In vitro characterization of the human ABC transporter MDR3/ABCB4

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"Jeder Tag, an dem du nicht lächelst, ist ein verlorener Tag."

– Charlie Chaplin

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Abstract

The canalicular membrane of hepatocytes of the liver is an example of transverse lipid asymmetry in a biological membrane that can respond to environmental changes. Here, an increased phosphatidylcholine (PC)-lipid content in the outer leaflet protects the membrane from the harsh detergent activity of bile salts. The ABC transporter ABCB4 is responsible for PC-lipid accumulation in the outer leaflet through the transverse flop of cytosolic PC-lipids. It has been shown that an ABCB4 deficiency can even lead to several clinically relevant diseases. A superordinate symptom is the reduction or complete absence of PC-lipids in bile. Without PC-lipids, cholesterol and bile salts are no longer soluble, which can lead to the formation of gallstones and cholestasis. However, it is still unclear, which property of ABCB4 provides this protection against harsh bile salts, what attributes a substrate must have in order to interact with ABCB4, and how ABCB4 functions mechanistically. Two key discoveries were made in this thesis.

First, ABCB4 ensures an asymmetric membrane with a 3 to 1 excess of PC in the outer leaflet of the canalicular membrane. However, this PC fraction includes several PC-lipid variants, more than just those that end up in bile. Thus, the membrane remains intact even after some PC-lipids have been solubilized by bile salts. A substrate of ABCB4 must have four properties to interact productively with ABCB4. A phosphatidylcholine head group must be bound to a glycerol core, on which two fatty acids are esterified, with a minimum length of 12 carbon units. This is needed to provide sufficient amphipathic properties. 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), an 18-C long PC-lipid with one monounsaturated fatty acid each, proved to be ideal.

Secondly, there seems to be a secondary property of ABCB4 that has already caused controversy in the literature. This secondary property refers to the high degree of sequence identity between the prototypical multidrug exporter ABCB1 and the PC-floppase ABCB4. Some substrates and inhibitors of ABCB1 such as vinblastine or digitoxin also seem to interact with ABCB4, which could lead to a common principle of interaction between ABCB1 and ABCB4. Differences between these two proteins accumulate in transmembrane helix 1 (TMH1). Here, hydrophobic amino acids from ABCB1 are changed to hydrophilic amino acids in ABCB4. Three specific mutations (Q52L, S58V, S69A) led to a breakdown of the stimulation of ATPase activity by PC-lipids in a mutant ABCB4 protein, in which all three amino acids of ABCB1 were inserted. Remarkably, the characteristics of modulators were not changed with respect to ATPase activity of ABCB4. This indicates that the protein is still intact, but TMH1 can no longer interact with PC-lipids. A transport assay confirmed that PC-lipids labelled with a fluorophore were no longer transported by this ABCB4 mutant. Further insights into a possible transport mechanism of ABCB4 were provided by molecular dynamics simulations. A transport principle seemed to be favoured, in which the hydrophobic tail of a PC-lipid does not have to penetrate the protein's interior. It was sufficient that the hydrophilic head group of a PC-lipid was shielded by the TMH1 of ABCB4, while the hydrophobic tail is still embedded in the membrane. As a result, the PC-lipid only

migrates along the membrane-facing side of the protein without any thermodynamic disadvantage that would have to be paid to force an amphipathic molecule into a hydrophilic binding pocket.

Zusammenfassung

Die kanalikuläre Membran der Hepatozyten der Leber stellt ein Beispiel für eine transversale Lipidasymmetry in einer biologischen Membran, die auf Umweltreize reagieren kann, dar. Hier wird durch einen erhöhten Phosphatidylcholin (PC)-Lipid Anteil im äußeren Blatt die Membran vor der harschen Detergenz-Aktivität von Gallensalzen geschützt. Dabei sorgt der ABC-Transporter ABCB4 für die PC-Lipid Anreicherung im äußeren Blatt durch den transversalen *flopp* zytosolischer PC-Lipide. Es wurde gezeigt, dass eine ABCB4-Defizienz sogar zu mehreren klinisch relevanten Krankheitsbildern führen kann. Ein übergeordnetes Symptom ist die Verringerung oder das komplette Nichtvorhandensein von PC-Lipiden in der Galle. Ohne PC-Lipide liegen Cholesterin und Gallensalze nicht mehr gelöst vor, wodurch sich Gallensteine formieren und Cholestasen entstehen können. Ungeklärt ist aber noch, welche Eigenschaft von ABCB4 diesen Schutz vor harschen Gallensalzen bietet, welche Attribute ein Substrat aufweisen muss, um mit ABCB4 zu interagieren und wie ABCB4 mechanistisch funktioniert. Dazu konnten in dieser Arbeit zwei Schlüsselentdeckungen gemacht werden.

Erstens sorgt ABCB4 für eine asymmetrische Membran mit einem 3 zu 1 Überschuss an PC im äußeren Blatt der kanalikulären Membran. Dieser PC-Anteil umfasst aber diverse PC-Lipidvarianten, mehr als nur jene, die in die Galle sekretiert werden. Dadurch bleibt die Membran auch noch intakt, sobald einige PC-Lipide durch Gallensalze solubilisiert wurden. Ein Substrat von ABCB4 muss dabei vier Eigenschaften aufweisen, um produktiv mit ABCB4 zu interagieren. Eine Phosphatidylcholin Kopfgruppe muss an ein Glyceringerüst gebunden sein, an dem wiederrum zwei Fettsäuren verestert sind, die eine Mindestlänge von 12 Kohlenstoffen besitzen und dadurch für eine ausreichend amphipathische Eigenschaft sorgen. Als ideal erwies sich 1,2-Dioleoyl-sn-glycero-3-phosphocholin (DOPC) ein 18-C langes PC-Lipid mit je einer einfach ungesättigten Fettsäure.

Zweitens scheint eine sekundäre Eigenschaft in ABCB4 zu existieren, die in der Literatur bereits für Kontroversen gesorgt hat. Diese sekundäre Eigenschaft bezieht sich auf den hohen Verwandtschaftsgrad zwischen dem prototypischen Multidrogen Exporter ABCB1 und der PC-Floppase ABCB4. Einige Substrate und Inhibitoren des ABCB1 wie Vinblastin oder Digitoxin scheinen auch mit ABCB4 zu interagieren, wodurch ein geteiltes Interaktions-Grundprinzip zwischen ABCB1 und ABCB4 zugrunde liegen könnte. Unterschiede zwischen diesen beiden Proteinen häufen sich unter anderem in der Transmembrane Helix 1 (TMH1). Hier sind hydrophobe Aminosäuren aus ABCB1 durch hydrophile Aminosäuren in ABCB4 ersetzt. Drei konkrete Mutationen (Q52L, S58V, S69A) führten in einem mutierten ABCB4 Protein, in dem alle drei hydrophoben Aminosäuren Varianten des ABCB1 eingefügt wurden, zu einem Verlust der Stimulierbarkeit der ATPase Aktivität durch PC-Lipide. Bemerkenswert war, dass die Charakteristika der ABCB1 Modulatoren in ihrer Wirkung auf die ATPase Aktivität von ABCB4 nicht verändert wurden. Dies spricht dafür, dass das Protein zwar noch intakt ist, TMH1 aber nicht mehr mit den PC-Lipiden interagieren kann. Dies wurde durch einen Transportassay bestätigt, in dem PC-Lipide, die mit einem Fluorophor markiert waren, durch die

ABCB4 Mutante nicht mehr transportiert wurden. Weitere Einblicke in einen möglichen Transportmechanismus von ABCB4 lieferten molekular dynamische Simulationen. Hier erschien ein Transportprinzip naheliegend, bei dem die hydrophobe Acylkette eines PC-Lipids nicht in den Proteinkern eindringen muss. Es ist ausreichend, dass die hydrophile Kopfgruppe, durch TMH1 von ABCB4 abgeschirmt wird, während die hydrophobe Acylkette in der Membran eingebettet bleibt. Dies führte dazu, dass das PC-Lipid lediglich an der membranzugewandten Seite des Proteins entlangwandert, ohne dass ein thermodynamischer Nachteil entsteht, der „bezahlt“ werden müsste, um ein amphipathisches Molekül über eine hydrophile Bindetasche zu transportieren.

1 Introduction

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Structure and Function of Hepatobiliary ATP Binding Cassette Transporters

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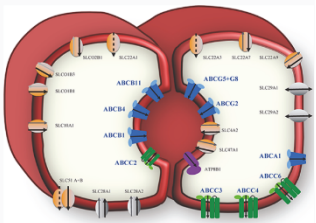
ABSTRACT: The liver is beyond any doubt the most important metabolic organ of the human body. This function requires an intensive crosstalk within liver cellular structures, but also with other organs. Membrane transport proteins are therefore of upmost importance as they represent the sensors and mediators that shuttle signals from outside to the inside of liver cells and/or vice versa. In this review, we summarize the known literature of liver transport proteins with a clear emphasis on functional and structural information on ATP binding cassette (ABC) transporters, which are expressed in the human liver. These primary active membrane transporters form one of the largest families of membrane proteins. In the liver, they play an essential role in for example bile formation or xenobiotic export. Our review provides a state of the art and comprehensive summary of the current knowledge of hepatobiliary ABC transporters. Clearly, our knowledge has improved with a breath-taking speed over the last few years and will expand further. Thus, this review will provide the status quo and will lay the foundation for new and exciting avenues in liver membrane transporter research.



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1. GENERAL INTRODUCTION

The biology and pathobiology of the liver are of central importance for human health as it is the major metabolizing and detoxifying organ of the human body. It is mainly composed of hepatocytes or parenchymal cells, which make up approximately 80% of the volume of the liver. Cholangiocytes, liver sinusoidal (LSEC), as well as macrovascular (MaVEC) endothelial cells, hepatic stellate cells (HSC), and Kupffer cells (KC), and immune cells form the remaining 20%.[1]

The liver represents a central metabolic hub for the regulation of glucose and lipid metabolism as well as protein synthesis. This metabolic function is performed specifically by hepatocytes, which are also responsible for the *de novo* synthesis of bile acids from cholesterol and their enterohepatic circulation, while metabolizing enzymes are mainly concentrated in the liver and intestine.[2]

Because of its physiological importance, hepatotoxicity or drug induced liver injury (DILI) is likely the most important factor in failure of drug development.[3] On top of this, transporters mainly of the ATP binding cassette (ABC) transporter[4] and solute carrier (SLC)[5] families play pivotal roles in absorption, distribution, targeting, or elimination. This obviously influences drug pharmacokinetics and -dynamics. For example, the liver mediates mainly detoxification reactions mediated by phase one (oxidation, reduction, hydrolyzation) and phase two biotransformations (conjugation with different chemical groups for higher solubility such as glutathione conjugation), and the final products of these reactions are often found as glucuronidated or sulfated conjugates, which are the substrates of efflux processes (phase three) mediated by ABC transporters of the C subfamily[6] (see section The ABCC Subfamily of the Liver - Drug and Toxin Efflux). This highlights how these processes interplay within the liver. Therefore, it should not come as a surprise that the international transport consortium (ITC)[5] has included hepatobiliary membrane transport proteins in drug screening studies. The ITC includes members of academia, industry, and the Food and Drug Administration (FDA). These transporters are of emerging clinic importance with respect to drug uptake and/or clearance. In 2013,[5] MATE (multidrug and toxin extrusion, SLC47A), the ABCC subfamily, more precisely ABCC2, ABCC3, ABCC4, and ABCB11 (bile acid export pump, BSEP), were added to the already existing list that was composed of the ABC transporters ABCB1 (or multidrug resistance protein 1 (MDR1)), ABCG2 (or breast cancer resistance protein, BCRP), and the SLCs (the organic anion transporter peptides OATP1B1 and OATP1B3, SLCO1B1, and SLCO1B3, respectively), OCT2 (organic cation transporter 2, SLC22A2), as well as OAT1 and OAT3 (organic anion transporter, SLC22A6 and SLC22A7, respectively). Furthermore, it is not only about the kinetics of drug−drug interactions that might be modulated by these transporters, it is also about the dynamics of these interactions, i.e., the interplay between drug concentration and biological effect. This emphasizes the importance of hepatobiliary membrane transporters in the physiology and pathophysiology of the human liver. Here, we will focus only on structural and functional aspects of hepatobiliary ABC transporters and try to summarize our current knowledge of the mode of action and how these primary active transporters coexist and function in the membrane.

1.1. Transport across the Plasma Membrane of the Liver

Over the last 25 years, cloning, transfecting cell lines, and functional characterization of hepatobiliary transporters has moved the field from a model of passive diffusion (import as well as export) of all liver relevant compounds across the plasma membrane to a rather detailed knowledge about the molecular players, the substrate spectrum, and mechanistic aspects of how liver cells communicate with their environment, a process mediated by active transport proteins. The importance of the liver is also reflected by the number of these membrane transport systems that are present in both the sinusoidal (basolateral) and the canalicular (apical) membranes of hepatocytes (Table 1 and Figure 1). Membrane import systems are enriched in the sinusoidal membrane, while the major drug efflux pumps and the transporters exporting the components of bile are localized in the canalicular membrane. Another way to classify these transport systems are the families of membrane transport proteins, to which they belong. These are either ABC transporters or SLCs and one member of the P-type ATPase family, ATP8B1.

Members of the first family are enriched in the canalicular membrane, while SLCs are predominately found in the basolateral membrane (Figure 1). Since hepatobiliary ABC transporters are the focus of this review, a detailed discussion of these transport systems will be given in the section An ABC of the Plasma Membrane of the Human Liver. The family of human SLCs covers more than 65 subfamilies with more than 450 different transporters. These include passive transporters (uniporter), ion-coupled transporters (symporters), and exchangers (antiporters). The liver is not only essential for metabolism, detoxification, and *de novo* bile acid synthesis, but also important with respect to enterohepatic circulation.[8] Here, bile acids are imported, recycled, and reused from the bloodstream into the hepatocyte predominantly by NTCP (sodium taurocholate cotransporting polypeptide, SLC10A1), a 56 kDa cotransporter, cloned in 1991,[9] which imports taurocholate (TC) at a stoichiometry of one TC: two Na^+. Nowadays, we know that not only TC, but all bile acids, steroid, and thyroid hormones as well as to a certain extend xenobiotics are substrates of NTCP.[10,11] NTCP also represents the entry point for the hepatitis B and D viruses[12] making it an interesting target for drug development. Subsequently, three OATPs (OATP1B1, OATP1B3, and OATP2B1) were cloned and functionally characterized.[13−17] Although they are also capable of functioning as bile acid import proteins, their substrate spectra are much broader and include a large variety of organic anions.[18] Important, however, is that all these compounds are transported together with bicarbonate.[19]

Pharmacokinetics, i.e., the relationship between drug concentration and resident time in the body, are strongly coupled to the capability of the liver to extracts drugs. This is mainly accomplished by SLCs of the OATP (SLCO, former SLC21), OAT (SLC22), and OCT (SLC22) subfamilies. OCTs are electrogenic uniporters that transport organic

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Table 1. Summary of Hepatobiliary Membrane Transport Proteins in Plasma Membrane of Humans, for Which the Presence on the Protein Level Was Verified According Referenced Papers or if Written in Italics to Human Protein Atlas (http://www.proteinatlas.org) or the UniProt Database (https://www.uniprot.org)[a]

member	substrate(s)	cell type	subcellular localization	function	PDB entry	disease	references
				ABC transporters			
ABCA1	phospholipids and cholesterol	hepatocytes	basolateral membrane	transport of phospholipids and excess of free cholesterol and HDL formation	5XJY	Tangier disease	92, 118
ABCB1	drugs	hepatocytes	canalicular (apical) membrane	decrease of intracellular drug levels	4M1M (Apo), 6QEE (inhibitor bound), 6C0V (ATP bound), 4Q9H, 5KO2, 5KPD (EQ mutant)	overexpression tumors, Alzheimer's disease	220, 234, 235, 520−523
ABCB4	phosphatidylcholine lipids	hepatocytes	canalicular (apical) membrane	protection of the biliary tree	6S7P (ATP bound)	mild chronical cholestasis, ICP, DILI, LPAC, PFIC-3	149, 240, 265, 270, 524−529
ABCB11	bile salts	hepatocytes	canalicular (apical) membrane	secretion of conjugated bile salts into the bile	6LR0	DILI, ICP, BRIC-2, PFIC-2, BSEP V444A linked to PBC, chronic hepatitis C, cholangiocarcinoma and sepsis-associated cholestasis	271, 328, 436−438, 451, 452, 452, 494, 525, 530
ABCC2	glucuronidated metabolites	hepatocytes	canalicular (apical) membrane	metabolite and toxin export		Dubin and Johnson disease, DILI	124, 172, 189
ABCC3	drugs and conjugated metabolites	hepatocytes	basolateral membrane	back-up system in liver cholestasis, export of metabolites and toxins		overexpression tumors	113, 193
ABCC4	drugs and conjugated metabolites	hepatocytes	basolateral membrane	back-up system in liver cholestasis, export of metabolites and toxins		overexpression in tumors	198, 199, 202
ABCC6	ATP	hepatocytes	basolateral membrane	unknown		*pseudoxanthoma elasticum* (PXE)	7, 128, 130
ABCC7[b]	chloride	cholangiocytes	apical membrane	electrolyte (Cl^-) transport in epithelial cells	5UAK (ATP-free), 6MSM (ATP-bound)	cystic fibrosis	86, 87, 90
ABCG2	drugs, cholesterol, urate, porphyrins and sulfated metabolites	hepatocytes	canalicular (apical) membrane	decrease of intracellular drug levels, urate excretion	6VXI (bound tomitoxantrone), 6VXJ (bound to SN38), 6VXH (bound to imatinib), 6HIJ (bound to cholesterol), 6HZM (bound to ATP Mg^{2+})	gout	364−368, 379,531, 532
ABCG5/G8	cholesterol, plant sterolins	hepatocytes	mainly canalicular membrane of	transport of cholesterol and plant sterols like sitosterol	5DO7	sitosterolemia, hypercholesterolemia, gallstone disease	389, 407, 498, 500, 509, 533−535
				P-type ATPases			
ATP8B1	phosphatidyl-serine lipids	hepatocytes and cholangiocytes	canalicular (apical) membrane	flip of PS lipids		PFIC1 (Byler's disease)	35, 36
				Solute Carrier Transporters (SLCs)			
SLC4A2 (AE2)	chloride vs bicarbonate	hepatocytes/*bile duct dells*	*plasma membrane*	secretion of bicarbonate in exchange for chloride ions			536
SLC22A7 (OAT2)	organic anions for unknown exchange	hepatocytes	basolateral membrane	import of organic anions in exchange for unknown ions			22, 537, 538
SLC22A9 (OAT7)	organic anions in exchange for short chain fatty acids	hepatocytes	basolateral membrane	organic anions in exchange for short chain fatty acids			20, 22

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Table 1. continued

member	substrate(s)	cell type	subcellular localization	function	PDB entry	disease	references
				Solute Carrier Transporters (SLCs)			
SLC47A1 (MATE1)	tetraethylammonium (TEA), 1-methyl-4-phenylpyridinium (MPP), cimetidine, metformin, guanidine, procainamide, cephalexin, cephradine	hepatocytes	*cell membrane*	The stated substrates are transported in exchange for protons.			539−541
SLC22A1 (OCT1)	organic cations	hepatocytes	basolateral membrane				22, 542
SLC22A3 (OCT3)	organic cations	hepatocytes, *bile duct cells*	*plasma membrane*				22
SLC51A (OSTα)	bile acids	hepatocytes	basolateral membrane, *ER membrane*			progressive cholestasis	24
SLC51B (OSTβ)	conjugated steroids	hepatocytes	basolateral membrane,*ER membrane*			progressive cholestasis	24
SLC10A1 (NTCP)	sodium and bile acids	hepatocytes	*basolateral membrane*	symport of sodium and bile acids; mainly for uptake into hepatocytes		hypercholanemia	543, 544
SLC10A2 (ASBT)	sodium dependent bile acid transport	(large) cholangiocytes	apical membrane	uptake of bile acids		Crohn's disease	545, 546
SLCO1B1 (OATP1B1)	bicarbonate, bile acids and organic anions	hepatocytes	basolateral membrane	Antiporter: import of bile acids and organic anions; export of bicarbonate		Involved in Rotor syndrome	547
SLCO1B3 (OATP1B3)	bicarbonate, bile acids and organic anions	hepatocytes	basolateral membrane	Antiporter: import of bile acids and organic anions; export of bicarbonate		Involved in Rotor syndrome	547
SLCO2B1 (OATP2B1)	bicarbonate, bile acids, organic anions and cations	hepatocytes	basolateral membrane	Antiporter: import of bile acids, organic anions and cations; export of bicarbonate		Involved in Rotor syndrome	547
SLC28A1 (CNT1)	concentrative 1:1 sodium/nucleoside	hepatocytes	apical and basolateral membrane	sodium and nucleoside import			548, 549
SLC28A2 (CNT2)	concentrative 1:1 sodium/nucleoside	hepatocytes	apical and basolateral membrane	sodium and nucleoside import			548, 550
SLC29A1 (ENT1)	nucleosides and nucleobases	hepatocytes	apical and basolateral membrane	facilitated transport, maintain equilibrium of nucleosides and nucleobases			548
SLC29A2 (ENT2)	nucleosides and nucleobases	hepatocytes	apical and basolateral membrane	facilitated transport, maintain equilibrium of nucleosides and nucleobases			548, 550

[a]SLC: Solute carrier transporters, OATP: organic anion transport peptides, OAT: organic anion transporters, OCT: organic cation transporters, NTCP: sodium taurocholate co-transporting polypeptide, MATE: multidrug and toxic compound extrusion. [b]ABCC7 or CFTR is included even though it is not an ABC transporter. ABCC1 is expressed in fetal liver, and expression is typically absent in adult liver, but often expressed again in hepatocellular carcinomas; therefore, ABCC1 was not included in the table. Please note that we have not included three dimensional structures of homologous proteins. As outlined in the section Concluding Remarks, these homologous structures might be suitable as templates in homology modelling to provide insights into the overall shape and conformation, but likely

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cations along their concentration gradient, a so-called facilitated diffusion. OATs, on the other hand, transport organic anions in a sodium ion independent manner. However, to reach the intracellular concentrations, active transporters are required. So far, sulfo-, but not glucuronide- or glutathione-conjugated anions have been identified as substrates of OAT7,[20] while for example prostaglandins or nonsteroidal drugs as well as creatine and cGMP are substrates of OAT2.[21] OCT1 and OCT3 are sodium ion independent, electrogenic facilitators (uniporters) that transport a large variety of structurally unrelated organic cations.[22,23] Finally, the heterodimeric organic solute transporter $\alpha-\beta$ (OST α/β, SLC51) catalyzes facilitated diffusion of bile acids and conjugated steroids across the apical membrane of hepatocytes. This process strictly depends on the gradient of these molecules.[24] Heterodimerization of SLC51 is mainly required for proper trafficking and stability. Interestingly, data bank searches identified so far no paralogues in other organisms. The three ABC transporters, ABCC3, ABCC4, and ABCC6, of the basolateral membrane will be discussed in more detail in sections ABCC3 (MRP3) − The Back-up System for ABCC2 in Liver Cholestasis, ABCC4 (MRP4) − The Little Brother of ABCC1 in the Liver, and ABCC6 − The ABC Transporter that Transports ATP, respectively.

Moving to the canalicular (apical) membrane of hepatocytes, two additional members of the SLC family are present, AE2 (anion exchanger isoform 2, SLC4A2) and MATE1 (SLC47A1). Isoform 1 of AE is better known as the band 3 protein found in erythrocytes. AE1 and AE2 both exchange chloride ions against bicarbonate ions. In cholangiocytes, AE2 participates in the so-called "biliary bicarbonate umbrella", a mechanism that protects these cells against damage by bile acids.[25] MATE1 like the bacterial prototype NorM[26,27] is a drug/H^+ antiporter that extrudes drugs and protects in concert with ABCB1 and ABCG2 (see sections The Multidrug Efflux Pump ABCB1 and ABCG2 - Brother in Arms of ABCB1) the canalicular membrane against a myriad of structurally unrelated xenobiotics. The last, non-ABC transmembrane protein of the canalicular membrane is ATP8B1 or FIC1. It belongs to the subfamily 4 of P-type ATPase[28,29] and flips lipids of the phosphatidylserine (PS) family from the outer to the inner leaflet of the membrane.[30,31] In *S. cerevisiae*, the functional homologue is Drs2 and Drs2-related ATPases,[32,33] well-studied lipid flippases.[34] Similar to Drs2, ATP8B1 plays a pivotal role in the initiation and maintenance of the transversal lipid asymmetry of the plasma membrane and mutations in ATP8B1 lead to PFIC1 (progressive familial intrahepatic cholestasis type I).[35,36] Likely, dysfunction of ATP8B1 results in a loss of membrane asymmetry and thereby loss of protection against the detergent action of bile acids.[37]

Cloning and functional analysis of hepatobiliary transporters are and were extremely important and represent powerful tools that increased our molecular knowledge about these systems exponentially. In the case of functional studies, transporters were either heterologously overexpressed or cell lines, which were transiently or stably infected with the respective transporter, were used in combination with various biochemical or biophysical approaches. However, these approaches assume that the transporter acts as an isolated entity. Only recently, these studies were expanded, and cell lines expressing two or more transporters at the same time were introduced.[38−49] This strategy arose in many unforeseen results that clearly demonstrated that hepatobiliary trans-

Table 1. continued

will be suitable to answer structure−function related questions. Even though P4-type ATPases were found in liver tissue of the mouse,[519] we only included ATP8B1 due to its characterized role in the human canalicular membrane.

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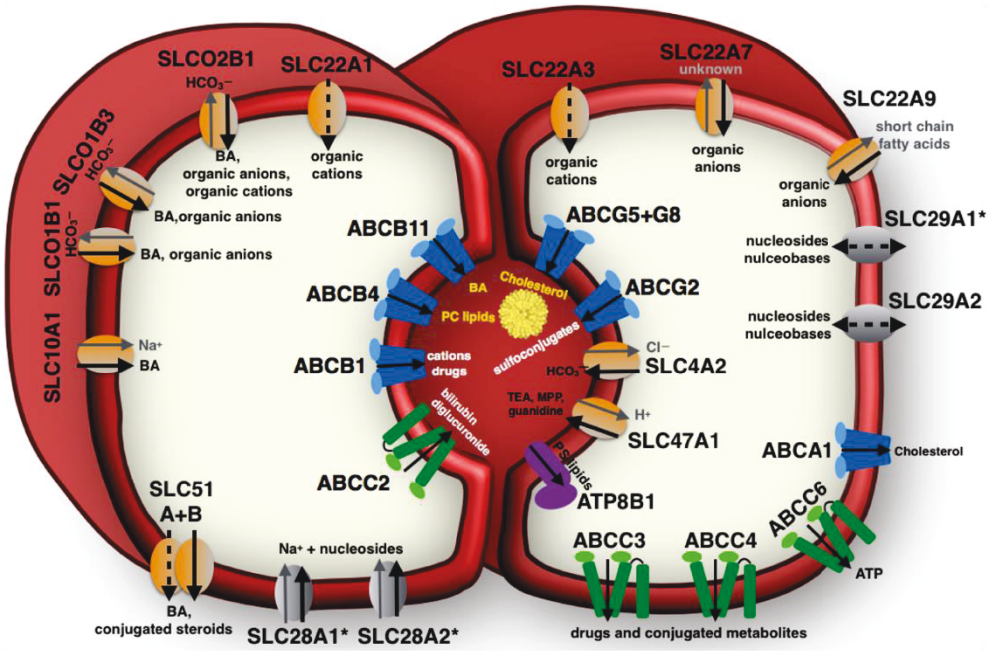

Figure 1. Scheme of two hepatocytes forming a bile canaliculi. All transporters, which are localized in the plasma membrane of human hepatocytes, are highlighted. ABC transporter with exception of the ABCC subfamily (colored in green) are illustrated in blue. ABCB4, ABCB11, and ABCG5/G8 translocate compounds, which form a mixed micelle (light yellow) in the bile canaliculus. ABCC transporters mostly secrete drugs and conjugated compounds such as bilirubin diglucuronide in the case of ABCC2. The exception is ABCC6, which most likely is an exporter for ATP.[7] The P-type ATPase ATP8B1 is shown in magenta. This transporter secretes phosphatidylserine (PS) lipids. The members of the solute carrier (SLC) superfamily are illustrated in orange and gray for the nucleoside transporters (SLC28 and SLC29), since those integrated in both the canalicular and basolateral membrane. Further SLCs can be divided into exchangers (SLC4A2, SLC22A7 SLC22A9, SLC47A1, SLCO1B1, SLCO1B3, SLCO2B1) indicated by a black and a gray arrow in opposite directions, facilitators (SLC22A1, SLC22A3, SLC29A1, SLC29A2, SCL51A+B) indicated by a dashed arrow and cotransporters (SLC10A1, SLC28A1, SLC28A2,) with black and gray arrows in the transport direction. For further information, see also Table 1. *SLC transporter marked in this are present in both membranes (canalicular and basolateral), but due to space limitations only shown in the basolateral membrane here.

porters are everything but not isolated entities. Rather an intense and functional important cross talk and interactions between transporters of the same (basolateral or canalicular) or cross talk between transporters of both membranes exist.[38,39,45,50−52] We believe that we have only seen the tip of the iceberg so far and will discuss these systems more in detail in section An ABC of the Plasma Membrane of the Human Liver as many times, these double or triple infected cell lines involved ABC transporters.

1.2. A General Introduction to ABC Transporters in Humans

In 1986, a seminal paper described the relation between bacterial nutrient uptake systems, nodulation systems, cell division proteins and hemolysin B from *Escherichia coli*.[53] This paper and modeling studies[54] coined the term ATP binding cassette (ABC) transporter. These primary active membrane transporters use the energy of binding and hydrolysis of ATP to translocate their substrates across biological membranes against a concentration gradient and are found in all kingdoms of life.

A functional ABC transporter is composed of two membrane spanning units, the transmembrane domains (TMD) and two copies of the power plant, the nucleotide binding domains (NBD), which harbor all conserved sequence motifs that give rise to this family of membrane transport proteins. In contrast to prokaryotes and archaea, in which the four modules are often encoded on separated genes, only fusion of one NBD or TMD (so-called half-size transporter) or a fusion of all four modules (so-called full-size transporter) are present in eukaryotes. Another important difference between ABC transporters of bacterial and archaeal origin and eukaryotic ABC transporters is the directionality of the transporter. With the exception of human ABCA4[55] and plant transporters,[56−59] only exporters have been described in the eukaryotic phyla. Since this review focuses on hepatobiliary ABC transporters, we will not discuss any ABC system that performs an import function.

As stated above, the NBD contains the conserved sequence motifs of ABC transporters. These include the Walker A and Walker B motifs[60] as found in all P-loop NTPases[61] and the hallmarks of ABC transporters, the signature motif and the D-loop. Additionally, two conserved amino acids are located in the so-called Q- and H-loops.[62,63] The consensus sequences of these motifs are summarized in Table 2. The Walker A motif, also called the P- or Gly-rich loop, coordinates the phosphate moiety of the bound nucleotide. This loop is located between a β-strand and an α-helix and is part of the mononucleotide fold.[64] The Walker B motif is formed by four hydrophobic residues forming one strand of the central β-strand of the catalytic subdomain of the NBD, which adopts a fold most similar to RecA[65] or the α subunit of the F_1 ATPase.[66] The β-strand is followed by an Asp and a Glu residue. The Asp

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Table 2. Conserved Sequence Motifs of ABC Transporters[a]

motif	consensus sequence	canonical	degenerated
Walker A	GXXGXGKST		NBD1 of ABCG8
C-loop	LSGGQ/R		NBD2 of ABCB11
			NBD2 of ABCC1
			NBD2 of ABCC2
			NBD2 of ABCC3
			NBD2 of ABCC4
			NBD2 of ABCC5
			NBD2 of ABCC6
			NBD2 of ABCC7
			NBD2 of ABCG5
Walker B	ΘΘΘΘDE		NBD1 of ABCC1
			NBD1 of ABCC2
			NBD1 of ABCC3
			NBD1 of ABCC4
			NBD1 of ABCC5
			NBD1 of ABCC6
			NBD1 of ABCC7
D-loop	SALD		
H-loop	XHX		
		ABCA1, ABCB1, ABCB4, ABCG2, ABCG5, ABCG8	

[a]A definition of canonical/degenerated is provided in the section A General Introduction to ABC Transporters. X: any amino acid, Θ: any hydrophobic amino acid. One has to stress that already a mutation in one of the conserved sequence motifs renders a transporter degenerated. For simplicity, the motif that is corrupted is indicated. If the transporter contains all catalytically relevant amino acids, it is listed at the bottom line of row "canonical".

residue coordinates the cofactor Mg^{2+} that is indispensable for hydrolytic activity of the NBD, while the following Glu residue forms the catalytic base[67] in the majority of systems.[68−70] This residue polarizes the attacking water molecule that results in nucleophilic hydrolysis of the anhydride bond of the $\beta-\gamma$ phosphate moiety. However, it has to be stressed that the histidine of the H-loop has been shown to play an identical role in for example the isolated NBD of the *E. coli* hemolysin B transporter and is proposed to act as the linchpin of ATP hydrolysis.[71] Here, the His and the Glu residues form a catalytic dyad, in which the His residue polarizes the attacking water molecule, while the Glu residue fixes the side chain of the His residue in a productive conformation.[72] Finally, the conserved Gln residue of the Q-loop connects the catalytic and the helical subdomain of the NBD and interacts with bound ATP.[68,70,73] The hallmarks of ABC transporters, the C- and D-loop are part of the so-called nucleotide binding site (NBS). In the presence of ATP, the NBDs form a head-to-tail dimer with two ATP binding sites. One NBS is formed by the Walker A and B motifs as well as the Q- and H-loop. The binding site is complemented by the C- and D-loop of the opposing dimer[68,70,71,73] (Figure 2). Thus, ATP acts as a "molecular glue"[70] that stabilizes the dimer. Here, the conserved Ser residue of the C-loop interacts specifically with the γ-phosphate of ATP, while the D-loop interacts with residues of the Walker A motif. This inter-NBD interaction within the two NBDs is furthermore strengthened by interactions of the C-terminal helices of the two NBDs.[68,70,73] This architecture therefore explains why preferably ATP binding induces formation of the NBD dimer and is proposed to act as the power stroke[74] of the catalytic cycle. In parallel, a processive clamp model[75] was proposed, which was subsequently refined by suggesting the constant contact model.[76]

ABC transporters are defined by four conserved sequence motifs and two conserved amino acids (Table 2). If an NBD harbors these catalytic essential amino acids, it is termed "canonical". If, however, one or multiple exchanges of conserved amino acids occur, the NBD is called "degenerated". Here, one has to stress that the degree of degeneration, i.e., the number of amino acids exchanged, is highly variable. However, very often a substitution of at least the Glu residue of the Walker B, the catalytic base, is observed. While degeneration of the NBD in bacteria and archaea is rather uncommon with LmrCD,[77] TmrAB,[78] Tm287/288,[79] or PatAB[80] being some of the few known exceptions, in eukaryotic organisms it is rather common. This is for example the case for entire ABCC subfamily of the hepatobiliary ABC transporters (Table 2). In these cases, the C-loop of NBD2 is corrupted. Because of the composite architecture of the NBS, these two exchanges will create an ATP binding site that might bind ATP, but is incapable of hydrolyzing the nucleotide on the biological time scale of a transport cycle. Thus, the entire ABCC subfamily can only hydrolyze ATP at one NBS. If one assumes that hydrolysis and substrate transport are strictly coupled in these transporters, the molecular mechanism will be different between canonical transporters that use two ATP per transport cycle compared to degenerated transporters.[81] For example, the drug efflux pump Pdr5 from baker's yeast, a full-size transporter and member of the G subfamily, contains one completely corrupted NBS; i.e., all catalytically relevant amino acids are exchanged and do not contribute to hydrolytic activity. More importantly, this transporter is uncoupled meaning that ATP hydrolysis and substrate transport are not linked on the molecular level.[82,83]

In humans, 48 ABC transporters have been identified that were grouped in seven subfamilies (ABCA to G) based on the phylogeny of the NBD.[84] As an exception from the "transporter rule", the ABCC subfamily contains ABCC7 or CFTR (cystic fibrosis conductance regulator), which is a chloride channel, and mutants of CFTR are the cause for the most inherited disease among Caucasians, cystic fibrosis.[85] While the structure of this important ABC channel is known by now[86−89] and ABCC7 is present in liver,[90] the second two nontransporter genes ABCC8/9 or SUR1/SUR2 (sulfonylurea receptor) are not expressed in the human liver. ABCC8 is a subunit of the beta cell ATP-sensitive potassium channel and acts as a regulator of these K^+ channels during insulin release, while ABCC9 performs the same function in nonpancreatic cells.[91]

Looking at the transporters, the ABCA subfamily covers 13 members representing the largest transporters with respect to molecular weight among all human ABC transporters. The full-

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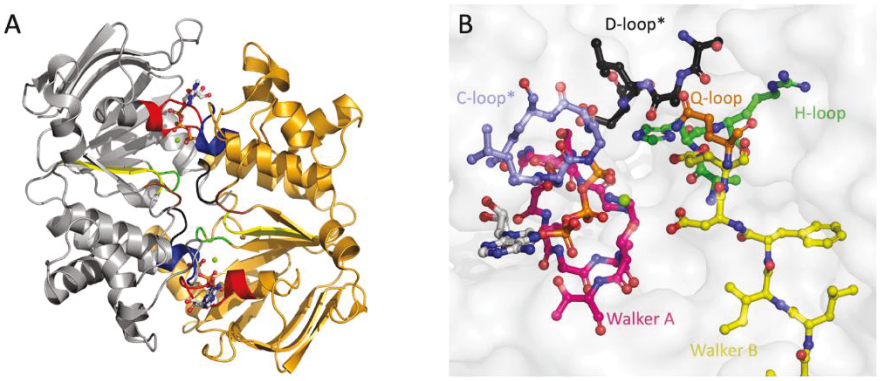

Figure 2. Structural overview of the NBD of ABC transporters. (A) Structure of the ATP-bound dimer of the isolated NBD of the *E. coli* hemolysin B ABC transporter (PDB entry 1XEF).[71] One NBD is shown in gray, and the other one is in orange cartoons. Color coding of the conserved sequence motifs is as follows: Walker A (red), Q-loop (brown), C-loop (blue), Walker B (yellow), D-loop (black), and H-loop (green). The bound ATP molecules are shown in ball-and-stick representation and the cofactor Mg^{2+} as green spheres. (B) Zoom-into into one NBS of the composite dimer. The NBD is shown as a light gray transparent solid. Color coding is as in (A). Water molecule within the NBS have been omitted for clarity. Please note that this is a model of the wild type NBD based on the crystal structure, in which the histidine residue (His 662) was mutated to Ala. * denotes conserved sequence motifs from the opposing NBD in the composite dimer. The interested reader is referred to ref 71 for a more detailed and schematic summary of the interactions of the NBD and bound ATP.

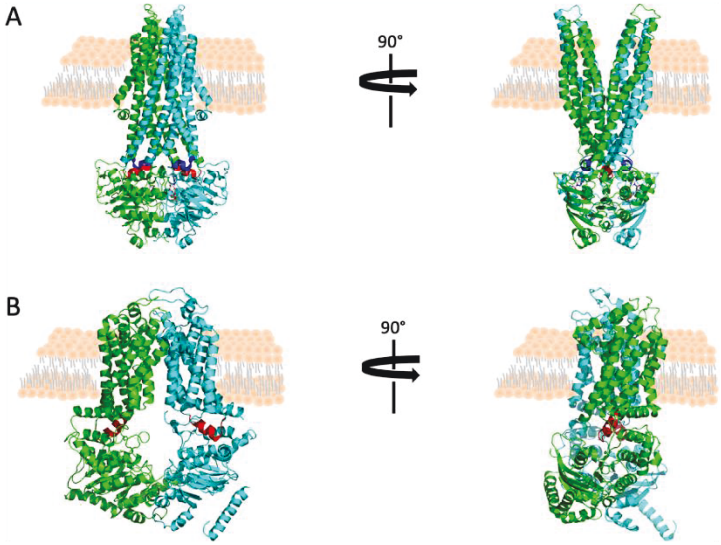

Figure 3. Crystal structures of Sav1866 and human ABCG5/G8 as blueprints for type I and type II exporters. (A) Crystal structure of Sav1866.[108] Monomers are shown in green and cyan cartoons. The CHs are highlighted in blue (CH1) and red (CH2), respectively. The bound ADP is shown in ball-and-stick representation. The putative position of the lipid bilayer is illustrated. (B) Crystal structure of human ABCG5/G8 heterodimer (PDB entry 5DO7).[111] ABCG5 is shown in the green carton and ABCG8 is the cyan cartoon. The single CH of each monomer is highlighted in red.

size transporter ABCA1, for example, whose structure has been determined by single particle electron cryomicroscopy (cryo EM),[92] is composed of 2261 amino acids, while ABCB1 the paradigm of a full-sized multidrug efflux pump is composed of "only" 1280 amino acids. ABCA transporters are involved in lipid and/or cholesterol transport; for example, ABCA1 transports cholesterol and is important in HDL (high density lipoprotein) formation.[93,94] The 11 members of the ABCB subfamily (seven half-sized and four full-sized) comprise various functions including drug, antigenic peptide, lipid, or bile acid transport and are expressed in different organs and cellular membranes. The ones present in liver cells will be discussed in later sections in more detail.

The 12 functional members of the ABCC subfamily are all full-size transporters. Interestingly, additional domains next to the NBDs and TMDs are present in this subfamily. First, ABCC7 contains the so-called R (regulatory) domain, which is located between the NBD1 and TMD2. The R-domain has to become phosphorylated by protein kinase C to act as an ATP-gated Cl^- channel.[95,96] Furthermore, ABCC1 harbors a third transmembrane domain (TMD0) composed of five transmembrane helices (TMH) at its N-terminus. Deletion of TMD0 does not result in impaired ATPase activity or substrate

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transport. TMD0 is also found in ABCC2, ABCC3, ABCC6, ABCC8, ABCC9, and ABCC10, respectively. However, there are two other ABC transporters of the B subfamily, TAP (transporter associated with antigen processing) or ABCB2/B3 as well as TAP-like (ABCB9) that contain a TMD0.[97,98] In this case and in the cases of ABCC8 and ABCC9,[99] TMD0 mediates protein−protein interactions, while the precise role of TMD0 for the other ABCC transporters remains elusive. In contrast, the linker connecting TMD0 and TMD1, the "lasso motif",[100] is essential for proper trafficking.[101−103] All four members of the D subfamily localize to peroxisomes and are involved in lipid metabolism. The ABCE and ABCF subfamilies do not contain a TMD and are referred to as ABC proteins. We will therefore not include ABC proteins in this review. Finally, the topology of the G subfamily with five half-size members out of which two (ABCG5/G8) form a heterodimer[104−106] is different from the classic one observed in all other ABC transporter subfamilies and often termed "inverse topology".[107] While the classic arrangement is $(TMD\text{-}NBD)_2$ in the case of half-size transporters or (TMD-NBD-TMD-NBD) in the case of full-size transporters, the NBD always precedes the TMD in the case of members of the G subfamily and is thus localized at the N-terminus of the NBD-TMD module.

The first crystal structure of an ABC exporter, the bacterial Sav 1866,[108] revealed the presence of long intracellular helices that continue directly from the TMHs, two coupling helices (CH) and a domain swapping (Figure 3A). This domain swapping subdivides each TMD into two parts. In the case of TMD1, TMH 1, 2, 3, and 6 belong to the first part, while TMH4 and TMH6 form the second part. For TMD2, the first part is composed of TMH10 and TMH11, while the second part is formed by TMH7, 8, 9, and 12. Thus, the first helical part is build up by TMH1, 2, 3, and 6 of TMD1 and TMH11 and 12 of TMD2. Accordingly, the second part is composed of TMH4 and 5 of TMD1 and TMH 7, 8, 9, and 12 of TMD2. This domain swap has important mechanochemical consequences. The intracellular loops (ICL) 1 and ICL2 adopt helical structures and are therefore also termed coupling helix (CH) 1 and 2. CH1 contacts primarily the *cis* NBD, while CH2 exclusive interacts with the *trans* NBD. On the side of the NBD, CH1 contacts a region preceding the C-loop that was termed the X-loop[108] as well as the helix following the Walker A motif. CH2, on the other hand, contacts the Q-loop region. This obviously has large mechanistical implications as the C- and Q-loop regions are the parts of the NBD that undergo the largest motions upon ATP-induced dimerization, suggesting that their motion is directly transmitted to the TMDs through the long and helical ICLs. This allows a direct coupling of NBD dimerization with conformational changes in the TMD. This coupling provides a molecular explanation for the two side access model proposed already in 1966[109] This proposal originally suggested for importers was extended to ABC transporters in 2004[74] and suggested how the NBDs catalyze the switch from the inward facing (IF) to the outward facing (OF) conformation. In the IF state, substrate binding site is accessible from the cytoplasm, while it is open to the extracellular space in the OF state. The X-loop furthermore was used as a diagnostic motif to identify multidrug exporters, as one laboratory could experimentally demonstrate that Sav1866 imposed drug resistance when overexpressed in *L. lactis*.[110] The results derived from the crystal structure of Sav1866 were not anticipated and provided a blueprint for human ABC exporters including ABCB1, which structure will be discussed in the section The Multidrug Efflux Pump ABCB1. It therefore came as a surprise when the crystal structure of the cholesterol exporter ABCG5/G8 (Figure 3B) was reported in the apo state.[111] ABCG5/G8 transports sterols including cholesterol and mutations cause sitosterolemia.

As shown in Figure 3B, the overall fold was more reminiscent of ABC import systems such as BtuCD[112] and contained only a single CH between TMH2 and TMH3, which is highlighted in red in Figure 3B. Thus, no domain swapping is present in this transporter. Additionally, a connecting helix (CnH) between the NBD and TMH1 and substantial extracellular domains (ECD) between TMH5 and TMH6 are evident. Missense mutations[105,106] causing sitosterolemia are located within the helical ECDs. Both the CH and the CnH are in proximity to the C-loop and might serve as a read-out platform for motions of the NBD due to ATP-induced dimerization. Furthermore, one has to stress that ABCG5/G8 is an asymmetric transporter; i.e., NBS1 formed by the Walker A and Walker B motifs as well as the Q- and H-loops of ABCG8 and the C- and D-loops of ABCG5 is deficient in hydrolyzing ATP. This is reflected by the architecture of the NBD-TMD interface. The CH, CnH, C-loop, and helix E form a three-helix bundle that present the communication pathway and appear to be more rigid in NBS1 than in NBS2.[111] Helix E, which is defined by the conserved Glu residue 146 (the E146Q mutation is disease causing[106]), packs against CnH and becomes part of the helical bundle that is supposed to transmit conformational changes from the NBD to the TMD.

Thus, structural biology provided us with a framework of structural information, and at least two different folds for human ABC export systems exist. Nevertheless, these two structures are not sufficient to allow detailed insights into the actual transport mechanism or the mode of substrate selection. The following section will now focus on human ABC exporters that are expressed in the liver on the protein level and summarize our current knowledge of their structure and function. Only when necessary, we will sidestep and include other ABC systems to explain more in detail the putative mode of action(s) of ABC transporters of the human liver.

1.3. An ABC of the Plasma Membrane of the Human Liver

Twelve[113] of the 48 human ABC transporters[84] have been identified to be functionally expressed in healthy human liver (Table 1), while 36 ABC transporter were only detected on the mRNA level. ABCB5[114] and B6[115] were predominantly detected in hepatocellular carcinomas. The latter is furthermore only expressed in mitochondria. This review focuses primarily on the healthy liver and transporters of the plasma membrane. Thus, we will not discuss both transporters any further.

Cellular cholesterol efflux and high density lipoprotein (HDL) formation is mediated by ABCA1.[116] On the mRNA level,[117] the protein was clearly detected, but so far it could only be visualized on the protein level in cell culture[118] and in rats.[119] Because of its importance for cholesterol homeostasis in mice[120] and the presence of structural information,[92] we will describe the liver relevant knowledge more in detail in section More hepatobiliary ABC transporters.

The expression of CFTR was so far only confirmed in cholangiocytes.[90,121] Structural information in different functional states is available[86−89] and presents obviously an

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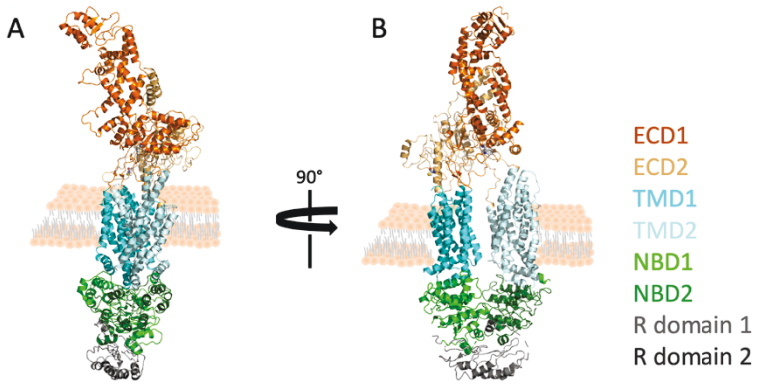

Figure 4. Moving cholesterol across a membrane - ABCA1. (A) Single particle cryo-EM structure of human ABCA1 (PDB entry 5XJY[92]). The R domains are shown in gray and light gray, the NBDs as green, the TMDS as cyan and light cyan, and the two ECDs as orange (ECD1) and light orange (ECD2) cartoons. The putative position of the lipid bilayer is illustrated. (B) As in (A) after a rotation of 90° perpendicular to the membrane as indicated.

important step forward in our understanding and the consequences of disease-causing mutations. However, CFTR is an ATP-gated chloride ion channel and will not be included in this review as already stated.

In hepatocytes, three ABC transporters are located in the basolateral membrane, ABCC3, ABCC4, and ABCC6, respectively. In the canalicular membrane of hepatocytes, six ABC transporters and only five SLCs are located. The functions of the two SCLs, MATE1 and AE2, have already been discussed in the previous section. The canalicular membrane also expresses three nucleoside transporters.[122,123] The ABC transporters can be functionally subdivided into a conjugate transporter (ABCC2), drug efflux pumps (ABCB1 and ABCG2, respectively), and the three transporters involved in bile formation ABCB11, ABCB4, and the heterodimer ABCG5/G8, respectively. Here, a delicate interplay between bile acid export (ABCB11), cholesterol secretion (ABCG5/G8), and PC lipid flop from the inner to the outer leaflet (ABCB4) is emerging. We will discuss the interplay of these three transporters more in detail in section The Bile Triumvirate – ABCB4, ABCB11, and ABCG5/G8. The drug efflux pumps, ABCB1 and ABCG2, respectively, possess sort of a complementary substrate spectrum, which together with ABCC2 imposes a maximal level of protection against xenobiotics, a topic that will be discussed in detail in the next sections. ABCC2 secretes conjugated metabolites, mainly in the form of glucuronide- or glutathione-conjugated.[124] This function is complementary to the function of ABCC3 and ABCC4 located in the basolateral membrane. In contrast, a pathophysiological role was assigned to ABCC6 as mutations to pseudoxanthoma elasticum (PXE),[125,126] a connective tissue disorder. ABCC6 is expressed in kidney and the basolateral membrane of hepatocyte.[127] Here, ATP was identified as the physiological substrate.[128] The extracellular hydrolysis of ATP to AMP and PP_i is a process that generates the calcification inhibitor pyrophosphate (PP_i).[129] Consequently, dysfunctional ABCC6 results in reduced levels of PP_i and misbalanced calcification of connecting tissue.[130]

Stable or transiently infected cell lines are probably the most powerful tools to study transport function, analyze amino acid exchanges, and determine the transport rates and kinetic parameters. The only "drawback", if at all, is the fact that the transporter is treated as an isolated entity in these cell lines. Thus, the laboratories of Dietrich Keppler,[39,41,43,44,47,48] Yuichi Sugiyama,[46,49] Bruno Stieger,[45] and other groups[40,42] developed double- or triple-stable transfected cell lines. In the first published example,[39] OATP1B3 and ABCC2 were stably infected in polarized MDCKII cells. As in hepatocytes, OATP1B3 localized to the basolateral and ABCC2 to the canalicular membrane. The obtained data clearly revealed that uptake and efflux are by no means separated transport processes. Rather, mathematical modeling[38] demonstrated that the cross-talk between both transporters was required to obtain intracellular transport rates that mirrored the situation in the human liver. This obviously imposes another level of complexity in the study of hepatobiliary transport processes. Since this review focuses on hepatobiliary ABC transporter, we will discuss one example[45] more in detail in section The ABC Bile Triumvirate – ABCB4, ABCB11, and ABCG5/G8.

We now turn to a detailed description of the structure and function of hepatobiliary ABC transporters grouped according to their functions outlined above.

