

Role of the N-terminal transmembrane domain in the endo-lysosomal targeting and function of the human ABCB6 protein

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Short title: Molecular dissection of the ABCB6 protein

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Summary statement: The intracellular localization of ABCB6 is a matter of debate. We show that ABCB6 is internalized from the plasma membrane to multivesicular bodies and lysosomes. Molecular dissection of the ABCB6 protein reveals a role of its N-terminal domain in targeting.

ABSTRACT

ABCB6 is a homodimeric ATP-binding cassette (ABC) transporter present in the plasma membrane and in intracellular organelles. The intracellular localization of ABCB6 has been a matter of debate, as it has been suggested to reside in the mitochondria and the endo-lysosomal system. Using a variety of imaging modalities including confocal and electron microscopy we confirm the endo-lysosomal localization of ABCB6 and show that the protein is internalized from the plasma membrane through endocytosis, to be distributed to multivesicular bodies and lysosomes. In addition to the canonical nucleotide binding (NBD) and transmembrane domains (TMD), ABCB6 contains a unique N-terminal transmembrane domain (TMD₀), which does not show sequence homology to known proteins. We investigated the functional role of these domains through the molecular dissection of ABCB6. We find that the folding, dimerization, membrane insertion and ATP binding/hydrolysis of the core ABCB6 complex devoid of TMD₀ is preserved. However, in contrast to the full-length transporter, the core ABCB6 construct is retained at the plasma membrane, and does not appear in Rab5-positive endosomes. TMD₀ is directly targeted to the lysosomes, without a passage to the plasma membrane. Collectively, our results reveal that TMD₀ represents an independently folding unit, which is dispensable for catalysis, but has a crucial role in the lysosomal targeting of ABCB6.

INTRODUCTION

ATP-binding cassette (ABC) transporters are large membrane-spanning multidomain proteins promoting the ATP-dependent transmembrane transport of a vast array of biological compounds, including drugs, bile acids, peptides, steroids, ions, and phospholipids [1]. The human genome encodes 48 ABC transporters, which have been grouped into subfamilies labeled A-G. Human ABC transporters are implicated in several diseases, including cystic fibrosis, retinal degeneration, cholesterol and bile transport defects, anemia, and drug response [2, 3]. Functionally active ABC transporters contain a minimum of two highly conserved hydrophilic cytosolic nucleotide-binding domains (NBD) and two hydrophobic transmembrane domains (TMD), which are encoded by the same gene in the case of “full transporters”. In contrast, “half-transporters” possess only a single NBD and a single TMD (together forming the “ABC-core”), and must form homodimers or heterodimers to generate a functional ABC transporter. Crystal structures of ABC transporters confirm that the two TMDs provide the substrate-binding sites and form the translocation pore; whereas a dimer of two NBDs bind and hydrolyze ATP [4]. In addition to the ABC-core, some ABC transporters contain further membrane-embedded regions, whose functions are less characterized. Several members of the ABCB and ABCC subfamilies contain an N-terminal extension to the ABC-core. Long MRPs (Multidrug Resistance Proteins, ABCC1-3, 6, 8, 10) share a domain arrangement of TMD₀-L₀-TMD₁-ABC₁-L-TMD₂-ABC₂, in which TMD₀ represents an N-terminal five transmembrane helix extension, while L₀ and L represent intracellular linker sequences [5]. Studies of truncated MRP-variants revealed that the intracellular L₀ (also present in short MRPs) is required for function, but the role of the TMD₀-domain remains to be clearly established [6, 7]. In addition to the well characterized ABCB1 (P-glycoprotein/P-gp), responsible for the multidrug resistance phenotype of cancer cells [8], the Bile Salt Export Pump (ABCB11/BSEP) regulating the excretion of bile salts from the liver, and Multidrug Resistance Protein 3 (ABCB4/MDR3) translocating phosphatidylcholine, the ABCB subfamily contains seven half transporters consisting of a single NBD and a TMD. ABCB half transporters are expressed in intracellular organelle membranes and must form homo- or heterodimers to transport their respective substrates [9]. Interestingly, the N-terminal regions of ABCB half transporters contain additional, relatively long and unique sequences, which have been implicated in protein-protein interactions and targeting.

The ATP binding cassette transporter subfamily member B6 (ABCB6) gene encodes a membrane protein of 842 amino acids, containing a unique N-terminal region followed by the ABC-core consisting of a TMD and an NBD [10]. ABCB6 was first identified as a porphyrin transporter present in the outer membrane of mitochondria [11]. Subsequent studies challenged this conclusion and suggested other localizations, including the plasma membrane [12] and the endolysosomal compartment [13, 14]. In line with the latter findings, ABCB6 was also found in red blood cells and in exosomes released from maturing reticulocytes [15]. Recently, ABCB6 was identified as the molecular basis of the rare blood group system Langereis (Lan) [16]. Given the uncertainty of the intracellular localization, the proposed role of ABCB6 in the direct mitochondrial uptake of heme synthesis intermediates is questionable. Although the initial findings indicated that loss of one *abcb6* allele in embryonic stem (ES) cells impairs porphyrin synthesis, mice derived from these stem cells were phenotypically normal [17]. While Lan-negative individuals (lacking ABCB6) are healthy, mutations in the *abcb6* gene have been associated with various conditions such as ocular coloboma [18], dominant familial pseudohyperkalemia [19] and dyschromatosis universalis hereditaria [20].

ABCB6 is presumed to form a homodimer with each subunit containing an N-terminal extension to the canonical ABC-core. This extra N-terminal segment (TMD₀) is unique to ABCB6 orthologs as it does not show sequence homology to any other protein. Based on

extensive sequence alignments and a consensus constrained transmembrane helix prediction algorithm [5], the N-terminal segment is predicted to contain five transmembrane helices. To study the role of the unique N-terminal TMD₀ domain of ABCB6, we compared the catalytic activity and intracellular targeting of the full-length ABCB6 protein and the N-terminally truncated ABCB6 core complex. We find that TMD₀ is essential and sufficient for lysosomal trafficking, but is dispensable for ATP binding and hydrolysis.

EXPERIMENTAL

Materials—Na-orthovanadate, N-ethylmaleimide (NEM), EDTA, EGTA, pheophorbide-A, hemin, prazosin, coproporphyrinogen-III and protoporphyrin-IX were purchased from Sigma-Aldrich. 8-azido-[α -³²P]ATP was obtained from Izinta.

Mutagenesis—cDNA encoding wild-type human ABCB6 (NM_005689) [12] was mutated by PCR to generate a variant bearing a human influenza hemagglutinin (HA) or FLAG-tag at its C-terminus. The truncated 206-ABCB6core variant was generated by PCR. Site-directed mutagenesis was performed to replace the conserved Walker A lysine at position 629 to methionine (K629M), as described previously [15].

Generation of recombinant baculoviruses—The human ABCB6 cDNA encoding the full-length 842 amino acid-protein was cloned into recombinant baculovirus transfer vector for expression in Sf9 insect (*Spodoptera frugiperda*) cells. Sf9 cells were infected and cultured as described [21]. Individual clones expressing high levels of the human ABCB6 were obtained by end-point dilution and subsequent amplification. The clone producing the highest yield of the ABCB6 protein was selected by immunoblotting.

Membrane preparation and immunoblotting—Sf9 cell membranes were isolated as described [22]. Three days after virus transfection, the Sf9 cells were harvested, their membranes were isolated, and the membrane protein concentrations were determined by the modified Lowry method [23]. Isolated Sf9 membranes were run on 7.5 % Laemmli-type SDS-gels and the proteins were electroblotted onto PVDF membranes (Bio-Rad). Immunoblotting was performed according to standard protocol, by using the anti-ABCB6-567 monoclonal antibody [12], in 1:1000 dilution, and an anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (1:20000 dilution, Jackson Immunoresearch). HRP-dependent luminescence was developed by the enhanced chemiluminescence technique (ECL, Amersham).

