The Yeast ATP-binding Cassette (ABC) Transporter Ycf1p Enhances the Recruitment of the Soluble SNARE Vam7p to Vacuoles for Efficient Membrane Fusion*

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Background: Ycf1p is a ABCC transporter that is localized to the vacuole and that was initially characterized as a cadmium transporter.

Results: Deletion of YCF1 inhibits vacuole fusion in part by excluding the soluble SNARE Vam7p.

Conclusion: The vacuole fusion machinery requires Ycf1p function for efficient fusion.

Significance: This is the first report that an ABCC protein affects fusion through the recruitment of a SNARE.

The Saccharomyces cerevisiae vacuole contains five ATP-binding cassette class C (ABCC) transporters, including Ycf1p, a family member that was originally characterized as a Cd²⁺ 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 Lys-669 to Met in the Walker A box of the first nucleotide-binding domain (Ycf1pK669M) 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 in vitro experiments. Thus, Ycf1p contributes in the recruitment of Vam7p to the vacuole for efficient membrane fusion.

Eukaryotic homeostasis requires the packaging, trafficking, and delivery of membrane-bound cargo between organelles. 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 (1). 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 (homotypic fusion and vacuole protein sorting), which tether membranes together. The final stage of fusion is catalyzed by SNARE proteins, which interact across the docking junction to form parallel four-helix 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 (2). These lipids and proteins interdependently form specialized membrane raft microdomains called vertices to create the site of fusion (3–5).

Studies that use reconstituted proteoliposomes as a model for fusion have identified the minimum 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 (6); the lipid modifiers Pah1p (7), Plc1p (8), and Vps34p (9); and the Na⁺/H⁺ exchanger Nhx1p (10). We recently found that the ATP-binding cassette class C (ABCC)³ transporter Ybt1p negatively regulates fusion through the control of Ca²⁺ transport across the vacuole membrane (11). Ybt1p also translocates phosphatidylcholine across the vacuole bilayer for degradation and choline recycling (12).

Ybt1p is one of the five confirmed ABC transporters that reside on the vacuole membrane and was initially discovered as an ATP-dependent bile acid transporter (13). The other yeast vacuole ABCC proteins include the yeast cadmium factor Ycf1p (14, 15), the bile acid transporter Bpt1p (16), and the less well characterized Vmr1p and Nft1p (17). 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 phosphatidylinositol 3-phosphate (PI3P) 5-kinase Fab1p (18). In this study, we examined the role of Ycf1p and other ABC transporters in vacuole fusion. We found that deletion of YCF1 strongly attenuated fusion by reducing the vacuole association of the soluble SNARE Vam7p.

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3 The abbreviations used are: ABCC, ATP-binding cassette class C; PI3P, phosphatidylinositol 3-phosphate; CBP, calmodulin-binding peptide; RH-PE, rhodamine-conjugated phosphatidylethanolamine; TAP, tandem affinity purification; CFTR, cystic fibrosis transmembrane conductance regulator.
TABLE 1

Yeast strains used in this study

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EXPERIMENTAL PROCEDURES

Reagents—Reagents were dissolved in PS buffer (20 mM PIPES-KOH (pH 6.8) and 200 mM sorbitol). The recombinant protiens GST-FYVE (19) and GST-Vam7p (20, 21) were prepared as described and stored in PS buffer with 125 mM KCl. The FYVE domain was labeled with Cy5 N-hydroxysuccinimide ester (GE Healthcare) according to the manufacturer’s protocol. The MARCKS (myristoylated alanine-rich C kinase substrate) effector domain peptide was prepared as described previously (3).

