Dissertation

Characterization of Aus1 protein – a putative yeast sterol transporter

zur Erlangung des akademischen Grades
doctor rerum naturalium
(Dr. rer. nat.)
in Fach Biophysik

eingereicht an der
Mathematisch-Naturwissenschaftlichen Fakultät I
der Humboldt-Universität zu Berlin

von
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Tag der mündlichen Prüfung: 05.10.2011

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Zusammenfassung

Sterine sind essentielle Komponenten der Zellmembran, deren Konzentration und Lokalisierung genau kontrolliert wird. Die Hefe *Saccharomyces cerevisiae* ist ein fakultativ anaerober Organismus, der in Abwesenheit von Sauerstoff auxotroph für Sterine wird, d.h. ohne Sauerstoff wächst Hefe nur wenn aufnahme von exogenen Sterinen möglich ist. Allerdings muss der genaue Mechanismus der Sterinaufnahme noch erforscht werden. Die Proteine Aus1p und Pdr11p gehören zur Familie der ABC (ATB-binding cassette) Proteine und spielen eine wichtige Rolle in diesem Prozess, da die gleichzeitige Deletion beider Protein die Aufnahme von Sterinen unter anaeroben Wachstumsbedingungen blockiert [1].

In dieser Arbeit wurde das Gen *AUS1* in voller Länge kloniert. Methoden für die Extraktion und Reinigung dieses Transporters wurden entwickelt, damit dieser detailliert charakterisiert werden kann. Mit Hilfe von Detergenzien wurde das Protein löslich gemacht und zeigte ATP-Bindung und -Hydrolyse. Die ATP-Hydrolyse konnte durch die Mutation eines konservierten Lysins zu Methionin im Walker A Motif verhindert. Genauso konnte die ATP-Hydrolyse auch durch klassische Inhibitoren von ABC Transportern inhibiert werden. Nach der Rekonstitution von Aus1p in Proteoliposomen wurde die ATPase Aktivität spezifisch durch Phosphatidylserin (PS) in einer stereoselektiven Weise stimuliert.

Zusätzlich konnte gezeigt werden, dass Änderungen im zellulären PS Spiegel die Aus1p-abhängige Aufnahme von Sterin, aber nicht die Expression und Verschickung an die Membran beeinflussen. Diese Ergebnisse schlagen eine für die Aktivität des Transporters wichtige, direkte Interaktion zwischen Aus1p und PS vor.

Da es sich bei der Aufnahme von Sterin um einen komplexen Prozess handelt, könnten Komponenten exisitieren, die mit Aus1p interagieren. Der Hefestamm, der die Immunpräzipitation von Aus1p mit seinem Interaktionspartner ermöglicht, wurde erzeugt und der Einfluß von Mannoproteinen auf Sterinaufnahme wurde getestet. Außerdem wurde eine Methode entwickelt, mit der Aus1p in Giant Unilamellar Vesicles (GUVs) rekonstituiert werden kann. Mit diesen Liposomen kann das Verhalten und die Aktivität von Aus1p in Membranen mit einer komplexen Lipidzusammensetzung untersucht werden.

Schlagwörter: ABC Protein, Aus1p, Sterinaufnahme, Proteoliposomen, Protein Aufreinigung, die Phosphatidylserin

Abstract

Sterols are essential components of cellular membranes and their concentration and localization are tightly controlled. *Saccharomyces cerevisiae* is a facultative anaerobic organism which becomes auxotrophic for sterols in the absence of oxygen. Under that condition yeast growth relays on the uptake of exogenous sterols. However, the precise mechanism of sterol uptake remains to be revealed. Two proteins belonging to ABC (ATP-binding cassette) protein family, Aus1p and Pdr11p were proposed to play a critical role in this process as simultaneous deletion of both of them blocks sterol uptake under anaerobic growth conditions [1].

In the present work, the full length *AUS1* gene was cloned. An extraction and purification procedures were then developed to allow for detailed characterization of the transporter. The detergent solubilized protein was shown to bind and hydrolyse ATP. Mutagenesis of the conserved lysine to methionine in the Walker A motif abolished ATP hydrolysis. Likewise, ATP hydrolysis was inhibited by classical inhibitors of ABC transporters. Upon reconstitution into proteoliposomes, the ATPase activity of Aus1p was specifically stimulated by phosphatidylserine (PS) in a stereoselective manner.

Furthermore, it was demonstrated that Aus1p-dependent sterol uptake, but not Aus1p expression and trafficking to the plasma membrane, was affected by changes in cellular PS levels. These results suggest a direct interaction between Aus1p and PS which is critical for the activity of the transporter.

Because of the complexity of sterol incorporation process efforts were made to identify additional components of the sterol uptake machinery that interact with Aus1p protein. The yeast strain allowing for immunopercipitation of Aus1p with its interaction partners was generated and previously proposed influence of mannoproteins [2] on the sterol uptake was tested. Additionally, method was developed to reconstitute Aus1p protein into Giant Unilamellar Vesicles (GUVs). These liposomes can be used further for testing of the behaviour and activity of Aus1p in the membranes with complex lipid composition.

Key Words: ABC protein, Aus1p, sterol uptake, proteoliposomes, protein purification, phosphatidylserine

List of abbreviations

Abbreviation	Meaning
ABC	ATP-binding cassette
ADP	Adenosine diphosphate
ALA	5-aminolevulinic acid
ATP	Adenosine triphosphate
AU	Arbitrary Units
BN-PAGE	Blue Native PAGE
CHOL	Cholesterol
cmc	Critical micellar concentration
CRAC	Cholesterol recognition/interaction amino acid consensus
CW	Calcofluor white
CWP	Covalently bound cell wall protein
DDM	n-dodecyl-β-maltoside
DIC	Differential interference contrast
DMSO	Dimethyl sulfoxide
DTT	Dithiothertiol
EDTA	Ethylenediaminetetraacetic acid
ERG	Ergosterol
ER	Endoplasmic reticulum
GUV	Giant Unilamellar Vesicle
HDL	High density lipoprotein
LDL	Low density lipoprotein
LTP	Lipid transport protein
LUV	Large unilamellar vesicle
MCS	Membrane contact site

NEM	N-ethylmaleimide
NBD	Nucleotide binding domain
25-NBD cholesterol	25-[N-[(7-nitro-2-1,3-benzoxadiazol-4-yl)methyl]amino]-27-norcholesterol
NBD (lipid)	7-nitrobenz-2-oxa-1,3-diazole (lipid)
OSBP	Oxysterol binding protein
ORP	OSBP related protein
PA	Phosphatidic acid
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PCR	Polymrase chain reaction
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidolinositol
PM	Plasma membrane
PMSF	Phenylmethylsulphonyl fluoride
PS	Phosphatidylserine
RFP	Red fluorescent protein
RT-PCR	Real time PCR
SPH	Sphingomyelin
TMD	Transmembrane domain
SEC	Size exclusion chromatography
SD	Standard deviation
SDS	Sodium dodecyl sulfate
WT	Wild type

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1. INTRODUCTION

Sterol homeostasis is critical for many cellular processes, including signaling and protein trafficking, as well as regulation of permeability and fluidity of cellular membranes [3]. Many cell types can synthesize sterols on their own but some have to relay on external sterol sources - permanently or under certain environmental conditions [4]. Understanding of the molecular mechanisms underlying sterol trafficking is extremely important since disturbances in that processes are connected with various diseases like obesity, artheriosclerosis and neurodegeneration [5].

In the yeast *Saccharomyces cerevisiae*, sterol uptake occurs only under anaerobic conditions (when the oxygen is inaccessible for the cells) and it is suggested to be mediated by two ABC transporters - Aus1p and Pdr11p [1]. It has been proposed that both proteins may transport sterol directly out of the plasma membrane to a cytosolic acceptor, such as soluble sterol-binding proteins, or closely apposed membranes of the endoplasmic reticulum [6]. Alternatively, they may indirectly facilitate sterol transport by catalyzing the transbilayer movement of other lipids (as suggested for other ABC transporters) or be required for the entry of external sterol into the plasma membrane [7,8,9]. Thus, direct biochemical proof of their function and key features of their activity remain to be elucidated. In this study several methods were employed to characterize Aus1p transporter and investigate its role the process of sterol uptake in budding yeast.

1.1 Lipid and sterol homeostasis in the cell

Cellular membranes are composed of lipid and protein molecules. They consist of a bilayer structure and separate functional compartments within the cell [10]. The most abundant lipids in eukaryotic membranes are: phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylinositol (PI), cholesterol [11] and sphingolipids (Figure 1) [12]. To fulfill properly their functions lipids have to be transported between organelles as well as between the inner and outer leaflet of the membranes (Figure 2). Since lipid molecules can move within the membrane (for example by lateral diffusion in the plane of the membrane or flip-flop across the membrane bilayer) it would be expected that they equilibrate among membranes connected by vesicular trafficking. However, such mixing is not observed and cells sustain different lipid composition between organelles, leaflets of some membranes and even within one leaflet [13]. It was proposed that generation of the transverse membrane asymmetry is mediated by flippases - a group of proteins that facilitate lipid movement between the leaflets, generating a symmetrical or asymmetrical lipid composition [14]. A symmetrical distribution of lipids occurs in endoplasmic reticulum (ER) membranes but the Golgi, plasma membrane (PM) and endosomal membranes display an asymmetric distribution, with sphingomyelin and glycosphingolipids on the non-cytosolic side and

phosphatidylserine and phosphatiylethanolamine enriched in the cytosolic part of the membrane [15].

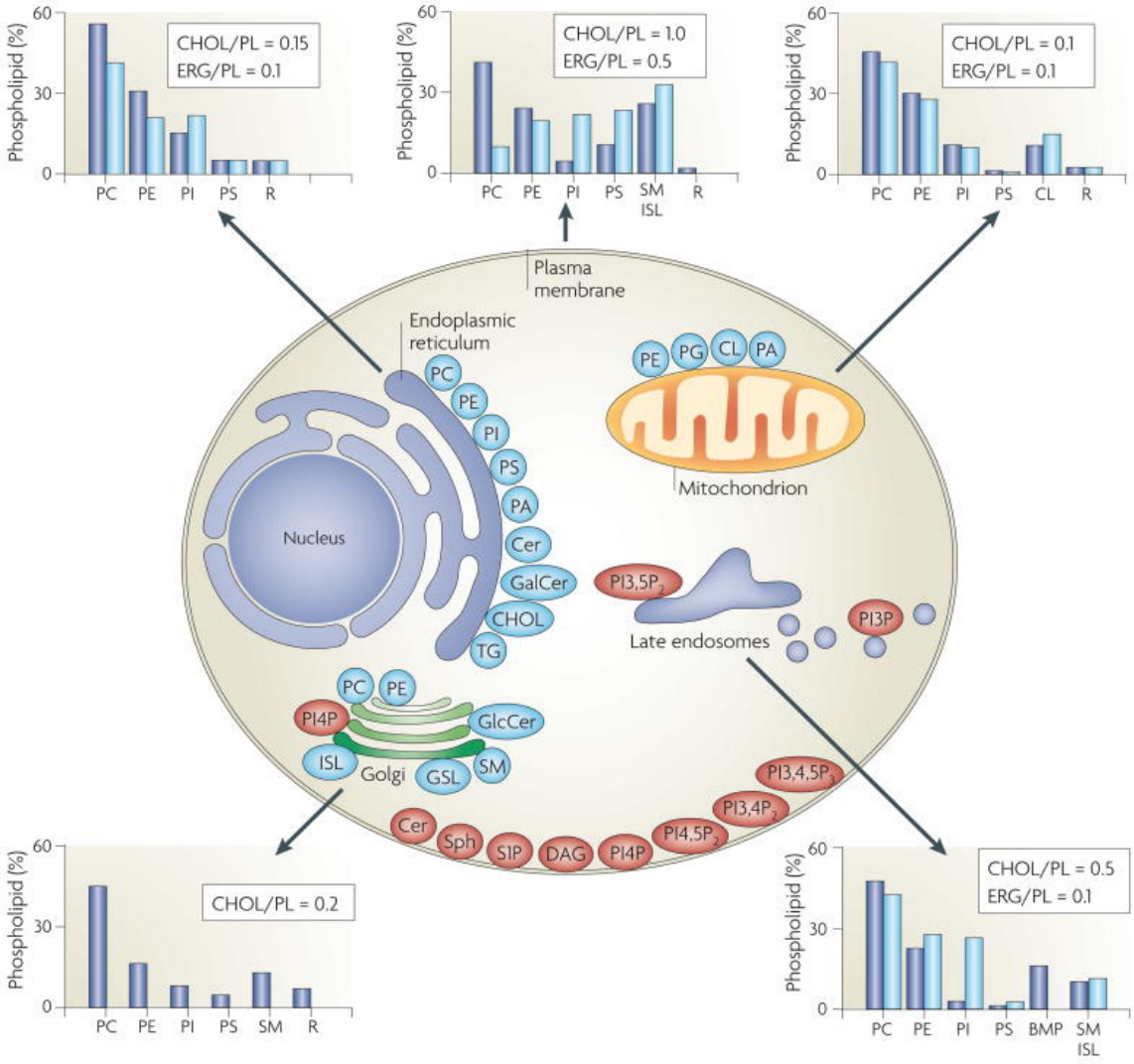


Figure 1: Lipid synthesis and steady-state composition of cell membranes. The lipid compositional data are expressed as a percentage of the total phospholipid (PL) in mammals (blue) and yeast (light blue). As a measure of sterol content, the molar ratio of cholesterol and ergosterol (ERG) to phospholipid is also included. The major glycerophospholipids assembled in the endoplasmic reticulum are phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine and phosphatidic acid (PA). In addition, the ER synthesizes ceramide (Cer), galactosylceramide (GalCer), cholesterol and ergosterol. The Golgi lumen is the site of synthesis of sphingomyelin (SM), complex glycosphingolipids (GSLs) and yeast inositol sphingolipid (ISL) synthesis. PC is also synthesized in the Golgi, and may be coupled to protein secretion at the level of its diacylglycerol (DAG) precursor. Approximately 45% of the phospholipid in mitochondria (mostly PE, PA and cardiolipin (CL)) is autonomously synthesized by the organelle. BMP (bis (monoacylglycero)phosphate) is a major phospholipid in the inner membranes of late endosomes. PI4P, phosphatidylinositol- 4-phosphate; R, remaining lipids; S1P, sphingosine-1-phosphate [12].

The asymmetric distribution of lipids has important functional consequences. For example phosphatidylserine acts as a susceptibility signal for phagocytosis when exposed on the cell surface

of mammalian cells, and as a propagation signal in blood coagulation [16]. Furthermore, lipid translocation to the cytosolic leaflet causes a lipid imbalance that can contribute to the membrane bending which is required for vesicle budding [12].

As mentioned before cellular organelles differ in their lipid composition. Especially sterols are not uniformly distributed throughout the cell and the amount of sterol in each subcellular organelle is unique [17]. For instance, the sterol level is low at its site of synthesis in the endoplasmic reticulum but high in the plasma membrane reaching up to 30 mol% of lipid molecules [18]. Therefore, mechanisms must exist which regulate the transport and distribution of sterols and that maintain a distinct level of sterol in each membrane. The importance of such transport and sorting mechanisms is highlighted in several human diseases. For instance, accumulation of cholesterol in endosomal compartments is associated with Niemann Pick Disease type C and possibly with Alzheimer's disease [19,20]. The molecular mechanisms underlying sterol biosynthesis and esterification are relatively well known. However, how the non-homogenous distribution of sterols within the cell is maintained and how sterols move between and within cellular membranes is not fully understood in molecular terms [3].

Sterols play essential role in eukaryotic cells by modulating membrane properties and taking a part in various cellular processes [21,22,23]. The endoplasmic reticulum produces the bulk of the structural phospholipids and sterols. Newly synthesized sterol is rapidly transported to other organelles and the excess of cholesterol is delivered to acyl-CoA cholesterol acyl transferase (ACAT) for esterification and subsequent storage in lipid particles [12]. The ER is a key site where the sterol level is monitored and feedback regulatory cascades are initiated to control sterol biosynthesis and uptake so that cellular sterol homeostasis is maintained [4].

In mammalian cells, cholesterol is obtained either through *de novo* synthesis in the ER or by the uptake of lipoproteins [3]. Recently it was also suggested that NPC1L1 – a protein expressed in intestine and liver - can mediate sterol uptake through clathrin mediated endocytosis [24]. *Saccharomyces cerevisae* relays on sterol synthesis under aerobic and on sterol uptake under anaerobic conditions whereas *Drosophila melanogaster* and *Caenorhabditis elegans* are sterol auxotrophs [4].

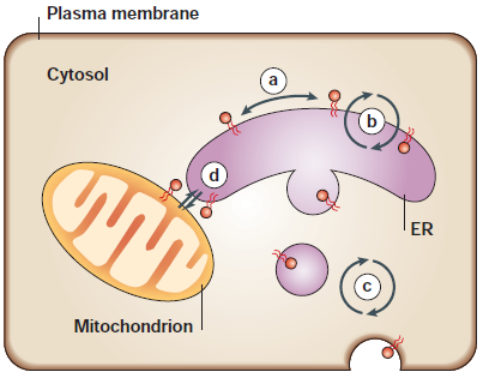


Figure 2: Transport of lipids within the cell. Lipids can diffuse laterally (step a; lateral movement) or transversely between the two leaflets of each organelle membrane (step b; transbilayer movement or flip–flop). As lipids form the backbone of a membrane, they are an integral part of vesicular carriers (step c; vesicular transport) that connect, for example, the endoplasmic reticulum and the plasma membrane indirectly through the Golgi (not shown). At the same time, lipids can be exchanged as monomers between the cytosolic surfaces of organelle membranes (step d; monomeric exchange). For simplicity in this figure, scaled-up lipids (red) are shown on top of single lines that represent the various membrane bilayers [25].

The ratio of particular lipid compounds in the cell is also highly regulated and interestingly the amount of sterols in the cell influences the amount of other lipids. It was shown that in yeast the pathways of ergosterol biosynthesis and synthesis of sphingolipids are closely connected – decrease in the amount of ergosterol leads to down-regulation of ceramide (sphingolipid precursore) production. Additionally, decreased amount of sphingomyelin in plasma membrane enhances the level of sterol estrification and subsequent storage in lipid droplets. Other cross talk has been observed between ergosterol and fatty acids biosynthesis pathways namely blocking the ergosterol synthesis results in decrease in the transcription level of genes responsible for the novo synthesis of long chain fatty acids (ELO1, OLE1, FAS1) [24].

A few mechanisms have been proposed to explain sterol transport between organelles. Sterols can be moved by membrane transport vesicles, by diffusible carrier proteins or by putative multi-protein scaffolding complexes between donor and acceptor membranes (Figure 2) [18,26]. Vesicular transport requires ATP and intact cytoskeleton and although lipids and sterols can be transported by endocytic and secretory vesicles, it is still not clear if this is the main transport pathway, especially for newly synthesized or incorporated molecules. Another possibility is that sterols are transported by carrier proteins called LTP (Lipid Transport Protein). LTPs are supposed to be peripheral membrane proteins, with two targeting signals – one for donor and one for acceptor compartment, so that the protein can shuttle substrate between the two [25]. It is known that CERT protein can transport ceramide from ER to Golgi and StarD1 is required for delivery of cholesterol to the inner mitochondrial membrane [25,27]. Recently, also OSBPs (Oxysterol Binding Protein) and ORPs (OSBP Related Proteins) have been shown to transport sterols [28,29]. It was also suggested that sterol transport could occurs at the contact sites between two organelles (MCS, membrane contact

site). Such domains, connecting for example plasma membrane and ER or ER and mitochondrium are well known in yeast cells [30]. At MCS, ER membrane comes close to the other membrane but does not fuse with it. Instead of that, protein-protein bridges are formed between organelles contact sites. Therefore endoplasmic reticulum could theoretically acts as a conduit for lipids. Increasing evidences suggest that lipid transfer proteins can work at MCSs [25].

Although several studies have been performed the maintenance of non-random lipid distribution in the cell is still a key question in cell biology. Different pathways and protein families are involved in regulation of lipid metabolism, uptake and extrusion. According to the results obtained during the last years also proteins belonging to the ABC family influence cellular lipid homeostasis. The precise mechanism of lipid transport is still under investigation but ABC transporters seem to be crucial components of the lipid uptake/export system in eukaryotes.

1.2 The superfamily of ABC transporters

ABC transporters constitute a large superfamily of membrane proteins involved in various cellular processes. They are present in both eukaryotes and prokaryotes and share a set of conserved domains as well as similar molecular architecture. ABC proteins can work as importers or exporters for a great amount of substrates like ions, lipids, peptides, sugars and a range of chemically unrelated xenobiotics [31]. Interestingly, they can also act as regulators of other proteins. For example SUR (sulfonylurea receptor) assembles with Kir subunit of potassium channel and by that modulate the activity of the potassium chanel complex ($K_{ATP)}$ [32,33]. Because of their involvement in many crucial cellular functions (eg. nutrient uptake, cell division, antigen processing), mutations affecting these proteins are often associated with a wide range of disorders including hypercholesterolemia, cystic fibriosis or diabetes [34].

1.2.1 Structure and architecture of ABC transporters

ABC proteins have a characteristic architecture that consists minimally of four domains: two transmembrane domains (TMDs) localized in the lipid bilayer and two nucleotide binding domains (NBDs) that are located outside the membrane. In contrast to the highly conserved structure of NBDs, there is considerable variation in the appearance and arrangements of TMDs between different ABC subfamilies [35].

NBD domains contain motifs characteristic for ABC transporters like the Walker A motif (or P loop, GXXGXGK(S/T)), the Walker B motif (xxxxD where x is a hydrophobic molecule), a Q loop, H motif (or switch region) and more structurally diverse helical domain, which contains ABC signature

motif LSGGQ (Figure 3) [36]. In ABC transporters hydrolysis of ATP is coupled to conformational changes in the TMDs which effect substrate translocation. Structural and biochemical studies on isolated NBD have demonstrated that they form dimers in the ATP-bound state. NBD dimers have a head-to-tail configuration, in which the Walker A motif (P-loop) of one monomer and the LSGGQ signature motif of the other monomer form a composite active site via their interaction with the bound ATP [37].

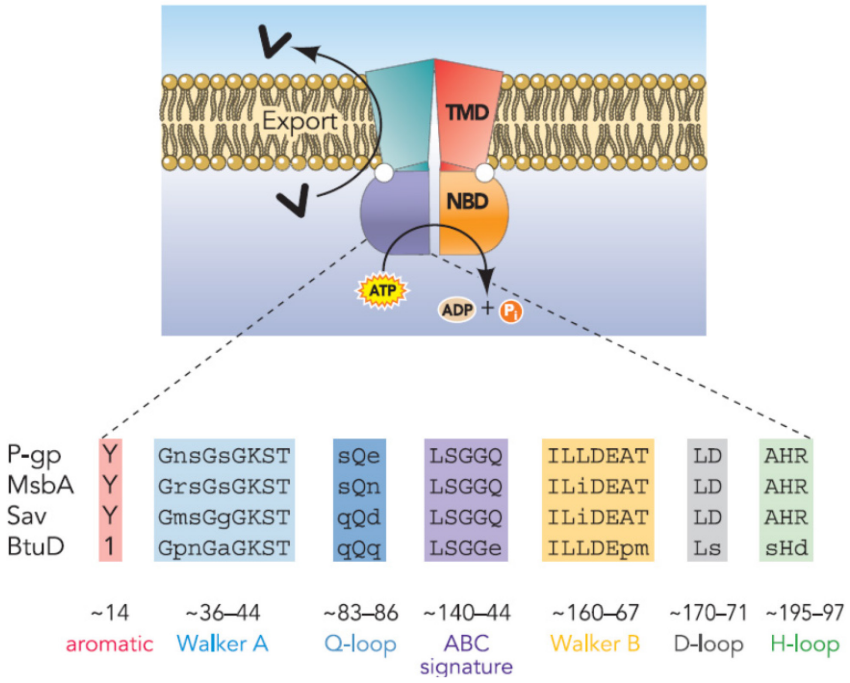


Figure 3: The minimal composition of ABC transporters. Classical ABC transporters are formed by two transmembrane domains which contain substrate binding site, and two nucleotide binding domains responsible for ATP binding and hydrolysis. The TMDs from different subfamilies of ABC transporters are not necessarily homologous. The NBDs are homologous throughout the family. Each NBD contains highly conserved, but not invariant, motifs [37].

The ABC proteins encoding genes can be divided into subfamilies, based on a similarity in their gene structure (eg. order of domains or the sequence homology in the NBDs and TMDs domains). There are seven mammalian ABC gene subfamilies and five of them can be found in *Saccharomyces cerevisiae* genome (Figure 4).

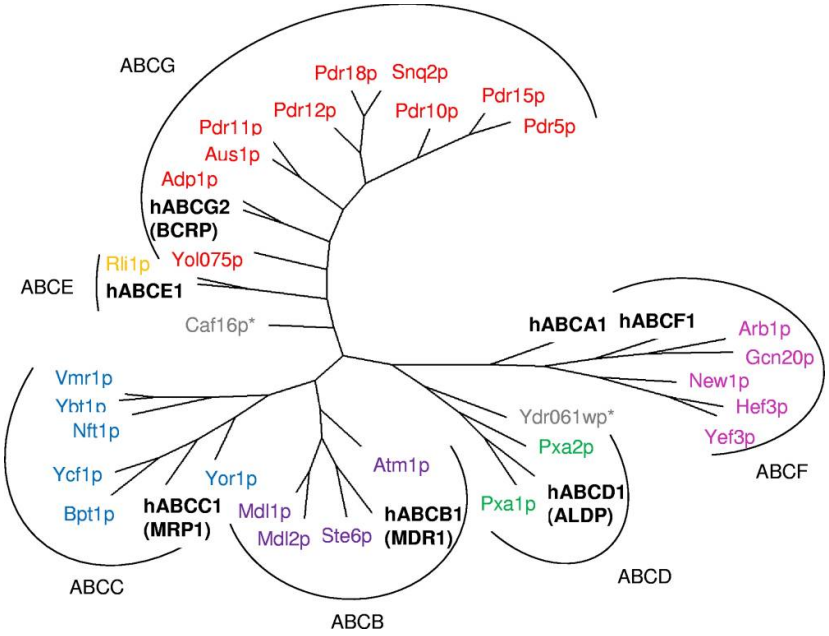


Figure 4: Yeast ABC phylogenetic tree. The protein sequences of the yeast ABC transporters have been subjected to a multiple-sequence alignment using CLUSTALW and phylogenetic analysis, and the resulting data are depicted in a radial-tree format (PHYLO). Subfamilies have been highlighted and grouped by black lines and arcs. The nomenclature ABCB to ABCG is used to assign the yeast ABC proteins to their homologous subfamilies. For each subfamily, a mammalian member (boldface type) was included in the analysis as a point of reference [38].

The yeast PDR, MRP/CFTR, MDR and ALDp family members contain at least six predicted TMDs when YEF3/RLI protein lacks any obvious TMD. The ABC proteins are organized either as full transporters containing two TMDs and two NBDs or as half transporters containing only one TMD and one NBD. Half transporters can further form homo or heterodimers (Figure 5). Full size ABC transporters, have a tandemly duplicated organization with usually six predicted helices in each half, arranged either in forward $(TMD6\text{-}NBD)_2$ or reverse $(NBD\text{-}TMD6)_2$ configuration. Yeast MRP/CFTR proteins carry an additional TMD at the N-terminus called an N terminal extension [39].

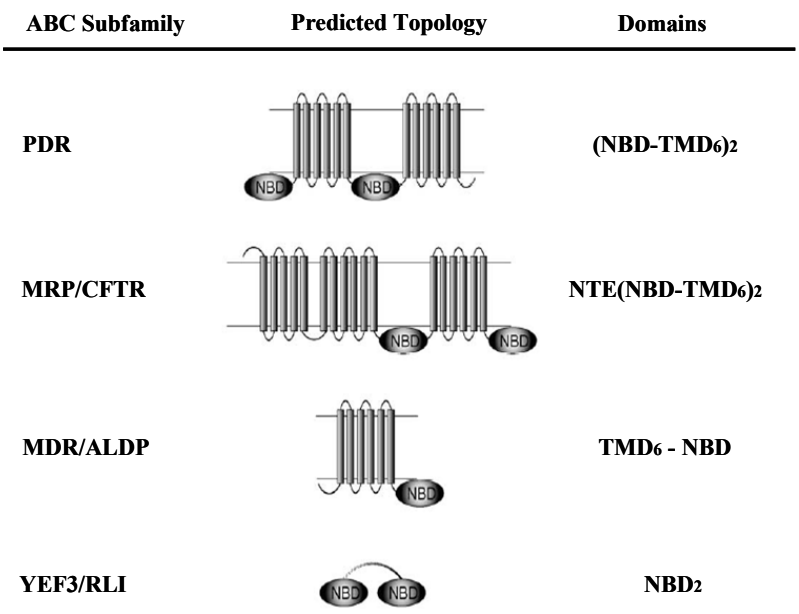


Figure 5: Predicted topology and domain organization of ABC protein subfamilies. The cartoon depicts the predicted membrane topology and domain organization of ABC proteins. ABC proteins can be organized as full transporters containing two NBD and two TM domains or as half transporters containing only one NBD and one TMD domain. Half transporters can further form homo or heterodimers. Proteins from MRP/CFTR family carry additional transmembrane domain called N terminal extension while proteins from YEF3/RLI family lack any transmembrane domain [39].

1.2.2 Working mechanism of ABC transporters

Translocation of substrate molecules by ABC transporters requires energy which comes from hydrolysis of ATP molecules. The switch model of action of ABC proteins assumes that transporter must cycle between high and low-affinity states for ligands on different sides of the membrane. The ATP switch mechanism describes how these states are coupled to the ATP catalytic cycle in a way that is consistent with the available structural data. During the first step ligand binds to the TMDs in inducing increased affinity for ATP within NBDs. Subsequently ATP binding induces formation of the closed NBD dimer, which in turn induces a large conformational change in the TMDs sufficient to translocate ligand. ATP hydrolysis initiates dissolution of the closed NBD dimer and at the end of the cycle phosphate and ADP is released to complete the transport cycle and restore the protein to a high - affinity state for ligand (Figure 6) [37].

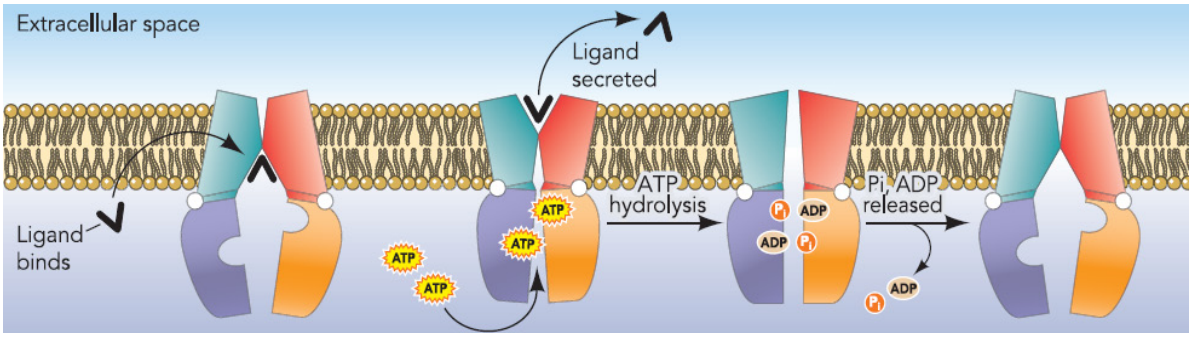


Figure 6: A simple ATP-switch mechanism powers ABC transporters. Ligand binding to a high-affinity pocket formed by the TMDs induces a conformational change in the NBDs resulting in a higher affinity for ATP. Two molecules of ATP bind to the NBDs. The energy released by the formation of the closed NBD dimer causes conformational change in the TMDs. ATP hydrolysis triggers dissolution of the closed NBD dimmer resulting in further conformational changes in the TMDs. Finally, phosphate and then ADP release restores the transporter to the open NBD dimer conformation ready for the subsequent cycle [37].

The detailed mode of substrate transport by ABC proteins seems to be more complex process. The flippase translocation model (Figure 7) proposes that the substrate binding sites are located within the TMDs and that the main criterion for transport is the partitioning of hydrophobic substrate into the membrane. After energy consuming conformational changes transported molecule is released into the opposite half of the bilayer. In this case relatively hydrophilic molecules could easily partition into external environment. However, this model does not explain how hydrophobic lipids substrate reach acceptor [40]. According to the vacuum cleaner model the substrate enters membrane localized binding site and subsequently is expelled into the medium on the other site. This model assumes complete hydration of the substrate after transport event what poses a problem from energetical point of view – since ABC transporter cannot produce enough energy during its power stroke to transport a lipid molecule [40,41]. The third, so called activation model assumes that the substrate is shifted by the transporter to the location of intermediate hydrophobicity and presented to the acceptor molecule [40]. It is also speculated that intra and extracytosolic loop domains can take a part in substrate site construction and/or filtering [42].

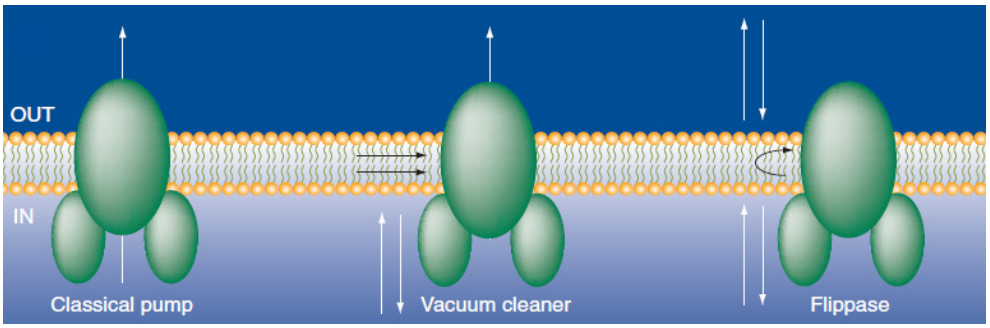


Figure 7: Models of substrate translocation by ABC transporters. Pumps transport polar substrates from one side of the membrane to the other, through a hydrophilic path formed by the transmembrane regions of the protein [43]. In the vacuum cleaner model, substrates partition into the lipid bilayer, interact with the transporter within the membrane, and are subsequently effluxed extracelluarly. In the flippase model, substrates partition into the membrane, interact with the drug-binding pocket localized within transporter and are translocated, or flipped, to the outer membrane leaflet [44].

Interestingly, for some prokaryotic ABC transporters that function as importers, substrate translocation is also dependent on another protein component - a high affinity binding protein that specifically associates with the ligand in the periplasm for the delivery of the substrate to the transporter. An example is the bacterial B12 vitamin transporter BtuCD which associates with the BtuF protein during the process of substrate translocation (Figure 8).

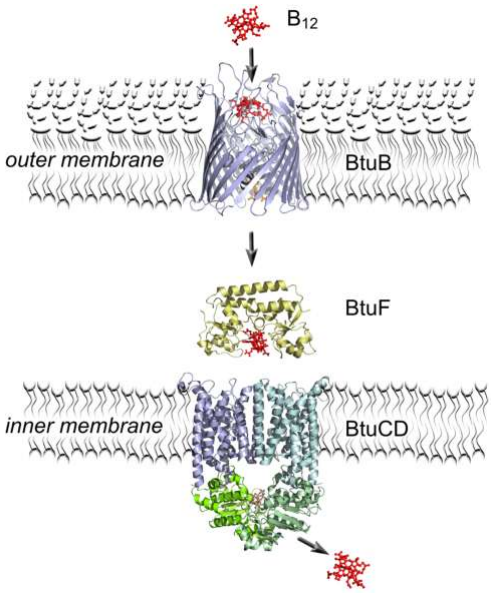


Figure 8: Vitamin B12 uptake system. Schematic cartoon of the vitamin B_{12} uptake system in *Escherichia coli*. BtuB transfers vitamin B_{12} across the outer membrane into the periplasmic space, where the vitamin binds to BtuF. Once the loaded form of BtuF docks to the ABC transporter BtuCD, transport across the inner membrane is initiated. This process is powered by ATP hydrolysis, taking place at the interface between the NBDs, shown in green, on the cytoplasmic side of the inner membrane. To date the B12 uptake system is the only ABC transporter system where the structures of all the key players are known [45].