2. THE ABCA SUBFAMILY MEMBER OF THE LIVER - ABCA1

Human ABCA1, first cloned in 1994 as ABC1,[131] is essential for cholesterol homeostasis and HDL formation. It transports excess of cholesterol and phospholipids to a proteinaceous acceptor, apolipoprotein A1 (apoA-1).[132] This secretion represents the rate limiting step of HDL biogenesis. Here, HDL absorbs cholesterol and carries it back to the liver. The secretion performed by ABCA1 is therefore crucial to reabsorb cholesterol and necessary for a healthy circulation. In macrophages, an additional anti-inflammatory activity was observed.[133] Thus, these two functions, cholesterol homeostasis and anti-inflammatory actions, protect pancreatic cells and regulate insulin secretion. A deficiency in ABCA1 is the basis of a rare genetic disorder, Tangier disease, that goes in hand with a severe reduction in HDL levels and increased levels of triacylglycerides.[134] As a consequence of the impaired function of ABCA1, cholesterol remains intracellular, the size of many body tissues increases significantly, and the risk of coronary artery diseases rises. As a result of increased intracellular cholesterol levels, cholesterol reacts with a fatty acid to form an ester that is deposited intracellular. These depositions are mainly found in tonsils, but also lymph nodes,

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Table 3. Sequence Identity of Human ABCC Subfamily Members Which Are Present in the Liver and Liver Excluded ABCC1 (MRP1)

	symbol	identifier	name	amino acids	long				short	
					ABCC1	ABCC2	ABCC3	ABCC6	CFTR	ABCC4
long	ABCC1	P33527-1	MRP1	1531	100					
	ABCC2	Q92887-1	MRP2	1545	48	100				
	ABCC3	O15438-1	MRP3	1527	58	47	100			
	ABCC6	O95255-1	MRP6	1503	45	38	43	100		
short	ABCC7	P13569-1	CFTR	1480	28	28	27	27	100	
	ABCC4	O15439-1	MRP4	1325	39	37	36	33	35	100

bone marrow, liver, and spleen and complicate blood circulation due to a reduction of the diameter of the blood vessels. The highest mRNA levels of human ABCA1 were detected in adrenal glands, fetal tissue, liver, lung, and placenta.[117] However, the subcellular location in human liver has not been demonstrated on the protein level. The situation is different in rats. Here, high levels of human ABCA1 were detected in endothelial cells and hepatocytes, while the level was lower in Kupffer cells.[119] In knockout mice, hepatic ABCA1 is apparently involved in very-low-density lipoprotein (VLDL) as increased levels of triacylglycerides-rich VLDL particles were detected. This pointed toward a new relationship between increases in triacylglyceride levels and reduced amounts of HDL.[135,136]

The structure of human ABCA1, determined by single particle cryo-EM, at 4.1 Å resolution (PDB entry 5XJY and Figure 4) suggested the presence of a lateral access mechanism for cholesterol transport,[92] which deviated from the classical two side-access model.[109] Similar to the ABC transporters of the C subfamily, only one coupling helix and six TMHs are present per TMD. Next to the two NBDs and two TMDs, two small R domains following the NBDs are present. The most prominent feature of ABCA1, however, is two large extracellular domains (ECD) located between TMH1 and TMH2 (ECD1) and TMH7 and TMH8 (ECD2). The ECDs are suggested to interact with apoA1[137,138] and/or substrate[139] in the ABCA subfamily. All structures of ABC transporters determined in the nucleotide-free state adopted the inward facing conformation with separated NBDs. In striking contrast, the nucleotide-free structure of human ABCA1 is in a conformation most similar to the outward-facing conformation.[92] ECD1 (583 amino acids) and ECD2 (270 amino acids) adopt a twisted conformation, in which ECD1 is placed above TMD2, while ECD2 is placed above TMD1. Both adopt a novel protein fold and are stabilized by three (ECD1) and one (ECD2) disulfide bond, respectively. Interestingly, a hydrophobic tunnel is present in the ECDs, which might serve as an existing tunnel for cholesterol or phospholipids. This proposal is supported by the fact that many of the mutations identified in Tangier disease map to the ECD, especially the two well-characterized ones, W590S and C1477R.[140,141]

3. THE ABCC SUBFAMILY OF THE LIVER - DRUG AND TOXIN EFFLUX

3.1. The ABCC Subfamily - Similarities and Differences

The ABCC subfamily consists of 12 functional members. Next to an ATP-dependent chloride anion channel, CFTR/ABCC7, and two sulfonylurea receptors, SUR1/ABCC8 and SUR2/ABCC9, nine multidrug resistance-associated proteins (MRPs) belong to the ABCC family.[142] A 13th member of the ABCC family (ABCC13) is a pseudogene for which no functional properties are known.[143]

The members of the ABCC subfamily can be further divided into short and long members. The term long ABCCs derives from an additional N-terminal domain called TMD0 present in these transporters.[144] Five ABCCs, namely, ABCC1 (MRP1), ABCC2 (MRP2), ABCC3 (MRP3), ABCC6 (MRP6), and ABCC7 (CFTR), belong to the long MRPs. The others, including ABCC4 (MPR4), belong to the short MRPs (other examples are ABCC5, ABCC11, and ABCC12). For the sake of completeness, ABCC8 and -9 are no MRPs, but are long members of the ABCC subfamily. ABCC10 or MRP7 is not expressed in the liver, but also belongs to the long MRPs.

All members of the ABCC family consist of at least four domains typical for ABC transporters, namely, two TMDs and NBDs. The NBDs, although highly conserved in all ABC transporters, are degenerated in the ABCC subfamily (Table 2). This means that the first NBS has little or no ATPase activity, whereas the second NBS (NBS2) has a much higher ATPase activity.[6,145] Interestingly, NBD1, which forms NBS1 with the exception of the C- and D-loops, is characterized by a high affinity for ATP, suggesting a coordinating and structurally stabilizing function.[145]

There are also differences in the tissue-specific localization of the individual members of the ABCC family, which influence the function and substrate spectrum of these transporters (Table 1). For example, an ABCC transporter may be found in the apical membrane in one organ and in the basolateral of another organ. Importantly, specific physiological functions are linked to localization.[146]

This section deals with the function and localization of ABCC transporters of the liver. There are five ABCC proteins that are expressed on protein level in the liver.[113] Besides ABCC7 (CFTR), these are ABCC2, ABCC3, ABCC4, and ABCC6. Additional information on the structure, function, localization and medical applications of ABCC7, which is not discussed here, are described in refs 86, 87, and 147.

We have implemented a short part to the structure of ABCC1, although it is not expressed in a healthy liver. The reason is the recently published structure,[148] which represents probably the closest structural information for the liver ABCC subfamily. ABCC1 shares approximately 50% sequence identity with other liver ABCCs. A cross sequence alignment of the different liver ABCCs are provided in Table 3 and demonstrate the relation within this subfamily. However, we have examples from nature, in which an even higher degree of identity results in physiologically different functions, for example, the 79% amino acid identity between ABCB1 and ABCB4.[149,150] ABCB1 transports hydrophobic compounds often with aromatic properties, whereas ABCB4 transports lipids of the phosphatidylcholine family. Therefore, we would

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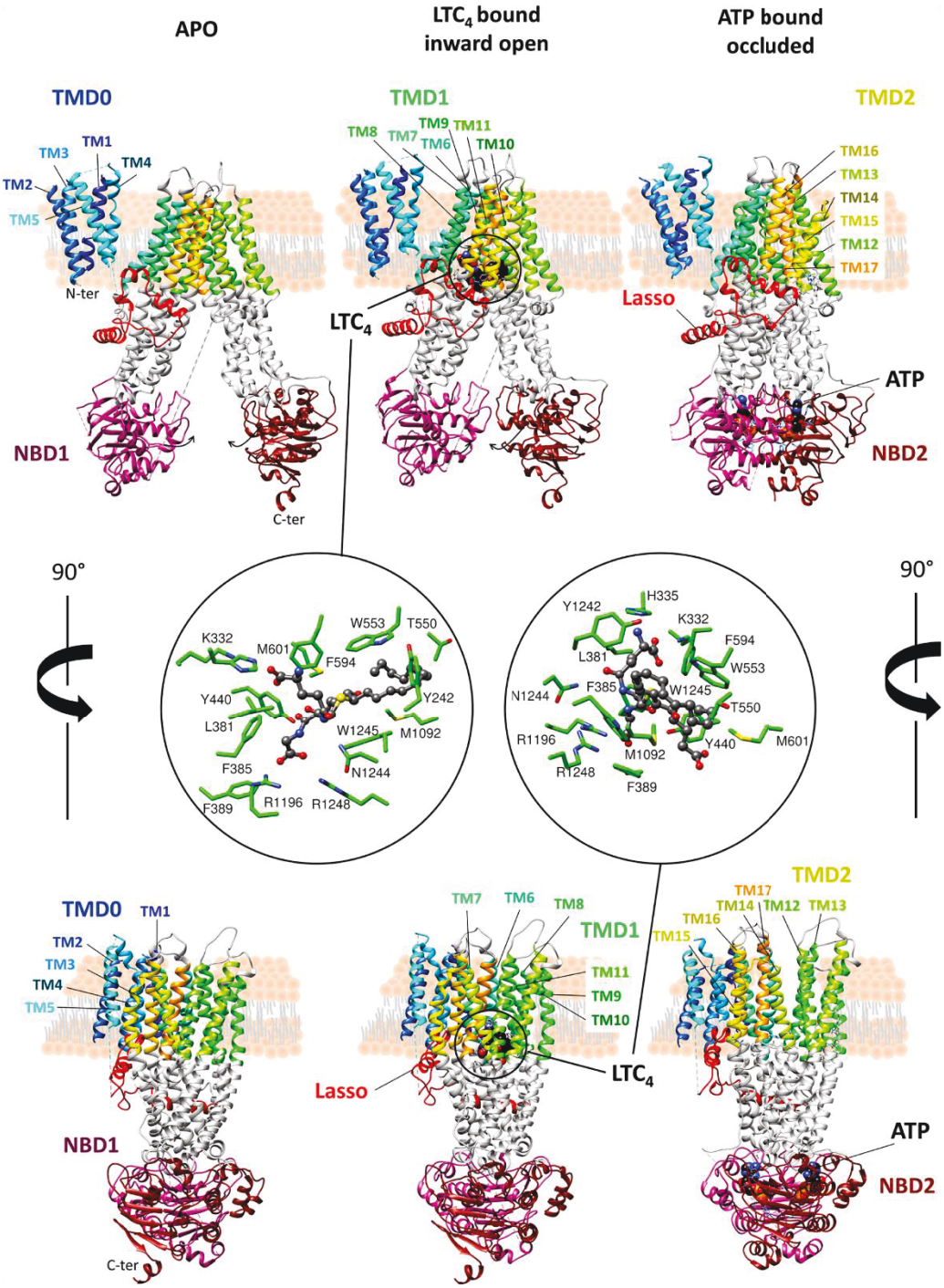

Figure 5. Structures of ABCC1. Apo (bovine, PBD entry 5UJ9), LTC_4-bound inward open (bovine, PDB entry 5UJA), and ATP-bound occluded (bovine, PDB entry 6BHU) conformations of ABCC1. The TMD0 is colored in blue, the TMD1 in turquoise, and the TMD2 in yellow. NBD1 is pink, and NBD2 is dark red. The typical lasso motif of the long ABCCs is highlighted in red. Substrates such as ATP and LTC_4 have a black background. The upper panel offers a frontal view. The lower panel is rotated by 90°. The N- and C-terminus are marked. The dashed lines in the upper left and upper middle picture are flexible regions of the protein with no structural information. Information about flexible areas in ABC transporters may be obtained here.[163]

like to point out that molecular differences between the different structures of the liver ABCCs may be crucial and can only be elucidated if structures are determined. However, the structure of ABCC1 may act as a prototype for this subfamily.

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In general, the ABCC subfamily transports a variety of substances across the corresponding plasma membranes including drugs, such as cancer therapeutics, but also conjugated metabolites. Characteristic for the ABCC family are substrates that are conjugated with glutathione (GSH), sulfate or glucuronate prior to transport. However, these substrate characteristics are not found to the same extent in all ABCCs. ABCC6 for example shows only a low affinity to GSH conjugated substrates.[151] The varying degree of redundancy of the substrate spectra of liver ABCCs is controlled under physiological conditions via different *in vivo* export kinetics. This means that although two ABCCs may have an overlapping substrate spectrum, in practice only one transporter will export the substrate[152,153] thereby regulating the detoxification pattern. It is therefore possible that the function of defective ABCCs is compensated by other ABCCs. For example, an ABCC2 deficiency can be counteracted by the upregulation of other transporters of the ABCC subfamily such as ABCC3 or ABCC4 as it could be shown in r*Abcc2* deficient rats.[154−156] However, since these ABCC members are localized in different membranes of the hepatocyte, ABCC2 substrates that would normally be exported to bile would now, compensated by ABCC3 or ABCC4, appear in the sinusoidal bloodstream.[157,158]

3.2. General Lessons Learned from Structures of the ABCC Family

First, we have no direct structural information for the ABCC transporters of the liver. Thus, information on the characteristics such as the TMD0 of long ABCC transporters must be derived from other members of the ABCC subfamily.

So far, structures of three members of the ABCC family have been published. These include CFTR, SUR1, and ABCC1.[100,159,160] From a functional as well as mechanistic point of view, the information gained from CFTR and SUR1 for the four liver ABCCs (ABCC2, ABCC3, ABCC4, and ABCC6) are limited, but might provide hints for the role of TMD0. In SUR1 for example, TMD0 interacts with a potassium channel to form a larger macromolecular complex.[98,159,160]

Additional information can be derived from the structure of ABCC1 (PDB entries 5UJ9, 6BHU, 5UJA)[100,161] and CFTR. First, ABCC1 provides structural information for long ABCC transporters, since it is the closest-related structure based on sequence identity (Table 3). TMD0 of ABCC1 as a prototype of long ABCC subfamily members of the liver such as ABCC2, ABCC3, and ABCC6 is highlighted in Figure 5. When the protein core of TMD1 and TMD2 undergoes a conformational change due to substrate binding and/or upon ATP binding the NBDs come closer together, it seems as if the conformation of TMD0 is nearly identical within all three structurally described states of ABCC1. This indicates a function prior and independent of substrate transport. Strikingly, deletions of TMD0 in the case of ABCC1, does not impair its export function,[102] which explains the rigid structure of this TMD0. Note that the connection to the core protein is not visible due to missing electron densities. This indicates a highly flexible area and might be explained by the fact that some parts of the protein have to be flexible in order to make up for the rigid TMD0. Taken together this suggests that TMD0 is involved in protein−protein interactions for ABCC members belonging to the long subfamily, but lack an involvement in substrate translocation.

A so-called lasso motif appears to be structurally highly conserved within the ABCC family.[148] Confirmatively, the lasso motif from ABCC1 differs from the analogous lasso motif of ABCC7 only by an rmsd of 1.1 Å over amino acids 1−64 from ABCC7 and 205−268 from ABCC1,[148] even if both proteins provide physiologically diverse functions. The assumption that this motif is also conserved in the more closely related ABCCs found in the liver seems convincing. It is a motif following TMD0 but is more closely positioned to the core protein and is connecting the flexible regions derived from TMD0 with TMD1 (highlighted in red in Figure 5). The function of this lasso motif is essential for the correct folding and trafficking of ABCC members.[87,148] Deletions led to misfolded proteins that exhibited impaired functionality.[162]

The substrate binding pocket, on the other hand, appears to show larger differences between the individual ABCCs of the liver. Substrate binding pockets are known to be localized in the TMD of ABC transporters. The ABCC subfamily is no exception. Again, highlighting the point, that up to now we have no direct structural data of any of the liver ABCC transporters making ABCC1 the closest relative. In the ABCC1 structure two binding pockets are present, the positively charged (P) and the hydrophobic (H) pocket. The prototype binding pocket of LTC_4 bound to ABCC1 and the engaged amino acid residues are depicted in Figure 5. Obviously, the binding pockets of the liver ABCCs must harbor different residues and/or folds in order to explain the substrate diversity of this family. However, there are also overlaps in the substrate spectrum within the liver ABCCs. It seems that ABCC transporters in general have two binding pockets for ligands, first substrates and second modulators.[164−166] Interestingly, potential nutrient-drug interactions in ABCC2 as observed for genistein and sorafenib[167] in the context of hepatocellular carcinoma (HCC) point to a possibility of two binding pockets. Substantially different is the binding pocket of ABCC1 and ABCC2 even if they share common substrates such as leukotriene C4 (LTC_4). Studies, in which the highly conserved W1254 and P1158 of ABCC2 and the homologues residues of ABCC1 and ABCC3 were exchanged, led to changed transport efficiencies of these three substrates.[168−170] Furthermore, particularly charged amino acids in TMD1 and TMD2 seem to be important for the substrate specificity of ABCC2. Estradiol, for example, is a prominent substrate of ABCC2, but it also has a regulatory influence *via* the allosteric binding pocket.[171] To investigate this dual character in more detail, *de novo* structures of ABCC2 or even other liver ABCC transporters are required.

However, the general conformational changes that occur due to substrate binding, followed by ATP hydrolysis, are key characteristics for all ABC transporters (Figure 5); thus, they may also appear in the other liver ABCC transporters as the prototype structure of ABCC1 exemplifies. We therefore show the known conformations of ABCC1 (Figure 5). More details about the liver ABCCs regarding biological function in the liver can be found in the following chapters (ABCC2, 3, 4, and 6).

3.3. ABCC2 (MRP2): The Only ABCC Transporter of the Canalicular (Apical) Membrane

Originally, ABCC2 was referred to as canalicular multidrug resistance-associated protein (cMRP)[172] or canalicular multispecific organic anion transporter (cMOAT),[173] because it was initially found in the canalicular membrane of hepatocytes. However, ABCC2 can also be found in the apical membrane of

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other polarized cell types.[124,174,175] TMD0 and the cytoplasmic helix 3 of ABCC2 are apparently responsible for the specific apical localization.[176] ABCC2 functions at the final stage of the bioelimination process. Potential toxins or metabolites diffused though the cell, where they were conjugated, are finally eliminated by ABCC2, as indicated by the apical localization in the membrane.[153,177] The substrate spectrum of ABCC2 includes conjugated endogenous or xenobiotic substances, which are exported into the bile duct rather than into the blood system, unlike other multidrug exporters. The substrate spectrum of ABCC2 is defined by its lipophilic properties and contains mainly organic anions. These substrates are often conjugated with glutathione, sulfate or glucuronate. Endogenous metabolites include bilirubin, bile acids[178−180] and LTC_4.[179,181] Additionally, ABCC2 also has a broad spectrum of cancer drugs such as *vinca* alkaloids, tamoxifen, cisplatin, or anthracyclines[182−186] as substrates. Cisplatin, for example, is an anticancer drug with a broad response spectrum and is often used in cancers for which no specific targets are known.[187] Accordingly, overexpression of ABCC2 is a frequently observed problem in antitumor treatment.[188]

ABCC2 deficiency, on the other hand, can lead to insufficient elimination of bilirubin from hepatocytes into the bile, a phenomenon described as Dubin and Johnson disease.[189] It is an autosomal recessive disease and occurs in 0.08% of Iranian Jews[190] and in 0.0003% of the Japanese population.[142,191] The liver appears black in these people, because the dark dye of the elevated bilirubin glucuronides in the hepatocytes cannot be exported.

3.4. ABCC3 (MRP3): The Back-up System for ABCC2 in Liver Cholestasis

ABCC3 (MRP3) is transported to the basolateral membrane in the same way as ABCC1 in polarized cell types, but in contrast to ABCC1 it is also found in the hepatocytes of the liver.[113] Previously, it was thought that ABCC3 is highly expressed in the liver, but immunohistochemistry of frozen sections has revealed that in healthy liver, there are only modest levels of ABCC3, especially in the basolateral membranes of cholangiocytes and hepatocytes surrounding the portal tracts.[192] Nevertheless, ABCC3 and ABCC1 show the highest sequence homology (Table 3). However, the substrate spectrum of ABCC3 differs substantially from that of ABCC1 and is rather limited. Particularly noteworthy is the low affinity to the tripeptide glutathione (GSH), which is often conjugated to other substances for ABCC1 mediated transport.[193] Although ABCC3 has been studied mostly in the context of bile acid transport, there are also other drugs and metabolites that are part of the substrate spectrum. These include for example acetaminophen glucuronide, methotrexate, and sorafenib.[194]

Even if ABCC3 deficiency does not lead to a specific phenotype,[193,195] it was detected that ABCC3 was up-regulated in a cholestatic liver. Currently, it is assumed that ABCC3 is sort of "back-up system", especially when transport of toxic metabolites by ABCC2 is impaired. Of pharmacological importance is the observation that in $Abcc3^{-/-}$ mice conjugated morphine accumulates in the liver and bile, which suggests a definitive role of ABCC3 in its elimination.[195] Furthermore, different ABCC3 single nucleotide polymorphisms (SNPs) may explain the differences in morphine metabolite elimination.[196] However, these ABCC3 variants were located on intronic regions of chromosome 17 (4871392 and 48744612, spanning 13221 bp) and hence do not alter the protein sequence. The authors speculated about regulatory factors that may alter DNA binding or noncoding regulatory sequences. Additionally, an impairment of liver regeneration could be observed in mice with deleted ABCC3 ($Abcc3^{-/-}$) after partial hepatectomy. Here, the authors concluded that ABCC3 plays again a nonredundant role in regulation of bile acid efflux during liver regeneration.[197] Altogether, these findings highlight the role of ABCC3 in injured liver systems to help to recover through the export of toxins and toxic metabolites such as bilirubin.[193]

3.5. ABCC4 (MRP4): The Little Brother of ABCC1 in the Liver

ABCC4 is expressed in several human tissues; high levels have been reported in prostate glandular, and in contrast to ABCC1, ABCC4 is also detected in the liver, but at rather low expression levels.[198,199] Here, ABCC4 exports chemically diverse substrates, signal molecules, as well as xenobiotics *via* the basolateral membrane of the hepatocytes. Beyond the liver, ABCC4 is particularly well-known for being not limited to the basolateral membrane, thus exporting substrates through both membrane types of polarized cells.[200,201]

Although ABCC4 expression is low in hepatocytes, it is enhanced in cholestatic livers.[198,202] In fact, the up-regulation of ABCC4 in cholestatic liver of humans provides an adaptive mechanism to eliminate excess bile acids.[202,203] ABCC4 appears to be a clinically relevant drug transporter, along with ABCC1, that makes cancer treatment more difficult.[146] Moreover, xenobiotics may increase the expression of hepatic ABCC4.[203,204] The substrate profile of ABCC4 includes cAMP and cGMP, leukotrienes (such as LTC_4), sphingosine 1-phosphates (S1P)[205] and pesticides.[206] However, the transport efficiency and affinity to the important signal transduction molecules such as cAMP and cGMP is low, which means that although no direct intracellular regulation by ABCC4 is considered possible, an indirect regulation by local microdomains cannot be excluded.[207]

ABCC4, as many ABC proteins, has more than one substrate binding site to account for its diverse substrate spectrum.[165] This assumption is based on homology modeling.[166] Amino acids involved in substrate binding, namely, F368, F369, E374, R375, E378, W995, and R998 are located at the surface of intramembrane water pore in between TMHs. The aromatic residues can selectivity bind aromatic substrates by $\pi-\pi$ stackings.[164,200,208] Additional theoretical work on ABCC4, also based on homology models generated by molecular dynamic simulations, provided insights in clinically relevant mutations, especially G187W. This SNP affected the tertiary structure of ABCC4 massively and provides an explanation for its transport disrupting effect.[209] Furthermore, it was shown, that certain substrates have been found to act in a concentration-dependent manner.[164]

In conclusion, these findings point to a role of ABCC4 in detoxification and liver damage, and suggests that its modulation could influence hepatocyte repair and regeneration during hepatocellular injury especially in cholestatic liver situations.[210,211]

3.6. ABCC6 - The ABC Transporter That Transports ATP

ABCC6 is mainly expressed in the liver and kidney, and deficiencies are linked to a rare disease of hereditary mineralization disorder, *pseudoxanthoma elasticum* (PXE).[126] ABCC6 is thought to mediate the secretion of ATP into blood,

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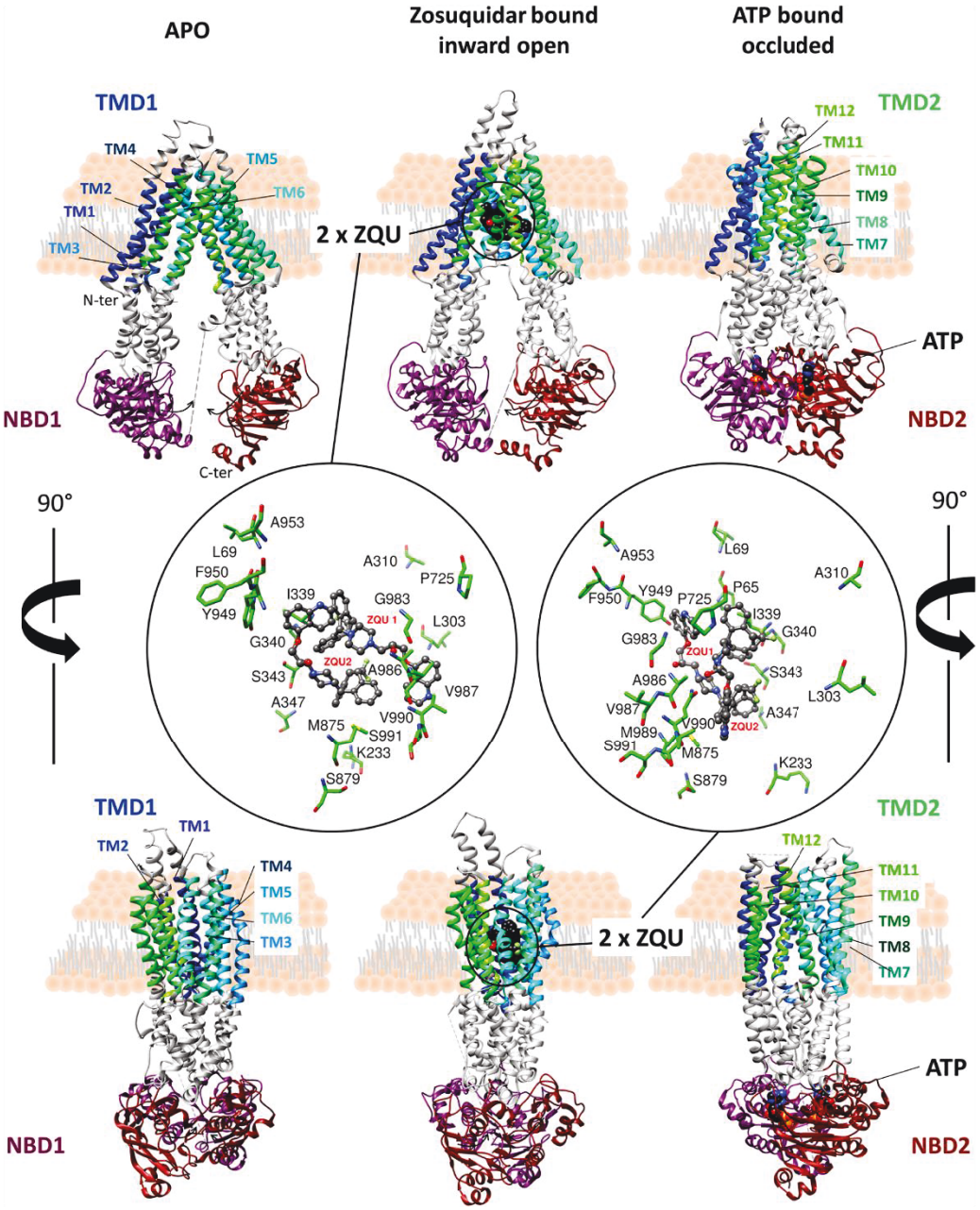

Figure 6. Structures of ABCB1. An apo (*Mus musculus*, PDB entry 4M1M),[233] zosuquidar-bound, inward open (human-mouse chimera, PDB entry 6QEE)[234] and ATP-bound outward facing (human, PDB entry 6C0V)[235] conformations of ABCB1. Blue colors are used for the TMD1, and TMD2 is held in greenish colors. NBD1 is pink and NBD2 is dark red. The binding pocket of the inhibitor bound structure is highlighted. The amino acids interacting with the inhibitor are visible in an enlarged harvester. The upper panel offers a frontal view. The lower panel is rotated by 90°. The N- and C-termini are marked. The dashed lines in the upper left and upper middle picture are flexible regions of the protein with no structural information. Information about flexible areas in ABC transporters may be obtained in ref 163.

where ATP is rapidly converted into AMP and the calcification inhibitor PP_i.

The absence of ABCC6 leads to a substantial decrease of plasma PP_i.[7,128,130,212] PP_i would act as a counter reagent to ectopic calcification that occurs in PXE patients. This theory of a substrate export via ABCC6 into blood circulation and transport through the whole body is known as metabolic hypothesis.[130,144] However, whether ATP is a direct substrate of ABCC6 remains to be proven. ABCC6 could also be an activator of other ATP export systems of the cell. A problem for the export of ATP by an ABC transporter is the millimolar concentration of ATP in the cells. ABC transporters have a relatively low affinity to this highly abundant molecule in the

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cell, whose hydrolysis provides the energy for substrate transport.

ABCC6 seems to have evolved from a gene duplication event of ABCC1. Both genes are located on chromosome 16 (*ABCC1* 16p13.11 and *ABCC6* 16p13.12).[213]

Like all ABCC transporters, substrates of the organic anions and GSH derivatives and conjunctions were tested for the substrate spectrum of ABCC6. Although an overlapping substrate spectrum of many ABCC typical substrates such as LTC_4 was found, it is worth to mention that the transport efficiency of ABCC6 is significantly lower.[151,214] A K_m value of 600 nM and a V_{max} value of 50 pmol/mg of membrane protein/min for LTC_4 transport were estimated.[151] In comparison the kinetic parameters of ABCC1 are a K_m of 77 nM for LTC4 and a V_{max} of 291 pmol/mg/min.[215] This clearly supports the theory that ABCC6 is mainly an ATP exporter *in vivo*.

4. THE ABCB SUBFAMILY - STRUCTURALLY CLOSE BUT DIFFERENT SUBSTRATES

4.1. The Multidrug Efflux Pump ABCB1

ABCB1 was first described in 1976 by Ling[216] as a permeabilization glycoprotein (P-gp). Later, the name was linked to its function, and it was called multidrug resistance protein 1 (MDR1). According to the nomenclature of human ABC transporters, MDR1 is now termed ABCB1. ABCB1 is ubiquitously expressed in various tissues[217] and also in organs associated with elimination processes in liver, kidney, or the blood−brain barrier. In the liver, ABCB1 does not yet perform one specific task; rather, it exports a large number of substances from the hepatocyte to bile and contributes considerably to the first defense against drugs.[218] Interestingly, the laboratory of Yin demonstrated in 2018 that ABCB1 can transport bile acids. Here, a model is suggested, in which rapamycin rescues BSEP-deficient phenotypes by activating alternative excretion of bile acids via ABCB1.[219]

Accordingly, ABCB1 is located in hepatocytes in the canalicular membrane and reduces the drug concentration in the cell by continuous export. The substrate spectrum mainly comprises hydrophobic substances, some with aromatic ring systems and slightly amphiphilic character.[220−222] ABCB1 is one of the best studied MDR exporters and is often considered as prototype of human ABC transporters[223] and directly associated with cancer.[224] As little is known about a specific task within the liver, as much is known about the over-expression of ABCB1 in cancer cells.[225] For example, in mice that possess two orthologous genes (*Abcb1a* and *Abcb1b*), knockout does not lead to a direct phenotype; however, several drugs did accumulate in these mice.[226] Additionally, investigations indicated that ABCB1 is involved in the export of short chain lipids albeit with low efficiencies.[222] So far, more than 300 substrates, modulators, and inductors have been described that interact with ABCB1. For a summary of these compounds, see refs 227−230. Additionally, polymorphisms appear to be medically relevant in ABCB1, as different variants can sometimes lead to altered drug excretion kinetics.[231] This is one reason why personalized medicine as treatment approach might also be an option for ABCB1.

The investigations, which led to structural insights into the transport mechanism of ABC transporters, were pioneering work for the entire family. ABCB1 was not the first ABC transporter, for which structural information became available, but was also next to Sav1866, a prototype of an ABC transporter.[108,232] Besides the structures of ABCC1, the structures of ABCB1 cover most of the individual states of a substrate transport cycle in humans, in the sense of different conformations including bound ligands. These structures comprise besides several structures of the apo state, the structurally more important ATP-bound and substrate-bound structures. To illustrate the different conformations of ABCB1, we chose some exemplary structures in Figure 6 (PDB entries 4M1M, 6QEE, 6C0V).[233−235] Table 1 contains more structures of ABCB1. The compendium of these structures allows to follow the complete transport cycle of an ABC transporter. The various conformations observed in these structures might also serve as a template for other members of the ABCB family. Again, the structure comprises a four-domain structure of two NBDs and two TMDs encoded on one gene in the canonical order of TMD1−NBD1−TMD2−NBD2. In the case of ABCB1, the NBDs harbor all conserved sequences motives in a canonical fashion and both NBS are active with respect to ATP hydrolysis.[236,237] Equally important, the two NBS alternately hydrolyze ATP. This mechanistic harmony is also reflected in the high ATP turnover of ABCB1 in the presence of a substrate.[235]

Unlike the NBDs, the TMDs are much more diverse in the ABC transporter family. They represent the transport pathway for substrates, and this is also where the substrate binding sites are located. In ABCB1, more than 30 amino acids have been identified to interact with substrates, although the exact number strongly depends on the substrate. However, they provide a flexible binding pocket, which ABCB1 requires to accommodate such a broad substrate spectrum.[238] Three-dimensional insights are provided by the two structures of ABCB1, in which one substrate (taxol, PDB entry 6QEX) or two inhibitor molecules (zosquidar, PDB entry 6QEE) are bound[234] (Figure 6, middle panel).

Because of these different structures of ABCB1, a detailed transport cycle can be proposed. In the ground state, the lateral gate between TMH 10 and 11 is flexible and allows substrates of different sizes to bind to the proteins interior.[239] Subsequent binding of ATP allows the transporter to switch to the outward-facing conformation. This conformation possesses a lower affinity to the substrate as in the inward-facing ground state. The parts of the transporter that do not interact with the membrane and point toward the extracellular medium are flexible and allow the final release of the substrate.[234] As a result, the export path is closed from the outside. The two ATP molecules stabilize the dimerized NBDs in the outward facing conformation[235] and ensure the release of the substrate. Subsequent ATP hydrolysis resets the entire system to the inward-facing ground state.

4.2. The PC-Lipid Floppase ABCB4

Although the structure of ABCB4 has been published only in 2019,[240] the function of ABCB4 was investigated since its finding in the late 1980s,[241,242] since ABCB4 is involved in a number of liver diseases (see section The ABC of Liver Diseases). ABCB4 was first identified as a membrane protein highly identical to ABCB1.[241,242] As stated above, both proteins share 76% identity and 86% homology, respectively. The highest degree of differences lies within the N-terminus and the linker region connecting both halves of this full-size ABC transporter. Therefore, it was initially assumed that both proteins perform the same physiological function. An

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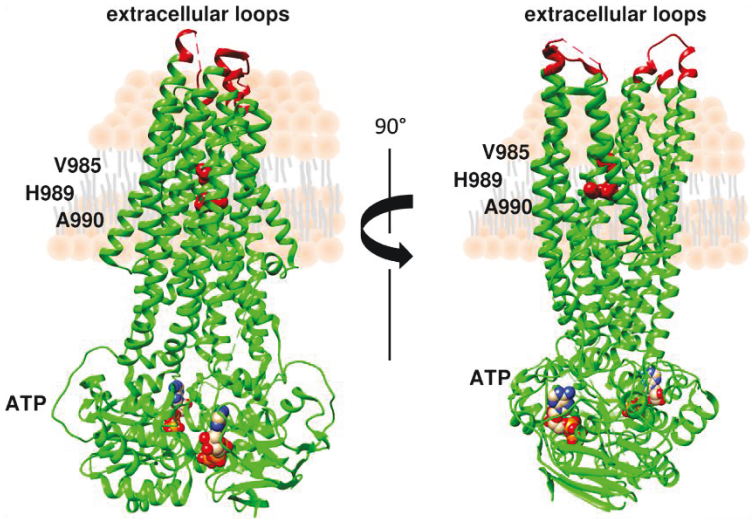

Figure 7. Structure of ABCB4 (human, PDB entry 6S7P,[266]). Left panel: frontal view, right panel: view after rotation by 90° as indicated. The structural areas, whose mutation led to a loss of function are marked in red.

assumption that turned out to be incorrect. While ABCB1 possesses a broad substrate spectrum and represents a classic multidrug resistance protein,[243] ABCB4 specifically but not exclusively, recognizes lipids of the PC family.[150,244] *In vitro* studies demonstrated that substrates of ABCB1 such as aureobasidin A, digoxin, paclitaxel, vinblastine, or ivermectin are recognized by ABCB4 heterologous expressed in yeast or pig cell lines.[245,246] Additionally, the ABCB1 inhibitors verapamil and cyclosporine also inhibited ABCB4 in cell culture.[246] This overlap in substrate spectrum suggests at least an artificial structural resemblance of some features of both proteins. However, *in vivo* studies demonstrated beyond any doubt that ABCB4 localized at the canalicular membrane is essential for the transport of PC lipids into the bile duct.[242,247−249] Especially for mouse ABCB4 (mABCB4, also termed mdr2), many studies investigated the function of (m/r)ABCB4.[250−257] For example, homozygous knockout mice did not contain cholesterol and PC lipids in their bile. Furthermore, bile composition changes in patients suffering from PFIC-3. In the heterozygous mutant mABCB4$^{+/-}$, approximately 60% of wildtype levels of PC lipid and nearly normal cholesterol levels were observed in bile. Subsequently, human ABCB4 was expressed in Abcb4$^{-/-}$ mice, which resulted in even higher phospholipid levels,[258] demonstrating that both perform the same function. It is now well accepted that ABCB4 recognizes PC lipids, i.e. choline headgroup.[150,259] In other words, lipids of different subfamilies bearing a different headgroup are not translocated[253,260] and showed no stimulation of ATPase activity in the case of purified ABCB4.[259] In contrast, a preference for a specific acyl chains are only poorly understood. One study demonstrated that short chain PC lipids are favorized in pig kidney epithelial cells transfected with human ABCB4.[150] In contrast to this study, another study indicated that ABCB4 might be a low affinity transporter and recognizes long chain acyl chains as well as unsaturated lipids.[261] Connecting both views a characterization of the ATPase activity of ABCB4 was performed concluding that every PC lipid productively interacts with ABCB4, while C 18 fatty acid esterification's with unsaturated chains do best.[244]

Is ABCB4 is "just" flopping PC lipids from the inner to the outer leaflet or it is lifting the PC lipid for subsequent extraction into the bile? So far, no experimental data exist that addresses this important question; however, the nearly total absence of PC lipids in *abcb4* knockout mice[255,262] and PFIC3 patients[263] suggests that ABCB4 may do much more than simply flopping PC lipids, but an extraction via bile salts is also plausible.[244] However, further investigations are necessary to understand ABCB4 in detail.

A study, expressing mABCB4 in yeast indicated that glycosylation is not necessary for expression, proper location, and function, respectively.[264] The two glycosylation sites for human ABCB4 are located between TMH1 and TMH2 at position 91 and 97.[241] For mABCB4, three glycosylation sites are known. These are also located in the first extracellular loop, but located at amino acid positions 83, 87 and 90.[251] In contrast to glycosylation, phosphorylation is essential for the function of ABCB4. Mutations with an altered phosphorylation pattern resulted in markedly decreased PC lipid translocation, despite the fact that the protein showed minor defects in maturation and localization.[265]

Recently, the closed conformation of the ABC transporter ABCB4 structure was published (PDB entry 6S7P).[266] ABCB4 has the characteristics of most eukaryotic ABC transporters.[267] The topology comprises a four-domain structure of 2 NBDs and 2 TMDs. The TMDs are defined by six α-helices, each. The typical "domain swap arrangement" is also present in ABCB4. TMH 4 and 5 of TMD1 reach over to the other half of the transporter and *vice versa* (TMH 10 and 11 for TMD2). In the two NBDs, two ATP molecules were observed in the typical head-to-tail dimer of the NBDs. Interestingly, only in the NBS1 an electron density for Mg^{2+} was found. Since Mg^{2+} is obligatory for ATP hydrolysis, the presence of Mg^{2+} may indicate an active NBS1 and a (temporarily) inactive NBS2. This structural asymmetry in the two NBS could not be shown for ABCB1. Whether ABCB4, like ABCB1, has two functional NBS that alternately hydrolyze ATP could not be deduced.[236] However, the structural asymmetry of the NBDs was extended by the H-loop, which differs in the two NBDs of ABCB4. The

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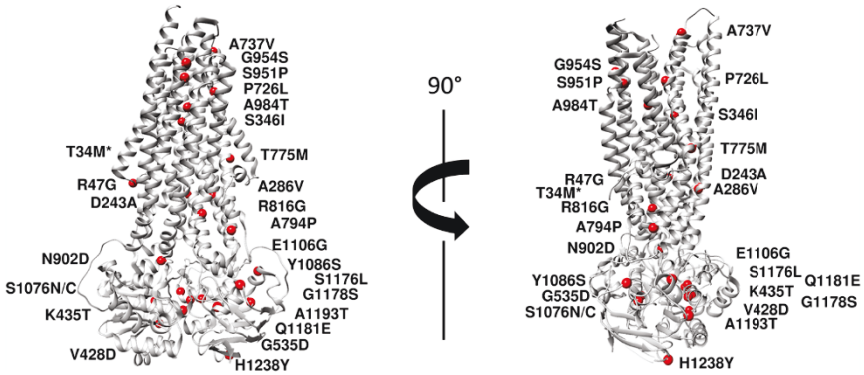

Figure 8. Structure of ABCB4. Disease-causing and potentially disease-causing mutations are highlighted in the structure of ABCB4 (human, PDB entry 6S7P,[266]) with refs 265, 266, 271−282. On the left, a frontal view, on the right the view after a rotation by 90°. Only the C_α atoms of the mutants are shown in red spheres. T34M* is not part of the structure. For the position S1076, two mutations (S1076N or S1076C) are described.[266,271]

H-loop was shown to be essential for ATP hydrolysis in the NBD of the bacterial ABC transporters HlyB.[268]

Olsen et al. proposed an "alternating access" mechanism for ABCB4.[109] This requires a nucleotide-bound outward conformation starting from an apo structure (without substrate) and resulting in an inward occluded structure (with substrate). Intermediate stages of ABCB4 could not been captured structurally so far. The ABCB4 structure is an exception in that it varies from the known conformations of ABCB1 as it resembles a closed conformation without bound substrate. Mechanistically, this conformation seems to represent a state immediately after substrate release.

The authors also tried to derive a mechanism from the high degree of relationship between ABCB1 and ABCB4. For this purpose, the substrate binding in ABCB1 was first examined. From the substrate and inhibitor bound conformations of ABCB1, 20 amino acids of the substrate binding pocket were defined. Most of them are also conserved in ABCB4,[234] except three (Figure 7). These residues, V985M, H989Q, and A990V, are located in the hydrophilic core of the protein. A triple mutant of these residue exchanging the three amino acid residues to the ones present in ABCB1 had a strong influence on substrate binding and inactivated the transporter.[266] The authors concluded that the hydrophobic cavity in the core of the proteins in ABCB4, analogous to ABCB1, is the substrate binding site. In another chimera, the extracellular loops of ABCB4 were exchanged with the extracellular loops of ABCB1. This chimeric transporter led to a loss of function as observed in whole cell extrusion assays (Figure 7).[240] Accordingly, the extracellular loops could support a reorientation of the PC lipids.

Mechanistically, however, there are still unresolved questions. How does the lipid from the inner leaflet enters the central cavity and leaves this hydrophobic binding pocket and how it ends up in the extracellular leaflet? A floppy state does not seem to be completely answered by a single conformation of ABCB4. A hydrophilic cavity was also observed between TMH 10 and 11 in the ABCB4 structure. Although sufficiently large for PC lipids, it is located slightly below the membrane interface and thus does not provide an optimal transfer pathway for a PC lipid to the cytoplasmic leaflet. This cavity might represent an allosteric modulation binding pocket for cytoplasmic ligands but is different to the lateral gate of ABCB1. Because of its localization at the cytoplasm-membrane interface, amphipathic substances would be particularly suitable to modulate the activity of ABCB4.

A preselection mechanism to select PC-lipids from the pool of phospholipids of the canalicular membrane has not yet been determined. A selection of lipids, which just takes place in the substrate binding pocket, i.e., in the hydrophobic cavity within the core protein, appears to be thermodynamically disadvantageous and could possibly lead to complications that slow down or even completely block the translocation process. In contrast, a mechanism of selection of phospholipids was reported for a P4-type ATPase[269] specific for PS lipids and might represent the starting point for further investigations to unravel how ABCB4 achieves specificity for PC lipids.

In general, however, the structure provides a basis to study functional ABCB4 deficiencies that lead to known clinical pictures associated with ABCB4 deficiency such as LPAC, ICP, and PFIC3.[149,270,271] Therefore, we summarized the known functional impairing mutations in Figure 8 and Table 4.

Originally, the mRNA of ABCB4 was detected especially in the liver hepatocytes (~70%) with low levels in the adrenal gland (~5−10%), lymph node (~7%), spleen (~6%), and heart (~4%), respectively.[283,284] On a protein level, mature ABCB4 is exclusively found in the canalicular membrane of hepatocytes.[285] Intuitively, this makes sense, since *m/rABCB4* expression in rodents is linked to bile acids.[256,286] Later, it was demonstrated that exclusive expression of ABCB4 in hepatocytes is due to the regulation of ABCB4 by the nuclear hormone receptor FXR (farnesoid X receptor).[287] Ligands of this receptor are mainly chenodeoxycholic and cholic acid. While the first one acts as a strong activator, the latter one is only a weak one. This could also answer the question of why ursodeoxycholic acid, a bile used as treatment in ABCB4 related diseases, did not modulate the transcription of ABCB4. In 2003, an FXR response element was identified in the human *ABCB4* gene.[288] In addition to FXR, the expression of ABCB4 is also under the control of a second nuclear hormone receptor, PPAR-α (peroxisome proliferator-active receptor alpha). Two studies demonstrated that fibrates induce the expression of mABCB4 in mice,[254,289] while PPAR-α double knockout mice displayed no response.[290] However, in humans, PPAR-α displays a different activation pattern toward fibrates than in mice. In a clinical trial, no induction of ABCB4

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Table 4. Overview of Disease-Causing and Potentially Disease-Causing (see also Figure 8) Mutations of ABCB4[b]

disease	mutation	ref
drug induced liver injury (DILI)	T34M	265
intrahepatic cholestasis of pregnancy (ICP)	T34M	265
	G535D	273, 282
low phospholipid- associated cholelithiasis (LPAC)	T34M	265
	R47G	265, 277
	A286V	275, 278
	S1176L	282
	G1178S	282
biliary cirrhosis (BC)	T34M	265
	D243A	274, 281
	K435T	276
	G535D	273, 282
progressive familial intrahepatic cholestasis type 3 (PFIC3)	S346I	272, 279
	P726L	279
	A737V	280
	T775M	275, 279
	G954S	272, 275, 279
	S1076N/C	266, 271
	A1193T	275
impaired nucleotide binding[a]	V428D	271
	E1106G	271
impaired TMD-NBD communication[a]	A794P	271
	N902D	271
	Y1086S	271
	Q1181E	271
impaired linker phosphorylation[a]	R816G	271
TMD instability[a]	S951P	271
impaired substrate translocation[a]	A984T	271
impaired catalytic activity[a]	H1238Y	271

[a]Potentially disease-causing mutations. [b]Mutations may appear more than once if mutations are linked to more than one disease. DILI - drug-induced liver injury, ICP - intrahepatic cholestasis of pregnancy, LPAC - low phospholipid associated cholelithiasis, BC - biliary cirrhosis, PFIC3 - progressive familial intrahepatic cholestasis type 3.

expression was observed in patients treated with bezafibrate (a fibrate known for lowering lipid levels).[291] Another study performed in HepG2 cells supported these results, but on the other hand observed a significant increase in the excretion of NBD-labeled PC into the pseudocanaliculi of these cells.[292] Thus, a hypothesis was put forward proposing the presence of an intracellular pool of ABCB4, which is mobilized to the canalicular membrane in the presence of bezafibrate. This is in line with the intracellular pools of ABCB11 and ABCG5/G8.[293] Interestingly, two studies demonstrated that the parenteral nutrition (TPN) affects the expression of ABCB4. In mice, TPN reduced *abcb4* expression, while *abcb11* expression was increased.[294] In contrast, in humans the absence of TPN caused a reduction in bile secretion and a particular dysfunction of ABCB4.[295]

A number of interaction partners are known for ABCB4, which are mainly responsible for trafficking.[285] The HS1-associated protein X-1 (HAX-1) is well studied in the case of endocytosis of ABCB11[296] and might perform the same function in the case of ABCB4. Furthermore, the myosin II regulatory light chain (MLC2) was identified as an interaction partner of ABCB4. MLC2 was necessary for proper trafficking of ABCB11,[297] but whether or not it performs the same function in the case of ABCB4 is currently unknown. A third interaction partner, the receptor for activated C-kinase 1 (RACK1), was identified and demonstrated as an essential protein for expression and targeting of ABCB4.[298] Furthermore, a PDZ domain-binding motif was identified at the C-terminus of ABCB4. This motif is known in CFTR and ABCC2 to link these transmembrane proteins to the cytoskeleton.[299−301]

4.3. The Bile Salt Export Pump ABCB11

Hepatocytes not only synthesize bile acids, they also take up bile acids, which underwent enterohepatic circulation.[302,303] This needs a coordinated action of distinct hepatocellular transporters located in both the sinusoidal and canalicular membrane of hepatocytes. Bile acid uptake occurs either in a sodium ion dependent manner mainly by NTCP or to a minor extent in a sodium-independent manner by members of the organic anion transporting polypeptides family.[304−308] In contrast, bile acid secretion across the canalicular membrane is mediated by an ATP-binding cassette (ABC) transporter, ABCB11, or bile salt export pump (BSEP). Additionally, this transporter represents the rate limiting step of hepatocellular bile acid secretion.[309−311]

Mammalian ABCB11 was identified in 1995[312] and functionally described in 1998 as the sister of P-gp,[309] mediating the transport of conjugated bile acids. In the same year, the gene of *ABCB11* was identified as the causative reason for progressive familial intrahepatic cholestasis type 2 (PFIC-2).[313]

Early after the discovery of the *ABCB11* gene, Gerloff et al. demonstrated that this ABC transporter specifically transports bile acids with a preference for taurine conjugations.[309] However, it is also capable of transporting glycine conjugated bile acids, which are the major form of bile acids in humans. Additionally, the absence of certain bile acids in the bile of PFIC2 patients confirmed the function and significance of this transporter.[313,314] Importantly, findings obtained in rodents can be extrapolated to humans since mice, rat, and human ABCB11 possess similar kinetic parameters.[315] However, one always has to keep in mind that rodent and human ABCB11 have different substrate specificity,[316] probably due to different ratios of bile acid conjugations present in both organisms. For example, human ABCB11 prefers taurine conjugated over glycine conjugated bile acids, although glycine is the major form of bile acid conjugation in humans. Furthermore, ABCB11 distinguishes between the primary bile acids: chenodeoxycholic acid containing two hydroxyl groups is preferred over cholic acid containing three hydroxyl groups.[315] Additionally, human ABCB11 transports taurolithocholate, while rat BSEP does not.[316] *In vitro* studies, on the other hand, demonstrated that ABCB11 transports also substances such as taxol,[317] pravastatin,[318] vinblastine, or calcein AM,[319] which are prominent substrates of ABCB1. Transport of sitosterol was however not observed, although it is structurally related to cholesterol and bile acids.[320] Nevertheless, the physiological function of ABCB11 remains the ATP dependent export of bile acids into the bile duct, which is emphasized by the absence of nearly 100% of bile acids in the bile of patients with severe forms of intrahepatic cholestasis due to mutations of the *BSEP* gene.[314] To address the impact of clinically relevant mutations in ABCB11 that affected the transport function, Stindt et al. developed an overexpression protocol in the

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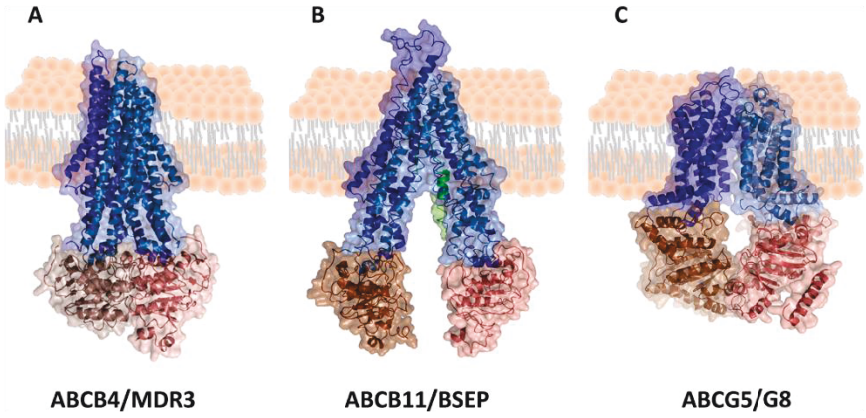

Figure 9. Structures and surface representation of the ABC transporter of the bile triumvirate colored by their domains. (A) The full-size transporter ABCB4/MDR3 (PDB entry 6S7P[266]) in the outward facing conformation with dimerized NBDs. (B) ABCB11/BSEP (PDB entry 6LR0[328]) in the inward facing conformation and totally separated NBDs. Both are colored according to their four domains: TMD1 in blue, NBD1 in brown, TMD2 in sky-blue, and NBD2 in salmon. Additionally, the unusual positioned N-terminus is highlighted in green in ABCB11. (C) AGCG5/G8 (PDB entry 5DO7[111]) are shown in using the following color code: TMD and NBD of G5 in blue and brown, respectively. TMD and NBD of G8 in sky-blue and salmon, respectively.

methylotrophic yeast *Pichia pastoris*.[321] This was subsequently employed to established a purification protocol[322] and determined the membrane interactome of ABCB11. Additionally, the impact of mutations within ABCB11 were analyzed,[323] and a transport rate of at least eight bile acids per second and transporter was determined.[324]

The ATP-dependent excretion of bile acids across the canalicular membrane and the post-translational modification of this ABC transporter is modulated by two different compounds that are not substrates. The first compound is cholesterol.[325] Cholesterol is an integral part of the cellular membrane as well as bile. Additionally, it is the substrate of ABCG5/G8, which is the third ABC transporter within the bile ABC triumvirate. The transport efficiency for human, rat, and mouse ABCB11 were analyzed in vesicles derived from baculovirus-infected Sf9 cells overexpressing ABCB11. The membrane of Sf9 insect cell contain less cholesterol compared to the canalicular membrane of hepatocytes. Overall, the kinetic parameters (v_{max} and K_m) of ABCB11 were determined for four different bile acids at endogenous cholesterol levels of Sf9 plasma membrane vesicles and after the addition of 1 mM cholesterol. v_{max} values for all four bile acids increased in the presence of additional cholesterol, while K_m values remained unchanged, and the general preference toward these four bile acids was preserved for human and mouse ABCB11. Quantitatively, cholesterol showed the largest effect for rat ABCB11 and the smallest effect for human ABCB11,[325] while expression levels were not influenced in the presence of additional cholesterol. Moving one step further, Guyot et al. fine-tuned the concentration of cholesterol in the membrane via depletion with cyclodextrins and also observed a correlation between the concentration of cholesterol and transport efficiency of ABCB11.[326] The second compound, which affects the function of ABCB11, is lipopolysaccharide (LPS).[327] It is a component of the cell wall of Gram-negative bacteria and can cause cholestasis, which is accordingly called endotoxin- or sepsis-induced cholestasis. Normally, LPS activates macrophages by binding to the CD14 receptor. In contrast, Kupffer cells are the primary targets of LPS in the liver. Kupffer cells respond to LPS with the secretion of different proinflammatory cytokines such as TNF-alpha, IL-1beta, IL-6, and IL-10. These cytokines bind to receptors on the sinusoidal membrane of hepatocytes and initiate a complex intracellular signaling pathway. Elferink et al. investigated the effect of LPS on the level of mRNA and correct trafficking of ABCB11, NTCP, and ABCC2 in rats and human, respectively. In humans, mRNA levels of NTCP were downregulated, while this was not the case for ABCB11 and ABCC2. In contrast, none of the three transporters was detected in the membrane of liver slices after LPS treatment, suggesting that LPS effects cellular trafficking of ABCB11 and ABCC2.[327]

Recently, the structure of ABCB11 determined by single particle cryo-EM was published in the inward facing state without any bound nucleotide or substrate (PDB entry 6LR0)[328] (Figure 9). The topology and overall structure correlate well with structures of other members to the ABCB subfamily. The full-size transporter ABCB11 harbors the four typical domains of ABC transporters, two NBDs and two TMDs. Each TMD is composed of six TMHs. First observed in the crystal structure of Sav1866[108] (Figure 3A) and subsequently also in all structures of members of the ABCB subfamily, the TMDs are split into two parts as already described above for other members of the B subfamily. Another important aspect of the ABCB11 structure concerns the N-terminus, which is different from all known structures of ABCB transporters.[329−331]

The N-terminus (highlighted in green in Figure 9) is extended and locates to the region of the putative substrate binding pocket of ABCB11. This is in line with the ABCB1 homologue from *Caenorhabditis elegans*.[332] Here, the N-terminal region adopts a helical hairpin, which inserts into the translocation cavity. However, and in contrast to ABCB1 from *C. elegans*, the N-terminus of ABCB11 adopts an α-helix followed by a loop that is located at the top of the putative substrate binding cavity. To analyze the function of the N-terminus in more detail, the authors created a truncated ABCB11 version lacking the N-terminus and determined the ATPase activity in the absence and presence of a bile acids for both wildtype and truncated ABCB11. Interestingly, no difference in ATPase activity was observed, but the expression

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level of ABCB11 was reduced 2-fold for the truncated version. The remaining part of the structure of ABCB11 is highly similar to the canonical structures of the ABCB subfamily. It possesses a large first extracellular loop, and both NBDs harbor the classical signature motives (see section A General Introduction to ABC Transporters in Humans). It is safe to assume that NBS1 is inactive, since the highly conserved glutamate of the Walker B motive is substituted by a methionine. The second NBS contains all catalytically important amino acid residues and is active. Since their structure represents a first snapshot, one has to assume that ABCB11 uses the "alternating access" mechanism[109] to translocate bile acids from one side of the canalicular membrane to the other. To deduce mechanistic insights, more structural snapshots such as a nucleotide-bound conformation or substrate bound structures are required.