Strains—BJ3505 and DKY6281 were used for fusion assays (Table 1) (22). YCF1 was deleted from BJ3505 and DKY6281 by homologous recombination with the kanMX6 cassette using PCR products amplified from pFA6a-kanMX6 (23) 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 (yeast extract/peptone/dextrose) medium containing G418 (250 μg/liter) to generate BJ3505 ycf1Δ::kanMX6 (RY32) and DKY6281 ycf1Δ::kanMX6 (RY33). Similarly, BPT1 was deleted from BJ3505 and DKY6281 to generate BJ3505 bpt1Δ::kanMX6 (RY34) and DKY6281 bpt1Δ::kanMX6 (RY35). To generate ycf1Δ::bpt1Δ strains, YCF1 was deleted from BJ3505 and DKY6281 with the IRA3 cassette. BPT1 was then deleted with TRP1 to generate RY36 or with HIS3 to generate RY37. YBT1 was deleted from RY36 and RY37 with kanMX6 to generate RY38–39. Deletions of NFT1, VMR1, and YOL075c were generated using PCR products amplified from pAG32 to generate nft1Δ::hphMX4 (RY40–41), vnr11Δ::hphMX4 (RY42–43), and yol075cΔ::hphMX4 (RY44–45) (24). For vacuole localization studies, YCF1, BPT1, NFT1, VMR1, and YOL075c were fused in frame to GFP by homologous recombination. DKY6281 was transformed with a PCR product amplified from pFA6a-GFP-kanMX6 (23) with homology flanking the stop codon of the gene to generate RY46 (DKY6281 YCF1::GFP), RY47 (DKY6281 BPT1::GFP), RY48 (DKY6281 NFT1::GFP), RY49 (DKY6281 VMR1::GFP), and RY50 (DKY6281 YOL075c::GFP). For complementation studies, WT YCF1 and YCF1Δ566/569 were subcloned from pRS424 vectors (a gift from Dr. W. Scott Moye-Rowley, University of Iowa) into pRS416 using KpnI and Scal. RY32 and RY33 were transformed with pYCF1 or pYCF1Δ566/569 to generate RY51–54. YCF1 was deleted from BJ3505 CBP-Vam3p ycf1ΔΔ with the hghMX4 cassette using PCR product amplified from pAG32 with homology flanking the YBT1 coding sequence. The PCR product was transformed into BJ3505 CBP-Vam3p ycf1ΔΔ by standard lithium acetate methods and plated on YPD medium containing hygromycin (250 μg/ml) to generate BJ3505 CBP-Vam3p ycf1ΔΔ ybt1Δ::hphMX4 (RY55). WT and ycf1Δ strains were transformed with the pRS424-GFP-FYVE plasmid (Addgene) and grown on selective medium lacking uracil to generate RY56–59.

Vacuole Isolation and in Vitro Vacuole Fusion—Vacuoles were isolated by flotation as described (22). Standard in vitro fusion reactions (30 μl) contained 3 μg each of vacuoles from the BJ3505 and DKY6281 backgrounds, fusion reaction buffer (20 mM PIPES-KOH (pH 6.8), 200 mM sorbitol, 125 mM KCl, and 5 mM MgCl2), and ATP-regenerating system (1 mM ATP, 0.1 mg/ml creatine kinase, and 29 mM creatine phosphate), 10 μM CoA, and 29 mM creatine phosphate), 10 μM CoA, and 29 mM creatine phosphate), 10 μM CoA, and 29 mM creatine phosphate). 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, and 1
Ycf1p and Vacuole Fusion

mm p-nitrophenyl phosphate. Fusion units were measured by determining the p-nitrophenolate produced per min/μg of pep4A 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 (7). 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% (w/v) Ficoll in PS buffer 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 reaction buffer and were transferred to a black half-volume 96-well flat-bottom microtiter plate with a nonbinding surface (Corning). Rhodamine fluorescence was measured using a POLARstar Omega fluorescence plate reader (BMG Labtech) at 27 °C. Measurements were taken every minute for 75 min, yielding fluorescence values at the onset (F₀) and during the reaction (Fₜ). The final 10 measurements of reactions after the addition of 0.33% (v/v) Triton X-100 were averaged and used as a value for the fluorescence after infinite dilution (Fₓ₁₀₀). The relative total fluorescence change (ΔF/Fₓ₁₀₀ = (Fₜ - F₀)/Fₓ₁₀₀) was calculated.

Microscopy—Vacuole morphology was monitored by incubating yeast cells with YPD broth containing FM® 4-64 (Invitrogen) as described previously (11). Images were acquired using a Zeiss Axio Observer Z1 inverted microscope equipped with an X-Cite 120XL light source, a Plan Apochromat 63× oil objective (numerical aperture of 1.4), and an AxioCam CCD camera. Vertex assembly reactions were performed as described (3). Docking reactions (30 μl) contained 6 μg of vacuoles. PI3P was labeled with 0.2 μM Cy5-FYVE and used as a marker for vertex microdomains (7). Reactions were incubated at 27 °C for 30 min, placed on ice, and stained with 3 μM MDY-64. Reactions were next mixed with low melting 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 analysis of variance. Significant differences were determined using t test and corrected for multiple comparisons using the Dunn-Sidak method (25). p values < 0.05 were considered significant.

