Developing BirA fusion constructs of human ABC transporters (ABCB1, ABCB9, ABCB10, ABCBD4, ABCF1) for proximity BioID studies in protein-protein interactions studies

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ABSTRACT

The ATP-binding cassette transporters (ABC transporters) are one of the oldest and largest gene families. In humans, this superfamily comprises 48 ABC transporters divided into seven sub-families labeled from A to G and serve vital function in selective movement of normal cell metabolites, along with the transport of xenobiotics, hence cell detoxifications. Altering of ABC transporters' expression and subcellular localizations can lead to major phenotypic changes in normal and diseased tissues and organs. Thus, to understand the molecular mechanisms of ABC transporter proteins regulation along with the role of interacting proteins in such regulatory mechanisms, we have focused on using a promiscuous biotin ligase (BirA) fused to ABC transporters. Our approach aimed to identify interacting as well as proximal proteins in mammalian cells. This latter approach, proximity-dependent biotin identification (or BioID), provide important insights into ABC transporters trafficking, subcellular localisation, and transient protein interactions in-vivo in the absence of, or presence of drug treatments.

Consequently, it is anticipated that knowledge of ABC-transporters interactomes can lead to: a) understanding their mechanisms of regulations in normal versus diseases; and b) identifying novel targets for the development of therapeutics to modulate their functions. My thesis work focuses on developing five constructs with BirA fused to the C-termini of ABCB1, ABCB9, ABCB10, ABCD4 and ABCF1.

ABRÉGÉ

Les transporteurs de cassettes de liaison à l'ATP (transporteurs ABC) sont l'une des plus anciennes et des plus grandes familles de gènes. Chez l'homme, cette superfamille comprend 48 transporteurs ABC répartis en sept sous-familles marquées de A à G et assurent une fonction vitale dans le mouvement sélectif des métabolites cellulaires normaux, ainsi que le transport des xénobiotiques, d'où les détoxifications cellulaires. La modification de l'expression et des localisations subcellulaires des transporteurs ABC peut entraîner des changements phénotypiques majeurs dans les tissus et organes normaux et malades. Ainsi, pour comprendre les mécanismes moléculaires de la régulation des protéines de transport ABC ainsi que le rôle des protéines en interaction dans ces mécanismes de régulation, nous nous sommes concentrés sur l'utilisation d'une biotine ligase promiscuité (BirA) fusionnée aux transporteurs ABC. Notre approche visait à identifier les protéines en interaction ainsi que les protéines proximales dans les cellules de mammifères. Cette dernière approche, l'identification de la biotine dépendante de la proximité (ou BioID), fournit des informations importantes sur le trafic des transporteurs ABC, la localisation sous-cellulaire et les interactions protéiques transitoires in vivo en l'absence ou en présence de traitements médicamenteux.

Par conséquent, il est prévu que la connaissance des interactomes des transporteurs ABC peut conduire à : a) comprendre leurs mécanismes de régulation dans les conditions normales versus pathologiques ; et b) identifier de nouvelles cibles pour le développement de thérapeutiques afin de moduler leurs fonctions. Mon travail de thèse porte sur le développement de cinq constructions avec BirA fusionné aux extrémités C-terminales de ABCB1, ABCB9, ABCB10, ABCD4 et ABCF1.

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CONTRIBUTION TO ORIGINAL KNOWLEDGE

Many extensive studies have been happening around ABC transporters protein-protein interactions using different techniques and very less information was known till date and some of the information interactions are known were just assumptions. Study of PPIs in different cell lines has the potential to serve as a promising therapeutic target for different diseases including cancer and Alzheimer's disease.

In this thesis, five ABC transporter proteins including ABCB1, ABCB9, ABCB10, ABCD4 and ABCF1 were fused into BirA construct to study protein-protein interactions.

CONTRIBUTION OF AUTHORS

The experimental work presented in this thesis was conceived and executed by the author, Venkata Manikanta Bellam, with the supervision and guidance of Professor Elias Georges. The thesis was written by the author, Venkata Manikanta Bellam and Professor Georges provided invaluable assistance in experimental design and thesis editing, contributing to the overall quality of the research conducted in this study.

LIST OF ABBREVATIONS

ABC	ATP-binding cassette
ABCB1	ATP-binding cassette transporter, family B, member 1
ABCB9	ATP-binding cassette transporter, family B, member 9
ABCB10	ATP-binding cassette transporter, family B, member 10
ABCD4	ATP-binding cassette transporter, family D, member 4
ABCF1	ATP-binding cassette transporter, family F, member 1
APCs	Antigen Presenting Cells
ALD	Adrenoleukodystrophy
ATP	Adenosine triphosphate
BCRP	Breast Cancer Resistance Protein
BirA	Bifunctional ligase/repressor
BioID	Promiscuous biotin ligase
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
DEER	Double electron-electron resonance
GOI	Gene of interest
HEK293F	Human embryonic kidney cells 293F

HDL	High Density Lipoprotein
HIV	Human Immunodeficiency Virus
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
MDR	Multidrug resistance
MDR1	Multidrug resistance protein 1
MHC	Major histocompatibility complex
NBD	Nucleotide Binding Domain
PCR	Polymerase chain reaction
POSs	Photoreceptor outer segments
P-gp	P-glycoprotein
PPIs	Protein-protein interactions
RBCs	Red Blood Cells
RPE	Retinal pigment epithelial
TAP	Transporter associated antigen processing
TLR	Toll-like receptors
TMD	Trans Membrane Domain

TNF	Tumour necrosis factor

INTRODUCTION

ABC Transporters

ABC Transporters are amongst oldest and largest gene families capable of transporting a range of substances including xenobiotics, proteins, and amino acids, by ATP hydrolysis across biological membranes in different cellular locations of the body [1–3]. They are ubiquitous intrinsic membrane transporter proteins found in both eukaryotes, prokaryotes that are involved in different biological processes and physiologies of cells [2, 4]. They are estimated to be more than 1000 members from microorganisms to humans [5]. The study of ABC transporters emerged in the 1970's when the uptake of nutrients was observed in bacteria. Further studies of microbial ABC transporters helped to explain, at least one mechanism of drug resistance, virulence, and synthetic biology systems [6, 7]. Bacterial ABC transporters help transport sugars, primary metabolites, antibiotics and proteins, whereas these transporter proteins in plants serve vital function in mediating movement of secondary metabolites, phytohormones, metals and xenobiotics [8, 9].

Active ABC transporters require two transmembrane binding domains (TMDs) and two nucleotide-binding domains (NBDs). TMDs span across cell/organelle membranes with 5–6 hydrophobic α-helices to facilitate transportation as well as identify specificity of a substrate. Cytoplasmic NBDs are participated in binding of ATP and hydrolysis, essential for active transport. NBDs consist of ABC signature, Walker A, Walker B, Q loop, and H loop motifs that are highly conserved.

ABC transporters can function as either importers that allow nutrients and macromolecules into the cell or exporters that efflux lipids, toxic substances, xenobiotics, and drugs [4, 2, 10, 11]. Most ABC transporters are unidirectional. They regulate the movement of heterogeneous substrates

against their concentration gradient through conformational changes within the transporter protein [2]. ABC genes encodes a full transporter with two TMDs and two NBDs or a half transporter with only one TMD and one NBD as shown in figure 1. Half transporters bind together and form either homodimers or heterodimers to be a functional transporter [12].

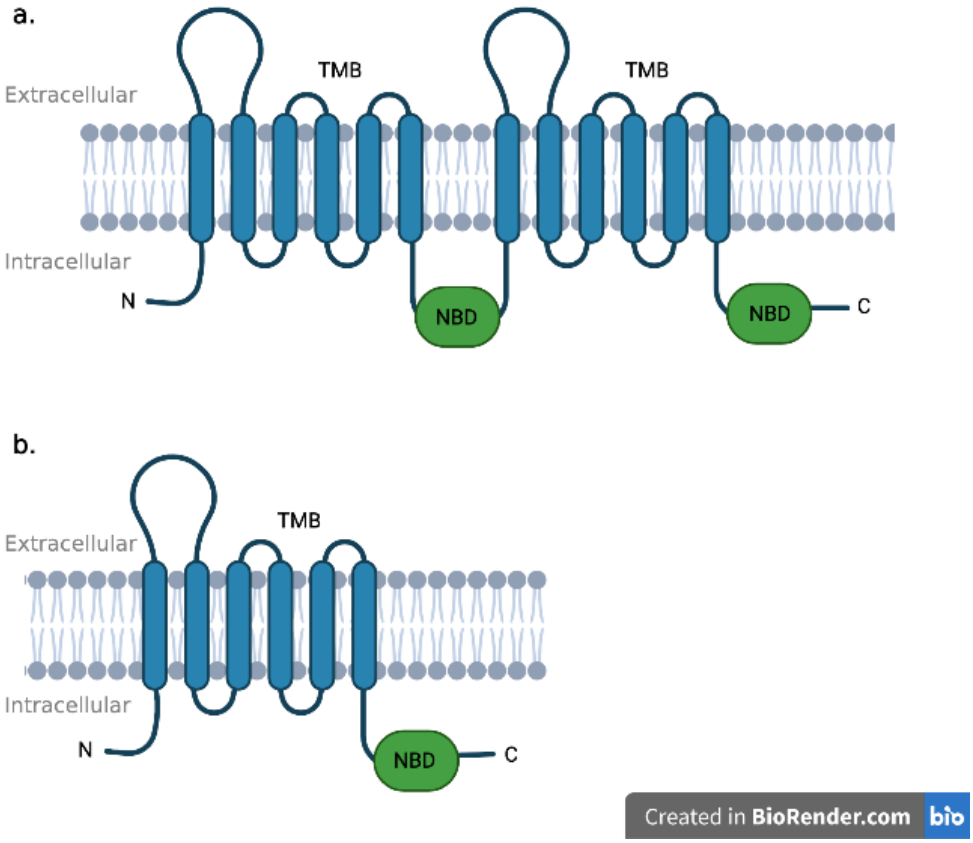


Figure 1. Topology of ABC transporters in which blue bars represents α-helices of Trans Membrane Domains (TMBs) and green oval represents Nucleotide Binding Domains (NBDs). Panel (a) shows an example of full transporter with two TMDs & two NDBs. Panel (b) shows an example of half-transporter with each of TMD and NBD.

Mechanism of ABC Transporter Proteins

ABC transporter proteins facilitates transport through ATP-powered mechanism and involves several steps. Firstly, ATP binding to the NBD of the transporter protein triggers a configurational modification in the protein that opens up the binding site of substrate in TMD. Substrate then binds to the transporter protein and is ready for transport. Then, ATP hydrolysis produces the energy required for transporting the substrate across the plasma membrane against its concentration gradient. Transported substrate is then ejected on another side of the cell membrane and ATP by-products are released [13–15]. The transporter protein then re-closes, allowing it to bind more ATP and cycle back to its original state, ready for another transport cycle.

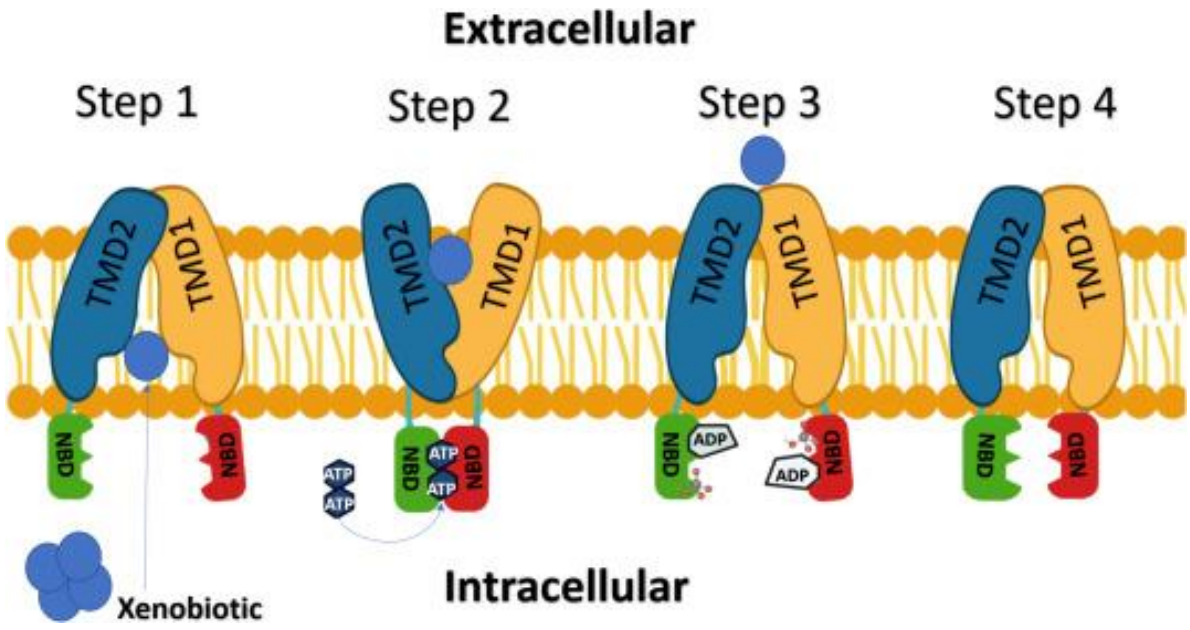


Figure 2. Mechanism of ABC transporter protein [16].

The schematic representations depict four required steps for substrate efflux via an ABC-transporter. Step 1 – Substrates binds to the TMDs of the ABC transporter. Step 2 – The NBDs of the ABC transporter binds ATP molecules causing conformational change in NBDs. Step 3 – Hydrolysis of ATP by the NBDs provides energy for the conformational change required to

transport substrate across membrane. Step 4 – NBDs release the products of ATP hydrolysis and revert to their original conformation.

Human genome consists of 48 ABC transporters and based on their genetic similarities they are categorised into seven subfamilies (A-G) and each subfamily has many members ranging from 1-13 as shown in table 1 [12, 17]. Each member of a subfamily is essential for different cellular processes.

Subfamily	Members
ABCA	ABCA1, A2, A3, A4, A5, A6, A7, A8, A9, A10, A12, A13
ABCB	ABCB1, B2, B3, B4, B5, B6, B7, B8, B9, B10, B11
ABCC	ABCC1, C2, C3, C4, C5, C6, C7, C8, C9, C10, C11, C12, C13
ABCD	ABCD1, D2, D3, D4
ABCE	ABCE1
ABCF	ABCF1, F2, F3
ABCG	ABCG1, G2, G4, G5, G8

Table 1. The human ABC transporters family is made up of 48 members classified into seven subfamilies (ABCA to ABCG), with each subfamily consisting of multiple members, save ABCE1. The ABCA1 consists of 13 members, ABCB harbours 11 members, 13 members in ABCC, 4 members in ABCD, 3 and 5 members in ABCF and ABCG respectively [5].

Functions of ABC Transporters

Typically, ABC transporters entail the movement of different substances from the cell cytoplasm to the inner compartments of cell organelles and/or extracellular fluid. They help maintain the homeostatic cellular function by the uptake of nutrients and efflux of different substrates like metabolites, lipids and toxins from a cell [18]. Transporters are primarily found in different tissues of brain, heart, breast, testis, ovary, kidney, and intestine. They play vital roles in the absorption, metabolism, distribution, and elimination of different substrates [19]. In addition, they are expressed in blood-placenta, blood-testis, and blood-brain barriers, which serve as protective functions and offer selective transport of nutrients in tissues [2, 20, 21].

ABC proteins transport a wide range of endogenous substances through the plasma and intracellular membranes including lipids, such as cholesterol and phospholipids, to maintain their fluidity and integrity [22, 23]. Some ABC transporters transfer metal ions, such as iron and copper, contributing to the proper balance of metal ions inside the cell and the function of enzymes requiring metal ions as cofactors [24, 25]. They also regulate the movement of proteins and peptides across the membrane, essential for the immune system's function and the proper functioning of the Golgi apparatus and the endoplasmic reticulum (ER) [26, 27]. The ABC transporter CFTR is involved in transport of chloride ions across the cell membrane in the epithelial cells of different organs, such as the lungs, reproductive tracts and reproductive tracts [28, 29].

They also play a crucial role in the removal of waste products from cells. In kidneys, they pump out waste products, such as creatinine, into the urine [30, 31]. Expression of ABC transporter in normal tissues exhibits circadian rhythm; depending on the time of day, the activities of these transporters vary. This helps in understanding the toxicity and efficacy of drugs based on application time of drugs [32, 33]. The ABC transporter proteins (ABCB, ABCC, and ABCG

subfamilies) have a crucial function in placental cells, as they contribute to the growth of the fetus and facilitate the transport of metabolites and nutrients through the placenta [34].

Multiple studies show that ABC transporters significantly provide resistance to antiviral, anticancer, and anti-inflammatory drugs [35]. Increased expression and efflux activity of certain transporters are frequently associated with chemotherapeutic drug resistance to cancer cells [35, 36]. Based on the efflux activity, nine ABC transporter proteins have been shown to impart multidrug resistance, in which ABCB1, ABCG2, and ABCC1 exhibit the strongest association [36, 37]. These proteins of the ABC family serve a critical function in detoxifying drugs, metabolites and pharmacokinetics [35].

ABCB1, ABCC1 and ABCG2 transporters are suggested to show a defense mechanism in hematopoietic stem cells, which could pose a challenge in treating hematological cancers, because cancer stem cells that express these proteins can evade treatment by chemotherapy [38, 39]. ABCB1 and ABCG2 transporter proteins are also linked to tissue regeneration in various organs including skeletal muscles, liver and heart [40–42].

ABCA1 and ABCG1 have been associated with the homeostasis of monocytes and macrophages, cholesterol efflux from peripheral tissues to the liver, and the migration and differentiation of dendritic cells [43]. ABCA1 is also known to engulf apoptotic bodies and biogenesis of high-density lipoprotein (HDL) [44]. ABCA4 (ABCR) transporter is found in photoreceptors and is reported to involve in transfer of retinol and phospholipid derivatives from the outer segment discs of photoreceptors into the cytosol [45]. ABCA8, also known as lipophilic drug transporter, regulates lipid metabolism and is involved in developing and maintaining myelin [46].

