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Vacuolar import of phosphatidylcholine requires the ATP-binding cassette transporter Ybt1

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Abstract

ATP-binding cassette (ABC) transporters are well-known for their roles as multidrug resistance determinants but also play important roles in regulation of lipid levels. In the yeast *Saccharomyces cerevisiae*, the plasma membrane ABC transporter proteins Pdr5 and Yor1 are required for normal rates of transport of phosphatidyethanolamine to the surface of the cell. Loss of these ABC transporters causes a defect in phospholipid asymmetry across the plasma membrane and has bee linked with slowed rates of trafficking of other membrane proteins. Four ABC transporter proteins are found on the limiting membrane of the yeast vacuole and loss of one of these vacuolar ABC transporters, Ybt1, caused a major defect in the normal delivery of the phosphatidylcholine (PC) analogue NBD-PC (7-nitro-2,1,3-benzoxadiazol-PC) to the lumen of the vacuole. NBD-PC accumulates on cytosolic membranes in a *ybt1Δ* strain. We demonstrated that Ybt1 is required to import NBD-PC into vacuoles in the presence of ATP *in vitro*. Loss of Ybt1 prevented vacuolar remodeling of PC analogues. Turnover of Ybt1 was reduced under conditions in which function of this vacuolar remodeling pathway was required. Our data describe a novel vacuolar route for lipid remodeling and reutilization in addition to previously described enzymatic avenues in the cytoplasm.

Keywords

ABC transporter; Phosphatidycholine; Vacuole; NBD-PC; Biosynthesis

Introduction

Control of lipid content of membranes is a feature that defines the many different membranes in a eukaryotic cell. Phospholipids represent the major component of membranes and are highly regulated in terms of their content in and distribution across these membranes (reviewed in (1)). The differential distribution of phospholipids across a membrane is referred to as asymmetry and is generated through the balanced movement of phospholipids from the outer leaflet to the inner leaflet (flip) and the inverse of this directional transport (flop) (see (2, 3) for reviews). The best characterized contributor to this vectorial movement of phospholipids are P-type ATPases such as Drs2 from *Saccharomyces cerevisiae* (4). Drs2 is a Golgi-localized enzyme that acts as a flippase moving phospholipids like phosphatidylethanolamine (PE) and phosphatidyserine (PS) into the cytoplasm (5, 6). Less is known about enzymes catalyzing the flop activity but a number of

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studies implicate ATP-binding cassette transporters as participants in this directional transport of phospholipids.

The first example of an ABC transporter protein exhibiting a potential floppase activity came from the demonstration that mouse Mdr2 (Abcb4) was required for secretion of phosphatidylcholine (PC) into biliary circulation (7). Later work in *S. cerevisiae* has demonstrated the role of the plasma membrane ABC transporters Pdr5 and Yor1 in flop of PC and PE (8, 9). A strain lacking Pdr5 and Yor1 exhibits a reduction in levels of exofacially exposed PE (10), indicating the normal plasma membrane asymmetry has been lost in response to loss of these two genes and their products. Importantly, analysis of a *pdr5Δ yor1* strain demonstrated that the membranes of this double mutant strain were unable to support normal plasma membrane accumulation of the tryptophan transporter Tat2, consistent with asymmetry being required to maintain wild-type protein trafficking (11).

Along with the ABC transporters found on the plasma membrane, *S. cerevisiae* typically expresses 4 of these proteins localizing to the limiting membrane of the vacuole. These include the cadmium resistance determinant Ycf1 (12, 13), two different transporters shown to transport bile pigments and bile acids: Bpt1 and Ybt1 (14, 15) and a recently characterized protein that exhibits a multidrug resistance phenotype, Vmr1 (16). All of these proteins are members of the ABCC class of ABC transporters (http://www.ncbi.nlm.nih.gov/books/NBK31/). Yor1 is the only known plasma membrane representation of the *S. cerevisiae* ABCC family (17, 18). Since Yor1 has been shown to be involved in flop of phospholipids at the plasma membrane, we wanted to examine if these vacuolar membrane-localized ABC transporters might also be involved in regulation of phospholipid homeostasis. To examine this possibility, we used the fluorescent PC analogue, NBD-PC, to follow trafficking of this compound in cells engineered to lack different combinations of genes encoding these vacuolar ABC transporter proteins.

Genetic analysis indicated that loss of the *YBT1* gene alone was sufficient to completely eliminate the usual import of NBD-PC into the vacuolar lumen. Earlier work had found that loss of the endosomal trafficking protein Vps27 also blocked luminal delivery of NBD-PC. Ybt1 localization, expression and function were undisturbed in a *vps27Δ* strain indicating that these two components of NBD-PC transport operated independently. Using an in vitro assay, we found that isolated vacuoles containing Ybt1 were capable of transporting NBD-PC into the lumen. Removal of Ybt1 from a strain constructed to lack other routes of PC analogue remodeling, caused a severe growth defect. Together, these data support the view that Ybt1 is a key participant in a novel vacuolar pathway required for phospholipid remodeling.

Results

Ybt1 is required for NBD-PC transport across vacuolar membrane in yeast

S. cerevisiae expresses 5 ABCC (Mrp/CFTR subfamily) type ABC transporter proteins (recently reviewed in (19)). While this type of ABC transporter is often found localized to the periphery of mammalian cells, only the Yor1 ABCC transporter is directed to the *S. cerevisiae* plasma membrane (18). The other 4 members of this group of ABC transporters are believed to localize to the limiting membrane of the vacuole. These proteins include Ycf1, Ybt1, Bpt1 and Vmr1. The best-studied of these transporters is Ycf1, a protein required for resistance to cadmium that acts as a glutathione conjugate transporter (20). To explore the function of these related vacuolar membrane transporters, we took a genetic approach and constructed a series of isogenic strains that varied in the dosage of the genes encoding these ABC transporters. These strains were examined for a variety of phenotypes

and a particularly interesting result was obtained when evaluating the influence of these mutations on vacuolar accumulation of the phosphatidylcholine analog, NBD-PC

NBD-PC has been widely used to follow traffic of phospholipids through cells. Previous experiments in *S. cerevisiae* established that NBD-PC is taken up via a Dnf1/2-dependent flip across the plasma membrane and eventually trafficked through a endosomal pathway into the lumen of the vacuolar (21) (22) (23) (10). Loss of the endosomal protein Vps27 prevented NBD-PC entry into the vacuolar lumen (24) while a strain lacking normal endocytosis (*end3Δ*) exhibited no defect in NBD-PC localization (23). The various vacuolar ABC transporter deletion strains were labeled with NBD-PC and then visualized by fluorescence microscopy to determine the subcellular distribution of this fluorescent phosphatidylcholine analog (Figure 1). These strains were also labeled with the endocytic probe FM4-64 to test for potential endocytosis defects.

A highly specific failure to accumulate luminal NBD-PC was observed when any strain lacking Ybt1 was tested in this assay. Even a strain lacking the other three vacuolar transporter loci (*ycf1Δ bpt1Δ vmr1Δ*) and expressing only Ybt1 was found to normally accumulate NBD-PC inside the vacuole. Loss of the P-type ATPase protein Dnf2 reduced NBD-PC uptake and *end3Δ* mutants were normally labeled as seen before (23). In all cases, labeling of cells by FM4-64 was undisturbed. These results strongly suggested that Ybt1p is the sole NBD-PC translocator present on the yeast vacuolar membrane. To determine the specificity of Ybt1-dependent phospholipid transport, the effect of loss of Ybt1 was also evaluated using other NBD-labeled phospholipids (NBD-PE, NBD-PS). As seen earlier (reviewed in (25)), these compounds were transported to the nuclear envelope and mitochondrial membrane in a Ybt1 independent manner, suggesting that trafficking defect seen in the *ybt1Δ* delete cells is specific toward NBD-PC.

