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The yeast vacuolar ABC transporter Ybt1p regulates membrane fusion through Ca^{2+} transport modulation

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Abstract

Ybt1p is a class C ABC transporter (ATP-binding cassette 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^{2+} homeostasis. In the present 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 (soluble *N*-ethylmaleimide-sensitive factor-attachment protein receptors) or recruitment of factors from the cytosol such as Ypt7p and the HOPS (homotypic fusion and vacuole protein sorting) 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. As vacuole fusion can be inhibited by high Ca^{2+} concentrations, we suggest that the delayed efflux in *ybt1Δ* vacuoles leads to the enhanced SNARE function.

Keywords

calcium efflux; fusion; homotypic fusion and vacuole protein sorting (HOPS); soluble *N*-ethylmaleimide-sensitive factor-attachment protein receptor (SNARE); vertex

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 [1]. The delivery of cargo to its destination is finalized by the fusion of two membranes that is driven through a series of regulated stages. In the present study we used vacuoles (lysosomes) from *Saccharomyces cerevisiae* to examine the regulation of membrane fusion. Yeast vacuole fusion requires numerous proteins, including the Q-SNAREs {SNAP [soluble NSF (*N*-ethylmaleimide-sensitive factor)-attachment protein] receptors} Vam3p, Vti1p, Vam7p and the R-SNARE Nyv1p. In addition, vacuole fusion requires the Rab GTPase Ypt7p and its effector complex HOPS (homotypic fusion and vacuole protein sorting), actin, as well as the lipids ergosterol, diacylglycerol, phosphatidic acid and phosphoinositides [2].

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AUTHOR CONTRIBUTION

All three authors designed the research. Terry Sasser and Mark Padolina performed the experiments. Terry Sasser and Rutilio Fratti analysed the data and wrote the paper.

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 [3]. Membranes with activated SNAREs then tether and dock with partner membranes in a mechanism driven by Ypt7p and its effector complex HOPS [4,5]. 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 the formation of a specialized membrane-raft microdomain called the vertex ring where the proteins and lipids that catalyse fusion laterally accumulate to create the site of fusion [6–8].

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 [9] and the lipid modifiers Pah1p [10] and Vps34p [11], as well as the Na⁺/H⁺ exchanger Nhx1p [12]. Additional regulation may occur through controlling Ca²⁺ 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 [13]. Some of the putative flip/floppases belong to the ABC transporter (ATP-binding cassette transporter) superfamily [14].

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 [15]. ABC transporters are formed from two homologous halves that contain an NBD (nucleotide-binding domain) and an MSD (membrane-spanning domain) consisting of six transmembrane spans. Human ABC transporters are divided into seven sub-families (ABCA–ABCG). Many of the best characterized ABC transporters belong to the Class C subfamily (ABCC). With the exception of CFTR (cystic fibrosis transconductance regulator), ABCC family members, also known as MRPs (multidrug resistance-associated proteins), contain an N-terminal extension composed of five transmembrane motifs and a short cytosolic loop. The yeast vacuole harbours five ABCC transporters: the yeast cadmium factor, Ycf1p [16,17]; two bile acid and pigment transporters, Bpt1p and Ybt1p [18,19]; and two less well-characterized transporters, Vmr1p and Nft1p [15]. Ybt1p was first described as an ATP-dependent bile acid transporter [19] and is similar in structure to the human MRP transporter. Ybt1p is highly expressed on the yeast vacuole [20] and also plays a part in *ade2* pigment transport [21]. 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 [14].

MATERIALS AND METHODS

Reagents

Reagents were dissolved in PS buffer (20 mM Pipes/KOH, pH 6.8, and 200 mM sorbitol). Antibodies against Vam3p [22], Sec17p [23], Nyv1p [24], Sec18p [25] and Ypt7p [4] were described previously. The recombinant proteins His₆-Gyp1-56 [8], GDI (guanine-nucleotide-dissociation inhibitor) [26], GST (glutathione transferase)-FYVE [27], MED [MARCKS (myristoylated alanine-rich C-kinase substrate) effector domain] [6], His₆-

MTM1 (myotubularin 1) [28] and GST–Vam7p [29,30] were prepared as described previously and stored in PS buffer with 125 mM KCl.