1.3 Involvement of the mammalian ABC transporters in the sterol transport

In mammalian cells specific proteins mediate sterol entrance and exit from the cells in order to maintain the balance between sterol synthesis, absorption and excretion (Figure 9). The entrance of dietary cholesterol into enterocytes is facilitated by NPC1L1 protein which localizes to the apical part of the plasma membrane. It is thought that the protein senses the high level of sterol in the membrane environment and upon endocytosis, is transported to the ERC (Endocytic Recycling Compartment) together with high amounts of cholesterol. When the cholesterol level is low the NPC1L1 cycles back to plasma membrane [46].

An important role in sterol homeostasis is played also by ABC transporters. One example is the ABCA1 protein which is expressed in many tissues but the highest amount can be detected in liver and in the macrophages. The absence of functional ABCA1 protein results in Tangier disease, with characteristic very low level of HDL (High Density Lipoprotein) in the blood. The exact function of ABCA1 is not known, although it is proved to be involved in the removal of cholesterol from the cells [42,47].

Proteins from ABCG family seem to be especially involved in the maintenance of the cellular sterol balance. ABCG5/G8 acts as a heterodimer and is expressed only in liver and intestine where localizes to the plasma membrane. Mutations affecting that protein lead to the sitosterolemia - a disease characterized by accumulation of high amounts of dietary phytosterols accompanied by higher intestinal cholesterol absorption. Currently there are two hypotheses explaining the mode of action of ABCG5/B8. The first one assumes that the protein acts as an extruder exposing sterols in the extracellular leaflet of plasma membrane that would facilitate the extraction into the intestine lumen by bile acids. The second one proposes that ABCG5/G8 is a flippase which moves phospholipids from inner to the outer leaflet of the apical membrane of enterocytes [48]. ABCG1 and ABCGG4 are involved in cholesterol efflux to HDL and regulation of sterol accumulation in the brain and lack of them leads to accumulation of sterol precursors (desmosterol, lathosterol and lanosterol) in primary astrocytes [49]. Also one of the multidrug resistance proteins – ABCG2 was implicated in the transport of sterols. This protein (known also as BCRP (Breast Cancer Resistance Protein)) is a plasma membrane transporter which is able to extrude a wide variety of drugs from the cell. Because overexpression of ABCG2 in *L.lactis* increases the uptake of estradiol, and estradiol stimulates ATPase activity of the protein, it was proposed that ABCG2 plays a role in the transport of sterols and steroids [50].

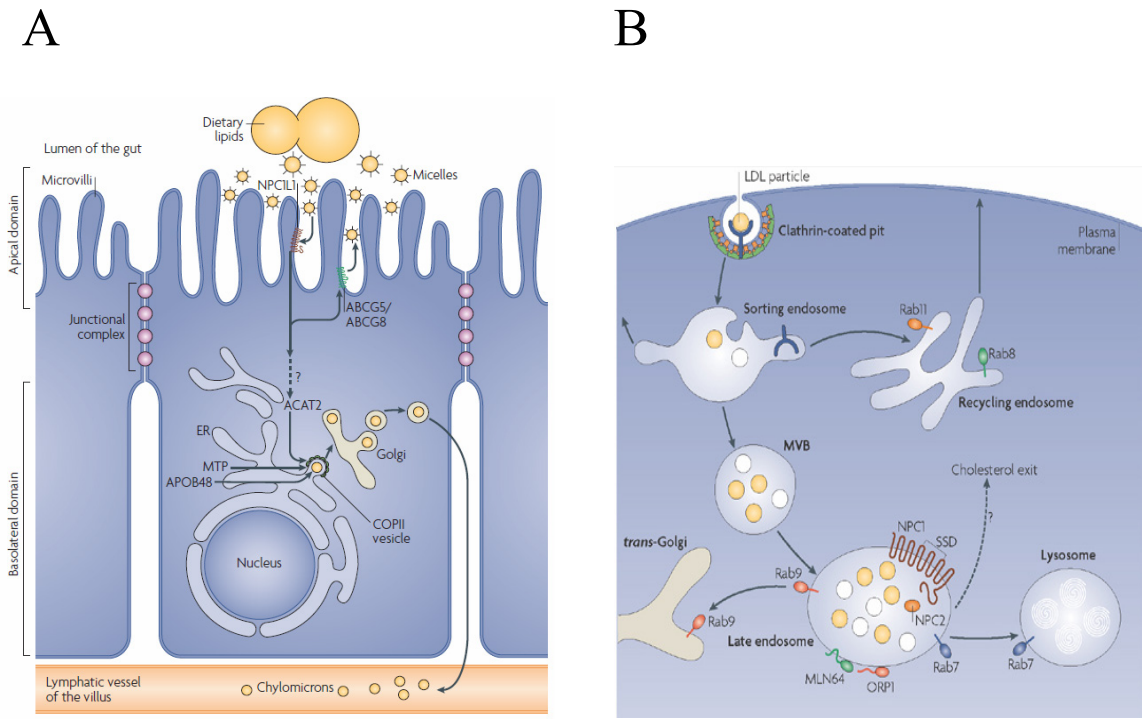


Figure 9: Sterol transport in mammalian cells. (A) Micellar solubilization of dietary sterols by bile acids allows them to move through the diffusion barrier overlying the luminal surface of enterocytes. The NPC1L1 protein (dark red) is located at the apical membrane of enterocytes and facilitates the uptake of cholesterol across the brush border membrane. In contrast, the ABCG5/G8 transporter (green) promotes the active transfer of cholesterol and plant sterols back into the intestinal lumen for excretion. Acyl CoA cholesterol acyltransferase isoform-2 (ACAT2) esterifies the absorbed cholesterol, which becomes incorporated into nascent chylomicron particles. Chylomicrons are synthesized around the APOB48 apoprotein in the endoplasmic reticulum (ER). Dietary fatty acids are used for triglyceride synthesis in the smooth ER and MTP (microsomal triglyceride transfer protein) transfers triglycerides and cholesteryl esters to APOB48. The nascent chylomicrons leave the ER in COPII-coated vesicles and are secreted through the Golgi complex to the basolateral side of the enterocyte and reach the venous circulation through lymphatic vessels. (B) Low density lipoprotein (LDL) receptors bind LDL particles (yellow) in clathrin-coated pits for delivery into early sorting endosomes. The LDL receptor recycles back to the plasma membrane and LDL is delivered to later endocytic compartments for hydrolysis. Acid lipase hydrolyses cholesteryl esters (and triglycerides) and the free cholesterol can exit the endosomal system for delivery to other compartments, including the PM and endoplasmic reticulum. Efflux from late endosomal compartments is not well characterized despite the fact that the process is inhibited by ablation of Niemann–Pick C1 (NPC1) or NPC2 proteins. Cholesterol is enriched in the internal membranes of multivesicular endosomes and depleted from lysosomes, which implies that cholesterol efflux takes place before cargo delivery to lysosomes [4].

1.4 Yeast as a model organism for studying sterol transport

The budding yeast *Saccharomyces cerevisiae* is widely used for studying different aspects of cell physiology and genetics. The yeast genome is sequenced and the cells are genetically and physiologically characterized. In addition, tools allowing for genetic manipulations on *S.cerevisiae* are well established. These features make yeast an excellent organism for biological studies as well as for industrial applications [51].

One of the processes widely studied in yeast is the uptake and metabolism of sterols. In contrast to mammalian cells yeast do not synthesize cholesterol but its analogue – ergosterol (Figure 10). The differences between ergosterol molecule in comparison to cholesterol comprise additional methyl group at C24 of the side chain and double bonds at C7 and C22. Both molecules influence membrane fluidity and have tendency for association with sphingolipids [52]. However, the amount of sterol that becomes associated with sphingolipid-rich domains (rafts) in the plasma membrane varies widely depending on the sort of sterol incorporated in PM [43]. In yeast, internalized ergosterol remains largely raft-associated while cholesterol does not. Presumably, newly incorporated cholesterol in the PM is out-competed by endogenous ergosterol for raft association. Interestingly, there exist an inverse correlation between the ability of sterol to become raft associated in the PM and the rate at which it is transported to the ER since sterols with low raft affinity are more rapidly transferred. In addition, depleting cells of sphingolipids (and thus raft associated sterols) decrease the differences in the rates at which exogenous ergosterol and cholesterol moved from PM to ER. Taken together these observations suggest that only non raft associated sterol in the PM is available for non vesicular transfer to ER [28].

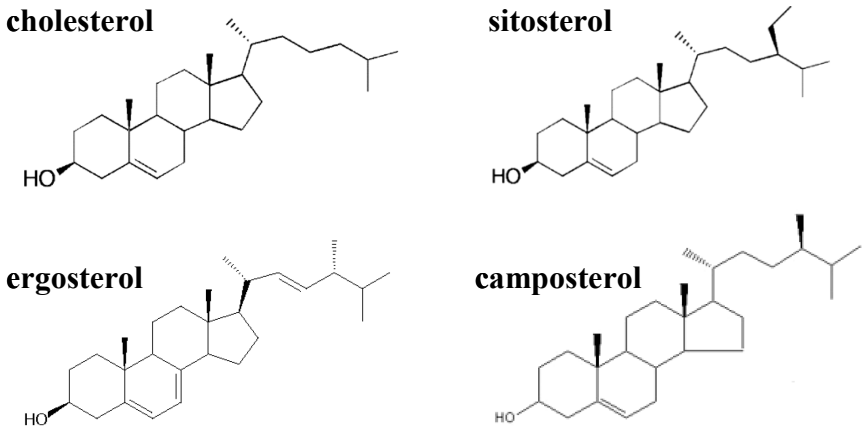


Figure 10: Chemical structures of different sterols. The main sterol in mammalian cells is cholesterol while fungi produce ergosterol. The main sterols of plant cells are stigmasterol and camposterol.

1.4.1 Sterol exclusion process in *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is a facultative anaerobe and under aerobic conditions can synthesize sterols from acetyl-CoA. Proteins responsible for ergosterol biosynthesis (so called ERG proteins) are localized in endoplasmic reticulum and only five of them functioning in the final steps of the pathway are encoded by nonessential genes [23]. However, under anaerobic conditions yeast growth relies on the uptake of sterols. This fact is called aerobic sterol exclusion and is a result of requirement of molecular oxygen in several steps of the ergosterol biosynthesis pathway (Figure 11A). Since ERG proteins require heme as a prosthetic group, blocking the mitochondrial heme synthesis pathway leads to the inactivation of sterol biosynthesis in the cell, and thus mimic hypoxic conditions. Aerobic sterol exclusion is so effective that most sterol auxotorphs without heme deficiency cannot grow under aerobic conditions even with sterols provided in the medium [53]. Cells with deleted *HEM1* (5-aminolevulinate synthase) gene express genes responsible for sterol uptake. Addition of 5 aminolevulinic acid (ALA) [50] – a product of 5-aminolevulinate synthase activity - overcomes the heme synthesis block and restores the sterol synthesis pathway (Figure 11B). In addition some transcription factors like SUT1 or UPC2-1 were shown to enable the sterol influx in heme-competent, aerobic cells. UPC2-1 (uptake control) cells express mutated version of UPC2 zinc finger transcription factor which is thought to be gain of function mutation in terms of activation of sterol influx (since deletion of UPC2 gene do not influence sterol uptake). Not mutated version of UPC2 binds to sterol regulatory elements of ergosterol biosynthesis genes [2]. In contrast to UPC2, SUT1 does not appear to bind to DNA directly. This protein acts rather at transcriptional level by relieving hypoxic genes from Cyc8-Tup1 repression through its physical interaction with Cyc8. Both SUT1 and UPC2 are upregulated under anaerobic conditions. Interestingly, among UPC2-1 responsive and anaerobic genes are two ABC transporters, *AUS1* and *PDR11* which are required for yeast anaerobic growth and sterol uptake [54].

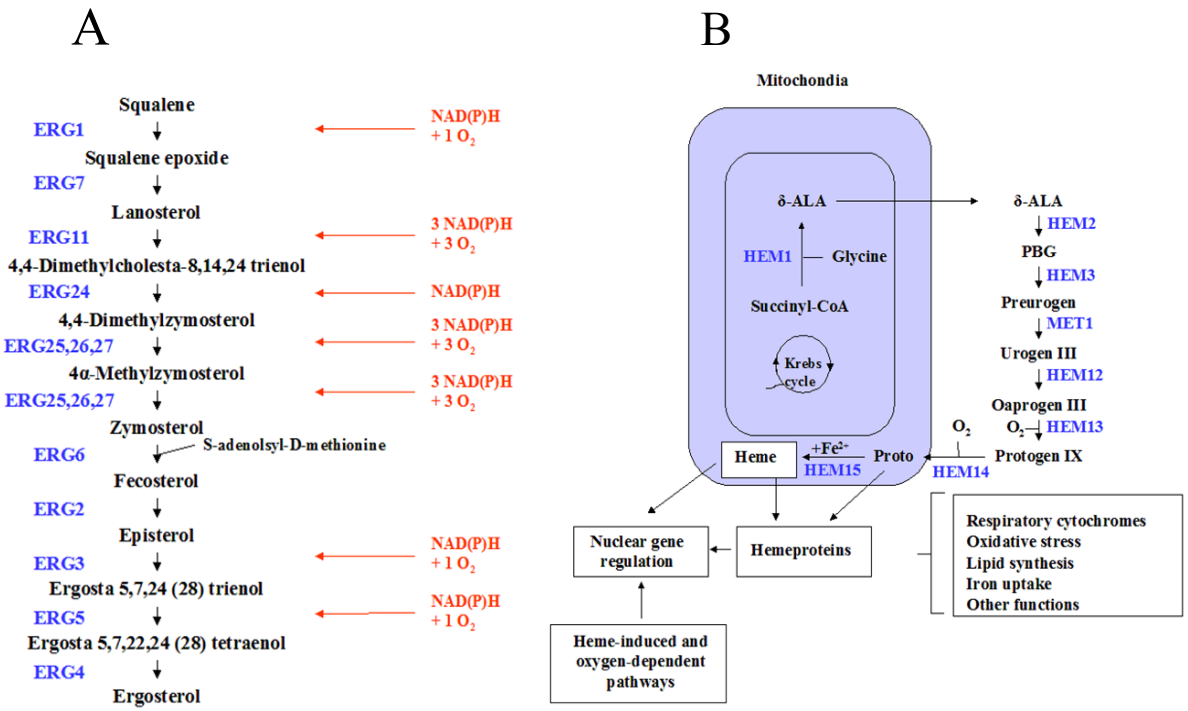


Figure 11: Biosynthesis of ergosterol and heme in yeast cells. (A) Sterol biosynthesis pathway is accomplished by ERG proteins and requires molecular oxygen on several steps. (B) Many of ERG proteins have heme prosthetic group which is required for theirs activity. Deletion of *HEM1* (5-aminolevulinate synthase) gene prevents heme synthesis by blocking conversion of succinly-CoA into 5-aminolevulinate. Under that condition ergosterol biosynthesis is blocked, and cells relay on sterol uptake. ERG enzymes are marked in blue. Oxygen dependent reactions are labelled in red [54].

1.4.2 Role of the cell wall in sterol uptake process in *Saccharomyces cerevisiae*

For being internalized into yeast cell sterol molecules have to pass the barrier of the cell wall which surrounds the cell and overlay plasma membrane. The fungal cell wall is composed of mannoproteins, β-glucans and chitin forming a macromolecular complex (Figure 12). With respect to their biochemical characteristics two groups of yeast cell wall proteins can be discriminated. The first group includes proteins disulphide-linked or non-covalently attached to the wall components (SEP—SDS-extractable proteins). The second group consists of proteins which are covalently linked to the glucan framework (CWP—covalently bound cell wall proteins) [55]. During adaptation to anaerobiosis budding yeast remodel the content of the cell wall [56]. Previous studies suggested that some of CWP proteins (Dan1p, Dan3p, Dan4p and Tir4p) could play a role in anaerobic sterol influx since their expression is significantly upregulated in anaerobiosis. Moreover, it was shown that coexpression of Aus1p together with Dan1p (but not each of the proteins alone) increases sterol influx around 1.7-fold under aerobic conditions [2]. However, the exact role of those proteins in sterol incorporation process is not clear [1]. One possibility is that they act in a way similar to FIT

proteins (FIT1, FIT2, FIT3) which take a part in iron uptake process. These group of mannoproteins has been shown to be responsible for binding of iron within the cell wall and although FITs are not directly involved in transport of iron through the plasma membrane, their abscence greatly reduces the amount of internalized iron [56].

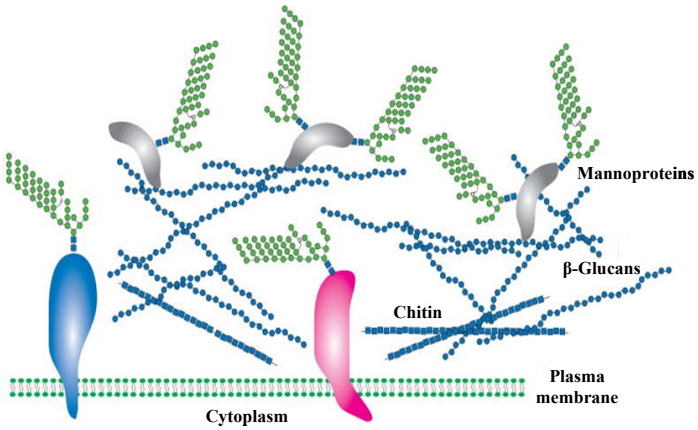


Figure 12: The architecture of yeast cell wall. Fungal cell wall is composed of mannoproteins, β-glucans and chitin forming a macromolecular complex. The cell wall polysaccharides that have been described so far are composed of polymers of mannose, glucose, galactose, N-acetylglucosamine, and/or rhamnose, and these include mannans, glucans, chitin, galactomannans, glucomannans, rhamnomannans, and phosphomannans. Fungal cell walls also contain covalently and noncovalently linked glycoproteins that bear N- and O-glycans.

1.4.3 Transport of sterol molecules from plasma membrane to endoplasmic reticulum in yeast

The cell wall and ABC transporters seem to play a crucial role in sterol uptake. How sterols are intracellulary transported remains unknown. Theoretically, they could be carried by vesicular transport or carrier proteins. However, it was shown that treatment with Brefeldin A (a chemical compound which disrupts ER to Golgi vesicular trafficking) only partially blocks the transport of newly synthesized cholesterol [57]. Transport from PM to ER also seems to be a non-vesicular process since estrification of incorporated sterol is not blocked in mutants defected in vesicular trafficking [29].

Yeast lack the homologs of known mammalian sterol binding proteins like START (Steroidogenic Acute Regulatory Protein), caveolin or SCP2 (Sterol Carrier Protein 2) [30]. However, they express proteins belonging to the conserved ORP family which were shown to bind lipids and oxysterols. The yeast ORP family comprises of seven proteins (OSH1-OSH7) which possess at least one overlapping function, since deletion of single OSH protein does not have physiological consequences, but removal of all of them is lethal [58]. The crystal structure of OSH1 (Kes1p) was solved displaying existence of hydrophobic binding pocket covered by a lid forming domain. Kes1p was shown to bind sterol (as well as PS and PI) directly, however it is suggested that OSH proteins

function more as lipid/sterol sensors than transporters. Although sterols can be taken up in mammalian cells by endocytosis, it seems that in yeast cells other, non-vesicular process is responsible for sterol uptake [59] (Figure 13).

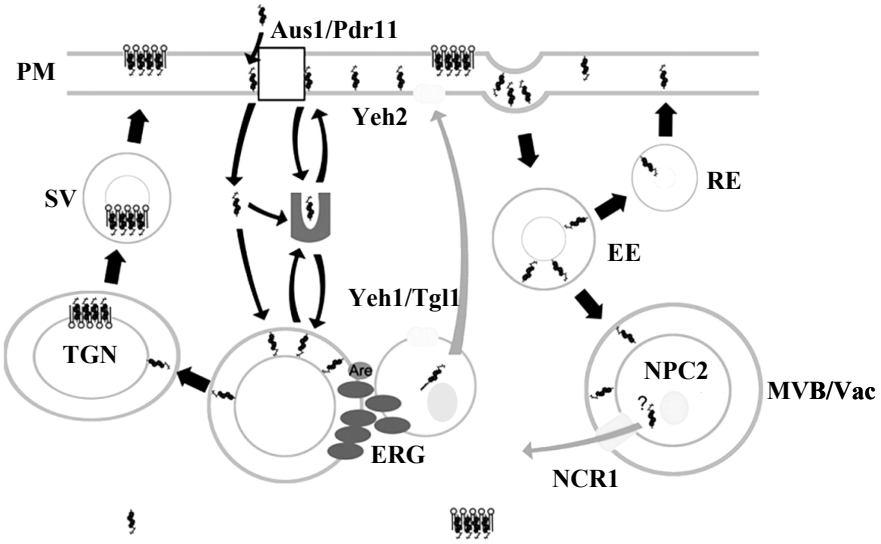


Figure 13: Sterol transport pathways in yeast. Straight arrows represent vesicular routes, curved arrows represent non-vesicular routes. Brown arrows indicate routes for which there is no direct evidence. Ergosterol is synthesized by proteins located in the ER and in lipid particles [60]. Sterol carrier proteins (Osh) might transport ergosterol to the cell surface. Vesicular transport to the plasma membrane occurs via the trans Golgi network (TGN) and secretory vesicles (SV). Ester hydrolases (Yeh1/2, Tgl1) can mobilize ergosterol from steryl esters in the LPs and the PM.The ABC transporters Aus1p/Pdr11p are involved in the uptake of extracellular sterols and might transfer them to carrier proteins (Osh). Vesicle budding can lead to internalization of sterols to early endosomes (EE). They can either immediately be back-transferred in recycling endosomes (RE) or be sent to the multivesicular body (MVB) or the vacuole (Vac). Ncr1/Npc2 might be involved in transfer of sterols from the MVB/Vac to other organelles [30].

1.4.4 Role of ABC transporters in sterol uptake in yeast

Saccharomyces cerevisiae exhibit many aspects of sterol homeostasis in common with higher eukaryotes but the process of sterol uptake mechanism seems to be different. Yeast cells do not express homologs of mammalian LDL receptor and most probably pathway other than endocytosis is involved in internalization of sterols. As mentioned before studies on sterol influx revealed the involvement of two ABC transporters - Aus1p and Pdr11p since deletion of both proteins abolished sterol internalization under anaerobic conditions (Figure 14). Additionally, Aus1p/Pdr11p mediated sterol uptake was inhibited by vanadate, indicating that classical inhibitor of ABC proteins can blocks the sterol import [1]. It was also proven that coexpression of Aus1p with cell wall mannoprotein Dan1p is sufficient to promote sterol in aerobiosis but only to a certain extent [2]. Other studies identified a protein called Det1p – a phosphatase which is suggested to be involved in the non-vesicular transport of sterols between the endoplasmic reticulum and plasma membrane

[61]. Deletion of *DET1* disturbs proper trafficking of Aus1p to plasma membrane and leads to a decrease in the amount of incorporated sterols.

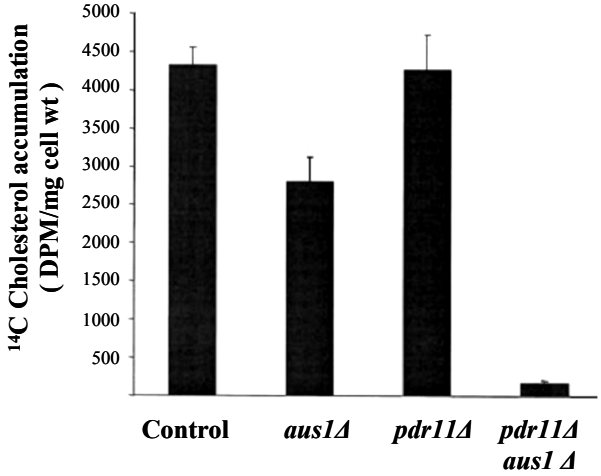


Figure 14: Sterol uptake in *AUS1* and *PDR11* deletion mutants under anaerobic conditions. *AUS1* and *PDR11* were deleted from the SCY325 control strain and uptake of radioactive cholesterol was investigated. Sterol uptake affected by single deletion of *AUS1* but not *PDR11* gene. Deletion of both genes abolished internalization of exogenously supplied cholesterol. Results are the means of ± SD of three independent determinations [1].

It is well known that cells sustain a gradient of sterol concentration across the secretory system with the highest amount in the plasma membrane and the lowest at the endoplasmic reticulum where the biosynthesis of sterols takes place [30]. Interestingly, there exists a similar gradient of sphingolipids among cellular membranes [62]. Since sterols and sphingolipids have a high affinity to each other, it has been proposed that raft association is one of the primary determinants of the intracellular distribution of those compounds, especially that raft domains are enriched in the plasma membrane and absent from endoplasmic reticulum [6,63]. The model in which Aus1p and Pdr11p mediate nonvesicular transfer of sterols from plasma membrane to endoplasmic reticulum was proposed by Li and Prinz [6]. They showed that the propensity of PM sterols to be moved to the ER is largely determined by their affinity for sterol sphingolipid-enriched microdomains (rafts) what suggests that raft association is a primary determinant of sterol accumulation in the PM and that Aus1p and Pdr11p facilitate sterol uptake by increasing the cycling of sterol between the PM and ER (Figure 15).

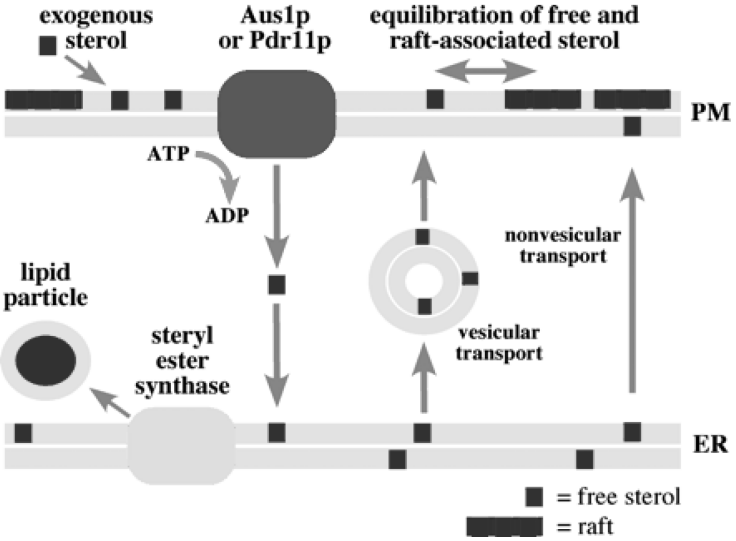
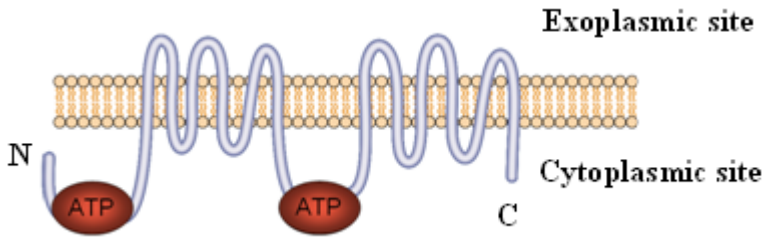


Figure 15: Influence of Aus1p, Pdr11p and raft association on sterol uptake and intracellular distribution. Sterol diffuses into the PM from the medium. Aus1p and Pdr11facilitate nonvesicular sterol transport to the ER. Sterol (not estrified in the ER) is rapidly moved back to the PM and concentrated there by a process that is likely driven by raft association [6].

1.4.5 Aus1p protein as a putative sterol transporter

Aus1p (encoded by YOR011W/*AUS1*) is predicted to be a transmembrane protein localized to the plasma membrane. Analysis of its amino acid sequence reveals existence of 12 transmembrane domains with topology NBD-TM-NBD-TM (Figure 16A). Sequence alignment of yeast ABC proteins with similar topology allowed for identification of crucial motifs characteristic for ABC proteins within the sequence of Aus1p (Figure 16B). Analysis of similarity and identity degree between the amino acid sequence of Aus1p and other yeast ABC transporters shows that the protein of interest has one homolog in *Saccharomyces cerevisiae* called Pdr11p (YIL013C). Other organisms do not possess Aus1p homologs.

A



B

Gene	NBD1 Walker A	NBD1 Signature motif	NBD1 Walker B	NBD 2 Walker A	NBD 2 Signature motif	NBD 2 Walker B
Pdr15	GRPGSGCT	VSGGERKRVSIA	WDNA	GASGAGKT	LNVEQRKRLTIGV	LVFLDE
Pdr5	GRPGSGCT	VSGGERKRVSIA	WDNA	GASGAGKT	LNVEQRKRLTIGV	LVFLDE
Pdr10	GRPGAGCT	VSGGERKRVSIA	WDNA	GASGAGKT	LNVEQRKRLTIGV	LVFLDE
Pdr18	GRPGAGCT	VSGGERKRVSIA	WDNA	GESGAGKT	LNVEQRKKLSIGV	LLFLDE
Snq2	GRPGAGCS	VSGGERKRVSIA	WDNA	GESGAGKT	LNVEQRKKLSIGV	LLFLDE
Pdr12	GRPGAGCS	VSGGERKRVSLV	WDNA	GESGAGKT	LNVEQRKKLSIGV	LLFLDE
Aus1	GYP---TS	VSGGERKRISII	WDNS	GESGAGKT	LNPTQRKLLSIGV	LLFLDE
Pdr11	GNP---TS	VSGGERKRISII	WDNS	GESGAGKT	LSPTQRKLLSIGV	LLFLDE
YOL075C	GGSGSGKT	LSGGEKRRLSIG	LDEP	GPSGSGKS	ISGGEKRRVTMGV	ILLLDE
Adp1	QDK----T	LCGGLSPDESGN	CHCD	GGSGAGKT	ISGGEKRRVSIAC	VLFLDE

Figure 16: Aus1p protein is a full size ABC transporter. (A) Schematic structure of Aus1p protein. Aus1p is a plasma membrane protein containing 12 transmembrane helices and displaying topology NBD(TMD)-NBD(TMD). (B) Alignment of yeast ABC proteins with inverted topology reveals the existence of conserved domains within both NBD regions.

AUS1	PDR11
VDRCLTYFR	VGNDYVR
LTTTGSYSR	LWIQSPYYKHWK
VFDYCFK	**LAHLQYNAA**
LAHLQYKAA	**VIYMK**
VIYLK	**LGNDYVRGR**
LSNELYNLK	**LKYTYHHVW**
LGNDYVRGR	LLRWNNYLKR
LSYTYHHVW	LPNACDYFVAHD
LVYDK	VWSPPSYMEQI
VDYTTSLWK	LQSKEVYIAREAR
LITRRQYICTK	VMQPYSLFPR
LTYVAR	VGFYLTYIK

Figure 17: Analysis of the protein sequences of Aus1p and Pdr11p. (A) CRAC (Cholesterol Recognition/Integration Amino Acid Consensus) motifs identified within the amino acid sequence of Aus1p and Pdr11p. Four marked motifs are conserved between both proteins.

Additionally, 12 putative CRAC (Cholesterol Recognition/Interaction Amino acid Consensus) motifs can be found in the Aus1p amino acid sequence and four of them are identical between Aus1p and Pdr11p (Figure 17). CRAC motifs are supposed to interact with sterols and this [64] the only sort of sterol interacting motif which can be detected *in silico* within Aus1p protein sequence. Although as much as 12 putative CRACs exist in the sequence, only two of them (CRAC 4 and CRAC 8) are localized on the border of transmembrane domains. Such localization is characteristic for functional CRAC motifs and the sequences which fulfill both conditions – fit to the CRAC - motif algorithm and localize at the border of TM domains and intra/extracellular loops can be considered to be functional CRACs.

As mentioned in previous chapter deletion of *AUS1* gene decreases sterol uptake during anaerobiosis and deletion of *AUS1* and *PDR11* abolishes the sterol influx almost completely. Sterols are crucial elements of the cellular membranes so their transport and metabolism are tightly regulated. Initial steps of sterol incorporation depend on the activity of ABC transporters and their role seems to be indispensable for cell viability. Although several studies have been done, still the exact function of Aus1p and Pdr11p proteins remains unclear.

2. AIM OF THE THESIS

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Equipment

Incubator	IG-150 Incubator, Thermo Electron Corporation
SLM Aminco Spectrofluorymeter	Thermo Fisher Scientific
Confocal Microscope Fluo View-1000	Olympus
Centrifuge Avanti J-20XP (Rotor JLA10.500)	Beckmann Coulter GmbH
Ultracentrifuge Optima L-100K (Rotors: 45Ti, 70.1Ti, SW40Ti, SW60)	Beckmann Coulter GmbH
Table top centrifuge Biofuge	Heraeus
Gel Electrophoresis Mini Protean System	Bio Rad
Gel-dryer	Uniequip
FPLC System AKTA	Amersham Biosciences
pH-Meter 761 Climatic	Knick
Biophotometer Plus Spectrophotometer	Eppendorf
Phosphoimager Image Analyser FLA3000 BASReader Software	FujiFilm
Semi-Dry Transfer cell TransBlot SD	BioRad
Thermal Cycler MyCycler	BioRad
Agarose Gel Electrophoresis System	BioRad
Scintillation Counter	Pacard, Canberra Company
Flow Cytometer FACSCalibur	Becton Dickinson
ABI Prism 7000	Applied Biosystem
FACS analysis software Cyflogic	Cyflogic

3.1.2 Chemicals

8-Azido-[α-32P] ATP (12.5 Ci/mmol) was purchased from ALT bioscience (Lexington, KY, USA); [γ-32P]ATP from Hartmann Analytic (Braunschweig, Germany). The detergent n-dodecyl-β-maltoside (DDM) was obtained from GLYCON Biochemicals (Luckenwalde, Germany). POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine), POPE (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanol- amine), POPS (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine), SM (sphingomyelin), and NBD (7-nitrobenz-2-oxa-1,3-diazole)-lipids including 25-NBD-cholesterol (25-{N-[NBD-methyl]amino}-27-norcholesterol), C_6-NBD-PS (1-palmitoyl-2-NBD-hexanoyl-*sn*-glycero-3-phosphoserine) and C_{12}-NBD-PS (1-palmitoyl-2-NBD-lauroyl-sn-glycero-3-phosphoserine) were purchased from Avanti Polar Lipids (Alabaster, AL). POPS stereoisomers were a gift from David Daleke (Department of Biochemistry and Molecular Biology, Indiana University, Bloomington, Indiana). All restriction enzymes, dNTPs, polymerase and ligase were ordered from New England Biolabs (Frankfurt, Germany). M2-Anti FLAG antibody, M2-anti Flag resin, ssDNA and SYBR Safe DNA gel stain were purchased from Invitrogen (Darmstadt, Germany). GAMPO antibody, molecular weight standards and SM-2 BioBeads were purchased from BioRad (Munich, Germany). Bacto Agar, Bacto Tryptone and Yeast Extract were purchased from BD Becton and Dickinson company (Lepont de Claix, France). Hybond ECL nitrocelullose membrane and autoradiography film Hyperfilm ECL were obtained from Amersham Bioscience. Nycodenz was purchased from Axis-Schield (Oslo, Norway). All other chemicals and reagents were obtained from Sigma-Aldrich (Munich, Germany) unless otherwise indicated.

3.1.3 Kits

QIAprep Spin Miniprep kit	Quiagen, Hilden, Germany
QIAquick gel extraction kit	Quiagen, Hilden, Germany
Micro BCA protein assay kit	Pierce, Rockford, USA
ProFound HA Tag IP/Co-IP Kit	Pierce, Rockford, USA
High Pure mRNA Isolation Kit	Roche, Berlin, Germany
Transcriptor High Fidelity cDNA Synthesis Kit	Roche, Berlin, Germany
TAMRA labelling Kit (Fluoro Spin 557)	Princeton Separations, Adelphia, USA
QuikChange™II XL Site-Directed Mutagenesis Kit	Stratagene, Waldbroon, Germany
ECL plus western blotting detection system	Amersham Biosciences, Freiburg, Germany

3.1.4 Yeast strains

Table 1: Summary of yeast strains used in this study

Yeast strain	Relevant phenotype	Source
W303	MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100	Roland Lill, Philipps-Universität Marburg
BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0	Michal Surma, MPI Dresden
MMY28	W303 hem1Δ:: LEU2	Roland Lill, Philipps-Universität Marburg
GKY22	W303 hem1Δ :: LEU2 pdr11Δ :: loxP aus1Δ :: loxP-HIS5Sp- loxP	This study
MMY112	W303 hem1Δ :: LEU2 cho1Δ :: loxP-HIS5Sp-loxP	This study
MMY115	W303 cho1Δ :: loxP-HIS5Sp-loxP	This study
MMY116	W303 AUS1::HA	This study
YPL1	BY4741 hem1Δ :: KAN dan1Δ :: loxP-HIS5Sp-loxP	This study
YPL2	BY4741 hem1Δ :: KAN dan4Δ :: loxP-HIS5Sp-loxP	This study
BJ 1991	MATa leu2 trp1 ura3-52 prb1-1122 pep4-3 gal2	Ralf Erdmann, Ruhr Universität Bochum
YPH500	MATα ura3-52 lys2-801_amber ade2-101_ochre trp1-Δ63 his3-Δ200 leu2-Δ1	Agilent Technologies

3.1.5 Bacteria

DH5α (*E. coli*) F$^-$ endA1 recA1 hsdR17(r_k^- m_k^+) supE44 λ$^-$ thi-1 gyrA(Na1) relA1 Φ80 lacZΔM15Δ(lacZY A-argF)

XL10-Gold Tetr Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lacHte [F′ proAB lacIqZΔM15 Tn10 (Tetr) Amy Camr]

3.1.6 Oligonucleotides and plasmids

Primers and oligonucleotides used for the PCR reactions are described in Table 1. All constructs used in this study are listed in Table 2.

