambda_{ex} = 544$ nm; $\lambda_{em} = 590$ nm) was measured using a POLARstar Omega fluorescent plate reader (BMG Labtech) at 27°C. Measurements were taken every minute for 75 minutes, yielding fluorescence values at the onset ($F_0$) and during the reaction ($F_t$). The final 10 measurements of a sample after adding 0.33% (vol/vol) Triton X-100 were averaged and used as a value for the fluorescence after infinite dilution ($F_{TX100}$). The relative total fluorescence change $\Delta F_t/F_{TX100} = (F_t - F_0)/F_{TX100}$ was calculated.
**Microscopy**

Vacuole morphology was monitored by incubating yeast cells with YPD broth containing the vital dye FM4-64 (Invitrogen). Cultures were grown overnight to saturation, diluted to ~0.2 OD_{600} in YPD containing 5 µM FM4-64, grown for 1 hour in 30°C shaker, washed with PBS, resuspended in YPD, incubated at 30°C for 3 hour, washed in PBS, concentrated by centrifugation, mixed with 0.6% agarose, and mounted on glass slides for observation. Images were acquired using a Zeiss Axio Observer Z1 inverted microscope equipped with an X-Cite 120XL light source, Plan Apochromat 63X oil objective (NA 1.4), and an AxioCam CCD camera. For vertex assembly detection, docking assays were performed. Docking reactions (30 µL) contained 6 µg of Ybt1p-GFP vacuoles, docking buffer (20 mM PIPES-KOH pH 6.8, 200 mM sorbitol, 100 mM KCl, 0.5 mM MgCl₂), ATP regenerating system (0.3 mM ATP, 0.7 mg/mL creatine kinase, 6 mM creatine phosphate), 20 µM CoA, and 283 nM IB2. Phosphatidylinositol 3-phosphate (PI3P) was labeled with 0.2 µM Cy5-FYVE and used as a marker for vertex microdomains (Sasser et al., 2012a). Reactions were incubated at 27°C for 30 minutes and placed on ice and stained with 3 µM FM4-64. Reactions were next mixed with 50 µl of 0.6% low-melt agarose (in PS buffer), vortexed to disrupt spurious clustering, and mounted on slides for observation by fluorescence microscopy.

**Ca^{2+} efflux assay**

SNARE-dependent Ca^{2+} efflux was measured as described (Robinson and Dixon, 2006) with some modifications. Fusion reactions (60 µL) contained 20 µg of vacuoles isolated from BJ3505 backgrounds, fusion reaction buffer with 10 µM CoA,
and 283 nM IB₂. In lieu of the luminescent Aequorin Ca²⁺ detection system we used the low affinity fluorescent probe Fluo-4-dextran at 200 µM (Invitrogen). Reaction mixtures were transferred to a black, half-volume 96-well flat-bottom microtiter plate with nonbinding surface (Corning). ATP regenerating system was added and reactions were incubated at 27°C while monitoring Fluo-4 fluorescence (λₚₓ = 488 nm; λₑᵣᵢₙ = 520 nm).

**Statistical analysis**

Significant differences were calculated using Student’s t test and *P*-values less than 0.05 were considered significant.
RESULTS

Ybt1p is localized to the vacuole

Ybt1p has been reported to transport bile acids into the vacuole lumen of *Saccharomyces cerevisiae* in an ATP-dependent manner (Ortiz et al., 1997). To verify the localization of Ybt1p in yeast, we constructed a strain expressing Ybt1p-GFP. We incubated the strain with the vital dye FM4-64, which accumulates in the vacuole (Vida and Emr, 1995), and found that Ybt1p-GFP primarily localized to the vacuole (Figure 2.1 a-d), which is consistent with previous findings (Gulshan and Moye-Rowley, 2011). We next examined the vacuole morphology in *ybt1Δ* cells and found that the morphology and size of the vacuoles were similar to those in wild type cells (Figure 2.1 e-g). Because fusion regulators often accumulate at the vertices of docked vacuoles (Fratti et al., 2004; Wang et al., 2003; Wang et al., 2002), we next examined the distribution of Ybt1p-GFP on isolated vacuoles during docking. Figure 2.2 shows that Ybt1p-GFP was evenly dispersed throughout the limiting membrane of the vacuole along with FM4-64. To probe for vertex microdomains, docking reactions were incubated with Cy5-conjugated FYVE domain. The FYVE domain specifically binds to the regulatory lipid PI3P (Gillooly et al., 2000), a critical lipid that accumulates at the site of fusion in vertex microdomains (Fratti et al., 2004). In Figure 2.2c the vertices of docked vacuoles were marked with Cy5-FYVE.

Ybt1p is a negative regulator of vacuole fusion

To determine the possible role of this ABCC transporter in the fusion process, we
next examined the effect of deleting \textit{YBT1} on homotypic vacuole fusion. Fusion was measured by a content mixing assay in which proPho8p (pro-alkaline phosphatase) is cleaved by the protease Pep4p to yield a mature alkaline phosphatase. The level of fusion directly correlates with Pho8p activity. Equal quantities of the strains DKY6281 (\textit{PEP4 \textit{pho8}}) and BJ3505 (\textit{pep4} \textit{PHO8}) were mixed to assay content mixing. We deleted \textit{YBT1} from these strains to generate RFY28 and RFY29 and found that fusion was augmented and showed an approximate 50\% increase compared to WT vacuoles (Figure 2.3a). This suggests that Ybt1p acts as a negative regulator for vacuole fusion.

Because changes in fusion could be due to dysregulated Pho8p reporting we also used a real-time lipid-dequenching assay that is independent of Pho8p activity (Qiu and Fratti, 2010; Reese et al., 2005; Reese and Mayer, 2005). Here, the outer leaflets of vacuole limiting membranes were labeled with Rh-PE, which is self-quenched at high concentrations. When labeled donor vacuoles were incubated with an 8-fold excess of unlabeled acceptor vacuoles, Rh-PE was dequenched. WT vacuoles showed characteristic rapid dequenching that was inhibited with anti-Vam3 IgG (Figure 2.3b, circles). When \textit{ybt1}\textit{Δ} vacuoles were examined, we found that the initial dequenching was identical to WT after which the \textit{ybt1}\textit{Δ} vacuoles surpassed WT fusion starting at 15 minutes (Figure 2.3b). This illustrates that the initial rates of fusion were the same for WT and \textit{ybt1}\textit{Δ} vacuoles yet the \textit{V}_{\text{max}} of \textit{ybt1}\textit{Δ} fusion was significantly higher. To determine if the increase in fusion of \textit{ybt1}\textit{Δ} vacuoles was due to elevated levels of the fusion machinery (e.g. SNAREs), western blot analysis was performed. No significant differences were observed in the fusion machinery of mutant vacuoles relative to their WT counterparts (Figure 2.3c). It should also be noted that the increased fusion was not
due to defects in trafficking of Pho8p or Pep4p (Figure 2.3d). To further verify that the deletion of *YBT1* caused an increase in fusion, visual measurements of vacuole diameters were measured. Fusion reactions containing either WT or *ybt1Δ* vacuoles were carried out as in Figure 2.3a. After 90 minutes of incubation vacuoles were placed on ice and stained with FM4-64. Aliquots of each reaction were transferred to microscope slides and images were captured for analysis. Shown in Figure 2.3e is a box plot of vacuole diameter ranges for reactions treated with either buffer or anti-Sec17 IgG to inhibit priming, and incubated on ice or at 27°C. We found that *ybt1Δ* vacuole diameters (7.13 ± 0.19 μm) were markedly larger than those of WT vacuoles (4.88 ± 0.23 μm).

To determine if the core fusion machinery regulated the augmented fusion seen in *ybt1Δ* vacuoles, we examined the sensitivity of fusion to a panel of well characterized fusion inhibitors that targeted SNAREs, Ypt7p and phosphoinositides. SNARE function was inhibited with antibodies against Nyv1p, Vam3p, Sec18p and Sec17p. Ypt7p function was inhibited with anti-Ypt7p IgG, the GTPase activating protein Gyp1-56p, or the Rab GDP-dissociation inhibitor GDI. PI3P function was inhibited by ligation with the FYVE domain or modification with the phosphoinositide 3-phosphatase, MTM1. The MARCKS effector domain (MED) was used to bind multi-phosphorylated phosphoinositides, including phosphatidylinositol 4,5-bisphosphate. We found that both WT and *ybt1Δ* fusion were similarly sensitive to the panel of inhibitors indicating that the enhanced fusion seen with *ybt1Δ* vacuoles was not due to an undefined mechanism (Figure 2.4).
YBT1 deletion does not alter SNARE complex formation

Dysregulation of fusion is often coupled with changes in SNARE complex formation. Thus, we tested whether the absence of Ybt1p altered the SNARE/HOPS interactions by two methods. First, we performed a Vam7p bypass assay when SNARE priming was inhibited with anti-Sec17p IgG (Fratti et al., 2007; Fratti and Wickner, 2007). Fusion was then rescued with the addition of 400 nM GST-Vam7p and incubated for an additional 70 minutes. Selected reactions were treated with anti-Vam3p prior to adding Vam7p. Fusion was restored when WT and mutant vacuoles were treated with exogenous GST-Vamp7p (Figure 2.5a). GST-Vam7p complexes were isolated and examined by immunoblotting. GST-Vam7p complexes included the SNAREs Nyv1p and Vam3p, the HOPS subunit Vps33p, the SNARE chaperone Sec17p, and actin (Figure 2.5b). As a control we also probed for the presence of Ypt7p, which does not co-purify with the SNARE/HOPS complexes. SNARE complex formation was blocked by anti-Vam3p. We found that GST-Vam7p complexes from ybt1Δ vacuoles contained similar SNARE makeup relative to WT vacuoles.

We next determined if endogenous SNAREs could form trans-complexes when priming is not blocked (Collins and Wickner, 2007; Jun and Wickner, 2007). YBT1 was deleted from the trans-SNARE reporter strain BJ3505 CBP-Vam3p nyv1Δ to create RFY30. Vacuoles isolated from RFY29 (pho8Δ ybt1Δ VAM3 NYV1) and RFY30 were incubated and trans-SNARE complexes were isolated through the calmodulin binding peptide (CBP) integrated into Vam3p. CBP-Vam3p complexes were isolated using calmodulin Sepharose beads and examined by immunoblotting. As shown previously, ybt1Δ vacuoles show increased fusion relative to WT vacuoles (Figure 2.6a). Both WT
and ybt1Δ fusion reactions were sensitive to anti-Vam3p IgG and to the inactivation of Ypt7p with the combination of Gyp1-56p and GDI. We found that ybt1Δ vacuoles formed trans-SNARE complexes in a similar manner as their WT counterparts indicating that the enhanced fusion was not to do an augmented number of SNARE complexes (Figure 2.6 b-c). Similarly, the association of SNAREs with the HOPS subunit Vps33p was not altered on ybt1Δ vacuoles. The lack of a notable difference between WT and mutant vacuoles suggest that fusion was not altered due to the efficiency of complex formation.