Strains

BJ3505 [*MATa pep4::HIS3 prb1-Δ 1.6R his3-200 lys2-801 trp1Δ 101 (gal3) ura3-52 gal2 can1*] and DKY6281 (*MATa leu2-3 leu 2-112 ura3-52 his3-Δ200 trp1-Δ 901 lys2-801*) were used for fusion assays [31]. BJ3505 CBP (calmodulin-binding peptide)–Vam3 *nyv1Δ* was used for *trans*-SNARE complex isolation [32] (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 [33] with homology flanking the *YBT1* coding sequence with the forward primer 5′-GTGTGCGCATCTGCAAAGAACGTACGTTGTGACTAATGAACGGATCCCCGGGTTAATTAA-3′ and the reverse primer 5′-TCAGTAAAAGTTCATTGGATCAGATTTTCCTTCAAAGACGCGAATTCGAGCTCGTTTAAAC-3′. The PCR product was transformed into BJ3505 and DKY6281 by standard lithium acetate methods and plated on YPD medium [1% (w/v) yeast extract, 2 % (w/v) peptone and 2 % (w/v) glucose] containing G418 (250 μg/μl) to generate BJ3505 *ybt1Δ::kanMX6* (RFY28) and DKY6281 *ybt1Δ::kanMX6* (RFY29). *YBT1* was deleted from BJ3505 CBP–Vam3 *nyv1Δ* with the *hghMX4* cassette using PCR product amplified from pAG32 [34] with homology flanking the *YBT1* coding sequence with the forward primer 5′-GTGTGCGCATCTGCAAAGAACGTACGTTGTGACTAATGAAATAGGCCACTAGTGATCTG-3′ and reverse primer 5′-TCAGTAAAAGTTCATTGGATCAGATTTTCCTTCAAAGACGCTCAGCTGAAGCTTCGTACGC-3′. The PCR product was transformed into BJ3505 CBP–Vam3 *nyv1Δ* by standard lithium acetate methods and plated on to YPD medium containing hygromycin (250 μg/ml) to generate BJ3505 CBP–Vam3 *nyv1Δ ybt1::hghMX4* (RFY30). For vacuole localization studies *YBT1* was fused in-frame to GFP (green fluorescent protein) by homologous recombination. DKY6281 was transformed with a PCR product amplified from pFA6a-GFP-kanMX6 [33] with the forward primer 5′-TAAAAAAGCCTTTGTGGAAAAATTGAACTCTAAAAAGGACCGGATCCCCGGGTTAATTAA-3′ and the reverse primer 5′-ATATATATATATATATATATATACTTTAGCATCGAAACAGGAATTCGAGCTCGTTTAAAC-3′ with homology flanking the stop codon of the *YBT1* gene to make RFY31 (DKY6281 *YBT1::GFP*).