A vector carrying N terminally Flag-tagged *AUS1* sequence was created by ligating the PCR-generated open reading frame sequence of *AUS1* (primer set F-Aus1 and R-Aus1) into the Pac1 and Not1 restriction sites of pESC-URA vector (Stratagene, La Jolla, CA). A vector carrying RFP tagged *AUS1* gene was generated by ligating PCR amplified monomeric RFP (primer set F-Aus1-RFP and R-Aus1-RFP) into the PacI restriction site of pESC-URA containing *AUS1* gene cloned into Not1/Pac1 restriction sites. A mutant unable to hydrolyze ATP was generated by site-directed mutagenesis (primer set F-Aus1-MM and R-Aus1-MM) following the manufacturer's (Stratagene) instructions for mutagenesis and was verified by sequencing. Low copy vectors carrying *AUS1* gene under the control of 1000bp endogenous promoter or under the control of GALs promoter were generated by homologous recombination method as described by Oldenburg et al. [65] using the primers Prom-F/Prom-R and F-GALs /R-GALs respectively.

Table 1: Summary of primers used in this study

Primer	Sequence from 5´ to 3`
F-Aus1	GAGAGCGGCCGCAATGGATTACAAGGATGACGACGATAAAATCTCAATTTCAAAGTACTTCACT
R-Aus1	GAGAATTAATTAATTAGTTGTGTACAGGCTT
F-Aus1-RFP	TTAATTAAATGGCCTCCTCCGAGGAC
R-Aus1-RFP	TTAATTATTTAGGCGCCGGTGGAGTG
F-Aus1-MM	ATGGGTGAGTCCGGTGCAGGTATGACTACTTTGCTGAATGTC
R-Aus1-MM	TGATAAGACATTCAGCAAAGTAGTCATACCTGCACCGGACTC
F-CHO1	GAGAGCGGCCGCAATGGATTACAAGGATGACGACGATAAAATCGTTGAATCAGATGAAGATTTCG
R-CHO1	GAGATTAATTAATTACTATGGCTTTGGAATTTTCAAG
1DF-AUS1	AGCTGAATAGTAAAGACTGCTGTAATTCATCTCTCAGTCCTTGCAGTCTGCTTTTTCTGGAATTCCTAGTCT AGAAGCTT
1DR-AUS1	CTGTACAGGCTTCTTCCCTCTGTGTGGAATTACTTTGGTGATAATTTTAAAGACCTTTGGCCATACACACCT CGAGGGCCC
2DF-PDR11	AGCTCTCTGCTCTTTTAGCTATTAATAGTATTATCACACACTTAAACCCTTTTCTCATTAGTTCTCCTAGTCT AGAAGCT
2DR-PDR11	AGTAGATATGGGGACATTTCAAAATTAAAAAGGATATATGATGCTTCGTTGAACGTAGAGGGACCACACC TCGAGGGCCC
3DF-CHO1	TGTCATTTTTAGTTGTCTATTTGATTCAATCAAAAAACAAAAATAAAACTATATATTAAAAA CTCCTAGTCTAGAAGCTT
3DR-CHO1	AAAGTAGAATAAAAAGTTATATGTACAAATTTTTTTTGACGCCAGGCATGAACAAAAACTA CACACACCTCGAGGGCCC
4DF-DAN1	ATGTCTAGAATTAGTATATTAGCTGTCGCCGCAGCATTAGTGGCAAGTGCAACCGCCGCATCCTCCTAGTC TAGAAGCTT
4DR-DAN1	CTATAACAATAGAGCGGCGGCACCAGCAATAGCAGCGGCACCGAAAACACCGTTATTGAACCACACACCT CGAGGGCCC
5DF-DAN4	CAAACTGAAATAAGTACCAGTTCCTGTCGAATCTGCGATATCCGAAAAACTCCGAAGAAAGCACTCCTAG TCTAGAAGCTT
5DR-DAN4	TCAATTTAAATTATTTTACAATTGTTTATATGCTTGTGAAGATTATATTGTATCACTATAGCACACACCTCG AGGGCCC
6DF-HEM1	ATGCAACGCTCCATTTTTGCGAGGTTCGGTAACTCCTCTGCCGCTGTTTCCACACTGAATAGCTCCTAGTCT AGAAGCTT
6DR-HEM1	TTACTGCTTGATACCACTAGAAACCTCTAGTTGTTTAACGATGGGGTCTCTAACATTAGGGCACACACCTC GAGGGCCC
Prom-F	CCAAGCGCGCAATTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTCATTTCAGGAAGCGATCAAAAAG
Prom-R	CACCTTGAAGCGCATGAACTCCTTGATGACGTCCTCGGAGGAGGCCATGTTCTGTACAGGCTTCTTCCCT

Primer	Sequence from 5´ to 3`
F-GALs	ATACCTCTATACTTTAACGTCAAGGAGAAAAAACCCCGGATTCTAGAATGTCAATTTCAAAGTACTTCAC
R-GALs	CACCTTGAAGCGCATGAACTCCTTGATGACGTCCTCGGAGGAGGCCATGTTCTGTACAGGCTTCTTCCCT
7F-AUS1-HA	AAGGTCTTTAAAATTATCACCAAAGTAATTCCACACAGAGGGAAGAAGCCTGTACAGAACGGAGCAGGGG CGGGTGC
7R-AUS1-HA	TCAAATCCGATTGGAAGCATTTTTTGTCGTGCACTTTTGCTATATAAATATTTTTTGAGCGAGGTCGACGGT ATCGATAAG
8F-ACT-RT	GGCATCATACCTTCTACAACG
8R-ACT-RT	TACCGGAAGAGTACAAGGAC
9F-AUS1-RT	TGAGGCTGCACTAACAATCG
9R-AUS1-RT	TCGTAAACGAGCGTGTTGTC
10F-PDR11-RT	GAAGAAGAAGCCCGGAAATC
10R-PDR11-RT	TAGCATCTTGCAGACCGTTG

1-6 Primer sets used to amplify the respective gene disruption cassette containing loxP-HIS5Sp loxP.

7-10 Primer sets used for RT-PCR

Prom-F/Prom-R primer set used for generation of pRS416 vector carrying *AUS1* gene under control of 1000bp endogenous promoter

F-GALs/R-GALs primer ser used for generation of pRS416 vector carrying *AUS1* gene under the control of GALs promoter

Table 2: Summary of plasmids used in the studies

Name	Marker	Description	Source
pESC-ura	URA	Empty vector	Agilent Technologies
pRS416	URA	Empty vector	Michal Surma, MPI Dresden
pESC-AUS1	URA	pESC-ura vector containig N terminally Flag tagged *AUS1* gene under the control of GAL10 promoter	This study
pESC-AUS1-RFP	URA	pESC-ura vector containing AUS1 gene tagged at N terminus with Flag tag and at C terminus with monomeric RFP; gene is placed under the control of GAL10 promoter	This study
pESC-AUS1 ^{K788M}	URA	pESC-ura vector containig N terminally Flag tagged *AUS1* carrying the mutation within Walker A motif of NBD2; gene under the control of GAL10 promoter	This study
pESC-AUS1 ^{K788M} - RFP	URA	pESC-ura vector containing *AUS1* gene tagged at N terminus with Flag tag and at C terminus with monomeric RFP and carrying the mutation within Walker A motif of NBD2; the gene is placed under the of GAL10 promoter	This study

Name	Marker	Description	Source
GalS-AUS1-RFP	URA	pRS 416 vector containing *AUS1* gene tagged at the C terminus with monomeric RFP; the gene is placed under the control of GALs promoter	This study
Endo-AUS1-RFP	URA	pRS 416 vector containing *AUS1* gene tagged at the C terminus with monomeric RFP; the gene is placed under the control of *AUS1* endogenous promoter	This study
pESC-CHO1	URA	pESC-ura vector containig N terminally Flag tagged *CHO1* gene under the control of GAL10 promoter	This study
pESC-PDR11	URA	pESC-ura vector containig N terminally Flag tagged *PDR111* gene under the control of GAL10 promoter	This study

3.1.7 Media and buffers

Yeast medium and plates

SD	0.17% Yeast Nitrogen Base, 0.5% Ammonium sulfate, 0.0055% Adenine, 0.0055% L-Thyrosin, 0.0055% Uracil, 2% glucose, 1% aminoacides stock
SG	0.17% Yeast Nitrogen Base, 0.5% Ammonium sulfate, 0.0055% Adenine, 0.0055% L-Thyrosin, 0.0055% Uracil, 2% galactose 1% aminoacides stock
Sterol containing medium	For a sterol supplementation selected medium (SD, SG or YPD) was supplemented with sterol mixture (20 μg/ml of sterol and 0.5% Tween 80), and 0.01 mg/ml methionine
ALA containing medium	For supplementation with 5-aminolevulinic acid (ALA) selected medium (SD, SG or YPD) was supplemented with 20 μg/ml aminolevulinic acid
Media for growth of *CHO1* mutants	Yeast mutants lacking *CHO1* gene were grown in selected medium (SD or SG) was supplemented with 600mM ethanolamine, 0.01 mg/ml myoinositol
YPD	1% BactoYeast Extract, 2% BactoPepton, 2% sugar (glucose or galactose)

For preparation of solid media, the liquid media were supplemented with 2% agar.

Bacteria medium

LB-medium	1% Bacto™ Tryptone, 0.5% Bacto™ Yeast Extract, 0.5% NaCl, in ddH_2O, pH 7. Optionally ampicilin was added to the medium in concentration of 100 µg/ml.

Buffers

Buffer	**Content**
10x PBS	40 g NaCl, 1 g KCl, 7.1 g Na_2HPO_4 $2H_2O$, 1 g KH_2 PO_4 in 500 ml ddH_2O
TBS	50 mM Tris-HCl pH 7.4, 150 mM NaCl
TBST	100 ml 10xTBS, 10 ml 10% Tween 20, 890 ml H_2O,
10x PAGE running buffer	30 g Tris-Base, 144 g Glycin, 10 g SDS in 1 l ddH_2O
4x SDS-PAGE-sample buffer	25% β-mercaptoethanol, 5% SDS, 0.05% Bromophenol Blue , 25% Glycerin, 12.5% 1 M Tris-HCl buffer pH 6.8
Blocking buffer	0.1% Tween-20, 5% milk, 1x PBS in ddH_2O
Transfer buffer	40 ml PAGE-Buffer, 20 ml methanol, 0.6 ml SDS (10%), in 100 ml ddH_2O
IP buffer	50mM Tris, 100 mM KCl, 1xPIC, 1mM PMSF
TK buffer	100 mM KCl, 50 mM Tris-HCl, pH 7.4
TKG buffer	100 mM KCl, 50 mM Tris-HCl, 20% glycerol, pH 7.4
Reconstitution buffer	20 mM HEPES, 150 mM KCl, pH 7.4

<u>Composition of SDS-PAGE gels</u>

Resolving gel (8%)	4.6 ml ddH2O, 2.7 ml Acrylamid/Bisacrylamid (30%), 2,5 ml 1.5 M Tris-HCl pH 8.8, 100 µl SDS (10%), 100 µl APS (10%), 4 µl TEMED,
Stacking gel (5%)	3.4 ml ddH_2O, 1 ml Acrylamid/Bisacrylamid (30%), 1.5 ml 0.5 M Tris-HCl pH 6.8, 60 µl SDS (10%), 60 µl APS (10%), 6 µl TEMED

3.2 Methods

3.2.1 Polymerase Chain Reaction (PCR)

The PCR (polymerase chain reaction) technique allows for amplification of specific DNA sequence *in vitro*. The reaction requires: the nucleotide mixture (dNTPs), the forward and reverse primers (namely F and R), the enzyme DNA polymerase and a buffer, specific for a type of used polymerase. The denaturation of the DNA template is the first step of reaction and leads to separation of DNA strands. During the second step – so called annealing – primers anneal with template what allows for generation of new DNA strands during the third, extension step. The Table 3 represents the standard conditions recommended for PCR reaction carried with Phusion Polymerase.

Table 3: Standard PCR reaction mixture and amplification program

PCR reagents (final concentration)		**PCR scheme**		
DNA template	50 ng	**CYCLE**	**T (°C)**	**Time**
dNTPs	200 μM	Initial Denaturation	98	1 min
Polymerase	1 U	*Denaturation	98	10 sek
F-primer	0.5 μM	*Annealing	primer dependent	30 sek
R-primer	0.5 μM	*Extension	72	1min/1kB
PCR Buffer	1x	Final extension	72	15 min
ddH_2O	to final volume of 50 μl	Cooling	4	30 min

* The denaturation-annealing-extension steps were repeated 30 times

3.2.2 Quick Change Mutagenesis

The Quick Change mutagenesis strategy allows for insertion of point mutation into the dsDNA plasmid. The reaction was carried out according to the Stratagene kit (QuikChange™II XL Site-Directed Mutagenesis Kit) manual. Generally, the mutation was inserted during PCR step followed by digestion of parental (not mutated) DNA with DpnI enzyme (1 h, 37ºC) - an endonuclease that digests methylated DNA. The product of mutagenesis – a plasmid carrying mutated fragment DNA was then transformed into *E.coli* XL10-Gold cells for amplification. Plasmids were recovered from bacteria and sequenced.

3.2.3 DNA purification, cleavage and ligation

To control the size of PCR products or products obtained after restriction digestions, the DNA fragments were run on 1% agarose gel supplemented with SYBR Safe gel stain for visualization. If necessary, DNA was extracted from agarose gel using QIAquick Gel Extraction Kit. Enzymatic cleavage of DNA fragments with restriction enzymes was carried out in a final volume of 30 µl (1-2 µg of DNA, 1xbuffer, 1U of selected enzyme, water) for 3 h at 37ºC. Before ligation, all DNA fragments were run on agarose gel and purified from the gel. Ligation was carried out using T4 ligase with following rations of vector to insert: 1:3, 1:6, 1:9 (w/w). Reaction was run for 10 min at 25ºC and 16 h at 16ºC. The product of ligation was used to transform DH5α *E.coli* competent cells.

3.2.4 Transformation of bacteria

50 µl of *E.coli* DH 5α chemically competent cells was incubated for 10 min with ligation product on ice. Afterwards, 50 sek of heat shock at 42ºC was applied, followed by 2 min incubation on ice. About 700 µl of LB medium was added to the mixture and the cell solution was shaken for 1 h at 37ºC. Transformed cells were plated on LB plates supplemented with appropriate antibiotics.

3.2.5 Plasmid purification from bacteria

Plasmid DNA was purified from 10 ml overnight bacterial cultures growing on LB medium (supplemented with appropriate antibiotic) using QIAprep Spin miniprep kit. The DNA was eluted from columns with 50 µl of elution buffer and its concentration was determined with a Biophotometer Plus Spectrophotometer. New generated plasmids were sequenced by Invitek company (Invitek, Berlin, Germany).

3.2.6 Transformation of yeast cells

Yeast cells were transformed with DNA (in form of plasmid or PCR product) by lithium-acetate method. Shortly, yeast was cultivated to the 0.6-1 OD_{600} and subsequently 2 ml of the cell culture were harvested by centrifugation (5 min, 700 g, 23ºC) and washed once with 500 µl of 0.1 M lithium acetate. Cell pellet was dissolved in 250 µl of 50% PEG 3350 and mixed with 37.5 µl 1M lithium acetate, 10 µl of boiled ssDNA (11 µg/ml) and 45 µl of DMSO. Cells were incubated 30 min at 30ºC and then heat shocked by 15 min incubation at 45ºC. Pelleted cells were plated on plates with appropriate selective medium and incubated for 3-4 days at 30ºC.

3.2.7 Gene deletion

A PCR generated deletion strategy was used to replace selected yeast genes with HIS5 deletion cassette [66]. A specific primers (HPLC purified) overlapping around 60 bp from upstream and downstream of deleted DNA fragment were designed and used to amplify deletion cassette by PCR

method. PCR products were run on agarose gel, purified and transformed into yeast cells according to the previously described methods. Plates were incubated at 30ºC for 5-7 days and obtained mutants were analysed by PCR method.

3.2.8 RNA isolation from *Saccharomyces cerevisiae*

RNA was isolated from the exponentially growing cells (0.8 OD_{600}) with a High Pure RNA Isolation Kit. 10 ml of cells were harvested and diluted in 200 µl of PBS. The cell wall was removed by treatment with 10 µl of lyticase (0.5 mg/ml) for 15 min at 30ºC. The further steps of RNA isolation were done according to the kit manual. The concentration of RNA was evaluated by measurement of absorbance at 260 nm.

3.2.9 cDNA synthesis and evaluation of transcript amount by RT-PCR

cDNA was synthesized on mRNA template (5 µg) using Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Germany). Amplification of cDNA was done on ABI Prism 7000 cycler and the data were normalized to the amount of actin (*ACT1*) as a housekeeping gene. The reaction conditions are depicted in the Table 4.

Table 4: RT-PCR reaction mixture and amplification program

PCR reagents		PCR scheme		
cDNA template	0.5 µl	**CYCLE**	**T (°C)**	**Time**
Sybr Green Mix	10 µl	Pre heating I	50	30 min
F-primer	2.4 µl (20µM)	Pre-heating II	95	15 min
R-primer	2.4 µl (20µM)	* Deanturation	94	15sek
ddH_2O to final volume = 20 µl		*Annealing	60	30 sek
		*Extension	72	30sek

*The denaturation-annealing-extention steps are repeated 45 times

The level of the transcript amount was evaluated by $\Delta\Delta C_t$ method:

Fold difference = $2^{-\Delta\Delta Ct}$ where $\Delta\Delta C_t = \Delta\Delta C_{t\ sample} - \Delta\Delta C_{t\ calibrator}$

* C_t threshold cycle

3.2.10 Protein overexpression and purification

Saccharomyces cerevisiae strain BJ1991 carrying a vector expressing tagged protein was grown in selective SD media to 1-1.5 OD_{600} (30°C, 170 rpm). Cells were harvested (700 g, 5 min, 24°C), and inoculated into selective SG media to induce protein expression. Growth was continued at 30°C and cells (around 15 g wet weight) were harvested 16 h after induction, lysed by vortexing 5 times for 1

min with acid-washed glass beads (10 g, 0.5 mm) and 10 ml ice-cold TK buffer containing the protease inhibitors and 1 mM phenylmethylsulfonyl fluoride (PMSF). The lysate was clarified by centrifugation (500 g, 10 min, 4°C). Membranes were collected from the precleared lysates by centrifugation (50.000 g, 45 min, 4°C) and detergent-solubilized at a protein concentration of 1 mg/ml in TKG buffer supplemented with 1% (w/v) n-dodecyl-maltoside (DDM) on an end-over-end rotator for 2 h at 4°C. Insoluble material was removed by centrifugation (100.000 g, 45 min, 4°C) and the supernatant was incubated with 14 µl/ml M2 anti-Flag affinity resin for 12 h at 4°C. The resin was washed three times for 10 min at 4°C with TKG buffer containing 0.05% (w/v) DDM. Proteins were eluted with 100 µg/ml triple FLAG peptide in TKG buffer containing 0.05% (w/v) DDM. Proteins were concentrated using Amicon Ultra with molecular weight cutoff of 50 kDa. Elution fraction was analyzed by SDS-PAGE and Coomassie staining. Concentrations of purified proteins were determined using Micro BCA protein assay kit.

3.2.11 SDS PAGE

For analysis of protein samples SDS-PAGE gels were prepared. Samples were mixed with 4x concentrated loading buffer before application on the gel. Gels were run at 140 V for 40 min and subsequently proteins were visualized by Coomassie Blue or were used for Western Blot analysis. Blue Native PAGE (BN-PAGE) was prepared as described by Witting et al.[67] using stacking gel containing 3.5% acrylamide and gradient resolving gel containing 4% to 13% acrylamide.

3.2.12 Western Blot

For detection of tagged proteins with specific antibodies, proteins resolved on SDS-PAGE gels were transferred on the nitrocellulose membranes for 40 min at 20 V. The membranes were blocked with 5% not-fat dry milk supplemented with 0.05% Tween 20, for at least 2 h at room temperature. Afterwards membranes were washed twice with TBST buffer for 10 min and incubated with primary antibody (diluted according to the supplier instructions) for 12 h at 4ºC. Subsequently membranes were washed twice with TBST and incubated 1 h with secondary antibody (GAMPO 1:1000) diluted in milk. After washing with TBST Western blots were developed with an ECL Western Blotting Kit on photofilm.

3.2.13 Protein Mass Spectrometry

The protein preparation containing the recombinant Aus1p protein was separated on 8% SDS-PAGE gel. After staining with Coomassie Blue the gel lane was cut in 12 slices. Proteins in each of the slices were converted to peptides by in-gel digestion with trypsin. The recovered peptides were separated on a 20 cm reverse-phase column (Waters, Milford, MA, USA) using a 10-50% acetonitrile linear gradient on a nonoUPLC system (Waters, nanoAcquity, Milford, MA, USA). The

separated peptides were sprayed directly into a Q-TOF premier (Waters, Milford, MA, USA) mass spectrometer. The recorded spectra were analysed using the proteinLynx (Waters, Milford, MA, USA) and MASCOT (Matrix Science, London, UK) software packages.

3.2.14 Size Exclusion Chromatography

Size exclusion chromatography was conducted using an AKTA Purifier 900 fast protein liquid chromatography (FPLC) system equipped with a Superdex 200 Hi Load 16/60 size exclusion column and a UV detector, all obtained from Amersham Pharmacia Biotech (Little Chalfont, United Kingdom). Protein was injected onto the column preequilbrated with buffer liquid phase (TKG plus 0.05% DDM) at a flow rate 1 ml/min.

3.2.15 Preparation of liposomes and protein reconstitution

For protein reconstitution, lipids were dissolved in chloroform and evaporated. The lipid film (2.5 mg) was rehydrated in 1 ml reconstitution buffer (150 mM KCl, 20 mM HEPES, pH 7.5). The resulting multilamellar liposomes were extruded 31 times through a polycarbonate filter of a pore size of 200 nm (Avanti Polar Lipids). The obtained unilamellar liposomes (LUVs) were destabilized by incubation with 1.5 mM DDM. Proteoliposomes were prepared by addition of purified protein to destabilized liposomes in a lipid to protein ratio of 20:1 (w/w) followed by a 15 min incubation at room temperature. Protein-free liposomes were prepared similarly by replacing purified protein with elution buffer. DDM was removed by incubation for 16 h at room temperature with 0.3 g/ml SM-2 Adsorbent Bio-Beads (Bio-Rad, Hercules, CA). The resulting proteoliposomes were collected by centrifugation (100.000 g, 1 h, 4°C), resuspended in reconstitution buffer and used for assays immediately.

3.2.16 Density flotation

Density flotation was prepared as described by Chen at al. [68]. Shortly, 250 µl of proteoliposomes was mixed with an equal volume of 80% Nycodenz in an 11 x 60 mm centrifuge tube (Beckman, Fullerton, CA) and overlaid with 0.5 ml 30%, 0.5 ml 20%, 0.5 ml 10%, 0.5 ml 5%, 0.5 ml 2.5% (w/v) Nycodenz, and 0.5 ml reconstitution buffer. After centrifugation (130.000 g, 4 h, 4°C), 14 fractions (0.25 ml) were collected from the top of centrifuge tubes and analyzed for RFP fluorescence and total phosphate determination. Phosphate determination was prepared as described by Bottcher [69].

3.2.17 Collisional quenching assay

To elucidate the orientation of reconstituted Aus1p-RFP, collisional quenching of RFP fluorescence with $CuCl_2$ was performed. Vesicles in the absence and presence of 0.5% (w/v) Triton X-100 were

titrated with $CuCl_2$ (0 to 30 µM, as indicated) until RFP fluorescence intensities did not decrease further. Parallel samples were measured in which $MgCl_2$ was used instead of $CuCl_2$ showing that Mg had no detectable effect on RFP fluorescence. Data were corrected for volume changes and scattering. The fraction of RFP that is accessible to the quencher was calculated as $(F_b - F_{Cu})/(F_b - F_0)$ x 100, where F_b is the initial fluorescence of vesicles in buffer without $CuCl_2$, F_{Cu} is the fluorescence plateau value of vesicles titrated with $CuCl_2$, and F_0 is the fluorescence plateau value of Triton X-100-permeabilized vesicles titrated with $CuCl_2$.

3.2.18 ATPase assay

Purified Aus1p (2-10 µg) was mixed with 45 µl reconstitution buffer containing 2 mM dithiothreitol and incubated for 20 min at 23°C for protein activation. ATPase activity was analyzed by measuring the release of inorganic phosphate (Pi) using two methods: (i) a spectrophotometric assay following the manufacturer's instructions (EnzChek® Phosphate Assay Kit) (ii) an assay with [γ-^{32}P]ATP as described by Gorbulev et al. (26). Briefly, for the [γ-^{32}P]ATP assay, 5 µl ATP mix (1 mM ATP, 5 mM $MgCl_2$, 2 µCi [γ-^{32}P]ATP) was added to the mixture and the reaction was carried on for 40 min at 27°C. The reaction was stopped by placing samples on ice and the addition of 1.5 ml of reagent A (10 mM ammonium molybdate in 1 N HCl), 15 µl of 20 mM H_3PO_4 and 3 ml of reagent B (isobutanol, cyclohexane, acetone and reagent A in a ratio of 5:5:1:0.1, v/v). The mixture was mixed vigorously for 30 s. After phase separation, 1 ml of the organic phase was mixed with scintillation fluid (Ultima Gold XR, Perkin Elmer, Wiesbaden, Germany) and the release of inorganic phosphate was determined by β-counting on Packard Liquid Scintillation Analyzer. A similar protocol was used to determine the ATPase activity of reconstituted Aus1p. Inhibition was assayed in cocktails containing ATP mix supplemented with either 1 mM orthovanadate, 1 mM $BeSO_4$ and 5 mM NaF (BeF_x), or 1 mM AlF_3). Orthovanadate solutions (100 mM) were prepared from Na_3VO_4 (Fisher Scientific GmbH, Schwerte, Germany) at pH 10 and boiled for 15 min before each use to break down polymeric species.

3.2.19 Nucleotide Binding Assay

Nucleotide binding was measured by 8-azido-[α-^{32}P]ATP photo cross-linking experiments. Reactions were performed in a 96-well microtiter plate in a final volume of 25 µl per reaction. Purified wild-type or mutant Aus1p (about 2 µg of protein) was incubated for 5 min on ice with 8-azido-[α-^{32}P]ATP (0.01- 20 µM) in reaction buffer (100 mM KCl, 2.5 mM $MgCl_2$, 50 mM Tris/HCl, pH 7.4). For competition experiments, 0.1 µM to 20 mM unlabeled ATP was included in the buffer. Subsequently, samples were irradiated with UV light (254 nm, 8 W) for 5 min at 4°C, separated by SDS–PAGE, Coomassie Blue stained, dried and exposed to a phosphor screen. Samples were visualized with a FLA-3000 Fuji Imaging System and bands were quantified using Aida Image

Analyser 3.24 software. Apparent K_d for 8-azido-[α-^{32}P]ATP values were obtained from the best fit of the data to a hyperbolic curve using SigmaPlot software (Systat Software, Inc) and the equation $Y = B_{max}X/(K_{d(azidoATP)} + X)$, where B_{max} is the maximal binding, X is the concentration of 8-azido nucleotide, and $K_{d(azidoATP)}$ is the concentration of the 8-azido-^{32}P-nucleotide required to reach half-maximal binding. The half maximal inhibitory concentration (IC_{50}) for ATP was derived by plotting labeling intensities corresponding Aus1p as a function of unlabeled ATP concentrations. The $K_{D(azidoATP)}$ values and the IC_{50} values were used to calculate the $K_{D(ATP)}$ for ATP applying the Cheng-Prusoff equation [70].

3.2.20 Spotting assay

Yeast cells were inoculated from the plate to 5 ml of appropriate medium and cultivated up to 0.8-0.9 OD_{600} at 30 ºC. Two ml of cells were then harvested (700 g, 5 min, 23ºC) and washed once with PBS buffer. Cells were diluted to 0.3 OD_{600} and five, five fold serial dilution was prepared. 3µl of each dilution was spotted on the testing plates and the plates were incubated 3-5 days at 30 ºC.

3.2.21 Lipid and Sterol Uptake Assay

Uptake of 25-NBD-cholesterol was analyzed on cells cultured for 16 h in minimal medium containing 5 mg/ml Tween 80 and 20 µg/ml cholesterol mix (cholesterol : 25-NBD-cholesterol; 1:1, w/w). Before analysis by flow cytometry or confocal microscopy, cells were washed twice with ice-cold PBS buffer containing 0.05% (w/v) Nonidet P-40 and finally cells were resuspended in PBS. Uptake of C_6-NBD-PS was analyzed as described before [71] with small modifications. Briefly, cells were grown to 0.6-0.8 OD_{600} on SD medium and subsequently harvested by centrifugation (3.000 g, 5 min, room temperature). Cells ($5x10^7$) were incubated in 250 µl SD medium with 60 µM C_6-NBD-PS for 30 min at 30°C with periodic mixing. Prior to analysis by flow cytometry, cells were washed twice in ice-cold medium containing 3% (w/v) bovine serum albumin to extract C_6-NBD-PS from the cell surface. Flow cytometry was performed on FACSCalibur flow cytometer equipped with an argon laser using Cell Quest software. One µl of 1 mg/ml propidium iodide in water was added to 10^7 cells in 1 ml PBS just before flow cytometry analysis. Cells were analyzed without gating during the acquisition and the data were analyzed by Cyflogic software (www.cyflogic.com). A histogram of the red fluorescence (propidium iodide) was used to set the gate that excluded dead cells from the analysis. Green fluorescence (C_6-NBD-PS) of living cells was plotted on a histogram and the geometric mean of the fluorescence intensity was calculated.

3.2.22 Microscopy

Microscopy was performed using FluoView 1000 microscope (Olympus, Tokio, Japan) and a 60x (N.A. 1.35) oil-immersion objective. Fluorescence of NBD was excited with a 488 nm argon laser and recorded between 500 and 530 nm. RFP was excited using a 559 nm laser and emission was recorded between 570 nm and 670 nm. For staining with Calcofluor White cells (around 40µl OD 0.5) were placed on the glass slide. Subsequently 5 µl of CW solution (1mg/ml) and 5 µl of 10% potassium hydroxide were added. After 1 min incubation the fluorescence of Calcofluor White was examined by microscopy using excitation at 405 nm and recording the emission between 420 nm and 460 nm.

3.2.23 Measurement of phosphatidylserine transport in liposomes

Proteoliposomes with desired lipid composition were labelled with C_6NBD-PS. For labelling of outer leaflet, short chain NBD-PS diluted in buffer was added directly to the liposomes and incubated 10 min at 27ºC. Labelling on both leaflets was done by integration of 0.6 mol % of fluorescent lipid during formation of liposomes. For the assay 20 µl of liposomes and proteoliposomes (12 mg/ml of lipid) were incubated 40 min at 27ºC with a buffer containing 0.5 mM ATP, 1mM $MgCl_2$, 2 mM DTT and optionally 1 mM vanadate as an inhibitor of protein activity. The fluorescence intensity (ex: 470 nm, em: 540 nm) was recorded 30 sec before addition of dithionite (50 mM final concentration) and 540 sek afterwards. At the last step 0.5% Triton X-100 was added and fluorescence was recorded for additional 30 sec.

3.2.24 Preparation of Giant Unilamellar Vesicles (GUVs)

GUVs were prepared by electroformation method. The desired lipid composition (100 nmol of lipid in total) was dissolved in chloroform and dried on metal parts of electroformation chamber under vaccum for at least 1h. Subsequently, the chamber was filled with 250 µl of swelling buffer and closed tightly. The chamber was connected to the power supply and AC field was applied with stepwise increasing voltage from 20 mM up to 1.1 V in one step per 6 min. Frequency at 10 Hz was constant for at least 3 h and was followed by 30 min at 4Hz and 1.3 V to detach the GUVs. For generation of proteo-GUVs (GUVs containing protein) proteoliposomes were dried 24 h on metal parts of the electroformation chamber in NaCl saturated chamber at 4ºC and the further steps were the same as in case of GUV formation. For microscopy samples were diluted 1:1 with PBS and incubated 10 min before visualization.

3.2.25 Measurment of the liposomal membrane order

The measurement of liposomal membrane order was done as described by Kaiser et al. [72]. Shortly, to determine the GP (Generalized Polarization) values, 200 μM LUVs were stained with 200 nM Laurdan for 12 h at room temperature. Samples were measured using Aminco spectrofluorymeter and excitation wavelength of 385 nm. Emission spectra were recorded between 400 nm and 560 nm. GP values were calculated according to the formula:

$GP = (I_{440}-I_{490)} /(I_{440}+I_{490})$

3.2.26 Immunoprecipitation

To induce the expression of HA tagged Aus1 protein, 250 ml of yeast culture was grown overnight to 1 OD_{600} on selective medium in the presence of sterol mixture (20 μg/ml of cholesterol, 0.5% Tween 80) at 30ºC. 25 ml of 11% formaldehyde was added directly to the culture and incubated at room temperature for 20 min. Subsequently 37.5 ml of Glycine Stop solution (3 M glycine, 20 mM Tris) was added and the mixture was incubated for 5 min at room temperature. Cells were pelleted (700 g, 5 min, 4ºC) and washed one with cold water supplemented with 1mM PMSF. Cell pellet was redissolved in IP buffer in ratio 10 ml of buffer per 5 g of cells. Cells were disrupted by vortexing with glass beads 5 times with 1 min break and incubation on ice. Cell debris were removed by centrifugation (500 g, 5 min, 4ºC) and the supernatant was centrifuged 100.000 g 45 min 4ºC. The pellet was redissolved in TKG buffer to the final protein concentration of 1 mg/ml and incubated with 1% digitonin for 2 h at 4ºC with rotation. Sample was centrifuged 100.000g 45 min 4ºC. The supernatant was recovered and further steps were done using Pierce Immunoprecipitation Kit following manufacturer instructions. Final eluate was analyzed by mass spectrometry as described before.

4. RESULTS

4.1 Cloning and expression of *AUS1* gene from *Saccharomyces cerevisiae*

To characterize the putative sterol transporter Aus1p in details the full length *AUS1* gene was cloned and expressed in yeast *Saccharomyces cearevisiae*. Two versions of protein were overexpressed: Aus1p with Flag tag at N terminus (159 kDa) and Aus1p with Flag tag at N terminus and RFP tag at C terminus (184.5 kDa).

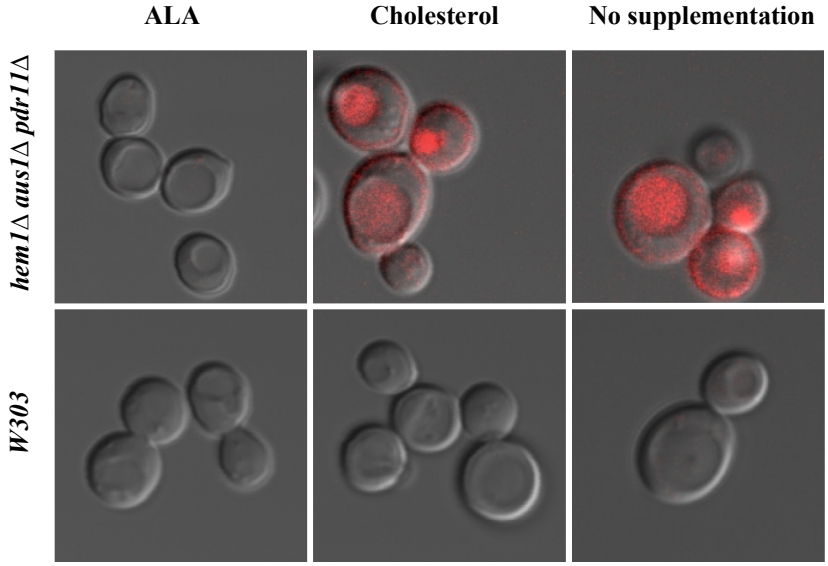


Figure 18: Expression of RFP tagged Aus1p protein under different conditions. Yeast cells *hem1Δaus1Δpdr11Δ* and W303 wild type cells were transformed with a centromeric vector carrying Aus1p-RFP sequence under the control of *AUS1* endogenous promoter (Endo-AUS1-RFP) and were cultivated in the presence of ALA, cholesterol or without any supplementation. Subsequently cells were visualized by fluorescence microscopy. Phase/fluorescent overlay images show that blocking of the sterol synthesis (by removing aminolevulinic acid from media) leads to activation of Aus1p protein expression in *hem1Δaus1Δpdr11Δ* yeast strain and that this activation is independent of the presence of sterol in the culture medium. No protein expression was detected in wild type W303 cells.