Quantitative PI3P ELISA—Total levels of PI3P were determined using a quantitative ELISA (Echelon, Inc.). Large-scale 10× reactions (300 μl) were prepared using DKY6281 background vacuoles (60 μg) and incubated at 27 °C for 60 min. Neutral lipids were first extracted from vacuoles by the addition of 3 ml of MeOH/CHCl₃ (2:1) and vortexing three times over 10 min at room temperature. Insoluble lipids were collected by centrifugation (1500 × g, 5 min), and the supernatant was discarded. Next, acidic lipids were extracted by the addition of 2.25 ml of MeOH, CHCl₃, and 12 M HCl (80:40:1) and vortexing four 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 of CHCl₃ and 1.35 ml of 0.1 M HCl, vortexed, and phase-separated by centrifugation as described above. The lower organic phase was collected, transferred into a new 1.5-ml microcentrifuge tube, and dried down in a SpeedVac system for 1 h. The dried lipids were resuspended in 190 μl of PBS/Tween 20 with 3% protein stabilizer. Samples were vortexed for 1 min and centrifuged prior to use in ELISA. ELISA was performed according to the manufacturer’s instructions.

trans-SNARE Complex Assay—Analysis of trans-SNARE complex formation was performed as described previously (7, 26, 27). Complex formation was compared in reactions containing vacuoles from RYF33 and RYF55 relative to vacuoles from BJ3505 CBP-Vam3p yvy1Δ and DKY6281. The trans-SNARE assays were performed using 16× large-scale reactions (480 μl) containing 48 μg of vacuoles each from BJ3505 CBP-Vam3p yvy1Δ and DKY6281 backgrounds. Reactions were incubated at 27 °C for 60 min and then placed on ice for 5 min prior to collecting 30 μl from each sample to test Pho8p activity. The remaining samples were centrifuged (13,000 × g, 15 min, 4 °C), and the supernatants were discarded. Vacuole pellets were overlaid with 200 μl of ice-cold solubilization buffer (20 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM MgCl₂, 0.5% Nonidet P-40 alternative, and 10% glycerol) with protease inhibitors (0.46 μg/ml leupeptin, 3.5 μg/ml pepstatin, 2.4 μg/ml Pefabloc SC, and 1 mM PMSF) and resuspended. Solubilization buffer was added to a final volume of 600 μl, and extracts were mixed for 20 min at 4 °C with nutating, followed by centrifugation (16,000 × g, 20 min, 4 °C) to remove detergent-insoluble material. Supernatants were transferred to new tubes, and 10% of the extract was removed for input samples. The extracts were brought to 2 mM CaCl₂ and incubated with 50 μl of equilibrated calmodulin-Sepharose 4B (GE Healthcare) at 4 °C for 12 h with nutating. Beads were collected by centrifugation (4000 × g, 2 min, 4 °C) and washed with 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 min. The samples were used for SDS-PAGE analysis and immunoblotting.

