

Yeast Sec14-like lipid transfer proteins Pdr16 and Pdr17 bind and transfer lanosterol in addition to phosphatidylinositol.

Dominik Šťastný¹, Lívia Petrisková¹, Dana Tahotná¹, Jacob Bauer², Lucia Pokorná¹, Roman Holič¹, Martin Valachovič¹, Vladimír Pevala², Shamshad Cockcroft³, Peter Griač^{1*}

¹ Centre of Biosciences, Institute of Animal Biochemistry and Genetics, Slovak Academy of Sciences, Bratislava, Slovakia

² Institute of Molecular Biology, Slovak Academy of Sciences, Bratislava, Slovakia

³ Department of Neuroscience, Physiology and Pharmacology, Division of Biosciences, University College London, U.K.

*Corresponding author Peter Griač (Peter.Griac@savba.sk)

Abstract

Yeast Sec14-like phosphatidylinositol transfer proteins (PITPs) contain a hydrophobic cavity capable of accepting a single molecule of phosphatidylinositol (PI) or another molecule in a mutually exclusive manner. We report here that two yeast Sec14 family PITPs, Pdr16p (Sfh3p) and Pdr17p (Sfh4p), possess high affinity binding and transfer towards lanosterol. To our knowledge, this is the first identification of lanosterol transfer proteins. In addition, a *pdr16Δpdr17Δ* double mutant had significantly increased level of cellular lanosterol compared to the corresponding wild-type. Based on the lipid profiles of wild-type and *pdr16Δpdr17Δ* cells grown in aerobic and anaerobic conditions we suggest that PI-lanosterol transfer proteins are important predominantly for the optimal functioning of the post lanosterol part of sterol biosynthesis.

Keywords:

Phosphatidylinositol transfer, lanosterol, ergosterol, cholesterol, azol, *Saccharomyces cerevisiae*, PITP, lipid binding

1. Introduction

Lipids can be moved inside the cells *via* vesicular or nonvesicular modes of trafficking. Nonvesicular lipid trafficking is carried out by lipid transfer proteins (LTPs), which shield the hydrophobic parts of lipids and thus enable them to be moved between membranes across an aqueous environment [1]. One class of soluble LTPs are the

phosphatidylinositol transfer proteins (PITPs) [2]. The importance of PITPs is demonstrated by their presence in all eukaryotic organisms studied and by the severe biological consequences associated with their absence. These include neurological defects, loss of phototransduction, defective intracellular trafficking and secretion, developmental defects, and sensory changes [3-6]. PITPs are tightly associated with phosphoinositide signaling [7]. Understanding the cellular function of PITPs is often facilitated by the use of model organisms. In the unicellular eukaryotic yeast model organism *Saccharomyces cerevisiae*, the Sec14 family represents the best studied PITPs [8,9]. In addition to the founding member of the family there are five Sec14p homologues in this yeast. All of them bind and transfer phosphatidylinositol (PI) and one another molecule in a mutually exclusive manner [8,10]: Sec14p and Sfh1p transfer PI and phosphatidylcholine (PC) [10]; Csr1p (Sfh2p) binds PI and squalene [9]; Pdr16p (Sfh3p) and Pdr17p (Sfh4p) bind PI and a sterol molecule [11,12]; and Sfh5p binds PI and a non-lipid molecule heme [13].

In this paper we present results on the lipid binding and transfer properties of the Pdr16 and Pdr17 proteins. Pdr16p localizes predominantly to lipid particles and the cell periphery [14]. The function of Pdr16p in the cell is not entirely clear, however, all available data point to the processes connected with the homeostasis of lipid particles and sterol metabolism [15,16]. The most distinct phenotype observed due to the absence of Pdr16p is an increased sensitivity to inhibitors of the post-lanosterol step of the sterol biosynthetic pathway, azole antifungals and amorolfine [11,17,18]. Importantly, the increased sensitivity of *pdr16Δ* cells to commonly used azole-based antifungal drugs was observed not only in *S. cerevisiae* but also in the pathogenic yeasts *Candida albicans* [19,20], *Candida glabrata* [21], and *Cryptococcus neoformans* [22]. It is thus of immense importance to understand in molecular detail the mechanism by which Pdr16p performs its role in yeast and fungal cells.

Pdr17p is highly homologous to Pdr16p [23]; it plays a role in phospholipid biosynthesis as an essential component in the decarboxylation of phosphatidylserine (PS) to phosphatidylethanolamine (PE) catalyzed by PS decarboxylase 2 [24,25]. Overexpression of Pdr17p can substitute for the absence of Pdr16p in *pdr16Δ* cells in relation to azole sensitivity [14,18], however, overexpression of Pdr16p does not substitute for the missing Pdr17p in the process of PS to PE decarboxylation [26].

Despite considerable effort [24,27] it is not clear which sterol molecules are preferentially bound and transferred by Pdr16p and Pdr17p or how the binding and transfer of

sterol molecules relate to the function of Pdr16p and Pdr17p. Here we present evidence that both Pdr16p and Pdr17p preferentially bind and transfer lanosterol in addition to PI. Simultaneous deletion of the *PDR16* and *PDR17* genes leads to a significant increase in lanosterol, both in the form of free lanosterol and also in the form of lanosteryl esters. Our data indicate a novel connection between these two homologous yeast PITPs and sterol metabolism.

2. Materials and methods

2.1. Media and chemicals

Media components were obtained from Becton-Dickinson (USA), BioLife (Italy) or Formedia (United Kingdom). [^{14}C] sodium acetate (1 mCi/ml) was purchased from American Radiolabeled Chemicals (USA). [^{14}C] lanosterol and [^{14}C] ergosterol were extracted from yeast cells (FY1679-28c) grown in a yeast extract/peptone/dextrose (YEED) medium containing [^{14}C] sodium acetate (30 μl [^{14}C] sodium acetate (1 mCi/ml) per 100 ml of media) and a sub-inhibitory concentration of miconazole (50 ng/ml). Both lipids were resolved on TLC, scraped off the TLC plate and re-extracted from silica gel using chloroform:methanol (2:1). Fine chemicals were mostly from Sigma-Aldrich (USA) or MP Biomedicals (USA).

2.2. Strains and culture conditions

Wild-type *S. cerevisiae* strain FY1679-28c and the *pdr16 Δ* (Mat a, *ura3-52*, *leu2 Δ 1*, *his3 Δ 200*, *trp1 Δ 63*, *GAL2*, *pdr16::hisG*) and *pdr17 Δ* (Mat a, *ura3-52*, *leu2 Δ 1*, *his3 Δ 200*, *trp1 Δ 63*, *GAL2*, *pdr17::HIS3*) mutant strains derived from it, originally prepared in the A. Goffeau laboratory (Catholic University Louvain, Belgium) [18], were kindly provided by G. Daum (Technical University, Graz, Austria). The double deletion strain *pdr16 Δ pdr17 Δ* (Mat a, *ura3-52*, *leu2 Δ 1*, *his3 Δ 200*, *trp1 Δ 63*, *GAL2*, *pdr16::hisG*, *pdr17::HIS3*) in the same genetic background was prepared by integrative transformation of a *pdr17::HIS3* cassette prepared by PCR from the WWY62 strain (kindly donated by D. Voelker, National Jewish Medical and Research Center, Denver, USA) into the *pdr16 Δ* strain.

Pdr16-His tag and Pdr17-His tag recombinant proteins were prepared in *Escherichia coli* Rosetta 2 (DE3) strain (F^- *omp T hsdS $_B$* (r_B^- m_B^-) *gal dcm* pRARE2 (*Cam R*)) from plasmids pET26-*PDR16* and pET26-*PDR17*, respectively. Construction of the pET26-*PDR16* and pET26-*PDR17* plasmids and recombinant protein purification is described in [11,12].

