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Study of the subcellular localization of the human ATP-binding cassette transporter isoform,ABCB5β, expressed in melanoma

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Study of the subcellular localization of the human ATP-binding cassette transporter isoform, ABCB5β, expressed in melanoma

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ABSTRACT

Human ATP-binding cassette (ABC) transporter superfamily comprises 44 membrane transporters classified into five families (A, B, C, D, and G). There are also 4 non-transporter ABC proteins that belong to family E and F. ABC transporters are responsible for translocating an array of endogenous and xenobiotic substances across cellular membranes. Many of these transporters are involved in cancer multidrug resistance. Among these transporters, ABCB5 has been described as a marker of skin progenitor cells, melanoma stem cells, and as a marker of limbal stem cells. *ABCB5* encodes a full transporter (ABCB5FL) and a half transporter (ABCB5β). *ABCB5FL* was cloned from testis and *ABCB5β* from melanocytes. There are also many additional transcript variants, including *ABCB5α*, but they are too short to form functional transporters. Recently, *ABCB5β* has attracted substantial attention due to its roles in promoting proliferation, metastasis, and invasive capacities in melanoma cells. Given the high expression of *ABCB5β* in melanoma, we directed our research toward this isoform. In this study, we explored the subcellular localization of ABCB5β using a combination of subcellular fractionation and immunofluorescence analyses. To conduct this investigation, we tested commercial anti-ABCB5 antibodies and engineered several tagged *ABCB5β* cDNA constructs. Our study focuses on two cell lines, HeLa (cervical cancer) and MelJuSo (melanoma) and revealed that GFP- and HA-tagged ABCB5β is primarily located in the endoplasmic reticulum. Transferring HA-ABCB5β to a low-expression plasmid yielded similar results, as well as treating the cells with a known inducer of chaperone activity (SAHA). In summary, our findings support that ABCB5β predominantly resides in the ER under normal cellular conditions. Additionally, we observed that the expression of the close homolog *ABCB1* increases after *ABCB5* knock-down in MelJuSo cells, raising the possibility that the former could compensate for the latter. However, it appears unlikely that this compensation would cover all ABCB5-dependent functions in these cells, considering that MelJuSo cells with a knocked-down *ABCB5* gene exhibited decreased proliferation. In summary, we demonstrated that ABCB5β is predominantly localized to the ER even after the treatment with SAHA, which restored the folding and increased the total level of ABCB5β expression to some extent. However, remains unclear the role of ABCB5β in the ER of melanoma cells and whether it can function either as a homodi-mer or as heterodimer.

LIST OF ABBREVIATIONS

ABC	ATP Binding Cassette
AC	Acral Melanoma
ADME	Absorption, Distribution, Metabolism, And Excretion
ADP	Adenosine Diphosphate
apoA-I	Apolipoprotein A-I
ATP	Adenosine Triphosphate
BRAF	Member Of RAF: Rapidly Accelerated Fibrosarcoma
BSA	Bovine Serum Albumin
CDKN2A	Cyclin Dependent Kinase Inhibitor 2A
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CRC	Colorectal Cancer
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
crRNA	Crispr RNA
CSC	Cancer Stem Cells
CXCR1	C-X-C Chemokine Receptor Type 1
DAPI	4’,6-Diamidino-2-Phenylindole
DKK1	Dickkopf-1
DMEM	Dulbecco’s Modified Eagle’s Medium
ECDs	Extra-Cytoplasmic Domains
ER	Endoplasmic Reticulum
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GFP	Green Fluorescent Protein
gRNA	Guide RNA
GSK3	Glycogen Synthase Kinase 3
HA	Hemagglutinin
HDL	High-Density Lipoprotein
HDR	Homology Directed Repair
IL	Interleukin
KD	Knockdown
KO	Knockout

LSCs	Leukemia Stem Cells
MCF7	Michigan Cancer Foundation - 7
MDR	Multidrug Resistance
MHCI	Major Histocompatibility Complex Class I
MITF	Microphthalmia-Associated Transcription Factor
MMIC	Malignant Melanoma Iniating Cell
MRP	Multidrug Resistance-Associated Protein
NBD	Nucleotide-Binding Domain
NF-κB	Nuclear Factor-Kappa B
NHEJ	Non-Homologous End-Joining
NRAS	Neuroblastoma RAS Viral Oncogene Homolog
OSCC	Oral Squamous Cell Carcinoma
PAM	Protospacer Adjacent Motif
PGK	Phosphoglycerate Kinase
RNP	Ribonucleoprotein
RSS	Relative Specific Signal
SAHA	Suberoylanilide Hydroxamic Acid
shRNA	Short Hairpin RNA
siRNA	Small Interfering RNA
SRA	Specific Relative Activity
SSM	Superficial Spreading Melanoma
ssODN	Single-Stranded Oligo Donor
SUR	Sulfonylurea Receptor
TAP	Transport Associated with Antigen Processing Transporters
TGN	Trans-Golgi Network
TM	Transmembrane Segments
TMD	Transmembrane Domain
TracrRNA	Trans-Activating Crispr RNA
WFDC1	Wap Four-Disulfide Core Domain 1
X-ALD	X-Linked Adrenoleukodystrophy
ZEB1	Zinc Finger E-Box Binding Homeobox 1

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Publications

Research paper submitted in the International Journal of Molecular Sciences (IF 5.6).

Diaz Anaya A., Gerard L., Albert M., Gaussin JF., Boonen M., Gillet JP. (2023). **The β Isoform of Human ATP-Binding Cassette B5, ABCB5β, is a Microsomal Protein.**

Commissioned review paper accepted for publication to Trends in Cancer (IF 18.4):

Duvivier L., Gerard L., Diaz Anaya A., Gillet JP. (2023). **Multifaceted Biological Functions of ABC Transporters.**

Oral and poster presentations

3rd BELSACT scientific meeting. University of Leuven, Belgium. Diaz Anaya A., Boonen M., Gillet JP. (2023). Poster presentation: Intracellular localization of ABCB5, a transmembrane protein expressed in melanoma.

Women and girls in science, 3rd edition. University of Namur, Belgium. Diaz Anaya A., Boonen M., Gillet JP. (2023). Poster presentation: Intracellular localization of ABCB5, a transmembrane protein expressed in melanoma.

3rd Symposium on Current trends in membrane protein biophysics. University of Namur, Belgium. Diaz Anaya A., Boonen M., Gillet JP. (2022). Poster presentation: Study of the subcellular localization of ABCB5.

PhD student day UCLouvain-UNamur. Louvain la Neuve, Belgium. Diaz Anaya A., Boonen M., Gillet JP. (2021). Poster and short talk: Study of the subcellular localization of the ATP-binding cassette transporter ABCB5β isoform in melanoma cell lines.

Unamur research day: Showcasing UNamur's research in development cooperation. Namur, Belgium. Diaz Anaya A., Boonen M., Gillet JP. (2021). Poster and short talk: Study of the subcellular localization of the ATP-binding cassette transporter ABCB5β isoform in melanoma cell lines.

INTRODUCTION

1. MULTIDRUG RESISTANCE MECHANISMS

Multidrug resistance (MDR) refers to the intrinsic or acquired resistance of microorganisms or cancer cells towards multiple drugs that may be structurally unrelated and have different mechanisms of action. This resistance is often the result of the overexpression of a variety of proteins that can limit the drugs entry into the cell, extrude them from the cell cytoplasm, lower association of the drugs with their target(s) and/or inactivate the drugs themselves. MDR raises substantial alarm due to its potential to result in treatment ineffectiveness and the progress of diseases, ultimately leading to elevated rates of morbidity and mortality, which is a prominent public health issue (Gottesman, Lavi, Hall, & Gillet, 2016).

Resistance is present against every potent anticancer drug and can emerge through various means, including decrease in the drug absorption, increase in the expulsion of drugs, initiation of detoxification processes, activation of DNA repair pathways, and avoidance of drug-induced apoptosis (**Figure 1**). Among these mechanisms is the activation or overexpression of members of the ATP-binding cassette (ABC) family of drug efflux transporters, especially P-glycoprotein (Pgp; ABCB1). They mediate MDR enabling the expulsion of a wide range of drugs from the cell cytoplasm or their sequestration in the endolysosomal compartment. They represent a very large superfamily of proteins, which mediate the transport of a multitude of molecules across phospholipid bilayers (Muriithi et al., 2020).

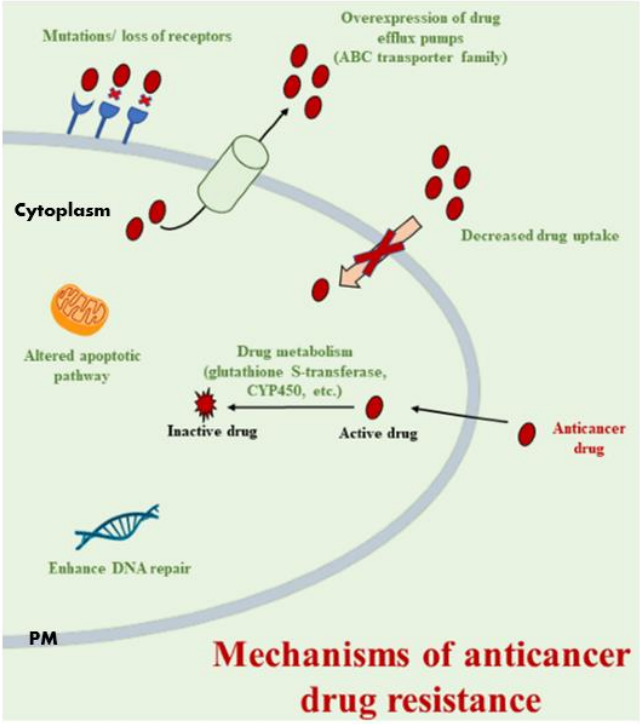

Figure 1. *Some of the mechanisms of anticancer drug resistance. Modified from Catalano et al. (2022).*

2. ABC TRANSPORTERS

ATP-binding cassette (ABC) transporters are mainly transmembrane proteins expressed ubiquitously in various organisms (Vasiliou, Vasiliou, & Nebert, 2009). They mechanistically link ATP-Mg^{2+} binding, ATP hydrolysis, and ADP/phosphate release to facilitate the translocation of a diverse range of substrates across membranes. These transporters are relevant in diverse physiological processes, such as lipid homeostasis, nutrient import, antigen presentation, signal transduction or xenobiotic detoxification. Depending on the direction of substrate translocation they catalyze, ABC transporters are generally categorized as importers, predominantly found in bacteria, and exporters (Thomas & Tampe, 2018).

2.1 Structural features of ABC transporters

These transporters are composed of two symmetric halves, each including a transmembrane domain (TMD), and a highly conserved nucleotide-binding domains (NBDs, also named the

ATP binding cassettes domains). TMDs are responsible for substrate binding and then to form the framework of the translocation pathway, while NBDs bind and hydrolyze ATP (Theodoulou & Kerr, 2015). In most cases, each TMD has from five to six transmembrane α-helical segments. They are expressed either as full transporters containing two nonidentical halves within a single polypeptide or as half transporters that have to homo- or heterodimerize to be functional (Thomas & Tampe, 2020).

Despite this shared basic architecture, ABC transporters display tremendous variability in substrate specificity, transport mechanisms, and regulation (Alam & Locher, 2023). Humans have 48 ABC transporter genes encoding 44 membrane transporters. Domain organization and phylogenetic analysis have defined eight major subfamilies of eukaryotic ABC proteins, two of which (E and F) are not membrane associated because they lack TMDs and have functions other than transport (Dean, Rzhetsky, & Allikmets, 2001). In members of the ABCA and C families, all four domains are fused in a single polypeptide chain (full transporters), while ABCD and G members are "half-transporters" where one TMD is fused to one NBD (**Figure 2**).

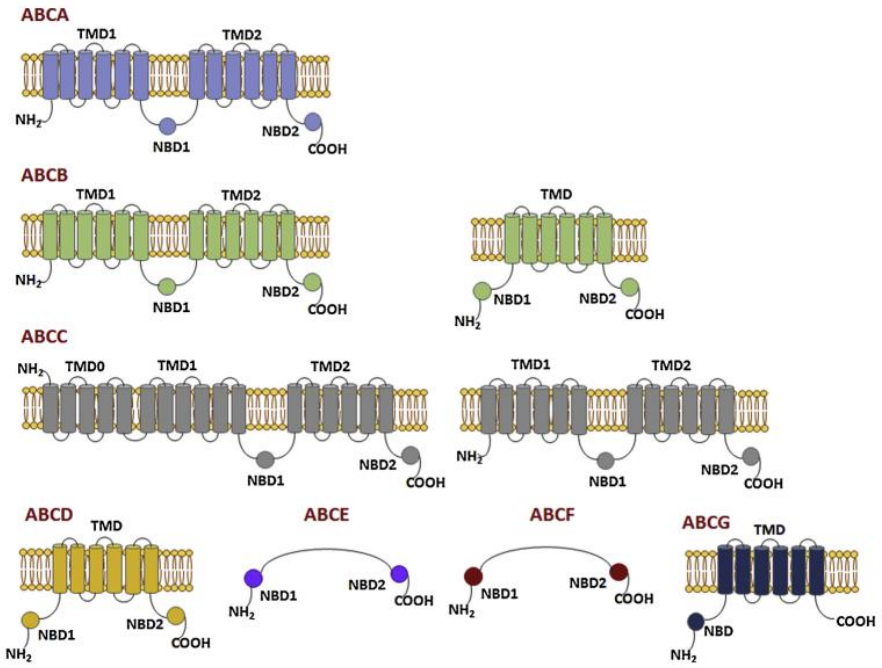

Figure 2. *Diagram illustrating the predicted domain organization within ABC transporter subfamilies. Barrels represent transmembrane domains (TMDs), while circles depict nucleotide-binding domains (NBDs); TMD0, composed of 5 hydrophobic α-helices; while TMD, TMD1 and TMD2 is composed of 6 hydrophobic α-helices* (Nobili et al., 2020).

2.1.1 Nucleotide-binding domains (NBDs) in ABC transporters

The NBDs are domains located in the cytoplasm, where they play indispensable roles in the catalytic cycle of ATP binding, hydrolysis and ADP/Pi release. During the ATP binding there is association of the two nucleotide binding sites (NBSs) using critical motifs from both NBDs: the Walker A, Walker B, and signature motif (C-loop); the A-, D-, H-and Q-loop (Seeger & van Veen, 2009) (**Figure 3a**). Motifs sequences and residues involved in the ATP binding and hydrolysis processes are briefly detailed in **Table 1**.

The walkers A, B and the signature motif form a large pocket for ATP binding, and it is usually believed that both NBDs are required for ATP hydrolysis. The NDBs are able to form a "nucleotide-sandwich dimer". ATP is then bound along the dimer interface between the walker A and B motifs of one subunit, and the signature motif and the D-loop of the other subunit. The adenine ring of ATP interacts with the A-loop while phosphate and magnesium interact with the Waker A and B (Ambudkar, Kim, Xia, & Sauna, 2006) (**Figure 3b**).

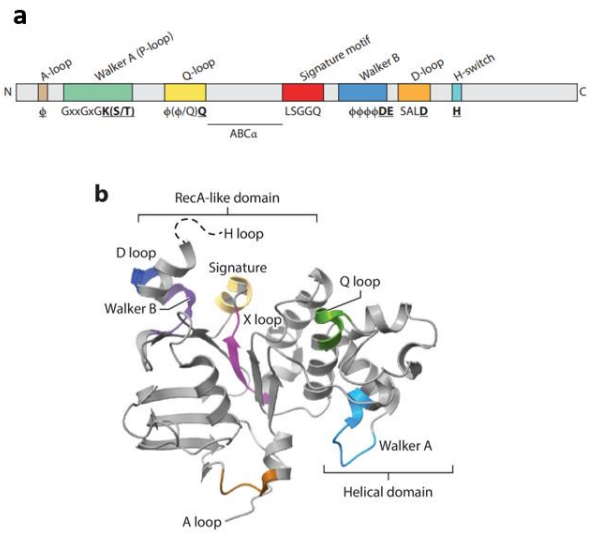

Figure 3. *Nucleotide binding domain structure. a) Linear arrangement of sequence motifs in the ABC. b) NBD from ABCB2/TAP1, view of the catalytic core domain (RecA-like domain) and the α-helical subdomain. The sequenced motifs are labeled and colored. The H loop, missing in this structure, is depicted as a dashed black. Adapted from: Thomas and Tampe (2020) and Alam and Locher (2023).*

Motif	*Sequence*	*Functions*
A-loop	Conserved aromatic (A) residue of 25 amino acids, (usually tyrosine)	Interaction between the aromatic ring of a tyrosine and the adenine moiety of ATP
Walker A (P-loop)	G*xx*G*x*GKS/T (where '*x*' is any residue)	Binds the alpha and beta phosphates of ATP
Q-loop	Conserved glutamine (Q) residue	Contacts the TMDs and contains a conserved glutamine that coordinates the bound Mg^{2+}ion
Signature motif (C-motif)	Consensus sequence LSGGQ	Located between the walker A and B. This motif is crucial for the hydrolysis of ATP and the interaction with the substrate binding site
Walker B	Consensus sequence *hhhh*DE, with *h* denoting a hydrophobic residue	Provides the catalytic glutamate residue and aspartate residue to coordinate the required Mg^{2+}ion
D-loop	Consensus sequence SALD, has a conserved aspartate (D) residue	Plays a role in NBD dimerization
H-switch	Conserved histidine (H) residue	Features a conserved histidine involved in ATP hydrolysis
X-loop	TxVGExG (where 'x' is any residue)	Provides contact between the TMDs in a closed conformation

Table 1. *Sequences and functions of the various conserved motifs of NBDs. Extracted from Ambudkar et al. (2006) and (Linton & Higgins, 2007).*

2.1.2 Transmembrane domains (TMDs) in ABC transporters

In contrast to the NBDs, which are highly conserved between ABC transporters, the TMDs display significant structural differences. They form substrate-binding sites (SBS), which contribute to substrate specificity and transport. The TMDs domains generally have 6–12 membrane-spanning alpha-helices, determining substrate specificity (**Figure 4a**). These α-helices are embedded in the membrane and traverse it several times in a zigzag fashion (Dean, Moitra, & Allikmets, 2022).

One common structural feature in transporters is the presence of the coupling helices (CH), which are short helices in the TMDs that interact with the NBDs (**Figure 4b**). These coupling

helices are the only part of the TMD-NBD interface that is structurally conserved between the many different TMD folds. It has been proposed that these helices constitute part of the transmission interface, where the binding and hydrolysis of ATP in NBDs is coupled to conformational changes in TMDs that influence solute translocation (Thomas & Tampe, 2020).

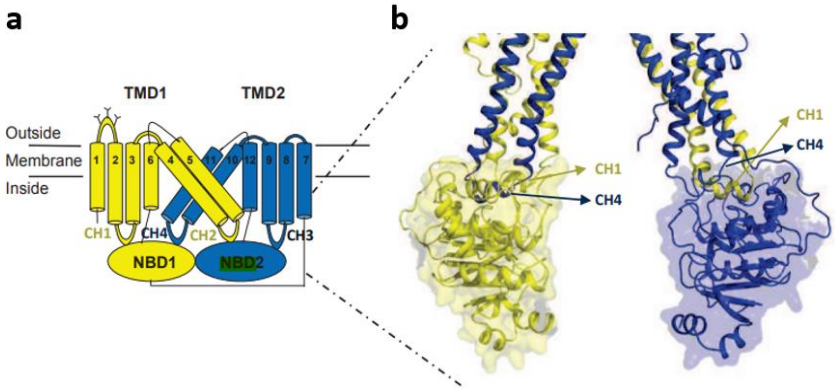

***Figure 4**. The coupling helices of P-glycoprotein P-gp/ABCB1. a) Topological model of P-gp. b) Structure of P-gp (code PDB: 4KSC). The two halves are colored blue and yellow. The NBD is shown as a transparent solid. CH: coupling helix; PDB: protein data bank. Modified from: Loo and Clarke (2014).*

2.1.3 Accessory domains in ABC transporters

In addition to the ubiquitous four core-domains, i.e. the two TMDs and two NBDs, accessory domains can be found in several ABC transporters. They are often involved in regulation or protein-protein interactions. Extra domains can be found separated, attached or integrated into parts of the core ABC transporter (Biemans-Oldehinkel, Doeven, & Poolman, 2006) (**Figure 5**).

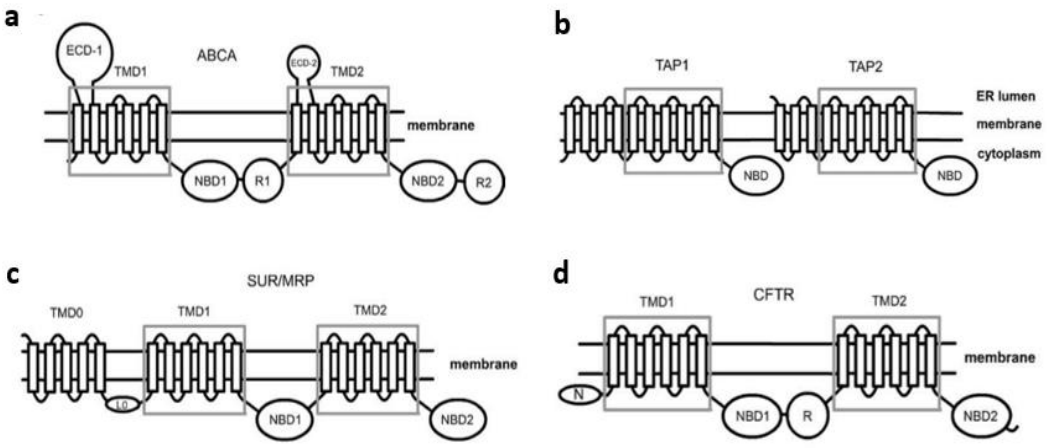

Figure 5. *Accessory domains in ABC transporters. a) Topology of members of the ABCA family. Extracellular loops (ECD-1 and ECD-2) and cytosolic regulatory domains (R1 and R2) are shown. b) TMD topology of the two half-transporters ABCB2/ABCB3 (TAP1/TAP2). c) Topology of the ABCC family. The extra TMD (TMD0) and L0-loop are indicated. d) Topology of CFTR. The N-tail and regulatory (R) domain is indicated. Adapted from Biemans-Oldehinkel et al. (2006).*

I. Extra-cytoplasmic domain

One example is the large extra-cytoplasmic domains (ECDs) found in members of the ABCA family. It comprises two parts, ECD1 and ECD2, located in external loops linking transmembrane segments (TM) 1 and TM2 or TM7 and TM8, respectively (**Figure 5a**). Each ECD establishes contacts with both TMDs, adding an element of domain exchanging. ABCA1 mediates the delivery of phospholipids and cholesterol from the membrane to the apolipoprotein A-I (apoA-I) and the ECD of ABCA1 is responsible to load apoA-I. The ECDs of other ABCA family members vary in size, and their physiological roles remain to be established (Qian et al., 2017).

II. Additional membrane embedded domain

The majority of ABC transporters are predicted to have six transmembrane segments (alpha-helices) per TMD core and thus there are twelve alpha-helices for a typical full transporter. However, some ABC transporters contain extra transmembrane segments that are not part of

the core TMD and are considered as accessory domains (Koch, Guntrum, Heintke, Kyritsis, & Tampe, 2004). In ABCB2/ABCB3 (TAP1/TAP2), an extra N-terminal transmembrane domain can be found (**Figure 5b**). This N-terminal region is not essential for substrate transport (peptide transport in this case). However, this domain is essential for binding to a tapasin chaperone (Koch et al., 2004). This specialized chaperone facilitates class I Major Histocompatibility Complex (MHCI) folding and thus enhances peptide transfer from TAP (the peptide transporter) to these MHCI molecules in the process of antigen presentation (Procko, Raghuraman, Wiley, Raghavan, & Gaudet, 2005).

Another example of additional membrane embedded domains are the TMD0s found in some ABCC (except ABCC7/CFTR) and ABCB family members. These are thought to be involved in the transport, folding, subcellular trafficking, and formation of oligomeric complexes with associated membrane proteins (Alam & Locher, 2023). ABCC8 (SUR1) and ABCC9 (SUR2) and the multidrug-resistance proteins ABCC1, 2, 3, 6 and 7, have 5 accessory transmembrane segments that may form a domain by itself call it TMD0. This TMD0 is linked via a cytoplasmic loop (L0) to the N-terminus of TMD1 (**Figure 5c**) (Bakos et al., 1998).

III. Cytosolic regulatory domains

Regulation of many ABC transporters takes place at the level of gene expression. However, the activity of ABC transporters, once expressed, may also be regulated. For instance, additional (autoregulatory) domains can be found in the transporter itself. Examples are the L0 linker in ABCC1 (MRP1) and ABCC8/9 (SUR1/2), or the R-domain of ABCC7 (CFTR).

ABCC7 (CFTR) is an ABC-type chloride channel regulated by protein kinase A-mediated phosphorylation of its cytoplasmic regulatory (R) domain. This R-domain, located between NBD1 and TMD2, contains multiple phosphorylation sites (**Figure 5d**). Under an unphosphorylated state, the R-domain inhibits channel activity by interacting with the CFTR core channel. Phosphorylation of the R-domain is a prerequisite for channel gating. However, evidence for the phosphorylation-dependent release of the R-domain (thereby promoting transporter activity) remains inconclusive. Some studies suggest that phosphorylation enhances (rather than repress) the association of the R-domain with the transporter core (Li & Naren, 2005).

The N-terminal tail of CFTR (N-tail) alongside its R-domain, also regulates CFTR channel activity. It influences the rate of channel openings, possibly through interactions with CFTR components (R-domain or NBD) or the pore itself. The N-tail and C-terminal tails of CFTR bind to various inhibitors and stimulators of CFTR activity, often facilitated by PDZ domain-containing proteins (Li & Naren, 2005).

In the case of ABCA1 (involved in lipid transport across membranes), the R-domains (R1 and R2) are located C-terminally of the NBDs (**Figure 5a**). Phosphorylation of the R1 domain reduces phospholipid flip-flop across the membrane, apolipoprotein binding, and cholesterol/lipid extrusion activities of this transporter (Qian et al., 2017).

2.2 Mechanism of transport by ABC transporters

ABC transporters allow an active transport of the substrate across the cellular membranes against a concentration gradient. The basic steps in the ATP hydrolysis cycle include the direct binding of a substrate to the TMDs, binding of two Mg^{2+} ATP molecules to the NBDs resulting in the dimerization of the NBDs. This step switches the TMDs between the in- and outward facing conformations by ATP hydrolysis. Subsequently, phosphate (Pi), ADP and the transport substrate are released, concomitant with NBD dissociation to reset the transporter to the ground state for the next cycle (**Figure 6**) (Wilkens, 2015).

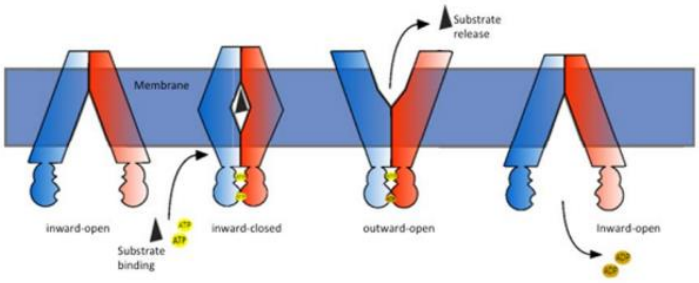

Figure 6. *Conformational states of the ABC transporter during the transport cycle. The inward-open conformation is shown by the dimer of the ABC transporter in blue and red. Substrate and ATP binding drives the transporter to the closed-state. ATP hydrolysis triggers the substrate release and the outward-open state of the transporter. The transporter is then reset back with the release of ADP and Pi.*

Two models for this catalytic cycle have been proposed. Although they share common basic steps, including ATP-dependent NBDs dimerization and the transition of TMDs between outward- and inward-facing conformations, they differ in some specific mechanistic aspects. It is relevant to note that there is limited evidence supporting the idea that all ABC transporters operate using precisely the same mechanism (Wilkens, 2015).

The first model is the ATP-switch model, also known as “Tweezers-Like” and “Processive Clamp” model (Linton & Higgins, 2007). It proposes that transport is a multifaceted and bidirectional process involving communication via conformational changes, in both directions, between the NBDs and TMDs (**Figure7a**). The resting state of the transport is when NBDs monomers are separated and nucleotide-free. Once the monomers are loaded progressively with ATP, it triggers formation of a sandwich dimer. Then, hydrolysis of ATP occurs progressively in each NBD site, followed by a sequential release of Pi and ADP. Thus, the dimer returns to the nucleotide-free open or reset state, which completes the cycle (George & Jones, 2012).

The second model is the “constant contact model” and proposes that NBDs are always in contact, with opening and closing of the binding sites occurring with conformational changes within the NBD monomers (**Figure 7b**). For each catalytic site there are two distinct substates, either occluded (closed) or open (allowing nucleotide exchange). The NBDs remain in contact during the cycle with opening and closing of the sites. During the initial step, one of the ATP-bound active sites is closed and the opposite is empty, and the hydrolysis of the site charged with ATP occurs and Pi is released. Next, the empty site switches to a high affinity state, enabling ATP binding. After Pi is released from the occluded site, it promotes the opening of the ADP-bound site. The same hydrolysis will then occur in the opposite active site with a release of ADP and the process thus repeats in alternating cycles (Jones & George, 2009).

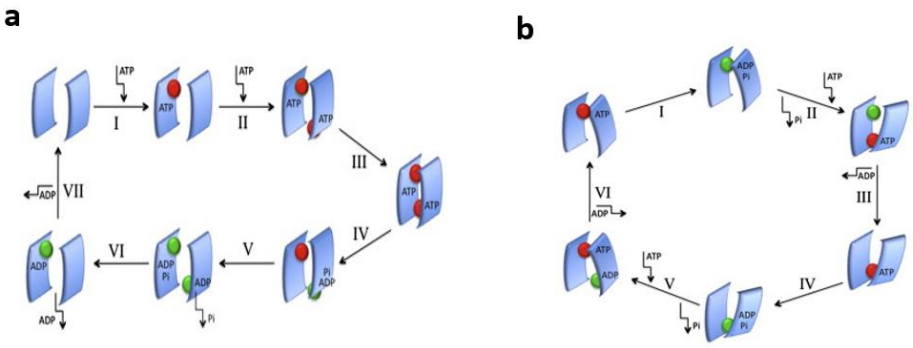

Figure 7. *Models for the catalytic cycle of ATP binding and hydrolysis in the ABC transporter NBD dimer. a) Switch Model. b) Constant contact model Adapted from George and Jones (2012).*

2.3 ABC transporters: subcellular localizations and roles

As mentioned above, the human ABC transporter superfamily lists 48 members distributed into seven subfamilies (ABCA-G). They are active in many cell types and tissues (as illustrated in **Figure 8**) and have been found, depending on the transporter, at the plasma membrane, in the limiting membrane of mitochondria, lysosomes, peroxisomes, Golgi apparatus and endoplasmic reticulum (**Figure 8**) (Alam & Locher, 2023; Gillet, Boonen, Jadot, & Gottesman, 2020).

The substances they transport encompass hormones, vitamins, lipids, sterols, fatty acids, peptides, xenobiotic compounds, and more (Domenichini, Adamska, & Falasca, 2019). Human ABC transporters have significant importance in both biomedical and pharmacological contexts. Furthermore, numerous severe medical conditions result directly from the malfunction of these transporters, underscoring their significance as valuable targets for therapeutic intervention (Borst & Elferink, 2002).

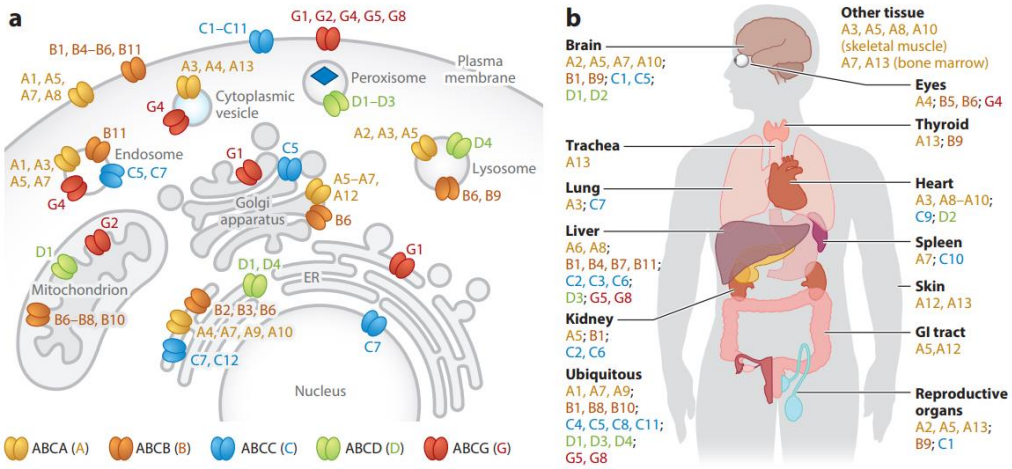

Figure 8. *Distribution of human ABC transporters within cells and various tissues/organs. a) Illustration of a human cell indicating the distribution of ABC transporters across organelles and the plasma membrane. Different colors represent distinct subfamilies of membrane transporters. (b) Diagram of the human organ system, emphasizing the presence of ABC transporters. Abbreviations: ER-endoplasmic reticulum, GI-gastrointestinal (Alam & Locher, 2023).*

The **ABCA transporter family** is composed of 12 members, most of which are involved in lipid transport and cholesterol homeostasis in many organs and cell types (**Figure 8b**) (Pasello, Giudice, & Scotlandi, 2020). Mutations in specific *ABCA* genes lead to genetic disorders, such as Tangier disease T1, a disorder associated with the accumulation of cholesterol in peripheral tissues (Quazi & Molday, 2013). Due to the role of ABCA4 in the transport of vitamin A and derivates in photoreceptor cells, mutations in ABCA4 are causal for the autosomal recessive inheritance of Stargardt's disease and are associated with multiple retinal degenerative conditions (Wiszniewski et al., 2005).

The **ABCB family** is the most diversified, composed of 11 members structurally heterogeneous, as it includes both full length (ABCB1, ABCB4, ABCB5 and ABCB11), as well as hetero- and homodimeric half transporters (ABCB2/B3, ABCB5β and ABCB6-10). They transport a wide range of substrates such as iron, peptides and drugs (Dean, Rzhetsky, et al., 2001). ABCB1 (also known as P-glycoprotein or multidrug resistance protein 1, MDR1), is the best-characterized ABC transporter, widely expressed with a broad spectrum of substrates. It is a multidrug transporter with a role in cellular detoxification that can be exploited by cancer

cells to acquire multidrug resistance in response to chemotherapeutic intervention. The transporter is also expressed at several blood–organ barriers, and hence often plays a role in the absorption, tissue distribution and/or elimination of some pharmacological drugs (i.e. in drug pharmacokinetics) (van Assema et al., 2012). Moreover, genetic variants in *ABCB1* have been linked to development of inflammatory bowel disease (Brinar et al., 2013). Mutations in other *ABCB* genes have been implicated in ankylosing spondylitis, diabetes type 2, coeliac disease, lethal neonatal syndrome, X-linked sideroblastic anaemia with ataxia, and several cholestatic liver diseases of infancy (Vasiliou et al., 2009).

The other full transporters from the ABCB family such as ABCB4 or ABCB11 are hepatocellular transporters that exhibit higher substrate specificity, transporting phosphatidylcholine and bile salts. ABCB4 helps move phosphatidylcholine from liver cell membranes to bile ducts. This action lowers the harmful effects of bile salts (Nosol et al., 2021). ABCB11, the canalicular bile salt export pump, is localized to the canalicular membrane and is the primary transporter responsible for the continuous excretion of bile acids from hepatocytes to the bile duct (Trauner & Boyer, 2003).

Three ABCB transporters are linked to antigen processing. This TAP (transporter associated with antigen processing) consists of ABCB2 (TAP1) and ABCB3 (TAP2), which are half-transporters that dimerize to form the TAP1/2 (ABCB2/ABCB3) heterodimer. This heterodimer transports a wide array of antigenic peptides into the ER lumen, allowing their loading onto the major histocompatibility complex class I molecules. This is an essential process for antigen presentation (Procko et al., 2005). Another member is the homodimeric TAP-like (TAPL/ ABCB9). In contrast with the other TAP proteins who are resident of the ER, TAPL is localized in lysosomes where it is translocating polypeptides (Graab et al., 2019). Moreover, a recent study highlighted that ABCB9 (TAPL) plays a role in regulating the movement of lipids between the inner and outer leaflets of the lysosomal membrane. It appears to employ distinct mechanisms to act both as a peptide transporter and a phosphatidylserine floppase (J. Park et al., 2022).