**Ybt1p regulates the release of luminal Ca\(^{2+}\) stores**

From Figure 2.3a we found that mutant fusion was increased after 15 minutes that coincides with the formation of trans-SNARE pairing (Collins and Wickner, 2007). The formation of trans-SNARE complexes is linked to the release of luminal Ca\(^{2+}\) stores from the vacuole (Merz and Wickner, 2004; Peters and Mayer, 1998). Because Ybt1p interacts with the Ca\(^{2+}\) ATPase Pmc1p (Tarassov et al., 2008), we next examined the effect of deleting YBT1 on Ca\(^{2+}\) transport. Fusion reactions (2X) were prepared containing 20 µg of either BJ3505 (WT) or RFY28 (ybt1Δ) in the presence of standard fusion reaction components and the fluorescent calcium indicator Fluo4-Dextran. Reactions were started by the addition of buffer or ATP regenerating system. Priming was blocked by addition of anti-Sec17p IgG where indicated and fluorescence was monitored. Upon the addition of ATP, Ca\(^{2+}\) was transported into the vacuole causing a drop in fluorescence. Ca\(^{2+}\) is transported into the vacuole via the Ca\(^{2+}\)-ATPase Pmc1p and the Vcx1p H\(^+\)/Ca\(^{2+}\) exchanger (Miseta et al., 1999). After approximately 15 minutes, when trans-SNAREs have formed, we observed an increase in fluorescence indicating
that Ca\(^{2+}\) was being effluxed from the vacuole lumen (Figure 2.7a, black circles), which is in keeping with previous studies using the Aequorin reporter system (Merz and Wickner, 2004). When \(ybt1\Delta\) vacuoles were observed we found that there were four differences relative to WT reactions. First, there was a prolonged influx of Ca\(^{2+}\) early in the reaction, suggesting that Pmc1p and/or Vcx1p activity may be augmented. Second, we found that the trans-SNARE complex-dependent efflux was markedly delayed in \(ybt1\Delta\) vacuoles (Figure 2.7b). These observations suggest that Ybt1p regulates Ca\(^{2+}\) transport in vacuoles and that the enhanced fusion may be linked to alterations in Ca\(^{2+}\) homeostasis. In addition, the relative amount of Ca\(^{2+}\) in \(ybt1\Delta\) vacuoles was nearly twice as much as seen with wild type vacuoles. We also found that wild type vacuoles reabsorb Ca\(^{2+}\) after docking resulting in a return to basal, “pre-docking” levels of extraluminal Ca\(^{2+}\) while \(ybt1\Delta\) fail to reabsorb the effluxed Ca\(^{2+}\).
DISCUSSION

In this study we found that the ABCC transporter Ybt1p acts as a negative regulator of vacuole fusion. This was evident by the enhanced fusion observed with vacuoles from YBT1 deleted strains. Using a battery of experiments that tested different stages of the fusion pathway, we found that the defect in fusion occurred after the docking stage when trans-SNARE complexes are formed. The enhancement in fusion correlated with a delay in Ca$^{2+}$ efflux, an event that is triggered by the formation of trans-SNARE complexes (Merz and Wickner, 2004). This suggested that Ybt1p might regulate the transport of ions across the membrane. Although Ybt1p itself does not transport Ca$^{2+}$, the observed changes might be attributed in part to the interaction of Ybt1p with Pmc1p, a Ca$^{2+}$-ATPase that transports Ca$^{2+}$ ions into the vacuole (Tarassov et al., 2008). Although the SNARE-dependent vacuolar Ca$^{2+}$ efflux channel remains undefined, the connections between Ybt1p, Pmc1p and the delayed efflux during fusion suggests that the import and export of Ca$^{2+}$ is linked to the fusion machinery.

Ybt1p and the SNARE machinery

The direct connection between Ybt1p and the fusion machinery remains unknown, however, the link between Ybt1p and Pmc1p suggests that an indirect mechanism is possible. Others have shown that Pmc1p interacts with free Nyv1p, the required R-SNARE in vacuole homotypic fusion (Takita et al., 2001). The interaction between Pmc1p and Nyv1p inhibits the transport of Ca$^{2+}$ into the vacuole lumen. After priming, the Q-SNARE complexes compete for Nyv1p binding to form trans-SNARE
complexes, thus de-repressing Pmc1p activity and leading to the uptake of extraluminal Ca\(^{2+}\). In the absence of Ybt1p we observed the prolonged uptake of Ca\(^{2+}\) upon the addition of ATP, suggesting that Pmc1p activity is augmented and that changes in activity might be due to reduced interactions Nyv1p.

There are several possible mechanisms by which the efflux of Ca\(^{2+}\) might contribute to the regulation of fusion. We have found that vacuole fusion strictly depends on the formation of membrane microdomains that are enriched in the proteins and lipids that regulate fusion (Fratti et al., 2004; Wang et al., 2003; Wang et al., 2002). Thus, any perturbation to the assembly pathway needed for the formation of vertices would undoubtedly alter fusion. Others have found that Ca\(^{2+}\) at sub-micromolar concentrations induces clustering of plasma membrane SNAREs (Zilly et al., 2011). This is thought to occur through the neutralization of negatively charged side chains. It is also likely that Ca\(^{2+}\) relieves the electrostatic repulsion between negatively charged phospholipids (Boettcher et al., 2011; Ellenbroek et al., 2011). Interestingly, Zilly et al., also found that elevated Ca\(^{2+}\) concentrations (≥10 µM) inhibited SNARE pairing. While the bulk measurement of Ca\(^{2+}\) efflux only measures sub-micromolar concentrations, it is possible that local concentrations of Ca\(^{2+}\) at the site of efflux may reach micromolar levels. Thus, a delay in Ca\(^{2+}\) efflux might allow more trans-SNARE pair formation and enhanced fusion.
### TABLES AND FIGURES

**Table 2.1.** Yeast strains used in this study

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<tr>
<th>Strain</th>
<th>Genotype</th>
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<td>(Jones et al., 1982)</td>
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</table>
Figure 2.1. **Ybt1-GFP is localized to the vacuole.** Wild type yeast cells harboring Ybt1p-GFP were incubated with 5 μM FM4-64 to label vacuoles. Cells were washed with PBS and grown for 1 h in labelfree YPD to chase the dye into the vacuole. Cells were washed with PBS and mounted for microscopy. (a,b) GFP images were acquired using a 38 HE Green Fluorescent Protein shift-free filter set and FM4-64 images were acquired using a 43 HE CY 3 shift-free filter set. (c) Cells were photographed using differential interference contrast (DIC). (c) Images were merged using Photoshop. (e-f) ybt1Δ yeast were incubated with FM4-64 as described above and imaged using DIC and CY 3 filters. (g) Quantitation and box plot of vacuole size in wild type and ybt1Δ cells. Bar, 4 μm.
Figure 2.2. Ybt1-GFP is not enriched at the vertex ring microdomain of docked vacuoles. Purified vacuoles isolated from cells harboring Ybt1p-GFP (a) were incubated at 27°C for 30 min. The limiting membranes were stained with 5 µM FM4-64 (b) and vertex microdomains were labeled with Cy5-conjugated FYVE domain to label PI3P (c). (d) Images were merged using Photoshop. Bar, 4 µm.
**Figure 2.3. Vacuoles from ybt1Δ yeast show enhanced fusion.** Vacuoles were harvested from WT and ybt1Δ yeast and tested for fusion activity. (a) Standard fusion reactions used equal amounts of reporter (PHO8 pep4Δ) and effector (pho8Δ PEP4) vacuoles for WT (closed circles) and ybt1Δ (open circles). Error bars represent S.E. (n=3). (b) Lipid mixing assays measuring fusion were employed by labeling isolated vacuoles with Rh-PE. Labeled vacuoles were incubated with 8-fold excess of unlabeled vacuoles and dequenching was measured using a fluorescence plate reader. (c) Analysis of core fusion components. Vacuoles were isolated from WT and ybt1Δ BJ3505 and DK6281 strains. Vacuoles (5 mg by protein) were mixed with 2X SDS loading buffer, separated by SDS-PAGE and transferred to nitrocellulose. Immunoblotting was performed using the indicated antibodies and bands were detected using ECF. (d) Quantitation of Pep4p and Pho8p on wild type and ybt1Δ vacuoles. (e) Measurements of vacuole diameters after incubation (27°C, 90 min). Error bars represent S.E. (n=3). * P<0.0001
Figure 2.3 continued. Vacuoles from \textit{ybt1}\textsuperscript{Δ} yeast show enhanced fusion.
Figure 2.4. The augmented fusion of $ybt1\Delta$ vacuoles is dependent on the standard fusion machinery. Fusion reactions were performed using WT or $ybt1\Delta$ vacuoles. Individual reactions were treated with PS, 14 µg/ml anti-Nyw1p IgG, 27 µg/ml anti-Vam3p IgG, 12 µg/ml anti-Sec18p IgG, 67 µg/ml anti-Sec17p IgG, 8 µg/ml anti-Ypt7p, 0.5 µM Gyp1-56, 0.5 µM GDI, 2 µM GST-FYVE, 10 µM MED or 2 µM His6- MTM1. Reactions were incubated for 90 min at 27°C and tested for fusion by content mixing and Pho8p activity. Error bars represent S.E. (n=3).
Figure 2.5. Vam7p forms complexes with SNAREs and HOPS on ybt1Δ vacuoles.
(a) Large-scale fusion reactions (6 X) were incubated with anti-Sec17p IgG to block priming. After 15 min, anti-Vam3 IgG was added to the indicated reactions and incubated for 5 min prior to adding 400 nM GST-Vam7p. Reactions were incubated for an additional 70 min. After incubation, reactions were placed on ice and 30 µl was removed to measure Pho8p activity. (b) The remaining fractions were extracted with solubilization buffer with protease inhibitors for 20 min while on ice. Insoluble debris was sedimented by centrifugation and supernatants were removed and incubated with equilibrated glutathione Sepharose. Beads were washed with solubilization buffer and protein complexes were eluted with 2X SDS-PAGE buffer. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose and probed with antibodies against Nyv1p, Vam3p, Vam7p, Sec17p, Vps33p, actin, and Ypt7p. Error bars represent S.E. (n=3).
**Figure 2.6. Trans-SNARE complex formation.** Vacuoles harboring CBP-Vam3p in a *nyv1Δ* background were incubated with vacuoles harboring a full complement of SNAREs. Large-scale reactions (16X) were incubated at 27°C for 60 min. After incubating, reactions were placed on ice for 5 min and 30 µl was withdrawn from each sample to assay Pho8p activity (a). (b) Membranes were pelleted and then resuspended in solubilization buffer containing protease inhibitors. CBP-Vam3p complexes were isolated with calmodulin Sepharose beads and protein complexes were resolved by SDS-PAGE. Complexes were probed for Nyv1p, Vam3p, Vps11p, and Vps33p by immunoblot. (c) Quantitation of efficiency of Nyv1p binding to CBP-Vam3p. Error bars represent S.E. (n=3).
Figure 2.7. Calcium efflux is delayed in $ybt1\Delta$ vacuoles. Vacuoles were harvested from WT BJ3503 and RFY28 ($ybt1\Delta$). Fusion reactions (2X) were prepared containing 20 mg of either WT or $ybt1\Delta$ vacuoles and 200 mM Fluo-4-dextran. Immediately after addition of ATP or PS buffer, reactions were incubated at 27°C and fluorescence was measured for 90 min. (a) A representative time course of Ca$^{2+}$ efflux. (b) The average times at which the start and peaks of efflux occurred in wild type and $ybt1\Delta$ vacuoles. Error bars represent S.E. (n=3).
BIBLIOGRAPHY