Although FM4-64 endocytosis was normal in all the strains examined, including the *ybt1Δ* mutant, we tested several other measures of vacuolar function to ensure that the failure to accumulate NBD-PC was not due to some more general issue with vacuolar function.

Ste3 and Npc2 are membrane proteins that are targeted to the vacuolar lumen (26, 27). Plasmids expressing fusions between each of these proteins and GFP were introduced into isogenic wild-type and *ybt1Δ* cells (Figure 2). Transformants were examined by fluorescence microscopy and both fusion proteins were found to be unaffected in their delivery to the vacuole by the presence of the *ybt1Δ* allele.

An important parameter of the vacuole is the maintenance of the pH gradient that is developed across its limiting membrane by the vacuolar ATPase complex (reviewed in (28)). The pH sensitive dye quinacrine was used to stain vacuoles from wild-type and *ybt1Δ* cells (Figure 2). This analysis indicated that Ybt1 did not influence formation of the characteristic pH gradient. Together, these results argue that *ybt1Δ* cells possess vacuoles that exhibit a specific defect in accumulation of NBD-PC.

Specific role of Ybt1 in NBD-PC uptake into the vacuolar lumen

Earlier work has indicated the presence of redundancy and overlapping substrate specificity among the vacuolar membrane ABC transporters. For example, substrates of the ABC transporter Ycf1, such as cadmium, adenine pigment, bile-pigment and glutathione can also be transported by Bpt1p (29). Similarly, Ybt1 is also involved in transportation of the adenine pigment like Ycf1p and Bpt1p (30). To determine if the close Ybt1p homologues have the ability to transport NBD-PC, we tested the ability of Ycf1p, Bpt1p and Vmr1p to bypass the defective NBD-PC trafficking of a *ybt1Δ* delete strain by introducing each of these transporters into *ybt1Δ* null background on a high-copy-number plasmid. We believe

these transporters are all overproduced relative to wild-type gene dosage on the basis of increased adenine pigmentation using a sensitive color assay ((30), data not shown).

Transformants of isogenic wild-type or *ybt1Δ* cells carrying an empty high-copy-number vector plasmid (pRS426) or the same plasmid containing a gene encoding one of the vacuolar ABC transporters were grown to mid-log phase and tested for accumulation of NBD-PC. These transformants were also labeled with FM4-64 to label the vacuolar membrane (Figure 3)

Only introduction of the high-copy-number plasmid containing the *YBT1* gene was able to correct the NBD-PC vacuolar accumulation defect of the *ybt1Δ* mutant strain. FM4-64 labeling was unaffected in all these strains. These data argue that Ybt1 provides a specific route of a PC analogue, NBD-PC, that cannot be conferred by any of the other 3 vacuolar ABC transporters.

To provide support for the argument that Ybt1 was acting as an ABC transporter to drive vacuolar uptake of NBD-PC, a mutant form of this gene was constructed. A lysine residue corresponding to K735 in the first nucleotide-binding domain of Ybt1 is critical for function of the analogous region present in ABC transporters. This lysine residue is in the Walker A domain of the nucleotide-binding domain (NBD) of these transporter proteins and is involved in ATP-binding and hydrolysis (reviewed in (31)). Mutation of this lysine to a methionine residue has been found to inactivate other ABCC-type transporters like multidrug resistance protein (32) or cystic fibrosis transmembrane conductance regulator (33). A K735M *YBT1* mutation was constructed in the context of the high-copy-number plasmid and introduced into the *ybt1Δ* strain (Figure 3). This mutant form of Ybt1 lost the ability to complement the NBC-PC uptake defect of the *ybt1Δ* mutant, consistent with Ybt1 acting as a typical ABC transporter in driving the accumulation of this PC analogue. To ensure that plasmid copy number was not influencing these results, we cloned the wild-type and K735M forms of *YBT1* into the low-copy-number vector pRS316. Again, the wild-type *YBT1* gene complemented the ybt1Δ strain while the K735M YBT1 gene did not (data not shown).

To confirm that the defect in NBD-PC accumulation of the K735M Ybt1 derivative was due to loss of activity, the level of expression and appropriate localization of this protein was compared to that of the wild-type transporter. An amino-terminal HA tag was inserted in plasmids expressing either the wild-type or K735M form of Ybt1 and whole cell protein extracts analyzed for expression of the epitope-tagged transporters (Figure 4A).

Two different forms of HA-Ybt1 proteins could be seen that were equivalently expressed in extracts from either wild-type or K735M forms of Ybt1: 180 or 115 kD. The defect in NBC-PC transport in cells containing K735M Ybt1 as their sole source of this protein was not due to defective expression of this mutant transporter.

Similarly, a C-terminal GFP cassette was inserted into plasmids expressing the wild-type or K735M forms of Ybt1. Transformants were visualized by fluorescence microscopy and both forms of Ybt1 were found on the limiting membrane of the vacuole (Figure 4B). These data indicate that localization of both forms of Ybt1 is normal and support the view that the K735M mutant is defective in transporter activity.

Vps27 acts upstream of Ybt1 in NBD-PC trafficking

Previous studies have characterized the itinerary followed by NBD-PC as it travels to the lumen of the vacuole. NBD-PC and other fluorescent phospholipid analogues are transported into the cell via the action of P-type ATPases such as Dnf1/2 at the plasma

membrane (10). These analogues then transit to the vacuolar lumen via a route that depends on function of the late endosomal sorting determinant Vps27 (34). Mutants lacking Vps27 were found to accumulate NBD-PC in the mitochondria and endoplasmic reticulum membranes. Vps27 is a late-acting endosomal sorting determinant known to influence protein trafficking to the vacuole (34). Since we have found that mutants lacking Ybt1 exhibit NBD-PC trafficking defects that resemble those previously described for *vps27Δ* and other class E mutant strains (23, 24), we explored the possible interactions between *vps27Δ* and *ybt1Δ* strains in terms of trafficking this fluorescent PC analogue.

An isogenic set of strains lacking *VPS27, YBT1* or both genes was prepared. These strains were grown along with the parental wild-type cells and then labeled with NBD-PC or DAPI. Labeled cells were then visualized by fluorescence microscopy (Figure 5).

As described earlier (23, 24), NBD-PC was found to accumulate in the mitochondrial and ER membranes of the *vps27Δ* strain. Both the *ybt1Δ* and *ybt1Δ vps27Δ* strains exhibited this same localization defect.

A possible unifying explanation for the failure to accumulate NBD-PC in the vacuolar lumen in *vps27Δ* cells could be provided by a defect in expression, localization or function of Ybt1. To determine if Ybt1 localization required Vps27, a GFP cassette was placed at the C-terminus of the chromosomal *YBT1* locus in wild-type and *vps27Δ* cells. Appropriate clones were grown to early log phase and localization of Ybt1-GFP assessed by fluorescence microscopy (Figure 6A). A TAP-tagged form of Ybt1 was prepared by integrating a cassette encoding the TAP tag into the C-terminus of the chromosomal *YBT1* gene in isogenic wild-type and *vps27Δ* cells. Transformants were grown to mid-log phase and levels of Ybt1-TAP assessed by western blotting using the anti-TAP antiserum (Figure 6B). Expression of Ybt1-TAP was unaffected by removal of the *VPS27* gene.