Nevertheless, the first structure of human ABCB11 provides important insights into the three-dimensional architecture of ABCB11 and represents an excellent starting point for further structure−function analysis aimed to understand the mechanism of bile acid translocation.

The *ABCB11* gene is located on the chromosomes 2q24−31[313] and encodes for a glycosylated protein of 1321 amino acids,[309] which is spread on 28 exons.[315,333] Its expression is regulated by a complex network of stimuli acting directly or indirectly on the promoter of ABCB11. The predominant regulator is the bile acid sensor FXR.[334−336] FXR regulates not only the expression of ABCB11, but also the expression of NTCP and the bile acid biosynthesis system.[335−337] FXR regulates the expression of target genes by binding as a heterodimer together with the retinoid X receptor alpha (RXRα). RXRα is activated by the vitamin A derivative 9-cis retinoic acid. In a recent study, cholate feeding of mice with vitamin A deficiency resulted in a marked induction of mABCB11 expression at the protein and mRNA level.[338] In other studies, it was observed that vitamin D3 repressed mABCB11 expression,[339] and in contrast ursodeoxycholic acid upregulated mABCB11 expression as it functions as an agonist for FXR.[340,341] Finally, the canalicular P-type ATPase ATP8B1 (FIC1) activates the promoter of BSEP in parallel with an upregulation of FXR.[342]

However, mature ABCB11 is localized in the canalicular membrane of hepatocytes in humans. It either derives from the Golgi, which is regulated by the p38 MAP kinase,[343] or for short-term regulation from rapid inserting transporter-containing vesicles.[344] Interestingly, ABCB11 was also detected in the testis on mRNA level in humans,[345] but Northern blotting of ABCB11 in pig,[312] rat,[309] and mouse[319,346] revealed a predominant expression in the liver.[345]

Recent studies have presented evidence for the presence of distinct microdomains in the canalicular membrane of rat hepatocytes.[347] These microdomains are characterized by a high cholesterol and sphingolipid content. ABC transporters including ABCB11 were found to partition into caveolin-1 enriched microdomains.[348] There is also evidence for an intracellular pool of ABCB11 that can be mobilized if higher secretion rates of bile acids are required.[293]

5. THE ABCG SUBFAMILY - THE REVERSE TOPOLOGY HALF-SIZE TRANSPORTER SUBFAMILY

5.1. ABCG2 - Brother in Arms with ABCB1

ABCG2, also known as breast cancer resistance protein (BCRP) or methotrexate resistance protein, was discovered about 20 years ago.[349,350] It is a half-size ABC transporter that requires homodimerization to form a functional unit[351] and is part of the body's chemo immunity defense system.[352−356] It is not only expressed in tissues with detoxifying function such as the liver, but also in stem cells, fulfilling a protective role.[357] In addition to ABCB1 and the MRPs, ABCG2 is the major multidrug exporter. Accordingly, it is not surprising that ABCG2 is on the US Food and Drug Administration list for drug−drug interactions, as a transporter to be monitored.[2] Especially stem cells, but also cancer cells, overexpress these drug exporters, whereby the apparent drug concentration within the cell is reduced by continuous export. As a consequence, many compounds are exported before they reach their destination, the cytoplasm, or other cellular organelles.

On the basis of studies with stem cells, ABCG2 was considered as a stem cell marker, which led to the development of a diagnostically used antibody (5D3)[358] for rapid identification of stem cells. However, only with the elucidation of the structure of ABCG2,[359] it was clarified how the 5D3 antibody affects protein activity: the 5D3 antibody binds to one extracellular loop (ECL3) of each monomer.[359] However, the binding of one monomer is sufficient to limit the conformational-switch from the inward-facing conformation to the outward-facing conformation, thus arresting ABCG2 in the inward-facing conformation.

Independent of its role in stem cells, ABCG2 plays a central role in the detoxification function of the human body. ABCG2 is complemented in the liver by the functional relatives, ABCB1 and MRP2, as mentioned above.[352−356] ABCG2 is localized to the canalicular membrane of hepatocytes.[360] Here, it was shown in primary human hepatocytes that exposure to certain nuclear receptor ligands results in increased levels of ABCG2 mRNA similar to many other hepatobiliary transporters.[361,362] Especially, the constitutive androstane receptor (CAR) seems to transactivate ABCG2 expression.[363]

Furthermore, ABCG2 has an even broader substrate spectrum than ABCB1. The spectrum of substrates of ABCG2 covers hydrophobic, partially water-soluble, but also charged substances. Physiologically, it is now assumed that ABCG2 plays a minor role in uric acid metabolism.[364,365] Genome studies have shown that ABCG2 variants that lead to ABCG2 deficiency are associated with gout.[366,367] Gout is characterized by the crystallization of uric acid in joints, which can lead to joint hardening. On the other side, hormone derivatives such as estradiol sulfate or estradiol glucuronides are very poor substrates of ABCG2.[368] Cholesterol, a precursor of these hormones, seems to be a decisive modulator of the transporter. Depletion of cholesterol or the reconstitution of ABCG2 in artificial membrane systems without cholesterol led to a reduced ATPase and transport activity.[369−371] Experimental evidence that ABCG2 is indeed a sterol transporter including cholesterol and estradiol stems from the observation of a biphasic activation pattern of the ATPase activity of ABCG2 similar to substrates interacting with ABCB1 and the identification of a functional estrogen response element in the human *BCRP* promoter.[372,373] However, cholesterol, bile acids,

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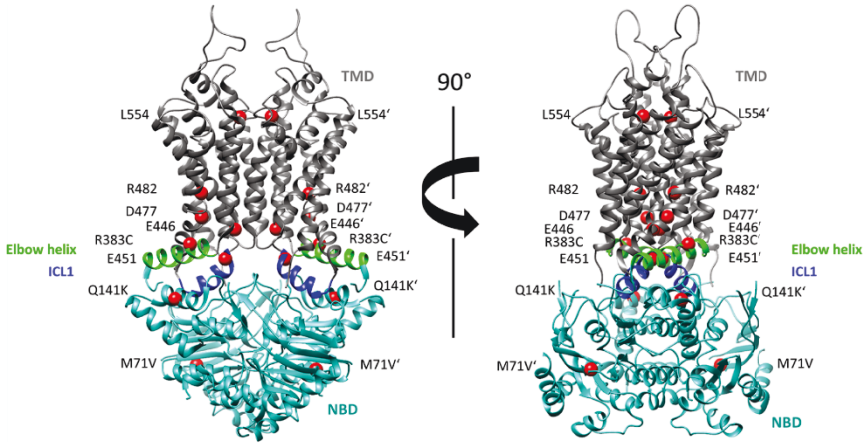

Figure 10. Structure of ABCG2 (PDB entry 6HZM)[359] Highlighted are crucial amino acids necessary for proper function (R482, E451, E446, and D477), prominent disease-causing mutations (M71V, Q141K, R383C) and the "plug" leucine L554 which separates the two cavities. The right panel is the same functional homodimer of ABCG2 after a 90° rotation. Amino acids with primes indicate the opposing monomers amino acids.

or detergents such as CHAPS may also just modulate the activity of ABCG2 without being true substrates.[371,374] Further contradictory observations were made, fluorescence-labeled bile salts accumulated in cells expressing ABCG2,[375] but this observation was not confirmed in studies using radioactive-labeled bile acids.[374] Nevertheless, the detergent effect of bile salts may separate the homodimer into monomers, and this can already have a detrimental effect on the function of ABCG2.[371,374]

Moreover, ABCG2 belongs to the class of type II exporters (Figure 10). In contrast to the ABCB subfamily, its fold is characterized by the absence of a domain swap.[359,376] Another striking feature is the proximity of the two NBDs in the apo state, which is likely due to the shortened dimensions of the TMDs compared to type I exporters.[359,376]

In summary, the ABCG family in general is characterized by a more compact form (exemplary ABCG2 in Figure 10), which is more reminiscent of bacterial import systems than its human functional relatives such as ABCB1 as the prototype of the B subfamily.[377,378]

The cryo-EM structure (PDB entry 6HZM) provided strong evidence that ABCG2 substrates can already access the transporter from within the membrane.[359,376] This model was supported by mutational and functional studies of the transporter.[364] Binding of substrates directly from the membrane was structurally confirmed by the observation of a central cavity (central cavity 1), which is accessible from the cytoplasm as well as from the inner leaflet of the membrane.[359,376] It is still a matter of debate whether substrates are more likely to be flopped, i.e., leave the protein on the extracellular half of the membrane, or whether the extracellular loops allow direct export into the extracellular space.[379] In addition to cavity 1, a second cavity closer to the extracellular space was identified in the structure. Here, an undefined electron density was observed, which was interpreted as two cholesterol molecules.[359] The two cavities are separated by leucine residues from opposing monomers (L554, Figure 10), which serve as a plug.[359] This second cavity is located directly beneath ECL3 and is not accessible from the extracellular site. It appears to be a cavity with lower substrate affinity, which becomes accessible by a conformational change and allows substrate release. Finally, cavity 2 is thought to be a shared feature of the ABCG subfamily.[359]

ABCG2 is stabilized by intra- and intermolecular disulfide bridges are present, intramolecular between C592−C608 within one monomer, and intermolecular C603−C603′ of opposing monomers.[359] Other stabilizing elements are the salt bridges between E451-K473 and E458-R383 in the so-called elbow helix.[376] Mutations in this region led to an almost complete loss of function.[376] The amphipathic elbow helix anchors the protein in the membrane and brings the NBD in close proximity to the water−membrane interface.[376] ICL1 can be seen as the equivalent of the classical coupling helix because it plays a crucial role in the regulation of NBD dimer formation.[376] Important for the function are the amino acids R482, E451, E446, and D477 (Figure 10).[376] R482 mutations alter the substrate specificity, but presumably by allosteric interactions, because it is located about 9 Å away from the binding pocket. E451 can be mutated to E451D and thus show increased ATPase activity, but loses transport efficiency. This mutation may be a first indication of decoupled transport; i.e., ATPase activity independent of substrate presence may build a functional bridge from ABCG2 to its close relative Pdr5 from *S. cerevisiae*, where decoupled ATPase activity occurs.[376,380] Indeed, the authors classified ABCG2 as an uncoupled transporter.[359,376]

Mutations of E446 and D477 also lead to an impaired ATPase and transport activity. While the amino acids discussed so far were artificial, the mutations discussed next have been identified in patients. We have made a selection of medically relevant ABCG2 variants and refer the interested reader to a recent publication for a more comprehensive collection of ABCG2 mutations.[381] The most well-known ABCG2 variant is Q141K, which is associated with hyperuricaemia.[382] This mutant leads to decreased ABCG2 expression, misfolding, and subsequent degradation by endoplasmic reticulum-associated proteins. Although the variant is still functional *in vitro*, a repulsion of the positive charge of lysine and TMH1 of the opposing monomer leads to misfolding, and the quality control of the cell degrades this variant.[376,383] Interestingly, the deficiency of this variant led to the discovery of the physiological substrate uric acid in a genome-wide study.

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Less relevant is the M71V variant, which is also characterized by misfolding and trafficking problems, which again led to degradation.[384] Another group of variants results in truncations that lead to a complete absence of ABCG2 from the plasma membrane. Examples for this effect are Q126X[385] in Asian populations or Q236X and R385C. Here, X implies a translation termination codon, which leads to a truncated and unfunctional polypeptide. This lack of ABCG2 in the plasma membrane of red blood cells leads to the rare blood type Jr^{-}.[386]

5.2. ABCG5/G8-Cholesterol Efflux into the Bile

Cholesterol is a key component of mammalian cell membranes and makes up to ~40% of the total lipid content of the plasma membrane.[387] Additionally, cholesterol serves as the precursor molecule for steroid hormones that modulate gene regulation and for bile acids. Since only a few cells have the capacity to metabolize cholesterol, elimination through biliary and intestinal secretion is essential to maintain homeostasis. Sterols (especially cholesterol) in peripheral tissues are mobilized to high density lipoproteins (HDL) and delivered to the liver for elimination by the reverse cholesterol transport (RCT) pathway as described in detail by Tall et al.[388] Therefore, the elimination of cholesterol is essential for proper function of liver. ABC transporters are major sterol exporters responsible for both cholesterol efflux from peripheral cells and the elimination of excess cholesterol and dietary sterols.[242,389] The human ABC transporter subfamily G is mostly responsible for sterol (especially cholesterol) transport. ABCG1 and ABCG4 mediate cholesterol trafficking in the plasma membrane and endosomes[390,391] and are believed to regulate cholesterol homeostasis in the brain and the macrophage-rich tissues. ABCG5 and ABCG8 function as a heterodimer and are mainly responsible for cholesterol transport across the canalicular membrane of hepatocytes. Nevertheless, there is evidence for expression of ABCG5/G8 in the intestine and gallbladder in addition to its expression in the liver.[104,392,393]

ABCG5/G8 was discovered in the context of sitosterolemia, a disease in which plant sterols accumulate, while cholesterol levels remain normal (see section The ABC of Liver Diseases). In 1998, the so-called sitosterolemia gene locus (STSL) was localized.[392,394] Subsequently, two teams simultaneously identified not one but two genes located in the STSL locus.[104−106] These genes encoded for the two highly homologous half-size ABC transporters, ABCG5 and ABCG8.

Similar to ABCB4 and ABCB11, mouse models were used to pinpoint the function of this heterodimeric ABC transporter. mABCG5- or mABCG8-deficient mice showed the same phenotype as human patients.[393,395] Loss of (m)ABCG8 in mice and men resulted in increased intestinal absorption of noncholesterol sterols and cholesterol. In addition, knockout of either m*Abcg8* or both genes led to a failure to secrete these sterols into bile. It was unclear at that time whether or not mABCG5 or mABCG8 might have independent function. Overexpression of human ABCG5 and ABCG8 in mice led to supersaturation of cholesterol in bile, with excretion of large quantities of neutral sterols, reduced plasma plant sterol levels, and a compensatory up-regulation of the cholesterol synthesis rates.[396] Furthermore, in these animals lower levels of HDL plasma cholesterol and lower cholesterol absorption rates were observed, probably due to an increased bile efflux by human ABCG5/G8. Subsequently, a study demonstrated that overexpression of both genes in hepatocytes was associated with a reduction in the production of apoB-lipoproteins and atherosclerosis.[397] Surprisingly, overexpression of ABCG5/G8 did not lead to artificial gall stone disease, although supersaturation of cholesterol was measured in the bile of these mice. Therefore, it was concluded that the heterodimer of both half-size transporters functions as a sterol efflux of both cholesterol and xenosterols, in liver and the small intestine. Unfortunately, the exact mechanism of how these transporters provide efflux is yet not completely understood. However, it is known that bile acids play a major role. Bile acid-containing micelles were found to be an acceptor of these sterols.[398−400] Furthermore, bile acids are essential for the function of ABCG5/G8 in the hepatobiliary system.[401] In the case of the purified transporter, ATP hydrolysis was only observed if bile acids were present.[402] This suggested that bile acids directly interact with ABCG5/G8 or that the transporter somehow recognizes that cholesterol acceptors are present. The latter hypothesis was proposed by Small, who postulated that ABCG5/ABCG8 may act as "extruder", exposing sterols to the outer leaflet of the membrane for facilitated extraction into the lumen of sterol acceptors such as bile acid: phospholipid complexes.[403] Others have suggested that ABCG5/G8 acts as a floppase similar to ABCB4, just flipping cholesterol from the inner to the outer leaflet of canalicular membranes.[404,405] To support or refute one of these models, a study was conducted, which demonstrated that forced biliary secretion of very hydrophobic bile acids failed to increase biliary cholesterol secretion in $Abcg8^{-/-}$ mice since it was not possible to extract cholesterol from the rigid canalicular membrane.[406] Although these data support the "flop hypothesis", a subsequent lifting of cholesterol from the outer leaflet is not disproven by this study.

The crystal structure of heterodimeric ABG5/G8 was published in 2016 at a resolution of 3.9 Å (PDB entry 5DO7, see Figures 3 and 12).[407] Surprisingly and as already elaborated in section A General Introduction to ABC Transporters in Humans, the expected domain swap arrangement was not present. Rather, the structure resembled the fold of type I ABC importers.[408] Each TMD composed of six TMH resembled a more or less isolated entity with the putative substrate-binding pocket at the interfaces. This moderate degree of amino acid conservation is contrasted by a high degree of structural conservation reflected by a root mean squared deviation (rmsd) of only 2.0 Å. Interestingly, ABCG5/G8 harbors more α-helices in its extracellular loops, which again reflect the higher similarity to type I ABC importer systems. The lack of electron density for nucleotides and the separation between opposing NBDs indicates that the ABCG5/G8 structure represents a nucleotide-free state in the inward facing conformation, which is similar to other nucleotide-free structures of ABC exporters[79,409,410] and importers.[145,411] Nonetheless, the two NBDs contact each other at the extreme cytoplasmic end to interact through a pair of NPXDF motifs. These motifs are conserved in the ABCG family and are required for cholesterol efflux by ABCG1.[412] Another feature of ABCG5/G8 is the asymmetry of the NBDs. As stated in section A General Introduction to ABC Transporters in Humans, each transporter comprises two NBS, in which the Walker A motif of one NBD with the ABC signature motif of the other NBD[413,414] come together to interact with ATP. ABCG8 contains a degenerate Walker A motif (GSSGCGRAS, see Table 2). Additionally, ABCG5 possesses a degenerated ABC signature motif (ISTGE, see Table 2). As a consequence, one NBS is degenerated, while the

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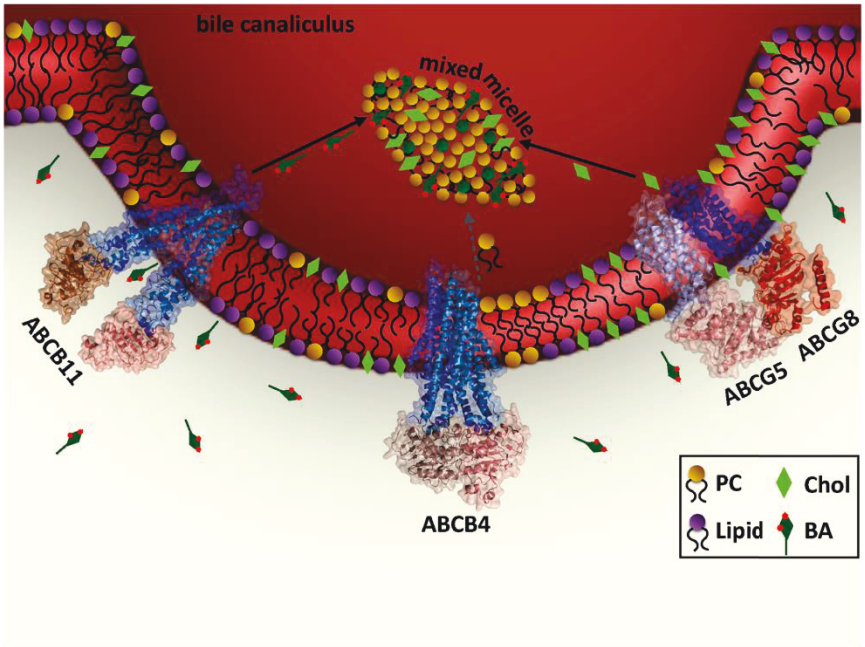

Figure 11. Bile triumvirate of the canalicular membrane. The ABC transporter ABCB11 and ABCG5/G8 are involved in the secretion (black arrows) of the bile compounds bile acids (BA) and cholesterol (Chol) across the canalicular membrane (violet lipid can represent any other kind than PC), respectively. In concert with PC-lipids (PC), which are flopped by ABCB4 from the inner to the outer leaflet, these three compounds from mixed micelles in the bile canaliculus. This prevents bile acids from solubilizing cell membranes and cholesterol from crystallization. However, it is not yet known how PC-lipids are taken up into the mixed micelles (gray dashed arrow). The full-size transporter ABCB4 (PDB entry 6S7P) and ABCB11 (PDB entry 6LR0[328]) are colored by their four domains: TMD1 in blue, NBD1 in brown, TMD2 in sky-blue and NBD2 in salmon, respectively. AGCG5 and G8 (PDB entry 5DO7) are shown as dimer in corresponding colors: TMD and NBD of G5 in blue and brown, TMD and NBD of G8 in sky-blue and salmon, respectively.

other is canonical, i.e., actively hydrolyzing ATP. This catalytic asymmetry is supported by functional studies using recombinant ABCG5/G8 mutants in ABCG5/G8-deficient mice.[415] In cases in which the Walker A or Walker B motif in ABCG5 was mutated, biliary sterol secretion was abolished. On the other hand, mutations of the corresponding residues in ABCG8 showed no effect on the biliary sterol secretion. For the ABC signature motif, the opposite effect was observed. Mutations on the ABC signature motif of ABCG5 had no effect on the biliary sterol secretion, while mutations of the corresponding residues in ABCG8 abolished sterol secretion.[416]

As elaborated above, this functional asymmetry is mirrored by a structural asymmetry. In brief, the degenerated signature motif of ABCG5[416] is located next to the CnH/CpH/E-helix bundle. In contrast, the canonical signature motif of ABCG8 is positioned near the three-helix bundle of G8. This arrangement suggested that the stable three-helix bundle of ABCG5 acts as an inflexible body, whereas the three-helix bundle of ABCG8 exhibits greater flexibility. With the proximity of G8-CpH to the catalytically active NBD, these conformational changes could allosterically link ATP hydrolysis to sterol transport.[407]

The structure of ABCG5/G8 harbors additional electron density, which might represent cholesterol.[407] Assuming that this electron density represents indeed cholesterol, an efflux mechanism was proposed. According to this proposal, the binding pocket of ABCG5/G8 is located at two symmetrical "vestibules" on opposing faces of the TMD dimer. Additionally, a lateral gate toward the lipid bilayer, which extends to the center of the dimer interface is present. One of the two vestibules is flanked by TMH1 and TMH2 of ABCG5 as well as TMH4, TMH5, and TMH6 of ABCG8. The other vestibule is formed by the inverse arrangement (TMH1 and TMH2 of ABCG8 as well as TMH4, TMH5, and TMH6 of ABCG5). Additionally, a kind of "ceiling" is formed in each vestibule by an α-helix from an extracellular loop pointing into the membrane. Several residues of the vestibule are highly conserved and may line an entry site for sterols to access the core of the heterodimer interface. To prove this proposal, a conserved amino acid of ABCG5 was mutated (Y432A). Although no complete absence of cholesterol transport was observed in mice, it was drastically decreased compared to the wild type transporter.

On the basis of a molecular dynamics (MD) simulation of ABCG5/G8, an upward movement of the TMDs and an inward movement of the NBDs occur simultaneously.[407] This result was supported by an evolutionary analysis. Here, several coevolved pairs of amino acids were identified, which are conserved but more than 8 Å apart in the apo structure. During a transport cycle, these residues might come in contact. However, several studies of ABCG5/G8 indicated an asymmetric use of the two NBDs,[415,416] the requirement of bile acids for sterol efflux[400,401] and ATP hydrolysis.[402] Thus, more structural information is required for this cholesterol transporter to understand the transport cycle on the molecular level.

The genes of ABCG5 and ABCG8 are in close proximity (only approximately 370 bp apart) and arranged in a head-to-

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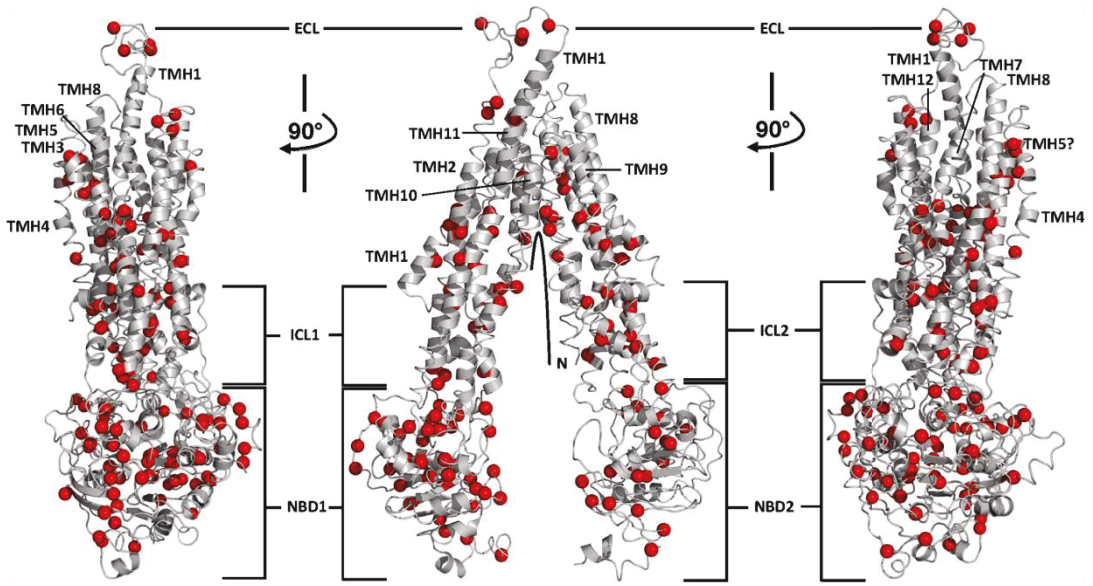

Figure 12. ABCB11 structure with highlighted mutations. The red spheres highlight the positions of disease-causing mutations in ABCB11, which impair function. Because of the large number of identified mutations in ABCB11, mutations, which affect expression, degradation, splicing, and trafficking were not taken into account. This lasso resulted, for reasons of clarity, that not every position was labeled with the corresponding mutation. Rather only the TMHs, ICDs, and NBDs were labeled. According to this scheme, a complete list of the mutations is provided in Table 6. 1: these amino acids are in a flexible region (the loop connecting NBD1 with TMD2) and therefore not resolved in the single particle cryo-EM structure (PDB 6LR0[328]). Left panel: the transporter was rotated by 90° with respect to the left panel for better visualization of all mutants.

tail orientation.[104] Both indicate that they share a common promotor and regulatory elements. The latter have been identified within the intergenic region, including the nuclear transcription factor hepatocyte nuclear factor 4 alpha (HNF4α), GATA4, GATA6,[417] and liver receptor homologue-1 (LRH-1).[418] HNF4 α seems to be the major reason for tissue specific expression of ABCG5/G8 and can be partially explained by epigenetic regulation. In tissues that do not express ABCG5/G8, chromatin in the regulatory region is methylated and histones are acetylated.[419] Although no specific response elements have been identified for FXR, FXR plays a prominent role in transcriptional control of ABCG5/G8 via bile acid signaling.[420] Additionally, administration of cholic acid in mice enhances ABCG5/G8 transcription. One study demonstrated that FXR may be partially mediated by fibroblast growth factor 15/19 (FGF15/19), while the other observed an inhibition of nuclear factor kappa-light-chain-enhancer of active B cells (NF-κB).[421] Two additional liver receptor response elements have been identified in the *ABCG5/G8* genes. Interestingly, one study demonstrated that insulin signaling can regulate expression of ABCG5/G8 via forkhead box protein O1 (FOXO1).[422]

Recombinant expression revealed that both transporter halves (ABCG5 and ABCG8) are glycosylated, depend on calnexin and calreticulin chaperones proteins for folding, and require dimerization to exit the endoplasmic reticulum.[423,424] Trafficking of the complex to the canalicular membrane is poorly understood, but there is evidence that bile acids, sterols,[395,425] and cAMP[426] are important factors. Similar to ABCB11, an intracellular pool of ABCG5/G8 transporters might exist for cases in which cholesterol secretion has to be increased. In the case of the latter, these transporters can be mobilized to increase cholesterol efflux as it was suggested by Guyot and Stieger.[293]

5.3. The Bile Triumvirate ABCB4, B11, and ABCG5/G8

Primary bile mainly consists of bile acids, PC lipids, cholesterol, which form mixed micelles, bilirubin glucuronides, and organic anions. Analysis of bile from healthy humans revealed that the bile in the gallbladder consists of bile acids (~70%), PC lipids (~20%), and cholesterol (~8–9%).[427] Each of these substances is transported by an individual ABC transporter into the canaliculus. Bile acids by ABCB11, PC-lipids by ABCB4, and cholesterol by ABCG5/G8 (Figure 11).

In the previous chapters, we provided detailed information about these liver ABC transporters. Here, we will briefly summarize details about the interplay of these three ABC transporters. Already in 2003, Small concluded that these three ABC transporters interplay in a specific order to ensure the proper formation of bile.[403] Bile acids in the canaliculi promote the release of PC lipids from the membrane to form mixed micelles, which are necessary for solubilization of cholesterol to prevent its crystallization.[403] It was reported that the reduction of PC lipids in primary bile leads to an increase of the toxic activity of bile acids against the ducts, which can result in cholestatic liver diseases.[428] This study emphasized how important a balanced ratio of PC lipid to bile acid and cholesterol is. For ABCG5/G8, one study demonstrated that *in vitro* ATPase activity is absent without any bile acids present.[402] This suggests that ABCG5/G8 sense the substrate of ABCB11 and becomes transport competent. Following the same line, ABCB11 responds to the concentration of cholesterol in the membrane.[325,326] Elegant studies by the Stieger laboratory demonstrated that administration of taurocholate increases the rate of PC secretion in canalicular vesicles derived from rats.[309] These examples clearly suggest a

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Table 5. Diseases Caused by ABCB11 Mutations in Correlation with Figure 12[a]

disease	localization	mutations			refs
PFIC2	N-terminus	G19R	L50S		271, 310, 313, 314, 432−436, 438, 442, 551−579
	TMH1	M62K	C68Y		
	ECL	C107R	I112T	W114R	
		Q121L			
	TMH2	Y157C	A167V	A167T	
	ICL1	I182K	M183V	M183T	
		G188W	V284L	E297G	
		R303G	R303K	Q312H	
		R313S	A390P		
	TMH3	M217R	R223C	S226L	
		G238V			
	TMH4	T242I	A257G		
	TMH5	G327E	W330R	C336S	
		Y337H	W342G		
	TMH6	A382G	R387H		
	NBD1	G410D	L413W	I420T	
		D440E	G455E	K461E	
		T463I	R470Q	V481E	
		R487H	R487P	N490D	
		I498T	I512T	N515T	
		R517H	R517T	F540L	
		I541L	I541T	F548Y	
		D549V	G556R	A570T	
		L581F	A588V	S593R	
		I627T	E636G		
	Connecting Loop[1]	R698C	R698H	S699P	
		E709K			
	TMH7	G758R	G766R		
	TMH8	Y818F			
	ICL2	R832C	R832H	T859R	
		A865V	Q869R	R948C	
		A1044P	L1055P		
	TMH9	G877R			
	TMH10	S901R			
	TMH11	N979D	G1004D		
	TMH12	T1029K	G1032R		
	NBD2	C1083Y	A1110E	S1114R	
		G1116E	G1116F	G1116R	
		S1120N	R1128C	S1144R	
		S1154P	N1173D	N1211D	
		V1212F	L1242I	D1243G	
		A1283V	G1292V	G1298R	
BRIC	TMH2	E135L	E137L		271, 310, 430−435, 438, 451, 454, 553, 557, 558, 560−563, 565, 566, 570, 571, 576, 578, 580−582
	ICL1	R181I	E186G	L198P	
		A277E	E297G		
	TMH6	G374S			
	NBD1	R432T	V444A	I498T	
		A570T	T586I	G648V	
		T655I			
	TMH10	T923P			
	ICL2	A926P	R948C	R1050C	
	TMH11	G1004D			
	NBD2	G1116R	R1128H	L1197G	
		R1231Q			
ICP	ICL1	V284D			271, 274, 430, 432, 433, 435, 438, 485, 563, 572, 581−585
	NBD1	R415Q	V444A	N591S	
		T655I			
hypothyroidism (elevated TSH)	TMH1	L71H			553

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Table 5. continued

disease	localization	mutations		refs
pediatric hepatocellular carcinoma	ICL1	A389P		454, 586
	ICL2	R1050C		
DILI	NBD1	V444A	N591S	271, 430, 433, 571

[a]Listed are only these mutations, which impair protein function and sorted according to the disease that they cause. Mutations, which affect expression, degradation, splicing, and trafficking were not taken into account.

crosstalk between the transporters of the ABC triumvirate. However, we still lack molecular insights into a concrete crosstalk, and many more details are completely unknown.

6. THE ABC OF LIVER DISEASES

6.1. Diseases Related to ABCB11

Over 300 mutations and polymorphisms in the ABCB11 gene have been identified so far, which cause hereditary liver diseases. Fortunately, an up-to-date database provides the available information on mutant variants of ABC transporters including ABCB11 (see http://abcmutations.hegelab.org/).[429] Additionally, a number of reviews focused on ABCB11 mutations and provide huge lists of the known disease-causing ABCB11 variants or tried to map these in different models.[271,430−438] Although all regions of ABCB11 are affected, an apparent hot spot for mutations are the NBDs and cytoplasmic loops (Figure 12 and Table 5).[271,436]

Progressive familial intrahepatic cholestasis (PFIC) represents a number of inherited cholestatic liver diseases, which typically reveal during early childhood[434,439] and likely lead to liver cirrhosis, often resulting in the need of liver transplantation (LTX).[440] As stated above, PFIC2 is based on mutations in the ABCB11 transporter (see section Progressive Familial Intrahepatic Cholestasis (PFIC2) and Benign Recurrent Intrahepatic Cholestasis Type 2 (BRIC2)). Interestingly, it was shown in 2009 that children who are transplanted because of PFIC-2 have a special risk for disease recurrence. The reversion of the symptoms is caused by the *de novo* appearance of anti-BSEP antibodies.[441,442] The risk of disease recurrence after LTX depends on the cause of liver failure. In the case of hepatitis C infection, liver damage always recurs, although the severity can be altered due to the host immune system, the virulence of the virus, or the regimen of immunosuppression.[443,444]

About 10 years ago, a recurrence of PFIC2 phenotypes after LTX was described.[441,442] After a patient underwent a second LTX, a cholestatic liver situation again developed after one year, although no evidence of rejection or other causes of cholestasis were detected. Finally, analysis of the posttransplant serum revealed the presence of antibodies directed against ABCB11, which were absent in the pretransplant serum. Obviously, *de novo* anti-ABCB11 antibodies developed after LTX,[445] which impair the efflux of bile acids by binding to the first extracellular loop of ABCB11.[441,446] As a therapy, reduction of serum antibodies by plasmapheresis and anti-CD20 antibodies (rituximab), which temporarily alleviated cholestasis,[441] was achieved. Subsequently, further cases of anti-BSEP antibodies were reported,[447−450] and it is estimated that up to 8% of transplanted PFIC-2 patients develop anti-BSEP antibodies.[448]

These findings raised the question of why no autoimmune disease for ATP8B1 and ABCB4 (recurrence of PFIC1 or PFIC3) has been detected so far. One hypothesis is that, due to the low abundance of ABCB11 in PFIC2 patients,[434] they do not achieve autotolerance. After LTX, however, the massive availability of ABCB11 may stimulate BSEP-reactive T lymphocytes, which lead to clonal expansion of anti-BSEP antibody-producing B cells. Consequently, the absence of ABCB11 in the patients is a requirement or indication to develop an autoimmune disease against ABCB11.[441,451]

6.2. Progressive Familial Intrahepatic Cholestasis Type 2 (PFIC2) and Benign Recurrent Intrahepatic Cholestasis Type 2 (BRIC2)

These two diseases have in common that they are hereditary and caused by loss of function of ABCB11. In both diseases, ABCB11 fails to export bile acids due to a complete lack or insufficient amount of functional ABCB11 in the canalicular membrane of hepatocytes. This leads to intrahepatic cholestasis characterized by reduced bile synthesis, less bile flow, and accumulation of bile acids within the hepatocyte.[452] Several mutations in ABCB11 are the causative reason for PFIC2 and/or BRIC2. These include the relatively frequent E297G and D482G variants, which are present in 58% of European PFIC2 patients.[434]

PFIC is an early onset disease with a rapid progression leading to end stage liver damage before adulthood. Defective ABCB11 can lead to impaired bile acid secretion, accumulation of bile acids in hepatocytes, and subsequent hepatocellular injury, apoptosis, or necrosis. Missense mutations cause less severe disease forms, whereas the risk of hepatobiliary malignancy is increased in the case of mutations leading to truncations of ABCB11.[453] PFIC patients often develop pediatric hepatocellular carcinoma and subsequently cholangiocarcinoma.[454,455] BRIC, in contrast, is the milder form of PFIC and comes with recurrent episodes of cholestasis and consequently pruritus without substantial liver damage in most cases.[431]

The clinical features of the patients usually include cholestatic jaundice in the neonatal period. Pruritus is usually the dominant feature. Clinical features and disease progression may be more severe in PFIC2 than in PFIC1. Other features may include growth failure and deficiency of fat-soluble vitamins. These patients are at considerable risk of hepatobiliary malignancy, hepatocellular carcinoma, or cholangiocarcinoma, and thus close surveillance is important for these patients.[434] The diagnosis is based on elevated bile acids, normal serum GGT, electron microscopy showing amorphous, or filamentous bile. Light microscopy shows nonspecific giant cell hepatitis. Immunohistochemical staining for canalicular BSEP is negative in majority of patients.[453] In general, these patients have poor response to ursodeoxycholic acid. Partial external biliary diversion (PBED), which interrupts the enterohepatic circulation by partially diverting bile from the gallbladder through a loop of jejunum connecting the gallbladder to abdominal skin, may be useful and if performed early enough may delay or interrupt hepatic injury. Liver

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transplantation is usually successful in this group of patients, although recurrence after transplantation has been reported.[442]

6.3. Acquired BSEP Deficiency Syndrome (Intrahepatic Cholestasis)

Inhibition of ABCB11 by endogenous metabolites such as aberrant bile acids[456] or drugs (see section Drug Induced Liver Injury (DILI)) leads to the so-called acquired BSEP deficiency syndrome. People with genetic or acquired defects in the biosynthesis of bile acids may suffer from cholestasis. In patients with a deficiency of steroid dehydrogenase/isomerase, aberrant bile acids are observed in urine and bile. Other non-natural bile acids are detected in the urine of patients with a deficiency of SP-reductase. It was observed that aberrant bile acids can inhibit the apical, ATP-dependent transport system for cholyltaurine in a competitive manner. These unusual bile acids act as cholestatic agents by inhibiting the canalicular ATP-dependent transport system for bile acids, which constitutes the rate-limiting step in the overall process of bile acid transport across hepatocytes and thereby leads to intrahepatic cholestasis[456]

6.4. Drug Induced Liver Injury (DILI)

Drug induced liver injury is different to most of the other liver diseases, in which ABC transporters are involved. While in the other cases the transporter is mutated or expression, proper trafficking, or membrane insertion is reduced, it is commonly accepted that DILI affects the wildtype situation. Additionally, patients who suffer from DILI normally also suffer from a more prominent disease, for which a drug is administered. This drug, especially if it is given orally and thereby taken up in the duodenum, perfuses the liver as the first organ. If this drug is taken up into the hepatocytes, it can potentially act as an inhibitor for the ABC transporter in hepatocytes. Especially if one of the ABC transporters involved in bile formation is affected, the delicate equilibrium between bile acids, PC lipids, and cholesterol is changed. This can result in symptoms as if the patient suffers from a liver disease related to one of these ABC transporters. That is the reason why a lot of drugs and treatments are tested for their effect on different ABC transporter, for example, ABCB11. For a more detailed investigation to DILI, we refer the interested reader to two recent reviews.[457,458]

6.5. ABCB4 - A Transporter with Trending Relevance for Hepatobiliary Diseases

In a sequence analysis of an Icelandic population, ABCB4 was the only hepatobiliary transporter correlating to an increased risk for a liver disease if deficient.[459] In general, diseases linked to ABCB4 deficiency can be sorted based on altered expression levels, misfolding, stability, or activity.[282] Here, the reduction in PC lipid presence in the bile correlates with the severity of the underlying ABCB4 mutation. As a result, free bile salts that would be otherwise safely packed into mixed micelles may lead to bile duct injuries, a principle that is known as the "toxic bile concept".[460] Mild forms of cholestasis related to ABCB4 deficiency are indicated by elevated serum levels of γ-GT.[270] Intermediate forms of cholestasis, which involve ABCB4 deficiencies are intrahepatic cholestasis of pregnancy (ICP) and low phospholipid associated cholelithiasis (LPAC). Even though the genetic background may be diverse (see next chapter - Intrahepatic Cholestasis of Pregnancy (ICP)) common fetal as well as maternal symptoms like pruritus can occur.[461,462] The fetus may be at risk of preterm birth or even fetal death. Even though ICP is reversible, patients may be at higher risk for subsequent hepatobiliary diseases.[463] Symptoms for LPAC are intrahepatic hyperechogenic foci, sludge, or microlithiasis probably via an increased biliary-cholesterol to phospholipid ratio resulting in cholesterol crystallization.[464] A disease of modern pharmaceutic treatment could lead to drug-induced liver injuries (DILI), in which drugs administered to cure even liver-unrelated diseases negatively interact with hepatobiliary transporters such as ABCB4.[465] Last but not least, progressive familial intrahepatic cholestasis type 3 (PFIC-3) is the most severe form of ABCB4 deficiency. It is described by a complete absence of biliary phospholipids and injured bile canaliculi. Symptoms are jaundice, discolored stool, hepatomegaly, splenomegaly, and pruritus.[466]

In fact, the severity of ABCB4 deficiency is not only based on the mutation itself, but also on the status of zygosity. Admittedly, heterozygous mutations are less severe than homozygous ones. One key to fight genetic diseases is therefore a comprehensive summary of mutants linked to empirical data of patients or even better to their molecular basis. A genetic investigation for example connected homozygous, compound heterozygous, and heterozygous mutations of ABCB4 with molecular reasons based on MD simulation.[271] In recent years, several drugs were investigated to cure ABCB4 deficiency; the success however is strongly linked to the underlying severity of the mutation.[279,282,462,467,468]

6.6. Intrahepatic Cholestasis of Pregnancy (ICP)

Liver dysfunction during pregnancy represents a unique challenge to health care providers and is detected in approximately 3% of all pregnancies.[469] However, severe liver injury is encountered in only a small subset of these patients.[470] Early recognition and appropriate management are warranted to avoid potentially serious consequences for both the mother and the fetus. Other major considerations are the teratogenic effect of certain medications and the risk that pregnancy may impose on any underlying liver condition. Symptoms may vary according to the severity of liver injury from abdominal pain, nausea, and vomiting to signs of acute liver failure. Common physical examination findings in pregnancy are palmar erythema and spider angiomas, and their presence may not indicate an underlying chronic liver disease. Normal changes seen in pregnancy should be considered in the interpretation of liver function test (LFT).[469] Alkaline phosphatase can be elevated in the third trimester due to placental production. Alpha fetoprotein (AFP) is not routinely obtained in this group of patients; however, it is produced by the fetal liver and commonly elevated.[471] Differential diagnosis include diseases unique to pregnancy, pregnancy in the setting of underlying chronic liver diseases, and coincidental conditions to pregnancy such as viral hepatitis, and Budd-Chiari.[472] Gallstones are commonly seen during pregnancy and liver ultrasound remains the best initial diagnostic imaging modality, followed by magnetic resonance imaging without gadolinium[473] and liver biopsy.[474,475]

ICP is the most common liver disease to appear during pregnancy and typically arise during the fourth to six week of pregnancy.[476] The incidence of ICP is higher among pregnant women infected with hepatitis C virus, which raises the concern for the need to screen all patients with ICP for HCV.[477,478] The other risk factors for ICP include personal or family history, multiple pregnancies, advanced maternal age,

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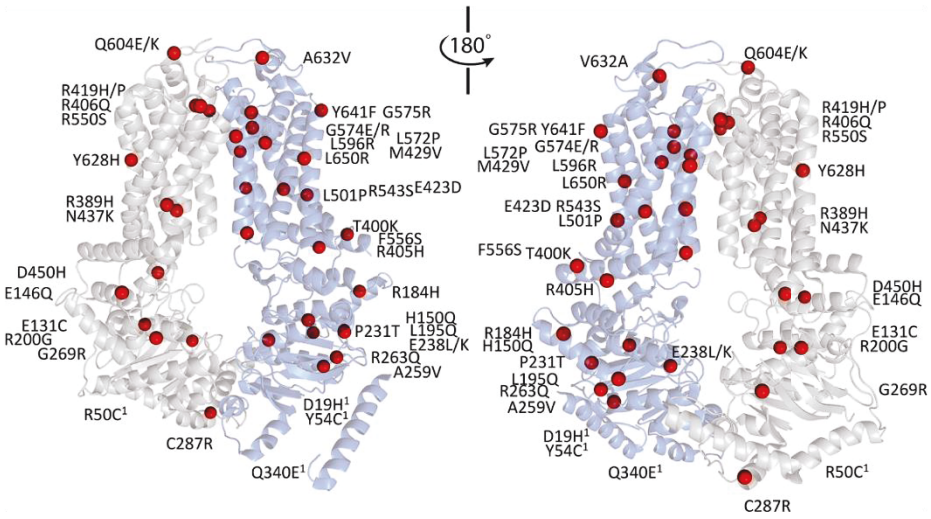

Figure 13. ABCG5/G8 structure with highlighted mutations. Left panel of the central structure of the ABCG5/G8 transporter is the monomeric ABCG5 structure (gray), in the right panel is the structure of monomeric ABCG8 (sky-blue). Red spheres correspond to positions of disease-causing mutations. 1: these amino acids are in flexible regions such as the two termini or loops and therefore not resolved in the X-ray structure (PDB 5DO7[111]). The transporter was rotated by 180° for better visualization of all mutants.

and cholelithiasis. Estrogen and progesterone, genetic mutations in bile acid transport proteins (such as ABCB11), and environmental factors play a role in the pathogenesis of ICP.[479−484] In the case of ABCB11, the well-known V444A polymorphism is a predisposition for ICP.[485] Additionally, mutations in ABCB4 are often observed in ICP.[486,487]

A study from 2015 demonstrated that ICP (due to an *Abcb11* deficiency) is associated with adverse neonatal survival and is estimated to impact between 0.4 and 5% of pregnancies worldwide. The neonatal death occurs within 24 h due to atelectasis-producing pulmonary hypoxia, which recapitulates the neonatal respiratory distress of human ICP. In neonates of Abcb11-deficient mothers elevated pulmonary bile acids could be obtained, which altered the pulmonary surfactant structure. These findings have important implications for neonatal respiratory failure, especially when maternal bile acids are elevated during pregnancy.[488]

As treatment so far only the application of ursodeoxycholic acid (UDCA) is successful. It consistently reduces the symptoms and improves LFTs.[489,490] The mechanism of action is not completely understood, but studies have demonstrated that after treatment there is a reduction in total serum bile acids in both, maternal and umbilical cord serum, and a qualitative change in the serum bile acid pool.[341,491,492] For more detailed information we recommend excellent reviews such as refs 469, 484, 493−496.

6.7. Diseases Related to ABCG5/G8

Abnormal elevations in plasma cholesterol contributes to hyperlipidemia, a critical factor leading to cardiovascular diseases and other metabolic disorders.[497] Twenty-four variants are known for ABCG5, which are related mostly to sitosterolemia. In ABCG8, 40 mutations have been identified so far. Again, the majority of these variants is related to sitosterolemia. The review by Tada et al. provides a recent and excellent overview of the localization of ABCG5/G8 mutations and polymorphism on DNA level[498] and we mapped these mutations on the crystal structure of ABCG5/G8 (Figure 13).

For ABCG5, it is obvious that there are three hot spots next to the NBD (Table 6). The first one covers amino acid residues E146 and D450 in the coupling helices. Since the coupling helix of ABCG5 are supposed to be inflexible due to the asymmetry in the NBDs (see section "Structure of ABCG5/G8") it can hardly suggest how these mutations affect the transporter. The second hot spot (R389 and N437) is positioned at the lower end of the TMD. But they are in the polar relay of ABCG5 and conserved polar residues that form the TMD polar relay proximal to CpH and CnH.[111] The third one comprises the three arginines R406, 419, 550, and glutamate Q604, which are located at the extracellular part of the TMD and the external loops. They might be important for cholesterol release. In the case of ABCG8 (Table 7), the majority of the mutations, which are not located in the NBD,

Table 6. Diseases Caused by ABCG5 Mutations in Correlation with Figure 13[a]

disease	mutations		refs
sitosterolemia	E146Q	R419H	105, 106, 502, 503, 587−594
	G269R	N437K	
	C287R	D450H	
	R389H	R550S	
	R406Q	Q604E	
	R419P	Q604K	
familial hypercholesterolemia[b]	Y131C		595
	R200G		
	Y628H		
	E146Q		
gallstone disease	R50C		105, 502, 503, 593, 594
	Q604E		
	Q604K		
low HDL-C level	Q604E		594

[a]Mutations may appear more than once if mutations are linked to more than one disease. [b]Only the pathogenic are listed.

