Yeast phosphatidylinositol transfer protein Pdr17 does not require high affinity phosphatidylinositol binding for its cellular function.

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Highlights:

- Mutated PI transfer protein Pdr17$^{E237A, K269A}$ is defective in high affinity PI binding
- Pdr17p binds a sterol in addition to PI in an assay using permeabilized cells
- PI binding defective Pdr17$^{E237A, K269A}$ retains the ability to transfer PI *in vitro*
- PI binding is not required for Pdr17p function in inter-membrane transfer of PS

ABSTRACT

Yeast phosphatidylinositol transfer protein (PITP) Pdr17 is an essential component of the complex required for decarboxylation of phosphatidylserine (PS) to phosphatidylethanolamine (PE) at a non-mitochondrial location. According to current understanding, this process involves the transfer of PS from the endoplasmic reticulum to the Golgi/endosomes. We generated a Pdr17$^{E237A, K269A}$ mutant protein to better understand the mechanism by which Pdr17p participates in the processes connected to the decarboxylation of PS to PE. We show that the Pdr17$^{E237A, K269A}$ mutant protein is not capable of binding phosphatidylinositol (PI) using permeabilized human cells, but still retains the ability to transfer PI between two membrane compartments *in vitro*. We provide data together with molecular models showing that the mutations E237A and K269A changed only the lipid binding cavity of Pdr17p and not its surface properties. In contrast to Pdr16p, a close homologue, the ability of Pdr17p to bind PI is not required for its major cellular function in the inter-membrane transfer of PS. We hypothesize that these two closely related yeast PITPs, Pdr16p and Pdr17p, have evolved from a common ancestor. Pdr16p fulfills those role(s) in which the ability to bind and transfer PI is required, while Pdr17p appears to have adapted to a different role which does not require the high affinity binding of PI, although the protein retains the capacity to transfer PI. Our results indicate that PITPs function in complex ways *in vivo* and underscore the need to consider multiple PITP parameters when studying these proteins *in vitro*.

ABBREVIATIONS:

PI, Phosphatidylinositol; PC, Phosphatidylcholine; LPA, Lysophosphatidic acid; LPC, Lysophosphatidylcholine; PI(4)P, Phosphatidylinositol (4) phosphate; PI(3)P, Phosphatidylinositol (3) phosphate; PI(5)P, Phosphatidylinositol (5) phosphate; PE, Phosphatidylethanolamine; S1P, Sphingosine 1-Phosphate; PI(3,4)P_2, Phosphatidylinositol (3,4) bisphosphate; PI(3,5)P_2, Phosphatidylinositol (3,5) bisphosphate; PI(4,5)P_2, Phosphatidylinositol (4,5) bisphosphate; PI(3,4,5)P_3, Phosphatidylinositol (3,4,5) trisphosphate; PA, Phosphatidic acid; PS, Phosphatidylserine; GT, Glyceryl tripalmitate; DAG, Diacylglycerol; PG, Phosphatidylglycerol; CL, Cardiolipin; CHOL, cholesterol; PITP, phosphatidylinositol transfer protein; HL60, human promyelocytic leukemia cells; Etn, Ethanolamine; Etn-P, Ethanolamine phosphate; CDP-Etn, Cytidine diphosphate ethanolamine; ER, Endoplasmic reticulum

1. INTRODUCTION

Lipid transfer proteins (LTPs) were originally characterized by their ability to transfer lipid molecules between two membrane compartments in cell-free experiments [1]. LTPs are increasingly recognized to shuttle lipids between membrane compartments in cells, or act as lipid sensing or lipid presenting proteins, which could lead to signaling or to lipid modification, respectively [2-3]. Many LTPs operate at membrane contact sites (MCS) where cellular membranes come close together (10-30 nm apart) to facilitate the flow of material and information, including lipids [4-8]. Despite enormous progress in recent years, the mechanism of operation of many LTPs at membrane contact sites is still unknown or at least uncertain.

Twenty three protein families capable of trafficking bilayer lipids have been identified so far [3]. Among them is the Sec14 (CRAL/TRIO) family of LTPs [9]. Members of this family typically bind and transfer one or two of the following ligands: PI, PC, or sterol.They also transfer small non-bilayer forming hydrophobic molecules such as tocopherol, retinaldehyde etc. [10]. CRAL/TRIO domain proteins contain a hydrophobic pocket that can accommodate hydrophobic ligands, thereby shielding them from the aqueos environment *en route* between two membranes (crystal structures of some yeast CRAL/TRIO family members' crystal structures have been published [11-14]). In the yeast *Saccharomyces cerevisiae*, nine proteins containing the CRAL/TRIO domain have been described [3], six of which belong to the Sec14 group of phosphatidylinositol transfer proteins (PITPs) [15-18]. All members of the yeast Sec14 group transfer PI *in vitro*; only Sec14p is also capable of transferring PC [15]. One of these proteins, Pdr17p (also known as Sfh4p, Iss1p, and PstB2p) is required for the transfer of phosphatidylserine (PS) from the ER to the membrane, where decarboxylation of PS to PE by phosphatidylserine decarboxylase 2 (Psd2p) takes place [19-21]. In the current model, PS is decarboxylated to PE by Psd2p in the Golgi/endosomes by a multi-subunit complex consisting of Psd2p itself, the phosphatidylinositol transfer protein Pdr17, the PSTB2 interacting protein Pbi1p, an integral ER membrane protein Scs2, and the phosphatidyinositol-4-kinase Stt4p. Genetic and biochemical studies identified Psd2p, Pdr17p and Stt4p as essential components of this complex with Pbi1p and Scs2p serving as interacting and tethering partners [19, 22]. While many details of this process are known, the basic question, what is the molecular mechanism by which PS is transported between the two membranes, remains unanswered. All the available evidence shows that Pdr17p does not act as a soluble carrier of PS and does not bind or transfer PS *in vitro* [20, 23]. It has been proposed that at least some PITPs may serve as PI presenting proteins for regulating the outcome of inositol lipid kinases rather than for transferring lipids between membranes *in vivo* [24]. Phosphatidylinositol transfer protein Pdr17p and PI-4-kinase Stt4p seem to be ideally suited for this. Both are present in the same complex, both are essential for the non-mitochondrial decarboxylation of PS to PE and the requirement for Pdr17p is highly specific: none of the other yeast PITPs can substitute for Pdr17p in this process [25].

In the course of this work we studied the role of the yeast PITP Pdr17p in the process of PS to PE conversion at the non-mitochondrial location. Transfer of PS to the endosomes/Golgi where PS decarboxylation takes place is a process which allows the role of a lipid transfer protein to be effectively studied. First, Pdr17p is an essential component of this

process and second, the inter-membrane transfer of PS can be easily monitored by the *de novo* production of non-mitochondrial PE. We generated a mutant version of Pdr17p unable to bind PI. We show that wild-type Pdr17p and this PI-binding deficient Pdr17p mutant can both still bind also a sterol molecule in lipid binding experiments. Surprisingly, the Pdr17p mutant unable to bind PI is indistinguishable from wild-type Pdr17p in supporting non-mitochondrial decarboxylation of PS to PE. This provides evidence that the high affinity PI binding of Pdr17p does not play a role in its major cellular function. Examination of its PI transfer activity demonstrated that it retains significant transfer activity despite the loss of its high affinity PI binding.

2. MATERIALS AND METHODS

2.1 Media and chemicals

Media components were obtained from Becton-Dickinson (USA) or BioLife (Italy). Miconazole was from MP Biomedicals (USA), [1-^{14}C] acetic acid was purchased from American Radiolabeled Chemicals (UK), [^{3}H] serine was from Hartmann Analytic (Germany). Fine chemicals were mostly from Sigma-Aldrich (USA) or MP Biomedicals (USA).

2.2 Strains and culture conditions

S. cerevisiae psd1::TRP1, pdr17::HIS3 strain WWY62 (Mat a**,** *lys2, ura3, his3, leu2, trp1, met, psd1::TRP1, pdr17::HIS3*) was kindly provided by D. Voelker (National Jewish Health, Denver, CO, USA) [26]. The yeast strain with temperature sensitive allele of *SEC14*, *sec14-1ts* (Mat α, *leu2, trp1, lys2, ura3, his3, sec14-1^{ts}*), is a spore from genetic cross and sporulation of CTY1-1A (donated by V. Bankaitis, Texas A&M College of Medicine, College Station, TX, USA) [27] and PGY59 (Mat α, *his3, leu2, trp1, ura3*). Wild type *S. cerevisiae* strain FY1679-28c and from its derived *pdr16Δ* strain (Mat a, *ura3-52, leu2Δ1, his3Δ200, trp1Δ63, GAL2, pdr16::hisG*) originally from A. Goffeau laboratory (Catholic University Louvain, Belgium) [28] were kindly provided by G. Daum (Technical University, Graz, Austria).

An episomal plasmid containing *PDR17* under its own promoter was constructed based on the 2 μm plasmid YEplac181 [29]. The details of its construction are given in [17]. To create a *PDR17* allele encoding a protein defective in PI binding, part of the wild type allele of *PDR17* was replaced in the plasmid YEplac181-*PDR17* by a custom synthetized *PDR17*(*E237A, K269A*) DNA String (Thermo Scientific; Supplementary material) using *Sex*AI and *Sac*I restriction enzymes. Centromeric plasmids containing wild type *PDR17* or the PI-binding deficient mutant allele of *PDR17* were constructed by subcloning *PDR17* or *PDR17*(*E237A, K269A*) from episomal YEplac181-based plasmids into a centromeric YCplac111 plasmid [29]. To create plasmids for recombinant protein production in *E. coli*, ORFs *PDR17* and *PDR17* (*E237A, K269A*) were amplified from plasmids YEplac181-*PDR17* and YEplac181-*PDR17* (*E237A, K269A*) using the following primers: 5`-

GTACCATATGGGTCTTTTTTCAAGAAAACGG -3` and 5`-GACGTCTCGAGGCGGCCGCAATGACCATATCAACAGGATAT -3`; *Nde*I and *Not*I sites are underlined. Amplified ORFs were cut with *Nde*I and *Not*I restriction enzymes and inserted into the corresponding sites of a pET26b(+) vector (Merck, USA) to create plasmids pET26-*PDR17* and pET26-*PDR17*(*E237A*, *K269A*). All constructs containing PCR amplified DNA fragments were verified by DNA sequencing. *Escherichia coli* Rosetta 2 (DE3) strain (F^- omp^T $hsdS_B$ (r_B^- m_B^-) *gal dcm* pRARE2 (Cam^R) was from Merck.