Ybt1-GFP was found on the limiting membrane of the vacuole, irrespective of the presence of Vps27. We also carried out this same experiment in a vps4Δ strain and again found that Ybt1-GFP localized to the vacuolar membrane (data not shown). FM4-64 trafficking was disturbed in the *vps27Δ* strain as detailed before (24). Lack of an effect of loss of Vps27 on a polytopic vacuolar integral membrane protein like Ybt1 is not without precedent as other membrane proteins can be delivered to the vacuolar limiting membrane in *vps27Δ* cells while lumenal, soluble proteins fail to appropriately traffic (35).

To determine if Ybt1 was non-functional in *vps27Δ* cells, we used an additional phenotype that has been associated with Ybt1. Resistance to the heavy metal nickel has been linked with the presence of Ybt1 (36). We tested our series of single vacuolar transporter disruption mutant strains for their level of nickel resistance (Figure 6C). As seen with NBD-PC, only cells lacking Ybt1 were sensitive to nickel, consistent with the data reported by another group. Importantly, a *ycf1Δ bpt1Δ vmr1Δ* triple mutant exhibited wild-type nickel resistance, fully agreeing with the conclusion that Ybt1 represents the sole important vacuolar ABC transporter required for nickel tolerance (data not shown).

Having linked Ybt1 activity to nickel tolerance, we used this phenotype to examine the functional status of this vacuolar ABC transporter in *vps27Δ* cells. Isogenic wild-type, *ybt1Δ* or *vps27Δ* cells were transformed with a high-copy-number vector plasmid or the same plasmid containing the *YBT1* gene. Transformants were then placed on medium containing nickel (Figure 6D).

The presence of wild-type *YBT1* on the high-copy-number plasmid consistently elevated nickel tolerance in all three strains when compared to the empty vector plasmid alone. Importantly, loss of Vps27 did not significantly alter the level of nickel resistance seen.

Together, these data support the view that Vps27 influences vacuolar accumulation of NBD-PC at a step other than Ybt1, expression, localization or function.

Ybt1 transports NBD-PC into the vacuolar lumen in vitro

While the data above support the idea that Ybt1 acts to transport NBD-PC into the vacuolar lumen, many possible explanations are consistent with these findings. To strengthen the argument that Ybt1 acts directly on NBD-PC, we developed a cell-free NBD-PC translocation assay using isolated vacuoles. This was accomplished by growing wild-type and *ybt1Δ* cells to early log-phase in rich medium in the presence of the fluorescent compound monochlorobimane (MCB). MCB has previously been described as a substrate of the Ycf1 vacuolar ABC transporter that accumulates in the lumen of the vacuole (20). After confirming the vacuolar localization of MCB, vacules were prepared from MCB-labeled wild-type and *ybt1Δ* cells using Ficoll density gradient centrifugation as described (13). Isolated vacuoles were then incubated with NBD-PC in the presence or absence of ATP. The limiting membrane of the vacuoles were also stained with FM4-64 to provide a measure of the relative intact nature of these isolated organelles along with their luminal MCB fluorescence. After incubation at 30°C for 30 minutes, vacuoles were washed with cold buffer and fatty acid-free BSA to remove excess dyes and then inspected under a fluorescence microscope (Figure 7).

Vacuoles isolated from wild-type cells accumulated NBD-PC in the lumen but only in the presence of ATP. When the same reaction was performed on vacuoles isolated from *ybt1Δ* cells, not luminal uptake of NBD-PC could be detected. These data are consistent with the idea that vacuoles containing Ybt1 in their limiting membrane are capable of importing NBD-PC into their lumen.

Ybt1 is required for growth under PC depletion

While NBD-PC represents a useful model for trafficking of PC in cells, this compound is still only an analogue of PC. To confirm that Ybt1 transport of NBD-PC into the vacuolar lumen faithfully reflected a facet of endogenous PC metabolism, we examined the role of this vacuolar ABC transporter in utilization and remodeling of PC analogues that can be used to support cell growth. To genetically isolate the presumptive vacuolar role of Ybt1 in PC metabolism, we constructed a strain lacking the de novo (*PEM1* and *PEM2*) and remodeling pathways (*ALE1* and *NTE1*) for PC biosynthesis. Pem1 and Pem2 are phosphatidylethanolamine methyltransferases required for the methylation pathway of PC biosynthesis from PE (37). ALE1 (Acyltransferase for Lyso-phosphatidylethanolamine) is highly enriched at mitochondria-associated endoplasmic reticulum membranes and accounts for the majority of acyl-CoA dependent LPEAT (lyso-PtdEtn acyltransferase) activity in yeast (38). Nte1p is an ER-localized phospholipase B that degrades PC to generate glycerophosphocholine and free fatty acids (39). A quadruple *pem1Δ pem2Δ ale1Δ nte1Δ* strain was constructed with the aim of eliminating all currently described routes of PC de novo biosynthesis (*pem1Δ pem2Δ*) as well as remodeling of PC analogues (*ale1Δ nte1Δ*). A *ybt1Δ* allele was introduced into this quadruple disruption strain to determine the contribution of Ybt1 to PC production in this strain. This strain lacking 5 genes (*pem1Δ pem2Δ ale1Δ nte1Δ ybt1Δ*) will be referred to as the pentuple disruption. We also evaluated the growth of *pem1Δ pem2Δ* and *pem1Δ pem2Δ ybt1Δ* strains to assess the possible role of Ybt1 in the presence of the remodeling pathways but absence of de novo biosynthesis.

To impose choline limitation on these strains, cultures were grown to saturation overnight in minimal media lacking choline. The next morning, cultures were diluted into fresh minimal media containing various sources of choline and growth measured over the next 48 hour period while incubating at 30°C (Figure 8A).

Supplementation of minimal media with the PC analogues lyso-PC or short acyl chain PC (diC_8-PC) supported growth of all mutant strains except the pentuple disruption mutant. The best growth for each PC analogue was shown by the *pem1Δ pem2Δ* strain. Introducing either the *ybt1Δ* or the *ale1Δ nte1Δ* alleles into this background reduced growth roughly equally. Only the pentuple mutant failed to resume growth on media containing these PC analogues. This same general trend was seen when these strains were incubated in the absence of choline. This is likely reflective of the relative internal pools of PC in each of these 4 different genetic backgrounds. Finally, addition of 1 mM choline to the media allowed equal and robust growth of all these strains.

These data support the view that Ybt1 is required for normal metabolism of PC analogues in cells, possibly through supporting a vacuolar PC analogue remodeling pathway. Given this metabolic link between Ybt1 and PC analogue utilization in cells, we examined if Ybt1 might exhibit a regulatory response when cells were in need of its role in vacuolar PC reclamation. First, a chromosomal TAP-tagged version of Ybt1 was constructed in wild-type and *pem1Δ pem2Δ ale1Δ nte1Δ* cells. This Ybt1-TAP tag was fully-functional as assessed by nickel resistance and NBD-PC uptake (data not shown). The wild-type and quadruple disruption Ybt1-TAP strains were grown in minimal media containing limiting choline and then resuspended in media containing or lacking exogenous choline. After two hours of growth, cycloheximide was added to each culture, time points removed and whole cell protein extracts prepared. Equal aliquots of these extracts were analyzed by western blotting using antibodies against TAP or the ER marker Kar2 (Figure 8B).

Imposition of choline starvation led to the dramatic stabilization of Ybt1-TAP as this protein was not detectably degraded over an 8 hour time course in a quadruple mutant background. Addition of choline returned the degradation of Ybt1-TAP back to a rate equivalent to that seen in wild-type cells. Ybt1-TAP exhibited no significant stabilization in wild-type cells, irrespective of choline supplementation, as these cells contained their full complement of choline biosynthetic enzymes. Kar2 degradation was unaffected by these regimens. These data strengthen the view that Ybt1 represents a normal component of the machinery cells use to maintain normal phospholipid levels.