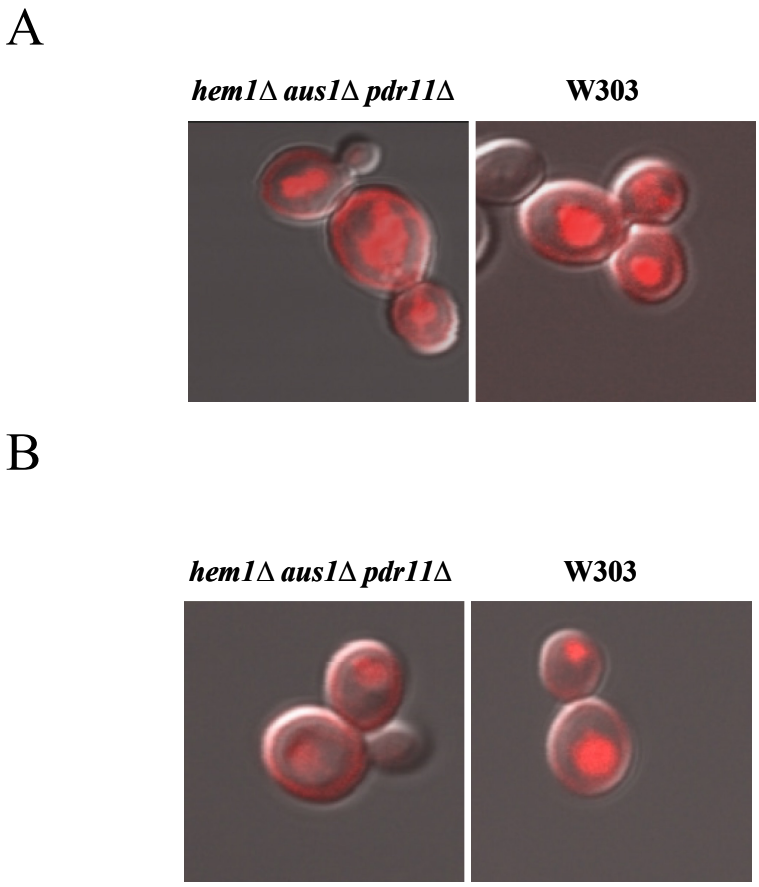


Figure 19: Expression of RFP tagged Aus1p protein in *Saccharomyces cerevisiae*. Yeast cells were growing on media supplemented with sterol/Tween mixture and 2% galactose until the logarithmic phase. Aus1p protein was overexpressed from (A) strong galactose promoter (pESC-AUS1-RFP) or (B) from weak GAL_S promoter (GalS-AUS1-RFP) in *hem1Δaus1Δpdr11Δ* and W303 yeast strains. Subsequently cells were visualized by fluorescent microscopy. Phase/fluorescent overlay images show that overexpressed Aus1p-RFP protein was able to reach plasma membrane in both strains when expressed from weak and strong promoter.

The full length Aus1p protein was effectively expressed upon 16 h of induction with 2% galactose or after induction of expression from natural promoter for the same time. The endogenous promoter containing 1000bp upstream of the *AUS1* ATG starting codon was long enough to mediate activation of *AUS1* expression in *hem1Δaus1Δpdr11Δ* cells upon starving for sterols or in medium containing sterol/Tween mixture (20μg/ml of sterol, 0.5% Tween 80) (Figure 18). In contrast, no expression from endogenous promoter was observed in aerobically growing cells with active ergosterol biosynthesis pathway. Those results are in line with the previous reports that *AUS1* is an anaerobic gene [1]. The RFP tagged protein localized to plasma membrane when expressed under the control of endogenous promoter in *hem1Δaus1Δpdr11Δ* yeast strain (Figure 18) and in *hem1Δaus1Δpdr11Δ* and wild type W303 strain when expression was driven from galactose inducible vectors (Figure 19A and B). In all cases upon induction of protein expression fluorescent signal was detected also

from other cell compartments like vacuole or endoplasmic reticulum. The endoplasmic reticulum is the site of protein synthesis and detected signal most likely corresponded to the newly synthesized molecules of Aus1p-RFP protein. Plasma membrane proteins designed for degradation are endocytosed and delivered to the vacuole for degradation [73]. The fluorescent signal obtained from that compartment after induction of *AUS1* expression corresponded probably to the protein molecules that undergo terminal degradation.

Since two types of constructs were used in this study: containing Flag tagged or Flag and RFP tagged Aus1p it was necessary to test if the fused reporter molecules do not disturb protein function. To test functionality of the constructs yeast cells lacking only *HEM1* gene or *HEM1* and *AUS1/PDR11* were transformed with the appropriate plasmid expressing tagged Aus1p and grown on media supplemented with sterol (survival only in the presence of functional sterol importer) or aminolevulinic acid (survival independent of the sterol uptake process) (Figure 20).

Overexpression of Aus1p (as well as Aus1p-RFP) protein partially rescued the growth of *hem1Δaus1Δpdr11Δ* yeast strain on the media supplemented with sterol. Therefore was concluded that the tagged protein reaches the proper destination place and is able to fulfill its function. To obtain a good negative control for further experiments an ATPase inactive version of Aus1p protein was created for both – Flag tagged protein (called later Aus1p^{K788M}) and its RFP tagged version (called later Aus1p^{K788M} RFP). Inactive mutants of tagged Aus1p were generated by introducing a lysine to methionine substitution in the Walker A region of NBD2 (Aus1^{K788M}) which is known to block the ATPase activity of ABC proteins [74]. Mutated proteins were expressed upon induction with 2% galactose and detected in the membrane fraction obtained from yeast cells (Figure 21A). The tagged Aus1p^{K788M} was able to reach plasma membrane although ER and vacuolar staining were also detected (Figure 21B). To confirm that introduced mutation inactivates protein, *hem1Δaus1Δpdr11Δ* yeast strain was transformed with plasmid expressing Aus1p^{K788M} or Aus1p^{K788M}-RFP proteins and tested for its ability to grow on media supplemented with cholesterol. Despite overexpression and plasma membrane localization (Figure 21A and B) the mutated proteins were not able to survive on sterol containing medium (Figure 20).

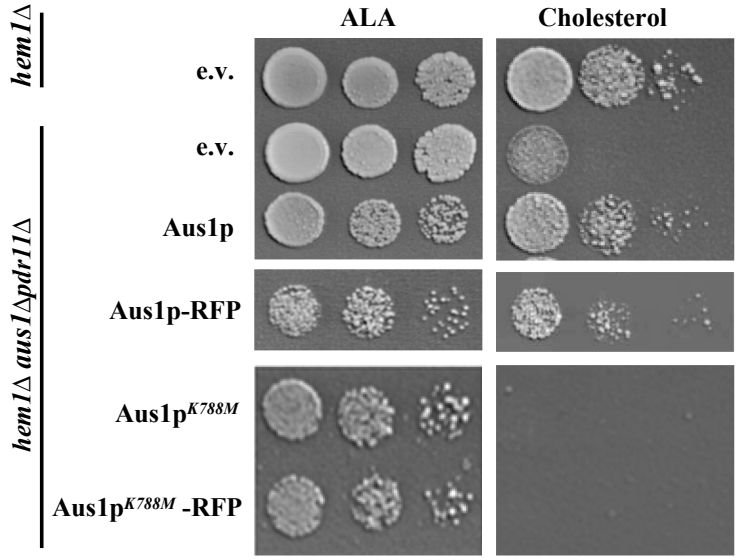


Figure 20: Growth rescue after overexpression of Aus1p or Aus1p-RFP protein in a triple mutant background (*hem1Δaus1Δpdr11Δ).* Five fold dilutions of the yeast cells were spotted on plates supplemented with galactose and incubated 3 days at 30ºC. Mutant lacking Aus1p and Pdr11p proteins (*hem1Δaus1Δpdr11Δ)* was unable to synthesize sterols due to the heme deficiency and could not grow on media supplemented with cholesterol. However, it survived in the presence of aminolevulinic acid which restores internal sterol synthesis. Overexpression of Aus1p (from pESC-AUS1 vector) or Aus1p tagged with RFP (from pESC-AUS1-RFP vector) restored partially the growth defect on sterol containing medium, confirming that fluorescently tagged version of protein was active. Overexpression of mutated versions of Aus1p (pESC-AUS1^{K788M} and pESC-AUS1^{K788M}-RFP) did not restore the growth of the triple mutant.

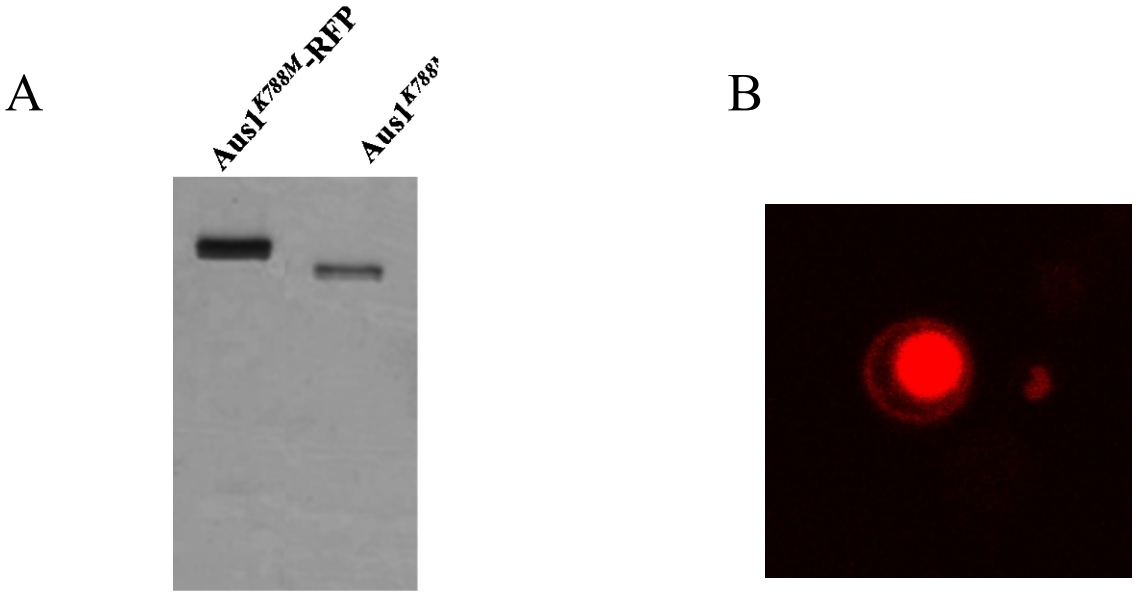


Figure 21: Expression of mutated Aus1p^{K788M} –RFP protein in *hem1Δ* yeast strain. (A) Aus1p^{K788M} and Aus1p^{K788M} –RFP were overexpressed in *hem1Δ* yeast strain upon 16 h of induction. Yeast total membranes were collected and subjected to analysis by Western Blot with anti-Flag antibody. (B) Aus1p-RFP protein carrying a mutation in Walker A motif was expressed upon induction with galactose in *hem1Δ* yeast strain. Protein expression was visualized by fluorescence microscopy. Aus1p^{K788M} –RFP localized to the plasma membrane, however additional staining of ER or vacuole could be also detected.

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4.2 Influence of putative sterol transporters on sterol uptake *in vivo*

Under anaerobic conditions incorporation of exogenous sterols is crucial for survival of yeast cells. To measure the sterol uptake, yeast cells were incubated with fluorescently labeled sterol (25-NBD cholesterol) for 16 h and subsequently subjected to flow cytometry.

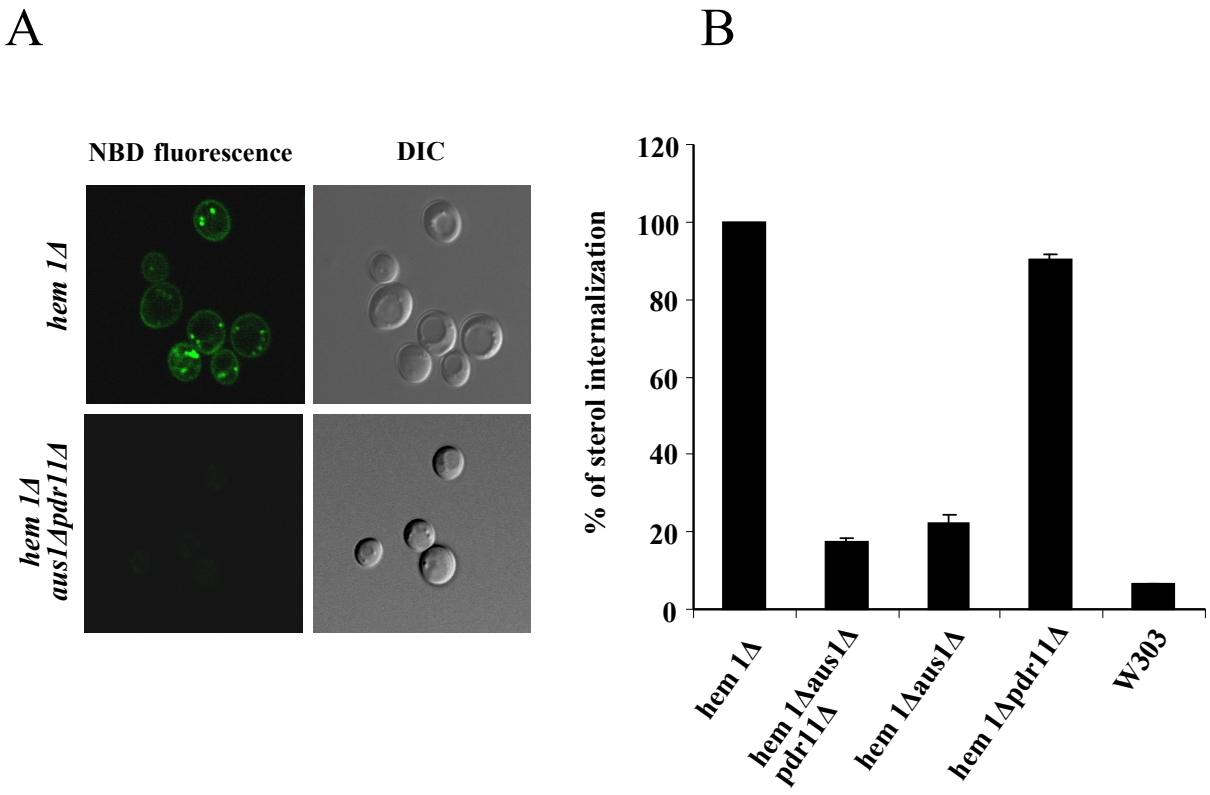


Figure 22: **Uptake of 25-NBD cholesterol by *Saccharomyces cerevisiae* mutants.** (A) Sterol auxotrophic cells (*hem1Δ* and *hem1Δaus1Δpdr11Δ*) were cultivated in the presence of 25-NBD cholesterol for 16 h, washed and subsequently visualized by DIC (Differential Interference Contrast) and fluorescent microscopy. Sterol auxotrophic cells lacking *AUS1* and *PDR11* genes were not able to accumulate exogenously applied sterol (B) Yeast cells were grown for 16 h in media supplemented with 20 μg/ml of sterol (25-NBD cholesterol:cholesterol, 1:1) and 0.5% Tween 80. Subsequently the fluorescent signal (corresponding to the amount of accumulated fluorescent sterol) was evaluated by flow cytometry. As depicted sterol internalization was blocked in the wild type W303 cells as well as in the sterol auxotropic cells lacking Aus1p protein. Results are the means ± SD of three independent determinations, relative to the value obtained for *hem1Δ* cells.

Yeast mutants lacking *HEM1* gene displayed high degree of fluorescence upon cultivating on media supplemented with 25-NBD cholesterol (Figure 22A). Additional deletion of *AUS1* and *PDR11* genes greatly diminished the NBD signal, lowering it to the level displayed by sterol prototrophic cells (W303) which do not take up sterols (Figure 22 B). Surprisingly, deletion of *AUS1* gene had much stronger effect on the sterol internalization than deletion of *PDR11*. Since overexpression of both proteins in *hem1Δaus1Δpdr11Δ* background rescued the cell growth on plates supplemented with sterol (Figure 20 and 23) both proteins can support sterol-dependent growth of yeast. However, measurement of endogenous expression level of *AUS1* and *PDR11* genes revealed around 40 fold

lower amount of *PDR11* transcript in comparison to the amount of *AUS1* mRNA (Figure 24). Altogether obtained data indicate that both proteins can participate in sterol internalization but under natural conditions expression of *AUS1* gene is much stronger.

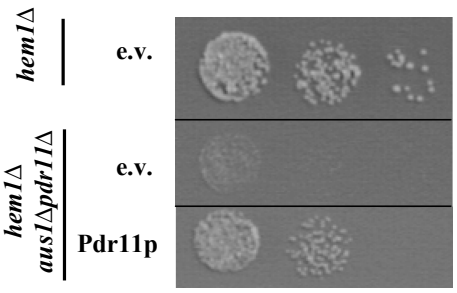


Figure 23: Growth rescue of *hem1Δaus1Δpdr11Δ* yeast strain overexpressing Pdr11p protein on medium supplemented with cholesterol. Five fold dilutions of the yeast cells transformed with vector pESC-Pdr11, spotted on the plate containing 2% galactose and incubated 3 days at 30ºC. Overexpression of Pdr11p protein partially rescued the growth of the triple mutant on the medium supplemented with sterols confirming that Pdr11p protein can assists in the sterol uptake process.

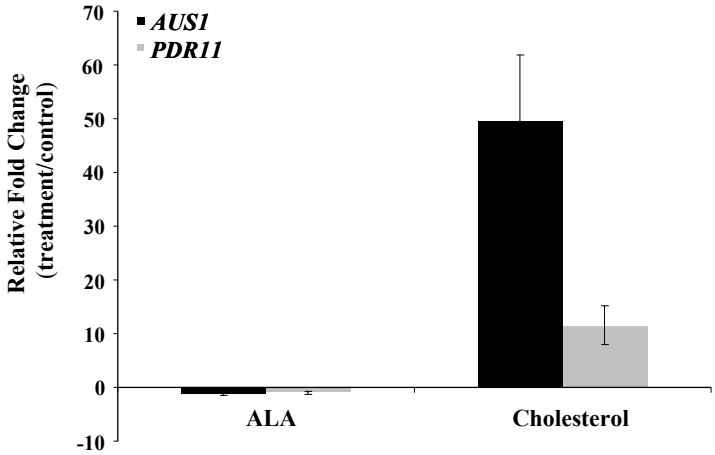


Figure 24: Measurement of transcript level of *AUS1* and *PDR11* genes. Yeast cells (*hem1Δ)* were cultivated either in the presence of 5-aminolevulinic acid or cholesterol. Subsequently the mRNA was isolated and the amount of transcript was evaluated by RT-PCR. Tested genes were not expressed when yeast cells were cultivated in the presence of ALA however, high amount of *AUS1* transcript was detected upon cultivation of sterol auxotrophic cells in the presence of sterol in medium. Although both genes were upregulated in hypoxic state *AUS1* expression level was much higher than *PDR11*. Results are the means ± SD of three independent determinations.

To test if Aus1p alone can mediate sterol incorporation the protein was overexpressed in *hem1Δaus1Δpdr11Δ* and W303 cells. Under aerobic conditions (W303) presence of elevated amounts of Aus1p did not lead to accumulation of 25-NBD cholesterol indicating that other components are necessary for efficient sterol uptake. Deletion of *AUS1* and *PDR11* in *hem1Δ* background with subsequent overexpression of Aus1p leads to generation of separate population of the cells accumulating high amounts of 25-NBD cholesterol. No uptake was observed in cells expressing mutated version of the protein (Figure 25). These results prove that Flag tagged Aus1p can support sterol uptake under anaerobic conditions.

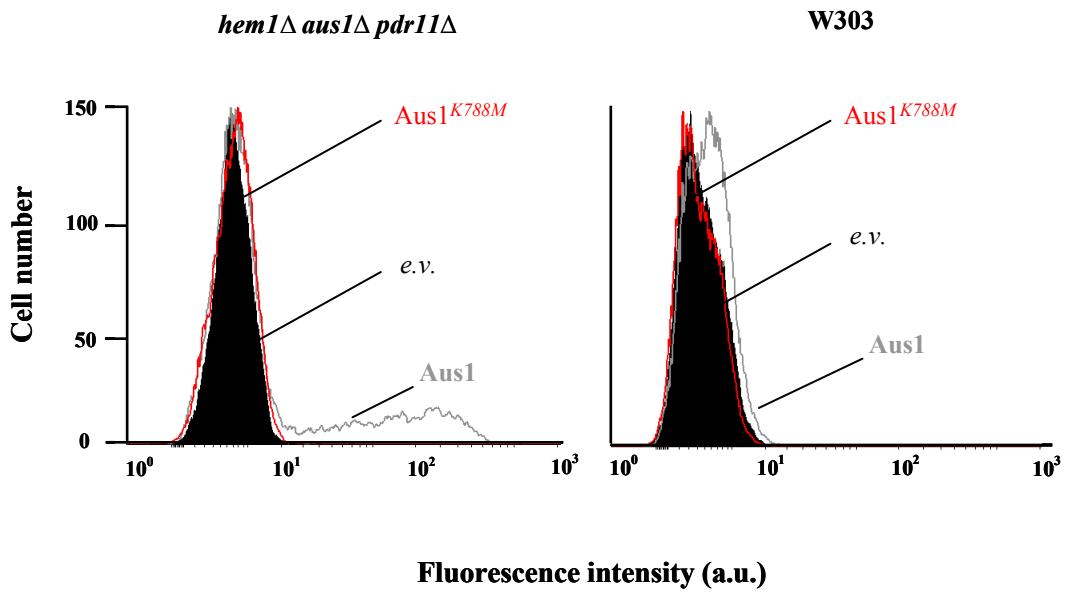


Figure 25: Measurment of 25-NBD cholesterol uptake in yeast strains overexpressing Aus1p or Aus1p^{K788M} proteins. Cells were cultivated for 16 h in the presence of fluorescently labeled sterol and 2% galactose as an induction factor and subsequently analyzed by flow cytometry. Overexpression of Aus1p wild type protein in *hem1Δaus1Δpdr11Δ* increased the amount of accumulated sterol while overexpression of Walker A mutant did not have any influence. No sterol uptake was detected in cells with active sterol biosynthesis pathway (W303). Grey line – Aus1p, red line Aus1p^{K788M}, black-e.v. (empty vector). An representative experiment of two independent measurements.

4.3 Optimization of cultivation conditions for overexpression of Aus1p protein

In order to overexpress high amount of protein the cultivation conditions were optimized. The expression level of Aus1p was compared in two strains – BJ1991 and YPH500 transformed with a multicopy vector in which Aus1p expression was under the control of GAL10 promoter. BJ1991 strain lacks endogenous Pep4 protease, what ensures higher stability of expressed protein and increases the yield of purified product. The growth of YPH500 strain was weaker on media supplemented with 2% galactose indicating that cell viability was affected by protein overexpression (Figure 26). Therefore the BJ1991 strain was selected for further studies.

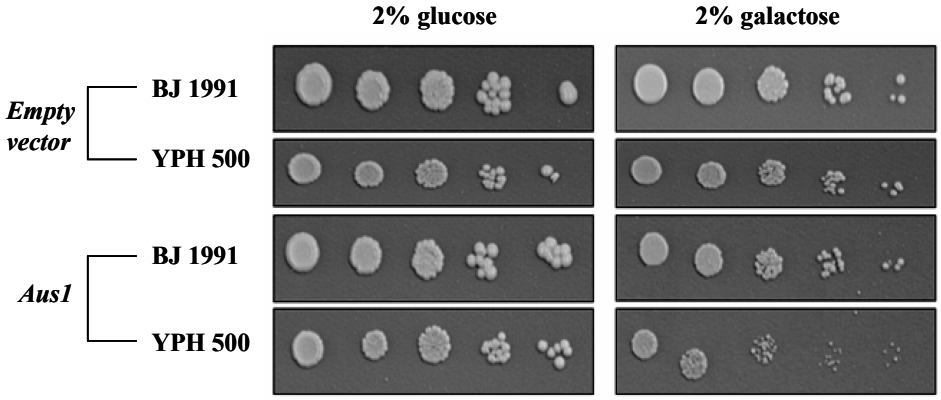


Figure 26: Comparison of growth of yeast cells upon induction of Aus1p protein overexpression. Yeast cells (BJ1991 and YPH 500 strains) were transformed with a vector carrying Flag tagged Aus1p protein under the control of galactose inducible promoter (GAL10). Five fold serial dilutions of cells were spotted on the plates containing minimal medium supplemented with either 2% glucose or galactose. Growth of EHY500 yeast strain was impaired in comparison to the cells transformed with empty vector when expression of Aus1p protein was induced by galactose.

From several clones obtained after transformation of BJ1991 strain with *AUS1* containing plasmid, four were tested for their ability to express the protein of interest. As shown in Figure 27, clone 4 produced the highest amount of protein and was selected for further experiments. In the next step, different induction times were tested to determine at which time the overexpression starts and reaches maximum level. To test that, cells were cultivated in a total volume of 20 ml media supplemented with 2% galactose as an induction factor. Upon indicated time the cultures were harvested and total membranes were isolated. Twenty µg of total protein was loaded on the SDS PAGE gel from each sample and the signal was detected with anti-Flag antibody. Product of overexpression was detected after 12 h of induction and was stable until 20 h. For further analysis the time of 16 h of induction was selected. To improved cultivation conditions the expression of Aus1p was tested in the cells growing on media with different pH, in range from 5 to 8 (Figure 27).

The optimum expression was achieved when the cells were cultivated on the minimal selective medium with pH between 6 to 7. Taken together, cultivation conditions were set up for growth on SD-ura medium for 16 h of induction with 2% galactose at pH 6.

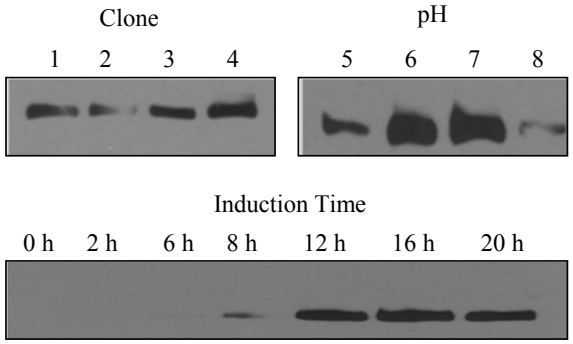


Figure 27: Expression of Aus1p protein. Western blot analysis of membranes prepared from protease-deficient *Saccharomyces cerevisiae* BJ1991 strain expressing FLAG tagged Aus1p. Several yeast clones, the effect of medium pH and induction time on Aus1p expression were tested. Flag tagged Aus1p protein was detected by immunodetection with M2 anti-Flag antibody.

4.4 Purification of Aus1p protein overexpressed in *S.cerevisiae* cells

To obtain a pure protein for *in vitro* studies Aus1p was overexpressed in BJ1991 *Saccharomyces cerevisiae* cells. The purification procedure (Figure 28) was done as described in Materials and Methods after induction of protein expression by 2% galactose for 16 h.

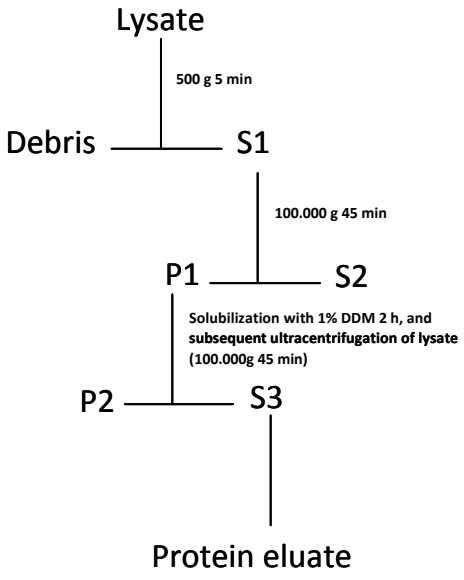


Figure 28: Schematic representation of steps required during Aus1p protein purification. Upon lysis of cells, cellular membranes (P1) were collected by ultracentrifugation of total lysate (S1). Membranes were solubilized with 1% DDM and insoluble material (P2) was removed by ultracentrifugation. Soluble fraction (S3) which contained Aus1p protein released from membranes was subjected to affinity chromatography step with anti-FLAG gel. Protein was released from affinity matrix by washing of the resin with a FLAG peptide.

Since Aus1p is a plasma membrane protein, a membrane solubilization step was necessary during the procedure to release the protein from its natural membrane environment and to make it competent for subsequent affinity chromatography step. Membrane solubilization is achieved upon treatment with detergent and the selection of correct one is a crucial step during purification. In this studies two detergents – DDM and Triton X-100 were tested. Both detergents were able to release the protein from membrane after 2 h of incubation although with different efficiency (Figure 29).

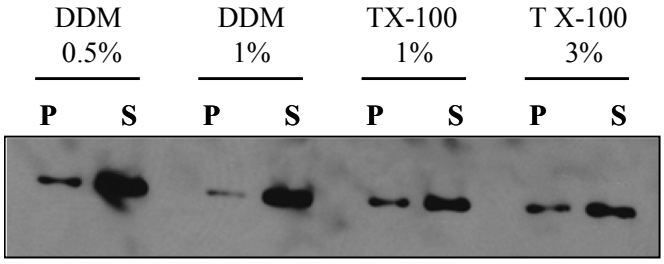


Figure 29: Solubilization of Aus1p protein from cellular membranes. Membranes (0.5 mg total protein) were resuspended in 100 µl of IP buffer supplemented with different detergents as indicated. Solubilized (S) and non-solubilized proteins (pellet, P) were separated by ultracentrifugation (100.000 g) and analyzed by immunoblotting using the FLAG M2 monoclonal antibody.

DDM at concentration of 1% solubilized Aus1p at the highest degree and therefore was selected for further experiments. The protein released from membranes was used for affinity chromatography in order to separate it from impurities. During that step Flag tagged protein interacted with anti-Flag resin composed of agarose beads covered with covalently bound anti-Flag antibody. After washing steps which remove unbound components the protein was eluted from the resin by treatment with FLAG peptide which has much higher affinity for anti-Flag beads than the Flag-tagged protein itself. After affinity chromatography, unbroken protein product was detected (Figure 30A). Densitometric analysis of the gels of purified Aus1p revealed at least 90% degree of protein purity. This high purity of the product is necessary if the protein is used subsequently for reconstitution. To ensure that the eluate did not contain contaminations the final sample was analyzed by mass spectrometry. According to mass spectrometry results only minor contaminations were present in the final product of purification procedure (Table 5).

Table 5: **Mass spectrometry analysis of Aus1p purification product**. After purification, Aus1p protein was run on SDS page gel and subsequently the gel line was cut into 12 pieces. Proteins were isolated from each fragment and analysed by mass spectrometry. As depicted in the table most of the peptides identified in the sample belong to Aus1p protein.

Protein name	Peptides recovered	Protein name	Peptides recovered
Aus1	175	**GIP3**	1
Pdr11	11	**Lic4**	1
Kin4	5	**RPL1A**	1
Trp2	4	**YER184C**	1
Stp3	2	**YOR248W**	1

The efficiency of purification was in range of 70 μg of pure product per 1g of cell pellet. This corresponds to high purification yield in comparison to other membrane proteins purified from yeast - Atm1p, a mitochondrial ABC transporter was purified with efficiency of 0.5 μg per liter of culture and Mdl1p, an yeast peptide transporter, gave the yield of 34 μg per liter of culture [75,76]. Exactly the same purification efficiency as for Aus1p protein was obtained after heterologous expression of plant H-ATPase in yeast [77].

Protein purified by Flag affinity chromatography can be purified further eg. by size exclusion chromatography (SEC) (Figure 30B). This technique allows for separation of molecules depending of their size and can be used also for determination of oligomeric state of the protein and/or detection of impurities. During SEC procedure molecules bigger than the pores of the resin elute faster since they cannot enter the pores and the way they travel through the column is shorter. Gel filtration could improve the purification of Aus1p by elimination of the small amount of impurities and/or oligomers from the sample. However, Aus1p recovered after SEC did not display ATPase activity (data not shown). Since no degradation was observed after gel filtration other factors eg. loss of structural lipid or other cofactors could to be responsible for protein inactivation.

To confirm that purified protein was mainly in a monomeric state Aus1p (and additionally Aus1p-RFP) were run on a Blue Native PAGE (BN-PAGE) which is often used to determine native protein masses and oligomeric state [67]. As depicted on Figure 30C, both proteins were detected as single bands with a size corresponding to the expected values.