Vacuole isolation and *in vitro* vacuole fusion

Vacuoles were isolated by floatation as described previously [31]. 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 and 5 mM MgCl₂), ATP-regenerating system (1 mM ATP, 0.1 mg/ml creatine kinase and 29 mM creatine phosphate), 10 μM CoA and 283 nM IB₂ (inhibitor of protease B). Reactions were incubated at 27 °C and Pho8p activity was assayed in 250 mM Tris/HCl, pH 8.5, 0.4 % Triton X-100, 10 mM MgCl₂ and 1 mM *p*-nitrophenyl phosphate. Fusion units were measured by determining the *p*-nitrophenolate produced in min⁻¹ · μg⁻¹ *pep4Δ* 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 [29,30]. Briefly, large-scale 6× 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 μ l aliquots were removed to measure Pho8p activity. The remaining 150 μ l reactions were sedimented (11 000 g, 10 min, 4 °C), and the supernatants were removed before extracting vacuoles with SB (solubilization buffer: 20 mM Hepes/KOH, pH 7.4, 100 mM NaCl, 2 mM EDTA, 20 % glycerol, 0.5 % Triton X-100 and 1 mM dithiothreitol) with protease inhibitors (1 mM PMSF, 10 μ M Pefabloc-SC, 5 μ M pepstatin A and 1 μ M leupeptin). Vacuole pellets were overlaid with 100 μ l of SB and resuspended gently. An additional 100 μ l of SB was added, gently mixed, and incubated on ice for 20 min. Insoluble debris was sedimented (16 000 g, 10 min, 4 °C) and 176 μ l of supernatants were removed and placed in chilled tubes. Next, 16 μ l was removed from each reaction as 10 % total samples, mixed with 8 μ l of 3 $\times$ SDS-loading buffer and heated (95 °C, 5 min). Equilibrated glutathione-Sepharose 4B beads (30 μ l) were incubated with the remaining extracts (15 h, 4 °C, nutation). Beads were sedimented and washed five times with 1 ml of SB (4000 g, 2 min, 4 °C), and bound material was eluted with 40 μ l of 1 $\times$ 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 previously with some modifications [32,35]. Complex formation was compared between reactions containing vacuoles from RFY29 and RFY30 relative to those with BJ3505 CBP-Vam3p *nyv1* Δ and DKY6281 vacuoles. The *trans*-SNARE assays were performed using 16 $\times$ large-scale reactions (480 μ l) containing 48 μ g of vacuoles each from BJ3505 CBP-Vam3 *nyv1* Δ and DKY6281 backgrounds and incubated at 27 °C for 60 min. After incubation, reactions were placed on ice for 5 min and 30 μ l was withdrawn from each sample to assay Pho8p activity. The remaining 450 μ l samples were centrifuged (13 000 g, 15 min, 4 °C) and the supernatants were decanted. Vacuole pellets were overlaid with 200 μ l of ice-cold vacuole solubilization buffer (20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM MgCl₂, 0.5 % Nonidet P40 alternative and 10 % glycerol) with protease inhibitors (0.46 μ g/ml leupeptin, 3.5 μ g/ml pepstatin, 2.4 μ g/ml Pefabloc®-SC and 1 mM PMSF) and gently resuspended. Vacuole solubilization buffer was added to a final volume of 600 μ l and extracts were mixed (20 min, 4 °C, nutation). Detergent-insoluble debris was removed by centrifugation (16 000 g, 20 min, 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 μ l of equilibrated calmodulin-Sepharose 4B (GE Healthcare) (4 °C, 12 h, nutation). Beads were collected by centrifugation (4000 g, 2 min, 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 min. The samples were used for SDS/PAGE and immunoblotting.

Lipid mixing

Lipid mixing assays were conducted using Lissamine rhodamine B (Rh-PE; Invitrogen) as described previously with minor modifications [10]. Briefly, BJ3505 background vacuoles (300 μ g) were incubated in 400 μ l of PS buffer containing 150 μ M Rh-PE (10 min, 4 °C, nutating). Samples were mixed with 15 % (w/v) Ficoll in PS buffer and transferred to a polyallomer tube (11 mm $\times$ 60 mm). Samples were overlaid with 1.0 ml each of 8 %, 4 % and 0 % Ficoll. Labelled vacuoles were re-isolated by centrifugation using a SW60 Ti rotor (105 200 g, 25 min, 4 °C) and recovered from the 0–4 % Ficoll interface. Lipid mixing assays (90 μ l) contained 2 μ g of Rh-PE-labelled vacuoles and 16 μ g of unlabelled vacuoles in fusion buffer. Reaction mixtures were transferred to a black half-volume 96-well flat-bottom microtitre plate with non-binding surface (Corning). Rhodamine fluorescence (λ_{ex} = 544

nm; $\lambda_{em} = 590$ nm) 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 (F_0) and during the reaction (F_t). The final ten measurements of a sample after adding 0.33 % 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 medium containing the vital dye FM4-64 (Invitrogen). Cultures were grown overnight to saturation, diluted to $\sim 0.2 D_{600}$ in YPD containing 5 μ M FM4-64, grown for 1 h in a 30 °C shaker, washed with PBS, resuspended in YPD, incubated at 30 °C for 3 h, washed in PBS, concentrated by centrifugation, mixed with 0.6 % agarose, and mounted on to 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 63 $\times$ oil objective (numerical aperture 1.4), and an AxioCam CCD (charge-coupled-device) camera. For vertex assembly detection, docking assays were performed. Docking reactions (30 μ l) contained 6 μ g of Ybt1p-GFP vacuoles, docking buffer (20 mM Pipes/KOH, pH 6.8, 200 mM sorbitol, 100 mM KCl and 0.5 mM MgCl₂), ATP-regenerating system, 20 μ M CoA and 283 nM IB₂. PtdIns3P was labelled with 0.2 μ M Cy5 (indodicarbocyanine)-FYVE and used as a marker for vertex microdomains [10]. Reactions were incubated at 27 °C for 30 min and placed on ice and stained with 3 μ M FM4-64. Reactions were next mixed with 50 μ l of 0.6 % low-melt agarose (in PS buffer), vortex-mixed to disrupt spurious clustering, and mounted on to slides for observation by fluorescence microscopy.