ABCB4 mediates the release of phospholipids and phosphatidylcholine from liver hepatocytes into bile [47]. ABCB6 transporter moves precursor of iron-sulphur (Fe/S) cluster from mitochondria to the cytoplasm [48]. ABCC11 has been shown to help know the type of earwax, further revealing the function of apocrine glands in humans. Additionally, it is linked to axillary osmidrosis, mammary gland colostrum secretion, and the potential for mastopathy [49]. ABCC3 involves in the efflux of organic anions, conjugated steroids, bile salts, glutathione conjugates and glucuronides [50].

ABCB11 is involved in transporting bile acids out of the liver and into the small intestine, thereby playing a crucial role in the digestion and absorption of fats and other lipids [51]. ABCB7 is known to carry antigens through the membrane of cell, where they are presented to the immune system [52]. Very long-chain fatty acids are transported into peroxisomes for metabolism by ABCD1 [53]. It is believed that ABCC1 transporter protein protects cells from chemical toxicity and oxidative stress as well as controls inflammatory responses that involve cysteinyl leukotrienes [54]. ABCG2 helps in excretion of xenobiotics, toxins in tissue barriers [55].

Role of ABC Transporters in diseases

Over the past few years, the role of ABC transporters in diseases including but not limited to gastrointestinal diseases, cardiovascular diseases, neurological diseases, infectious diseases has been widely studied. [56].

ABC transporters also engage in several mechanisms that leads to resistance of drugs in carcinoma cells. Several experiments suggested that presence of ABC proteins has been correlated to poor prognosis in different types of carcinoma [57]. ABCC1 and ABCC4 transporters are predominantly present in breast cancer cell lines and perform an vital function in cell proliferation

and migration respectively [58]. ABCB1 (MDR 1) confers drug resistance in different cancer cell lines. ABCB1 are expressed highly in several cancers causing limited efficacy of chemotherapy drugs due to accelerated drug efflux [59]. ABCG2 also known as Breast Cancer Resistance Protein (BCRP) also imparts drug resistance to different cancer cell lines by showing limited efficacy to drugs as well as certain tyrosine kinase inhibitors. It's elevated levels of expression is associated with a poor prognosis in different types of cancer patients [60]. ABCB4 is heavily located in cancer cells of breast and show resistance to doxorubicin by boosting drug efflux [61].

Several neurological disorders, such as Alzheimer's disease, and Parkinson's disease, have been linked to ABC transporter proteins. These proteins serve a crucial function in transporting neurotoxic molecules through the barriers of blood-brain, such as β -amyloid and alpha-synuclein, which could lead to the development of neurodegenerative diseases [62]. Accumulation of β -amyloid in parenchymal cells of brain leads to Alzheimer's disease and expression of ABCB1 at the blood-brain barrier is linked to β- amyloid clearance [63]. ABCG2 protein has been indicated to be essential in the movement of antiepileptic drugs, and defects in ABCG2 function can alter the pharmacokinetics and efficacy of these drugs [64]. Defect in ABCC1 have been associated with the decreased brain dopamine levels and the development of Parkinson's disease [65]. ABCA7 was involved in clearance of damaged proteins and the elimination of toxin protein aggregates from cells and thus defect in ABCA7 leads to an increased risk of Huntington's disease [66].

ABC transporter proteins also involved in the pathogenesis of number of infectious diseases, including tuberculosis, HIV, and malaria. ABCB1 serves crucial function in the efflux of antibiotics from infected cells, leading to resistances to drugs in tuberculosis [67]. ABCG2 (BCRP) imparts cellular resistance to HIV nucleoside reverse transcriptase inhibitors, and it has also shown

reduced efficacy of antiretroviral drugs, such as efavirenz, and contributes to the progressionof drug resistance [68].

ABC transporters are associated to the development of several cardiovascular infections, such as hypertension, heart failure, and atherosclerosis. They're also linked with regulation of blood pressure, vascular endothelial homeostasis, production and aggregation of platelets, and thus affect different cardiovascular diseases [69]. ABC transporters are involved in bile acid transport, which regulates gut motility and may contribute to IBS [70]. Expression of ABCC2 and ABCB11 are found to be altered in liver cirrhosis patients, leading to the development of cholestasis, a condition characterized by the buildup of bile acids in the liver [71].

Together, most of the ABC transporter proteins are crucial in many physiological functions of different human tissues and organs and play a vital role in many diseases. This thesis focuses on establishing tools to study ABCB1, ABCB9, ABCB10, ABCD4, and ABCF1 transporter proteins regulation through protein-protein interactions.

ABCB1

ABCB1 transporter protein also called as the P-glycoprotein (P-gp) or Multidrug resistance protein 1 (MDR1), is one of the earliest identified proteins in ABC transporter family in mammalian cells. In the year 1974, Juliano and Ling have isolated colchicine resistant (CHO^R) mutant Chinese Hamster Ovary tissue culture cells, which exhibited reduced permeability to colchicine. The cell lines on being exposed to different drugs did not involve in microtubule formation, this behavior strongly correlates to the pleotropic cross-resistance to different drugs [74, 75]. Further research demonstrated that this drug resistance mechanism was energy-dependent where they observed uptake of colchicine and other drugs in the presence of cyanide, and azide metabolic inhibitors and uptake of drugs are inhibited in the presence of non-metabolized sugars such as 2D-deoxyglucose [76].

This led to the discovery of 170kDa P-glycoprotein in the year 1976 by Dr. Victor Ling group, and further research explained it as the causative resistant protein in different cell lines. Thereafter, in gel electrophoresis and immunoblotting examination, ABCB1 was identified as responsible protein for multidrug resistance in different cancer cell lines [77]. Further research revealed that P-gp and MDR1 gene expression are linked to multidrug resistance, indicating that MDR1 gene encodes for ABCB1 (P-gp) [78]. The clinical significance of ABCB1has been extensively studied, and its expression has been linked to poor prognosis in various diseases. ABCB1 serves a vital role in efflux of drugs from cells, leading to reduced drug efficacy, as demonstrated by multiple studies.

ABCB1 is a unidirectional efflux pump dependent on ATP hydrolysis for its transporter function. The efflux function has been observed in various microorganisms, and similar transporter proteins

have been associated with antibiotic resistance [79]. In normal tissue, P-glycoprotein regulates the rate of cellular intake, distribution, and elimination of foreign substances, thereby impacting the absorption, distribution, metabolism, excretion, and toxicity properties of drugs, which can affect their efficacy and bioavailability [80].

Structure of ABCB1/P-gp

The molecular structure of a ABCB1 in a transport cycle have been studied using various techniques, including tryptophan fluorescence, luminescence, antibody binding, double electron-electron resonance (DEER), x-ray crystallography and electron microscopy [81]. The human P-glycoprotein is trans-membrane protein with 1280 amino acids long, arranged as 610 amino acids into two homologous parts with a linker region of approximately 75 amino acids [82].

Each homologous part of ABCB1 is composed of the N-terminal TMD with six hydrophobic transmembrane α-helix segments, followed by NBD with several conserved domains, including two active ATPase sites; Walker A or P loop (GXGKST; X is a non-specific amino acid), Walker B (DEATSALD) on one end and a signature motif LSGGQ at the other end [81], in addition to A-D- H- Q- loops [82]. Cytosol has N- and C- termini as shown in Figure 4,5,6.

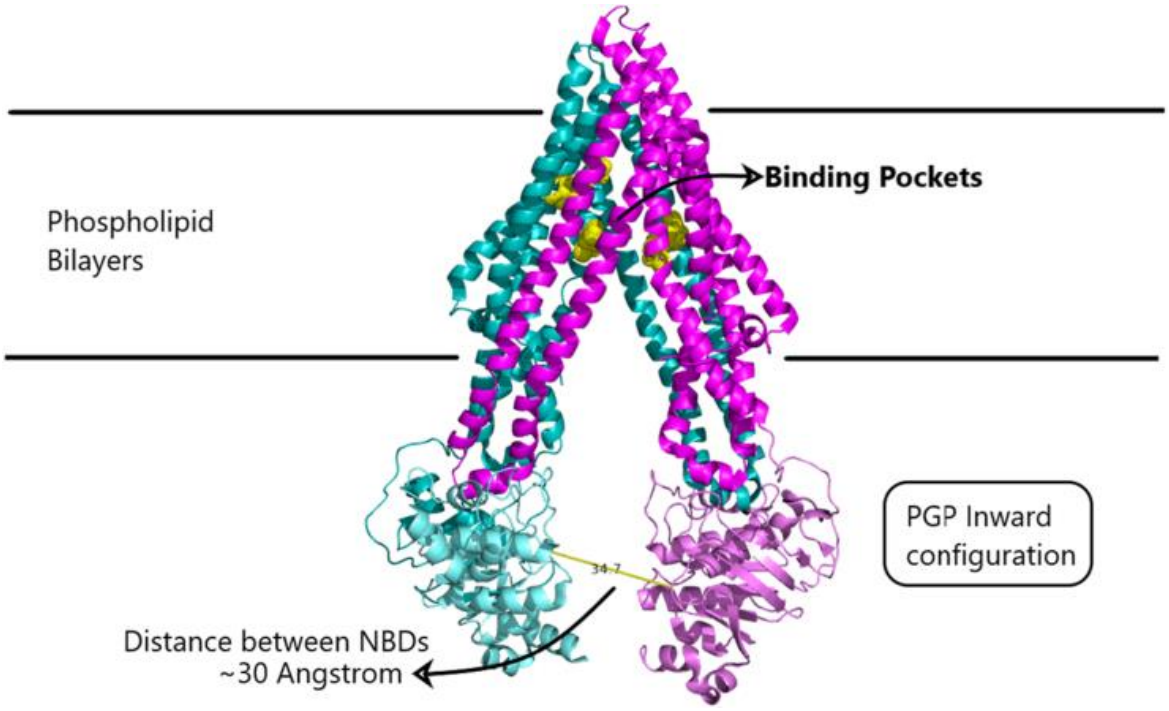


Figure 4: Three-dimensional structure of ABCB1showing active binding pockets [83].

The separated NBDs of P-gp1 (or ABCB1) in the inward configuration are depicted by yellow spheres, with their active binding pockets also shown in yellow. The yellow dots illustrate the distance between the NBDs.

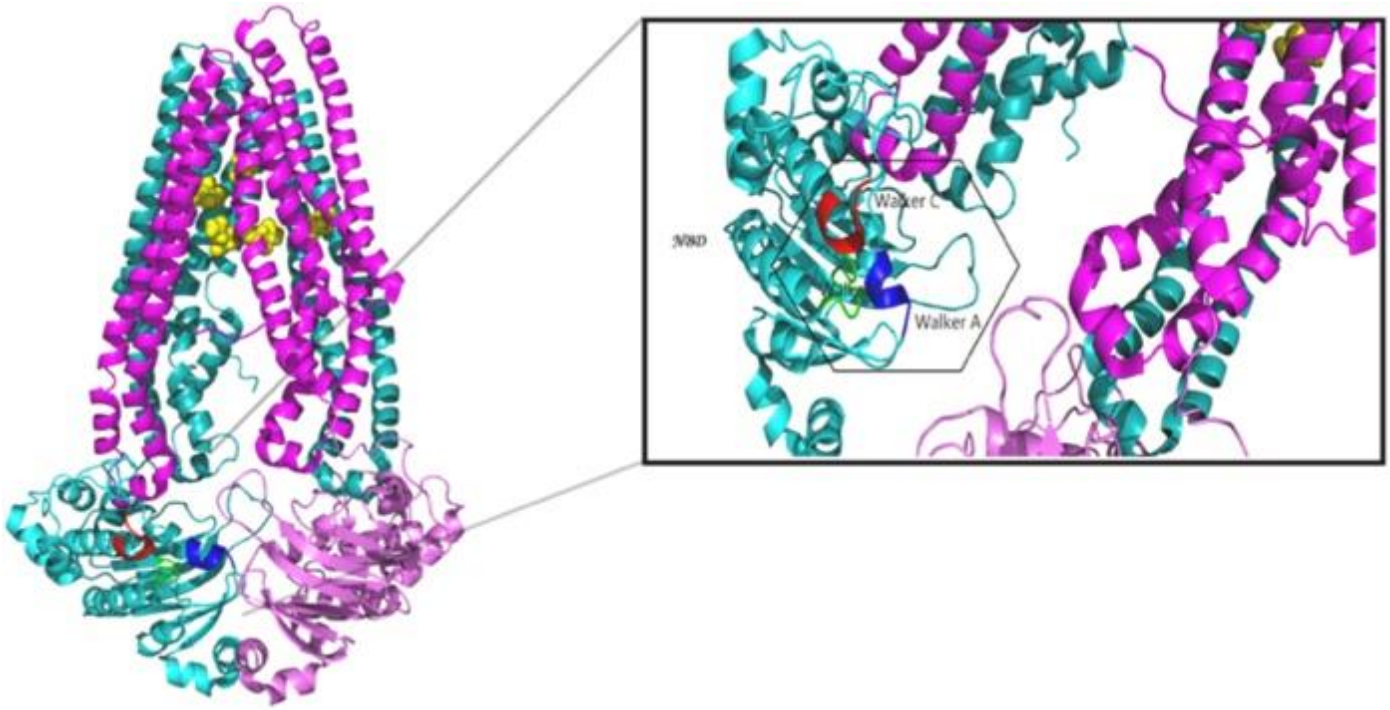


Figure 5. NBDs with walker regions [83]**.**

Enclosed by a black hexagon shape, the three distinct regions, coloured blue, green, and red, facilitate ATP binding and hydrolysis. The ribbon representation of the overall structure is coloured differently based on the TMD and NBD.

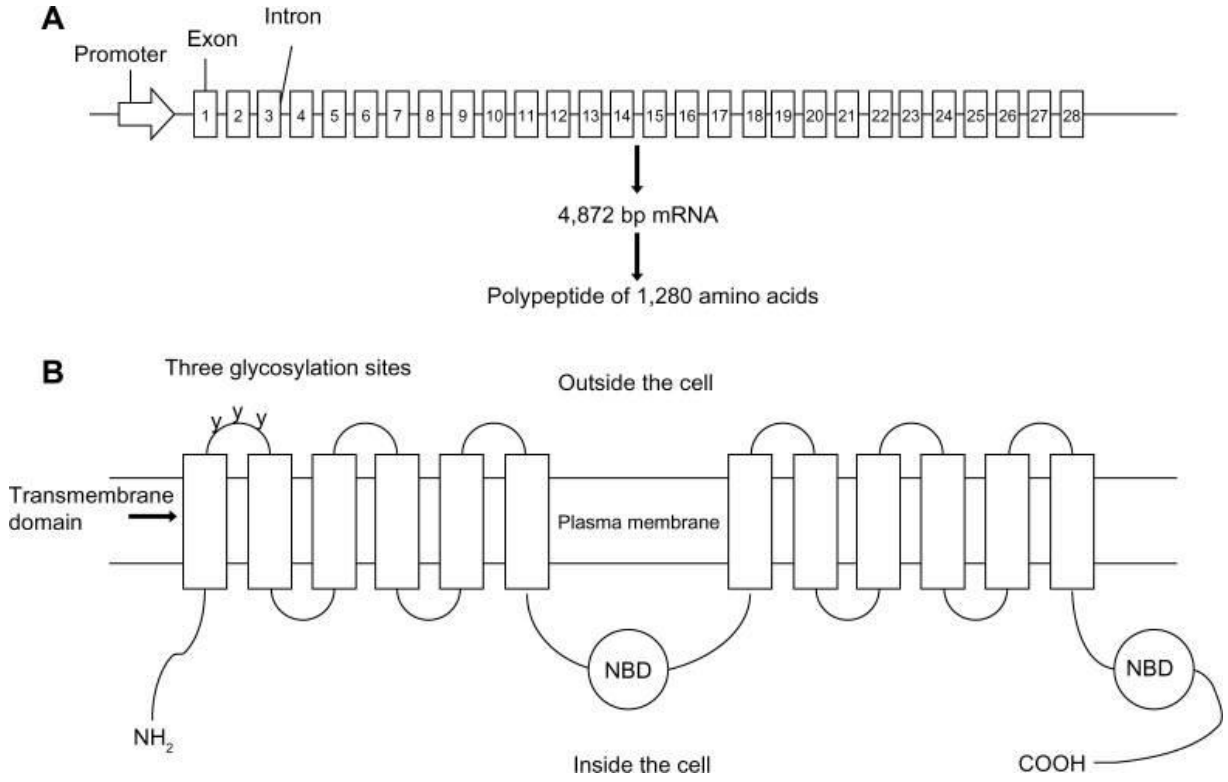


Figure 6. The genomic organization of ABCB1 (P-gp1) [84]**.**

Panel (A) shows the molecular structure of P-gp1 with 28 exons, introns and 1280 amino acids. Panel (B) shows secondary structure of ABCB1 (or P-gp1) with C- and N- termini present in cytosol region and 12 TMDs are inside plasma membrane.

A highly conserved glutamate residue present on each ATPase site serves as the catalytic basis for ATP hydrolysis [81]. Earlier drug transport is not observed in the ABCB1 mutant with defective N-linked glycosylation and was assumed to serve efflux function, but later experiments confirmed that it does not a play a role in function [85, 86]. Subsequent studies have validated that ABCB1 function is regulated by phosphorylation, and it is dynamic because of the involvement of multiple kinases and phosphorylation sites [87]. Pim-1 phosphorylation consensus sequence is found in the ABCB1 and it is said to regulate ABCB1 expression by protecting it from proteasomal and proteolytic degradation and also ensuring its glycosylation and cell-surface expression [88]. Unlike

proteins that has induced-fit model or lock and key model, ABCB1 lacks a set ligand binding pocket and so, different compounds with varying molecular weights are identified as substrates. This adaptability enables ABCB1 to transport a diverse range of molecules across cell membranes, underscoring its versatility as a transporter protein [89].

Mechanism of ABCB1 Transporter Protein

Like any other ABC transporter, ABCB1 transporter protein transports substrates based on ATP-dependent in which the NBDs mediates the ATP hydrolysis, while TMDs confer substrate binding and transport. Despite extensive research to elucidate the mechanisms of P-gp, the details and order of process involved in the efflux of drugs or transport of different substrates remains controversial.