CHAPTER 3: THE YEAST ABC TRANSPORTER Ycf1p REGULATES VACUOLE FUSION THROUGH THE RECRUITMENT OF THE SOLUBLE SNARE Vam7p

ABSTRACT

The *Saccharomyces cerevisiae* vacuole contains five class C ABC transporters including Ycf1p, a family member that was originally characterized as a Cd$^{2+}$ transporter. Ycf1p has also been found to physically interact with a wide array of proteins including factors that regulate vacuole homeostasis. In this study we examined the role of Ycf1p and other ABCC transporters in the regulation of vacuole homotypic fusion. We found that deletion of *YCF1* attenuated *in vitro* vacuole fusion by up to 40% relative to wild type vacuoles. Plasmid-expressed wild type Ycf1p rescued the deletion phenotype; however, Ycf1p containing a mutation of the conserved Lys669 to a Met in the Walker A box of the first nucleotide binding domain (Ycf1p$^{\text{K669M}}$) was unable to complement the fusion defect of *ycf1Δ* vacuoles. This indicates that the ATPase activity of Ycf1p is required for its function in regulating fusion. In addition, we found that deleting *YCF1* caused a striking decrease in vacuolar levels of the soluble SNARE Vam7p, whereas total cellular levels were not altered. The attenuated fusion of *ycf1Δ* vacuoles was rescued by the addition of recombinant Vam7p to *in vitro* experiments. Thus, Ycf1p regulates fusion through the recruitment Vam7p to vacuolar membranes.
INTRODUCTION

Eukaryotic homeostasis requires the packaging, trafficking and delivery of membrane-bound cargo. The final stage of these pathways occurs through the fusion of two membranes, which is catalyzed by a core set of machinery that is conserved throughout eukaryotes (Jahn and Sudhof, 1999). To study the regulation of membrane fusion we used vacuoles (lysosomes) from *Saccharomyces cerevisiae*. Vacuole fusion requires the Rab GTPase Ypt7p and its effector complex HOPS that tether membranes together. The final stage of fusion is catalyzed by SNARE proteins, which interact across the docking junction to form parallel four-helical bundles that deform and destabilize membranes to trigger fusion. The function of these proteins is regulated by a group of lipids that include ergosterol, diacylglycerol, phosphatidic acid, and phosphoinositides (Wickner, 2010). These lipids and proteins interdependently form specialized membrane-raft microdomains called vertices to create the site of fusion (Fratti et al., 2004; Wang et al., 2003; Wang et al., 2002).

Studies that use reconstituted proteoliposomes as a model for fusion have identified the minimal fusion machinery; however, in biological systems the fusion machinery has to be highly regulated through various pathways. Vacuole homotypic fusion is regulated by various factors including the casein kinase Yck3p (Cabrera et al., 2009), the lipid modifiers Pah1p (Sasser et al., 2012a), Plc1p (Jun et al., 2004) and Vps34p (Schu et al., 1993), and the Na\(^+\)/H\(^+\) exchanger Nhx1p (Qiu and Fratti, 2010). We recently found that the class C ABC transporter (ABCC) Ybt1p negatively regulates fusion through the control of Ca\(^{2+}\) transport across the vacuole membrane (Sasser et
Ybt1p also translocates phosphatidylcholine across the vacuole bilayer for degradation and choline recycling (Gulshan and Moye-Rowley, 2011).

Ybt1p is one of the five confirmed ABCC transporters that reside on the vacuole membrane and was initially discovered as an ATP-dependent bile acid transporter (Ortiz et al., 1997). The other yeast vacuole ABCC proteins include the yeast cadmium factor, Ycf1p (Szczypka et al., 1994; Wemmie and Moye-Rowley, 1997), the bile acid transporter, Bpt1p (Petrovic et al., 2000) as well as the less well-characterized Vmr1p and Nft1p (Paumi et al., 2009). Aside from the transport of cadmium, Ycf1p has been reported to physically interact with various vacuolar proteins that are linked to membrane trafficking and fusion including the PI3P kinase Fab1p (Paumi et al., 2007). In this study we examined the role of Ycf1p and other ABCC transporters in vacuole fusion. We found that deletion of YCF1 strongly attenuated fusion by reducing the vacuole association of the soluble SNARE Vam7p.
EXPERIMENTAL PROCEDURES

Reagents

Reagents were dissolved in PS buffer (20 mM PIPES-KOH, pH 6.8, 200 mM sorbitol). The recombinant proteins GST-FYVE (Gillooly et al., 2000) and GST-Vam7p (Fratti et al., 2007; Fratti and Wickner, 2007) were prepared as described and stored in PS buffer with 125 mM KCl. FYVE domain was labeled with Cy5 NHS ester (GE Healthcare) according to the manufacturer’s protocol.

Strains

BJ3505 and DKY6281 were used for fusion assays (Table 3.1) (Haas et al., 1995). YCF1 was deleted from BJ3505 and DKY6281 by homologous recombination with the kanMX6 cassette using PCR products amplified from pFA6a-kanMx6 (Longtine et al., 1998) with homology flanking the YCF1 coding sequence. The PCR product was transformed into BJ3505 and DKY6281 by standard lithium acetate methods and plated on YPD media containing G418 (250 μg/L) to generate BJ3505 ycf1Δ::kanMX6 (RFY32) and DKY6281 ycf1Δ::kanMX6 (RFY33). Similarly BPT1 was deleted from BJ3505 and DKY6281 to generate BJ3505 bpt1Δ::kanMX6 (RFY34) and DKY6281 bpt1Δ::kanMX6 (RFY35). To generate ycf1Δ bpt1Δ strains, YCF1 was deleted from BY3505 and DKY6281 with the URA3 cassette. BPT1 was then deleted with TRP1 to generate RFY36 or HIS3 to generate RFY37. YBT1 was deleted from RFY36 and RFY37 with kanMx6 to generate RFY38-39. Deletions of NFT1, VMR1 and YOL075c were generated using PCR products amplified from pAG32 to generate nft1Δ::hphMX4.
(RFY40-41), \textit{vmr1}\Delta::hphMX4 (RFY42-43), and \textit{yol075c}\Delta::hphMX4 (RFY44-45) (Goldstein and McCusker, 1999). For vacuole localization studies \textit{YCF1}, \textit{BPT1}, \textit{NFT1}, \textit{VMR1} and \textit{YOL075c} were fused in frame to GFP by homologous recombination. DKY6281 was transformed with a PCR products amplified from pFA6a-GFP-kanMX6 (Longtine et al., 1998) with homology flanking the stop codon of the gene to generate RFY46 (DKY6281 \textit{YCF1}::GFP), RFY47 (DKY6281 \textit{BPT1}::GFP), RFY48 (DKY6281 \textit{NFT1}::GFP), RFY49 (DKY6281 \textit{VMR1}::GFP), and RFY50 (DKY6281 \textit{YOL075c}::GFP). For complementation studies WT \textit{YCF1} and \textit{YCF1}^{K699M} were subcloned from pRS424 vectors (a gift from W.S. Moye-Rowley, University of Iowa) into pRS416 using \textit{KpnI} and \textit{Scal}. RYF32 and RFY33 were transformed with p\textit{YCF1} or p\textit{YCF1}^{K699M} to generate RFY51-54.

\textbf{Vacuole isolation and in vitro vacuole fusion}

Vacuoles were isolated by floatation as described (Haas et al., 1995). Standard \textit{in vitro} fusion reactions (30 \textmu L) contained 3 \textmu g each of vacuoles from BJ3505 and DKY6281 backgrounds, fusion reaction buffer (20 mM PIPES-KOH pH 6.8, 200 mM sorbitol, 125 mM KCl, 5 mM MgCl$_2$), ATP regenerating system (1 mM ATP, 0.1 mg/mL creatine kinase, 29 mM creatine phosphate), 10 \textmu M CoA, and 283 nM IB$_2$. Reactions were incubated at 27°C and Pho8p activity was assayed in 250 mM Tris-Cl, pH 8.5, 0.4% Triton X-100, 10 mM MgCl$_2$, 1 mM \textit{p}-nitrophenyl phosphate. Fusion units were measured by determining the \textit{p}-nitrophenolate produced min$^{-1}$\textmu g$^{-1}$ \textit{pep4}\Delta vacuole and absorbance was detected at 400 nm.
Lipid mixing

Lipid mixing assays were conducted using rhodamine B-conjugated phosphatidylethanolamine (Rh-PE; Invitrogen) as described (Sasser et al., 2012a). Briefly, BJ3505 background vacuoles (300 µg) were incubated in 400 µl of PS buffer containing 150 µM Rh-PE (10 min, 4°C, nutating). Samples were mixed with 15% Ficoll in PS buffer (wt/vol) and transferred to an ultracentrifuge tube. Samples were overlaid with 1.0 ml each of 8%, 4%, and 0% Ficoll. Labeled vacuoles were re-isolated by centrifugation (105,200 g, 25 min, 4°C) and harvested from the 0 - 4%-Ficoll interface. Lipid mixing assays (90 µl) contained 2 µg of Rh-PE-labeled vacuoles and 16 µg of unlabeled vacuoles in fusion buffer and transferred to a black, half-volume 96-well flat-bottom microtiter plate with nonbinding surface (Corning). Rhodamine fluorescence was measured using a POLARstar Omega fluorescence plate reader (BMG Labtech) at 27°C. Measurements were taken every min for 75 min, yielding fluorescence values at the onset (F₀) and during the reaction (Fₜ). The final 10 measurements of reactions after adding 0.33% (vol/vol) Triton X-100 were averaged and used as a value for the fluorescence after infinite dilution (Fₓ₁₀₀). The relative total fluorescence change ∆Fₜ /Fₓ₁₀₀ = (Fₜ - F₀)/Fₓ₁₀₀ was calculated.