ATPase activity measurements—Membrane ATPase activity was measured by colorimetric detection of inorganic phosphate liberation as described [24], with minor modifications. The reaction mixture contained 40 mM MOPS-Tris (pH 7.0), 50 mM KCl, 500 mM EGTA-Tris, 5 mM Na-azide, 1 mM ouabain and 1 μ g membrane protein. The reaction was started with the addition of 3.3 mM MgATP. The vanadate sensitive fraction was determined in the presence of 1 mM Na-orthovanadate. The indicated drugs (obtained from Sigma) were added in dimethyl sulfoxide (final concentration 1 %). ATP binding and nucleotide occlusion/trapping was measured as described [25]. In brief, in the ATP binding assay isolated Sf9 membranes (100 μ g) were incubated under nonhydrolytic conditions in a Tris-EGTA buffer (50 mM Tris-KCl, pH 7.0; 0.1 mM EGTA; 2 mM MgCl₂) for 5 min at 4 °C in the presence of 8-azido-[α -³²P]ATP containing 0.2 MBq ³²P isotope, in a final volume of 50 μ l. After incubation, the membranes were irradiated with UV light in the presence of the labeled nucleotide for cross-linking. Membranes were washed (centrifuged at 4 °C for 20 min, 15000 g and resuspended) twice in ice-cold Tris-EGTA buffer. The final pellet was resuspended in 20 μ l Tris-EGTA for SDS-PAGE. In the nucleotide trapping assays isolated Sf9 membranes (100 μ g) were

incubated under conditions allowing ATP hydrolysis for 5 min at 37 °C in the presence of trapping agents (500 µM sodium-orthovanadate or AlF₄) and 5-50 µM of 8-azido-[α-³²P]ATP, containing 0.2 MBq ³²P isotope, in a final volume of 50 µl. The reaction was stopped by the addition of 500 µl of ice-cold Tris-EGTA buffer containing 10 mM unlabeled MgATP and 500 µM vanadate. The membranes were washed (centrifuged at 4 °C for 20 min, 15000 g and resuspended) three times in ice-cold buffer. The final pellet was resuspended in 20 µl Tris-EGTA and UV-irradiated at 4 °C for 10 min using a Vilber-Lourmat T-15 UV lamp (312 nm, 15 Watt) positioned 5 cm above the samples. The labeled membrane proteins were separated by gel-electrophoresis and electroblotted to PVDF membranes. Quantitative ³²P-labeling was determined by a Phospho-Imager (Bio-Rad). The identity of the 8-azido-[α-³²P]ATP-labeled bands was verified by immunostaining of the same blot.

Generation of cell lines—Wild type ABCB6 and its variants were cloned into pBabe retroviral vectors (AddGene plasmid 1764) and stably expressed in K562 and HeLa cells by retroviral transduction. Briefly, the Phoenix-eco packaging cell line was transfected by using the ExGene transfection system (Fermentas). The cell-free viral supernatant was collected at 48 hours after transfection, and was immediately used to transduce retrovirus producing PG13 cells. The Phoenix-eco cell line [26] was a gift from G. Nolan (Department of Pharmacology, Stanford University, Stanford, CA); PG13 cells were obtained from ATCC (Manassas, VA). For transient transfection, wild type ABCB6, 206-ABCB6core and the N-terminal part of the protein were subcloned into a 3×FLAG-CMV-14 vector (ABCB6-FLAG, 206-ABCB6core-FLAG and TMD₀-FLAG, respectively). To obtain GFP-fusion proteins, wild type ABCB6, 206-ABCB6core and the N-terminal part of the protein were subcloned into a pEGFP-N1 vector (AdGene). Cells were transfected according to the manufacturer's instructions (Fugene HD Transfection Reagent, Promega). The vectors containing GFP-tagged Rab5(Q79L) variant were kindly provided by M. Vidal (Université Montpellier II, Montpellier, France).

Antibodies and dyes—The following primary antibodies were used: ABCB6-567 [12], anti-transferrin receptor (TfR) (H68.4, Invitrogen), anti-HA (H6908, Sigma) and anti-FLAG (F3165, Sigma). HRP-conjugated secondary antibodies were purchased from Jackson Immuno Research Laboratories Inc. (323-005-024 and 223-005-024). Fluorescently labeled secondary antibodies (goat anti-mouse IgGs conjugated with Alexa Fluor 647 A21235, Alexa Fluor 594 A11005 or Alexa Fluor 488 A10667; goat anti-rabbit IgGs conjugated with Alexa Fluor 647 A21244, Alexa Fluor 594 A11012 or Alexa Fluor 488 A11008) were purchased from Invitrogen. LysoTracker and DAPI dyes were from Invitrogen. The OSK43 antibody was a kind gift from Dr. Yoshihiko Tani (Japanese Red Cross Osaka Blood Center, Osaka, Japan). To label cellular organelles, the following dyes were used: LysoTracker Red DND-99 (Invitrogen, L7528), DAPI (Sigma D9564). As isotype controls reagent grade IgG from mouse and rabbit serum were used (Sigma).

Glycosidase treatment—ABCB6-HA and 206-ABCB6core-HA overexpressing K562 cell lysates were treated with Peptide-N-glycosidase F (PNGase-F) enzyme (P0704L, New England BioLabs) to remove N-glycans. 20 µg total protein was analyzed by SDS-PAGE, ABCB6 was visualized by the ABCB6-567 antibody.

Cell Culture Conditions—The K562 cell line (American Type Culture Collection, ATCC) was grown in RPMI medium (Roswell Park Memorial Institute Medium) supplemented with 10 % (v/v) fetal bovine serum (Invitrogen Cat.No. 10106-169) and with 2 mM glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin (Lonza, Belgium) at 37 °C in humidified air/CO₂ (19:1) atmosphere. The HeLa cell line (ATCC) was grown in DMEM (Dulbecco's modified Eagle's medium), supplemented with 10 % (v/v) fetal bovine serum (Invitrogen Cat.No. 10106-169) and with 2 mM glutamine, 100 units/ml penicillin, and 100 units/ml

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streptomycin (Lonza, Belgium) at 37 °C in humidified air/CO₂ (19:1) atmosphere. Cell lines were regularly screened with the Mycoplasma Detection Kit (Lonza, Belgium), and the assays were carried out in mycoplasma-negative cells.

Co-immunoprecipitation—For immunoprecipitation, 80 µl Protein A/G Agarose beads (Pierce, 20421) were loaded to on a column (Pierce, 69705) and coated with antiFLAG antibody (10 µg) for 2 h at RT, then at 4 °C overnight. For each sample, 10⁶ transfected cells (72 h after transfection) 70 % confluency were solubilized in 1 ml freshly made lysis buffer (45 mM Tris, 135 mM NaCl, protease inhibitor cocktail (Roche), 0.5 % NP-40 (nonyl phenoxypolyethoxyethanol), 1 mM PMSF and 10 mM NEM for 5 min on ice. Cells were harvested and sonicated for 10 sec. Solubilized cells were centrifuged for 10 min at 13000 g at 4 °C. The supernatant was incubated with the precoated beads for 2.5 h at 4 °C, then for 30 min at RT. After washing the beads with PBS containing 0.5 % NP-40, the bound protein was eluted with 60 µl Laemmli buffer for 2 h at RT and 2 h for 4 °C. Eluted proteins were separated by SDS-PAGE, transferred onto PVDF membranes (BioRad) and visualized by immunodetection.

Confocal Microscopy—Cells transfected by GFP-fusion proteins were incubated with LysoTracker for 30 min at 37 °C and living cells were analysed by confocal microscopy. Where indicated, chloroquine was added to the cells at 100 nM concentration for 2 h in serum free medium. Rab5(Q79L)-GFP and the FLAG-tagged protein co-transfected cells at 70-80 % confluency were gently washed with Dulbecco's modified phosphate-buffered saline (DPBS), fixed with 4 % paraformaldehyde (PFA) in DPBS for 10 min, and permeabilized in methanol for 90 sec at RT. The samples were then blocked for 1 h at RT in DPBS containing 2 mg/ml BSA (Sigma, A7030), 1 % fish gelatin from cold water fish skin (Sigma, G7765), 0.1 % Triton-X 100 and 5 % goat serum (Sigma, G9023). After blocking, the cells were incubated for 1 h at RT with the primary antibody diluted in blocking buffer. After washing with DPBS, the cells were incubated for 1 h at RT with the respective Alexa Fluor-conjugated secondary antibody diluted at 1:250 in blocking buffer. Where indicated, DAPI was diluted in DPBS and added to the cells after the incubation with the secondary antibody, for 10 min at RT. HeLa cells treated with Dyngo were incubated for 4 h at 37 °C in the presence of 30 µM Dyngo-4a (Abcam ab120689). After fixation and permeabilization the cells were labeled by the OSK43 and TfR antibodies. Surface expression of ABCB6 variants expressed in K562 cells was performed as described previously [15]. Samples were studied with an Olympus IX-81/FV500 laser scanning confocal microscope, using an Olympus PLAPO 606 (1.4 NA) oil immersion objective (Olympus Europa GmbH) [27, 28].

FACS analysis— For FACS analysis K562 cells were labeled by OSK43 anti-ABCB6 antibody with or without permeabilization. Permeabilized cells were first fixed by 4 % PFA (10 min at RT) and then permeabilized by 0.1 % Triton-X (10 min at RT). Antibody staining was performed for 45 min at 4 °C by using the OSK43 monoclonal antibody (100× dilution, 0.5 µl/tube) specifically recognizing ABCB6 protein, or without primary antibody (negative staining). After washing out the unbound primary antibodies, secondary antibodies corresponding to the IgG type and labeled with Alexa Fluor 488 were added to the cells (Goat F(ab')₂ fragment anti-human IgG (H+L)-Alexa Fluor 488 (Invitrogen), in 200× dilution, incubated for 45 min at 4 °C, washed and resuspended in PBS containing 0.5 % BSA. The labeled samples were subjected to FACS; intact cells were gated based on the forward scatter (FSC) and side scatter (SSC) parameters. Intact cells were analyzed for antibody staining by a FACS Attune® Acoustic Focusing Cytometer, Blue/Violet (excitation wavelength: 488 nm for Argon ion laser; emission filters: 530/15 nm for Alexa Fluor 488).