Ca²⁺ efflux assay

SNARE-dependent Ca²⁺ efflux was measured as described previously [36] with some modifications. Fusion reactions (60 μ l) contained 20 μ g of vacuoles isolated from BJ3505 backgrounds, fusion reaction buffer with 10 μ M 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 μ M (Invitrogen). Reaction mixtures were transferred to a black half-volume 96-well flat-bottom microtitre plate with non-binding surface (Corning). ATP-regenerating system was added and reactions were incubated at 27 °C while monitoring Fluo-4 fluorescence ($\lambda_{ex} = 488$ nm; $\lambda_{em} = 520$ 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 [19]. 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 [37], and found that Ybt1p-GFP primarily localized to the vacuole (Figures 1a–1d), which is consistent with previous findings [14]. We next examined the vacuole morphology in *ybt1Δ* cells and found that the morphology and size of the vacuoles were similar to those in WT (wild-type) cells (Figures 1e–1g). As fusion regulators often accumulate at the vertices of docked vacuoles [6–8], we next examined the distribution of Ybt1p-GFP on isolated vacuoles during docking. Figure 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 a Cy5-conjugated FYVE domain. The FYVE domain specifically binds to the regulatory lipid PtdIns3P [27], a critical lipid that accumulates at the site of fusion in vertex microdomains [6]. In Figure 2(c), 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 *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 (*PEP4 pho8Δ*) and BJ3505 (*pep4Δ PHO8*) were mixed to assay content mixing. We deleted *YBT1* from these strains to generate RFY28 and RFY29 and found that fusion was augmented and showed an approximate 50 % increase compared with WT vacuoles (Figure 3a). This suggests that Ybt1p acts as a negative regulator for vacuole fusion.

As 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 [12,38,39]. In the present study, the outer leaflets of vacuole-limiting membranes were labelled with Rh-PE, which is self-quenched at high concentrations. When labelled donor vacuoles were incubated with an 8-fold excess of unlabelled acceptor vacuoles, Rh-PE was dequenched. WT vacuoles showed a characteristic rapid dequenching that was inhibited with anti-Vam3 IgG (Figure 3b, circles). When *ybt1Δ* vacuoles were examined, we found that the initial dequenching was identical with WT, after which the *ybt1Δ* vacuoles surpassed WT fusion starting at 15 min (Figure 3b). This illustrates that the initial rates of fusion were the same for WT and *ybt1Δ* vacuoles, yet the $V_{\max}$ of *ybt1Δ* fusion was significantly higher. To determine whether the increase in fusion of *ybt1Δ* 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 3c). It should also be noted that the increased fusion was not due to defects in trafficking of Pho8p or Pep4p (Figure 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 3(a). After 90 min 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. Figure 3(e) shows 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 ($7.13 \pm 0.19 \mu\text{m}$) were markedly larger than those of WT vacuoles ($4.88 \pm 0.23 \mu\text{m}$).

To determine whether the augmented fusion seen with *ybt1Δ* vacuoles was regulated by the core fusion machinery, 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 GDI. PtdIns3P function was inhibited by ligation with the FYVE domain or modification with the phosphoinositide 3-phosphatase, MTM1. MED was used to bind multi-phosphorylated phosphoinositides, including PtdIns(4,5) P_2 . 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 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 [29,30]. Fusion was then rescued with the addition of 400 nM GST-Vam7p and incubated for an additional 70 min. Selected reactions were treated with anti-Vam3p antibody prior to adding Vam7p. Fusion was restored when WT and mutant vacuoles were treated with exogenous GST-Vam7p (Figure 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 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 the anti-Vam3p antibody. We found that GST-Vam7p complexes from *ybt1Δ* vacuoles contained a similar SNARE makeup relative to WT vacuoles.