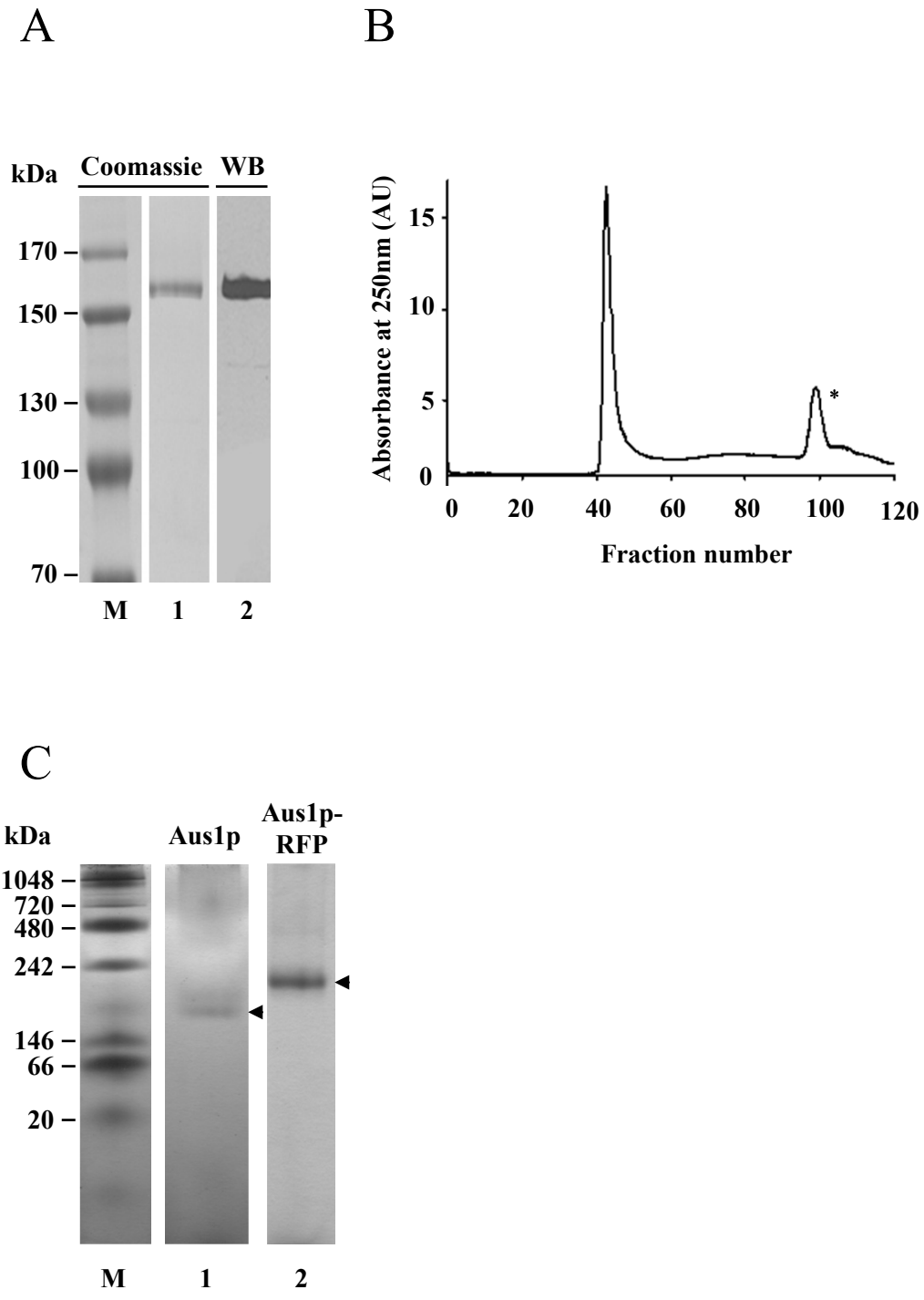
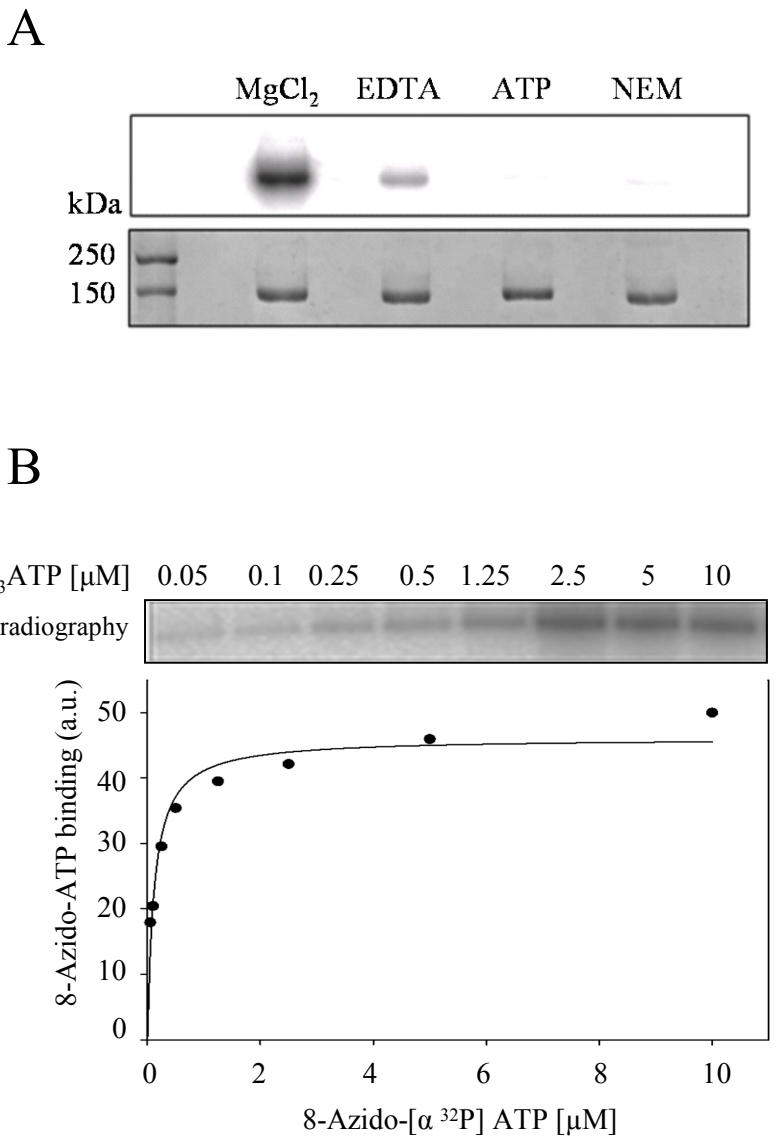


Figure 30: Validation of Aus1p purity state. (A) The purified protein was visualized on SDS-PAGE gel by Coommasie Blue staining (lane 1) and by immunodetection (Western Blot) with anti-Flag antibody (lane 2). (B) Protein obtained after affinity chromatography step were a subject of size exclusion chromatography. Main signal depicted on chromatogram corresponded to Aus1p protein revealing high degree of purification. Second peak (*) corresponded to the FLAG peptide used during purification process for elution of protein from the resin. (C) Analysis of Aus1p protein by native PAGE**.** Purified Aus1p (lane 1) and Aus1p-RFP (lane 2) were analyzed by BN-PAGE and Coomassie Blue staining and their position is indicated by arrows. Soluble molecular mass standards (M) are shown for size comparison. AU; arbitraty units.

4.5 Nucleotide binding by Aus1p protein

Functional ABC transporters are able to bind and hydrolyze ATP molecules and use the released energy for conformational changes which in turn lead to the transport of substrate molecule. Although it is not clarified how ATP hydrolysis drives substrate transport, active ABC transporters require ATP binding and hydrolysis to fulfill their functions. To examine the interaction of Aus1p with ATP, the detergent-solubilized, purified protein was incubated at 4°C with 8-azido-[α-^{32}P]ATP and the protein complex was exposed to UV light followed by analysis by SDS-PAGE. The SDS-PAGE gels were subject to autoradiography for detection of 8-azido-[α-^{32}P]ATP labelling and to Coomassie Blue staining for determination of protein content (Figure 31A).



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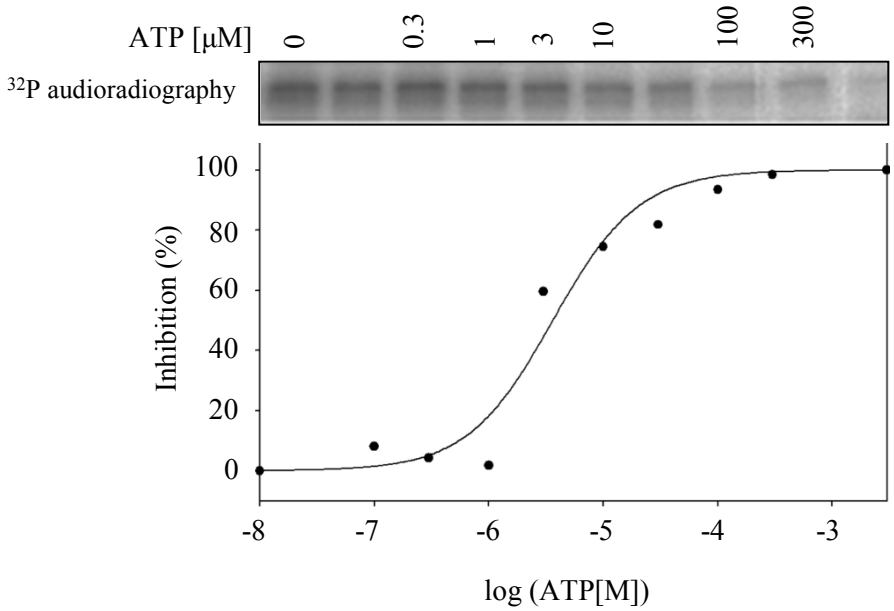


Figure 31: Binding of ATP by pure Aus1p protein. (A) Binding of 8-Azido-(α ^{32}P) ATP to purified Aus1p protein, in the presence of magnesium chloride, EDTA, excess of cold ATP or N-methylmaleimide (NEM) respectively. As expected for active ATPase strong signal was obtained in the presence of magnesium which could be quenched by the excess of cold ATP. EDTA and NEM prevented binding of azido-ATP to the pure protein. (B) Binding affinity of pure Aus1p to 8-Azido-(α^{32}P) ATP was determined by incubation of the protein with increasing amounts of photoreactive probe and subsequent UV irradiation. Samples were analyzed on SDS-PAGE gel and obtained autoradiography signal was evaluated. (C) Determination of half maximal inhibition (IC 50) value by competition experiment with non-radioactive ATP. Aus1p protein was incubated with constant amount of 8-Azido-(α^{32}P) ATP and increasing amounts of cold ATP. After UV cross-linking, protein was separated on SDS-PAGE gel and autoradiography signal was evaluated.

A major band of about 160 kDa, consistent with the molecular weight of Aus1p, was identified by Coomassie Blue staining and found to be photoaffinity-labeled by 8-azido-[α-^{32}P]ATP. Labelling of Aus1p by 8-azido-[α-^{32}P]ATP required magnesium and was strongly inhibited by addition of EDTA (1 mM), excess of cold ATP (10 mM) or pretreatment with 1 mM N-ethylmaleimide (NEM), a covalent SH-group reagent (Figure 31A). Taken together, these data indicate that the protein had retained its ability to bind ATP through out the purification process. The apparent affinity constant for 8-azido-ATP was determined to be 0.12 µM (Figure 31B). Photoaffinity labeling of Aus1p by 8-azido-[α-32P]ATP was inhibited by ATP with an IC_{50} value of 3.6 µM (Figure 31C). Based on the apparent affinity of 8-azido-ATP, a dissociation constant for ATP of 0.2 µM was calculated.

4.6 Reconstitution of Aus1p into liposomes

Some properties of membrane proteins can be determined using purified detergent solubilized protein, however the studies on transport activity can be done only in the native, membrane environment. Although it is possible to use crude cell membranes that system is often avoided due to the undefined lipid composition and undetermined amount of other proteins in it. To overcome those disadvantages the protein of interest can be reconstituted into liposomes – an artificial lipid membranes which serve as an excellent model for studying membrane properties.

The first step during the reconstitution procedure is the insertion of the protein into liposomes. At this point preformed liposomes are destabilized with detergent and subsequently mixed with the purified, detergent solubilized protein. After short equilibration the detergent is removed leading to the insertion of the hydrophobic membrane protein into liposomes (Figure 32A). There are several factors influencing the efficiency of this process (eg. the sort of used detergent, protein:lipid ratio or the method of detergent removal) and no general method for protein reconstitution has been developed so far. Because of that the reconstitution conditions have to be adjusted for each protein individually.

For reconstitution of Aus1p (and Aus1p-RFP) liposomes preformed by extrusion through 200 μm filter were destabilized with 1.5 mM DMM. DDM was choosen for destabilization of liposomes because it is generally recommended to use for reconstitution the same detergent as was used for purification of the protein. The protein and lipids were mixed at the ratio 1:20 (w/w) and equilibrated for 15 min at 25ºC. Detergent was removed by treatment with BioBeads - a polysterene beads which are able to absorb detergent on their surface. The BioBeads were added in excess (300 mg/ml of reconstitution mixture) and incubated for 16 h at 4ºC to assure complete removal of detergent from the sample. The proteoliposome formed after treatment with BioBeads were collected by ultracentrifugation and dissolved in buffer. The successful reconstitution was confirmed by detection of unbroken protein on Coomassie stained polyacrylamide gel (Figure 32 B) and by flotation assay. During the flotation assay liposomes containing protein migrate to the top fractions of the gradient whereas pure protein stays at the bottom after ultracentrifugation. Since the protein posses a fluorescent tag (RFP) it can be easily detected in particular fractions by measurement of red fluorescence. As shown in Figure 33 Aus1p-RFP fluorescence was detected in the upper fractions of the Nycodenz gradient together with the lipids, confirming successful reconstitution of Aus1p into liposomes.

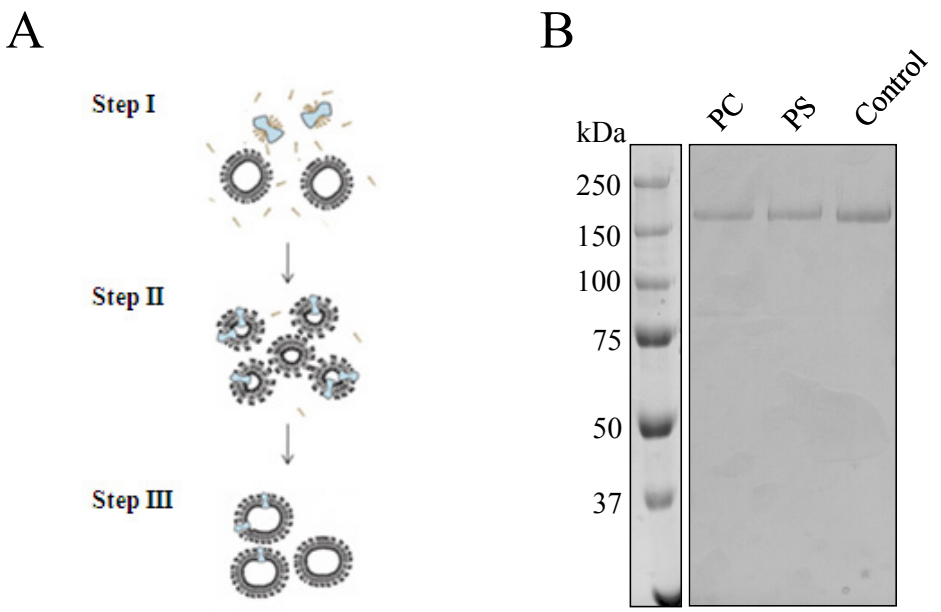


Figure 32: Reconstitution of Aus1p protein. (A) Main steps of protein reconstitution into liposomes. Detergent destabilized liposomes are mixed with pure protein (Step I) and subsequently the detergent is removed (Step II) what leads to formation of proteoliposomes (Step III). (B) Coomassie Blue stained SDS-PAGE gel of purified, solubilized Aus1-RFP (control) and Aus1-RFP reconstituted in proteoliposomes composed of different lipids. PC: POPC only; PS: POPC/POPS (7:3). Molecular mass is indicated on the left.

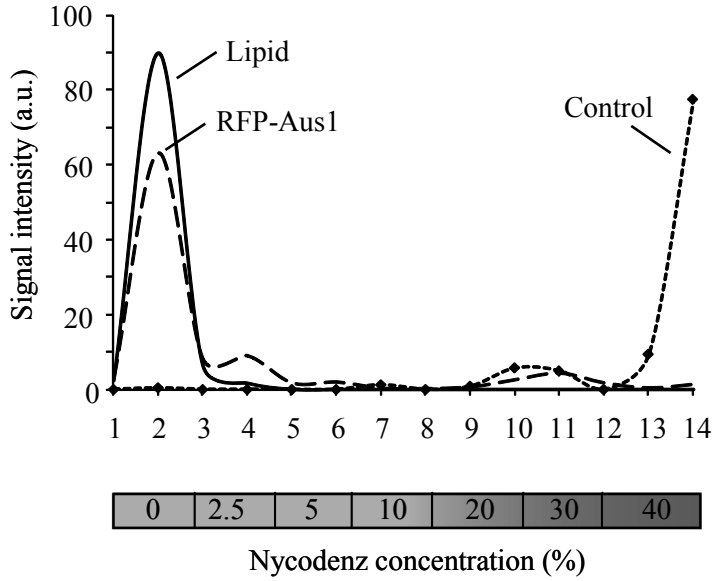


Figure 33: Flotation of Aus1p containing proteoliposomes on Nycodenz gradient. Purified Aus1–RFP was reconstituted into preformed detergent-destabilized liposomes as described in Materials and Methods. Aus1-RFP containing liposomes were applied to a Nycodenz gradient, centrifuged, and the obtained fractions were analyzed for RFP fluorescence (dashed line) and phospholipid content (solid line). Reconstitution was evident from co-migration of phospholipid and Aus1-RFP. A control sample containing only purified, solubilized Aus1p in the absence of lipids was mock-treated in 0.05% n-dodecylmaltoside-containing buffer at the same temperatures as the reconstitution samples (dotted line).

To determine the orientation of the protein in the liposomal membrane RFP quenching method was used. It was described previously [78] that red fluorescent protein can be effectively quenched by the copper ions and the effect was also reproduced during titration of Aus1p-RFP with Cu^{+2}. The copper binding constant was calculated from a Stern-Volmer Plot giving the quenching constant K_q 1.8μM^{-1} and copper disassociation constant K_d 0.6 µM. K_d values were lower in case of Aus1p-RFP in comparison to previously reported disassociation constant for monomeric RFP molecule (1.7 µM), indicating higher affinity of Aus1p-RFP to the Cu^{+2} ions.

To prove the specificity for copper ions the control experiments were done with magnesium chloride showing no considerable quenching effect. According to assumptions addition of saturating amounts of $CuCl_2$ should quench the RFP fluorescence from all the protein molecules with C terminus outside the liposome and the remaining signal would correspond to the molecules with the opposite orientation for which fluorescent C terminus is protected from copper ions. Comparison of the quenching data on intact and detergent permeabilized vesicles revealed that about 50-60% of Aus1p was inserted into POPC vesicles with the NBDs facing outward (Figure 34). Proteoliposomes composed of POPC/POPS (7:3) and POPC/POPE (7:3) were also analyzed for Aus1p orientation. Here, about 70-80% of Aus1p was inserted into these vesicles with the NBDs facing outward (data not shown). However, an exclusive asymmetric orientation of Aus1p towards one side of the vesicles was never observed.

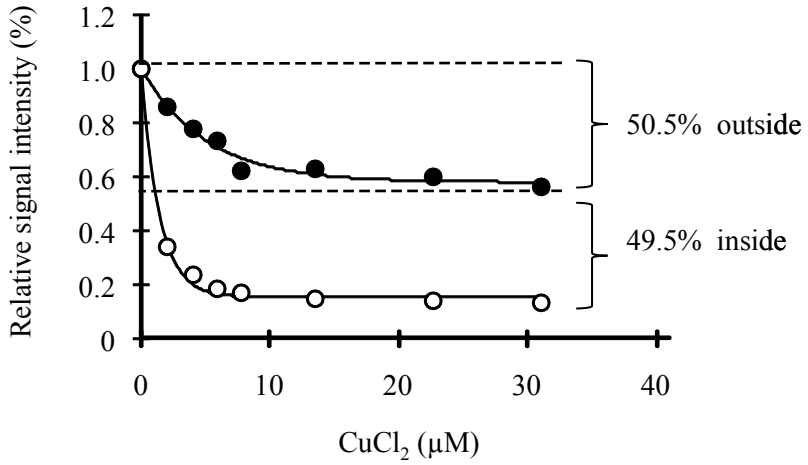


Figure 34: Determination of protein orientation in liposomes. Protein molecules reconstituted into proteoliposomes can display different orientations - toward the interior or exterior of vesicles. To determine the direction in which RFP tagged Aus1p was incorporated in liposomes the RFP-copper quenching method was used. As depicted above the method relies on the fact that RFP fluorescence can be efficiently quenched upon addition of copper ions – because of that – the fluorescence of C terminally tagged Aus1p molecules which display their C terminus outside the vesicle, will be quenched after addition of Cu^{+2} ions. The remaining signal will correspond to the molecules with opposite orientation, thereby allowing to calculate the ratio of molecules directed inside and outside the vesicle. Orientation of Aus1p-RFP in POPC proteoliposomes was determined to be symmetrical with half of the reconstituted molecules orientated outside the liposome and half orientated inside.

4.7 ATPase activity of pure protein and its sensitivity to inhibitors

Aus1p protein is a yeast specific plasma membrane ABC transporter containing two ATP binding domains which are expected to bind and hydrolyze ATP molecules during the catalytic cycle in order to deliver energy for transport of substrates. However some ATPases (eg. P-glycoprotein) can hydrolyze ATP without the presence of substrate displaying so called basal activity which can be further stimulated by the presence of the substrate [79]. The ATPase activity of the purified protein in detergent-containing buffer was determined using a spectrophotometric assay for released phosphate. ATP was hydrolyzed by the protein in a time-dependent manner at 27ºC as revealed by the linear increase of released phosphate ions during the first 30 min. From that, a specific ATPase activity of the purified, detergent-solubilized Aus1p in the range of about 56 nmol ATP/min/mg of protein was estimated (Figure 35).

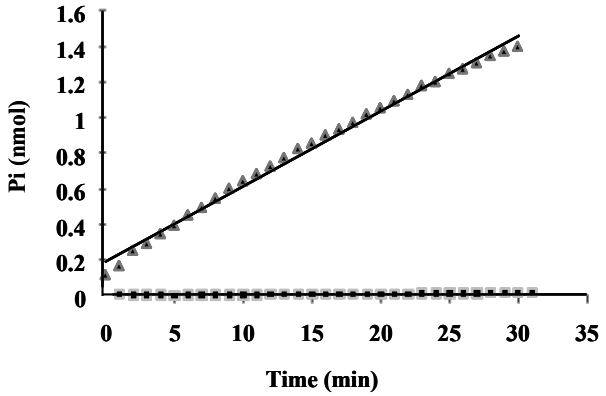


Figure 35: ATPase activity of pure Aus1p protein in buffer solution. The ATPase activity of pure Aus1p protein was measured over 30 min at 27ºC by EnzCheck Assay. During the reaction, enzyme PNP (purine nucleoside phosphorylase) convertes the substrate MESG (2-amino-6-mercapto-7-methylpurine riboside) to robose 1-phosphate and 2-amino-6mercapto-7-methylpurine. The accompanying change in absorption at 360 nm is measured and allows for quantification of inorganic phosphate consumed in the reaction (▲ Aus1p protein ■ buffer).

Further, ATPase activities of wild type protein and mutated version (Aus1p^{K788M}) were compared using radiolabeled ATP. As shown in Figure 36 protein mutated in Walker A motif of NBD2 was inactive. This result confirmed that observed ATP hydrolysis was a result of Aus1p activity and that the lysine at position 788 is crucial for the protein catalytic cycle (Figure 36).

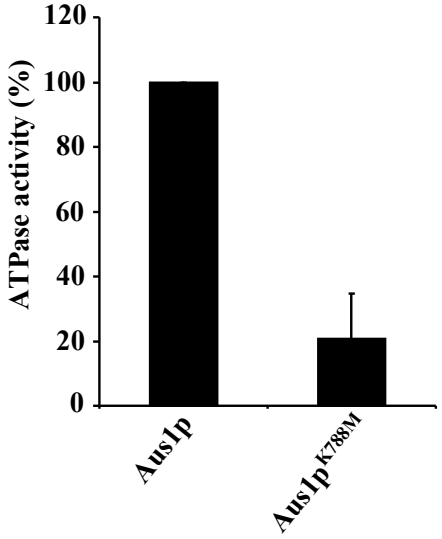


Figure 36: ATPase activity of pure Aus1p protein and its mutated version Aus1p^{K788M}. Equal amounts of pure proteins were evaluated for its ability to hydrolyze γ-P^{32}- ATP over 40 min at 27ºC and the amount of inorganic phosphate released during ATP hydrolysis was measured. Activity of wild type protein was set up as 100% and compared with activity of the mutated protein. Substitution of lysine (K) to methionine (M) at position 788 inactivated the protein. Results are the means ± SD of three independent determinations, relative to the value obtained for the purified detergent-solubilized WT protein.

Many ABC proteins display sensitivity to the transition state analogue orthovanadate. Vanadate acts as a structural analog of the inorganic phosphate transition state and arrests the hydrolytic cycle at an intermediate point by forming complex with MgADP at the catalytic site [80,81]. Aus1p was sensitive to the vanadate displaying around 90% of reduction in activity after treatment with this inhibitor.

ABC transporters show also sensitivity to metal fluorides like beryllium (BeF_x) and aluminium fluoride (AlF_3) which block the ATP hydrolysis by trapping the nucleotide in the catalytic centre. However, they differ in the range of sensitivity to these compounds and some of them (eg.TAP, ALDP) seem to be even insensitive to vanadate treatment [82]. As shown in Table 6, all classical inhibitors of ABC proteins could efficiently block the basal activity of pure Aus1p confirming that the measured release of inorganic phosphate was a result of activity of ABC protein.

Additionally, three other inhibitors were tested namely sodium azide, ouabain and verapamil. Sodium azide is a strong inhibiotor of the cytochrom oxidase and mitochondrial proton pump [83], but Aus1p (like all known ABC transporters) was insensitive to this compound. Ouabain (g-strophantin) inhibits Na^+/K^+ ATPases [84] but as in case of sodium azide Aus1p was insensitive to this inhibitor. ATPase activity was effectively blocked by elevated concentrations of verapamil – a calcium channel blocker which can also block some other ABC proteins (eg. ABCB1) [85,86] (Table 6).

Table 6: Determination of ATPase activity of pure Aus1p protein in the presence of inhibitors. ATPase activity of most of ABC transporters is inhibited by vanadate, beryllium fluoride and aluminium fluoride. Pure Aus1p protein displayed expected sensitivity and additionally was inhibited by verapamil - although only at elevated concentration. Results are the means ± SD of three independent determinations, relative to the value obtained for the purified detergent-solubilized protein.

Compound	Final conc. (mM)	% ATPase activity
Control	-	100.00
Vanadate	1	2.64 ± 0.03
BeSO4 /NaF (BeFx)	1 / 5	21.63 ± 0.18
AlF3	1	14.17 ± 0.06
Sodium azide	5	108.04 ± 0.51
Ouabin	5	108.12 ± 0.38
Verapamil	1	4.16 ± 0.30
Verapamil	0.1	86.74 ± 8.67
Verapamil	0.01	111.07 ± 6.77

As the exact substrate of Aus1p is unknown, the ATPase activity of pure protein was tested in the presence of different lipids to evaluate if any of them would have positive impact. As shown in Figure 37A all tested lipids stimulated the activity in a similar manner although phosphatidylserine displayed a higher impact. Also sterols (putative Aus1p substrates) were tested, however none of them was able to significantly influence the activity of the transporter (Figure 37B). These results however do not exclude the possibility that sterols or other lipids are Aus1p substrates since the presence of substrate not always have a stimulatory effect [44]. Because of the poor solubility of sterols in aqueous solutions under the experimental conditions sterol was delivered in form of liposomes. This sort of delivery system might be not optimal and it is possible that in this form the lipids are not recognized by the protein.

4.8 Influence of lipids on ATPase activity of reconstituted Aus1p

The functionality of membrane proteins is often regulated by the lipid bilayer composition [87]. It was shown previously that changes in physical properties of membrane influence the energetic costs of bilayer deformation during conformational changes of protein, and finally can change both - the function and conformational distribution of the protein [69]. In case of some ABC transporters surrounding lipid environment and membrane fluidity can modulate the activity of the protein and its interactions with the substrate [88,89]. To test the influence of membrane fluidity on the ATPase activity of Aus1p the protein of interest was reconstituted into fluid POPC liposomes, partially fluid

POPC:cholesterol liposomes or into rigid liposomes composed of PC:sphingomyelin:cholesterol. (1:1:1).

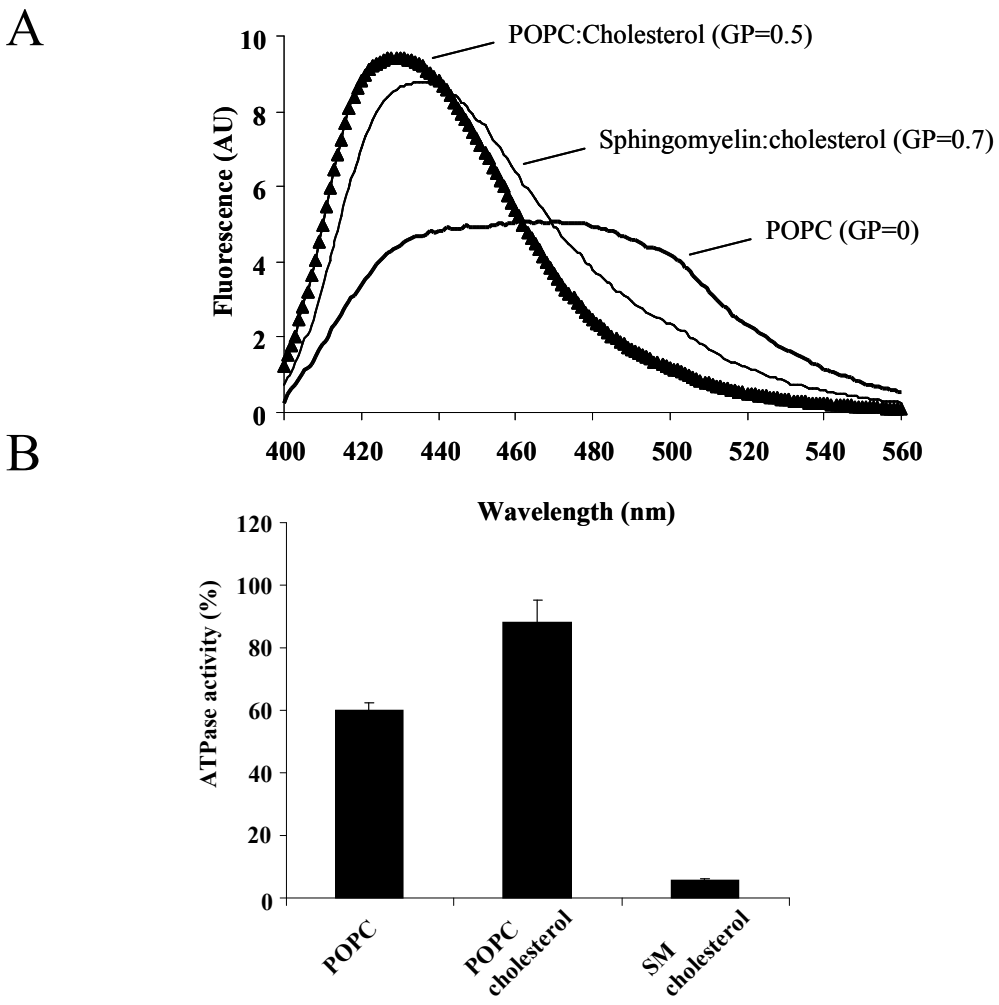


Figure 38: Influence of membrane fluidity on activity of Aus1p protein reconstituted into liposomes. (A) Spectra of liposomes with different lipid composition after membrane labeling with Laurdan, showed that three tested lipid compositions display different membrane fluidity. (B) The ATPase activity of protein reconstituted into liposomes with different membrane fluidity was measured using radiolabeled ATP. Aus1p ATPase activity was slightly stimulated upon addition of cholesterol to the membrane, however the protein was inactive in rigid membrane containing high amount of sterol. Results are the means ± SD of three independent determinations, relative to the value obtained for the purified detergent-solubilized protein.

The fluidity of the membrane was determined by measurement of anisotropy after the staining with Laurdan. Laurdan is a fluorescent probe, sensitive to membrane phase transitions and other alternations in membrane fluidity (Figure 38A) [90]. Changes in the degree of lipid packing in the membrane lead to the shifts in the Laurdan emission spectrum and allow to calculate so called GP (Generalized Polarization) value. GP can assumes values from -1 (what corresponds to not ordered state) to +1 (corresponding to the most ordered state) [72]. As depicted on Figure 38B activity of Aus1p in fluid environment was diminished in comparison to the pure protein and the rigid membrane surrounding blocked almost totally the ability of protein to hydrolyze ATP. Interestingly,

addition of cholesterol to POPC membranes slightly stimulated the activity what can be due to the better protein arrangement in this mixture or by the fact that cholesterol is a substrate for Aus1p.

To test influence of other lipids on Aus1p activity, the protein was reconstituted into liposomes containing POPC and 30 mol% of selected lipid. Upon reconstitution into liposomes the protein did not show any stimulation neither by addition of phosphatidylethanolamine or phosphatidylinositol. However, addition of 30 mol% phosphatidylserine stimulated the activity about 6 fold (Figure 39).

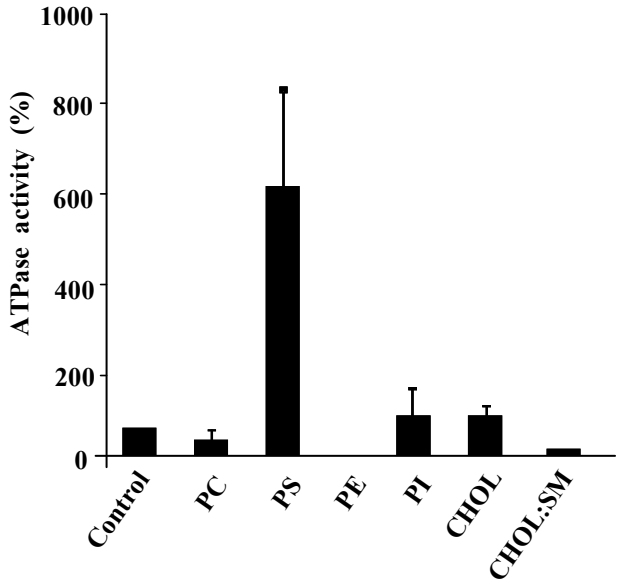


Figure 39: Influence of membrane composition on ATPase activity of Aus1p protein reconstituted into liposomes. ATPase activity was measured for Aus1p protein reconstituted into POPC proteoliposomes containing 30 mol% of selected lipid was evaluated using radiolabelled ATP. In the presence of phosphatidylserine 6 fold stimulation was obtained in comparison to the activity of pure protein in buffer. Results are the means ± SD of three independent determinations, relative to the value obtained for the purified, detergent-solubilized protein (Control).

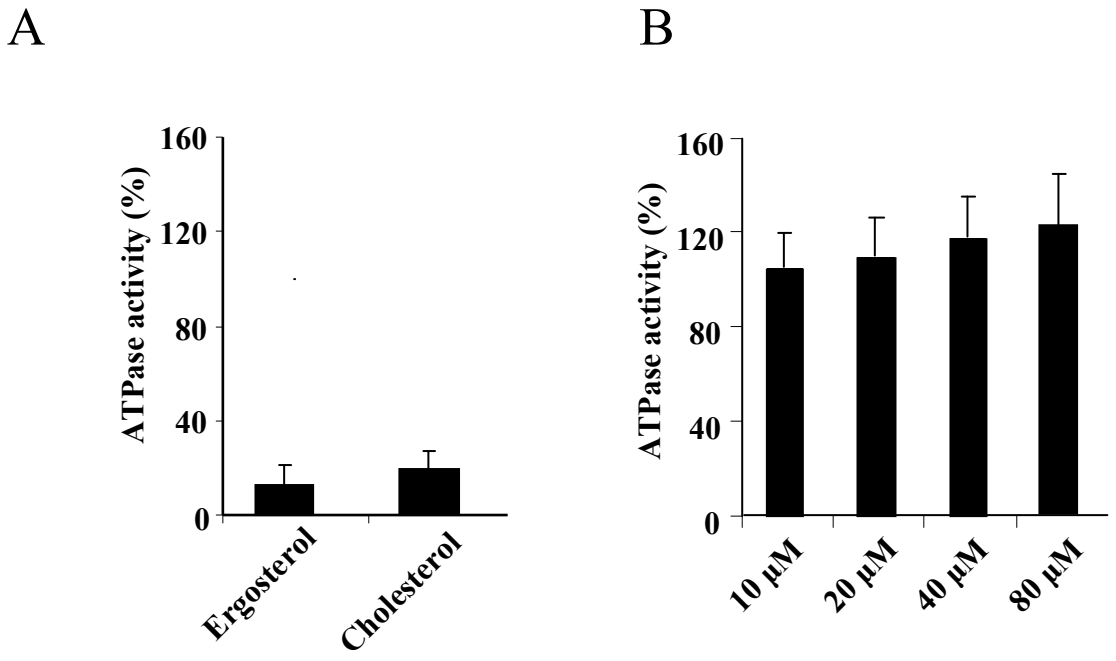


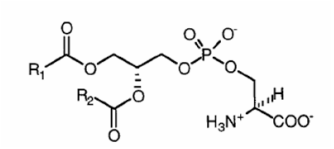
Figure 40: Influence of cholesterol on ATPase activity of Aus1p reconstituted into liposomes. (A) ATPase activity of Aus1p reconstituted into POPC liposomes in the presence of sterol was determined using radiolabelled ATP. Cholesterol and ergosterol (50 µM) were applied as a sterol::cyclomaltodextrin complex. Results are the means ± SD of three independent determinations, relative to the value obtained for the purified detergent-solubilized protein. (B) Aus1p was reconstituted into POPC/POPS liposomes. ATPase activity was evaluated using radiolabelled ATP in the presence of increasing amount of cholesterol complexed with cyclomaltodextrin. Results are the means ± SD of three independent determinations, relative to the value obtained for protein reconstituted into POPC/POPS vesicles without supplementation with cholesterol complexed with cyclomaltodextrin.

To test the influence of exogenously applied sterols on the ATPase activity of reconstituted Aus1p proteoliposomes were incubated with the sterol (cholesterol or ergosterol) encapsulated into β-methylcyclodextrin. Cyclodextrins are cyclic oligosaccharides with internal cavity capable to encapsulate normally hydrophobic compounds what allows them to became soluble in aqueous solution [91]. Upon reconstitution of Aus1p into POPC liposomes no stimulatory effect was observed after addition of 50 µM cyclodextrin complexed with sterol. Instead the ATPase activity decreased even further (Figure 40A). When the protein was reconstituted into vesicles composed of POPC/POPS mixture addition of cholesterol complexed with β-methylcyclodextrin did not influence ATPase activity of Aus1p and only the stimulatory effect of PS was detected (Figure 40B). This lack of stimulation by the putative substrate might be explained by the fact that - although useful for solubilization of hydrophobic compounds - cyclodextrins are not optimal donors of sterol molecules for Aus1p protein.