ABCB6, ABCB7, ABCB8 and ABCB10, are speculated to be localized in the mitochondria, though it was later demonstrated that ABCB6 rather localizes to lysosomes (Gillet et al., 2020; Kiss et al., 2012). They are involved in the transport of metals, especially iron metabolism and homeostasis. Some of these transporters also translocate peptides, proteins and heme across

mitochondria membranes (Dean et al., 2022). For example, ABCB10 is important in early steps of heme synthesis in the heart. Its expression is highly induced during erythroid differentiation and its overexpression increases hemoglobin synthesis in erythroid cells (Bayeva et al., 2013). Some studies suggest being involved in the protection from oxidative stress (Liesa, Qiu, & Shirihai, 2012). One of the most recently discovered members of the ABCB subfamily is ABCB5, which was first identified in 2001 based upon its sequence homology to the other ABCB family members (Dean, Rzhetsky, et al., 2001). We will discuss this transporter more specifically in section 3 as this is the protein of interest in our research.

The **ABCC family** is composed of 12 transporters, 9 of which transport structurally diverse lipophilic anions and function as drug efflux pumps (Kruh & Belinsky, 2003). The exploration of this family has contributed to our understanding of cellular resistance mechanisms related to chemotherapeutic agents, antifolates, and nucleotide analogs. Based on their versatile behavior and the overlapping patterns in substrate recognition, these transporters establish a functional network with the capacity to extrude a broad spectrum of xenobiotic substances (Szakacs, Varadi, Ozvegy-Laczka, & Sarkadi, 2008).

ABCC transporters are also involved in lipid trafficking. This is the case of ABCC1 which exports lysolipids, such as sphingosine-1-phosphate (S1P) and lysophosphatidilinositol (LPI), both important signaling molecules and intracellular messengers in tumor proliferation (Tan, Ramesh, Toh, & Nguyen, 2020). Of note, several members of this ABCC subfamily are not classified as transporters and exert a role of ion channel or act as channel regulators. It is the case of the cystic fibrosis transmembrane conductance regulator (CFTR/ ABCC7). In contrast to other family members, CFTR is responsible for the passive transport of chloride ions down an electrochemical gradient and operates as a typical ion channel (Aleksandrov & Riordan, 1998). ABCC8 which functions as a modulator of ATP-sensitive potassium channels and insulin release (Bonfanti et al., 2015), and ABCC9 encodes regulatory SUR-2A subunit of cardiac K+ channel and modulates the extra-pancreatic ATP-sensitive potassium channels (Bryan et al., 2007). Mutations in one or more of the ABCC genes have been implicated in multidrug resistance, Dubin–Johnson syndrome (Siddiqui et al., 2023), congenital bilateral aplasia of the vas deferens, diabetes type 2 and paroxysmal kinesigenic choreoathetosis, as well as autosomal recessive diseases such as cystic fibrosis (Veit et al., 2016).

The **ABCD family** also known as the peroxisomal transporters, contains four half-transporters that form homodimers or heterodimers. ABCD1, ABCD2 and ABCD3 mainly localize in the peroxisomal membrane and are known to translocate very long chain fatty acids (VLCFA) into peroxisomes (Genin et al., 2011). By contrast, ABCD4 have been reported localized to endoplasmic reticulum and lysosomes, where it plays an important role in the release of Vitamine B12 into the cytosol (Kashiwayama et al., 2009). Mutations in *ABCBD* genes are known to cause X-linked adrenoleukodystrophy (X-ALD), which results in the toxic accumulation of VLCFA in tissues (Wiesinger, Eichler, & Berger, 2015).

To date, little is known about the **ABCE and ABCF subfamilies**. However, ABCE1 is preserved across both prokaryotic and eukaryotic organisms. Characterized by only two NBDs and lacking the TMD, ABCE1 does not function as a transporter. Rather, its significance lies in pivotal contributions to cell division and the initiation of protein translation (Mancera-Martinez, Brito Querido, Valasek, Simonetti, & Hashem, 2017).

Similarly, **ABCF members** do not have reported transporter roles, instead appearing to participate in the regulation of translation processes (Dean, Hamon, & Chimini, 2001). No diseases have been associated, so far, with either the ABCE or ABCF genes.

The **ABCG subfamily** stands out due to their distinctive domain arrangement, where an N-terminal NBD leads followed by the TMD. This subfamily comprises at least 5 genes that encode reverse “half-transporters”, where four out of five are able to move lipids, sterol and urea across membranes, as well as several drugs (Kerr, Haider, & Gelissen, 2011). The mammalian *ABCG1* gene is involved in cholesterol transport regulation (Klucken et al. 2000). Other *ABCG* genes include *ABCG2*, a drug resistance gene, as well as *ABCG5* and *ABCG8* which code for transporters of sterols in the intestine and liver. Mutations in one or the other of these two genes lead to sitosterolaemia (Shen et al., 2022). ABCG4 functions as a lipid exporter and localizes mainly in the central nervous system (Tarr & Edwards, 2008).

Of note, several ABC transporters, including the ABCB1 transporter (P-glycoprotein) already mentioned above, also play important roles in drug pharmacokinetics (ADME- Absorption, Distribution, Metabolism and Excretion) (Szakacs et al., 2008). Roughly 50% of drug candidates fail to achieve a therapeutical effect due to factors like inadequate absorption and unfavorable metabolism in the organism. ABC transporters significantly influence drug pharmacokinetics,

affecting oral bioavailability, tissue permeation, cellular accumulation, and excretion. They are prominent in critical pharmacological barriers, including the blood-brain barrier and intestinal epithelium. Specifically, ABC transporters like ABCB1, ABCG2, ABCC1, and ABCC2 have dual roles, offering neuroprotection against the drugs they export back to the blood circulation, while restricting their therapeutic entry in the brain, contributing to central nervous system pharmacoresistance (Qosa, Miller, Pasinelli, & Trotti, 2015). These transporters are also present in excretory sites in the liver and kidneys where they contribute to drug elimination from the body, as well as in absorption barriers in intestinal cells, limiting gastrointestinal absorption of many drugs. In essence, ABC transporters must be taken into account in drug development and clinical pharmacology (Szakacs et al., 2008).

Lastly, it is worth noting that several research groups have also highlighted roles of ABC transporters in the process of tumorigenesis. Upon invitation by the Journal Trends in Cancer, we and our colleagues reviewed the published findings on this topic and wrote the attached review article (see **Annex 2**). Hence, we will not develop this part here. However, we will come back to this topic in a later section, when discussing the specific roles of our protein of interest, ABCB5, in tumorigenesis.

2.4 Therapeutic modulation of ABC transporters

ABC transporters have been considered for their potential role in all the phases of cancer development from cancer susceptibility, tumor initiation, tumor progression and metastasis (Nobili et al., 2020). Despite the advances in the development of novel chemotherapeutic drugs, the emergence of multidrug resistance (MDR) in tumor cells, remains a major complex phenomenon in which tumor cells develop resistance to a wide range of anticancer drugs with different chemical structures and mechanism of action (Gottesman et al., 2016).

Diverse strategies for overcoming this adverse phenomenon have been proposed in the last decades. The strategy based on the development of MDR-modulating compounds used as augmenting agents in combination with antitumor therapy was one of the most promising ones. The goal was to block ABC transporters using inhibitors, also known as modulators,

chemosensitizers or reversal agents, depending on their type of action (irreversible, reversible, competitive or noncompetitive) (Borowski, Bontemps-Gracz, & Piwkowska, 2005).

Most of the modulator drugs used for this purpose are basically transport substrates of ABC drug transporters that inhibit efflux from cancer cells of anti-cancer agents in both *in vitro* and *in vivo* systems (Shukla, Ohnuma, & Ambudkar, 2011). Since ABC transporter modulators can be used in combination with chemotherapeutics to increase the effective intracellular concentration of anticancer drugs, the possible effect of modulators of ABC drug transporters has been target of clinical interest. An ideal inhibitor can be effective at low concentration, specific to the ABC transporter, do not have pharmacokinetic interaction with the co-administrated drug, and do not have toxicity. However, as we already mention, ABC transporters have essential physiological roles and their inhibition in normal tissues may affect healthy cells (Shukla et al., 2011).

Over the years, three generations of ABC transporter inhibitors have developed. First-generation inhibitors like verapamil and cyclosporine A had unpredictable side effects. Second-generation inhibitors, like dexverapamil and valspodar, were less toxic but interfered with cytochrome P450 activities. Third-generation inhibitors, like zosuquidar and tariquidar, have shown disappointing clinical benefits (Srivalli & Lakshmi, 2012). More recent inhibitors have been developed from natural sources, through peptides or antibodies, and even using compounds like surfactants and lipids, though non-selective inhibition in non-target organs remains a limiting factor (Binkhathlan & Lavasanifar, 2013).

When looking at the data from clinical trials involving ABC transporter inhibitors, it becomes evident that even with efforts to enhance their specificity for ABCB1 (one of the main ABC transporter responsible for drug resistance) and to make concurrent chemotherapy adjustments, the issue of toxicity remains a substantial challenge. This toxicity issue is likely to overshadow any potential advantages that these ABC transporter-targeting drugs might offer (Shaffer et al., 2012).

2.5 Subcellular trafficking of ABC transporters

Transmembrane proteins are translated by ribosomes located at the surface of several intracellular compartments (mitochondria, peroxisomes and the endoplasmic reticulum), and are concomitantly inserted in their membrane by specific sorting machineries. The proteins synthesized in the endoplasmic reticulum may remain at this site or may be transported to other compartments of the secretory pathway, including the Golgi apparatus, endosomes, lysosomes and the plasma membrane.

For instance, the ABCC7 (CFTR) transporter can reach the plasma membrane after its synthesis in the endoplasmic reticulum, by traveling through the Golgi apparatus (main pathway). This trafficking is notably dependent upon the glycosylation of the protein in the ER, which allows interaction and folding by the calnexin chaperone. This step is critical since failed folding by calnexin (or calreticulin, which plays a similar chaperone role towards glycoproteins) will result in sending the glycoprotein for degradation by proteasome complexes, after its retrotranslocation towards the cytosolic side of the endoplasmic reticulum (Farinha, Matos, & Amaral, 2013; McClure, Barnes, Brodsky, & Sorscher, 2016). Wild-type CFTR that passes the endoplasmic reticulum quality control exits from this compartment and traffics through the Golgi apparatus, where glycan processing occurs. After this point, the mature and fully glycosylated CFTR exits the trans-Golgi network (TGN) in exocytic vesicles, ultimately leading to the insertion of the channel into the plasma membrane (PM) (Farinha et al., 2013).

On the other hand, misfolded proteins are retained in the endoplasmic reticulum by the ER quality control system, and then degraded by the proteasome system, or through autophagy mechanisms including ER-phagy, chaperone-mediated autophagy (mostly for soluble proteins exposing KFERQ motifs when misfolded) and macroautophagy. The latter is predominantly involved in degradation of “aggresomes” resulting from the aggregation of misfolded proteins in the cytosol (Assaye & Gizaw, 2022; Lamark & Johansen, 2012).

Folded CFTR proteins expose a diacidic motif (DAD located in the first NBD), which allows packaging in coat protein II (COPII)-coated vesicles that bud from endoplasmic reticulum exit sites and transport their cargo to the Golgi apparatus, from where CFTR can reach the plasma membrane (Roxo-Rosa et al., 2006). This transport step does not occur for misfolded F508del-CFTR because its misfolding results in the exposure of endoplasmic reticulum retention motifs

(four arginine-rich (RXR) motifs). Of note, it has also been reported that CFTR can reach the cell surface by the GRASP55 pathway, which sends membrane proteins from the ER to the plasma membrane by a process that bypasses the Golgi apparatus (Gee, Noh, Tang, Kim, & Lee, 2011).

Once at the plasma membrane, CFTR will play its role in chloride transport and may, upon time, exhibit signs of unfolding. This will trigger interaction with chaperones, leading to its ubiquitination. Ubiquitinated CFTR molecules will then be endocytosed in a Rab5-dependent manner. Though processes that have not been entirely elucidated, the protein will then either be refolded and recycled to the plasma membrane (a process involving RhoA), of sent for degradation in lysosomes (a process dependent on Rab7). It has notably been shown that Rab4 and Rab27a limit recycling of CFTR to the plasma membrane by maintaining this channel in endosomes (Brusa et al., 2022).

The ABCB9/TAPL protein is also synthesized in the endoplasmic reticulum and has been reported to travel, via the Golgi apparatus and endosomes, to lysosomes. Graab et al. (2019), demonstrated by synchronizing intracellular trafficking using the RUSH assay (Pacheco-Fernandez, Pakdel, & Von Blume, 2021), that TAPL chooses the direct route to lysosomes via the Golgi and early endosomes but not the indirect route via the PM (i.e., it does not go to the plasma membrane before entering the endosomal system leading to lysosomes). Interestingly, the subcellular localization of ABCB9/TAPL depends on its N-terminal transmembrane domain, TMD0, though it lacks conventional lysosomal targeting sequences (such YXXΦ or acidic dileucine motifs of the D/EXXXLL/I-type). Instead, it has been found that charged residues within the TMD0 transmembrane helices play a crucial role in determining the subcellular localization of ABCB9/TAPL. Substitutions of these residues cause retention of the protein in the endoplasmic reticulum or Golgi apparatus (Demirel, Bangert, Tampe, & Abele, 2010; Graab et al., 2019).

TAP1 and TAP2 are subunits of the transporter associated with antigen processing (TAP) complex, responsible for translocating peptides across the endoplasmic reticulum membrane for presentation by MHC class I molecules (Koch et al., 2004). It has been found that the first transmembrane alpha-helices of TAP1 and TAP2 are responsible for endoplasmic reticulum localization, though they do not contain canonical ER-retention motifs (such as di-lysine or di-arginine signals)(Vos, Spee, Momburg, & Neefjes, 1999).

Intriguingly, ABCD4 does not localize in peroxisomes like its homologues ABCD1 and ABCD3. ABCD4 was first reported as an endoplasmic reticulum resident-protein that lacks the NH_2-teminal hydrophobic region that is responsible for targeting to peroxisomes (Kashiwayama et al., 2009). More recently a ABCD4-LMBD1 complex was shown to be involved in the export of cobalamin from lysosomes to the cytosol. As mentioned above, the TAPL protein contains an extra N-terminal transmembrane domain (TMD0) which is essential for the targeting of this ABC transporter to the lysosomal membrane. By contrast, ABCD4 does not possess accessory domains. In this case, the transport of ABCD4 from the endoplasmic reticulum to lysosomes depends on its association with LMBD1. This protein contains a least one functional endocytic motif (YERL) that, once mutated, prevents transport of the ABCD4-LMBD1 complex to lysosomes (Kawaguchi, Okamoto, Morita, & Imanaka, 2016). The complex accumulates at the plasma membrane, suggesting that it travels to this site (at least partly) before its endocytosis and sorting to lysosomes.

Among human ABC transporters that are reported to be in mitochondria, ABCB7, ABCB8, and ABCB10 possess long N-terminal hydrophilic sequences. The number of positive charges in these regions and their length are similar, except for ABCB10, which contains a longer N-ter cytosolic region. It has been proposed that positive charges in such sequences might mask the hydrophobic segments and prevent their recognition for targeting to the endoplasmic reticulum (Miyazaki, Kida, Mihara, & Sakaguchi, 2005). These N-ter sequences also appears to contain mitochondrial targeting information (S. A. Graf, Haigh, Corson, & Shirihai, 2004).

Interestingly, ABCG5 and ABCG8 homodimers are localized to the endoplasmic reticulum. However, when they are expressed together and form a heterodimer, they localize to the plasma membrane. The N-terminal cytoplasmic domains of ABCG5 and ABCG8 are responsible for their retention in the endoplasmic reticulum, though specific motifs have not been identified so far (Hirata, Okabe, Kobayashi, Ueda, & Matsuo, 2009). It is possible that their heterodimerization would mask these motifs. Of note, several membrane proteins that function as a multiple subunit complex are retained in the endoplasmic reticulum until they correctly assemble. Such is the case of ABCC8, ABCC9 and Kir6.x, subunits of K_{atp} channels. They contain endoplasmic reticulum-retrieval (RXR) motifs in their sequences (i.e., sequences that promote their recycling back to endoplasmic reticulum in case their escape to the cis-Golgi). Once ABCC8, ABCC9 and Kir6.x form a mature complex, these motifs are masked,

allowing transport to the plasma membrane where they carry out their function. Otherwise, they are degraded rapidly (Zerangue, Schwappach, Nung Jan, & Yeh Jan, 1999).

3. ABCB5

ABCB5 is an ATP-binding cassette (ABC) transporter that is a member of one of the largest, most ancient superfamilies of proteins found in all living organisms. ABCB5 is predominantly expressed in pigmented cells such as melanocytes and retinal epithelial cells, although the physiological relevance in these cells remains poorly understood (Chen, Valencia, Gillet, Hearing, & Gottesman, 2009).

The *ABCB5* gene is located on chromosome 7p21, has 28 exons, and spans 141.8 kb of the DNA. The origin of ABCB full transporters can be traced back to a single gene, originating from a shared precursor in non-vertebrate organisms, evident in species as ancient as *Caenorhabditis elegans* (*C. elegans*) and yeast. The only non-mammalian *ABCB5* is present in Xenopus, implying the possibility of gene loss within specific lineages (Moitra et al., 2011).

3.1 ABCB5 isoforms

According to the AceView program, which provides a strictly cDNA-supported view of the human transcriptome, *ABCB5* gene transcription gives rise to at least 11 different transcript variants (Thierry-Mieg & Thierry-Mieg, 2006). Among these, three variants have been documented.

- Full length: ABCB5FL; 1257aa (NP_001157413.1), which is a full transporter predominantly expressed in the human prostate and testis (Kawanobe et al., 2012)
- Beta isoform: ABCB5β; 812 aa (NP_848654.3), which was first described by Frank et al. (2003). This is a half-transporter expressed in melanocytes and melanoma.
- Alpha: ABCB5α; 131 aa (NP_001157414.1), which is the smallest predicted protein form (15 kDa), containing one walker B and one C motif which has been hypothesized to have a regulatory rather than a transport role (Chen et al., 2005).

Of these three isoforms (**Figure 9**), the ABCB5β and full-length isoforms have been identified to function as drug efflux pumps by in vitro studies (Kawanobe et al., 2012).

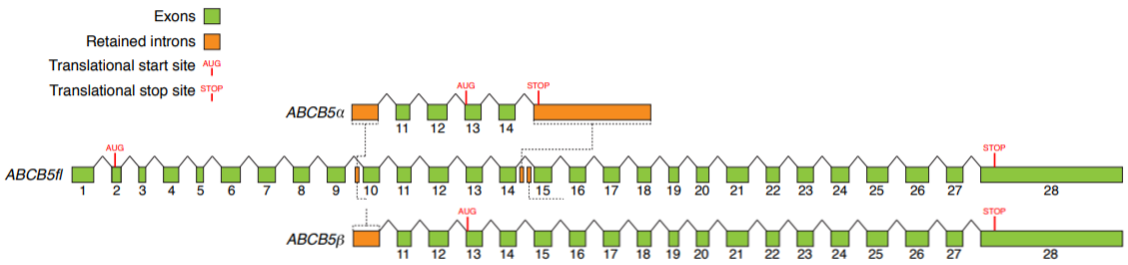

Figure 9. *Exon and intron arrangement of the four ABCB5 isoforms. The model shows the relative sizes of each of the 28 main exons (green) giving rise to ABCB5 FL, and the mixture of introns (orange) and exons that comprise ABCB5α and ABCB5β. Translational start sites are denoted by (AUG), translational stop sites are marked by (STOP). Distance between exons is not indicative of intron length (Braker, 2009).*

3.1.1 ABCB5FL

ABCB5FL has 28 exons that code for a protein of 1257 amino acids (aa). The topology of ABCB5FL is described as the conventional structure of the full transporter; it consists of two TMDs, each of them composed of six α-helices, and two NBDs where binding and hydrolysis of ATP molecules occurs (**Figure 10**). In silico analysis identified 14 putative N-glycosylation sites (not yet validated experimentally) and showed that the full-length isoform has retained the conservative domains common to all ABC transporters throughout the entire mammalian evolution, suggesting that the ABCB5FL isoform corresponds to a functional form of ABCB5 (Moitra et al., 2011). Kawanobe et al. isolated the full-lenght human *ABCB5* cDNA from a human testis cDNA library. They performed an analysis of the tissue expression level of the full-length isoform using specific primers localized in the 5' region of ABCB5FL (not presented in the *ABCB5β* mRNA), they reveal that the ABCB5FL transporter is predominantly expressed in prostate and testis (Kawanobe et al., 2012).

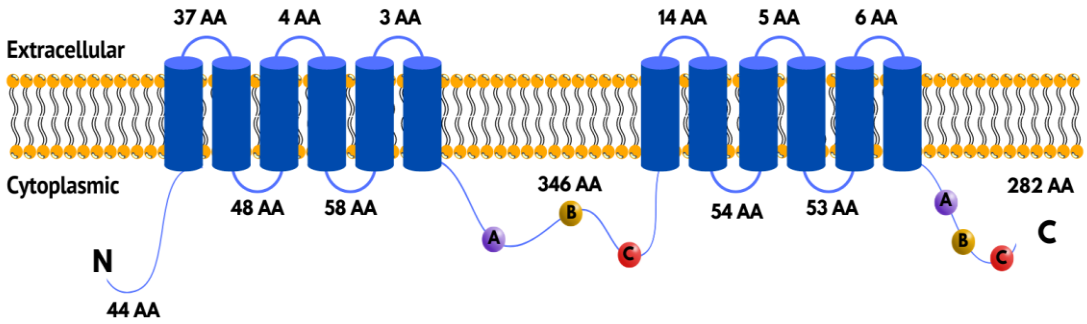

Figure 10. *Predicted structural topology of full-length human ABCB5 isoform. The topology of the full-length isoform is comprised of two TMDs and two cytosolic NBDs interconnected by a linker region.*

P-glycoprotein (Pgp, MDR1, ABCB1) and ABCB5 are close homologues (homology of 73%). Pgp is the most studied transporter that acts as a multidrug resistance mediator in cancer. Tangella and colleagues generated a precise, atom-level model of the complete human ABCB5 transporter, using the crystal structure of the mouse Pgp in its open-inward conformation. This model comprises two TMDs and two NBDs, all adopting an open-inward conformation (**Figure 11**). The transporter composition is divided into two segments: TMD1-NBD1 at the N-terminus (highlighted in yellow in **Figure 11**) and TMD2-NBD2 at the C-terminus (depicted in blue), bridged by a linker region (shaded in green) (Tangella, Arooj, Deplazes, Gray, & Mancera, 2021).

The structural model proposes eight β-sheets in the NBDs, incorporates the signature motif, Walker A and Walker B motif, and predicts twelve α-helices (six in each TMD), consistent with earlier topology predictions. The Walker A motif in the ABCB5 model contains a coiled loop and a glycine-rich α-helix (P-loop), while the Walker B motifs adopt a β-sheet conformation. These motifs are largely conserved between Pgp and ABCB5 in NBD2, but with slight variations compared to NBD1 of ABCB5 (Tangella et al., 2021).

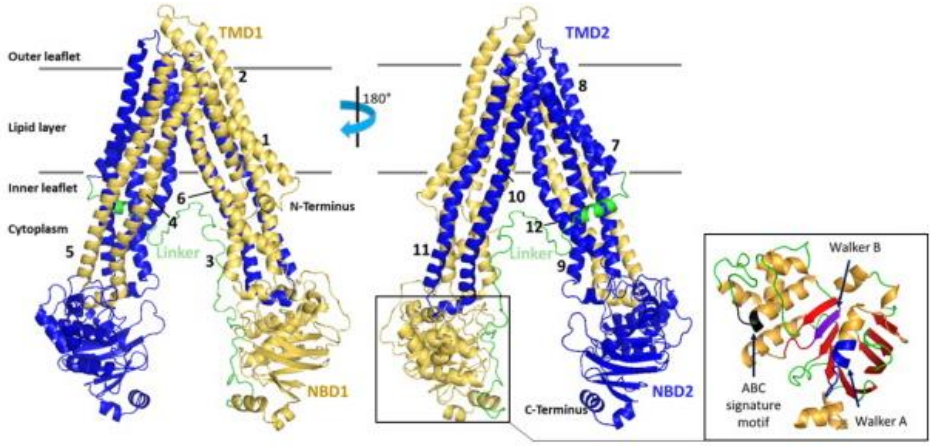

Figure 11. *Structure of the human full-length ABCB5 model. Representative ABCB5 model generated by SWISS-MODEL. The TMD1 and NBD1 are coloured in yellow, TMD2 and NBD2 are coloured in blue. The linker region is coloured in green. The α-helices are shown as cartoons. A close-up view of NBD1 in the box shows the secondary structure of NBD1, comprising of α-helices (golden yellow), β-strands and sheets (red) interconnected by coils (green). The Walker A, Walker B and ABC signature motifs are coloured in blue, purple and black, respectively (Tangella et al., 2021).*

3.1.2 ABCB5α

ABCB5α is produced after a splicing event from intron 9 in ABCB5FL to exons 14 (**Figure 9**). This splicing event creates an mRNA of 2247 nucleotides, which could then be translated to produce a protein of 131 amino acids. The resultant protein would contain the Q-loop, ABC signature motif, Walker B, and D-Loop from NBD1, but it would lack transmembrane helices. ABCB5α has no Walker A consensus sequence and shows 60 to 70% homology with ABCB1. The absence of transmembrane helices renders the protein incapable of functioning as a transporter or even anchoring to a membrane. The potential role of the small predicted protein could be transport regulation, through it remains to be seen (Y. Huang et al., 2004).

3.1.3 ABCB5β

ABCB5β is slightly more than half the size of ABCB5FL. It is encoded by exons 11 to 28 of the full-length isoform. The resulting mRNA sequence encodes an 812 amino acid protein corresponding to a C-terminal segment of the complete/full-length protein. The predicted

topology of the β isoform is composed of one TMD flanked by two NBDs, which is a unique feature for a half transporter. Conventional half-transporters possess only one NBD, either on the N- or on the C-terminal region. Of note, the N-terminal NBD of ABCB5β lacks the Walker A motif, preventing the binding of one ATP molecule. It has been proposed that ABCB5β could potentially establish a dimeric structure to form a functional transporter (**Figure 12**). This is supported by the presence of potential dimerization motifs (i.e. coil-coiled regions) in its N-terminal region (Moitra et al., 2011). The same group has identified six putative N-glycoslytation sites, similar to those found in ABCB5FL.

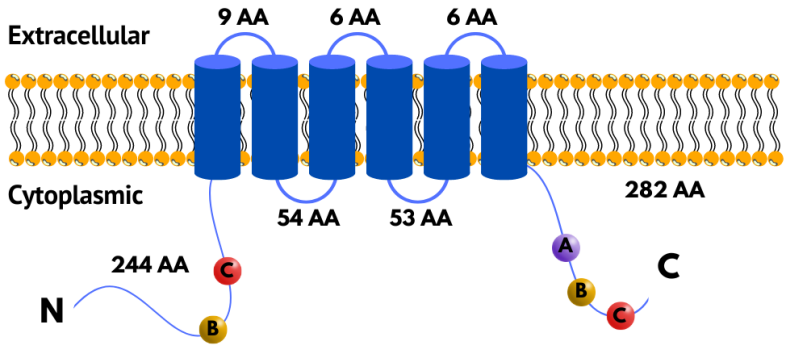

Figure 12. *Predicted structural topology of the human ABCB5β isoform. The proposed model by Moitra et al. (2011) shows a topology of ABCB5β consisting of one TMD with 6 α-helices and two NBD located on the intracellular surface. The walker A is lacking in the NBD located on the N terminal side of ABCB5β.*

3.2 ABCB5 tissue expression pattern

ABCB5 was cloned and characterized for the first time by Frank and colleagues in 2003 (Frank et al., 2003). They amplified a 2906-base-long *ABCB5* cDNA after retrotranscription of mRNAs isolated from primary human epidermal melanocytes or G3361 melanoma cell lines. This sequence encodes the β isoform of *ABCB5*.

Later on, *ABCB5* was found in tissues derived from the neuroectodermal lineage, including melanocyte progenitors (Frank et al., 2005). Additionally, mRNA expression of *ABCB5* was reported in healthy human tissues, such as testis, colon, stomach, mammary gland and retina

tissues. It is important to mention that in this study, the oligonucleotides targeting the *ABCB5* gene used to assess mRNA expression by RT-qPCR amplification, did not allow to distinguish between the *ABCB5FL*, *ABCB5β*, or *ABCB5α* isoforms in any of the analyzed tissues (Frank et al., 2005). Actually, Chen et al. (2005) reported low or no expression of the *ABCB5β* and *ABCB5α* isoforms in normal tissues such as liver, spleen, thymus, kidney, lung, colon, small intestine or placenta. Based on sequence analysis indicating that both *ABCB5β* and *ABCB5α* share common exonic regions (exon 1 to exon 6), they used an oligonucleotide probe (B5P102S) for the detection of both isoforms by Northern blotting with a human tissue mRNA-blot (data not shown). However, this probe is not specific for this two isoforms because they share this region from exon 1 to exon 6 with *ABCB5FL,* which makes it difficult to draw conclusions about the specific expression between the different isoforms. In 2009 and 2012, additional studies showed that the *ABCB5FL* isoform was the one predominantly expressed in prostate and testis (testis-specific; GenBank Accession Numbers AY766239), based on PCR analyses targeting either the 5'UTR region only found in *ABCB5FL*, or 3' UTR region found in both FL and β isoforms. Targeting the 5'UTR region only resulted in amplification in testis and prostate, whereas the second resulted in amplification in many other tissues (Frank & Frank, 2009; Kawanobe et al., 2012).

High expression of *ABCB5* in melanoma cells has been reported multiple times. Many studies mention that ABCB5β and ABCB5α are the most expressed isoforms in melanomas, as FL (which can be identified with primers hybridizing to the first exons, that are not part of the other isoforms) was below detection level in all tested samples (Frank et al., 2005; Frank et al., 2003; Lin et al., 2013; Schatton et al., 2008). An RT-qPCR analysis of all ABC transporter gene expression in the NCI-60 human cancer cell panel also showed that *ABCB5* is selectively expressed in melanoma-derived cells such as UACC-257, SK-MEL-5, MALME-3M, SK-MEL-2, UACC-62 and SK-MEL-28. These are pigmented cell lines. *ABCB5* was also found highly expressed in MDA-MB435 and MDA-N, which are cell lines that were originally thought to originate from breast cancer, but are rather melanoma-derived or at least melanoma-like (Szakacs et al., 2004). Again, this study did not specify which *ABCB5* isoform was detected, but combined with the studies mentioned above (Frank et al., 2005; Frank et al., 2003; Lin et al., 2013; Schatton et al., 2008), in which FL could not be found, it appears likely that they actually focused on the β isoform.

The expression of ABCB5β was assessed later by RT-qPCR amplification in normal pigmented cells (i.e. melanocytes) and one melanoma cell line (UACC-257). The results showed that the level of expression are similar between melanocytes and this melanoma cell, suggesting that the expression is not specific for malignant cells, but specific to pigmented cells (Chen et al., 2005). In the same study, *ABCB5* mRNAs were not detectable in two amelanotic melanomas, M14 and LOX-IMVI, characterized by a deficiency in melanin synthesis (Chen et al., 2005; Szakacs et al., 2004).

Within melanoma subtypes, Vasquez-Moctezuma et al. (2010) documented, using in situ hybridization (ISH) assays and RT-qPCR analysis (which did not distinguish isoforms), that acral melanoma (AM) exhibited a higher and more intense expression than superficial spreading melanoma (SSM). A direct correlation was observed between *ABCB5* mRNA expression and the level of pathogenicity in acral melanoma, whereas intermediate stages of superficial spreading melanoma displayed lower expression (Vasquez-Moctezuma et al., 2010). Notably, *ABCB5* expression in AM cases was over five times greater than that observed in healthy skin (Vasquez-Moctezuma et al., 2010).

Saeed et al. (2022) have also published a recent study reporting tissue expression pattern for ABCB5 at the protein level. The monoclonal antibody (clone 5H3C6; MA5-17026, Thermo Fisher Scientific, Inc), they used for their immunohistochemistry analyses supposedly allows the recognition of ABCB5β and ABCB5FL isoforms, but specificity was not demonstrated. For this study, they included healthy tissues from different species, i.e., *Homo sapiens*, *Mus musculus* (mouse), *Rattus norvegicus* (rat), *Sus scrofa domesticus* (pig), *Gallus gallus* (chicken), *Anser anser* (goose), *Lumbricus terrestris* (earthworm), and *Poecilia reticulata* (Guppy fish). ABCB5 was found in excretory organs of the gastrointestinal tract like the esophagus, stomach, colon, liver, and kidney, raising the possibility that this protein could play a role in excreting molecules, metabolites, xenobiotics and/or toxic compounds from the body. Expression in respiratory organs (bronchi, lung alveoli) could link to detoxification of inhaled xenobiotics and protection against harmful airborne substances (Saeed et al., 2022).

ABCB5 was also observed in pancreas and gallbladder. This suggests secretion of compounds into pancreatic juice and/or bile. ABCB5 expression has also been found in the placenta, uterus, testis, and thyroid. While present in the central nervous system, ABCB5 is absent in brain blood vessels. Instead, it's found in spinal cord neurons. Whether it would play a role in

neurotransmitter transport is an intriguing hypothesis. ABCB5 immunostaining is also observed in organs with smooth and striated muscles like the heart, blood vessels, tongue, and muscle strands (Saeed et al., 2022).

In summary, ABCB5 diverse expression patterns suggest possible involvement in multiple processes, including excretion and detoxification. However, it should be noted that antibody specificity remains questionable (we will discuss this point further in the results section), and that ABCB5 full-length and beta isoforms (i.e. the isoforms with putative transport activity) are rarely distinguished in these published studies, preventing the differentiation of expression patterns, and possibly functions, between the two (Duvivier & Gillet, 2022). Nevertheless, several groups have started to gather functional data about ABCB5, as we will now present.

3.3 ABCB5 in normal and cancer stem cells (CSCs)

3.3.1 ABCB5 as a marker of stem cells found within melanocytes and melanoma cancer cell populations

Stem cells are characterized by the capacity for self-renewal and the ability to differentiate into diverse specialized cell types. Cancer Stem Cells (CSCs) are a small subpopulation of cells within tumors with capabilities of self-renewal, differentiation, and tumorigenicity (Yu, Pestell, Lisanti, & Pestell, 2012). Initially, Frank et al., described in 2003 that 56% of $CD133^+$ expressing Human epidermal melanocytes (HEM), isolated by flow cytometry also co-express *ABCB5*. It marks a strong enrichment compared to total HEM, in which only 11% of the cells expressed ABCB5. Based on the cDNA extracted from these cells, they postulated that they express the ABCB5β isoform. As $CD133^+$ is a progenitor cell marker, it led them to suggest that ABCB5β might also mark a specific subset of progenitor cells and could act as a negative regulator of cell differentiation. The study also indicated that 13% of melanoma cells resistant to cisplatin (G3361/CDDP) express *ABCB5*, compared to the 3% of the parental cell line that express it. This suggests that *ABCB5* may confer chemoresistance, possibly by promoting stemness (Frank et al., 2003).

Following this initial report, a significant part of the research has focused around the expression of *ABCB5* as marker for cancer stem cells (CSCs) in various tumors including malignant melanoma (Schatton et al., 2008), colorectal cancer (Wilson et al., 2011), malignant pleural mesothelioma (Milosevic et al., 2020), hepatocellular carcinoma (Cheung, Cheung, Cheng, Wong, & Fan, 2011), breast cancer (Yang, Ha, Yang, & Kim, 2010), glioblastoma (Lee et al., 2020), head and neck, oral squamous cell and merkel cell carcinomas (Grimm et al., 2012; Jongkhajornpong et al., 2016).

In melanoma Fang et al. (2005), ABCB5β expression is particularly elevated in malignant melanoma initiating cells (MMICs). These MMICs are malignant stem cell subpopulations in which clinical virulence is a consequence of unlimited self-renewal capacity, leading to inevitable tumor progression and metastasis (Wilson et al., 2014). ABCB5β+ MMICs were found to have the ability to evade the host anti-tumor immune response, thereby promoting tumor growth (Schatton & Frank, 2009). These MMICs have also been found to circulate in the peripheral blood of melanoma patients, associating the presence of MMICs with disease recurrence and progression (Reid et al., 2013).

3.3.2 ABCB5 as a marker of dermal mesenchymal stem cells

ABCB5 has also been described as a surface marker (based on flow cytometry and immunofluorescence analyses) for the identification of a novel subset of dermal Mesenchymal stem cells (MSCs) (Schatton et al., 2015). MSCs are stromal cells that have the ability to self-renew and also exhibit multilineage differentiation. MSCs can be isolated from a variety of tissues, such as umbilical cord, endometrial polyps, menses blood, bone marrow, adipose tissue, etc. (Ballikaya et al., 2020). The isolated ABCB5+ MSC subpopulation demonstrates the ability for clonal self-renewal and differentiation in vitro. Injecting these ABCB5+ MSCs near skin wounds can shift pro-inflammatory M1 macrophages to anti-inflammatory wound-healing M2 macrophages at the wound site, through paracrine IL-1RA release, thereby accelerating healing of chronic wounds in humans (Vander Beken et al., 2019).