Yeast strains were grown in YEPD media. Anaerobic cultivation was performed in YEPD media in thick-walled flasks closed by special gas-tight rubber stoppers on a rotary shaker at 30 °C for 24 h. Media were degassed by short boiling and then extensively flushed with deoxygenated nitrogen. Traces of oxygen remaining in the medium were removed using L-cysteine (final concentration 400 µg/ml). The media were supplemented with Tween 80 (source of unsaturated fatty acids, final concentration 0.134 %) and ergosterol (final concentration 20 µg/ml) using a gas-tight Hamilton syringe. In order to eradicate any endogenously synthesized ergosterol, 24-hr anaerobic inoculum was used to inoculate the experimental culture for anaerobic experiments.

2.3. Binding of cellular lipids using HL60 cells

The interactions of eukaryotic cellular lipids with the yeast Pdr16 and Pdr17 PITPs were analyzed as described previously [12,28]. Briefly, HL60 cells were labeled with 0.5 µCi/ml [¹⁴C] sodium acetate in RPMI 1640 medium supplemented with 12.5 % heat-inactivated (v/v) fetal bovine serum, 4 mM glutamine, 50 IU/ml of penicillin and 50 µg/ml of streptomycin with or without 2 µg/ml of ketoconazole for 72 h. The cells were permeabilized with streptolysin O, and the leaked cytosol was removed by centrifugation. Permeabilized cells (~10⁷ cells in 500 µl) were incubated with 120 µg of the respective recombinant protein for 20 min at 37 °C in the presence of 2 mM Mg²⁺-ATP. At the end of incubation, the cells were removed by centrifugation, the recombinant proteins in the supernatant were captured on nickel beads, eluted with 500 µl of 500 mM imidazole and desalted. Lipids extracted from re-purified proteins were resolved by thin layer chromatography using a Whatman silica gel 60 TLC plate using a two phase solvent system. The lipids were first separated in Petroleum spirit: Diethyl ether: Acetic acid (70:30:2 v/v) solvent for 20 minutes, followed by Petroleum spirit: Diethyl ether (49:1 v/v) solvent for a further 30 min. Lipids extracted from the permeabilized HL60 cells (approximately 20,000 dpm) were analyzed alongside for comparison. The TLC plates were exposed to Fuji phosphorimaging screens and analyzed using a Bio-Rad Personal Molecular Imager FX system. TLC images were quantified using Quantity One v4.4 (Bio-Rad, US) software.

2.4. Ergosterol and lanosterol transfer assays

Ergosterol and lanosterol *in vitro* transfer was monitored by measuring the transfer of radiolabeled ergosterol or lanosterol from liposomes containing 75 mol % PC, 14 mol % PE, and 11 mol % ¹⁴C ergosterol or ¹⁴C lanosterol to heat inactivated yeast mitochondria using a

slight modification of the method described in [29]. For competition experiments donor liposomes contained 75 mol % PC, 14 mol % PE, 5.5 mol % ^{14}C lanosterol, and either additional 5.5 mol % PC or 5.5 mol % lanosterol or 5.5 mol % zymosterol. Liposomes of defined size and composition were prepared by the extrusion of hydrated lipids (in a buffer containing 20 mM HEPES, 100 mM NaCl, 1 mM EDTA, pH 7.3) through a 100 nm membrane (Mini-extruder, Avanti Polar Lipids, US). Mitochondria were heat-inactivated at 85 °C for 20 min, sedimented at $23,100 \times g$ for 15 min at 4 °C and resuspended in the same buffer as used to prepare liposomes. Liposomes, mitochondria (200 μg of protein) and defined amounts of Pdr16 or Pdr17 proteins were incubated for 40 min at 37 °C. The reaction was terminated by sodium acetate followed by sedimentation of mitochondria at $23,100 \times g$ for 15 min at 4 °C. The rate of transfer was calculated from the decrease of ^{14}C label in the supernatant after correction for nonspecific sterol transfer (determined in the absence of externally added protein). The concentration of donor liposome lipids per assay was 262 μM .

2.5. Yeast lipid extraction and analysis

Total lipids, including free sterols and non-hydrolyzed steryl esters, were extracted by a modified method of Bligh and Dyer [30]. 1.2 ml suspension of 1×10^9 cells in distilled water was disrupted in FastPrep-24TM homogenizer (MP Biomedicals) with glass beads and incubated with 3 ml of methanol for 15 min at 70 °C. 1.5 ml of chloroform was added and lipids were extracted for 2 h at laboratory temperature. The homogenate was centrifuged to sediment cell debris which were re-extracted with a chloroform:methanol:water mixture (1:2:0.8). The collected supernatants were mixed with 1.3 ml of chloroform and 2.6 ml of water. The mixture was centrifuged to separate the phases and the lipids were recovered from the organic phase. Isolated lipids were separated on SPE columns (CHROMABOND® NH₂, Macherey-Nagel) in order to separate free sterols from esterified sterols. The sterol fractions were recovered following Macherey-Nagel application procedure no. 302970 with the following modifications. Steryl esters and squalene were eluted by 3 ml of n-hexane followed by 3 ml of n-hexane : chloroform (9:2, v/v). Both eluents were combined and labeled as the “steryl ester fraction”. Free sterols (together with triacylglycerols and phospholipids) were eluted in two subsequent elutions: 4 ml of n-hexane : diethyl ether (1:1, v/v) and 3 ml of methanol. Both eluents were combined and labeled as the “free sterols fraction”. In order to determine the sterol composition of the steryl esters, the steryl ester fraction was hydrolyzed in methanolic KOH as described previously [31]. Prior to the hydrolyzation procedure, the steryl esters were solubilized in 3% Triton X-100 at 50 °C for 2 h. Sterols in both fractions

(steryl esters and free sterols) were determined separately by reversed phase HPLC on an Agilent 1100 instrument equipped with an Eclipse XDB-C18 column (particle size 5 μm , column size 4.6 \times 250 mm; Agilent Technologies, USA), a diode array detector (Agilent Technologies, USA), and a Corona Charged Aerosol Detector (ESA Inc., USA). The sterols were eluted at 37 $^{\circ}\text{C}$ with 95% methanol at a 1.5 ml/min flow rate. The identities of individual sterol peaks were determined from the retention time of corresponding sterol standards and, in case of ergosterol and ergosta-5,7-dienol, verified by characteristic UV-VIS spectrum. The amounts of sterols were quantified from the values collected from Corona Charged Aerosol Detector based on the calibration curve for ergosterol. Lipids from anaerobically growing yeasts were isolated using the same method but without separation on SPE columns. Purified lipids were resolved by TLC using a two phase solvent system as described above (Section 2.3). Relative quantification of lipids was performed using a CAMAG Linomat 3 TLC scanner with the help of winCAT software.

2.6. Modeling and computational docking

To date, four structures of Pdr16 are available in the PDB (PDB ID 4FMM, 4J7P, 4J7Q, and 4M8Z) [16,32,33]. All four structures are present as dimers, and the eight available monomeric structures are all very similar to one another, with the greatest divergence of 0.337 \AA between chain A of 4FMM and chain B of 4J7P. The greatest differences are in the position of residues 244–267, which form the gating helix and may potentially alter the conformation of the binding site slightly. The two chains within each structure were very similar to one another in all four cases. In light of all these considerations, we chose to take only one chain from each structure (chain A in all cases) for computational docking studies.

At present, there is no Pdr17 crystal structure available. For computational docking, we therefore used a Pdr17 homology model prepared from chain A of 4J7P [33] using Modeller v9.17 as described earlier [12].

Computational docking was carried out using AutoDock Vina 1.2.2 [34,35] in two stages. In the first stage, the ligand was allowed to be flexible while the protein was kept rigid and the docking search area was over the entire lipid binding cavity. In the second stage, the search area was reduced to cover the region containing the highest affinity binding site and the side-chains of all residues that came within 4 \AA of the ligand were allowed to be flexible. The ligands were taken from the PDB structures 6UEZ (lanosterol) [36] and 2AIB (ergosterol) [37]. The structures of the ligands and receptors were prepared for docking using

MGLtools 1.5.7 [38]. Visualization of the results was done using PyMOL Molecular Graphics System Ver. 2.5.0 (Schrödinger LLC.).