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Table 7. Diseases Caused by ABCG8 Mutations in Correlation with Figure 13[a]

disease	mutations		refs
sitosterolemia	R184H	L501P	104, 106, 498, 591, 593, 594, 596−603
	L195Q	R543S	
	P231T	L572P	
	E238L	G574E	
	E238 K	G574R	
	A259V	G575R	
	R263Q	L596R	
	Q340E	Y641F	
	R405H	L650R	
	E423D		
familial hypercholesterolemia[b]	H150Q	R543S	595
	E238L	F556S	
	R263Q	G574E	
	R405H	G575R	
	E423D		
gallstone disease	D19H	M429V	498−503, 591, 594, 602, 604−606
	Y54C	V632A	
	T400K		
high LDL-C levels	D19H		502, 503, 604, 498, 499, 501, 591, 594
	T400K		
	V632A		
renal disease in type 2 diabetic patients	T400K		607

[a]Mutations may appear more than once if mutations are linked to more than one disease. [b]Only the pathogenic mutations are listed.

are spread throughout the entire TMD. Nevertheless, they can be divided into four groups. First, there are amino acids of the coupling helices (H150 and R184), which possibly abolish the communication between NBD and TMD. Other amino acids (E423, L501, and R543) are located at the polar relay of ABCG8.[111] The third hot spot is located at the upper end of the TMHs facing toward the possible gate and ABCG5. It is compromised of the amino acids M429, L572, G574, and G575, G574, and G575 are part of the heterodimer interface and abolish dimerization, if mutated. The last group of mutations are located in the TMD facing the lipid bilayer. It includes the amino acids T400, Y641, and L650. The T400K mutation sits on the tip of the CnH helix and is a risk factor for gallstone disease.[499,500] Interestingly, only one mutation (V632) occurs in the external loops of ABCG8, but is localized at the same height and position as Q604 in ABCG5. Mutation in both lead to gall stone disease.[500−502] Nevertheless, it was shown that only Q604 is associated with cholesterol kinetics.[503]

6.8. Sitosterolemia

Sitosterolemia is a rare, monogenic, autosomal recessive disease characterized by the presence of plant sterols (the major type of xenosterols) and premature coronary artery disease in the face of relatively normal plasma cholesterol level.[504] When plant sterols are ingested, they compete with the bulk cholesterol for solubilization, thereby reducing dietary absorption of cholesterol and lowering plasma cholesterol.[505] However, the majority of xenosterols that enter enterocytes are immediately excreted via ABCG5/G8 back into the intestinal lumen. Human subjects that fail to actively prevent xenosterol absorption develop sitosterolemia named after the most abundant dietary xenosterol, sitosterol. The disease was first described by Bhattacharyya and Connor.[506] In addition, sitosterolemia is also characterized by hypercholesterolemia, premature cardiovascular disease, hematologic manifestations, arthritis and in rare cases hepatic failure. Hypercholesterolemia-induced premature cardiovascular disease can manifest as premature coronary heart disease or sudden cardiac death.[506] Hemolytic anemia, splenomegaly, bleeding disorders, and macrothrombocytopenia can result from the accumulation of plant sterols in platelet membranes, producing hypertrophic and hyperplasic dysfunctional platelets.[392,394,507,508] Recent studies in tissue-specific ABCG5/G8-knockout mice revealed that activity in either organ is sufficient to protect from sitosterolemia; however, cholesterol elimination is partially compromised.[509] ABCG5/G8 is the primary sterol transporter responsible for the elimination of dietary neutral sterols through transintestinal cholesterol efflux.[510] What remains unclear is whether ABCG5/G8 possesses a selectivity between cholesterol and xenosterols.

6.9. Gallstone Disease

Gallstones have a prevalence of 10−15% in adults[511] in the United States and Europe. However, about 75% of these adult patients have no symptoms. Nevertheless, gallstone disease generates major economic and social burdens if symptoms or complications occur.[511−513] In Western countries, gallstones are comprised mainly of cholesterol in 75−80% of the cases, and are often associated with systemic abnormalities.[514] There are five primary defects, which play a critical role in the pathogenesis of cholesterol gallstones:[513] (1) predisposing genetic factors, (2) hepatic hypersecretion of cholesterol (resulting in supersaturated gallbladder bile), (3) rapid phase transitions of cholesterol in bile, with the precipitation of solid cholesterol crystals, (4) impaired gallbladder motility with hypersecretion and accumulation of mucin gel in the gallbladder lumen and immune-mediated gallbladder inflammation, and (5) intestinal factors involving absorption of cholesterol, slow intestinal motility, and altered gut microbiota

Lifestyle and dietary factors influence the pathogenesis of gallstone disease because the mean energy intake may be higher in gallstone patients than in control subjects.[515] Thus, diet and lifestyle have a potential role in primary prevention of cholesterol gallstones. The European Society for the Study of the Liver panel concludes that healthy lifestyle and food, regular physical activity, and maintenance of an ideal body weight might prevent cholesterol stones and symptomatic gallstones.

7. CONCLUDING REMARKS

The liver is, beyond any doubt, the central organ for glucose and lipid metabolism, *de novo* bile acid synthesis, and detoxification mechanisms. This of course requires an intense communication with the "surrounding cells, tissues, and organs". Thus, membrane transport proteins perform a pivotal role. Interestingly, hepatobiliary transporters of the ABC superfamily are concentrated in the canalicular membrane (Figure 1). Here, they perform the expected export function, e.g., drug export (ABCB1 and ABCG2), the bile triumvirate (ABCB4, ABCB11, and ABCG5/G8), or transport of metabolites (by the members of the ABCC subfamily).

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Tremendous progress was made over the last few decades in our molecular understanding of the structure and function of hepatobiliary ABC transporters. This includes among many other things, the expression levels, environmental clues regulating these levels, the precise role of these transporters in many diseases of the liver (Table 1), their substrates or spectrum of substrates, and in particular even in the last five years their three-dimensional structure (Figure 14). We have

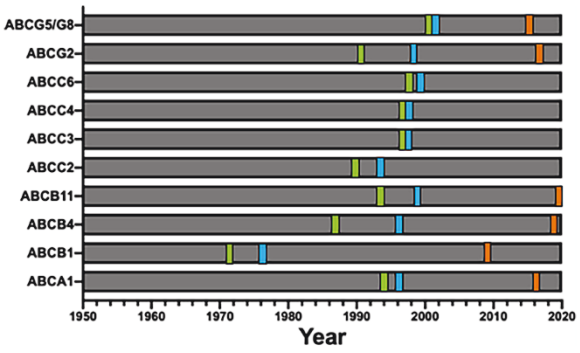

Figure 14. Timeline of important insights into the structure and function of hepatobiliary ABC transporters. The identification of the genes is highlighted in green, while the disease caused by mutations in the ABC transporter genes is given in blue. The year, in which the first structure of a hepatobiliary ABC transporters was determined, is highlighted in orange. Please note that the *x* axis is on year scale.

tried in this review to summarize this knowledge. However, we are fully aware that such an endeavor cannot cover every aspect and every detail. We thus apologize to all our colleagues whose work was not cited appropriately.

Obviously, structural information is a prerequisite for a molecular understanding of the entire transport cycle (Figure 14). Especially, the developments in single particle cryo-EM have had an important impact on membrane protein structure determination. Thus, it does not come as a surprise that most of the structures of hepatobiliary ABC transporters have been determined in the last five years. However, with the exception of ABCB1, we do not have sufficient structures to define an entire transport cycle. It is mandatory to capture all conformations of the different states of a membrane transporter at appropriate resolution. Only than a molecular picture will emerge that defines the changes in space that underlie transport of a compound across a biological membrane. Here, homology models will not necessarily be helpful since many of the transporters do have significant sequence identities, which would make these systems ideal candidates for homology modeling. However, the function is often very different as exemplified by ABCB1 and ABCB4 (76% sequence identity), and it is likely that such models will provide a correct overall picture, but fail to answer the important functional questions.

Structural data however have to be complemented with knowledge about the molecular transitions in time. How fast is a substrate recognized? How fast does occur translocation? How is the hydrolysis of ATP in the NBD coupled to substrate transport in the TMDs? These answers require *in vitro* systems, in which biophysical techniques such as single molecule Förster resonance energy transfer (FRET) or electron paramagnetic resonance (EPR) can be applied. And these approaches should be complemented by molecular dynamics simulations. Examples of such approaches exist for ABC transporters,[516−518] but have to be applied to the ABC transporter present in liver. But also, classic biochemical transport assays are needed to provide quantitative data for these systems. To complicate the situation even further, the elegant studies generating and using double and/or triple transfected cell line have demonstrated that hepatobiliary ABC transporters are not isolated entities. Rather, molecular communication and networks exist that fine-tune their function to the physiological demands of the liver. Only if we obtain a molecular understanding covering space, time, and networks, we will be able to efficiently fight liver diseases and promote liver regeneration when necessary. In summary, we have witnessed an impressive increase of knowledge on hepatobiliary ABC transporters, but the future will still hold many surprises as well as exciting but also unexpected insights.

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Notes

The authors declare no competing financial interest.

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Martin Prescher studied Biochemistry in Germany at the Heinrich Heine University in Düsseldorf and obtained his bachelor's degree in 2014 and his master's degree in 2016, respectively. Currently, he is a Ph.D. candidate, and his doctoral thesis deals with the in vitro characterization of ABCB4 a human ABC transporter of the liver.

Sander Smits obtained his Ph.D. from the Heinrich Heine University in 2008. Since 2008, he has been a senior scientist at the Institute of Biochemistry and initiated and founded the Center for Structural Studies in 2018 of which he is the managing director.

Lutz Schmitt obtained his Ph.D. from Technical University Munich in 1996 and moved as a postdoctoral fellow to Stanford University. In 1999, he started his independent research group (Emmy Noether program of the DFG) first at the Philipps University Marburg and since 2001 at the Institute of Biochemistry at the Goethe University

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Frankfurt. In 2004, he obtained a Heisenberg fellowship of the DFG and was appointed as Associate Professor at Heinrich Heine University Düsseldorf. In 2008, he was promoted to Full Professor of Biochemistry.

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ABBREVIATIONS

ABC	ATP-binding cassette
ATP	adenosine triphosphate
BCRP	breast cancer resistance protein
bp	base pairs
BRIC-2	benign recurrent intrahepatic cholestasis type 2
BSEP	bile salt export pump
cAMP	cyclic adenosine monophosphate
CAR	constitutive androstane receptor
CFTR	cystic fibrosis transmembrane conductance regulator
cGMP	cyclic guanosine monophosphate
CH	coupling helix
cMOAT	canalicular multispecific organic anion transporter
cMRP	canalicular multidrug resistance-associated protein
CnH	connecting helix
DILI	drug induced liver injury
ECL	extracellular loop
EPR	electron paramagnetic resonance
FGF15/19	fibroblast growth factor 15/19
FOXO1	forkhead box protein O1
FRET	Förster resonance energy transfer
FXR	farnesoid X receptor
GATA4/6	GATA binding protein 4/6
GSH	gluthathione
HAX-1	HS1-associated protein X-1
HCC	hepatocellular carcinoma
HCC	hepatocellular carcinoma
HDL	high density lipoproteins
HepG2	hepatoma G2 cell line, human carcinoma cell line
HlyB	hemolysin B
HNF4α	hepatocyte nuclear factor 4 alpha
HSC	hepatic stellate cells
ICL	intracellular loop
ICP	intrahepatic cholestasis of pregnancy
IF	inward facing
KC	Kupffer cells
LPAC	low phospholipid-associated cholelithiasis
LPS	lipopolysaccharide
LRH-1	liver receptor homologue-1
LSEC	liver sinusoidal
LTC4	leukotriene C4
LTX	liver transplantation
MATE	multidrug and toxin extrusion
MaVEC	macrovascular
MD simulation	molecular dynamics simulation
MDR	multidrug resistance protein
MLC2	myosin II regulatory light chain
MRP	multidrug resistance-associated proteins
NBD	nucleotide binding domain
NBS	nucleotide binding site
NF-κB	nuclear factor kappa-light-chain-enhancer of active B cells
NTCP	sodium taurocholate cotransporting polypeptide
OATP	organic anion transporter peptides
OF	outward facing
PC lipids	phosphatidyl choline lipids
PDZ	combining the first letters of the first three proteins: PSD95, Dlg1, zo-1
PDZ	combining the first letters of the first three proteins: PSD95, Dlg1, zo-1
PFIC1/2/3	progressive familial intrahepatic cholestasis
PPAR-α	peroxisome proliferator-active receptor alpha
PP_i	pyrophosphate
PS lipid	phosphatidylserine lipid
PXE	pseudoxanthoma elasticum
RACK1	receptor for activated C-kinase 1
RCT	reverse cholesterol transport
rmsd	root-mean-square deviation
RXRα	retinoid X receptor alpha
SLC	solute carrier
STSL	sitosterolemia gene locus
SUR1/2	sulfonylurea receptor1
TAP	transporter associated with antigen processing
TMD	transmembrane domain
TMH	transmembrane helix
TPN	the parenteral nutrition
VLDL	very-low-density lipoprotein

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2 Aims

In the literature, ABCB4 has been described as an ATP binding cassette transporter that translocates PC-lipids from the inner to the outer leaflet of the canalicular membrane. Thus, ABCB4 creates the typical accumulation of PC-lipids in the outer leaflet of the canalicular membrane of hepatocytic cells of the liver. Despite this knowledge, it is not clear how ABCB4 ensures that the membrane is sufficiently resistant against the harsh detergent activity of bile salts which are present in the bile canaliculus at millimolar ranges.

To investigate isolated ABCB4 a suitable *in vitro* test system was required. Preliminary work was already achieved by Ellinger *et al.* and Kluth *et al.*, who used *Pichia pastoris* as an overexpression host and established a protein purification protocol. The task of this thesis was to use these tools and to further characterize ABCB4 biochemically in order to put the results into a medically relevant context.

Van Helvoort *et al.* observed that while ABCB1 showed no phospholipid preference and transported all tested fluorophore or radioactively labelled short-chain phospholipids, ABCB4 could only transport fluorophore labelled PC-lipids. This indicated that although ABCB4 is now confirmed as a specific PC-lipid floppase, ABCB4 was not able to transport the short-chain 8:0-8:0 PC-lipid. To clarify this observation, two main objectives were proposed for this work.

First, it had to be clarified what is the minimum necessity for a productive interaction between potential substrates and ABCB4. To put it into a medical context, this finding should provide information on whether different substrate affinities of different PC-lipid species could explain the resistance of the canalicular membrane. Therefore, a system had to be established that overcame the limitation that ABCB4 is a phospholipid floppase whose substrate represents its own environment in the *in vivo* or reconstituted situation.

The second goal was to specify the transport mechanism of ABCB4. Here, the high degree of sequence identity between ABCB4 and the multidrug exporter ABCB1 should help to show parallels and / or differences between the transport mechanisms. The knowledge should be used to explain the partially overlapping, but nevertheless different substrate spectrum of ABCB4 and ABCB1. A translocation mechanism for ABCB4 should be developed which would be more suitable to translocate amphipathic molecules such as phospholipids without the thermodynamic disadvantage of forcing a lipid from the native membrane into the hydrophilic interior of a proteins binding pocket.

3 Publications

3.1 Chapter I – ABCB4 in health and disease

Title:	**ABCB4/MDR3 in health and disease – at the crossroads of biochemistry and medicine**
Authors:	Martin Prescher, Tim Kroll, Lutz Schmitt
Published in:	Biological Chemistry
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Review

Martin Prescher, Tim Kroll and Lutz Schmitt*

ABCB4/MDR3 in health and disease – at the crossroads of biochemistry and medicine

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Abstract: Several ABC transporters of the human liver are responsible for the secretion of bile salts, lipids and cholesterol. Their interplay protects the biliary tree from the harsh detergent activity of bile salts. Among these transporters, ABCB4 is essential for the translocation of phosphatidylcholine (PC) lipids from the inner to the outer leaflet of the canalicular membrane of hepatocytes. ABCB4 deficiency can result in altered PC to bile salt ratios, which led to intrahepatic cholestasis of pregnancy, low phospholipid associated cholelithiasis, drug induced liver injury or even progressive familial intrahepatic cholestasis type 3. Although PC lipids only account for 30–40% of the lipids in the canalicular membrane, 95% of all phospholipids in bile are PC lipids. We discuss this discrepancy in the light of PC synthesis and bile salts favoring certain lipids. Nevertheless, the *in vivo* extraction of PC lipids from the outer leaflet of the canalicular membrane by bile salts should be considered as a separate step in bile formation. Therefore, methods to characterize disease causing ABCB4 mutations should be considered carefully, but such an analysis represents a crucial point in understanding the currently unknown transport mechanism of this ABC transporter.

Keywords: ABC transporter; ABCB4 deficiency; bile acids; liver disease; phosphatidylcholine.

Introduction: ABC transporters in general

The human genome contains 48 genes encoding for ABC transporters (Dean et al., 2001) and several of these are expressed in human hepatocytes. ABC transporters are by definition ATP hydrolyzing, primary transporters, which possess two copies of an ATP binding cassette, known as the nucleotide binding domain (NBD), and two copies of a transmembrane spanning segment, known as the transmembrane domain (TMD; Figure 1). Furthermore, the NBDs of ABC transporters harbor conserved sequence motifs (Figure 1B, D), which form the foundation of this superfamily of membrane transport proteins: the Walker A (consensus sequence: GxxGxGKT/S, where x can be any amino acid), the Walker B (consensus sequence: $\phi\phi\phi\phi$D, where ϕ represents any hydrophobic amino acid), the C-loop (consensus sequence: LSGGQ), the Q-loop and the D-loop (consensus sequence: SALD) (Schmitt and Tampe, 2002). The C-loop and the D-loop are the hallmarks of the protein family, with specific locations between the Walker A and B motifs (C-loop) and downstream of the Walker B motif (D-loop). These five motifs serve as a diagnostic tool to identify new ABC proteins. The Q-loop and a highly conserved histidine (~30 amino acids downstream of the D-loop, also called the H-loop) are essential for the ATP hydrolysis (Zaitseva et al., 2005a). Additionally, a Pro-loop connects the two subunits of NBDs (Schmitt et al., 2003).

The TMDs form the translocation pathway across a biological membrane. In the case of eukaryotic exporters each TMD in general consists of six membrane spanning α-helices. Two TMDs form the functional unit that is required together with the energy, which is provided by ATP binding and/or hydrolysis within the NBDs to allow substrate transport against a concentration gradient (Schmitt and Tampe, 2002). However, there are cases where the number of domains of single ABC transporters vary from the canonical number of six. In humans, for example, ABCA4 has two additional extra cellular domains (Illing et al., 1997; Bungert et al., 2001). Additionally, a number of transporters of the ABCC subfamily harbor a third TMD, which is generally termed TMD0 (Kruh and Belinsky, 2003).

ABC transporters can be divided into importers and exporters. Prokaryotes and plants have both kinds of ABC transporters, while all other eukaryotes mainly harbor exporters, with the often-discussed exception of ABCA4

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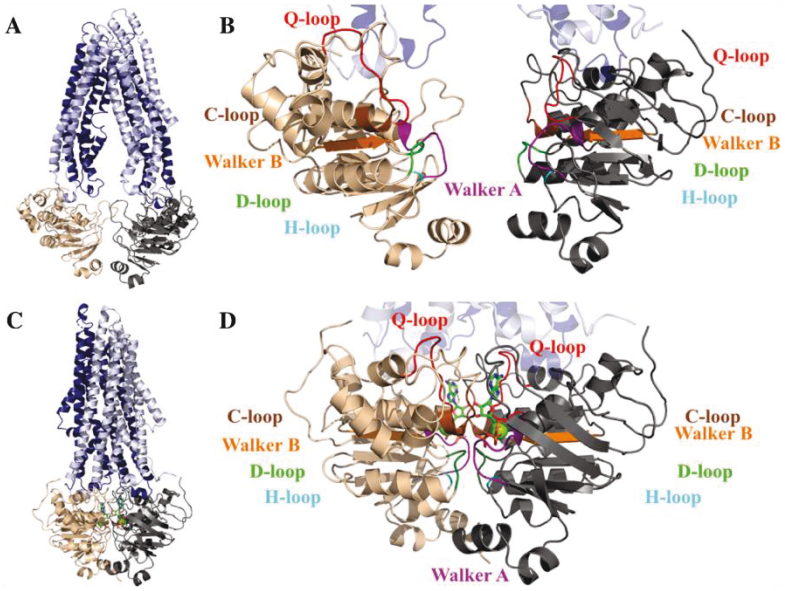

Figure 1: Homology model of ABCB4 in the inward and outward facing conformation.
(A) The inward facing conformation of the ABCB4 homology model was calculated by the bioinformatic tool Phyre2 (Kelley et al., 2015). The two TMDs are highlighted in blue and light blue, while the two NBDs of ABCB4 are displayed in gray and light brown. (B) Zoom-in into the NBDs of the inward facing conformation with the five motifs and the conserved sequence motifs highlighted. (C) The outward facing structure of the ABCB4 model is based on the single particle cryo-EM structure of human ABCB1 in the ATP bound state [PDB # 6C0V (Mi Sun Jin et al., 2012)]. Color-coding corresponds to panel (A). (D) Zoom-in into the NBDs in their head-to-tail orientation of the ATP-induced dimeric state. The two bound ATP molecules are depicted in balls-and-spheres representation and Mg^{2+}-ions as a green sphere.

(Beharry et al., 2004; Molday, 2007). Additionally, eukaryotic ABC transporters can be divided in full-size and half-size transporters. A gene of the former encodes all four modules, while a gene coding for a half size transporter harbors only one copy of the TMD and NBD. They can form homodimers (two copies of the same gene) or heterodimers (two different copies containing one of each domain) (Hyde et al., 1990).

To get insights in the mode of action, it is essential to obtain structural information in combination with biochemical analysis. This has led to an understanding of the mechanism of ATP hydrolysis and several models of transport. All structurally analyzed NBDs are L-shaped and can be divided into a catalytic and a helical subunit (Chen et al., 2016; Li et al., 2016; Locher, 2016). The first one contains both Walker motifs, while the latter one harbors the C-loop. Analysis of isolated NBDs in the presence of ATP demonstrated that the two NBDs form a dimer: the adenosine and the phosphate moiety of one ATP molecule are coordinated by the Walker A and B motifs, while the γ-phosphate interacts with the C-loop of the opposing NBD and *vice versa* for the second ATP. Here, ATP acts as a molecular glue inducing the formation of a head-to-tail dimer of the NBDs (Smith et al., 2002; Chen et al., 2003; Zaitseva et al., 2005b).

The knowledge of ATP-induced dimerization derived from structures of isolated NBDs and subsequently supported by structures of full-length ABC transporters led to one of the most favorized models of substrate transport for ABC exporters: the continuous ATP-switch model (Higgins and Linton, 2004; Locher, 2016). For ABC exporters in the resting or ground state, the so-called inward-facing conformation, the substrate binding site in the TMDs is accessible from the cytosol. The NBDs are far apart in an apparent 'monomeric state'. ATP binding induces dimerization of the NBDs, which results in a conformational change of the TMDs. This exposes the substrate binding site to the extra cellular space (the so-called outward-facing conformation). Additionally, the substrate affinity is reduced, and the substrate can be released. After ATP hydrolysis, the transporter switches back to the ground state (inward-facing conformation) (Higgins and Linton, 2004). Nevertheless, some conformations of the transport cycle of ABC transporters are still unknown. Furthermore, the coordination and timing of substrate and ATP binding as well as the control of futile ATP hydrolysis are still open questions.

The human ABC transporter ABCB4 (MDR3)

Structure and function of MDR3

The complete cDNA sequence of a gene highly homologous to human *ABCB1* [also called multidrug resistance protein 1 (MDR1) or P-glycoprotein (P-gp)] was published in 1987 (Van der Bliek et al., 1987) and subsequently termed ABCB4 (or MDR3). Sequence analysis of this gene identified two halves of an ABC transporter consisting of six putative transmembrane helices (TMHs) and one NBD, in each half of the gene (van der Bliek et al., 1988). Additionally, the sequences of the NBDs as well as the TMDs were highly identical to human ABCB1. Both proteins share an overall 76% identity and 86% homology, respectively. The highest discrepancy is within the N-terminus and the linker region connecting both halves (Van der Bliek et al., 1987).

ABCB4 mRNA is mainly transcribed in the liver especially in hepatocytes (68–69%) with low mRNA levels also found in the adrenal gland (5–10%), heart (4%), lymph node (7%) and spleen (4–7%) (Nishimura and Naito, 2005; Fagerberg et al., 2014). In the hepatocytes, ABCB4 is exclusively localized in the apical (canalicular) membrane. The intracellular trafficking of canalicular ABC transporters was discussed in detail by Kipp and Arias (2002). Briefly summarized, three interaction partners of ABCB4 are known, which ensure intracellular trafficking. Pulldown assays revealed the HS1-associated protein X-1 (HAX-1), the myosin II regulatory light chain (MLC2) and the receptor for activated C-kinase 1 (RACK1) as interaction partners for ABCB4. HAX-1 is known to be involved in the apical endocytosis of ABCB11 and might also regulate ABCB4 endocytosis (Ortiz et al., 2004). It has to be further verified whether MLC2 is required for ABCB4 trafficking similar to ABCB11 (Chan et al., 2005). On the other hand, RACK1 is essential for the expression and localization of ABCB4 (Ikebuchi et al., 2009). Additionally, ABCB4 contains a C-terminal motif that resembles the PDZ domain-binding motif. This PDZ motif might interact with scaffolding proteins as it could be shown in the cystic fibrosis transmembrane conductance regulator (CFTR) (Swiatecka-Urban et al., 2002) and the multidrug-regulated protein 2 (MRP2) (Kocher et al., 1999; Hegedus et al., 2003) in order to link transmembrane proteins to the cytoskeleton.

ABCB4 contains characteristic posttranslational modifications. Two N-glycosylation sites, which are located between TMH1 and TMH2 at position 91 and 97 (van der Bliek et al., 1988) as well as several phosphorylation sites. In the mouse homologue, Mdr2, of human ABCB4 three N-glycosylation sites have been identified, which are located in the first extracellular loop at positions 83, 87 and 90, respectively (Devault and Gros, 1990). Even though, the heterologous expression of mouse Mdr2 in the yeast *Pichia pastoris* did not contain N-glycosylations (Urbatsch et al., 2001), it had no effect on expression, nucleotide binding or catalytic characteristics indicating that glycosylation is not necessary for function. On the other hand, phosphorylation sites of ABCB4 do have an influence. Mutations with an altered phosphorylation pattern resulted in markedly decreased phosphatidylcholines (PC)-secretion, despite the fact that the protein showed minor defects in maturation and targeting to the apical membrane (Gautherot et al., 2014).

First indications for a function of the protein were published in 1993 in a study, in which *Mdr2* (the mouse homologue of *ABCB4*) was knocked out. The homozygous knock-out mice did not contain cholesterol and PC lipids in their bile. In contrast, in the heterozygous mutant $Mdr2^{-/+}$ 40% of wildtype PC lipid and nearly normal cholesterol levels in the bile were observed (Smit et al., 1993). Furthermore, complementation of mouse Mdr2 by human ABCB4 demonstrated that both ABC transporters mediated the same function. Here, $ABCB4^{+/+}$ mice possessed even higher phospholipid levels (Smith et al., 1998). Most importantly, the use of [^{3}H] choline-labeled PC proved that human ABCB4 expressed at the surface of transgenic mice fibroblasts acted as a PC flippase (Smith et al., 1994). Additionally, van Helvoort et al. demonstrated the specific translocation of a short-chain PC lipid in polarized pig kidney epithelial cells transfected with human *ABCB4* (van Helvoort et al., 1996).

The high amino acid identity of ABCB1 and ABCB4 initially led to speculations that both ABC transporters are multidrug resistance proteins and to the misjudgment classifying ABCB4 as a transporter providing multidrug resistance. Even today, there are data in favor of and against this scenario. For example, ABCB4 heterologously expressed in yeast resulted in resistance against the antifungal aureobasidin A (Kino et al., 1996). In another study, transport of several ABCB1 substrates (digoxin, paclitaxel, vinblastine or ivermectine) was demonstrated in polarized pig kidney monolayers transfected with the cDNA of *ABCB4*. Additionally, verapamil and cyclosporine, well-known ABCB1 inhibitors, inhibited the ABCB4 mediated transport of digoxin (Smith et al., 2000). In contrast to these studies, experiments with *ABCB4* or *Mdr2* transfected cells showed no drug resistance (Van der Bliek et al., 1987; Buschman and Gros, 1991, 1994; Gros and Buschman, 1993). Additionally, ABCB4 has not been detected in MDR cell lines (Raymond et al., 1990).

In summary, it is now well documented and accepted that ABCB4 specifically translocate lipids of the PC family from the inner to the outer leaflet of the canalicular membrane of hepatocytes and has at the most an artificial function as a multidrug resistance protein.

Bile formation

Bile is synthesized and secreted by hepatocytes and plays a key role in digestion and absorption of fat and fat-soluble vitamins. Primary bile mainly consists of mixed micelles formed by PC lipids, bile salts and cholesterol, bilirubin glucuronides and organic anions. Different ABC transporters are involved in this process, for example, bilirubin glucuronides are excreted into bile by ABCC2 (MRP2) (Kamisako et al., 2000) and transport of glutathione, the most common organic anion, is facilitated by ABCC1 (MRP1) (Cole and Deeley, 2006), while bile salts are secreted by ABCB11 [bile salt export pump (BSEP)] (Gerloff et al., 1998b), PC lipids are translocated by ABCB4 (Smit et al., 1993) and cholesterol is a substrate of the heterodimeric ABC transporter ABCG5/G8 (Graf et al., 2003). In 2003, Small concluded that the latter three ABC transporters interplay in a specific order to ensure the proper formation of bile. Bile salts in the canaliculi promote the release of PC lipids from the membrane to form mixed micelles, which are necessary for solubilization of cholesterol to prevent its crystallization (Small, 2003). It was reported that the reduction of PC lipids in primary bile leads to an increase of the toxic activity of the bile salts against the ducts, which can result in cholestatic liver diseases (Trauner et al., 2008). This study pointed out how important a healthy ratio of PC lipid to bile salt and cholesterol concentration is. Variations in ABCB11 (Carlton et al., 1995; Bull et al., 1998) and ABCB4 (Deleuze et al., 1996; de Vree et al., 1998) can lead to altered ratios and therefore to different kinds of liver diseases. Since then, the interest to understand these proteins and their role in liver diseases has increased dramatically.

Phosphatidylcholine – from biosynthesis to protection

Most biological membranes such as the plasma membrane are asymmetric, i.e. they consist of different lipid compositions in the cytosolic (inner) and the exoplasmic (outer) leaflet. Lipids in eukaryotic plasma membranes are distributed with a majority of sphingomyelin (SM) and PC in the outer leaflet. On the other hand, amino phospholipids such as phosphatidylethanolamine (PE) and phosphatidylserine (PS) are the major lipid components of the inner leaflet, respectively (Devaux, 1991). However, passive transbilayer lipid movement especially against a concentration gradient is a relatively slow event, which occurs on the time scale of minutes to hours and depends on charge, shape and length of the lipid. In general, the more amphipathic and bulky a lipid becomes the more unfavorable is the passive flip-flop. An unassisted flip-flop of a single 1,2-dioleyl-sn-glycero-3-phosphocholine (DOPC) lipid in a DOPC bilayer occurs, for example, once per 25 h (McConnell and Kornberg, 2002). Thus, a protein mediated lipid flip-flop is required to build up this asymmetry and the superfamily of ABC transporters is involved in such transbilayer movement of lipids. In contrast to scramblases, the transport of ABC transporters is unidirectional and can be further subdivided into inward (flip) or outward (flop) transport (Borst et al., 2000).

Many ABC transporters [e.g. ABCA1 (Oram and Lawn, 2001), ABCB1 (Higgins and Gottesman, 1992), ABCB4 (Smit et al., 1993), ABCC1 (Deeley and Cole, 2006), ABCG1 (Klucken et al., 2000) and ABCG2 (Doyle and Ross, 2003)] have been described to be involved in lipid homeostasis. ABCB4 is maintaining this asymmetry in the canalicular membrane of hepatocytes with PC lipids.

PC can be synthesized by either the cytidine diphosphate (CDP)-choline pathway or the PE methylation pathway (PEMP). The CDP-choline pathway uses choline as the initial substrate, while the PEMP methylates PE in three sequential steps to PC.

Although PC is mainly synthesized by the CDP-choline pathway (Kent, 1995), PEMP is highly active in hepatocytes (Yao and Vance, 1988). Two-thirds of PC originates from the CDP-choline pathway, while one-third is synthezised via the PEMP. Both choline synthesis pathways have characteristic acyl-chain preferences. The CDP-choline uses mainly medium-chain, saturated acyl-chains, for example, 16:0, 18:0 variants, whereas the PEMP uses more diverse, longer and poly-unsaturated acyl-chains, for example, 18:1, 18:2, 20:4 (DeLong et al., 1999). PC products derived from PEMP seem to be more metabolically active and involved in cellular regulations [e.g. arachidonic acid (20:4) (Needleman et al., 1986)] compared to the CDP-choline synthesis products.

The PC proportion of the canalicular membrane is only about 30–40% (Yousef and Fisher, 1976; Meier et al., 1984), but lipids of the PC family build up the majority (95%) of all phospholipids in bile (Boyer, 1986; Puglielli et al., 1994) with hardly any other phospholipid detectable. This raises the question of how this discrepancy can

be explained. It is unlikely that an unknown protein is involved in mediating the 'lipid exposure' process (Pohl et al., 2005) of PC lipids, however, there is evidence for bile acids actively extracting phospholipids from the canalicular membrane (Elferink et al., 1997). 'Lipid exposure' explains the uptake of lipids out of the membrane into or through a specific acceptor. In the case of ABCB4 the acceptor seems to be the mixed micelles, while bile salts act as extractors. Considering the detergent nature of bile salts and the fact that nearly exclusively it is PC lipids that are solubilized from the extracellular leaflet in the bile duct, it is surprising to see that bile salts discriminate between lipids of different acyl chains (Wustner et al., 2000), as shorter and more hydrophilic lipids are more favorably taken up into mixed micelles independently of their headgroup (Nibbering et al., 2001). Studies investigating the characteristics of the acyl-residues of biliary lipids found that mainly PC lipid variants with 16:0–18:2 (50%) and 16:0-20:4 acyl-chains (20%) (Boyer, 1986; Puglielli et al., 1994; Nibbering et al., 2001) were secreted. These findings are supported by the fact that SM variants found in bile are mainly the more hydrophilic, short-chain ones [e.g. 16:0 acyl-chain lipids (Carey and Carey, 1998)] and membranes with a high sphingomyelin proportion make membranes highly detergent resistant (Guyot and Stieger, 2011).

The puzzling enrichment of PC as the major lipid component in bile can be explained with (i) the asymmetry of the canalicular membrane and (ii) with the insertion capabilities of bile salts, which can just integrate into the outer leaflet of the canalicular membrane. It is important to point out that for ABCB4 the substrate selection is not characterized. Indications are present, in which the choline head group seems to be the part of the lipid, which is recognized (Smith et al., 1994; van Helvoort et al., 1996; Kluth et al., 2015). However, a complete substrate specificity of ABCB4 with insights on preferences for the nature of the acyl chains has so far not been investigated. Therefore, the 'lipid exposure' step which follows the lipid flop from the inner to the outer leaflet of the canalicular membrane may also be part of the ABCB4 translocation process.

However, enrichment of PC lipids in bile may also be explained by the regulation of PC synthesis and/or the discrimination between specific medium-chain lipids by acceptors like bile salts. The fact that PC lipids are highly abundant in the outer leaflet of hepatocytes due to the ABCB4-mediated PC lipid flop may not be omitted. This could be the reason why the hepatocytes utilize PC lipids derived from the CDP-choline synthesis pathway as well as the ATP-consuming translocation by ABCB4 to protect itself from solubilization by bile salts. This is supported by the fact that ABCB4 is suggested to be a low affinity transporter (Ishigami et al., 2013), as ABCB4 also recognizes PC-variants of the PEMP, i.e. longer acyl-chains and poly unsaturated lipids, the integrity of the membrane remains intact even in the presence of bile acids reflected by the relatively high activity of the PEMP in hepatocytes.

'Lipid exposure' is generally a crucial step in lipid translocation. However, it is often not included in mechanistic considerations and little is known about the implications on ABCB4. ABCB4 might not have to expose lipids out of the outer leaflet itself. Rather, the transporter creates a high abundance of various PC lipid variants without the discrimination by the choice of its substrates. Bile acids, on the other hand, discriminate and extract due to a higher affinity the abundant 16:0–18:1 PC-variants, which were derived from the CDP-choline pathway. Therefore, ABCB4 protects the biliary tree from the harsh detergent activity of bile salts, while bile salts preferably solubilize PC-variants with a characteristic nature. ABCB4 may do more than merely flop PC lipids. If lipid extraction would only be based on bile salt selectivity at least some phospholipids should remain in the bile of patients with ABCB4 deficiency, probably with a changed lipid composition away from the PC majority. Supported by the observations that the bile salt to phospholipid and cholesterol to phospholipid ratio in PFIC-3 patients is five times higher (Davit-Spraul et al., 2010). These findings together with experiments of *Mdr2* knock-out mice, which show nearly no remaining phospholipids in bile (Smit et al., 1993; Crawford et al., 1997), point towards a crucial role of ABCB4 itself in the uptake of PC lipids into bile. On the contary, it could be observed that bile acids largely release PC lipids from isolated canalicular vesicles, demonstrating the need for further investigations (Gerloff et al., 1998a).

It remains unclear which part contributes the most to the enrichment and uptake of PC in bile. It might be (i) *de novo* PC synthesis via CDP-choline or PEMP, (ii) the accumulation or even lateral tension in the canalicular leaflet mediated by ABCB4 translocation, (iii) the statistical abundance of PC in the outer leaflet of the canalicular membrane by the active flop of PC by ABCB4, (iv) ABCB4 selection of certain PC lipids or (v) the bile acid composition and its concentration.

What we can learn from structurally-related proteins such as ABCB1

The amino acid sequence of the ABC transporter ABCB4 is highly homologous to other transporters of the ABC subfamily B especially ABCB1. Their substantial sequence

identity covers not only the conserved NBDs, but also the TMDs. ABCB1, probably one of the most investigated ABC transporter, allows extracting information regarding the key features of ABCB4, as, for example, the three-dimensional structure through homology modeling (Figure 1). Even though ABCB4 and ABCB1 share up to 76% identity (86% homology), they transport structurally, physically and physiologically unrelated substrates. The difference between these two transporters likely hides in the molecular mechanisms of substrate recognition. However, homology models of ABCB4 derived from the known structures [inward-facing conformation, (Li et al., 2014)] or Kim et al., [outward-facing conformation (Kim and Chen, 2018)], obviously remain structural models of ABCB4. This is, for example, emphasized by the fact that for ABCB1 at least 40 residues are known to interact with substrates (Loo and Clarke, 2001; Vahedi et al., 2017), while key residues in ABCB4 involved in substrate binding and translocation remain unknown.

ABCB4 in disease

Liver diseases related to ABCB4 deficiency – PFIC3, ICP, LPAC, DILI

Liver diseases related to dysfunctional ABCB4 are very diverse (Figure 2). They include progressive familial intrahepatic cholestasis type 3 (PFIC3), intrahepatic cholestasis of pregnancy (ICP), low phospholipid associated cholelithiasis (LPAC), drug-induced liver injury (DILI) and some forms of chronic cholangiopathy with biliary fibrosis (for further details see Table 1). They can be divided into inherited (PFIC3, ICP, LPAC) and acquired (DILI) diseases (recently reviewed by Reichert and Lammert, 2018). The interested reader can obtain further information about jaundice as a symptom of liver diseases in a recent review from Chen and colleagues (Chen et al., 2018).

PFIC is caused by variations, for example, in hepatocanalicular transporters disease-causing mutations (DCM) in ATP8B1 result in PFIC-1, DCM in ABCB11 result in PFIC-2 and DCM in ABCB4 result in PFIC-3 and is the most severe form of all 'ABCB4-related' liver diseases. ABCB4 deficiency can be sorted into ABCB4 variants causing altered expression levels, folding, stability or activity (Delaunay et al., 2017). All cases therefore reduce or abolish net PC lipid translocation. Not only the mutation itself, but also the status of zygosity can cause different severities of diseases like ICP, LPAC or even PFIC-3 (Figure 2). The accumulation of free bile salts not safely stored in mixed micelles may lead to bile duct injuries, which is known as the 'toxic bile concept' (Trauner et al., 2007).

Mild forms of cholestasis are indicated by elevated serum cholestasis marker such as alkaline phosphatase (ALP), alanine transaminase (ALT) and aspartate transaminase (AST). An indicator for liver diseases which are related to ABCB4 deficiency, is an elevated γ-GT serum level (Gudbjartsson et al., 2015).

Intermediate forms of severity of liver diseases are ICP and DILI. ICP typically occurs during the third trimester of pregnancy and is the most common primary liver disease in pregnancy (Lammert et al., 2000; Williamson

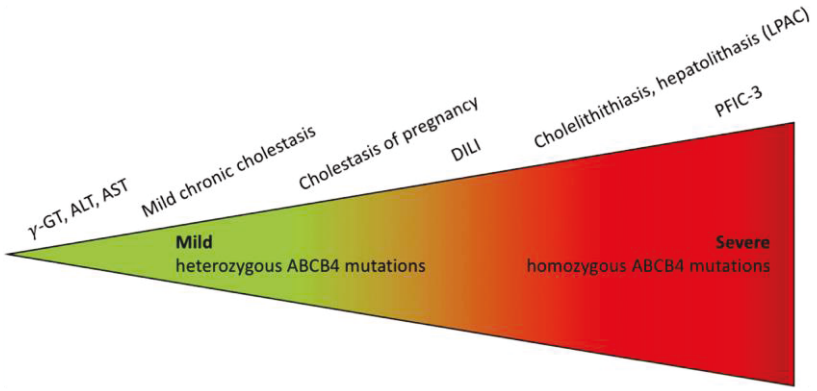

Figure 2: Spectrum of ABCB4-related diseases.
From left to right increasing severity of ABCB4 related diseases (from mild forms to severe forms). γ-GT, γ-glutamyl transpeptidase; ALT, alanine transaminase; AST, aspartate transaminase; DILI, drug induced liver injury; PFIC-3, progressive familial intrahepatic cholestasis type 3. Adopted from Reichert and Lammert (2018).

Table 1: ABCB4-related liver diseases.

Disease	Symptoms	Physiopathology
PFIC-3	Jaundice, discolored stool, hepatomegaly, splenomegaly, pruritus	Absence of biliary phospholipids, injury of bile canaliculi and epithelium, high serum γ-GT activity
LPAC	Intrahepatic hyperechogenic foci, sludge or microlithiasis	Increased biliary cholesterol to phospholipid ratio, lithogenicity of bile with crystallization of cholesterol
ICP3	Slightly elevated serum γ-GT activity in contrast to 'classic ICP' with no elevated serum γ-GT	Likely heterozygous state for an ABCB4 defect, associated with nongenetic factors, such as female sex hormones and metabolites during pregnancy, cholestasis derives from the toxicity of bile/reduced biliary phospholipids
DILI	Cholestasis with high serum γ-GT activity caused by oral contraceptive pill treatment	Xenobiotic inhibits one or a number of hepatic transporters

The table summarizes the symptoms and physiopathology for the four most common ABCB4-related liver diseases: progressive familial intrahepatic cholestasis (PFIC-3), low phospholipid associated cholelithiasis (LPAC) intrahepatic cholestasis of pregnancy type 3 (ICP-3) and drug induced liver injury (DILI), respectively (Davit-Spraul et al., 2010). γ-GT, γ-glutamyl transpeptidase.

and Geenes, 2014). Fetal as well as maternal symptoms can occur. Typical symptoms for pregnant women are pruritus and increased bile acid concentrations in the serum. Fetuses may be at risk for fetal distress, preterm birth or even fetal death, if the bile salt concentration in serum exceeds 40 µmol/l (Chappell et al., 2012; Bacq et al., 2017). ICP is reversible and ends after pregnancy, nevertheless, ICP patients may be at higher risk for subsequent hepatobiliary diseases (Wikstrom Shemer et al., 2015).

DILI can result from a variety of drugs due to the inhibition of hepatobiliary transporters, for example, ABCB4, ABCB11 or ATP8B1 and is an acquired liver disease (Stieger et al., 2000; Schuster et al., 2005; Smith and Schmid, 2006; Bleibel et al., 2007; Stieger, 2010; Bhamidimarri and Schiff, 2013). Next to ABCB11 little attention was paid to ABCB4. Nevertheless, both are listed in the Food and Drug Administration (FDA) list of candidate transporters for drug-induced inhibition (http://bts.ucsf.edu/fdatransportal). Inhibition of BSEP, for example, can trigger elevations in serum bile salt concentration because of increased basolateral transporters mediating the efflux of bile acids into the serum (Rodrigues et al., 2014; Aleo et al., 2017).

LPAC is already classified as a severe liver disease. It occurs in young adults and can be diagnosed with findings of cholesterol crystals in the hepatobiliary duct system. The low concentration of PC in bile is attributed to ABCB4 deficiency (Poupon et al., 2013). LPAC has an overlapping molecular background to PFIC-3, heterozygous ABCB4 variants tend to manifest in LPAC, whereas homozygous or compound heterozygous variants are more likely to confer PFIC-3 (Poupon et al., 2010).

PFIC occurs in most patients in the first year of life (Droge et al., 2017). It rapidly progresses to liver cirrhosis in childhood. The main symptoms are jaundice, pruritus, gallstones, and in case of PFIC-3 elevated γ-GT levels are measurable together with reduced PC-levels in bile. It is caused by both alleles and typically results in the need for liver transplantation.

Possible treatment before liver transplantation is necessary

Drugs applied to ABCB4 deficiency can be classified based on their mode of action. Drugs may stimulate ABCB4 expression, its intracellular transport from the Golgi apparatus to the canalicular membrane, the ATPase activity or they may stimulate the translocation process in general (Table 2).

Ursodeoxycholic acid (UDCA) has been the only treatment in patients with ABCB4-related diseases and provided benefit in patients with ICP or LPAC syndrome. UDCA, a natural bile acid found in bears is used to recover liver function with the aim of preventing liver cirrhosis. The success of the therapy depends on the severity of ABCB4 deficiency (Gordo-Gilart et al., 2015). Indeed, UDCA treatment unfortunately fails in approximately 50% of the patients with PFIC-3. Non-responders often lack ABCB4 in the canalicular membrane due to truncated variants of the protein and subsequent degradation (Jacquemin et al., 2001). Patients with at least one third of functional ABCB4 are favorable to respond to UDCA treatment (Gordo-Gilart et al., 2015).

The UDCA derivative NorUDCA is a shorter, C_{23} version of UDCA (C_{24}). It undergoes cholehepatic shunting and resembles bile acids to lower the harsh detergent activity, making it a promising drug against primary sclerosing cholangitis (Halilbasic et al., 2017).

Patients with known genotypes can be treated with more specific drugs, for example, drugs that stimulate

Table 2: Drug treatment and treatment options of ABCB4-related diseases.

Drug	Clinical trials[a]	Physiological function	Mode of action	Reference
UDCA	In use	Bile salt	Reduction of the harshness of bile, stimulates secretion of bile salts	(Gordo-Gilart et al., 2015; van der Woerd et al., 2017)
Rifampicin	In use	Semisynthetic antibiotic	Upregulation of detoxification enzymes and export pumps	(van Dijk et al., 2015; van der Woerd et al., 2017)
Nor-UDCA	Phase II study completed 2016	Half synthetic bile salt	Reduction of the harshness of bile	(Trauner et al., 2015; Halilbasic et al., 2017)
Obiticholic acid	Phase I study completed 2015	FXR agonist	Stimulating the ABCB4 expression	(Hirschfield et al., 2015)
Bezafibrate	Phase III study completed 2012 (Bezafibrate + UDCA)	PPAR alpha agonist	Down regulating the bile acid synthesis	(Honda et al., 2013)
Ivacaftor	In use for CFTR Phase I completed for moderate hepatic impairment	Potentiator	Recover weakened ATP binding	(Delaunay et al., 2017)
Cyclosporin A Cyclosporin C	No clinical study for ABCB4 several studies after liver transplantation	Chemical chaperone	Rescuing impaired ABCB4 trafficking	(Gautherot et al., 2012; Delaunay et al., 2016; Andress et al., 2017)
4-phenylbutric acid	No studies found	Chemical chaperone	Rescuing impaired ABCB4 trafficking	
Curcumin	No clinical study for ABCB4 Two studies for NAFLD	Chemical chaperone	Rescuing impaired ABCB4 trafficking	

The table briefly compiles the information about the different drugs: their current status in clinical phase ([a]taken from clinicaltrails.gov), mode of action and physiological function.

the mRNA expression level in cases where the presence of ABCB4 is marginal. *ABCB4* is under transcriptional regulation of the farnesoid X receptor (FXR), peroxisome proliferator-activated receptor α (PPARα) and presumably the liver receptor homolog 1 (LRH1) (Marrapodi and Chiang, 2000; Hochrath et al., 2012). Transcriptional receptors may be promising drug targets for patients with low ABCB4 expression levels, with the idea of finding agonists stimulating ABCB4 mRNA expression. FXR induces ABCB4 mRNA expression due to binding and therefore activation in the promotor region of ABCB4 (Huang et al., 2003). PPARα transactivates a range of additional mechanisms including cholesterol homeostasis, by down-regulation the bile acid synthesis through cytochrome P450 (CYP) enzyme 7A1. Studies in clinical phase I have shown that targeting the nuclear receptors with the FXR agonist obeticholic acid (Hirschfield et al., 2015) or with the peroxisome proliferator-activator receptor-α agonist bezafibrate (Honda et al., 2013) are promising in the treatment of chronic liver diseases with poor response to ursodeoxycholic acid.

ABCB4 variants with mutations in the NBD may be targeted differently. First experimental evidence observed for ivacaftor. Ivacaftor is a potentiator, which is therapeutically approved in patients suffering from cystic fibrosis with defective gating mutations. In ABCB4 it should recover deficiencies attributed to weakened ATP binding (Delaunay et al., 2017).

Chemical chaperones such as cyclosporin A, C, 4-phenylbutyric acid or curcumin, which lack specificity, but are able to rescue impaired ABCB4 trafficking in an *in vitro* set up, provide proof of principle that protection of misfolded proteins and/or prevention of degradation is a feasible approach (Gautherot et al., 2012; Delaunay et al., 2016; Andress et al., 2017). These examples lay the path for highly specific and tailor-made pharmaceutics.

How molecular mutations can cause dysfunction in MDR3

Databases for ABCB4 [e.g. Genome Aggregation Database (gnomAD; dbSNP Short genetic variation NCBI, 11.2018)] list approximately 250 synonymous, 550 missense and 30 loss of function variants. About 300 of these are distinct disease-causing ABCB4 variants (Pauli-Magnus et al., 2004; Gotthardt et al., 2008; Meier et al., 2008; Ziol et al., 2008; Dixon et al., 2009; Davit-Spraul et al., 2010; Krawczyk et al., 2010; Kubitz et al., 2011; Lammert and Hochrath, 2015; Dixon and Williamson, 2016; Keitel et al., 2016; Delaunay et al., 2017; Droge et al., 2017). However, most ABCB4 variants are family-derived and therefore very likely unique

to this specific genetic background. Additionally, none of these databases combine all the sequencing data and databases are growing every day, making it challenging to track precise statistics. The variants in ABCB4 are distributed largely over the entire *ABCB4* gene, making the identification of the molecular basis of the disease ambitious. The impact of synonymous variants is also challenging, as they do not result in an altered amino acid sequence. However, they may potentially modulate mRNA processing, affect splicing or modulate protein folding as demonstrated for ABCB1 (Kimchi-Sarfaty et al., 2007). Disease causing mutations of ABCB4 can be categorized in mutations which lead to the total absence of ABCB4, protein trafficking or altered function. Nonsense mutations can result in truncated protein variants where stop codons occur within the gene (de Vree et al., 1998; Wendum et al., 2012; Vij et al., 2018). Other variations may not lead to the total absence of ABCB4 but can cause mis-trafficking or intracellular accumulation of ABCB4 (Andress et al., 2014; Gordo-Gilart et al., 2015). For example, the ABCB4 G68H variant remained in the endoplasmic reticulum (Gordo-Gilart et al., 2015). Mutations which do not affect expression or trafficking can affect the functionality of ABCB4. Some of these mutations which can be pinpointed to a conserved motifs of ABC transporters (see Introduction) often result in an inactive ABCB4 variant, as these motifs are necessary to bind and hydrolyze ATP (Dzagania et al., 2012; Kluth et al., 2015; Droge et al., 2017). One example would be a substitution in the Walker A motif (K435T) (Andress et al., 2017). Mutations located in the TMDs are more difficult to interpret. For example, the ABCB4 T715I (TMH7) variant had still 80% of its activity (Park et al., 2016), while ABCB4 T201M (TMH3) showed ~30% activity compared to the wild type (Gordo-Gilart et al., 2015). With an unknown lipid translocation mechanism, the underlying significance of certain ABCB4 variants especially in the case of mutations of non-conserved areas in the protein are more challenging to interpret therefore it becomes inevitable to characterize these ABCB4 variants in adequate *in vivo* or *in vitro* methods.

Challenges in characterization of ABCB4 and its variants

ABCB4 variants with changes in their functionality, expression or trafficking have different molecular origins. Nevertheless, the characterization demands various *in silico*, *in vivo* and *in vitro* methods. We want to highlight crucial challenges which cannot be easily addressed by a single method.

A crucial pitfall in ABCB4 research is the interpretation of data collected from different species. Data derived from animal models, cell culture models or from humans need to be combined, for example, the bile salt pool composition differs between the species with consequences for the pathogenesis (Kararli, 1995; Takahashi et al., 2016). This raises a fundamental question of the different phenotypes occurring after homozygous knockout of *Abcb4* in mice and hereditary dysfunction of ABCB4 in humans (Jacquemin et al., 2001). However, ABCB4 similarities of different species allowed more than a basic understanding (van Helvoort et al., 1996).

Due to *in silico* databases with a focus on conserved amino acid sequences between different species, the importance of ABCB4 variants can be assessed (Droge et al., 2017). Tools from the Berkeley Drosophila Genome Project (Reese et al., 1997) and NetGene2 Server (http://www.cbs.dtu.dk/services/NetGene2/) help to predict altered splicing-sites. However, limits of this *in silico* analysis are obvious for especially non-conserved mutations localized in the TMD.

In vitro studies allow the characterization of ABCB4 variants of all three categories (see the previous section). Absence and mistrafficking can be studied by either immunostaining or expression of GFP fusion proteins (Delaunay et al., 2009; Ellinger et al., 2013; Andress et al., 2014; Gordo-Gilart et al., 2015). Naturally polarized cell lines like MDCK-II or HepG2 should be considered for studying ABCB4 and other biliary transporters. However, PC efflux or secretion assays are not suitable for all of these cell lines. An alternative to investigate the effect of drugs on ABCB4 and ABCB11 would be the use of Transwell plates to generate a basal and apical membrane (Mahdi et al., 2016).