Vacuoles were isolated from yeast harboring YCF1 fused to a tandem affinity purification (TAP) tag. Protein complexes were isolated as described (28) with some modification. 10% of the extract was removed for input samples. For each strain, 1 ml of vacuoles at 1 mg/ml was incubated with 500 μl of IgG-Sepharose 6 Fast Flow (GE Healthcare) equilibrated with Nonidet P-40 alternative buffer (15 mM Na₂HPO₄, 10 mM NaH₂PO₄, H₂O, 1.0% Nonidet P-40 alternative, 150 mM NaCl, and 2 mM EDTA) containing a protease inhibitor mixture (1 mM PMSF, 0.46 μg/ml leupeptin, and 3.5 μg/ml pepstatin) and incubated at 4 °C for 2 h with nutating. After incubation, the beads were washed twice with 10 ml of buffer containing 25 mM Tris-Cl (pH 8.0), 300 mM NaCl, and 0.1% Nonidet P-40 alternative and once with buffer containing 25 mM Tris-Cl (pH 8.0), 150 mM NaCl, and 0.1% Nonidet P-40 alternative. Next, the beads were washed with 10 ml of tobacco etch virus protease buffer (25 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.1% Nonidet P-40 alternative, and 0.5 mM EDTA), resuspended with 1 ml of tobacco etch
virus protease buffer containing 5 μg of tobacco etch virus protease, and incubated at 4°C for 2 h with nutating. Released proteins were collected, mixed 1:1 with calmodulin binding buffer (25 mM Tris-Cl (pH 8.0), 150 mM NaCl, 1 mM magnesium acetate, 1 mM imidazole, 2 mM CaCl₂, and 10 mM β-mercaptoethanol), incubated with 300 μl of calmodulin-Sepharose 4B equilibrated in calmodulin binding buffer, and incubated for 1 h at 4°C with nutating. Protein-bound beads were washed twice with calmodulin binding buffer with 0.1% Nonidet P-40 alternative and three times with calmodulin binding buffer containing 0.02% Nonidet P-40 alternative. Protein complexes were eluted with 1 ml of calmodulin elution buffer (25 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.02% Nonidet P-40 alternative, 1 mM magnesium acetate, 1 mM imidazole, 20 mM EGTA, and 10 mM β-mercaptoethanol) into a siliconized microcentrifuge tube. Eluted proteins were TCA-precipitated. Dried proteins were solubilized with SDS-PAGE buffer and processed for immunoblotting.

Ca²⁺ Efflux Assay—Vacuole lumen Ca²⁺ efflux was measured as described (11). Fusion reactions (60 μl) contained 20 μg of vacuoles isolated from BJ3505 backgrounds, fusion reaction buffer with 10 μM CoA, 283 nM inhibitore of protease B (IBu), and the fluorescent Ca²⁺ probe Fluo-4 dextran (200 μM; Invitrogen). Reaction mixtures were transferred to a black half-volume 96-well flat-bottom plate with a nonbinding surface. An ATP-regenerating system or buffer was added, and reactions were incubated at 27°C while monitoring Fluo-4 fluorescence.

RESULTS
ABCC Transporter Localization to Vacuoles—The yeast vacuole harbors multiple ABCC 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 FM 4-64, which is endocytosed and accumulates on the vacuole membrane. Colocalization analysis showed that Ycf1p-GFP and Bpt1p-GFP were highly enriched on the vacuole relative to the other transporters tested (Fig. 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 Attenuate Vacuole Fusion—Although ABCC transporters function primarily in detoxification of yeast cytoplasm, new studies have shown that some family members perform additional functions. For example, Ybt1p regulates calcium homeostasis and vacuole fusion and has been shown to translocate phosphatidylcholine to aid in choline recycling (11, 12, 29). Others have shown that Ycf1p physically interacts with Fab1p, a PI3P 5-kinase that modifies PI3P, a lipid that is essential for vacuole fusion (3, 9, 18). 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 strain 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. 2, A and B). Interestingly, vmr1Δ, nft1Δ, and yol075cΔ vacuoles showed no defects in vacuole fusion (data not shown). This suggests that the effects of deleting YCF1 and BPT1 are specific to functions of the individual proteins and not to general characteristics of ABCC family members. To determine whether the effects of deleting YCF1 and BPT1 were additive, we generated double deletion strains (ycf1Δ/bpt1Δ) and examined fusion. We found that deletion of both genes did not inhibit fusion further (Fig. 2C), suggesting that there was some redundancy in their function. Previously, we found that deletion of the ABCC transporter YBT1 stimulated fusion up to 50% above wild-type fusion (11). To examine whether the absence of Ybt1p would compensate for the fusion defect seen with ycf1Δ/bpt1Δ vacuoles, we produced triple deletion fusion 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 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 (27). Labeled vacuoles were incubated with an excess of unlabeled vacuoles, and fusion was measured by the dilution and dequenching of Rh-PE. Fig. 2E shows that ycf1Δ/bpt1Δ vacuoles were inhibited for fusion by fluorescence dequenching at levels similar to those shown in Fig. 2C. Because the double deletion did not further reduce fusion, the remainder of the study was performed with YCF1 single deletion strains. Attenuated fusion is often linked with vacuole fragmentation; thus, we examined the vacuole morphology of ycf1Δ cells as described above. Although ycf1Δ vacuoles were attenuated for fusion, we did not observe a defect in vacuole morphology relative to the wild-type parent strain (Fig. 2F). We attribute the lack of a fragmentation defect to the
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FIGURE 2. Deletion of YCF1 and BPT1 attenuates vacuole fusion. 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). D, inset: vacuoles were isolated from WT and ycf1Δ/bpt1Δ strains and examined for Vam7p content. Blotting for Ypt7p served as a loading control. E, lipid mixing assays measuring fusion were performed by labeling isolated vacuoles with Rh-PE. Labeled vacuoles were incubated with an 8-fold excess of unlabeled vacuoles, and dequenching was measured using a fluorescence plate reader. F, WT and ycf1Δ yeast cells were stained with FM 4-64, and vacuole morphology was analyzed by fluorescence microscopy. DIC, differential interference contrast.