3. Results and discussion

3.1. Lipid binding

In our previous studies [11,12] we observed that Pdr16p and Pdr17p bind a neutral lipid, most likely cholesterol, in addition to PI. The method used was an *in situ* lipid binding assay using human HL60 cells [28,39]. The assay is based on co-incubating the LTP of interest with radiolabeled, permeabilized HL60 cells followed by re-purification of the LTP and subsequent extraction of the lipids associated with it. The extracted lipids are then analyzed by thin layer chromatography (TLC) and autoradiography. As we were originally interested in the PI bound by Pdr16p and Pdr17p, the extracted lipids were previously resolved in conditions favorable for the TLC resolution of phospholipids [11,12]. To better analyze bound neutral lipids, the extracted lipids are now resolved in conditions that separate different neutral lipids. We observed that Pdr16p and Pdr17p bind cholesterol and lanosterol (Fig. 1, lanes “Pdr16 DMSO” and “Pdr17 DMSO”; a small amount of DMSO was used in untreated HL60 cells because ketoconazole was dissolved in this solvent). Lanosterol is a sterol precursor in the biosynthetic pathway leading to cholesterol (to ergosterol in yeast). It is present in very limited amounts in HL60 cells (Fig. 1, line “HL60 DMSO”). Despite the low presence of lanosterol in HL60 cells, Pdr16p and Pdr17p bind approximately the same amount of lanosterol as cholesterol (Fig. 1, lanes “Pdr16 DMSO” and “Pdr17 DMSO”). To increase the amount of lanosterol in HL60 cells, the cells were grown in the presence of a sub-inhibitory concentration of ketoconazole, an inhibitor of lanosterol 14- α demethylase, which is responsible for conversion of lanosterol to 4,4-dimethylcholesta-8, 14, 24-trienol [40]. Under these conditions HL60 cells accumulate lanosterol at the expense of cholesterol, as expected (see lanes “HL60 KETO” and “HL60 DMSO”). Comparing the amounts of cholesterol and lanosterol bound to Pdr16p and Pdr17p and the cholesterol and lanosterol present in HL60 cells, we concluded that lanosterol is the preferred neutral lipid bound by Pdr16 and Pdr17 proteins and represents the preferred counter ligand of these LTPs.

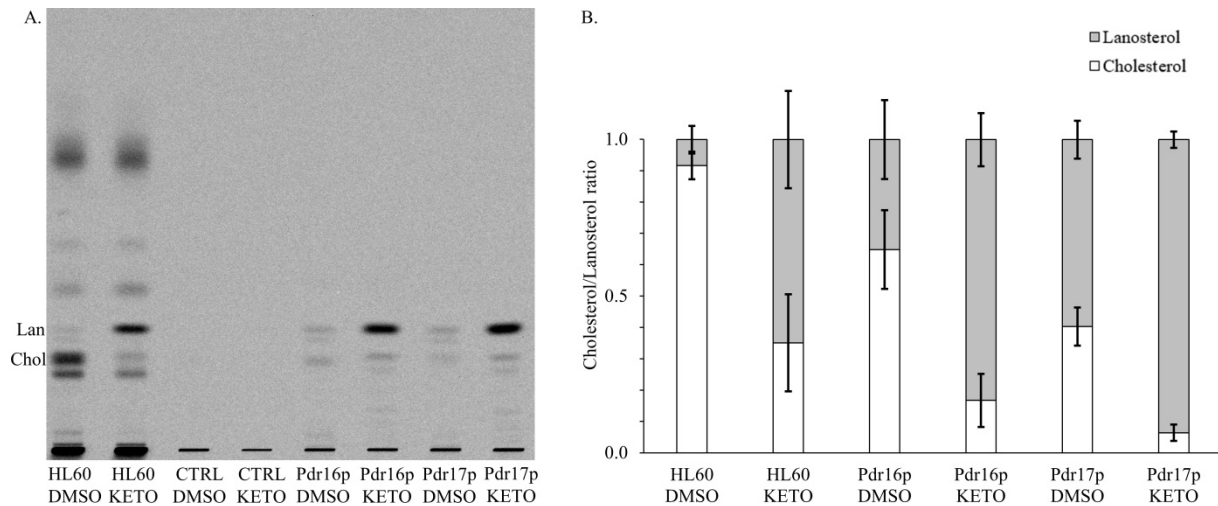


Fig. 1 Lipid binding of Pdr16 and Pdr17 proteins. (A) Recombinant Pdr16 and Pdr17 proteins (at 120 μg) were incubated with permeabilized HL60 cells pre-labeled with [^{14}C] sodium acetate for 72 h in the presence of ketoconazole (data labeled KETO) or in the absence of ketoconazole (data labeled DMSO). Proteins were re-isolated using nickel beads following this co-incubation. The lipids bound to re-isolated proteins were extracted and separated by TLC (data labeled Pdr16p or Pdr17p). In the “CTRL” sample, no protein was added to the HL60 cells. (B) The ratio between radiolabeled cholesterol to lanosterol in HL60 cells or bound to Pdr16p or Pdr17p is shown. The data represent the mean \pm S.D. from three independent experiments; one of which is depicted in panel A. Abbreviations: Chol, Cholesterol; Lan, Lanosterol; DMSO, HL60 cells were grown in the presence of 0.02 % DMSO – the solvent used to dissolve ketoconazole; KETO, HL60 cells were grown in the presence of 2 $\mu\text{g}/\text{ml}$ ketoconazole.

3.2. Lipid transfer

Based on the lipid binding results in HL60 cells we decided to explore the *in vitro* ergosterol and lanosterol lipid transfer activity of Pdr16p and Pdr17p. The method used is a minor modification of published procedures (for details see Materials and methods). Briefly, 100 nm small unilamellar vesicles containing ^{14}C ergosterol or ^{14}C lanosterol served as donor membranes for the lipids to be transferred. Lipid transfer assays were then conducted using heat inactivated yeast mitochondria as acceptor membranes [29]. We decided to compare the ergosterol and lanosterol transfer activity of Pdr16p and Pdr17p because ergosterol is the major yeast sterol and lanosterol shows high binding affinity to both Pdr16p and Pdr17p in HL60 cells. As can be seen from Fig. 2A, both proteins transfer some ergosterol, but less than 10% of the ^{14}C ergosterol input. For Pdr17p (Sfh4p), the ergosterol transfer results agree with the published data [9]; for Pdr16p (Sfh3p), the ergosterol transfer in our experiments is lower

than that reported previously [9,16]. The lower ergosterol transfer for Pdr16p in our conditions might arise from the different composition of donor liposomes between the two studies: ours was optimized for Pdr17p transfer. Nevertheless, the data show that both LTPs possess massive lanosterol transfer activity which manifests itself even at low protein concentrations. Thus, the overall conclusion from lipid binding and transfer assays is that Pdr16p and Pdr17p prefer lanosterol to ergosterol as their second ligand in addition to PI. Especially noteworthy is the very effective lanosterol transfer activity that both these proteins possess. To compare the relative transfer of lanosterol to other ergosterol biosynthesis precursors, competition experiments were performed in which zymosterol was added to donor liposomes together with radioactive ^{14}C lanosterol (Fig. 2B ^{14}C -Lan:Zym). In control samples no non-radioactive sterol was added (Fig. 2B ^{14}C -Lan) or in addition to ^{14}C lanosterol also non-radioactive lanosterol was added (Fig. 2B ^{14}C -Lan:Lan). The competition experiments were performed at protein concentration of 5 $\mu\text{g}/\text{mL}$. As expected, addition of non-radiolabeled lanosterol competes with transfer of ^{14}C lanosterol facilitated by Pdr16p and Pdr17p. Addition of the same amount of non-radioactive zymosterol to donor liposomes decreases ^{14}C lanosterol transfer in the same way as addition of non-radioactive lanosterol. This indicates that zymosterol (and possibly other structurally similar ergosterol biosynthesis precursors) can also be accommodated into the lipid binding cavity of Pdr16p and Pdr17p.