Microscopy

Vacuole morphology was monitored by incubating yeast cells with YPD broth containing FM4-64 (Invitrogen) as previously described (Sasser et al., 2012b). Images were acquired using a Zeiss Axio Observer Z1 inverted microscope equipped with an X-Cite 120XL light source, Plan Apochromat 63X oil objective (NA 1.4), and an AxioCam
CCD camera. Vertex assembly reactions were performed as described (Fratti et al., 2004). Docking reactions (30 µl) contained 6 µg of vacuoles. Phosphatidylinositol 3-phosphate (PI3P) was labeled with 0.2 µM Cy5-FYVE and used as a marker for vertex microdomains (Sasser et al., 2012a). Reactions were incubated at 27°C for 30 min and placed on ice and stained with 3 µM MDY64. Reactions were next mixed with low-melt agarose, vortexed to disrupt spurious clustering, and mounted on slides for observation by fluorescence microscopy. Statistical analysis of Cy5-FYVE enrichment at vertices was done using JMP 5 (SAS Institute, Inc.). Ratio data were log transformed before analysis to yield near-normal distributions with comparable variances. Ratio means and 95% confidence intervals were analyzed using one-way ANOVAs. Significant differences were determined using t-test and corrected for multiple comparisons using the Dunn-Sidak method (Sokal and Rohlf, 1994). P-values less than 0.05 were considered significant.

Quantitative PI3P ELISA

Total levels of PI3P were determined by using a quantitative ELISA (Echelon, Inc). Large-scale 10X reactions (300 µl) were prepared using DKY6281 background vacuoles (60 µg) and incubated at 27°C for 60 minutes. Neutral lipids were first extracted from vacuoles by adding 3 ml MeOH:CHCl₃ (2:1) and vortexing 3 times over 10 min at room temperature. Insoluble lipids were collected by centrifugation (1,500 g, 5 min) and the supernatant was discarded. Next, acidic lipids were extracted by adding 2.25 ml MeOH:CHCl₃:12 M HCl (80:40:1) and vortexing 4 times over 15 min at room temperature. Insoluble lipids were collected by centrifugation and the supernatant was
transferred to a 15 mL centrifuge tube. The acidic lipid fraction was treated with 0.75 ml CHCl₃ and 1.35 ml of 0.1 M HCl, vortexed, and phase separated by centrifugation as above. The lower organic phase was collected and transferred into a new 1.5 ml microfuge and dried down in a SpeedVac for 1 h. The dried lipids were re-suspended in 190 µl PBS-T with 3% protein stabilizer. Samples were vortexed for 1 min and centrifuged prior to use in the ELISA assay. The ELISA was performed according to the manufacturer’s instructions.

**Ca²⁺ efflux assay**

Vacuole lumen Ca²⁺ efflux was measured as described (Sasser et al., 2012a). Fusion reactions (60 µL) contained 20 µg of vacuoles isolated from BJ3505 backgrounds, fusion reaction buffer with 10 µM CoA, and 283 nM IB₂ and the fluorescent Ca²⁺ probe Fluo-4-dextran at 200 µM (Invitrogen). Reaction mixtures were transferred to a black, half-volume 96-well flat-bottom plate with nonbinding surface. ATP regenerating system, or buffer was added and reactions were incubated at 27°C while monitoring Fluo-4 fluorescence.
RESULTS

**ABCC transporters localized to the vacuole**

The yeast vacuole harbors multiple class C ABC transporters including Ycf1p, Bpt1p, Vmr1p and Nft1p. Here we compared the relative abundance of each transporter in our yeast tester strains by expressing GFP fusions. We also examined the distribution of the putative ABCC transporter YOL075c. Yeast cells were grown with the vital dye FM4-64, which is endocytosed and accumulates on the vacuole membrane. Colocalization analysis showed that Ycf1p-GFP and Bpt1p-GFP were highly enriched at the vacuole relative to the other transporters tested (Figure 3.1). Although Vmr1p-GFP, Nft1p-GFP and YOL075c-GFP were detected on vacuoles, these proteins were also present in other membrane pools yielding a hazy cytoplasm.

**YCF1 and BPT1 deletions attenuated vacuole fusion**

Although ABCC transporters primarily function in detoxification of yeast cytoplasm, new studies have shown that some family members perform additional functions. For example, Ybt1p translocates phosphatidylcholine to aid in choline recycling, calcium homeostasis and vacuole fusion (Gulshan and Moye-Rowley, 2011; Sasser et al., 2012b; Tarassov et al., 2008). Others have shown that Ycf1p physically interacts with Fab1p, a PI3 5-kinase that modifies PI3P, a lipid that is essential for vacuole fusion (Fratti et al., 2004; Paumi et al., 2007; Schu et al., 1993). Here we examined the possible role of ABCC transporters in the fusion process. *YCF1, BPT1,*
VMR1, NFT1, and YOL075C were deleted from our fusion tester strains and examined for abnormalities in the fusion pathway. These experiments showed that ycf1Δ and bpt1Δ vacuoles were attenuated for fusion by 30-40% relative to wild type parent strains (Fig. 3.2A, B). Interestingly, vmr1Δ, nft1Δ, and yol075cΔ vacuoles showed no defects in vacuole fusion (not shown). This suggests that the effects of deleting YCF1 and BPT1 were specific to functions of the individual proteins and not to general characteristics of ABCC family members. To determine if the effects of deleting YCF1 and BPT1 were additive, we generated double deletion strains (ycf1Δ/bpt1Δ) and examined fusion. We found that the deletion of both genes did not further inhibit fusion, suggesting that there is some redundancy in their function (Fig. 3.2C). Previously we found that the deletion of the ABCC transporter YBT1 stimulated fusion up to 50% above wild type fusion (Sasser et al., 2012b). To examine whether the absence of Ybt1p would compensate for the fusion defect seen with ycf1Δ/bpt1Δ vacuoles we produced fusion triple deletion tester strains (ycf1Δ/bpt1Δ/ybt1Δ). We found the triple deletion restored fusion to wild type levels. This suggests that the fusion defect seen in ycf1Δ/bpt1Δ vacuoles was not due to irreversible deleterious effects and that the two effects offset each other. To verify that the inhibited fusion was not due to an inhibition of the Pho8p reporter system, we also examined the effects of deleting YCF1 and BPT1 using a non-enzymatic reporter system. Here, vacuoles were labeled with Rh-PE at self-quenching levels (Jun and Wickner, 2007). Labeled vacuoles were incubated with an excess of unlabeled vacuoles and fusion was measured by the dilution and de-quenching of Rh-PE. Figure 3.2E shows that ycf1Δ/bpt1Δ vacuoles were inhibited for fusion by fluorescence dequenching at levels similar to Figure 3.2C. Because the effect of the double deletion
did not further reduce fusion the remainder of the study was performed with \textit{YCF1} single deletion strains. Attenuated fusion is often linked with vacuole fragmentation, thus we examined the vacuole morphology of \textit{ycf1Δ} cells as described above. Although \textit{ycf1Δ} vacuoles were attenuated for fusion we did not observe a defect in vacuole morphology relative to its wild type parent strain (Fig. 3.2F). We attribute the lack of a fragmentation defect to the redundant functions of ABCC transporters and to the supply of the soluble SNARE Vam7p in the cytoplasm that will be elucidated below.

\textbf{Ycf1p supported fusion requires ATPase activity}

To determine whether the role of Ycf1p in vacuole fusion was dependent on its transporter activity we complemented \textit{ycf1Δ} cells with plasmid-expressed wild type Ycf1p or mutant Ycf1p$^{K669M}$. The point mutation was in the Walker A motif of the first nucleotide binding domain. The conserved lysine is required for ATP hydrolysis and growth in the presence of cadmium (Wemmie and Moye-Rowley, 1997). Complementation of \textit{ycf1Δ} strains with wild type p\textit{YCF1} fully rescued fusion indicating that the inhibition was directly due to the absence of Ycf1p (Fig. 3.3A). However, when \textit{ycf1Δ} strains were complemented with the ATPase-deficient mutant p\textit{YCF1}$_{K669M}$ fusion remained attenuated (Fig. 3.3B), suggesting that the transport function of Ycf1p is important for the regulation of fusion.

\textbf{Ycf1p regulates Vam7p recruitment to vacuoles}

Changes in vacuole fusion can be due to alterations in the trafficking of fusion regulators to the vacuole, thus we examined the protein profile of \textit{ycf1Δ} vacuoles.
Figure 3.4A shows the levels of SNAREs (Vam3p, Vam7p, Vti1p, and Nyv1p), their chaperones Sec18p and Sec17p, HOPS subunits (Vps11p, Vps33p, and Vps41), Ypt7p, and other fusion regulators in wild type and ycf1Δ vacuoles. Most proteins were equally abundant on wild type and ycf1Δ with the prominent exception of the soluble SNARE Vam7p. Mutant ycf1Δ vacuoles contained 30-40% less Vam7p relative to wild type vacuoles (Fig. 3.4B), suggesting that the defect in fusion was due in part to the depletion of a SNARE protein. Levels of Pho8p and Pep4p were also analyzed and there were no significant changes in either component of the reporter system further indicating that the attenuated fusion of ycf1Δ vacuole was due to changes in the fusion machinery and not Pho8p activation. To determine whether the reduction in vacuole associated Vam7p was due to defective recruitment or degradation of the protein we examined Vam7p levels in whole cell lysates. There was no observable difference in Vam7p levels between WT and ycf1Δ cells indicating that mutant vacuoles were unable to recruit sufficient Vam7p to support fusion (Fig. 3.4C).