Reducant functions of ABCC transporters and to the supply of the soluble SNARE Vam7p in the cytoplasm, which will be elucidated below.

Ycf1p-supported Fusion Requires ATPase Activity—To determine whether the role of Ycf1p in vacuole fusion is dependent on its transporter activity, we complemented ycf1Δ cells with plasmid-expressed wild-type Ycf1p or mutant Ycf1pK669M. 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 (15). Complementation of ycf1Δ strains with wild-type pYCF1 fully rescued fusion (Fig. 3A), indicating that the inhibition was directly due to the absence of Ycf1p. However, when ycf1Δ strains were complemented with the ATPase-deficient mutant pYCF1K669M, fusion remained attenuated (Fig. 3B), suggesting that the transport function of Ycf1p was important for the regulation of fusion. It was previously shown that Ycf1pK669M was properly trafficked to the vacuole (15), indicating that the inability of Ycf1pK669M to rescue fusion was linked to its ATPase activity and not a sorting defect.

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 ycf1Δ vacuoles. Fig. 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 vacuoles, with the prominent exception of the soluble SNARE Vam7p. Mutant ycf1Δ vacuoles contained 30–40% less Vam7p relative to wild-type vacuoles (Fig. 4B), suggesting that the defect in fusion was due in part to depletion of a SNARE protein. The 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 vacuoles 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 (Fig. 4C), indicating that mutant vacuoles were unable to recruit sufficient Vam7p to support fusion. Actin levels were probed as a loading control.

To further examine the distribution of Vam7p in WT and ycf1Δ cells, we performed cellular fractionation as described (10). Vam7p distribution was examined by immunoblotting (Fig. 5A). We observed an increase of Vam7p in the cytosolic fraction of ycf1Δ relative to WT cytosol. We also probed for...
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Vps11p as a marker for vacuoles and as a loading control. These data are in accord with the idea that Ycf1p plays a role in the recruitment of Vam7p. However, it is unclear whether Vam7p recruitment was direct or indirect.

In this study, we determined that deleting YBT1 in ycf1Δ/ ybt1Δ cells restored fusion to WT levels. Because of the role of Ycf1p in Vam7p recruitment, we examined Vam7p recruitment to ycf1Δbpt1Δybt1Δ vacuoles. Vacuoles were isolated from WT and ycf1Δbpt1Δybt1Δ cells and immunoblotted for Vam7p and Ypt7p. We found that ycf1Δbpt1Δybt1Δ cells harbored similar levels of Vam7p relative to WT vacuoles (Fig. 2D, inset). Ypt7p levels were measured as a loading control. These data suggest that the triple deletion mimicked the conditions necessary for Vam7p recruitment.

The role of Ycf1p in fusion was not limited to its physical presence and potential interactions with the fusion machinery. This was demonstrated by the inability of Ycf1pK669M to support fusion (Fig. 3B). When vacuoles from Ycf1pK669M yeast were immunoblotted for Vam7p, we found that these vacuoles contained elevated levels of Vam7p relative to ycf1Δ vacuoles or those from ycf1Δ yeast complemented with WT Ycf1p (Fig. 5B). Actin levels were measured as a loading control. This suggests that Ycf1p-dependent recruitment of Vam7p was insufficient to restore fusion. Furthermore, this indicates that the ATPase-dependent transport activity of Ycf1p played a role in regulating fusion.