Yeast strains were grown on yeast extract/peptone/dextrose (YEPD; 2% glucose) media unless otherwise stated. Yeast strains containing episomal plasmids were maintained and pre-grown on standard synthetic minimal medium (0.67% YNB without amino acids, 2% glucose) supplemented with essential amino acids and nucleotide bases as required for plasmid maintenance.

2.3 Growth complementation testing

Yeast cultures were pre-grown overnight in YNB-LEU media, diluted and spotted as 10-fold dilutions onto indicated solid media. Miconazole-containing plates were prepared as follows: miconazol was added to the growth media before plate pouring from 1000 × stock solution in DMSO to the final concentration 5 ng/ml. The growth was scored after 2-3 days of incubation at 28°C or 35°C, as indicated.

2.4 Protein expression and purification

C-terminally 6×His tagged Pdr17 and Sfh1 proteins were purified from *E. coli* Rosetta2 (Merck) cells transformed with plasmids pET26-*PDR17*, pET26-*PDR17*$^{E237A,\ K269A}$, and pET26-*SFH1*, respectively. Bacteria were grown at 37°C in 1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl, with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol and induced with 20 μM isopropylthio-β-D-galactoside for 18 h at 18°C. The cells were harvested and suspended in buffer A (25 mM Tris-HCl pH 8.0, 300 mM KCl, 10% glycerol, 0.5% Tween 20, 10 mM imidazole) for Pdr17p or in buffer B (25 mM Tris-HCl pH 7.5, 300 mM NaCl, 10% glycerol, 0.5% Tween 20, 10 mM imidazol) for Sfh1p with protease inhibitors (Roche) and sonicated on ice. The cell lysates were centrifuged 30 min at 15,000× g and the supernatants were loaded onto Ni-NTA agarose columns (Qiagen). The columns were washed with 5 column volumes of buffer A or B containing 40 mM imidazole and 0.1% Tween 20. The protein bound to the column was then eluted in 3 steps with buffer C (25 mM MES pH 6.0, 300 mM KCl, 10% glycerol and 0.05% Tween 20) or buffer D (25 mM Tris-HCl pH 7.5, 300 mM NaCl, 10% glycerol, 0.02% Tween 20) containing 0.1, 0.3 and 0.5 M imidazole. Elution fractions containing the Pdr17 and Sfh1 proteins were desalted from imidazole using a PD Midi Trap G-25 (GE Healthcare) and then gel filtered on a Superdex 75 10/300 GL column or a Superdex 200 Increase 10/300 GL column (GE Healthcare) equilibrated in buffer C or D, respectively. The Pdr17 protein was then purified on a Mono S HR 5/5 column (GE Healthcare) equilibrated in buffer E (25 mM MES pH 6.0, 10% glycerol and 0.05% Tween 20), by eluting in a linear gradient from 200 to 500 mM KCl. For the PI transfer assay Pdr17 protein buffer was exchanged using a PD Midi Trap G-25 (GE

Healthcare) to SET buffer (250 mM sucrose, 1 mM EDTA, 10 mM Tris-HCl pH 7.4). The purified proteins were analyzed by SDS-PAGE followed by Coomassie Brilliant Blue staining. Pdr16p was purified from *E. coli* Rosetta2 (Merck) transformed with plasmids pET26-*PDR16* and pET26-*PDR16*$^{E235A, K267A}$ according to [42].

2.5 Lipid-protein overlay assay

Echelon PIP 6001 and 6002 strips with lipids immobilized on nitrocellulose membranes were first incubated for 1 h in 3% (w/v) fatty acid-free BSA (Sigma-Aldrich) in TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% (v/v) Tween 20) to block non-specific interactions. The PIP 6001 and 6002 strips were then incubated overnight at 4^{o}C in 4 ml TBS buffer containing 0.1 μg/ml of recombinant purified Pdr17-6×His and Pdr17$^{E237A, K269A}$-6×His proteins, respectively. The membranes were then incubated for 2 h with a 1:1000 dilution of anti-His antibody (Qiagen) followed by a 2 h incubation with a 1:20000 dilution of secondary alkaline phosphatase conjugated anti-mouse IgG (Sigma-Aldrich). Repeated washing steps with TBS containing 0.05% Tween 20 were inserted between all incubation steps.

2.6 Binding of cellular lipids using HL60 cells

The interactions of eukaryotic cellular lipids with the yeast PITPs Pdr17, Pdr17$^{E237A, K269A}$, and as a control, Sfh1p, were analyzed as described previously [30-31]. Briefly, HL60 cells were labeled with 1 μCi/ml [^{14}C]acetate in RPMI 1640 medium for 48 h. The cells were permeabilized with streptolysin O, and the leaked cytosol was removed by centrifugation. Permeabilized cells (~10^7 cells in 500μl) were incubated with 120 μg of the respective recombinant protein in 100 μl volume for 20 min at 37 °C in the presence of 2 mM Mg^{2+}-ATP and 100 nM Ca^{2+} buffered with 3mM EGTA. Samples of the proteins were saved and run on 12% SDS-PAGE. At the end of the incubation, the cells were removed by centrifugation, and the recombinant proteins in the supernatant were captured on nickel beads. An aliquot of the recovered proteins was run on SDS-PAGE to assess their recovery, and the rest of the sample was used for lipid extraction. The lipids were resolved by thin layer chromatography using a Whatman silica gel 60 TLC plate using a chloroform/methanol/acetic acid/water (75:45:3:1, v/v) mobile phase. Lipids extracted from the permeabilized HL60 cells (approximately 100,000 dpm) were analyzed alongside for comparison. The TLC plates were exposed to Fuji phosphorimaging screens and analyzed using a Fuji BAS1000 phosphorimaging system. Both the SDS-PAGE and TLC images were analyzed using AIDA software.

2.7 PI transfer assay

PI transfer was monitored by measuring the transfer of radiolabeled PI from labeled rat liver microsomes to liposomes as described in [32]. The percentage of lipid transferred was calculated as a percentage of the counts transferred from the microsomes to the liposomes. 100% represents the total radioactivity in the assay.

2.8 Phospholipid extraction and analysis

S. cerevisiae strains were grown in YNB-LEU media to late logarithmic phase. Cells were disintegrated by glass beads using repeated vortexing with cooling on ice in between. Phospholipids were extracted by a modification of the method of Blight and Dyer [33]. Extracted lipids were spotted onto a Silica-gel 60 TLC plate (Merck, Germany) together with lipid standards. The chromatogram was run twice in chloroform/ethanol/H_2O/triethylamine (30:35:7:35, v/v). Phospholipids were visualized by iodine vapor staining and compared to standards under UV light. Phospholipid-containing spots were scraped off the TLC plate and quantified using the method of Broekhuyse [34]. Inorganic phosphate was used as standard. Proteins in the cell extracts were quantified by the method of Bradford [35].

3.9 [^{3}H] serine incorporation into aminoglycerophospholipids

Yeast aminoglycerophospholipids were labeled by [^{3}H] serine according to Achleitner *et al.* [23]. Briefly, *S. cerevisiae psd1::TRP1, pdr17::HIS3* WWY62 strains containing either the wild-type *PDR17* gene or a PI-binding deficient *PDR17* (*E237A, K269A*) mutant on the centromeric plasmid YCplac111were grown overnight in YEPD medium containing 2 mM ethanolamine. The cultures were washed with water and used to inoculate 20 ml of YNB-LEU media to a cell density of 2 x 10^6 cells/ml. When control cells containing an empty cloning plasmid were used, YNB-LEU medium containing 1 mM ethanolamine was used. Myriocin was added to a final concentration of 10 μg/ml when cells reached a density of 10^7 cells/ml (approximately 6 hours at 30^{o}C). 30 minutes later, 10 μCi of [^{3}H] serine was added to the cultures and incubated for another 30 min. After labeling, the cultures were cooled on ice, and washed twice with ice cold water. 2×10^9 unlabeled carrier cells were added to each culture, and lipids were extracted and analyzed by TLC as described in Section 2.8. The radioactivity associated with PS and PE was measured using a liquid scintillation counter. The percentage of PS converted to PE was calculated using the formula: percent PS converted to PE = 100 × {cpm [^{3}H]PE/(cpm [^{3}H]PS + cpm [^{3}H]PE)}[36].

3.10 Construction of homology models

The Pdr17 homology model was prepared using chain A of the 2.00 Å uncomplexed Pdr16 structure (PDB ID 4J7P, chain A) [13] using Modeller v9.17 [37]. A slow optimization protocol was used for model refinement and four loops (residues 23–40, 64–74, 202–220, and 330–340) were separately refined to reduce their local DOPE score [38]. The geometry of the final model was optimized against the AMBER99sb-ILDN force field [39] using steepest descent and conjugate gradient minimization as implemented in GROMACS 5.1.3. Two models were prepared for the Pdr17$^{E237A,\ K269A}$ mutant from the Pdr17 homology model: a simple one created by changing the two residues from lysine and glutamate to alanine and a slightly more sophisticated one using Modeller v9.17 and a fast optimization protocol with no additional loop remodeling. Electrostatic surfaces for all models were calculated using APBS [40] and visualized using PyMOL 1.8.6 (Schrödinger) using the APBS Tools 2.1 plugin. The sizes of the binding cavities were calculated using Caver 3.0.1 [41].

3. RESULTS AND DISCUSSION

3.1 Generation of the PI binding defective Pdr17$^{E237A, K269A}$ protein.

We generated a mutated Pdr17p that is unable to bind PI to study whether PI binding is required for the functioning of Pdr17 in the complex that transports PS to Psd2p. To do so, we changed two Pdr17p amino acids, glutamic acid 237 and lysine 269, to alanine. Amino acids corresponding to the E237 and K269 of Pdr17p are absolutely conserved in all yeast Sec14 homologues (Fig. 1). In addition, they correspond to the E235 and K267 of Pdr16p, a close homologue of Pdr17p [16, 28]. When Pdr16p E235 and K267 were changed to alanine, the resulting Pdr16$^{E235A, K267A}$ protein was not able to bind PI, and the compromised Pdr16$^{E235A, K267A}$ protein was non-functional as evidenced by the increased sensitivity of the yeast cells to azole antifungals [42].