Vam7p directly interacts with the HOPS complex as well as other SNAREs (Stroupe et al., 2006; Ungermann and Wickner, 1998); however, the levels of these proteins were not affected on ycf1Δ vacuoles. Vam7p also interacts with the lipid PI3P (Cheever et al., 2001), which is made on the vacuole during fusion by the PI kinase Vps34p (Thorngren et al., 2004). Yet levels of this lipid kinase were also unaffected on mutant vacuoles. These data however, do not show whether Vam7p association to its binding partners was affected on mutant vacuoles. To test whether the simple lack of Vam7p affected ycf1Δ vacuole fusion, we added exogenous GST-Vam7p to fusion reactions containing either wild type or ycf1Δ vacuoles. We found that the direct addition
of Vam7 during the fusion reaction rescued ycf1Δ vacuole fusion to wild type levels (Fig. 3.4D). This indicates that Vam7p can function with its binding partners during fusion and that the defect in ycf1Δ vacuoles fusion is due to the inability to recruit wild type levels of Vam7p. We also observed that high levels of Vam7p reduce fusion. This is consistent with previous studies showing a biphasic curve of the effects of Vam7p on fusion (Fratti et al., 2007; Fratti and Wickner, 2007).

Ycf1p regulates PI3P accumulation at vertex microdomains

In addition to binding HOPS and SNAREs, Vam7p binds the regulatory lipid PI3P. This lipid is required for fusion in part by its function in the assembly of vertex microdomains as well as the direct recruitment of Vam7p (Cheever et al., 2001; Fratti et al., 2004). Although the lipid kinase Vps34p was present on ycf1Δ vacuoles, it remained possible that PI3P levels were insufficient to recruit Vam7p to the vacuole. To examine the levels and distribution of PI3P on wild type and ycf1Δ vacuoles we used ratiometric fluorescence microscopy and quantitative ELISA assays. To track PI3P accumulation into vertex microdomains we incubated reactions containing either wild type or ycf1Δ vacuoles with the specific PI3P ligand FYVE domain (Gillooly et al., 2000). The FYVE domain was conjugated with the fluorophore Cy5. After incubation, the docking reactions were placed on ice and stained with MDY-64 to label the limiting vacuole membrane (Fratti et al., 2004; Wang et al., 2003). We found that ycf1Δ vacuoles contained elevated levels of PI3P at the vertices of docked vacuoles relative to wild type vacuoles (Fig. 3.5A). Cy5-FYVE accumulation at vertices relative to the outer edge of membranes was measured as a ratio of Cy5-FYVE to MDY-64. Vertex and outer edge
ratios were plotted on cumulative distribution plots. The right-shift of the \( ycf1\Delta \) vertex curve relative to the wild type vertex curve indicated that there was more PI3P at the vertices of \( ycf1\Delta \) vacuoles. Figure 3.5B shows the geometric means and 95% confidence intervals of the curves in Figure 3.5A and shows that the enhanced vertex enrichment of PI3P on \( ycf1\Delta \) vacuoles was statistically significant \((P<0.0001)\). To distinguish whether the difference in PI3P distribution was due to absolute changes in lipid concentrations or enhanced microdomain formation we used a quantitative ELISA assay to measure total levels of PI3P in solubilized wild type and \( ycf1\Delta \) vacuoles. Figure 3.5C shows that the total levels of PI3P in \( ycf1\Delta \) vacuoles were not significantly different relative to wild type vacuoles. Taken together, these data suggest that PI3P accumulates at vertices with more efficiency in the absence of Ycf1p. It was also possible that the absence of Vam7p on \( ycf1\Delta \) vacuoles resulted in more PI3P available for Cy5-FYVE staining.

**Exogenous Vam7p restored Ca\(^{2+}\) efflux in \( ycf1\Delta \) vacuoles**

Thus far we have observed that the fusion defect in \( ycf1\Delta \) vacuoles was due in part by the exclusion of Vam7p from the vacuole, which could lead to a reduction in \textit{trans}-SNARE pairing. Since the formation of \textit{trans}-SNARE complexes triggers the release of lumenal Ca\(^{2+}\) stores from the vacuole prior to fusion (Merz and Wickner, 2004; Peters and Mayer, 1998), we next determined if the deletion of \textit{YCF1} altered Ca\(^{2+}\) efflux. Fusion reactions containing wild type or \( ycf1\Delta \) vacuoles were incubated in standard fusion reaction components in the presence of the fluorescent calcium indicator Fluo4-dextran (Sasser et al., 2012b). Reactions were started by the addition of
buffer or ATP regenerating system. In the absence of ATP, Fluo4 fluorescence remained stable while fluorescence decreased in the presence of ATP during the first 20 min of the reactions (Fig 3.6A), indicating that Ca\(^{2+}\) was taken up by the vacuoles. As a negative control we inhibited selected reactions with Gyp1-46p to inhibit Ypt7p-dependent docking and Fluo4 fluorescence continued to decrease until the inhibited reactions reached a baseline. Interestingly, the initial uptake of Ca\(^{2+}\) was measurably greater in ycf1\(^{Δ}\) reactions relative to wild type. This could be attributed to differences in relative amounts of Vam7p and trans-SNARE complexes on mutant vacuoles. After 15-20 min of incubation, the uninhibited reactions released Ca\(^{2+}\) that correlated with the formation of trans-SNARE complexes. Reactions containing ycf1\(^{Δ}\) vacuoles released Ca\(^{2+}\) at the same time as wild type vacuoles, but the external concentration of cations did not reach wild type levels. It should be noted that there was no difference in the net amount of released Ca\(^{2+}\) in mutant reactions. To determine if the levels of Vam7p were linked to the changes in Ca\(^{2+}\) we performed experiments in the presence or absence of exogenous Vam7p. We found that adding supplemental Vam7p to reactions eliminated the differences in Ca\(^{2+}\) efflux between ycf1\(^{Δ}\) and wild type vacuoles. This was consistent with the effect of exogenous Vam7p on the fusion of ycf1\(^{Δ}\) vacuoles in Figure 3.4D and suggests that the primary defect in these vacuoles is the defective recruitment of Vam7p.
**DISCUSSION**

Ycf1p regulates the transport of cadmium, mercury and other toxins into the vacuole lumen to detoxify the cytoplasm (Gueldry et al., 2003; Li et al., 1996; Szczypka et al., 1994). However, a role for Ycf1p in the regulation of fusion had not been directly examined. Interestingly, an integrated split-ubiquitin membrane yeast two-hybrid (iMYTH) analysis has shown that Ycf1p physically interacts with the PI3P 5-kinase Fab1p, which uses PI3P to produce PI(3,5)P₂, as well as the Rho1p nucleotide exchange factor, Tus1p (Paumi et al., 2007). Ycf1p has also been found to physically interact with Rho1p, a GTPase associated with actin dynamics (Lee et al., 2011). Since Rho1p, actin and PI3P have been found to play important roles in vacuole fusion we examined the effect of deleting YCF1 and other ABCC transporters on vacuole fusion. We found that ycf1Δ and bpt1Δ vacuoles were each attenuated for fusion, yet the double deletion did not exhibit additive effects. The defect in fusion was relieved by also deleting YBT1, an ABCC transporter previously reported to negatively regulate vacuole fusion (Sasser et al., 2012b). The mechanism(s) for the rescued fusion remain unclear and will be further explored in future studies. The rest of the study focused on the effect of deleting YCF1 alone. We found that the attenuated fusion seen with ycf1Δ vacuoles was due to the exclusion of Vam7p, a defect in fusion that was rescued by the addition of recombinant Vam7p. The exclusion of Vam7p from ycf1Δ vacuoles was not due to the lack of known binding partners that include SNAREs, HOPS and the regulatory lipid PI3P, which were all at, or near, wild type levels. Moreover, the exogenous Vam7p
readily associated with the vacuoles to trigger fusion, illustrating that neither Vam7p-protein, nor Vam7-PI3P interactions were deleteriously affected by the lack of Ycf1p.

In this study we also found that the ATPase activity of Ycf1p was important for vacuole fusion. A point mutation that changed the conserved Lys with a Met of the Walker A box motif of NBD1 prevented the complementation of the ycf1Δ fusion defect. Although physical interactions between Ycf1p and trafficking proteins have been documented by iMYTH, the interactions were independent of ATPase activity or in the context of the vacuole. Because the addition of recombinant Vam7p rescued ycf1Δ vacuole fusion, we posit that the direct transport activity of Ycf1p is not related to Vam7p recruitment to the vacuole membrane. One possible mechanism for the link between these two proteins is the putative “flippase” activity of Ycf1p and other ABCC transporters. Although flippase activity has not been reported for Ycf1p, its parologue Ybt1p has been reported to translocate (or flip) phosphatidylcholine from the outer-to-inner leaflets of the vacuole membrane (Gulshan and Moye-Rowley, 2011). Thus, it is not unlikely that Ycf1p may also function as a lipid translocation enzyme for other lipids. This is important because Vam7p binding to the vacuole is regulated by the composition of the outer leaflet vertex microdomains of vacuole (Fratti et al., 2004), where it was shown that disruption of the vertex microdomain by binding or modifying various lipids other than PI3P inhibited the binding of Vam7p to vacuoles. Therefore, if Ycf1p modifies the lipid composition of the vacuole outer leaflet, inactivating its ATPase-dependent translocation activity could alter the steady state association of Vam7p.
FOOTNOTES

We thank Dr. William Wickner for generous gifts of antisera and Dr. W. Scott Moye-Rowley for plasmids. We also thank members of the Fratti Lab for critical reading of the manuscript. This research was supported by a grant from the University of Illinois Research Board awarded to RAF and a grant from the National Institutes of Health (GM101132) to RAF.

The Abbreviations used are: SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptors; CBP, Calmodulin binding peptide; HOPS, homotypic fusion and vacuole protein sorting complex; PI, phosphatidylinositol; PI3P, phosphatidylinositol 3-phosphate; YPD, yeast extract/peptone/dextrose; Rh-PE, rhodamine phosphatidylethanolamine.
Table 3.1. Yeast strains used in this study.