Early studies suggested that ABCB1 transporter protein may function through a both active movement and passive diffusion mechanisms. The 'hydrophobic vacuum cleaner' model proposes that P-gp binds and pumps hydrophobic compounds out of the cell by exploiting the hydrophobic mismatch between the interior of the protein and the polar membrane environment. This model is supported by structural studies that have identified hydrophobic cavities within the ABCB1 transporter protein that may serve as substrate-binding sites [90, 91].

The 'flippase model' proposes that ABCB1 transporter protein acts as a flippase, by actively pivoting substrates from the internal leaflet to the external leaflet of the lipid bilayer, and thereby expelling such substrates from the cell. ABCB1 transporter is confined to cholesterol-rich microdomains of the cell membrane, and it may communicate with lipid substrates [91]. Further, studies showed that ABCB1 is energy dependent where binding of ATP and it's hydrolysis induces conformational changes and allow TMDs to efflux the drugs or substrates out of the cells. But, by

then it is remains unclear if movement of substrates is driven by hydrolysis of ATP or binding of ATP [90].

Other well-known study is the ABCB1 switching, in which ABCB1 transporter protein switches from the inward- to the outward-facing orientation i.e., from drug recognition and binding at the NBDs to release of the drugs from the TMDs [83, 92]. The stabilization of ABCB1 conformation is reliant on ATP, and multiple experiments have demonstrated a link with the ATPase function of ABCB1 and the efflux of substrates, which results in drug resistance [93]. Studies shows that although the binding of ATP helps to establish ABCB1 outward-facing configuration, it is the efflux of drugs that leads to hydrolysis of ATP [81].

Despite having two active sites for ATPase activity, 'alternating catalytic site' model suggests only single ATP molecule is hydrolysed at a time by NBDs of ABCB1 and both NBDs alternate at each catalytic cycle [94]. The 'ATP switch model' shows that binding and hydrolysis of ATP, induces the formation and dissociation of an NBD dimer respectively [95] and that the drug binds to a high-affinity site in TMD in inward-facing configuration, increasing ATP affinity at the NBDs. This causes dimerization and a configurational change that transports the drug to a low-affinity site on the extracellular portion of the membrane, resulting in the outward-facing configuration. Molecule further regains its original conformation by the hydrolysis of the two ATP molecules [95]. Second model suggests that drug movement is fueled by the ATP hydrolysis. Latter model demonstrates the two unique roles for hydrolysis of ATP in one catalytic cycle of ABCB1, one involved in the substrate movement whereas other involved in configurational changes to regain the original conformation of the transporter for the next catalytic cycle [96].

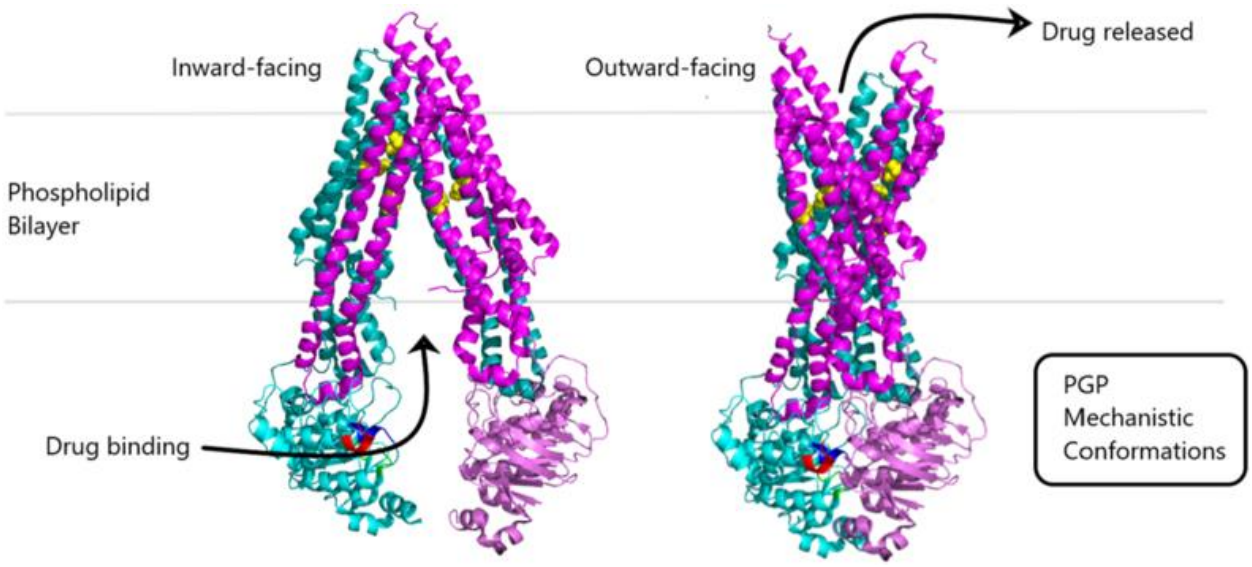


Figure 7. ABCB1 transporter in two configurations in the membrane [83]**.**

The inward-facing configuration (on the left) is responsible for drug binding, whereas the outward-facing configuration (on the right) promotes the efflux activity.

Role of ABCB1 in physiological functions

ABCB1 protein is located in various parts of the human body, such as adrenal, kidneys, colon, brain, heart, testis, digestive tract, lung, and placenta as shown in figure 8. It is also present in barrier of tissues including, blood-brain barrier, blood-testis barrier, and blood-placenta barrier.

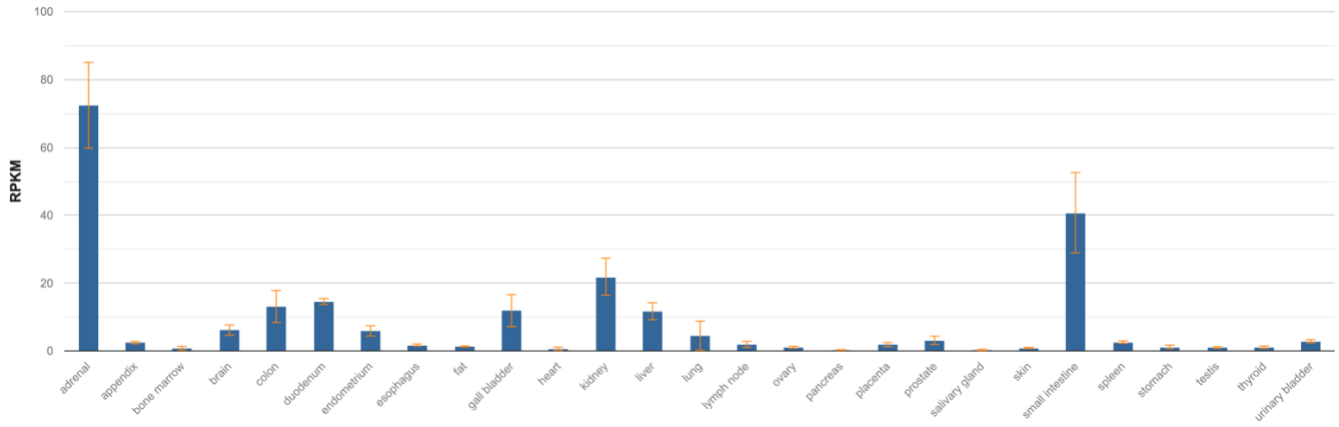


Figure 8. Expression of human ABCB1 in tissues according to reads per kilobase per million reads placed (RPKM) in RNA-seq experiment. [97].

In blood-brain barrier, it is present at the capillary endothelial cells apical surface and/or at astrocytic end-feet, blocking the entrance of foreign toxic compounds into the brain, thus it plays as one of the key components in blood-brain barrier [98]. ABCB1 additionally serves a vital function in regulating brain homeostasis by modulating the floe and/or efflux of substances into the brain, helping to maintain their level in the blood and other peripheral tissues [99]. Expression of ABCB1 is upregulated in response to stress and inflammation in the brain as a protective response to limit the movement of toxins during times of strain or inflammation [100]. It is also expressed apically in epithelium of choroid plexus and prevents the entry of toxic substrates from cerebrospinal fluid into the blood [101]. Study shows that increase in BMI are correlated with a significant reduction of ABCB1 in the prefrontal cortex and have been linked to higher rates of obesity [102, 103].

ABCB1 is present in Sertoli cells (supporting cells of seminiferous tubules) and involves in crucial function of the blood-testis barrier by pumping out any toxic substances from Sertoli cells into the interstitial compartment and prevents the passage of toxins, thus it maintains integrity of the blood-

testis barrier and ensures the developing spermatozoa are not exposed to harmful substances [104]. ABCB1 also located in the Leydig cells and involves the transport of testosterone from the Sertoli cells into the interstitial compartment in blood-testis barrier to ensure that the developing spermatozoa are exposed to the appropriate levels of testosterone that are necessary for their development [104].

In blood-placenta barrier, ABCB1 is exists in the syncytiotrophoblasts, the outermost layer of the placenta villi, where it pumps out xenobiotics from fetus into the maternal circulation [105, 106]. It also protects the fetus from exposure of drugs during the pregnancy [107, 108]. ABCB1 is one of the transporters that is involved in the transfer of hormones, nutrients through the mother to the developing fetus that helps in fetal development [106]. ABCB1 is also present in high levels in hematopoietic stem cells, and it shields those cells from harmful toxic substances [109].

ABCB1 is expressed on the proximal tubule epithelial cell apical surface in kidneys where it is involved in the efflux function [110]. For example, it helps in the removal of drugs into the urine which is an important function to prevent accumulation of drugs and renal toxicity [111]. In different animal studies it is observed that the levels of ABCB1 increased due to ischemia reperfusion injury [112] and endotoxemia [113]. In another study, during endotoxemia, upon exposure of proinflammatory cytokine tumour necrosis factor- α (TNF- α), an endotoxin lipopolysaccharide either individually or combination of both, there is an elevated levels of *de novo* ABCB1 synthesis in kidneys [114]. This study suggests that accumulation of toxic substances during injuries is prevented and protected by ABCB1.

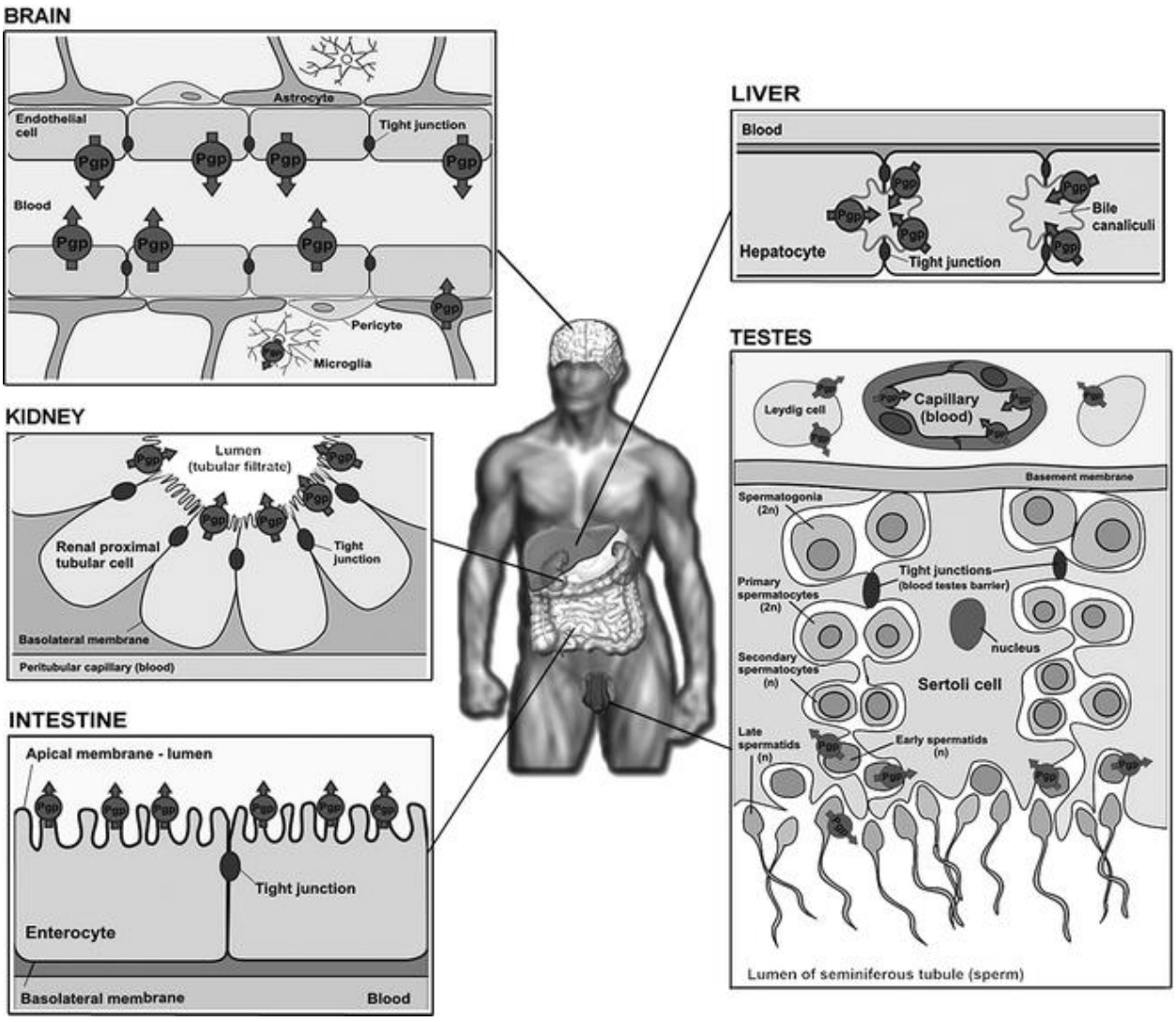


Figure 9. **Location of ABCB1 expression in different human organs/tissues and its function** [115]**.**

ABCB1 is expressed mainly on the biliary canalicular front of hepatocytes and on epithelial cells apical surface in small biliary ductules of liver. Its primary function is to transport drugs and xenobiotics out of the hepatocytes into the bile, which is then excreted from the brain. They also serve crucial function in the transfer of bilirubin and other endogenous substances [116].

ABCB1 is also located in small intestine, at top villi of enterocytes, where it's involved in absorption of drugs and excretion by pumping out substrates into the intestinal lumen [117]. It plays crucial role in the disposition of many xenobiotics and drugs that are absorbed in the small intestine. After being absorbed into the enterocytes, these substrates are actively transported by ABCB into the gut lumen, where they can be eliminated from the body via feces [118]. There is an increased expression of ABCB1 from the stomach to the colon along the entire gastrointestinal tract [119]. ABCB1 can influence the pharmacokinetics of many drugs that are metabolized in the liver by actively transporting these drugs back into the intestinal lumen [120]. It prevents entry of toxic substances into the gut mucosa [121]. In a study, it is also shown to limit the absorption of HIV protease inhibitors in the intestine [122].

ABCB1 involves in the migration of dendritic cells [123]. Few studies suggested that ABCB1 plays vital role in cholesterol trafficking [124] and homeostasis of lipids [125].

Role of ABCB1 in diseases

ABCB1 transporter protein has been implicated in several diseases. It contributes to multidrug resistance in many diseases, which limits the efficacy of chemotherapy drugs by increasing the risk of disease progression.

ABCB1 is expressed in elevate levels in different types of cancers that leads to multidrug resistance (MDR) by effluxing anticancer drugs from cancer cells, consequently reducing the effectiveness of chemotherapy. It is correlated with resistance to many drugs in different types of cancer including, leukemia, prostate, ovarian, lung, and breast cancers. Different *in vitro*, *in vivo* and clinical studies have shown ABCB1 overexpression in breast cancer cells is associated to resistance to chemotherapeutic drugs including doxorubicin, paclitaxel, vincristine, and

mitoxantrone [126, 127]. Immunohistochemical experimental studies concludes that elevated expression of ABCB1 indicates shorter survival rates, and increased risk of elapse in breast cancer [128]. Mutation of ABCB1 is linked to higher neurotoxicity of taxane-based chemotherapy in patients treated for lung cancer. Paclitaxel-based treatment in non-small cell lung cancer has shown shorter progression-free survival [129]. Studies in small cell lung cancer have shown that ABCB1 is involved in acquired resistance to the topoisomerase inhibitors, etoposide [130]. Elevated ABCB1 expression in ovarian cancer patients is linked with poor response to paclitaxel, and unfavourable clinical outcome [131]. However recent studies have shown that combination of poziotinib and lapatinib can overcome the paclitaxel resistance in ovarian cancer [132].

Recent studies reported that miRNAs modulate drug resistance in different types of cancer by regulating ABCB1 expression. miR-451 and miR-331-5p regulates ABCB1 expression by directly binding to its 3'-UTR in breast cancer and leukemic cells [133, 134]. miR-27a positively regulates ABCB1 and is associated with ovarian, cervical and oesophageal squamous cell carcinoma [135, 136].

Overexpression of ABCB1 in cancer patients has led to poor diseases prognosis. Identifying ABCB1 gene expression during chemotherapy enhances the efficacy of drug treatment by guiding the selection of the most effective drugs, resulting in better disease prognosis [137]. Several small molecule inhibitors (verapamil, quinine, and cyclosporine) were used to reverse MDR but inhibition of ABCB1 function proved to be toxic to cancer cells but has not proven to be an effective approach in patients due to inhibition of its function in normal cells leading to high normal tissue toxicity [138].

Expression of ABCB1 is considered as a major factor for prolonged inflammation and altered steroid efficacy in autoimmune diseases, such as, hepatic cirrhosis, rheumatoid arthritis, IBD, and thrombocytopenic pupura, causing resistance to different drugs [139, 140]. ABCB1 is involved in modulating the immune response by regulating the movement of various immunomodulatory agents across cell membranes. ABCB1 is upregulated in the multiple sclerosis patients, which contributes to the progression of resistance to drugs by regulating the transport of immunomodulatory agents, including glucocorticoids [141]. Studies suggest that overexpression of ABCB1 reduces the buildup of protease inhibitors and antiviral drugs during the replication site of virus and accelerate the disease in the HIV patients [142, 143].