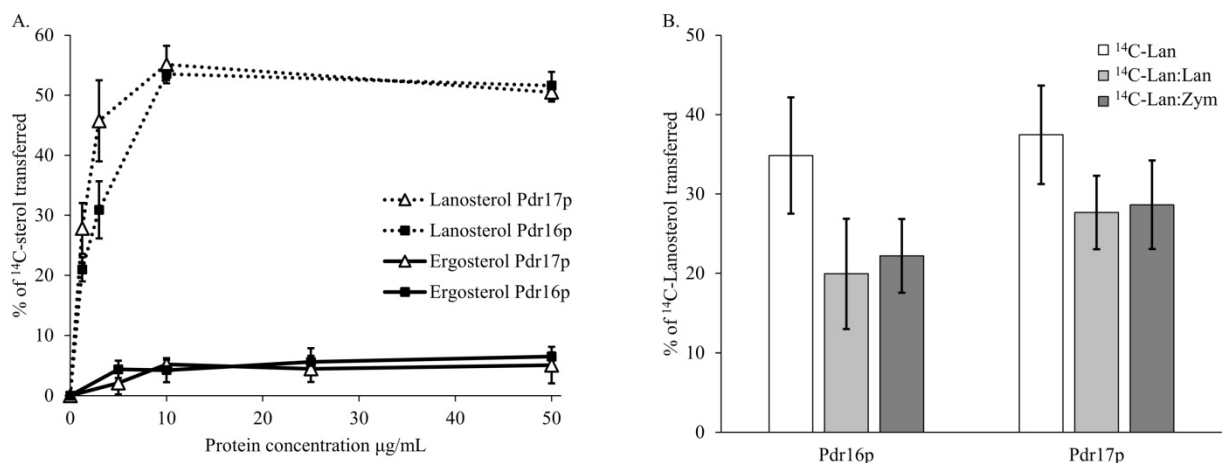


Fig. 2 *In vitro* Pdr16p and Pdr17p ergosterol and lanosterol transfer activity. (A) Purified recombinant Pdr16 and Pdr17 proteins were used in the transfer process between liposomes containing either radiolabeled ergosterol or lanosterol and heat inactivated acceptor mitochondria. The composition of donor liposomes was 75 mol % PC 14 mol % PE 11 mol % ^{14}C Ergosterol or ^{14}C Lanosterol. 100% represents the total radioactivity in the assay. The results show the average from three independent experiments, each performed in duplicates \pm

S.D. **(B)** ^{14}C lanosterol transfer competition experiment. ^{14}C lanosterol transfer was measured as described in legend to Panel A with the following modifications: composition of donor liposomes was 75 mol % PC 14 mol % PE 5 mol % ^{14}C Lanosterol + additional 5 mol % PC (data marked ^{14}C -Lan) or 5 mol % lanosterol (data marked ^{14}C -Lan:Lan) or 5 mol % zymosterol (data marked ^{14}C -Lan:Zym). The amount of lipid transfer proteins in the assay was 5 $\mu\text{g}/\text{mL}$. The results show the average from four independent experiments \pm S.D.

3.3. Structural features of Pdr16p and Pdr17p with bound ergosterol and lanosterol

To determine the possible structural features of lanosterol and ergosterol binding to Pdr16p and Pdr17p, we carried out molecular docking on all presently known Pdr16p structures and a homology model of Pdr17p. Unfortunately, both lanosterol and ergosterol showed similar behavior during docking, so these results cannot be used to describe structurally why either protein prefers lanosterol to ergosterol. For this reason, only lanosterol binding will be described. Pdr16p and Pdr17p contain a single large lipid-binding cavity. In one of the available Pdr16p crystal structures, 4J7Q [33], this cavity contains PI. The cavity has a more polar or charged edge where the inositol and phosphate groups are bound and a large hydrophobic cleft where the fatty-acid chain is found. Since Pdr16 and Pdr17 are known to bind and transfer PI and a sterol in a mutually exclusive manner [11,12], it was assumed that PI and the sterol occupy the same binding cavity. The docking analysis showed that for both Pdr16p and Pdr17p, two different conformations had nearly the same affinity (Fig. 3). For Pdr16p, the preferred conformation has an affinity of 25.9 kcal/mol and places lanosterol into the central, more hydrophobic part of the lipid binding cavity where there is the fatty-acid part of PI in case of PI binding (Supplementary Fig. 1). In this conformation, residues Leu-158, Leu-160, Leu-177, Leu-181, Leu-195, Leu-197, Ile-199, Leu-229, Tyr-233, Arg-236, and Leu-237 form contacts with the bound sterol, and it is fairly clear that the L158Y and L160Y mutations [9] would prevent sterol binding (Fig. 3A). The second conformation has slightly higher affinity (26.1 kcal/mol) and places the hydroxyl-containing A ring of the sterol into a position close to the phosphate and inositol binding pocket. In this conformation, residues Lys-98, Arg-107, Glu-235, Pro-234, and Glu-230 make additional contacts while residues Leu-160, Leu-177, Leu-181, Leu-195, and Ile-199 become less important (Fig. 3B). In particular, Leu-160 no longer directly contacts the sterol, and a L160Y mutation may not prevent lanosterol from binding (Fig. 3B).

The affinities of both Pdr17p conformations are significantly lower (14.8 kcal/mol for the preferred conformation and 14.9 kcal/mol for the secondary one). In the preferred conformation, lanosterol binds at the end of the cavity away from the phosphate and inositol binding site, in an area involving residues Lys-148, Gln-149, Asn-166, Gln-169, Gln-177, Leu-181, Phe-205, Ser-207, Pro-219, Ile-221, Ile-223, and Cys-227. Pdr17 may also bind lanosterol in a location similar to that of Pdr16p (Fig. 3D). In this position, residues Arg-100, Met-231, Gln-232, Glu-237, Leu-239, Cys-242, Thr-266, and Lys-269 appear to be involved in binding the sterol, while none of the residues in the previous conformation are.