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Figure 3.1. Ycf1-GFP is localized to the vacuole. Wild type yeast cells harboring either Ycf1p-GFP, Bpt1-GFP, Nft1-GFP, Vmr1-GFP or YOL075c-GFP were incubated with 5 μM FM4-64 to label vacuoles. Cells were washed with PBS and grown for 1 h in label-free YPD to chase the dye into the vacuole. Cells were washed with PBS and mounted for microscopy. GFP images were acquired using a 38 HE Green Fluorescent Protein shift-free filter set and FM4-64 images were acquired using a 43 HE CY3 shift-free filter set. Cells were photographed using differential interference contrast (DIC). Images were merged using Photoshop. Bar, 4 μm.
Figure 3.2. Deletion of YCF1 and BPT1 attenuates vacuole fusion. (A) Vacuoles were harvested from WT, ycf1Δ (A), bpt1Δ (B), ycf1Δ/bpt1Δ (C), and ycf1Δ/bpt1Δ/ypt1Δ (D) yeast and tested for fusion activity. Standard fusion reactions used equal amounts of reporter (PHO8 pep4Δ) and effector (pho8Δ PEP4) vacuoles. Error bars represent S.E. (n=3). (E) Lipid mixing assays measuring fusion were employed by labeling isolated vacuoles with Rh-PE. Labeled vacuoles were incubated with 8-fold excess of unlabeled vacuoles and dequenching was measured using a fluorescence plate reader. (F) WT and ycf1Δ yeast were stained with FM4-64 and vacuole morphology was analyzed by fluorescence microscopy.
Figure 3.2 continued. Deletion of *YCF1* and *BPT1* attenuates vacuole fusion.
Figure 3.3. Ycf1p supported fusion requires ATPase activity. Vacuoles were harvested from WT, ycf1Δ + pYCF1 (A), and ycf1Δ pYCF1<sup>R669M</sup> (B) yeast and tested for fusion activity. Standard fusion reactions used equal amounts of reporter (PHO8 pep4Δ) and effector (pho8Δ PEP4) vacuoles. Error bars represent S.E. (n=3).
Figure 3.4. Vam7p depletion on ycf1Δ vacuoles regulates vacuole fusion. (A) Analysis of core fusion components. Vacuoles were isolated from WT and ycf1Δ BJ3505 and DK6281 strains. Vacuoles (5 µg by protein) were mixed with 2X SDS loading buffer, separated by SDS-PAGE and transferred to nitrocellulose. Immunoblotting was performed using the indicated antibodies and bands were detected using ECF. (B) Quantitation of Vam7p, Pho8p and Pep4p levels on WT and ycf1Δ vacuoles. (C) Western blot of Vam7p in whole cell lysates of WT and ycf1Δ yeast. (D) Fusion reactions containing WT or ycf1Δ were incubated with recombinant GST-Vam7p and tested for fusion as described above. Error bars represent S.E. (n=3).
Figure 3.5. PI3P levels are increased at the vertices of docked ycf1Δ vacuoles. (A) Cumulative distribution plots show the percentile values of Cy5-FYVE to MDY-64 ratios for each vertex (V) and outer edge (O). Each curve is compiled from at least 10 vacuole clusters where the maximum pixel intensity was determined for every vertex and midpoint of the outer edge membrane. Pixel intensities were measured in both fluorescence channels at each subdomain and expressed as a ratio of Cy5-FYVE to MDY-64. Outer edge ratios were normalized to a value of 1 and the enrichment of Cy5-FYVE at vertices were expressed relative to outer edge intensities. Each ratio in a dataset is ordered and plotted versus the percentile rank of the values. (B) Geometric means with their 95% confidence intervals for the data in (A). (C) Quantitative ELISA analysis of PI3P levels on WT and ycf1Δ vacuole fusion reactions after 60 min of incubation at 27°C. Error bars represent S.E. (n=3)
Figure 3.6. Calcium efflux is altered in ycf1Δ vacuoles. Vacuoles were harvested from WT BJ3503 and RFY32 (ycf1Δ). Fusion reactions (2X) were prepared containing 20 µg of either WT or ycf1Δ vacuoles and 200 µM Fluo4-dextran. Immediately after addition of ATP or PS buffer, reactions were incubated at 27°C and fluorescence was measured for 90 min. (A) Reactions were treated with buffer or Gyp1-56. (B) Reactions were treated with buffer or GST-Vam7p. Shown are representative experiments of 3 repeats.
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the ATP-dependent vacuolar transport of unconjugated bilirubin in *Saccharomyces cerevisiae*. *Yeast*. 16:561-71.


CHAPTER 4: A REAL-TIME FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET) ASSAY TO MEASURE THE TRANSLOCATION OF RHODAMINE-LABELED PHOSPHATIDYLETHANOLAMINE IN YEAST VACUOLES

ABSTRACT

Maintenance of eukaryotic cellular homeostasis requires fusion of vesicle membranes. Membrane lipids are organized in an asymmetric fashion across the vesicle bilayer and this asymmetry must be maintained through lipid translocation. The translocation or flippase activity can be monitored using fluorescently labeled lipids. In this study, we describe a FRET-based assay to follow the translocation of the rhodamine-labeled phosphatidylethanolamine (RH-PE) in a real-time assay. We found that there is an ATP-dependant Rh-PE flippase on the vacuole and the ABC transporters Ycf1p, Bpt1p and Ypk9p are not responsible for the translocation activity. We also report that pH and osmolyte concentration do not affect the lipid translocation activity. However, modifying the bilayer fluidity with the addition of chlorpromazine (CPZ) abolished lipid translocation activity and propranolol inhibition increases the rate of lipid translocation.
INTRODUCTION

Maintenance of eukaryotic cellular homeostasis requires fusion of vesicle membranes. Membrane fusion is the merger of two lipid bilayers into one continuous membrane. Multiprotein complexes that have been conserved in eukaryotes carry out the basic reactions of fusion. In *Saccharomyces cerevisiae*, homotypic vacuole fusion occurs in experimentally defined phases. Fusion *priming* does not involve contact between vacuoles but includes the disassembly of complexes of SNAREs on the same membrane (cis) by Sec18p (NSF) and its cochaperone Sec17p (a-SNAP). *Tethering* requires Ypt7p (a Rab GTPase) and the HOPS effector complex. SNARE complexes, including one R SNARE from a donor vacuole and three Q SNAREs from the acceptor vacuole, are formed in trans during *docking* of vacuoles. The membranes of the docked vacuoles are drawn together to form the “boundary domain” that resembles flat discs. The “outer membranes” are not in contact and come together at the boundary membrane to form the “vertex ring.” The vertex microdomain, enriched in fusogenic lipids and proteins, is the origin of fusion where the outer membranes are joined. Fusion culminates with the internalization of the luminal vesicle and mixing of luminal contents.

Membrane lipids are organized in an asymmetric fashion across the bilayer and this asymmetry must be maintained through lipid translocation. The cytoplasmic surface of the eukaryotic bilayer contains an enrichment of phosphoinositides (PI), phosphatidic acid (PA), phosphatidylethanolamine (PE), and phosphatidylserine (PS) while the extracytoplasmic leaflet contains primarily phosphatidylcholine (PC) and
sphingomyelin (Bretscher, 1972; Op den Kamp, 1979). The thermodynamic barrier prevents passive transverse movement of the phospholipids from the inside to the outside (Kornberg and McConnell, 1971). Transmembrane transporters maintain lipid asymmetry by flipping or flopping phospholipids (Daleke, 2007). Translocation of lipids is executed by membrane proteins that are referred to as lipid “flippases” and “floppases”, which establish lipid asymmetry between the inner and outer leaflets of a membrane (van Meer et al., 2008). Scramblase, an energy-independent transporter, eliminates lipid asymmetry. Translocation of lipids can cause changes in membrane shape that leads to fission or fusion (Papadopulos et al., 2007). Gram negative bacteria have ABC transporter systems that maintain lipid asymmetry in the outer membrane (Malinverni and Silhavy, 2009). Some of the putative flip/floppases in eukaryotes belong to the ABC transporter superfamily (Gulshan and Moye-Rowley, 2011).

Flippase activity has been measured by a variety of techniques that generally measure the amount of a labeled lipid at an endpoint. NBD-labeled lipids are added to the vesicles or liposomes of interest, back-extracted with BSA at a specified time to remove lipids not translocated and then the remaining NBD-labeled lipid that has been translocated inward can be measured (Dekkers et al., 2000). A similar method monitors translocation by quenching the NBD label in the outer leaflet with an impermeant reagent such as dithionite (Vehring et al., 2007). Both methods require multiple reactions for control, mutant or inhibitor experiments to generate activity data, which therefore consumes valuable vesicle preparations and lipid reagents. In addition, the anion dithionite is impermeable to liposomes (McIntyre and Sleight, 1991) but is highly permeable in cellular membranes due to anion transporters (Pomorski et al., 1994) and
other undefined mechanisms (Pomorski et al., 1995). To overcome some of the shortfalls with the endpoint assays, we have developed a real-time fluorescence resonance energy transfer assay that uses a GFP-labeled membrane protein and a rhodamine-labeled lipid to monitor the translocation of a phospholipid.
MATERIALS AND METHODS

Reagents

Reagents were dissolved in PS buffer (20 mM PIPES-KOH, pH 6.8, 200 mM sorbitol). Antibodies against Vam3p [22] and Sec17p [23] were previously described. Lissamine rhodamine B (Rh-PE; Invitrogen) was dissolved in ethanol to a final concentration of 2.5 mM.

Strains

YCF1, NHX1 and YPK9 were deleted from BJ3505 by homologous recombination with the ura3 marker using PCR products amplified from pRS406 (Sikorski and Hieter, 1989). The PCR product was transformed into BJ3505 (MATα pep4::HIS3 prb1-Δ1.6R his3–200 lys2–801 trp1Δ101 (gal3) ura3–52 gal2 can1) by standard lithium acetate methods to generate BJ3505 ycf1Δ::URA3 (TSY1), BJ3505 nhx1::URA3 (TSY2), and BJ3505 ypk9Δ:URA3 (TSY3). Transformants were selected using complete synthetic media lacking uracil. BPT1 was then deleted from TSY1 by homologous recombination with the trp1 marker using a PCR product amplified from pRS404 (Sikorski and Hieter, 1989). The PCR product was transformed into TSY1 to generate BJ3505 ycf1Δ::URA3 bpt1::TRP1 (TSY4). To create the translocation tester strains, PHO8 was fused in-frame to GFP by homologous recombination. BJ3505, TSY1, TSY2, TSY3, and TSY4 were transformed with a PCR product amplified from pFA6a-GFP-kanMX6 (Longtine et al., 1998) with homology flanking the stop codon of PHO8 to generate TSY5 (BJ3505 PHO8::GFP), TSY6 (BJ3505 ycf1::URA3...
PHO8::GFP), TSY7 (BJ3505 ycf1::URA3 bpt1::URA3 PHO8::GFP), TSY8 (BJ3505 nhx1::URA3 PHO8::GFP), and TSY9 (BJ3505 ypk9::kanMX6 PHO8::GFP).

**Vacuole isolation and lissamine B labeling**

Vacuoles were isolated by floatation as described (Haas, 1995). BJ3505 background vacuoles (300 µg) were incubated in 400 µL of PS buffer (20 mM PIPES-KOH pH 6.8, 200 mM sorbitol) containing 10 µM Lissamine rhodamine B (Rh-PE; Invitrogen) for 10 minutes at 4°C with nutation. Samples were mixed with 15% Ficoll in PS buffer (wt/vol) and transferred to a polyallomer tube (11 x 60 mm). Samples were overlaid with 1.0 mL each of 8%, 4%, and 0% Ficoll. Labeled vacuoles were re-isolated by centrifugation using a SW60 Ti rotor (105,200 g, 25 minutes, 4°C) and recovered from the 0 - 4%-Ficoll interface.