Electron Microscopy—In electron microscopy studies, ABCB6 was revealed by the monoclonal antibody (Santa Cruz sc-135726); the antibody recognizing CD63 was kindly provided by E. Rubinstein (Université Paris) [29]. For ultrathin cryosectioning and immunogold labeling, cells were fixed with 2 % PFA or with a mixture of 2 % PFA and 0.2 % glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Cells were processed for ultracryomicrotomy and single or double immunogold labeled using PAG10 or PAG15 as [30]. All samples were analyzed using a FEI CM120 electron microscope (FEI Company), and digital acquisitions were made with a numeric camera (Keen View; Soft Imaging System, SIS, Germany). The definition of the distinct compartments was based on their morphology and by correlation with immunogold labeling for CD63 as marker of late endosomes/lysosomes. Multivesicular bodies were defined as compartments delimited by a membrane with numerous internal vesicles. Electron-dense compartments with vesicular or lamellar membranes were classified as mixed lysosomes. Electron-dense compartments with no internal membranes were classified as dense lysosomes.

RESULTS

The ABCB6 core shows membrane insertion, dimerization, ATP binding and hydrolysis— ABCB6 is composed of a core ABC-unit (containing a nucleotide binding domain followed by six transmembrane helices) and an extra N-terminal transmembrane segment (TMD₀) predicted to contain 5 transmembrane helices. Except for the ABCB6 orthologs, the N-terminal segment does not appear in any other proteins. In order to characterize the role of these domains, we created an N-terminally truncated construct lacking TMD₀. As the cytosolic loop between the extra N-terminal domain and the core ABC complex was shown to be crucial for functionality in other ABC transporters [7, 31], the ABCB6-core was designed to include the entire cytoplasmic loop connecting the fifth and sixth transmembrane helices (Figure 1).

As shown in Figure 2A, the full length and the N-terminally truncated ABCB6-core (206-ABCB6core) were expressed in insect cells with equal efficiency. Despite our significant experience with measuring the catalytic activity of various ABC transporters [6, 22, 24, 32], the colorimetric assay did not detect any measurable ATPase activity associated with insect cell membranes containing high levels of full-length ABCB6. Thus, we performed additional experiments to verify that the protein is nevertheless capable of binding and hydrolyzing ATP [25]. By using magnesium-8-azido-[α -³²P]ATP, covalent photoaffinity labeling of the ABCB6 variants could be achieved. Experiments performed under non-hydrolytic and hydrolytic conditions proved that both the full-length protein and the N-terminally truncated ABCB6 core are capable of binding and hydrolyzing 8-azido-[α -³²P]ATP, suggesting that loss of the first 205 amino acids does not impede folding, membrane insertion and ATP hydrolysis (Figure 2 B,C). In contrast, mutation of a crucial Walker A lysine moiety (ABCB6-K629M) is compatible with ATP binding, but abolishes nucleotide trapping/hydrolysis (Figure 2 D).

ATP hydrolysis requires the cooperation of two NBDs, suggesting that the core-ABCB6 complex can efficiently dimerize. The ability of the ABCB6-core to form dimers was verified by coimmunoprecipitation of HA- and FLAG-tagged ABCB6 variants transiently coexpressed in HeLa cells; the proteins were solubilized from the membranes and analyzed by means of a pull-down assay using A/G agarose beads. In control experiments, the dimerization of the full-length proteins was analyzed. In agreement with published data, full-length ABCB6 was found to form homodimers (ABCB6-HA was precipitated with anti-FLAG coated beads) [11]. Similarly, the N-terminally truncated ABCB6 core formed homodimers, suggesting that TMD₀ is not required for dimerization (Figure 3).

*Role of the N-terminal segment in intracellular targeting—*The ACBB6 core is sufficient to promote ATP hydrolysis – additional transmembrane helices may be important for the regulation of function or intracellular targeting. To elucidate the role of TMD₀ in intracellular targeting, we followed the localization of the core ABCB6 domain. Human HeLa and K562 cell lines were stably transduced by retroviral vectors encoding the full-length or the N-terminally truncated core domain of ABCB6. Expression of the core ABCB6 domain was confirmed by Western-blotting, which showed a single band that could not be shifted by PNGase-F treatment (Figure 4). This is consistent with the glycosylation of ABCB6 on its N-terminal domain, as suggested by a recent study showing that ABCB6 is glycosylated on its amino terminus [33].

We next investigated the subcellular localization of GFP-tagged ABCB6 variants in transiently transfected HeLa cells (Figure 5 A,B). In accordance with its endolysosomal expression, full-length ABCB6 appeared in LysoTracker-positive intracellular compartments. This colocalization persisted after treating the cells with the lysosomotropic agent

chloroquine, which resulted in a characteristic change in lysosome number and morphology. Strikingly, the core-ABCB6 domain showed a distinct localization, appearing dominantly in the plasma membrane. In sharp contrast, TMD₀ directed the GFP protein to the endolysosomal compartment. As seen with the full-length protein, colocalization of TMD₀ with LysoTracker was enhanced by chloroquine treatment.

To substantiate the plasma membrane localization of the ABCB6 core domain, we repeated the experiments with K562 cells stably expressing untagged forms of the full-length ABCB6 protein and the core-ABCB6 variant. In this experimental setup we benefited from the use of the OSK43 antibody, which recognizes an extracellular epitope of the core ABCB6 domain [16]. FACS analysis of intact cells demonstrated that the core-ABCB6 domain was predominantly found in the plasma membrane (note that the cells are heterogeneous with respect to the cell-surface expression of the core-ABCB6 domain), whereas the full-length ABCB6 protein could be revealed only upon permeabilizing the cells (Figure 6). These results confirm that ABCB6 is mainly intracellular, and the core ABCB6 domain is predominantly localized in the plasma membrane.

Finally, the intracellular localization of ABCB6 was analyzed by electron microscopy. The electron microscopy pictures of ultrathin cryosections of HeLa cells overexpressing ABCB6 confirmed the localization of ABCB6 in CD63-positive endosomal compartments including the membranes of multivesicular bodies, multilamellar lysosomes and dense lysosomes (Figure 7).

ABCB6 is rapidly internalized through dynamin-mediated endocytosis—Newly synthesized lysosomal proteins can be directly shuttled to the endosomal system from the trans-Golgi network. Alternatively, endolysosomal localization may be preceded by a transit at the plasma membrane compartment. If ABCB6 was to follow the latter route, a relatively rapid internalization of its plasma membrane pool would be expected. To verify this possibility, we followed the redistribution of ABCB6 in K562 cells that express detectable levels of the protein at the plasma membrane [15, 34]. Surface labeling of ABCB6 in K562 cells at the initial time point revealed a faint signal corresponding to the plasma-membrane-resident endogenous ABCB6 pool. Similarly to the redistribution TfR molecules, at 37 °C, plasma membrane ABCB6 was completely internalized within 30 min (Figure 8A). To investigate the involvement of dynamins and clathrin-dependent coated vesicle formation in the internalization of ABCB6, the pulse-chase experiments were conducted in the presence of Dyngo that acts as a potent inhibitor of endocytic pathways mediating the internalization of plasma membrane proteins [35]. Dyngo treatment significantly reduced the internalization of both ABCB6 and TfR (Figure 8A). Next, we characterized the effect of Dyngo in HeLa cells, which do not express detectable ABCB6 levels on the cell surface. Dyngo treatment blocked the internalization of TfR. Similarly, treatment of cells with Dyngo seemed to completely block the internalization of endogenous ABCB6, as ABCB6 remained on the surface throughout the entire 30-min time course (Figure 8B). Thus, blocking the clathrin-coated, dynamin-dependent internalization of endosomes resulted in a marked increase of cell surface ABCB6 in HeLa cells, suggesting the absence of plasma membrane pool in these cells is due a rapid internalization.

In contrast to the full-length ABCB6, the core domain does not appear in Rab5-positive endosomes—Overexpression of the constitutively active Rab5 mutant (Rab5 Q79L) results in the formation of enlarged cytoplasmic vesicles that exhibit many characteristics of early endosomes including immunoreactivity for Rab5 and transferrin receptor [36]. To determine if internalization of ABCB6 is mediated through early endosomes, ABCB6-expressing cells

were transiently transfected with plasmids encoding a GFP-tagged form of Rab5(Q79L). As expected, the enlarged vesicles were positive for GFP and the internalized TfR (Figure 9). Similarly, increased Rab5 function resulted in the redistribution of both endogenous and overexpressed ABCB6 into the membranes of GFP-positive intracellular vesicles. In sharp contrast, the N-terminally truncated ABCB6 core labeled the plasma membrane and did not show accumulation in the enlarged vesicles corresponding to early endosomes, suggesting that in lack of TMD₀, the ABCB core is not recognized by the endocytic machinery. TMD₀ did not accumulate in the giant vesicles or the plasma membrane, suggesting that it is directly targeted to the lysosomes, without a detour in the plasma membrane.