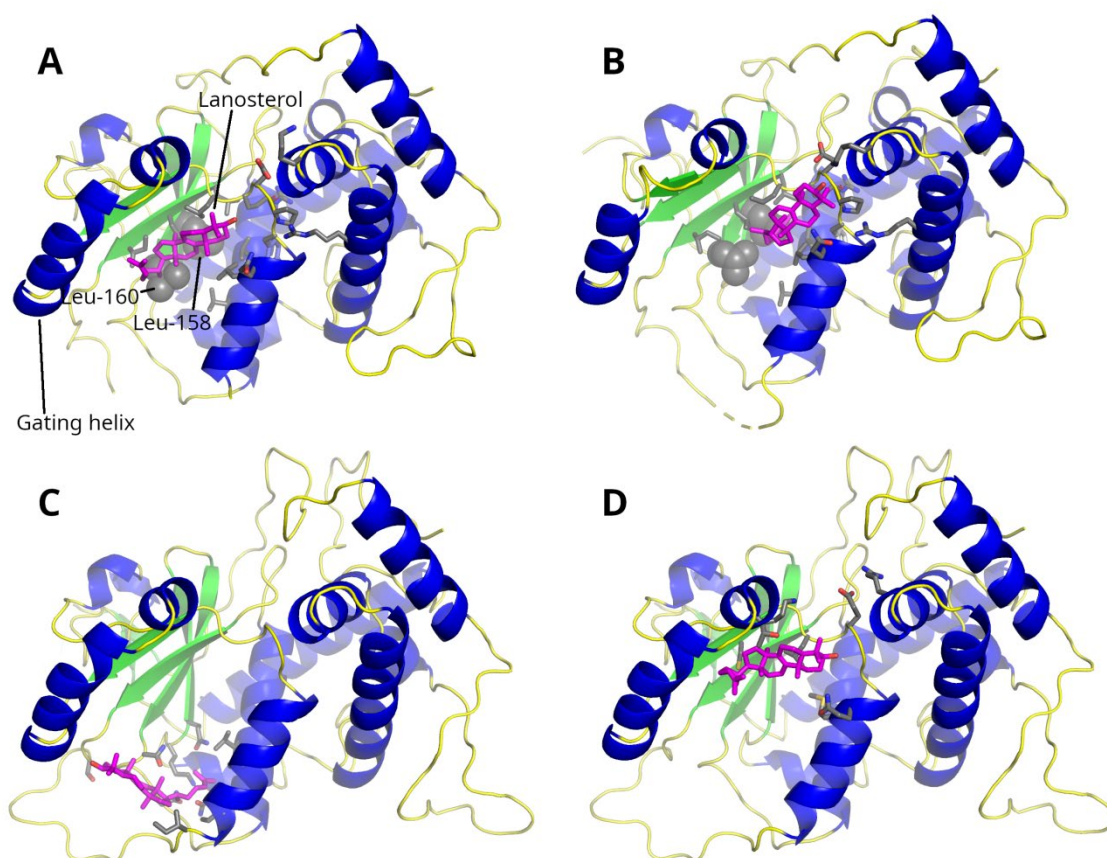


Fig. 3 The most likely binding sites for lanosterol in Pdr16p and Pdr17p. (A) The most likely binding site for lanosterol in Pdr16p based on molecular docking calculations. Lanosterol (magenta) binds to the hydrophobic fatty-acid binding pocket, making contact with residues Leu-158, Leu-160, Leu-177, Leu-181, Leu-195, Leu-197, Ile-199, Leu-229, Tyr-233, Arg-236, and Leu-237 (gray). Residues Leu-158 and Leu-160, whose mutation to tyrosine abolishes sterol transfer as referred to in [9], are shown as van der Waals spheres. The gating helix containing residues 245–267 is indicated. The Pdr16p structure shown is PDB ID 4M8Z [32]. (B) A secondary conformation in which lanosterol binds higher up in the binding cleft,

placing the hydroxyl-containing ring A of the sterol near the phosphate and inositol binding pocket. In this conformation, residues Lys-98, Arg-107, Glu-235, Pro-234, and Glu-230 make contacts, while residues Leu-160, Leu-177, Leu-181, Leu-195, and Ile-199 become less important. The Pdr16p structure shown here is 4FMM [33]. **(C)** The most likely binding site for lanosterol in Pdr17p. Lanosterol is predicted to bind at the end of the cavity distal from the phosphate and inositol binding pocket. The residues which may be involved in binding include Lys-148, Gln-149, Asn-166, Gln-169, Gln-177, Leu-181, Phe-205, Ser-207, Pro-219, Ile-221, Ile-223, and Cys-227 (gray). **(D)** A secondary conformation in which lanosterol binds in a position similar to that predicted for Pdr16p. Here residues Arg-100, Met-231, Gln-232, Glu-237, Leu-239, Cys-242, Thr-266, and Lys-269 appear to be involved in binding.

3.4. Sterol composition of yeast strains in the absence of Pdr16p and/or Pdr17p

Next, we determined the changes in sterol composition of the *pdr16Δ*, *pdr17Δ*, and *pdr16Δ pdr17Δ* mutants compared to wild-type yeast cells. Yeast cells contain sterols in two distinct forms, as free sterols and as esterified sterols (sterols conjugated with fatty acids). Most free sterols are localized to the plasma membrane, while the esterified sterols are stored in the core of lipid droplets (Fig. 4). Yeast cells esterify sterols especially when sterol biosynthesis exceeds their actual need to incorporate sterols into membranes, as, for example, during early stationary phase of growth. In this case the sterol esters serve as a depot for future needs. Yeast also esterify and store in lipid particles sterols that are not suitable for maintaining optimal membrane properties [41,42]. In order to analyze these two sterol fractions separately, we first extracted the total lipids and subsequently separated the sterols and sterol esters by solid phase extraction. Following the hydrolysis of sterol esters, the two pools of sterols were analyzed using HPLC. These analyses showed some decrease in ergosterol in all three mutant strains analyzed. The double mutant *pdr16Δ pdr17Δ* exhibits a high increase in cellular lanosterol in the form of free lanosterol and also in the form of lanosterol esters (Fig. 5A). Quite surprisingly, about one fifth of the total cellular ergosterol is also esterified in the *pdr16Δ pdr17Δ* mutant. A similar increase in lanosterol level in the subcellular fraction of the purified plasma membrane of a *pdr16Δ pdr17Δ* mutant was also reported previously by van den Hazel et al. [18]. None of the other ergosterol precursors accumulates in cells lacking Pdr16p and Pdr17p (Supplementary Fig. 2).

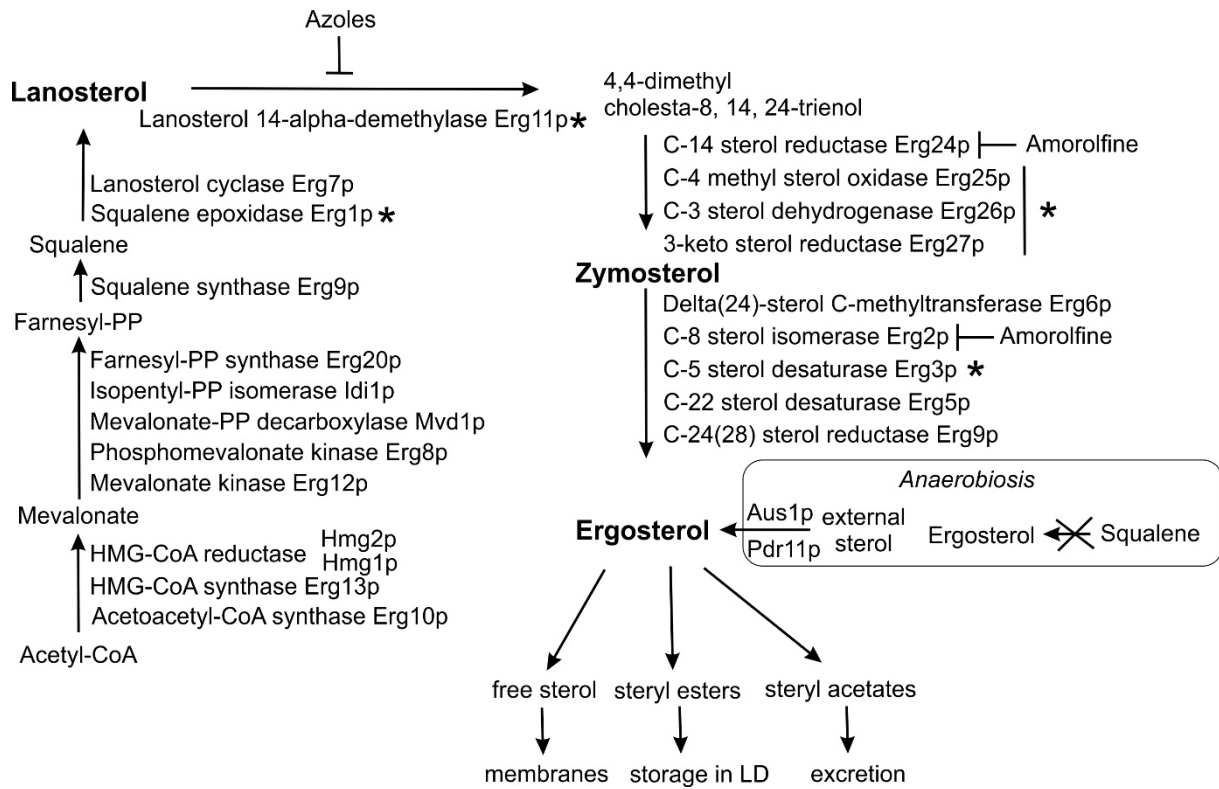


Fig. 4 Ergosterol metabolism in the yeast *Saccharomyces cerevisiae*. The biosynthesis of ergosterol starts with two molecules of acetyl-CoA and requires oxygen / heme at the enzymatic steps marked with asterisks. In anaerobiosis no ergosterol is internally produced and all sterols are imported from external sources with the help of the plasma membrane transporters Aus1p and Pdr11p. In addition to incorporating sterols into membranes, yeast cells are able to store them in the form of steryl esters in lipid droplets or to excrete them as steryl acetates. The targets of two inhibitor classes of the sterol biosynthetic pathway, azoles and morpholines, are also indicated. CoA, Coenzyme A; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; P, phosphate; LD, Lipid droplets. Based on [42] and [41].