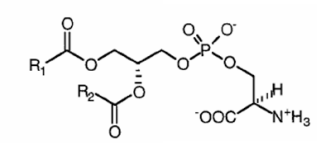
To provide further insight into Aus1's specific requirement for PS, the ability of PS stereoisomers to stimulate ATPase activity of Aus1p was explored (Figure 41A). Maximal activation occurred in the

presence of the naturally occurring 1-palmitoyl-2-oleoyl-*sn*-phosphatidyl-L-serine (1,2-*sn*-POP-L-S). Altering the stereochemistry in the serine headgroup (1,2-*sn*-POP-D-S) or in the glycerol backbone (2,3-*sn*-POP-L-S, 2,3-*sn*-POP-D-S) caused a decrease in ATPase activity. Maximum activation of Aus1p in the presence of natural PS and the ability of the enzyme to discriminate between various PS stereoisomers indicates that the activation results from the association of PS with a specific binding site on the enzyme.

A



1,2-sn-phosphatidyl-L-S



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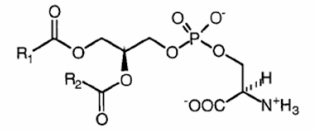
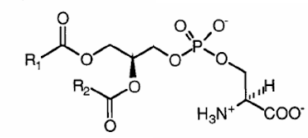


2,3-sn-phosphatidyl-D-S



2,3-sn-phosphatidyl-L-S

B

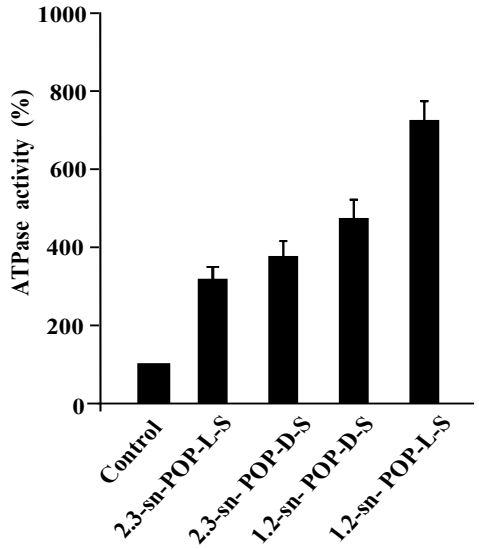


Figure 41: Influence of phosphatidylserine isomers on ATPase activity of Aus1p protein in liposomes. (A) Schematic models of four phosphaidylserine stereoisomers. (B) ATPase activity of Aus1p protein reconstituted into liposomes containing 30 mol% of selected phosphatidylserine isomer. Results are the means ± SD of three independent determinations, relative to the value obtained for the purified detergent-solubilized protein in the absence of inhibitors (control).

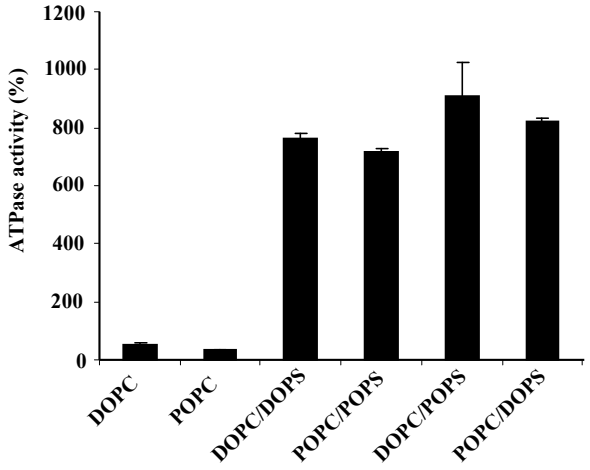


Figure 42 : ATPase activity of Aus1p reconstituted into different lipid mixtures. Aus1p was reconstituted into liposomes containing DOPC or POPC or mixtures DO/PO-PC and 30 mol% of DO/PO-PS and the ATPase activity of protein was evaluated using radioactively labeled ATP. Results are the means ± SD of three independent determinations, relative to the value obtained for the purified detergent-solubilized protein. No significant differences were observed between tested mixtures.

Additionally, there were no differences in protein activity when DOPC was replaced by POPC and/or DOPS by POPS (Figure 42).

To test if the influence of PS is a dose dependent effect Aus1p protein was reconstituted into liposomes containing different amounts of phosphatidylserine.

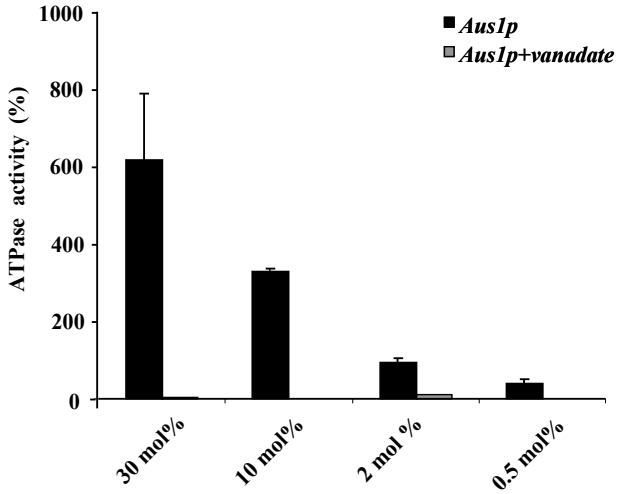
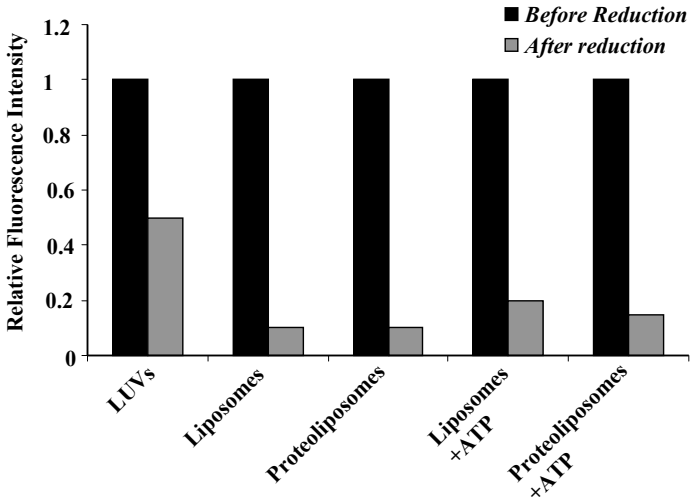


Figure 43: ATPase activity of Aus1p protein reconstituted into POPC membranes containing different amount of POPS. Aus1p protein was reconstituted into liposomes containing various amount of phosphatidylserine. ATPase activity of the protein was evaluated by radioactive ATPase assay. Samples containing 1mM vanadate were used as a negative control. Results are the means ± SD of three independent determinations, relative to the value obtained for the purified detergent-solubilized protein.

As shown in Figure 43 the ATPase activity diminished with decreasing content of PS in the liposomal membrane. To exclude the possibility that PS is a substrate for Aus1p, the protein was reconstituted into two kinds of liposomes: liposomes with the outer liposomal leaflet labeled with C_6NBD PS or with both leaflets labeled with fluorescent lipids.

A



B

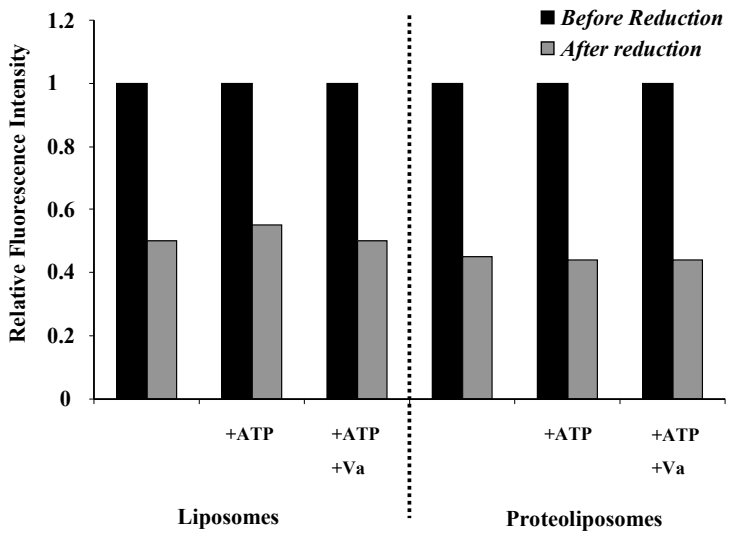


Figure 44: Measurement of PS transport across membranes containing Aus1p. (A) The outer leaflet of liposomes was labeled with 0.6 mol% C_6NBD PS and the samples were incubated at 27ºC for 1 h. Subsequently, dithionite was added to quench the fluorescene of accessible C_6NBD PS molecules. In all tested samples (empty liposomes, proteoliposomes - with and without ATP mixture), almost all signal was destroyed indicating that Aus1p protein under tested conditions could not transport C_6NBD PS. (B) Liposomes were labeled on both leaflets with 0.6 mol% C_6NBD PS and subsequently were incubated with ATP mixture (1 mM ATP, 10 mM $MgCl_2$) for 1 h at 27ºC. 1mM vanadate was used as an inhibitor of Aus1p ATPase activity. After addition of dithionite half of the initial fluorescence was quenched in all samples indicating that protein does not transport C_6 NBD PS molecules from inner liposomal leaflet.

Subsequently samples were incubated with ATP mixture to activate Aus1p. After 60 minutes of reaction, dithionite was used to quench the fluorescence of lipid molecules which remained in the outer layer. If Aus1p would be a PS exporter, after quenching, the signal from ATP induced sample would be higher than from the control one. If Aus1p would be an importer for PS, after quenching of fluorescence, the signal from ATP induced sample would be lower than from the control one. However, in both cases, activated and not activated samples displayed the same quenching level. This suggests that PS is not a substrate for Aus1p protein (Figure 44A and B).

To test if fluorescently labeled phosphatidylserine has the same effect on ATPase activity as POPS Aus1p was reconstituted into liposomes containing 30 mol% C_6NBD PS or C_{12}NBD PS. Both fluorescent lipids were able to increase ATPase activity of the protein several folds (Figure 45). Taken together, those data show that PS is able to enhance ATPase activity of reconstituted Aus1p in a dose dependent and stereoselective manner. At the same time, PS is not a substrate for the investigated transporter. This leads to the conclusion that PS could be structural lipid for Aus1p or it could modify membrane properties making the membrane environment more convenient for Aus1p.

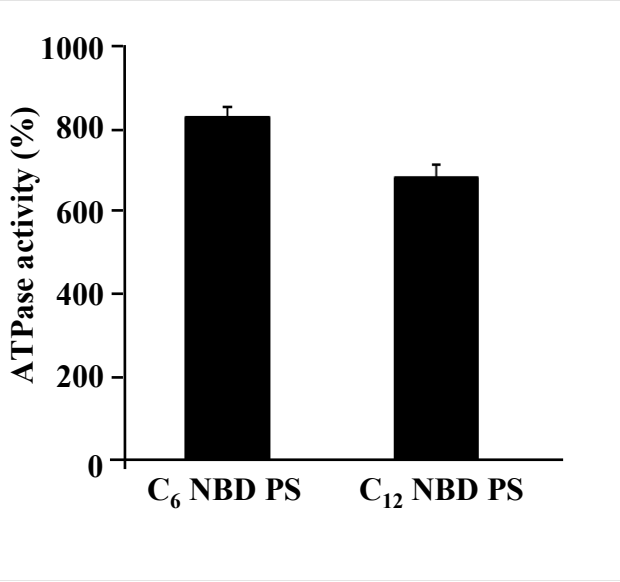


Figure 45: ATPase activity of Aus1p reconstituted into liposomes containing fluorescently labeled phosphatidylserine. Aus1p was reconstituted into vesicles containing 30 mol% of C_6NBD PS NBD or C_{12}NBD PS. The ATPase activity of the protein was stimulated by fluorescently tagged lipids in the same

manner as by natural PS. Results are the means ± SD of three independent determinations, relative to the value obtained for the purified detergent-solubilized protein.

4.9 Influence of putative sterol transporters on phosphatidylserine uptake and cellular distribution

The specific stimulation of Aus1p ATPase activity by PS suggests that this phospholipid might be a substrate for the ABC transporter. Therefore the uptake of C_6-NBD-PS was measured by flow cytometry in *hem1Δ* cells containing or lacking both, *AUS1* and *PDR11* genes. As shown in Figure 46, no significant differences in the internalization of C_6-NBD-PS were observed between cells containing or lacking these two ABC proteins.

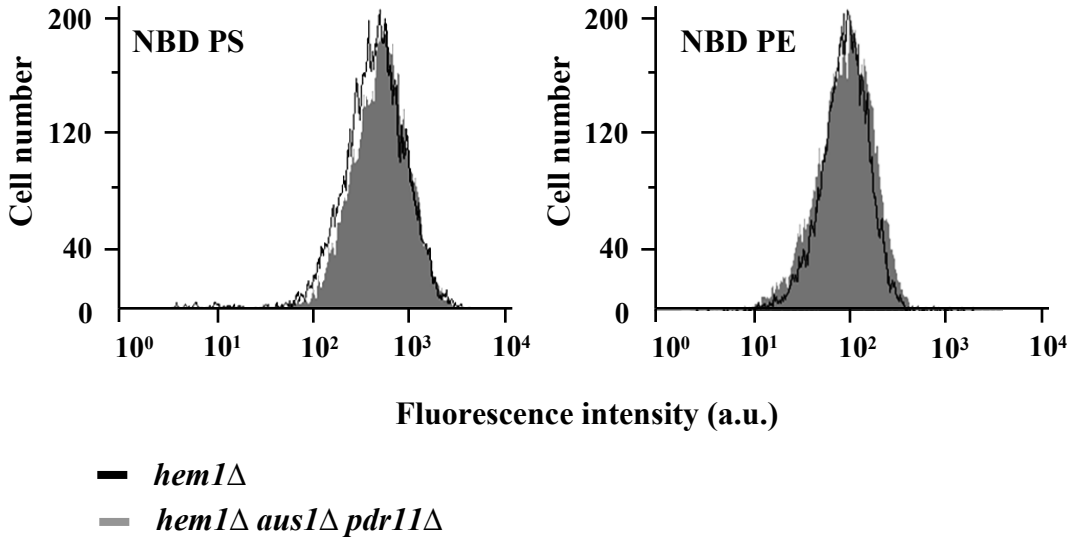


Figure 46: Lipid internalization in yeast sterol uptake mutants. *hem1Δ* cells containing or lacking both, *AUS1* and *PDR11* genes were analyzed for uptake of fluorescently tagged PS and PE. Cells were grown on media supplemented with fluorescently labeled cholesterol to the early logarithmic phase and then were tested for uptake of C_6NBD labeled PS or PE by flow cytometry. No significant differences between the samples were observed indicating that *in vivo* Aus1p is not involved in the uptake of PS and PE.

C_6NBD-PS has been shown previously to be predominantly internalized by transbilayer transport across the plasma membrane resulting in intracellular labeling of various organelles [11]. This result was confirmed for both *hem1Δ* cells containing or lacking *AUS1* and *PDR11* by examining the intracellular localization of C_6NBD-PS by fluorescence microscopy. As shown in Figure 47 localization of investigated lipid was not changed in the absence of Aus1p and Pdr11p when cells were growing in the presence of ALA or cholesterol. However, cells growing on media supplemented with sterol had more dispersed fluorescent signal in comparison to the cells with

active sterol biosynthetic pathway. That could be due to the changes in metabolism and trafficking between aerobic and anaerobic state - but these differences seemed to be independent of the action of Aus1p.

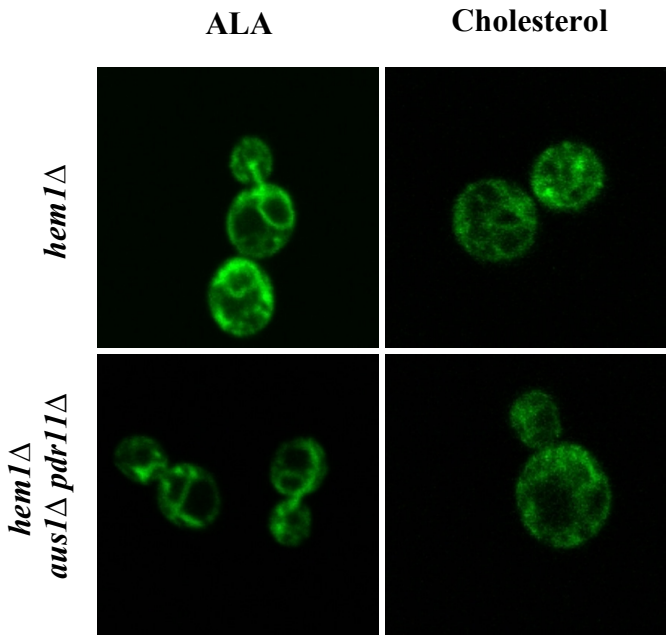


Figure 47: Localization of C_6NBD phosphatidylserine in yeast. Yeast was grown on media supplemented with aminolevulinic acid. Subsequently cells were labeled with 60 µM C_6NBD-PS, washed with medium containing BSA to extract not internalized C_6-NBD-PS and visualized by fluorescent microscopy. Both mutants displayed no differences in term of cellular localization of internalized C_6NBD PS.

4.10 Influence of PS on sterol uptake *in vivo*

As described in the previous chapter the presence of phosphatidylserine greatly enhances the ATPase activity of Aus1p protein reconstituted into liposomes. However, PS was not a substrate for Aus1p *in vitro* and deletion of *AUS1* gene had no effect on accumulation of C_6NBD PS in yeast cells. To further investigate the role of phosphatidylserine in sterol uptake process the gene encoding phosphatidylserine synthase (*CHO1*) was deleted and the mutant was evaluated for its ability to accumulate 25-NBD cholesterol.

Phosphatidylserine synthase is a crucial enzyme in PS synthesis pathway which specifically transfers the phosphatidyl group from CDP-diacylglycerol or dCDP-diacylglycerol to l-serine [92]. Newly synthesized PS is conversed to PE and indirectly serves as a substrate in PC synthesis pathway. Deletion of *CHO1* gene completely blocks phosphatidylserine synthesis in the cell. However the mutation is not lethal and the cells are still able to synthesize PE and PC through Kennedy pathway when supplemented with choline or ethanolamine (Figure 48) [93]. As depicted in Figure 49A cells lacking phosphatidylserine were still able to express Aus1p-RFP when expression was driven from *AUS1* natural promoter and the protein was able to reach plasma membrane.

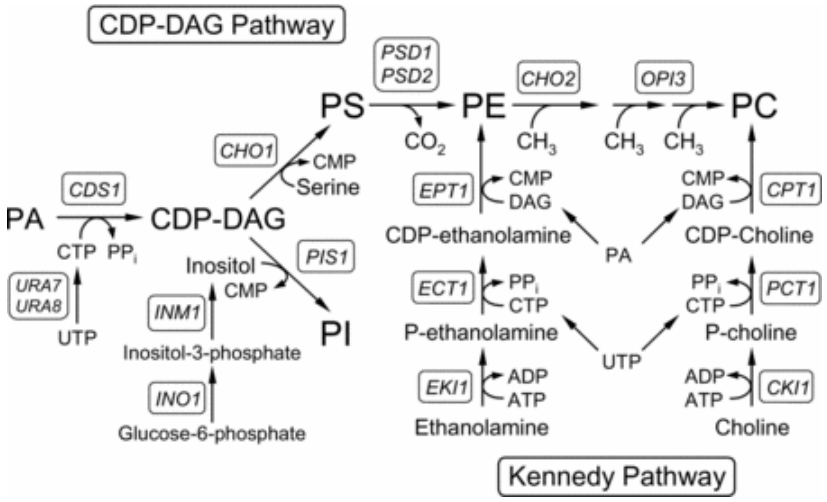


Figure 48: Lipid synthesis in yeast. Phosphatidylserine synthase encoded by *CHO1* gene catalyse transformation of CDP-DAG into PS. When *CHO1* gene is deleted PS synthesis is completely blocked. Under this conditions lipids like PC and PE are synthesized through Kennedy pathway [93].

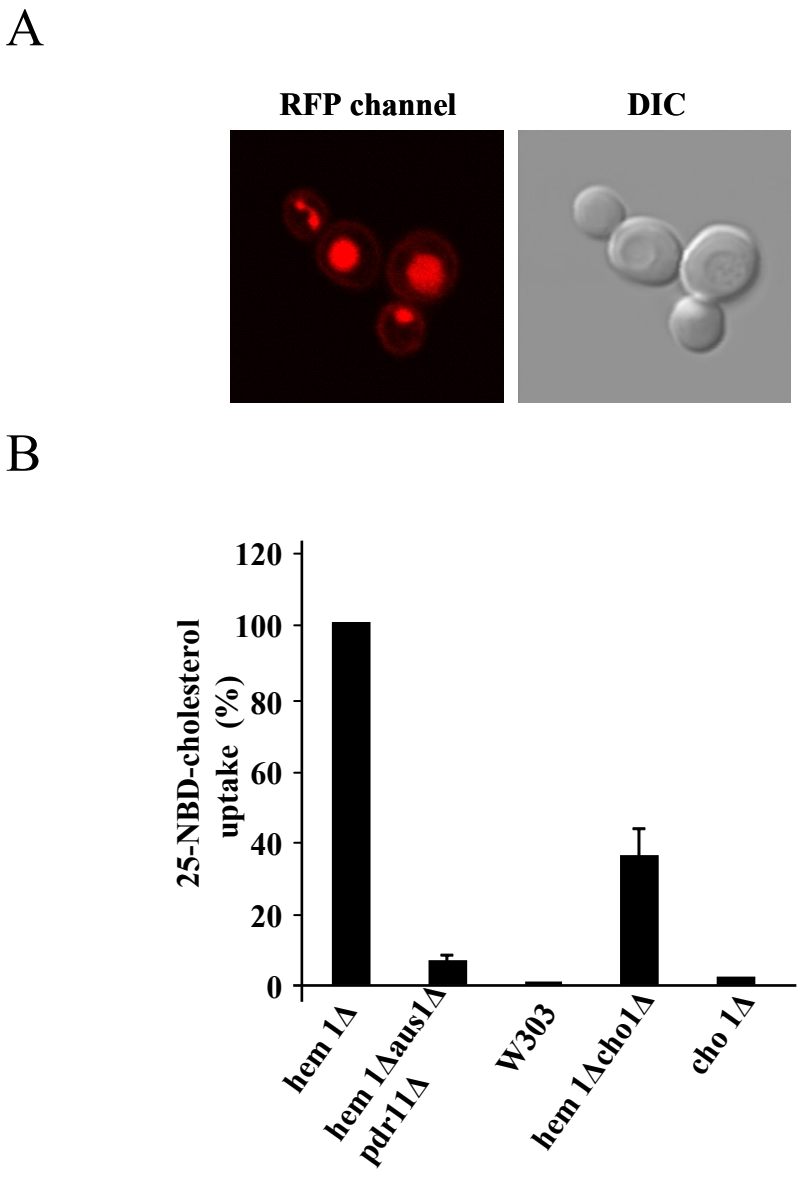


Figure 49: Influence of phosphatidylserine on sterol uptake *in vivo*. (A) Expression of Aus1p-RFP protein in *hem1Δcho1Δ*. Yeast cells transformed with vector carrying construct containing Aus1p-RFP (vector Endo-AUS1-RFP) and cultivated for 16 h in media supplemented with 2% galactose and sterol/Tween mixture. Subsequently cells were visualized by DIC and the RFP tagged protein expression was examined by fluorescence microscopy. (B) 25-NBD cholesterol uptake was measured by flow cytometry as described in Materials and Methods. Cells lacking phosphatidylserine synthase (*hem1Δcho1Δ*) incorporated less 25-NBD cholesterol than the control *hem1Δ* strain. Results are the means ± SD of three independent determinations, relative to the value obtained for *hem1Δ* yeast strain.

As shown previously, cells with active sterol synthesis pathway do not incorporate sterols and removal of *CHO1* gene from those cells do not change this state. However, sterol accumulation in cells that are sterol competent and at the same time lack phosphatidylserine was greatly reduced (around 50%) (Figure 49B). This fact supports the idea that phosphatidylserine is necessary for

effective sterol uptake process in yeast. In the next step *CHO1* gene encoding phosphatidylserine synthase was cloned and overexpressed in yeast. N terminally Flag tagged Cho1p protein was functional since it was able to rescue the growth of *Δcho1* mutant on media not supplemented with choline (Figure 50A). Overexpressed protein was detected by immunodetection in two forms - phosphorylated (30 kDa) and non phosphorylated (27 kDa) (Figure 50B). Although only the non-phosphorylated form is active both versions of protein are known to co-exist in the equal amount in the growing cells [94]. Generated construct can be further used to study the effects of phosphatidylserine synthase overexpression on sterol uptake in yeast.

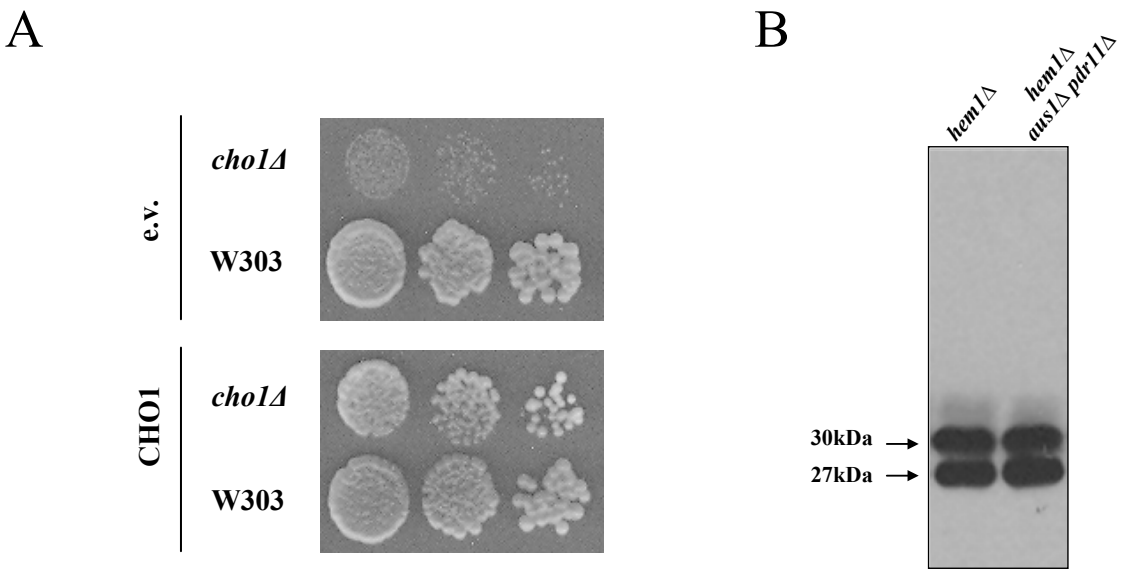


Figure 50: Overexpression of phosphatidylserine synthase and its influence on sterol uptake in yeast. (A) Serial dilutions of *CHO1* deficient strain and WT transformed with empty vector (pESC-ura) or vector carrying *CHO1* gene (pESC-CHO1) were spotted on plates supplemented with 2% galactose. Overexpression of CHO1 rescued the growth of *cho1Δ* mutant on media not supplemented with choline. (B) Immunodetection of N terminally Flag-tagged phosphatidylserine synthase overexpressed in *hem1Δ* and *hem1Δ aus1Δpdr11 Δ*. In both strains protein was detected in two forms phosphorylated (30 kDa) and not phosphorylated (27 kDa).

5. PERSPECTIVE

5.1 Influence of the cell wall on the sterol uptake process

Sterol influx is restricted to the anaerobiosis or to heme deficiency. The cell wall surrounding the yeast cell seems to play an important role in the process of sterol uptake. Upon anaerobiosis cell wall undergo general remodeling [95] and increase its affinity for sterols. 25-NBD cholesterol binds efficiently to the cell wall of sterol competent cells but not to cell wall of wild type W303 cells (data not shown).

Among the genes, which expression is up regulated in anaerobiosis are also genes encoding cell wall mannoproteins belonging to DAN/TIR family. It was suggested by Alimardani *et al*. [2] that one of those proteins, Dan1p can assists Aus1p in sterol uptake process. To test that hypothesis yeast mutants lacking *DAN1* gene were generated and tested for its ability to accumulate fluorescent sterol analog, 25-NBD cholesterol. Deletion of *DAN1* but not *DAN4* significantly decreased sterol incorporation in *hem1Δ* background, confirming specific influence of tested mannoprotein on the sterol uptake and excluding the possibility that removal of any of the mannoproteins would have impact on sterol internalization (Figure 51A and B).

Since the cell wall seems to play a crucial role in sterol incorporation it was tested if there are changes in the cell wall structure between mutants expressing or lacking *AUS1*, *PDR11* and *DAN1* genes (Figure 52A and B). Cells were grown in the presence of sterol or ALA and subsequently were labeled with calcofluor white – a non specific fluorochrome that binds to cellulose and chitin. In the presence of ALA and cholesterol sterol competent cells (*Δhem1*), sterol uptake mutant (*Δhem1Δaus1Δpdr11*) and wild type cells (W303) did not display any differences. However sterol competent cells lacking *DAN1* gene were labeled differently than when grown in the presence of ALA or cholesterol (Figure 52C). The punctature labeling could correspond to the specific depositions of chitin and cellulose and indicate perturbations in the cell wall integrity what in turn could affect sterol transport.

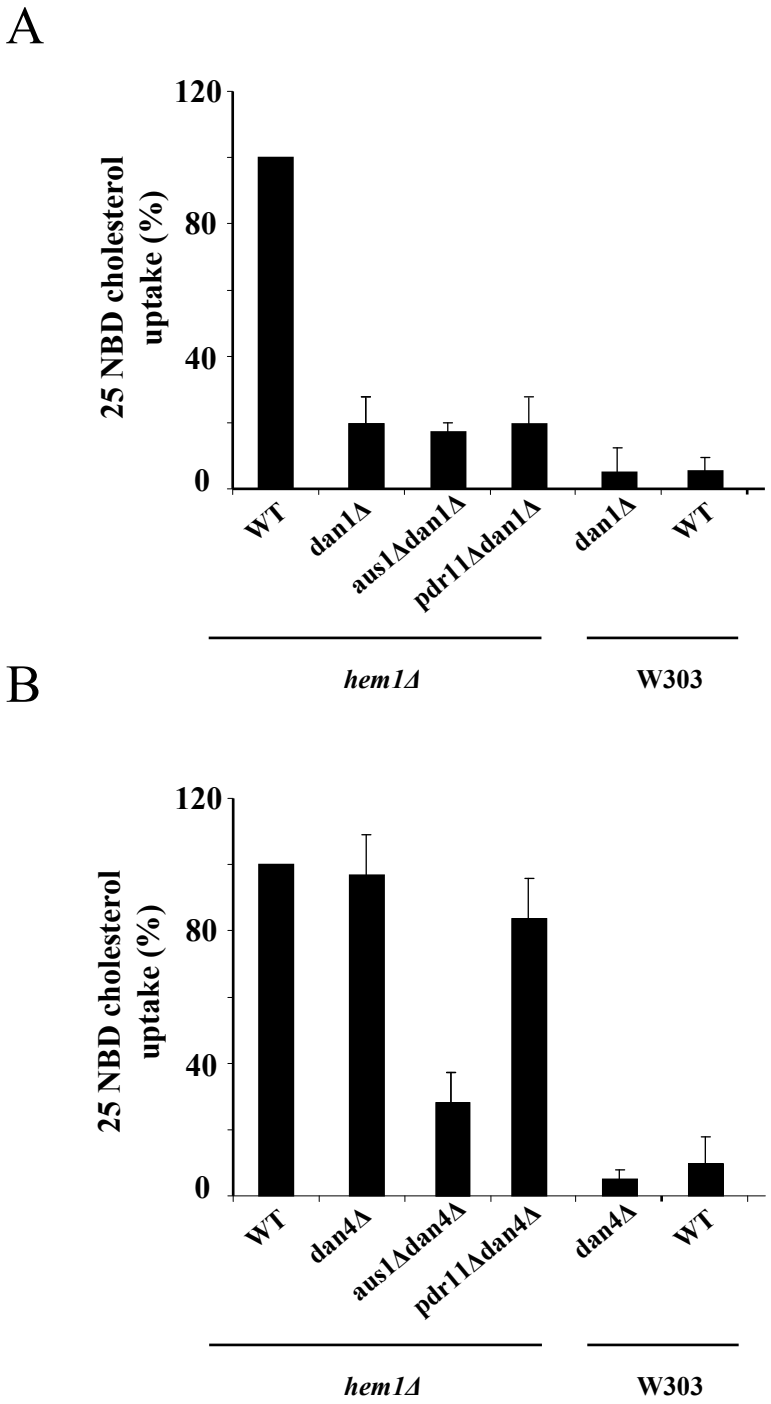


Figure 51: Influence of cell wall mannoproteins on 25-NBD cholesterol uptake. Uptake of 25-NBD cholesterol in yeast mutants was measured by flow cytometry as described in Materials and Methods. Results are the means ± SD of three independent determinations, relative to the value obtained for *hem1Δ* yeast strain. (A) Measurement of sterol uptake in cells lacking *DAN1* gene in *hem1Δ* background and in wild type background. Cells unable to express Dan1p could not incorporate exogenously applied sterols. (B) Incorporation of 25-NBD cholesterol in yeast mutants lacking *DAN4* gene in *hem1Δ* background and in wild type background. Deletion of *DAN4* did not have influence on sterol uptake confirming particular role of Dan1p in the process of sterol uptake.

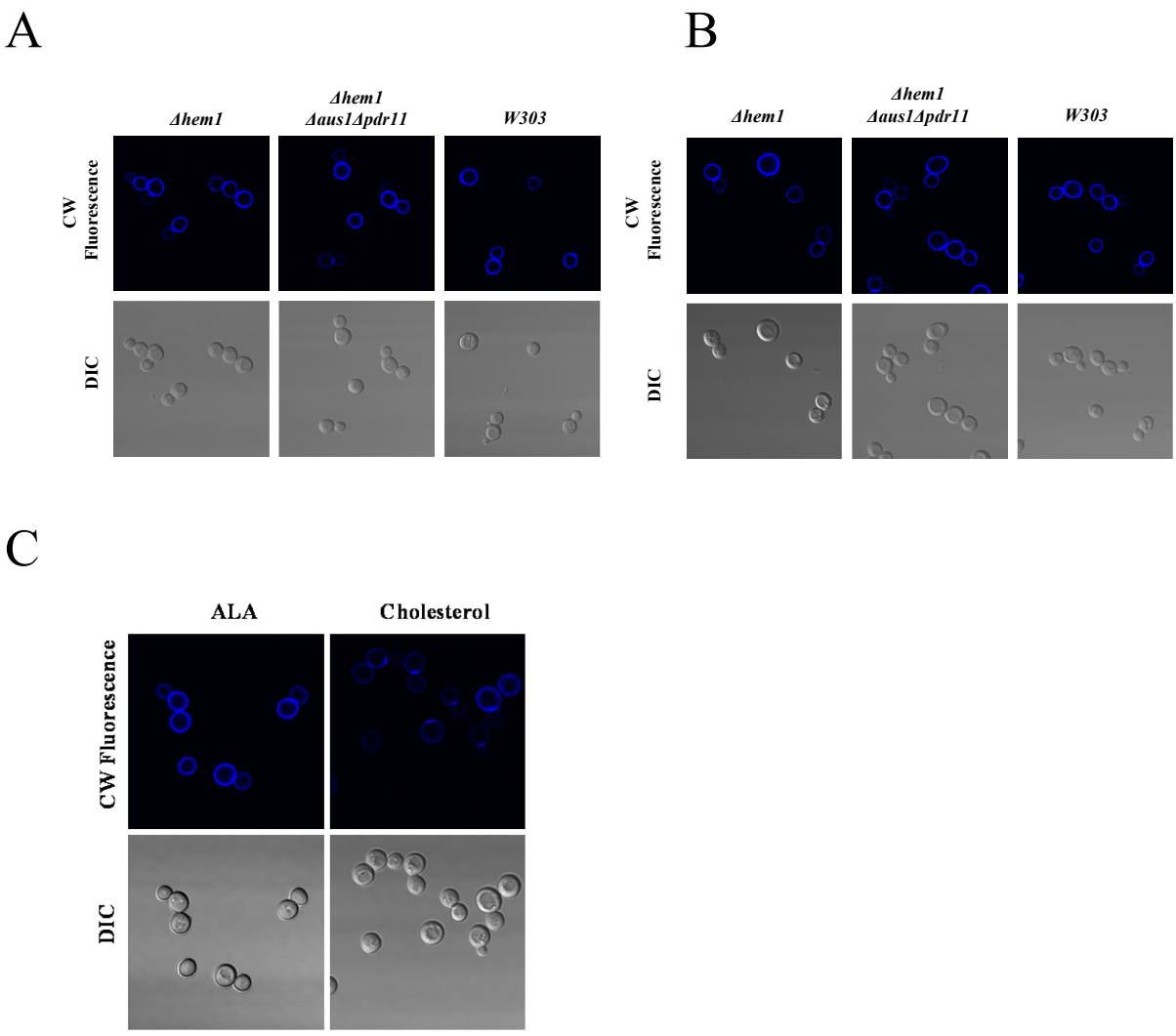


Figure 52: Stainig of the yeast cell wall with calcofluor white. Yeast mutants were cultivated in the presence of (A) cholesterol/ Tween mixture or (B) aminolevulinic acid for 16 h. Subsequently cells were stained with calcofluor white (CW) and visualized by DIC and fluorescence microscopy. No differences in term of cell wall integrity and structure was observed between sterol auxotrophic cells lacking DAN1 gene and control cells when cultivated in the presence of ALA or sterols. (C) Yeast mutant *hem1Δdan1Δ* was cultivated in the presence of ALA or cholesterol/Tween mixture and subsequently labeled with calcofluor white. The punctature structures within the cell wall were present when the cells were growing on media supplemented with sterol.