Studying PC efflux or secretion bring other challenges. It is undisputed that ABCB4 translocates lipids of the PC family, however, the molecular basis of this translocation is unknown. To address this problem many transport assays use stable transfectants of common cell culture models to measure ABCB4 translocation (Roulier et al., 1990; van Helvoort et al., 1996; Hojjati and Jiang, 2006; Gautherot et al., 2014). However, it appears to be challenging to monitor a translocation event of certain PC lipids in a membrane full of other lipids. Labels in the form of fluorescence or radioactivity are therefore required. However, labels should not interfere with the substrate recognition machinery (e.g. currently unknown binding site) of ABCB4. Therefore, the use of NBD-labeled PC lipids is controversially discussed, as ABCB1 is also able to flop NBD-labeled PC (van Helvoort et al., 1996), but an *in vivo* proof for this function is still missing. Nevertheless, the fact that,

ABCB4 translocation of NBD-PC lipids can be stimulated by adding taurocholate (TC) in the extra cellular media (Ruetz and Gros, 1995) or that NBD-PC lipids can be found in the pseudocanaliculi of HepG2 cells only if transfected with *ABCB4* (Shoda et al., 2004), suggest using these lipids may be valid. This problem can be circumvented by radioactive assays. A more indirect method is the extraction of total lipids, where PC can be distinguished between others via cleavage by the phospholipase D (Gautherot et al., 2014; Delaunay et al., 2016; Khabou et al., 2017).

Mostly unaddressed is the fact that the flop of PC lipids from the inner to the outer leaflet of a membrane (either native or synthetic) should be distinguished from the extraction process performed by an acceptor. Many transport assays measure the PC concentration in the medium and directly assign this concentration to ABCB4 translocation without considering the discrimination of acceptors for certain lipids or address the velocity of this process (see section 'Phosphatidyl choline – from biosynthesis to protection'). *In vitro* acceptors may be albumin, together with certain bile acids like TC or liposomes and all of them may act differently (Ruetz and Gros, 1994, 1995; van Helvoort et al., 1996). For example, without the acceptor TC no PC translocation by active ABCB4 can be measured (Morita et al., 2007).

One way to overcome this issue is the measurement of the ATPase activity as an indirect evidence for transport, which in general is used for ABC transporters. However, the measurement of ATPase activity in native membranes is challenging due to background activity of other ATP hydrolyzing proteins. Without knowledge of specific inhibitors, the effect of the mutation in ABCB4 can only refer to the difference of their stimulation (Park et al., 2016). A detergent solubilized and purified ABCB4 offers a solution. The detergent solubilized and purified ABCB4 permits direct measurements of kinetic parameters (Ellinger et al., 2013; Kluth et al., 2015). It is feasible to conclude that ATPase stimulation by a certain substrate is linked to its transport, because most substrates do indeed stimulate its ATPase activity as it was shown for ABCB1 (Chufan et al., 2013).

In conclusion an assay is required with a substrate which is easily identified but does not alter the recognition or binding features of ABCB4.

The future of ABCB4 research

Here, we have tried to summarize the current knowledge of the ABCB4 structure, function and its part in bile formation, but also its contribution to different liver diseases. We also described methods that are mainly used to analyze ABCB4 variants. So far, the mechanism of lipid translocation and uptake into bile canaliculi remains unclear or is controversially discussed. Structural information on ABCB4 as well as functional studies on a molecular level are required to understand ABCB4 mechanistically. A molecular mechanism would provide more accuracy for *in silico* tools to predict the effect of mutations in ABCB4. However, many more studies are required to understand the function of ABCB4 on a molecular level.

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3.2 Chapter II – ABCB4 requires a double acylated PC lipid

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Stimulation of ABCB4 /MDR3 ATPase activity requires an intact phosphatidylcholine lipid

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M.P. performed the experiments, M.P., S.H.J.S. and L.S. designed the experiments, M.P., S.H.J.S. and L.S. evaluated the data and all authors wrote the manuscript

Abstract

ABCB4/MDR3 is located in the canalicular membrane of hepatocytes and translocates phosphatidylcholine (PC) lipids from the cytoplasmic to the extracellular leaflet. ABCB4 is an ATP-dependent transporter that reduces the harsh detergent effect of the bile salts by counteracting self-digestion. To do so ABCB4 provides PC-lipids for extraction into bile. PC-lipids account for 40% of the entire pool of lipids in the canalicular membrane with an unknown distribution over both leaflets. Extracted PC lipids end up in so-called mixed micelles. Mixed micelles are composed of phospholipids, bile salts and cholesterol. 90-95% of the phospholipids are member of the of the PC family, but only a subset of mainly 16.0-18:1 PC and 16:0-18:2 PC variants are present. To elucidate if ABCB4 is the key discriminator in this enrichment of specific PC-lipids, we used *in vitro* studies to identify crucial determinants substrate selection. We demonstrate PC-lipid moieties alone are insufficient for stimulating ABCB4 ATPase activity, and that at least two acyl chains and the backbone itself are required for a productive interaction. The nature of the fatty acids like length or saturation, has a quantitative impact on the ATPase activity. Our data demonstrate a two-step enrichment and protective function of ABCB4 to mitigate the harsh detergent effect of the bile salts, since ABCB4 can translocate more than just the PC-lipid variants found in bile.

Introduction

Bile salts are synthesized in the liver and translocated across the canalicular (apical) membrane. This membrane forms a meshwork between hepatocytes, which are connected via tight junctions and several adjacent hepatocytes lead to the formation of the small bile duct (1-3). This area is exposed to relative high bile salt concentrations, which can reach the millimolar range, and must therefore resist the detergent action of bile salts at these elevated levels (4). Protection is provided by the canalicular membrane itself that contains especially medium to long-chain fatty acids as building blocks of the phospholipids. The canalicular membrane has also a high cholesterol content which increases the thickness of the bilayer (5). Additionally, cholesterol increases the packing density of fatty acids, through smoothing the kinks of unsaturated fatty acids, and therefore reduces packing defects (6). As a consequence, a more densely packed membrane decreases the probability for detergents to incorporate (7, 8). This obviously requires a strict regulation and continuous supply of the components. The latter function is mainly carried out by three hepatobiliary transporters. One of them, the bile salt export pump (BSEP, ABCB11), is an ABC transporter that translocates conjugated bile salts across the canalicular membrane (1-3). To reduce the detergent action of these bile acids, the other two ABC transporters translocate additional compounds to intercept free bile salts in so-called mixed micelles. They consist mainly of phospholipids, cholesterol and the bile salts. A balanced ratio of these three components not only reduces the detergent action of bile salts, but also exports cholesterol for excretion without the formation of cholesterol crystals, so-called gallstones. Cholesterol is transported by ABCG5/G8 (9, 10), a heterodimer consisting of two half-size ABC transporters ABCG5 and ABCG8. ABCB4/MDR3 flops phospholipids, more precisely lipids of the phosphatidylcholine (PC) family, from the cytosolic to the extracellular leaflet of the membrane. ABCB4 is a so-called full-size ABC exporter composed of two transmembrane domains (TMD) and nucleotide binding domains (NBD) localized on a single gene (11, 12). Historically, it was termed a multidrug resistance (MDR) efflux pump due to its high amino acid identity (76 %) to P-gp/MDR1/ABCB1 (12). However, the physiological role of ABCB4 is clearly different (13-15).

The exact composition of the human canalicular membrane as well as the composition of the two leaflets is currently unknown. However, the overall composition of the rat canalicular membrane has been experimentally determined. It harbors 44 % PC-lipids, 25 % sphingomyelin (SM), 22 % phosphatidylethanolamine (PE) lipids and 8 % phosphatidylserine (PS) lipids, respectively (16). The phospholipids are also mainly esterified with one saturated and one unsaturated fatty acid (6, 17). Looking at the phosphatidylcholines of the canalicular membrane and the bile in more detail, PC-lipids account for 44 % of the total lipids of the canalicular membrane (18, 19). In striking contrast, bile, or more precisely the mixed micelles, is composed of approximately 90-95 % PC-lipids. Especially, two variants, 16:0-18:1 and 16:0-18:2 PC, are drastically enriched (17). This phenomenon has not been sufficiently addressed so far.

A process, which is often underestimated, but might explain this enrichment is the extraction of phospholipids from the membrane. In this model, extraction of lipids out of the membrane occurs into or through a specific acceptor. In the case of ABCB4, the acceptor seems to be the mixed micelle itself. Specifically for bile salts, there is evidence that they actively extract phospholipids from the canalicular membrane (20) and it is unlikely that an unknown protein mediates this 'lipid extraction' process (21). Considering the detergent action of bile salts and the fact that exclusively PC-lipids are solubilized from the extracellular leaflet, it is counterintuitive that bile salts discriminate between lipids of different acyl chain composition (22) and favorably extract lipids independent of their headgroup (16).

In such a scenario, ABCB4 translocates every PC-lipid variant presented to create a diverse pool of PC-lipids in the outer leaflet. Bile salts subsequently extract or transfer, dependent or independent of ABCB4, only a subset of PC-lipid variants, namely those PC-lipid variants, which build up bile. Thus, ABCB4 protects the biliary tree from self-destruction by bile salts. Until now, it is however unknown whether ABCB4 possesses a selectivity for certain PC variants, e.g. different fatty acid composition and / or degree of unsaturation, or whether it flops all PC lipids with similar efficiencies.

Therefore, we demonstrated previously that the ATPase activity of ABCB4 is specifically modulated by PC-lipids *in vitro* reflecting its *in vivo* functionality (23). Here, we moved one step further and used an *in vitro* set-up to analyze the physiological function of ABCB4 (24) with respect to the determinants of substrate recognition. The detergent solubilized and purified ABCB4 permits direct measurements of kinetic parameters and it is feasible to conclude that ATPase stimulation by a certain substrate might be linked to its transport, because most substrates do indeed stimulate the ATPase activity of for example ABCB1 (25). Thus, we employed total liver lipid extracts, synthetic lipid mixtures and pure PC-lipids as well as the building blocks of PC-lipids to characterize the activity in more detail. This resulted in the understanding that both, the lipid chain as well as the headgroup, are recognized by ABCB4 to stimulate ATP hydrolysis as a prerequisite for its transport function.

Material and Methods

Transformation of *Pichia pastoris* and test expression of ABCB4

All steps were performed as described in (24).

Protein production

X33 *Pichia pastoris* cells carrying the pSGP18-2µ-MDR3 plasmid were grown on a selection YPD plate containing 200 µg/ml zeocin for two days. 1 L precultures were grown in 2 L baffled flasks for 18 h in MGY media and used for inoculation of a 6 l benchtop fermenter (I&L) containing minimal glycerol medium (26.7 ml/l phosphoric acid; 0.93 g/l calcium sulfate; 18.2 g/l potassium sulfate; 14.9 g/l magnesium sulfate heptahydrate; 4.13 g/l potassium hydroxide; 40 g/l glycerol). After batch glycerol was consumed, cells were fed by glycerol for 5 h to increase the biomass. Protein expression was induced by a methanol feed for 24 h. Afterwards, cells were harvested, washed with 50 mM Tris-HCl pH 8.0 and re-pelleted. Usually cell mass reaching 1- 1.3 kg were obtained, flash frozen in liquid nitrogen and stored at -80 °C until further use.

Crude membrane vesicle preparation

All following purification steps were performed at 4 °C as described in Kluth *et al.* (23). Usually 100 g cells were thawed on ice and crude membranes were prepared. Therefore, cells were resuspended in lysis buffer (0.33 M sucrose; 100 mM 6-aminohexanoic acid;1 mM EGTA; 1 mM EDTA; 50 mM Tris-HCl pH 8.0, 75 mM NaCl) in a 1:5 ratio. Cells were ruptured at 2.7 kbar using a cell disruptor system (I&L). Differential centrifugal steps separated cell debris from crude membrane vesicles. Crude membrane vesicles were collected at 138000 x *g* and resuspended in membrane buffer (50 mM Tris pH 8.0, 150 mM NaCl; 30 % (v/v) glycerin, protease inhibitor cocktail (Roche). Total protein concentration was determined with a Bradford assay following the standard protocol.

Solubilization

Crude membrane vesicles were diluted to 10 mg/ml total protein concentration and supplemented with 1 % (w/v) Fos-Choline-16 (FC-16, Anatrace). Protein solubilization was performed for 1 h at 18 °C. Non-solubilized parts were removed at 138000 x*g* for 1 h.

Tandem affinity purification

20 mM imidazole was added to the solution of solubilized membranes to increase binding

selectivity. The solution was loaded on a 5 ml HiTrap chelating column (GE healthcare) loaded with Ni^{2+} ions, washed with washing buffer (50 mM Tris HCl pH 8.0; 50 mM NaCl; 30 % glycerin (v/v); 0.0011 % (w/v) FC-16; 20 mM imidazole) to baseline level and eluted in one step with an elution buffer containing 50 mM Tris HCl pH 8.0; 50 mM NaCl; 30 % (v/v) glycerin; 0.0011 % (w/v) FC-16; 200 mM imidazole. Fractions of the immobilized metal ion affinity chromatography (IMAC) with the highest absorbance at 280 nm were pooled and transferred onto a calmodulin affinity resin (GE healthcare) and incubated over night with gentle rotation at 4 °C. Calmodulin affinity purification (CAC) was conducted according to GE healthcare standard protocol. Aliquots with the highest protein concentration were pooled and either directly used for ATPase activity measurements or flash frozen in liquid nitrogen and stored at –80 °C until further use.

Liposome preparation

Lipids were purchased from Avanti Polar Lipids and resuspended in chloroform. Chloroform was removed in a vacuum oven at 40 °C with stepwise increasing vacuum. After 30 min at lowest pressure levels, lipids were hydrated in suspension buffer (50 mM Tris-HCl pH 8.0; 150 mM NaCl) 6 A sonication step for 5–10 min was needed to decrease the turbidity of the solution. Dilution series for ATPase measurements were prepared with suspension buffer.

Enzyme coupled ATPase assay

ATPase activity of detergent purified ABCB4 was determined by an enzyme coupled assay. Measurements were performed at 37 °C in a 96 well plate. A standard ATPase reaction volume was 200 µl in total with 50 mM Tris-HCl pH 7.4 at 37 °C; 5 mM $MgCl_2$; 4 mM phosphoenolpyruvate, 0.6 mM NADH (reduced); 13 units pyruvate kinase (PK); 16 units lactate dehydrogenase (LDH); 0.0011 % (w/v) Fos-Choline 16 and 35 µg/ml (239 nM) detergent-solubilized protein. Substrate stock solutions were prepared and 10 µl solution was added to the solution. The reaction was started by addition of 5 mM ATP. The absorbance of NADH was followed for 60 min. Since NADH oxidation is stoichiometric to ATP hydrolysis, the ATPase activity of ABCB4 was expressed as nmol ATP consumption per mg of ABCB4 protein and minute. No influence on PK or LDH performance due to compound or solvent concentrations was observed. A background measurement was subtracted from all data points. Therefore, separate ATP hydrolysis measurements in which the ABCB4 cysteines in the Walker A motives were labeled with the fluorophore FL-maleimide-Bodipy were conducted. This steric hindrance inhibits ABCB4 specifically (23). Prior to the Bodipy labeling both samples (labeled and not labeled) were reduced with tris(2-carboxyethyl) phosphine (TCEP) at 10 times the molarity of the protein for 1 min at 22 °C. For the labeling reaction, a Bodipy amount 50 times of the molarity of the protein was chosen and incubated for 20 min at 22 °C. The unlabeled sample was treated in the same way with the only exception that DMSO was used instead of the maleimide-fluorophore.

To account for batch-to-batch variations in the basal ATPase activity of ABCB4, we decided to present the data as normalized stimulation. To ensure reproducibility and comparability, each batch was analyzed for its basal ATPase activity and in the presence of highest 18:1-18:1 PC (DOPC) concentration (250 µM). While we observed variations in the absolute activities, the fold of stimulation was always within the margin of 5.31 ± 0.58.

The physical state of the liposomes

Lipids may adopt different physical states in solution, where the molar ratio between lipids and detergents changes. This solution could contain pure detergent micelles, a mixed species of detergent/lipid micelles up to pure liposomes. To exclude any artifacts evoked by lipid to detergent interactions, the absorption of each ATPase reaction was determined at 492 nm to obtain

insights in the physical state of putative liposome formation (26, 27). A molar ratio of the constant detergent concentration against the increasing lipid was calculated. The resulting curve resembles an inverse titration curve as one obtains in liposome reconstitution experiments, comparable to (28).
We also performed a dynamic light scattering (DLS) measurement with a selection of PC lipids to see if any change in physical state of the PC-lipid variants is correlated to the fold of stimulation. Here, we used 250 µM of lipids diluted with the sample buffer and measured 3 times for 200 s at 37 °C. The measurements were taken with a Malvern Zetasizer Nano ZS (Malvern Panalytical, Kassel, Germany) equipped with a He-Ne-Laser (wavelength of 633 nm) as a light source. The scattered light was detected with a scattering angle of 173° (backscattering).

Thin layer chromatography

We dripped 10 µL of each sample on a silica gel. The phospholipid standards had a concentration of 1 mg/ml. Total buffer as well as buffer isolated components were dripped on the silica gel, next to a sample of purified ABCB4. The running phase consists of 95 parts chloroform, 55 parts methanol, 5.5 parts ammonia and 5.5 parts double distilled water. For detection a primuline solution (0.1 % (w/v) in a 80 % acetone, 20 % water) was used. For fluorescence image we excited with a short wavelength light and emitted at long wavelength at a ChemiGenius2 Bioimaging System from Syngene.

Data evaluation

For analysis the background activity of ABCB4-Bodipy was directly subtracted from the data of ABCB4 in the absence of Bodipy (23). ATPase activities were normalized relative to the basal ATPase activity of ABCB4 (57.7 ± 20.3 nmol·min^{-1}·mg^{-1}) Kinetic parameters were analyzed with Prism 7 (Graphpad) and plotted using Michaelis Menten kinetics (equation 1) or a dose response fit (equation 2). Here, $v_{(c)}$ represents the ATPase activity at a given compound concentrations. v_0 is the basal ATPase activity in absence of any compound. v_1 is the maximal ATPase activity in the absence of inhibition. c defines the compound concentration. k_1 is defined as the compound concentration at half-maximal stimulating conditions (at half v_1). k_2 is the compound concentration at half-maximal inhibition of ATPase activity from the value v_1. v_{max} denotes the ATPase activity in the starting plateau, while v_{min} denotes the ATPase activity in the maximal reduction plateau.

(Eq. 1) $$v_{(c)} = \frac{v_1 \cdot c}{k_1 + c} + v_0$$

(Eq. 2) $$v_{(c)} = v_{min} + \frac{v_{max} - v_{min}}{1 + 10^{([\log(k_2)] - c) \cdot slope)}}$$

Results

Yield and purity of human ABCB4

A purification starting with 100 g wet cell weight pellet of X33 *P. pastoris* cells expressing wildtype ABCB4 as described in experimental procedures typically yielded approximately 2-5 mg FC-16 solubilized protein. The homogeneity of the sample was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot (Figure 1A). A dominant signal at approximately 135 kDa was clearly visible, which according to Western blot analysis corresponded to ABCB4 (calculated theoretical molecular weight including the two affinity tags: 146 kDa). The signals ranging from 45 – 110 kDa correspond to degradation products of ABCB4 as determined by mass spectrometry (23). To determine the specific ATPase activity of ABCB4, labeling with a Bodipy-FL-maleimide was performed. The modification was visualized by UV excitation

(Figure 1B). (24).

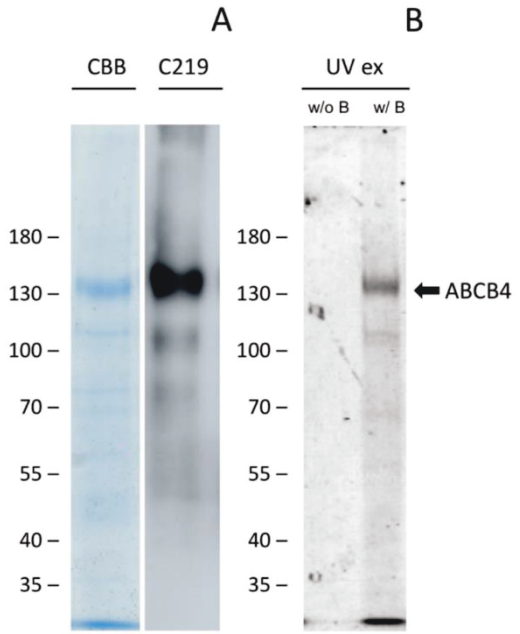


Figure 1. [A] Human ABCB4 was purified by tandem affinity purification (TAP) in FC-16 micelles. Panel "CBB" displays 2 µg of the elution fraction after TAP stained with Coomassie brilliant blue. Panel "C219" shows the same purification on a Western blot with immunostaining using the monoclonal antibody C219. [B] The same purification was loaded onto a different SDS gel without (w/o B) or after covalent modification with the thiol reactive maleimide-Bodipy fluorophore (w/ B) and exposed to UV light. The arrow indicates the position of ABCB4.

ATPase dependent characterization of PC-lipid variants

Previously, we demonstrated that ABCB4 was purified in detergent-solubilized state in an active form. Importantly, ATPase activity could be stimulated with 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), a member of the PC-lipid family or crude liver PC-lipids, but not by phospholipids bearing other headgroups (23, 24). To analyze the stimulation in more detail, we chose different PC-lipids to account for the natural variation of PC-lipids with respect to length and degree of unsaturation. Thus, we plotted the ATPase activity expressed as percent of stimulation, where 100 % represents the basal activity. Overall, the proteins basal activity was 57.7 ± 20.3 nmol·min^{-1}·mg^{-1}. Each activity was defined as 100 % and all PC concentrations were displayed relative to their basal activity. For example, the k_1 of 16:0-16:0 PC on ABCB4 ATPase activity was 62.9 ± 11.5 µM and the $v_{1\_absolute}$ was 174.0 ± 6.5 nmol·min^{-1}·mg^{-1}. Here, $v_{1\_absolute}$ corresponds to the maximal ATPase activity calculated without a reference. While k_1 remains unchanged the v_1 was calculated to 268.8 ± 10.5 % (Figure 2).

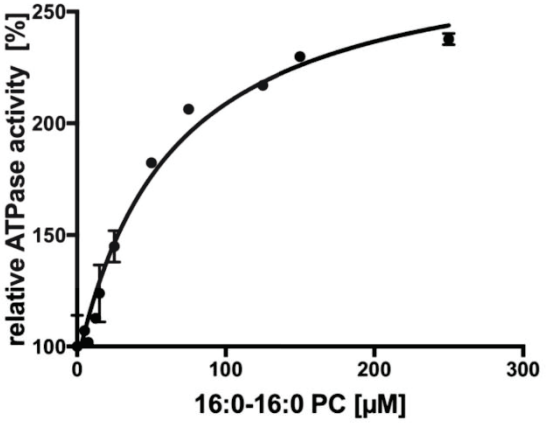


Figure 2. Relative ATPase activity measurements of ABCB4 in the presence of the substrate 16:0-16:0 PC. 100 % is defined as ATPase activity of ABCB4 in the absence of any lipid (basal activity). Data point represent the average of three independent experiment with the errors reported as S.D.

Furthermore, we performed an experiment inverse to a typical liposome destabilization experiments. Here, we kept the detergent concentration constant while the lipid concentration excelled. The light scattering of liposomes at 492 nm changes due to variations in the lipid to detergent ratio used in the ATPase assay. This change in the physical state of lipids might have an impact on the stimulation of ABCB4 (28). Therefore, we measured and plotted the change of light scattering at 492 nm against an increasing lipid to detergent ratio (Figure 3A). Figure 3B shows the slope of the curves. However, no correlation could be shown between the physical property of the PC-lipids and the ATPase activity,

like liposome formation or intermediate species like mixed detergent-lipid micelles.

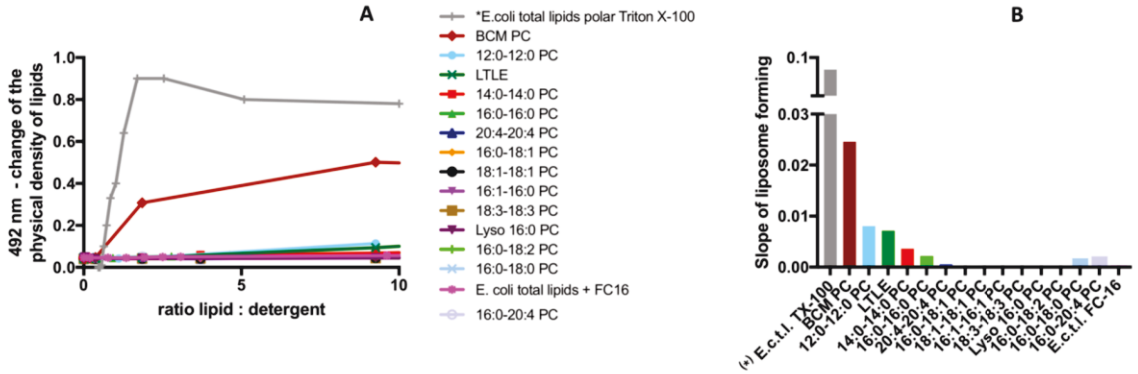


Figure 3. [A] Change in the physical state of lipids with an increasing lipid to detergent molar ratio. *E. coli* polar lipids treated with Triton X-100 in gray were taken from (28) and corresponds to a typical titration curve for destabilization of liposomes with detergent. All different PC-lipid variants were measured at 492 nm. [B] Slopes of graph A, where a change in the physical state of lipids can be visualized at 492 nm with an increased lipid to detergent molar ratio.

Additionally, a DLS measurement also showed no correlation between the physical state of PC-liposomes and the stimulation capability of the PC-lipid (Supplemental Figure S1).

In the next step, the influence of liver total lipid extract (LTLE) from bovine liver on the activity of detergent solubilized ABCB4 was analyzed. LTLE is composed of 42 % PC-lipids, 22 % PE-lipids, 8 % PI-lipids, 1 % lyso-PI-lipids, 7 % cholesterol and 20% neutral unspecified lipids (see pie chart in Figure 4A). The relative ATPase activity of ABCB4 in percent was plotted against increasing concentrations of LTLE in Figure 4A. This lipid mixture stimulated ABCB4 with a v_I of 315.2 ± 30.4 % and a k_I of 54.9 ± 26.1 µM. Additionally, we used a synthetic PC-lipid mixture (Figure 4B), which resembles the composition of the fatty acids distribution of PC-lipids in the canalicular membrane more likely (29). Note that we have no direct information about the concrete composition of the human canalicular membrane. We took our information out of a rat canalicular membrane, for which only the overall fatty acids composition is known. Therefore, we concentrated on the nature of the fatty acids even if artificial lipids like double unsaturated lipids such as 20:4-20:4 PC had to be used. The composition was visualized as a pie chart and composed of 1.8 % 14:0-14:0 PC, 20.6 % 16:0-16:0 PC, 0.9 % 16:1-16:1 PC, 25.2 % 18:1-16:0 PC, 27.5 % 18:1-18:1 PC, 19.5 % 18:3-18:3 PC and 4.4 % 20:4-20:4 PC, respectively. Here, an even higher stimulation with a v_I to 375.5 ± 13.6 % and a k_I of 26.9 ± 6.2 µM was evident. This suggested higher flop rates resulting in a higher turnover rate of ATP (Figure 4B). A detailed analysis of single PC-lipids is of course necessary to characterize a possible lipid preference of ABCB4. Therefore, we chose a subset of PC-lipids covering representatives of this family that differed in their acyl chain length, their degree of unsaturation and the natural occurrence. The main PC-lipids of bile are 16:0-18:1 PC as well as 16:0-18:2 PC and next to them 16:0-18:0 PC and 16:0-20:4 PC are also present in bile (Figure 4C). The chosen bile acids were constant in their *sn1* position with 16:0, but varied in their *sn2* position, highlighting the importance of the *sn2* esterification. Their ATPase activities were in the same range as other PC-lipid variants with a 16-C fatty acid esterification at their *sn1* position. 16:0-18:1 PC stimulated ABCB4 with v_I of 262.1 ± 12.5 % and 16:0-18:2 PC with 236.8 ± 9.1 %.

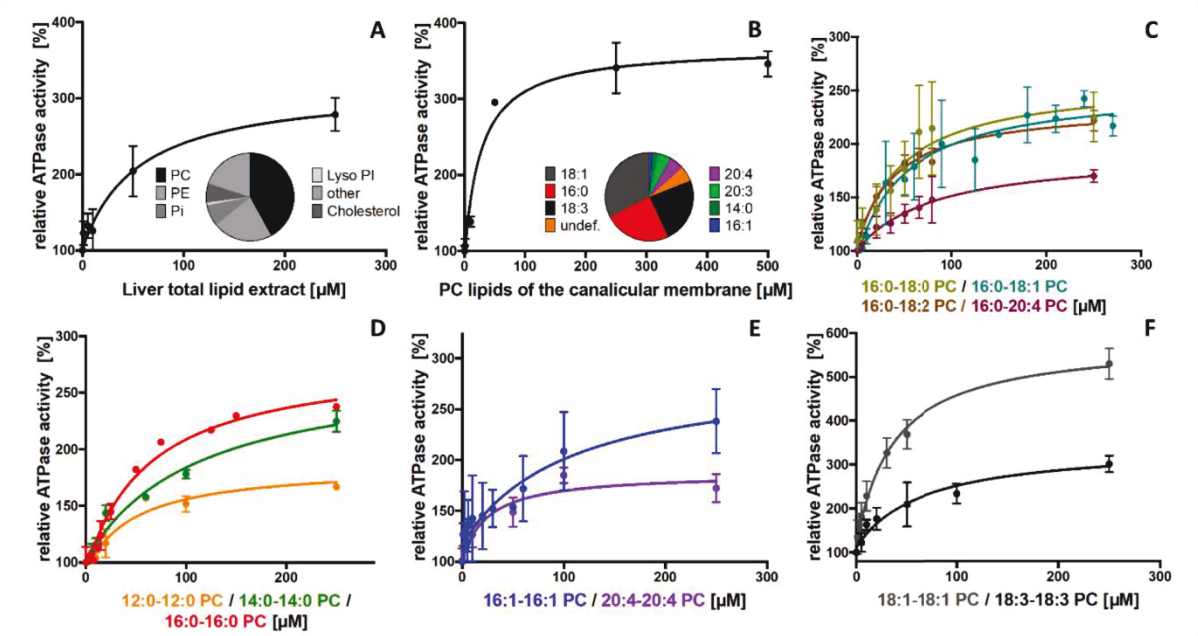
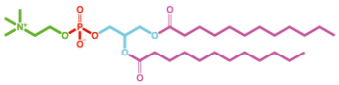
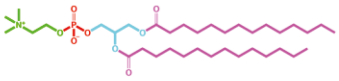
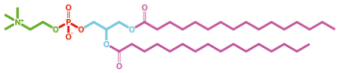
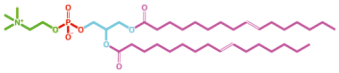
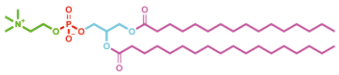
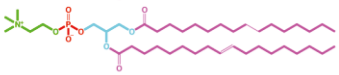
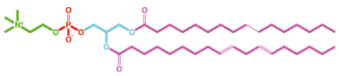


Figure 4. Relative ATPase activities of detergent-solubilized and purified ABCB4 in the presence of different PC-lipids with increasing concentrations. [A] Activity in the presence of LTLE. The composition of LTLE of bovine liver is shown as a pie chart in the inset. [B] Activity in the presence of a synthetic PC composition that resembles the natural PC composition of the bile canalicular membrane (shown as pie chart in the inset). [C] The influence of the main bile PC-lipids 16:0-18:1 PC (POPC, turquoise) and 16:0-18:2 PC (mocha) on the ATPase activity as well as 16:0-18:2 PC in brown and 16:0-20:4 PC in pink. [D] ATPase activity in the presence of PC-lipids with increasing chain length: 12:0-12:0 PC (orange), 14:0-14:0 PC (green) and 16:0-16:0 PC (red). [E] ATPase activity in the presence of PC-lipids with an increasing degree of unsaturation: 16:1-16:1 PC (blue) and 20:4-20:4 PC (purple). [F] ATPase activity in the presence of symmetric PC-lipids, 18:3-18:3 PC (black) and 18:1-18:1 PC (grey). All data points were analyzed using equation 1 and represent the average of three independent experiment with the errors reported as S.D (18:1-18:1 PC; n= 6). Each kinetic is the result of approximately one independent protein purification.

Also, their affinity constant k_l were comparable with 53.3 ± 14.3 µM and 44.7 ± 9.9 µM, respectively. Additionally, 16:0-18:0 PC showed similar ATPase activities of 260.8 ± 25.5 % and with a k_l of 51.2 ± 25.3 µM. The 16:0-20:4 PC variant was an outlier showing more similarities to the 20:4-20:4 PC variant different from the other variants with a 16:0 fatty acid in the *sn1* position. Figure 4D summarizes the influence of the acyl chain length with three different representatives. The 12:0-12:0 PC-variant, which does not occur naturally in the canalicular membrane was chosen to analyze the biological significance of the observed modulation of ATPase activity. Indeed, this PC-lipid was the one, which had the least stimulating effect on ABCB4 ATPase activity with a k_l of 47.2 ± 20.5 µM and a v_l of 194.4 ± 12.6 %. PC-lipids with longer acyl chains such as 14:0-14:0 or 16:0-16:0 PC-lipid activated the protein to a similar degree with a v_l of 272.1 ± 19.0 % for 14:0-14:0 PC and 268.8 ± 10.5 % for 16:0-16:0 PC and k_l of 110.5 ±3 0.9 µM and 62.9 ± 11.5 µM, respectively (Figure 4D). Figure 4E addresses the degree of unsaturation for PC-lipids with one or four double bonds per acyl chains. The 16:1-16:1 (blue curve) and the 20:4-20:4 PC-variant (purple curve) possessed v_l values of 272.1 ± 49.0 % and 186.1 ± 14.8 %, respectively, indicating a preference for a smaller degree of unsaturation. Even if the fatty acid 18:3 is highly abundant (17 % of total fatty acids) in the canalicular membrane of hepatocytes, which content is summarized as pie chart (Figure 4B), we were only able to test the double unsaturated version 18:3-18:3 PC, which is

not naturally part of the canalicular membrane. Together with the 18:1 (24 % abundance) and the 16:0 (18 % abundance) PC-lipid, they make up 59 % of the fatty acid composition of the PC-lipids of the canalicular membrane (29). We determined the highest stimulation for 18:1-18:1 PC (DOPC, v_I = 564.2 ± 19.7 %, k_I = 37.4 ± 5.3 µM, Figure 4F). The second highest stimulation of pure PC-lipids was observed for the 18:3-18:3 PC-lipid-variant with 332.8 ± 31.9 % (67.4 ± 32.3 µM). All kinetic parameter of the ATPase activities of ABCB4 determined for the different PC-lipids are summarized in Table 1.

Table 1. Summary of the kinetic parameter of the ATPase activity of ABCB4 in the presence of different PC-lipids varying in acyl chain length and degree of unsaturation. The transition temperature was taken from (30). The ratio in the canalicular membrane was calculated according to (29). MW – molecular weight, Tt – transition temperature, k_I – concentration at half maximal stimulation and mod. – nature of modulation on ABCB4.

name	structure or lipid proportion	MW [Da]	Tt [°C]	k_I [µM]	v_I [%]	Mod.
LTLE	42 % PC, 22 % PE, 8 % PI, 1 % Lyso PI, 7 % Chol., 20 % n.d.	~830		54.9 ± 26.1	315.2 ± 30.4	Stimulation
BCM	1.8 % 14:0-14:0 PC, 20.6 % 16:0-16:0 PC, 0.9% 16:1-16:1 PC, 25.2 % 16:0-18:1 PC, 27.5 % 18:1-18:1 PC, 19.5 % 18:3-18:3 PC and 4.4 % 20:4-20:4 PC	~773		26.9 ± 6.2	375.5 ± 13.6	Stimulation
12:0-12:0 PC		622	-2	47.2±20.5	194.4±12.6	Stimulation
14:0-14:0 PC		678	24	110.5±30.9	273.0±19.0	Stimulation
16:0-16:0 PC		734	41	62.9±11.5	268.8±10.5	Stimulation
16:1-16:1 PC		730	-36	104.8±69.8	272.1±49.0	Stimulation
16:0-18:0 PC		762	49	51.3±25.2	260.8±25.5	Stimulation
16:0-18:1 PC		760	-2	53.3±14.3	262.1±12.5	Stimulation
16:0-18:2 PC		757	-	44.7±9.9	236.8±9.1	Stimulation
16:0-20:4 PC		782	-	84.5±29.9	193.2±12.4	Stimulation
18:1-18:1 PC		786	-17	37.4±5.3	564.2±19.7	Stimulation
18:3-18:3 PC		788	-60	67.4±32.3	332.8±31.9	Stimulation
20:4-20:4 PC		830	-69	25.2±17.8	186.1±14.8	Stimulation

9

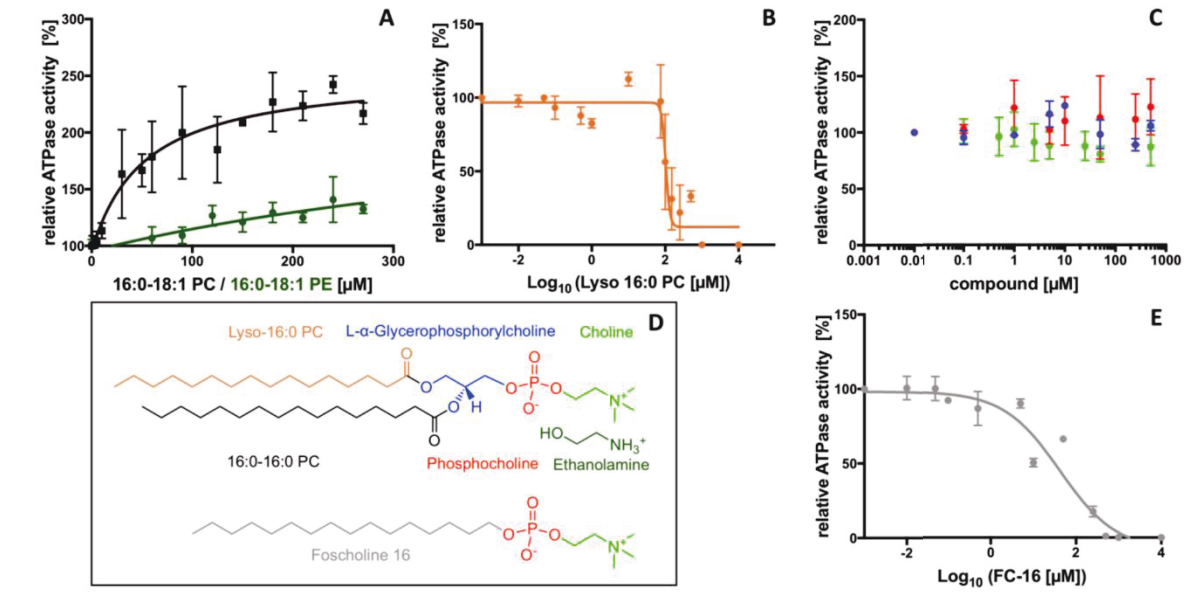


Figure 5. Relative ATPase activity of detergent-solubilized and purified ABCB4 in the presence of different building blocks ranging from choline to a phosphatidylcholine lipid POPC (16:0-18:1 PC). [A] 16:0-18:1 PC (black) and 16:0-18:1 PE (POPE, moss green), [B] lyso-16:0 PC, [C] Lαgpc (blue), ppc (red) and choline (green). [D] Chemical structures of the above-mentioned compounds. Choline moieties are shown in green, phosphate in red, the glycerin backbone in blue, fatty acids in orange and black, and alkyl chains in grey. [E] The zwitterionic detergent Fos-choline-16 (FC-16) is depicted in grey. Data points represent the average of two independent experiments with the errors reported as S.D (16:0 – 18:1 PC; n=3). Each kinetic is the result of approximately one independent protein purification.

The influence of single PC-lipids moieties on the ATPase activity of ABCB4

The ATPase activity of ABCB4 supplies the energy to mediate the flop of PC-lipids from the inner to the outer leaflet. Since ABCB4 displays a clear specificity for PC-lipids *in vivo* (15, 31, 32) and *in vitro* ((23) and this study) it would be plausible to assume that the isolated choline headgroup is capable of stimulating the ATPase activity as well. As a control, 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), an example for a PE-lipid (16:0-18:1 PE) was tested (Figure 5A). Here, no significant modulation of activity was observed as it was already described (23). In clear contrast, 16:0-18:1 PC stimulated the ATPase activity of ABCB4 with a v_1 of 262.1 ± 12.5 % and a k_1 of 53.3 ± 14.3 µM (Figure 5A). Figure 5B shows the influence of a lyso-PC-lipid - 16:0, which lacks one acyl chain. Surprisingly, a large shift in modulation was observed. Now, an inhibition became apparent with a k_2 of 103.9 ± 7.7 µM, note that a $\log_{10}$ representation was used. This value is distinct from the detergent's *cmc* value of 4 - 8.3 µM and thus indicates molecular interactions and not simple solvent effects (33). Note that, we cannot directly exclude effects of lyso-PC which change the solvation of ABCB4 or on perturbations to annular lipids. However, we have indications from thin layer chromatography (Supplemental Figure S2) that no annular lipids are co-purified with ABCB4 most likely due to extensive washing with detergent during IMAC and CAC (34). The detection limit of primuline staining was 0.1 mmol. Assuming at least a one lipid to one ABCB4 transporter ratio we would expect to detect lipids if present since the sample size of ABCB4 on the TLC was 3.4 mmol. Detergents, which share the PC headgroup but lack the core glycerol unit, are Fos-cholines such as FC-16 (Figure 5E). FC-16 also inhibited the ATPase activity at micromolar ranges with an k_2 of 41.9 ± 11.3 µM, which corresponded

to approximately three-times the critical micelle concentration (*cmc*). It is important to emphasis that FC-16 is the detergent of choice for ABCB4 solubilization and purification. However, all ATPase activity measurements were performed at concentrations of twice the *cmc* (26 µM). This concentration was present in all other experiments so that the actual concentration of FC-16 was approximately fivefold above the *cmc.* Only under these conditions, an inhibition was determined. We analyzed the interaction of FC-16 in more detail in Figure 6. Here, we performed a FC-16 titration experiments against ascending POPC concentrations. Additional 25 µM (on top of the 2 times *cmc* of FC-16 which are always present) are tolerable for the transporter. Increasing the concentration near to the k_2 value of approximate 50 µM the k_1 as well as the v_1 values are dampened. At concentrations of 100 µM the transporter is inactive until POPC concentration exceeds 100 µM, here POPC is able to "rescue" the transporters ATPase activity. But at 500 µM FC16 the transporter remains inactive due to unspecific detergent effects. To gain further insights into compounds that might directly interact with the transporter, L-α-glycero-phosphocholine (Lαgpc) lacking both acyl chains (Figure 5C) or phosphocholine (ppc) lacking both acyl chains and the glycerol backbone (Figure 5C) and choline (c) itself, which lacks even the phosphate group (Figure 5C), were analyzed. All three, Lαgpc, ppc and c, did not interfere with the ATPase activity of ABCB4 and are likely not recognized by ABCB4. Table 2 summarizes the kinetic parameters of the ATPase activity of ABCB4 in the presence of c, ppc, Lαgpc, FC-16, lyso-16:0 PC and 16:0-16:0 PC.

Table 2. Summary of the kinetic parameter of the ATPase activity of ABCB4 in the presence of different building blocks ranging from choline to phosphatidylcholine lipid. (c) Choline, (ppc) phosphocholine, (Lαgpc) L-α-glycerophosphorylcholine, (LPC) Lyso-16:0 PC, (FC-16) Fos-choline 16 and (16:0-18:1 PC) 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine. The PC proportion of the canalicular membrane was calculated according to (29). MW – molecular weight, k1 – concentration at half maximal stimulation, k2 – concentration at half maximal inhibition, v1 – maximal stimulation, mod. – nature of modulation on ABCB4.

name	MW [Da]	k_1[%]	k_2 [µM]	v_1 [%]	Modulation
LPC	495	-	103.9 ±7.7	-	Inhibition
FC-16	407	-	41.9 ±11.3	-	Inhibition
16:0-18:1 PC	760	51.3±25.2	-	260.8±25.5	Stimulation

Table 3. Summary of the kinetic parameters of figure 6 a FC-16 titration against ascending POPC concentration. k1 – concentration at half maximal stimulation, v1 – maximal stimulation, ATPase activity w/o FC-16 being the Y-intercept of the curves and n.d. not determined.

name	k_1[%]	v_1 [%]	ATPase activity w/o POPC
POPC – 0µM FC-16	36.9±8.3	234.7±7.4	100
POPC – 25µM FC-16	49.8±9.5	249.9±9	74.5
POPC – 50µM FC-16	542±385.5	181.1±36.2	49
POPC – 100µM FC-16	376.7±209.3	329.9±24	0
POPC – 500µM FC-16	n.d.	n.d.	0

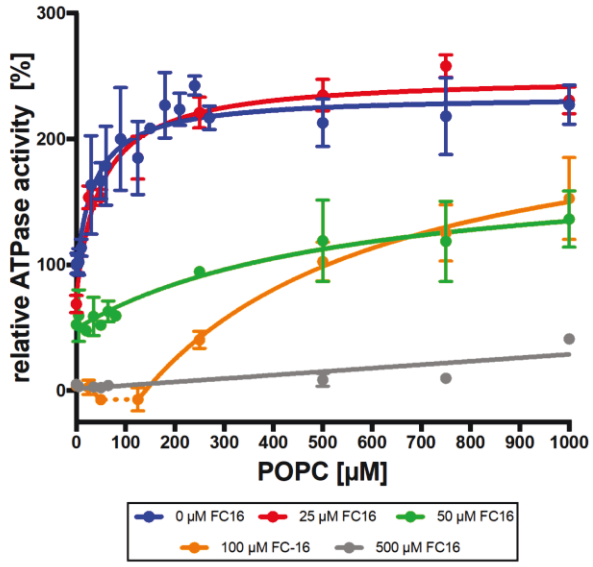


Figure 6. A titration experiment with ascending the concentrations of POPC (16:0-18:1 PC) from 0 to 1000 µM with 4 different but constant FC-16 concentrations and an exclusive kinetic without detergent. Each kinetic is plotted as Michaelis-Menten (MM) kinetics. The MM fit for orange kinetic of 100 µM FC-16 had to ignore the first three data points in order to be fitted accordingly. Data points which are not used for the MM fit of the 100 µM FC-16 are connected via a dashed line. Each kinetic is the result of approximately one independent protein purification.

Discussion

A two-step enrichment of the PC-lipid variants of the canalicular membrane – ABCB4 protects the biliary tree through lipid preferences

Since ABCB4 is confronted with unknown concentrations of substrate in *in vivo* or in reconstituted systems, the regulation of the effective concentration might be challenging. Therefore, we chose to use an *in vitro* environment of a detergent solution as fine-tuning of composition and concentration of various compounds is possible. ABCB4 was stimulated by a lipid mixture derived from bovine liver (Figure 4A). This provided us with a starting point for the analysis of specificity of the protein towards the substrate(s) (Figure 4A). We used the two most abundant phospholipid family's PC and PE to support this fact. In the case of POPE, no stimulation of the ATPase activity of ABCB4 was observed (Figure 5A). In clear contrast, 16:0-18:1 PC stimulated the activity of ABCB4. This again highlighted the specificity of ABCB4 for PC-lipids. Kluth *et al.* provided first insights for this stimulation of the ATPase activity with PC-lipids in contrast to other abundant phospholipids of the canalicular membrane (23). Even though, an ATPase activity assay is not directly measuring substrate translocation, it is now generally accepted, that most substrate indeed increase the proteins ATPase turnover numbers, since the NBDs change conformation and are temporally in close proximity (35). In this study, the phospholipids SM, PE and PS were analyzed. Neither of these stimulated ABCB4 above background. The stimulation we observed for LTLE (v_I 315.2 ± 30.4 %) was larger than the stimulation of POPC suggesting that the nature of the fatty acid had a significant influence on the magnitude of stimulation. Thus, we mimicked the fatty acids distribution of the PC composition of the bile canalicular membrane according to Northfield *et al.* (29). Here, an even higher stimulation was observed (v_I 375.5 ± 13.6 %). The sum of all maximal stimulations of the individual lipids of the mixture multiplied with the amount present in the mixture results in a theoretical stimulation of 357 % strongly suggesting that the effects of PC-lipids on the ATPase activity of ABCB4 are additive.

The canalicular membrane contains 40 % PC-lipids that vary in the nature of their fatty acids. While the canalicular membrane harbors PC-lipid variants of every combination of a saturated and an unsaturated fatty acid of the categories 16:0 (18 %), 18:0 (13 %), 18:1 (24 %), 18:2 (13 %), 18:3 (18 %), and 20:4 (4 %) (29), bile PC-lipid content differs drastically.

In order to understand the two-step enrichment of specifically 16:0-18:1 and 16:0-18:2 PC-lipids, which end up in bile, ABCB4 (36) and bile salts perform a crucial interplay (Figure 7). First, the PC-lipid content increases from 40 % in the canalicular membrane to 95 % in bile.

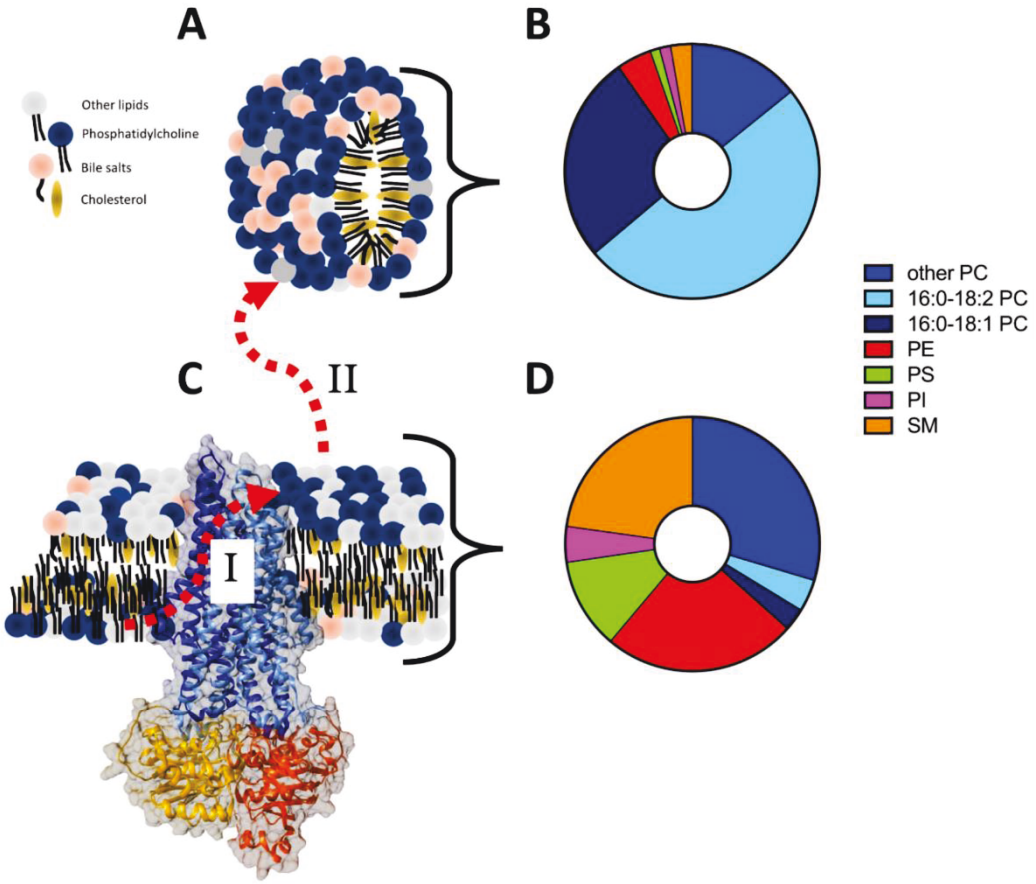


Figure 7. Two-step enrichment of PC-lipid variants into the mixed micelles. [A] A schematic view on mixed micelles [B] with a ratio of 14.2 % other PC-lipid variant, 49.7 % 16:0-18:2 PC, 26.4 % 16:0-18:1 PC, 4.4 % PE, 1.2 % PS, 1.4 % PI and 2.7 % SM (37). [C] Schematic view on the floppase ABCB4 (structure taken from (38)) actively generating a high abundance of PC-lipids in the outer leaflet of the canalicular membrane (I). The second step of PC enrichment (II) may be performed by headgroup-independent bile salt selection. The canalicular membrane consists of [D] 29.6 % other PC-lipid variants, 4.1 % 16:0-18:2 PC, 2.9 % 16:0-18:1 PC, 24.5 % PE, 11.5 % PS, 4.5 % PI and 22.8 % SM (37).

Second, the apparent PC-lipid variants change. For example, ABCB4 creates the asymmetry in the canalicular membrane resulting in a canalicular membrane of mainly PC-lipids in the outer leaflet and PS-lipids in the inner leaflet (Figure 7CI). Here, ABCB4 flops PC-lipids from the inner to the outer leaflet and ATP8B1 predominantly PS-lipids from the outer to the inner leaflet of the canalicular membrane. Confirmatively, we could show that ABCB4 was indeed stimulated by the analyzed PC-lipid-variants, indicating that ABCB4 can translocate a broad spectrum of PC-lipids. The high abundance of PC-lipid in the outer leaflet may be directly related to the amount found in bile (approximately 90-95 %) and answers the first step of PC enrichment. The level of stimulation may go back to a dual recognition of ABCB4 itself or even different accessibilities due to differences in the tightness of packing through fatty acids with a certain level of unsaturation. The second enrichment of specifically 16:0-18:1 PC and 16:0-18:2 PC-lipids cannot be explained on the basis of the stimulation of ATPase activity of ABCB4, since these PC-lipid variants did not stimulate ABCB4 significantly stronger than other PC-lipids (Table 1 and Figure 4C). Rather, bile salts tend to extract bile-PC-lipids out of the canalicular membrane independent of the headgroup, but related to the

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nature of fatty acids (22, 39). Accordingly, bile salts extract mainly phospholipids with 16:0-18:1 and 16:0-18:2 fatty acid chains (22). Since this pool in the outer leaflet of the canalicular membrane consists mainly of PC-lipids created by ABCB4, specifically 16:0-18:1 PC and 16:0-18:2 PC are extracted (Figure 7CII). Consequently, this would protect the biliary tree of lipids from self-digestion (Figure 7). This selectivity from the site of bile salts is also manifested by the resistance of membranes of sphingomyelin/cholesterol rich membranes against the detergent actions of bile salts (37). Following this hypothesis, 16:0-18:1 PC and 16:0-18:2 PC would be completely extracted in ABCB4 deficient systems, because no continuous replenishment of the extracted PC-lipid variants could occur. Analysis of the phospholipid content of bile in mdr2$^{-/-}$ knockout mice showed indeed a full absence of PC-lipids in bile (31), but the lipid composition of the canalicular membrane is still insufficiently studied to validate this hypothesis further.