3.3.3 ABCB5 as a marker of limbal stem cells

As ABCB5 was proposed to be a marker of skin progenitor cells, a subsequent study hypothesizes that ABCB5 might also identify slow cycling limbal stem cells (LSC). Limbal stem cells represent a quiescent cell population with proliferative capacity residing in the basal epithelial layer of the limbus within a cellular niche. In addition to LSC, this niche consists of various cell populations such as limbal stromal fibroblasts, melanocytes and immune cells as well as a basement membrane, all of which are essential for LSC maintenance and LSC-driven regeneration (Gonzalez, Sasamoto, Ksander, Frank, & Frank, 2018).

Bromodeoxyuridine (BrdU)-based 'pulse-chase' experiments on mice identified slow cycling cells in mouse limbus, and these cells were found to express ABCB5. More specifically, ABCB5 was found preferentially expressed in LSC (limbal stem cells). Thus, ABCB5 requirement for LSC maintenance was investigated. An C57BL/6 *Abcb5* knockout mouse that lacks exon 10 of the murine gene was generated (GenBank accession number JQ655148). Abcb5 protein loss was demonstrated by western blotting using monoclonal antibodies targeting either the region that was lost after KO, a N-terminal section, or a loop region within the Abcb5 sequence. Although the molecular weight was not shown (preventing verification of ABCB5 size and discrimination between FL and β isoforms), a decrease of signal intensity was observed on these blots. Interestingly, this loss caused, according to flow cytometry, IHC and IF analyses, a depletion of quiescent Limbal Stem Cells (LSC) in the retina. It was proposed that this results from enhanced proliferation, and thus differentiation of these cells (loss of their stemness phenotype). Lastly, it was found that this loss of LSC explains the defective corneal differentiation in the KO mice (decreased cellularity within the apical epithelial layer, disorganization within the basal and wing cell layers, and decrease number of epithelial cells in the central cornea). This was accompanied by defective wound healing after debriding of a central area in the cornea, demonstrating that Abcb5 expressed in LSC is required for LSC functions, including tissue regeneration (Ksander et al., 2014).

In the same study, expression of Abcb5 in mouse retinal pigment epithelium progenitor cells (RPE) was also assessed by IHC and flow cytometry. They found a small Abcb5+ subpopulation among those cells (2-8% of total RPE cells). After KO of *Abcb5*, there was an age-related loss of the normal RPE morphology, including a significant thinning and attenuation of overlaying

photoreceptor outer and inner segments, which was associated with a loss of cells in the outer nuclear layer. Moreover, RPE cells were found enlarged and distended due to the accumulation of cytoplasmic vacuoles, which may suggest an intracellular role of Abcb5. Hence, Abcb5+ RPE cells appear required to maintain the normal morphology of the RPE layers (Ksander et al., 2014; Ksander et al., 2015). Of note, these *Abcb5* KO mice did not exhibit any other detectable/obvious phenotypes. They could not be distinguished from their wild-type littermates based on physical examination as they matured into adulthood (Ksander et al., 2014).

3.3.4 Mechanism of stemness regulation by ABCB5

Wilson et al. postulated that ABCB5 might maintain MMIC (melanoma stem cells) stemness (i.e. non differentiated state), by acting in IL-1β secretion (Wilson et al., 2014). Indeed, among genes upregulated after knock-down of *ABCB5* in these cells, was the whey acidic protein 4-disulphide core domain 1 (WFDC1, tumor suppressor expressed in differentiated melanocytes), while IL8 (a cytokine that promotes tumorigenesis) was among the most downregulated genes. It was confirmed with an ELISA test that IL8 secretion is also decreased. WFDC1 is a known repressor of the WNT signaling in melanoma, and IL8 is actually a target gene of WNT. Hence, this group progressively uncovered that ABCB5 is a negative regulator of WFDC1, that controls IL8 expression via the WNT pathway. Furthermore, the authors identified the presence of IL8 receptors (CXCR1) on the MIMIC cells and demonstrated that IL8 secretion in ABCB5 expressing cells can promote their stemness via the activation of this receptor, i.e. they remain slow cycling (**Figure 13**) (Wilson et al., 2014).

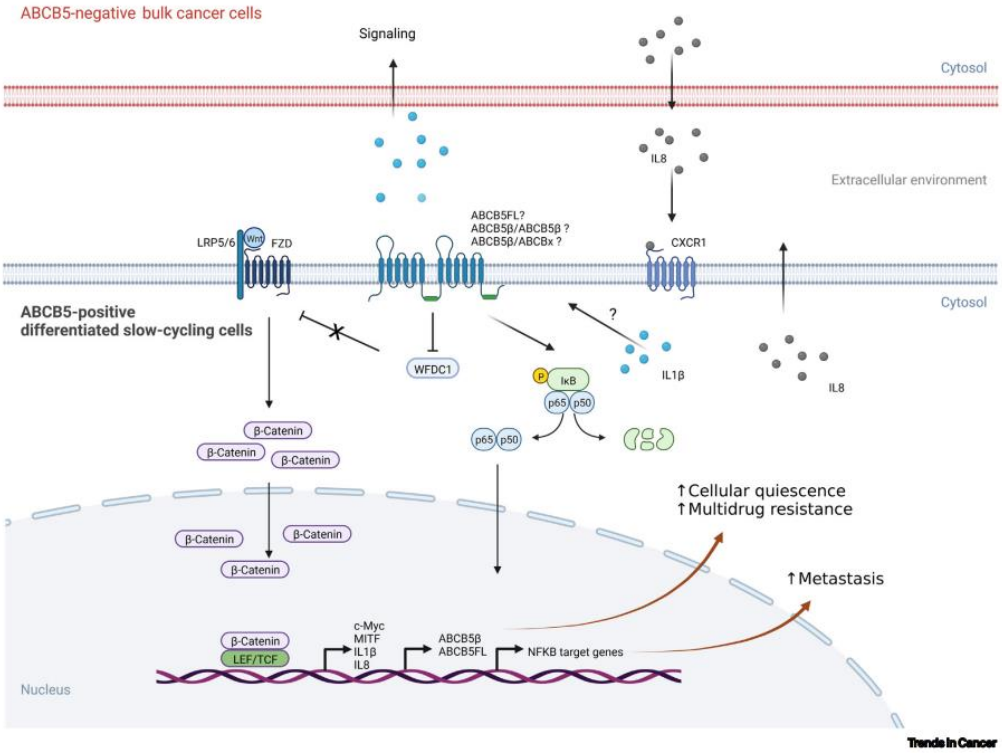

Figure 13. *A proposed working model to illustrate ABCB5-regulated signaling pathways in human melanoma. In the proposed model ABCB5 identifies differentiated slow-cycling cells and it is a putative resident of the plasma membrane. The ABCB5 transporter (ABCB5FL, ABCB5β homodimer, or ABCB5β/ABCBx heterodimer) downregulates WFDC1, indirectly suppressing the WNT pathway by elevating Dickkopf-1 (DKK1). This inhibition prevents β-catenin proteasomal degradation by initiating disassembly of the β-catenin destruction complex through WNT binding to LRP5/6 and FZD receptors. Stabilized β-catenin then accumulates in the cytoplasm and migrates to the nucleus, binding to LEF/TCF proteins to regulate WNT target gene expression, including c-MYC, MITF, interleukin (IL)1β, and IL8 (Duvivier & Gillet, 2022).*

Interestingly, Milosevic et al. (2020) also showed that ABCB5 controls the secretion of IL-1β, which serves to maintain the undifferentiated phenotype of malignant pleural mesothelioma (MPM). Indeed, KO-ABCB5-AC (adherent cells) clones exhibited reduced stemness characteristics despite being grown in a medium that normally supports stem cell growth. They also found that ABCB5 is transcriptionally activated by the Wnt/GSK3β/β-catenin/c-myc axis and also that IL-8/c-myc and IL-1β/c-myc axis upregulate ABCB5 (Milosevic et al., 2020). In contrast to data previously shown by Wilson et al. (2014) in melanoma, where that requires a paracrine cooperation between ABCB5-positive and ABCB5-negative cells to maintain high

levels of IL-1β and IL-8, and chemoresistance (Wilson et al., 2014), MPM IC adopted a completely autocrine system. ABCB5-KO clones had reduced secretion of IL-8 and IL-1β, suggesting that ABCB5 controls the secretion of both cytokines, and reduced binding of c-myc to the ABCB5 promoter, which was restored by exogenous IL-8 and IL-1β (Milosevic et al., 2020).

3.4 Role of ABCB5 in cancer development and progression

Melanoma has the highest mutation rate of all common cancers (Lawrence et al., 2013). *ABCB5* was found to exhibit high mutation burdens in melanoma. Sana et al. found 109 mutations within coding sequences, in 88 out of 640 clinical melanoma samples (13.75%). These mutations comprise 77 missense, 10 nonsense, 1 splice-site variant, and 21 silent mutations, which were localized in regions coding for nucleotide-binding domains and the second transmembrane domain. *ABCB5* mutations were found in samples carrying NRAS, CDKN2A, or BRAF mutations in 75%, 62.5%, and 25% of the samples analyzed, respectively, but not in samples harboring PTEN mutations. Examination of four melanoma cell lines with diverse genetic profiles revealed an increase in proliferation and enhanced migration abilities in cells expressing mutant ABCB5FL. This implies that ABCB5FL may function as a tumor-suppressor gene in melanoma development (Sana et al., 2019). Of note, the deleterious effect of four representative mutations in *ABCB5* (one nonsense and three missense) on transporter activity was demonstrated (Sana et al., 2019).

In contrast, ABCB5β has demonstrated oncogenic behavior. Establishing stable knockdown of ABCB5 in two melanoma cell lines led to a notable reduction in their proliferation compared to the ABCB5-expressing counterparts (Wilson et al., 2014). In vitro experiments conducted by Wang et al. (2017), indicated that the $ABCB5^{+}$ in melanoma population exhibited greater cell migration and invasion when compared to the $ABCB5^{-}$ group. ABCB5-positive melanoma-initiating cells (MMICs) exhibited a higher metastatic potential compared to their ABCB5-negative counterparts. Silencing ABCB5 led to a reduction in melanoma cell migration and invasion in vitro, along with decreased pulmonary metastasis in tumor xenograft mice. Furthermore, it was revealed that ABCB5 promotes melanoma metastasis by activating the

nuclear factor kappa B (NF-κB) pathway through the inhibition of p65 ubiquitination, which results in increased p65 protein stability in melanoma tissues and cell lines. This correlation between ABCB5 and NF-kB p65 expression was evident in both melanoma tissues and cell lines(Wang et al., 2017).

Lin et al. (2013), identified three SNPs in the *ABCB5* gene associated with melanoma risk and showed that one of them results in a nonsynonymous ABCB5 amino acid change (K115E, within the FL isoform). This variant genotype decreased melanoma risk associated with lower ABCB5 protein function, which was concluded based on a reduction of rhodamine efflux measured by flow cytometry. This suggests a molecular role of ABCB5 in driving tumorigenic growth (Wilson et al., 2011). Actually, the ABCB5 K115E polymorphism was associated with human pigmentation phenotype and melanoma risk and point to potential functional roles of ABCB5 in melanomagenesis (Lin et al., 2013).

There is little mechanistic insight into the role of ABCB5 in cancer cell proliferation reported in the literature, but some evidence can be found. For instance, the bioinformatic analysis between ABCB5 expression and melanoma phenotype RNA expression in melanoma cell lines from the Cancer Cell Line Encyclopedia (CCLE) database revealed a strong association between *MITF* expression and *ABCB5* expression at the mRNA level. Specifically, *ABCB5* exhibited higher expression in cell lines with elevated MITF levels and a proliferative gene expression signature. Given the role of MITF in promoting melanoma differentiation and proliferation (Kawakami & Fisher, 2017), this study also demonstrated that both MITF and β-catenin contribute to the upregulation of ABCB5, which is associated with melanoma cells displaying a more differentiated phenotype (Louphrasitthiphol, Chauhan, & Goding, 2020). Additionally, a correlation between ABCB5 and CD133 expression was found in the TCGA melanoma cohort, particularly in slow-cycling $MITF^{High}$ tumor-initiating cells with a partially differentiated phenotype. Most importantly, they are intrinsically resistant to conventional treatments and retain the ability to undergo self-renewal (Louphrasitthiphol et al., 2020).

Aside from melanogenesis, Grimm et al. (2012) have shown that ABCB5 expression is associated with tumor progression and metastasis in human oral squamous cell carcinoma (OSCC). The patients with positive ABCB5 expression in their OSCC have a survival rate lower in comparison to the subgroup of patients with low ABCB5 expression. This simple distinction may guide ABCB5 as a biomarker in the clinic (Grimm et al., 2012).

Lastly, epithelial–mesenchymal transition (EMT) has been shown to play a critical role in enabling cancer tissue invasion through transitioning from a less aggressive, epithelial, to a more aggressive, mesenchymal, phenotype (Mani et al., 2008). ABCB5 was found to promote colorectal cancer (CRC) cell invasion through maintenance of the mesenchymal phenotype (Guo et al., 2018). More specifically, it was shown that the invasion potential (within the vascular system of a xenograft mouse model), was increased for cells positive for ABCB5. Similar findings were also shown in breast cancer where ABCB5 contribution to oncogenesis has also been outlined. The study revealed that *ABCB5* expression is higher in metastatic tissues compared to healthy tissues. Overexpression of ABCB5 in MCF7 breast cancer cells, increased their migration and invasion potential. By contrast, silencing of *ABCB5* in the breast cancer cell line BT549 (an aggressive cell line), a decrease in cell migration, invasion and fewer metastatic tumor were observed in mice injected with these cells. This effect was found mediated by a downstream factor, zinc finger e-box binding homeobox 1 (ZEB1), which is upregulated in *ABCB5* knocked-down cells according to a microarray analysis. Knocking down *ZEB1* had similar effects on tumorigenicity compared to knocking down *ABCB5*, increasing the metastatic capacity. Interestingly, while the short hairpin RNAs (shRNAs) targeted both *ABCB5FL* and *ABCB5β* mRNAs, it was found that the resulting decrease in cancer cell "aggressivity" could be rescued by ABCB5β overexpression, indicating that the ABCB5β isoform is the one that has a oncogenic activity (Yao et al., 2017).

3.5 ABCB5 and multidrug resistance (MDR)

Given their homology with other ABCB family transporters, especially ABCB1, there was a postulation that ABCB5FL and/or ABCB5β might be able to transport chemotherapeutic substrates, with ABCB5β potentially forming dimers to create a complete transporter, as proposed by Moitra et al. (2011). In support of this idea, *ABCB5* expression has been shown to exhibit statistically significant and strong positive correlations with drug resistance (towards paclitaxel, teniposide, Vinka alkaloids, docetaxel, etoposide, doxorubicin and vincristine) in melanoma and other malignancies (Wilson et al., 2014).

Another study was conducted using MCF-7 cells (i.e. breast cancer cells) transfected with ABCB5β cDNA, and G3361 melanoma cells, which naturally express ABCB5β but lack ABCB5FL (as determined by antibody antigen) (Frank et al., 2003). The authors found an elevated uptake of doxorubicin in ABCB5β $^{+}$ in G3361 melanoma cells. This transport appeared driven by membrane hyperpolarization, as evidenced by the reduction in early doxorubicin uptake upon membrane depolarization. These findings align with prior research that established a correlation between intracellular doxorubicin accumulation may vary with membrane potential, though ABCB5 involvement was not identified at the time (Frank et al., 2005). It is noteworthy that hyperpolarization induced by ABCB5 triggers a significant initial uptake of doxorubicin, while the use of a membrane depolarizing agent like KCl eliminates it. The positive charge of doxorubicin may explain this, as it is attracted to the negatively charged interior of cells.

After downregulation of ABCB5 by using siRNAs in SK-MEL-28 (using siRNAs sequences that do not discriminate between FL, β and α isoforms), the cells were 2–3 times more sensitive to several drugs, including camptothecin, 10-OH camptothecin, and 5-fluorouracil (5FU), compared to control cells transfected with non-targeting siRNAs (Y. Huang et al., 2004). These results support the hypothesis that ABCB5 represents a novel chemoresistance gene. Yet, whether the resistance process involves enhanced drug efflux or another mechanism, remains to be determined (Y. Huang et al., 2004).

The specific function of the ABCB5FL isoform in multidrug resistance was initially investigated in transfected HEK-293 (transfection of this selected isoform). The cells showed 1.5-times increase of resistance to doxorubicin, a 2.3-times increase of resistance to paclitaxel, and threefold increase in resistance to docetaxel compared with ABCB5FL non-expressing cells. This transporter was also shown to increase 1.2- to 1.5-times the resistance to daunorubicin, vincristine, etoposide, and actinomycin D but did not mediate resistance to methotrexate or 5-fluorouracil (Kawanobe et al., 2012).

Using a Saccharomyces cerevisiae model in which ABCB5FL and ABCB5β were exogenously expressed separately, it was found that, despite ABCB5β being more abundantly expressed compared to the FL isoform, only the latter conferred resistance to rhodamine 123, daunorubicin, tetramethylrhodamine, tacrolimus, and clorgyline in this model (Keniya et al., 2014).

Taken together, the data highlight that ABCB5FL can confer multidrug resistance. Whether the β isoform is also involved in this process is less clear but not excluded, considering that in the G3361 melanoma cells, which exhibit ABCB5-dependent resistance to doxorubicin, ABCB5β is the predominantly expressed isoform (Chen et al., 2005). However, where they function in the cells remains elusive (whether plasma membrane, Lysosomes, or another compartment).

3.6 Subcellular localization of ABCB5: an open question

To date, the intracellular localization of ABCB5 remains controversial. Moitra et al. (2011) detected the presence of several putative phosphorylation and glycosylation sites, which may be important for targeting of the protein to a membrane organelle. However, this was never demonstrated.

Actually, there are few publications in the literature that address the possible subcellular localization of ABCB5. Using flow cytometry, Frank et al. (2003) found expression of ABCB5β at the plasma membrane in a subpopulation of human epidermal melanocytes (HEM) that co-express the CD133 progenitor phenotype marker. They also documented an intracellular staining using the same antibody when the permeabilised cells were examined by immunofluorescence microscopy, which suggested that a significant portion of the protein could be located intracellularly. For both analyses, they used a mouse antibody directed against a 16-mer sequence located in a predicted extracellular loop of ABCB5. A western blotting was conducted as well, showing a band around 90kDa (i.e., the theoretical molecular mass of the β isoform) in several cancer cell lines. A positive control was included (cells overexpressing ABCB5). However, there was no negative control shown (no knock-down of knock-out of *ABCB5* to check antibody specificity).

Similar distribution patterns have been shown in other studies when they are using immunostaining (IF), which further supports the hypothesis of an intracellular distribution of ABCB5 to some extent in malignant melanoma cells (Linley et al., 2012; Mathieu et al., 2014).

It should be noted, however, that the research of Louphrasitthiphol et al. (2020), as well as our own findings (as will be presented in the results section), raise questions regarding the ability of commercial antibodies to detect ABCB5 expressed at an endogenous level. The same

concern was also expressed by Chen et al. (2009). They could not identify specific anti-ABCB5 antibodies, preventing them from assessing ABCB5 expression at the protein level in melanoma cells, and to discriminate between ABCB5 isoforms.

None of the studies available in the literature have confirmed the presumed localization of ABCB5 at the plasma membrane or conducted comprehensive research to detect its subcellular localization. Hence, the exact localization, as well as the physiological roles, of the two ABCB5 transporter isoforms (FL and β) remain elusive. It is worth considering that they could possess distinct subcellular localizations and that they could potentially be present in multiple compartments. Indeed, the two isoforms appear to differ in their tumorigenic activities and involvement in drug transport, as discussed above (Duvivier & Gillet, 2022).

OBJECTIVES OF THE THESIS

ABCB5β has attracted substantial attention due to its roles in promoting proliferation, metastasis, and invasive capacities in melanoma cells. Nevertheless, our understanding of this protein remains limited, and several unresolved questions persist regarding its localization, function, and mechanisms of action.

The primary objective of this thesis was the characterization of the subcellular localization of the ABCB5β isoform, as a first step towards the elucidation of its function. To accomplish this, our strategy included testing anti-ABCB5 antibodies to detect the protein endogenously expressed in MelJuSo (melanoma) cell line. We also generated multiple ABCB5β cDNA constructs with different tags either in N- and C- term position to perform subcellular fractionation experiments and a series of immunofluorescence analyses to explore the localization of ABCB5β within various cellular compartments in two different cells models, HeLa (cervical cancer) and MelJuSo cell lines.

On the side, following the validation of *ABCB5* knock-down (KD) in MelJuSo cells, we worked on a secondary objective which was to gather functional information through a search for candidate genes that could exhibit decreased or increased expression upon *ABCB5* KD. This exploration was related to several pathways (including cell cycle regulation, Interleukins, Wnt pathway and transcriptions factors), based on the current literature pointing out a putative involvement of ABCB5 in these processes. We also assessed the expression of some of these candidates at protein level. Lastly, we determined whether *ABCB5* knock-down affects MelJuSo cell proliferation.

MATERIAL AND METHODS

1. Cell culture

The human melanoma cell line MelJuSo (cutaneous melanoma, CVCL_1403), HeLa cells (human cervix adenocarcinoma, ATCC® CCL-2) and HEK293T (human embryonic kidney, ATCC® CRL-3216™), were grown at 37 °C under 5% CO_2 in high-glucose DMEM containing L-glutamine and sodium pyruvate (VWR, 392-0416), supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin (Lonza, DE17-602E).

2. Transfection and treatments

Cells were transfected with a mix containing Opti-Mem (Gibco, 31985062) or serum-free medium, plasmid and FuGENE® HD Transfection Reagent (Promega, E2311) for HeLa cells or jetPRIME® (Polyplus, #114-15) for MelJuSo cells according to the manufacturer's instructions. Experiments were conducted 24 or 48 h after transfection (as indicated in Figure legends). The inhibition of the proteasome was achieved by treating cells with 1 µM of MG132 (Sigma Aldrich, M7449) during 16h.

After 48h of transfection with ABCB5β-mCherry, HEK293T were treated during 16h either with bafilomycin (15nM), U18666A (1 µg/ml) and vehicle DMSO (Sigma Aldrich, 200-664-3), prior to fractionation experiments.

Transfected Hela cells were treated overnight with 2.5 or 5 µM Suberoylanilide Hydroxamic Acid-SAHA (Cayman chemicals, #10009929), before western blotting or immunofluorescence experiments.

3. Antibodies

Primary and secondary antibodies used for western blotting and immunofluorescence experiments are described in **Table 2**.

Antibodies for western blotting				
Antibodies	**Species**	**Clonality Source**	**Working Dilution**	**Source/Reference**
Anti-ABCB5	Rabbit	Polyclonal	1:500	Rockland Immunochemicals; 600-401-A775
Anti-ABCB5	Rabbit	Polyclonal	1:100	Abcam; ab80108
Anti-ABCB5	Rabbit	Polyclonal	1:500	Atlas antibodies; HPA026975
Anti-GFP	Goat	Polyclonal	1:1000	Rockland; 600-101-215
Anti-HA (6E2)	Mouse	Monoclonal	1:100	Cell signaling; #2367
Anti- calnexin	Rabbit	Polyclonal	1:1000	Abcam; ab22595
Anti-tdTomato	Goat	Polyclonal	1:1000	Origene; AB8181-200
Anti-α-Tubulin	Mouse	Monoclonal	1:1000	Sigma; T5168
Anti-p21 Waf1/Cip1(12D1)	Rabbit	Monoclonal	1:1000	Cell signaling; 2947
Anti-p27 Kip1 (D69C12)	Rabbit	Monoclonal	1:1000	Cell signaling; 3686
Anti-Rb (4H1)	Mouse	monoclonal	1:2000	Cell signaling; #9309
Anti phosphor-Rb (Ser807/811) (D20B12)	Rabbit	Monoclonal	1:1000	Cell signaling; #8516S
Anti-CDK2 (78B2)	Rabbit	Monoclonal	1:1000	Cell signaling; #2546S
Anti-β-Catenin [15B8]	Mouse	monoclonal	1:1000	Abcam; ab6301
Anti-MITF [D5]	Mouse	monoclonal	1:1000	Abcam; ab3201
Anti-NFKβ-p65 (D14E12)	Rabbit	Monoclonal	1:1000	Cell signaling; #8242S
Anti-p44/42 MAPK (Erk1/2)	Rabbit	Polyclonal	1:1000	Cell signaling; #9102S
Anti-Anti-Melanoma 100gp [EP4863(2)]	Rabbit	Monoclonal	1:1000	Abcam; ab137078
Secondary antibodies for western blotting				
IRDye 680RD donkey	Mouse	Polyclonal	1:10000	Li-cor Biosciences; 926-68072

IRDye 680RD donkey	Rabbit	Polyclonal	1:10000	Li-cor Biosciences; 926-68071
IRDye 800CW donkey	Goat	Polyclonal	1:10000	Li-corBiosciences; 926-680724
Primary antibodies for immunofluorescence				
Anti-ABCB5	Rabbit	Polyclonal	1:100	Rockland Immunochemicals; 600-401-A775
Anti-HA (C29F4)	Rabbit	Monoclonal	1:100	Cell signaling; #3724
Anti-HA (6E2)	Mouse	Monoclonal	1:100	Cell signaling; #2367
Anti-calnexin	Rabbit	Polyclonal	1:100	Abcam, ab22595
Anti-GM130 [EP892Y]	Mouse	Monoclonal	1:50	Abcam, ab52649
Anti-TGN46	Rabbit	Polyclonal	1:100	ProteinTech, 13573-1-AP
Anti-LAMP1 [H4A3]	Mouse	Monoclonal	1:50	DSHB, AB_2296838
Anti-melanoma [HMB45+M2-7C10+M2-9E3]	Mouse	Monoclonal	1:50	Abcam, ab732
Secondary antibodies for immunofluorescence				
Alexa Fluor™ 568 goat	Mouse	Polyclonal	1:250	Invitrogen, A11004
Alexa Fluor 594™ donkey	Rabbit	Polyclonal	1:250	Invitrogen, A21207
Alexa Fluor 488™ goat	Rabbit	Polyclonal	1:250	Invitrogen, A11034

Table 2. *List of primary and secondary antibodies used for western blotting and immunofluorescence experiments. Sources and working dilutions are listed.*

4. Western blotting

Cell lysates prepared in RIPA buffer (10mM Tris-HCL, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140mM NaCl, 1x complete Mini protease inhibitors from Merck, P5726) were mixed with Laemmli's sample buffer (with DTT), heated for 30 min at 37°C, and resolved in 8% SDS polyacrylamide gel. Proteins were resolved using a 1x migration buffer, diluted from a stock of Migration buffer 10x (250mM TRIS-HCl; 1,92M glycine, 1% SDS and H_2O, pH8.3). After migration, proteins were transferred onto PVDF membranes (Immobilon®-FL) that has been previously hydrated with methanol, washed with H_2O and incubated in transfer buffer (20mM TRIS-base, 150mM glycine, H_2O and 20% of methanol).

The membrane containing the proteins was then incubated in 5% free fat milk diluted in PBS-Tween 0.1% prior to detection of the proteins of interests using antibodies and dilutions listed above (in **Table 2**). After an incubation overnight (O/N) with the primary antibody, the membrane was washed in PBS-Tween 0.1% (5 times for 10 min) and incubated with the secondary antibody for 45 min. Infrared signals were detected using an Odyssey infrared imaging system (LI-COR Biosciences, USA). PageRuler™ Prestained Protein Ladder was purchased from Thermo Fisher Scientific (Cat. 26617).

5. Immunofluorescence

Cells were grown on round glass coverslips placed in 12-well plates. The cells were washed 3 times with Phosphate Buffer Saline (PBS) and then fixed in 4% paraformaldehyde in PBS for 10 min at room temperature (RT). After 3 washes, cells were permeabilized with a solution of 0.2% Triton and 1% BSA (Bovine Serum Albumin) in PBS for 10 min and blocked with 3% BSA for 20 min. Next, the cells were incubated with the primary antibody diluted in BSA solution for 1h at RT (dilutions are documented in **Table 2**). After 3 washes, cells were incubated with secondary antibodies diluted in BSA solution for 45 min at RT. Fixed samples were imaged with a Leica SP5 confocal microscopy, using a 40x or 63x objective (1.3 and 1.4 numerical aperture, respectively) or LSM 900 confocal microscope equipped with an Airyscan detector and with a Plan Apo 63× numerical aperture (NA) 1.4 oil immersion objective.

6. Image analyses and quantifications

All image quantifications were performed using (Fiji Is Just) ImageJ 1;53t (NIH). Quantification of co-localization between two channels was performed using the JACoP plugin in ImageJ software (Cordelieres & Bolte, 2006). After setting a threshold for the signal of interest for each channel, the JACoP plugin was used to obtain the Manders' coefficient and calculate the co-localization.

7. RNA interference

To silence *ABCB5* expression we purchased ONTARGETplus human ABCB5 siRNA SMARTpool from Dharmacon Inc. (Dharmacon™, Horizon Discovery Ltd; L-007303-01-0020). We conducted reverse transfection with Lipofectamine™ RNAiMAX Transfection Reagent according to the manufacturer's instructions. ON-TARGET plus non-targeting pool (Dharmacon™; D-001810-10-20) served as a reference point. We used a final concentration of 90nM. Proteins and mRNA were collected from 24h up to 96h post-transfection.

8. RT-qPCR

Total RNA was isolated using NucleoSpin RNA plus kit (Machenerey-Nagel, #740984.50). The quantity and quality of extracted RNA were assessed by spectrophotometry using the NanoDrop™ One/One^{C} Microvolume UV-Vis (Thermo Scientific™ Acclaro™). Retro-transcription of mRNAs into cDNAs was conducted with RevertAid Minus First Strand cDNA synthesis kit (Thermo scientific, #K1631).

Primers for qPCR were designed to hybridize sequences found in *ABCB5β* (i.e. in the second half of the *ABCB5FL* sequence), excluding detection of the rest of the *ABCB5* isoforms. Primer sequences are shown in **Table 3**. Gene expression was determined using Takyon NoRox Sybr Mastermix Blue (Eurogentec, UF-RTAD-D0701) according to the manufacturer's recommendations on a BioRad CFX96 system (BioRad). Thermocycling conditions were: 10 min 95 °C, and followed by 40 cycles of amplification (95 °C for 10 s, 60 °C for 10 s and 72 °C for 10 s. To normalize the expression levels, Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as a housekeeping gene.

The primers used to analyze the expression of genes coding for Interleukins, Retinoblastoma (Rb), Wnt proteins (WNT), NF-KB, other ABC transporters, etc., after knock-down of *ABCB5* are presented in **Table 3**.

Target	Primer	Sequence (5’ - 3’)
ABCB5	F	GCAGATTTGATTGTGACCCT
	R	GACTCCATCTGTTCATCAGC
GAPDH	F	ACCAGGTGGTCTCCTCTGAC
	R	TGCTGTAGCCAAATTCGTTG
IL6	F	GAGTGGACCACACTGCGCCAA
	R	GTGCCCATGCTACATTTGCC
IL8	F	GAGTGGACCACACTGCGCCAA
	R	TCCACAACCCTCTGCACCCAGTT
IL-1β	F	ATGATGGCTTATTACAGTGGCAA
	R	GTCGGAGATTCGTAGCTGGA
IL10	F	GGTTGCCAAGCCTTATCGGA
	R	ACCTGCTCCACTGCCTTGCT
IL13	F	AACGGCAGCATGGTATGGAGTG
	R	TGGGTCCTGTAGATGGCATTGC
IL4	F	ACAGGAGAAGGGACGCCAT
	R	GAAGCCCTACAGACGAGCTCA
Rb	F	ACCTCAGCCTTCCAGACCCA
	R	GGGTGCTCAGACAGAAGGCG
p21	F	ACTGGAGGGTGACTTCGCCT
	R	TCCACCTGGGGACCCTTCAG
p16	F	CTAGAGCGGGGACATCACGA
	R	AGCAGAAGTTATGCCTGTCGGT
p73	F	TGCAGGCCCAGTTCAATCTGC
	R	TCGGTGTTGGAGGGGATGACA
CDK2	F	ATGGAGAACTTCCAAAAGGTGGA
	R	CAGGCGGATTTTCTTAAGCG
WNT1	F	ATGAACCTTCACAACAACGA
	R	TTGGGCGATTTCTCGAAGTA
WNT2B	F	ATGGATCCGAGAGTGTCAGC
	R	GCGACCACAGCGGTTATTAT
WNT3	F	TGGAACTGCACCACCATAGA
	R	GGACACTAACACGCCGAAGT
WNT3A	F	GTTTGGTGGGATGGTGTCTC
	R	GCGCTGTCGTACTTGTCCTT
WNT5A	F	ATTCTTGGTGGTCGCTAGGTA
	R	CGCCTTCTCCGATGTACTGC
WNT7A	F	GCCTGGACGAGTGTCAGTTT
	R	CTGGCCTTGCTTCTCTTTGT
WFDC1	F	AACCTCGATGGCTTGGTGGCAA
	R	TCAGGATGTGGCACTCATAGCC
TNF-α	F	CCCAGGGACCTCTCTCTAATCA
	R	GCTACAGGCTTGTCACTCGG
ABCB6	F	CGCCGACAATATCCGTTAC
	R	CATCCAGCAGAATGATGCC
ABCB9	F	GCTCTGGGAGAGACCTTCCT
	R	GAGCGGAAGAGACAGTTTCG
ABCB1	F	TTGCTGCTTACATTCAGGTTTCA
	R	AGCCTATCTCCTGTCGCATTA
ABCB11	F	GGGTCCCAGGGGTCTCAACTCTCTAG
	R	AACCTGCACCGTCTTTTCACTTTCTG

NF-κB p50	F R	GGATTTCGTTTCCGTTATGT TGTCCTTGGGTCCAGCAGTT
NF-κB p65	F R	CCCCACGAGCTTG TAGGAAAG CCAGGTTCTGGAAACTGTGGAT
β-catenin	F R	CACAAGCAGAGTGCTGAAGGTG GATTCCTGAGAGTCCAAAGACAG
GSK3-β	F R	GGCAGCATGAAAGTTAGCAGA GGCGACCAGTTCTCCTGAATC
MITF	F R	CATCCGTGGACTATATCCGAAAG CGAGCCTGCATTTCAAGTTC

Table 3. *List of primers used for RT-qPCR.*

9. Cell viability assay with 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT test)

After transfection of MelJuso with siRNAs targeting *ABCB5* (24h up to 96h), 100 µL of MTT solution (5 mg/mL) was added to each well of the 96-well plate. After 2h incubation at 37 °C, the medium was removed and 100 µL of DMSO was added. After incubating the plate for 30 min in an orbital shaker at room temperature, absorbance was measured in a Spectramax i3x (Molecular Devices, USA) using a wavelength of 595 nm.

10. Cell proliferation evaluation using a CYTONOTE

We assessed cell proliferation up to 96 hours following transfection of MelJuSo with siRNAs targeting *ABCB5* using a CYTONOTE 1W (IPRASENSE, France) through time-lapse imaging. For this measurement, the cells were seeded in 35mm petri dishes and cultured in an incubator at 37 °C with 95% humidity. The CYTONOTE carried out continuous measurements during 4 days from plating (day 0). Grown curves were determined using the percentage of confluence and number of cells per well.

11. Plasmid constructions

The cDNA encoding the *ABCB5*β isoform (NP_ 848654.3) has been inserted via BamHI and EcoRV in a pcDNA3.1(+)N-eGFP plasmid using an In-Fusion® HD Cloning Kit (Takara Bio Inc.,

638910). Similarly, the C-terminus tagged version of this construct was prepared by inserting *ABCB5β* cDNA upstream of the GFP sequence using a KpnI restriction site.

HA (Hemagglutinin)-tagged constructs of *ABCB5β* were obtained by insertion of a linker coding for HA via the NheI and KpnI restriction sites located upstream of the *ABCB5β* cDNA sequence in pcDNA3.1(+). Finally, *ABCB5β* cDNA was amplified by InFusion PCR with primers containing a EcoRI restriction site and inserted into a EcoRI digested PGK plasmid (Addgene plasmid # 35094) to obtain the HA-ABCB5β construct with a low expression promoter.

Additional constructs were engineered using a myc-tag or the monomeric red fluorescent protein (mCherry), to tag *ABCB5FL* and *ABCB5β*. The myc-tagged construction was obtained by insertion of a linker coding for myc via the BamH1 restriction site at the N- terminus of *ABCB5β*, and via EcoRV for C-terminal tagging, in pcDNA3.1(+). Finally, cDNA encoding the *ABCB5β* and *ABCB5FL* were inserted via EcoRI in a pcDNA3.1(+)C-mCherry plasmid.