Altogether, obtained results confirm that Dan1p protein is important for sterol uptake and open the possibility that it could interact with Aus1p eg. acting as a sterol donor for ABC transporter, which deliver the sterol molecules directly to the transporter or assists its incorporation into the plasma membrane (Figure 53). However further experiments are necessary to confirm this hypothesis and to elucidate the further steps of sterol transport.

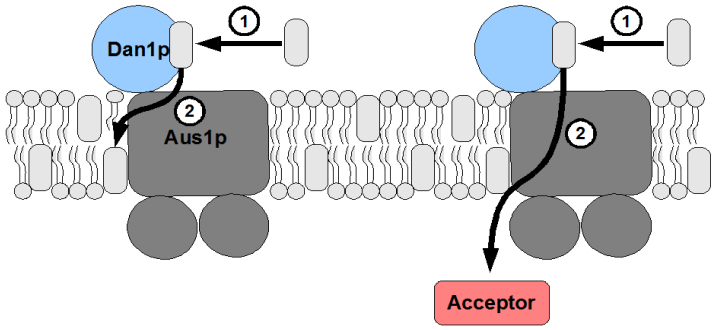


Figure 53: Theoretical mechanism of sterol uptake mediated by Dan1p and Aus1p proteins. Assuming that Dan1p mannoprotein could act as a sterol donor, two mechanisms of further sterol transport are possible: (A) Dan1p could bind sterol and delivers it to Aus1p protein which subsequently could insert the sterol molecule into the membrane (B) Dan1p could deliver sterol to Aus1p which transport it through the membrane and release it to the acceptor molecule

5.2 Immunoprecipitation of Aus1p protein with interacting partners

As suggested in the previous chapter Aus1p could interact with other proteins eg. Dan1p. Therefore attempts were made to detect putative interaction partners for Aus1p which could act as direct regulators of protein activity or as partners in sterol transport process (acceptors of donors of sterol molecules). For detection of interactors, the gene of interest was genomically tagged with 10xHA tag on C terminus, immunoprecipitated and the obtained eluate was analyzed by mass spectrometry. The expression of protein was confirmed by immunodetection with anti-HA antibody showing that full size protein was generated upon cultivation of the cells on the media supplemented with sterol. Upon tagging the protein expression was detected only in cells auxotrophic for sterols (*hem1Δ* background)). In the W303 strain, which is able to synthesize its own sterols no signal was detected (Figure 54A). Immunoprecipitation procedure was done according to the method described in Materials and Methods section. Although the protein was detected by Western Blot procedure (Figure 54B), the amount of immunoprecipitaed Aus1p was too low to be detected by Silver or Coommasie staining on SDS-PAGE gels, indicating that the sample contain less that 1 ng of the protein. Preliminary analysis by mass spectrometry revealed low amount of peptides copurified with Aus1p (Table 6).

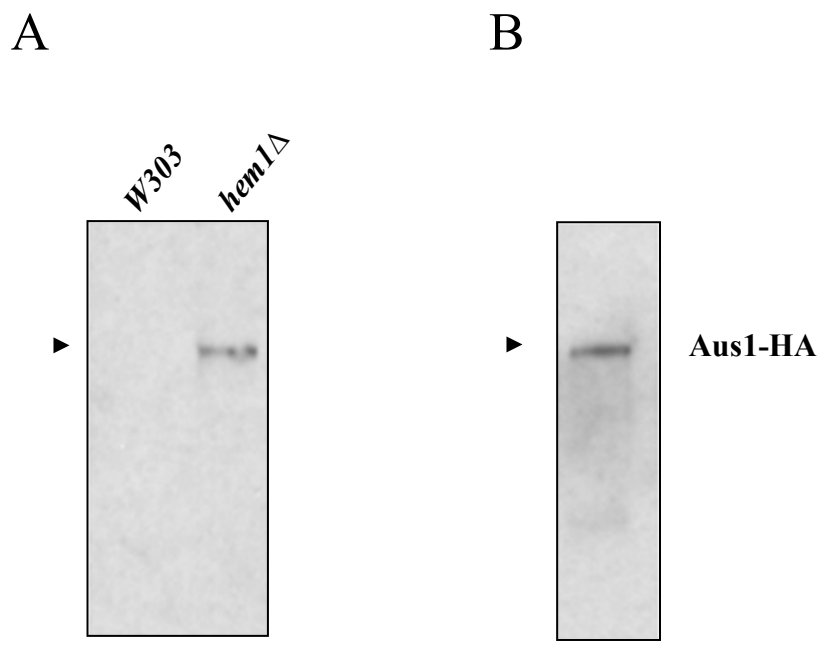


Figure 54: Detection of Aus1p-HA expression (A) *AUS1* was genomically tagged with 10xHA tag at C terminus in *hem1Δ* and W303 yeast strains. Cells were cultivated in the presence of 20 μg/ml of cholesterol and 0.5% Tween80 for 16h and subsequently total cellular membranes were isolated. Protein expression was evaluated by immunodetection with anti HA antibody. Signal was detected in the membranes obtained from *hem1Δ* cells in which *AUS1* was tagged with HA tag, confirming that *AUS1* gene expression is not activated when the cells are able to synthesize sterols. (B) HA tagged Aus1p protein was immunoprecipitated as described in Materials and Methods. Signal corresponding to the Aus1-HA protein was detected by Western Blot in the eluate.

Interestingly, among detected proteins is a components of eisosomes - Pil1 and actin (End7) which are important for uptake of cargo from that structures. Eisosomes are large cytoplasmic protein assemblies that localize to specialized domains on the plasma membrane which are supposed to be sites of endocytosis [96]. Since LDL mediated sterol uptake in mammalian system relays on endocytosis it would be especially interesting to investigate if such a connection would exist also in yeast. However, due to the poor number of identified peptides it is necessary to confirm that data and to optimize the process of immunoprecipitation to obtain more clear results.

Table 6 List of peptides detected after Aus1-HA immunoprecipitation. Peptides present in the control (cells growing in the presence of ALA) sample were excluded.

Gene name	Gene description	Number of identified peptides
AUS1	Transporter of the ATP-binding cassette family, involved in uptake of sterols and anaerobic growth	42
PDC1	Major of three pyruvate decarboxylase isozymes, key enzyme in alcoholic fermentation, decarboxylates pyruvate to acetaldehyde; subject to glucose-, ethanol-, and autoregulation; involved in amino acid catabolism	13
TDH2	Glyceraldehyde-3-phosphate dehydrogenase, isozyme 2, involved in glycolysis and gluconeogenesis; tetramer that catalyzes the reaction of glyceraldehyde-3-phosphate to 1,3 bis-phosphoglycerate; detected in the cytoplasm and cell wall	12
YDL055C	GDP-mannose pyrophosphorylase (mannose-1-phosphate guanyltransferase), synthesizes GDP-mannose from GTP and mannose-1-phosphate in cell wall biosynthesis; required for normal cell wall structure	2
HSP82	Hsp90 chaperone required for pheromone signaling and negative regulation of Hsf1p; docks with Tom70p for mitochondrial preprotein delivery; promotes telomerase DNA binding and nucleotide addition	2
HSC82	Cytoplasmic chaperone of the Hsp90 family, redundant in function and nearly identical with Hsp82p, and together they are essential;	2
GRP78	ATPase involved in protein import into the ER, also acts as a chaperone to mediate protein folding in the ER and may play a role in ER export of soluble proteins; regulates the unfolded protein response via interaction with Ire1p	2
END7	Actin, structural protein involved in cell polarization, endocytosis, and other cytoskeletal functions	1
YNL135C	Peptidyl-prolyl cis-trans isomerase (PPIase), binds to the drugs FK506 and rapamycin; also binds to the nonhistone chromatin binding protein Hmo1p and may regulate its assembly or function	1
PIL1	Primary component of eisosomes, which are large immobile cell cortex structures associated with endocytosis; null mutants show activation of Pkc1p/Ypk1p stress resistance pathways	1

5.3 Reconstitution of Aus1p into Giant Unilamellar Vesicles (GUVs)

Giant Unilamellar Vesicles serve as a model system for studying aspects of biological membranes. Although during last years a lot of effort was done to optimize generation of GUVs, it is still difficult to obtain proteo-GUVs (GUVs with a protein inserted into the membrane), especially ones containing large proteins. Here attempts were made to reconstitute Aus1p into such vesicles in order to investigate later the impact of ABC transporter on sterol transport. Several conditions were tested for effective formation of giant vesicles containing Aus1p-RFP varying in the composition of reconstitution buffer (low versus high salt concentration) and electroformation parameters (short versus long electrofrmation). Initially the Aus1p-RFP protein was used during the experiments, in order to confirm protein incorporation into GUVs by RFP fluorescence. However, in most cases no fluorescence was detected from formed GUVs.

When the GUVs were generated from proteoliposmes prepared in reconstitution buffer (20mM HEPES, 150 mM KCl) aggregates of RFP tagged Aus1p were visible on the surface of liposomes (Figure 55A). It is possible that RFP tag (around 20kDa) leads to the protein aggregation during electroformation. To overcome that problem, protein of interest (without RFP tag) was labeled with TAMRA dye (5-(and-6)- carboxytetramethylrhodamine succininmidyl ester) using Fluoro Spin 557 TAMRA labeling kit. The TAMRA labeling reagent undergoes a cross-linking reaction between the NHS ester on the dye and primary amines on the protein that results in the formation of a stable, covalent amide bond. Finally, the labeled protein was successfully reconstituted under following conditions: upon reconstitution in LUVs composed of POPC the proteoliposomes were dried in a salt chamber at 4ºC on the platinium electrodes. Subsequently, the electrodes were placed in electroformation buffer (250 mM sucrose) for 3 h. After preincubation, electroformation was applied for 6 h with 1 V and 10 Hz. GUVs were deatached from the electrodes for 30 min with 1.2 V. Although formation results in low amount of GUVs, the obtained material was fluorescent confirming successful reconstitution of protein (Figure 55B). Obtained GUVs with Aus1p protein incorporated into membrane can be used to test the impact of the ABC protein on the membrane lipid organization (by using for example GUV shape assay) or for examination of the protein behavior in the membrane.

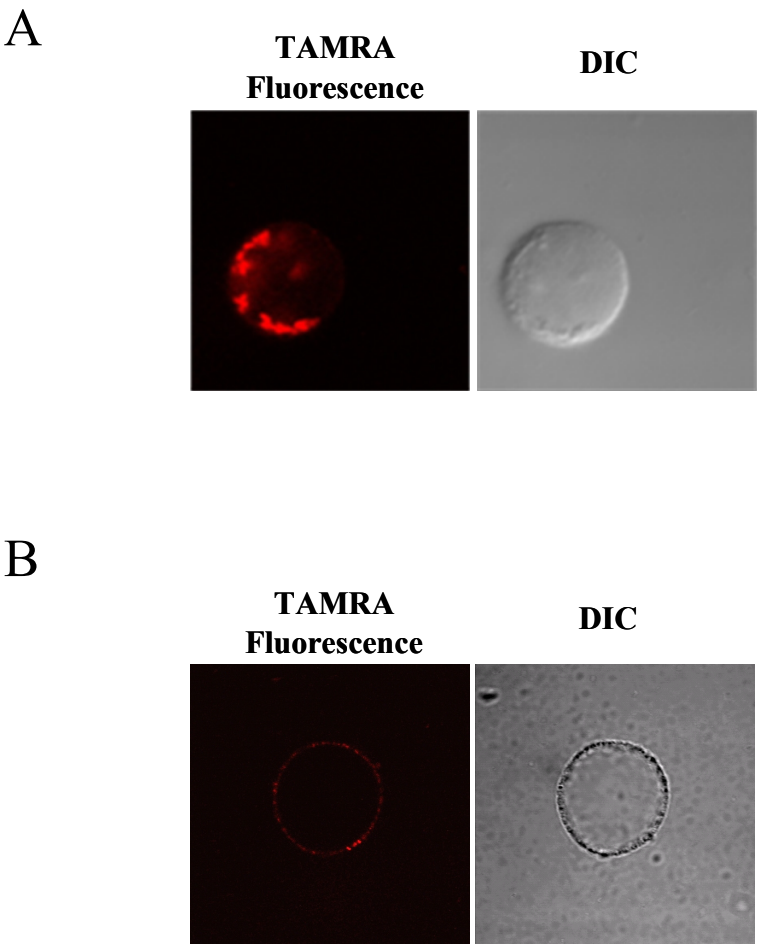


Figure 55: Giant Unilamellar Vesicles (GUV) with Aus1p protein. Formation of Giant Unilamellar vesicles was examined by DIC and fluorescence microscopy. (A) Aus1-RFP protein was reconstituted into liposomes, which were subsequently dried and electroformed into GUVs. Although GUVs were formed the protein was aggregated. (B) TAMRA labeled Aus1p protein were reconstituted into liposomes and used for formation of GUVs. Although low yield of GUV formation, some vesicles were properly labeled with the fluorescent protein.

6. DISCUSSION

6.1 Influence of Aus1p protein on sterol uptake process in vivo

Aus1p protein and its homolog Pdr11p are among the genes induced by anaerobiosis in and have great influence on sterol uptake process in yeast. Both ABC transporters were expressed under anaerobic conditions and mutants with double deletions (*aus1Δ pdr11Δ)* were found to be unable to take up radiolabeled cholesterol. During this studies yeast mutants lacking *AUS1* and/or *PDR11* genes in a *hem1Δ* background were generated. Deletion of *HEM1* gene mimics physiologically anaerobic state – blocking, among others, sterol biosynthesis, inducing the expression of *AUS1/PDR11* and probably others, undefined elements of sterol-uptake machinery [97].

25-NBD cholesterol was used to follow the sterol uptake in yeast *in vivo*. Although this fluorescent sterol analog posses a large reporter group attached to the aliphatic side chain, it was efficiently internalized, estrified and stored in lipid droplets. Although some studies report that localization of 25-NBD cholesterol in the cell can differ than localization of natural sterols [98], this analog is widely used in studies concerning sterol transport [99,100].

Removal of *AUS1* and *PDR11* blocked internalization of 25-NBD cholesterol – although the internal sterol biosynthesis was inactive and cells were competent for sterol uptake (due to the lack of *HEM1* gene). External sterol molecules were trapped within cell wall and were not internalized into the cell. That could suggests that Aus1p and Pdr11p act on the level of incorporation of sterol from periplasmic space into the plasma membrane.

Deletion of *PDR11*, (an *AUS1* homolog that displays around 70% identity with Aus1p within protein sequence), did not influence the amount of 25-NBD cholesterol uptake. However when overexpressed in *hem1Δ* background, Pdr11p rescued the growth of triple mutant (*hem1Δaus1Δpdr11Δ*) as efficiently as overexpressed Aus1p. One explanation of that fact is that under physiological conditions both genes are expressed at different levels although, as transporters, still have the same efficiency. To confirm that assumption, the amount of *AUS1* and *PDR11* transcript was evaluated in the cells that internalize sterol and in cells with active ergosterol biosynthetic pathway. Real Time PCR results confirmed that both genes were induced when internal sterol synthesis was blocked and that the amount of *AUS1* mRNA was much higher than *PDR11*. That would suggest that Aus1p is a main player in sterol uptake and Pdr11p support its action or has other, undefined functions.

To characterize Aus1p transporter *in vitro,* the gene of interest has been cloned, tagged with fluorescent RFP tag at the C terminus and placed under the control of either galactose or endogenous promoter. When expressed under the control of strong inducible promoter, Aus1p protein was

produced in the cells with both active and inactive ergosterol biosynthesis pathway. The red fluorescent signal was detected in plasma membrane, endoplasmic reticulum and vacuole. However, since strong overexpression can leads to mislocalization of protein product, Aus1p-RFP construct was placed under the control of its own endogenous promoter. In this case the protein expression took place only in the cells lacking *HEM1* gene when cultivated in the presence or absence of sterol. That indicates that the signal which induces *AUS1* expression does not depend on the presence of sterol in the environment, but on the availability of oxygen. However, when expressed under the control of endogenous promoter, Aus1p-RFP has been detected in the same cellular compartments as when expressed under the control of galactose promoter. Since Aus1p is predicted to be a plasma membrane protein and other studies confirmed that [20,61], obtained signal most probably corresponds to the newly synthesized molecules in ER and degraded Aus1p-RFP molecules in vacuole. That would suggest that the turnover of Aus1p is very rapid. It has to be also considered that the tagging with RFP or Flag tag could be the reason of mislocalization and/or faster protein turnover. However, since tagged protein was able to support the growth of triple mutant, it was assumed that tagging did not disrupt its function.

6.2 Expression and purification of Aus1p protein

In this study for the first time a successful purification protocol and the biochemical characterization of the yeast putative sterol transporter Aus1p are described. Expression of a FLAG tagged version of Aus1p from a high copy plasmid under the control of the strong inducible promoter (GAL10) allowed for effective purification to homogeneity in yields high enough for subsequent biochemical studies.

Yeast cells were used as a host organism for protein production because bacterial expression system (theoretically able to produce more product than yeast one) would not ensure correct posttranslational modifications which can be crucial for protein activity. Moreover, the prokaryotic lipid environment differs from eukaryotic (eg. bacteria produce hopanoids instead of sterols) what could influence the amount and quality of expressed protein [101].

To make the purification possible, *AUS1* was tagged on the N terminus with Flag tag which allowed for purification of this protein by affinity chromatography. Moreover, adding a small soluble tag (like a Flag tag) at N terminus can efficiently decrease protein degradation level, as shown already for yeast H-ATPase Pma1p. Protein purification is a multistep process during which the protein of interest is isolated from a complex mixture of all cellular components. Several factors influence the amount and quality of the obtained product – therefore purification protocol has to be optimized for each protein separately. Since protein proteolysis can be a serious problem during purification, Aus1p has been overexpressed in a protease deficient strain (BJ1991) and high amount of protease

inhibitors were added during purification process. Additionally, cultivation conditions were optimized to ensure that the protein is expressed with high efficiency. Since galactose promoters are the most efficient for protein overexpression, *AUS1* was placed under the control of GAL10 and its expression was induced for 16h. Although this induction time seems to be long, detection of full length Aus1p protein was possible only after 8 h and was constant up to 20 h. Since prolonged induction (around 20 h) can affect cell viability, 16 h of cultivation in presence of galactose has been selected as an effective induction time.

In the cell, membrane proteins account for around 30% of all proteins and they vary in size and physio-chemical properties. Purification of these components is more difficult than purification of soluble proteins since membrane proteins are highly hydrophopbic and have to be released from membrane environment by solubilization with detergents. To purify Aus1p, cellular membranes from the induced cells were collected and solubilized with 1% dodecylmaltoside (DDM). DDM was detergent of choice firstly because it is a non ionic detergent widely used during purification of ABC transporters and secondly, because it was able to efficiently release Aus1p from membranes. Although, Triton X-100 was also efficient in terms of Aus1p solubilization, it inactivated protein in term of ATP hydrolisis.

Solubilized protein was subjected to affinity chromatography which allowed for purification of around 70 μg of Aus1p protein per 1 g of yeast cells. Affinity chromatography was in this case very efficient and selective process, since mass spectrometry analysis of the obtained product showed only minor contaminations. Theoretically, additional steps of purification can be included into protocol (eg. size exclusion chromatography) however it does not seem to be necessary in that case since the final product already displayed more than 90% of purity. Moreover, one has to take into account that any additional step can decrease the amount of purified product and can lead to decrease or even lost of activity.

6.3 Reconstitution of Aus1 protein into liposomes

Since Aus1p is a membrane protein, it was necessary to reconstitute it into membrane for further analysis. Reconstitution into liposomes is a well known strategy for studying the properties of membrane proteins. Again, as in the case of purification, the reconstitution procedure has to be set up for each protein separately since there is no general protocol. Several reconstitution methods exist, which differ in terms of insertion of membrane proteins into liposomes (eg. freez-thaw method or liposome destabilization) or detergent removal (dilution, dialysis, size exclusion chromatography or adsorption on polystyrene beads). Most common strategy for proteoliposome formation involves the use of detergents. It requires co-micellization of protein and preformed liposomes with detergent. Subsequently, during detergent removal, when its concentration decreases to a critical level, protein

molecules spontaneously associate with phospholipids to form proteoliposomes [102]. Since Aus1p belongs to the family of ABC transporters, which are frequently reconstituted with use of dodecylmaltoside, DDM was the detergent of choice for reconstitution of putative sterol flippase. It efficiently in solubilized of Aus1p from cellular membranes during purification and preserved ATPase activity of the protein. Additionally, it is also advised, to use for reconstitution the same detergent as for purification. Extremely important is also the complete removal of detergent from reconstitution mixture, as traces of detergent can negatively influence further assays eg. due to the higher membrane permeability in the presence of detergent leftovers [102]. Because dodecylmaltoside has low critical micellar concentration the only efficient method for removal of the detergent from lipid-protein-detergent micellar solutions is incubation with BioBeads (in contrast to detergents with high cmc which can be easily removed by dialysis). The polystyrene beads efficiently adsorb organic materials from aqueous solutions. In case of Aus1p the reconstitution mixture was incubated with high amount of adsorptive material for 16 h at 4ºC. Addition of high amount of BioBeads resin leads to fast detergent removal. That method of treatment was shown result in formation of smaller but unilamellar proteoliposomes (DDM is a detergent which, when removed with slow rate leads to formation of multilamellar vesicles). Although it was shown that the detergent adsorption rate of Bio Beads strongly depends on temperature (it doubles every 15ºC) [103] the reconstitution procedure was carried at 4ºC (to minimalize protein degradation) what in turns forced longer incubation time (16 h in contrast to 5 h which would be sufficient at 25ºC). After removal of detergent proteoliposomes were collected by ultracentrifugation giving a clearly visible pellet and confirming that vesicles were formed since solubilized lipid-protein-detergent mixture cannot be pelleted at 100.000g.

To prove that Aus1p protein was reconstituted, preparation was floated on the step nycodenz gradient. During gradient ultracentrifugation liposomes migrate from the bottom to the top fractions, so if the protein is reconstituted into the liposomal membrane it will be detected also at the top of the gradient. Indeed, RFP fluorescence was clearly detected in the same fractions as liposomes confirming that upon applied reconstitution conditions Aus1p can be reconstituted. Moreover, although prolongated incubation at 4ºC, the protein was not degraded and only one band corresponding to Aus1p protein was detected by SDS-PAGE and Western Blot analysis. Although successful, the reconstitution protocol still could be optimized. Under given conditions protein was reconstituted with orientation dependent on the used lipid mixture. It is possible that manipulations with reconstitution conditions eg. lipid/protein ratio, resin amount and incubation time can improve the efficiency of protein incorporation and influence the orientation of Aus1p in the membrane, theoretically giving even symmetrical orientation with both NBD domains directed outside vesicle. That would be useful for further studies since only molecules with that orientation are able to hydrolyze ATP which is applied from outside.

6.4 ATPase activity of Aus1p protein

Purified, solubilized Aus1p was able to bind ATP in the presence of Mg^{2+} and exhibited an orthovanadate-, BeFx- and AlF_3 sensitive ATPase activity even before reconstitution in liposomes, consistent with the properties of other purified ABC transporters such as the human multidrug transporter P-glycoprotein [57,73]. ATPase activity was also effectively blocked at higher concentration (1 mM) of verapamil. Conceivably, binding of numerous verapamil molecules to Aus1p might start to induce structural perturbation in the protein and block the transport cycle. A specific activity of the purified protein of 56 nmol ATP per minute and mg of protein has been determined. This is within the range of values reported for a number of eukaryotic ABC transporters that have been purified and biochemically characterized such as Ste6p, ABCR, TAP and ABCA1 [2,78,90,92]. This activity did not show any specific increase upon addition of lipids or sterols. Some ABC proteins are known to increase their activity upon addition of the substrate (eg. BCRP in the presence of prazosin [104]), however it is not a rule. Lack of stimulation could also be caused by the fact that the protein was in a detergent solution and not in its native membrane environment.

Upon reconstitution into proteoliposomes with different lipid composition, Aus1p ATPase activity was reduced by PC and PE but specifically stimulated several fold in the presence of PS. Phosphatidylserine is an anionic lipid and the presence of this type of lipids was proven to be necessary for function of acetylcholine receptor (AChR) [105,106] and for the opening of potassium channel (KcsA) [107,108]. However, opposite to the effect observed for Aus1p, the positive impact was observed for mentioned proteins only in the presence of small amounts of anionic lipids, and its elevated concentrations inhibited protein activity. Stimulation of Aus1p by PS cannot simply be explained by the presence of the negatively charged lipid, since the anionic lipid PI failed to enhance Aus1p ATPase activity. Furthermore, the stimulation of Aus1p ATPase activity by PS was stereoselective; the natural stereoisomer of PS was more potent than its enantiomers in enhancing the ATPase activity. Although other lipids may also be important for Aus1p function, obtained data suggest that the direct, and stereospecific, interaction between the phospholipid PS and Aus1p regulates the activity of the transporter.

Many ABC transporters have been shown to possess intrinsic ATPase activity that is stimulated in the presence of transported substrates. The best investigated example is the mammalian Mdr1p (P-glycoprotein), which possesses an ATPase activity stimulated by various drugs which are known to be transported [109]. Hence, the specific stimulation of Aus1p ATPase activity by PS suggests that this phospholipid might be the primary substrate for the ABC transporter, and translocating PS across the plasma membrane could be the physiological function of Aus1p. However, present results rule out this possibility; the presence or absence of Aus1p has no effect on the internalization and

localization of NBD-PS and other phospholipid analogues across the yeast plasma membrane. Instead, Aus1p-dependent sterol uptake was drastically reduced in PS-deficient cells. Those *in vivo* data support a model in which PS is required for the full activity of the transporter to drive sterol uptake.

Biochemical and structural information obtained over recent years has highlighted a relationship between membrane proteins and lipids that is important for full functional and structural integrity of the protein. Membrane proteins are embedded in the lipid environment and surrounded directly by so called annular lipids which cover the hydrophobic protein surface and form a ring around it [109]. This group (composed of approximately 30 molecules) of loosely bound lipids interacts with a protein and undergo very fast ($2x10^7$ s^{-1}) exchange with bulk membrane lipids [110]. Proteins by itself display in most cases only a small specificity towards the kind of annular lipids, mostly preferring anionic ones [111]. However, it has been demonstrated that some lipid molecules interact with protein much stronger and cannot be removed even by detergent treatment. These so called non-annular lipids acts more as a cofactors and influence strongly protein activity. One example is the Ca^{+2} ATPase which binds tightly a cholesterol molecule [112,113]. Likewise, the activity of the yeast cytochrome bc1 complex requires cardiolipin and specific lipid binding sites have been identified in the X-ray structures of the protein complex [110,111]. Changes in PE levels have recently been shown to affect the activity of the vacuolar membrane-localized ABC transporter Ycf1 [112]. Similarly, Aus1p activity might be regulated by the PS levels in the cell. As this lipid is enriched along the secretory pathway, constituting ~13% of the glycerophospholipids in late secretory vesicles and ~34% in the plasma membrane, respectively [113], Aus1p activity would increase as it transits the secretory pathway *en route* to its functional residence in the plasma membrane. Notably, a requirement for PS has also been reported for the activity of the tryptophan transporter in the plasma membrane of *S. cervisiae* [107]. The specificity of lipid stimulated Aus1p ATPase activity is reminiscent of the activation of lipid transporting ATPases from the P4-ATPase family. Like Aus1p, the ATPase activity of Atp8a1 [108] and Atp8a2 [105] are selectively activated by PS and, at least for Atp8a1, the enatiomeric specificity is qualitatively similar; enzyme activation is greatest in the presence of the 1,2-sn-PS with some preference for the L-serine stereoisomers. These data indicate that the binding sites for PS on these very different classes of proteins may be similar or may reflect some other common features of their interactions with lipids.

The lipid composition of the membrane determines its phase state which can greatly influence the protein. Many membrane proteins prefer liquid crystalline state over rigid gel phase. Reconstituted Aus1p followed this pattern of behavior, displaying lack of activity in rigid membranes composed of cholesterol and sphingomyelin. However, also in pure fluid POPC liposomes the activity of protein was reduced in comparison to the activity of purified protein in buffer. Addition of small amount of cholesterol restored the activity to the basal level suggesting that cholesterol serves as a substrate,

structural lipid or that the protein prefers a lipid environment with middle range rigidity. However the stimulation by cholesterol addition was much smaller than observed by addition of phosphatidylserine. Interestingly, it was reported that in the presence of calcium PS is able to form gel like domains ([114,115] of (PS)2 Ca from which some protein (eg. Ca^{2+} ATPase [116] or gramidicin [117]) are excluded. Therefore, it is possible that stimulation of Aus1 activity can come from partition into such regions.

6.5 Influence of phosphatidylserine on sterol uptake *in vivo*

Specific stimulation of Aus1p ATPase activity by PS suggested that phosphatidylserine can play an important role in sterol uptake process. Indeed, yeast mutants lacking phosphatidylserine displayed strongly reduced accumulation of 25-NBD cholesterol although Aus1p expression and trafficking were not affected by PS deficiency. Overexpression of phosphatidylserine synthase (*CHO1*) – an enzyme responsible for PS synthesis in yeast, rescued the growth of *Δcho1* mutant on media not supplemented with choline and at the same time lead to elevated accumulation of fluorescent sterol in sterol uptake competent cells. This data confirmed the suggestions obtained from *in vitro* studies that phosphatidylserine plays an important role in sterol uptake process. Presented data suggest that PS modulates the activity of Aus1p transporter and by that influence sterol uptake. However, it is still possible that PS plays additional roles – eg. by modulation of plasma membrane properties, influence on protein arrangement in membrane or by affecting the extractability of sterol from the membrane. Unfortunately, there is no reliable study about the lipid composition of yeast plasma membrane under oxygen limited conditions. It would be therefore extremely interesting to investigate further the role of phosphatidylserine in sterol uptake.

7. CONCLUSION

In this work Aus1p protein belonging to ABC family was characterized. The protein of interest is a yeast specific plasma membrane transporter involved in the uptake of sterols, however the precise mechanism of its action remains to be revealed.

Here, a full length gene encoding Aus1p was cloned and protocols for protein overexpression and purification were developed. Pure, detergent solubilized protein was shown to bind and hydrolyze ATP, and its ATPase activity was efficiently blocked by classical inhibitors of ABC transporters (eg. vanadate). Moreover, a mutated version of the protein (carrying a lysine to methionine substitution within Walker A motif) was generated and it was shown to be unable to hydrolyze ATP.

Purified protein was successfully reconstituted into liposomes and its ATPase activity was stimulated by phosphatidylserine in a steroloselective manner. At the same time PS was not a substrate for Aus1p. These results suggest a direct interaction between Aus1p and PS.

Additionally, PS deficient yeast strain (*Δcho1*) was generated in order to test the influence of phosphatidylserine on sterol uptake *in vivo*. In the absence of PS sterol competent yeast cells incorporated significantly less exogenously applied sterol than cells capable to synthesize PS. At the same time expression and trafficking of Aus1p transporter to the plasma membrane were not affected.

Moreover, materials allowing for further studies of Aus1p were prepared. Aus1p was reconstituted into Giant Unilamellar Vesicles, which can be used further for testing the behaviour of the protein in the membranes with complex lipid compositions. The attempts were also made to detect interaction partners for Aus1p by immunoprecipitation as well as influence of a cell wall protein Dan1p on sterol uptake. Deletion of Dan1p significantly decreased sterol uptake process by sterol competent cells suggesting that this mannnoprotein can act as a sterol donor for Aus1p.