ABCB1 transporter protein inolves in modulating and safeguarding the intra-cerebral concentrations caused by the neuro-toxicants and thus expression and functional activity in CNS is linked to disease progression and pharmacological treatment of neurological diseases [144]. ABCB1 plays a role in the clearance of beta-amyloid from the brain into the blood circulation, and its dysfunction has been implicated in the buildup of β- amyloid and the progression of the Alzheimer's disease. Studies show that expression and activity of ABCB1 is reduced in the brains of Alzheimer's patients, which contribute to the β- amyloid accumulation and neuroinflammation [145]. Dysregulation and antipsychotic tolerability of ABCB1 contributes to the altered neurotransmitter levels and leads to the development of schizophrenia, a complex psychiatric disorder characterized by altered cognition, perception, and affect [146].

ABCB9

ABCB9 transporter protein is a member of MDR/TAP subfamily and referred as TAPL. It is located on chromosome 12q24.31 containing 12 exons and has a predicted molecular weight of 82 kDa [147]. ABCB9 transporter is highly homologous to transporter proteins linked with antigen

presenting/processing proteins, TAP1 and TAP2 which suggests that they could be derived from a common ancestral gene [148]. It is a highly conserved gene with around 95% sequence identity between humans and rodents (rat/mouse) [149].

ABCB9 is an ATP dependent half transporter and it works as a homodimer [150, 151]. Half transporter ABCB9 is divided into N-terminal TMD0 (grey box), coreTAPL (light and dark blue) composed of six transmembrane helices and a C-terminal cytoplasmic NBD as shown in figure 10 [152].

Initial studies predicted that ABCB9 transporter protein is localized to mitochondrial membranes [153] or plasma membranes [154], later immunofluorescence double staining and subcellular fractionation studies has shown that human ABCB9 transporter protein is localized to lysosomes [155].

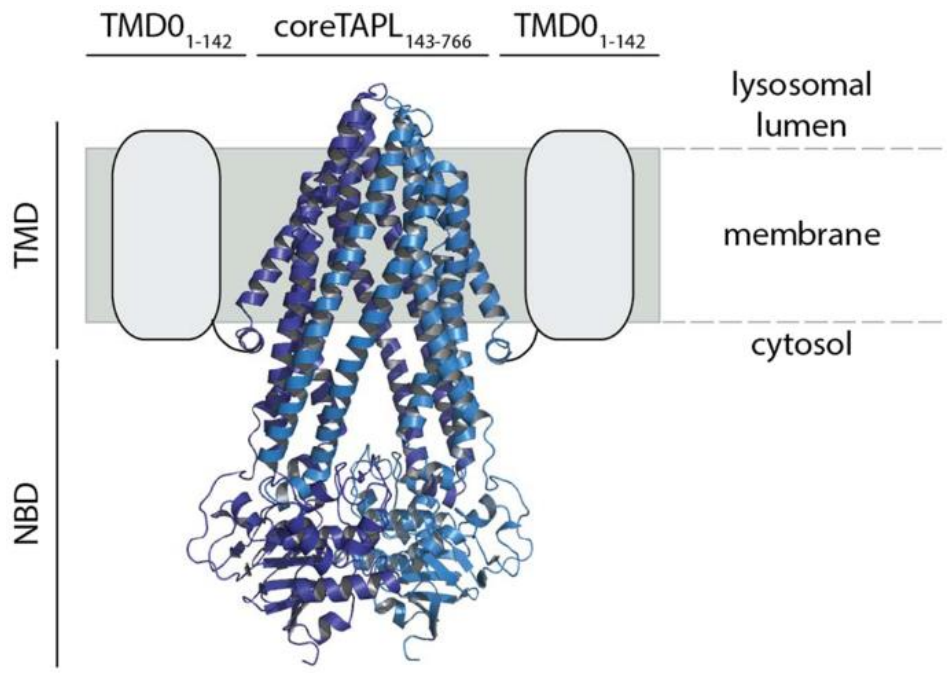


Figure 10. Structure of ABCB9 complex shown schematically with its N-terminal TMD0, core TAPL and C-terminal NBD domains localized to the lysosomal membrane [152].

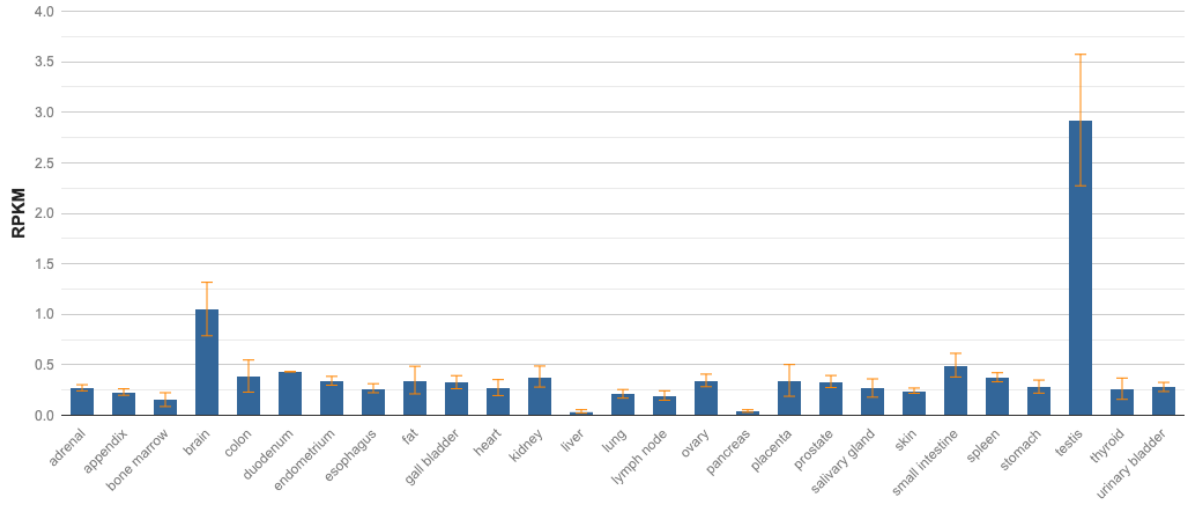


Figure 11. Expression of human ABCB9 in tissues according to reads per kilobase per million reads placed (RPKM) in RNA-seq experiment. [156]**.**

Role of ABCB9 in physiological functions and diseases

ABCB9 transporter protein is expressed in tissues of spleen, kidney, heart, spinal cord and immune cells [148, 156]. It is abundantly present in Sertoli cells of testis, endothelial cells of BBB [157] and in APCs including macrophages and dendritic cells [158]. ABCB9 is not present in monocytes but its expression is elevated during the monocyte differentiation into macrophages and dendritic cells [158].

ABCB9-mediated peptide transport was first demonstrated in the crude membranes of insect cells [151]. It pumps range of peptides from the cytoplasm to the lumen of lysosome for degradation [151]. ABCB9 protein exhibits a wide range of peptide length specificity, from 6-mer to 59-mer peptides with an optimum range of 23-mers [151]. ABCB9 is likely to favour the transport of positively charged, hydrophobic or aromatic residues at the both C- and N-terminal whereas it disfavours negatively charged residues as well as methionine and asparagine [159].

Based on its widespread tissue distribution and presence of several GC boxes in its promoter region for binding of the ubiquitous transcription factor Sp1 [147, 160], it is likely to work as a housekeeping factor that prevents the storage of potential toxic or stressful peptides in the cytoplasm [161].

Like other MDR ABC transporters, ABCB9 is also known to show chemoresistance and independent prognostic factor in different cancer patients [162]. Some studies have shown that it might be a target for various microRNAs that involve in chemoresistance in different malignancies. *In-vivo* experiments have shown that ABCB9 was upregulated by miR-24 in paclitaxel-resistant breast cancer cells and it could be a target for reducing drug resistance [163]. Similar to miR-24, miR-31 shows an anti-apoptotic effect in cisplatin resistance through the

inhibition of ABCB9 in non-small cell lung cancer [164]. ABCB9 also linked with the modulation of oxaliplatin-based chemosensitivity by miR-31-5p, that inhibits nuclear localization of PARP1 in hepatocellular carcinoma [165]. Low expression of ABCB9 have shown poor prognosis in ovarian cancer and suggests it might be a helpful biomarker [162].

ABCB10

ABCB10 transporter protein is also referred to as mitochondrial erythroid ABC protein located in the inner membrane of the human mitochondria, where NBDs are found within the matrix of mitochondria [166, 167]. It was identified when investigating, the genes up-regulated during erythroid differentiation by the GATA-1, transcription factor where ABCB10 was hypothesised to facilitate crucial mitochondrial transport functions associated with the heme biosynthesis [168]. It is a homodimeric half transporter with short N-terminal α-helix and six long Transmembrane helices (TMH) which is connected to the C-terminal by an extended linker [166].

ABCB10 is expressed in high levels in erythroid tissues of adults as well as embryo [168]. It is also found to be present at the primitive sites of hematopoiesis like yolk sac blood islands [168]. It is expressed highly in bone marrow, at intermediate levels in heart, pancreas, liver, brain ovary, small intestine, and testis, but expressed at low levels in spleen, lung, colon and kidney [169].

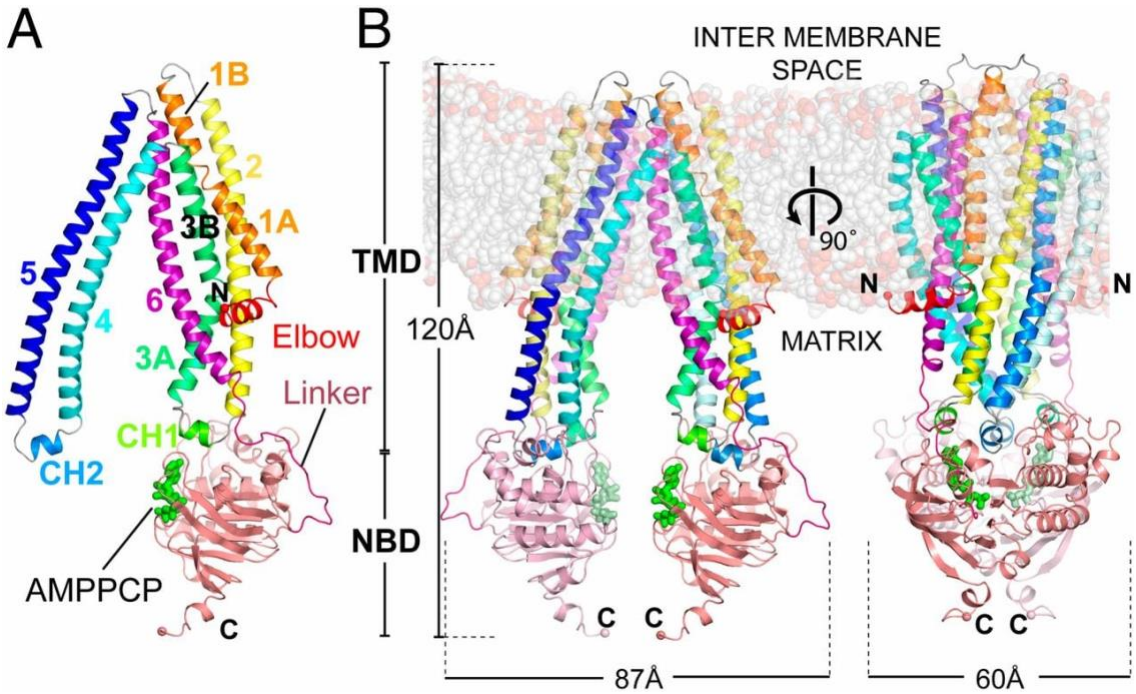


Figure 12. Structure of ABCB10 complex with the nucleotide analog AMPCP [166]**.**

The monomer (A) and homodimer (B) of the ABCB10/AMPPCP complex are depicted in figure, based on the rod-form B crystal structure. The asymmetric unit contains only one monomer, with the dimer being created by a crystallographic two-fold [166].

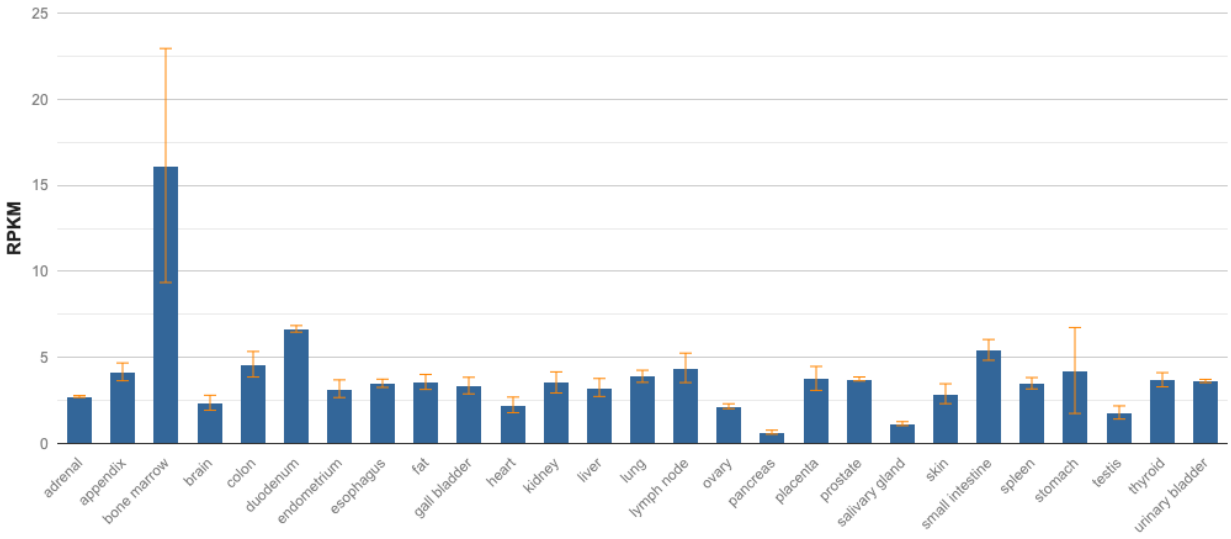


Figure 13. Expression of human ABCB9 in tissues according to reads per kilobase per million reads placed (RPKM) in RNA-seq experiment. [169].

Role of ABCB10 in physiological functions and diseases

ABCB10 transporter protein is known to export different physiological substrates from the matrix of mitochondria to the cytoplasm in an ATP-dependent manner [170]. Experimental studies have shown that downregulation of ABCB10 have been linked to decrease in mitoferrin1 protein levels and influx of iron into mitochondria, resulting in a decrease in biosynthesis of heme [171]. The study also suggested that the hydrolysis of ATP is an essential factor for hemoglobinization, and the transported substrate by ABCB10 serves as a signal to enhance the process of hemoglobinization [171]. ABCB10 plays and important role in the biosynthesis of heme process when the incorporation of iron into protoporphyrin IX (PPIX) [170]. Later studies found that ABCB10 may be involved in transport of heme analog Zn (II) mesoporphyrin (ZnMP) but can't transport the heme precursor 5-aminolevulinic acid from mitochondria [170].

ABCB10 has crucial function in erythropoiesis and in relieving oxidative stress, making it a promising target to explain a common side effect of clinical inhibitors and drugs that inhibit RBC development, leading to anemia or reduced cardiac function recovery after ischemia/reperfusion [172]. Mutations or inactivation of ABCB10 may leads to congenital and/or drug-induced anemias and defects in other human tissues, including liver, heart, kidney and brain where the tissues are sensitive to mitochondrial oxidative stress associated with heme metabolism [172].

Circular RNA originating from exons of ABCB10 known as Circ-ABCB10, expressed in various tissues in different developmental stages, has been shown to stimulate breast cancer cell proliferation and migration by sponging miR-1271 [173–175]. Different studies have shown that circ-ABCB10 is linked to various cancer characterstics [176] where it could stimulate tumor growth [175, 177] and insulin resistance [178, 179]. Circ-ABCB10 has the potential to serve in the future as a diagnostic and prognostic biomarker, in addition to a therapeutic target.

ABCD4

ABCD4 also known as peroxisomal membrane protein or lysosomal cobalamin transporter ABCD4, is a member of adrenoleukodystrophy (ALD) subfamily [180]. It's a 69 kDa transmembrane protein present on the membranes of endoplasmic reticulum and lysosomes [181]. Early studies suggested that ABCD4 was localized to peroxisomes [182] but later studies found it resides on endoplasmic reticulum, not on peroxisomes [181].

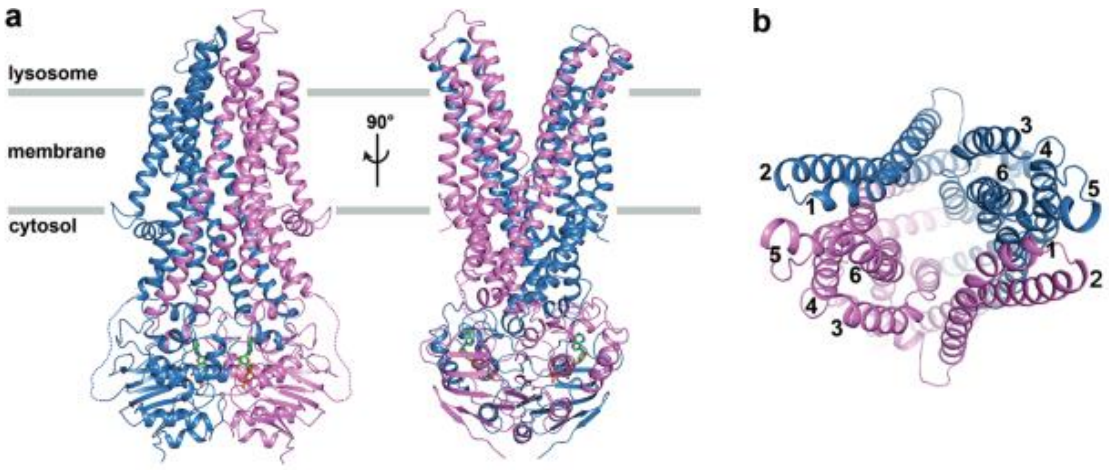


Figure 14. Cryo-EM structure of ABCD4 transporter protein [183]. Panel

a) Shows ABCD4 structure in a lysosome-open state in two perpendicular views. Panel

b) Shows the same ABCD4 structure, but in top view from inside of lysosome.