12. Cell fractionation

Differential centrifugation was performed following the protocol of De Duve, Pressman, Gianetto, Wattiaux, and F (1955). We prepared a nuclear fraction (N) and a cytoplasmic extract (E) using a low-speed centrifugation. This latter fraction was then further fractionated into M, enriched in mitochondria, however this is mainly described for rat liver, L (light mitochondrial fraction), P (microsomal fraction) and S (cytosolic) fractions.

More specifically, HEK293T cells were cultured in 0.55 dm^2 petri dishes and transfected with *ABCB5β*-mCherry for 48 hours. When the cells reached 90-100% confluence, fractionation was conducted using six petri dishes. The cells were rinsed three times with ice-cold 0,25M sucrose (isotonic solution). We scraped the cells (using 500 µL of isotonic sucrose per petri dish) and homogenized them (i.e. broke down their plasma membrane) using a Dounce homogenizer (12 passages). The homogenate was transferred in a 15 mL Falcon tube and the volume was adjusted to 4 mL with 0.25M sucrose. The sample was then centrifuged in an Allegra centrifuge for 8 minutes at 1000g at 4°C (SX4400 rotor). The supernatant was collected up to 0.5 cm from the pellet and called E1. The pellet was then suspended in 2 mL (final volume) of 0.25M sucrose and homogenized using a Dounce homogenizer a second time (12 passages).

The 2 mL of sample was then centrifuged in the Allegra centrifuge for 7 minutes and 32 seconds at 1000g at 4°C.

The supernatant (E2) was collected up to 0.5 cm above the pellet then pooled with E1. The pellet is the **N fraction** and is suspended in 1 mL of 0.25M sucrose. The E supernatant (E1+E2, minus a small aliquote conserved for future analyses) was transferred in a thick-wall opaque tube and centrifuged using a L7-35 centrifuge (Beckman Coulter) rotor 75.1 Ti at 8000 rpm at 4°C. The time of centrifugation is determined by the volume of the sample E (see in **annex 2** Table with centrifugation times).

The supernatant, called LPS1, was collected. The pellet called M was suspended in 2mL of 0.25M sucrose. Then it was centrifuged for 2 minutes and 24 seconds at 8000 rpm. The supernatant, called LPS2, was collected and pooled with LPS1. The pellet was suspended again in a maximum of 0.8ml of 0.25M sucrose. This is the final **M fraction**.

The pool of LPS supernatants was centrifuged at 25000 rpm at 4°C in the 75.1Ti rotor for a time determined by its volume (see **annex 1**). The supernatant was collected and named PS1. The pellet, called L, was suspended again in 0.2 mL of 0.25M sucrose and centrifuged for 2 minutes and 52 seconds. The supernatant was then collected and pooled with PS1, while the pellet was suspended in a maximum of 100 µL of sucrose. This is the **L Fraction**.

PS was centrifuged at 35000 at 4°C for 40 minutes in the 70.1Ti rotor. The supernatant was collected and transferred in a 15 mL falcon; this is the **S Fraction**. The pellet was suspended in 0.8 mL of sucrose; this is the **P Fraction.**

Of note, after each fraction was suspended in sucrose, it was weighted in order to determine the relative dilution of the material found in this fraction relative to the starting material (e.g. the volume of each fraction is divided by the total cell surface, in cm^2, that was engaged at a given step). The dilution of each fraction is used for “bookkeeping”, i.e. we check at the end of the experiment that the amount of proteins and marker proteins recovered in the sum of fractions N-M-L-P-S corresponds to the amount that was engaged in the experiment (sum of E + N). We considered a recovery between 85% and 115% acceptable.

13. Centrifugation in a sucrose gradient density

In order to separate the different organelles based on their density, we prepared a sucrose gradient with density limits between 1.09 and 1.26 g/mL. 4.5 mL of the solution was placed in a 5 mL thin wall polyallomer tube. 500 µL of a pooled M + L+ P fraction (1/3 each if they have the same dilution factor relative to the starting material, see above) was loaded on top of the 4.5 mL of sucrose gradient. The tube was then centrifuged in the LE-80k centrifuge using the SW55ti rotor at 35000 rpm O/N at 4°C. After centrifugation, the tube was then cut every 6.4 mm to collect a total of 12 fractions. Enzymatic assays or western blotting analyses were then conducted on these fractions. Of note, in one experiment, the cells were treated with U18666A (1 µg/mL) overnight prior to fractionation and separation of the organelles in the sucrose density gradient.

14. Enzymatic assays

The activity of three marker enzymes was measured in subcellular fractions: β-hexosaminidase (lysosome), alkaline α-glucosidase (endoplasmic reticulum) and alkaline phosphodiesterase (plasma membrane).

14.1 Alkaline α-glucosidase

The substrate of alkaline α-glucosidase, 4-Methylumbelliferyl α-D-glucoside (Sigma-Aldrich, M9766) was freshly prepared before performing the assay. A substrate stock at 10 mM was prepared in DMSO. The diluted fractions were then incubated with a mix of the substrate (1x) in a buffer containing glycine-NaOH (0.5M, pH 9), 0,06% of Triton-X-100 and H_2O. For every test 10 µL of sample was incubated with 90 µL of the mix. After an incubation of 4 hours, the reaction was stopped by the addition of 1 mL of Glycine-NaOH (0.1M, pH 10). The fluorescence was measured at 495 nm using fluorimeter VersaFluor™ (BioRad, Hercules, CA, USA).

14.2 Alkaline phosphodiesterase

The activity of alkaline phosphodiesterase was measured using the substrate Thymidine 5'-monophosphate p-nitrophenyl ester sodium salt, (Sigma-Aldrich, T4510). The substrate was dissolved in a solution of glycine-NaOH (0.5M, pH 9,6), zinc acetate 0.01 M, 1M NaOH and H_2O to obtain a final concentration of substrate of 1,5 mM. The 10 µL of diluted fractions were added in 110 µL of substrate solution and incubated at 37°C until this solution turned yellow. The reaction was then stopped by adding 1 mL of glycine-NaOH (0.1M, pH 10) and the absorbance was measured at 400 nm using a Genesys 50 UV-visible Spectrophotometer (Thermo Fisher Scientific).

14.3 β-hexosaminidase

The substrate of β-hexosaminidase, 4-MU-N-acetyl-β-D-glucosaminide (Sigma-Aldrich, 69585), was freshly prepared (50 mM stock in DMSO) before performing the assay. The diluted fractions were incubated with a mix of the substrate (1x final) in citrate buffer pH 4.5 + 0.05% of Triton-X-100. For every test 10 µL of sample was incubated with 90 µL of the mix. After incubation for 2 hours, the reaction was stopped by the addition of 1 mL of Glycine-NaOH (0.1M, pH 10). The fluorescence was measured at 495 nm using fluorimeter VersaFluor™ (BioRad, Hercules, CA, USA).

15. Insertion of a hemagglutinin (HA) tag in N-terminal position of *ABCB5β* within the genome of MelJuSo cells using a CRISPR-Cas9 method

15.1 Design of a guide RNA (gRNA) and of the single-stranded oligodeoxynucleotide (ssODN)

To use a CRISPR-Cas9-based method of gene editing, we first designed a guide RNA (gRNA) to recruit the Cas9 nuclease enzyme close to the translation initiation codon of *ABCB5β*. The reference genome sequence found in databases (NCBI Reference Sequence: NM_178559.6) can serve as a reference point to design this gRNA; however, the presence of any genetic variations or polymorphisms may impact the hybridization of this gRNA and thus the efficiency of the CRISPR-Cas9 cleavage. Hence, we first sequenced the region around the ATG site of

ABCB5β in the genome of MelJuSo cells specifically. Then, we designed and ordered the synthesis (by Integrated DNA Technologies, Inc) of two individual sequences to prepare the gRNA: the crispr RNA (crRNA) part, which is a 17-20 nucleotide sequence complementary to a sequence upstream of the ATG codon of *ABCB5β* (ATG codon that initiates ABCB5β translation is in exon 4 of the gene sequence coding for this isoform) (**table 4**), and a typical tracr RNA part, which serves as a binding scaffold for the Cas9 nuclease. Of note, the crRNA hybridizes to a sequence that contains a PAM site, which is composed of 3 nucleotides (TGG in this case). This sequence is required for cleavage, a few nucleotides upstream of it, by the Cas9 enzyme.

Target	***Sequence (5' - 3')***	***PAM sequence***	***Locus***
Sequence in EXON 4	TTCTTTGGATTGGCAGATCA	TGG	chr7:+20651403

Table 4. *crRNA designed to target a sequence in exon 4 of ABCB5β by the CRISPR/Cas9 system. PAM=protospacer adjacent motif.*

The crRNA and tracrRNA sequences were then assembled with the Cas9 enzyme (IDT) as shown on **Figure 14**. This constitutes the ribonucleoprotein (RNP) complex. Practically, we prepared the gRNA complex at a final concentration of 150 pmol of crRNA:tracrRNA using quantities listed in **Table 5**. Then, for annealing, we placed the tubes in a thermocycler (BioRad) and cooled down from 95 to 30°C at a rate of 5°C per minute. Lastly, we mixed the gRNA complex with 1 µl of 10 µg/µL Cas9 and let it incubate for 10 min at RT to get our RNP.

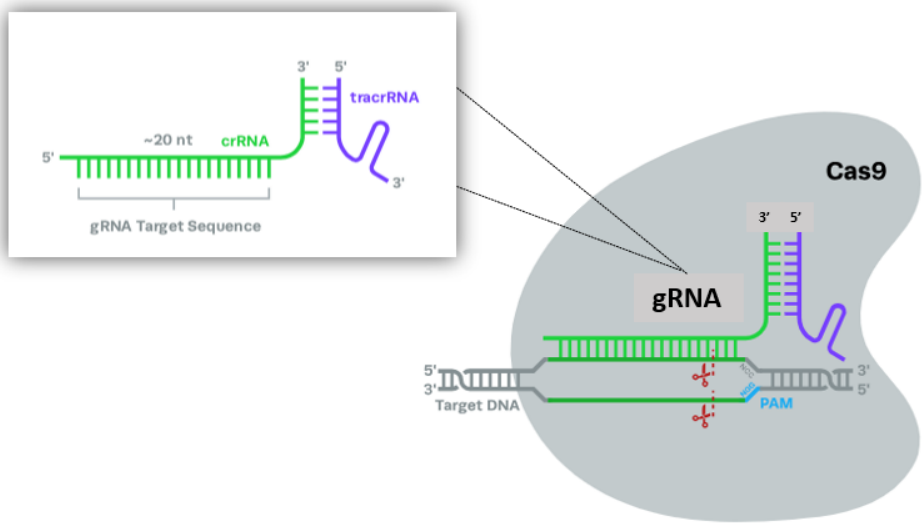

Figure 14. *Description of the CRISPR-Cas9 System. The CRISPR-Cas9 system consists of two essential components: a guide RNA (gRNA) in green and the Cas9 nuclease in grey, which together assemble into a ribonucleoprotein (RNP) complex. To initiate the process, a specific protospacer adjacent motif (PAM) in blue, allow the gRNA to attach itself to the target sequence. Subsequently, the Cas9 nuclease acts like molecular scissors in red, creating a double-strand break in the DNA.*

Component	*Amount (µL)*
120 µM Alt-R CRISPR-Cas9 crRNA	1.25 µl
120 µM Alt-R CRISPR-Cas9 tracrRNA	1.25 µl
Nuclease-free duplex buffer	7.5 µl
Total volume	10 µl

Table 5. *List of components to make the gRNA complex at a final concentration of 150 pmol.*

Next, we designed a 190 bp single-stranded oligo donor (ssODN) encoding the HA tag, flanked with 80 nucleotide homology arms either at 5' or 3' of the HA sequence. ABCB5β was tagged at the N-terminus position right after its ATG start codon. In **Figure 15,** the whole ssODN sequence that was manufactured by IDT (Integrated DNA Technologies, Inc) is shown.

5’tgctacttctcagcttatattttggtctagtatgaaaaaccctaaaatcaatacagtaaa
aggcatcacaacatggttcattctttggattggcagATCATGTACCCATACGATGTTCCAGA
TTACGCTgtggatgagaatgacatcagagctttaaatgtgcggcattatcgagaccatattg
gagtggttagtcaagagcctgttttgttcgggaccaccatcagta 3’

Figure 15 . *Whole ssODN used for HA-KI at the start codon (ATG in red bold) of ABCB5β in MelJuSo. The ssODN consists of HA-coding sequence (red) and two 80-bp homology sequences, homology arm 5’ (green) and homology arm 3’ (blue) of the HA.*

15.2 Electroporation

The day before the electroporation, MelJuSo cells were seeded in a 6-well plate in a density of 1 x 10^6 cells/well with 2 mL of complete growth medium. We mixed the ssODN and the RNP complex, using three different concentrations of ssODN: 20 µM, 40 µM and 80 µM.

The protocol for electroporation includes the codelivery of the HDR donor oligo ssODN and the RNP complex (crRNA:tracrRNA and Cas9), using Nucleofactor system (Amaxa kit electroporation, Lonza). We harvested the cells and rinsed them 1x with PBS. We prepared 100 µL of supplemented nucleofector solution following the instructions of the manufacturer. This 100 µL of nucleofector solution was mixed with the 11 µL of RNP complex which already contain the ssODN. The cell suspension was then placed in the Amaxa Nucleocuvette™ and we proceeded to electroporation using program C-09.

After electroporation, we added 500 µL of pre-warmed medium to the cuvette, transferred the electroporated cells, and divided them (200 µL each) into 3 wells of a 6-well plate containing prewarmed medium. To each well, we added 1.5 µL of NHEJ inhibitor SCR7, to inhibit Ligase IV and effectively disrupted nonhomologous DNA end joining (NHEJ), which is the major DNA double-strand break repair pathway in mammals. This is done to promote HDR repair (i.e. insertion of our tag). Lastly, the cells were allowed to recover for 48 hours (with a medium change after 24 hours).

15.3 Limiting dilution

The generation of monoclonal cell populations positive for HA tagged ABCB5β was performed by cell counting (Vi-Cell XR Analyzer, Life Sciences) and seeding transfected cells in 96-well plates at a density of one cell per well. When colonies were detected, cells were harvested and seeded again to expand their culture in 24- and 12-well plates. When confluence was around 100% half of the cells were used for genomic DNA extraction to perform a screening by PCR and the other half suspension was conserved at -80°C after addition of cell freezing medium (70% DMEM, 20% DMSO, 10% FBS, 0.1% Pen/Strep).

15.4 Genomic DNA and PCR screening

The culture medium (DMEM, 10% FBS, 0.1% Pen/Strep) was removed and cells washed with PBS. Cell suspension was centrifuged at 1,200 rpm for 10 min (Rottina 380 R, Hettich). The supernatant was removed and 200 µL of lysis buffer 1X (Tris 0,1M pH 8, EDTA 5mM, NaCl 0,2 M and SDS 0,2%) was added to the pellet. The sample was incubated at 95 °C during 5 min, before adding 5 µL of proteinase K (100µg/ml) (Promega, EO0491). The tube was then incubated at 55 °C for 30 min and afterwards centrifuged at 15,000 g for 2 min (Sigma 1-16K). The supernatant was recovered and 200 µL of isopropanol were added to precipitate DNA. Centrifugation was performed at 15,000 g for 2 min, before removing isopropanol. Then, 200 µL of ethanol 70% were added to wash the DNA pellet. The sample was centrifuged again at 15,000 g for 2 min, and ethanol removed. The pellet was dried for 15 min at RT and suspended in 30 µL H_20 before incubation at 65 °C for 1 h, and under agitation. DNA was quantified using a spectrophotometer (Spectramax I3, Molecular Devices). DNA was conserved at – 20 °C before screening by PCR.

After DNA extraction, PCR were carried out to detect the insertion of the HA tag at the beginning of *ABCB5β*. The conditions for the PCR we used were: initial denaturation at 95 °C for 3 min, and 35 cycles of 95 °C denaturation temperature for 30 s, annealing temperature depending on the primers at 62 °C for 30 s, and 1 min of extension at 72 °C followed by another 5 min of final extension at 72 °C. PCR products were conserved at 4 °C before visualization in 1% agarose (Carl Roth, Germany) gel stained with ethidium bromide, after electrophoresis in

a 1X TAE buffer at 120 V for 30 min. GeneRuler 1 kb DNA Ladder (Thermo Scientific, SM0311) was used as size standard.

PCR primers flanking the HA tag were designed to generate ~400-600 bp PCR amplicons (**Table 6**). One of the primers from each pair matches the sequence of HA tag, either in forward or reverse orientation, and the other primers ("ATG primers") were designed to be far further away in the homology arms of the ssODN used for the CRISPR-Cas9 strategy.

Primer	***Forward Sequence*** *(5' - 3')*	***Primer***	***Reverse Sequence*** *(5' - 3')*	*Ta (∘C)*	***Product size***
HA_F	ATGTACCCATACGATGTTCC	***ATG_R***	TGGCCTTTCAATGATGCATTTCTT	62	~600
ATG_F	TGAGATGTTGAAATAATGGCGGC	***HA_R***	GGAACATCGTATGGGTACAT	62	~422

Table 6: *List of primers used for the PCR genotyping.*

RESULTS

1. Study of subcellular localization of ABCB5β

Here in this first section, we present the results obtain from the characterization of the subcellular localization of the ABCB5β isoform, the main objective of this thesis. We investigated the localization of ABCB5β using a combination of subcellular fractionation and immunofluorescence analysis. A research paper containing the results of the localization of ABCB5β based on immunofluorescence analyses has been written and submitted for publication. We included this manuscript below. We notably tested different anti-ABCB5 antibodies hoping to detect the protein endogenously expressed in MelJuSo (melanoma) cell line. Additionally, we generated several ABCB5β cDNA constructs, each tagged at either the N-terminal or C-terminal positions. These constructs were utilized in subcellular fractionation experiments and a series of immunofluorescence analyses aimed at investigating the cellular localization of ABCB5β in several models: HeLa (cervical cancer), HEK293T (Human embryonic kidney cells) and MelJuSo cell lines.

Of note, we selected HeLa cells because it is an easily transfectable cell line well suited for subcellular localization analyses by immunofluorescence methods. HEK293T cells were used in subcellular fractionation experiments, as a high expression model allowing detection of the mCherry-tagged construct. MelJuSo is a melanoma cell line in which ABCB5β is endogenously expressed at relatively high levels and higher transfection rates compared to other melanoma cells. This was demonstrated by RT-qPCR. CT cycles were lowest (indicative of highest expression) in MelJuSo and UACC cells. We inferred that the amplification product was the beta isoform considering that Chen et al. demonstrated that FL is not detected in melanoma cell lines (Chen et al., 2005).

	Sample	18S
	Average Ct value	
SK-MEL-28	36,65	6,51
UACC-257	23,91	6,09
Mel Ju	26,05	6,04
MelJuSo	26,37	6,73
MNT-1	30,48	6,63

Table 7. *Analysis of the expression of ABCB5 mRNA in five different melanoma cell lines. Average Ct values obtained after RT-qPCR using a probe that recognizes the three main transcript of ABCB5. 18S Ct are shown as controls.*

In the last part of the results section of our thesis manuscript, we will present additional data collected during our work, including some consequences of *ABCB5* knock-down in MelJuSo cells.

1.1 Investigation of the subcellular localization of an mCherry-tagged ABCB5β in HEK293T cells.

1.1.1 Engineering and expression of the mCherry construct in HEK293T cells

We first engineered mCherry-tagged ABCB5 (FL or β) constructs. The mCherry tag was fused to the C-terminus. HEK293T cells were transfected for 48h and the tagged proteins were detected by western blotting using a tdTomato (tandem dimer tomato) polyclonal antibody. Of note, this antibody recognizes mCherry and Tomato tags; the latter containing two mCherry sequences. Consistent with the theoretical molecular weight of the fusion proteins, ABCB5FL-mCherry was detected at 170 KDa and ABCB5β-mCherry at 120KDa (**Figure 15**). It is worth noting the presence of a 55 KDa band on the gel that appears to be a C-terminal fragment of these proteins since it was detected with the anti-tdTomato antibody.

Other *ABCB5* constructs (with ABCB5 fused to a Myc tag placed either in C- or N-ter position) were also tested but, for a reason that remains unclear at this stage, these fusion proteins

could not be detected by western blotting nor by immunofluorescence labeling after transfection in HEK293T cells or melanoma cell lines (SK-MEL-28 and MelJuSo). Hence, we continued working with the mCherry-tagged constructs, focusing on ABCB5β, the topic of this thesis.

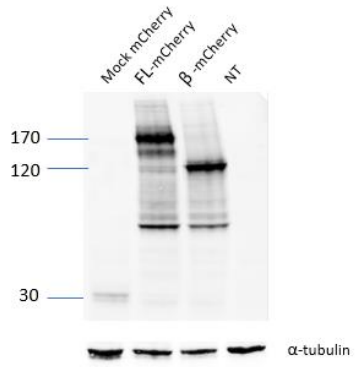

Figure 16. *Detection of ABCB5FL-mCherry (FL-mCherry) and ABCB5β-mCherry (β-mCherry) in HEK293T cells. The cells were transiently transfected with these constructs, or with a mock pcDNA3.1-mCherry plasmid as a control, for 48h prior to the detection of the tagged proteins by western blotting using anti-tdTomato antibody (NT= non-transfected cells).*

1.1.2 Analysis of the mCherry-tagged ABCB5β localization by subcellular fractionation using a differential velocity centrifugation method.

During our study of the subcellular localization of *ABCB5β*, we fractionated the HEK293T transfected cells in five organelle-enriched fractions using a differential centrifugation method, following the protocol described by De Duve et al. (1955). After homogenization of the cells (i.e. plasma membrane breakdown) in isotonic sucrose using a Dounce homogenizer, we prepared a nuclear fraction (N) and a cytoplasmic extract (C). This latter fraction was then further fractionated into M (heavy mitochondrial fraction), L (light mitochondrial fraction), P (microsomal fraction) and S (cytosolic) fractions. C. de Duve's protocol, when applied to rat liver as source material, gives rise to the following organelle enrichment pattern in these fractions (**Figure 16**):

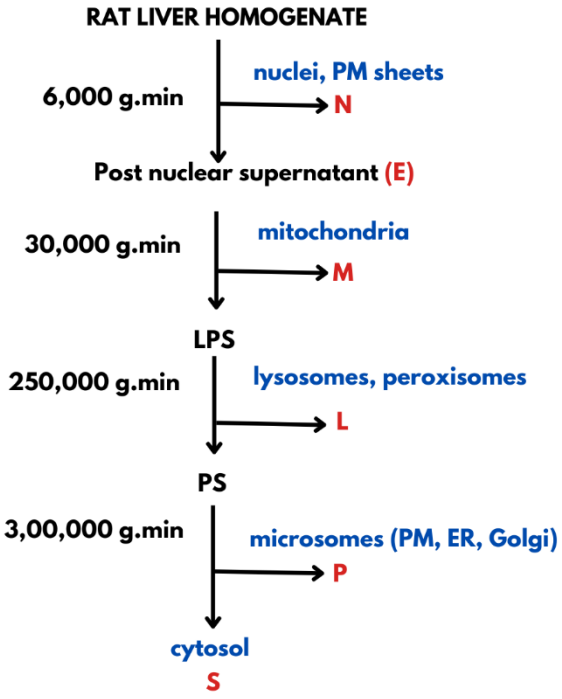

Figure 17. *Schematic representation of the fractionation protocol described by de Duve and applied to rat liver as source material. PM= plasma membrane, N=nuclear fraction, E= cytoplasmic extract, M= heavy mitochondria fraction, L= light mitochondrial fraction, P= microsomal fraction and S= cytosolic fraction. The organelles that are most enriched in each fraction are indicated in blue.*

To analyze the distribution of subcellular organelles in the fractions obtained from transfected HEK293T cells, we conducted enzymatic assays to measure the activity of different markers. The distribution profiles are shown in **Figure 17**. The relative enrichment of each marker in a given fraction (Specific Relative Activity, SRA, i.e. the % of activity of the marker divided by the % of total proteins in this fraction) is plotted against the total protein amount in the fractions. The associated tables summarize the total activity (in %) measured in each fraction. As indicated in **Figure 17a** for β-hexosaminidase, lysosomes were largely recovered in the M and L fractions (22 and 32%, respectively), with a enrichment peak (high SRA) in the L fraction, which is typical for lysosomal proteins. Large amounts of alkaline phosphodiesterase, a marker of plasma membrane, were detected in M, L and P fractions (22, 29 and 36 %, respectively), with an enrichment peak in the L fraction (SRA~ 6) and a slightly higher enrichment in the P fraction (SRA~2.2) compared to the lysosomal marker (SRA~0.8 for β-hexosaminidase in this fraction) (**Figure 17b**). To analyze the distribution of the endoplasmic reticulum (ER), an alkaline α-glucosidase assay was conducted. It was found to be slightly enriched in the L and

P fractions, but without the large SRA peak seen in the L fraction for the other markers (**Figure 17c**).

Next, ABCB5β-mCherry was detected by immunoblotting. Equal amounts of proteins were loaded on the gel, meaning that the signal intensities directly reflect the ABCB5β-mCherry enrichment level in the different fractions (Specific Relative signal, SRR). This enrichment level (SRR) was plotted against total protein amounts in the fractions to establish the distribution profile. **Figure 17d** shows that the distribution profile of this protein is reminiscent of the α-glucosidase distribution profile, suggesting that ABCB5β may localize in the ER. However, the distribution profiles of the organelle markers showed significant overlapping after application of this differential centrifugation protocol. Hence, the presence of ABCB5β in other compartments could not be excluded based on this first experiment.

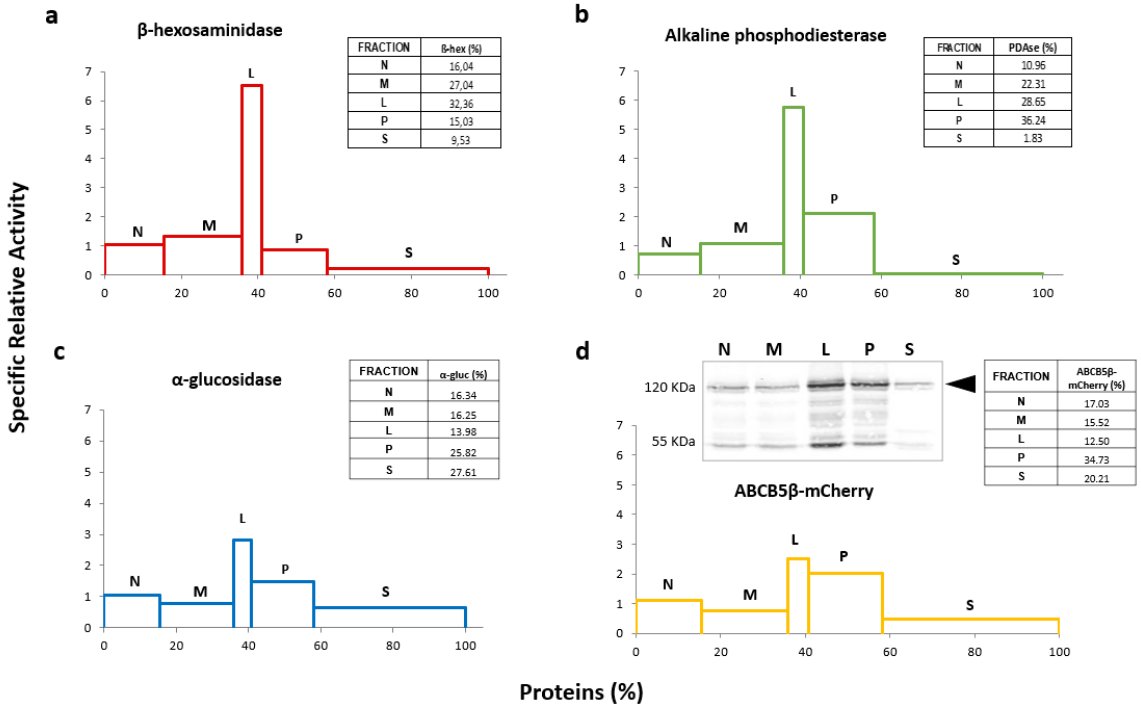

Figure 18. *Distribution of ABCB5β-mCherry, alkaline α-glucosidase, β-hexosaminidase and alkaline phosphodiesterase after fractionation of transfected HEK293T cells by differential centrifugation. HEK293T cells were fractionated into five fractions (N, M, L, P and S) following de D'ye's fractionation scheme. The activities of β-hexosaminidase (a), alkaline phosphodiesterase (b) and alkaline α-glucosidase (c) were detected by fluorometric assays to establish the distribution of lysosomes, ER and plasma membrane, respectively. The graph shows the relative specific activity (SRA; ratio of the percentage of activity of the enzyme in a*

given fraction to the percentage of proteins in this fraction), which is indicative of the enrichment factor of the enzyme in the fractions, plotted against the percentage of proteins in each fraction. The distribution of ABCB5β-mCherry (~120 kDa, see arrow) was analyzed by western blotting (reducing conditions). Equal amounts of proteins were loaded for each fraction (d).

1.1.3 Analysis of mCherry-tagged ABCB5β localization by subcellular fractionation using an isopycnic centrifugation method.

As the majority of ABCB5β-mCherry proteins were recovered in the M, L and P fractions, we subjected a pooled MLP fraction to isopycnic centrifugation in a linear sucrose density gradient (with 1.05 and 1.26 g/mL density limits) (**Figure 18**). Lysosomes, represented by the β-hexosaminidase activity, exhibited a median equilibrium density of 1.1499 g/mL (**Figure 11a**), while the plasma membrane marker, alkaline phosphodiesterase, was found to be around 1.1149 g/mL (**Figure 18b**). The alkaline α-glucosidase, marker of the endoplasmic reticulum, showed a bimodal distribution. Most of this marker was recovered around 1.1896 g/mL whereas a smaller peak was detected around 1.1289 g/mL (**Figure 18c**). This likely represents the presence of α-glucosidase in both the rough ER (in the denser region) and smooth ER (less dense due to the absence of ribosomes).

Figure 18d shows the distribution of ABCB5β–mCherry, which co-distributes to a large extent with alkaline α-glucosidase. The distribution of the 120 KDa band also shows two peaks, which are found in the same density regions as the ER marker, consistent with the presence of ABCB5β in this compartment. Of note, the 55 KDa ABCB5 band did not show the same distribution pattern. It was found in a lower density region, where other organelles, including plasma membrane and lysosomes were found, at least to some extent.

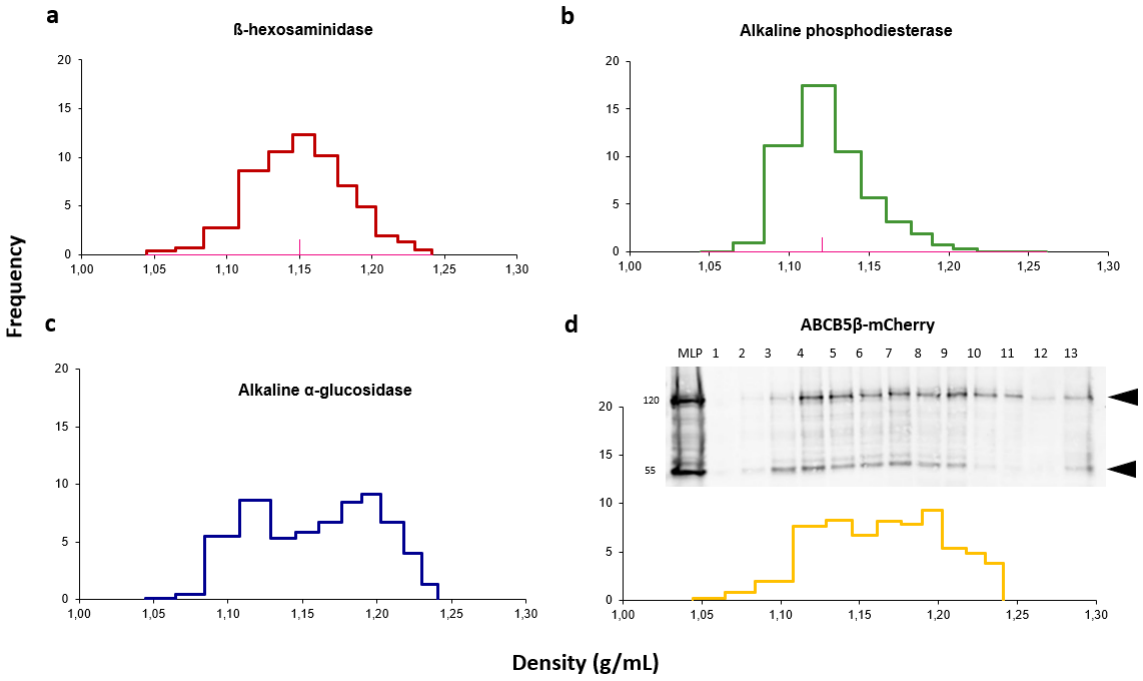

Figure 19. *Distribution of ABCB5β-mCherry, β-hexosaminidase, alkaline phosphodiesterase and α-glucosidase, after isopycnic centrifugation of a MLP fractions in a linear sucrose density gradient. HEK293T cells transfected for 48h with ABCB5β-mCherry were fractionated into five fractions (N, M, L, P and S) by differential centrifugation. Next, a MLP pooled fraction was centrifuged at 35.000 rpm OVN in a linear sucrose density gradient using a Beckman SW55.Ti rotor. The graphs show the distributions of β-hexosaminidase (a), alkaline phosphodiesterase (b) and alkaline α-glucosidase (c) in the 13 fractions collected from the gradient, and thereby highlight the distribution of lysosomes, plasma membrane and ER, respectively. The frequency of each marker (i.e. the activity found in the fraction, normalized to the total activity recovered in the sum of the fractions, and to the increment of density from top to bottom of the fraction) is plotted against the densities in the gradient. (d) The distribution of ABCB5β-mCherry was analyzed by western blotting (reducing conditions) in 13 collected fractions.*

To test if the 55 KDa band is located in lysosomes, we treated the cells with U18666A (1 µg/ml) prior to fractionation, with the aim to block the export of cholesterol from lysosomes and, consequently, reduce the equilibrium density of the lysosomes in the sucrose gradient (**Figure 19**). If the 55KDa band is truly in lysosomes, its distribution in the gradient should be modified by the treatment, as seen for the lysosomal marker, which was recovered in fractions 1, 2 and 3 of the gradient after treatment (**Figure 19b**).

The distributions of the ER and plasma membrane markers were unaffected by the treatment, indicating that the density shift induced by U18666A was specific of lysosomes (**Figure 19c** and **19d**). However, the distributions of the 120KDa and 55 KDa ABCB5 bands were also found unaffected by the treatment (**Figure 19a**), indicating that ABCB5β and its putative fragment are not located in lysosomes.