BIBLIOGRAPHY

[1] Wilcox, L. J.; Balderes, D. A.; Wharton, B.; Tinkelenberg, A. H.; Rao, G. and Sturley, S. L. (2002): Transcriptional profiling identifies two members of the ATP-binding cassette transporter superfamily required for sterol uptake in yeast, J Biol Chem 277 [36], pp. 32466-72. URL: **http://www.ncbi.nlm.nih.gov/pubmed/12077145**

[2] Alimardani, P.; Regnacq, M.; Moreau-Vauzelle, C.; Ferreira, T.; Rossignol, T.; Blondin, B. and Berges, T. (2004): SUT1-promoted sterol uptake involves the ABC transporter Aus1 and the mannoprotein Dan1 whose synergistic action is sufficient for this process, Biochem J 381 [Pt 1], pp. 195-202. URL: **http://www.ncbi.nlm.nih.gov/pubmed/15035656**

[3] Yang, H. (2006): Nonvesicular sterol transport: two protein families and a sterol sensor?, Trends Cell Biol 16 [9], pp. 427-32. URL: **http://www.ncbi.nlm.nih.gov/pubmed/16876994**

[4] Ikonen, E. (2008): Cellular cholesterol trafficking and compartmentalization, Nat Rev Mol Cell Biol 9 [2], pp. 125-38. URL: **http://www.ncbi.nlm.nih.gov/pubmed/18216769**

[5] Henneberry, A. L. and Sturley, S. L. (2005): Sterol homeostasis in the budding yeast, Saccharomyces cerevisiae, Semin Cell Dev Biol 16 [2], pp. 155-61. URL: **http://www.ncbi.nlm.nih.gov/pubmed/15797826**

[6] Li, Y. and Prinz, W. A. (2004): ATP-binding cassette (ABC) transporters mediate nonvesicular, raft-modulated sterol movement from the plasma membrane to the endoplasmic reticulum, J Biol Chem 279 [43], pp. 45226-34. URL: **http://www.ncbi.nlm.nih.gov/pubmed/15316012**

[7] Hamon, Y.; Broccardo, C.; Chambenoit, O.; Luciani, M. F.; Toti, F.; Chaslin, S.; Freyssinet, J. M.; Devaux, P. F.; McNeish, J.; Marguet, D. and Chimini, G. (2000): ABC1 promotes engulfment of apoptotic cells and transbilayer redistribution of phosphatidylserine, Nat Cell Biol 2 [7], pp. 399-406. URL: **http://www.ncbi.nlm.nih.gov/pubmed/10878804**

[8] Zarubica, A.; Plazzo, A. P.; Stockl, M.; Trombik, T.; Hamon, Y.; Muller, P.; Pomorski, T.; Herrmann, A. and Chimini, G. (2009): Functional implications of the influence of ABCA1 on lipid microenvironment at the plasma membrane: a biophysical study, FASEB J 23 [6], pp. 1775-85. URL: **http://www.ncbi.nlm.nih.gov/pubmed/19151332**

[9] Kohut, P.; Wustner, D.; Hronska, L.; Kuchler, K.; Hapala, I. and Valachovic, M. (2011): The role of ABC proteins Aus1p and Pdr11p in the uptake of external sterols in yeast: dehydroergosterol fluorescence study, Biochem Biophys Res Commun 404 [1], pp. 233-8. URL: **http://www.ncbi.nlm.nih.gov/pubmed/21110944**

[10] Edidin, M. (2003): Lipids on the frontier: a century of cell-membrane bilayers, Nat Rev Mol Cell Biol 4 [5], pp. 414-8. URL: **http://www.ncbi.nlm.nih.gov/pubmed/12728275**

[11] Stevens, H. C.; Malone, L. and Nichols, J. W. (2008): The putative aminophospholipid translocases, DNF1 and DNF2, are not required for 7-nitrobenz-2-oxa-1,3-diazol-4-yl-phosphatidylserine flip across the plasma membrane of Saccharomyces cerevisiae, J Biol Chem 283 [50], pp. 35060-9. URL: **http://www.ncbi.nlm.nih.gov/pubmed/18931395**

[12] van Meer, G.; Voelker, D. R. and Feigenson, G. W. (2008): Membrane lipids: where they are and how they behave, Nat Rev Mol Cell Biol 9 [2], pp. 112-24. URL: **http://www.ncbi.nlm.nih.gov/pubmed/18216768**

[13] Devaux, P. F. and Morris, R. (2004): Transmembrane asymmetry and lateral domains in biological membranes, Traffic 5 [4], pp. 241-6. URL: **http://www.ncbi.nlm.nih.gov/pubmed/15030565**

[14] Pomorski, T. and Menon, A. K. (2006): Lipid flippases and their biological functions, Cell Mol Life Sci 63 [24], pp. 2908-21. URL: **http://www.ncbi.nlm.nih.gov/pubmed/17103115**

[15] Daleke, D. L. (2003): Regulation of transbilayer plasma membrane phospholipid asymmetry, J Lipid Res 44 [2], pp. 233-42. URL: **http://www.ncbi.nlm.nih.gov/pubmed/12576505**

[16] Tang, X.; Halleck, M. S.; Schlegel, R. A. and Williamson, P. (1996): A subfamily of P-type ATPases with aminophospholipid transporting activity, Science 272 [5267], pp. 1495-7. URL: **http://www.ncbi.nlm.nih.gov/pubmed/8633245**

[17] Liscum, L. and Munn, N. J. (1999): Intracellular cholesterol transport, Biochim Biophys Acta 1438 [1], pp. 19-37. URL: **http://www.ncbi.nlm.nih.gov/pubmed/10216277**

[18] Maxfield, F. R. and Wustner, D. (2002): Intracellular cholesterol transport, J Clin Invest 110 [7], pp. 891-8. URL: **http://www.ncbi.nlm.nih.gov/pubmed/12370264**

[19] Chang, T. Y.; Reid, P. C.; Sugii, S.; Ohgami, N.; Cruz, J. C. and Chang, C. C. (2005): Niemann-Pick type C disease and intracellular cholesterol trafficking, J Biol Chem 280 [22], pp. 20917-20. URL: **http://www.ncbi.nlm.nih.gov/pubmed/15831488**

[20] Vance, J. E.; Hayashi, H. and Karten, B. (2005): Cholesterol homeostasis in neurons and glial cells, Semin Cell Dev Biol 16 [2], pp. 193-212. URL: **http://www.ncbi.nlm.nih.gov/pubmed/15797830**

[21] Carvalho, M.; Schwudke, D.; Sampaio, J. L.; Palm, W.; Riezman, I.; Dey, G.; Gupta, G. D.; Mayor, S.; Riezman, H.; Shevchenko, A.; Kurzchalia, T. V. and Eaton, S. (2010): Survival strategies of a sterol auxotroph, Development 137 [21], pp. 3675-85. URL: **http://www.ncbi.nlm.nih.gov/pubmed/20940226**

[22] Simons, K. and Ikonen, E. (1997): Functional rafts in cell membranes, Nature 387 [6633], pp. 569-72. URL: **http://www.ncbi.nlm.nih.gov/pubmed/9177342**

[23] Munn, A. L.; Heese-Peck, A.; Stevenson, B. J.; Pichler, H. and Riezman, H. (1999): Specific sterols required for the internalization step of endocytosis in yeast, Mol Biol Cell 10 [11], pp. 3943-57. URL: **http://www.ncbi.nlm.nih.gov/pubmed/10564282**

[24] Betters, J. L. and Yu, L. (2010): NPC1L1 and cholesterol transport, FEBS Lett 584 [13], pp. 2740-7. URL: **http://www.ncbi.nlm.nih.gov/pubmed/20307540**

[25] Holthuis, J. C. and Levine, T. P. (2005): Lipid traffic: floppy drives and a superhighway, Nat Rev Mol Cell Biol 6 [3], pp. 209-20. URL: **http://www.ncbi.nlm.nih.gov/pubmed/15738987**

[26] Levine, T. (2004): Short-range intracellular trafficking of small molecules across endoplasmic reticulum junctions, Trends Cell Biol 14 [9], pp. 483-90. URL: **http://www.ncbi.nlm.nih.gov/pubmed/15350976**

[27] Soccio, R. E. and Breslow, J. L. (2004): Intracellular cholesterol transport, Arterioscler Thromb Vasc Biol 24 [7], pp. 1150-60. URL: **http://www.ncbi.nlm.nih.gov/pubmed/15130918**

[28] Raychaudhuri, S.; Im, Y. J.; Hurley, J. H. and Prinz, W. A. (2006): Nonvesicular sterol movement from plasma membrane to ER requires oxysterol-binding protein-related proteins and phosphoinositides, J Cell Biol 173 [1], pp. 107-19. URL: **http://www.ncbi.nlm.nih.gov/pubmed/16585271**

[29] Lehto, M.; Laitinen, S.; Chinetti, G.; Johansson, M.; Ehnholm, C.; Staels, B.; Ikonen, E. and Olkkonen, V. M. (2001): The OSBP-related protein family in humans, J Lipid Res 42 [8], pp. 1203-13. URL: **http://www.ncbi.nlm.nih.gov/pubmed/11483621**

[30] Schulz, T. A. and Prinz, W. A. (2007): Sterol transport in yeast and the oxysterol binding protein homologue (OSH) family, Biochim Biophys Acta 1771 [6], pp. 769-80. URL: **http://www.ncbi.nlm.nih.gov/pubmed/17434796**

[31] Altenberg, G. A. (2003): The engine of ABC proteins, News Physiol Sci 18, pp. 191-5. URL: **http://www.ncbi.nlm.nih.gov/pubmed/14500797**

[32] Bryan, J. and Aguilar-Bryan, L. (1999): Sulfonylurea receptors: ABC transporters that regulate ATP-sensitive K(+) channels, Biochim Biophys Acta 1461 [2], pp. 285-303. URL: **http://www.ncbi.nlm.nih.gov/pubmed/10581362**

[33] Chan, K. W.; Zhang, H. and Logothetis, D. E. (2003): N-terminal transmembrane domain of the SUR controls trafficking and gating of Kir6 channel subunits, EMBO J 22 [15], pp. 3833-43. URL: **http://www.ncbi.nlm.nih.gov/pubmed/12881418**

[34] Gottesman, M. M. and Ambudkar, S. V. (2001): Overview: ABC transporters and human disease, J Bioenerg Biomembr 33 [6], pp. 453-8. URL: **http://www.ncbi.nlm.nih.gov/pubmed/11804186**

[35] Jones, P. M.; O'Mara, M. L. and George, A. M. (2009): ABC transporters: a riddle wrapped in a mystery inside an enigma, Trends Biochem Sci 34 [10], pp. 520-31. URL: **http://www.ncbi.nlm.nih.gov/pubmed/19748784**

[36] Rees, D. C.; Johnson, E. and Lewinson, O. (2009): ABC transporters: the power to change, Nat Rev Mol Cell Biol 10 [3], pp. 218-27. URL: **http://www.ncbi.nlm.nih.gov/pubmed/19234479**

[37] Linton, K. J. (2007): Structure and function of ABC transporters, Physiology (Bethesda) 22, pp. 122-30. URL: **http://www.ncbi.nlm.nih.gov/pubmed/17420303**

[38] Paumi, C. M.; Chuk, M.; Snider, J.; Stagljar, I. and Michaelis, S. (2009): ABC transporters in Saccharomyces cerevisiae and their interactors: new technology advances the biology of the ABCC (MRP) subfamily, Microbiol Mol Biol Rev 73 [4], pp. 577-93. URL: **http://www.ncbi.nlm.nih.gov/pubmed/19946134**

[39] Jungwirth, H. and Kuchler, K. (2006): Yeast ABC transporters-- a tale of sex, stress, drugs and aging, FEBS Lett 580 [4], pp. 1131-8. URL: **http://www.ncbi.nlm.nih.gov/pubmed/16406363**

[40] van Meer, G.; Halter, D.; Sprong, H.; Somerharju, P. and Egmond, M. R. (2006): ABC lipid transporters: extruders, flippases, or flopless activators?, FEBS Lett 580 [4], pp. 1171-7. URL: **http://www.ncbi.nlm.nih.gov/pubmed/16376334**

[41] Abreu, M. S.; Moreno, M. J. and Vaz, W. L. (2004): Kinetics and thermodynamics of association of a phospholipid derivative with lipid bilayers in liquid-disordered and liquid-ordered phases, Biophys J 87 [1], pp. 353-65. URL: **http://www.ncbi.nlm.nih.gov/pubmed/15240470**

[42] Oram, J. F. (2000): Tangier disease and ABCA1, Biochim Biophys Acta 1529 [1-3], pp. 321-30. URL: **http://www.ncbi.nlm.nih.gov/pubmed/11111099**

[43] Xu, X.; Bittman, R.; Duportail, G.; Heissler, D.; Vilcheze, C. and London, E. (2001): Effect of the structure of natural sterols and sphingolipids on the formation of ordered sphingolipid/sterol domains (rafts). Comparison of cholesterol to plant, fungal, and disease-associated sterols and comparison of sphingomyelin, cerebrosides, and ceramide, J Biol Chem 276 [36], pp. 33540-6. URL: **http://www.ncbi.nlm.nih.gov/pubmed/11432870**

[44] Sharom, F. J. (2008): ABC multidrug transporters: structure, function and role in chemoresistance, Pharmacogenomics 9 [1], pp. 105-27. URL: **http://www.ncbi.nlm.nih.gov/pubmed/18154452**

[45] Oloo, E. O.; Kandt, C.; O'Mara, M. L. and Tieleman, D. P. (2006): Computer simulations of ABC transporter components, Biochem Cell Biol 84 [6], pp. 900-11. URL: **http://www.ncbi.nlm.nih.gov/pubmed/17215877**

[46] Ge, L.; Wang, J.; Qi, W.; Miao, H. H.; Cao, J.; Qu, Y. X.; Li, B. L. and Song, B. L. (2008): The cholesterol absorption inhibitor ezetimibe acts by blocking the sterol-induced internalization of NPC1L1, Cell Metab 7 [6], pp. 508-19. URL: **http://www.ncbi.nlm.nih.gov/pubmed/18522832**

[47] Neufeld, E. B.; Stonik, J. A.; Demosky, S. J., Jr.; Knapper, C. L.; Combs, C. A.; Cooney, A.; Comly, M.; Dwyer, N.; Blanchette-Mackie, J.; Remaley, A. T.; Santamarina-Fojo, S. and Brewer, H. B., Jr. (2004): The ABCA1 transporter modulates late endocytic trafficking:

insights from the correction of the genetic defect in Tangier disease, J Biol Chem 279 [15], pp. 15571-8. URL: **http://www.ncbi.nlm.nih.gov/pubmed/14747463**

[48] Wang, J.; Sun, F.; Zhang, D. W.; Ma, Y.; Xu, F.; Belani, J. D.; Cohen, J. C.; Hobbs, H. H. and Xie, X. S. (2006): Sterol transfer by ABCG5 and ABCG8: in vitro assay and reconstitution, J Biol Chem 281 [38], pp. 27894-904. URL: **http://www.ncbi.nlm.nih.gov/pubmed/16867993**

[49] Wang, N.; Yvan-Charvet, L.; Lutjohann, D.; Mulder, M.; Vanmierlo, T.; Kim, T. W. and Tall, A. R. (2008): ATP-binding cassette transporters G1 and G4 mediate cholesterol and desmosterol efflux to HDL and regulate sterol accumulation in the brain, FASEB J 22 [4], pp. 1073-82. URL: **http://www.ncbi.nlm.nih.gov/pubmed/18039927**

[50] Janvilisri, T.; Venter, H.; Shahi, S.; Reuter, G.; Balakrishnan, L. and van Veen, H. W. (2003): Sterol transport by the human breast cancer resistance protein (ABCG2) expressed in Lactococcus lactis, J Biol Chem 278 [23], pp. 20645-51. URL: **http://www.ncbi.nlm.nih.gov/pubmed/12668685**

[51] Raab, A. M.; Gebhardt, G.; Bolotina, N.; Weuster-Botz, D. and Lang, C. (2010): Metabolic engineering of Saccharomyces cerevisiae for the biotechnological production of succinic acid, Metab Eng 12 [6], pp. 518-25. URL: **http://www.ncbi.nlm.nih.gov/pubmed/20854924**

[52] Arora, A.; Raghuraman, H. and Chattopadhyay, A. (2004): Influence of cholesterol and ergosterol on membrane dynamics: a fluorescence approach, Biochem Biophys Res Commun 318 [4], pp. 920-6. URL: **http://www.ncbi.nlm.nih.gov/pubmed/15147960**

[53] Parks, L. W. and Casey, W. M. (1995): Physiological implications of sterol biosynthesis in yeast, Annu Rev Microbiol 49, pp. 95-116. URL: **http://www.ncbi.nlm.nih.gov/pubmed/8561481**

[54] Rosenfeld, E. and Beauvoit, B. (2003): Role of the non-respiratory pathways in the utilization of molecular oxygen by Saccharomyces cerevisiae, Yeast 20 [13], pp. 1115-44. URL: **http://www.ncbi.nlm.nih.gov/pubmed/14558145**

[55] Abramova, N. E.; Cohen, B. D.; Sertil, O.; Kapoor, R.; Davies, K. J. and Lowry, C. V. (2001): Regulatory mechanisms controlling expression of the DAN/TIR mannoprotein genes during anaerobic remodeling of the cell wall in Saccharomyces cerevisiae, Genetics 157 [3], pp. 1169-77. URL: **http://www.ncbi.nlm.nih.gov/pubmed/11238402**

[56] Lesage, G. and Bussey, H. (2006): Cell wall assembly in Saccharomyces cerevisiae, Microbiol Mol Biol Rev 70 [2], pp. 317-43. URL: **http://www.ncbi.nlm.nih.gov/pubmed/16760306**

[57] Heino, S.; Lusa, S.; Somerharju, P.; Ehnholm, C.; Olkkonen, V. M. and Ikonen, E. (2000): Dissecting the role of the golgi complex and lipid rafts in biosynthetic transport of cholesterol to the cell surface, Proc Natl Acad Sci U S A 97 [15], pp. 8375-80. URL: **http://www.ncbi.nlm.nih.gov/pubmed/10890900**

[58] Fairn, G. D. and McMaster, C. R. (2008): Emerging roles of the oxysterol-binding protein family in metabolism, transport, and signaling, Cell Mol Life Sci 65 [2], pp. 228-36. URL: **http://www.ncbi.nlm.nih.gov/pubmed/17938859**

[59] Hapala, I. and Hunakova, A. (1996): Mechanism of sterol uptake in yeast, Folia Microbiol (Praha) 41 [1], pp. 95-6. URL: **http://www.ncbi.nlm.nih.gov/pubmed/9090837**

[60] Lishko, P. V.; Procko, E.; Jin, X.; Phelps, C. B. and Gaudet, R. (2007): The ankyrin repeats of TRPV1 bind multiple ligands and modulate channel sensitivity, Neuron 54 [6], pp. 905-18. URL: **http://www.ncbi.nlm.nih.gov/pubmed/17582331**

[61] Sullivan, D. P.; Georgiev, A. and Menon, A. K. (2009): Tritium suicide selection identifies proteins involved in the uptake and intracellular transport of sterols in Saccharomyces cerevisiae, Eukaryot Cell 8 [2], pp. 161-9. URL: **http://www.ncbi.nlm.nih.gov/pubmed/19060182**

[62] Souza, C. M. and Pichler, H. (2007): Lipid requirements for endocytosis in yeast, Biochim Biophys Acta 1771 [3], pp. 442-54. URL: **http://www.ncbi.nlm.nih.gov/pubmed/16997624**

[63] Holthuis, J. C.; Pomorski, T.; Raggers, R. J.; Sprong, H. and Van Meer, G. (2001): The organizing potential of sphingolipids in intracellular membrane transport, Physiol Rev 81 [4], pp. 1689-723. URL: **http://www.ncbi.nlm.nih.gov/pubmed/11581500**

[64] Epand, R. M. (2006): Cholesterol and the interaction of proteins with membrane domains, Prog Lipid Res 45 [4], pp. 279-94. URL: **http://www.ncbi.nlm.nih.gov/pubmed/16574236**

[65] Oldenburg, K. R.; Vo, K. T.; Michaelis, S. and Paddon, C. (1997): Recombination-mediated PCR-directed plasmid construction in vivo in yeast, Nucleic Acids Research 25 [2], pp. 451-2. URL: **http://www.ncbi.nlm.nih.gov/pubmed/9016579**

[66] Gueldener, U.; Heinisch, J.; Koehler, G. J.; Voss, D. and Hegemann, J. H. (2002): A second set of loxP marker cassettes for Cre-mediated multiple gene knockouts in budding yeast, Nucleic Acids Research 30 [6], pp. -. URL: <Go to ISI>://000174467900018

[67] Wittig, I.; Braun, H. P. and Schagger, H. (2006): Blue native PAGE, Nat Protoc 1 [1], pp. 418-28. URL: **http://www.ncbi.nlm.nih.gov/pubmed/17406264**

[68] Chen, X.; Arac, D.; Wang, T. M.; Gilpin, C. J.; Zimmerberg, J. and Rizo, J. (2006): SNARE-mediated lipid mixing depends on the physical state of the vesicles, Biophys J 90 [6], pp. 2062-74. URL: **http://www.ncbi.nlm.nih.gov/pubmed/16361343**

[69] Lundbaek, J. A.; Birn, P.; Hansen, A. J.; Sogaard, R.; Nielsen, C.; Girshman, J.; Bruno, M. J.; Tape, S. E.; Egebjerg, J.; Greathouse, D. V.; Mattice, G. L.; Koeppe, R. E., 2nd and Andersen, O. S. (2004): Regulation of sodium channel function by bilayer elasticity: the importance of hydrophobic coupling. Effects of Micelle-forming amphiphiles and cholesterol, J Gen Physiol 123 [5], pp. 599-621. URL: **http://www.ncbi.nlm.nih.gov/pubmed/15111647**

[70] Cheng, Y. and Prusoff, W. H. (1973): Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction, Biochem Pharmacol 22 [23], pp. 3099-108. URL: **http://www.ncbi.nlm.nih.gov/pubmed/4202581**

[71] Lopez-Marques, R. L.; Poulsen, L. R.; Hanisch, S.; Meffert, K.; Buch-Pedersen, M. J.; Jakobsen, M. K.; Pomorski, T. G. and Palmgren, M. G. (2010): Intracellular targeting signals and lipid specificity determinants of the ALA/ALIS P4-ATPase complex reside in the catalytic ALA alpha-subunit, Mol Biol Cell 21 [5], pp. 791-801. URL: **http://www.ncbi.nlm.nih.gov/pubmed/20053675**

[72] Kaiser, H. J.; Lingwood, D.; Levental, I.; Sampaio, J. L.; Kalvodova, L.; Rajendran, L. and Simons, K. (2009): Order of lipid phases in model and plasma membranes, Proc Natl Acad Sci U S A 106 [39], pp. 16645-50. URL: **http://www.ncbi.nlm.nih.gov/pubmed/19805351**

[73] Egner, R.; Mahe, Y.; Pandjaitan, R. and Kuchler, K. (1995): Endocytosis and vacuolar degradation of the plasma membrane-localized Pdr5 ATP-binding cassette multidrug transporter in Saccharomyces cerevisiae, Mol Cell Biol 15 [11], pp. 5879-87. URL: **http://www.ncbi.nlm.nih.gov/pubmed/7565740**

[74] Gao, M.; Cui, H. R.; Loe, D. W.; Grant, C. E.; Almquist, K. C.; Cole, S. P. and Deeley, R. G. (2000): Comparison of the functional characteristics of the nucleotide binding domains of multidrug resistance protein 1, J Biol Chem 275 [17], pp. 13098-108. URL: **http://www.ncbi.nlm.nih.gov/pubmed/10777615**

[75] Kuhnke, G.; Neumann, K.; Muhlenhoff, U. and Lill, R. (2006): Stimulation of the ATPase activity of the yeast mitochondrial ABC transporter Atm1p by thiol compounds, Mol Membr Biol 23 [2], pp. 173-84. URL: **http://www.ncbi.nlm.nih.gov/pubmed/16754360**

[76] Hofacker, M.; Gompf, S.; Zutz, A.; Presenti, C.; Haase, W.; van der Does, C.; Model, K. and Tampe, R. (2007): Structural and functional fingerprint of the mitochondrial ATP-binding cassette transporter Mdl1 from Saccharomyces cerevisiae, J Biol Chem 282 [6], pp. 3951-61. URL: **http://www.ncbi.nlm.nih.gov/pubmed/17150958**

[77] Villalba, J. M.; Palmgren, M. G.; Berberian, G. E.; Ferguson, C. and Serrano, R. (1992): Functional expression of plant plasma membrane H(+)-ATPase in yeast endoplasmic reticulum, J Biol Chem 267 [17], pp. 12341-9. URL: **http://www.ncbi.nlm.nih.gov/pubmed/1534807**

[78] Rahimi, Y.; Goulding, A.; Shrestha, S.; Mirpuri, S. and Deo, S. K. (2008): Mechanism of copper induced fluorescence quenching of red fluorescent protein, DsRed, Biochem Biophys Res Commun 370 [1], pp. 57-61. URL: **http://www.ncbi.nlm.nih.gov/pubmed/18348863**

[79] Al-Shawi, M. K.; Polar, M. K.; Omote, H. and Figler, R. A. (2003): Transition state analysis of the coupling of drug transport to ATP hydrolysis by P-glycoprotein, J Biol Chem 278 [52], pp. 52629-40. URL: **http://www.ncbi.nlm.nih.gov/pubmed/14551217**

[80] Tombline, G.; Bartholomew, L.; Gimi, K.; Tyndall, G. A. and Senior, A. E. (2004): Synergy between conserved ABC signature Ser residues in P-glycoprotein catalysis, J Biol Chem 279 [7], pp. 5363-73. URL: **http://www.ncbi.nlm.nih.gov/pubmed/14638679**

[81] Davidson, A. L. (2002): Mechanism of coupling of transport to hydrolysis in bacterial ATP-binding cassette transporters, J Bacteriol 184 [5], pp. 1225-33. URL: **http://www.ncbi.nlm.nih.gov/pubmed/11844750**

[82] Nyathi, Y.; De Marcos Lousa, C.; van Roermund, C. W.; Wanders, R. J.; Johnson, B.; Baldwin, S. A.; Theodoulou, F. L. and Baker, A. (2010): The Arabidopsis peroxisomal ABC transporter, comatose, complements the Saccharomyces cerevisiae pxa1 pxa2Delta mutant for metabolism of long-chain fatty acids and exhibits fatty acyl-CoA-stimulated ATPase activity, J Biol Chem 285 [39], pp. 29892-902. URL: **http://www.ncbi.nlm.nih.gov/pubmed/20659892**

[83] Gyurko, C.; Lendenmann, U.; Troxler, R. F. and Oppenheim, F. G. (2000): Candida albicans mutants deficient in respiration are resistant to the small cationic salivary antimicrobial peptide histatin 5, Antimicrob Agents Chemother 44 [2], pp. 348-54. URL: **http://www.ncbi.nlm.nih.gov/pubmed/10639361**

[84] Artigas, P. and Gadsby, D. C. (2006): Ouabain affinity determining residues lie close to the Na/K pump ion pathway, Proc Natl Acad Sci U S A 103 [33], pp. 12613-8. URL: **http://www.ncbi.nlm.nih.gov/pubmed/16894161**

[85] Cai, J.; Daoud, R.; Alqawi, O.; Georges, E.; Pelletier, J. and Gros, P. (2002): Nucleotide binding and nucleotide hydrolysis properties of the ABC transporter MRP6 (ABCC6), Biochemistry 41 [25], pp. 8058-67. URL: **http://www.ncbi.nlm.nih.gov/pubmed/12069597**

[86] Urbatsch, I. L.; Tyndall, G. A.; Tombline, G. and Senior, A. E. (2003): P-glycoprotein catalytic mechanism: studies of the ADP-vanadate inhibited state, J Biol Chem 278 [25], pp. 23171-9. URL: **http://www.ncbi.nlm.nih.gov/pubmed/12670938**

[87] Gustot, A.; Smriti; Ruysschaert, J. M.; McHaourab, H. and Govaerts, C. (2010): Lipid composition regulates the orientation of transmembrane helices in HorA, an ABC multidrug transporter, J Biol Chem 285 [19], pp. 14144-51. URL: **http://www.ncbi.nlm.nih.gov/pubmed/20223819**

[88] Kimura, Y.; Kioka, N.; Kato, H.; Matsuo, M. and Ueda, K. (2007): Modulation of drug-stimulated ATPase activity of human MDR1/P-glycoprotein by cholesterol, Biochem J 401 [2], pp. 597-605. URL: **http://www.ncbi.nlm.nih.gov/pubmed/17029589**

[89] Smriti; Krishnamurthy, S. S. and Prasad, R. (1999): Membrane fluidity affects functions of Cdr1p, a multidrug ABC transporter of Candida albicans, FEMS Microbiol Lett 173 [2], pp. 475-81. URL: **http://www.ncbi.nlm.nih.gov/pubmed/10227177**

[90] Harris, F. M.; Best, K. B. and Bell, J. D. (2002): Use of laurdan fluorescence intensity and polarization to distinguish between changes in membrane fluidity and phospholipid order, Biochim Biophys Acta 1565 [1], pp. 123-8. URL: **http://www.ncbi.nlm.nih.gov/pubmed/12225860**

[91] Christian, A. E.; Haynes, M. P.; Phillips, M. C. and Rothblat, G. H. (1997): Use of cyclodextrins for manipulating cellular cholesterol content, J Lipid Res 38 [11], pp. 2264-72. URL: **http://www.ncbi.nlm.nih.gov/pubmed/9392424**

[92] Yamashita, S. and Nikawa, J. (1997): Phosphatidylserine synthase from yeast, Biochim Biophys Acta 1348 [1-2], pp. 228-35. URL: **http://www.ncbi.nlm.nih.gov/pubmed/9370337**

[93] Choi, H. S. and Carman, G. M. (2007): Respiratory deficiency mediates the regulation of CHO1-encoded phosphatidylserine synthase by mRNA stability in Saccharomyces cerevisiae, J Biol Chem 282 [43], pp. 31217-27. URL: **http://www.ncbi.nlm.nih.gov/pubmed/17761681**

[94] Choi, H. S.; Han, G. S. and Carman, G. M. (2010): Phosphorylation of Yeast Phosphatidylserine Synthase by Protein Kinase A IDENTIFICATION OF SER46 AND SER47 AS MAJOR SITES OF PHOSPHORYLATION, Journal of Biological Chemistry 285 [15], pp. 11526-11536. URL: <Go to ISI>://000276286200059

[95] Snoek, I. S. I. and Steensma, H. Y. (2007): Factors involved in anaerobic growth of Saccharomyces cerevisiae, Yeast 24 [1], pp. 1-10. URL: <Go to ISI>://000243918900001

[96] Walther, T. C.; Brickner, J. H.; Aguilar, P. S.; Bernales, S.; Pantoja, C. and Walter, P. (2006): Eisosomes mark static sites of endocytosis, Nature 439 [7079], pp. 998-1003. URL: **http://www.ncbi.nlm.nih.gov/pubmed/16496001**

[97] Ferreira, T.; Regnacq, M.; Alimardani, P.; Moreau-Vauzelle, C. and Berges, T. (2004): Lipid dynamics in yeast under haem-induced unsaturated fatty acid and/or sterol depletion, Biochem J 378 [Pt 3], pp. 899-908. URL: **http://www.ncbi.nlm.nih.gov/pubmed/14640980**

[98] Mukherjee, S.; Zha, X.; Tabas, I. and Maxfield, F. R. (1998): Cholesterol distribution in living cells: fluorescence imaging using dehydroergosterol as a fluorescent cholesterol analog, Biophys J 75 [4], pp. 1915-25. URL: **http://www.ncbi.nlm.nih.gov/pubmed/9746532**

[99] Reiner, S.; Micolod, D.; Zellnig, G. and Schneiter, R. (2006): A genomewide screen reveals a role of mitochondria in anaerobic uptake of sterols in yeast, Mol Biol Cell 17 [1], pp. 90-103. URL: **http://www.ncbi.nlm.nih.gov/pubmed/16251356**

[100] Reiner, S.; Micolod, D. and Schneiter, R. (2005): Saccharomyces cerevisiae, a model to study sterol uptake and transport in eukaryotes, Biochem Soc Trans 33 [Pt 5], pp. 1186-8. URL: **http://www.ncbi.nlm.nih.gov/pubmed/16246078**

[101] Tate, C. G. (2001): Overexpression of mammalian integral membrane proteins for structural studies, FEBS Lett 504 [3], pp. 94-8. URL: **http://www.ncbi.nlm.nih.gov/pubmed/11532439**

[102] Rigaud, J. L.; Levy, D.; Mosser, G. and Lambert, O. (1998): Detergent removal by non-polar polystyrene beads - Applications to membrane protein reconstitution and two-dimensional crystallization, European Biophysics Journal with Biophysics Letters 27 [4], pp. 305-319. URL: <Go to ISI>://000074651400001

[103] Levy, D.; Bluzat, A.; Seigneuret, M. and Rigaud, J. L. (1990): A systematic study of liposome and proteoliposome reconstitution involving Bio-Bead-mediated Triton X-100 removal, Biochim Biophys Acta 1025 [2], pp. 179-90. URL: **http://www.ncbi.nlm.nih.gov/pubmed/2364077**

[104] Rosenberg, M. F.; Bikadi, Z.; Chan, J.; Liu, X.; Ni, Z.; Cai, X.; Ford, R. C. and Mao, Q. (2010): The human breast cancer resistance protein (BCRP/ABCG2) shows conformational changes with mitoxantrone, Structure 18 [4], pp. 482-93. URL: **http://www.ncbi.nlm.nih.gov/pubmed/20399185**

[105] Sunshine, C. and McNamee, M. G. (1994): Lipid modulation of nicotinic acetylcholine receptor function: the role of membrane lipid composition and fluidity, Biochim Biophys Acta 1191 [1], pp. 59-64. URL: **http://www.ncbi.nlm.nih.gov/pubmed/7512384**

[106] Rankin, S. E.; Addona, G. H.; Kloczewiak, M. A.; Bugge, B. and Miller, K. W. (1997): The cholesterol dependence of activation and fast desensitization of the nicotinic acetylcholine receptor, Biophys J 73 [5], pp. 2446-55. URL: **http://www.ncbi.nlm.nih.gov/pubmed/9370438**

[107] Valiyaveetil, F. I.; Zhou, Y. and MacKinnon, R. (2002): Lipids in the structure, folding, and function of the KcsA K+ channel, Biochemistry 41 [35], pp. 10771-7. URL: **http://www.ncbi.nlm.nih.gov/pubmed/12196015**

[108] Heginbotham, L.; Kolmakova-Partensky, L. and Miller, C. (1998): Functional reconstitution of a prokaryotic K+ channel, J Gen Physiol 111 [6], pp. 741-9. URL: **http://www.ncbi.nlm.nih.gov/pubmed/9607934**

[109] Lee, A. G. (1977): Annular Events - Lipid-Protein Interactions, Trends in Biochemical Sciences 2 [10], pp. 231-233. URL: <Go to ISI>://A1977DW95800020

[110] Marsh, D. and Horvath, L. I. (1998): Structure, dynamics and composition of the lipid-protein interface. Perspectives from spin-labelling, Biochim Biophys Acta 1376 [3], pp. 267-96. URL: **http://www.ncbi.nlm.nih.gov/pubmed/9804973**

[111] Lee, A. G. (2003): Lipid-protein interactions in biological membranes: a structural perspective, Biochim Biophys Acta 1612 [1], pp. 1-40. URL: **http://www.ncbi.nlm.nih.gov/pubmed/12729927**

[112] de Foresta, B.; le Maire, M.; Orlowski, S.; Champeil, P.; Lund, S.; Moller, J. V.; Michelangeli, F. and Lee, A. G. (1989): Membrane solubilization by detergent: use of brominated phospholipids to evaluate the detergent-induced changes in Ca2+-ATPase/lipid interaction, Biochemistry 28 [6], pp. 2558-67. URL: **http://www.ncbi.nlm.nih.gov/pubmed/2525049**

[113] Froud, R. J.; East, J. M.; Rooney, E. K. and Lee, A. G. (1986): Binding of Long-Chain Alkyl Derivatives to Lipid Bilayers and to (Ca-2+-Mg-2+)-Atpase, Biochemistry 25 [23], pp. 7535-7544. URL: <Go to ISI>://A1986F008500042

[114] Hauser, H. and Shipley, G. G. (1984): Interactions of divalent cations with phosphatidylserine bilayer membranes, Biochemistry 23 [1], pp. 34-41. URL: **http://www.ncbi.nlm.nih.gov/pubmed/6691965**

[115] Dluhy, R. A.; Mendelsohn, R.; Casal, H. L. and Mantsch, H. H. (1983): Interaction of dipalmitoylphosphatidylcholine and dimyristoylphosphatidylcholine-d54 mixtures with glycophorin. A fourier transform infrared investigation, Biochemistry 22 [5], pp. 1170-7. URL: **http://www.ncbi.nlm.nih.gov/pubmed/6687692**

[116] Florine, K. I. and Feigenson, G. W. (1987): Influence of the calcium-induced gel phase on the behavior of small molecules in phosphatidylserine and phosphatidylserine-phosphatidylcholine multilamellar vesicles, Biochemistry 26 [6], pp. 1757-68. URL: **http://www.ncbi.nlm.nih.gov/pubmed/3036210**

[117] Dibble, A. R. G. and Feigenson, G. W. (1992): Nonideal Mixing of Gramicidin-a' and Phosphatidylserine in Fluid Phase Bilayers, Faseb Journal 6 [1], pp. A85-A85. URL: <Go to ISI>://A1992GY44000489

ACKNOWLEDGEMENTS

I would like to thank many people for their contribution to this work.

First of all I would like to thank Prof. Thomas Günther-Pomorski for encouragement and motivation as well as discussions and practical help.

I also want to thank Sigrid Milles for a lot of work on the project, amazing scientific ideas and friendship.

I would like to express my gratitude to Dr. Peter Müller and Prof. Andreas Herrmann for their support and help.

I want to thank our collaborators – Prof. David Daleke and Dr. Dittmar for their input to the project.

Many thanks to all wonderfull members of AG Molecular Biophysics for creating great atmosphere in the group!

Special thanks to Marta for all the discussions during my PhD time and to Michal for sharing knowledge about liposomes and reconstitution.

Olbrzymie podziekowania dla moich kochanych rodzicow i brata za wsparcie i pomoc.

PUBLICATIONS

Talk

"Characterization of yeast ABC transporters involved in sterol transport", Annual Meeting of the RTN Network "Flippases", Budapest, Hungary (07-09.02.2008)

Posters

Marek, M., Pomorski, T.G., Müller, P., Herrmann, A. Purification of yeast ABC transporters Pdr11p and Aus1p involved in sterol transport", FEBS Special Meeting "ATP Binding Cassette (ABC) Proteins: From Genetic Disease to Multidrug Resistance", Innsbruck, Österreich (01-08.03.2008)

Marek, M., Milles, S., Pomorski, T.G., Müller, P., Herrmann, A. "Characterization of yeast ABC transporters involved in sterol transport", Final Meeting of the RTN Network "Flippases", Ascona, Switzerland (02-06.11.2008)

Marek, M., Milles, S., Pomorski, T.G., Müller P., Herrmann, A. "Purification and reconstitution of Aus1p a putative yeast sterol transporter", FEBS Special Meeting "ATP Binding Cassette (ABC) Proteins: From Genetic Disease to Multidrug Resistance" Innsbruck, Österreich (01-05.03.2011)

Manuscripts

Marek, M., Milles, S., Schreiber, G., Daleke, D.L., Dittmar, G., Herrmann, A., Müller, P., Pomorski, TG. (2011) "The Yeast Plasma Membrane ATP Binding Cassette (ABC) Transporter Aus1: PURIFICATION, CHARACTERIZATION, AND THE EFFECT OF LIPIDS ON ITS ACTIVITY" J Biol Chem. 2011 Jun 17; 286 (24):21835-43

Eidesstattliche Erklärung

Hiermit erkläre ich, die vorliegende Arbeit selbständig ohne fremde Hilfe verfasst und nur die angegebene Literatur verwendet zu haben.

Ich besitze keinen entsprechenden Doktorgrad und habe mich anderwärts nicht um einen solchen beworben.

Die dem Promotionsverfahren zugrunde liegende Promotionsordnung ist mir bekannt.

Magdalena Marek