Discussion

ABC transporters are well-known for their actions as drug transporters and arguably, this is likely their best known role in eukaryotic cells. A role of growing importance is the activity of these proteins to traffic lipids in the cell. The importance of ABC transporters in lipid trafficking is illustrated by the observation that of the 18 ABC transporters implicated in human disease, 8 of these diseases represent defects in lipid transport (reviewed in (40)). One of the puzzles that remains unsolved in the study of ABC transporter activity is their astounding substrate range, which can reach into the hundreds of different substrates (see (41) for example). Recent structural information suggests that their active sites may have unprecedented capacity for accommodation of different substrates (reviewed in (42)) but understanding of this feature is still a work in progress. An attractive speculation is that these transporters might exert their final phenotypic influence in part through direct drug transport but also as a consequence of their activity to control membrane composition.

Transport of phospholipids across the yeast plasma membrane has been extensively studied (reviewed in (25)). Outward movement or flop of phospholipids has been linked to the function of two ABC transporters that are also involved in drug resistance: Pdr5 and Yor1 (10). Interestingly, Yor1 is the only ABCC type ABC transporter in that is found in the yeast plasma membrane (18). All other members of this class of transporters are represented by the 4 vacuolar ABC transporters. Yor1 (along with Pdr5) is required for movement of

phospholipids out of the cytoplasm which is the same direction we propose Ybt1 transports PC. In the case of Ybt1, PC is localized inside the lumen of the vacuole. We suspect that the other vacuolar ABC transporters may also participate in lipid trafficking, perhaps by regulating the uptake of other phospholipids into the vacuole.

Previous studies on trafficking of NBD-PC provided clear evidence that Vps27 function is required for delivery of this PC analogue into the vacuolar lumen (43). Since Vps27 also plays important roles in trafficking of membrane proteins to the vacuole (34), we examined if the NBD-PC trafficking defect of cells lacking this protein might be due to compromised Ybt1. The data reported here argue that Ybt1 has a selective defect in transport of NBD-PC into the vacuolar lumen but other substrates of this transporter (like nickel) are unaffected in *vps27Δ* cells. From this result, we suggest that Vps27 is required to deliver PC and its analogues in an appropriate form to support Ybt1-dependent translocation into the vacuole. No difference could be detected in delivery of Ybt1 to the vacuolar limiting membrane in comparison of wild-type and *vps27Δ* strains (Figure 6) indicating that Ybt1 traffic is normal even in this strong vacuolar mutant background. We conclude that loss of Vps27 was unlikely to globally interfere with Ybt1 action and was restricted to phospholipid transport.

A simple model for vacuolar accumulation of NBD-PC comes from the role of Vps27 in formation of multivesicular bodies (43). As vesicles and their cargo arrive at the late endosome, Vps27 participates in a process through which material destined for delivery into the lumen of the vacuole is sorted from other components. A straightforward route for delivery of NBD-PC into the vacuolar lumen would be to passively follow membranes into the intralumenal vesicles forming in the late endosome. These intralumenal vesicles enter the vacuolar lumen upon fusion with the vacuole. This mechanism is unlikely to explain luminal accumulation of NBD-PC as even in cells with wild-type Vps27 and no apparent defects in overall protein traffic to the vacuole, loss of Ybt1 prevents NBD-PC uptake. We suggest that NBD-PC is sorted in a Vps27-dependent fashion allowing this phospholipid derivative to be transported by Ybt1 when NBD-PC reaches the limiting membrane of the vacuole. Our findings are most consistent with a model in which Vps27 (and other class E Vps proteins) are necessary but not sufficient for accumulation of NBD-PC into the vacuolar lumen.

While our data have provided an explanation for vacuolar accumulation of PC, important questions still remain concerning remodeling of these phospholipids. Ybt1 provide the route of entry of PC analogues but the enzyme(s) responsible for either release of the choline headgroup or reconstruction of appropriate acyl chains have yet to be determined. One candidate gene that was examined was the phospholipase B homologue Plb3. Use of a GFP fusion provided evidence that this protein is localized to the vacuolar lumen (44). We constructed a *plb3Δ* disruption mutant but could find no effect on recycling of PC analogues (data not shown), suggesting that either this phospholipase is not involved in this process or that redundant enzymes exist. Finally, the protein(s) involved in extracting the remodeled PC/choline from the vacuolar lumen must be identified.

Our finding of the growth defect caused by loss of *YBT1* from the *pem1Δ pem2Δ ale1Δ nte1Δ* strain was critical in establishing that Ybt1-dependent uptake of NBD-PC into the vacuolar lumen represents a physiologically relevant feature of phospholipid metabolism. Other members of the ABCC class of ABC transporters, such as multidrug resistance protein (Mrp1), are known to act as broad specificity drug transporters (45, 46). This raises the concern that the action of Ybt1 on NBD-PC might simply be an illustration of its ability to sequester drugs into the vacuole, much as Mrp1 can efflux drugs out of mammalian cells. The requirement of Ybt1 for vacuolar reutilization of naturally occurring PC analogues like lyso-PC, coupled with Ybt1 stabilization in choline-limited cells, supports the belief that this

function of Ybt1 reflects a fundamental role for this ABC transporter in phospholipid homeostasis.

Control of the distribution of derivatized phospholipids across organellar membranes has previously been described in two different human diseases: respiratory distress syndrome and Stargardt's disease. RDS occurs upon loss of an ABC transporter normally found in the limiting membrane of lamellar bodies in alveolar cells in the lung (47). This ABC transporter, called ABCA3, is involved in exporting surfactant which contains large amounts of dipalmitoyl-PC into airspaces of the lung (48). Stargardt's disease is caused by defects in the ABCA4 gene (49). Loss of this ABC transporter protein causes the accumulation of a PE derivative in the luminal leaflet of the disc membrane in eye photoreceptor cells (50). Normally, this PE-based compound is flipped into the cytoplasmic leaflet out of the lumen of the disc. Interestingly, this activity of ABCA4 is in the opposite direction (into the cytoplasm) than described for either ABCA3 or Ybt1. Coordination of the activities of these organellar ABC transporters with their plasma membrane counterparts is critical for establishing normal physiology. Ybt1 and the other vacuolar ABC transporters provide an important model system for understanding how eukaryotic cells regulate phospholipids and their derivatives.

Material and Methods

Yeast Strains and Media

Yeast strains were routinely grown in rich YPD liquid media containing 2% yeast extract, 1% peptone, and 2% glucose for nonselective conditions or CSM media lacking desired amino acids (51). The choline auxotrophic strains were grown in presence of 1 mM choline, unless otherwise indicated. The drug resistance assays were performed on solid agar plates containing indicated concentration of nickel sulfate. Yeast transformation was performed by using the lithium acetate technique (52).