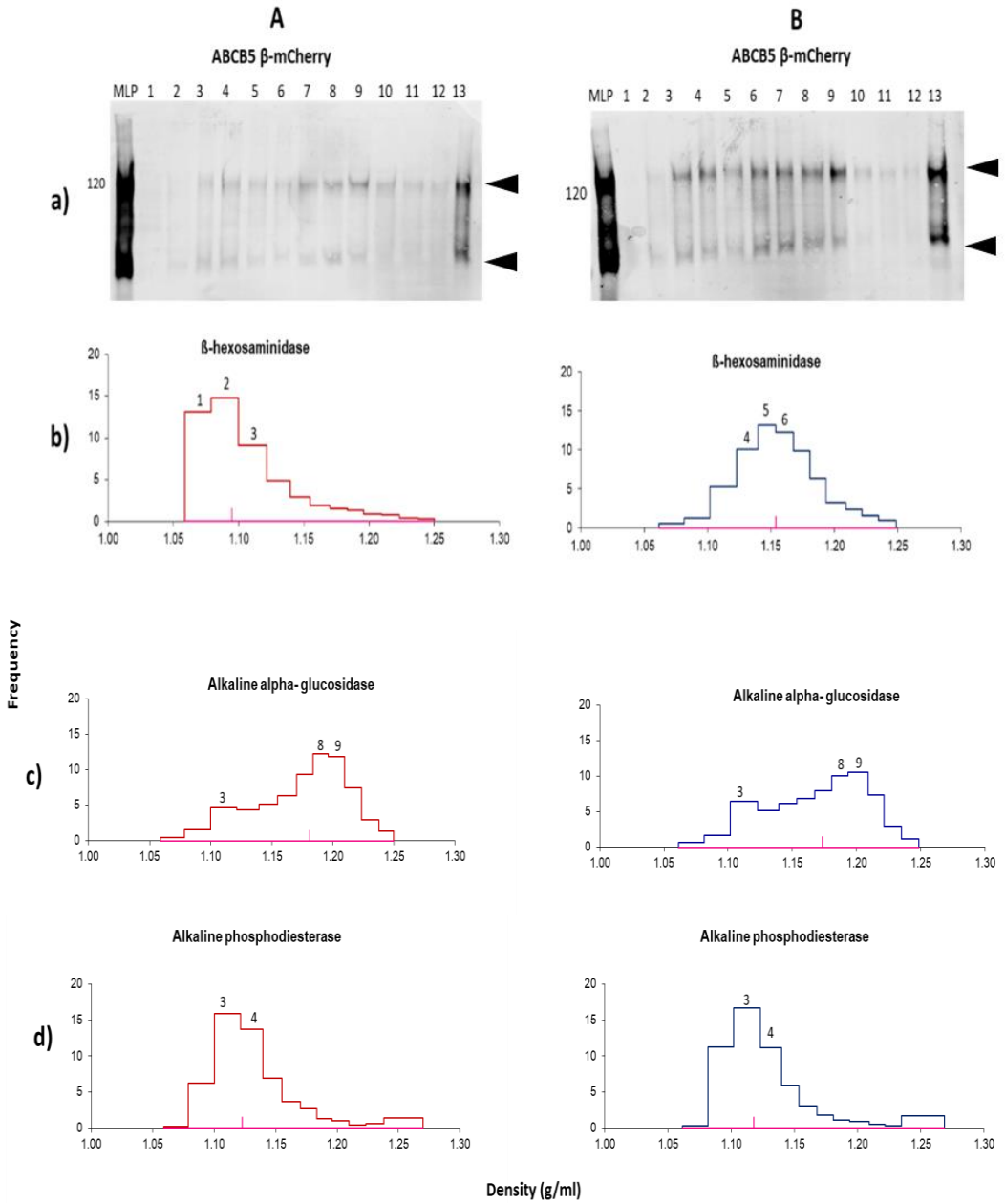

Figure 20. *Distribution of ABCB5ß-mCherry, ß-hexosaminidase, alkaline α-glucosidase and alkaline phosphodiesterase, after isopycnic centrifugation of a MLP fraction in a linear sucrose density gradient. MLP fractions obtained by differential centrifugation from HEK293T transfected cells treated (B) or not (A) with the inhibitor of lysosomal cholesterol export U18666A were layered on top of a sucrose gradient and centrifuged at 35.000 rpm OVN in a Beckman SW55.Ti rotor. The distributions of ABCB5ß-mCherry (a), ß-hexosaminidase for lysosomes (b), alkaline α-glucosidase for endoplasmic reticulum (c) and alkaline phosphodiesterase for plasma membranes (d), among the 13 collected fractions, were detected by enzymatic assays. Histograms represent the frequency (i.e. the activity found in the fraction, normalized to the total activity recovered in the sum of the fractions, and to the increment of density from top to bottom of the fraction) as a function of the density.*

The observation that treatment of the transfected HEK293T cells with Bafilomycin (an inhibitor of the lysosomal vATPase) did not prevent formation of the 55 KDa band (**Figure 20**) further supports that it does not result from a proteolytic cleavage taking place in lysosomes.

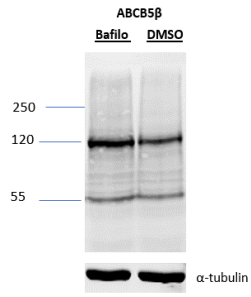

Figure 21. *Treatment of HEK293T cells transfected with ABCB5β-mCherry with Bafilomycin (an inhibitor of the lysosomal vATPase). HEK293T cells transfected with ABCB5β-mCherry for 48h were treatment with bafilomycin (15nM) or vehicle (DMSO) during the last 16 h. Detection by western blotting (reducing conditions) using an anti-tdTomato antibody.*

Unexpectedly, the ABCB5β-mCherry construct could not be detected by fluorescence microscopy, due to the absence of fluorescence emitted by the tag. This is surprising, possibly accounted for by a conformation defect of the tag when attached to the C-terminus of ABCB5. Thus, we decided to engineer GFP and HA-tagged constructions, to study ABCB5β localization using microscopy and additional biochemical methods. These analyses were conducted using HeLa cells (adapted for microscopy analyses) and MelJuSo cells (that express ABCB5 endogenously). Indeed, in addition to studying the localization of the tagged proteins, we also aimed at detecting the endogenous ABCB5β protein. The results of these analyses have been compiled in the research article included in the next pages. It was recently submitted for publication to the International Journal of Molecular Sciences.

1.2 Investigation of the subcellular localization of GFP and HA-tagged ABCB5β in HeLa and MelJuso cells: research article

The β Isoform of Human ATP-Binding Cassette B5, ABCB5β, is a Microsomal Protein

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ABSTRACT

ABCB5β is a member of the ABC transporter superfamily cloned from melanocytes. It has been reported as a marker of skin progenitor cells and melanoma stem cells. ABCB5β has also been shown to exert an oncogenic activity and to promote cancer metastasis. However, this protein remains poorly characterized. To elucidate its subcellular localization, we tested several anti-ABCB5 antibodies and prepared several tagged *ABCB5β* cDNA constructs. We then used a combination of immunofluorescence and biochemical analyses to investigate the presence of ABCB5β in different subcellular compartments of HeLa and MelJuSo cell lines. Treatment of the cells with the proteasome inhibitor MG132 showed that part of the population of newly synthesized ABCB5β is degraded by the proteasome system. Interestingly, treatment with SAHA, a molecule that promotes chaperone-assisted folding, largely increased the expression of ABCB5β. Nevertheless, the overall protein distribution in the cells remained similar to control conditions; the protein extensively colocalized with the endoplasmic reticulum marker calnexin. Taken together with cell surface biotinylation studies demonstrating that the protein does not reach the plasma membrane (even after SAHA treatment), the data indicate that ABCB5β is a microsomal protein predominantly localized to the ER.

INTRODUCTION

ABCB5 is a member of the ATP-binding cassette (ABC) transporter superfamily (Duvivier & Gillet, 2022). Humans have 48 ABC genes encoding 44 membrane transporters classified into five families (A, B, C, D, and G). These transporters are composed of two symmetric halves, each including a transmembrane domain (TMD) that facilitates substrate export (for most of them) or import (for *ABCA4* and *ABCD4*), and a nucleotide binding domain (NBD) responsible for ATP binding and hydrolysis. They are expressed either as full transporters containing two nonidentical halves within a single polypeptide, or as half transporters that have to homo- or heterodimerize to be functional. There are also 4 non-transporter ABC proteins that belong to family E and F. They exist as twin NBDs without any TMDs (Thomas & Tampe, 2020).

The ABC transporters translocate a wide variety of endogenous and xenobiotic substrates across membranes, including peptides, polysaccharides, glutathione conjugates, antibiotics, and anticancer drugs, among many others (Alam & Locher, 2023). They have been extensively studied for their roles in cancer multidrug resistance and more recently in tumorigenesis (Gottesman et al., 2016; Muriithi et al., 2020). Moreover, mutated ABC genes cause several monogenic diseases (including cystic fibrosis) and may increase susceptibility to complex diseases such as coronary artery disease and Alzheimer's disease (Dean et al., 2022; Moore, Bell, Hughes, & Garfield, 2023).

ABCB5 is one of the most frequently mutated genes in melanoma (Krauthammer et al., 2015; Sana et al., 2019). This is the deadliest type of skin cancer, which represents a significant challenge in clinical oncology due to intrinsic and acquired mechanisms of resistance to treatments (Garbe et al., 2022; Mishra et al., 2018). ABCB5 was also reported to be a marker of skin progenitor cells (Frank et al., 2003) and melanoma stem cells (Schatton et al., 2008), and to mediate anthracycline resistance (Frank et al., 2005).

ABCB5 encodes a full transporter (ABCB5FL) and a half transporter (ABCB5β). *ABCB5FL* was cloned from testis (Kawanobe et al., 2012) and *ABCB5β* from melanocytes (Frank et al., 2003). There are also many additional transcript variants, including ABCB5α, but they are too short to form functional transporters (Chen et al., 2005). ABCB5FL was shown to confer low-level multidrug resistance. The drugs transported by this full-length transporter include anthracyclines, taxanes, vinca-alkaloids, and epipodophyllotoxin (Kawanobe et al., 2012; Keniya et al., 2014). Drugs transported by *ABCB5β* have not been identified thus far, but only a limited number of molecules have been tested. ABCB5β might transport some other drugs, and/or act as a drug-efflux transporter either as a homo- or heterodimer.

It has been shown that several mutations in *ABCB5* promote proliferation and invasive capacities of melanoma cells (9). It has also been reported that melanoma cells with a high *ABCB5* expression exhibit an elevated metastatic potential both *in vitro* and *in vivo* (Wilson et al., 2014). However, the underlying mechanisms and to what extent both *ABCB5* isoforms interplay have yet to be unraveled. Intriguingly, Chen et al. reported that *ABCB5FL* was undetectable in a melanotic cDNA library, by contrast to α and β isoforms. These were also found enriched in melanoma cells compared to several normal tissues (Chen et al., 2005).

Hence, we decided to focus on the ABCB5β isoform that, by contrast to the α isoform, has a predicted transport function. Our aim here was to answer a question that remains unresolved, and that is an important requirement in the search for the molecular role of this protein, i.e. its subcellular localization. To address this, we tested anti-*ABCB5* antibodies and prepared several tagged ABCB5β cDNA constructs. We then used a combination of immunofluorescence analyses to investigate the presence of ABCB5β in different cell compartments of HeLa (cervical cancer) and MelJuSo (melanoma) cell lines.

RESULTS

Western blotting signals detected with three different commercial anti-*ABCB5* antibodies do not decrease after knock-down of *ABCB5* in MelJuSo cells

First, we tested whether we could detect the endogenous expression of ABCB5β at protein level in MelJuSo cells. We used three different polyclonal anti-*ABCB5* antibodies commercially available. The Abcam antibody was raised against the first 99 amino acids of ABCB5β, whereas the Rockland and Atlas antibodies recognize overlapping epitopes located between amino acids 192-208, and 145-234, respectively (**Table S1**). All of them detected human ABCB5β by western blotting when overexpressed in membranes of High-5 insect cells (**Fig. 1A**). To assess their specificity for detection of endogenously expressed ABCB5, we silenced *ABCB5* expression at mRNA level in MelJuSo cells using a pool of 4 siRNAs and collected the samples up to 96h post-transfection (**Fig. 1B**). The 4 siRNAs recognize sequences that are found in both *ABCB5FL* and *ABCB5β*. However, albeit RT-qPCR revealed a ≥ 90 % decrease of *ABCB5* expression up to 72h post-transfection (most likely of the β isoform since FL is barely expressed, if at all, at basal level according to Chen et al.(Chen et al., 2005)), none of the signals detected by western blotting close to the expected molecular weight for ABCB5β (90 kDa) decreased after siRNA transfection, nor any other bands for that matter (**Fig 1C**).

We also tested the three commercial antibodies in an immunofluorescence application. Only the Rockland anti-*ABCB5* antibody gave rise to a detectable fluorescent signal, which did not decrease in MelJuSo cells transfected with siRNAs targeting *ABCB5* (**Fig. 1D**).

Taken together, these results highlight that several commercial antibodies can detect large amounts of ABCB5β, e.g. after overexpression in High-5 insect cells, but fail to detect the protein expressed endogenously in melanoma cells. Of note, similar results were reported by Louphrasitthiphol et al. who tested three additional commercial anti-*ABCB5* antibodies to analyze *ABCB5* expression in the 501mel cell line (Louphrasitthiphol et al., 2020). These data motivated the use of tagged-*ABCB5* constructs to address the localization of ABCB5β proteins in the cell.

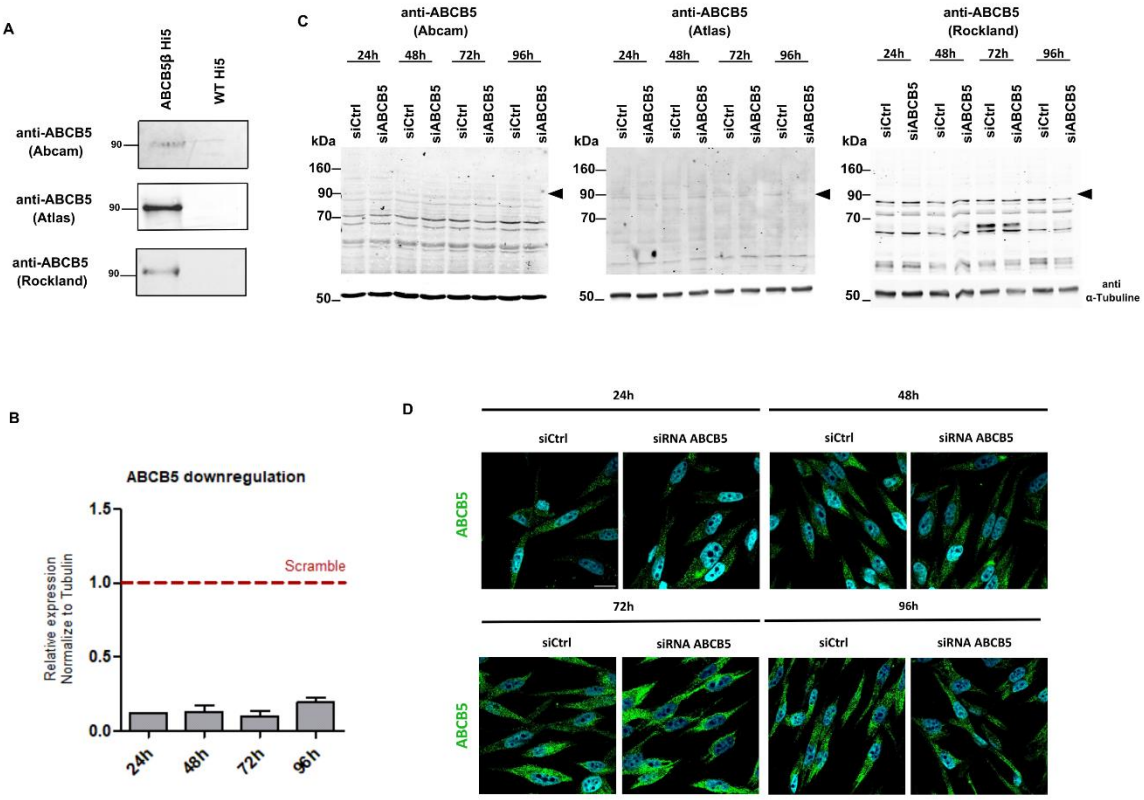

Figure 1. Western blotting signals detected with three different commercial anti-ABCB5 antibodies do not decrease after knock-down of ABCB5 in MelJuSo cells. *A) Western blotting detection of human ABCB5β in membrane preparations of High Five insect cells infected or not with ABCB5β-containing baculoviruses. Microsomal proteins (1µg) were resolved by SDS-PAGE prior to transfer on a PVDF membrane. 3 different commercial anti-ABCB5 antibodies were tested: rabbit polyclonal anti-ABCB5 (Rockland Immunochemicals, 600-401-A775), rabbit anti-ABCB5 (Abcam, ab8010889) and rabbit anti-ABCB5 (Atlas antibodies, HPA026975). B) RT-qPCR analysis of ABCB5 mRNA expression in MelJuSo cells after transfection with a scrambled siRNA pool (siCtrl) or with a pool of 4 siRNAs directed against ABCB5 (siABCB5) during 24h, 48h, 72h or 96h.*

Primers recognize a sequence located in the beta isoform. mRNA expression levels normalized to GAPDH and relative to siCtrl conditions are shown on the graph. C) Western blotting detection of endogenous ABCB5 protein expression in MelJuSo cells transfected with siCtrl or siABCB5 during 24h, 48h, 72h and 96h, using the 3 different commercial anti-ABCB5 antibodies anti-ABCB5 (Rockland Immunochemicals, 600-401-A775), rabbit anti-ABCB5 (Abcam, ab8010889) and rabbit anti-ABCB5 (Atlas antibodies, HPA026975). D) Immunofluorescence micrographs taken at different time points after transfection of siCtrl and siABCB5 in MelJuSo cells. Cells were stained with anti-ABCB5 Rockland antibody followed by an AlexaFluor 488 (green) rabbit secondary antibody. Nuclei were stained with DAPI. Scale bar= 50 µm.

GFP-ABCB5β localizes to the endoplasmic reticulum in HeLa and MelJuSo cells

As we cannot study the subcellular localization of the endogenous ABCB5β with commercial antibodies, we decided to engineer GFP-tagged ABCB5β constructs, with the GFP either fused at the N-terminus or C-terminus of the protein (GFP-ABCB5β or ABCB5β-GFP, respectively). The chimeric proteins were then expressed in HeLa or MelJuSo cells using a pcDNA3.1(+) plasmid with a conventional CMV promoter. As shown in **Fig. 2A**, a protein band with a molecular mass of approx. 120 kDa was detected by western blotting 48h post-transfection of the GFP-ABCB5β construct in HeLa and MelJuSo cells, using an anti-GFP antibody. This band corresponds to the molecular weight of ABCB5β (90 kDa) combined with the eGFP tag (~30 kDa). ABCB5β-GFP (C-ter) was also detected but at a very low level.

Treatment of the cells with the proteasome inhibitor MG132 increased ABCB5β-GFP expression, though it remained quite low in MelJuSo cells (**Fig. 2B**). Of note, this treatment also increased the amount of N-terminally tagged GFP-ABCB5 in both cell types, suggesting partial degradation of the newly synthesized ABCB5β protein population.

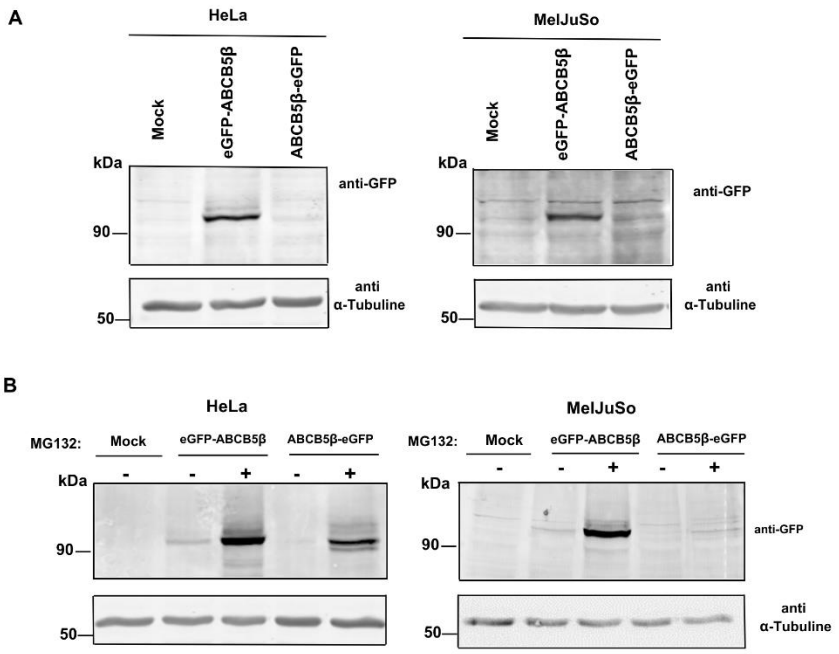

Figure 2. Expression of N-ter and C-ter GFP-tagged ABCB5β constructs in HeLa and MelJuSo cells. *A) pcDNA3.1 plasmids containing GFP-ABCB5β or ABCB5β-GFP cDNAs were transfected in HeLa or MelJuSo cells. A mock condition was included as control (transfection with an empty pcDNA3.1 vector). Proteins were extracted 48h post-transfection and resolved by SDS-PAGE. An anti-GFP antibody was used to detect the chimeric proteins. α-tubulin detection was used as loading control. B) HeLa and MelJuSo cells were transfected with GFP- ABCB5β and ABCB5β-GFP constructs for 48h and treated for the last 6h with 10 µM MG132. The proteins of interest were then detected as described in A.*

Next, we examined the subcellular localization of GFP-tagged ABCB5β in HeLa and MelJuSo cells using confocal microscopy. Although transfection efficiency was quite low, the N-terminally tagged protein could be detected, by contrast to the C-terminally tagged protein. Since ABCB5 is highly homologous with ABCB1, which is primarily located at the plasma membrane of cells, but also found in endosomes, lysosomes, endoplasmic reticulum and the Golgi apparatus (Bendayan, Ronaldson, Gingras, & Bendayan, 2006; K. Katayama et al., 2015), we analyzed putative co-localization between GFP-ABCB5β and markers for these organelles.

In the HeLa cell line, the majority of ABCB5β exhibited cytoplasmic localization within an extensive tubular network, which is a characteristic feature of the endoplasmic reticulum (ER) (**Fig. 3A**). The protein exhibited a similar distribution in MelJuSo cells (**Fig. 4A**). Interestingly, we detected co-localization in both cell types with the endoplasmic reticulum marker calnexin, but with none of the other markers analyzed: GM130 for cis-Golgi, TGN46 for Trans-Golgi and LAMP1 for late endosomes/lysosomes (**Fig. 3B-3D** and **Fig. 4B-4D,** SP5 Leica confocal microscope, quantifications are shown in **Fig. 3E** and **4F**). **Fig. 3F** and **Fig. 4E** show high-resolution imaging of the ABCB5β-calnexin colocalization using the Zeiss LSM900 with Airyscan 2 confocal microscope. We also analyzed a marker of melanosomes (lysosomes-like organelles only found in melanoma cells) in MelJuSo cells but did not find any co-localization either (**Figure S1A**).

Keeping in mind that a large GFP-tag may sometimes result in protein mislocalization, we conducted a series of controls and additional experiments. We notably studied the localization of ABCB9, another half transporter from the same subfamily, by using a GFP-tagged ABCB9 protein. As a half-transporter, ABCB9 and ABCB5β have similar molecular weights (84 kDa and 90 kDa, respectively). Importantly, adding a GFP tag at the N-ter position of ABCB9 did not prevent the transporter from reaching lysosomes, i.e. its reported residence site in the cells (Graab et al., 2019). Indeed, GFP-ABCB9 was found to colocalize with LAMP1 (**Fig. 3G**).

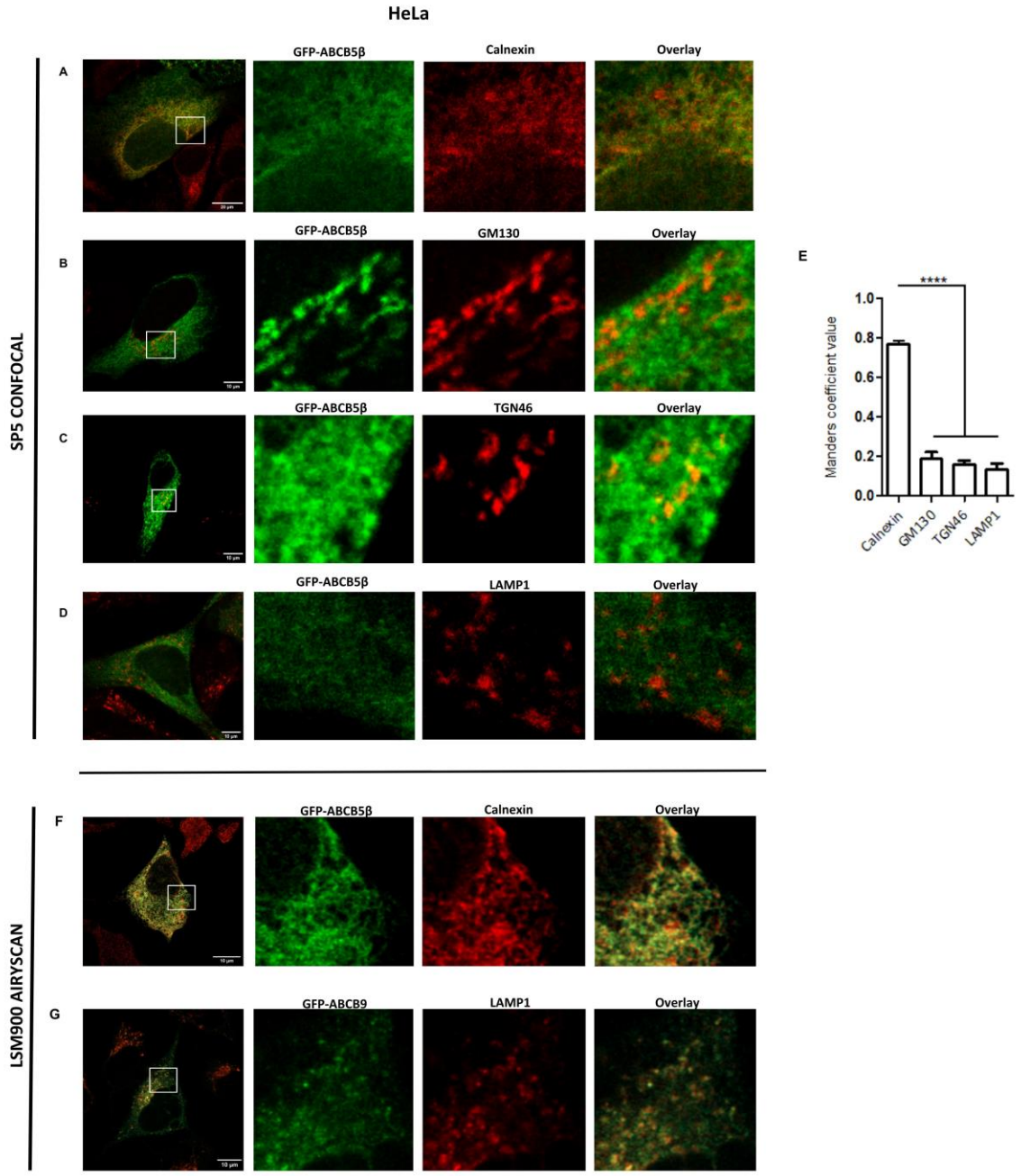

Figure 3. GFP-ABCB5β localizes to the endoplasmic reticulum in HeLa cells*. After 48h of transfection, cells were fixed with 4% paraformaldehyde and processed for detection of eGFP-ABCB5β (green) and of different organelle markers (red): A) calnexin for the ER, B) GM130 for the cis-Golgi apparatus, C) TGN46 for the trans-Golgi apparatus, D) LAMP1 for late endosomes and lysosomes. Micrographs were obtained using an SP5 confocal microscope. E) Graph shows quantification of co-localization extent by using Mander's coefficient. n = 10 cells were analyzed, from at least three independent experiments. ****P<0.001 (one-way ANOVA test). F) Higher resolution micrographs of ABCB5-calnexin co-localization taken using a Zeiss LSM900 microscope. G) Analysis of the presence of GFP-ABCB9 (with the tag in N-ter position) in the lysosomes of HeLa cells detected using an anti-LAMP1 antibody. Imaging with SP5 Leica microscope.*

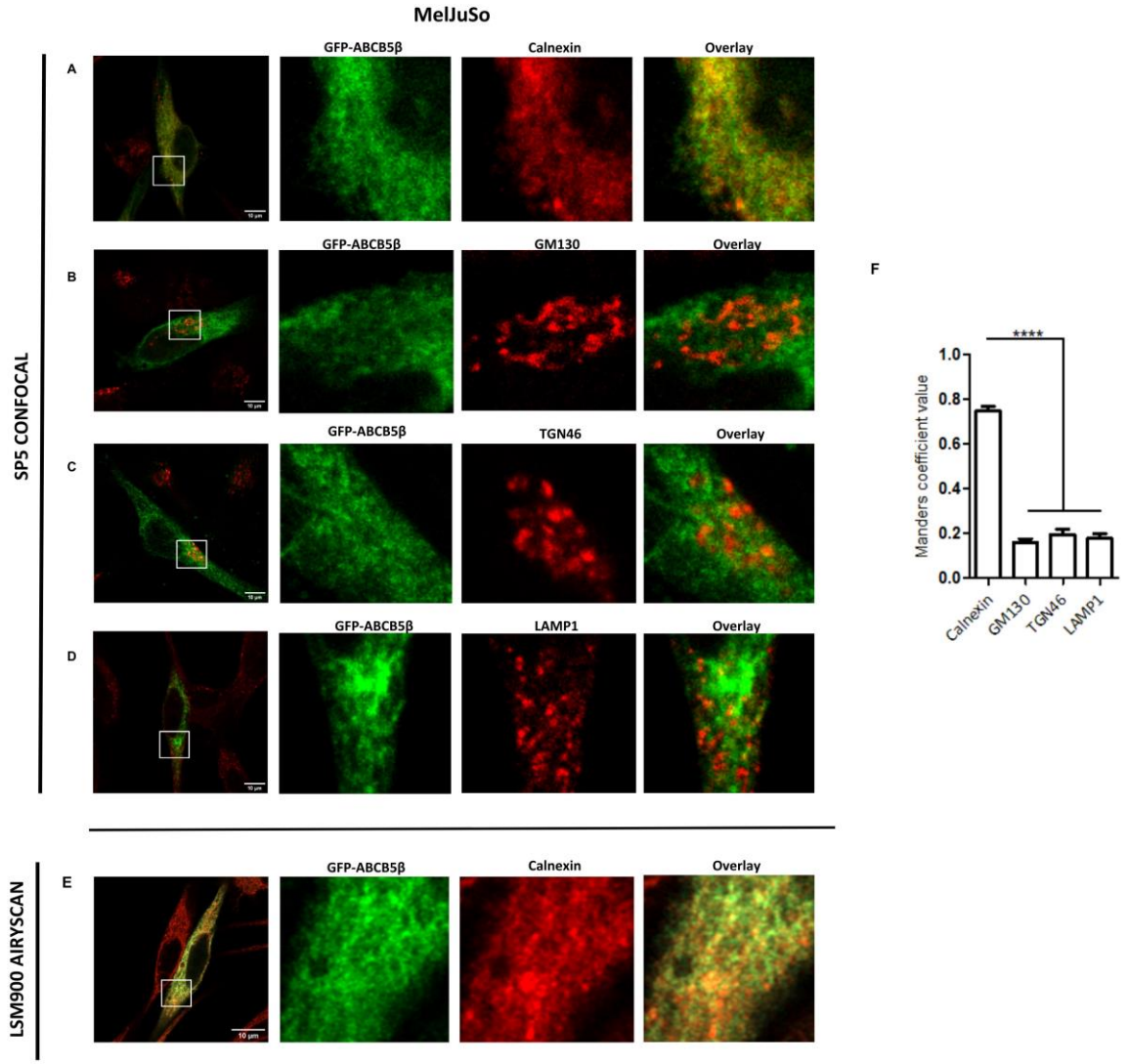

Figure 4. GFP-ABCB5β localizes to the endoplasmic reticulum in MelJuSo cells*. After 48h of transfection, cells were fixed with 4% paraformaldehyde and processed for detection of eGFP-ABCB5β (green) and of different organelle markers (red): A) calnexin for the ER, B) GM130 for the cis-Golgi apparatus, C) TGN46 for the trans-Golgi apparatus, D) LAMP1 for late endosomes and lysosomes. Micrographs were obtained using an SP5 confocal microscope. E) Graph shows quantification of co-localization extent by using Mander's coefficient. n = 10 cells were analyzed, from at least three independent experiments. ****P<0.001 (one-way ANOVA test). F) Higher resolution micrographs of ABCB5-calnexin co-localization taken using a Zeiss LSM900 microscope.*

HA-ABCB5β localizes to the endoplasmic reticulum in HeLa and MelJuSo cells

We engineered an additional construct in which ABCB5β was fused to smaller tag in N-ter position. Hemagglutinin (HA) is only composed of nine amino acids (YPYDVPDYA). No signal overlap was detected between this tagged protein and other organelle markers, including GM130 for cis-Golgi, TGN46 for trans-Golgi, LAMP1 for lysosomes (**Fig. 5A-5D** for HeLa and **Fig. 6A-6D**) and anti-Melanoma antibody for melanosomes in MelJuSo (**Fig. S1B**). GFP-ABCB5β

and HA-ABCB5β co-localized when co-transfected, suggesting that these chimeric proteins exhibit the same localization (**Fig. 5G**). Indeed, when we conducted colocalization analyses as described above, we only observed colocalization between the HA-ABCB5β and the ER marker calnexin (Quantifications are shown **Fig. 5E** for HeLa and **Fig. 6E** for MelJuSo, **Fig. 5F and 6F** for higher resolution).

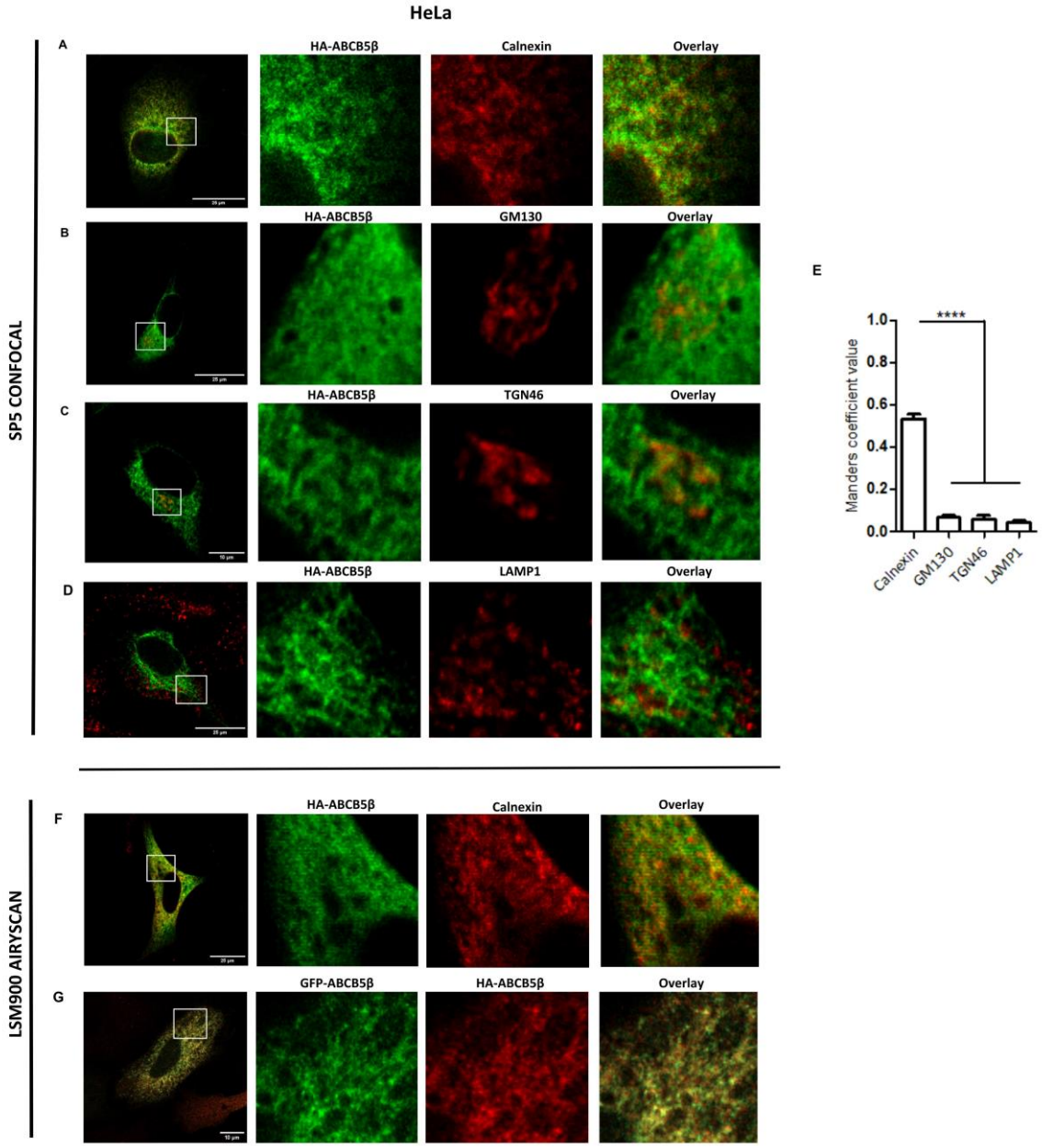

Figure 5. HA-ABCB5β localizes to the endoplasmic reticulum in HeLa and cells*. After 48h of transfection, cells were fixed with 4% paraformaldehyde and processed for detection of HA-ABCB5β (green) and of different organelle markers (red): A) calnexin for the ER, B) GM130 for the cis-Golgi apparatus, C) TGN46 for the trans-Golgi apparatus, D) LAMP1 for late endosomes and lysosomes. E) Graph shows quantification of co-localization extent by using Mander's coefficient. n = 10 cells were analyzed, from at least three independent experiments. ****P<0.001 (one-way ANOVA test). F) Higher*

resolution micrographs of HA-ABCB5 - calnexin co-localization taken using a Zeiss LSM900 microscope. Scale bar= 25 or 10 µm. Micrographs were obtained using an SP5 confocal microscope. G) Analysis of co-localization between GFP-ABCB5β (green) and HA-ABCB5β (red, detected using an anti-HA antibody) 48h after co-transfection in HeLa cells. Micrographs were obtained using an SP5 confocal microscope.