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Sec14  195 REASYISQNYYPERMGKFYIINAPFGFSTAFRLFKPFLDPVTVSKIFILGSSYQKELL 252
Sfh1   197 KDVADISQNYYPERMGKFYIIHSPFGFSTMFKMVKPFLDPVTVSKIFILGSSYKKELL 254
Csr1   242 KFLITCFEAHYPESLGHLLIHKAPWIFNPIWNIIKNWLDPVVASKIVFTKN--IDELH 297
Pdr16  223 KEVLHILQTHYPERLGKALLTNIPWLAWTFLKLIHPFIDPLTREKLVFD-----EPFV 275
Pdr17  225 RMCLNVMQDHYPERLAKCVLINIPWFAWAFLKMMYPFLDPATKAKAIFD-----EPFE 277
Sfh5   192 KTVIGIFQKYYPELLYAKYFVNVPTVFGWVYDLIKKFVDETTRKKFVVLTD---GSKL 246
                      E237A                          K269A
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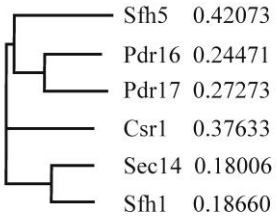

Fig. 1. Alignment of yeast Sec14 PITP proteins. Regions close to the C-termini of yeast PITPs were aligned using ClustalW2 (http://www.ebi.ac.uk/). Asterisks (*) indicate absolutely conserved residues, colons (:) and periods (.) indicate strongly and loosely homologous residues, respectively. Amino acids E237 and K269 (in bold) were changed to alanine to create a Pdr17 mutant defective in PI binding. Sequence numbering begins from the first methionine of each respective protein. Sfh1p in this figure is encoded by ORF YKL091c. The phylogenetic tree of yeast PITPs alignment produced by Clustal W2 is also shown.

C-terminally His-tagged Pdr17 and Pdr17$^{E237A, K269A}$ proteins were expressed in *E. coli* and purified on Ni-NTA affinity columns followed by gel filtration and ion exchange chromatography (for details see Section 2.4). The purified recombinant proteins were subsequently tested for lipid binding using permeabilized HL60 cells [30-31]. In this assay, radiolabeled HL60 cells were co-incubated with either wild-type Pdr17p or with Pdr17p$^{E237A, K269A}$. Purified recombinant Sfh1p was used as a control. During co-incubation of HL60 cells with lipid transfer proteins, radiolabeled lipids to which the respective lipid transfer protein has affinity can be incorporated into the protein's lipid binding cavity. Following co-incubation, the proteins were re-purified using their C-terminal 6×His-tags, and the bound lipids were extracted and analyzed by TLC (see Section 2.6 for details). The results show that

wild-type Pdr17p binds preferentially PI and cholesterol together with a small amount of an unidentified neutral lipid (Fig. 2). Mutant Pdr17p$^{E237A, K269A}$ binds almost exclusively cholesterol (90% of the recovered lipid-associated radioactivity) and the unknown neutral lipid (close to 10% of the recovered lipid-associated radioactivity) with very little PC and almost no PI. Cholesterol binding by the PI-binding defective mutant is similar to that observed for Pdr16p PI-binding deficient mutant [42]. In summary, the *in vitro* lipid binding assay demonstrated that the Pdr17p$^{E237A, K269A}$ mutant is unable to bind PI at high affinity and can therefore be used to test whether Pdr17 high affinity PI binding is required for supporting PS transfer from the ER to the Golgi/endosomes. Notably, no binding of PS or PE was observed using either wild-type Pdr17p or mutated Pdr17p$^{E237A, K269A}$ in this assay. This result is fully consistent with the previous observation that Pdr17p does not support transfer of PS as a soluble carrier [20].

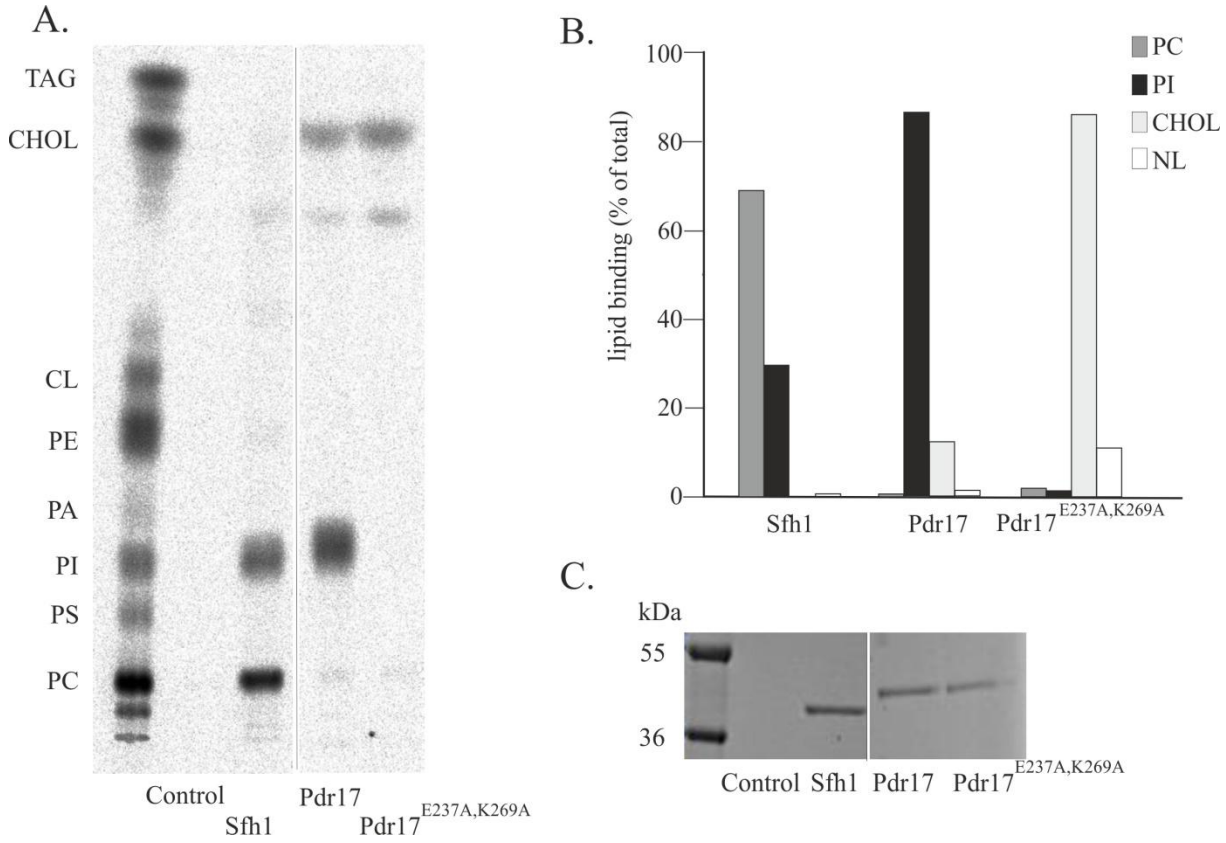

Fig. 2. Lipid binding of Pdr17p and Pdr17p $^{E237A, K269A}$. A. Wild-type Pdr17p, Pdr17p $^{E237A, K269A}$, and Sfh1p (all at 120 μg) were incubated with permeabilized HL60 cells prelabeled with [^{14}C]-acetate for 48 h. Proteins were re-isolated using nickel beads following this co-incubation. The lipids bound to the re-isolated proteins were extracted and separated by TLC. In the "control", no protein was added to the HL60 cells. **B.** Quantification of the lipid bound to each protein expressed as a percentage of total lipid bound (i.e. the total recovered radioactivity from PC + PI + CHOL + NL). The data represent the mean from two independent experiments, one of which is depicted in panels A and C. Both experiments showed no binding of PI to Pdr17p $^{E237A, K269A}$. Quantitatively, the differences in lipid binding in these two experiments were less than 10%. **C.** A representative SDS-PAGE analysis of protein (2% of total) captured by the nickel beads following co-incubation with HL60 cells.

Proteins were stained with Coomassie Blue. Abbreviations: PC, Phosphatidylcholine; PS, Phosphatidylserine; PI, Phosphatidylinositol; PA, Phosphatidic acid; PE, Phosphatidylethanolamine; CL, Cardiolipin; CHOL, Cholesterol; TAG, Triacylglycerol; NL, unknown neutral lipid.

To analyze the surface properties of Pdr17p and the Pdr17p$^{E237A, K269A}$ PI-binding deficient mutant, we used both purified proteins in protein-lipid overlay assays (Fig. 3).

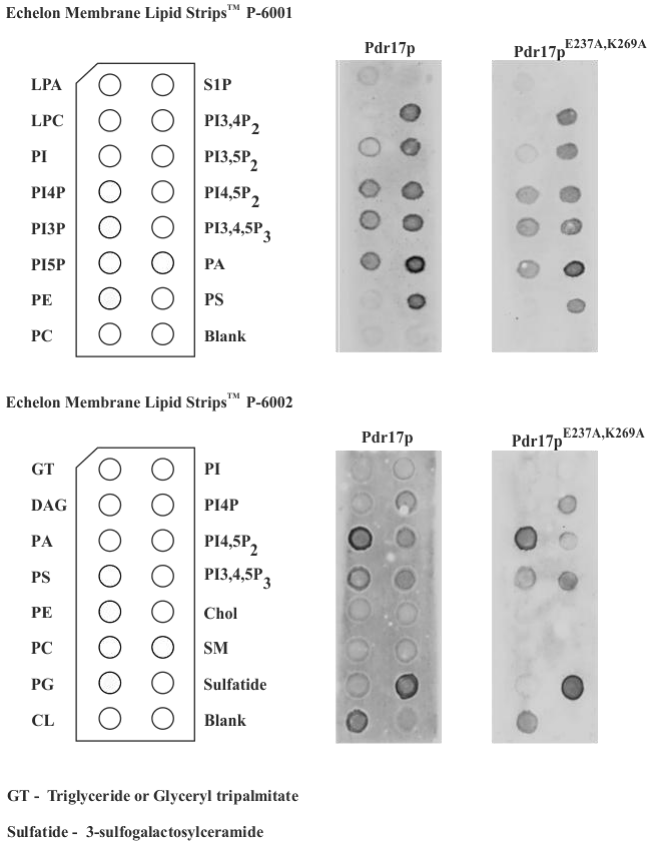

Fig. 3. Protein-lipid overlay assays. Protein-lipid overlays using purified 6×His tagged Pdr17p and Pdr17p$^{E237A, K269A}$ show that both proteins preferentially associate with the anionic phospholipids PA, PS, and CL, and all phosphatidylinositol phosphates. Abbreviations: LPA, Lysophosphatidic acid; LPC, Lysophosphatidylcholine; PI, Phosphatidylinositol; PI(4)P, Phosphatidylinositol (4) phosphate; PI(3)P, Phosphatidylinositol (3) phosphate; PI(5)P, Phosphatidylinositol (5) phosphate; PE, Phosphatidylethanolamine; PC, Phosphatidylcholine; S1P, Sphingosine 1-Phosphate; PI(3,4)P_2, Phosphatidylinositol (3,4) bisphosphate; PI(3,5)P_2, Phosphatidylinositol (3,5) bisphosphate; PI(4,5)P_2, Phosphatidylinositol (4,5) bisphosphate; PI(3,4,5)P_3, Phosphatidylinositol (3,4,5) trisphosphate; PA, Phosphatidic acid; PS, Phosphatidylserine; GT, Glyceryl tripalmitate; Blank, no lipid spotted.