Strain Construction

Yeast strains are listed in Table 1. Generation of strains carrying single deletions of vacuolar ABC transporters in SEY6210 was accomplished by transformation with PCR disruption cassettes constructed as follows: *YBT1* disruption cassettes with KanMX6 and His3MX6 markers were amplified from plasmids pFA6a-KanMX6 and pFA6a-His3MX6 (53) by using primers Ybt1 Del-For and Ybt1-Del-Rev to generate KGS69 and KGS70. For disruption of *VMR1, vmr1::KanMX4* and *vmr1::TRP1* cassettes were amplified from plasmids pFA6a-KanMX6 and pFA6a-TRP1 by using primers Vmr1 Del-For and Vmr1 Del-Rev to generate KGS72 and KGS73. The *bpt1::KanMX4* deletion cassette was amplified by using primers Bpt1-Del-For and Bpt1-Del-Rev and transformed in SEY6210 to generate KGS71. The *ybt1::His3MX6, ale1::hphMX4* and *nte1::HIS3MX6* deletion cassettes were transformed in SEY6210 *pem1::natR-MX4 Δpem2Δ::KanMX4* strain to generate KGS75, KGS80 and KGS78 respectively. To generate the single *nte1Δ* deletion strain, *nte1::HIS3MX6* deletion cassettes were transformed in SEY6210 to generate KGS77. Construction of the quadruple knock out strain was accomplished by using the SEY6210 *pem1::natR-MX4 Δpem2Δ::KanMX4* strain and then sequentially transforming with deletion cassettes *ale1Δ::hphMX4* and *nte1Δ::HIS3MX6* to generate KGS79. The strain KGS79 was then transformed with a *ybt1Δ::URA3* deletion cassette yielding KGS81. The strain KGS79 was marker swapped by changing *nte1Δ::HIS3MX6* to *nte1Δ::TRP1* to generate KGS84. The *YBT1* C-terminal tandem affinity purification (TAP) tag fusion construct *YBT1*-TAP was chromosomally integrated in BY4742 *vps27Δ::KanMX4*, SEY6210 *pem1Δ::natR-MX4 pem2Δ::KanMX4, and* KGS84 strains yielding KGS76, KGS83 and KGS85 respectively.

In vitro NBD-PC translocation assay

The WT and *ybt1Δ* null mutant strains were grown in YPD containing 500 μM monochlorobimane (MCB) for 6 hrs. MCB is imported into the vacuolar lumen (20) and its accumulation inside the vacuoles was observed under fluorescence microscope in intact cells. Vacuoles were prepared from the MCB loaded cells using a Ficoll density gradient as described earlier (13) and kept at 4°C until initiation of the assay. The NBD-PC uptake assay was performed on freshly prepared vacuolar membranes at 30°C in TS buffer (250 mM sucrose, 25 mM Tris-MES, pH 8.0) containing either no ATP or 5 mM ATP, 10 mM creatine phosphate, 20 units/ml creatine kinase, 2.5 μM NBD-PC and 50 μM FM4-64 dye. The uptake reaction was initiated by addition of isolated vacuoles (25 μg of protein) and allowed to proceed. Time points were typically taken every 30 minutes. Vacuoles were then washed twice by ice-cold TS buffer containing 3% fatty acid free BSA. Washed vacuoles were then visualized under fluorescence microscope. Intact vacuoles were selected on the basis of MCB fluorescence and FM4-64 labeling. Only vacuoles with an intact structure as judged by positive luminal staining for MCB and limiting membrane staining with FM4-64 were used for observing NBD-PC accumulation.

Cycloheximide Chase

Wild-type and *pem1Δ pem2Δ ale1Δ nte1Δ* null mutant strains carrying chromosomally integrated C-terminal *YBT1-TAP* tagged alleles were grown in rich media until saturation. These cells were then extensively washed with water and transferred to fresh SD media without choline and allowed to grow for 12 hrs to deplete the intracellular pools of PC. These cultures were then shifted to limiting choline conditions (0.1 mM choline) and again grown to saturation. Cells from these cultures were then diluted to an OD 600_{nm} of 0.1 in fresh media containing 0.05 mM choline and allowed to reach OD 600_{nm} of 0.8. Equal samples from these cultures were then placed in SD media containing or lacking choline and allowed to grow for 2 hrs before addition of 100 μg/ml cycloheximide. Samples were collected at indicated time points, protein extracts were made and subjected to western analysis using anti-TAP and anti-Kar2 antibodies.

Growth Assays

The strains carrying indicated genomic deletion backgrounds were first grown in YPD media overnight. These cells were then extensively washed with water and transferred to fresh SD media without choline and allowed to grow for 12 hrs to deplete the intracellular pools of PC. The cultures were reinoculated in fresh media containing no choline, 1mM choline or 0.2 mM lyso-PC to an OD_{600nm} of 0.2. The growth rates of these strains were measured by taking OD_{600nm} at different time-periods over 27 hrs. Averaged OD_{600nm} values from four independent experiments were plotted against the indicated time period.

NBD-PC transport assay

To label cells with NBD-PC, cultures were grown to early log phase in SD medium. Cells were incubated with NBD-PC solubilized in DMSO to a final concentration of 10 μM for 25 min at 30°C. Cells were harvested and washed twice with SD media at room temperature. For visualization of nuclear and mitochondrial DNA, cells were resuspended in 2 ml of SD media and 2 μl of a 1 mg/ml stock of DAPI (4′,6-diamidino-2-phenylindole) was added. Cells were incubated at 30°C for 15 min, washed twice with ice-cold SC+NaN_3 and visualized under fluorescence microscope.

Fluorescence Microscopy

Yeast strains carrying GFP-tagged proteins were grown to early log phase, washed twice with water and analyzed for GFP fluorescence. For FM4-64 labeling, cells were incubated

with FM4-64 for 20 min or the indicated time period, washed twice with dye free media and then analyzed by fluorescence microscopy. Isolated vacuoles were visualized at 2.5X aperture settings. Fluorescent images were taken by placing a few μl of a culture or isolated vacuoles on a glass slide, overlaying with a cover slip, and visualizing under the 100X oil objective of an Olympus BX60 fluorescence microscope.

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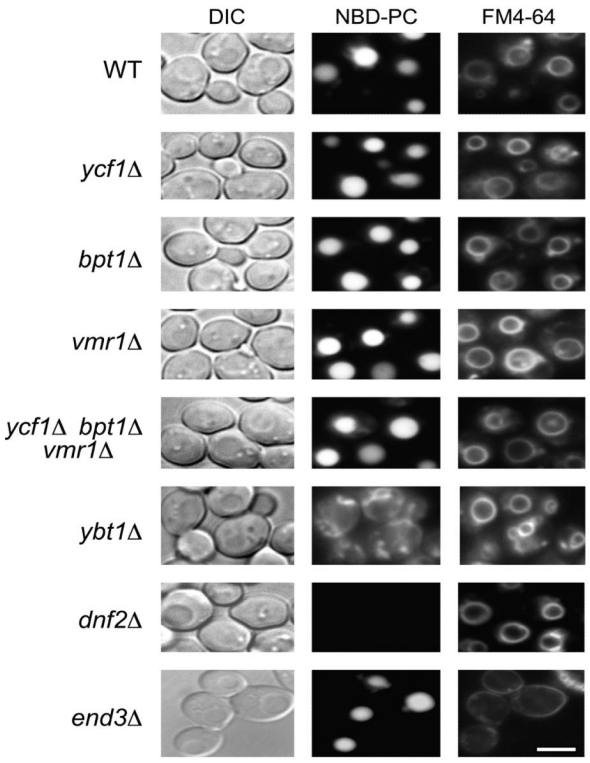

Figure 1. ***In vivo*** **NBD-PC transport analysis**

Wild-type and the indicated deletion mutant strains were grown to early log phase. Cells were incubated with 10 μM NBD-PC in DMSO for 40 minutes at 30°C. During the last 25 minutes, FM4-64 dye was added. Cells were then washed twice with ice-cold minimal medium containing NaN_3 and visualized by fluoresce visualized by Nomarski optics (DIC) and fluorescence microscopy. Scale bar represents 5 μm.