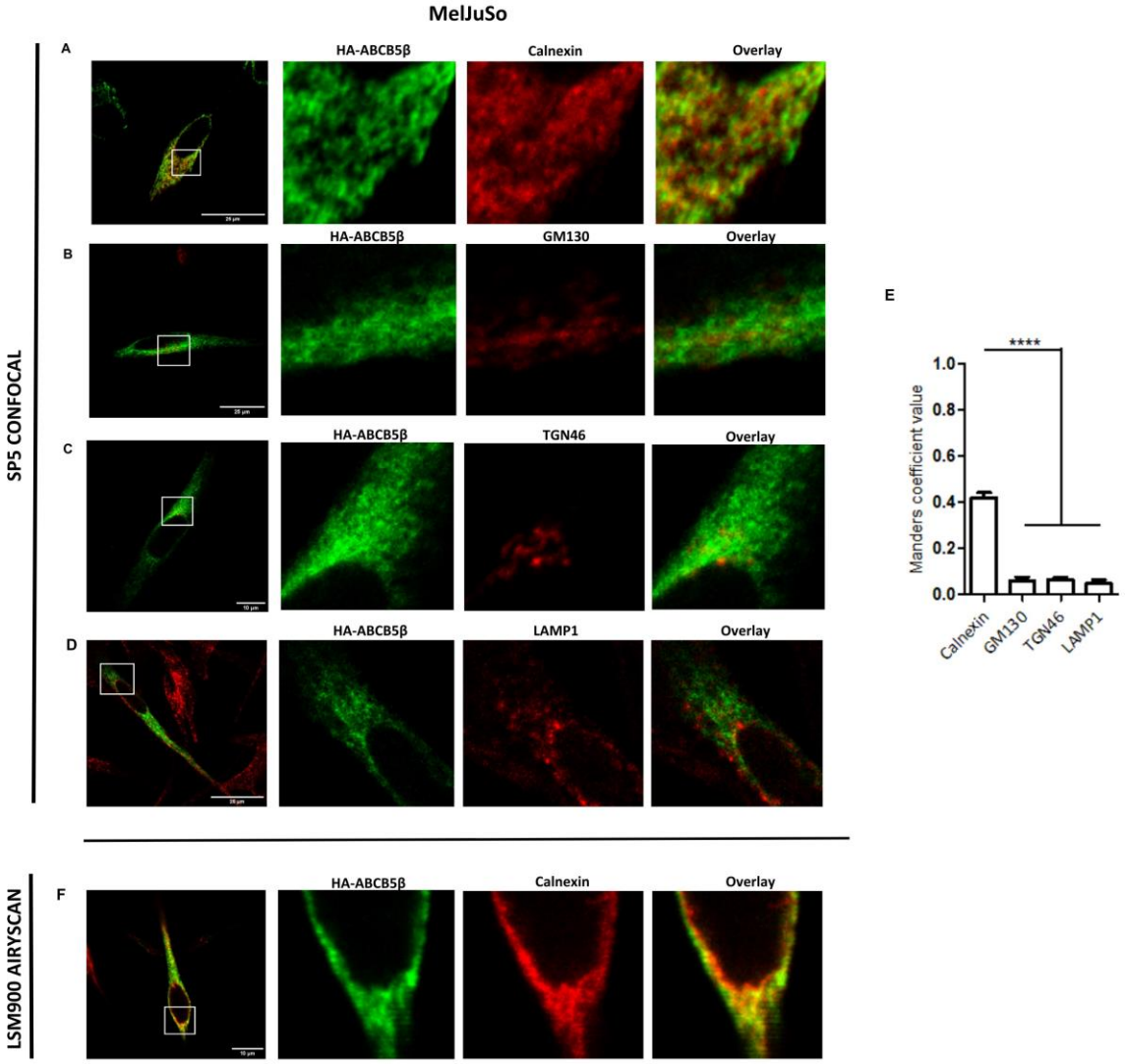

Figure 6. HA-ABCB5β localizes to the endoplasmic reticulum in MelJuSo cells*. After 48h of transfection, cells were fixed with 4% paraformaldehyde and processed for detection of HA-ABCB5β (green) and of different organelle markers (red): A) calnexin for the ER, B) GM130 for the cis-Golgi apparatus, C) TGN46 for the trans-Golgi apparatus, D) LAMP1 for late endosomes and lysosomes. E) Graph shows quantification of co-localization extent by using Mander's coefficient. n = 10 cells were analyzed, from at least three independent experiments. ****P<0.001 (one-way ANOVA test). F) Higher resolution micrographs of HA-ABCB5 - calnexin co-localization taken using a Zeiss LSM900 microscope. Scale bar= 25 or 10 µm. Micrographs were obtained using an SP5 confocal microscope.*

As an additional control, we transferred the HA-ABCB5β construct from the pcDNA3.1 plasmid used in previous experiments, i.e., a plasmid that contains a high expression CMV promoter, to a plasmid containing a lower PGK expression promoter. GFP-ABCB5β and pPGK-HA-ABCB5β co-localized when co-transfected, suggesting that these chimeric proteins exhibit the same localization as well. Again, we only found colocalization between the HA-ABCB5β protein (expressed from the low expression plasmid) and the endoplasmic reticulum marker calnexin (**Fig. 7A, Fig.7F** high resolution picture). No signal overlap was detected between this tagged protein and the other organelle markers (**Fig. 7B-7D,** quantifications of co-localization are shown in **Fig. 7E**). Lastly, we conducted a co-localization analysis with another ER marker, the tail-anchored TA-GFP, which is an ER membrane protein. We also found an important co-distribution with pPGK-HA-ABCB5β (**Fig. S1C-D**).

Taken together, these results support that ABCB5 resides, to a large extent, in the ER under basal conditions.

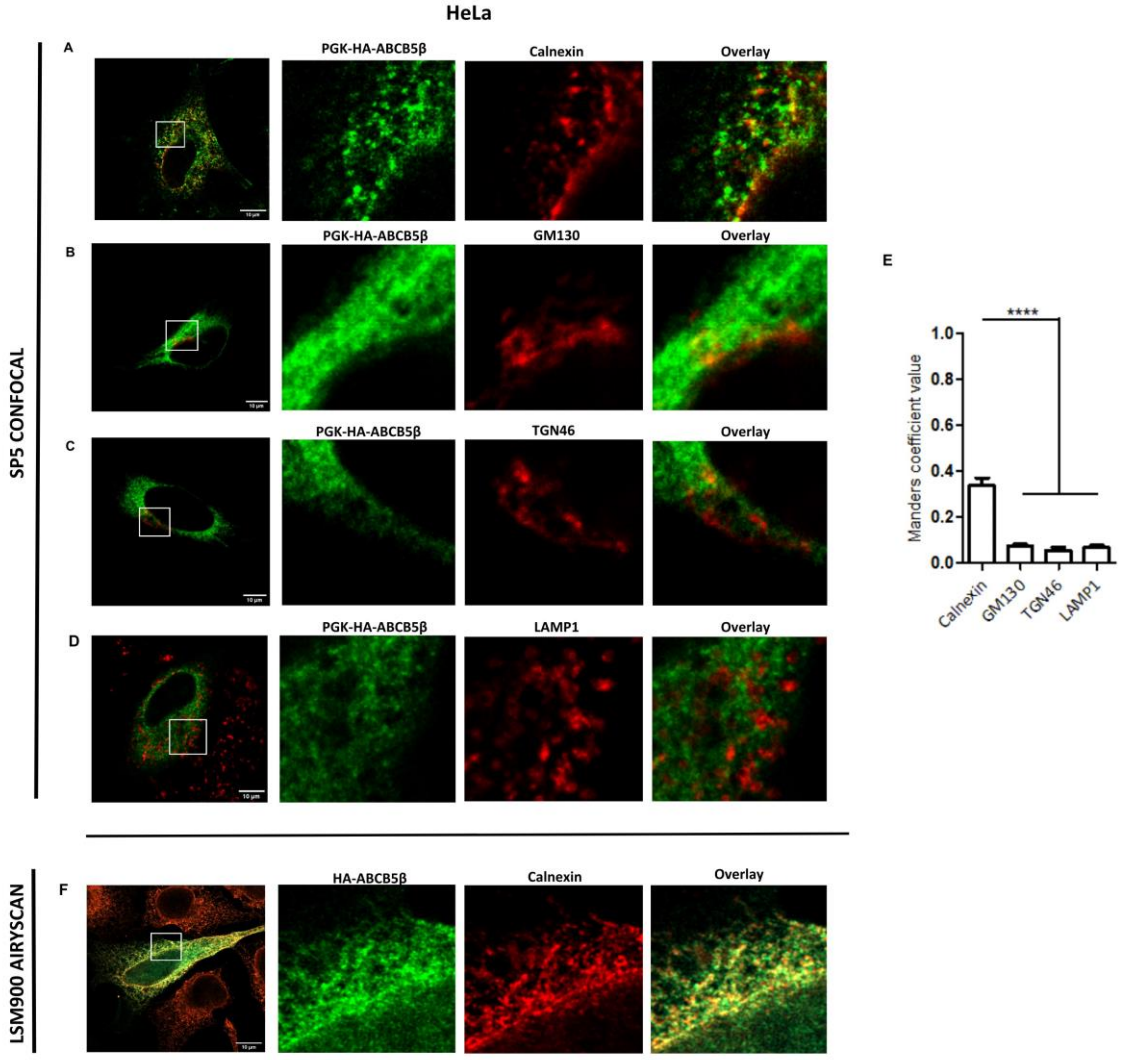

Figure 7. ***PGK-HA-ABCB5β localizes to the endoplasmic reticulum in HeLa cells.*** *After 48h of transfection, cells were fixed with 4% paraformaldehyde and processed for detection of PGK-HA-ABCB5β (green) and of different organelle markers (red): A) calnexin for the ER, B) GM130 for the cis-Golgi apparatus, C) TGN46 for the trans-Golgi apparatus, D) LAMP1 for late endosomes and lysosomes. E) Graph shows quantification of co-localization extent by using Mander's coefficient. n = 10 cells were analyzed, from at least three independent experiments. ****P<0.001 (one-way ANOVA test). F) Higher resolution micrographs of PGK-HA-ABCB5β - calnexin co-localization taken using a Zeiss LSM900 microscope. Scale bar= 25 or 10 µm. Micrographs were obtained using an SP5 confocal microscope.*

ABCB5β remains localized in the ER after treatment of the cells with SAHA

Since part of the population of newly synthesized ABCB5β is degraded by the proteasome system (**Fig. 2**), we also investigated whether treatment of transfected cells with a small molecule that promotes chaperone-assisted folding, i.e. SAHA (suberoylanilide hydroxamic acid) would modify ABCB5β expression and/or localization in the cells. It has notably been reported that SAHA increases the presence of the ΔF508 mutant of CFTR (ABCC7), which is largely misfolded under basal conditions, at the plasma membrane (i.e. the residence site of wild-type CFTR) (Hutt et al., 2010).

Interestingly, incubation of HeLa cells with 2.5 or 5 µM SAHA largely increased the expression of HA-ABCB5β (by 13 and 25-fold, respectively), consistent with an enhancement of its folding (**Fig. 6A**). Nevertheless, the overall protein distribution in the cells remained similar to control conditions; the protein extensively colocalized with the endoplasmic reticulum marker calnexin (**Fig. 6B**).

A few cells exhibited ABCB5β labeling at the cell periphery (see arrows in **Figs. 6B and C**; N.B. cell limits were highlighted with an actin staining in panel C). Considering that ABCB1, a protein with 70% of sequence similarity with ABCB5, localizes at the plasma membrane, we biotinylated all cell surface proteins and assessed the presence of HA-ABCB5β among them by western blotting (**Fig. 6D**). However, the fraction of total HA-ABCB5β proteins recovered in the biotinylated fraction was almost null (0.3 +/- 0.6 %) and did not increase after treatment with SAHA (marginal fractions of 0.4 +/- 0.5% and 0.3 +/- 0.5% of total HA-ABCB5β proteins were biotinylated after treatment with 2.5 or 5 µM of this molecule, respectively). We infer that the ABCB5β signal detected at the cell periphery is not accounted for by a presence of this protein within the plasma membrane. It is possible that this signal results from proximity between ER tubules and the plasma membrane, though not all of these ABCB5β-containing tubules were positive for calnexin.

Taken together, our findings support that ABCB5β is a microsomal protein, mostly found in the endoplasmic reticulum.

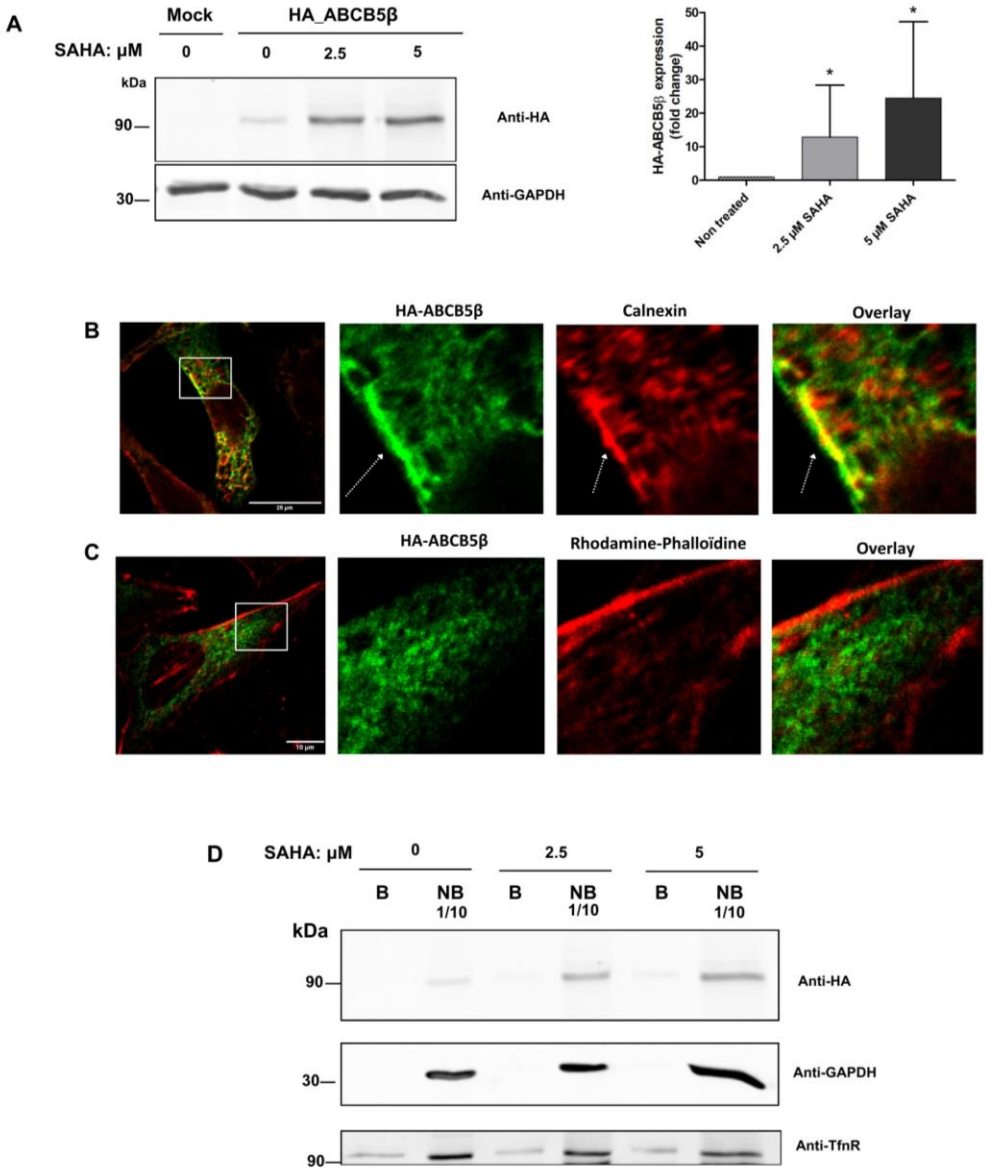

Figure 6. ABCB5β remains localized in the ER after treatment of the HeLa cells with SAHA. *A) Effect of an overnight SAHA treatment on the expression of HA-ABCB5β after transfection in HeLa cells. Proteins were extracted 24h post-transfection and resolved by SDS-PAGE. An anti-HA antibody was used to detect the chimeric proteins. GAPDH detection was used as loading control. Graph shows quantification of expression in n=4 independent experiments. * p<0.05. B) HeLa cells were transfected with HA-ABCB5β and treated overnight with SAHA (2.5 µM), then processed for immunofluorescence detection of calnexin (B) or for detection of the actin cytoskeleton using Rhodamine-Phalloïdin (C). Scale bar= 25 or 10 µm. Micrographs obtained using a SP5 confocal microscope. D) HeLa cells transfected with HA-ABCB5β were treated overnight with DMSO (control) or SAHA (2.5 or 5 µM). Proteins located at the cell surface were then biotinylated and separated from non-biotinylated (i.e., intracellular proteins) using streptavidin agarose beads. The presence of HA- ABCB5β was then analyzed in the B (biotinylated) fraction and in the NB (non-biotinylated) fraction). Of note, 1/10th of total NB fraction was loaded on the gel. GAPDH detection was used as a negative control. As a cytosolic*

protein, it is detected in the NB fraction. Detection of the transferrin receptor (TfnR) served as a positive control, since this protein cycles between the plasma membrane and endosomes.

DISCUSION

The subcellular localization of ABCB5β, which appears to be the main *ABCB5* isoform with putative transport activity expressed in melanoma cells (Chen et al., 2005), has remained unclear, primarily due to concerns about the reliability of anti-ABCB5 antibodies. Indeed, we (**Fig. 1**) and others (19) found that commercial antibodies may be used to detect the overexpressed protein but fail to detect ABCB5 expressed endogenously in melanoma cells, at least by classical western blotting and immunofluorescence methods. It may be that the protein expression level is simply below detection level. Louphrasitthiphol et al. considered that the *ABCB5* protein could exhibit a very long half-life. Hence, the temporal knockdown achieved through siRNA might not be sufficient to obtain a substantial reduction in ABCB5 protein expression. However, we observed ≥80% decrease of *ABCB5* expression at the mRNA level up to 96h post-transfection of the siRNAs, with no change of signal intensities in western blotting experiments. Though it cannot be excluded, it seems unlikely that ABCB5 half-life would be longer than 96h. For instance, the half-life of ABCB1, which shares ~55% identity with ABCB5FL and β, is approximately 26h (K. Katayama et al., 2015).

Several mammalian ABC transporters have been reported to localize at the plasma membrane and to mediate the transport of substances from inside the cell to the external environment (Wu, Wojtowicz, Savary, Hamon, & Trombik, 2020). However, approximately half of all ABC transporters are localized to intracellular compartments such as peroxisomes, lysosomes and endosomes, endoplasmic reticulum, mitochondria and Golgi apparatus (Tarling, de Aguiar Vallim, & Edwards, 2013). As we could not rely on antibodies to analyze ABCB5β localization in the cells, we then made use of GFP and HA tagged proteins. All chimeras were found located in the endoplasmic reticulum, even when using a low expression promoter.

Newly synthesized membrane and secretory proteins must undergo correct folding and sometimes oligomerization, for proper export from the endoplasmic reticulum. Polytopic membrane proteins, like ABC transporters, often encounter challenges in the folding process and/or trafficking from the ER. For instance, studies have shown that a substantial portion, up to 85%, of newly synthesized CFTR (wild-type ABCC7) undergoes degradation via the proteasome system (Ward & Kopito, 1994). Nonetheless, the correctly folded molecules effectively make their way to the plasma membrane to carry out their intended functions. In our study, we found that adding a N-ter GFP tag to ABCB9 (a half-transporter) did not prevent sorting, at least to some extent, to the lysosomes (which is the expected localization site for this protein according to (Fujimoto, Kamakura, Motohashi, Ohashi-Kobayashi, & Maeda, 2011) (Demirel et al., 2012)). Since ABCB5β was only detected in the ER of HeLa and MelJuSo cells, in all tested conditions, it is worth considering that this might be its main localization site under basal conditions.

Several ABC transporters are located, at least partly, in the ER membrane. They can exercise some important functions at this site. For instance, it has been reported that the *ABCA9* transporter localizes to the ER in Hek293 cells and in some breast cancer cell lines, and that it is involved in cholesterol import into this compartment (Hwang, Lee, & Cho, 2023). When overexpressed, it decreases cholesterol synthesis due to its inhibitory effect on SREBP-2 activation and translocation to the nucleus, and it decreases breast cancer cell lines proliferation. Within the ABCB subfamily, the ABCB2/B3 heterodimer associated with antigen processing resides in the ER. The heterodimer transports peptides from the cytosol into the endoplasmic reticulum (ER), thereby selecting peptides for binding to MHC class I molecules (Geng, Sivaramakrishnan, & Raghavan, 2013).

The function of ABCB5β remains elusive and the role of ABCB5β in melanoma has not been fully understood yet. Studies have investigated the potential role of *ABCB5* in cancer multidrug resistance. However, most of them do not specify which isoform was investigated, and the data provided do not allow discrimination between ABCB5FL and ABCB5β. This is a recurrent concern in many of the studies involving ABCB5 (Duvivier & Gillet, 2022). Keniya and colleagues have shown that ABCB5FL confers resistance to several drugs including anthracyclines as opposed to ABCB5β homodimer. Another study strictly focusing on ABCB5FL, showed that this transporter mediates resistance to paclitaxel, docetaxel and anthracyclines (Frank et al., 2005; Keniya et al., 2014).

Conventional half transporters typically have only one NBD, either at the N- or C-terminal region. However, ABCB5β is predicted to possess a transmembrane domain (TMD) composed of six α-helices, flanked by two intracellular NBDs (one of them complete, the other truncated). ABCB5β might form a dimer to create a functional transporter, as potential dimerization motifs have been identified in its N-terminal region (Moitra et al., 2011). Whether homo- or heterodimers of ABCB5β (involving association of this protein with other half-transporters of the ABCB family) confer drug resistance remains an open question.

Our experimental data indicate that ABCB5β expressed by itself localizes to the ER. An intriguing idea is that ABCB5β could travel to other compartments by becoming part of an heterodimer, similarly to what was observed for the obligate ABCG5/G8 heterodimer. These two half transporters are dependent on one another for trafficking to the plasma membrane (G. A. Graf et al., 2003). Under homodimer forms, they remain in the ER.

It has also been reported, for several transmembrane multimeric proteins, that heterodimerization can lead to the masking of ER retention signals. It is notably the case for the NMDA receptor, a molecule that is essential for neurotransmission. The major NR1 splice variant (NR1-1) and the NR2 subunits of NMDA are retained in the ER when expressed alone in heterologous cells and neurons, but when expressed together, they form functional receptors on the cell surface. These receptors are likely heterotetramers, assembled from NR1, NR2, and NR3 subunits (Horak, Chang, & Wenthold, 2008).

Additionally, some proteins may attach themselves to carrier proteins to reach their final destination. It is notably the case of *ABCD4,* which localizes to the ER when expressed alone

but traffics to LAMP1 positive compartments (late endosomes/lysosomes) when co-expressed with the lysosomal membrane protein LMBD1 (Kawaguchi et al., 2016).

In summary, we demonstrated that ABCB5β is predominantly localized to the ER even after the treatment with SAHA, which restored the folding and increased the total level of ABCB5β expression to some extent. However, remains unclear the role of ABCB5β in the ER of melanoma cells and whether it can function either as a homodimer or as heterodimer.

MATERIALS AND METHODS

Antibodies

Three different polyclonal anti-ABCB5 antibodies were used in western-blotting analyses: rabbit anti-ABCB5 (Rockland Immunochemicals, 600-401-A775, 1:500), rabbit anti-ABCB5 (Abcam, ab80108, 1:100); rabbit anti-ABCB5 (Atlas antibodies, HPA026975, 1:500). Goat polyclonal anti-GFP (Rockland, 600-101-215, 1:1000); mouse monoclonal anti-α-Tubulin (Sigma-Aldrich, T5168, 1:1000); rabbit polyclonal anti-GAPDH (Sigma-Aldrich, G9545, 1:1000); mouse monoclonal anti- transferrin receptor (H68.4) (Invitrogen,1:500,13-6800). IRDye 680RD donkey anti-mouse secondary antibodies (Li-cor Biosciences, 926-68072, 1:10000); IRDye 680RD goat anti-rabbit secondary antibodies (Li-cor Biosciences, 926-68071, 1:10000); IRDye 800CW donkey anti-goat secondary antibodies (Li-cor Biosciences, 926-680724, 1:10000). PageRuler™ Prestained Protein Ladder (26617) was purchased from Thermo Fisher Scientific.

The following antibodies were used in immunofluorescence applications: rabbit polyclonal anti-ABCB5 (Rockland Immunochemicals, 600-401-A775, 1:100); rabbit polyclonal anti-HA [C29F4] (Cell Signaling Technology; 3724; 1:100); rabbit anti-calnexin (Abcam, ab22595, 1:100); rabbit anti-GM130 [EP892Y] (Abcam, ab52649, 1:50); rabbit anti-TGN46 (ProteinTech, 13573-1-AP, 1:100); mouse anti-LAMP1 [H4A3] (DSHB, AB_2296838,1:50); mouse anti-melanoma [HMB45 + M2-7C10 + M2-9E3] (Abcam, ab732, 1:50). Anti-mouse conjugated IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 568 (Invitrogen, A11004, 1:250), anti-rabbit Alexa Fluor 594 (Invitrogen, A21207, 1:250), or Alexa Fluor 488 (Invitrogen, A11034, 1:250).

Cell culture, transfection and treatment

The human melanoma cell line MelJuSo (ATCC® CVCL_1403) and HeLa cells (human cervix adenocarcinoma, ATCC® CCL-2) were grown at 37 °C under 5% CO_2 in DMEM with 4,5 g per L glucose, with L-glutamine, with sodium pyruvate (VWR, 392-0416) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin (Lonza, DE17-602E). For transfection, cells were transfected with a mix containing Opti-Mem (Gibco, 31985062), plasmid and FuGENE® HD Transfection Reagent (Promega, E2311) for HeLa cells and jetPRIME® (Polyplus, #114-15) for MelJuSo cells according to the manufacturer's instruction. Experiments were performed 48 h after transfection unless otherwise specified. The inhibition of the proteasome was achieved by treating cells with 1 µM of MG132 (Sigma-

Aldrich, M7449) during 16h. When indicated, the cells were transfected for 6h then treated overnight with 2.5 or 5 µM of SAHA (suberoylanilide hydroxamic acid).

RNA interference

To silence *ABCB5* expression we purchased ONTARGETplus human ABCB5 siRNA SMARTpool from Dharmacon Inc. (Dharmacon™, Horizon Discovery, L-007303-01-0020,) and transfected with Lipofectamine™ RNAiMAX Transfection Reagent according to the manufacturer's instructions. ON-TARGET plus non-targeting pool (D-001810-10-20, Dharmacon™) served as a reference point. Using a total final concentration of 90nM, transfection of the siRNAs was performed at different times points of transfection from 24h up to 96h, where protein depletion efficiency was maximal as shown by RT-qPCR.

RT-qPCR

Total RNA was isolated from MelJuSo cells using NucleoSpin RNA plus kit (Machenerey-Nagel). The quantity and quality of extracted RNA were assessed by spectrophotometry using the NanoDrop™ One/One^{C} Microvolume UV-Vis (Thermo Scientific™ Acclaro™). *cDNA* synthesis was performed using RevertAid Minus First Strand cDNA synthesis kit (Thermo scientific #K1631).

Specific primers for qPCR were designed to target *ABCB5FL* and *ABCB5β*, excluding the rest of the ABCB5 isoforms (forward primer: 5´-GCAGATTTGATTGTGACCCT-3´; reverse primer: 5´-GACTCCATCTGTTCATCAGC-3´). Gene expression was determined using Takyon NoRox Sybr Mastermix Blue (Eurogentec, UF-RTAD-D0701) according to the manufacturer's recommendations on a BioRad CFX96 system (BioRad, Hercules). To normalize the expression analysis, Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (forward primer: 5´-ACCAGGTGGTCTCCTCTGAC-3´; reverse primer: 5´-TGCTGTAGCCAAATTCGTTG-3´) was used as a housekeeping gene.

Plasmid constructs

The cDNA of the *ABCB5β* isoform (NP_ 848654.3) has been inserted via BamHI and EcoRV in a pcDNA3.1(+)N-eGFP plasmid using an In-Fusion method (Takara Bio Inc., 638910). Similarly, the C-terminus tagged version of this construct was prepared by inserting *ABCB5β* in front of the GFP sequence using KpnI. HA-tagged constructs of *ABCB5β* were obtained by insertion of a linker coding for HA via the NheI and KpnI restriction sites located upstream of the *ABCB5β* sequence in pcDNA3.1(+). Finally, ABCB5β was amplified by In-Fusion PCR with primers containing a EcoRI restriction site and inserted into a EcoRI digested pPGK plasmid (Addgene plasmid # 35094) to obtain the HA- *ABCB5β* construct with a low expression promoter.

Western blotting

Cell lysates prepared in RIPA buffer (10mM Tris-HCL, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140Mm NaCl, 1x complete Mini protease inhibitors (Merck, P5726)) were mixed with Laemmli's sample buffer (with DTT) and resolved in 8% SDS polyacrylamide gel. Proteins were transferred onto PVDF membranes (Immobilon®-FL) prior

to detection of the proteins of interests using antibodies and dilutions listed above. Infrared signals were detected using an Odyssey infrared imaging system (LI-COR Biosciences).

Trichoplusia ni (high-five) insect cells were infected with a recombinant baculovirus carrying on the sequence of either ABCB5FL or ABCB5β. Cells were harvested after 58-72 hours, washed with PBS containing 1% aprotinin, and stored at -80°C. Total membrane vesicles were prepared with hypotonic lysis and differential centrifugation as detailed in (Nandigama, Lusvarghi, Shukla, & Ambudkar, 2019). Total membrane vesicles (1µg) were heated for 30 min at 37°C and resolved in 8% SDS polyacrylamide gel, then transferred onto PVDF membranes (Immobilon®-FL) for 1h10 at 150 V, prior to detection of the *ABCB5* isoforms using antibodies and dilutions listed above.

Biotinylation of cell surface proteins

Transfected HeLa cells were washed twice with ice-cold PBS and five times with PBS supplemented with 0.7 mM CaCl2 and 0.25 mM MgSO4 (PBS++, pH 8). Cell-surface proteins were labelled by incubation with 1 mg/ml sulfo-NHS-SS-biotin [sulfosuccinimidyl 2-(biotinamido) ethyl-1,3-dithiopropionate] in PBS++ (Pierce, 79378) for 45 min on ice. Biotinylation was stopped by washing five times with ice-cold 50 mM glycine/PBS++ and cells were lysed with RIPA buffer [50 mM Tris/HCl (pH 7.4), 120 mM NaCl, 1%(v/v) Triton X-100, 0.1%SDS and 1%deoxycholate] containing protease inhibitors (Complete Mini Protease inhibitor cocktail tablets, Roche, 11836153001). Biotinylated proteins were precipitated with streptavidin–agarose beads (Pierce, 20353) by centrifugation at 4◦C for 1.5 min at 8 000 rpm in a bench top centrifuge and then eluted by incubation for 40 min at room temperature in Laemmli's buffer containing 200 mM fresh DTT (dithiothreitol). One-tenth of the supernatant obtained after centrifugation, containing proteins unbound to streptavidin beads, and proteins eluted from the beads (bound), were separated on SDS/PAGE (8% gel) and the proteins of interest were detected by western blotting. Of note, GAPDH was detected as a control. As a cytosolic protein, it should be recovered in the non-biotinylated fraction.

Immunofluorescence

Cells were fixed in 4% paraformaldehyde for 10 min. Cells were then permeabilized with a solution of 0.2% Triton and 1% BSA (Bovine Serum Albumin) in PBS for 10 min and blocked with 3% BSA. Then the cells were incubated with primary and secondary antibodies diluted in BSA solution for 1h. To visualize actin filaments, rhodamine-conjugated phalloidin (ThermoFisher, R415) was used. Lastly, coverslips were incubated for 10 minutes in DAPI (Merck, #28718-90-3) to label nuclei and mounted with Mowiol mounting medium (Sigma-Aldrich, 81381). Fixed samples were imaged with a Leica SP5 confocal microscopy, using a 40x or 63x objective (1.3 and 1.4 numerical aperture, respectively) or LSM 900 confocal microscope equipped with an Airyscan detector and with a Plan Apo 63× numerical aperture (NA) 1.4 oil immersion objective.

Image analyses and quantifications

All image quantifications were performed using (Fiji Is Just) ImageJ 1;53t (NIH). Quantification of co-localization between two channels was performed using the JACoP plugin in ImageJ

software (Cordelieres & Bolte, 2006). After setting a threshold for the signal of interest for each channel, the JACoP plugin was used to obtain the Manders' coefficient and calculate the co-localization.

Data availability

All data used in this article are included within the figures and Supporting information files.

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Author contributions— A.M.D.A., L.G., M.A., J.-F. G. and M.B. conducted the experiments. A.M.D.A., M.B. and J.-P.G. analyzed the data. A.M.D.A. wrote the original draft; M.B. and J.P.G. contributed to writing and revised the manuscript. A.M.D.A., M.B. and J.-P.G. designed the experiments. M.B. and J.-P.G. supervised the project.

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Conflict of interest— The authors declare that they have no conflicts of interest with the contents of this article.

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SUPLEMENTARY DATA

Antibody	Type	Epitope Position in ABCB5FL isoform	Epitope Position in ABCB5β isoform	Epitope sequence	Source
Anti-ABCB5 600-401-A77	Rabbit PC	637-653	192-208	YSTERKTNSLPLHSVKSIKS	Rockland Immunochemicals
Anti-ABCB5 HPA026975	Rabbit PC	590-679	145-234	DLIVTLKDGMLAEKGAHAELMAKRGLYYSLV MSQDIKKADEQMESMTYSTERKTNSLPLHS VKSIKSDFIDKAEESTQSKEISLPEVSLL	Atlas antibodies
Anti-ABCB5 Ab80108	Rabbit PC	446-545	1-99	MVDENDIRALNVRHYRDHIGVVSQE PVLFGTTISNNIKYGRDDVTDEEMERAARE ANAYDFIMEFPNKFNTLVGEKGAQMSGGQ KQRIAIARALVRNPK	Abcam

Table S1. *Protein sequence for the specific epitope of the three anti-ABCB5 antibodies selected for the screening. PC: polyclonal*

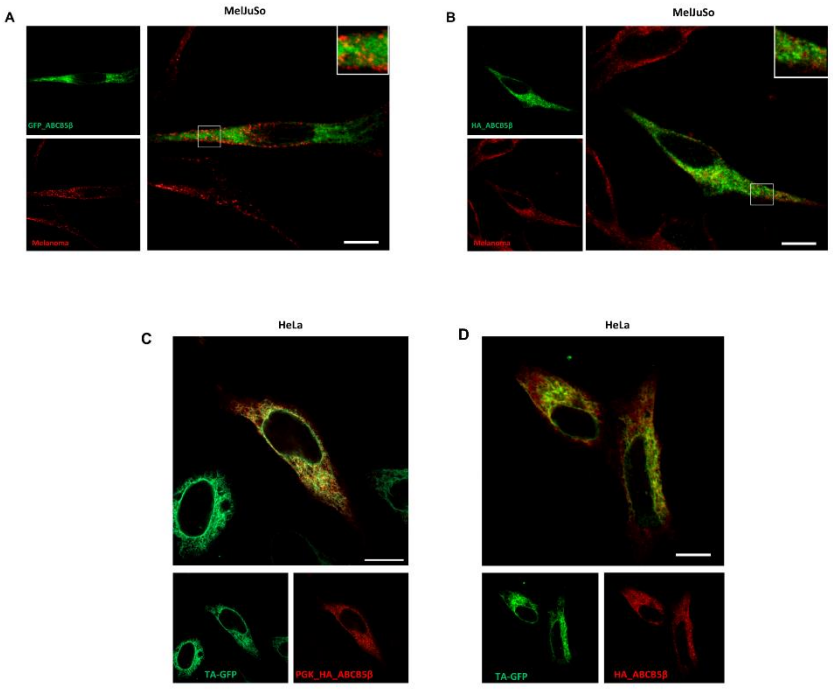

FIGURE S1. Co-localization analysis of the different ABCB5 constructions in transfected HeLa and MelJuSo cells*. A) MelJuSo cells were transfected during 48h with GFP-ABCB5β and processed for detection of melanosomes, B) co-localization between HA-ABCB5β and melanosomes marker. C) Analysis of co-localization between TA-GFP and pPGK_HA-ABCB5β (red, detected using an anti-HA antibody) 48h after co-transfection in HeLa cells. D) Colocalization between TA-GFP and HA-ABCB5β construction. Scale bar= 20 µm.*

2. Additional data not included in the research article

2.1 CRISPR-Cas9-based strategy for insertion of a hemagglutinin (HA) tag in N-terminal position of *ABCB5* within the genome of MelJuSo cells

We established a strategy for implementing CRISPR/Cas9-mediated endogenous tagging of ABCB5β within MelJuSo cells using a HA tag.