The lipid binding assay using permeabilized HL60 cells and the protein-lipid overlay assay monitor two different properties of the proteins. In the lipid binding assay using permeabilized HL60 cells, the lipid that "fits" into the lipid binding cavity of a lipid transfer protein is extracted from the membrane of the HL60 cells and becomes inserted into the lipid

binding pocket. In this assay, only tightly bound lipids are detected due to the lengthy purification process. The protein-lipid overlay assay shows with which lipids the external protein surface can interact with. The results from the protein-lipid overlay on wild-type Pdr17 are in good agreement with published results from a liposome-protein binding assay [22], thus validating the use of this method for studying the surface properties of Pdr17p$^{E237A, K269A}$.The experiment shows that the E237A and K269A mutations changed only the lipid binding properties of the hydrophobic cavity (Fig. 2), but not the surface properties of the Pdr17 mutant protein (Fig. 3).

3.2 Functional characterization of PI binding defective Pdr17p$^{E237A, K269A}$

PE can be synthesized by three major pathways in *S. cerevisiae* (Fig. 4): (i) by decarboxylation of PS through the action of phosphatidylserine decarboxylase 1 (Psd1p) in the mitochondria [43-45], (ii) by decarboxylation of PS catalyzed by phosphatidylserine decarboxylase 2 (Psd2p) in the Golgi/endosomes [21, 46], and (iii) by the incorporation of ethanolamine via the CDP-ethanolamine pathway [47]. Ethanolamine phosphate, an intermediate in the CDP-ethanolamine pathway, can also be derived from sphingolipids in a reaction catalyzed by the dihydrosphingosine-1-phosphate lyase Dpl1p [48].

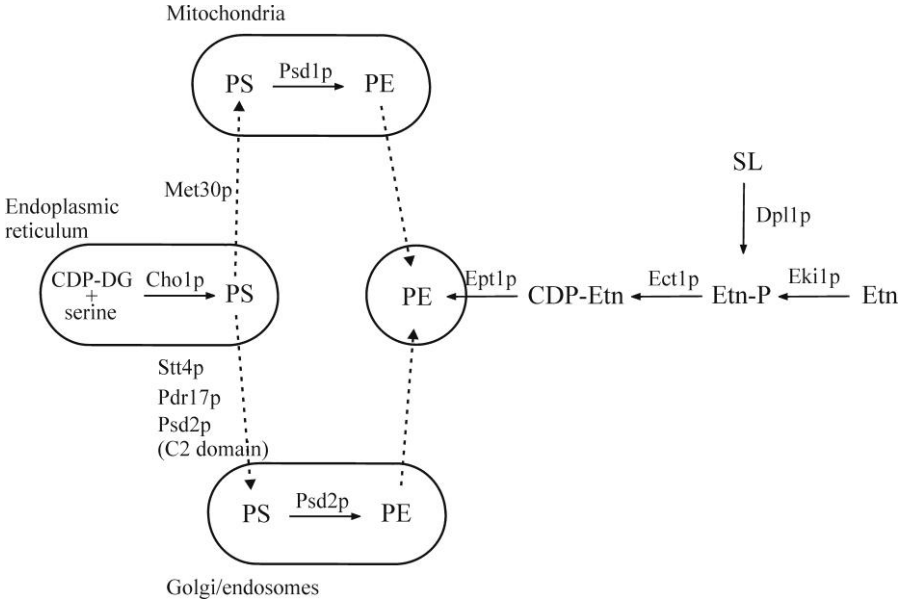

Fig. 4. Biosynthetic pathways of PE in *S. cerevisiae*. Three pathways contribute to the biosynthesis of PE in *S. cerevisiae*: (i) conversion of PS to PE in the mitochondria catalyzed by Psd1p, phosphatidylserine decarboxylase 1; (ii) conversion of PS to PE in the Golgi/endosomes, catalyzed by Psd2p, phosphatidylserine decarboxylase 2; and (iii) incorporation of ethanolamine or ethanolamine phosphate derived from sphingolipids via the CDP-ethanolamine (Kennedy) pathway. PI-4-kinase Stt4p, phosphatidylinositol transfer protein Pdr17p, and the C2 domain of Psd2p were identified as essential components of the transport machinery delivering PS from the ER to the Golgi/endosomes [19]. Abbreviations are: CDP-DG, cytidine diphosphate diacylglycerol; PS, phosphatidylserine; PE,

phosphatidylethanolamine; SL, sphingolipid; Etn, ethanolamine; Etn-P, ethanolamine phosphate; CDP-Etn, cytidine diphosphate ethanolamine.

Disruption of both PS to PE decarboxylation pathways causes auxotrophy for ethanolamine which can still be converted to PE *via* the CDP-ethanolamine pathway [49]. Thus, to test the functionality of the PI-binding defective Pdr17$^{E237A, K269A}$ protein, we used the *psd1::TRP1 pdr17::HIS3* double disruption strain PGY285 (kindly donated by D. Voelker, National Jewish Health, Denver, CO, USA) [26]. In this strain, the mitochondrial PS decarboxylation pathway is inactivated by disruption of mitochondrial PS decarboxylase 1, Psd1p, and the Golgi/endosomal PS decarboxylation pathway is inactivated by disruption of the Pdr17p, an essential component of the PS transport machinery. As a result, *psd1::TRP1 pdr17::HIS3* strain iss auxotrophic for ethanolamine (Fig. 5A). The strain still exhibits very weak residual growth when transformed with the empty cloning plasmid in growth medium lacking ethanolamine due to the incorporation of Etn-P derived from SL degradation into PE [48]. After the introduction of wild-type *PDR17* on a plasmid (either multicopy or a centromeric) into this strain the Golgi/endosomal PS to PE decarboxylation pathway becomes functional and the strain no longer requires ethanolamine for growth. To test whether PI binding to Pdr17p is necessary for its function in the transfer of PS from the ER to the location of Psd2p, we introduced a PI-binding deficient Pdr17p mutant into the *psd1::TRP1 pdr17::HIS3* strain and tested the resulting strain for ethanolamine dependency. We observed that the Pdr17$^{E237A, K269A}$ protein supported cell growth in the absence of ethanolamine equally as well as the wild-type Pdr17p. In addition, the amount of PE in the *psd1::TRP1 pdr17::HIS3* strain transformed with the wild-type *PDR17* and grown in the absence of ethanolamine was the same as in the *psd1::TRP1 pdr17::HIS3* strain transformed with the PI-binding deficient mutant *PDR17* (*E237A, K269A*) (Fig. 5B). Moreover, the amount of PI was also the same regardless of whether the *psd1::TRP1 pdr17::HIS3* strain was transformed with wild-type *PDR17* or with the PI-binding deficient mutant *PDR17* (*E237A, K269A*) (Fig. 5B). Thus, the PI binding defect in the Pdr17p mutant does not affect the overall cellular PI homeostasis.