DISCUSSION

The homodimeric ABC transporter ABCB6 was initially identified in mitochondria [11, 12], and was later also found in the endolysosomal compartment [14, 15], the Golgi apparatus [37] and the plasma membrane [12, 15, 34, 38]. While we cannot exclude mitochondrial localization of ABCB6, in our hands, imaging with a variety of modalities of cells expressing endogenous or overexpressed ABCB6 failed to ascertain targeting to the mitochondria. Our results show that ABCB6 is internalized from the plasma membrane through endocytosis, to be distributed to multivesicular bodies and lysosomes. Using electron microscopy we show for the first time that ABCB6 is localized in the membranes of multivesicular bodies, multilamellar lysosomes and dense lysosomes.

Our major aim was to delineate the role of ABCB6's unique N-terminal domain in regulating ABCB6 function and intracellular targeting. The N-terminal extension of ABCB6 does not contain recognizable motifs or homology to other proteins. As the cytosolic loop between the N-terminal TMD₀ and the core transporter is crucial for functionality in some ABC transporters, we assigned the domain boundary between the core-ABCB6 and TMD₀ to residue 205, which is immediately proximal to the predicted five transmembrane helix bundle of TMD₀. The ABCB6 fragments were expressed both in Sf9 insect cells and mammalian cells. In the first system, due to the high levels of expression in a heterologous cell type, functional studies and convenient biochemical assays could be carried out on isolated membranes with less interference from other proteins, whereas the mammalian system offered the possibility to efficiently study membrane routing and intracellular trafficking.

Despite high expression levels, ABCB6 did not show a measurable ATPase activity in Sf9 membranes, as may have been expected based on the successful functional expression of several ABC transporters in insect cells, including ABCB1 (MDR1/P-gp), ABCC1-2 (MRP1, MRP2), ABCG1 or ABCG2, that have been extensively characterized in our laboratory [6, 7, 21, 24, 25, 39-41]. It may be argued that the lack of measurable ABCB6 ATPase activity is due to a defect in the insertion or folding in the insect cell membrane. The high-level expression of a core-glycosylated ABCB6 with a correct molecular mass, the specific, Mg²⁺-dependent ATP binding, and the formation of a catalytic intermediate strongly argue against this possibility. It is also possible that the ATPase turnover of the ABCB6 is too low to allow a direct detection of ABCB6-mediated ATP hydrolysis against the background of the insect ATPases present in isolated membrane preparations. However, a recent report demonstrated that the purified and reconstituted ABCB6 protein possesses a significant, substrate-stimulated ATPase activity [42]. Therefore we measured the vanadate-sensitive ATPase activity in the presence of various compounds suggested to be transported by ABCB6, but found no stimulation when pheophorbide-A (5-100 μM), hemin (5-100 μM), prazosin (5-100 μM), coproporphyrinogen-III (5-50 μM), or protoporphyrin-IX (5-100 μM) were added to the isolated membranes containing ABCB6 (data not shown).

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In the presence of ATP, a divalent cation and a transition state analogue such as vanadate, a nucleotide is trapped by ABC proteins, reflecting a drug-stimulated partial reaction of the ATP-hydrolytic cycle [9, 43]. Detailed analysis of the optimum conditions for 8-azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ trapping indicated that the formation of the stable (“trapped”) nucleotide-ABCB6 complex requires incubation at 37 °C and the presence of phosphate-mimicking transition state analogs, suggesting that the catalytic turnover does not allow the detection of natural nucleotide occlusion in the absence of inhibitory anions. Mutation of the conserved Walker A methionine residue impairs hydrolysis of several ABC transporters [21]. Here we show that the K629M mutation allows ATP binding, but prevents ATP hydrolysis, in agreement with the abolished ATPase activity of ABCB6 K629A [42]. In contrast, the core ABCB6 construct can hydrolyze ATP (Figure 2C). Quantification of the experiments revealed that the core ABCB6 form shows reduced trapping activity (50%). This result may suggest a drop in the affinity for ATP; a change in the catalytic cycle resulting in a decrease of the average time during which vanadate can replace the terminal phosphate; or an altered conformation in which the labeling efficiency of the azido group is decreased. Labeling was only observed in the presence of vanadate, indicating that the trapped nucleotide species is ADP. Thus, ABCB6 is able to hydrolyze ATP without its N-terminal domain. Since the ATPase activity of ABCB6 seems to be inhibited by a component of the insect cell membrane, a more detailed characterization of the influence of TMD₀ on the kinetic parameters of ATP hydrolysis will require purified and reconstituted proteins.

Similar results have been obtained with a variety of “long” ABC transporters possessing an N-terminal extension. TMD₀ was shown to be dispensable for the ATPase and/or transport activity of ABCC1 (MRP1) [6, 7] and ABCC2 (MRP2) [44]. Intriguingly, all of the ABCB half transporters are expressed in intracellular organelles, and all have N-terminal regions containing relatively long sequences [5]. Correct folding and preserved function of N-terminally truncated core-ABCB transporters were reported for ABCB9 [31] and ABCB2-3 [45]. It has to be noted that the functionality of the truncated ABC transporters is dependent on the domain boundaries of the constructs. The selection of appropriate boundaries can be difficult unless the structure is already known, since the deletion of critical amino acids may disturb the structure or folding of the neighboring membrane-associated regions, which may influence function and/or trafficking [7, 46]. Although the exact topology of ABCB6 is unknown, the domain boundary of the ABCB6 core was defined to include the entire cytoplasmic loop connecting the fifth and sixth transmembrane helices.

While the ATPase activity of the core domains of long ABC transporters was generally preserved, removal of TMD₀ had variable consequences on trafficking. Core-MRP1 still traffics to the basolateral membrane in polarized epithelial cells [7] and the core-TAP (Transporter Associated with antigen Processing) complex seems to be correctly retained in the endoplasmic reticulum [45, 47, 48]. In contrast, core-MRP2 is localized in intracellular compartments and is no longer directed to the apical membrane [6]. The core ABC domain of Ycf1p (Yeast cadmium factor 1 protein), the prototypical member of the ABCC subfamily of *Saccharomyces cerevisiae*, fails to reach the vacuoles and accumulates in intracellular compartments [49]. Remarkably, the N-terminally truncated ABCB9 behaves much like the ABCB6 core as it is also targeted preferentially to the plasma membrane [31]. Similarly to the results obtained with the TMD₀ of ABCB9 (containing only four transmembrane helices), the ABCB6 TMD₀ was able to direct GFP to the endolysosomal compartment of HeLa cells, suggesting that it also forms a folding domain containing sufficient information for lysosomal targeting [31].

Newly synthesized lysosomal proteins can be directly shuttled to the endosomal system from the trans-Golgi network. The best understood direct pathway is the mannose-6-phosphate

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receptor (M6PR)-mediated transport of lysosomal hydrolytic enzymes [50]. Alternatively, lysosomal membrane proteins are first targeted to the plasma membrane for subsequent endocytosis. Recently, we have shown that at high expression levels, ABCB6 accumulates in the plasma membrane, whereas under the same experimental conditions the canonical mitochondrial ABC transporters ABCB7, 8 and 10 remained confined to the mitochondria [15]. Here, we have followed the intracellular trafficking of ABCB6 in K562 cells that express low, but detectable levels of the protein in the plasma membrane. Our results indicate that ABCB6 is indeed rapidly internalized from the plasma membrane through the clathrin/dynamin-dependent pathway. Blocking dynamin-dependent internalization resulted in a marked increase of cell surface ABCB6 expression in HeLa cells, suggesting that the absence of plasma membrane pool in these cells is due a rapid internalization of ABCB6.

Internalized proteins traffic through the dynamic and adaptable continuum of the endolysosomal system to be recycled to the plasma membrane or sorted to the lysosomes. The small GTPase Rab5 controls the fusogenic properties of early endosomes through GTP-dependent recruitment and activation of effector proteins. Expression of a GTPase-defective mutant, Rab5(Q79L), is known to cause formation of enlarged early endosomes and later endocytic profiles [36]. Membrane components of early endosomes undergo stringent selection so that only a specific cohort is passed on the late endosomes and eventually to lysosomes, where longer lived proteins escaping degradation contribute to the maintenance and generation of lysosomes [51]. The localization of ABCB6 in Rab5-positive vesicles confirms the presence of ABCB6 in early endosomes, and the EM images convincingly demonstrate the localization of ABCB6 in late endosomes or multivesicular bodies and multilamellar lysosomes. These results suggest that ABCB6 is first targeted to the plasma membrane to be internalized by endocytosis.