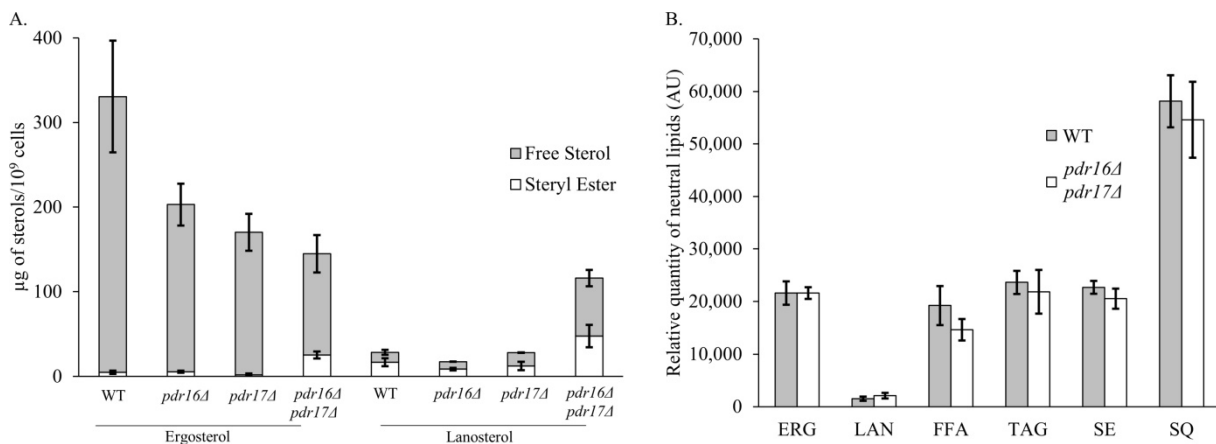


Fig. 5 Lipid analyses. (A) The ergosterol and lanosterol content of aerobically grown yeast cells were analyzed using reversed phase HPLC. Prior to HPLC analysis, steryl esters and free sterols were separated on SPE columns according to Materials and methods. **(B)** TLC lipid analysis of cells growing anaerobically in the presence of externally added ergosterol. Relative quantification of lipids was performed using a CAMAG Linomat 3 TLC scanner. The results show the average from three independent experiments, each performed in duplicates \pm S.D. Abbreviations: ERG – ergosterol; LAN – lanosterol; FFA – free fatty acids; TAG – triacylglycerols; SE – steryl esters; SQ - squalene

Sterols are essential components of eukaryotic cellular membranes. The yeast *S. cerevisiae* synthesizes ergosterol under aerobic conditions through a highly conserved and complex chain of reactions (Fig. 4) (reviewed in [42]). As sterol biosynthesis is strictly dependent on oxygen, the biosynthesis of ergosterol is blocked in anaerobiosis at the step where squalene is converted to squalene epoxide. Under low oxygen conditions, yeast cells cannot synthesize ergosterol and instead sterols are imported from the external environment [43,44]. Thus, growing yeast cells defective in Pdr16p and Pdr17p under anaerobic conditions and comparing the lipid composition of the *pdr16 Δ pdr17 Δ* double mutant in aerobic vs. anaerobic conditions enables to determine whether the absence of Pdr16p and Pdr17p disrupts ergosterol biosynthesis or if it predominantly influences sterol turnover and storage, as was suggested previously [16]. The results depicted in Fig. 5B show that the sterol profile of the *pdr16 Δ pdr17 Δ* double mutant is largely indistinguishable from that of the parental wild-type strain when both were grown anaerobically in the presence of external ergosterol, i.e. in conditions that effectively bypass internal ergosterol biosynthesis. In contrast, the sterol profile of *pdr16 Δ pdr17 Δ* double mutant cells in aerobiosis, when cells depend solely on the internal biosynthesis of ergosterol, is considerably different from that of the parental strain (Fig. 5A, Supplementary Fig. 2). It is also worth noting that the growth rate of the *pdr16 Δ pdr17 Δ* double mutant is much slower than that of its parental wild-type strain under aerobic conditions (the doubling time of an exponentially growing wild-type culture was approximately 1.5 h in contrast to 2.5 h for a *pdr16 Δ pdr17 Δ* culture). Under anaerobic conditions, however, the growth rate of the *pdr16 Δ pdr17 Δ* double mutant strain was exactly the same as the growth rate of its parental strain. Together, our data suggest that the effect of

the simultaneous absence of both PI-lanosterol transfer proteins Pdr16p and Pdr17p is predominantly manifested in the post-lanosterol part of the ergosterol biosynthetic pathway. The presence of esterified ergosterol in aerobically growing *pdr16Δ pdr17Δ* double mutant cells also indicates some defect in steryl ester homeostasis, as suggested earlier [16].

4. Conclusions

We show here that the second ligand of the yeast Sec14-like PITP proteins Pdr16 and Pdr17 is lanosterol, a precursor in the biosynthetic pathway leading to the main yeast sterol, ergosterol. To our knowledge, this is the first identification of a lanosterol transfer protein. Lanosterol transfer competition experiments indicate that, in addition to lanosterol, also other ergosterol precursors with the structure and properties similar to lanosterol (e. g. zymosterol) may be accommodated into the lipid binding cavity or be transferred by Pdr16p and Pdr17p. A comparative lipid analyses of *pdr16Δ pdr17Δ* double mutant cells grown aerobically (internal biosynthesis of ergosterol) and anaerobically (uptake of sterols from the environment) demonstrate that it is the actual biosynthesis of ergosterol that is affected by Pdr16p and Pdr17p. Our results suggest a new connection between PITPs and sterol biosynthesis. The precise mechanism by which these PITPs influence sterol metabolism remains to be established, however. It is possible that the actual lanosterol transfer activity is required for effective ergosterol biosynthesis, as the individual enzymes of the ergosterol biosynthetic pathway are localized not only to the endoplasmic reticulum but also to other organelles, mostly to lipid particles [45-47]. Alternatively, the PI transfer activity of these PITPs may be important in some regulatory aspects of ergosterol biosynthesis. Another possibility is that interplay between lanosterol and PI binding and/or transfer has a sensory or regulatory role in sterol biosynthesis.

Acknowledgements:

This research was supported by the Scientific Grant Agency of the Ministry of Education, Science, Research and Sport of the Slovak Republic and the Slovak Academy of Sciences grants VEGA 2/0027/19, VEGA 2/0106/20, and VEGA 2/0131/20; the Slovak Research and Development Agency contracts Nos. APVV-19-0094, APVV-20-0166, and the Interreg V-A Slovakia-Austria program (www.sk-at.eu) for the project StruBioMol, ITMS: 305011X666 and is co-financed by the European Regional Development Fund. We thank G. Daum (Technical University Graz, Austria) and D. Voelker (National Jewish Medical and Research Center, Denver, USA) for kindly providing yeast strains used in this study. Marta

Kostolanská, Petronela Melicherová and Katarína Nagyová are acknowledged for valuable technical help.