ABCD4 is a half-transporter which forms either homodimeric or heterodimeric with other transporter proteins to be a functional transporter. It has TMD with six transmembrane helices and one NBD [183]. Unlike other type 1 ABC exporters located on the cell membrane which allows both ATP and substrate from the cytosolic side (cis-acting exporters) [184], ABCD4 adopts a unique trans-acting transport mechanism [183]. It is highly expressed in the tissues of kidney, placenta, small intestine, spleen, duodenum, lung and in moderate levels in pancreas and salivary gland [185].

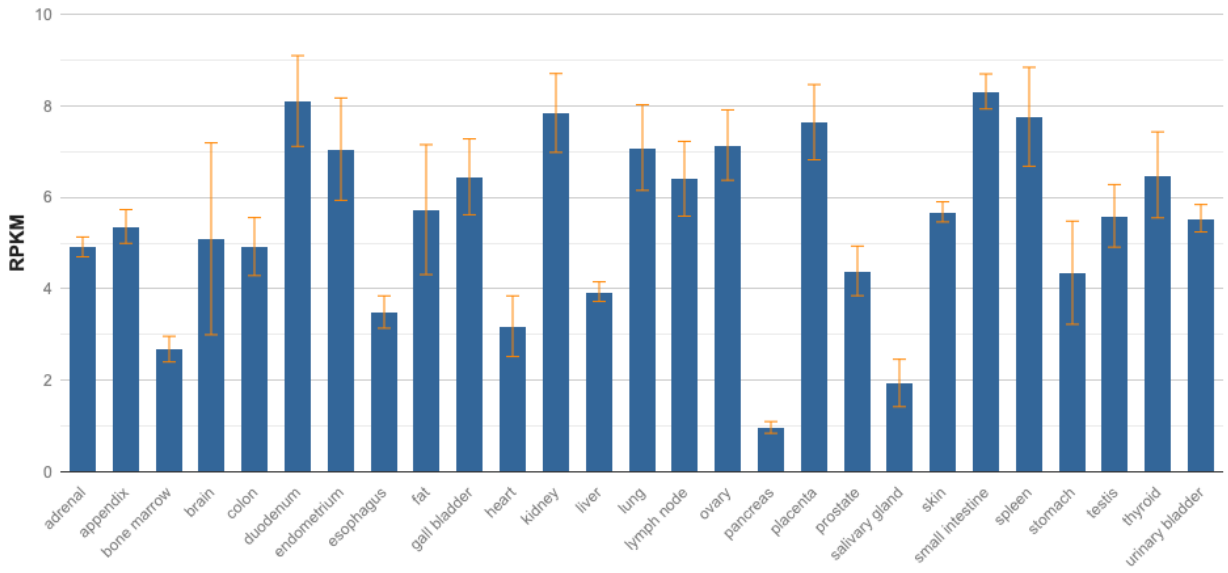


Figure 15. Expression of human ABCB9 in tissues according to reads per kilobase per million reads placed (RPKM) in RNA-seq experiment. [185].

Role of ABCB10 in physiological functions and diseases

ABCD4 forms a complex with LMBD1 protein for functioning and/or targeting. ABCD4 transporter protein translocated to lysosomes from endoplasmic reticulum in association with LMBD1via an unknown intracellular route [181, 186]. CD320 allows cobalamin to infiltrate cells bound to transcobalamin. As depicted in Figure 16, the latter complex is endocytosed, and the cobalamin/transcobalamin heterodimer dissociates from CD320 in early endosomes. Cobalamin is transported into the cytoplasm via reverse transport by the ABCD4/LMBD1 complex. The methylated and adenosylated forms of cobalamin are indispensable cofactors for methionine synthase and methylmalonyl-CoA mutase. [181, 186, 187].

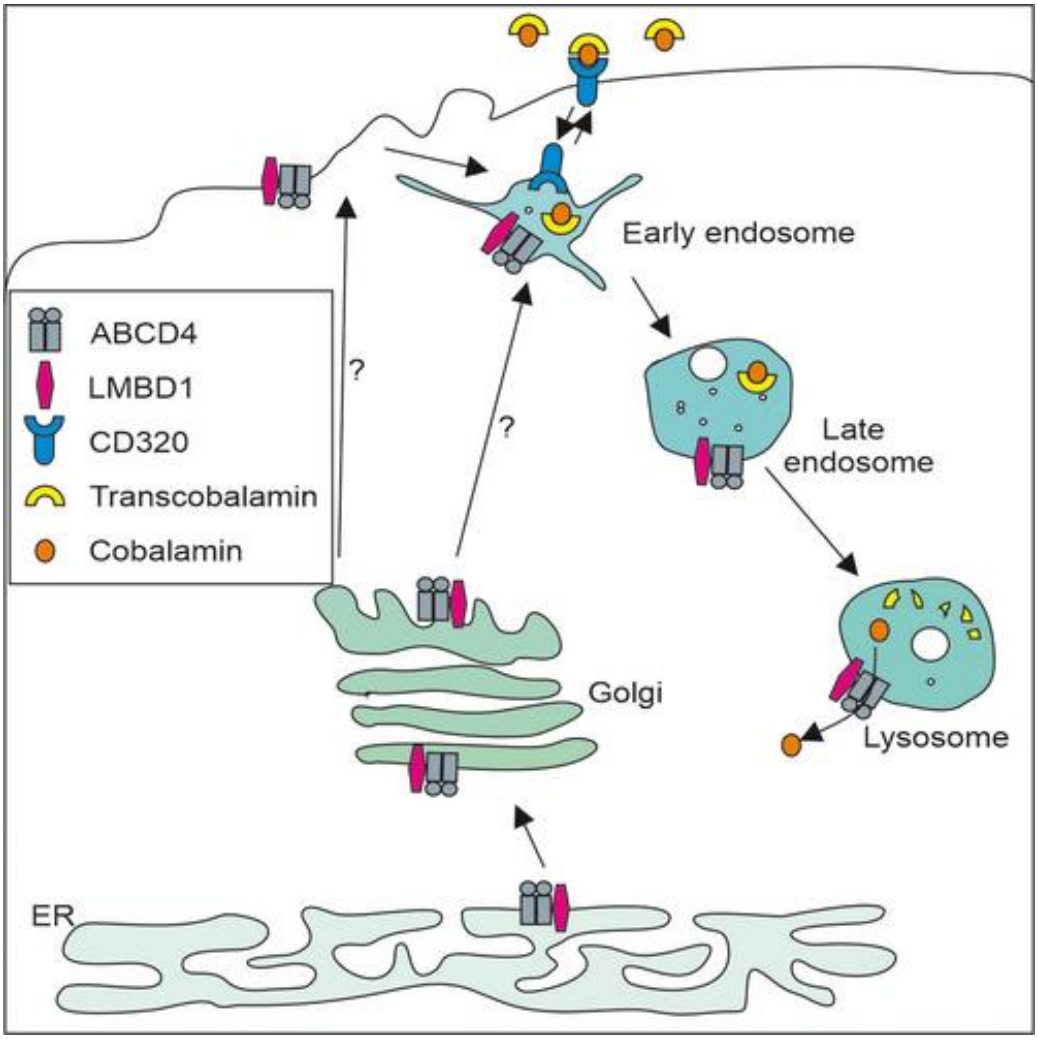


Figure 16. Simplified schematic representation of intracellular cobalamin transport and role of the ABCD4 transporter protein [161].

A study reported that mutation in ABCD4 is responsible for a novel inherited disorder affecting metabolism of vitamin B12 (cobalamin) [188]. ABCD4 malfunction prevents the release of cobalamin from lysosomes, mimicking the deficiency of cobalamin observed in the individuals with defects in the lysosomal membrane protein LMBD1 [189]. There are around nine known genetic disorders in the subcellular processing of cobalamin, named cblA to cblG, cblJ, and mut [188, 190]. These disorders cause the buildup of homocysteine and/or methylmalonic acid, leading to methylmalonic aciduria and/or isolated homocystinuria [191].

ABCF1

ABCF1, also known as MRP40, is a member of the GCN20 subfamily [192]. It was the first identified ABC transporter protein that lacks transmembrane domain but has two NBDs [193]. ABCF1 was identified in synoviocytes stimulated with TNFα using differential display PCR [193]. The expression of ABCF1 mRNA was found in both normal patients and patients with rheumatoid arthritis (RA), and it was upregulated upon treatment with TNFα [193]. Additionally, ABCF1 protein expression was increased in activated T cells stimulated with phorbol myristate acetate and ionomycin [194].

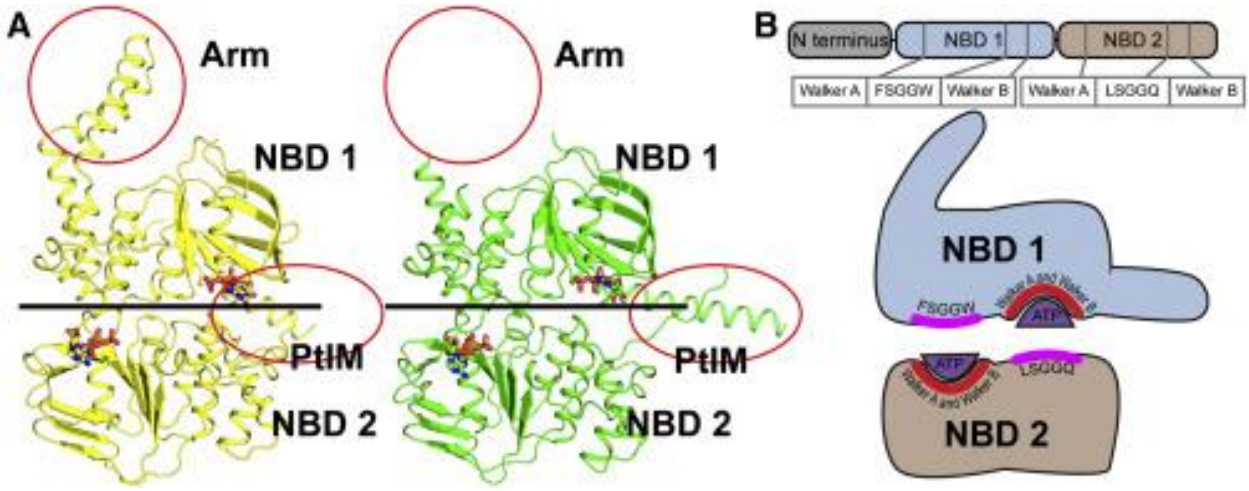


Figure 17. ABCD4 role in intracellular cobalamin transport [195]**.**

(A) The complex structure of the ABCF1-ATP is presented, where both molecules are observed in the lopsided unit. (B) The domain organization of ABCF1 is illustrated in a schematic representation, with NBD1 in blue and NBD2 in brown. The positions of conserved motifs are indicated. The ABCF1 dimer is shown below, with domains colored as in the top panel.

ABCF1 is ubiquitous and expressed high levels in testis, placenta, bone marrow, and lymph node and low levels in pancreas and salivary gland [192].

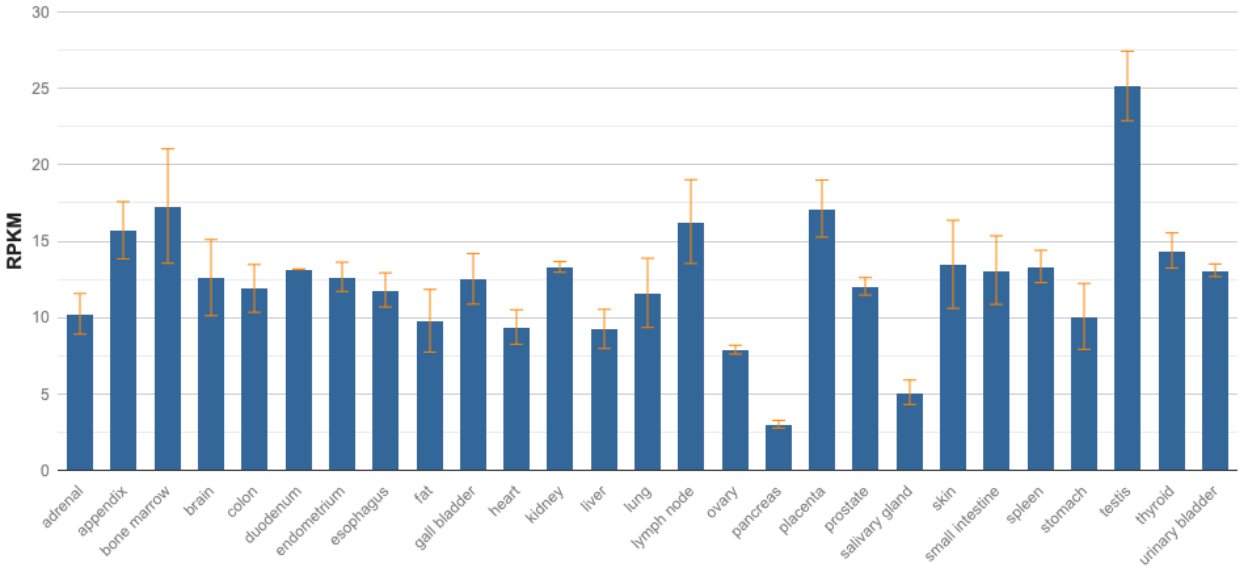


Figure 18. Expression of human ABCF1 in tissues according to reads per kilobase per million reads placed (RPKM) in RNA-seq experiment. [192].

Mechanism of function of ribosome in ABCF1 recycling can be summarized in four steps as shown in figure 19 [195]. Firstly, ATP attaches to the Walker A and Walker B motifs of ABCF1, causing configurational change bringing NBD1 and NBD2 closer together. Secondly, the ABCF1-ATP goes into the ribosome, and components of ribosomes cause the adenine to be squeezed, bringing the C motif nearer to the ATP. Thirdly, ATP is hydrolyzed by ABCF1. Finally, ADP is released, and it returns to its unliganded-protein configuration [195].

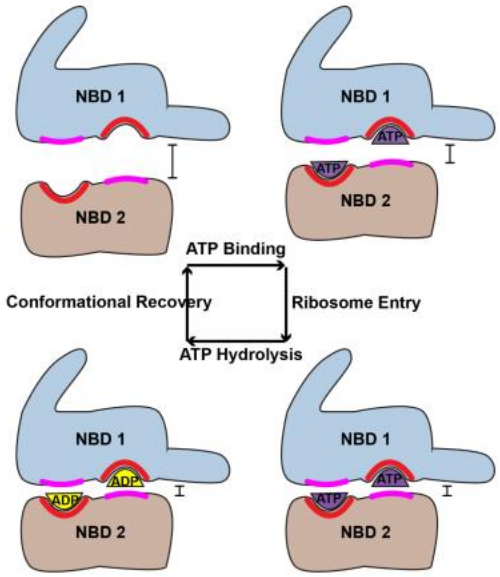


Figure 19. Recycling procedure of ABCF1 in ribosomal function shown in four steps [195]**.** The cycle of events begins with step 1) ATP-binding; 2) ribosome entry; 3) ATP hydrolysis; and 4) conformational recovery.

Role of ABCF1 in physiological functions and diseases

ABCF1 transporter inolves in regulation of immune response, protein synthesis and RNA processing [193, 194, 196]. ABCF1 expression is required for embryonic development, while in adults, it is closely linked with cells actively proliferating and differentiating [197]. ABCF1 gene is located in the class 1 region of the major histocompatibility complex (MHC) locus which is involved in the presentation of antigen to T-cells and serves vital function in the regulation of immune response and inflammation process [198]. Thus, ABCF1 has been linked or associated with different diseases including diabetes, psoriasis, RA and autoimmune pancreatitis [198, 199].

ABCF1 controls the transition from the inflammatory phase to the endotoxin tolerance phase in sepsis and can affect cytokine storm and IFN-β production, which are mediated by SIRT1 [200].

As a result, ABCF1 helps control sepsis-induced mortality by preventing hypotension-induced renal circulatory dysfunction [200].

Expression levels of ABCCF1 were increased in hepatocellular carcinoma upon acquiring chemoresistance, suggesting that it can function as a hepatic onco-fetal protein which may be a new target for treatment in hepatocellular cancer [201].

It is considered as potential cytoplasmic nucleic acid sensor and Toll-like receptors (TLRs) signalling modulator expressed in epithelial cells of humans [202]. It is mainly expressed in photoreceptor outer segments (POSs) and co-localizes with the POS marker rhodopsin, making it convenient to regulate the phagocytosis of retinal pigment epithelial (RPE) cells [203]. These findings indicates that the protein is ejected from POSs and attaches in an autocrine manner, playing a role in facilitating RPE phagocytosis via conserved pathway [203].

Protein-Protein Interactions (PPIs)

Proteins facilitate most biological and cellular functions, such as cell growth, proliferation, gene expression, morphology, metabolic and developmental control, nutrient uptake, intercellular communication, cell-cell interactions, and apoptosis [204]. Proteins rarely acts as an isolated species while carrying out their functions in an organism[205]. Typically, they carry out their physiological activities by directly interacting with other molecules or proteins including lipids, metabolites and nucleic acids [204]. It is suggested that more than 80% of proteins operate in groups and form interrelated systems to exhibit crucial role in larger mechanistic pathway [206, 207]. The extensive analysis of large number of proteins demonstrates that proteins communicate with one another, especially those participate in the similar biological/physiological processes [208].

Non-covalent contacts between amino acid residue sidechains are the foundation for protein assembly, folding, and these bonds cause myriad of associations and interactions among proteins [209]. Protein-protein interactions (PPIs) are crucial for performing various functions in the cell, including sensing the environment, regulating metabolic and signaling enzymes, transmitting signals, organizing the cellular structure and producing physical motion [204]. PPIs also play a vital function in trafficking and transport, cell-to-cell interactions, functioning of a protein, metabolic regulation, and control of developmental processes.

Based on their various structural and functional properties, PPIs can be categorised in different ways [213]a) they can be homo- or hetero-oligomeric (i.e., identical, or non-identical chains), b) they may be obligate or non-obligate, or c) they may be transient or permanent interactions [210].