Notably, the ABCB6 core was not endocytosed, suggesting a role for TMD₀ in cargo recognition during clathrin-mediated endocytosis [52]. Lysosomal membrane protein trafficking encompasses a complex network of sorting signals and cytosolic recognition proteins [53]. Sorting signals are typically located in cytosolic loops proximal to transmembrane helices. Most signals consist of short, linear sequences of amino acid residues including tyrosine-based motifs (NPXY or YXXØ, Ø being a bulky hydrophobic residue) or dileucine motifs (D/EXXXLL or DXXLL) [53]. Unconventional dileucine motifs missing the upstream acidic residue and split targeting motifs were also found to be sufficient for lysosomal localization [54, 55]. Finally, ubiquitination of cytosolic lysine residues may serve as a signal for sorting at various stages of the endosomal-lysosomal system [56]. The predicted intracellular loops of TMD₀ do not contain conventional lysosomal sorting motifs. Further studies will address the role of TMD₀ in trafficking to identify cryptic routing signals or protein interactions responsible for lysosomal targeting [57].

In summary, molecular dissection of ABCB6 revealed that the TMD₀ and the ABC core represent independently folding units, responsible for the lysosomal targeting and the ATPase activity of the full length protein, respectively. Based on the trapping experiments, the ability of the ABCB6 core to hydrolyze ATP seems to be preserved -further work is needed to establish whether TMD₀ influences the ATPase or the transport function of ABCB6..The physiological function of ABCB6 in the endolysosomal continuum remains to be clarified. While the trafficking pattern of ABCB6 revealed in this study may vary in a cell type-specific manner, our results suggest a function for ABCB6 in lysosomes and related organelles such as melanosomes [20, 58, 59]. Additional work, currently ongoing in our laboratory will be necessary to address this hypothesis.

Author contribution: KK, NK, AB performed the experiments; GET performed topology predictions; PB and GvN obtained EM data; KK, GvN and GS analyzed the data; KK and GS designed the study; GS wrote the paper.

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FOOTNOTES

The abbreviations used are: ABC, ATP-binding cassette; β -gal, β -galactosidase; BSEP, Bile Salt Export Pump; DMEM, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's modified phosphate-buffered saline; HA, human influenza hemagglutinin; HRP, horseradish peroxidase; L, dense lysosome; Lan, Langereis; M6PR, Mannose-6-Phosphate Receptor; MDR, multidrug resistance protein; MOPS, 4-morpholinepropanesulfonic acid; ML, multilamellar lysosome; MRP, multi-drug resistance associated protein; MVB, multivesicular body; NBD, nucleotide-binding domain; NEM, N-ethylmaleimide; NP-40, nonyl phenoxypolyethoxyethanol; PAG, Protein A Gold; PFA, paraformaldehyde; P-gp, P-glycoprotein; PNGase-F, peptide-N-glycosidase F; RPMI, Roswell Park Memorial Institute Medium; Sf9, *Spodoptera frugiperda* insect cells; TAP1-2, Transporter associated with Antigen Processing-1 and -2; TfR, Transferrin Receptor; TMD, transmembrane domain; Ycf1p, Yeast Cadmium Factor 1 protein.

FIGURE LEGENDS

FIGURE 1. Schematic illustration of the constructs characterized in this study

The core ABCB6 domain was designed to include the entire intracellular loop preceding the canonic transmembrane and nucleotide binding domains (residues 205-842). The complementary sequence encoding TMD₀ contains 5 predicted transmembrane helices (residues of 1-205).

FIGURE 2. Functional expression of ABCB6 variants in insect cells

A. Expression of the ABCB6 core domain in Sf9 insect cells. Isolated Sf9 membranes (2 μ g protein/lane) expressing β -galactosidase (β -gal, lane 1), ABCB6 (lane 2), and ABCB6core (lane 3) were separated by 7.5 % SDS-PAGE and were electroblotted onto PVDF-membranes. Immunoblotting was performed using monoclonal ABCB6-567 as described in the *Experimental* section. Membrane proteins are only core-glycosylated in insect cells [6], which is consistent with the apparent molecular mass of 95 kDa, corresponding to underglycosylated ABCB6.

B. TMD₀ is not required for ATP binding. Isolated Sf9 membranes expressing β -galactosidase (lane 1), ABCB6 (lane 2), and ABCB6core (lane 3) were incubated with 5 μ M 8-azido- $[\alpha$ -³²P]ATP under nonhydrolytic conditions (at 4 °C) for 5 min, followed by UV irradiation in the presence of the labeled nucleotide as described in the *Experimental* section.

C. TMD₀ is not required for ATP hydrolysis. Isolated Sf9 membranes expressing β -galactosidase (lane 1), ABCB6 (lane 2), and ABCB6core (lane 3) were incubated with 5 μ M 8-azido- $[\alpha$ -³²P]ATP and 0.4 mM Na-orthovanadate under catalytic conditions (at 37 °C) as described in the *Experimental* section. Both the full-length and the N-terminally truncated ABCB6 core are capable of ATP-binding and hydrolysis. The lower MW bands seen in lane 2 correspond to proteolytic fragments and products of vanadate-induced photocleavage [60, 61].

D. Mutation of the conserved Walker A lysine is compatible with ATP binding but abolishes nucleotide trapping of ABCB6. Isolated Sf9 membranes expressing ABCB6-K629M were

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incubated with 5-50 μM 8-u-[α - ^{32}P]ATP under nonhydrolytic (left) and hydrolytic (right) conditions as described in the *Experimental* section.

FIGURE 3. Coimmunoprecipitation of the full length and the N-terminally truncated ABCB6 core variants

Expression plasmids encoding full-length ABCB6 (ABCB6-FLAG and ABCB6-HA), the N-terminally truncated ABCB6 (coreABCB6-HA) were introduced into HeLa cells. The FLAG-tagged proteins were expressed with the HA-tagged proteins in each set of experiments. The solubilized protein complexes were adsorbed to protein A/G agarose beads coated with FLAG antibody. The eluted fractions were separated by SDS-PAGE; the identity of the protein bands was revealed by Western blotting using anti-HA antibodies (see Experimental section). The "input" lane contains 10 μg total protein, which corresponds to ~3% of the total sample. The "precipitated with anti-FLAG lane" also contains 10 μg total protein, which, however, corresponds to about 1/3 of the total eluted sample. Thus, there is a significant enrichment of ABCB6 in the immunoprecipitated sample.

FIGURE 4. Expression of ABCB6 variants in K562 cells

Total lysates of K562 cells transduced with full-length or N-terminally truncated ABCB6 were analyzed by SDS-PAGE (7.5 %, 20 μg protein per lane), followed by immunoblotting using the ABCB6-567 antibody. Lysates were treated with PNGase-F enzyme to remove N-glycans. While full-length ABCB6 shows a clear shift after the treatment, there was no change in case of the core-ABCB6 lacking the N-terminal part of the protein.

FIGURE 5. Characterization of the intracellular targeting of GFP-tagged ABCB6 variants in HeLa cells by confocal laser scanning microscopy

Cells transfected by GFP-fusion proteins were incubated with LysoTracker for 30 min at 37 $^{\circ}\text{C}$. Localization of ABCB6-GFP, core ABCB6-GFP, and TMD₀-GFP was revealed by GFP (green) in the context of the lysosomal marker LysoTracker (red). Panel B shows the effect of 100 nM chloroquine. Images were collected with an Olympus IX-81/FV500 laser scanning confocal microscope, using an Olympus PLAPO 606 (1.4 NA) oil immersion objective (Olympus Europa GmbH) [27, 28].

FIGURE 6. Detection of ABCB6 levels by flow cytometry

K562 cells were stably transduced with retroviral constructs encoding ABCB6 or its N-terminally truncated variant (ABCB6 core). Expression of the ABCB6 variants was analyzed by flow cytometry using the monoclonal anti-Lan OSK43 antibody. The left and the right panels show histograms corresponding to background fluorescence (secondary antibody only), endogenous ABCB6 expression, the overexpression of the wild-type or the truncated ABCB6 variants, measured in unpermeabilized and permeabilized cells, respectively.

FIGURE 7. Localization of ABCB6 by electron microscopy

HeLa cells overexpressing ABCB6 WT were processed for ultrathin cryosectioning and immunogold labeling. A. Electron micrographs showing the localization of ABCB6 (Protein A gold 10 nm, PAG10). B. Colocalization of ABCB6 (Protein A gold 10 nm, PAG10) with

CD63 (Protein A gold 15 nm, PAG15). Multivesicular bodies, multilamellar lysosomes and dense lysosomes are respectively annotated as MVB, ML and L (Scale bar, 500 nm).

FIGURE 8. Endogenous ABCB6 is rapidly internalized through dynamin-mediated endocytosis

A. K562 cells were pulsed on ice with the OSK43 antibody recognizing an extracellular ABCB6 epitope. Following the removal of unbound antibodies, the cells were placed to 37 °C and the OSK43-label (green) was “chased” for the indicated times by confocal microscopy in the presence or absence of 30 μM Dyngo-4a. In control experiments we followed the redistribution of the TfR (red) that is known to enter the cell through clathrin-mediated endocytosis.