We next determined whether endogenous SNAREs could form *trans* complexes when priming is not blocked [32,35]. *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 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 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 due to an augmented number of SNARE complexes (Figures 6b and 6c). 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 Ca²⁺ stores

From Figure 3(b) we found that mutant fusion was increased after 15 min, which coincides with the formation of *trans*-SNARE pairing [32]. The formation of *trans*-SNARE complexes is linked to the release of luminal Ca²⁺ stores from the vacuole [40,41]. As Ybt1p interacts with the Ca²⁺ ATPase Pmc1p [42], we next examined the effect of deleting *YBT1* on Ca²⁺ transport. Fusion reactions (2×) 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 the addition of anti-Sec17p IgG where indicated and fluorescence was monitored. Upon the addition of ATP, Ca²⁺ was transported into the vacuole, causing a drop in fluorescence. Ca²⁺ is transported into the vacuole via the Ca²⁺-ATPase Pmc1p and the Vcx1p H⁺/Ca²⁺ exchanger [43]. After approximately 15 min, when *trans*-SNAREs have formed, we observed an increase in fluorescence indicating that Ca²⁺ was being effluxed from the vacuole lumen (Figure 7a, ●), which is in keeping with previous studies using the aequorin reporter system [40]. When *ybt1Δ* vacuoles were observed, we found that there were four differences relative to WT reactions. First, there was a prolonged influx of Ca²⁺ early in the reaction, suggesting that Pmc1p and/or Vcx1p activity may be augmented. Secondly, we found that the *trans*-SNARE complex-dependent efflux was markedly delayed in *ybt1Δ* vacuoles (Figure 7b). These observations suggest that Ybt1p regulates Ca²⁺ transport in vacuoles and that the enhanced fusion may be linked to alterations in Ca²⁺ homeostasis. In addition, the relative amount of Ca²⁺ in *ybt1Δ* vacuoles was nearly twice as much as seen with WT vacuoles. We also found that WT vacuoles

reabsorb Ca^{2+} after docking, resulting in a return to basal 'pre-docking' levels of extraluminal Ca^{2+} , whereas *ybt1Δ* fails to reabsorb the effluxed Ca^{2+} .

DISCUSSION

In the present 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 [40]. 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 [42]. 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 [44]. 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 with 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 upon the formation of membrane microdomains that are enriched in the proteins and lipids that regulate fusion [6–8]. 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 [45]. 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 [46,47]. Interestingly, Zilly et al. [45] also found that elevated Ca^{2+} concentrations ($10\ \mu\text{M}$) inhibited SNARE pairing. Although 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.

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Abbreviations used

ABC transporter	ATP-binding cassette transporter
CBP	calmodulin-binding peptide
Cy5	indodicarbocyanine
GDI	guanine-nucleotide-dissociation inhibitor
GFP	green fluorescent protein
GST	glutathione transferase
HOPS	homotypic fusion and vacuole protein sorting
MRP	multidrug resistance-associated protein
MTM1	myotubularin 1
NSF	<i>N</i> -ethylmaleimide-sensitive factor
SB	solubilization buffer
SNAP	soluble NSF-attachment protein
SNARE	SNAP receptor
WT	wild-type

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Figure 1. Ybt1-GFP is localized to the vacuole

WT yeast cells harbouring Ybt1p-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. **(a and b)** GFP images were acquired using a 38 HE GFP shift-free filter set and FM4-64 images were acquired using a 43 HE Cy3 (indocarbocyanine) shift-free filter set. **(c)** Cells were photographed using differential interference contrast (DIC). **(d)** Images were merged using Adobe Photoshop. **(e and f)** *ybt1Δ* yeast were incubated with FM4-64 as described above and imaged using DIC and Cy3 filters. **(g)** Quantification and box plot of vacuole size in WT and *ybt1Δ* cells. Scale bar, 4 μm .

Figure 2. Ybt1–GFP is not enriched at the vertex ring microdomain of docked vacuoles
Purified vacuoles isolated from cells harbouring Ybt1p–GFP (a) were incubated at 27 °C for 30 min. The limiting membranes were stained with 5 μ M FM4-64 (b) and vertex microdomains were labelled with Cy5-conjugated FYVE domain to label PtdIns3P(c). (d) Images were merged using Adobe Photoshop. Scale bar, 4 μ m.