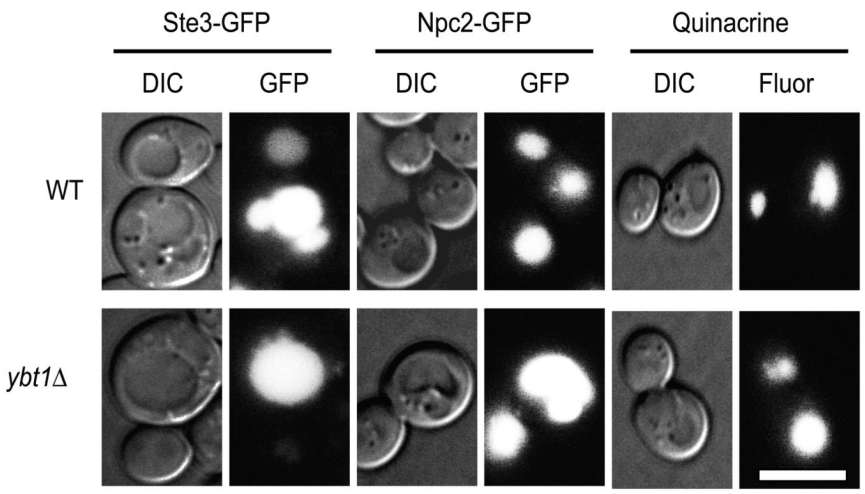

Figure 2. Loss of Ybt1 does not cause pleiotropic vacuolar defects

Wild-type and isogenic *ybt1Δ* cells were transformed with low-copy-number plasmids expressing Ste3- or Npc2-green fluorescent protein (GFP) fusion proteins. Transformants were grown to early log phase and visualized by fluorescence microscopy. Maintenance of the vacuolar pH gradient was ascertained by uptake of the fluorescent dye quinacrine. Cells grown to early log phase were resuspended in 500 μl YPD medium buffered with 50 mM Na_2HPO_4 (pH 7.6) containing 200 μM quinacrine. After a 5 minute incubation at room temperature, cells were pelleted and resuspended in 50 μl of 2% glucose buffered with 50 mM Na_2HPO_4 (pH 7.6). Labeled cells were immediately visualized by fluorescence microscopy (Fluor). Scale bar represents 5 μm.

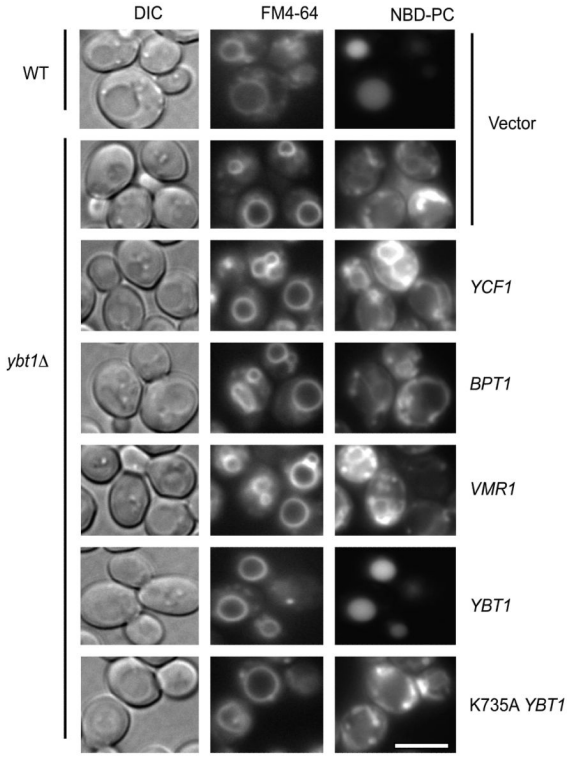

Figure 3. Specific complementation of NBD-PC transport defect by Ybt1

A *ybt1Δ* strain was transformed with empty high-copy-number plasmid pRS426 (Vector) or this same plasmid containing a wild-type copy of the vacuolar ABC transporter-encoding gene indicated. A site-directed mutant form of YBT1 lacking a key amino acid in the amino-terminal nucleotide binding domain (K735M) was also expressed using the same vector plasmid. Transformants were grown to mid-log phase, stained with FM4-64 and NBD-PC. After incubation with these dye probes, cells were photographed using Nomarski optics and fluorescence microscopy. Scale bar represents 5 μm.

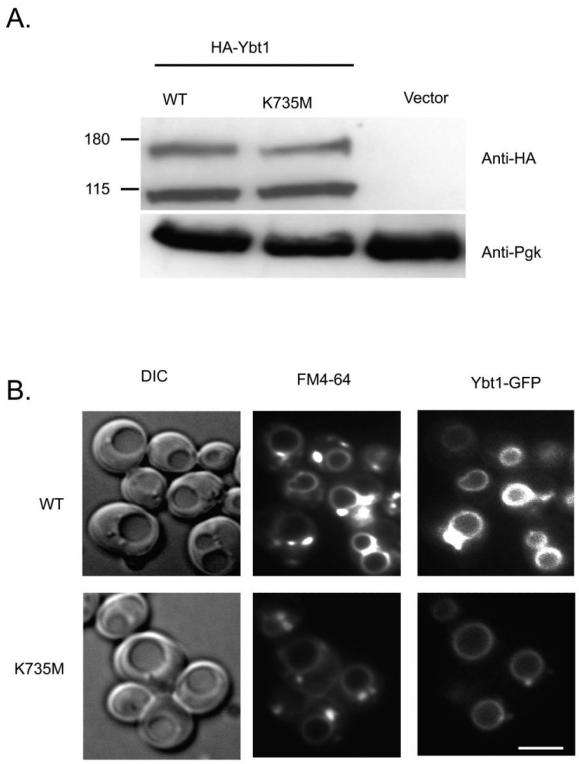

Figure 4. K735M Ybt1 exhibits wild-type expression and localization

(A) A *ybt1Δ* null strain was transformed with either empty high-copy-number plasmid pRS426 (Vector) or pRS426 carrying either the wild-type *YBT1* gene or the K735M variant of *YBT1*. The K735M mutant form of *YBT1* is located in nucleotide binding domain 1 and was generated by converting a lysine codon (AAG encoding residue 735) to methionine (ATG) by site directed mutagenesis. Protein extracts were made from these transformants after growing them to early log phase. Equal amounts of proteins were resolved on 10% SDS-PAGE gel and then probed with either anti-HA or anti-Pgk1 antibodies. Molecular mass standards are indicated on the left hand side in kD. (B) A *ybt1Δ* null strain was transformed with the pRS426 plasmid carrying C-terminal GFP fusions to either wild-type or the K735M *YBT1*. Transformants were grown to early log phase and incubated with FM4-64 dye for 25 minutes. These cells were then washed twice with fresh media lacking FM4-64 and visualized under fluorescence microscope. Scale bar represents 5 μm.