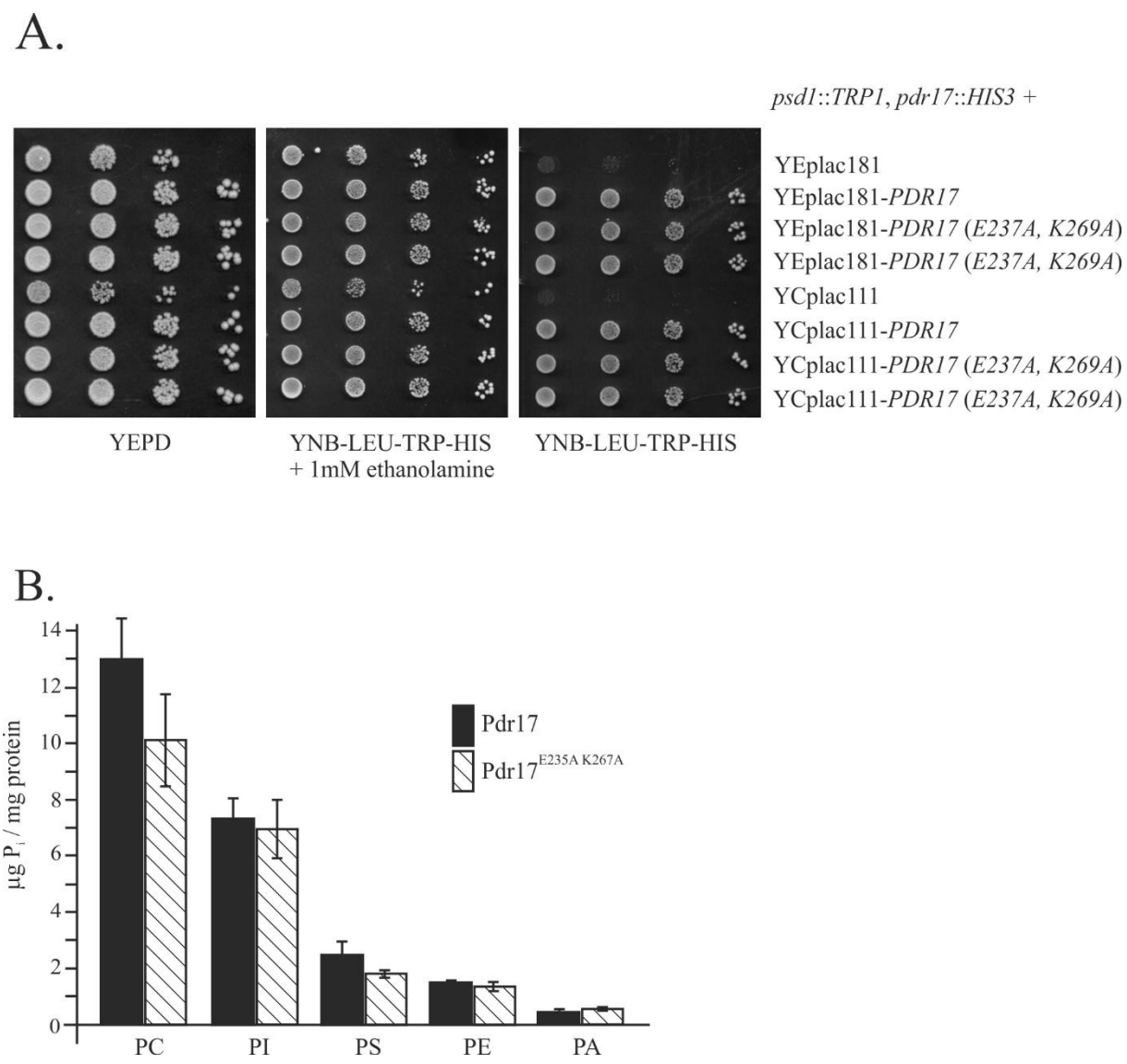

Fig. 5. Complementation of a disrupted *PDR17* gene by a cloned wild type *PDR17* or PI-binding deficient mutant *PDR17* (*E237A K269A*). A. A *psd1::TRP1 pdr17::HIS3* yeast strain auxotrophic for ethanolamine due to the disruption of both PS to PE decarboxylation pathways was transformed with either a YEplac181 multicopy plasmid or a YCplac111 centromeric plasmid containing either wild-type *PDR17* or a PI-binding deficient mutant *PDR17* (*E237A K269A*) (two individual transformants). The respective strains were pre-grown in selective -LEU media to early stationary phase and subsequently spotted at concentrations of 10^4, 10^3, 10^2, and 10 cells onto the YEPD medium, YNB-LEU-TRP-HIS medium containing 1 mM ethanolamine, and YNB-LEU-TRP-HIS medium without ethanolamine. The cells were allowed to grow for 3 days at 28^{o}C. **B.** Phospholipids were extracted from a *psd1::TRP1 pdr17::HIS3* yeast strain grown in the absence of ethanolamine transformed with YCplac111 centromeric plasmid containing either wild-type *PDR17* or a PI-binding defective mutant *PDR17* (*E237A K269A*). The extracted phospholipids were subjected to TLC, scraped of the TLC plate and the amount of phospholipid phosphate in each phospholipid was quantified. Phospholipid amounts are expressed as µg of phospholipid phosphate relative to the amount of protein in cell extracts. Inorganic phosphate was used as a

standard. The mean values of three independent experiments and standard deviations are shown.

The complementation of the lack of PS to PE decarboxylation in the *psd1::TRP1 pdr17::HIS3* strain, by either wild-type Pdr17p or the Pdr17$^{E237A, K269A}$ mutant was also followed by incorporation of radioactive serine into PS and PE *in vivo*. Yeast cultures containing the respective plasmids were incubated for 30 min with radioactive serine (details in Section 3.9), the lipids were extracte and analyzed by TLC, and the radioactivity associated with PS and PE was counted and plotted as percentage of PS converted to PE during the incubation period. We found no difference in conversion of radiolabeled PS to PE in the *psd1::TRP1 pdr17::HIS3* strain when transformed with either wild-type *PDR17* or the PI-binding deficient mutant (Fig. 6).

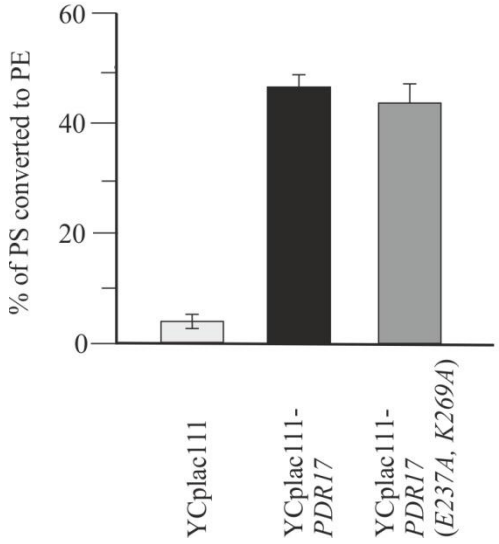

Fig. 6. Radioactive serine incorporation into PS and PE. A *psd1::TRP1 pdr17::HIS3* strain transformed with the indicated plasmids was labeled with [^{3}H]serine for 30 min and the percentage of [^{3}H]PS converted to [^{3}H]PE was determined (mean ± SD). The results show the mean ± SD of three independent experiments.

These results together provide clear evidence that the high affinity PI binding of Pdr17p is not required for Pdr17 to fulfill its role in the complex that transfers PS from the ER to the Golgi/endosomes where the non-mitochondrial decarboxylation of PS to PE takes place.

This was surprising, since PI transfer is the defining activity of PITPs. Our PI binding experiments essentially monitor high affinity binding as the protein undergoes rigorous manipulations during the protein purification process (see Section 2.6). We therefore also examined the PI transfer activity of the PI binding defective mutant Pdr17p$^{E237A, K269A}$ *in vitro* where radiolabeled PI is transferred between two membrane compartments. In this assay, the Pdr17$^{E237A, K269A}$protein was found to retain PI transfer activity. The Pdr17p$^{E237A, K269A}$ mutant

was less active compared to the wild-type at low concentrations, but had similar activity as the wild-type when added at high concentrations (Fig. 7).

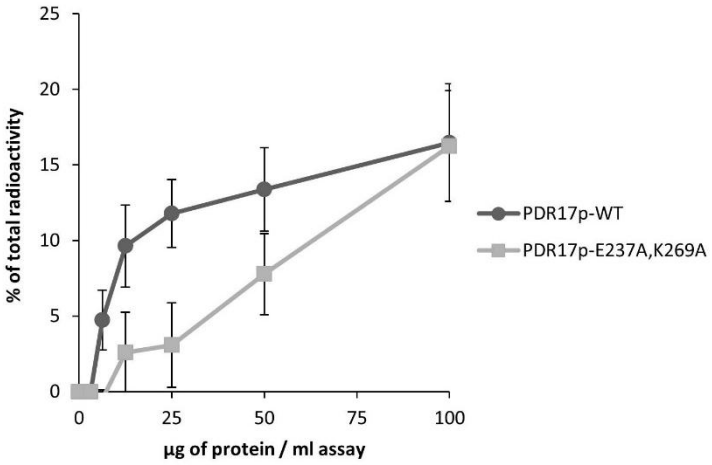

Fig. 7. *In vitro* Pdr17 phosphatidylinositol transfer activity. Purified recombinant Pdr17 and Pdr17$^{E237A, K269A}$ were used in the transfer process between radiolabeled microsomes and acceptor liposomes PC:PI:cholesterol (96:2:2). The results show the average ± SD (n = 6).

Previously, we made a *PDR16* mutant (E235A, K267A) which was unable to bind PI (42). This Pdr16 $^{E235A, K267A}$ protein was a non-functional Pdr16p based on azole sensitivity complementation assays. We now tested this mutant in the PI transfer assay and found it to be unable to transfer PI (Fig. 8).

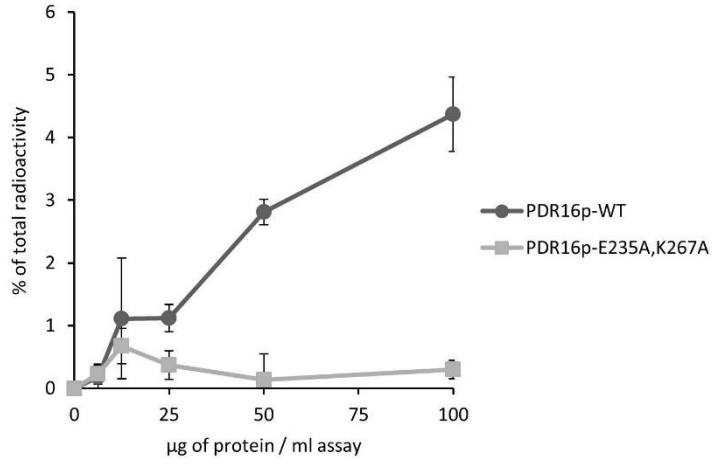

Fig. 8. *In vitro* Pdr16 phosphatidylinositol transfer activity. Purified recombinant Pdr16 and Pdr16$^{E235A, K267A}$ were used in the transfer process between radiolabeled microsomes and acceptor liposomes PC:PI (96:4). The results show the average ± SD (n = 4).

The ability to transfer PI was found to be essential for all previously tested yeast PITPs, Sec14p, Pdr16p, and Sfh5p, to be functional [12, 42, 50]. It was also shown previously that over-expressed Pdr17p suppressed the growth phenotype associated with deletion of the *SEC14* gene and the phenotype associated with inactivation of the temperature sensitive allele of *SEC14*, *sec14-1*^{ts}, at a non-permissive temperature [17]. It also suppressed the increased azole sensitivity arising from *PDR16* deletion [51]. In the next experiment, we tested the ability of the PI- binding defective Pdr17p$^{E237A, K269A}$ mutant to complement the phenotypes of the *sec14-1*^{ts} and *pdr16Δ* mutants. The results presented in Fig. 9 show that the Pdr17p$^{E237A, K269A}$ protein is not able to complement either Sec14p or Pdr16p, unlike the wild-type Pdr17p. These results indicate that lipid binding and lipid transfer are separable events for Pdr17p and that for some functions lipid binding is an important feature for functionality.