Exogenous Vam7p Restores Fusion of ycf1Δ Vacuoles—Vam7p directly interacts with the HOPS complex as well as other SNAREs (30, 31); however, the levels of these proteins were not affected on ycf1Δ vacuoles. Vam7p also interacts with the lipid PI3P (32), which is made on the vacuole during fusion by the phosphatidylinositol kinase Vps34p (33), yet the levels of this lipid kinase were also unaffected on mutant vacuoles. However, these data do not show whether the association of Vam7p with its binding partners was affected on mutant vacuoles. To test whether the simple lack of Vam7p affected ycf1Δ vacuole fusion, we added exogenous recombinant GST-Vam7p to fusion reactions containing either wild-type or ycf1Δ vacuoles.

We found that the direct addition of Vam7p during the fusion reaction rescued ycf1Δ vacuole fusion to wild-type levels (Fig. 6A). This indicates that Vam7p could function with its binding partners during fusion and that the defect in ycf1Δ vacuole fusion was due to the inability to recruit wild-type levels of Vam7p. We also observed that high levels of Vam7p reduced fusion. This is consistent with previous studies showing a biphasic curve of the effects of Vam7p on fusion (20, 21).

Thus far, we have found that ycf1Δ vacuoles harbored reduced levels of Vam7p and that the exogenous addition of this SNARE rescued the fusion phenotype. Accordingly, we posited that the levels of trans-SNARE complexes might also be reduced on ycf1Δ vacuoles. Here, we used a well characterized assay that measures bona fide trans-SNARE complex formation. This was performed using two distinct vacuole populations. One set of vacuoles was isolated from a nyv1Δ deletion strain that harbors Vam3p containing an internal calmodulin-binding peptide (CBP-Vam3p). These vacuoles were mixed with WT vacuoles (NYV1 VAM3). Thus, the isolable trans-SNARE complexes formed between CBP-Vam3p and Nyv1p were generated from the docking of separate organelles. In our experiments, we found that ycf1Δ vacuoles formed significantly less trans-SNARE complexes relative to those containing Ycf1p. The addition of exogenous Vam7p (50 nM) resulted in an increase in Nyv1p-CBP-Vam3p complex formation for both sets of vacuoles (Fig. 6, B and C). This indicated that the increase in fusion shown in Fig. 6A was due to increased SNARE complex formation. The MARCKS effector domain was used as an inhibitor of fusion (7). Quantitation of three experiments showed that the addition of recombinant Vam7p had a positive effect on SNARE complex formation on both WT and ycf1Δ vacuoles (Fig. 6C).
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Ycf1p Interacts with the Fusion Machinery—The reduction of Vam7p in ycf1Δ vacuoles led us to hypothesize that Ycf1p may directly interact with the fusion machinery. To test this notion, we used vacuoles harboring Ycf1p-TAP to isolate factors physically interacting with Ycf1p. We found that Ycf1p-TAP co-purified with the Vps33p subunit of HOPS as well as Vam3p (Fig. 6D). These results do not indicate, however, which protein was directly interacting with Ycf1p. We also observed a weak interaction with Ypt7p when reactions remained on ice. The interaction was lost when vacuoles were brought up to 27 °C, suggesting that the interaction was nonspecific. We also loaded 10% of the total extracts as a control for starting material.