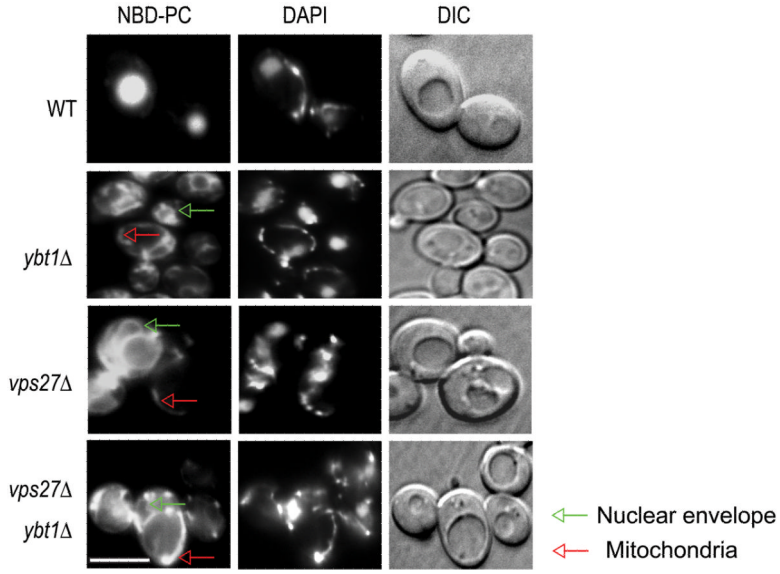

Figure 5. NBD-PC accumulation assay in *ybt1?* null and vps27? null strains

An isogenic series of strains corresponding to the wild-type, *ybt1?*, vps27? or the *ybt1?* vps27? double mutant were grown to early log phase in CSM. Cells were then labeled with DAPI for 20 minutes prior to incubation with 10 μM DMSO-solubilized NBD-PC for 40 minutes. Cells were then harvested and washed three times with ice-cold SCNaN3 and subjected to analysis and imaging by fluorescence microscopy. Green arrows indicate representative staining of the nuclear envelope while the red arrows denote mitochondrial fluorescence. DAPI was used to stain nuclear and organellar DNA. Scale bar represents 5 μm.

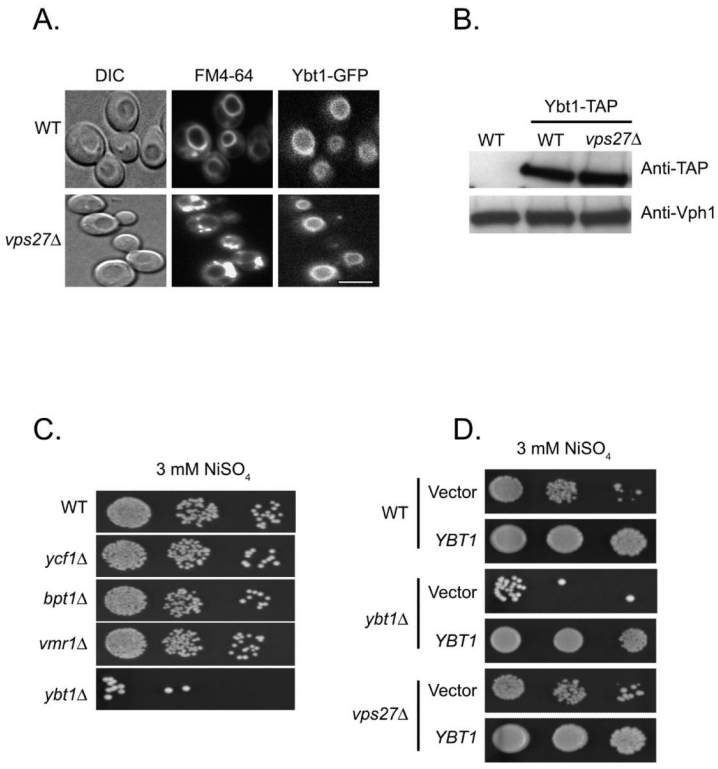

Figure 6. Ybt1 exhibits normal localization, expression and metal resistance in a *vps27?* null mutant

(A) The wild-type strain along with isogenic single *vps27?* null mutant carrying chromosomally integrated C-terminal *YBT1*- GFP tagged construct were grown to early log phase. The cells were incubated with FM4-64 for 30 min and then washed twice with water. Each strain was visualized under fluorescence microscope to observe Ybt1p-GFP and FM4-64. Scale bar represents 5 μm. (B) Protein extracts were prepared from isogenic wild-type strains and a *vps27?* null mutant carrying a chromosomally integrated C- terminal *YBT1*-TAP tagged construct. Equal amounts of protein from each sample was resolved on SDS-PAGE and subjected to western analysis using anti- TAP antibodies. The vacuolar ATPase subunit Vph1 was immunoblotted using anti-Vph1 antibodies as a loading control. (C) An isogenic series of strains of the indicated relevant genotype denoted on the left hand side of the panel were grown to mid-log phase. Serial dilutions of these cultures were spotted on YPD medium containing 3 mM of nickel sulfate. The plates were incubated at 30°C for two days and photographed. (D) The isogenic series of WT, *ybt1?* and *vps27?* null mutant strains carrying either high-copy-number plasmid, pRS426 (Vector), or the same plasmid containing the wild-type *YBT1* gene were grown to early log phase. Serial dilutions of these cultures were spotted on YPD medium containing 3 mM of nickel sulfate. The plates were incubated at 30°C two days and photographed.

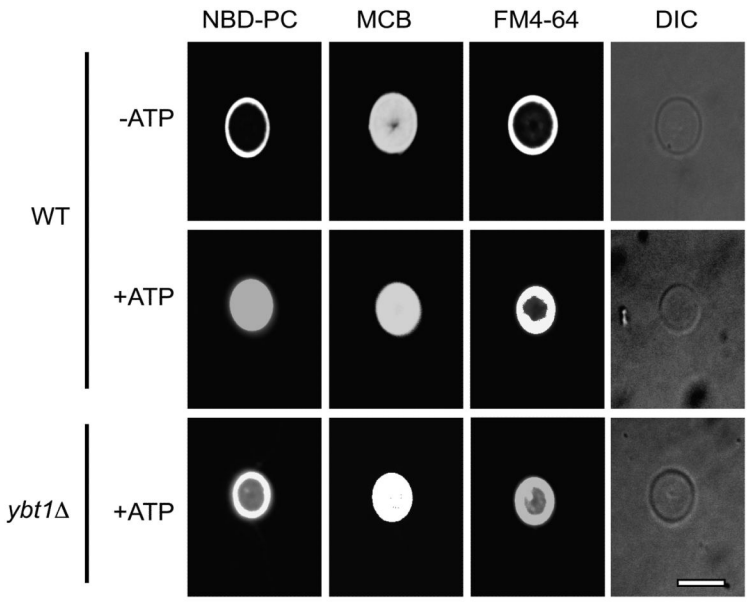

Figure 7. *In vitro* NBD-PC transport analysis

Wild type and *ybt1?* strains were grown in YPD medium containing 500 μM monochlorobimane (MCB) for 6 hours. Vacuoles were then isolated using a Ficoll density gradient. Isolated vacuoles (20 μg protein) were then incubated at 30°C in buffer containing 10 μM NBD-PC and 50 μM FM4-64. ATP was either included (+ATP) or omitted as a control (−ATP). Incorporation was allowed to proceed for 30 minutes, followed by washing in ice-cold TS buffer containing 3% fatty acid-free BSA. Washed vacuoles were then inspected by fluorescence microscopy. Vacuole integrity was evaluated by retention of MCB fluorescence and FM4-64 staining. 15-18% of isolated vacuoles from wild-type cells were positive for NBD-PC accumulation in the presence of ATP. No vacuoles were seen to accumulate NBD-PC in wild-type vacuoles in the absence of ATP or in the vacuoles isolated from *ybt1?* strain. The number of isolated vacuoles from wild-type and *ybt1?* strains found to be positive for both MCB fluorescence and FM4-64 staining were comparable. Scale bar represents 2.5 μm.