A guide RNA (gRNA) was designed to induce a Cas9-mediated cut proximal to the start codon of *ABCB5β* located in exon 4. A 190 bp single-stranded oligo donor (ssODN) encoding the HA tag flanked with 80 nucleotide homology arms, was then used to repair the cut and insert the tag (**Figure 22**).

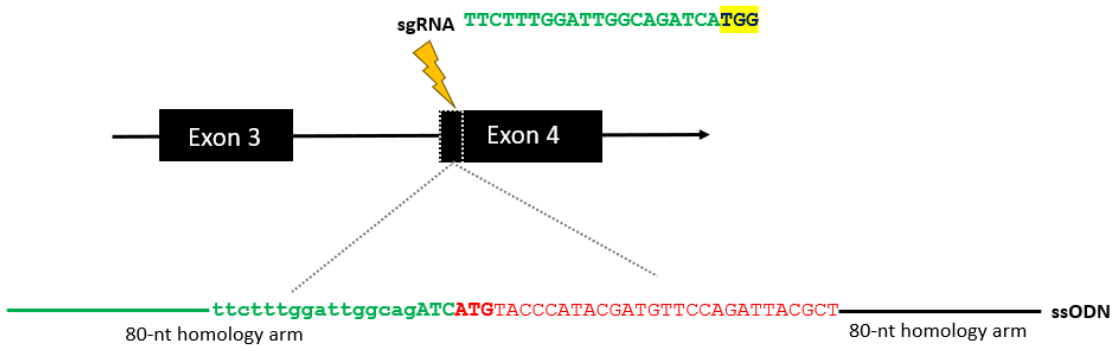

Figure 22. *Schematic representation of the HA-tag insertion strategy. A gRNA was designed to cut proximal to the ATG region in exon 4 of ABCB5β. A single stranded donor nucleotide containing the HA sequence, the beginning of ABCB5β sequence, and two 80-bp homology arms in both sides was used.*

2.1.1 Preliminary analysis of HA insertion in a non-clonal population

We first applied a PCR to detect the presence of the HA tag in the genome of three individual pools of transfected cells (non-clonal population). These three different pools were obtained using three different concentrations of ssODN (**Figure 23a**). The results show, for all three pools, a band at the expected size of 600 bp, the forward primer being located in the HA sequence and the reverse primer 600 bp downstream from this site, as shown on the

schematic, **Figure 23b**. Seeing no difference between the three pools, we randomly selected the pool of cells transfected with the intermediate concentration of ssODN to proceed with the clonal dilution of the cells.

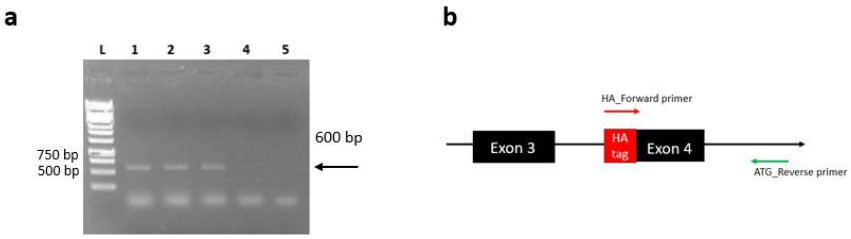

Figure 23. *Schematic representation of screening strategy of genomic DNA by PCR. a) Migration on agarose gel of amplification products obtained after PCR screening for genomic HA insertion in the pool of MelJuSo cells after 48h of electroporation of RNP complex. Ladder (L). Lane 1) 20µM ssODN, lane 2) 40µM ssODN, lane 3) 80µM ssODN, lane 4) no ssODN, lane 5) no genomic DNA (control with water). b) Location of the forward primer (HA_ATG) and reverse primer (ATG_R).*

2.1.2 Screening of the clones

For the screening of the clones, we applied the same PCR strategy as described above, but used an additional primer pair (as shown on the panel a and b, **Figure 24**). We found several positive clones in the 30 clones tested using the two different pair of primers (**Figure 24c**). We also used sanger sequencing to confirm in-frame and error-free in the insertion of the HA tag sequence at the N-terminus of *ABCB5* (Panel c in the **Figure 24**).

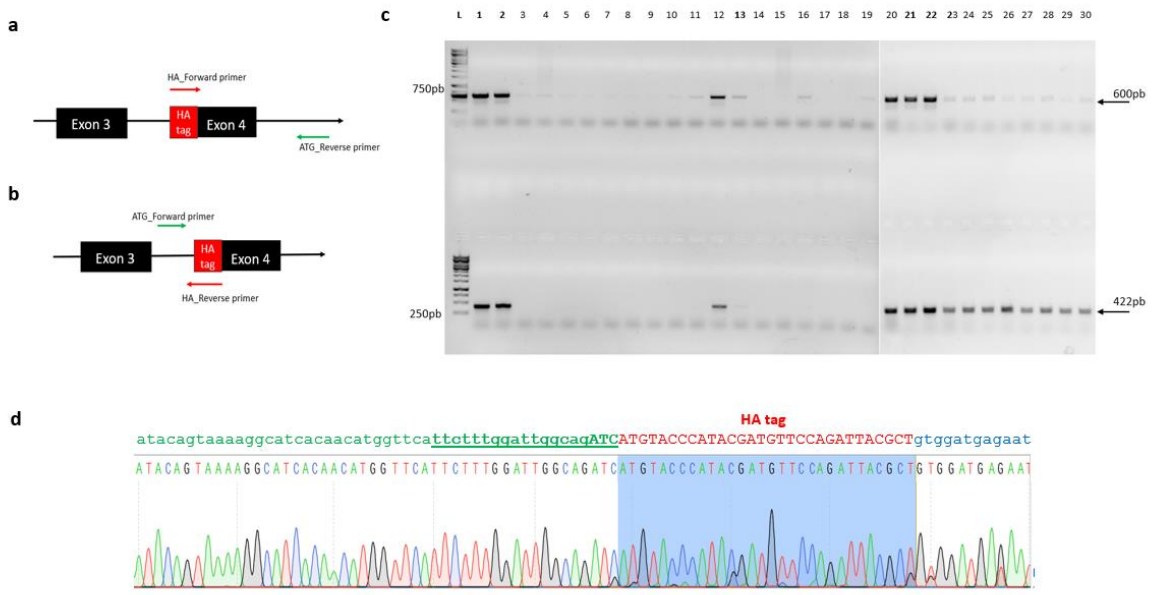

Figure 24. *CRISPR-Cas9-based strategy for insertion of a hemagglutinin (HA) tag in N-terminal position of ABCB5 within the genome of MelJuSo cells. a) HA_F/ ATG_R primers were used. They amplify a 600bp fragment if the HA tag is present. b) we used ATG_F/HA_R primers, which amplify a fragment of ~422bp if the HA tag is inserted in the genome. c) Migration on agarose gel of amplification products obtained after PCR screening of the HA insertion in gDNA extracted from 30 clones of MelJuSo. In the upper panel HA_F/ ATG_R primers, in the lower panel ATG_F/HA_R primers. Ladder (L). d) Sequencing traces from the respective PCR amplicons were aligned with the expected HA_ ABCB5β chimeric sequence.*

Additionally, we extracted total proteins from the clones that were positive by PCR. We tested them by western blotting using either a monoclonal or a polyclonal anti-HA antibody. However, no band were detected at the expected molecular size (91KDa) under basal conditions, nor after treatment with the proteasome inhibitor MG132. We included this control, to test for a possible rapid degradation of the HA-tagged protein.

We also tried to detect the tagged ABCB5β protein by immunofluorescence in the positive clones detected by PCR. The signal obtained in the clones was very low and not increased compared to control cells (without HA tag insertion), so it likely represents background noise (**Figure 25**).

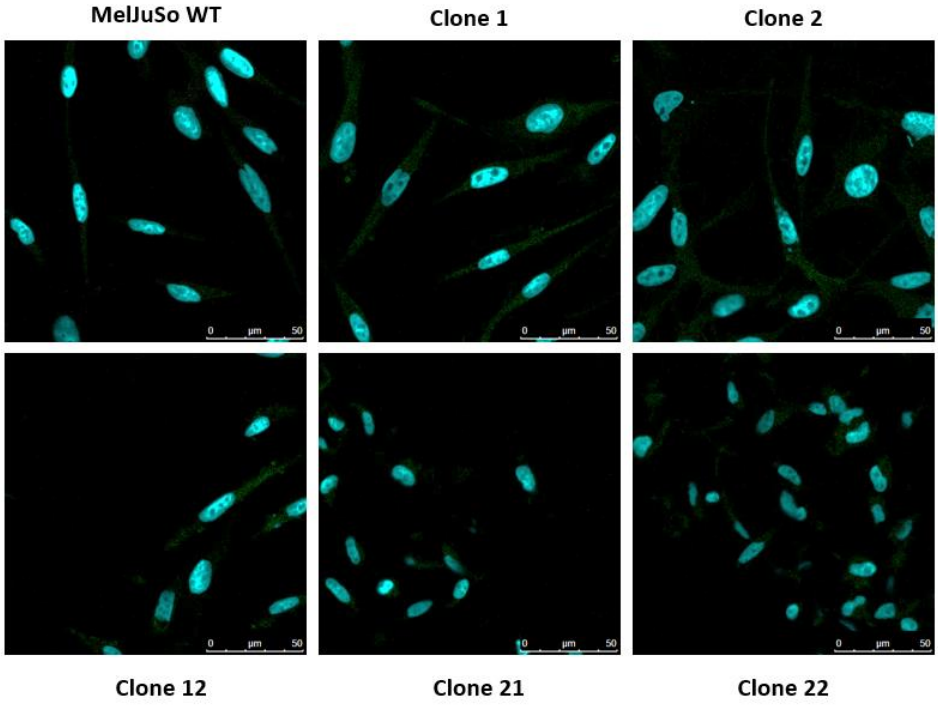

Figure 25. *Immunofluorescence micrographies in PCR-positive clones of HA-ABCB5β in MelJuSo cells. Cells were fixed with 4% paraformaldehyde and stained with anti-HA antibody followed by an AlexaFluor 488 (green) rabbit secondary antibody. Nuclei were stained with DAPI.*

Lastly, we used RT-qPCR to check whether the HA tagged-*ABCB5β* was expressed at the mRNA level, using a forward primer located in the HA sequence, and a reverse primer 150bp downstream. Four clones that were positive in the genomic DNA screen, gave rise to qPCR Cq values between 26-29 (**Figure 26a**). For comparison, Cq value was around 40 in the control clone (without HA insertion). Threshold cycle ratio ratios (CT gene-CT *GAPDH,* i.e. ΔCT) are shown in **Figure 26b**.

a

	Sample	GAPDH
	Average Cq value	
Control neg.	40,93	22,48
Clone 1	28,92	20,75
Clone 2	25,84	22,18
Clone 12	29,40	20,75
Clone 21	26,09	21,20
Clone 22	29,51	21,27

b

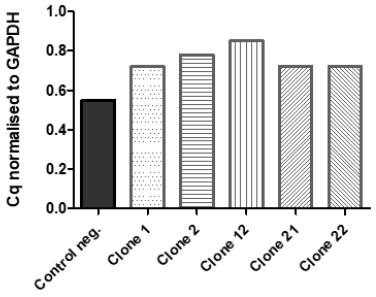

Figure 26. *Analysis of the presence of HA-ABCB5β mRNA in MelJuso clones. a) Average Cq values obtained after RT-qPCR using a primer that recognizes the HA sequence, and a downstream primer located in the ABCB5β sequence. GAPDH Cq are shown as controls. Negative control is a cell line that was negative by PCR screening. b) The graph shows the Cq mean values normalized to GAPDH Cq values for the clones compared with the negative control cells.*

2.2 Investigation of the putative consequences of *ABCB5* knock-down in MelJuSo cells.

After optimization of an siRNA transfection protocol in MelJuSo cells (see Materials and Methods for experimental conditions), the expression of *ABCB5 mRNA* could be decreased by ≥90% up to 72h of siRNA transfection (by 80% at 96h) in MelJuSo cells (as shown in our article). Of note, the siRNA pool used in these experiments does not discriminate between *ABCB5* full-length and its β isoform. However, considering that the latter is the main transporter-coding isoform expressed in MelJuSo cells, it is likely that the knock-down observed at the mRNA level mostly reflects a decrease of expression of ABCB5β.

2.2.1 Search for putative pathway(s) disrupted by *ABCB5* knock-down

Following the validation of *ABCB5* knock-down (KD) in MelJuSo cells, we decided to assess the gene expression level of several candidates potentially linked to *ABCB5*. This exploration was related to several pathways, based on the current literature and on datamining of the GEO profile database.

As mentioned in the introduction, ABCB5 has been shown to constitute a key element of stem cell maintenance by controlling the IL1-β/IL-8/CXCR1 signaling pathway in several melanoma cell lines (Wilson et al., 2014). Indeed, ABCB5 controls IL-8 expression, a required cytokine for malignant melanoma initiating cells maintenance, through WFDC1 repression and IL1-β secretion. Moreover, in melanoma-initiating cells ABCB5/WFDC1 control of IL-8 expression seemed to be exerted through the regulation of several Wnt members as ABCB5 KD and WFDC1 overexpression resulted in significant decreased mRNA expression of Wnt1, Wnt7B, Wnt9A, Wnt10A and Wnt11 while use of Wnt-mimicking drugs enhanced IL-8 expression (Wilson et al., 2011).

Based on this information, we decided to test IL1-β, IL-8 and WFDC1 expression at mRNA level in our *ABCB5*-KD MelJuSo cell model. It turned out that IL-8 expression significantly decreased by 57% (*$p < 0.05$; p value 0,0413), while IL1-β and WFDC1 expression remained stable or slightly increased (**Figure 27**, ns). As IL-8 has been shown to be a target gene of the Wnt pathway, Wnt1, Wnt2B, Wnt5, Wnt3/3A, Wnt7A and Wnt13 were also assessed, however most of them were below detection level. We tested Wnt members involved in both canonical (Wnt1, Wnt2, Wnt3/3A) and non-canonical (Wnt5A, Wnt7A) Wnt pathway (Zhu & Li, 2023). Moreover, Wnt5A has also been reported involved in IL-1β-mediated cell migration and differentiation (S. Y. Park, Kang, & Han, 2018). Other regulators within the Wnt pathway, such as GSK3-β and β-catenin, have also been tested. These elements are also involved in the regulation of melanogenesis through the GSK3-β/β-catenin signaling axis, which is regulated by ABCB6 (She et al., 2022). The RT-qPCR assay showed that downregulation of ABCB5 in MelJuSo cells, did not significantly change the expression of most of the selected genes (**Figure 27**).

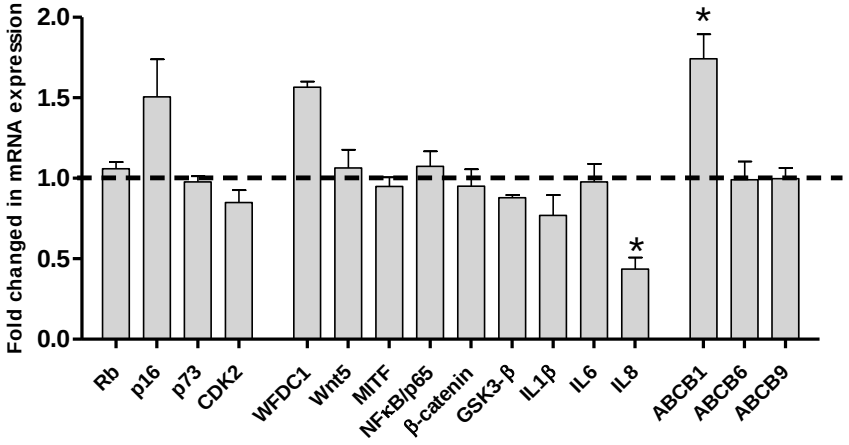

Figure 27. *Effect of ABCB5 know-down in MelJuSo cells over the expression of different genes. Levels of each gene were analyzed by real-time RT-qPCR. Relative expression normalized to GAPDH is shown on the graph. All values are mean ± SD of three independent experiments with triplicate measurement (n=3). *p < 0.05; unpaired student's t-test applied to ΔΔCT values.*

As ABCB5 regulates IL-8 expression and IL1-β secretion, we also assessed the consequences of its KD on the expression of several other proinflammatory (IL-6, TNF-α) and anti-inflammatory (IL-4, IL-10, IL-13) cytokines (usually associated with IL-1β and IL-8 inflammation). Only IL-6 could be detected in our MelJuSo model, and its expression remained unchanged after the silencing of *ABCB5*. The NF-κB signaling pathway is abnormally activated during melanoma metastasis, of note, ABCB5 has also been shown to stabilize NF-κB p65 in melanoma, however at the protein level (Wang et al., 2017). Therefore, NF-κB p65 but also NF-κB p50 expression after ABCB5 silencing were also assessed in MelJuSo. The former did not exhibit any change of expression, whereas the latter was below detection level in the qPCR analysis.

An interesting PhD thesis published in 2014, showed that p16 expression decreased after downregulation of ABCB5β in Human Mammary Epithelial Cells (HMEC). p16 is known as an important regulator of the normal cell cycle. This decrease in p16 induce an increase in proliferation (Braker, 2014). To further explore this link, we assessed the expression of several cell cycle regulators such as Rb, p16, p73, CDK2 and p21 in *ABCB5* KD MelJuSo cells. Only p16 and Rb were detected. The former exhibited an non-significant increase of expression.

Finally, we wanted to determine if *ABCB5* silencing could influence the expression of other ABC transporters from the same family, *ABCB1, ABCB4 and ABCB11* which code for full transporters, as well as *ABCB6* and *ABCB9*, which code for half transporters involved in the putative formation of two heterodimers in melanoma: ABCB5β/B6 and ABCB5β/B9 (Gerard et al., 2022).

From the screening by real-time RT-qPCR, we found an upregulation of *ABCB1* (*p < 0.05; p value 0,0198) at mRNA level. The other ABCB genes were either not detected at mRNA level (*ABCB1, ABCB4 and ABCB11*), or did not show any modification of their expression level (ABCB6 and 9).

Regarding ABCB1, we decided to analyze its expression at the protein level. Interestingly, silencing of *ABCB5* resulted in an upregulation of *ABCB1* expression at both the mRNA (As shown before in the **Figure 27**) and protein levels (**Figure 28**). These findings indicate a potential inverse relationship between *ABCB5* and *ABCB1* expression.

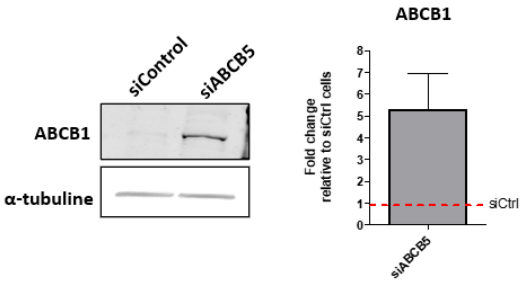

Figure 28. *Detection of ABCB1 in MelJuSo cells by western blotting after knock-down of ABCB5. Western blotting detection of ABCB1 using an anti-ABCB1 (C219) antibody. Tubulin was detected as loading control Quantification showing the increase in the expression of ABCB1 after ABCB5 knock-down is shown on the graph.*

We also assessed protein expression of several candidates that were tested at mRNA level previously. The target proteins chosen for this screening were *p21, p27, Rb, phospho-Rb, CDK2, β-catenin*, *MITF, NF-κB p65, p44/p2 MAPK* and *melanoma gp100*. *α-tubulin* was used as a loading control (**Figure 29**). While small changes of expression could be observed for some target proteins (e.g. for p27 and phosphor-Rb) after *ABCB5* KD, these were not reproduced in

a second experiment, suggesting that none of the tested protein is affected (in terms of expression) by *ABCB5* silencing.

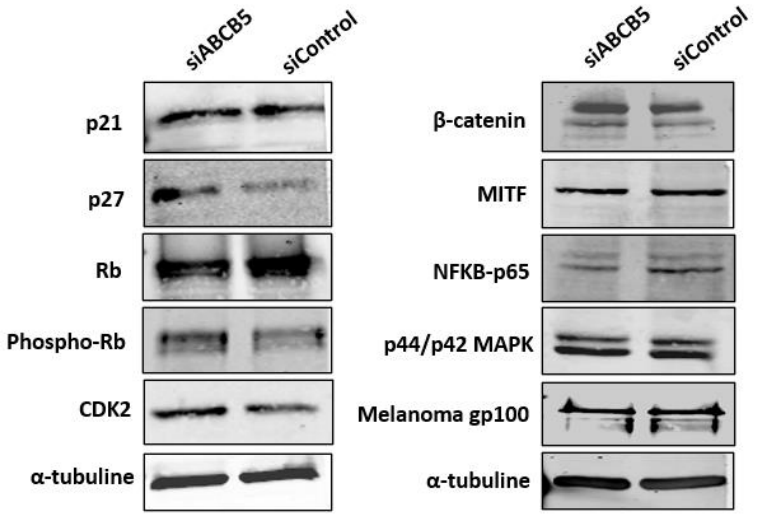

Figure 29. *Western blotting screening of putative changes of expression of selected proteins after knock-down of ABCB5 in MelJuSo cells. MelJuSo cell lysates were prepared 72h after transfection with control or ABCB5 siRNAs, then processed for western blotting using antibodies against p21, p27, Rb, phospho Rb, CDK2, β-catenin, MITF, NF-κB p65, p44/p42 MAPK and melanoma gp100. α-tubulin was used as a loading control.*

2.2.2 Analysis of the effect of *ABCB5* knock-down on MelJuSo cell proliferation.

To determine whether *ABCB5* knock-down affects MelJuSo cell proliferation, we used two methods. First, a MTT test, which is used to measure cellular metabolic activity and thus allows to follow the multiplication of living cells over time (**Figure 30a**). On the other hand, we carried out the experiment using the Cytonote, which is a cell imaging system placed in a CO_2 incubator. This system makes it possible to detect the number of cells present in the wells by automatic counting (**Figure 30b**). Similar proliferation results were obtained with the two methods used, with n=2 independent experiments conducted with each method. Although we did not have time to analyze additional biological replicates, these preliminary data, obtained by two different methods, indicate that the silencing of *ABCB5* results in a decrease of cell proliferation, suggesting that expression of this putative transporter is required to support cell survival and/or division.

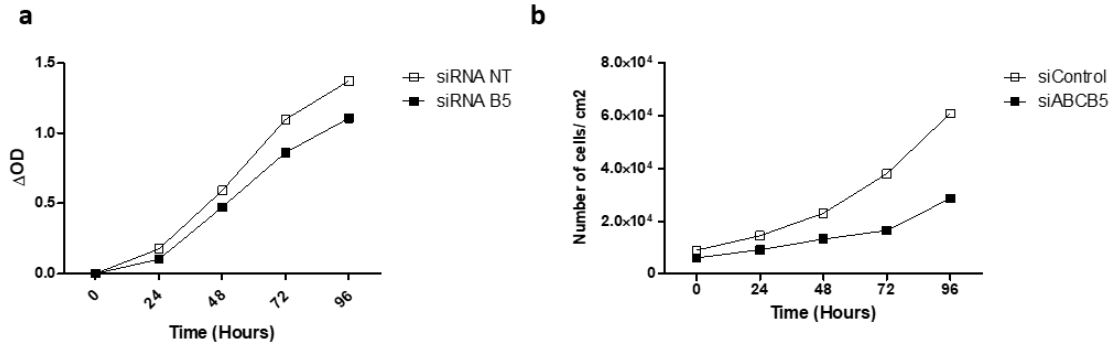

Figure 30. *Analysis of the effect of ABCB5 silencing on MelJuSo cell proliferation. MelJuSo cells were transfected with a scrambled siRNA pool (siCtrl) or with a pool of 4 siRNAs directed against ABCB5 (siABCB5) during 24h, 48h, 72h or 96h. a) Proliferation was monitored with the MTT assay. b) Proliferation was analyzed with Cytonote. N=2 for each experiment. ΔOD = Optical Density, this value is obtained by subtracting the optical density from the day by the measurement at day 0 in order to avoid considering the adhesion of the cells to the plastic.*

DISCUSSION AND PERSPECTIVES

ABCB5 has been reported for its roles as a marker of skin progenitor cells (Frank et al., 2003), melanoma stem cells (Schatton et al., 2008) and more recently as a marker of limbal stem cells (Ksander et al., 2014). It was additionally documented as a mediator of multidrug resistance in various cancers, including melanoma (Frank et al., 2005), colorectal cancer (Kugimiya et al., 2015; Wilson et al., 2011), hepatocellular carcinoma (Cheung et al., 2011) and in several haematological malignancies (Farawela, Khorshied, Kassem, Kassem, & Zawam, 2014). *ABCB5* expression is particularly strong in pigment-producing cells like melanocytes (Chen et al., 2005; Frank et al., 2003; Heimerla, Bosserhoffb, Langmanna, Eckera, & Schmitza, 2007). It has been proposed by Chen et al. (2009) that ABCB5 might be intricately linked to the differentiation and/or pigmentation processes. However, further research is needed to unravel the exact mechanisms through which ABCB5 might influence these processes, and also which ABCB5 isoform is involved in the studied biological process. Indeed, there are two ABCB5 isoforms, namely ABCB5FL and ABCB5β, and their expression is driven by independent promoters. ABCB5FL was shown to confer multidrug resistance in contrast to ABCB5β (Kawanobe et al., 2012). However, the studies focusing on ABCB5β only included a limited number of drugs and therefore we may not exclude that this transporter might be involved in drug resistance. It may also be involved in other biological functions that have yet to be unravelled.

Our research group recently discovered that ABCB5β forms a heterodimer with both ABCB6 and ABCB9 (manuscript under review Gerard et al. (2022)). These two heterodimers exert an ATPase activity and the screening of compounds libraries is ongoing to identify their substrates. There are many unanswered questions concerning ABCB5 transporters in melanoma. Are there specific regulatory pathways that drive the expression of each ABCB5 isoform? What are their physiological substrates? What are the respective functions of the two isoforms? What is their subcellular localization in the cells? Since clarifying the intracellular localization of a protein is essential to fully understand its function in the cells, we decided to focus specifically on this unresolved localization question in this thesis work.

Finding the localization of ABCB5 has been a challenge for many years due to concerns about the specificity of anti-ABCB5 antibodies produced by different groups or companies. Although the Frank group reported the presence of ABCB5 at the plasma membrane as well as in the cytoplasm of human epidermal melanocytes using flow cytometry and immunofluorescence approaches, the study did not include an assessment of the specificity of the antibody they generated (Frank et al., 2003). In addition, several subsequent studies using commercially available anti-ABCB5 antibodies, including our own work, cast doubt on the relevance of using antibody-based approaches to study ABCB5 localization. Indeed, Chen et al. (2009) failed to find specific antibodies for ABCB5 isoforms. Louphrasitthiphol et al. (2020), tested three additional anti-ABCB5 antibodies to analyze ABCB5 expression in a melanoma cell line using a western blotting method, but reported that the signals detected with these antibodies did not decrease in intensity after knockdown of *ABCB5*. In this thesis, our team tested some more commercial antibodies and found that they could detect the ABCB5 protein when overexpressed in insect cells, but failed to detect the endogenously expressed ABCB5 in melanoma cells. The signals detected by Western blotting and immunofluorescence did not diminish after successful knockdown of *ABCB5* expression. Louphrasitthiphol et al. (2020) hypothesized that the ABCB5 protein may have a very long half-life or that its expression may be below the detection level of the antibodies tested. However, it seems unlikely that ABCB5 protein levels would remain unchanged for up to 96 hours after transfection of siRNAs targeting *ABCB5* (a time point at which >80% of mRNA expression is still extinguished). Moreover, ABCB1, which shares around 55% of identity with ABCB5, has a half-life of approximately 26 hours (K. Katayama et al., 2015).

Since anti-ABCB5 antibodies do not allow to study the subcellular localization of endogenous ABCB5, we decided to use protein tagging. In our work, we wanted to investigate more specifically the subcellular localization of ABCB5 in melanoma cells, where it is predominantly expressed when compared to non-pigmented cells (Chen et al., 2005). We focused solely on the beta isoform of ABCB5 (ABCB5β) considering that Chen et al. reported that ABCB5FL was undetectable in a melanotic cDNA library, by contrast to α and β isoforms (Chen et al., 2005).

We generated GFP-tagged ABCB5β constructs, which had GFP fused to either the N-terminus or C-terminus of the protein. After overexpression of these constructions in MelJuSo and Hela

cells, we observed that N-ter tagged ABCB5β showed a higher level of expression compared to the C-ter construct, suggesting that the presence of GFP at the end of the protein disturbed its folding and resulted in its degradation by the proteasome system. We validated this interpretation using a MG132 proteasome inhibitor, which showed that large amounts of ABCB5β-GFP are indeed degraded by this system.

We pursued our investigations of ABCB5β localization using the N-ter tagged construct, and conducted a colocalization study using markers for different organelles. These analyses revealed that GFP-ABCB5β has a predominant ER distribution in both MelJuSo and Hela cells.

It is important to note that tagged proteins might differ from their natural state and experience reduced function, altered interactions, and incorrect folding, which may impact their localization, notably when using bulky tags (e.g. GFP) (L. Huang, Pike, Sleat, Nanda, & Lobel, 2014; Montecinos-Franjola, Bauer, Mears, & Ramachandran, 2020). In the case of ABCB5β, we observed that C-ter tagging with GFP has a major effect on the stability of the protein. In fact, the N-ter tagging did as well, although to a lesser extent. Whether the presence of this large tag also impacted ABCB5 localization was an open question at this stage. Thus, we decided to prepare constructions of ABCB5β fused to smaller tags, i.e., HA or myc. For reasons that are unclear at this stage, by contrast to the HA-ABCB5β construct (with HA in N-ter position), the myc-tagged ABCB5β constructs (with the tag placed in N-ter position) were not detectable by western blotting nor by immunofluorescence labeling after transfection in HeLa, HEK293T cells or melanoma cell lines.

For the construct in which ABCB5β was fused to HA in the N-ter position, ABCB5β showed predominant distribution in the ER of both MelJuSo and HeLa cells. It is notable that the transfection efficiency was higher for HA-ABCB5β compared to GFP-ABCB5β. This may be accounted for by the fact that the HA- plasmid is smaller, and/or by a better folding of the HA-tagged protein. Nevertheless, this remains an overexpression system and it has been reported that saturation of the ER machineries (involved in folding, co- and post-translational modifications,…) may occur due to overexpression of some proteins, including tagged proteins (Raden et al., 2005). As a result, these proteins may fail to exit from the ER. Considering that the CMV promoter in the HA- and GFP-ABCB5β constructs is quite strong, we decided to transfer HA-ABCB5β in a plasmid containing a low expression promoter (pGK). Reassuringly, ER localization was also observed for this chimeric protein.

Of note, it has been reported that tags can be cleaved in the cells upon expression of a chimeric protein, and that some fluorescent tags may aggregate and generate artefactual signals under a microscope (L. Huang et al., 2014; H. Katayama, Yamamoto, Mizushima, Yoshimori, & Miyawaki, 2008). However, it worth noting that we confirmed the ER localization of ABCB5β using biochemical methods as well. Subcellular fractionation experiments applied to transfected Hek293T cells showed that a C-ter mCherry tagged ABCB5β co-distributed with the ER marker alkaline glucosidase in subcellular fractions. In these analyses, ABCB5β-mCherry was detected by Western blotting, so the molecular weight of the tagged protein could be used as a control.

In summary, both N-terminally tagged constructs (GFP and HA) and the C-terminally tagged ABCB5β (with mCherry) were found in the ER. Taken together, these findings indicate that the presence and position of the tag does not affect the localization of the protein.

Importantly, the ABCB5β protein remained localized in the ER even after cells were treated with a chaperone "booster," further supporting that this compartment is the primary residence of this protein, despite the fact that a portion of the ABCB5β protein population is degraded by the proteasome system. This degradation is not unexpected for proteins with a high number of transmembrane domains, including ABC transporters. Indeed, it has been reported that only 15-30% of wild-type CFTR (ABCC7) correctly folds and reaches the plasma membrane, i.e., the site where this protein acts as a chloride channel. Folding of CFTR ΔF508 mutant (involved in the disease cystic fibrosis) is even more difficult, with almost no trafficking to the plasma membrane (Ward & Kopito, 1994).

Molecules that rescue misfolded proteins, also known as pharmacological chaperones, have been described to rescue the trafficking-defective mutants of ABCB family members. Gautherot et al. (2012), have shown that mutation I541F in ABCB1 and ABCB4 lead to a retention of this proteins in the ER/Golgi compartment. After testing potential pharmacological chaperones, they found that Thapsigardin and sodium 4-phenyl butyrate were inefficient, but glycerol improved the maturation and exit of the mutant from the ER. Opposite, Cyclosporin A which is a competitive substrate of ABCB1, restored the maturation, plasma membrane expression and activity of the ABCB1-I541F (Gautherot et al., 2012).

Interestingly, incubation of cells expressing this ΔF508 mutant with suberoylanilide hydroxamic acid (SAHA), a chaperone "booster", can promote its folding and transport to the cell surface (Hutt et al., 2010). It was also shown that histone deacetylase inhibitors (HDIs) including SAHA induce Q141K ABCG2 surface localization. Although the mechanism has not been fully uncovered, HDIs would act by an increase in ABCG2 transcription coupled to a trafficking/folding partner overexpression, and by inhibition of retrograde transport from cytoplasm to aggresome (Basseville et al., 2012). Hence, in our research, we tested whether treatment of transfected cells with ABCB5β would also affect its expression level and eventually modify its localization. Consistent with SAHA boosting the folding of transmembrane proteins, the total level of ABCB5β increased upon treatment. However, little change was observed in terms of subcellular localization. ABCB5β continued to co-localize to a large extent with the ER marker calnexin.

As ABCB5β has been described by others to have a plasma membrane localization in a flow cytometry experiment (Frank et al., 2003), and since its close homolog ABCB1 resides at the plasma membrane (as well as in the secretory pathway), we also conducted cell surface biotinylation experiments and checked for the presence of HA-ABCB5β at the plasma membrane directly. However, less than 1% of ABCB5β was detected among biotinylated proteins, in non-treated and SAHA-treated cells. Taken together, our findings support that the main residence site of ABCB5β in HeLa and MelJuSo cells is the ER.

The knowledge of the localization of ABCB5β represents an important step towards its characterization. The role of this protein in ER now has to be studied. One may hypothesize that ABCB5β could be retained within the ER in response to the activation of one of several mechanisms to protect the cells against a putative ABCB5-dependent proteotoxicity (Nadanaka, Yoshida, Kano, Murata, & Mori, 2004). Another hypothesis is that ABCB5β could exercise its function in the ER. Several other ABC transporters are active at this site. For example, half transporters from the same family, ABCB2 and ABCB3, are located in the membrane of the ER where they form a dimer specialized in the transport peptides from the cytoplasm into the ER lumen (Herget & Tampé, 2006). They also associate with the major histocompatibility complex (MHC) class I molecules, allowing peptide presentation to T-lymphocytes (Koch et al., 2004).

As mentioned in the beginning of this discussion, ABCB5β was shown to heterodimerize with ABCB6 and ABCB9 (manuscript under review, Gerard et al. (2022)). In terms of localization, it should not be excluded that the subcellular localization of homo- and heterodimers could differ. Both ABCB6 and ABCB9 have been located to lysosomes (Kiss et al., 2012). Hence, an intriguing idea is that lysosomal trafficking motifs found in these proteins could bring the ABCB5β/ABCB6 and ABCB5β/ABCB9 dimers to this organelle. ABCG5 and ABCG8, for example, exist as homo- or heterodimers. The former localizes to the ER, whereas the latter travels to the plasma membrane (G. A. Graf et al., 2003; Hirata et al., 2009). Of note, a couple of studies have demonstrated that ABCB5β homodimer cannot confer drug resistance, both in the yeast model *S. cerevisiae* and in mammalian cells (Kawanobe et al., 2012; Keniya et al., 2014). Further studies will be needed (1) to investigate the biological role of ABCB5β homodimer in the ER, (2) to elucidate the subcellular localization of the putative heterodimers ABCB5β/B6 and ABCB5β/B9 and (3) to assess their putative transport activities.