Author contributions

VP, SC, MV and PG conceived and supervised the study; RH, MV, SC and PG designed experiments; DS, LP, DT, LP, RH performed experiments; JB and VP performed molecular modeling; DS, LP, DT, LP, RH, MV, SC and PG analysed data; DS, JB and PG wrote the manuscript

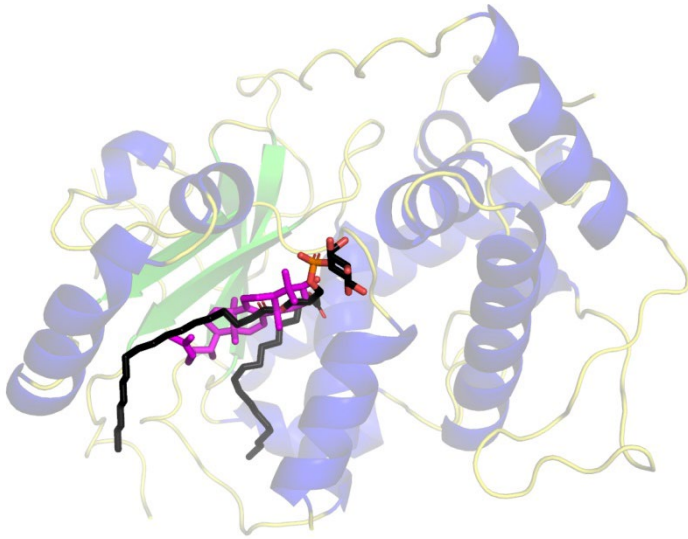
References:

- [1] Wong, L.H., Gatta, A.T. and Levine, T.P. (2019). Lipid transfer proteins: the lipid commute via shuttles, bridges and tubes. *Nat Rev Mol Cell Biol* 20, 85-101.
- [2] Ashlin, T.G., Blunsom, N.J. and Cockcroft, S. (2021). Courier service for phosphatidylinositol: PITPs deliver on demand. *Biochim Biophys Acta Mol Cell Biol Lipids* 1866, 158985.
- [3] Alb, J.G., Jr., Phillips, S.E., Wilfley, L.R., Philpot, B.D. and Bankaitis, V.A. (2007). The pathologies associated with functional titration of phosphatidylinositol transfer protein alpha activity in mice. *J Lipid Res* 48, 1857-72.
- [4] Cockcroft, S. (2012). The diverse functions of phosphatidylinositol transfer proteins. *Curr Top Microbiol Immunol* 362, 185-208.
- [5] Ghosh, R. et al. (2015). Sec14-nodulin proteins and the patterning of phosphoinositide landmarks for developmental control of membrane morphogenesis. *Mol Biol Cell* 26, 1764-81.
- [6] Hamilton, B.A. et al. (1997). The vibrator mutation causes neurodegeneration via reduced expression of PITP alpha: positional complementation cloning and extragenic suppression. *Neuron* 18, 711-22.
- [7] Pemberton, J.G., Kim, Y.J., Humpolickova, J., Eisenreichova, A., Sengupta, N., Toth, D.J., Boura, E. and Balla, T. (2020). Defining the subcellular distribution and metabolic channeling of phosphatidylinositol. *J Cell Biol* 219
- [8] Holic, R., Stastny, D. and Griac, P. (2021). Sec14 family of lipid transfer proteins in yeasts. *Biochim Biophys Acta Mol Cell Biol Lipids* 1866, 158990.
- [9] Tripathi, A. et al. (2019). Functional diversification of the chemical landscapes of yeast Sec14-like phosphatidylinositol transfer protein lipid-binding cavities. *J Biol Chem* 294, 19081-19098.
- [10] Li, X. et al. (2000). Identification of a novel family of nonclassic yeast phosphatidylinositol transfer proteins whose function modulates phospholipase D activity and Sec14p-independent cell growth. *Mol Biol Cell* 11, 1989-2005.
- [11] Holic, R. et al. (2014). Phosphatidylinositol binding of *Saccharomyces cerevisiae* Pdr16p represents an essential feature of this lipid transfer protein to provide protection against azole antifungals. *Biochim Biophys Acta* 1842, 1483-90.
- [12] Pevalova, Z. et al. (2019). Yeast phosphatidylinositol transfer protein Pdr17 does not require high affinity phosphatidylinositol binding for its cellular function. *Biochim Biophys Acta Mol Cell Biol Lipids* 1864, 1412-1421.