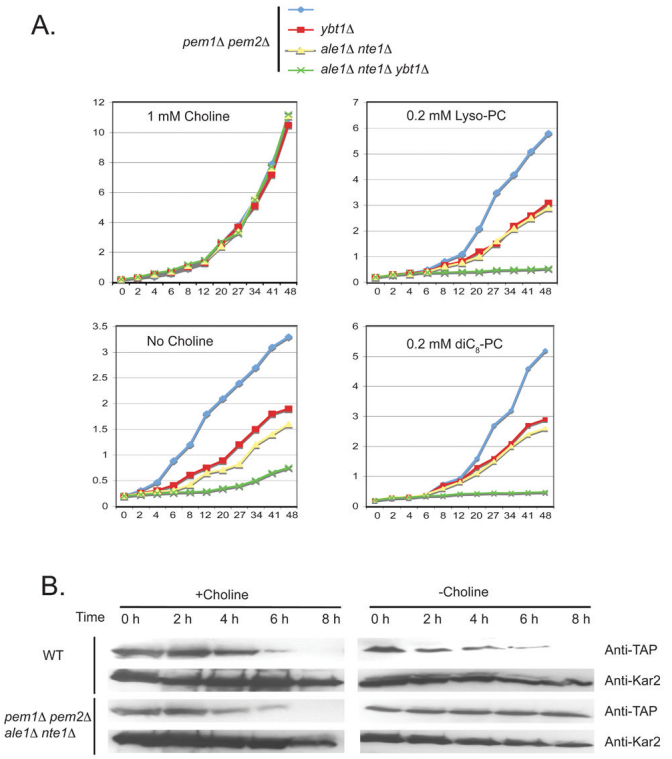

Figure 8. Integration of Ybt1 into PC metabolism

(A) Ybt1 is required for wild-type response to conditions of PC limitation. Isogenic *pem1? pem2?* strains lacking the indicated additional genes were diluted into minimal medium lacking choline and allowed to grow for 12 hours to partially deplete intracellular PC pools. Aliquots of these cultures were then inoculated into minimal medium containing the indicated supplements and incubated at 30°C with shaking. A_{600} measurements were performed to follow the growth pattern of these cells over time for a period of 48 hours. (B) Ybt1 is stabilized upon choline limitation. Wild-type or *pem1? pem2? ale1? nte1?* cells containing an integrated *YBT1-TAP* allele were grown to saturation in the presence of 0.1 mM choline. Liquots of these cultures were then diluted into media containing or lacking 0.1 mM choline and grown at 30°C with shaking for two hours. Cycloheximde (100 μg/ml) was then added and samples of these cultures withdrawn at the indicated times. Whole cell protein extracts were prepared and analyzed by western blotting using antibodies directed against the TAP epitope or the ER luminal marker protein Kar2.

Table 1

Strain list.

Strain	Description	Source
SEY6210	*MATα leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 Mel⁻*	Scott Emr
BY4742	*MATα his3-Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Open Biosystems
YPH499	*MATα ade2-101 ura3-52 his-3Δ200, trp1-Δ63 leu2Δ lys2-801*	(54)
YPH499 *ybt1Δ*	*MATα ade2-101 ura3-52 his-3Δ200, trp1-Δ63 leu2Δ1 lys2-801 ybt1Δ::URA3*	AK Bachhawat
BY4742 *ybt1Δ*	*MATα his3-Δ1 leu2Δ0 lys2Δ0 ura3Δ0 ybt1Δ::KanMX2*	Open Biosystems
BY4742 *vps27Δ*	*MATα his3-Δ1 leu2Δ0 lys2Δ0 ura3Δ0 vps27Δ::KanMX2*	Open Biosystems
BY4742 *dnf2Δ*	*MATα his3-Δ1 leu2Δ0 lys2Δ0 ura3Δ0 dnf2Δ::KanMX2*	Open Biosystems
BY4742 *end3Δ*	*MATα his3-Δ1 leu2Δ0 lys2Δ0 ura3Δ0 end3Δ::KanMX2*	Open Biosystems
KGS28	*MATα leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 Mel⁻ pem1Δ::natR-MX4 pem2Δ::kanMX2*	(55)
KGS52	*MATα leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 Mel⁻ ycf1Δ::kanMX2*	(55)
KGS53	*MATα leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 Mel⁻ ycf1Δ::natR-MX4 bpt1Δ::kanMX2*	(55)
YBT1-GFP	*MATα his3-Δ1 leu2Δ0 lys2Δ0 ura3Δ0 YBT1-GFP::HIS3MX6*	Invitrogen
YBT1-TAP	*MATα his3-Δ1 leu2Δ0 lys2Δ0 ura3Δ0 YBT1-TAP::HIS3MX6*	Open Biosystems
KGS69	*MATα leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 Mel⁻ ybt1Δ::KanMX2*	This study
KGS70	*MATα leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 Mel⁻ ybt1Δ::HIS3MX6*	This study
KGS71	*MATα leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 Mel⁻ bpt1Δ::KanMX2*	This study
KGS72	*MATα leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 Mel⁻ vmr1Δ::KanMX2*	This study
KGS73	*MATα leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 Mel⁻ ycf1Δ::natR-MX4 bpt1Δ::kanMX2 vmr1Δ::TRP1*	This study
KGS74	*MATα his3-Δ1 leu2Δ0 lys2Δ0 ura3Δ0 vps27Δ::KanMX2 YBT1-GFP::HIS3MX6*	This study
KGS75	*MATα leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 Mel⁻ pem1Δ:: natR-MX4 pem2Δ::kanMX2 ybt1Δ::HIS3MX6*	This study
KGS76	*MATα his3-Δ1 leu2Δ0 lys2Δ0 ura3Δ0 vps27Δ::KanMX2 YBT1-TAP::HIS3MX6*	This study
KGS77	*MATα leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 nte1Δ::HIS3MX6*	This study
KGS78	*MATα leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 Mel⁻ pem1Δ:: natR-MX4 pem2Δ::kanMX2 nte1Δ::HIS3MX6*	This study

Strain	Description	Source
KGS79	*MATα leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 Mel⁻ pem1Δ:: natR-MX4 pem2Δ::kanMX2 ale1::hphMX4 nte1Δ::HIS3MX6*	This study
KGS80	*MATα leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 Lys2-801 Mel⁻ pem1Δ:: natR-MX4 pem2Δ::kanMX2 ale1::hphMX4*	This study
KGS81	*MATα leu2-3, 11 2ura3-52 his3-Δ200 trp1-Δ901 lys2-801 Mel⁻ pem1Δ:: natR-MX4 pem2Δ::kanMX2 ybt1Δ::URA3 nte1Δ::HIS3MX6 ale1::hphMX4*	This study
KGS82	*MATα leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 Lys2-801 Mel⁻ YBT1-TAP::HIS3MX6*	This study
KGS83	*MATα leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 Mel⁻ pem1Δ:: natR-MX4 pem2Δ::kanMX2 YBT1-TAP::HIS3MX6*	This study
KGS84	*MATα leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 Mel⁻ pem1Δ:: natR-MX4 pem2Δ::kanMX2 ale1::hphMX4 nte1Δ::TRP1*	This study
KGS85	*MATα leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 Mel⁻ pem1Δ:: natR-MX4 pem2Δ::kanMX2 ale1::hphMX4 nte1Δ::TRP1 YBT1-TAP::HIS3MX6*	This study

Table 2

Plasmid list.

Name	Description	
pSR67	pRS426-HA-*YBT1*	This study
pSR69	pRS426-HA-*BPT1*	This study
pSR91	pRS426-HA-*VMR1*	This study
pKGE168	pRS426-*YCF1*	This study
pKGE237	pRS426-HA-K735M-*YBT1*	This study
pKGE238	pRS426-HA-*YBT1*-GFP	This study
pKGE239	pRS426-HA-K735M-*YBT1*-GFP	This study