Coming back to the putative function of ABCB5β, it is worth considering that it could play a role in lipid transport. Interestingly, ongoing research in our laboratory revealed a potential implication of mouse Abcb5 in fat metabolism. Indeed, the plasma level of triglycerides was found to decrease in ad libitum-fed *Abcb5* knockout mice, whereas total cholesterol and HDL cholesterol levels were elevated during fasting periods. Further analyses revealed that female *Abcb5*-knockout mice had decreased fat content and increased lean mass, along with a trend toward an increase of maximum oxygen consumption, while in males, no difference between genotypes was observed. Since ABCB5 has been found to be highly expressed in atherosclerotic plaques (Kotlyarov & Kotlyarova, 2021), we may speculate that ABCB5 could play a role in atherogenesis. It has been reported that several ABC transporters (ABCA1, G1, G5 and G8) transfer cholesterol to HDL particles (which are protective against cholesterol deposits in arteries), and that the loss of these transporters leads to the formation of atherosclerotic plaques (Fitzgerald, Mujawar, & Tamehiro, 2010). Based on the increase of HDL in the *Abcb5* KO mice, it would appear that, by contrast to these transporters, loss of ABCB5 would rather be protective against atherosclerosis. It is interesting to note that its close homolog, ABCB1, can transport cholesterol and other lipids (Neumann, Rose-Sperling, & Hellmich, 2017).

Another interesting finding is that the regulation of *Abcb5* gene expression can be influenced by hormones, providing a feasible explanation for the significant gender disparities in body composition. Notably, the *Abcb5* promoter region contains hormone response elements, with a particular emphasis on the estrogen-response element. The liver transcriptomic analysis unveiled connections between the circadian clock, lipid metabolism, and observed metabolic alterations and body composition changes. In contrast, the heart analysis highlighted molecular-level changes associated with inflammation, atherosclerosis, blood pressure, and cardiomyopathy, signaling significant disruptions in heart function. Further studies need to be conducted to strengthen the working hypotheses and to fully understand the role of ABCB5 in the cell metabolism.

The fact that ABCB5 could not be detected when expressed at endogenous level (including after insertion of an HA tag before *ABCB5*β in the genome of MelJuSo cells using a CRISPR-Cas9-dependent method) suggests that ABCB5 expression, under basal conditions, is quite low at the protein level. With this in mind, it might be worth investigating whether some consequences of *ABCB5* inactivation (decreased proliferation of melanoma cells, increased HDL level in blood samples of the KO mice, ….) could be accounted for by the loss of *ABCB5* mRNA molecules in the cells, rather than by some modification at the protein level. Indeed, it has been reported that some mRNAs may have additional cellular roles, beyond serving as template for protein synthesis. For example, it has been reported that some mRNAs can contribute to the formation of non-membranous structures, such as histone locus bodies (i.e., structures that regulate the transcription and maturation of mRNAs coding for histones), Cajal bodies (nuclear structures that modulate chromatin interactions and spliceosome activity, thereby affecting gene expression) or nuclear speckles (which are also interchromatin granules containing pre-mRNA splicing factors) (Kloc, Foreman, & Reddy, 2011). Some mRNAs may also play a role in organizing cytoskeleton elements in the cytoplasm, or regulate Ribonucleoprotein assemblies. Whether ABCB5 mRNA could be involved in some of these processes is currently unknown, but may also be worth exploring in the future.

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ANNEXES

Annexe 1: Centrifugation times in the 50Ti rotor depending on the volume of sample

	M	L	P
ml	8000RPM	25000RPM	35000RPM
2	2'24"	2'52"	18'30"
2,1	2'27"	2'54"	
2,2	2'30"	2'56"	
2,3	2'33"	2'59"	
2,4	2'36"	3'02"	
2,5	2'39"	3'04"	
2,6	2'42"	3'06"	
2,7	2'45"	3'09"	
2,8	2'48"	3'11"	
2,9	2'52"	3'13"	
3	2'55"	3'16"	21'40"
3,1	2'59"	3'19"	
3,2	3'03"	3'22"	
3,3	3'07"	3'25"	
3,4	3'11"	3'28"	
3,5	3'15"	3'31"	
3,6	3'19"	3'34"	
3,7	3'23"	3'37"	
3,8	3'27"	3'40"	
3,9	3'31"	3'42"	
4	3'34"	3'45"	24'20"
4,1	3'38"	3'48"	
4,2	3'42"	3'51"	
4,3	3'46"	3'54"	
4,4	3'50"	3'57"	
4,5	3'54"	4'	
4,6	3'58"	4'03"	
4,7	4'02"	4'07"	
4,8	4'06"	4'10"	
4,9	4'11"	4'13"	
5	4'15"	4'16"	28'20"
5,1	4'18"	4'19"	
5,2	4'21"	4'22"	
5,3	4'24"	4'25"	
5,4	4'26"	4'29"	
5,5	4'29"	4'32"	
5,6	4'31"	4'35"	
5,7	4'34"	4'38"	
5,8	4'37"	4'42"	
5,9	4'40"	4'45"	
6	4'43"	4'48"	32'13"

Annexe 2: Review paper submitted for publication to Trends in Cancer

Linking ABC Transporters to the Canonical and Prospective New Hallmarks of Cancer

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Keywords: ABC transporters, cancer drivers, hallmarks of cancer, multidrug resistance

Abstract

Human ATP-binding cassette (ABC) transporters are ubiquitously expressed and transport a broad range of endogenous and xenobiotic substrates across extra- and intracellular membranes. Mutations in ABC genes cause 21 monogenic diseases, and polymorphisms in these genes are associated with susceptibility to complex diseases. ABC transporters also play a major role in drug bioavailability, and they mediate multidrug resistance in cancer. At least 13 ABC transporters were shown to be involved in drug resistance *in vitro*. On the other hand, in the past decade, efforts have been made to elucidate their roles in tumor biology. Herein, we explore their involvement in tumorigenesis focusing on the hallmarks of cells as they make their way from normalcy to neoplastic growth states.

Introduction

Human ATP-binding cassette (ABC) transporters comprise 44 membrane transporters and four non-transporter ABC proteins (**Box 1**). ABC transporters translocate a wide variety of substrates, including lipids, peptides, sterols, and vitamins, across cellular membranes and have a broad range of cellular roles (**Box 2**). ABCB1 was the first ABC transporter found to confer multidrug resistance in cancer. This discovery led to almost 30 years of clinical investigations to test whether ABCB1, and later ABCC1 and ABCG2, could be modulated to increase tumor chemosensitivity. Although the modulation of multidrug resistance via competitive inhibitors was effective in cultured cancer cells, as a therapeutic strategy, this approach failed for the most part to improve outcomes in patients [1]. Further studies addressed the clinical relevance of ABC transporters in human cancer samples. Interestingly, many ABC transporters were shown not to be correlated with progression-free survival, a clinical surrogate for drug resistance, but to be correlated with overall survival, indicating that they may play a role in tumor biology. In this review, we explore the potential roles of ABC transporters in tumorigenesis using the hallmarks of cancer as a heuristic tool for representing cancer complexity. We also consider the potential roles of these transporters in light of prospective new hallmarks and enabling characteristics, which have been recently proposed (**Fig. 1**) [2].

ABC transporters: Beyond their roles as ATP-dependent drug-efflux transporters

Genomic instability and mutation

ABC transporters represent an important line of defense against carcinogens. Their expression at physiological barriers and excretory organs restricts exposure to environmental carcinogens and increases their clearance. For example, ABCB1, ABCC2, and ABCG2 transport the food-derived carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). Triple knockout *Abcb1*:*Abcc2*:*Abcg2*$^{(-/-)}$ mice exhibit markedly reduced fecal and biliary excretion of PhIP 30 minutes after its administration [3]. ABCC1 and ABCC2 transport the β-O-glucuronide conjugate of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), a metabolite of the tobacco-derived carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) [4]. *Abcc2*-deficiency in rats unraveled the role of Abcc2 in biliary excretion of NNAL-O-glucuronide and increased formation of CYP450-derived metabolites [5]. Interestingly,

CYP450 gene expression and activity are increased in *Abcc2* knockout rats when compared to their wild-type counterparts. We may speculate that in humans, mutations in the *ABCC2* gene that have deleterious effects on the function of the protein increase the cancer risk from NNK exposure. We can also speculate that ABC transporters provide protection against a wide range of genotoxic compounds.

ABC transporters also play a role in reactive oxygen species (ROS) homeostasis. It is accepted that a high level of ROS not only initiates DNA damage and carcinogenesis but also induces regulatory pathways, such as proliferation, angiogenesis, and metastasis. The tumorigenesis-promoting ROS level involves a delicate balance, as an excessively high concentration of ROS induces apoptosis and senescence of tumor cells [6]. ABCG2 plays a role in the redox balance and antioxidant production by modulating the intracellular concentration of glutathione. However, ABCG2 does not directly mediate glutathione transport, which indicates that modulation occurs through the induction of ABCG2-associated signaling pathways [7]. Similarly, ABCB5FL, the full transporter isoform, was also shown to upregulate cellular glutathione levels via STAT1 overexpression [8]. The interplay between both proteins has yet to be unraveled. ABCB7 overexpression reduces both cytosolic and mitochondrial ROS, likely by reducing the concentration of intracellular free iron (Fe), as the latter generates ROS via the Fenton reaction [9]. ABCB7 may be linked to genome instability via its involvement in iron-sulfur (Fe-S) protein biogenesis. Fe-S proteins have a role in central processes, such as DNA replication and repair, so malfunction of Fe-S proteins may affect genome integrity [10]. As ABCB7 plays a pivotal role in the transport of mitochondrial Fe-S proteins to the cytosol, we may hypothesize that mutations altering ABCB7 function affect genome integrity (**Fig. 1**).

Inducing vasculature

Tumor-induced angiogenesis is essential to supply oxygen and nutrients to sustain tumor survival and progression. Several ABC transporters, including ABCC1, modulate tumor angiogenesis (**Fig. 1**). Conditioned medium from MCF-7 human breast cancer cells overexpressing ABCC1 stimulates angiogenesis of human umbilical vein endothelial cells [11]. Conversely, treatment of these cells with the ABCC1 inhibitor MK571 prevented angiogenesis. Furthermore, ABCC1-overexpressing MCF-7 and 4T1 murine breast adenocarcinoma xenografts grow faster and have a higher microvessel density of blood vessels than controls

[11]. This was linked to an increase ABCC1-dependent secretion of sphyngosine-1-phosphate (S1P), a sphingolipid mediator involved in the tumor microenvironment and angiogenesis regulation.

In a B16-F10 melanoma model developed in CRP/CAST transgenic mice (**see Glossary**), probenecid-induced inhibition of ABCA1 activity reduced externalization of calpain, which, in turn, inhibited endothelial cell migration *in vitro* and neoangiogenesis. Probenecib addition did not impair endothelial cell death or endothelial cell proliferation but rather prevented neoangiogenesis by limiting calpain-mediated fibronectin cleavage and therefore endothelial cell migration [12].

ABCB1 or ABCC4 expression in rat brain microvessel endothelial cells (RBE4) and human retinal microvascular endothelial cells (HRECs), respectively, modulate capillary length, with possible implications for cancer. Specifically, RBE4 cells overexpressing ABCB1 exhibit increased migration capability and capillary-like tubular formation, modulated through an interaction with caveolin-1 [13]. In the second case scenario, siRNA against ABCC4 showed the alteration of HREC migration and capillary morphogenesis without affecting proliferation [14].

Avoiding immune destruction

Escaping immune surveillance is an important underlying mechanism in tumor development. ABCB2 (TAP1) and ABCB3 (TAP2) form a heterodimeric complex, which transports proteasomal degradation products into the endoplasmic reticulum. The peptides are loaded onto major histocompatibility complex class 1 molecules and presented on the cell surface to tumor-specific cytotoxic $CD8^+$ T cells [15]. ABCB2 and/or ABCB3 are downregulated in a large number of cancers, including head and neck cancer, colorectal carcinoma, and breast cancer [15]. ABCB3 expression, which is correlated with that of ABCB2, is linked not only with the infiltration of cytotoxic lymphocytes but also with the infiltration of T-helper lymphocytes, regulatory lymphocytes, and M1- and M2-type macrophages [16]. Interestingly, mice injected with a mixed population of $ABCB2^-$ and $ABCB2^+$ tumor cells developed tumors exclusively composed of $ABCB2^-$ cells, indicating the increased tumor potential of ABCB2-negative cells [17]. On the other hand, research has suggested that ABCBA1 may induce reprogramming of

tumor-related macrophages into anti-inflammatory M2-type macrophages by mediating the effects of 27-hydroxycholesterol secreted by cancer cells (**Fig. 1**) [18]. This draws some attention on ABC transporters functions in immune cells beside their roles in cancer cells.

Resisting cell death

ABCC1 was one of the first ABC transporters to be linked to apoptosis. Knockdown of ABCC1 in a mouse-human xenograft model of neuroblastoma increases tumor cell death [19]. ABCC1 overexpressing cells are less susceptible to Fas antibody- or staurosporine-induced apoptosis, which is attributed to the ability of ABCC1 to extrude reduced glutathione [20]. Other ABC transporters have been linked to cell death, with knockdown of ABCG2 significantly increasing apoptosis of gastric and laryngeal cancer cells, and knockdown of ABCC6 inhibiting cell cycle arrest and apoptosis [21-23]. As compared with ABCG1-nonfunctional mutant transfected cells, ABCG1-overexpressing cancer cell lines exhibit characteristics of apoptotic cells and higher cell death (**Fig. 1**) [24].

Deregulating cellular energetics

More than 60 years ago, Warburg reported that cancer cells preferentially use glycolysis over oxidative phosphorylation to produce energy, even in aerobic conditions. As demonstrated in two different urinary bladder cancer cell lines, ABCC3 knockdown decreases lactate dehydrogenase, a key rate-limiting enzyme of aerobic glycolysis, leading to a drastic reduction in glucose consumption and lactate production, pointing to an important role for ABCC3 in aerobic glycolysis (**Fig. 1**) [25].

ABCA1 may be an essential regulator of the mevalonate pathway. Numerous mevalonate-derived metabolites, including cholesterol, are linked to cancer. Cholesterol synthesis enables cancer cells to form the membranes of newly produced cells, and plays a pivotal role in cell signaling through lipid rafts [26]. ABCA1 is upregulated by p53, which, in turn, inhibits SREBP-2 maturation and mevalonate pathway activation. Further analysis showed that ABCA1 expression protects against liver tumorigenesis [27]

Tumor-promoting inflammation

ABCC7 defect is tightly linked to chronic pancreatitis, a known risk factor for pancreas cancer. Other studies have shown that ABCC7-inactivating mutations are associated with pulmonary inflammation and obstruction, and ABCC7 loss in cholangiocytes induces NF-κB activation and inflammation, ultimately increasing susceptibility to hepatic cancer [28]. Impairment of ABCB4, ABCB11, or ABCG5/G8 may promote hepatic inflammatory stress resulting in hepatocellular carcinoma or cholangiocarcinoma through bile acid-mediated toxicity [29]. ABCB1 polymorphisms are also linked to ulcerative colitis, which is also associated with colorectal carcinoma, thereby highlighting the importance of ABC transporters in pro-tumorigenic inflammation (**Fig. 1**) [30].

Sustaining proliferative signaling and evading growth suppressors

In gastric, lung, and mitoxantrone-resistant breast cancer cells, following ABCG2 knockdown, G_0/G_1 phase cells markedly decrease, and was associated with cyclin D3 expression decrease, and cyclin-dependent kinase inhibitor $p21^{cip1}$ expression increase [21, 31]. ABCB6 knockdown in hepatocellular carcinoma is associated with a decrease in cell proliferation and a delay in the G2/M phase [32]. Overexpression of these transporters sustains cell growth and proliferation. Other ABC transporters, including ABCB5β, ABCC4, and ABCC8, were also shown to exhibit pro-oncogenic characteristics [33-35].

On the other hand, some ABC transporters act as tumor suppressors. For example, ABCA8 overexpression reduces breast cancer cell proliferation [36], and mutations in the ABCB5FL transporter or loss of its expression increases the proliferative capacities of melanoma cells [37]. Interestingly, some ABC transporters seem to play a dual role as growth-sustaining proteins and tumor suppressors. For example, ABCC7 exerts antiproliferative effects in prostate cancer, while sustaining the growth of ovarian cancer cells (**Fig. 1**) [38, 39]. We may hypothesize that the transporter's substrates are different based upon the tissue in which the transporter is localized, and so impact differently the biology of the cells. We may also speculate that in some cases, the expression of different isoforms, localized in different cell compartments and involved in separate cell signaling pathways, might explain this dual role.

Activating invasion and metastasis

Primary cancer cells undergo phenotypic transformation via the epithelial-to-mesenchymal transition (EMT), allowing them to escape from the bulk tumor by reducing cell-cell adhesion and cell polarity while increasing cell motility. This increases their capacity to invade adjacent stroma and to migrate to other tissue via blood and lymphatic vasculature. ABC transporters were shown to inhibit or promote tumor metastasis formation. For example, knockdown or inhibition of ABCC6 in HepG2 cells leads to cytoskeleton rearrangement and impaired cell motility, thus hindering cell migration [40]. ABCA1 overexpression in colon cancer cells reduced the expression of E-cadherin, a well-known marker of the epithelial phenotype, and increased colon cancer cell invasion and migration capacities [41].

Cytoplasmic ABC proteins ABCE1 and ABCF1 play also a role in the metastatic process. ABCE1 was shown to be associated with advanced clinical stage of lung cancer and tumor lymph node metastasis. Overexpression of this cytoplasmic ABC protein in lung cancer cells promotes cell proliferation, invasion, and metastasis [42]. ABCF1 was shown to promote EMT, cell migration and stemness properties in hepatocellular carcinoma, underlining the interconnections existing between the different hallmarks (**Fig. 1**) [43].

Unlocking phenotypic plasticity

Cellular plasticity refers to the ability of cells to change their phenotype in response to external signals without affecting their DNA sequence. Cellular plasticity can occur through several processes. Cancer cells originating from fully differentiated normal cells may dedifferentiate back to progenitor-like cells, whereas those arising from progenitor cells may be maintained in a partially differentiated progenitor-like state. Cancer cells may also transdifferentiate from one cell type to another as observed in the EMT transition, enabling invasion, migration, and ultimately dissemination of cancer cells and metastasis.

Several ABC transporters have been linked to the EMT. Knockdown of ABCC7 in MCF-7 breast cancer cells reduces the expression of E-cadherin and occludin, two epithelial markers, while increasing the expression of the mesenchymal marker laminin-γ2 at the protein level [44]. Overexpression of ABCC7 in highly metastatic breast cancer MDA-MB-231 cells has the

opposite effects, highlighting the EMT-suppressive effect of this transporter. Defective F508 del-ABCC7 alone is sufficient to significantly upregulate in the expression of some mesenchymal markers, while significantly decreasing the expression of epithelial markers, therefore leading to a partial EMT [45]. In breast cancer, ABCB5β was shown to promote the EMT by regulating the expression of the transcription factor ZEB1, a well-known driver of the EMT [46]. Other ABC transporters including ABCA4, ABCC4, and ABCF1 promote the EMT in retinal pigment epithelial cells, pancreatic cancer, and hepatocellular carcinoma, respectively [43, 47, 48].

Although the mechanisms by which transdifferentiation promotes cancer are unclear, it has been hypothesized that the switch of gene expression induced by the passage from one cell identity to another enable the transcription of some usually locked oncogenic drivers. In mouse, Abca1 is linked to the suppression of the transition of cholesterol-loaded smooth muscle cells into foam cells [49]. This points to the possibility of ABCA1 playing a role in oncogenic-promoting transdifferentiation. In the same line, mutated ABCC7 is related to pancreatitis, the associated condition of acinar to ductal conversion that can drive pancreatic ductal adenocarcinoma [50]. ABCC7 is also associated with cystic fibrosis that can lead to mucus metaplasia in airways, without any known implication in cancer [51].

In addition to transdifferentiation, blocking cell differentiation can also account for cellular plasticity. Besides their roles as cancer stem cell (CSC) markers, ABCG2 and ABCB5 may play active roles in stemness maintenance (**Box 3**). Pharmacological inhibition of ABCG2 significantly impairs the expression of stem cell markers and spheroid formation in a side population of glioma cells [52]. In addition, ABCG2 blocks the differentiation of normal stem cells, raising the possibility of a similar role in malignant stem cells [53]. Furthermore, ABCB5 is hypothesized to maintain slow-cycling of differentiated cells in melanoma through a proinflammatory cytokine signaling circuit (**Fig. 1**) [54].

Senescent cells

Senescence is a cellular state characterized by an irreversible cell cycle arrest and functional and structural changes in response to diverse sources of stress. Despite high heterogeneity, which makes reliable identification difficult, senescent cells exhibit a senescent associated

secretory phenotype, which includes multiple proinflammatory chemokines and cytokines that communicate with surrounding cells. Although senescence was first considered a tumor suppressor process, it has been shown that failure to remove senescent cells leads to senescence-associated secretory phenotype (SASP)-induced inflammation, which exerts pro-tumorigenic effects. Thus, the influence of senescence on cancer progression is now considered as context and stage dependent [55].

As shown using engineered immortalized human epithelial cells as a model for oncogenic-induced senescence, ABCC3 knockdown rescues cell proliferation arrest, strongly decreases senescence-associated β-galactosidase (SA-β-gal) activity, and prevents the accumulation of IL-8, a component of the SASP [56]. Endogenous ABCC3 exerts pro-senescence effects, with these effects being transport dependent. In mice, knockout of Abcc3 displays signs of senescence escape, ultimately resulting in increased tumor formation [56]. Conversely, the cell cycle is impaired in HepG2 cells with ABCC6 knockdown, with the cells exhibiting a slower transition from the G1 to the S phase, together with an 8-fold increase of SA-β-gal-positive cells compared to wild-type HepG2 cells, suggesting a role for ABCC6 in senescence escape [57]. ABCB8 was shown to exert senescence-protective effects in endothelial cells, with ABCB8 knockdown in human microvascular endothelial cells increasing the number SA-β-gal-positive cells (**Fig. 1**) [58].

Polymorphic microbiome

A growing body of evidence points to a potential role for commensal microbiota in promoting cancer in their hosts, with some studies reporting a link between the expression of ABC transporters and microbial dysbiosis. Knockout of *Abcb1a* in mice leads to changes in cecal microbiota associated with inflammation development [59]. The same mice were shown to develop spontaneous colitis, a risk factor for colon cancer. Colitis could be prevented in germfree conditions and solved by a cocktail of antibiotics, demonstrating the essential role of microbiota in Abcb1a-associated colitis [60]. ABCC7 and ABCC11 drive active selection of gut and axillary microbiota, respectively, suggesting that other ABC transporters may play a role in microbiota homeostasis [61, 62]. However, the exact mechanisms by which these transporters mediate this selection remain to be determined.

ABC transporters are key elements of the remote sensing and signaling theory, which explains how the coordinated functions of transporters, metabolizing enzymes and regulatory proteins maintain homeostasis through tightly regulated inter-organ and inter-organismal communication (e.g., gut microbiome-host). Butyrate, a short chain fatty acid produced by gut microbiota during anaerobic fermentation, exerts antitumorigenic effects on hepatic and pancreatic cancers [63, 64]. This fatty acid is a substrate of ABCG2, which could potentially transport it back into the intestinal lumen and then modulate its effects on remote organs by controlling its access to the bloodstream. Furthermore, as butyrate provides protection against colon cancer, ABCG2 activity might regulate these protective effects by controlling the availability of butyrate in the gut (**Fig. 1**) [65].

ABCG2 also transports enterolactone, a metabolite derived from lignan metabolization by gut microbiota [66]. As documented, not only the plasma concentration of enterolactone but also the renal and testicular concentrations are increased in *Abcg2* knockout mice [67]. It is interesting to note that enterolactone displays antiproliferative activity in prostate cancer *in vitro* [68]. However, the beneficial effects of enterolactone on prostate cancer are disputed in clinical studies [69].

Nonmutational epigenetic reprogramming

Epigenetics is defined as heritable changes in gene expression, without modification of the DNA sequence and is usually divided into three mechanisms: DNA methylation, histone modification, and noncoding RNA. ABCC7 is linked to all three types of epigenetic mechanisms. This transporter suppresses prostate cancer development by regulating the tumor suppressor miR-193b [38]. It is also linked to H4 acetylation and to CpG methylation of the promoter of different genes [70, 71].

Substrates of the different ABC transporters may also influence epigenetic regulation. Folate and riboflavin, two ABCG2 substrates, contribute to DNA methylation through the regulation of the methyl donor S-adenosylmethionine (**Fig. 1**) [72-74]. Likely, other ABC transporters' substrates also exert control on epigenetic regulation. Metabolites and sensors potentially transported by ABC transporters within the framework of the remote sensing and signaling theory could activate different epigenetic pathways. The advent and widespread use of next-

generation sequencing to identify DNA methylation, noncoding RNA expression, and histone modification through Chip-seq, should soon provide additional data on the influence of ABC transporters on epigenetic regulation.

Concluding remarks

The discovery that ABCB1 mediates multidrug resistance in cancer has defined a druggable therapeutic target with the aim of resensitizing cancer cells to chemotherapy. This clinical objective led to more than 30 years of extensive research and the identification of at least 13 ABC transporters involved in multidrug resistance mechanisms and their drug substrates. These studies also contributed to highlight the potential roles of ABC transporters in tumor development. Since then, a growing body of evidence supports the role of ABC transporters in tumor biology. In this review, we used the revised list of hallmarks of cancer to map their roles in tumor development and metastasis. All these hallmarks of cancer are intertwined and are difficult to characterize as a sole entity. Many ABC transporters appear to be involved in several hallmarks of cancer, and for most of them, the underlying mechanisms that support the proposed cartography remain to be elucidated, see outstanding questions.

The alteration in the transport of endogenous metabolites and signaling molecules may contribute to the development of cancer. The time has come to direct more resources and efforts to the discovery of ABC transporters' endogenous substrates. It is also important to determine the mutational profiles of ABC genes in cancers and to assess the impacts of mutations on the activity of the transporters to reveal their exact roles in the mechanisms of tumorigenesis. Finally, the identification of transporters' substrates, together with the potential binding partners, might also shed light on new druggable targets, which could circumvent current limitations associated with the use of competitive inhibitors to modulate ABC transporter-mediated multidrug resistance.

Box 1. ABC transporters in humans

ABC proteins are found throughout all kingdoms of life and are divided into three classes: exporters, importers, and non-transporters, with all three classes found in humans. There are 48 human ABC proteins, which are classified into seven families from A to G based on sequence homology. A canonical ABC transporter is composed of two highly conserved nucleotide binding domains (NBDs), the hallmark of ABC proteins, and two highly variable transmembrane domains, which form the framework of the translocation pathway. They exist as full transporters encoded by a single polypeptide chain or as half transporters, which must either homo- or heterodimerize to be functional [75]. Although most ABC transporters are exporters, ABCA4 functions as an importer in photoreceptor cells, moving retinal from the disc lumen into the cytosol [76]. ABCD4 is another importer, which transports vitamin B_{12} from the lysosomal lumen to the cytosol [77]. Members of the ABCE and ABCF families do not function as transporters but rather as regulators of mRNA translation [78]. Finally, several members of ABCC family are not classified as transporters and serve as ion channels or channel regulators. These include the gated-chloride channel ABCC7 (also called cystic fibrosis transmembrane conductance regulator), and ABCC8 and C9, the regulatory subunits of the ATP-sensitive potassium channel [79].

Box 2. Physiological functions of ABC transporters

There are 12 members of the ABCA family, all of which play a significant role in cholesterol homeostasis and membrane lipid trafficking [80]. Several ABCA family members are linked to Alzheimer's disease (AD). ABCA-mediated maintenance of cholesterol homeostasis is linked to amyloid beta aggregation, which is thought to be involved in AD pathology [81]. Mutations in ABCA4 are associated with numerous retinal degenerative diseases [82]. Other disorders associated with these transporters include Tangier disease, cardiovascular diseases, and respiratory distress.

The ABCB family is unique in that it is composed of four full and eight half transporters. They transport a wide range of substrates, including endogenous molecules, heme, lipids, peptides, and drugs. They are implicated in antigen presentation to T-lymphocytes, heme processing, and bile salt and phospholipid transport. As a result, the ABCB family is linked to immune deficiency, sideroblastic anemia, ataxia, and intrahepatic cholestasis [83].

The ABCC family plays a role in lipid trafficking. ABCC1 transports lysolipids (S1P), lysophosphatidil-inositol, prostaglandins, and glutathione [84]. Other members of this family are implicated in genetic disorders, such as Dublin–Johnson syndrome, pseudoxanthoma elasticum, and persistent hypoglycemia in infancy.

The ABCD family is composed of four half transporters that predominantly function as homodimers in the peroxisome. They are involved in the transport of long and very long chain fatty acids, and their CoA-derivatives across the peroxisomal membrane. ABCD1 defects cause X-linked adrenoleukodystrophy, a neurodegenerative disease. ABCD3 transports bile acid precursors di- and trihydroxycholestanoic acid and long-chain dicarboxylic fatty acids. ABCD3 deficiency is linked to hepatosplenomegaly, and ABCD4 mutations are linked to disruption of vitamin B12 metabolism [85, 86].

The ABCE and ABCF families comprise soluble proteins composed of only two NBDs. In mammals, ABCE proteins contribute to the role of ribosomes in gene expression regulation, and ABCF family members function as regulators of translation [87].

There are five ABCG members, which are half transporters that form a functional complex either by homo- or heterodimerization. They are implicated in cholesterol, bile acid and steroid hormone transport. ABCG1 is thought to prevent lipid accumulation and is linked to arteriosclerosis and, cardiovascular and metabolic diseases. Partial or total loss of function of ABCG5/ABCG8 obligate heterodimer is involved in sitosterolemia, which is characterized by excess accumulation of plant sterols in the blood [88].

Box 3. Cancer stem cells (CSCs)

According to CSC models, only a small subset of malignant cells is able to self-renew and to recapitulate tumor heterogeneity. CSCs exhibit a multidrug resistance phenotype and therefore contribute to treatment failure and tumor relapse [89]. Many ABC transporters were found to be upregulated in CSCs. Although there is no universal molecular CSC signature among cancers, ABCG2 and ABCB1 are widely used to isolate a “side population” displaying enhanced self-renewal ability, spheroid formation, and *in vivo* tumor-initiating ability through their ability to transport Hoechst 33342, a known substrate of both transporters [90]. Interestingly, many additional ABC transporters including ABCA1, A3, B2, B5β, C1, C2, C3,

C4, C6, C11, G1, and G5 are found to be upregulated in the side populations of tumors from different origins, and are suggested, to different extents, to be putative CSC markers [91-99].

Recent research implicates ABC transporters in the stemness process, notably by blocking cell differentiation. In non-small cell lung cancer, knockdown and inhibition of ABCB1 reduces spheroid formation and the EMT, whereas ABCD4 knockdown reduces self-renewal ability [100, 101]. In breast cancer, knockdown of ABCC1 and ABCC3 is correlated with a decrease in the expression of stemness-related genes [102]. ABCC4 blockade resulted in leukemia stem cell differentiation, and ABCF1 overexpression increases colony and spheroid formation in hepatocellular carcinoma cell lines [43, 103]. Interestingly, ABCG1 overexpression was shown to correlate with an increase in the expression of the CSC markers CD133 and ALDH in lung cancer [104].

Glossary

B16-F10 melanoma model: The B16 melanoma cell line was established from a spontaneous C57BL/6 mouse-derived melanoma. The following B16-F1 melanoma cell line was derived from pulmonary metastases produced by intravenous injection of B16 cells into a syngeneic C57BL/6 mouse. B16F10 cell line corresponds to the tenth serial passage of this subclone in C57BL/6 mice.

CRP/CAST transgenic mice: The transgene construct includes the promoter region, the signal peptide-encoding region and the exon 1 of the human C-reactive protein (CRP) gene followed by the coding sequence of the mouse calpastatin (CAST) gene. CRP promoter drives CAST expression specifically in the liver, and the fusion of the signal peptide to CAST results in the secretion of the calpastatin protein into the circulation.

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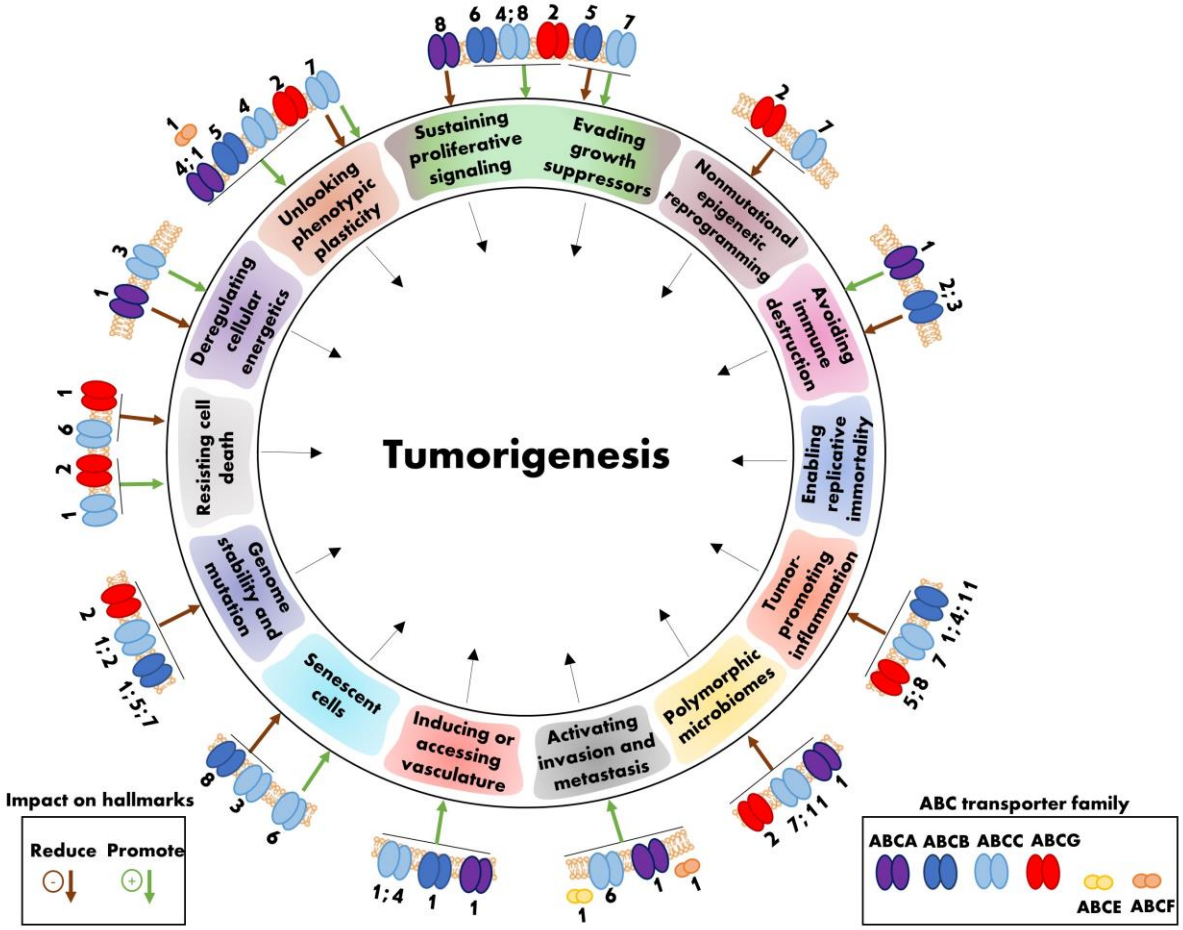

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633 **Figure 1.** The diverse roles of ABC transporters in tumorigenesis. A schematic representation
634 of the potential roles ABC transporters in different hallmarks of cancer is presented based on
635 Hanahan's publication entitled "Hallmarks of cancer: new dimensions." Each ABC transporter
636 family is represented by a color (A: purple, B: dark blue, C: light blue, G: red, E: yellow, and
637 F: orange), and the member of the family is highlighted by the number shown above each
638 transporter's schematic representation. ABC transporters can either promote (green arrows) or
639 interfere (brown arrows) with the mentioned hallmark.

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Highlights

- ABC proteins contribute to tumorigenesis in a number of ways. In addition to their roles in the detoxification of xenobiotics (e.g., food-derived carcinogens) and cellular protection from genomic instability and mutations, expression modulation of many ABC proteins impairs normal cell functions, triggers cancer hallmarks, and facilitates processes leading to cancer development and metastasis.
- Studies support a role for ABC proteins in the emerging hallmarks of cancer and enabling characteristics, which have been introduced to the heuristic tool for representing cancer complexity.
- The discovery of ABC transporters' endogenous substrates holds promise for a better understanding of the underlying mechanisms of the roles of these transporters in tumorigenesis.

Outstanding questions

- What are the endogenous substrates of ABC transporters and what are their role in tumorigenesis?
- There is a good knowledge of the ABC genes' mutational profiles in cancers. What is the impact of the mutations on the function of the ABC proteins?
- To what extent do mutation in ABC transporters affect cell communication including cell microenvironment?
- Are ABC transporters required for stabilizing a partner protein, leading to the activation or repression of a particular signaling pathway?
- Peroxisomal ABCD transporters are known to be differentially expressed in many cancers. What are their roles in the biology of tumors?