B. Treatment of HeLa cells with a highly potent dynamin inhibitor prevents internalization of ABCB6 protein. HeLa cells were incubated for 4 h at 37 °C in the presence or absence of 30 μM Dyngo-4a. After fixation and permeabilization the cells were labeled by the anti-ABCB6 OSK43 (green) and TfR (red) antibodies.

FIGURE 9. Effect of the constitutively active rab5 on the distribution of ABCB6 variants within the cell

HeLa cells were transiently cotransfected with GFP-Rab5-Q79L (green) and FLAG-tagged ABCB6 variants (full-length, ABCB6-core, TMD₀; red). Following fixation, the expression of the endogenous TfR and ABCB6 was detected by confocal microscopy, using anti-TfR and the ABCB6-567 antibodies, respectively; whereas the overexpressed ABCB6 variants were detected by an anti-FLAG antibody (red).

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Figure 1.

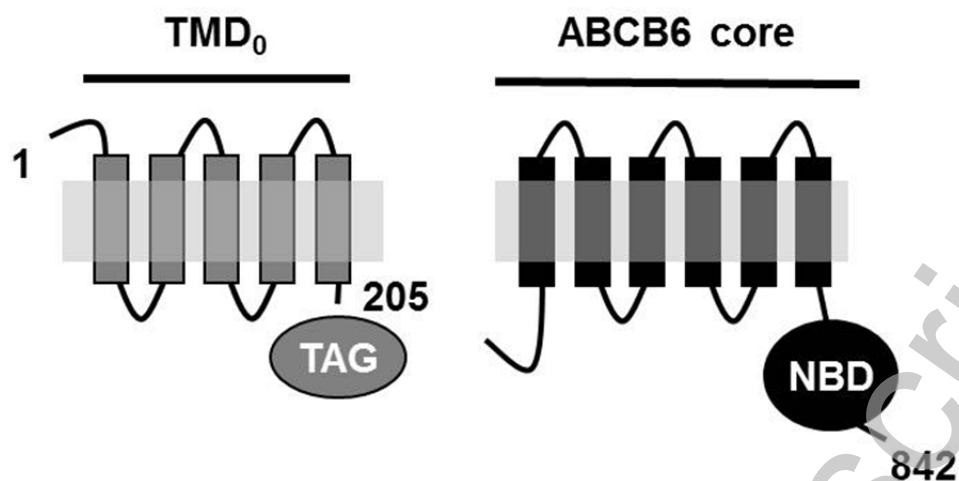


Figure 2.

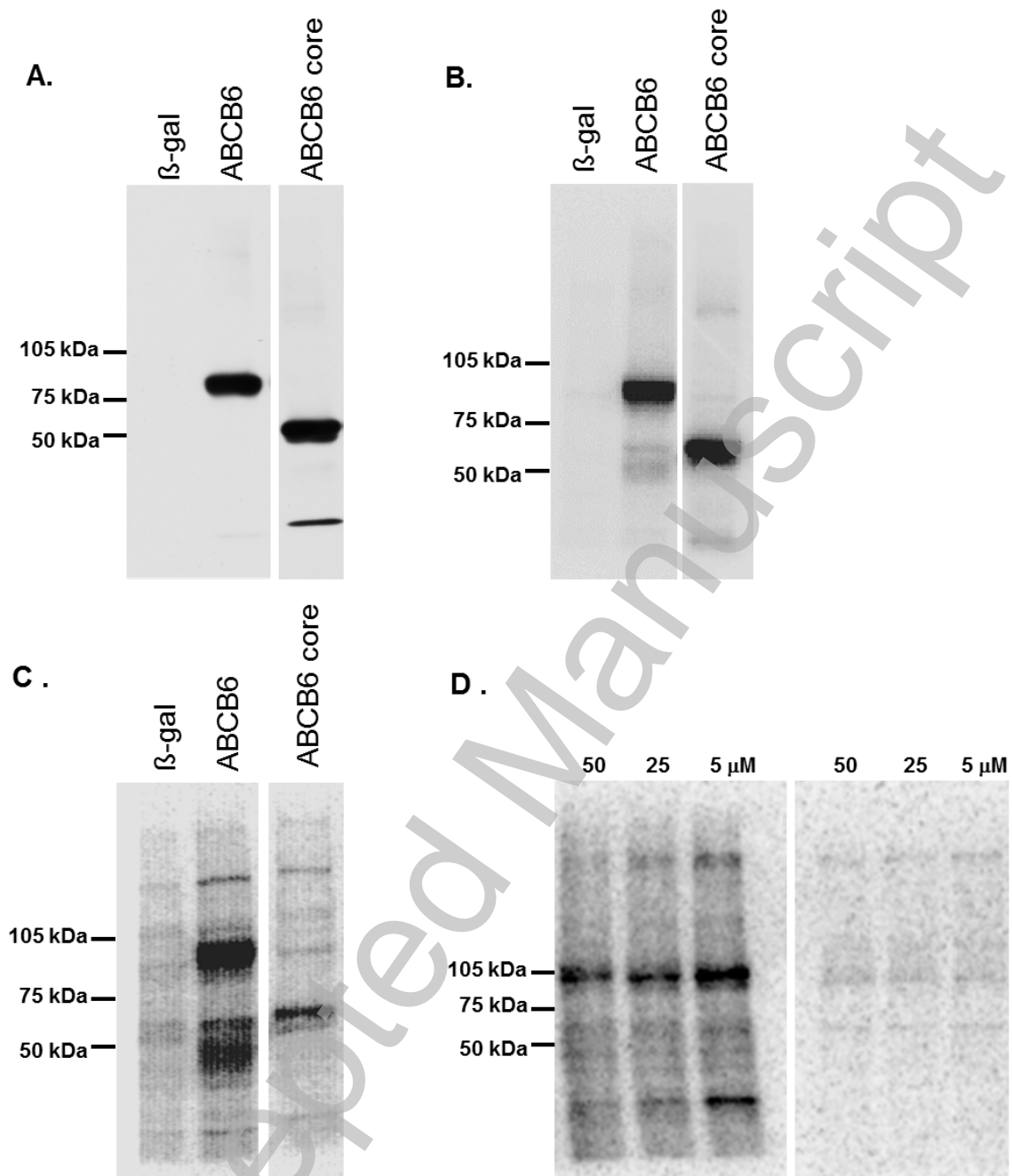


Figure 3.