**FRET assay to measure Rh-PE translocation**

Translocation assays (90 µL) contained 18 µg of purified Rh-PE-labeled vacuoles in reaction buffer (130 mM NaCl, 4 mM MgCl₂, 1 mM PIPES (pH 6.8), 20 mM Sorbitol). Reaction mixtures were transferred to a black, half-volume 96-well flat-bottom microtiter plate with nonbinding surface (Corning) containing inhibitors. Emission at 520 nm and 620 nm after excitation at 410 nm was measured in a POLARstar Omega fluorescent plate reader (BMG Labtech) at 27°C. Measurements were taken every minute for 5 minutes and then PS or ATP (final 4 mM) was added. Measurements were continued for an additional 60 minutes. FRET is expressed as the ratio of acceptor emission (rhodamine) at 620 nm to donor emission (GFP) at 520 nm.
RESULTS

Yeast vacuoles contain an ATP-dependent lipid translocator

During membrane fusion, two membrane bilayers are pulled tightly together to form a flattened boundary resulting in stress from lipid crowding at the vertices. To relieve this stress the phospholipids are remodeled which is likely to occur through lipid translocation across the bilayer. To determine if there is ATP-dependant translocation of lipids on the vacuole, vacuoles harboring a Pho8-GFP N-terminal fusion (on the inner leaflet of the vacuole) were labeled on the outer leaflet with Rhodamine-PE (Figure 4.1A). The labeled phospholipid was flipped to the inner leaflet by an ATP-dependent mechanism resulting in a FRET signal (Figure 4.1B). Reactions containing the ATPase inhibitor vanadate (Decottignies et al., 1998) or the MDR transporter inhibitor verapamil (Gottesman and Pastan, 1993; Ruetz and Gros, 1994) were inhibited resulting in similar to (verapamil) or less than (vanadate) the FRET activity seen in the no ATP reactions (Figure 4.1C). This result again suggests that an ATPase is responsible for translocation of lipids. To determine if the core fusion machinery had any effect on lipid translocation, antibodies to Sec17p and Vam3p were added to reactions (Figure 4.2). The antibodies had no effect on translocation of lipids indicating that SNAREs and subsequent membrane fusion are not required for the activity.

Ycf1p, Bpt1p and Ypk9p do not act as the lipid translocator

Multi-drug resistance associated protein (MRP) is a member of the ABCC family of transporters and has been shown to translocate NBD-labeled lipids from human
erythrocytes (Dekkers et al., 2000; Huang et al., 2004). To identify the transporter responsible for translocation activity, Pho8-GFP fusions were prepared in each of the previously prepared ABCC transporter knockouts. TSY6 and TSY7 were examined in the translocation assay to determine the effect of Ycf1p and Bpt1p on translocation activity. Figure 4.3A shows that translocation activity in ABCC mutant vacuoles was similar to activity in WT yeast vacuoles in the presence of ATP. Attempts to prepare a Pho8-GFP fusion in an Ybt1p mutant have been unsuccessful.

ATPase activity has already been shown to be necessary for translocation activity (Figure 4.1) so other ATPases on the vacuole were investigated. The P-glycoprotein multidrug transporter in humans has been shown to have flippase activity with fluorescently labeled short chain phospholipids (Romsicki and Sharom, 2001). The yeast vacuole contains one P-glycoprotein transporter, Ypk9p, that is known to transport metal ions into the lumen (Schmidt et al., 2009). TSY9 was used in the translocation assay to determine if it plays a role in the translocation of Rh-PE. Translocation activity was similar to that observed in WT strains (Figure 4.3B) indicating that Ypk9p is not responsible for the activity.

**Lipid translocation is not pH dependant**

Although we did not yet identify the ATPase responsible for lipid translocation, we next set out to examine possible influences on translocation activity. Nhx1p is a Na⁺(K⁺)/H⁺ antiporter that plays a role in acidifying the vacuole (Brett et al., 2005) and has been shown to regulate membrane fusion (Qiu and Fratti, 2010). To determine if the acidification of the vacuole alters the rate of lipid translocation, the Pho8-GFP fusion
was prepared in \textit{nhx1} mutant yeast (TSY8). Vacuoles isolated from this tester strain showed comparable activity to WT vacuoles with and without ATP (Figure 4.4A).

\textbf{Blocking the conversion of PA to DAG increases lipid translocation rate}

Phosphatidic acid phosphatase (PAP) activity is necessary for vacuole homeostasis and membrane fusion (Sasser et al., 2012a) and may also play a role in remodeling lipids during fusion. Phosphatidic acid and diacylglycerol are regulatory lipids in membrane fusion (Fratti et al., 2004; Jun et al., 2004) and also affect membrane curvature and surface charge. To test if the PAP activity is necessary for lipid translocation the β-adrenergic receptor antagonist propranolol (Morlock et al., 1991) was used in translocation assays. Addition of propranolol increased the initial rate of lipid translocation and then reached equilibrium similar to activity in WT vacuoles (Figure 4.4B) suggesting that conversion of phosphatidic acid to diacyl glycerol acts to decrease lipid translocation.

\textbf{Increasing bilayer fluidity abolishes lipid translocation}

In addition to the curvature of a membrane seeming to have an effect on lipid translocation activity, membrane fluidity may also be important. To test this correlation we used the drug chlorpromazine (CPZ) that has been shown to increase membrane fluidity (Ogiso et al., 1981). CPZ also lowers the activation energy required for membrane fusion and rescues fusion in vacuoles with non-canonical SNAREs (Karunakaran and Fratti, 2012). Addition of 150 μM chlorpromazine completely abolished Rh-PE translocation to levels similar to the no ATP reactions (Figure 4.4C).
**Lipid translocation is unaffected by osmolyte concentration**

Osmolyte concentration has been implicated in the regulation of vacuole membrane fusion (Brett and Merz, 2008). An increase in the extent of membrane fusion was observed when sorbitol concentration was reduced and a decrease was observed in high concentrations of sorbitol. Typical reactions contain 200 mM sorbitol so to increase or decrease osmolyte concentration, translocation assays were prepared with 600 mM sorbitol and 100 mM sorbitol, respectively. Although differences are observed in membrane fusion, there was no difference in lipid translocation with either higher or lower sorbitol concentrations (Figure 4.4D). Therefore, although increased osmolyte concentration tends to cause rupture of membranes in isolated vacuoles, this stress does not influence the translocation of Rh-PE.
DISCUSSION

In this chapter I have described a FRET-based assay to monitor the translocation of Rhodamine-labeled phosphatidylethanolamine on the yeast vacuole. As opposed to end-point assays that utilize short chain NBD-lipids to label liposomes and either dithionite quenching or BSA back extraction to determine the amount of lipid that has been translocated, this new assay uses a labeled-lipid that can be used to monitor the translocation of the lipid in real-time. The advantages of the FRET assay are that time and resources are conserved, and it eliminates the necessity of the anion dithionite that can enter prepared organelles through the nascent anion channels.

Many regulators of membrane fusion have been described including protein (Cabrera et al., 2009) and lipid modifiers (Sasser et al., 2012a; Schu et al., 1993), ion transporters (Qiu and Fratti, 2010) as well as several lipids (Fratti et al., 2004; Jun et al., 2004). Remodeling of membranes during membrane fusion suggests a requirement for a lipid translocator also known as a flippase. Observations of the ATP-dependant translocation of Rh-PE strongly suggest the presence of a flippase on the vacuole. We sought to identify the flippase by preparing a Pho8-GFP fusion in a variety of yeast strains deficient in the candidate protein to probe for flippase activity. Unfortunately, we were unable to identify the protein responsible but we did eliminate the possibility that Ycf1p, Bpt1p, and Ypk9 are the flippases. Of the ABCC transporters, Ybt1p remains as a candidate for translocation activity and has even been shown to translocate phosphatidylcholine in yeast vacuoles (Gulshan and Moye-Rowley, 2011). In addition, there is no evidence that PC is the lone substrate of Ybt1p leaving the possibility open
that it is responsible for the translocation of Rh-PE. There are additional ABC transporters located on the vacuole such as Ymr1p, Nft1p, and the uncharacterized YOL075c (Paumi et al., 2009). Although these are considered to localize to the vacuole, in our lab they appear to be evenly distributed throughout the yeast cell with some localization to the vacuole. However, future explorations to determine the Rh-PE flippase will require that all potential ATPases in the yeast genome be incorporated into the FRET-assay.

As in other fluorescent lipid assays, it should be noted that the flippase might recognize the fluorescent label as the substrate rather than the lipid itself. Therefore, it cannot be said with certainty that the flippase is capable of translocating endogenous lipids. One option to detect translocation of un-labeled lipids is to localize a fluorescently-labeled probe into the interior of the organelle (Yeung et al., 2008). For example, the FYVE domain specifically binds to phosphatidylinositol 3-phosphate (PI(3)P) (Gillooly et al., 2000), can be fused to GFP or another fluorescent protein and then localized to the lumen of the vacuole. Vacuoles can be monitored under a fluorescent microscope to determine the binding of the GFP-FYVE domain to PI(3)P. Although a dependence of membrane fusion on lipid translocation has yet to be described, identifying a vacuolar flippase and then characterizing the flippase activity and the associated membrane remodeling will be interesting for future investigations.
### Table 4.1. Yeast strains used in this study

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<td>TSY6</td>
<td>BJ3505, ycf1Δ::URA3 PHO8::GFP</td>
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<td>TSY9</td>
<td>BJ3505, ypk9Δ::URA3 PHO8::GFP</td>
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Figure 4.1. Yeast vacuoles show ATP-depandant lipid translocation. Vacuoles are incubated with lissamine rhodamine B (Rh-PE) and re-isolated through a ficoll gradient. The reaction is excited at the GFP wavelength ($\lambda_{\text{ex}} = 410$ nm). When ATP is added, the translocated Rh-PE absorbs energy emitted by GFP ($\lambda_{\text{em}} = 520$ nm) and fluoresces at its emission wavelength ($\lambda_{\text{em}} = 620$ nm). A. Cartoon representation of Pho8-GFP lipid translocation assay. B. ATP (closed circles) or PS buffer (open circles) was added to begin the reaction. Fluorescence was measured in a PolarStar platereader for 90 minutes. C. The inhibitors vanadate (x) and and verapamil (+) were added to reactions to block ATPase activity.
Figure 4.2. Lipid translocation proceeds when membrane fusion is blocked. Vacuoles were isolated from WT yeast (RFY55) and labeled with Rh-PE. Reactions were prepared in a 96-well plate and either PS, anti-Vam3p IgG (+, 27 μg/mL), or anti-Sec17p IgG (x, 12 μg/mL) were added. ATP or PS was then added and the fluorescence was measured for 75 minutes in a PolarStar plate reader.
Figure 4.3. The ABC transporters Ycf1p, Bpt1p and Ypk9p are not associated with Rh-PE translocation. Vacuoles were isolated from WT, ycf1Δ, and ycf1Δbpt1Δ yeasts in A and WT and ypk9Δ yeasts in B. Reactions were prepared as described and fluorescence was measured for 90 minutes.
Figure 4.4. Effects of membrane perturbation and osmotic pressure on lipid translocation activity. Vacuoles were prepared as previously described from WT (A-D) or nhx1Δ yeast. Phosphatidic acid phosphatase activity was blocked with 3 mM propranolol (B). Membrane fluidity was altered with the addition of 3 mM chlorpromazine (C). Osmolyte concentration was altered by using 100 mM or 600 mM sorbitol (D).
Figure 4.4 continued.