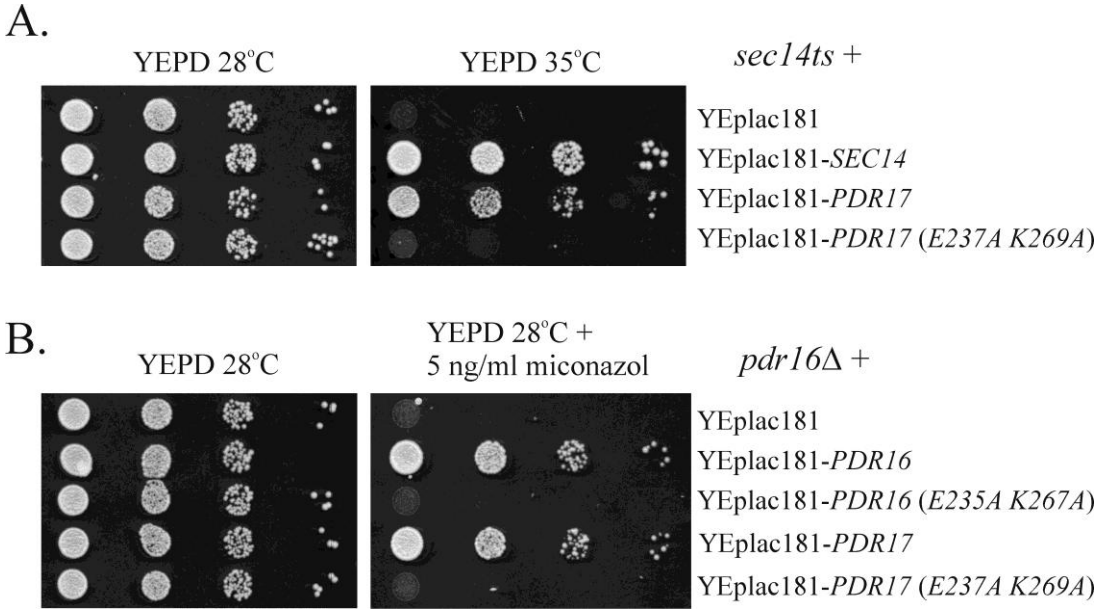

Fig. 9. Complementation of yeast PITPs phenotypes by Pdr17p. A. A yeast strain with a *SEC14* temperature sensitive allele, *sec14-1ts*, was transformed with a YEplac181 multicopy plasmid containing the *SEC14* gene, wild-type *PDR17*, or the PI-binding defective *PDR17* mutant *PDR17* (*E237A K269A*). The respective yeast strains were pre-grown in selective -LEU media to early stationary phase and subsequently spotted at concentrations of 10^4, 10^3, 10^2, and 10 cells onto YEPD medium. The cells were allowed to grow for 48 h at the indicated temperatures: 28°C is the permissive and 35°C is the non-permissive temperature for a yeast strain with the *sec14-1ts* allele. **B.** A *pdr16Δ* yeast strain was transformed with a YEplac181 multicopy plasmid containing wild-type *PDR16*; PI-binding defective *PDR16* mutant *PDR16* (*E235A K267A*); wild-type *PDR17*; and the PI-binding defective *PDR17* mutant *PDR17* (*E237A K269A*). The respective yeast strains were pre-grown in selective -LEU media to early stationary phase and subsequently spotted at concentrations of 10^4, 10^3, 10^2, and 10 cells onto YEPD medium with or without miconazol, an inhibitor of sterol biosynthesis.

3.3 Comparison of two closely related yeast PITPs, Pdr16p and Pdr17p

Our results provide evidence that high affinity PI binding is not required for Pdr17p to perform what is considered the major function of this PI transfer protein, namely to assist in the transfer of PS from the ER to the Golgi/endosomes. Our results show that retention of PI transfer activity is sufficient for Pdr17p functionality. We hypothesize, based on the high similarity between Pdr16 and Pdr17, that these two proteins may have evolved from a common PI transfer protein ancestor. One of the proteins, Pdr16p, fulfills those roles in which the ability to bind PI at high affinity and transfer it is required. The other one, Pdr17p, adapted to fulfill a different role in which the ability to bind PI at high affinity is not required. This scenario may also account for both the high similarity between these two proteins and the large divergence in their proposed roles in the yeast cell (Table 1).

Table 1. Comparison of the Pdr16 and Pdr17 proteins. These two proteins are 49% identical and 75% similar [28], yet they differ in many aspects, including the requirement for high affinity PI binding for their major function.

	Pdr16p (Sfh3p)	Pdr17p (Sfh4p, PstB2p, Iss1p)
Protein length	351 AA	350 AA
Identity/similarity to Sec14p	18%/35%	18%/35%
Classified as	PI transfer protein [15]	PI transfer protein [15]
Subcellular localization	lipid particles and the cell periphery [17] [52] [53] [54-55]	mainly cytosolic; cell periphery close to or at the plasma membrane [17]
Proposed cellular function	regulates lipid droplet utilization [54]; clearance of cytosolic inclusion bodies [56]; possible function as sterol sensor [42]; required for resistance to azoles [51, 57]	essential component of a complex required for transport of PS between the ER and the Golgi apparatus/endosomes [19-20]
PI binding required for protein's major function	Yes [42]	No (this work)
PI transfer required for protein's major function	Yes (this work)	Not known*
Complements non-functional Sec14p when over-expressed	No [15, 17]	Yes [15, 17]
Lipid binding	PI [15] and sterol [42]	PI [15, 58] and sterol (this work)
Affinity to lipids in lipid-protein overlay assay	PA; PI4P [42]	PA; all phosphorylated PIs; CL (this work)

*In addition to PI binding deficient mutant Pdr17^{E237A,K269A} we also prepared and tested Pdr17^{T266W}, Pdr17$^{E237A, T266W, K269A}$, and Pdr17$^{T266W, K269A}$ mutants. These additional Pdr17 mutants were chosen based on analogy with mutations in the homologous proteins Sec14, Pdr16, and Sfh5 known to abolish PI transfer [12, 42, 50]. All three of these Pdr17 mutants retain the ability to transfer PI *in vitro* and are functional in the non-mitochondrial PS to PE decarboxylation (data are presented in the Supplementary material).

In an attempt to account structurally for the behavior of Pdr17, we prepared a homology model of Pdr17 using a known structure of Pdr16 (4J7P; [13]) and calculated its electrostatic surface potential. The results showed that Pdr16 and Pdr17 are broadly similar, but do feature a few possibly significant differences. Although it is lined with hydrophobic residues, the binding cavity of Pdr16 actually has a modest overall positive charge (about 2.5 kT/e) with two pockets of greater positive charge near the phosphate and inositol binding area and in a pocket at the distal end of the binding cavity, centered on residue K144. On the opposite surface of the molecule there is a large, negatively-charged patch centered on residues 78–81, all of which are glutamates. Pdr17 also has a generally positively charged binding pocket, but its overall potential is clearly lower than Pdr16's (below 1.3 kT/e). This may arise from the replacement of H176 with E180, which introduces a negatively charged patch into the binding cavity. This substitution also has the effect of lowering the overall positive charge in the pocket at the end of the binding cavity from above +12 kT/e to around +5 kT/e. The phosphate and inositol binding region has also suffered a reduction in charge due to the replacement of R107 in Pdr16 with G112; in addition to making this area less positively charged, this change also makes this area more open. Finally, the binding cavity also has had a number of substitutions which change its overall shape. Many of these involve the substitution of a leucine with a methionine and include the replacement of L181 with M185, L229 with M231, L160 with M164, I256 with M259 and also P162 with N166, L158 with Y162, A240 with C242, and L248 with F250. The overall effect of these changes is to make the binding cavity slightly larger (from around 4.3 Å at the widest point to around 4.5 Å). The large negatively charged surface on the opposite side of the molecule does remain in Pdr17, and is still centered on the same physical region, but its area of extent and the site of greatest charge has shifted. The four glutamate residues at its center in Pdr16 have now become S83, D84, W85, and E86, thereby placing a hydrophobic Trp at the center of what was originally a highly charged region. As a consequence, the negatively charged area now has two centers which lie to either side of the original center. The first is now on residues 61–80, which are not conserved between Pdr16 and Pdr17, and the second is on residues 130–133, which are conserved. The C-terminal D347 lies adjacent to residues 130–133 and also forms part of this second center. There are also two new positively charged areas which are not present in Pdr16. One of these is located around residues 334–343 (not conserved) and is structurally adjacent to the negatively charged area. The other is located adjacent to the phosphate binding area and is separated from it only by the salt bridge formed by residues E237 and K269. It is formed by R159 and K198. No such pocket is present in Pdr16, where K198 is replaced with S194 and the counterpart to R159 (R155) is neutralized by D153 (which is A157 in Pdr17).