Figure 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 (●) and *ybt1Δ* (○). Error bars represent S.E.M. ($n = 3$). **(b)** Lipid mixing assays measuring fusion were employed by labelling isolated vacuoles with Rh-PE. Labelled vacuoles were incubated with an 8-fold excess of unlabelled 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 μg by protein) were mixed with 2× SDS loading buffer, separated by SDS/PAGE and transferred on to nitrocellulose. Immunoblotting was performed using the indicated antibodies and bands were detected using ECF. **(d)** Quantification of Pep4p and Pho8p in WT and *ybt1Δ* vacuoles. **(e)** Measurements of vacuole diameters after incubation (27 °C, 90 min). Error bars are S.E.M. ($n = 3$). * $P < 0.0001$

Figure 4. The augmented fusion of *ybt1Δ* vacuoles is dependent on the standard fusion machinery

Fusion reactions were performed using WT or *ybt1Δ* vacuoles. Individual reactions were treated with PS buffer, 14 $\mu\text{g/ml}$ anti-Nyv1p IgG, 27 $\mu\text{g/ml}$ anti-Vam3p IgG, 12 $\mu\text{g/ml}$ anti-Sec18p IgG, 67 $\mu\text{g/ml}$ anti-Sec17p IgG, 8 $\mu\text{g/ml}$ 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 are S.E.M. ($n = 3$).

Figure 5. Vam7p forms complexes with SNAREs and HOPS on *ybt1Δ* vacuoles

(a) Large-scale fusion reactions (6×) 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 μ l was removed to measure Pho8p activity. (b) The remaining fractions were extracted with SB with protease inhibitors for 20 min while on ice. Insoluble debris was sedimented by centrifugation and supernatants were removed and incubated with equilibrated glutathione-Sephadex. Beads were washed with SB and protein complexes were eluted with 2× SDS/PAGE buffer. Proteins were resolved by SDS/PAGE, transferred on to nitrocellulose and probed with antibodies against Nyv1p, Vam3p, Vam7p, Sec17p, Vps33p, actin and Ypt7p. Error bars are S.E.M. ($n = 3$).

Figure 6. *trans*-SNARE complex formation

Vacuoles harbouring CBP-Vam3p in a *nyv1Δ* background were incubated with vacuoles harbouring a full complement of SNAREs. Large-scale reactions (16×) were incubated at 27 °C for 60 min. After incubating, reactions were placed on ice for 5 min and 30 μ l was withdrawn from each sample to assay Pho8p activity (a). (b) Membranes were pelleted and then resuspended in SB 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) Quantification of efficiency of Nyv1p binding to CBP-Vam3p. Error bars are S.E.M. ($n = 3$).

Figure 7. Calcium efflux is delayed in *ybt1Δ* vacuoles

Vacuoles were harvested from WT BJ3503 and RFY28 (*ybt1Δ*). Fusion reactions (2×) were prepared containing 20 μg of either WT or *ybt1Δ* vacuoles and 200 μM 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 WT and *ybt1Δ* vacuoles. Error bars are S.E.M. ($n = 3$).

Table 1

Yeast strains used in the present study

Strain	Genotype	Source
BJ3505	<i>MAT a pep4::HIS3 prb1-Δ1.6R his3-200 lys2-801 trp1Δ101 (gal3) ura3-52 gal2 can1</i>	[48]
DKY6281	<i>MAT a pho8::TRP1 leu2-3 leu 2-112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801</i>	[49]
BJ3505 CBP-Vam3 <i>nyv1Δ</i>	BJ3505, <i>CBP-VAM3::Kan^r nyv1Δ::nat^r</i>	[32]
RFY28	BJ3505, <i>ybt1Δ::kanMX6</i>	The present study
RFY29	DKY6281, <i>ybt1Δ::kanMX6</i>	The present study
RFY30	BJ3505 CBP- <i>VAM3 nyv1Δ ybt1Δ::hghMX4</i>	The present study
RFY31	DKY6281, <i>YBT1::GFP</i>	The present study