Fatty acids matter – both *sn* positions matter

We analyzed the fatty acids with respect three categories, (I) acyl chain length, (II) degree of unsaturation and (III) natural occurrence. The data summarized in Figure 4D demonstrate that the stimulation of ATPase activity increased with increasing chain length until a peak was reached for a chain length of 16 carbon atoms. The degree of unsaturation of the acyl chains was investigated in Figure 4E and F. Here, no clear trend was evident, although the 18:3-18:3 PC-lipid was more active than the 16:1-16:1 PC-lipid and the 20:4-20:4 PC-lipid, but 18:1-18:1 PC had clearly the highest degree of stimulation. Whether unsaturation or chain length had a more pronounced impact cannot finally be answered, since both parameters were changed at the same time. However, a trend manifested, in which the 18 carbon atoms tend to stimulate ABCB4 more than others (Figure 4F). Table 1 illustrates that the tested PC-lipid variants with 16 carbon atoms at the *sn1* position activate ABCB4 to the same extent with the exception of 16:0-20:4 PC which is highly unsaturated at the *sn2* position, therefore the *sn2* position may not be as crucial as the *sn1* position, but should not be underestimated. Especially, C18 variants of the *sn1* position emerge out of the pool of PC-lipid variants. One plausible explanation for especially 18:1-18:1 PC activating ABCB4 most efficiently might be increasing packaging defects due to the introduction of the optimal amount of kinks in the PC-lipids. DOPC has two double bonds, hence two introduced kinks that might make the headgroup more accessible for the uptake process performed by ABCB4. In category three, we intended to analyze the natural occurrence of PC-lipids in bile. Since 16:0-18:1 PC and 16:0-18:2 PC are known as the most prominent bile PC, ABCB4 might be the discriminating factor and the molecular factor responsible for this enrichment. Unnatural PC-lipids, as well as PC-lipid variants exclusively found in the canalicular membrane had higher potencies in ATPase stimulation of ABCB4. The 18:1-18:1 PC-lipid excelled the bile PC-lipid variants with more than twice the maximal stimulation v_I.

Interestingly as the modulation of the 16:0-16:0 PC-lipid was identical to 16:0-18:1 and the 18:1-18:1 PC lipid again was the most potent PC-lipid, it might suggest a pivotal role for the *sn1* position. Further support of this notion comes from the results of lyso- 16:0 PC. Lyso-16:0 PC as well as FC-16 were the only compounds that resulted in inhibition of the ATPase activity of ABCB4. This might be related to either i) the detergent properties of lyso-16:0 PC and FC-16 or ii) the lack of the fatty acid of the *sn1* position (lyso-PC).

The lipid headgroup dictates whether transport occurs, the fatty acids regulates the level of ATPase stimulation

The beneficial effect of lipids on the ATPase activity of ABC transporters such ABCB1 is generally accepted (40). In the case of human ABCB1 lipids were used for stabilizing the protein and to diminish the detrimental effect of the detergent of choice, n-dodecyl β-D-maltoside (β-

DDM) (41). ABCB1 purified in detergent micelles often required higher drug concentrations than the reconstituted protein to obtain similar effects on ATPase activity. Although, ABCB4 and ABCB1 are highly homologous, ABCB4 is highly specific for PC-lipids (12, 15). Therefore, next to any stabilization effects of lipids, it translocates lipids of the PC family *per se*. However, a stabilizing effect of phospholipids on detergent solubilized and purified ABCB4 might also modulate the activity independent of the family of phospholipids. To exclude any changes in the ATPase activity due to the change in the physical state of lipid to detergent ratios, we demonstrated that even if liposomes or liposome-like structures behave differently, with increasing detergent concentrations, no correlation to stimulation was observed (Figure 3A, B, Supplemental Figure S1). We however like to emphasize that we cannot distinguish based on the observed stimulation of ATPase activity whether these lipids are indeed substrates of ABCB4 or whether they solely act as activators of ATPase activity, which is a prerequisite of substrate translocation of this primary active membrane transporter. Additionally, effects of annular lipids co-purified and still attached may also play a role in the protein's activity and even result in a change of the basal activity. However, no co-purified lipids could be detected on a thin layer-chromatography (Supplemental Figure S2) and are more likely washed off during the washing steps of the immobilized metal ion chromatography (34). We could previously demonstrate, that ABCB4 is specifically stimulated *in vitro* by lipids of the PC-family (23). This is in line with *in vivo* studies that only lipids of the PC family are translocated by ABCB4 (15, 31). Both studies imply that the recognition of the substrate occurs within the headgroup, since other phospholipid families share the glycerol core and fatty acids. Using our *in vitro* system, which displays a PC-lipid specific stimulation of ATPase activity as read out, we analyzed the ATPase activity of ABCB4 in the presence of the phosphatidylcholine headgroup (Figure 5C). Surprisingly, no stimulation was observed. The same holds true for choline or Lαgpc (Figure 5C). However, we cannot exclude that the highly water-soluble choline, ppc and Lαgpc may require greater than sub-millimolar concentrations to show any potential effects on the ATPase activity of ABCB4. Nevertheless, this of course pointed towards a pivotal role of the fatty acids. Interestingly, one fatty acid resulted in inhibition of ATPase activity (Figure 5B), namely lyso-16:0 PC, a detergent, inhibited the ATPase activity with a k_2 value of 103.9 ± 7.7 µM (Figure 5B and Table 2).The analysis of the nature of inhibition for FC-16 provided no indication for a competitive inhibition, rather a negative detergent effect is responsible for the inhibition of ABCB4 (Figure 6, table 3). Therefore, only a full choline headgroup with at least the glycerol core as to be found in lyso-16:0-PC needs to be present to be recognized by the binding site of ABCB4 and only in the presence of two fatty acids, a stimulation of the ATPase activity of ABCB4 was observed (Figure 5A). For example, stimulation occurred with 16:0-16:0 PC but was rather low in the context of other ABC transporters as it was only 268.8 ± 10.5 % (174.0 ± 6.5 $nmol \cdot min^{-1} \cdot mg^{-1}$) in comparison for example to chABCB1, which was stimulated up to 3 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ (42). The low activity of ABCB4 is however in line with studies from Ishigami *et al.* (43). The authors pointed out that ABCB4 needs to possess a low activity as the large pool of surrounding substrates would result in a waste of energy and a loss of membrane integrity if too many substrates are translocated (43).

With the knowledge that neither the headgroup nor the glycerol backbone were capable of stimulating ABCB4 accordingly, we provided evidence for the requirement of both features of a PC-lipid, the head and the tail of a lipid, need to be present to activate ABCB4. Either of both is not sufficient as PE with the same glycerol backbone core and two acyl chains did not stimulate ABCB4 (Figure 5A).

Conclusion

Every PC-lipid variant tested was able to stimulate the ATPase activity of ABCB4. ABCB4 seem to

prefer PC-lipids with 18 carbon units at the *sn1* position, while the building blocks of PC-lipids alone are not sufficient to stimulate ABCB4. The differences in the level of ATPase stimulation by PC-lipids regarding their fatty acid chain length and the degree of unsaturation provides an explanation for the protection of the canalicular membrane from the harsh detergent activity of bile salts. However, and equally important, only PC-lipids are capable of stimulation the ATPase activity of ABCB4 originating from the headgroup and the presence of two fatty acid chains.

Data availability

All data are contained within the manuscript.

Acknowledgments

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Conflict of interest statement

The authors declare that they have no conflicts of interest with the contents of the articles.

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3.3 Chapter III – Mechanism of ABCB4

Title:	**Evidence for a credit-card-swipe mechanism in the human PC-floppase ABCB4**
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Evidence for a credit-card-swipe mechanism in the human PC-floppase ABCB4

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1. Abstract

ABCB4 is described as an ABC transporter that primarily transports lipids of the phosphatidyl choline family but is also capable of translocating a subset of typical multidrug resistance-associated drugs. The high degree of amino acid identity of 76% for ABCB4 and ABCB1, which is a prototype multidrug resistance mediating protein, results in ABCB4's second subset of substrates, which are linked to ABCB1's substrates. This often leads to incomplete annotations of ABCB4, in which it was described as exclusively PC-lipid specific. Differences between both proteins are distributed over the entire protein, but significantly cluster in transmembrane helix 1. Especially Q52, S58 and S69 in transmembrane helix 1 of ABCB4 are essential for productive interaction with the primary substrate's in form of PC-lipids as demonstrated by ATPase activity measurements and molecular dynamic simulations. When the hydrophilic amino acids from ABCB4 are changed to the analogous, but hydrophobic ones from ABCB1, the stimulation of ATPase activity by DOPC as a prime example of PC-lipids is strongly diminished, while the modulation capability of ABCB1 substrates remains completely unchanged. This indicates two distinct and autonomous substrate binding sites in ABCB4.

2. Introduction

ABCB4 is a phosphatidylcholine floppase localized in the canalicular membrane of hepatocytes (A.M. vanderBliek, 1987; Ruetz and Gros, 1994; Smit et al., 1993; Van der Bliek et al., 1988). Historically, ABCB4 has been called multidrug resistance (MDR) protein 3 (MDR3) because of its high degree of similarity (76% identity, 86% similarity of the primary structure) to Pgp (MDR1; ABCB1) (Smith et al., 2000). ABCB1, in contrast to ABCB4, is a prototype MDR protein that exports hydrophobic xenobiotics out of the cell (Juliano and Ling, 1976). The small molecular differences between these two ABC transporters, however, lead to a large physiological difference. Therefore, it is very likely that the amino acid (aa) differences play a special role in substrate recognition and the translocation mechanism. Interestingly, sequence homology demonstrate that these differences are distributed largely over the entire protein, with only a few clusters. One such cluster lies in the N-terminal part and the subsequent transmembrane helix 1 (TMH1).

Molecular differences that influence the substrate spectrum of an ABC transporter are thought to be especially clustered in the transmembrane region, since here the substrate binding sites are often located, especially in MDR ABC transporters. Hence, the substrate binding pocket of ABCB1 is located in the hydrophilic core of the protein approximately at the location of the center of the membrane (Kim and Chen, 2018). It would therefore be easy to assume that amino acid exchanges, which alter the substrate spectrum (or are interchanged between two highly homologous proteins with different substrate spectra) are also present in the region of the central cavity within the TMD. Assuming that a hydrophilic cavity in the center of the protein as a common substrate-binding site comparable to ABCB1 (Alam et al., 2019) or ABCG2 (Manolaridis et al., 2018) is also true for ABCB4, lipid-transport, in general, would need to overcome a thermodynamic barrier, if a hydrophobic phospholipid molecule enters an aqueous cavity. The substrate binding site of ABCB4 is not yet described in detail, although recently, the structure of this PC-floppase was determined by single-particle cryo-EM (Olsen et al., 2019). Here, ABCB4 was indeed described as an ABC transporter to follow the alternating two-site access model with a substrate-binding site analogous to ABCB1 (Hollenstein et al., 2007). In contrast to the described mechanism for ABCB4 (Olsen et al., 2019), proteins translocating lipids or lipid-like substances, for which this information is present, apparently follow a different concept. For lipids or lipid-like substances with a polar headgroup (lipid headgroups or oligosaccharides, respectively) and hydrophobic tails in the form of polyprenyl or fatty acids, the tails do not necessarily have to enter the protein's core. It is sufficient to shield the headgroup in a translocation cavity at the protein-lipid bilayer interface, while the hydrophobic

tail is still embedded in the membrane. For ABCB4 to follow a translocation mechanism based on the classic alternative access model (like ABCB1 does) would contradict the general features used by other lipid transport systems (Brunner et al., 2014; Perez et al., 2015; Qian et al., 2017). For example, PglK is a bacterial transporter that translocates cell wall precursors employing an outward-only mechanism (Perez et al., 2015). Here, the headgroup of pyrophosphate-oligosaccharide linked lipid interacts with positively charged amino acids of PglK. The polyprenyl tail binds to a binding cavity and activates transport but remains embedded in the lipid bilayer (Perez et al., 2015). Even though the translocation process itself just requires one conformation, both states are present and connected through ATP hydrolysis. The inward-facing conformation is probably needed to load the substrate in close proximity to a cavity, which is only formed in the outward-facing conformation. Therefore, the inward-facing conformation presents the stage of loading (Perez et al., 2015).

TMEM16, on the other hand, is a Ca^{2+}-activated lipid scramblase (Brunner et al., 2014). Ca^{2+} binds in the hydrophobic core of the protein at the level of the membrane. Each monomer of the homodimer consists of 10 transmembrane helices. On both sites that face the lipid bilayer, lipid shuffling can take place. This transverse shuffling has no specificity and occurs without the need for ATP hydrolysis (Brunner et al., 2014).

Comparatively, ABCB4 and ABCB1 are each coupled, primary active transporters (Al-Shawi et al., 2003; Kluth et al., 2015; Shukla et al., 2017), which require ATP hydrolysis to ensure substrate translocation. Regardless of whether one or two ATP per transport cycle is required, transport *per se* cannot take place without ATP hydrolysis. Next to MD simulations, we used this coupling between ATP hydrolysis and substrate transport to gain insights into the transport mechanism of ABCB4.

3. Results

3.1. ABCB1 and ABCB4 share high amino acid sequence identity

ABCB4 and ABCB1 share a high amino acid sequence identity of 76%. Even if their substrate spectrum covers some overlaps, the physiological role and their evolutionary optimized substrates are remarkably different. ABCB1 is a prototype MDR protein and decreases the effective concentration of drugs through continuous export. ABCB4, on the other hand, is a PC-lipid-specific lipid translocase, which flops PC lipids from the inner to the outer leaflet with only a little multidrug resistance mediating propabilities. Substrate selection is determined by the transmembrane domain (TMD) of ABC transporter; therefore, we took a closer look on the differences of both proteins to unravel crucial determinants for the transport mechanism.

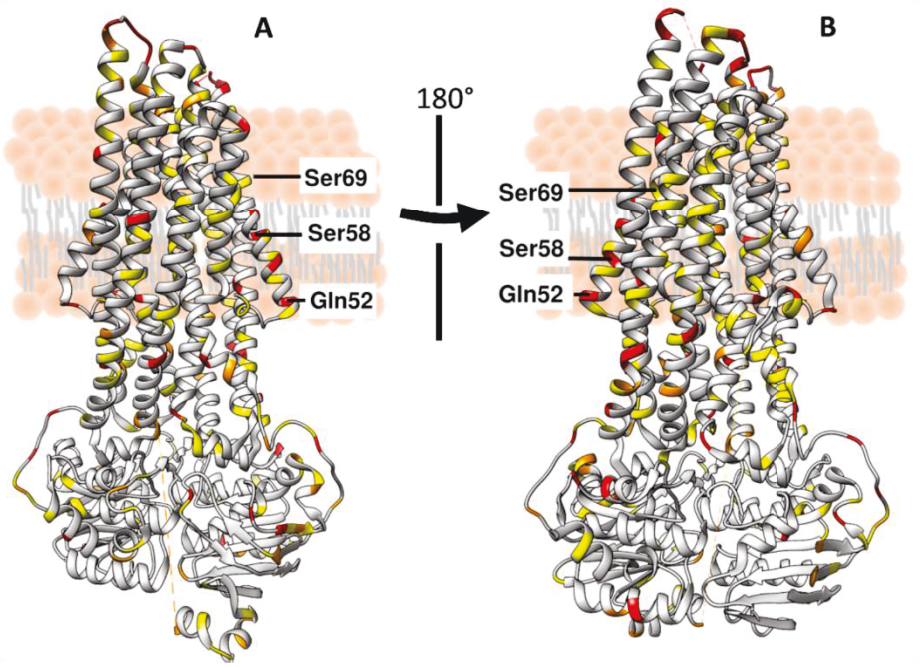

Figure 1. **Differences between ABCB1 and ABCB4 on a structural level.** [A] Structure of ABCB4 (PDB entry 6S7P, (Olsen et al., 2019)) colour-coded according to sequence conservation between ABCB1 and ABCB4 and based on BLOSUM62 scoring (Henikoff and Henikoff, 1992). Yellow corresponds to a moderate (e.g. R785K, positive BLOSUM62 score) change, orange to an intermediate change (e.g.V746T, neutral BLOSUM62 score), while red corresponds to no conservation (e.g. F760A, negative BLOSUM62 score). Three amino acids located in TMH1 are highlighted [B] View of A rotated by 180°.

The sequence alignment of ABCB1 and ABCB4 based on the structure of ABCB4 (Olsen et al., 2019) was color-coded to provide an overview of how amino acids at the respective positions differ (Figure 1). Yellow corresponds to moderate differences such as an exchange of an aliphatic side group to another aliphatic side group, while red represents a change of charge or polarity. It is worth noting that most of these amino acids are not found in the classical substrate binding pocket of ABC transporters, which is in the hydrophilic core of the protein as

identified for the prototype of an MDR exporter, ABCB1 (Alam et al., 2019; Sharom, 2014). There are 55 amino acid differences within the entire transmembrane domain (TMD). Only three amino acids are different in the classical binding pocket in comparison to ABCB1 V985, H989, and A990 (Olsen et al., 2019). The other amino acid exchanges clustered in other parts of the transmembrane domain. Surprisingly, one cluster was located in TMH1, which was not thought to be part of the substrate recognition due to its localization apart from the central cavity and proximity to the lipid bilayer (Figure 1). TMH1 itself harbors 18% of the exchanges (10 of the total 55). Three of them are of importance and are characterized by their polar nature (Q52, S58, and S69). We analyzed the degree of conservation of these three amino acids in 50 different species that possess ABCB4 homologues. The amino acid Q52 was conserved in 47 out of 50 cases (94%), S58 with 40/50 (80%) and S69 48/50 (96%) (Figure 2B). This indicated a high degree of conservation, especially for positions 52 and 69, respectively. Only amino acid position 58 was less conserved, although 80% is obviously significant.

On top of that, TMH1 in ABCB1, in contrast to ABCB4, was especially prominent with respect to sequence divergence as emphasized for two representative organisms (human and mouse, Figure 2B). In ABCB1, these positions are fulfilled by nonpolar counterparts (Q52L, S58V and S69A; Figure 2A). Note that, from a thermodynamic point of view, it seems unfavorable that ABCB4 places polar amino acids to the lipid facing part of TMH1.

Figure 2. **Level of conservation within ABCB4 genes and compared to ABCB1**. [A] Sequence alignment of ABCB4 and ABCB1 from mouse and human in the region of residues 50 to 74 of TMH1. [B] Conservation of positions 52, 58, and 69 of TMH1 in 50 different species with ABCB4 genes.

3.2. Evidence for ABCB4-mediated PC flop along TMH1

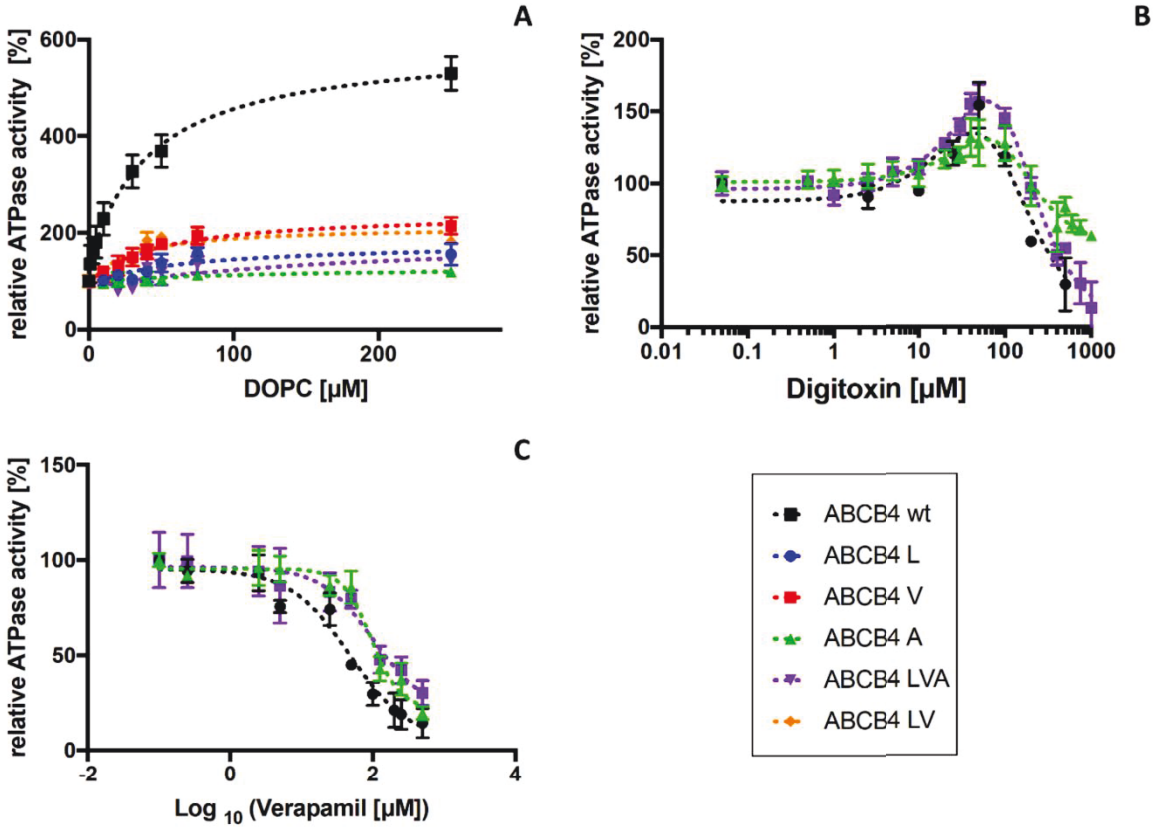

Figure 3. ***In vitro*** **data on the importance of TMH1 to PC-lipid binding**. [A] Relative ATPase activity of ABCB4 wt and the mutants Q52L (L), S58V (V), S69A (A), Q52L-S58V (LV) and Q52L-S58V-S69A (LVA) in the presence of increasing concentrations of the phosphatidylcholine lipid 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC). [B] Relative ATPase activity of ABCB4 wt and the mutants A and LVA in the presence of increasing concentrations of digitoxin. [C] Relative ATPase activity of ABCB4 wt and the mutants A and LVA in the presence of increasing concentrations of verapamil.

As a starting point to investigate the lipid transport mechanism of ABCB4, we chose the three amino acid residues (Q52, S58, and S69) in TMH1 that were identified in the sequence alignments (Figure 2). Thus, single, double, and triple mutations were introduced. Direct comparison of the ATPase measurements between the ABCB4 mutants and wt is facilitated by a normalization of the data to the amount of used protein and to the basal activity. WT ABCB4 showed DOPC dependent stimulation of ATPase activity (black curve in Figure 3A), which reached 500% of the activity in the absence of DOPC. In contrast, ABCB4 mutants with the introduced mutations L, V, A, LV, LVA did not show such a degree of stimulation. A residual stimulation was measured for ABCB4 V and ABCB4 LV, whereas ABCB4 A, in particular, was not stimulated above the basal level of ATPase activity. Surprisingly, this reduction of the DOPC stimulation is also reflected by the conservation (Figure 2B). Here, the less conserved S58 also shows a less, but still detected low stimulation in the presence of DOPC (190 %), while S69 seems to be crucial for ATPase activity (110 %). However, the loss of stimulation might be simply due to a partial misfolding of the protein. Therefore, we selected two

compounds that are able to modulate the ATPase activity of ABCB4, namely digitoxin and verapamil, respectively, to analyze the integrity of ABCB4 (Ishigami et al., 2013; Pauli-Magnus et al., 2001; Smith et al., 2000). Digitoxin, which is a well-known modulator of ABCB1 (Pauli-Magnus et al., 2001), has not been described as a secondary substrate of ABCB4 so far. Interestingly, the well-known bell-shaped dose-response curve was also observed for wt ABCB4 (Figure 3B, black curve). Most importantly, all mutants of ABCB4 displayed an identical behavior (Figure 3B). The shape of the curve is probably due to the affinity change of the substrate binding pocket after a conformational change. Here, substrates are bound in the inward-facing conformation with high affinity (high-affinity binding site). ATP is bound, and the outward-facing conformation is adopted. This, in turn, changes the substrate affinity. The affinity is reduced (low-affinity binding site), and as a result, the substrate is released into the extracellular space. If the concentration of substrate exceeds a threshold value, i.e., the affinity of the low-affinity binding site, the outward-facing conformation is arrested, and the transporter can no longer release the substrate. As a result, inhibition of the ATPase activity is observed. WT and ABCB4 mutants showed nearly identical dose-response curves in the presence of increasing concentrations of digitoxin. If we indeed modified the substrate binding pocket in these mutants, substrate-dependent ATPase activity should be changed or become even independent of substrate concentration. However, this was just the case for DOPC as a substrate, not for digitoxin. The ATPase activity for the modulator remained unaltered in the ABCB4 mutants.

Additionally, we analyzed the ATPase activity of ABCB4 and the mutants in the presence of verapamil (Figure 3C), a well-known inhibitor of ABCB1 (Smith et al., 2000), and ABCB4 (Duan et al., 2004; Smith et al., 2000). Here, again no significant difference in the inhibition of ATPase activity of wildtype protein and the mutants was observed. Thus, the architecture of the verapamil and digitoxin binding sites are not modified in the mutants. Since we observed no DOPC stimulation in the mutants, this clearly demonstrates that drug and lipid binding site(s) are different. Classical ABCB1 substrates, which have hydrophobicity in common, are recognized differently than PC-lipids.

3.3. Cavities within the proteins differ between ABCB1 and ABCB4

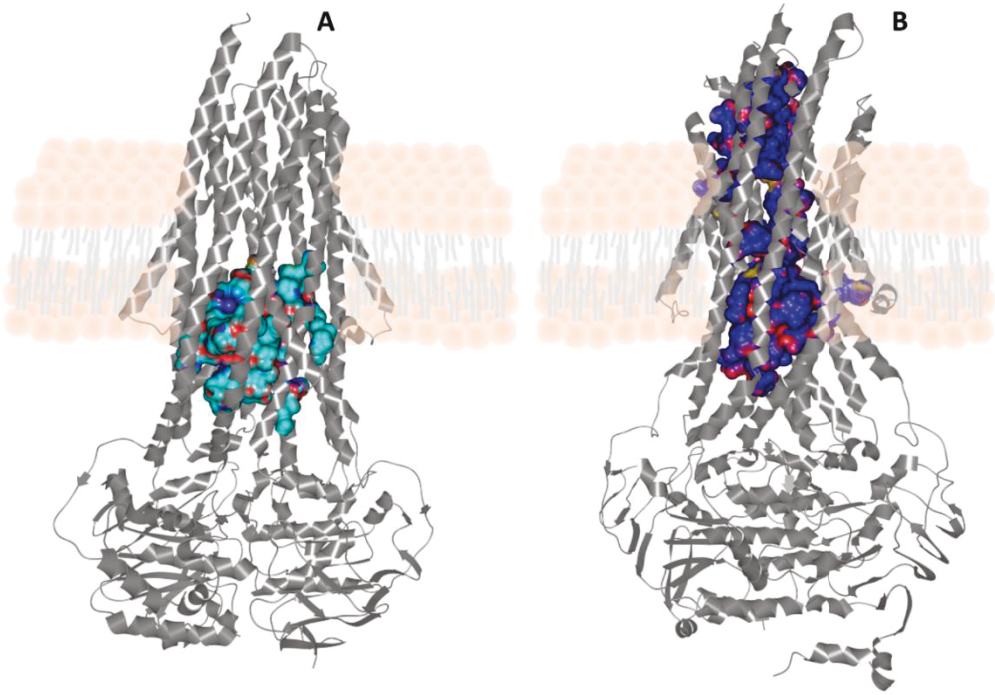

Figure 4. **Cavities of ABCB1 and ABCB4.** [A] Structure of ABCB1 (PDB entry 6C0V, (Kim and Chen, 2018)) highlighting the cavity (solid) with a volume of 5325 Å^3. ATP-bound ABCB1 adopts the outward-facing conformation. [B] Structure of ABCB4 (PDB entry 6S7P, (Olsen et al., 2019)) highlighting the cavity (solid) with a volume of 3756 Å^3 in an ATP-bound, closed-conformation. Both computations were performed with Caver, (Chovancova et al., 2012) using a probe size of 1.4 Å^3.

The structural comparison of ABCB1 and ABCB4 reveals that the cavity of ABCB4 has a smaller volume and is somewhat globular (Figure 4A), whereas that of ABCB1 is more pore-like (Figure 4B). Thus, in the case of ABCB4, the cavity spans the entire membrane, but it only covers the inner membrane leaflet in the case of ABCB1. However, note that the structure of ABCB4 represents an outward-facing but closed conformation (Olsen et al., 2019). Per definition no similar conformation has been described for ABCB1. The most similar conformation corresponds to the outward-facing but open conformation of ABCB1 (Kim and Chen, 2018), which is shown in Figure 4B and differs only 1 Å rmsd between their Cα atoms. Even though, it is also possible that the different sizes of the cavities originate from different conformations, a difference to a 40 % larger cavity in ABCB1 is somewhat surprising of such similar strucutres and goes in hand with the more promiscuous substrate spectrum.

3.4. ABCB4 restricts lipid access

To determine whether and to what extent ABCB1 and ABCB4 are able to take up and bind DOPC lipids in their substrate cavity, we conducted five independent all-atom MD simulations of each transporter starting from the inward-facing conformation embedded in an explicit DOPC membrane. MD Simulations were carried out using the GPU implementation of pmemd

(Le Grand et al., 2013; Salomon-Ferrer et al., 2013b) implemented in the Amber Suite of biomolecular simulation programs (Salomon-Ferrer et al., 2013a).

For the alternating two-site access model, in this conformation, a potential substrate in the inner leaflet must first traverse one of the two entry gates (front gate: TMH3/4/6, rear gate: TMH9/10/12) to enter the hydrophobic substrate cavity. Therefore, we first determined differences in the accessibility of the substrate cavities of ABCB1 and ABCB4. In all MD simulations of ABCB1, both entry gates were, on average, wide open (Figure 5A); by contrast, in all MD simulations of ABCB4, at least one of the entry gates was constricted or completely closed (Figure 5B). We furthermore counted the number of bound lipids for each simulation snapshot. ABCB1 accommodated, at least partially, up to four lipids in its substrate cavity (Figure 5C, left), but ABCB4 never took up more than one lipid (Figure 5C, right). Finally, we computed the average number of snapshots in which one lipid was bound. On average, in 64.8 ± 9.4 % of all snapshots of an ABCB1 simulation, at least one DOPC molecule crossed the boundary to the substrate cavity; by contrast, on average, only 18.8 ± 12.5 % of all snapshots of an ABCB4 simulation had at least one DOPC molecule bound in its analogous cavity in the center of the protein (see also Figure 4). Together, these results suggest that in ABCB4, unlike in ABCB1, lipid access to the substrate cavity is markedly restricted.

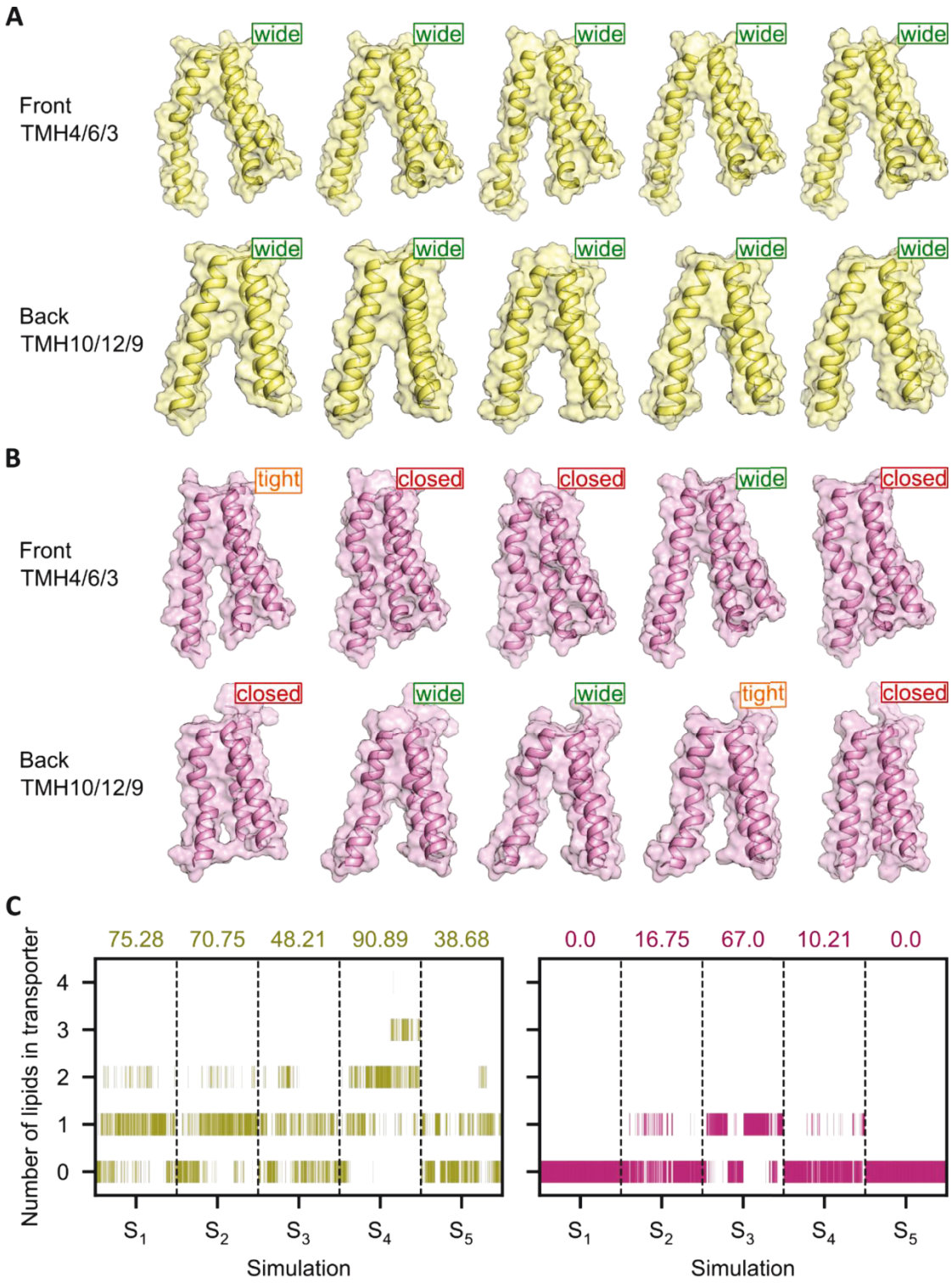

Figure 5. **Lipid binding to the substrate cavities of ABCB1 and ABCB4.** [A] Closest-to-average structure of the front gate (formed by TMH4, TMH6, and TMH3) and rear gate (formed by TMH10, TMH12, and TMH9) to the substrate cavity of ABCB1, extracted from five independent MD simulations of inward-facing ABCB1 embedded in a DOPC membrane. [B] Closest-to-average structure of the front gate (formed by TMH4, TMH6, and TMH3) and rear gate (formed by TMH10, TMH12, and TMH9) to the putative substrate cavity of ABCB4, extracted from five independent MD simulations of inward-facing ABCB4 embedded in a DOPC membrane. [C] Time course of DOPC binding into the substrate cavity of ABCB1 (left, golden) and ABCB4 (right, purple), determined from five independent MD-Simulations (S_1-S_5) of each system. The percentage of snapshots in which at least one DOPC molecule was bound to the substrate cavity is given above the plot for each simulation. A DOPC molecule is classified as bound if its phosphate atom lies within the bounding box around the position of the C-alpha atoms of TMH3, TMH4, TMH9, and TMH10, and if its phosphate atom is located between the innermost C-alpha atoms of the helix pairs TMH3/TMH4 and TMH9/TMH10.

3.5. Q52 in TMH1 of ABCB4 may pre-organize PC molecules for flip-flop

Next, using the same set of MD simulations, we probed whether TMH1 may be differently involved in the phospholipid translocation mechanism in ABCB1 and ABCB4. To do so, we computed the residence probability of DOPC molecules in the lower leaflet. The residence probability near TMH1 was higher in both simulation systems than near TMH7 (Figure 6A); TMH7 is the structural equivalent of TMH1 in the other pseudohalf of the transporter. The magnitude of the residence probability of DOPC molecules near TMH1 differed overall little between ABCB1 and ABCB4 (Figure 6B), except for the high probability close to Q52 in ABCB4. This result suggests that the residue helps in ABCB4 in pre-organizing DOPC molecules for flip-flop along TMH1.

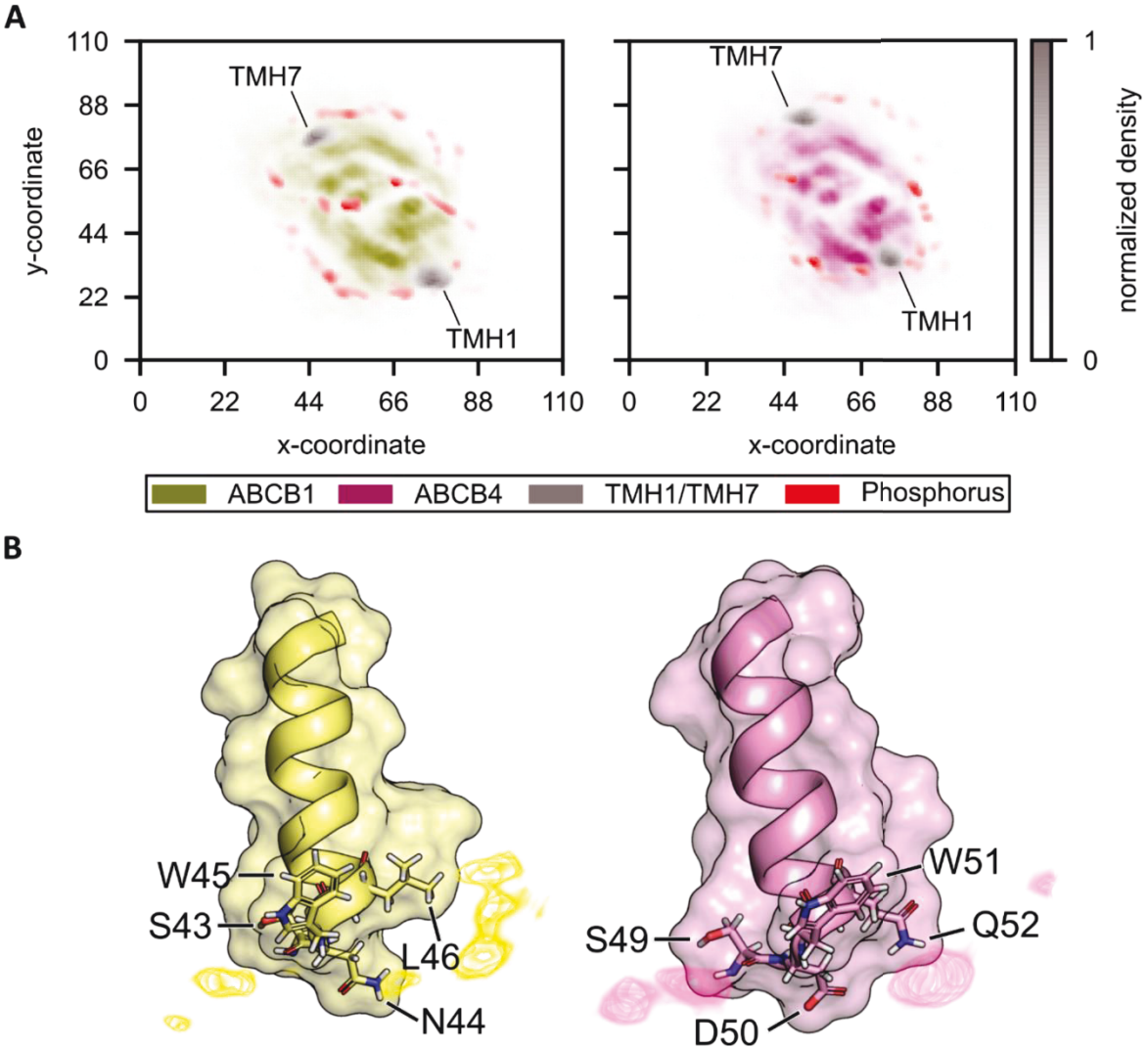

Figure 6. **Comparison of the residence probability of DOPC in simulation systems containing ABCB1 (yellow) and ABCB4 (purple).** [A] Z-axis-averaged residence probabilities of the backbone atoms of ABCB1 (left, yellow) and ABCB4 (right, purple), of the Cα atoms of the first helical turn of TMH1 and TMH7 (grey), and the phosphorus atoms of the DOPC molecules in the lower leaflet of the membrane (red). The values for each grid point were normalized to values between zero and one for each grid individually and are encoded through the opacity of the respective color (see color scale on the right). [B] Visualization of regions with high residence probabilities of DOPC near the origin of TMH1 in ABCB1 (yellow, left) and ABCB4 (purple, right). The volumes represented as wireframe enclose regions of high probability.

3.6. Free energy calculations of DOPC flip-flop and ABCB4-mediated DOPC translocation.

Using a combination of steered MD simulations, umbrella sampling (Kästner, 2011), and potential of mean force (PMF) calculations using the weighted histogram analysis method (Kumar et al., 1992), we next studied the differences in configurational free energy between ABCB4-mediated DOPC translocation, and DOPC flip-flop across a DOPC membrane. In total, we sampled four pathways along each of which the PMF was calculated: 1) DOPC flip-from from the inner to the outer membrane leaflet in a pure DOPC membrane, 2) DOPC translocation along TMH1 of inward-facing ABCB4 (Figure 7A, B), 3) DOPC translocation along TMH7 of inward-facing ABCB4, and 4) DOPC translocation along TMH1 of ABCB4 in the outward-occluded conformation. We calculated the energy barrier for an unassisted DOPC flip-flop in a pure DOPC membrane as ~20.4 kcal mol^{-1} (Figure 7C, top), which is in good agreement with the experimentally determined flip-flop rate for spin-labeled phosphatidylcholine (Kornberg and Mcconnell, 1971) (0.04–0.07 h^{-1}, corresponding to 19.2–19.5 kcal mol^{-1} according to the Eyring equation), and the calculated energy barrier for DPPC flip-flop in a pure DPPC membrane (Tieleman and Marrink, 2006) (~19.5 kcal mol^{-1}). This energy barrier decreased by 6.8 kcal mol^{-1} when DOPC was translocated along TMH1 of ABCB4 (Figure 7C, top). Notably, this difference corresponds to the gain in energy due to ATP hydrolysis, which is 7.3 kcal mol^{-1} under physiological conditions (Rosing and Slater, 1972). Among the three hydrophilic residues along the transmembrane segment of TMH1 – S58, T61, and S69 – S69 stood out since the most prominent differences in the free energy profile are found in the region of its location (Figure 7C, top). No major differences in the free-energy profile were detected in the region where S58 and T61 were located during the umbrella sampling simulations. Nevertheless, since the S58V mutant displayed a significantly reduced DOPC-stimulated ATPase activity, we assume that S58, and possibly T61, control the correct positioning of DOPC during translocation, and that ATPase activity is only stimulated when the lipid reaches the vicinity of S69. We further found evidence that the observed reduction of the free energy barrier is specific for translocation along TMH1, since translocation along TMH7 did not result in a reduction of the barrier (Figure 7C, middle). Since we detected a reduction of the free energy barrier when DOPC is translocated along TMH1 only considering the inward-facing conformation, we next attempted to account for the role of the different conformations of ABCB4 during translocation and the associated need for ATP hydrolysis. To do so, we recalculated the free energy profile

along TMH1 using the outward-occluded conformation of ABCB4 (Olsen et al., 2019). Comparing the free energy profiles for the inward-facing conformation and the outward-occluded conformation, the first half of the translocation pathway appears to be more favourable in the outward-occluded conformation, while the second half of the translocation pathway is more favourable in the inward-facing conformation (Figure 7C, bottom). This comparison suggests that binding, positioning and initial translocation of the hydrophilic lipid head group into the membrane occurs in the outward-facing or outward-occluded conformation, while the energetically most demanding step – the transfer of the head group across the centre of the membrane – occurs in the inward-facing conformation.

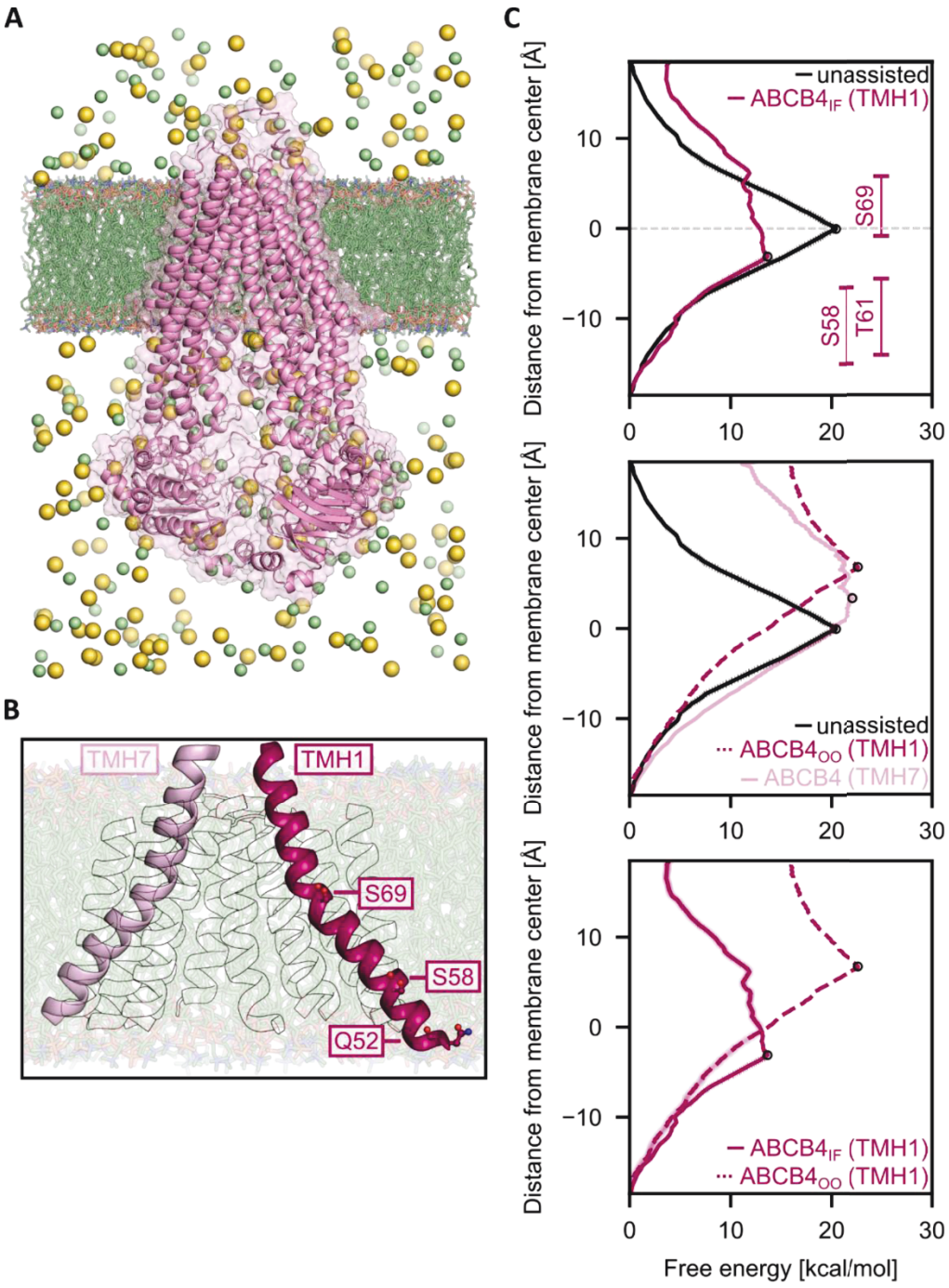

Figure 7. **Configurational free energy calculations of DOPC flip-flop and ABCB4-mediated DOPC translocation.** [A] Representation of the complete simulation system. ABCB4 (purple) is shown in cartoon and surface representation. The DOPC membrane (green) is shown as sticks. Na^+ and Cl^- ions are depicted as yellow and light green spheres, respectively. [B] Transmembrane region of ABCB4. TMH1 and TMH7 are highlighted in dark purple and pink, respectively. Hydrophilic residues in TMH1 that have been exchanged in mutation experiments are highlighted. [C] Free energy profiles of DOPC flip-flop in a DOPC membrane (black), DOPC translocation along TMH1 (dark purple), and DOPC translocation along TMH7 (pink). The free energy profile of DOPC translocation along TMH1 of outward-occluded ABCB4 is indicated with a dashed line. The locations of the side chain oxygen atoms of S58, T61, and S69 during the course of the simulation are displayed with two standard deviations.

4. Discussion

4.1. ABCB4 and ABCB1 – highly similar sequence, but different physiological roles

Despite their high sequence identity (76% for the entire protein), both membrane proteins fulfill different physiological roles. ABCB1 is a prototype drug exporter, while ABCB4 flops PC lipids in the canalicular membrane of the liver. Due to translocation efficiencies, ABCB1 and ABCB4 most likely do not interfere with each other *in vivo*, and each protein translocates only its own substrates despite a partly overlapping substrate spectrum under *in vitro* circumstances. ABCB1 was claimed to translocate phospholipids independently of their headgroup and ABCB4 was able to facilitate the export of hydrophobic drugs (Smith et al., 2000; van Helvoort et al., 1996). However, one has to keep in mind that some substances are exclusive for each of the ABC transporters pointing towards similarities but also significant differences (Smith et al., 2000; van Helvoort et al., 1996). For example, it might even be possible that ABCB4 transports its native substrates, PC-lipids, completely different, but possesses a second translocation pathway identical to the one proposed for ABCB1.

Despite a possible shared translocation pathway for hydrophobic drugs in ABCB1 and ABCB4, each protein is evolutionarily optimized to its specific subtype of substrate(s). In the case of ABCB4, the transporter might possess a drug binding site and utilize an export pathway that is used, if the apparent drug concentration exceeds certain levels and matches the size.

Both necessities are most likely not fulfilled under physiological conditions but may be achieved in *in vitro* circumstances. This hypothesis is supported by the observation that ABCB4 shows the same ATPase activation pattern as ABCB1 *in vitro* as demonstrated for the case of digitoxin (Figure 3B). A pattern described as a bell-shaped-curve most likely due to the accessibility of substrates to a high- and a low-affinity binding site and transporters that utilize the classical alternating two side access model (Hollenstein et al., 2007). If the low-affinity binding site, e.g., the substrate-release binding site, facing the extracellular space, is saturated with excess of substrate, the substrate cannot be released and occupies the binding site trapping the transporter in the outward-facing conformation. This leads to an inhibition of the ATPase activity, while stimulation is observed at lower substrate concentrations (Al-Shawi et al., 2003). If ABCB4 would only utilize the secondary pathway analogous to ABCB1 substrate uptake process must be highly conserved between both transporters. Instead, a different type of substrate uptake between ABCB4 and ABCB1 can be seen in Figure 5, where the substrate uptake through a lateral gate is clearly different and less pronounced in ABCB4. Together with

the different size and shape of the potential substrate binding pocket, it again points towards a different mode of operation of ABCB4. Here, the pore-like cavity in ABCB1 allows the substrates to reach the extracellular space, while the cavity of ABCB4 (Figure 4A), at least in the determined conformation, does not span the complete length of the membrane like the pore of ABCB1 does (Figure 4B). Again, this might explain the differences in the substrate spectrum and indicate that lipids might be transported with or along other regions. Together with the fact that most of the amino acids are different in the areas of the protein facing the membrane, this could explain why ABCB4 is able to transport PC lipids, while ABCB1 does not. Strikingly, we tried to identify the PC-lipid binding site of ABCB4 and introduced mutations in an area, where we assumed substrate-protein interactions. This area in TMH1 is crucially different in ABCB4 and ABCB1. It is precisely this change in polarity that provides ABCB4 with a concrete advantage, enabling it to translocate PC-lipids especially the polar headgroup without forcing the lipids into a water-filled cavity in the protein interior. The idea is that ABCB4 shifts the PC-lipids along TMH1 from the inner to the outer leaflet. Here, TMH1 serves as anchor point for the lipid head group. If the distance covered is more than half of the total distance, an energetic barrier and a conformational change prevents any back-transport, and the flopping process is completed. Additionally, the asymmetric energy landscape does not favour a back transport, a so-called PC-flip. Thus, the mutations abolished the DOPC stimulated ATPase activity, but not the basal activity nor the modulation by digitoxin or verapamil. We therefore strongly believe to have obtained indirect evidence that PC-lipids act as substrates of ABCB4 even in the detergent-purified state rather than acting as modulators. Additionally, no changes of ATPase activity of either the wt protein or the mutants was observed in the presence of the inhibitor (verapamil) or the modulator (digitoxin). This clearly indicates the presence of two distinct translocation pathways in ABCB4, one used for ABCB1 substrates and one for PC-lipids. It has been shown in whole cell experiments and cell culture-based assays that ABCB4 is capable of exporting classical ABCB1 substrates (Duan et al., 2004). However, this translocation is significantly slower than the transport rates of ABCB1. Duan et al., demonstrated that ABCB4 siRNA-treated cell lines showed minor reductions in paclitaxel resistance and is consistent with the observation of lower rates of transport of paclitaxel by ABCB4 as compared with ABCB1 (Duan et al., 2004; Smith et al., 2000).