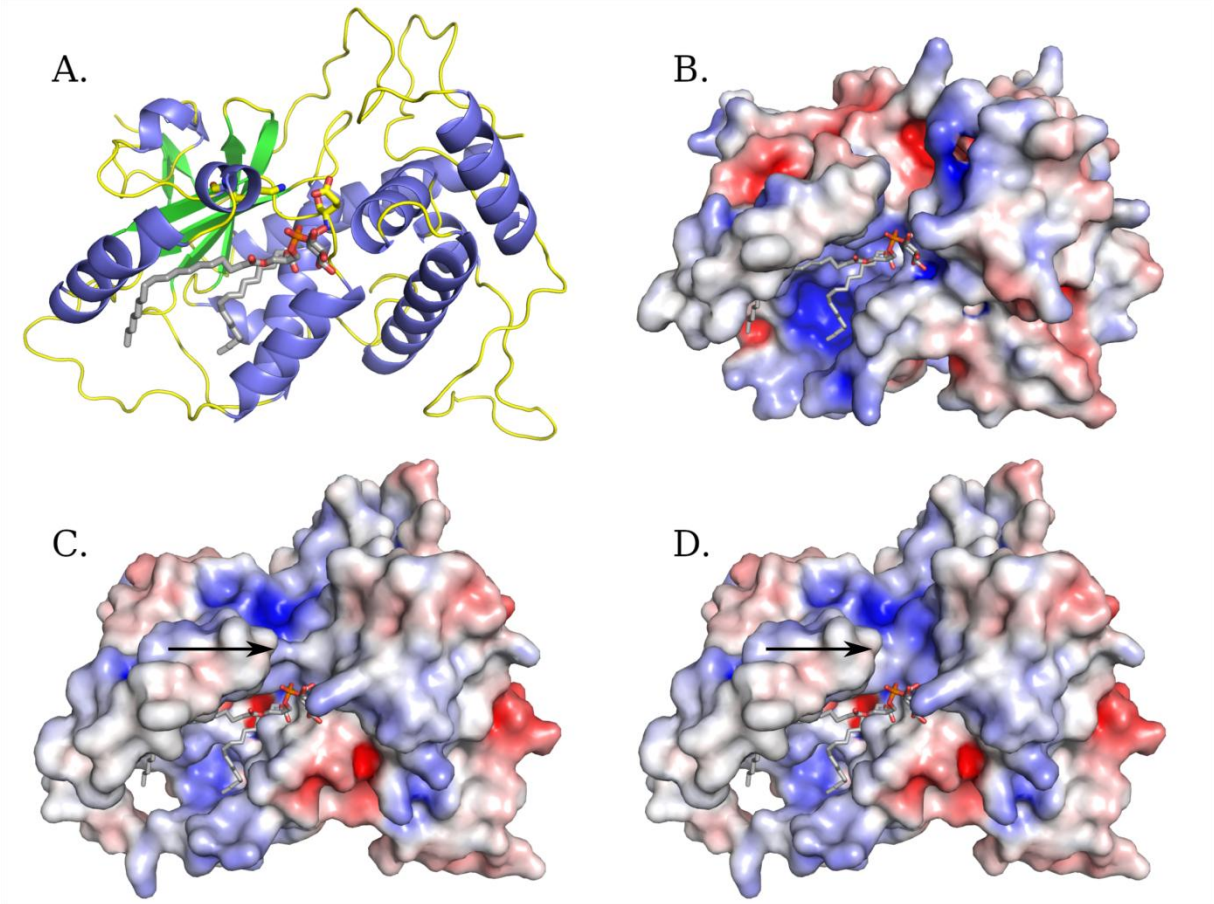

Fig 10 Pdr16 and the homology models of Pdr17 and Pdr17$^{E237A, K269A}$. All structures are viewed in the same orientation. A. A ribbon diagram of the Pdr17 homology model based on a Pdr16 template (PDB ID: 4J7P) [13]. E237 and K269 are shown as sticks; α-helices are blue, and β-strands are green. The binding cleft is marked using the phosphatidylinositol, colored gray, found in the bound Pdr16–PI complex (PDB ID 4J7Q) by superposition. B. The electrostatic surface of Pdr16 calculated by APBS [40]. Note the positive charge in the binding pocket. C. The electrostatic surface of the Pdr17 homology model. Note the lower charge on the binding pocket, the additional negatively charged area at the bottom of the binding pocket and the additional positively charged region above the PI phosphate. D. The electrostatic surface of the model of the Pdr17$^{E237A, K269A}$ mutant. The additional positively charged region in Pdr17 is now continuous with the binding pocket, suggesting that at least one additional binding mode may have become available. In C and D, the arrow indicates the location of the bridge (in C) which is removed by the double mutation (in D), thereby connecting the additional charged region with the binding pocket.

This additional pocket may be important for explaining the behavior of the Pdr17$^{E237A, K269A}$ mutant. The E237A,K269A mutations may very well abolish the ability of Pdr17 to bind PI using its binding cleft in much the same way that the corresponding mutations in Pdr16 abolish binding in that protein. In Pdr17, however, this double mutation may also allow this positively charged pocket to be used for binding the PI phosphate group. This second binding mode is not likely to be as strong or as specific as the original one, but it may still be sufficient for allowing the mutant to carry out PI transfer.

It may also be noted here that the existence of three positively charged sites on the surface of Pdr17 may account for the preference of this protein for negatively charged lipids. The presence of large positively and negatively charged areas on opposite sides of the molecule is also worth comment: In approaching a negatively-charged phospholipid, the polarity would help to orient the molecule in the correct orientation for phospholipid binding.

4. CONCLUSIONS

We have shown that the PI binding deficient Pdr17 $^{E237A, K269A}$ mutant is fully functional in the complex that is required for transfer of PS from the ER to the Golgi apparatus/endosomes and its subsequent decarboxylation to PE. At the same time this PI-binding deficient mutant is not able to complement a defective Sec14p or a missing Pdr16p. The Pdr17 $^{E237A, K269A}$ mutant retained, however, the ability to transfer PI *in vitro* between two membrane compartments. The observation that high affinity PI binding is essential for some features of Pdr17 (complementation of Sec14ts and azole sensitivity arising from *PDR16* deletion) but not for PS transfer to endosomes suggests that PITPs function in complex ways *in vivo,* and that the *in vitro* characterizations of these proteins should include studies of multiple parameters. In addition, the observed separation of Pdr17p functions in the conversion of PS to PE from its ability to complement Sec14p will enable the preparation of Pdr17p mutants specifically defective in non-mitochondrial PS to PE decarboxylation. We propose that these mutants will be instrumental in understanding the molecular mechanism of Pdr17 function and will add to our knowledge of the mechanism by which lipid transfer proteins operate.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY MATERIAL:

1. DNA String for constructing the Pdr17$^{E237A, K269A}$ PI-binding deficient mutant.

To construct the Pdr17$^{E237A,\ K269A}$ PI-binding deficient mutant we used the following DNA String (Thermo Scientific). Italicized are the *Sex*AI and *Sac*I restriction sites; underlined are the nucleotides for changing glutamic acid 237 and lysine 269 to alanine:

aaagaa*cctggt*attatcactgataaagctccacccatctctattgctaggatgtgtttgaacgtcatgcaagatcattaccctgcaaggtta
gctaagtgtgtcctgatcaatatcccatggtttgcgtgggctttttgaagatgatgtatccgttccttgaccctgccactaaggcggcagct
atctttgacgaaccatttgaaaaccacatagagccatctcaactagatgctctatacaatggtctgttagacttcaagtataaacatgaagtc
tattggccagatatggttaagaaagttgacgatttacgattgaaacgttttgatagatttttaaagtttggaggcattgtcggattaagtgaat
atgacaccaagggccaacatgacgaattaaaatatcctgttgatatggtcatttgataaactataattaagattttttttttttaacgtttcccgct
acacaaacgtatatatctagactcagatgtacgtttataatggctgcttatgcgcttatcgcattttatatacttttagaatcaactgaatcggc
tatccgcactctattgttttgtttcatgaaaaaaaaaacgccttctgaattccaaagggtgtgaaaattatatatactattagttccagctaatc
aatc*gagctc*gaattc

2. Construction of additional Pdr17 mutants.

To prepare the Pdr17^{T266W}, Pdr17$^{E237A,\ T266W,\ K269A}$, and Pdr17$^{T266W,\ K269A}$ mutants, two DNA Strings (Thermo Scientific) were designed, one containing a T266W change, the other one containing T266W and K269A changes. They are as follows:

DNA String PDR17_T226W:

caatatc*ccatgg*tttgcgtgggctttttgaagatgatgtatccgttccttgaccctgcctggaaggcgaaggctatctttgacgaaccatt
tgaaaaccacatagagccatctcaactagatgctctatacaatggtctgttagacttcaagtataaacatgaagtctattggccagatatgg
ttaagaaagttgacgatttacgattgaaacgttttgatagatttttaaagtttggaggcattgtcggattaagtgaatatgacaccaagggcc
aacatgacgaattaaaatatcctgttgatatggtcatttgataaactataattaagattttttttttaacgtttcccgctacacaaacgtatatat
ctagactcagatgtacgtttataatggctgcttatgcgcttatcgcattttatatacttttagaatcaactgaatcggctatccgcactctattgt
tttgtttcatgaaaaaaaaaacgccttctgaattccaaagggtgtgaaaattatatatactattagttccagctaatcaatc*gagctc*gaattc

Italicized are the *Nco*I and *Sac*I restriction sites; underlined are the nucleotides to change threonine 266 to tryptophan.

DNA String PDR17_T226W,K269A:

caatatc*ccatgg*tttgcgtgggctttttgaagatgatgtatccgttccttgaccctgcctggaaggcggcagctatctttgacgaaccatt
tgaaaaccacatagagccatctcaactagatgctctatacaatggtctgttagacttcaagtataaacatgaagtctattggccagatatgg
ttaagaaagttgacgatttacgattgaaacgttttgatagatttttaaagtttggaggcattgtcggattaagtgaatatgacaccaagggcc
aacatgacgaattaaaatatcctgttgatatggtcatttgataaactataattaagattttttttttaacgtttcccgctacacaaacgtatatat
ctagactcagatgtacgtttataatggctgcttatgcgcttatcgcattttatatacttttagaatcaactgaatcggctatccgcactctattgt
tttgtttcatgaaaaaaaaaacgccttctgaattccaaagggtgtgaaaattatatatactattagttccagctaatcaatc*gagctc*gaattc

Italicized are the *Nco*I and *Sac*I restriction sites; underlined are the nucleotides to change threonine 266 to tryptophan and lysine 269 to alanine.

By cloning these DNA Strings using the *Nco*I and *Sac*I restriction enzymes into YEplac181-*PDR17* or YEplac181-*PDR17* (*E237A, K269A*) we prepared the following mutation combinations:

1. YEplac181-PDR17_T266W
2. YEplac181-PDR17_T266W,K269A
3. YEplac181-PDR17_E237A,T266W,K269A

Centromeric plasmids containing these *PDR17* alleles were constructed by subcloning them from episomal YEplac181 based plasmids into a YCplac111 plasmid. To create plasmids for recombinant protein production in *E. coli*, their respective ORFs were amplified from YEplac181 based plasmids using the following primers: 5`-GTACCATATGGGTCTTTTTTCAAGAAAACGG -3` and 5`-GACGTCTCGAGGCGGCCGCAATGACCATATCAACAGGATAT -3`; the *Nde*I and *Not*I sites are underlined. The amplified ORFs were cut with *Nde*I and *Not*I restriction enzymes and inserted into the corresponding sites of a pET26b(+) vector (Merck, USA) to create the plasmids pET26-*PDR17* (*T266W*), pET26-*PDR17* (*T266W,K269A*), and pET26-*PDR17*(*E237A,T266W,K269A*). All constructs containing PCR amplified DNA fragments were verified by DNA sequencing. The *Escherichia coli* Rosetta 2 (DE3) strain (F^- omp^T $hsdS_B$ (r_B^- m_B^-) *gal dcm* pRARE2 (Cam^R) was from Merck.