Exogenous Vam7p Restores Ca2+ Efflux in ycf1Δ Vacuoles—Thus far, we have observed that the fusion defect in ycf1Δ vacuoles was due in part by the exclusion of Vam7p from the vacuole, which could lead to a reduction in trans-SNARE pairing. Because the formation of trans-SNARE complexes triggers the release of luminal Ca2+ stores from the vacuole prior to fusion (34, 35), we next determined if the reduced level of trans-SNARE complexes seen using ycf1Δ vacuoles correlated with a decrease in Ca2+ efflux. Fusion reactions containing wild-type or ycf1Δ vacuoles were incubated with standard fusion reaction components in the presence of the fluorescent calcium indicator Fluo-4 dextran (11). Reactions were started by the addition of buffer or an ATP-regenerating system. In the absence of ATP, Fluo-4 fluorescence remained stable, whereas fluorescence decreased in the presence of ATP during the first 20 min of the reactions (Fig. 7A), indicating that Ca2+ was taken up by the vacuoles. We inhibited selected reactions with Gyp1-46p to inhibit Ypt7p-dependent docking as a negative control. Gyp1-46p-inhibited reactions showed continued Fluo-4 fluorescence until a base line was reached. Interestingly, the initial uptake of Ca2+ was measurably greater in ycf1Δ reactions relative to wild-type reactions. This could be attributed to differences in the relative amounts of Vam7p and trans-SNARE complexes on mutant vacuoles. After 15–20 min of incubation, the uninhibited reactions released Ca2+ that correlated with the formation of trans-SNARE complexes. Reactions containing ycf1Δ vacuoles released Ca2+ at the same time as those containing wild-type vacuoles, but the external concentration of cations did not reach wild-type levels. Again, this was likely due to the reduced number of trans-SNARE complexes present on ycf1Δ vacuoles. It should also be noted that there was no difference in the net amount of released Ca2+ in mutant reactions. To determine whether the levels of Vam7p were linked to the changes in Ca2+, we performed experiments in the presence or absence of exogenous Vam7p. We found that the addition of supplemental Vam7p to reactions eliminated the differences in Ca2+ efflux between ycf1Δ and wild-type vacuoles (Fig. 7B). This was consistent with the effect of exogenous Vam7p on the fusion of ycf1Δ vacuoles in Fig. 6 and suggests that the primary defect in these vacuoles was the defective recruitment of Vam7p.

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 (3, 32). Although the lipid kinase Vps34p is present on ycf1Δ vacuoles, it remained possible that PI3P levels were insufficient to recruit Vam7p to the
together, these data suggest that PI3P accumulated at vertices with more efficiency in the absence of Ycf1p. It was also possible that the absence of Vam7p on ycf1Δ vacuoles resulted in more PI3P available for Cy5-FYVE staining.

To examine the distribution of PI3P in vivo, WT and ycf1Δ cells were transformed with plasmid-encoding GFP-FYVE (36). Cells were incubated with the vital dye FM 4-64 to label vacuoles as described previously (37), and cells were examined by fluorescence microscopy. We found that both WT and ycf1Δ vacuoles were similarly labeled with GFP-FYVE (Fig. 8D). This was in accord with the quantitative ELISA.

**DISCUSSION**

Ycf1p regulates the transport of cadmium, mercury, and other toxins into the vacuole lumen to detoxify the cytoplasm (14, 38, 39). However, a role for Ycf1p in the regulation of fusion had not been directly examined. Interestingly, an iMYTH (integrated split-ubiquitin membrane yeast two-hybrid) analysis showed that Ycf1p physically interacts with the PI3P 5-kinase Fab1p, which uses PI3P to produce PI(3,5)P2. It also interacts with the Rho1p nucleotide exchange factor, Tus1p (18). Others have found that Ycf1p physically interacts directly with Rho1p, a GTPase associated with actin dynamics (40). Because Rho1p (41), actin (42, 43), and PI3P (3) play important roles in vacuole fusion, we examined the effect of deleting YCF1 and other ABC 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 ABC transporter previously reported to negatively regulate vacuole fusion (11). The mechanism(s) for the rescued fusion remain unclear and will be further explored in future studies. The rest of this study focused on the effect of deleting YCF1 alone. We found that the attenuated fusion seen with ycf1Δ vacuoles was due to the diminished recruitment of Vam7p. The defect in fusion 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 (including SNAREs, HOPS, and the regulatory lipid PI3P), which were all at wild-type levels. Moreover, exogenous Vam7p readily associated with the vacuoles to trigger fusion, suggesting that neither Vam7p-protein nor Vam7p-PI3P interactions were adversely affected by the lack of Ycf1p.