4.2. Evidence for the TMH1 as an anchor point for lipid translocation

Families of other proteins interacting, and translocating lipids or lipid-like structures face the same problem of pulling an amphipathic substrate out of the membrane into a potentially hydrophilic cavity. Pglk is a bacterial transporter that translocates cell wall precursors with an outward-only mechanism (Perez et al., 2015). Here the pyrophosphate oligosaccharide-linked headgroup interacts with positive charges of Pglk. The polyprenyl tail binds initially to the protein facing the lipid bilayer and activates transport, but remains embedded in the lipid bilayer (Perez et al., 2015). Even though the translocation process itself needs only one conformation, both states can be achieved through ATP hydrolysis. The inward-facing conformation is probably needed to load the substrate in close proximity to a cavity which is only formed in the outward-facing conformation. Therefore, the inward-facing conformation exhibits a stage where loading, but no translocation takes place (Perez et al., 2015). The decrease in the expanded energy for the shift of a PC lipid along TMH1 was observed only with the inward-facing conformation of ABCB4. Is ABCB4 as it was shown for Pglk a lipid-transporter that only needs one conformation for lipid translocation and the other conformation is required for loading purposes (Perez et al., 2015)?

The TMEM16 scramblase on the other hand is a Ca^{2+}-activated lipid scramblase (Brunner et al., 2014). Ca^{2+} binds in the hydrophobic core of the protein in height of the membrane. Each monomer of the functional homodimeric protein consists of 10 transmembrane helices. Lipid shuffling can take place on both sides that are facing the lipid. This transverse shuffling has no specificity, and therefore, all phospholipids are shuffled equally without the need of ATP hydrolysis (Brunner et al., 2014).

In contrast to the unspecific scramblases two P4-type ATPases are described as being phospholipid specific. Drs2 is PS and Dnf1 PC specific, respectively (Baldridge and Graham, 2012). A shared feature between these P4-type-ATPases and lipid transporters of the ABC superfamily may be the substrate selection. Dnf1, the transporter, which is specific for PC lipids, has to distinguish between different phospholipids and the same task has to be accomplished by ABCB4. Dnf1 utilizes at least one phenylalanine at the protein membrane interface to select PC lipid over other phospholipids (Baldridge and Graham, 2012). In ABCB4 a phenylalanine is indeed present at the height of the inner leaflet which is completely conserved within 58 ABCB4 orthologs. The residue, Phe46, lays on the elbow helix, which becomes the first transmembrane helix spanning the membrane (Olsen et al., 2019). In theory, this position could reach the PC headgroups and trap them long enough to be translocated along and via the

whole TMH1. Again, the P4-type ATPases face the same challenges of translocating lipids as substrates.

Like the P-type ATPase ABCB4 as an ABC protein needs ATP for function, therefore, the outward conformation has to have a beneficial effect on the transport cycle. From the energy profiles gained from homology structures based on ABCB1 templates a cycle starting with the outward facing conformation may be plausible. Here, in the outward facing conformation a lipid is recognized and a lipid-shift along the TMH1is initiated. After ATP binding, ATP hydrolysis is required to reset the system to the inward conformation, while lipid translocation takes place (Figure 8).

ABCB4/ABCB1 hybrids revealed a crucial role for the extracellular loops and three residues in the protein core (Olsen et al., 2019). We believe a sorting and/or release function of the extracellular loops is still possible if substrates were flopped along the outside of the protein. The three amino acids V985, H989 and A990 in the protein's interior may be required for substrate translocation or are simply part of the substrate binding site following the alternating two site access model known form ABCB1 (Olsen et al., 2019). However, they may also only mediate a signal to the NBDs after substrate recognition at the TMH1 in order to dimerize the NBDs and change conformation. This would therefore influence the proteins activity, but not specifically interrupt protein substrate interactions. Additionally, the ATPase measurements we performed provided insights into the substrate protein interaction on a minute timescale, whereas the translocation assay from Olsen *et al.* required 24 h.

Our two different approaches provided further evidence for the role and function of TMH1. Sequence comparison of ABCB1 and ABCB4 (Figure 2) pointed to particular three positions, Q52, S58 and S69, respectively. MD simulation found a decrease in the expanded energy for the shift of a PC lipid along TMH1. This was exclusively found in TMH1 instead of TMH7 and was strikingly in the scale of energy gained from ATP hydrolysis (7.3 kcal/mol (Rosing and Slater, 1972)). To investigate the importance of TMH1, mutations at the three positions Q52, S58 and S69 were introduced, which corresponded to the non-polar analogous of ABCB1 (Q52L, S58V and S69A). These were investigated via *in vitro* ATPase measurements. We observed a loss of ATPase stimulation by DOPC, a prototype PC-lipid (Prescher et al., 2020). As a control, two compounds, digitoxin and verapamil, were employed, which in the wildtype as well as all mutants either inhibited (verapamil) or modulated (digitoxin) the ATPase activity (Figure 3). This supports the conclusion that the mutations did not impair protein stability and function. Furthermore, it suggests that two different binding modes exist, one for lipid translocation and another, where the modulatory or secondary substrates like drugs are located.

In summary, all data indicate a crucial role for TMH1 in the lipid transport cycle, which does not interfere with hydrophobic ABCB1 substrates.

In essence, we provided evidence that the common way of proteins interacting with lipids is located rather in membrane proximity than pulling a lipid into the protein's interior. For ABCB4 TMH1 may be utilized as a lipid anchor point and potential translocation path. This would follow the principle and reduce the thermodynamic penalty to pull out a lipid of its native bilayer and rather shift the lipid along the membrane facing parts of the protein, in ABCB4's case along TMH1.

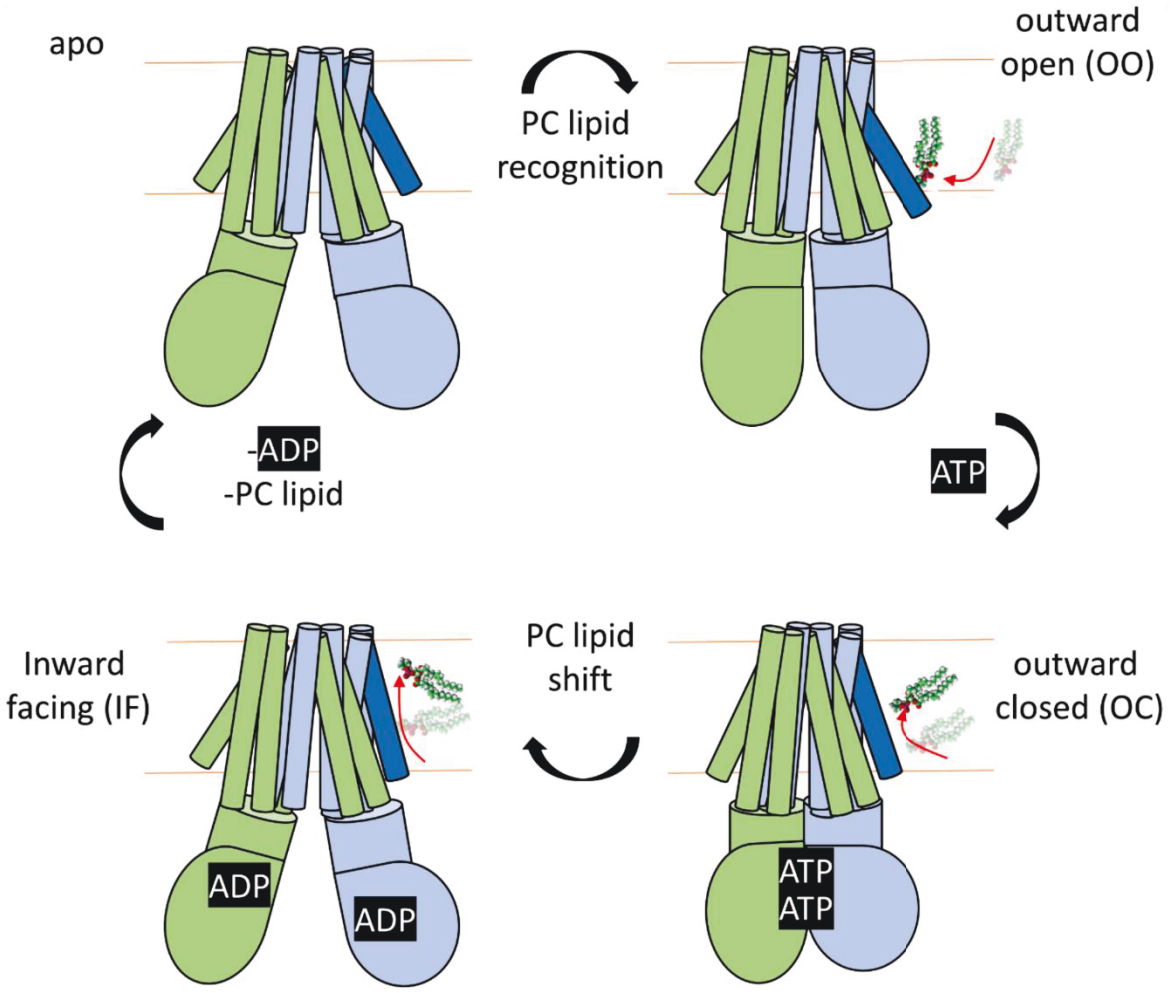

Figure 8. **Suggested transport mechanism of ABCB4 derived from energy landscapes of MD simulations.** The outward open (OO) structure may be needed for substrate loading. A transition to the outward closed (OC) may be initiated by substrate recognition and ATP binding. ATP hydrolyzation resets the transporter and may facilitate the substrate shift along the TMH1 (dark blue).

4.3. Data availability

All data are contained within the manuscript.

4.4. Acknowledgments

We thank all members of the Institute of Biochemistry for fruitful discussions. This work was supported by the Deutsche Forschungsgemeinschaft (Funder Id 10.13039/501100001659, CRC 974 project B03 to L.S.). The Center for Structural studies is funded by the DFG by Grant number 417919780 to S.S.

4.5. Conflict of interest statement

The authors declare that they have no conflicts of interest with the contents of the articles.

5. STAR methods

Chemicals were usually obtained from ThermoFisher (Germany) if not stated otherwise.

5.1. *In vitro* characterization of ABCB4 and its mutants

5.1.1. Cloning

The cloning procedures were described in detail in Ellinger et al., 2013 (Ellinger et al., 2013). Briefly, pSGP18 was cloned with a 2µ cloning site suitable for *S. cerevisiae, E. coli,* and *P. pastoris* cultivation, in which isoform two of the ABCB4 gene (databank NM_000443.3) was inserted. At the C-terminus, nucleotides coding for a hexa-His-tag and a calmodulin affinity tag were added. Mutations were introduced by site-directed mutagenesis according to (Edelheit et al., 2009).

5.1.2. Transformation and test expression

Linearized (cut with MssI) pSGP18-2µ-ABCB4 plasmid was transformed in 80 µl competent X33 *Pichia pastoris* cells via electroporation with a 1500 V pulse for 5 ms. Cell recovery was performed with 1 ml YPD for 1 h at 30 °C without shaking, followed by 2 h with shaking at 30 °C and 210 rpm. Cells were stroke on selection agar plates with 100 – 1000 µg/ml zeocin. Expression tests were performed in 50 ml MGY media (1.34 % yeast nitrogen base, 1 % glycerol, $4 \cdot 10^{-5}$ % Biotin) in 200 ml baffled flasks for 24 h. Protein expression was induced with a media change to MM Medium (1.34 % yeast nitrogen base, 0.5 % methanol, $4 \cdot 10^{-5}$ % biotin) for 24 h. Crude membranes were prepared prior to Western blot analysis. *P. pastoris* clones with the highest ABCB4 protein expression were selected for fermentation as described in detail in (Ellinger et al., 2013).

5.1.3. Fermentation

Verified X33 *Pichia pastoris* cells carrying the pSGP18-2µ-ABCB4(-wt ,-Q52L (L), -S58V (V), -S69A (A), -Q52L and S58V (LV), -Q52L, S58V and S69A (LVA)) plasmid were grown on a YPD selection plate containing 200 mg/ml zeocin for 2 days. 1 l precultures were grown in 2 l baffled flasks for 18 h in MGY media and completely used for inoculation of a 6 l benchtop fermenter containing minimal glycerol medium (26.7 ml/l phosphoric acid; 0.93 g/l calcium sulfate; 18.2 g/l potassium sulfate; 14.9 g/l magnesium sulfate heptahydrate; 4.13 g/l potassium hydroxide; 40 g/l glycerol). After the batch glycerol was consumed, cells were fed by glycerol for 5 h to increase biomass. Protein induction was accomplished by a methanol feed for 24 h. After harvesting, cells were washed with 50 mM Tris-HCl pH 8.0 and re-pelleted.

Usually, cell masses reaching 1-1.3 kg were obtained, aliquoted (100 g batches), flash-frozen in liquid nitrogen, and stored at -80 °C until further use.

5.1.4. Preparation of crude membrane vesicles

All purification steps were conducted at 4 °C. Usually 100 g frozen cells were thawed on ice, and crude membranes were prepared. Therefore, cells were resuspended in lysis buffer (0.33 M sucrose; 100 mM 6-aminohexanoic acid; 1 mM EGTA; 1 mM EDTA; 50 mM Tris-HCl pH 8.0; NaCl) in a 1:5 ratio. Cells were disrupted at 2.7 kbar with a cell disruptor system (I&L constant systems). Differential centrifugal steps separated cell debris from crude membrane vesicles. Crude membrane vesicles were collected at 138000 x *g* and resuspended in membrane buffer (50 mM Tris-HCl pH 8.0; 150 mM NaCl, 30 % (v/v) glycerol, protease inhibitor cocktail (Roche)). Total protein concentration was determined with a Bradford assay following the standard protocol (Roche).

5.1.5. Solubilization

Crude membrane vesicles were diluted to 10 mg/ml total protein concentration and supplemented with 1 % (w/v) Fos-Choline-16 (FC-16, Anatrace). Protein solubilization was performed for 1 h at 18 °C. Non-solubilized parts were removed at 138000 x *g* for 1 h.

5.1.6. Tandem affinity purification

20 mM imidazole was added to the solubilized membranes to increase binding selectivity. The solution was loaded on a 5 ml HiTrap chelating column (GE healthcare) loaded with Ni^{2+} ions, washed with washing buffer (50 mM Tris-HCl pH 8.0; 50 mM NaCl; 30 % glycerin (v/v); 0.0011 % (w/v) FC-16; 20 mM imidazole) to baseline level and eluted in one step with elution buffer (50 mM Tris-HCl pH 8.0; 50 mM NaCl; 30 % (v/v) glycerin; 0.0011 % (w/v) FC-16; 200 mM imidazole). Fractions of the immobilized metal ion affinity chromatography (IMAC) with the highest absorbance at 280 nm were pooled and transferred onto a calmodulin affinity resin (GE Healthcare) and incubated overnight with gentle rotation at 4 °C. Calmodulin affinity purification (CAC) was conducted according to a standard protocol (GE healthcare). Aliquots with the highest protein concentration were pooled and either directly used for ATPase activity measurements or flash-frozen in liquid nitrogen and stored at –80 °C until further use.

5.1.7. In vitro ATPase of ABCB4 and its mutants

5.1.8. Compound preparation

Lipids were purchased from Avanti Polar Lipids and resuspended in chloroform. Chloroform was removed in a vacuum oven at 40 °C by stepwise increasing the applied vacuum. After 30 min at the lowest pressure levels, lipids were hydrated in suspension buffer (50 mM Tris-HCl pH 8.0; 150 mM NaCl). A sonication step for 5– 10 min was included to decrease the turbidity of the solution. Dilution series for ATPase measurements were prepared with suspension buffer. Digitoxin was dissolved in DMSO, verapamil in ethanol. The amount of solvent used in the actual ATPase reaction did not exceed 5 %, and background measurements were performed in the presence of the same volume of solvent.

5.1.9. Enzyme coupled ATPase assay

The ATPase activity of detergent purified ABCB4 was determined by an enzyme coupled assay (Prescher et al., 2020). Briefly, a standard ATPase reaction volume was 200 µl in total composed of 50 mM Tris-HCl pH 7.4 at 37 °C; 5 mM $MgCl_2$; 4 mM phosphoenolpyruvate; 0.6 mM NADH (reduced); 13 units pyruvate kinase (PK); 16 units lactate dehydrogenase (LDH); 0.0011 % (w/v) FC-16 and 35 µg/ml (239 µM) detergent purified protein. Substrate stock solutions were compounded, and 5 or 10 µl solution was added to the ATPase reactions keeping the solvent concentration below 5 % (v/v). The reaction was started by the addition of 5 mM ATP. The absorbance of NADH was followed for 60 min. Since the NADH oxidation is stoichiometric to ATP hydrolysis, the ATPase activity of ABCB4 could be expressed as nmol ATP consumption per mg of ABCB4 protein and minute. A background measurement was always subtracted from the data points. Here, the cysteine residue in the Walker A motive of ABCB4 was labeled with the fluorophore FL-maleimide-bodipy and was conducted in a separate reactions (Kluth et al., 2015) [XXX: hier stimmt etwas nicht?]. The steric constraints imposed by the fluorophore prevent ATPase activity of ABCB4. No influences on the PK or LDH performance due to compound or solvent concentrations were observed.

5.2. *In silico* molecular dynamic Simulation

MD Simulations were carried out using the GPU implementation of pmemd (Le Grand et al., 2013; Salomon-Ferrer et al., 2013b) implemented in the Amber Suite of biomolecular simulation programs (Salomon-Ferrer et al., 2013a). The differences in configurational free energy between ABCB4-mediated DOPC translocation, and DOPC flip-flop across a DOPC membrane were studied using a combination of steered MD simulations of umbrella sampling

(Kästner, 2011), and potential of mean force (PMF) calculations using the weighted histogram analysis method (Kumar et al., 1992).

5.3. Data evaluation

For analysis, the background activity of ABCB4 labeled with bodipy (ABCB4-Bodipy) was subtracted. Kinetic parameters were analyzed using Prism 7 (GraphPad) and plotted either by Michaelis Menten kinetics (eq.1), Litman (eq.2) (Litman et al., 1997) or a dose-response fit (eq.3). Here, $V_{(S)}$ represents the ATPase activity at a given compound concentration. V_0 is the basal ATPase activity without any compound, V_1 is the maximal ATPase activity, at which no inhibition occurred. V_2 corresponds to the activity at infinite concentration of a compound, S the compound concentration, K_1 the compound concentration at half-maximal stimulating ATPase activity (at half V_1), and K_2 the compound concentration at half-maximal inhibitory concentration. B represents the plateau of the lowest ATPase activity, T the plateau of the highest ATPase activity, and H_S the Hill slope.

(eq.1) $V_{(S)} = \frac{V_1 \cdot S}{K_1 + S} + V_0$ Michaelis-Menten equation

(eq.2) $V_{(S)} = \frac{K_1 K_2 V_0 + K_2 V_1 S + V_2 S^2}{K_1 K_2 + K_2 S + S^2}$ Litman equation

(eq.3) $V_{(S)} = B + \frac{T - B}{1 + 10^{([\log(K_2)] - S) \cdot H_S)}}$ Dose-response equation

6. Literature

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7. Supplementary Data

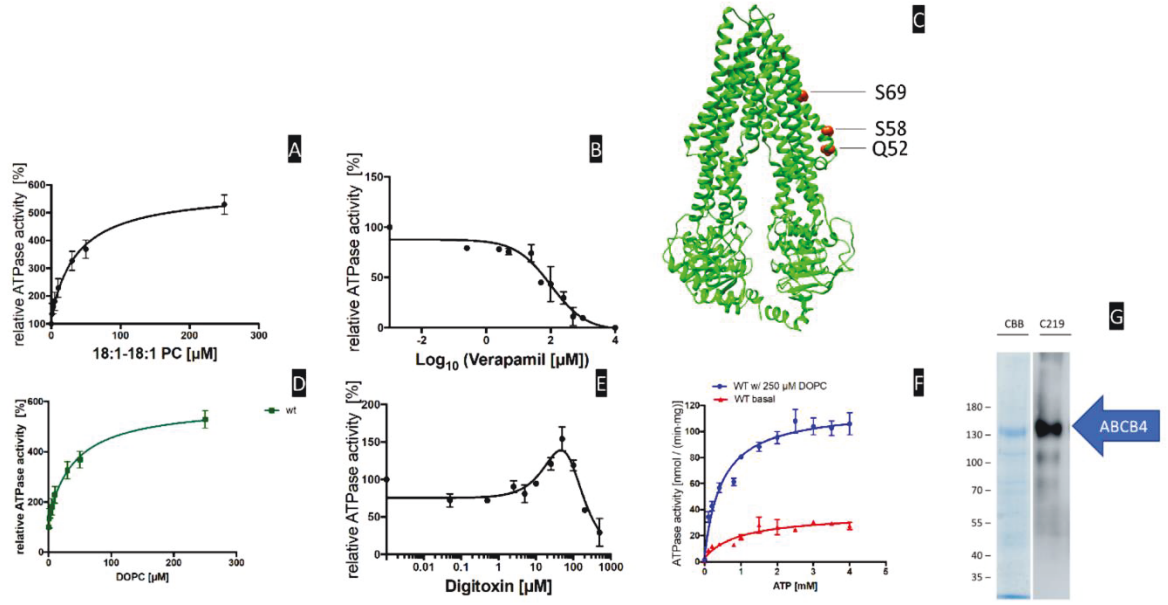

Figure SD 1 Collected ATPases of the ABCB4 wt protein. [A] Relative ATPase graph with increasing 18:1-18:1 PC (DOPC) concentration a prototype lipid out of the PC family. [B] Relative ATPase graph with increasing Verapamil concentrations, a known inhibitor of ABCB1 and ABCB4, note that the concentration was logarithmized. [C] Schematic figure of the position and mutation. Black stands for wildetype aminoacids, red for mutated versions. [D] Compression of the relative ATPase activity with the prototype PC lipid DOPC to the wildetype protein of ABCB4. [E] A new modulator of the ATPase activity of ABCB4 ; Digitoxin was characterized with increasing concentrations. [F] ATPase activity [nmol × min^{-1} × mg^{-1}] with and without 250 µM DOPC and ascending ATP concentrations up to 4 mM ATP. [G] SDS PAGE of the ABCB4 wt protein, the band at the height of 135 kDa belongs to the 146.5 kDa protein with His and Calmodulin tag.

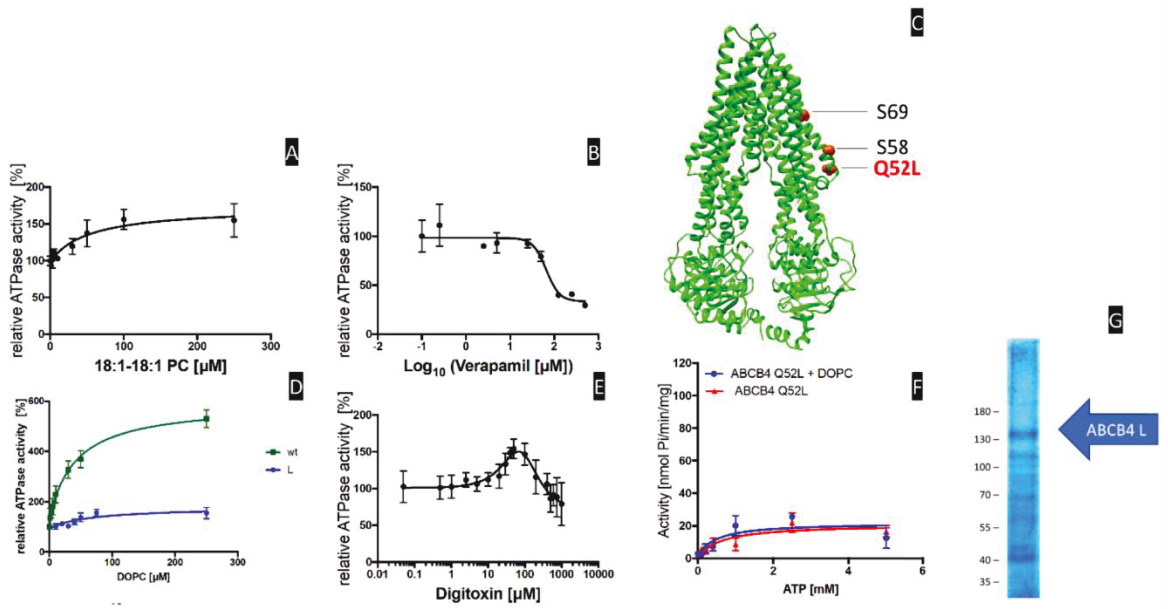

Figure SD 2 Collected ATPases of the ABCB4 L protein. [A] Relative ATPase graph with increasing 18:1-18:1 PC (DOPC) concentration a prototype lipid out of the PC family. [B] Relative ATPase graph with increasing Verapamil concentrations, a known inhibitor of ABCB1 and ABCB4, note that the concentration was logarithmized. [C] Schematic figure of the position and mutation. Black stands for wildetype aminoacids, red for mutated versions. [D] Compression of the relative ATPase activity with the prototype PC lipid DOPC to the wildetype protein of ABCB4. [E] A new modulator of the ATPase activity of ABCB4 ; Digitoxin was characterized with increasing concentrations. [F] ATPase activity [nmol × min^{-1} × mg^{-1}] with and without 250 µM DOPC and ascending ATP concentrations up to 4 mM ATP. [G] SDS PAGE of the ABCB4 L protein, the band at the height of 135 kDa belongs to the 146.5 kDa protein with His and Calmodulin tag.

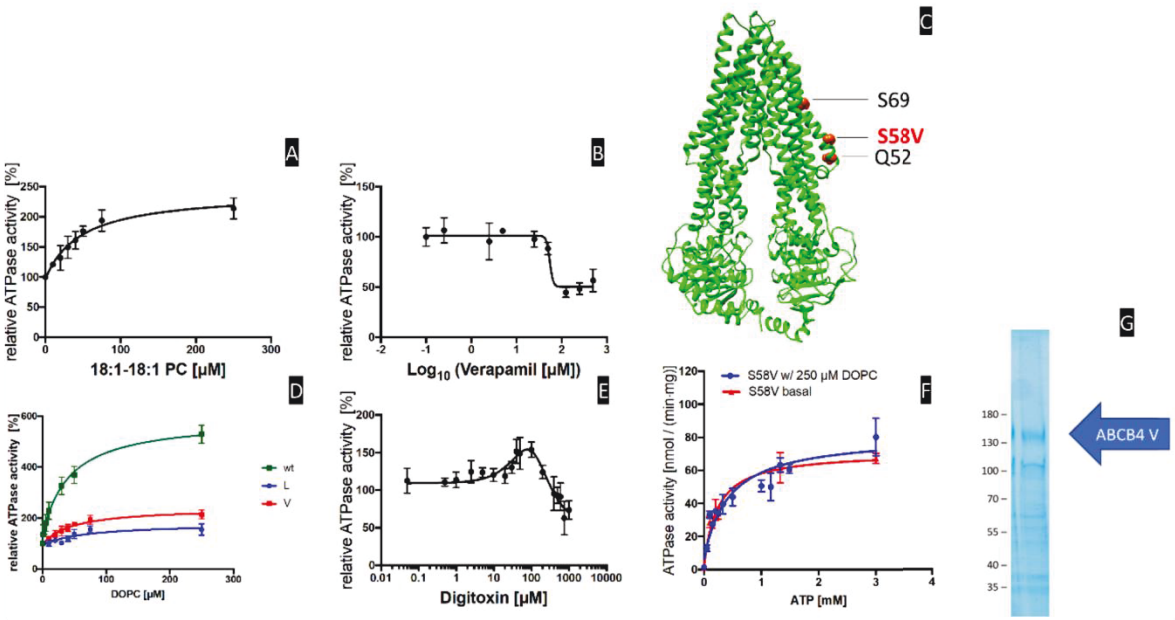

Figure SD 3 Collected ATPases of the ABCB4 V protein. [A] Relative ATPase graph with increasing 18:1-18:1 PC (DOPC) concentration a prototype lipid out of the PC family. [B] Relative ATPase graph with increasing Verapamil concentrations, a known inhibitor of ABCB1 and ABCB4, note that the concentration was logarithmized. [C] Schematic figure of the position and mutation. Black stands for wildetype aminoacids, red for mutated versions. [D] Compression of the relative ATPase activity with the prototype PC lipid DOPC to the wildetype protein of ABCB4. [E] A new modulator of the ATPase activity of ABCB4 ; Digitoxin was characterized with increasing concentrations. [F] ATPase activity [nmol × min^{-1} × mg^{-1}] with and without 250 µM DOPC and ascending ATP concentrations up to 4 mM ATP. [G] SDS PAGE of the ABCB4 V protein, the band at the height of 135 kDa belongs to the 146.5 kDa protein with His and Calmodulin tag.

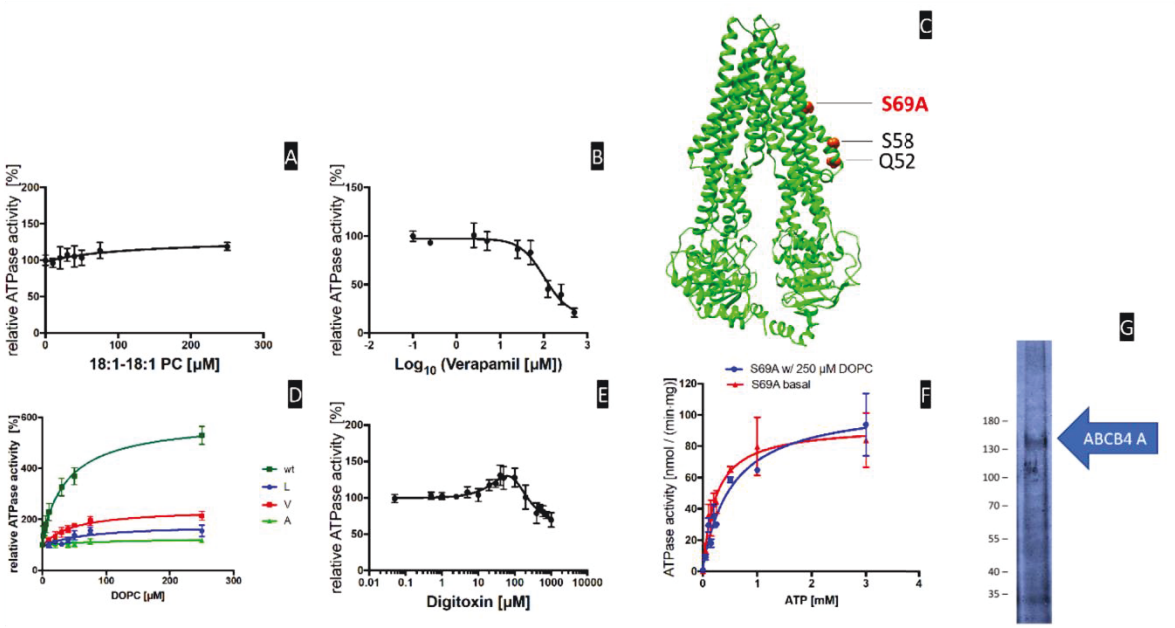

Figure SD 4 Collected ATPases of the ABCB4 A protein. [A] Relative ATPase graph with increasing 18:1-18:1 PC (DOPC) concentration a prototype lipid out of the PC family. [B] Relative ATPase graph with increasing Verapamil concentrations, a known inhibitor of ABCB1 and ABCB4, note that the concentration was logarithmized. [C] Schematic figure of the position and mutation. Black stands for wildetype aminoacids, red for mutated versions. [D] Compression of the relative ATPase activity with the prototype PC lipid DOPC to the wildetype protein of ABCB4. [E] A new modulator of the ATPase activity of ABCB4 ; Digitoxin was characterized with increasing concentrations. [F] ATPase activity [nmol × min^{-1} × mg^{-1}] with and without 250 µM DOPC and ascending ATP concentrations up to 4 mM ATP. [G] SDS PAGE of the ABCB4 A protein, the band at the height of 135 kDa belongs to the 146.5 kDa protein with His and Calmodulin tag.

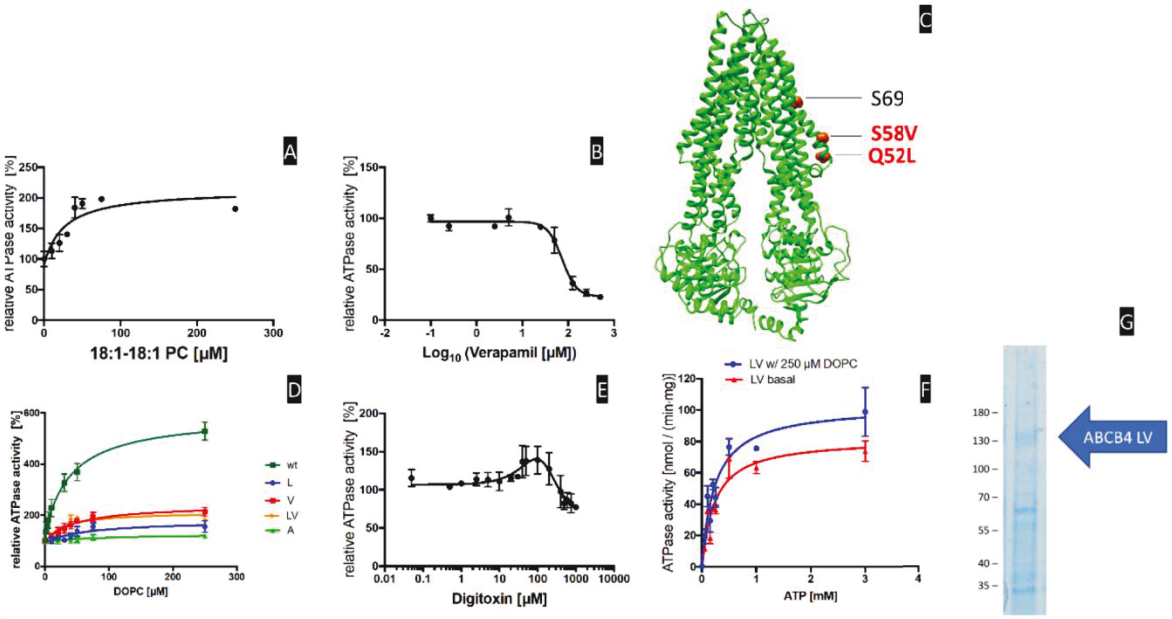

Figure SD 5 Collected ATPases of the ABCB4 LV protein. [A] Relative ATPase graph with increasing 18:1-18:1 PC (DOPC) concentration a prototype lipid out of the PC family. [B] Relative ATPase graph with increasing Verapamil concentrations, a known inhibitor of ABCB1 and ABCB4, note that the concentration was logarithmized. [C] Schematic figure of the position and mutation. Black stands for wildetype aminoacids, red for mutated versions. [D] Compression of the relative ATPase activity with the prototype PC lipid DOPC to the wildetype protein of ABCB4. [E] A new modulator of the ATPase activity of ABCB4 ; Digitoxin was characterized with increasing concentrations. [F] ATPase activity [nmol × min^{-1} × mg^{-1}] with and without 250 µM DOPC and ascending ATP concentrations up to 4 mM ATP. [G] SDS PAGE of the ABCB4 LV protein, the band at the height of 135 kDa belongs to the 146.5 kDa protein with His and Calmodulin tag.

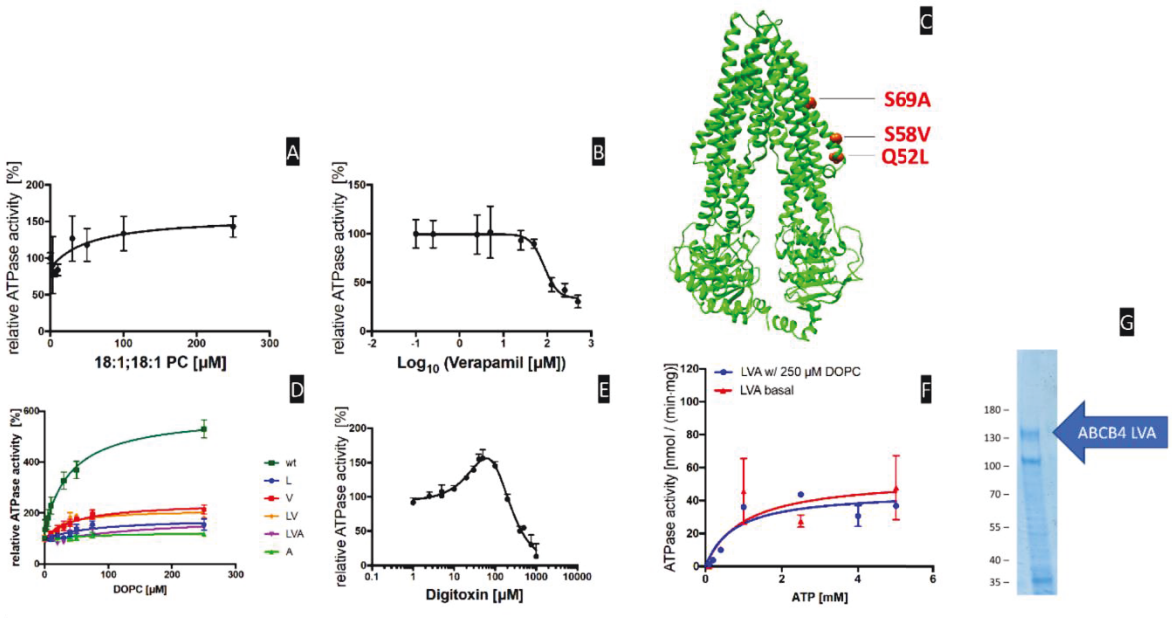

Figure SD 6 Collected ATPases of the ABCB4 LVA protein. [A] Relative ATPase graph with increasing 18:1-18:1 PC (DOPC) concentration a prototype lipid out of the PC family. [B] Relative ATPase graph with increasing Verapamil concentrations, a known inhibitor of ABCB1 and ABCB4, note that the concentration was logarithmized. [C] Schematic figure of the position and mutation. Black stands for wildetype aminoacids, red for mutated versions. [D] Compression of the relative ATPase activity with the prototype PC lipid DOPC to the wildetype protein of ABCB4. [E] A new modulator of the ATPase activity of ABCB4 ; Digitoxin was characterized with increasing concentrations. [F] ATPase activity [nmol × min^{-1} × mg^{-1}] with and without 250 µM DOPC and ascending ATP concentrations up to 4 mM ATP. [G] SDS PAGE of the ABCB4 LVA protein, the band at the height of 135 kDa belongs to the 146.5 kDa protein with His and Calmodulin tag.

4 Discussion

4.1 Summary

Literature describes ABCB4 as an ATP binding cassette transporter, which translocates lipids of the PC family from the inner to the outer leaflet of the canalicular membrane, to mitigate the harsh detergent activity of bile salts. How this task is performed in context of transport mechanism and protective function was the aim of this doctoral thesis. Two major findings were made:

I. ABCB4 creates an asymmetric canalicular membrane of diverse PC-lipid variants apart from the ones extracted which mitigates the harsh detergent activity of bile salts (Chapter II).
    i. ABCB4 needs a full intact phosphatidylcholine (PC) lipid to stimulate the ATPase activity. Moieties of the PC-lipid headgroup such as choline, phosphocholine or L-α-glycerophosphorylcholine are insufficient to productively interact with ABCB4 (Chapter II)
    ii. ABCB4 can be stimulated with every PC-lipid, but C18 fatty acids with unsaturated fatty acids in both chains do best. Ideal was 18:1-18:1 PC (Chapter II)

II. Substrates seem to interact with the TMH1 of ABCB4 and most likely do not enter the hydrophilic core of the protein. The classic ‘alternative access’ mechanism is insufficient to describe lipid translocation but may be true for artificial transport of hydrophobic drugs (Chapter III).
    i. ABCB4 might have more than one interaction site. One for modulatory ligands and another one for substrates (Chapter III).
    ii. Hydrophobic drugs, known from the substrate spectrum of ABCB1 productively interact with ABCB4 ATPase activity and follow the stimulation pattern of a bell-curved stimulation pattern despite a mutated TMH1 (Chapter III).

4.2 ABCB4 mitigates the harsh detergent activity of bile salts

4.2.1 The canalicular membrane and mixed micelles

ABCB4 is a liver membrane protein, which is exclusively found in the canalicular membrane of hepatocytes [1]. This membrane separates the cytosol from the bile ducts and forms the apical membrane of hepatocytes. Bile salts, lipids, and cholesterol are exported from the cytosol or the membrane itself into bile. The physical form of the sphere-shaped aggregates of the above-mentioned components of bile is called mixed micelles. 95% of the phospholipid content in humans is composed of PC-lipids, and in this case PC-lipid variants which do not correspond to the versatile spectrum found in the canalicular membrane [2]. Precisely, variants with the esterification pattern of 16:0-18:1 and 16:0-18:2 PC make up approximately 70% of the total PC-lipid content of bile [3]. Thus, the question arises how the mixed micelle contain only two PC-lipid variants whereas the canalicular membrane where it derives from contains a diverse spectrum of PC-lipids. One major obstacle in answering this question is that the exact composition of the canalicular membrane is unknown. There are only a handful of reference membranes from other organelles and a PC-lipid composition of the canalicular membrane from rats available [3-9]. In general, a plasma membrane such as the canalicular membrane harbors in total about 40% PC-lipids. Additionally, the proportion of Sphingomyelin (SM) is enriched in the outer leaflet, while cholesterol is distributed relatively evenly between both leaflets of the canalicular bilayer [8]. Nonetheless, exact ratios of the asymmetry of both leaflets are missing. It gets even more complex if one takes into account that the canalicular membrane is a fluid system, which can be altered due to chemical or physical stress [10]. Mainly active lipid transporters create this asymmetry [11]. ABCB4 as a PC-lipid floppase is not the only one to be considered, there are other transporters in the canalicular membrane, which alter the membrane composition. For example, the P type ATPase ATP8B1 flips PS lipids from the outer to the inner leaflet [12]. Thus, it helps to develop an asymmetrical membrane, in which PC and SM mainly occur in the outer leaflet and PS in the inner, respectively.

4.2.2 ABCB4 is stimulated by all PC-lipid variants but shows preferences anyway

ABCB4 contributes to the formation of asymmetry and prefers PC phospholipids over others. Recently, it was shown for the first time that ABCB4 could indeed interact with all tested PC variants, but shows different preferences [13]. As demonstrated in chapter II, ABCB4 needs, the phosphatidylcholine head group and two fatty acids esterified onto a glycerol core, to recognize a substance as a substrate [13]. Only if all three requirements are matched, ABCB4 will interact with the lipid on a molecular level which will then probably be transported in the following. Substances, however, which only partly resemble a PC-lipid, for example the lyso-PC variant, which lacks one fatty acid esterification, might cause the protein to be trapped in one conformation unable to release the ligand, resulting in an inhibition of the ATPase activity. Similarly, the detergent Foscholine-16 (FC-16) structurally resembles a lyso-PC-lipid without the glycerol core but with the same choline head group also acts as an inhibitor [13]. This effect, however, is most likely due to unspecific detergent effects as demonstrated in a competition assay in

which an increasing concentration of PC-lipid was added onto a constant concentration of FC-16 as inhibitor. The results indicated a non-specific detergent interaction, since neither the K_M nor the v_{max} could be reached after lipid saturation. Above a molar lipid:detergent ratio of 1:1, the ATPase activity could be restored, but this did not result in the typical enzyme kinetics known from Michaelis Menten (Chapter II, Figure 6). While higher concentrations of FC-16 were harmful for the protein and probably led to protein misfolding, lower concentrations could still be fully compensated and showed the typical Michaelis Menten kinetics as observed with a prototype PC-lipid. Taken together, it seems important for a proper molecular interaction between a potential ligand and ABCB4 that the ligand has a phospholipid-like structure with a choline headgroup. Note that other phospholipids which differ in their head group, such as PE lipids, do not stimulate the ATPase activity of ABCB4. Accordingly, the head group is crucial for recognition, but not sufficient to stimulate the protein's activity. The fatty acid esterification, on the other hand, is responsible for the magnitude of the stimulation. Both available esterifications are critical for the level of stimulation and presumably an interaction with both defines the strength of activation of ABCB4 by the respective PC-lipid. Artificial PC-lipids that do not have a physiological role in the canalicular membrane such as DOPC, a PC-lipid consisting of 18 C atoms, each carrying one unsaturated fatty acid, stimulate ABCB4's activity the strongest. It can be speculated that precisely these two double bonds ensure that the head group is particularly accessible to the transporter. The double bonds cause the lipid to form kinks, which may result in an appropriate distance to other adjacent lipid head groups. I also observed that as soon as both fatty acids are symmetrically prolonged, a preference for longer chain lipid exists. Accordingly, the lowest PC-lipid stimulation of all was measured with the PC-lipid variant 12:0-12:0. This is in accordance with the observation that 8:0-8:0 PC was not transported by ABCB4 in an *in vivo* setup with radio labeled lipids [14]. The shorter the lipids the less pronounced is the amphipathic character of a lipid. Hence, the authors needed precisely this loss in the typical lipid character of a short chain lipid to perform their transport assay. This could hint towards a fourth feature for ABCB4 substrates: the presence of a distinct amphipathic property. Here, a lipid longer than 8:0-8:0 PC is needed, while 12:0-12:0 PC is most likely already recognized.

If both fatty acid chains are installed, differences in the ability to stimulate ABCB4 may exist depending on the sn1 or sn2 position of the fatty acid. However, regardless of the fatty acid variation in the sn2 position, PC-lipids with palmitic acid (16:0) in the sn1 position stimulated the ATPase activity of ABCB4 to the same extend. Surprisingly, the PC-lipid variants extracted into the bile (16:0-18:1 PC and 16:0-18:2 PC) did not stimulate ABCB4 any better than the other tested PC-lipid variants.

In conclusion, the canalicular membrane consists mainly of PC-lipid variants that carry one saturated fatty acid in the sn1 and one unsaturated fatty acid and the sn2 position, respectively. ABCB4 does not discriminate between these different PC-lipid variants. In theory, an outer leaflet would develop an equal distribution of PC-lipid variants by constant, active PC-lipid flopping of ABCB4, directly depending on PC-lipid variants that were present in the inner leaflet. The membrane composition would correlate with

PC-lipids which were newly synthesized or incorporated *via* vesicular or non-vesicular systems like PC-lipid transferases. Regulatory processes would dictate which PC-lipid variants were supplied to the canalicular membrane and therefore which ones are presented to ABCB4 to be flopped. PC-lipid synthesis is indeed a coordinated system of the cytidine diphosphate pathway (CDP) and the PE methylation pathway (PEMP) [15,16]. Here, the CDP-choline pathway is three to four times more active in order to compensate the extracted variants (16:0-18:1 and 16:0-18:2 PC) with newly synthesized PC-lipid variants [16]. This overproduction of the 16:0-18:1 and 16:0-18:2 PC variants through the CDP may directly restore the lipids that were extracted from the canalicular membrane.

4.2.3 Second part of enrichment *via* bile salts requires proper ABCB4 function

Since there is a multitude of different PC-lipid variants in the canalicular membrane and that these are clearly different from the mixed micelle PC-lipids, a system or discriminatory factor is still needed that accounts for the accumulation of specific PC-lipid variants in bile.

Interestingly, Wüstner *et al*. proposed that bile salts themselves do not distinguish between the head group of phospholipids [6]. However, it has been shown that bile salts have a high affinity for phospholipids, which combine an unsaturated with a saturated fatty acid. Thus, bile salts would be able to solubilize all lipids out of the membrane that match this pattern independently of the head group. Since the abundance of phospholipids of the PC family in the outer leaflet is enriched through active transport of ABCB4 and other systems such as ATP8B1, the lipids which match the preference of bile salts and are actually present in the outer leaflet are16:0-18:1 and 16:0-18:2 PC.

It should be noted that a high proportion of SM in the outer leaflet of a membrane is known to be resistant to bile salt since only low amounts of SM (less than 1% of the mixed micelles) is extracted into bile [17].

Consequently, in patients with none functional ABCB4, no PC-lipids can be detected in bile. In a kinetic membrane model that does not re-deliver any extracted PC-lipids, these PC-lipid variants would simply deplete from the canalicular membrane leaving unextractable PC-lipid variants and SM in the outer leaflet.

Unfortunately, there is no study available that examined the canalicular membrane for its alteration or its wildtype condition in humans with a corresponding differentiation between the inner and outer leaflet. It would be interesting to see how the canalicular membrane changes due to ABCB4 activity or deficiency. It is also important to determine how the lipid or phospholipid content of the bile has changed. Are there any lipids that are extracted alternatively to the PC-lipids? Due to the severity of the diseases, especially in the case of PFIC3, a disease that is defined by the complete loss of ABCB4, there is apparently no compensatory mechanism in humans [18,19]. These patients lose the ability to proper solubilize cholesterol and bile salts. Crystallization of these two compounds is a common observation in patients suffering from ABCB4 deficiency. In summary, ABCB4 mitigates the harsh detergent effect of bile salts by providing the PC-lipids that are preferably extracted from bile salts. Other PC-lipids characterized by either a more complex fatty acid at the sn2 position or other types of esterification are

required to keep the integrity of the membrane. Nature has thus ensured that PC-lipids are replenished continuously as they are solubilized, making the membranes more resistant with only one protein, ABCB4 (**Figure D 1**).

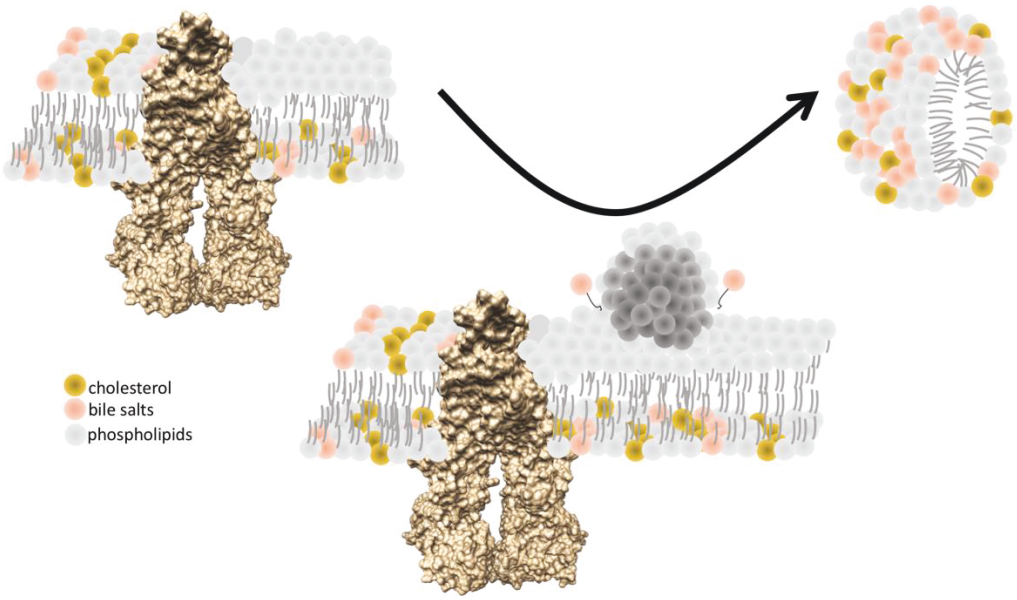

Figure D 1: Suggested model of bile salts extraction of PC-lipids that were flopped by ABCB4 from the cytoplasmic to the extracellular leaflet of the canalicular membrane. Bile salts tend to extract a specific subset of phospholipids, the ones with one saturated and one unsaturated fatty acid of medium length (C16-C18) independent of the headgroup. Phospholipids that match these criteria are the highly abundant PC-lipids 16:0-18:1 PC and 16:0-18:2 PC flopped by ABCB4.

The reason why it is necessary to sacrifice some lipids is often overlooked since in theory a membrane would be completely resistant to bile salts. With only SM or higher unsaturated PC-lipids in the membrane. Several points come here into play: First, a membrane made of lipids of purely one type is highly susceptible to crystallize and is not adaptable [20]. Second, it would be susceptible to temperature and possibly denature if the melting temperature is exceeded. In the context of the canalicular membrane, however, there is another factor to consider. Cholesterol must be released from the membrane. Cholesterol is exported by the heterodimer ABCG5/G8. Pure cholesterol would crystallize resulting in gallstones. To ensure the solubility of cholesterol, a sensitive balance of phospholipids and bile salts is required. Here the phospholipid portion is the decisive factor [21].

4.2.4 ABCB4 – a transporter regulated by lipids, drugs and bile salts

Even though everything mentioned above draws a quite sophisticated network of interactions between ABCB4, lipids and bile salts, the regulation of ABCB4 is probably even more complex. Bile salts may in fact directly interact with ABCB4. One could imagine bile salts function as a signal that a bile salt extraction will take place and therefore boost the ATPase activity and thereby the whole transport efficiency in order to compensate for the forthcoming loss of PC-lipids. There are already studies that showed an increased export of PC-lipids due to exposure to bile salts [22]. Experiments by my colleague saw in an *in vitro* setup a stimulation of the ATPase activity of ABCB4 if bile salts are present (unpublished data by Tim Kroll). I tend to believe the molecular interaction is modulatory rather than

bile salts being substrates of ABCB4. It is also difficult to determine from which side this interaction originated. The structure of ABCB4 [23] showed a cavity on the inner side of the membrane which could have an influence on the transport rate, preferably by modulators. The capability of ABCB4 to be modulated by chemical chaperones or drugs was already published. Chemical chaperones are small molecules such as Ivacaftor which stabilizes ABCB4 NBD dimerization, which recovers the ability to hydrolyze ATP, thus, being functional again. This provides the possibility to target specific ABCB4 deficiencies, a crucial step towards personalized medicine [24-26].

It is possible that a *de novo* synthesis, which takes place in the hepatocytes themselves, not only stimulates ABCB11, but also causes a co-activation of ABCB4. Or if bile salts which are exported by ABCB11 either incorporate into the mixed micelles or to diffuse back into the outer leaflet of the canalicular membrane where they can be sensed by ABCB4 for example *via* the extracellular loops. A double control of either present PC-lipids and/or the censoring of bile salts *via* extracellular loops or modulatory pocket would also save resources in form of chemical energy stored in ATP.