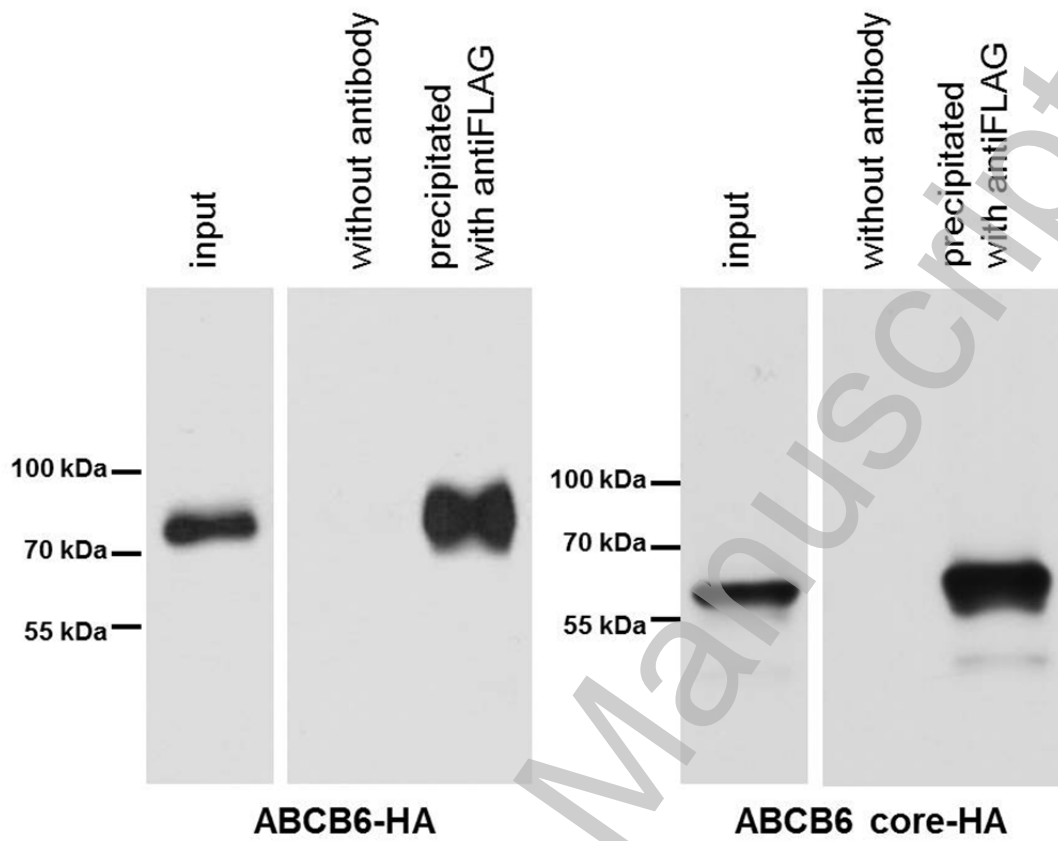


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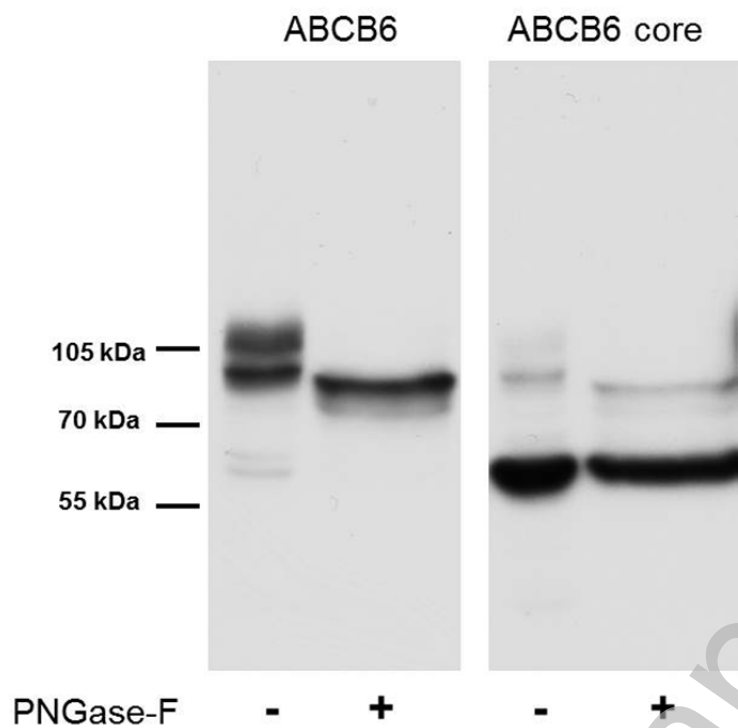


Figure 5.A.

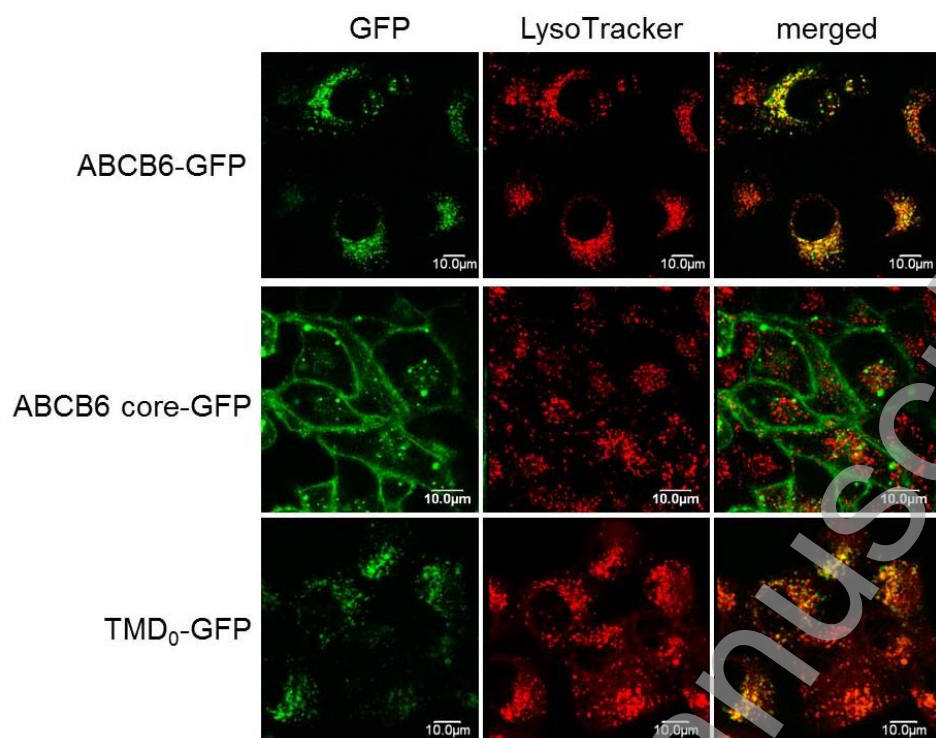


Figure 5B

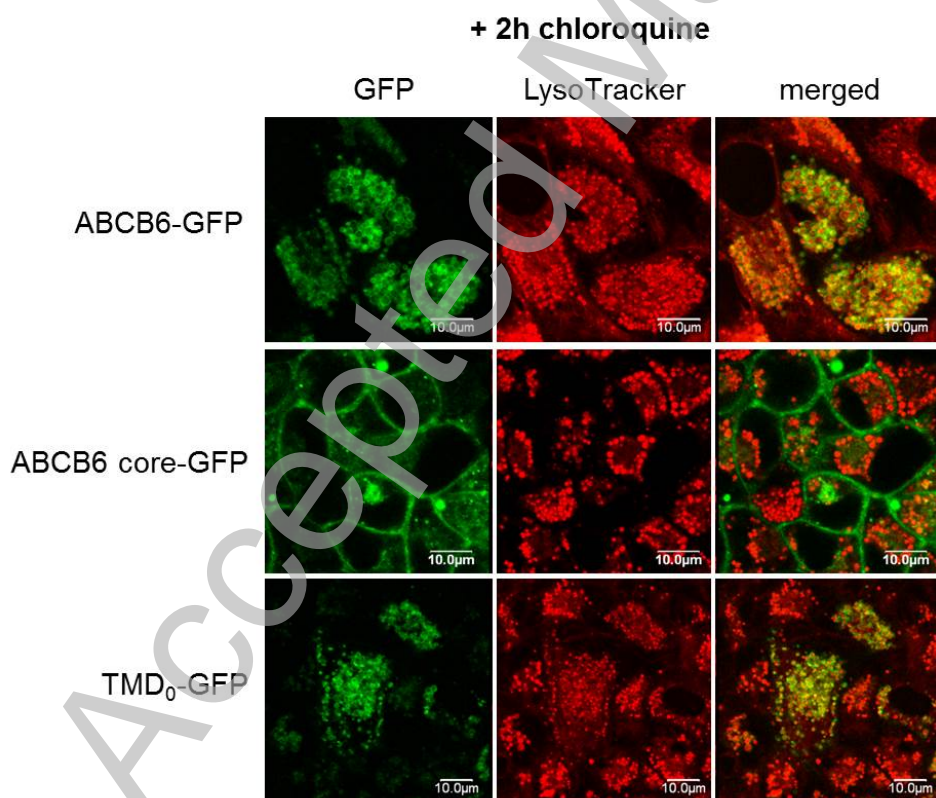
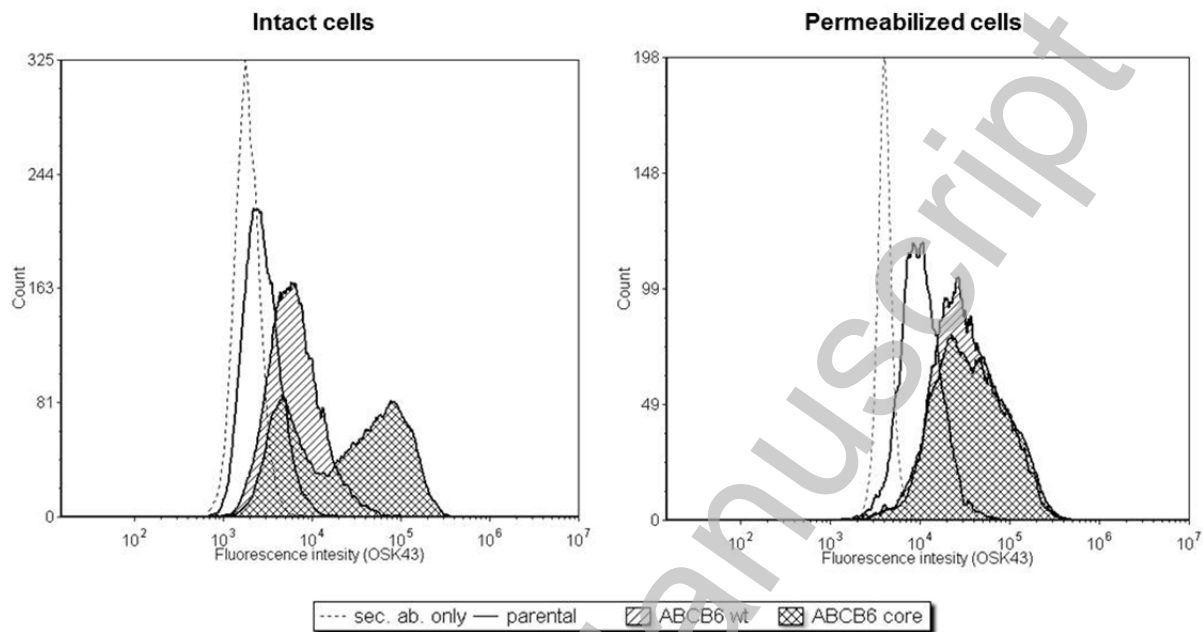


Figure 6.