3. **Tests on the additional Pdr17 mutants.**

Ren et al. [54] identified a $Pdr16^{T264W}$ mutant unable to transfer PI between two membrane compartments *in vitro* when studying a close homologue of Pdr17p. Thus, in an attempt to prepare a Pdr17 mutant defective in PI we also made a corresponding mutation (T266W) in Pdr17p and we combined this mutation with the two mutations previously identified to cause Pdr17 PI binding deficiency, $Pdr17^{E237A, K269A}$. We tested all three Pdr17 mutants, $Pdr17^{T266W}$, $Pdr17^{E237A, T266W, K269A}$, and $Pdr17^{T266W, K269A}$ for their ability to complement disruption of the *PDR17* gene using growth complementation assays (Fig. S1) and PS to PE conversion using radiolabeling (Fig. S2). According these criteria, all three mutants are fully functional in supporting non-mitochondrial PS to PE decarboxylation. None of these three mutants is able to replace wild-type Pdr17 in complementing either the *sec14ts* allele or deletion of the *PDR16* gene. We also examined the PI transfer activity of these Pdr17 mutants and found that all of them still possess PI transfer activity *in vitro*. Thus, despite all efforts we were not able to prepare a Pdr17 mutant that is fully functional in the non-mitochondrial PS to PE decarboxylation and also lacking PI transfer ability.

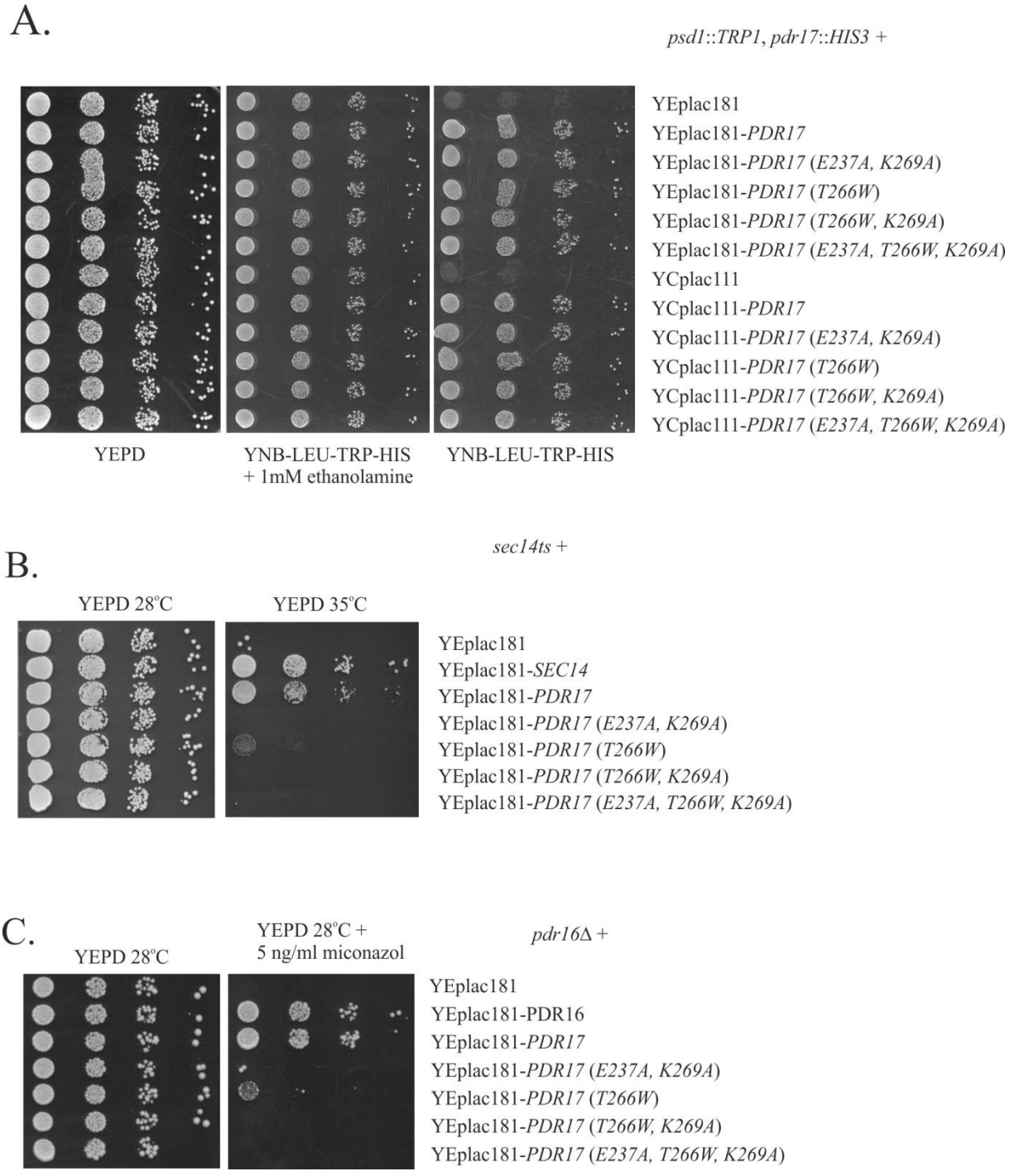

Fig. S1. Complementation of growth phenotypes by Pdr17 mutants. A. Complementation of the disrupted *PDR17* gene. A *psd1::TRP1 pdr17::HIS3* yeast strain auxotrophic for ethanolamine due to disruption of both decarboxylation pathways for PE was transformed with either a YEplac181 multicopy plasmid or a YCplac111 centromeric plasmid containing either wild-type or mutant *PDR17*. The respective yeast strains were pre-grown in selective -LEU media to early stationary phase and subsequently spotted at concentrations of 10^4, 10^3, 10^2, and 10 cells onto YEPD medium, YNB-LEU-TRP-HIS medium containing 1 mM ethanolamine, and YNB-LEU-TRP-HIS medium without ethanolamine. The cells were

allowed to grow for 3 days at 28^{o}C. **B.** A yeast strain with a *SEC14* temperature sensitive allele, *sec14-1ts*, was transformed with a YEplac181 multicopy plasmid containing the *SEC14* gene, wild-type *PDR17*, or the *PDR17* mutant alleles. The respective yeast strains were pre-grown in selective -LEU media to early stationary phase and subsequently spotted at concentrations of 10^4, 10^3, 10^2, and 10 cells onto YEPD medium. The cells were allowed to grow for 48 h at the indicated temperatures; 28^{o}C is the permissive and 35^{o}C is the non-permissive temperature for yeast strain with the *sec14-1ts* allele. **C.** A *pdr16Δ* yeast strain was transformed with a YEplac181 multicopy plasmid containing wild-type *PDR16*, wild-type *PDR17*, and the *PDR17* mutant alleles. The respective yeast strains were pre-grown in selective -LEU media to early stationary phase and subsequently spotted at concentrations of 10^4, 10^3, 10^2, and 10 cells onto YEPD medium with or without miconazol, an inhibitor of sterol biosynthesis.

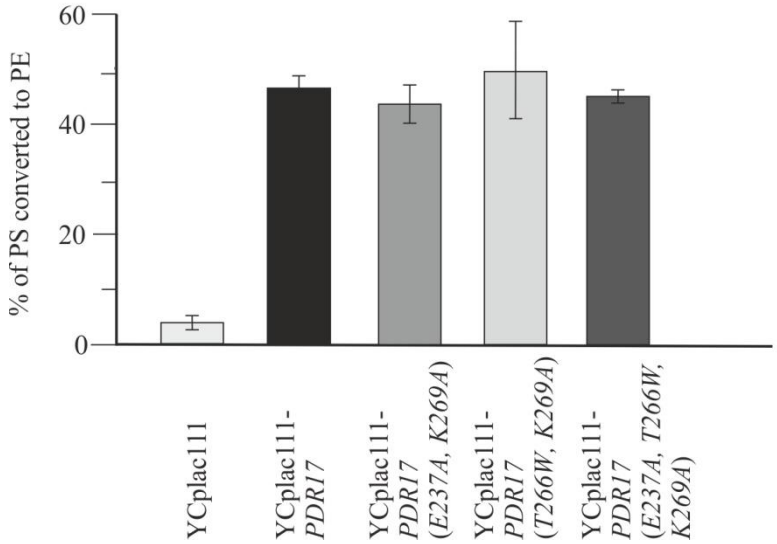

Fig. S2. Radioactive serine incorporation into PS and PE. A *psd1::TRP1 pdr17::HIS3* strain transformed with the given plasmids was labeled with [^{3}H]serine for 30 min and the percentage of [^{3}H]PS converted to [^{3}H]PE was determined (mean ± SD). Three independent experiments were performed.

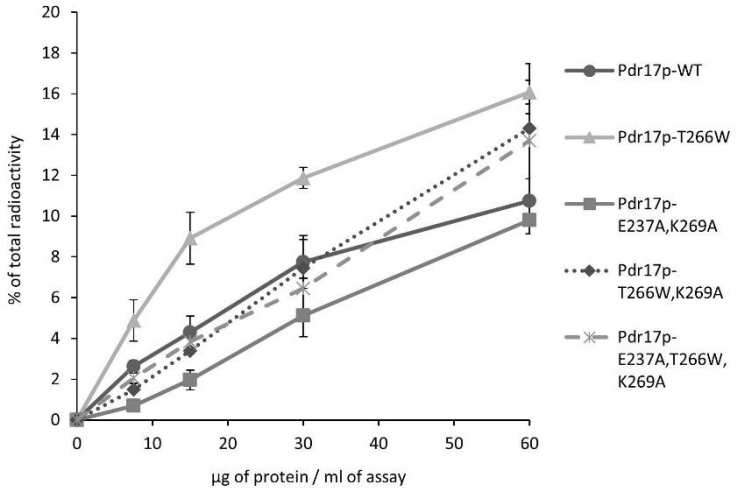

Fig. S3. ***In vitro*** **phosphatidylinositol transfer activity.** Purified recombinant Pdr17, $Pdr17^{E237A,\ K269A}$, $Pdr17^{E237A,\ T266W,\ K269A}$, $Pdr17^{T266W}$, and $Pdr17^{T266W,\ K269A}$ were used in the transfer between radiolabeled microsomes and acceptor liposomes PC:PI (96:4). The results show the average ± SD (n = 4).

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