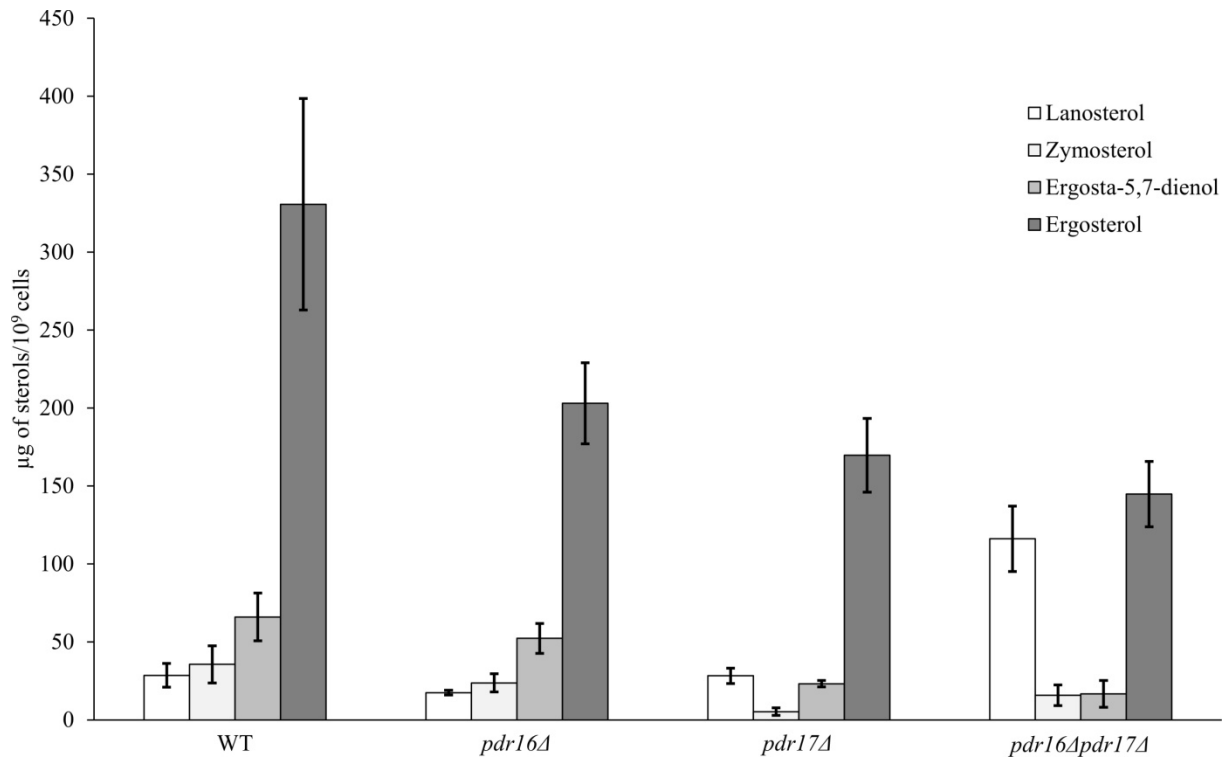
- [13] Khan, D. et al. (2020). A Sec14-like phosphatidylinositol transfer protein paralogue defines a novel class of heme-binding proteins. *Elife* 9
- [14] Schnabl, M. et al. (2003). Subcellular localization of yeast Sec14 homologues and their involvement in regulation of phospholipid turnover. *Eur J Biochem* 270, 3133-45.
- [15] Moldavski, O., Amen, T., Levin-Zaidman, S., Eisenstein, M., Rogachev, I., Brandis, A., Kaganovich, D. and Schuldiner, M. (2015). Lipid Droplets Are Essential for Efficient Clearance of Cytosolic Inclusion Bodies. *Dev Cell* 33, 603-10.
- [16] Ren, J. et al. (2014). A phosphatidylinositol transfer protein integrates phosphoinositide signaling with lipid droplet metabolism to regulate a developmental program of nutrient stress-induced membrane biogenesis. *Mol Biol Cell* 25, 712-27.
- [17] Simova, Z., Poloncova, K., Tahotna, D., Holic, R., Hapala, I., Smith, A.R., White, T.C. and Griac, P. (2013). The yeast *Saccharomyces cerevisiae* Pdr16p restricts changes in ergosterol biosynthesis caused by the presence of azole antifungals. *Yeast* 30, 229-41.
- [18] van den Hazel, H.B., Pichler, H., do Valle Matta, M.A., Leitner, E., Goffeau, A. and Daum, G. (1999). PDR16 and PDR17, two homologous genes of *Saccharomyces cerevisiae*, affect lipid biosynthesis and resistance to multiple drugs. *J Biol Chem* 274, 1934-41.
- [19] Bencova, A., Goffa, E., Morvova, M., Valachovic, M., Griac, P., Toth Hervay, N. and Gbelska, Y. (2020). The Absence of PDR16 Gene Restricts the Overexpression of CaSNQ2 Gene in the Presence of Fluconazole in *Candida albicans*. *Mycopathologia* 185, 455-465.
- [20] Saidane, S., Weber, S., De Deken, X., St-Germain, G. and Raymond, M. (2006). PDR16-mediated azole resistance in *Candida albicans*. *Mol Microbiol* 60, 1546-62.
- [21] Culakova, H., Dzugasova, V., Perzelova, J., Gbelska, Y. and Subik, J. (2013). Mutation of the CgPDR16 gene attenuates azole tolerance and biofilm production in pathogenic *Candida glabrata*. *Yeast* 30, 403-14.
- [22] Ngamskulrungraj, P., Chang, Y., Hansen, B., Bugge, C., Fischer, E. and Kwon-Chung, K.J. (2012). Characterization of the chromosome 4 genes that affect fluconazole-induced disomy formation in *Cryptococcus neoformans*. *PLoS One* 7, e33022.
- [23] Griac, P. (2007). Sec14 related proteins in yeast. *Biochim Biophys Acta* 1771, 737-45.
- [24] Wang, Y. et al. (2020). Noncanonical regulation of phosphatidylserine metabolism by a Sec14-like protein and a lipid kinase. *J Cell Biol* 219
- [25] Wu, W.I., Routt, S., Bankaitis, V.A. and Voelker, D.R. (2000). A new gene involved in the transport-dependent metabolism of phosphatidylserine, PSTB2/PDR17, shares sequence similarity with the gene encoding the phosphatidylinositol/phosphatidylcholine transfer protein, SEC14. *J Biol Chem* 275, 14446-56.
- [26] Routt, S.M., Ryan, M.M., Tyeryar, K., Rizzieri, K.E., Mousley, C., Roumanie, O., Brennwald, P.J. and Bankaitis, V.A. (2005). Nonclassical PITPs activate PLD via the Stt4p PtdIns-4-kinase and modulate function of late stages of exocytosis in vegetative yeast. *Traffic* 6, 1157-72.
- [27] Maeda, K., Anand, K., Chiapparino, A., Kumar, A., Poletto, M., Kaksonen, M. and Gavin, A.C. (2013). Interactome map uncovers phosphatidylserine transport by oxysterol-binding proteins. *Nature* 501, 257-61.
- [28] Cockcroft, S. (2009). Measurement of phosphatidylinositol and phosphatidylcholine binding and transfer activity of the lipid transport protein PITP. *Methods Mol Biol* 462, 363-77.
- [29] Bloj, B. and Zilversmit, D.B. (1977). Rat liver proteins capable of transferring phosphatidylethanolamine. Purification and transfer activity for other phospholipids and cholesterol. *J Biol Chem* 252, 1613-9.
- [30] Bligh, E.G. and Dyer, W.J. (1959). A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 37, 911-7.
- [31] Valachovic, M. and Hapala, I. (2017). Biosynthetic Approaches to Squalene Production: The Case of Yeast. *Methods Mol Biol* 1494, 95-106.
- [32] Yuan, Y., Zhao, W., Wang, X., Gao, Y.X., Niu, L.W. and Teng, M.K. (2013). Dimeric Sfh3 has structural changes in its binding pocket that are associated with a dimermonomer state

- transformation induced by substrate binding. *Acta Crystallographica Section D-Structural Biology* 69, 313-323.
- [33] Yang, H., Tong, J., Leonard, T.A. and Im, Y.J. (2013). Structural determinants for phosphatidylinositol recognition by Sfh3 and substrate-induced dimer-monomer transition during lipid transfer cycles. *FEBS Lett* 587, 1610-6.
- [34] Eberhardt, J., Santos-Martins, D., Tillack, A.F. and Forli, S. (2021). AutoDock Vina 1.2.0: New Docking Methods, Expanded Force Field, and Python Bindings. *J Chem Inf Model* 61, 3891-3898.
- [35] Trott, O. and Olson, A.J. (2010). AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J Comput Chem* 31, 455-61.
- [36] Hargrove, T.Y., Wawrzak, Z., Guengerich, F.P. and Lepesheva, G.I. (2020). A requirement for an active proton delivery network supports a compound I-mediated C-C bond cleavage in CYP51 catalysis. *J Biol Chem* 295, 9998-10007.
- [37] Rodrigues, M.L. et al. (2006). Crystal structures of the free and sterol-bound forms of beta-cinnamomin. *Biochim Biophys Acta* 1764, 110-21.
- [38] Sanner, M.F. (1999). Python: a programming language for software integration and development. *J Mol Graph Model* 17, 57-61.
- [39] Segui, B., Allen-Baume, V. and Cockcroft, S. (2002). Phosphatidylinositol transfer protein beta displays minimal sphingomyelin transfer activity and is not required for biosynthesis and trafficking of sphingomyelin. *Biochem J* 366, 23-34.
- [40] Nes, W.D. (2011). Biosynthesis of cholesterol and other sterols. *Chem Rev* 111, 6423-51.
- [41] Hapala, I., Griac, P. and Holic, R. (2020). Metabolism of Storage Lipids and the Role of Lipid Droplets in the Yeast *Schizosaccharomyces pombe*. *Lipids* 55, 513-535.
- [42] Klug, L. and Daum, G. (2014). Yeast lipid metabolism at a glance. *FEMS Yeast Res* 14, 369-88.
- [43] Lorenz, R.T. and Parks, L.W. (1987). Regulation of ergosterol biosynthesis and sterol uptake in a sterol-auxotrophic yeast. *J Bacteriol* 169, 3707-11.
- [44] Raychaudhuri, S. and Prinz, W.A. (2006). Uptake and trafficking of exogenous sterols in *Saccharomyces cerevisiae*. *Biochem Soc Trans* 34, 359-62.
- [45] Ott, R.G., Athenstaedt, K., Hrastnik, C., Leitner, E., Bergler, H. and Daum, G. (2005). Flux of sterol intermediates in a yeast strain deleted of the lanosterol C-14 demethylase Erg11p. *Biochim Biophys Acta* 1735, 111-8.
- [46] Mo, C. and Bard, M. (2005). A systematic study of yeast sterol biosynthetic protein-protein interactions using the split-ubiquitin system. *Biochim Biophys Acta* 1737, 152-60.
- [47] Jorda, T. and Puig, S. (2020). Regulation of Ergosterol Biosynthesis in *Saccharomyces cerevisiae*. *Genes (Basel)* 11

Supplementary figures:



Supplementary Fig. 1. The most likely lanosterol binding site in Pdr16p largely occupies the hydrophobic part of the PI binding cavity. The view is the same as in Figure 3A. Here PI is shown in black and lanosterol in magenta. Note that the majority of the lanosterol falls in the same part of the binding cavity as the fatty-acid part of PI.



Supplementary Fig. 2. Sterol analyses. Sterol content of aerobically grown yeast cells were analyzed using reversed phase HPLC. The amounts of sterols were quantified from the values collected from Corona Charged Aerosol Detector based on the calibration curve for ergosterol. The results show the average from three independent experiments, each performed in duplicates \pm S.D.