Many innovative techniques and methods are being used in different studies around the world for identification and characterization of protein-protein interactions. Some of most common methods/techniques used for detecting protein-protein interactions are; *In vitro* techniques, includes coimmunoprecipitation, affinity chromatography, tandem affinity purification, protein arrays, NMR spectroscopy and X-ray crystallography; *In vivo* methods such as, yeast two-hybrid (Y2H), synthetic lethality and BioID technique; *In silico* techniques like sequence-based and structure-based approaches, gene fusion, gene-expression based approaches, and chromosome proximity [211]. Most of the *in vitro* protein techniques helps to detect the strong protein interaction and fails to detect some of the weak and real-time interactions. In co-immunoprecipitation technique, protein interactions occur in non-denaturing conditions, but some interactions may not be detected if protein interaction or antibody is weak. In Y2H technique many true interactions are may not be traced, which leads to false negative results and other drawback is

its inability to detect indirect protein-protein interactions [212]. Most of these techniques cannot detect the protein interactions which contain post-translational modifications.

Promiscuous biotin ligase (BioID) is one of the novel approaches to detect PPIs. Biotin ligase, known as BirA is fused to the protein of interest and expressed in mammalian cells, to biotinylate to near-by proteins as shown in figure 20 [213]. Biotinylated proteins which have specific binding proteins along with other proteins within the vicinity are purified using streptavidin beads and identified by mass spectrometry method [214]. This technique has ability to identify and detect transient or weak interactions over a period of time with high sensitivity [215]. It also helps to detect vicinal proteins under relatively natural physiological cellular conditions.

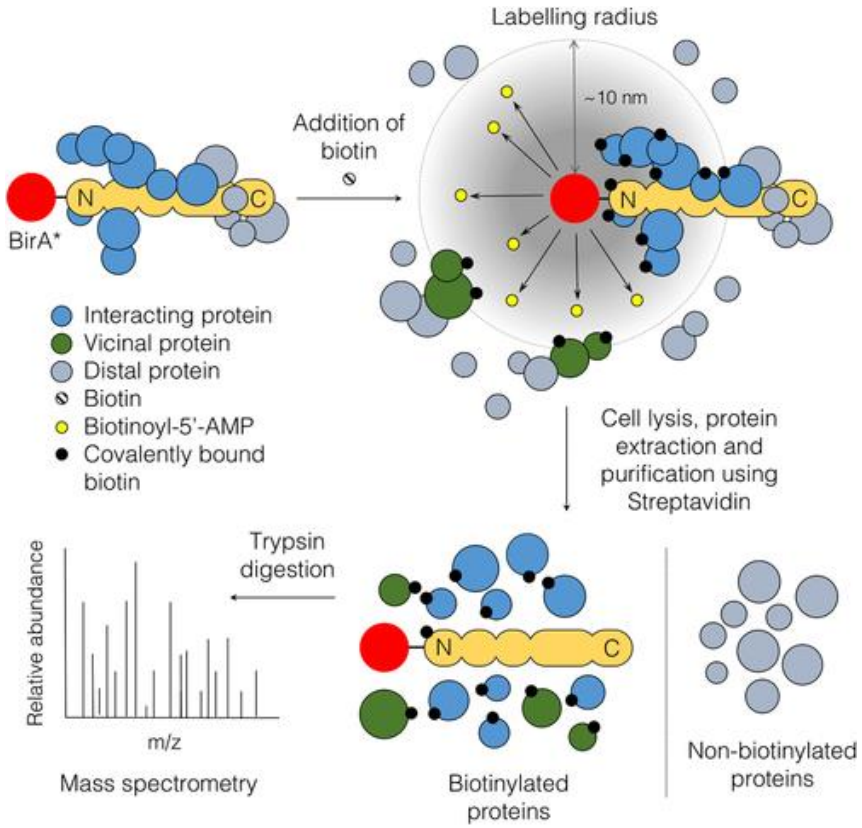


Figure 20. Proximity-dependent labeling with BioID [216]**.**

Potential Interacting Proteins with ABCB1, ABCB9, ABCB10, ABCD4, and ABCF1

The below interaction shown from string database includes known interactions i.e., from curated databases (light blue), experimentally determined (pink), and predicted interactions which are gene neighbourhood (green), gene fusions (red), gene co-occurrence (blue). Some of the other interactions include text mining (light green), co-expression (black), and protein homology (light slate blue) as shown in fig 21.

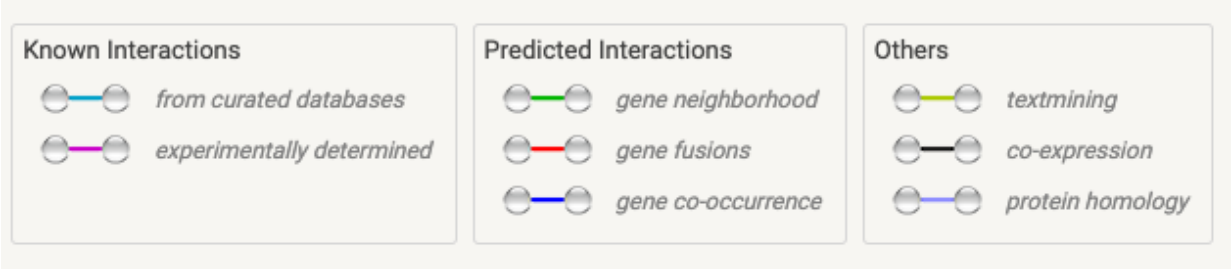


Figure 20. The above representations of protein-protein interactions obtained string database show the different sources used to predict protein interactions.

ABCB1

Many experiments have been conducted to comprehend and screen for the interacting proteins of ABCB1 to study it drug resistance and for other functions associated with ABCB1, over the years. Some of these studies have shown that ABCB1 interacts with cytochrome P450 enzymes CYP3A4 and CYP3A5, which are involved in drug metabolism. The functional consequences of the latter interactions, include altered drug disposition and increased drug resistance in cancer cells [217, 218]. ABCB1 interacts with tumour suppressor protein (TP53), known to play a role in cell cycle regulation and DNA repair in cancer biology [219]. As shown in figure 22, it is also predicted that ABCB1 may interact with TAF1, MRPS7 and RPA1 in different cellular functions but mechanism of these interactions are not fully understood [220].

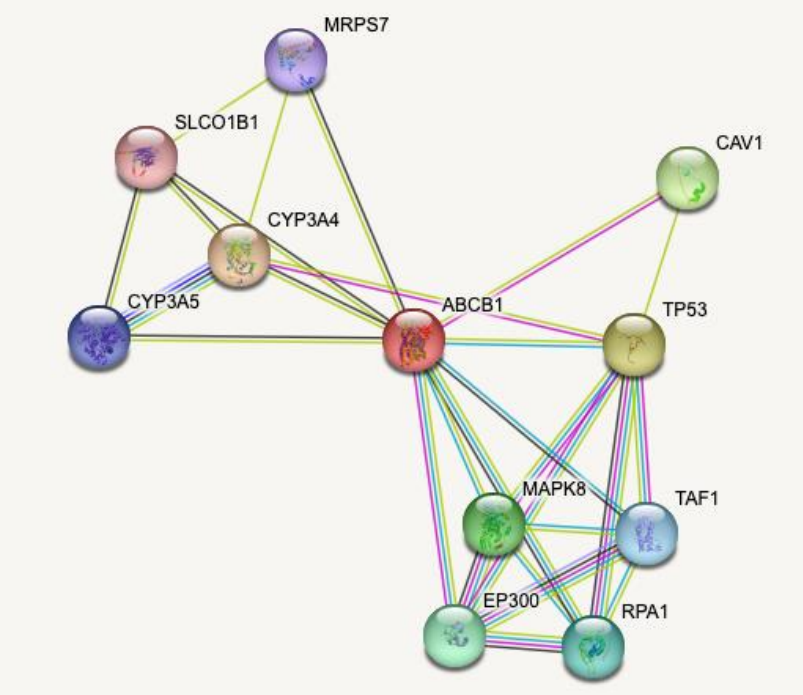


Figure 22. Protein interactions of ABCB1 identified till date (Retrieved from string-db.org)

ABCB9

ABCB9 protein interactions are one of most studied. ABCB9 interacts directly with ABCD4 and is involved in the transport of cobalamin from the lysosome to the mitochondria [161]. ABCB9 was shown to interact with both LAMP1 and LAMP2 through its transmembrane domains which are required for the efficient lysosome biogenesis and autophagy [155]. It is also suggested to interact with ABCF3 and ABCE1, however their interactions are not well characterised [221].

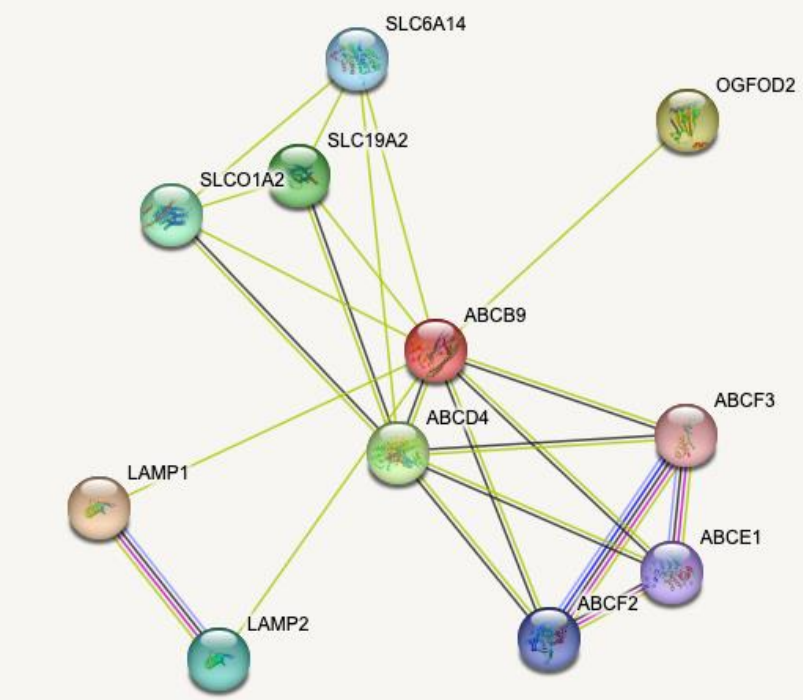


Figure 23. Protein interactions of ABCB9 identified till date (Retrieved from string-db.org)

ABCB10

ABCB10 has been shown to interact with several other proteins involved in heme metabolism and mitochondrial function. It interacts with ABCB7 and ABCD5 to regulate and transport Fe2+ and heme. It forms an oligomeric complex with ABCB7 to facilitate the transport of Fe2+ from cytosol into the mitochondria for incorporation into heme. This interaction is necessary for erythroid heme biosynthesis and is crucial for proper mitochondria function [222, 223]. ABCB10 also interacts with SLC25A38, SLCA25A28, and SLC25A37 where they play a role inn transport of heme intermediates [224].

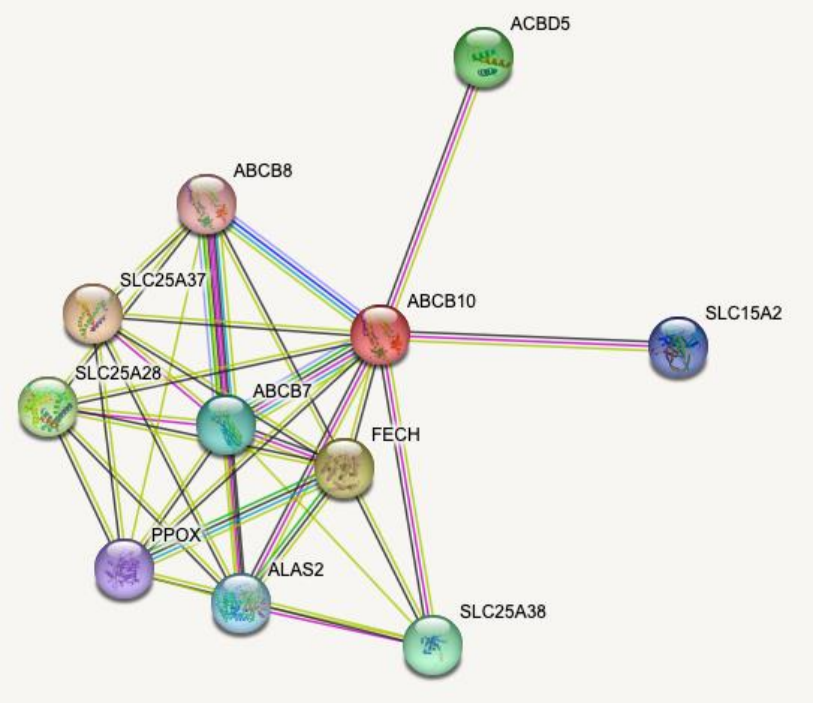


Figure 24. Protein interactions of ABCB10 identified till date (Retrieved from string-db.org)

ABCD4

ABCD4 is found to interact with other ABC transporter including ABCA2, ABCB6, ABCB8 and ABCG8 [225]. ABCA2, ABCB6, and ABCD4 are lysosomal proteins and are believed that they interact with each other to carryout different functions [226]. It is also believed to interact with ABCG5 but there's no clear experimental evidence. ABCD4 interacts with LMBRD1and is involved in the transport and metabolism of vitamin B12 (cobalamin) [187].

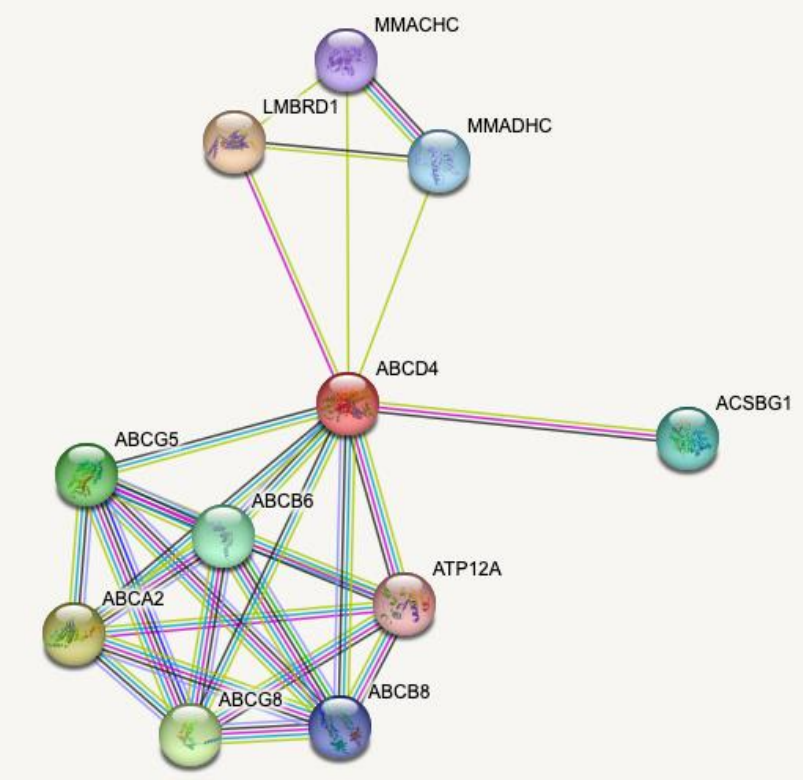


Figure 25. Protein interactions of ABCD4 identified till date (Retrieved from string-db.org)

ABCF1

ABCF1 has been to shown to interact with various proteins involved in RNA processing, translation, and stress granulation [197, 227]. ABCF1 is assumed to interact with ribosomal protein RPL8, RPL11, RPL19, RPL38 which play an important role in ribosome function and biogenesis [228].

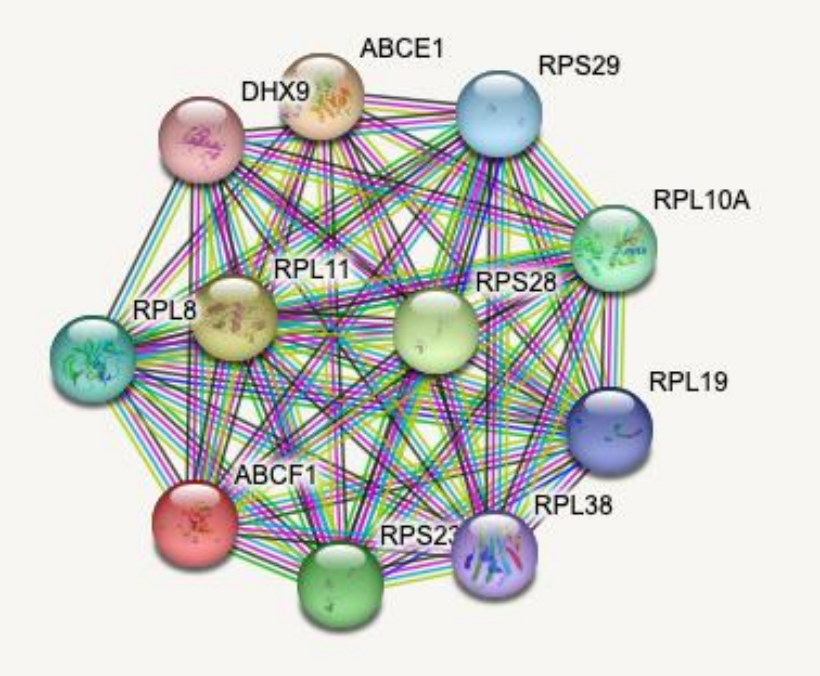


Figure 26. Protein interactions of ABCF1 identified till date (Retrieved from string-db.org)

METHODOLOGY

PCR Amplification of gene of interest (GOI)

ABC transporter proteins cDNA clones were commercially obtained from Sino Biological, expressed in one of the following vectors, pGEM, pUC19, or pCMV3. The gene of interest was amplified by PCR containing the appropriate restriction sites (NheI - GCTAGC and AflII - CTTAAG) at each end compatible with such sites in pcDNA 3.1 MCS-BirA(R118G) – HA vector as shown in fig 27. Primers were designed and ordered from IDT. Phusion Plus Green Master Mix (Thermofisher; F632S) was used to amplify PCR and following set of primers were used.