Of the published iMYTH results, the interaction between Ycf1p and Fab1p stands out with respect to vacuole fusion. PI3P is generated during fusion (33) by the phosphatidylinositol kinase Vps34p (9), and the absence or sequestering of PI3P has severe negative effects on the fusion machinery. This lipid is required for the recruitment of Vam7p as well as the formation of membrane raft domains and the interactions between SNAREs and HOPS (3, 44, 45). PI3P levels can be reduced through the phosphatase activity of Ymr1p or the kinase activity of Fab1p (46, 47), although it remains unknown whether either Ymr1p or Fab1p functions during vacuole homotypic fusion. Regarding vacuole homeostasis, the importance of PI(3,5)P2 has been linked to acidification, trafficking to the vacuole, and response to hypertonic shock (48–50). However, the role of PI(3,5)P2 in vacuole fusion remains unclear. Previous work has shown that deletion of FAB1 does not affect vacuole...
fusion under isotonic conditions (51), suggesting that formation of PI(3,5)P₂ is not essential for fusion to occur. Nevertheless, a regulatory role was not ruled out for the lipid. In this study, we found that PI3P levels were similar in WT and ycf₁/H₉₀₀₄ vacuoles, suggesting that any Ycf₁p-dependent Fab₁p activity had no effect on the steady-state levels of PI3P. Although the levels of PI3P did not change between the WT and ycf₁/H₉₀₀₄ strains, we did observe a marked increase in PI3P accumulation at the vertices of docked vacuoles. Although the mechanism for the altered distribution remains unclear, we are able to propose a link between the PI3P localization and the Ycf₁p-Tus₁p interaction. Tus₁p is the nucleotide exchange factor for Rho₁p, a GTPase involved in actin remodeling and vacuole fusion (41, 52). Moreover, Ycf₁p directly interacts with Rho₁p (40). Because actin remodeling is essential for vacuole fusion at different stages of the reaction, we can theorize that in the absence of Ycf₁p, Rho₁p might be inactive, resulting in a reduction in actin polymerization on vacuoles. Importantly, we previously reported a link between PI3P localization to vertices and the state of actin polymerization (3). When actin was depolymerized by latrunculin B, vertex-localized PI3P levels were dramatically increased on docked vacuoles. In contrast, when F-actin was stabilized by jasplakinolide, PI3P enrichment was completely inhibited. Together, these results suggest that Ycf₁p may play a role in the state of actin polymerization on vacuoles and the subsequent lateral mobilization of PI3P on docked vacuoles.

In this study, we also found that the ATPase activity of Ycf₁p was important for vacuole fusion. A point mutation that substituted the conserved Lys with Met in the Walker A box motif of the first nucleotide-binding domain prevented the complementation of the ycf₁Δ fusion defect. Although physical interactions between Ycf₁p and trafficking proteins have been doc-
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Interdependent assembly of specific regulatory lipids and membrane fusion proteins into the vertex ring domain of docked vacuoles. J. Cell Biol. 167, 1087–1098


ummented by iMYTH, the interactions were independent of ATPase activity and were not 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 “flipase” activity of Ycf1p and other ABC transporters. Although flipase activity has not been reported for Ycf1p, its paralog Ybt1p has been reported to translocate (or flip) phosphatidylcholine from the outer-to-inner leaflets of the vacuole membrane (12). 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 vacuoles (3), 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. Our unpublished preliminary studies have indicated that vacuoles flip Rh-PE.4 However, this activity was not dependent on Ycf1p.

Importantly, we found that Ycf1p formed stable complexes with the fusion machinery. Ycf1p-TAP was co-purified with SNAREs and the HOPS complex, suggesting that Ycf1p may play a role in regulating the core fusion machinery as evident by the defective Vam7p recruitment to ycf1Δ vacuoles. There is a growing body of work that links various ABC transporters to membrane trafficking pathways. For instance, the ABC mammalian chloride channel cystic fibrosis transmembrane conductance regulator (CFTR) interacts with the SNARE motif of syntaxin 1a at the plasma membrane, and disruption of the interaction inhibits CFTR-mediated currents (53). CFTR also interacts with EBP50 (ERM-binding protein 50), a protein that bridges CFTR to the actin cytoskeleton (54). Others have shown that ABCA1 interacts with syntaxin 13 and flotillin-1 in monocytes (55). In addition, we found that the ABC protein Ybt1p is linked to the regulation of vacuole fusion through affecting the transport of Ca2+ across the membrane (11).

Although the regulatory mechanisms driven by these interactions remain to be elucidated, it is clear that ABC transporters play important roles in membrane trafficking.

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The Yeast ATP-binding Cassette (ABC) Transporter Ycf1p Enhances the Recruitment of the Soluble SNARE Vam7p to Vacuoles for Efficient Membrane Fusion
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