4.3 How ABCB4 translocates PC-lipids

4.3.1 Other transporters which translocate lipids and lipid-like substances

ABCB4 is not the only transporter, which interacts with lipids or lipid-like molecules. This chapter will provide an idea of the general concepts of how transporters mediate lipid-transversion through the membrane. Many biological membranes have an asymmetrical membrane, meaning both leaflets harbor different amounts of phospholipids. Specific transport systems like lipid transporters or ion channels are needed to let lipids pass the membrane at biological relevant rates. An unassisted transversal flop of lipids happens between once each 30 minutes up to once per day but anyway considered irrelevant on a biological time scale [8,27]. Lipids undergo a constant cycle of non-vesicular and vesicular transport or removal from and to the membrane [7]. Strict regulation of the lipid asymmetry is needed as processes that lead to symmetry demonstrated. Scramblase-mediated exposure of PS for example generates physiological signals that can end in apoptosis [28]. The general assumption of lipid asymmetry has changed with new emerging methods to measure both membrane leaflets separately. Choline-containing lipids like SM or PC are thought to be mainly in the outer membrane, while the strictly controlled PS and other amino phospholipids like PE populate the inner leaflet. Initially, especially the distribution of PC was wrongfully determined for common plasma membranes in mammalian cell types. Knowledge was derived from human red blood cells and was extrapolated to all plasma membranes. However, in red blood cells the strictly exofacial (75 %) abundance of PC-lipids is explained by a currently unknown distributor, but may go back to an ABC transporter with PC floppase activity as ABCB4 does for the canalicular membrane [8]. Therefore, the lipid asymmetry in the canalicular membrane as well as the plasma membrane of red blood cells are even more asymmetrical in terms of PC-lipid ratios than other plasma membranes.

In contrast to the unspecific scramblases, two P4-type ATPases are described as being phospholipid specific. Drs2 is PS and Dnf1 PC specific, respectively [29]. A shared feature between these P4-type-ATPases and lipid transporters of the ABC superfamily may be the substrate selection. Dnf1, the transporter, which is specific for PC-lipids, has to distinguish between different phospholipids and the same task has to be accomplished by ABCB4. Dnf1 utilizes at least one phenylalanine at the protein membrane interface to select PC-lipid over other phospholipids [29]. In ABCB4 indeed a phenylalanine is present at the height of the inner leaflet which is completely conserved within 58 ABCB4 orthologs. The residue Phe46 lays on the elbow helix which is connected to the transmembrane 1 helix [23] (Figure D 2).

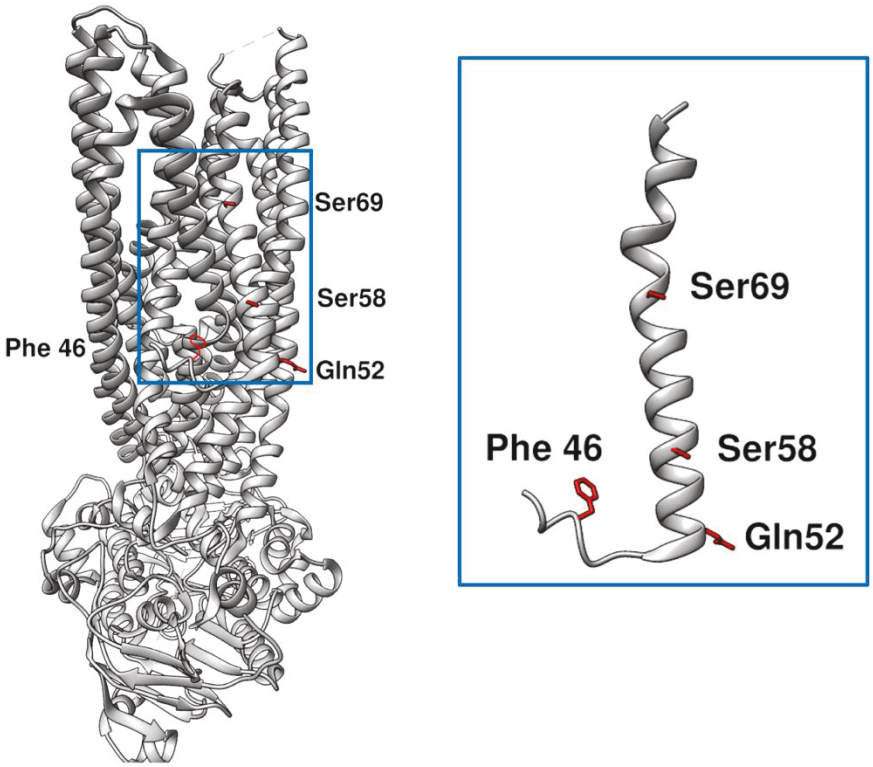

Figure D 2: Structure of ABCB4 based on 6S7P from Olsen et al. [23]. Highlighted are the three amino acids Gln52, Ser58 and Ser69 which are thought to be involved in PC-lipid translocation along TMH1. Next to these residues, Phe46 is located in the elbow helix proceeding TMH1 and is introduced as potential phosphatidylcholine discriminator. Phe46 is 100% conserved among 58 species, which have an ortholog ABCB4 gene.

In theory, this position could reach the PC headgroups and trap them long enough to be translocated along and *via* the entire TMH1. Again, the P4-type ATPases face the same challenges of translocating lipids as substrates. Lipid-transport has to overcome a thermodynamic penalty that has to be paid if hydrophobic phospholipid molecules enter a hydrophilic, aqueous cavity. The P4-type ATPases utilize a similar mechanism to PglK (ABC transporter) or even TMEM16 (Scramblase), where the two acyl chains (polyprenyl tail respectively) always remain embedded in the phospholipid bilayer. Only the polar lipid headgroup has to be transported through a hydrophobic environment. Common biocatalytic compensation principles help this translocation process. Here, the headgroup is stabilized by salt bridges or hydrophilic interactions.

Protein glycosylation K (PglK) is a bacterial transporter that translocates cell wall precursors with an outward-only mechanism [30]. Here, the pyrophosphate oligosaccharide-linked headgroup interacts with positive charges of Pglk. The polyprenyl tail binds initially to the protein facing the lipid bilayer and activates transport, but remains embedded in the lipid bilayer [30]. Even though the translocation process itself needs only one conformation, both states can be achieved through ATP hydrolysis. The inward-facing conformation is probably needed to load the substrate in close proximity to a cavity which is only formed in the outward-facing conformation. Therefore, the inward-facing conformation exhibits a stage where loading, but no translocation takes place [30]. Since PglK is a representative of an ABC transporter interacting with lipid-like structures general features may also be shared. It might be interesting if

ABCB4 also utilizes one structural conformation for the active translocation process of PC-lipids like PglK or needs more conformations like ABCB1. Or if ABCB4 shares the feature of lipid shifting along helices facing the lipid bilayer.

Another example for a lipid interacting protein but from a whole different categorical branch is TMEM16. TMEM16 is a Ca^{2+}-activated lipid scramblase [31]. Ca^{2+} binds in the hydrophobic core of the protein in height of the membrane. Each monomer of the functional homodimeric protein consists of 10 transmembrane helices. In contrast to PglK or Dnf1 lipid shuffling of TMEM16 can take place on two sides of protein parts that face the membrane. This transverse shuffling has no specificity, and therefore, all phospholipids are shuffled equally without the need of energetic input [31]. For ABCB4 the information gained are rather limited. ABCB4 is PC specific and is ATP-coupled but again a transport mechanism along the outside of the protein is used. A common feature of proteins translocating lipids tends to emerge.

ABCA1 is an outsider, since it is larger than any previous described lipid-interacting protein and accomplishes cholesterol and phospholipid efflux with the help of auxiliary proteins [32]. ABCA1 transports excessive cholesterol and phospholipids to a protein acceptor (apoA1). Large extracellular domains (ECDs) which interact with the substrate and ABCA1 itself are probably needed to mediate the substrate exchange. Therefore, the mechanism of ABCA1 may differ too drastically from the one of ABCB4 to gain mechanistic insights through shared features [33].

Even if the molecular mechanisms of transport are structurally and categorically distinct from ABCB4 a shared feature of lipid translocation in general emerges. In order to translocate lipids or lipid-like substances with a polar headgroup (lipid headgroups or oligosaccharides, respectively) and a hydrophobic tail in form of polyprenyl or fatty acids the tail does not necessarily have to enter the proteins core. Shielding of the headgroup in a translocation cavity within the protein which faces the lipid bilayer while the hydrophobic tail is still embedded in the membrane is apparently the common mechanism. If ABCB4 followed a translocation mechanism based on the classic alternative access model (like ABCB1 does), it would contradict the general features used by other lipid transport systems.

4.3.2 ABCB4 and ABCB1 – highly similar sequence, but different physiological niche

ABCB4 and ABCB1 are both expressed in the canalicular membrane of human hepatocytes and share a high sequence identity of 76% resulting in an equal molecular weight and overall architecture of typical ABC transporters [14] (Figure D 3). The sequence differences between the proteins are distributed over the whole structure, but cluster at the surface facing the membrane especially in TMH1.

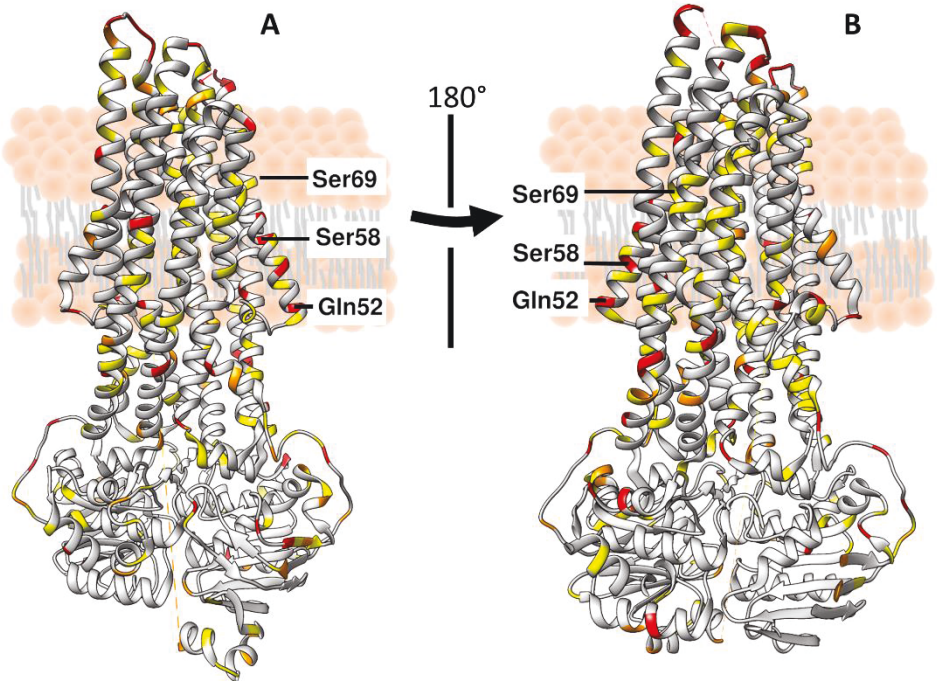

Figure D 3: (see chapter III) [A] Structure of ABCB4 (PDB entry 6S7P, [23]) colour-coded according to sequence conservation between ABCB1 and ABCB4 and based on Blosum 62 scoring [34]. Yellow corresponds to a moderate (e.g. R785K, positive Blosum62 score) change, orange to an intermediate change (e.g.V746T, neutral Blosum62 score), while red corresponds to no conservation (e.g. F760A, negative Blosum62 score). Three amino acids located in TMH1 are highlighted [B] View of A rotated by 180°.

Despite the high molecular homology, both proteins fulfill different physiological roles in the liver. ABCB1 is a prototype drug exporter while ABCB4 flops PC-lipids. Due to enzyme kinetics ABCB1 and ABCB4 *in vivo* most likely do not interfere in the same physiological processes. However, in some *in vitro* studies, an artificial overlapping substrate spectrum was demonstrated. Here, ABCB1 was claimed to translocate phospholipids independently of their headgroup and ABCB4 was able to facilitate the export of hydrophobic drugs [14,35]. Therefore, it can be assumed that both proteins share a secondary translocation pathway (primary for ABCB1), but each protein is evolutionary trained to its specific subtype of substrates. In the narrative of ABCB4, the transporter might have a drug export pathway that is used if the apparent drug concentration exceeds certain affinity levels and matches the size and shape of the secondary transport pathway. Both necessities are most likely not fulfilled in physiological context but may be achieved under *in vitro* conditions. This theory is matched by the observation that ABCB4 *in vitro* shows the same ATPase activation pattern as ABCB1. A pattern described as a bell-shaped-curve most likely due to the accessibility of substrates to a high and a low affinity binding site to be found in the classical alternative access model (Chapter III). If the low affinity binding site e.g. the substrate-exit binding site facing the extracellular space is saturated with excess of substrate, the substrate cannot be released and occupies the binding site trapping the transporter in one conformation. This leads to the inhibition effect after the initial stimulation in ATPase activity [36]. Strikingly, I tried to identify the PC-lipid binding site of ABCB4 and introduced mutations in an area, in which I assumed substrate-protein interactions. This area in the TMH1 is fundamentally different in ABCB4 to ABCB1.

Since the introduced mutations just altered the ATPase activity of ABCB4 in presence of PC-lipids I strongly believe to have a first indirect evidence that PC-lipids act as substrates of ABCB4 even in the detergent-purified state rather than acting as a modulator. On top of that, I saw no change in the ATPase activity of ABCB4 if either an inhibitor or the modulator is present even when the mutations were introduced indicating to two distinct translocation pathways or rather interaction sites (Chapter III).

4.3.3 Evidence for the TMH1 as an anchor point for lipid translocation

TMH1 and TMH7 are the helices of ABCB4 with the stiffest angle spanning the membrane. Thus, they appear the most expansive and might interact first with the membrane and sense changes of the surrounding. Sequence alignment studies showed that especially in THM1 the most and the most drastic amino acid differences between ABCB1 and ABCB4 are located. Nonpolar residues in ABCB1 are changed to polar residues in ABCB4 [13] (Chapter III, Figure 1). In chapter III I introduced three positions Q52, S58 and S69 as potentially interacting with lipids. First indication for a crucial function of these three residues was the cluster in differences of ABCB1 and ABCB4 in TMH1. Second, the fact that thermodynamically unfavored polar residues are conserved in the membrane and third the need for an alternative transport mechanism since the potential lateral gates, known from ABCB1 are found to be rather closed (Chapter III, Figure 5). Three different approaches were utilized to provide further evidence for the role and function of the TMH1 and particularly of the three positions Q52, S58 and S69. MD simulation found a decrease in the expanded energy for the shift of a PC-lipid along the TMH1. This was exclusively found for TMH1 and not for TMH7 and was within the scale of energy gained from ATP hydrolysis (7.4 kcal/mol [37]). Introducing mutations at the three positions Q52, S58 and S69 and changing these to the nonpolar ones of ABCB1 (Q52L, S58V and S69A) was investigated *via* a *pseudo-in vivo* transport assay and *in vitro* ATPase measurements. The transport assay was performed with NBD-labeled lipid analogue 14:0-6:0 NBD-PC and showed in the triple mutant drastic effects on the transport rates [14]. This observation goes in hand with the loss in ATPase stimulation by DOPC a prototype PC-lipid [13]. However, differences occurred in one specific single mutant in the ABCB4-S69A, here the ATPase measurement and the NBD-PC assay (Figure D 4) separated. While the ATPase activity measurements showed a complete loss of stimulation by DOPC, the transport rates were wildtype-like. Again, raising the question which part is recognized by ABCB4. Does the transporter recognize the fluorophore group more than the PC-lipid features? These observations clearly contradict each other, more so if considered that the triple mutant shows transport defects to the same extend the ATPase stimulability by PC-lipids do.

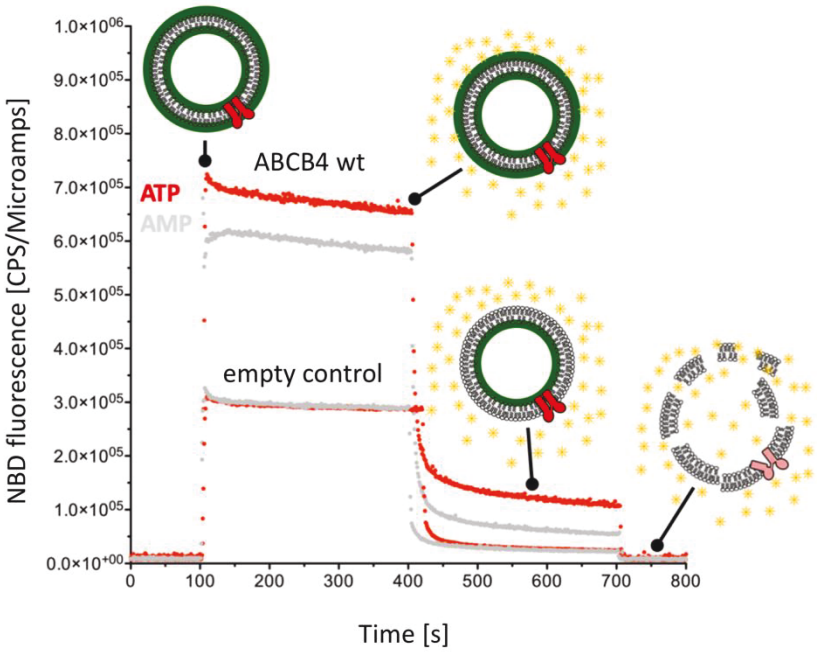

Figure D 4: Schematic illustration of the PC-NBD (1-myristoyl-2-(6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl)-sn-glycero-3-phosphocholine, Avanti Polar Lipids) Assay. Compared are two HEK293 cell membranes with and without ABCB4 transfected. The red line corresponds to active transport of ABCB4, where PC-NBD (yellow stars) is protected from dithionite quenching. A fluorescence drop is observed after solubilization of the membrane vesicles with Triton-X-100. The grey graph corresponds to AMP measurements of the unenergized membrane vesicles. Figure was taken with permission from Jan Stindt.

As a control other substances which in the wildtype either inhibited or modulated the ATPase activity did show the same activity in the ABCB4 mutants. This hints to a protein, which is still functional and utilizes two ligand binding sites, one for lipid translocation and another, where the modulatory or secondary transporting features are located. In summary, all data on TMH1 indicate that it is crucial for the transport cycle of lipid translocation. However, there are still open questions to be discussed. The decrease in the expanded energy for the shift of a PC-lipid along the TMH1 was observed only with the inward-facing conformation of ABCB4. Is ABCB4 like PglK, a lipid-transporter that only needs one conformation for lipid translocation and the other conformation is needed for loading purposes [30]? Since the need for ATP is undisputed the outward conformation has to have a beneficial effect on the transport cycle. From the energy profiles gained from homology structures based on ABCB1 templates a cycle starting with the outward facing conformation may be plausible. Here, in the outward facing conformation a lipid is recognized and a lipid-shift along TMH1is initiated. After ATP binding, ATP hydrolysis is needed to reset the system to the inward conformation, while lipid translocation takes place (Figure D 5). I found the necessity of a PC headgroup and two acyl chains for substances in order to be substrates of ABCB4. How does the recognition of substrates work? The residue Phe46 may play an important role, but this amino acid has not been investigated yet and would only answer the necessity of a headgroup-based selection. Both acyl chains may also interact with ABCB4 on a molecular level. The MD simulation, on the contrary only shows specific headgroup interactions. The different stimulation levels of ABCB4's ATPase activity by for PC-lipid variants with different acyl chain compositions may also have a molecular explanation. The fact that the headgroup alone does not

stimulate ABCB4 may simply go back to the inability of hydrophilic substances to enter the detergent micelle or the membrane in the first place. On top of that ABCB4/ABCB1 hybrids revealed a crucial role for the extracellular loops and three residues in the protein core [23]. I believe a sorting and/or release function of the extracellular loops is still possible, if substrates were flopped along the outside of the protein. The three amino acids V985, H989 and A990 in the protein's interior may be needed as described by the authors for substrate translocation or are simply part of the substrate binding site following the two site access model known form ABCB1 [23]. However, they may also only mediate a signal to the NBDs after substrate recognition at the TMH1 in order to dimerize the NBDs and change conformation.

Overall, I provided evidence for the TMH1 as lipid anchor point and potential translocation path in ABCB4 following the principles learned from other lipid-translocating systems bilayer and reduce therefore the thermodynamic penalty it would cost to pull out a lipid of its native bilayer. The lipid preselection may be performed by residue Phe46, which characterizes the spot where PC-lipids were preferred over other phospholipids. This would provide time for molecular interactions of the choline headgroup with the residues of TMH1 (at least Q52, S58 and S69) to slide the lipid along the helix. Here, the hydrophobic fatty acid tail would remain within the lipid bilayer. Upon ATP hydrolysis the ABC transporter would undergo a conformational shift and finish the last step of the translocation process. After the point where half the membrane has been overcome the lipid follows the thermodynamic relief and flops to the outer leaflet (see Figure D5).

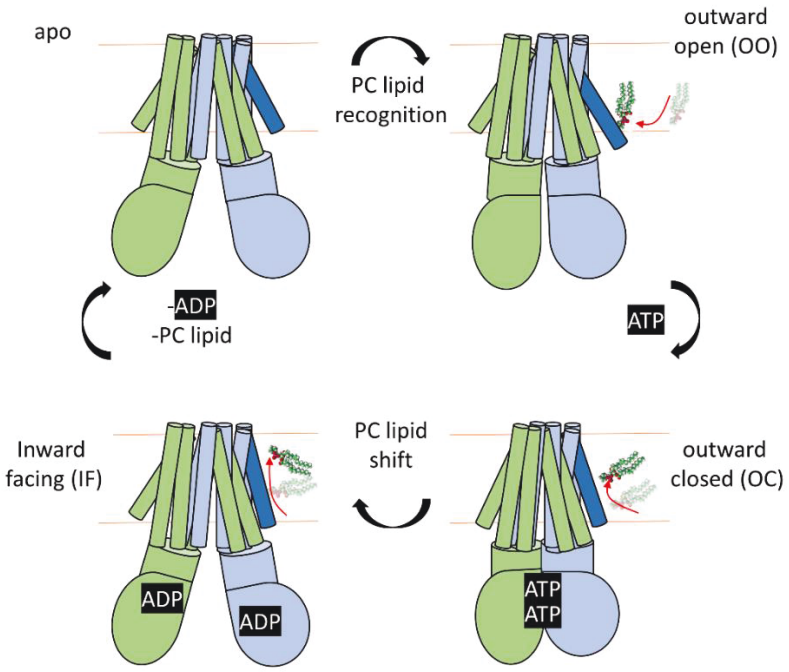

Figure D 5: Proposed transport mechanism of ABCB4 derived from energy landscapes of molecular dynamic simulation. The cartoon is based on only respective ABCB4 structure and other conformations from ABCB1 [23,38-43]. According to the model, the outward open (OO) structure is needed for substrate loading and a transition to the outward closed is initiated by substrate recognition and ATP binding. ATP hydrolysis resets the transporter and facilitates the substrate shift along TMH1.

4.4 Methodical outlook and pitfalls of the practical work with ABCB4

This section is intended to highlight the experience gained from working with ABCB4. It has been divided into different sections, ranging from strategy, cloning and purification to functional assays. The sections are more detailed and also include negative results with potential explanation that in this form cannot be used in scientific publications.

4.4.1 Expression host, cloning strategy, and promoter selection

The expression levels of human membrane proteins are rather low and have to be boosted *via* heterologous expression in order to perform *in vitro* assays [44]. The high biomass production of *Pichia pastoris* (*P. pastoris)* in particular is on a kg level rather than on a g level as it would be in the case of bacterial systems [44]. Alternatively, homologous expression in human cell culture models is rather expensive and only leads to sufficient bio material if high volume is used, e.g. on a liter scale for the purification of ABCB4 as described by Olsen *et al*. [23]. *P. pastoris* is a yeast strain that is widely used in biotechnology. The glycosylation properties as a form of posttranslational modifications (PTM) in eukaryotic yeast are more similar to the human glycosylation pattern than those of prokaryotic bacteria. Thus, Stindt and Ellinger decided to use the AOX1 promoter based *P. pastoris* system as expression host [45]. It is further characterized by its ability to utilize alcohol as a carbon-source and to express the downstream-encoded eponymous alcohol oxidase. Biotechnologically, this strong promoter is used for purposes of overexpressing heterologous proteins. The gene-of-interest is randomly inserted into the yeast genome by homologous recombination. The frequency with which the promoter region and the gene-of-interest are inserted into the *P. pastoris* genome is crucial for a good expression performance. A high number correlates with the success of the expression and the corresponding production quantity. Fermentation in a bioreactor is suitable since methanol induction in particular must be strictly controlled. To decide which clone is optimal to use for fermentation, three successive tests were performed as recommended by the *invitogen* fermentation guidelines concerning *P. pastoris*. First, the transformed electrocompetent *P. pastoris* cells were plated out on selection agar plates with different concentrations of Zeozin. These ranged from 200 µg/ml to 1000 µg/ml zeocin in the YPD agar. This was made possible by the co-transformed zeocin resistance genes. Again, the more frequently the genes were inserted, the greater the resistance against Zeocin. Afterwards, the clones with the highest resistance potential were picked for a colony PCR, which made it possible to test for the presence of the ABCB4 gene. The number of amplification rounds was set to 15 in order to determine which clone originally had the most template DNA (about 30 cycles are usually used to amplify a gene of interest in a PCR) [46]. Thus, the colony PCR not only provided information about the success but also quantitative data about the integration number. Clones that grew on the selection plates and had the highest amount of template DNA were then used for test expression. This was essential to detect the protein expression of ABCB4 by Western blotting. Only a clone that was positive in all three tests was fermented. The long pre-fermentation selection procedure meant that the expression of ABCB4 (and its mutants) clones remained

comparatively high and spared the experimenter from fermentation of false-positive clones. However, both the smaller test expressions in the shaking flask and the larger fermentation approaches may run into the problem of induction. As briefly mentioned above, methanol is used for induction, it serves as a carbon source of *P. pastoris* and activates the metabolism machinery through the AOX1 promoters. Problematic may be the dosing of the amount of methanol. Even though *P. pastoris* can survive on low level methanol basis; concentrations above 5% (v/v) [47] cause problems in protein stability and in the proliferation property of *P. pastoris*. It should also be mentioned that if there are residues of another carbon source such as glucose or glycerol in the medium, the AOX1 promoter regions are repressed (*invitrogen* guidelines). This means for the daily laboratory routine, especially for test expressions in shaking flasks the batch amount of carbon source has to be measured precisely to ensure that the expression in all flasks is simultaneous and comparable. Just as important is that the addition of methanol must be controlled. Especially the vitality parameters must be measured at regular intervals. These are the optical densities at 600 nm, which provide information about the division properties of the cells, as well as the oxygen content in the form of dO_2 measurements [48]. The generation time of *P. pastoris* with carbon sources such as glycerol is from 2-3 h to 1.5 h after an initial lag phase, while the generation time of *P. pastoris* that have successfully changed their metabolism to methanol is increased to about 5 h [49]. The oxygen measurement can be performed with reference measurements of 10 ml samples at the dO_2 electrode of the fermenter. It should be noted that *P. pastoris* falls to almost 0% dO_2 level after complete consumption of the batch carbon source (glycerol), but then shows a characteristic dO_2 spike when the energy source is fully metabolized [50]. When changing to the induction medium (methanol), to circumvent these above-mentioned problems I allowed the yeast a period of adaption. In fermentation, a low constant flow of methanol feeding was added to the culture. In the case of the test expression in the overnight flasks, it is advisable to add the same volume again to keep the actual, potentially toxic amounts of methanol as low as possible. An expression time of 24-36 hours has proven to be effective [45]. A shorter expression time did not deliver the desired protein amounts, while a longer expression time stressed the cells too much. This stress can be caused by a too long-lasting or too abrupt methanol supply before the metabolism could adapt completely [49]. As a result, these stressed cells may have expressed an adequate amount of protein, but the stress-induced thicker cell wall makes it more difficult to break *P. pastoris* cells in French-press and reduces the solubilization efficiency of detergents (see section 4.2). As an alternative to methanol induction for the AOX1 system, there is the possibility to express ABCB4 constitutively and thus avoid some problems. A system under the promoter which actually already exists in our group but has not been tested yet [46]. Potential advantages could be that the cell stress is significantly reduced, and a thinner cell wall facilitates membrane preparation and improves the solubilization properties. It is not clear whether the constitutively expressed amount of ABCB4 is sufficient. Test expressions would no longer be a blind flight and the protein amount would correlate with the expression duration and a prolonged fermentation would lead to an adequate protein amount.

4.4.2 Membrane isolation and solubilization of ABCB4 out of *Pichia pastoris* cells

A crucial step to purify membrane proteins is the necessity to obtain a crude isolation of membranes from other cell organelles [45]. It increases the abundance of the protein of interest *per se*. Cracking of cells can be performed with various principles. A beat-beater system as used for *S. cerevisiae* is insufficient for *P. pastoris* due to the differences in the membrane and cell wall of both yeasts [51]. Also, an ultrasound system did not show a sufficient cell disruption success. Membrane isolation in particular turned out to be the decisive step in determining whether and with what success the purification was accomplished. As described in the previous section, the problem of a thick cell wall was considered. This was conspicuous by fermentations in which expression was carried out for 48 h or longer (own observation). Normally, after cell disruption at the French press at 2.72 kbar, cell debris are sedimented in 3 successive centrifugation steps. The size of the pellet, its composition, and the color (same appearance as cells that were not disrupted and no cell debris typical black organelles) gave an indication of the disruption efficiency. Stressed cells that had to grow without nutrient medium for a longer period of time, whose oxygen saturation was reduced due to increased foam generation or that were exposed to too much methanol too quickly, *per se* produced less biomass at the end of the fermentation. As a reference 150 g/1 liter cell culture volume are considered normal [44] (<1 kg instead of the usual 1.3 kg per 7 L initial culture medium). Besides the lower number of cells, purification from cells with unusually high stress levels produced a lower yield *per se*, even if the same clone was used for both comparable fermentations. The user manual of the cell disruptor speaks of an exclusion rate of 40 % of the cells after one passage. Experience has shown that 3 passages provided the best cell-breaking results with minimal denaturation of the proteins and minimal foaming during the individual passages [52]. Especially for the purpose of stabilizing the protein-of-interest, it is very important to give the system the appropriate time to cool down between the individual shots. Also, both cell culture and lysate should be permanently cooled on ice. The homogenization of the membranes is also of decisive importance for the solubilization success. Recommendable based on own experience is the use of a homogenizer with two stamps, a thinner one for coarser comminution and a precisely fitting one for optimal shear force. The *Pichia pastoris* cell with its cell wall and stable cell membrane [53] could only be solubilized with a relatively harsh detergent. The best success was obtained with the Foscholine series [54]. Although DDM was used as a detergent of choice in other laboratories to purify ABCB4, it could not be used as an extraction detergent [23]. Although, I was able to show that FC-16 extracted ABCB4 proteins that were subjected to a detergent switch to DDM were still active, the improvement was not worth the effort. This suggests that partly detergent resistant membranes in *Pichia pastoris* make the extraction of ABCB4 more difficult. This extraction difficulty can be even higher if cells were exposed to an increased stress level during cultivation. In summary, a solubilization slightly below room temperature (18 °C) with 1% FC-16 showed the greatest success.

4.4.3 Tandem Affinity Chromatography (TAP)

Affinity chromatographies are most likely the most frequently used methods to purify membrane proteins. Several affinity chromatographies are known each relying on their own specific protein tag. Rather uncommon is the urge of two subsequent downstream affinity chromatographies as the purification protocol of ABCB4 requires, since one alone was not sufficient to purify ABCB4 to the demand of *in vitro* characterization [45,54].

After solubilization of membranes and sedimentation of non-solubilized components by centrifugation, immobilized metal ion affinity chromatography (IMAC) with nickel ions was performed on a HiTrap chelating column and competition with imidazole. Testing of another metal ion and histidine as competitor did not result in an improvement. The second purification *via* the calmodulin binding peptide (CBP) on immobilized calmodulin on a Sepharose matrix increased the purity of the sample. Instead of improving ABCB4's purity, it was bleeding from the columns and was found in the wash fractions. For this reason, a low concentration of competitor was chosen, and the elution was performed in a single step. Thus, the loss of ABCB4 remains low while maximizing the purification success. Together with the observation that ABCB4 cannot be separated from the much smaller impurities in a size exclusion chromatography, the impurities are most-likely located together with ABCB4 in the detergent micelles, however Olsen *et al.* succeeded in this task with a purification originating from human cell culture [23]. In a mass spectrometric analysis of the TAP eluate, mainly impurities were found that should differ significantly in their monomer molecular weight from the 145 kDa ABC transporter ABCB4 (Figure **D 6**).

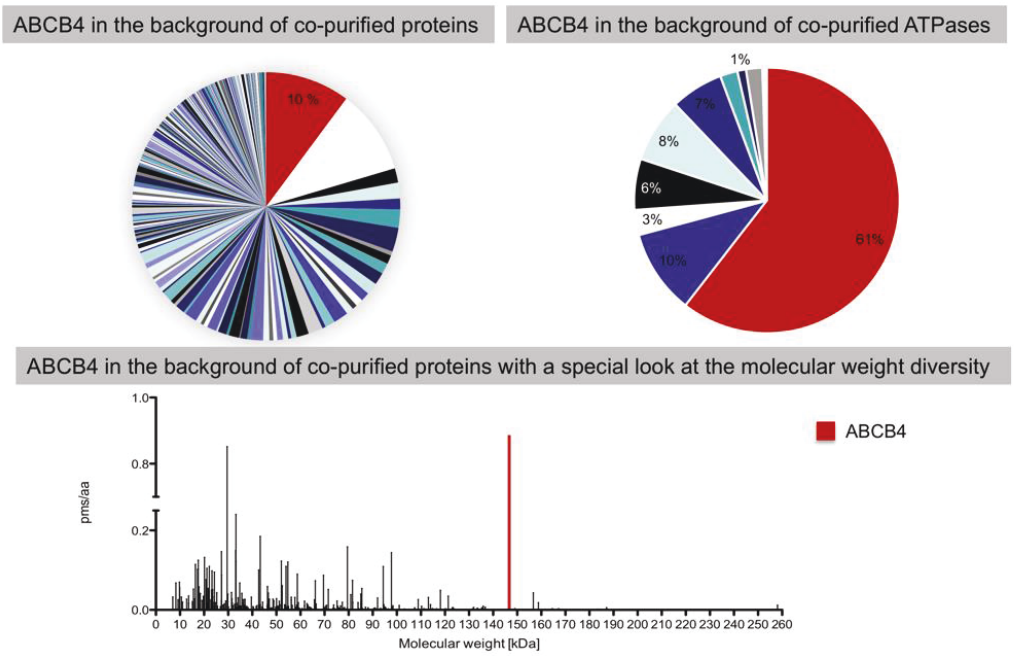

Figure D 6: Illustration of the mass spectrometric analysis of the TAP eluate. Even though, ABCB4 was separated via two affinity chromatography's impurities are measurable. ABCB4 in the background of co-purified ATPases explains the residual ATPase activity after ABCB4-specific Bodipy inactivation. The majority of impurities are in their monomeric form smaller than ABCB4 is but cannot be separated via size-exclusion chromatography.

However, if one assumes that the detergent micelles are large [55] enough for ABCB4 and impurities, it makes sense that after size exclusion chromatography ABCB4 eluted in several peaks over a comparatively large elution volume. It is not surprising that the impurities based on this theory could not be removed by extended washing steps. Surprisingly, the degree of purity could not be improved by a detergent exchange from FC-16 to DDM or LMNG bound to the IMAC column. An exchange of the detergent micelles, i.e. a change in the size, affinity, or polarity, would remove at least a high quantity of the impurities. Although admittedly the elution pattern on a SDS PAGE changed, it could not be concluded that the sample was purer than before. It is worth mentioning that other laboratories [23] were already able to purify ABCB4. The detergent of choice was DDM. Surprisingly ABCB4 showed no ATPase activity in the detergent solubilized state [23] and only a reconstitution in peptidiscs or liposomes restored the activity. A crucial difference is that this group solubilized the protein from a human cell line (T-Rex 293) and therefore did not need to work with a harsh detergent such as Foscholine 16. It should be noted that I was still able to measure ATPase activity when I solubilized the protein with FC-16 and switched to DDM during purification. It has to be admitted that despite intensive washing with the new detergent I cannot assume a complete exchange. As a conclusion, it should be tried to further optimize the purification by continuing to solubilize with FC-16 if necessary and switching to detergents that form the smallest possible micelles to provide space for only one ABCB4 per micelle with the hope that ABCB4 will continue to show detergent ATPase activity. Unfortunately, I could not test this theory. Admittedly, the purification together with ABCB4-specific Bodipy inactivation was perfectly suited to answer all demands raised in the *in vitro* system. In the longer term, however, the detergent-purified form of ABCB4 should be abandoned and used with a greater goal to reconstitute the ABC transporter into liposomes. Alternatively, finding the smallest possible micelles to make the detergent purification cleaner, a change of detergent should also be sought to facilitate a better starting point for reconstitution. FC-16 was presumably a critical reason why the reconstitution did not work. Should the constitutive expression be successful in providing less stressed and therefore more easily solubilized membranes with a comparable or even increased ABCB4 quantities, this would be a decisive improvement on the basis of the purification of ABCB4.

4.4.4 Bodipy labeling – targeting the cysteine in the Walker A motif of ABCB4

Commonly, purity and specificity of a purification is compared to a second purification of a dead-mutant of the respective ABC transporter (see EQ or HA mutants) [52,56]. However, Bodipy labeling circumvented the need for a second purification and additionally minimized the error bars and therefore increased the reproductivity of results. Thus, inactivation with Bodipy labeling was used to dampen the effects in ATPase activity when comparing two different purifications. The purity of ABCB4 in FC-16 detergent micelles is 60-70 % of the total protein concentration as quantified from SDS PAGE levels *via* Image J. To exclude that co-purified impurities, have an influence on the ATPase activity, it is necessary to specifically inactivate ABCB4. Bodipy is a fluorophore with a maleimide group as reactive residue [52,57].

The maleimide group forms a thioester bond to cysteines of the protein. There are 10 cysteines in the whole protein, but probably the ABCB4 characteristic cysteine of the Walker A motif makes it possible to irreversibly prevent ATP binding (Figure D 7). The Walker A motif in turn coordinates the phosphate moiety of the bound nucleotide. This loop is located between an α-helix and a β-strand and is part of the mononucleotide fold. If the nucleotide binding is occluded sterically, the ATPase activity seems to be disabled.

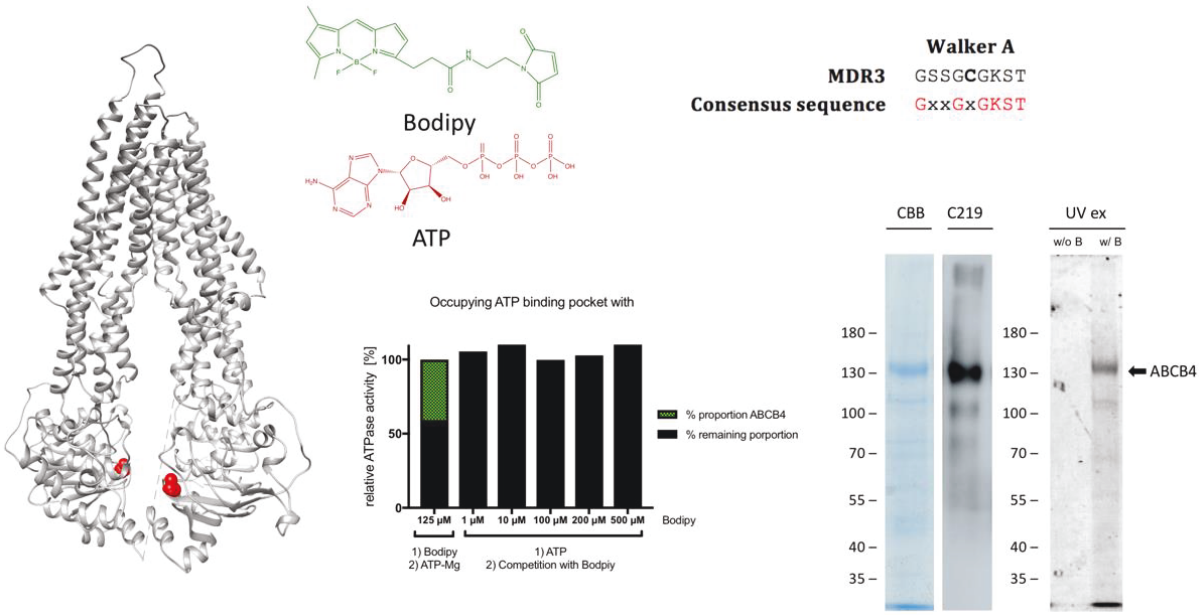

Figure D 7: From left to right. Red sphered cysteines are highlighted in a grey structure of ABCB4. Bodipy and ATP roughly share the same size, explain the possibility of how bound Bodipy sterically hinders ATP binding in the nucleotide binding sites of ABCB4. The Walker A motif of ABCB4 has a rather unique cysteine in its motif which is likely to be the explanation of the inactivation of Bodipy labeling. Coomassie brilliant blue (CBB) stained ABCB4 runs at a molecular weight of roughly 135 kDa even though it has a molecular mass of 146 kDa. Bodipy labeled ABCB4 can be exited under UV light (UV ex).

Initially the labeling reaction caused a low reproducibility of the Bodipy (ABCB4 specific) background. It was advisable to reduce the protein solution in advance to ensure that no unwanted oxidation prevented the labeling reaction. Reversible oxidization can occur during or due to purification and prevent the formation of thioester bonds [58]. For this purpose, a reducing agent was used, which itself does not have any sulfhydryl groups, TCEP. A 50-fold molar excess of Bodipy to protein ratio was henceforth ideal for reproductive background measurements, with a 20 up to a 100-fold excess causing no significant changes in activity [52]. As a precautionary measure, the excess was chosen so that statistically speaking, all ABCB4 transporters were actually deactivated in the control measurement. The additive volume of Bodipy dissolved in DMSO was kept as low as possible to minimize solvent effects. The non-Bodipy treated sample was prepared identically. As a result, the Bodipy labelled sample provided the perfect background, i.e. negative control. Auto-Hydrolysis of nucleotides, for example, or effects of the solvents used for drugs were automatically deducted.

4.4.5 A time -resolved ATPase assay increased reproductivity and throughput

The ATPase activity measurement is probably one of the most used activity assays in the field of ATPases. It is an indirect tool to quantify substrate selection and velocities, since substrate translocation is often strictly coupled to ATP hydrolysis [36]. Some proteins, however, are unable to be stimulated by their substrates above a basal level like the yeast ABC transporter Pdr5 [51]. ABCB4 on the other hand is an ABC transporter that is stimulated by various substances, especially PC-lipids [13]. Even though, the ATPase measurement in general is an indirect read-out for the protein's activity in the case of ABCB4 it came with an undeniable advantage as the *in vitro* characterization of ABCB4 demonstrated. Here, it was investigated which PC-lipids moiety and or which PC-lipid variants were preferred. It was unnecessary to reconstitute the protein. Features of liposome stability with an active floppase, size, shape or thickness differences between PC-lipid variants didn't have to be controlled [13]. Substrate selectivity and preferences are often mirrored by the ATPase activity activation or inhibition, a phenomenon sometimes overlooked [36,59]. The NADH oxidation assay was used for this task. It is time-resolved and allows higher throughput with less technical error in comparison to the colorimetric malachite-green assay [60,61]. From an efficiency point of view, it is possible to work with a buffer stock, which allows not only faster but also more accurate and reproducible addition of components. Fewer individual pipetting steps lead to statistically fewer errors. The interaction with the semi-automatic Tecan Reader allowed a delayed but reproducible start. In contrast to the endpoint determination from assays such as the malachite green assay, where the inorganic phosphate is determined colorimetrically, the NADH oxidation assay determines data points every 30 s. Especially for initial tests with new protein or new substrate the linear range of the reaction was always determined and used in the time-resolved assay. A potential initial lag phase was excluded, and it could be verified whether the reaction had already been completed. In contrast to colometry, the detection limit was not exceeded, because a decrease in intensity was measured instead of an increase in absorption with malachite green. A disadvantage of the NADH oxidation assay is the dependence of auxiliary enzymes such as lactate dehydrogenase or pyruvate kinase. For each substance it must first be tested that the auxiliary enzymes are not affected, and that the measurement does not represent an artifact. Regardless of this, any effect is also present in the Bodipy control and can be observed and strictly controlled.

4.4.6 *Status-quo* of the reconstitution of ABCB4

One of the main objectives when working with ABC transporters is to return membrane proteins to their natural environment, i.e. to integrate them into a synthetic membrane. Many proteins tend to behave differently in different physical environments. ABCB1 or LmrA for example change properties in detergent purified state compared to its reconstituted form in liposomes [62,63]. I tried to reconstitute ABCB4 into a synthetic membrane. I tested several lipid mixtures including different ratios of to DOPC, POPE and cholesterol concentration and *E. coli* total lipids [61]. I extruded the liposomes with 200, 400 and 800 nm pores. A proper pore size showed strong differences in activity and stability of the respective

proteins [64]. First of all, I tried to detect the presence of integrated ABCB4 in liposomes. For this purpose, I performed a density gradient centrifugation. Detergent-stabilized proteins appear in a different phase than empty liposomes and correctly reconstituted protein. Reconstituted protein could only be detected once in a sample and only in a weak band, unfortunately this success could not be reproduced. The low initial concentration of ABCB4 may be the underlying problem. The protein could be purified in average 0.5 mg/ml concentration and any attempts to artificially increase this protein concentration failed, a low concentration compared to heterologous expressed soluble proteins, but above average for human expression levels [65]. SDS gels did not show any improvement and the concentration at the nanodrop did not increase significantly, so that it must be assumed that the protein precipitated during the concentration step. Subsequent SDS analysis of the eluate, supernatant and rinsing of the filters showed that a large portion of the protein was indeed present in the filters. Essential for a correct reconstitution is the gentle, but complete removal of detergent. Probably FC-16 could not be removed by the bio beads due to its dense packing or this procedure led to early precipitation of ABCB4. Liposomes on the other hand were destabilized with a well-studied detergent: Triton-X-100 [61]. The liposomes behaved accordingly in the spectrometer analysis. Initially, swelling of the liposomes was observed and light scattering at 600 nm increased, and then the integrity of the liposomes was reduced, as shown by the reduction in light scattering. Triton-X-100 as a mild detergent with the ability to form large micelles is considered in the literature as an ideal detergent to be removed from the lipid-detergent emulsion by bio-beads. FC-16 as a harsh detergent with compact, smaller micelles can be a problem, why it was not possible to integrate the detergent purified protein successfully [66-69]. Alternatively, in the future it should be tried to exchange ABCB4 for reconstitution in the immobilized state of the IMAC column. In this case a detergent should be chosen that is either described in the literature and can stabilize ABCB4 like DDM [23] or alternatively, micelles should be used that form large micelles and tend to be more easily removed from ABCB4 by bio beads.

4.4.7 Transport assay – what has to be addressed how the future can look like

A transport assay for ABCB4 would be the ideal condition to characterize ABCB4 in general. However, there are several obstacles to overcome especially for ABCB4 as an PC-Floppase. A classic transport assay (substrate transport across the membrane), however, is not feasible for ABCB4 as the transported PC-lipid is transported from one leaflet of the bilayer to the other. One plausible assay that is currently available is based on colorimetric detection of PC after an extraction of lipids with BSA [14,23]. This is based on the assay from van Helvoort *et al.*, where ABCB4 translocation of PC is measured based on a cell culture model with NBD-labeled short-chain lipids. And in the third one, of Smith *et al.*, did also analyze cell culture-based translocation of ABCB4, but with radio labeled medium-chain PC-lipids. All the assays require ABCB4 to be reconstituted into a membrane system or are based on whole cells. To produce such a system from our detergent-purified protein, ABCB4 has to be reincorporated into liposomes. This alone is for membrane proteins a pitfall and requires optimization. The assay itself bears

some problems. First, lipid sinks are required to extract lipids out of the membrane system in order to be measured. Such a lipid sink may not show different affinities or velocities for different PC-lipid variants. Such a system may be a hydrophobic protein such as BSA or needs a mediator such as a PC-transfer protein. The PC-transfer protein itself adds an additional level of complexity in the read-out that again might show affinities or efficiencies for certain lipid variants. Secondly, it has to be addressed how a stable liposome can be created where ABCB4 can be reconstituted. This means, a reconstitution protocol has to be established with reconstituted ABCB4 in similar amounts. If the liposomes are stable enough the question of how to ensure that membrane curvature or size do not alter the proteins translocation velocities remains. ABCB4 as an isolated entity would probably destabilize the surrounding PC-liposome *via* active PC transport. This problem is most likely circumvented by compensation methods of the phospholipid flippases in whole-cell assay, but likely will be a problem for our *in vitro* system. This would most likely result in liposome shrinking when PC-lipids are constantly extracted. This gives the need for giant-uni-laminar vesicles (GUVs) where size differences can be neglected, but they are even more unstable [70]. Another task that has to be regulated and taken into consideration is the orientation of ABCB4. If the transporter is "right-side in" orientated to allow subsequent quantification with a lipid sink such as BSA, a ATP regenerating systems has to be incorporated.

One critical aspect of any transport assay for ABCB4 is the substrate itself. Since ABCB4 is able to be modulated or can even transport hydrophobic drugs; a transport assay based on lipid analogs to read out true lipid translocation always carries the doubt which part of the substrate is recognized. Our ATPase data add up and reinforce the arguments of at least two ligand recognition sites within ABCB4. One might be just modulatory, but it is also possible that two translocation pathways are used. The high level of sequence identity between ABCB1 and ABCB4 further supports this fact. *In vivo* assays demonstrated for example the export of digoxin, paclitaxel, daunorubicin, vinblastine and ivermectin out of monolayers of kidney cells [35]. Therefore, we thought of a transport assay which uses lipid analogues as similar as possible as read-out. PC-lipids with bromide groups might be the answer. PC-Br-lipids quenche the Trp fluorescence in proximity of ~8 Å [71]. With a membrane thickness of 30-40 Å [8] it may be possible to distinguish flopped PC-Br-lipids from non-flopped ones.

In a setup-up of reconstituted inside-out liposomes the outer leaflet could be supplied with PC-Br-lipids and ABCB4 would translocate PC-Br-lipids into the inner leaflet. Based on the structure of ABCB4 and the hypothesis of the TMH1 being the anchor point of substrate translocation mutants need to be introduced.

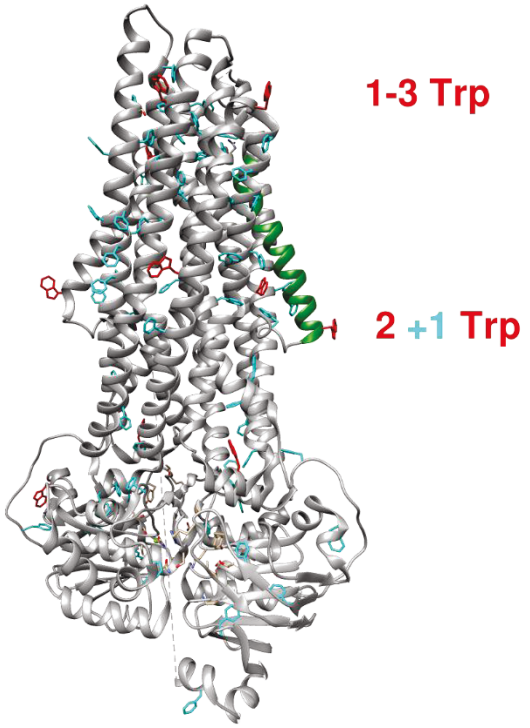

Figure D 8: Structure of ABCB4 with highlighted highly conserved tryptophan's residues in red and less conserved phenylalanine residues in cyan. In dark green the transmembrane helix 1 is highlighted. Structural data provide the possibility to predict an increased asymmetry in tryptophan's between the two leaflets if Phe in proximity are mutated to Trp. Within 8 Å of the outer leaflet minimum one and maximum three Trps are present. One mutated Phe to Trp in the inner leaflet would increase this asymmetry even further if needed.

It is needed to create a higher asymmetry of Trp residues to distinguish the inner from the outer leaflet. Since Trp are highly conserved within the ABCB4 orthologs of different species I looked for close Phe residues to be mutated into Trp (Figure D 8). The inner leaflet was more suitable to increase the amount of Trp residues. Only direct measurements will tell if an increased asymmetry is needed or if the observed Trp fluorescence is already sufficient. The outer leaflet even if predicted with 1-3 Trp may also have no Trp residue in 8 Å proximity depending on how flexible or rigid the structure especially the extracellular loops really are (not part of this structure from Olsen et al.). When all these issues are considered the introduction of ABCB4 mutants could be used to analyze the effect of each crucial position in TMH1.

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I. Curriculum Vitae

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Publications

2020	Structure and function of hepatobiliary ABC transporters
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	Chemical Reviews
2020	Stimulation of ABCB4/MDR3 ATPase activity requires an intact phosphatidylcholine lipid
	Martin Prescher, Sander Smits, Lutz Schmitt
	Journal of Lipid Research

2020	mGBP7 interacting proteins in *Toxoplasma gondii* infection and biochemical characteristics of mGBP7 Larissa Legewie, Jennifer Loschwitz, Sander Smits, **Martin Prescher**, Xue Wang, Nora Steffens, Daniel Degrandi, Birgit Strodel, Lutz Schmitt and Klaus Pfeffer The American Association of Immunologists, Inc. Vol 202, Issue 1
2019	ABCB4/MDR3 in health and disease – at the crossroads of biochemistry and medicine **Martin Prescher** *, Tim Kroll*, Lutz Schmitt Biological Chemistry Volume 400, Issue 10

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III. Declaration

Ich versichere an Eides Statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist. Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Fakultät eingereicht. Ich habe bisher keine erfolglosen und erfolgreichen Promotionsversuche unternommen.

(Ort, Datum) (Martin Prescher)