ABCB1

Forward Primer: AGCACG GCTAGC GCCGCCACC ATGGATCTTGAAGGGGACCG

Reverse Primer: AGCACG CTTAAG ATCTGGCGCTTTGTTCCAG

ABCB9

Forward Primer: AGCACG GCTAGC GCCGCCACC ATGCGGCTGTGGAAGGC

Reverse Primer: AGCACG CTTAAG ATGATGCAGAAAGGGCGGAGAAG

ABCB10

Forward Primer: AGCACG GCTAGC GCCGCCACC ATGCGAGGCCCCCCTG

Reverse Primer: AGCACG CTTAAG ATTGCTGAAATAAAACTTTGTTTGTTCATTAGTTTTCTG

ABCD4

Forward Primer: AGCACG GCTAGC GCCGCCACC ATGGCGGTCGCGGG

Reverse Primer: AGCACG CTTAAG TTCCACTTTGATTCTCATCAGCTCCCATC

ABCF1

Forward Primer: AGCACG GCTAGC GCCGCCACC ATGCCGAAGGCGCCCAAG

Reverse Primer: AGCACG CTTAAG ATCTCTCGGGGCCGGCTGAC

The following PCR conditions were used for amplification of gene of interests.

ABCB1

Cycle step	Temperature	Time	No. of Cycles
Initial Denaturation	98°C	1:15 mins	1
Denaturation	98°C	15 s	35 cycles
Annealing & Extension	72°C	15 s	
		2:00 mins	
Final Extension	72°C	5 mins	1
Hold	4°C	Hold	Hold

Table 2. PCR Conditions for the amplification of ABCB1 gene

ABCB9, ABCB10, ABCD4 and ABCF1

Cycle step	Temperature	Time	No. of Cycles
Initial Denaturation	98°C	1:15 mins	1
Denaturation	98°C	15 s	35 cycles
Annealing & Extension	72°C	15 s	
		1:00 min	
Final Extension	72°C	5 min	1

Hold	4°C	Hold	Hold

Table 3. PCR Conditions for the amplification of ABCB9, ABCB10, ABCD4, and ABCF1 genes

For positive controls, gene of interests were amplified using different set of primers, in such a way that only gene of interest is expressed without the pcDNA 3.1 MCS-BirA expression. The following set of primers were used for the PCR amplification.

ABCB1

Forward Primer: AGCAC GCTAGC ATGGATCTTGAAGGGGACCG

Reverse Primer: AGCACG CTTAAG TCATCTGGCGCTTTGTTCCAG

ABCB9

Forward Primer: TAAGCA GCTAGC ACC ATGCGGCTGTGGAAGGC

Reverse Primer: TAAGCA CTTAAG GATGCAGAAAGGGCGGAGAAG

ABCB10

Forward Primer: TAAGCA GCTAGC ACC ATGCGAGGCCCCCCTG

Reverse Primer: TAAGCA CTTAAG TGCTGAAATAAAACTTTGTTTGTTCATTAGTTTTCTG

ABCD4

Forward Primer: AGCACG GCTAGC GCCGCCACC ATGGCGGTCGCGGG

Reverse Primer: AGCACG CTTAAG ATTTCCACTTTGATTCTCATCAGCTCCCATC

The following PCR conditions were used for amplification of positive control gene of interests.

ABCB1

Cycle step	Temperature	Time	No. of Cycles
Initial Denaturation	98°C	1:15 mins	1
Denaturation	98°C	15 s	35 cycles
Annealing & Extension	72°C	15 s	
		2:00 mins	
Final Extension	72°C	5 mins	1
Hold	4°C	Hold	Hold

Table 4. PCR Conditions for the amplification of positive control for ABCB1 gene

ABCB9, ABCB10, ABCD4 and ABCF1

Cycle step	Temperature	Time	No. of Cycles
Initial Denaturation	98°C	1:15 mins	1
Denaturation	98°C	15 s	35 cycles
Annealing & Extension	72°C	15 s	
		1:00 min	
Final Extension	72°C	5 min	1
Hold	4°C	Hold	Hold

Table 5. PCR Conditions for the amplification of positive controls for ABCB9, ABCB10, ABCD4 and ABCF1 genes

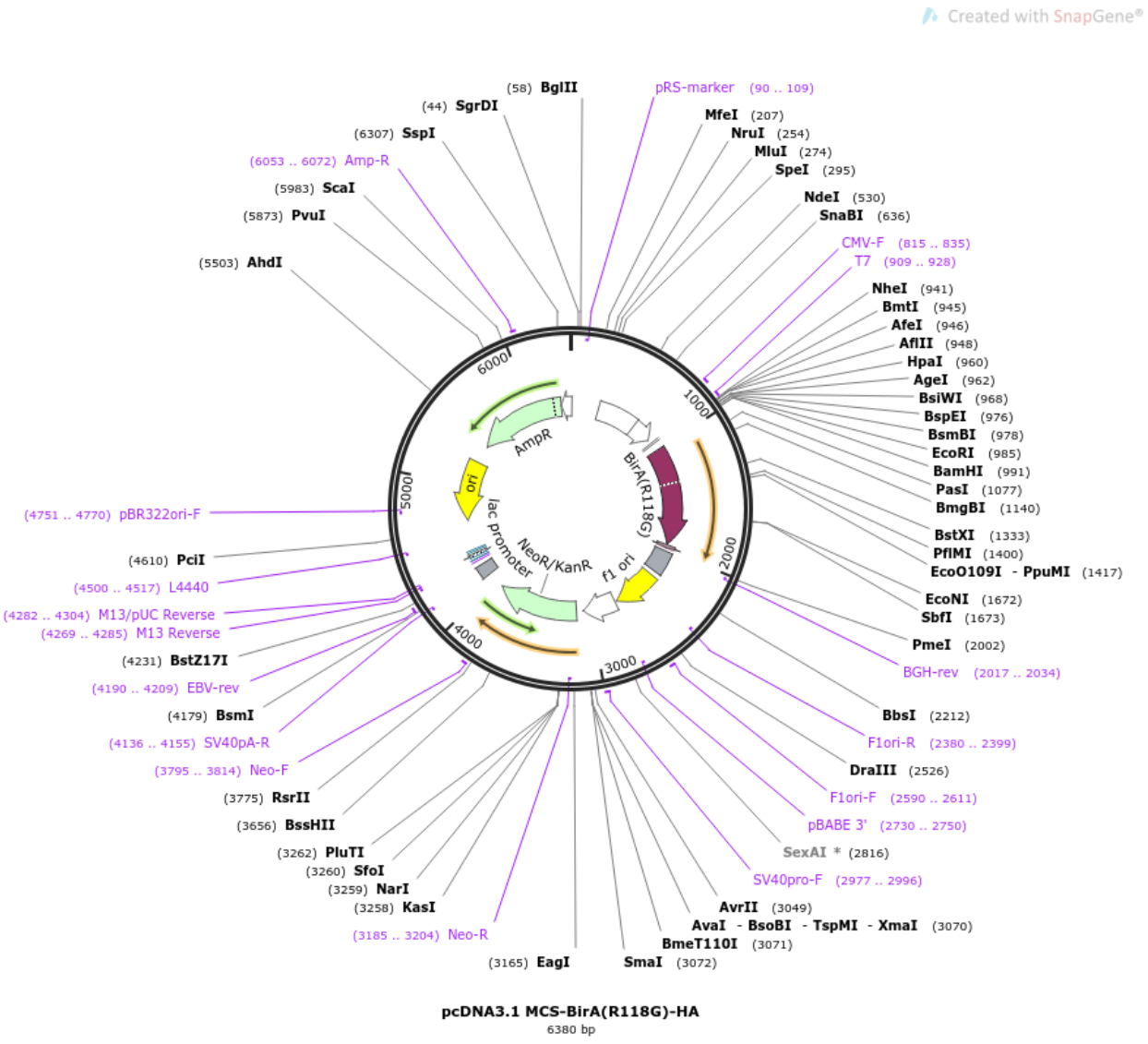


Figure 27. Vector diagram of pcDNA 3.1 MCS-BirA(R118G)-HA used for cloning [229]**.**

Restriction digestion and Ligation of vector and GOIs

pcDNA3.1 MCS-BirA(R118G)-HA was a gift from Kyle Roux (Addgene plasmid #36047 ; http;//n2t.net/addgene;36047 ; RRID;Addgene_36047) [230]. EZ-10 Spin column plasmid DNA miniprep kit (BioBasic; BS414) was used to isolate the plasmids. PCR amplified full length human ABC transporter proteins (ABCB1, ABCB9, ABCB10, ABCD4) and pcDNA3.1 MCS-BirA(R118G)-HA were digested using NheI and AflII restriction enzymes (NEB; R3131 and R0520). The digested vector (pcDNA3.1 MCS-BirA) and inserts (ABCB1, ABCB9, ABCB10, ABCD4) are ligated using T4 DNA ligase (NEB; M0202) as shown in fig 28.

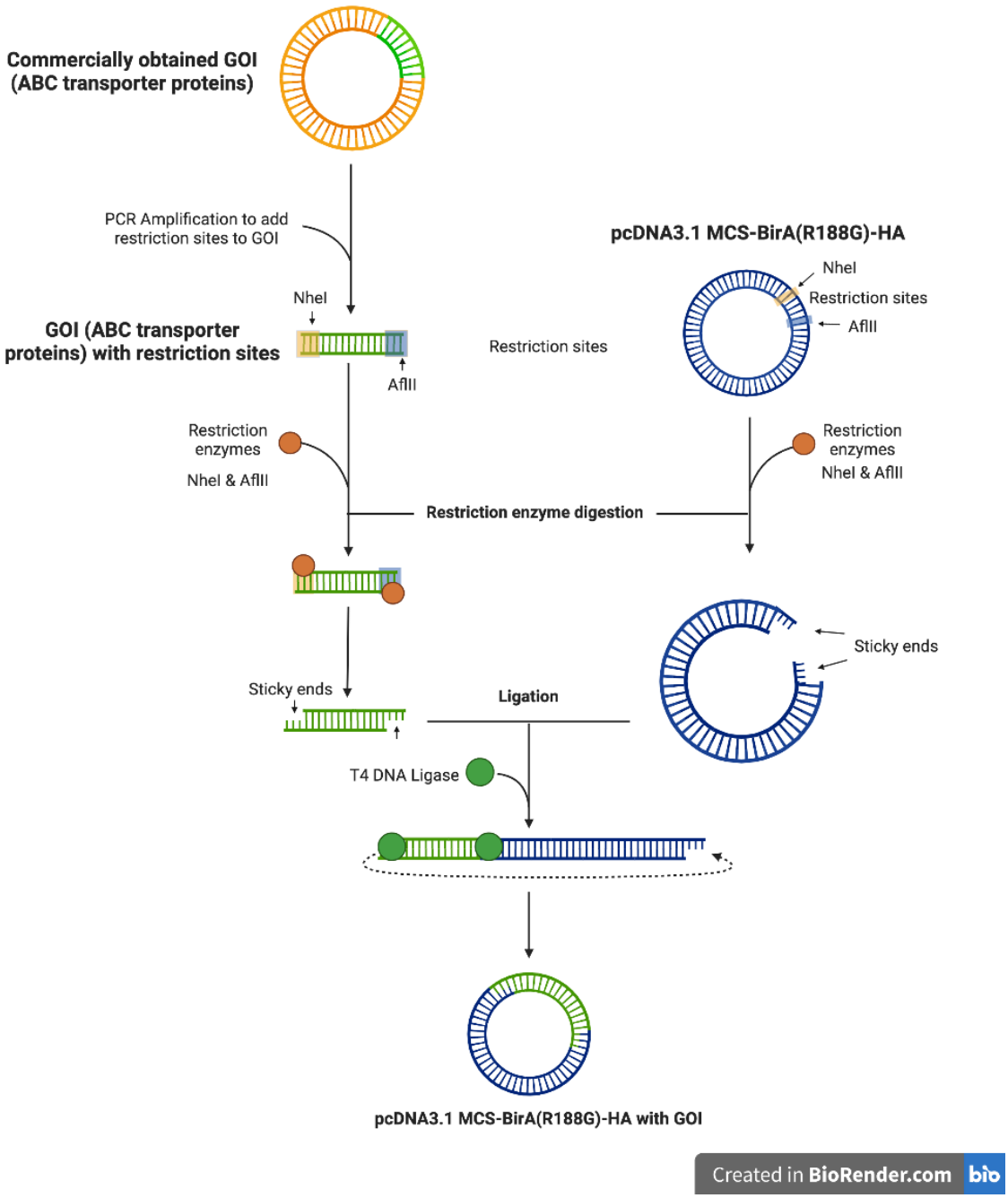


Figure 28. Schematic representation of cloning technique utilized in this study. PCR amplified GOI and the vector are digested using NheI and AflII, then the GOI with compatible restriction sites is ligated into the vector.

Transformation of ligated constructs

The ligated constructs were cloned into TOP10 *Escherichia coli* competent cells (NEB; C3019H) by heat shock transformation technique. pcDNA 3.1 MCS-BirA(R118G)-HA vector confers resistance to ampicillin. Transformed colonies were screened by plating on LB agar supplemented with 100 µg/mL ampicillin (BioBasic; AB0028). After overnight incubation, colonies were selected randomly for each of the gene of interest and colony PCR was performed using T7, BGH primers and the respective GOI primers mentioned above to confirm the cloning of GOI into pcDNA 3.1 MCS-BirA(R118G)-HA vector.

Positive colonies were isolated for each GOI and cultured further. Plasmid extraction was performed for each of the cloned GOI, and clones were further confirmed by doing PCR using respective ABC transporter primers and with T7 and BGH primers. It was also confirmed by doing restriction digestion using NheI and AflII as shown in figure 28. Clones were also sent for Sanger Sequencing (Genome Quebec) to make sure no mutations were present.

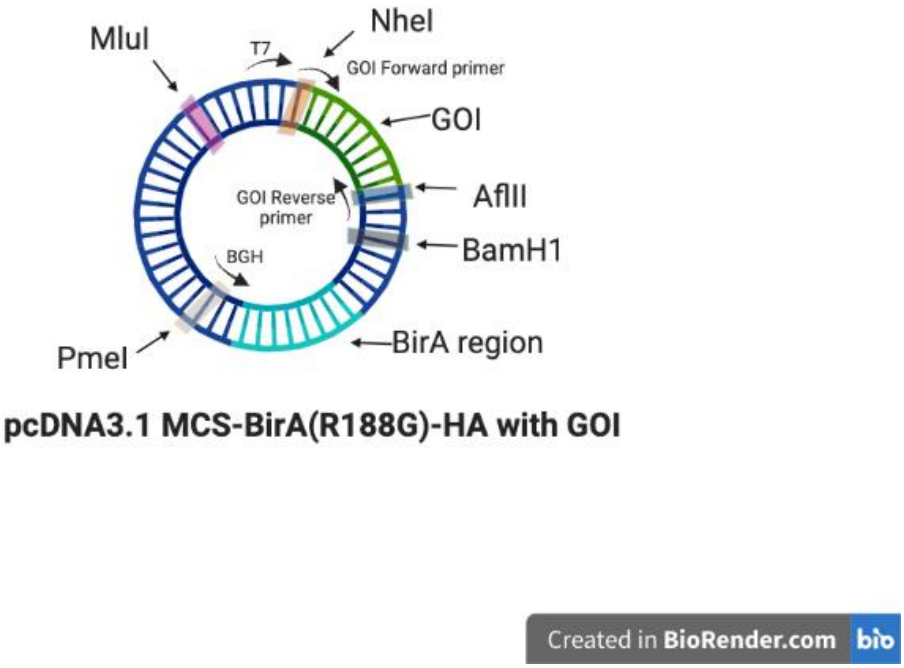


Figure 29. Schematic representation of clone with BirA construct showing primers and restriction sites used for confirmation of successful cloning.

RESULTS

PCR Amplification of gene of interest (GOI)

Commercially obtained ABC transporter proteins (GOI) was amplified to incorporate the desired restriction sites on either side of the full length GOI (see, fig 28,29). Figure 30 shows an agarose electrophoresis gel image demonstrating all amplified cDNAs for each of the transporters with restriction sites. Moreover, fig 29 shows amplified fragments encoding each ABC transporter (e.g., ABCB1, ABCB9, ABCB10, ABCD4 and ABCF1) migrating with expected/calculated molecular size.

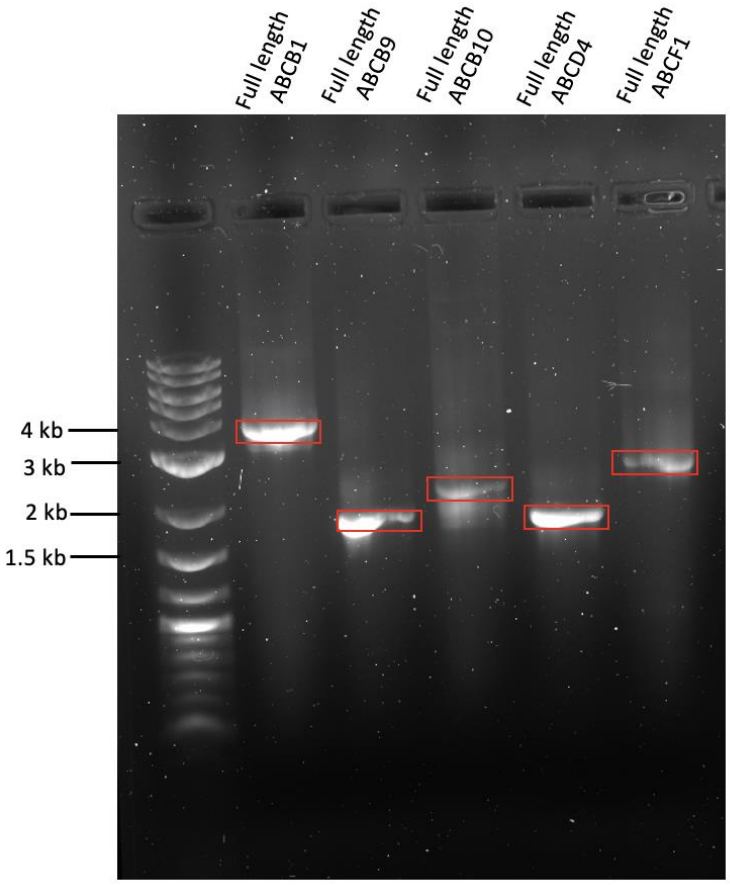
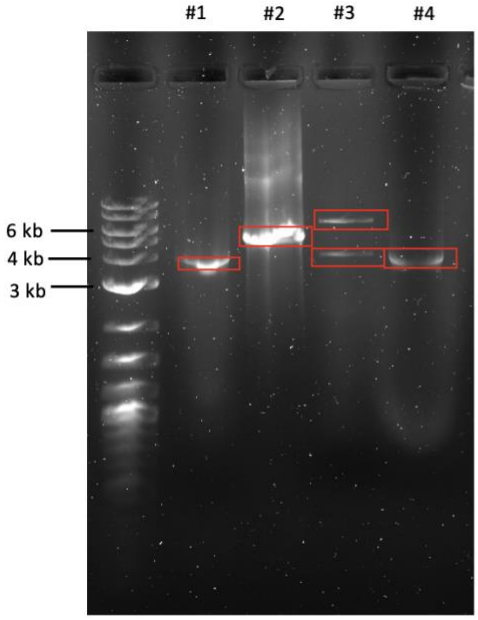


Figure 30. Agarose gel electrophoresis image showing PCR amplified ABC transporters using specific primer sets for each respective ABC transporter proteins.