CHAPTER 5: GENERAL DISCUSSION

All eukaryotic organisms require the process of trafficking for cellular cargo whose delivery is accomplished by a SNARE mediated mechanism known as membrane fusion. When two bilayers merge to become a single bilayer, membrane fusion has occurred and the process can be repeated. The process occurs in several stages that require a host of membrane proteins, soluble proteins, and regulatory lipids. The details of the mechanism have been studied extensively but there are many regulatory factors yet to be defined. In the research summarized in this dissertation, I have focused my explorations on a class of ABC transporters with a wide spectrum of substrates and activity to determine their role in the regulation of membrane fusion and maintenance of lipid asymmetry in the bilayer. In these studies, the following has been demonstrated: (i) yeast vacuoles lacking Ybt1p display a delay in Ca$^{2+}$ efflux resulting in enhanced fusion; (ii) Ycf1p regulates fusion through the recruitment of Vam7p to vacuolar membranes; (iii) an unidentified ATP-dependent lipid translocator resides on the yeast vacuole and is regulated by membrane fluidity.

**Ybt1p acts as a negative regulator of membrane fusion**

In Chapter 2 we found that the ABCC transporter Ybt1p acts as a negative regulator of vacuole fusion from the observation of the enhanced fusion in vacuoles from YBT1 deleted strains. Experiments testing the stages of the fusion showed that the defect in fusion occurred after the docking stage when trans-SNARE complexes are formed. The enhanced fusion coincided with a delay in Ca$^{2+}$ efflux that is normally
triggered by the formation of trans-SNARE complexes (Merz and Wickner, 2004). This suggested that Ybt1p might regulate the transport of ions across the membrane. The observed changes might be attributed in part to the interaction of Ybt1p with Pmc1p, a Ca\textsuperscript{2+}-ATPase that transports Ca\textsuperscript{2+} ions into the vacuole (Tarassov et al., 2008). Although the SNARE-dependent vacuolar Ca\textsuperscript{2+} efflux channel remains undefined, the connections between Ybt1p, Pmc1p and the delayed efflux during fusion suggests that the import and export of Ca\textsuperscript{2+} is linked to the fusion machinery. Although there is no direct connection between Ybt1p and the fusion machinery, the link between Ybt1p and Pmc1p suggests that an indirect mechanism is possible. In the absence of Ybt1p we observed the prolonged uptake of Ca\textsuperscript{2+} upon the addition of ATP, suggesting that Pmc1p activity is augmented and that changes in activity might be due to reduced interactions with Nyv1p.

There are several possible mechanisms by which the efflux of Ca\textsuperscript{2+} might contribute to the regulation of fusion. Vacuole fusion depends on the formation of membrane microdomains enriched in the proteins and lipids that regulate fusion (Fratti et al., 2004; Wang et al., 2003; Wang et al., 2002). Perturbations to the vertex formation pathway would undoubtedly alter fusion. Others have found that Ca\textsuperscript{2+} at sub-micromolar concentrations induces clustering of plasma membrane SNAREs by neutralizing negatively charged side chains (Zilly et al., 2011). Ca\textsuperscript{2+} could also relieve the electrostatic repulsion between negatively charged phospholipids (Boettcher et al., 2011; Ellenbroek et al., 2011). While the measurement of Ca\textsuperscript{2+} efflux only measures global concentrations, it is possible that local concentrations of Ca\textsuperscript{2+} at the site of efflux may reach micromolar levels thus inhibiting fusion. Thus, a delay in Ca\textsuperscript{2+} efflux might
allow more *trans*-SNARE pair formation and enhanced fusion.

**Ycf1, Bpt1p and fusion**

In Chapter 3 we saw that *ycf1* and *bpt1* vacuoles had reduced fusion. Ycf1p regulates the transport of cadmium, mercury and other toxins into the vacuole lumen to detoxify the cytoplasm (Gueldry et al., 2003; Li et al., 1996; Szczypka et al., 1994). However, a role for Ycf1p in the regulation of fusion had not been directly examined. An integrated split-ubiquitin membrane yeast two-hybrid (iMYTH) analysis has shown that Ycf1p physically interacts with the PI3P 5-kinase Fab1p, which phosphorylates PI3P to produce PI(3,5)P₂, as well as the Rho1p nucleotide exchange factor, Tus1p (Paumi et al., 2007). Ycf1p has also been found to physically interact with Rho1p, a GTPase associated with actin dynamics (Lee et al., 2011). Since Rho1p, actin and PI3P have been found to play important roles in vacuole fusion we examined the effect of deleting *YCF1* and other ABCC transporters on vacuole fusion. We found that *ycf1* and *bpt1* vacuoles each had reduced levels of fusion, but the double deletion did not show additive effects. The defect in fusion was relieved by also deleting *YBT1*, an ABCC transporter previously reported to negatively regulate vacuole fusion (Sasser et al., 2012b). The mechanism(s) for the rescued fusion remain unclear and will be explored in future studies. The rest of the chapter focused on the effect of deleting *YCF1* alone. We found that the attenuated fusion seen with *ycf1* vacuoles was due to the exclusion of Vam7p, a defect in fusion that was rescued by the addition of recombinant Vam7p. The exclusion of Vam7p from *ycf1* vacuoles was not due to the lack of known binding partners that include SNAREs, HOPS and the regulatory lipid PI3P, which were all at, or
near, wild type levels. Moreover, the exogenous Vam7p readily associated with the vacuoles to trigger fusion, illustrating that neither Vam7p-protein, nor Vam7-PI3P interactions were deleteriously affected by the lack of Ycf1p.

In this study we also found that the ATPase activity of Ycf1p was important for vacuole fusion. Whereas a WT YCF1 plasmid complemented the ycf1Δ fusion defect, a point mutation in NBD1 of YCF1 prevented complementation of the defect. Although physical interactions between Ycf1p and trafficking proteins have been documented by iMYTH, the interactions were independent of ATPase activity or in the context of the vacuole. Because the addition of recombinant Vam7p rescued ycf1Δ vacuole fusion, we hypothesize that the direct transport activity of Ycf1p is not related to Vam7p recruitment to the vacuole membrane. One possible mechanism for the link between these two proteins is the putative “flippase” activity of Ycf1p and other ABCC transporters. Although flippase activity has not been reported for Ycf1p, its paralogue Ybt1p has been reported to translocate (or flip) phosphatidylcholine from the outer-to-inner leaflets of the vacuole membrane (Gulshan and Moye-Rowley, 2011). In addition, another paralogue located on the plasma membrane, Yor1p, has been shown to translocate fluorescently labeled phosphatidylethanolamine (Decottignies et al., 1998). Therefore, it is quite possible that Ycf1p may also function as a lipid translocation enzyme for other lipids. This is important because Vam7p binding to the vacuole is regulated by the composition of the outer leaflet vertex microdomains of vacuole, where it was shown that disruption of the vertex microdomain by binding or modifying various lipids other than PI3P inhibited the binding of Vam7p to vacuoles (Fratti et al., 2004). Therefore, if Ycf1p modifies the lipid composition of the vacuole outer leaflet,
inactivating its ATPase-dependent translocation activity could alter the steady state association of Vam7p.

Membrane fusion and lipid asymmetry

In Chapter 4 I described a FRET-based assay to monitor the translocation of Rhodamine-labeled PE on the yeast vacuole. The assay is intended to help define a role for lipid translocation in membrane fusion. We used the assay to seek the mechanism by which rhodamine-PE is translocated across the membrane bilayer and to probe whether the translocation is required for membrane fusion. Remodeling of membranes during membrane fusion suggests a requirement for a lipid translocator also known as a flippase. Observations of the ATP-dependant translocation of Rh-PE strongly suggested the presence of a flippase on the vacuole. We sought to identify the flippase by preparing a Pho8-GFP fusion in a variety of yeast strains deficient in the candidate protein to probe for flippase activity. Unfortunately, we did not identify the transporter but we do know that blocking membrane fusion does not affect lipid translocation. To determine if lipid translocation is necessary for membrane fusion we need to identify the flippase and then determine if yeast with a deficiency in that flippase show aberrant fusion activity. Of the ABCC transporters, Ybt1p remains as a candidate for translocation activity and has even been shown to translocate phosphatidylcholine in yeast vacuoles (Gulshan and Moyer-Rowley, 2011). Since ABCC transporters have such a wide variety of substrates, it is possible that Ybt1p may be responsible for the translocation of Rh-PE. Other candidates are the less well characterized ABC transporters located on the vacuole such as Ymr1p, Nft1p, and the uncharacterized
YOL075c (Paumi et al., 2009). Other transporters such as Yor1p that are localized to the plasma membrane are trafficked through the yeast cytoplasm and may therefore be transiently associated with the vacuole. Therefore, all ABC transporters in the yeast genome should remain candidates to be included in future explorations to determine if they have Rh-PE flippase activity.

Summary

The research presented in this dissertation offers a beginning to understanding the role of ABCC transporters in the regulation of membrane fusion. The work provides evidence that Ybt1p regulates Ca$^{2+}$ levels and that Ycf1p affects recruitment of Vam7p to the vacuole. The discovery of an ATP-dependent lipid translocator on the vacuole shows that lipid remodeling could play an important role in organelle homeostasis. Many questions remain regarding the mechanisms involved, the identity of the lipid translocator and whether or not the flippase activity is required for fusion. However, this work has provided a solid foundation for future investigations into fusion regulators and flippase activity.


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