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Figure 7.

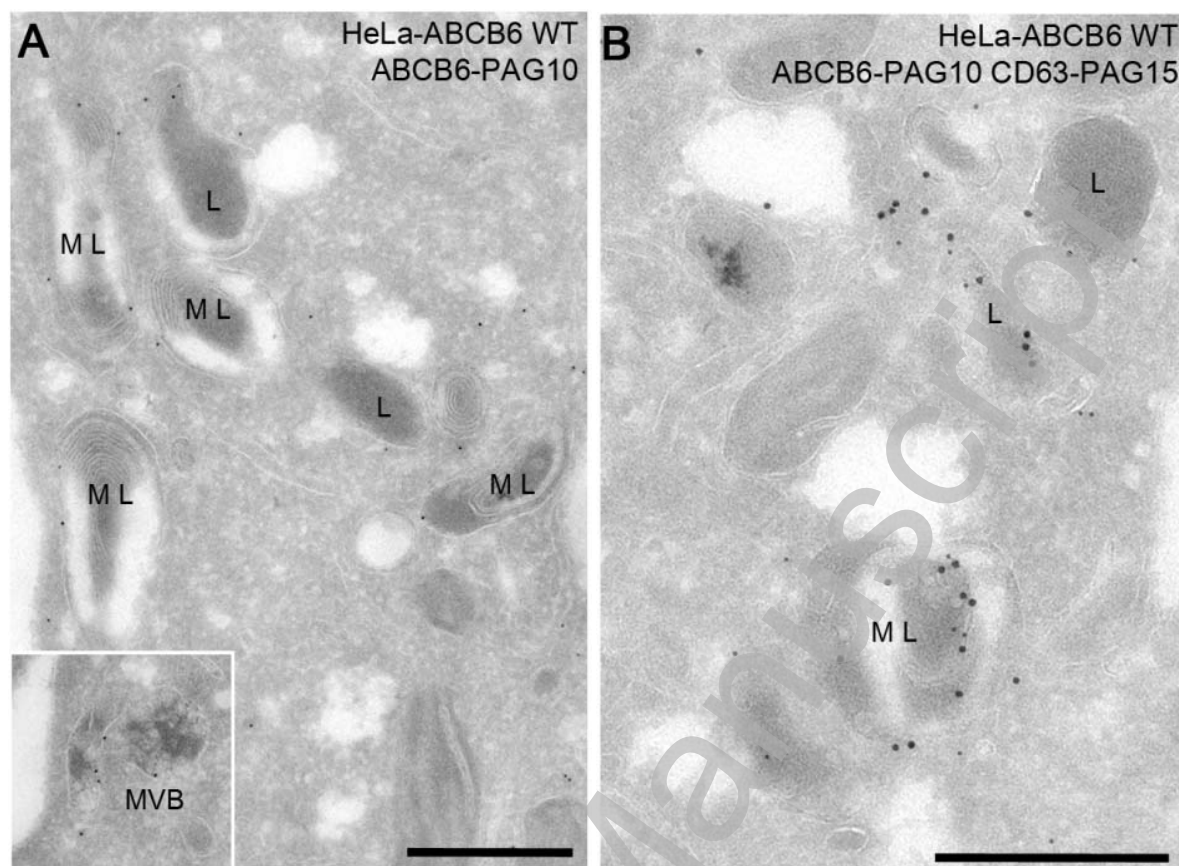


Figure 8.A.

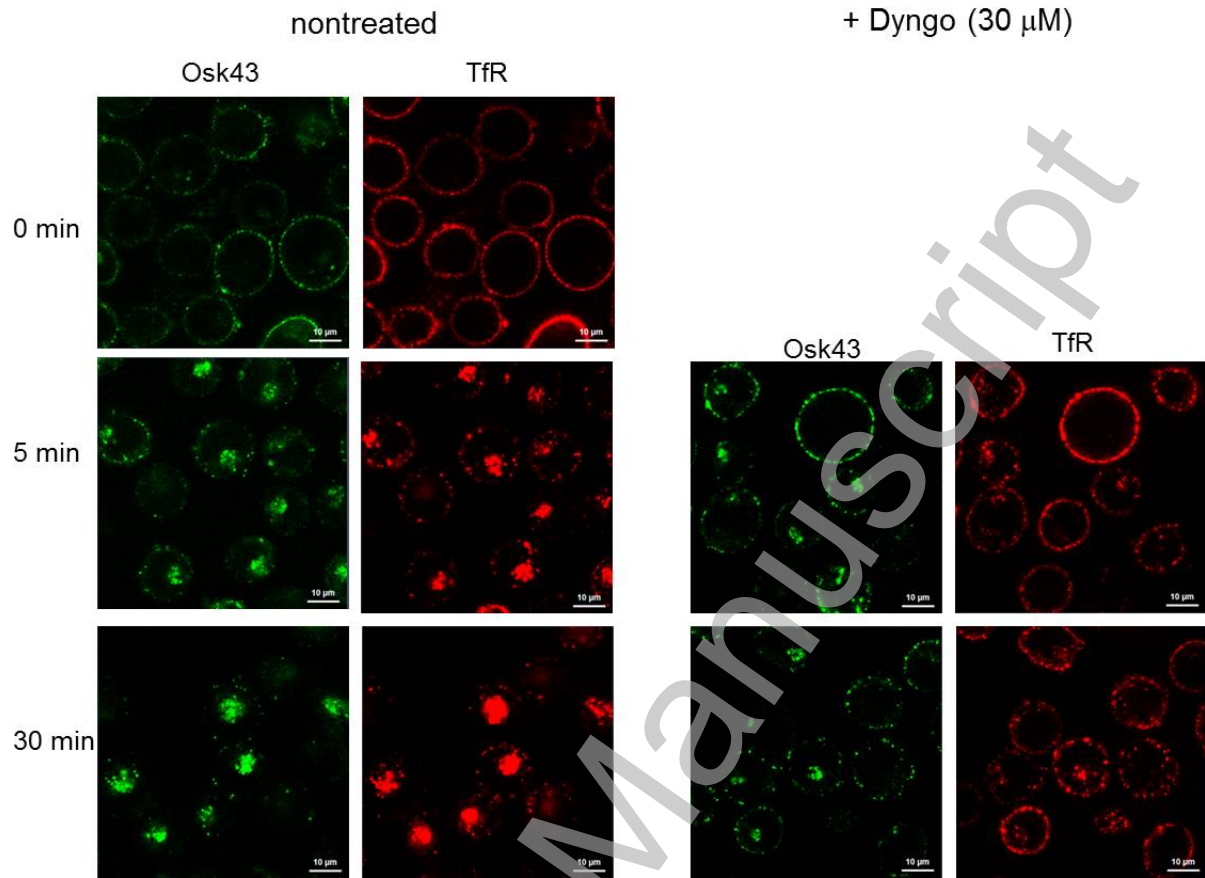


Figure 8.B.

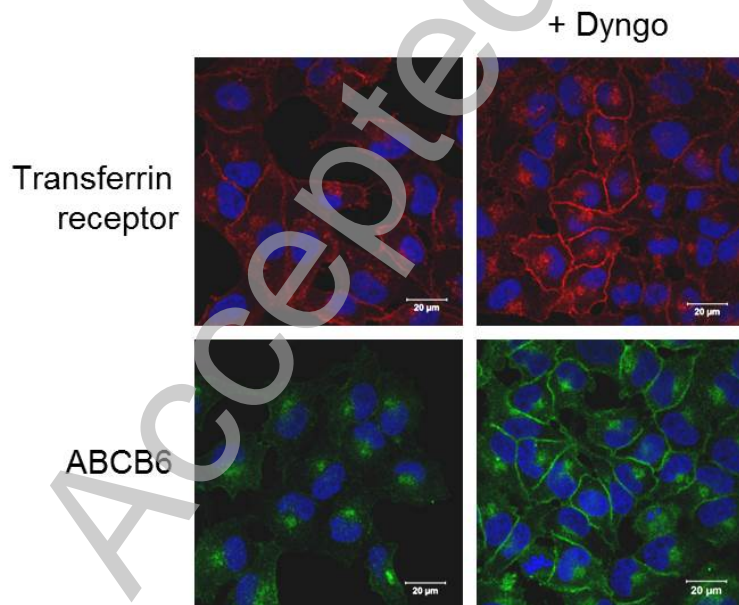


Figure 9.