Confirmation of successfully cloning

All the clones containing the full-length cDNA for each of the five ABC transporters were confirmed by PCR using different primers and by restriction digestion using NheI and AflII enzymes as shown in figure 29. Below are the agarose gel images showing confirmation of successful cloning.



Lane	Sample	Molecular weight
#1	PCR using ABCB1 Primers	3843 bp
#2	PCR of ABCB1 + BirA using T7 & BGH Primers	4968 bp
#3	Restriction digestion of ABCB1+BirA using NheI & AflII	6380 bp 3843 bp
#4	ABCB1 Positive Control	3843 bp

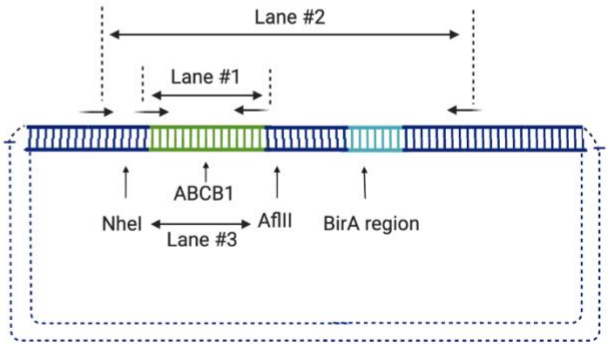
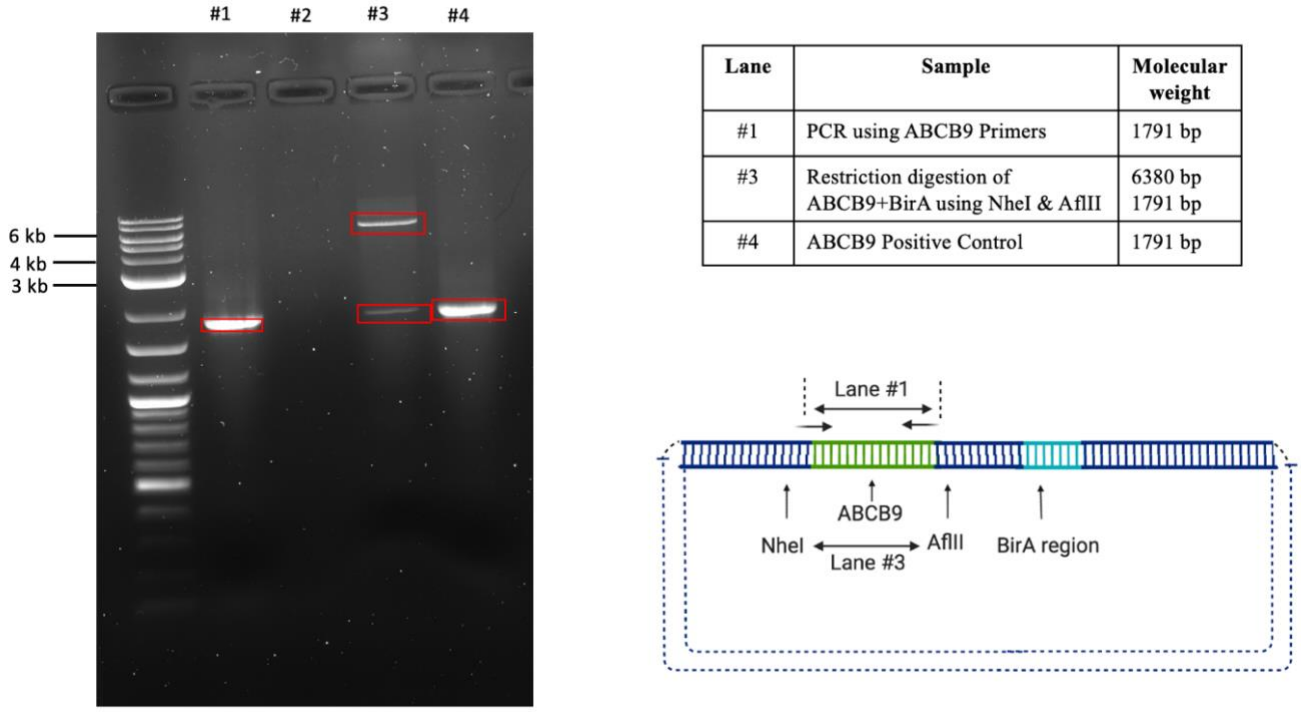
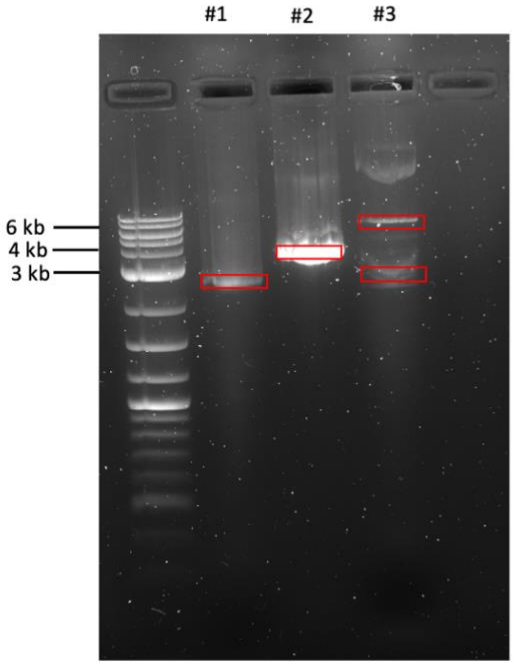


Figure 31. Agarose gel electrophoresis image showing confirmation of ABCB1 cloning into pcDNA3.1 MCS-BirA(R188G)-HA. Lane #1 is PCR using ABCB1 primers, lane #2 is PCR using T7 and BGH primers, Lane #3 is the digestion using NheI and AflII enzymes and lane #4 is ABCB1 positive control sample.



Lane	Sample	Molecular weight
#1	PCR using ABCB9 Primers	1791 bp
#3	Restriction digestion of ABCB9+BirA using NheI & AflII	6380 bp 1791 bp
#4	ABCB9 Positive Control	1791 bp

Figure 32. Agarose gel electrophoresis image showing confirmation of ABCB9 cloning into pcDNA3.1 MCS-BirA(R188G)-HA. Lane #1 is PCR using ABCB9 primers, lane #3 is the digestion using NheI and AflII enzymes and lane #4 is ABCB9 positive control sample.



Lane	Sample	Molecular weight
#1	PCR using ABCB10 Primers	2217 bp
#2	PCR of ABCB10 + BirA using T7 & BGH Primers	33342 bp
#3	Restriction digestion of ABCB10+BirA using NheI & AflII	6380 bp 2217 bp

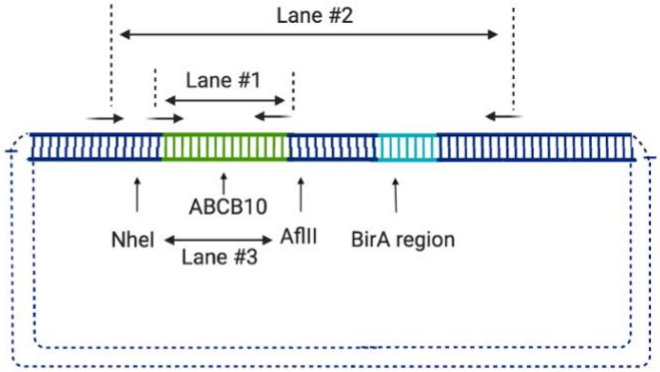
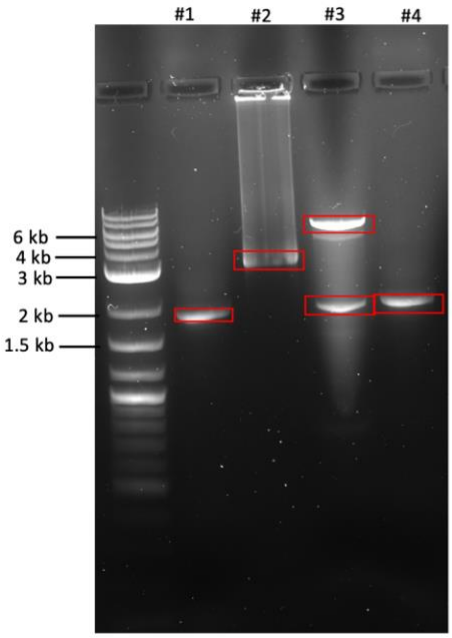


Figure 33. Agarose gel electrophoresis image showing confirmation of ABCB10 cloning into pcDNA3.1 MCS-BirA(R188G)-HA. Lane #1 is PCR using ABCB10 primers, lane #2 is PCR using T7 and BGH primers, and lane #3 is the digestion using NheI and AflII enzymes.



Lane	Sample	Molecular weight
#1	PCR using ABCD4 Primers	2217 bp
#2	PCR of ABCD4 + BirA using T7 & BGH Primers	3342 bp
#3	Restriction digestion of ABCD4+BirA using NheI & AflII	6380 bp 2217 bp
#4	ABCD4 Positive Control	2217 bp

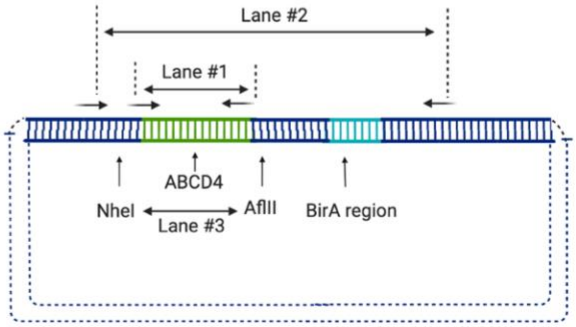
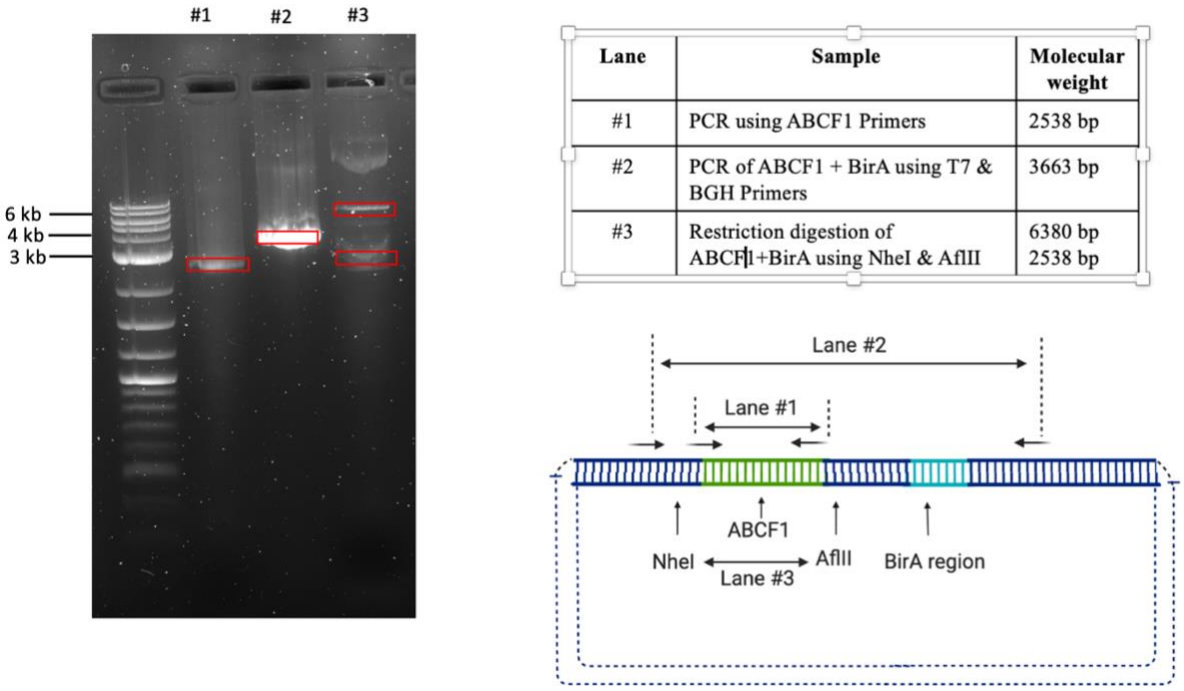


Figure 34. Agarose gel electrophoresis image showing confirmation of ABCD4 cloning into pcDNA3.1 MCS-BirA(R188G)-HA. Lane #1 is PCR using ABCD4 primers, lane #2 is PCR using T7 and BGH primers, lane #3 is the digestion using NheI and AflII enzymes and lane #4 is ABCD4 positive control sample.



Lane	Sample	Molecular weight
#1	PCR using ABCF1 Primers	2538 bp
#2	PCR of ABCF1 + BirA using T7 & BGH Primers	3663 bp
#3	Restriction digestion of ABCF1+BirA using NheI & AflII	6380 bp 2538 bp

Figure 35. Agarose gel electrophoresis image showing confirmation of ABCF1 cloning into pcDNA3.1 MCS-BirA(R188G)-HA. Lane #1 is PCR using ABCF1 primers, lane #2 is PCR using T7 and BGH primers, and lane #3 is the digestion using NheI and AflII enzymes.

The clones were also sent for Sanger sequencing at Genome Quebec for further confirmation. ABCB1, ABCB9 and ABCD4 sequences were found to be matching 100%. Sequencing results for other two clones are in progress.

DISCUSSION

As described above, ABC transporter proteins have different subcellular localization. The differential subcellular localization, in addition to their differential physiologic and pathologic functions were reasons behind this selection panel of ABC transporters. Hence, knowledge of their near-by and direct interactors, using the approach described in this thesis, is expected to confirm, or modify our knowledge about their subcellular localization. Moreover, given that one can examine both near-by and direct interactors under in-vivo (in HEK-293F) cells, in the absence and presence of normal physiologic stimuli or inhibitors, will further our knowledge and possibly understanding of the mechanisms that regulate and are regulated by this panel of ABC transporters.

The objective of this thesis work was to establish BirA fusion constructs whereby BirA is localized to the C-terminal of five different ABC transporter proteins (e.g., ABCB1-BirA, ABCB9-BirA, ABCB10-BirA, ABCD4-BirA, and ABCF1-BirA), in addition to the same but without the BirA fusion. The latter constructs (without BirA fusion) will serve as controls. ABCX-BirA and ABCX HEK-293F stable transfectants will be compared to rule out the potential effect of BirA fusion on ABCX-transporters functions and subcellular localization. The construction of BirA fusion to the C-terminal of all ABC transporters in this thesis are based on earlier work whereby constructs with BirA N-terminal to ABCC1, ABCG2 and ABCG1 were not functional (Lee, K and Krishner H., manuscripts in preparation). We recognize that different approaches can be utilized to identify interacting protein with a given X-bait protein [231, 206, 211], each method or approach has its advantages and disadvantages. In this case, the BirA-fusion and biotinylation approach is likely to identify several non-interacting proteins, however this fact can yield useful information regarding the subcellular localization of the bait protein and its cellular trafficking to its site of function. Furthermore, the presence of ABC-X-BirA (or bait-BirA) protein stably expressed

protein in vivo, provides a consistent system whereby protein interactions can be investigated and assessed under modifiable experimental conditions.

FUTURE WORK

All the ABC transporter clones with BirA construct will be transfected stably into HEK293F cell lines and confirm by western blotting, using two different antibodies, ABC-specific antibody and an anti-HA tag localized to BirA C-terminal sequence. Stable HEK-293F clones (typically three clones) expressing each ABC transporter will be characterized according to the best-established function of each transporter (e.g., drugs or ligand transport). Following the functional characterization of each ABC-expressing HEK-293F clones, the identity of nearby or directly interaction proteins, through their biotinylation with BirA fused domain, will be determined by mass spectrometry as previously demonstrated for ABCG2, ABCG1 (Lee, K, manuscripts in preparation), and ABCC1 (Krishnar, H. manuscript in preparation). For each ABC-transporter described in this thesis, identified interacting proteins using aforementioned approach will be compared to those identified earlier and shown in each of the STRING diagrams above.

SUMMARY AND CONCLUSION

ABC transporter protein plays a crucial role in different physiological functions and diseases. We believe that these proteins do not function alone but rather interact with different proteins either directly or indirectly. There were different experiments performed around the world to identify protein interactions of ABC transporter proteins but most of those techniques has significant drawbacks. Compared to other techniques, BioID technique is one of the best techniques for identifying potential protein interactors of a target protein or for identifying proteins localized to a specific cellular compartment based on proximity-dependent biotinylation. This technique helps to identify direct interacting proteins and as well as the vicinity proteins that could be a part of different functions or pathways. Limitations of this techniques includes, because of the long incubation, some of the random proteins due to Brownian motion within the cell which may come in radius with BirA will be labelled. So, to ensure protein interactions, overlapping peptide approach can be performed. Studying the protein interactions using this technique in different cell lines including diseased cell lines help in better understanding their interactions which may lead to the development of improved therapeutics by targeting these transporter proteins.

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