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Leishmania tarentolae as a host for heterologous expression of functional human ABCB6 transporter

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ABSTRACT

The need for large amounts of reproducibly produced and isolated protein arises not only in structural studies, but even more so in biochemical ones, and with regard to ABC transporters it is especially pressing when faced with the prospect of enzymatic/transport activity studies, substrate screening etc. Thus, reliable heterologous expression systems/model organisms for large and complex proteins are at a premium. We have verified the applicability of the recently established novel eukaryotic expression system, using *Leishmania tarentolae* as a host, for human ABC protein overexpression. We succeeded in overexpressing human ABCB6, a protein with controversial subcellular localization and multiple proposed cellular functions. We were able to demonstrate its efficient expression in the expected subcellular locations as well as biochemical activity of the overexpressed protein (ATPase activity and porphyrin-like substrate transport). This activity was absent in cells overexpressing the catalytically inactive variant of ABCB6 (K629M). We demonstrate the possibility of applying a cost-effective expression system to study the activity of membrane transporters from the ABC superfamily.

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1. Introduction

ATP-**b**inding **c**assette (ABC) transporters move structurally diverse compounds across membranes: in humans, among other functions, they protect normal tissues against toxins and confer multidrug resistance on malignant cells [1–3]. ABC proteins are divided into seven subfamilies, based on domain organization and sequence homology. ABCB6 belongs to the ABCB subfamily, which includes P-glycoprotein (ABCB1/ MDR1) causing multidrug resistance of cancer cells. Despite extensive studies, the physiological function of several ABC transporters remains unknown until now — one of more controversial proteins in this respect is ABCB6 [4]. It was demonstrated that the putative mitochondrial/endosomal ABC transporter ABCB6 activates *de novo* heme and porphyrin biosynthesis and elevates intracellular protoporphyrin IX (PPIX) levels [5–7], whereas the loss of one *Abcb6* allele in embryonic stem cells impairs porphyrin synthesis [8]. ABCB6 is also expressed in red blood cells: after upregulation during erythroid differentiation, it is expressed in cell membranes of mature erythrocytes at relatively high levels [4]. ABCB6 protein was identified as the antigen of Langereis (Lan) blood group. Lan-negativity is believed to be very rare and manifests in approximately 0.005% of the Caucasian population [9], although recent populational and genetic studies show that missense polymorphisms in ABCB6 gene can be more common than previously expected [10]. Individuals that are Lan-negative do not show any apparent clinical phenotype, arguing against a vital role of ABCB6 in porphyrin synthesis. However, *ABCB6* mutations have unexpectedly been identified as causal for a number of non-hematological genetic diseases including coloboma [11], dyschromatosis universalis [12] and pseudohyperkalemia [13].

Leishmania tarentolae is a trypanosomatid protozoan parasite of the Mauritanian gecko, easily cultured in vitro as a protein expression host [14]. There are established vectors and procedures for transfection as well as selection of transgenic strains that allow efficient production of recombinant proteins. *L. tarentolae* cells are rich in glycoproteins, which may constitute even >10% of the total protein. Moreover, oligosaccharide structures are similar to those appearing in mammalian cells, with N-linked galactose and fucose residues [14,15]. This may make them especially suitable for expression of ABC transporters which are often strongly glycosylated. The advantages of the *L. tarentolae* expression system include facile handling of cultures, an optimal culture temperature of 26 °C, rapid growth rate (with a doubling time of approximately 6 h in agitated culture), high cell densities (up to 10^9 cells/ml) with promising potential for scale-up as well as the stability of transgenic strains [16–18].

The aim of this study was to investigate the possibility of expression of human ABCB6 protein (as a model ABC transporter) using *L. tarentolae* as a heterologous host and to characterize localization and function of the transporter in a stable transgenic strain. To evaluate the potential role of ABCB6 and other transporters in cellular physiology, it is important to

Abbreviations: NTC, nourseothricin; BHI, brain-heart infusion; HRP, horseradish peroxidase.

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establish reliable models of functional protein. While other heterologous expression systems were used in the past to biochemically characterize ABC transporters, including *Pichia pastoris* [19], insect [20] or mammalian [6] cell lines, their main disadvantages (which partially led to limited success in their application to ABCB6 functional studies) are respectively: complicated membrane isolation procedure, complex transgenic strain derivation and relatively high cost. In all these respects, the *L. tarentolae* system was shown to be superior [21,22], also for human membrane proteins [23]. Here, we present a proof of concept for applying this convenient and cost-effective Eukaryotic host to study the biochemistry of ABCB6, a clinically relevant and poorly characterized membrane transporter.

2. Materials and methods

2.1. Materials and cell culture

L. tarentolae cells, empty expression vector (pLEXSY-sat2) and all culture materials were purchased from Jena Bioscience (Jena, Germany). Cells were grown at 26 °C in static suspension cultures in LEXSY BHI liquid medium supplemented with porcine hemin (5 μg/ml), penicillin/streptomycin (Pen-Strep, 100 U/ml) and nourseothricin (NTC, 100 μg/ml).

Genomic DNA from transgenic *L. tarentolae* strains was isolated using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). For ABCB6 immunostaining, anti-ABCB6 antibody (clone 24.39; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used. An antibody derived against the *Leishmania major* major surface glycoprotein Gp63 (clone [96-126], Abcam, Cambridge, UK) was used as a plasma membrane marker since it was previously demonstrated in the literature [24] to detect the *L. tarentolae* ortholog (which is a larger protein migrating at ca. 75 kDa MW). As secondary antibodies, HRP-conjugated goat anti-mouse IgG (ThermoFisher Scientific, Waltham, MA, USA) was used for Western blotting and Alexa Fluor 555-conjugated goat anti-mouse IgG_{2a} antibody (Life Technologies, Carlsbad, CA, USA) was used for immunofluorescence.

Sf9-ABCB6 membranes, overexpressing human ABCB6 from a baculoviral vector, were a kind gift from Dr. G. Szakács (Budapest) [20].

2.2. Construction of L. tarentolae transgenic strains

pLEXSY-sat2-ABCB6 expression plasmid for human ABCB6 protein was constructed using commercial pLEXSY-sat2 vector backbone and coding sequence of human ABCB6 protein, amplified by high-fidelity PCR from commercial cDNA (TrueClone vector; Origene, Rockville, MD, USA) and cloned using BglII/NotI restriction sites. Use of internal translation termination codon from the pLEXSY-sat2 vector caused the addition of a 33-AA C-terminal protein tag. Other than that, ABCB6 cDNA in pLEXSY-sat2-ABCB6 construct was proven to be identical to NCBI Reference Sequence NM_005689.1 by Sanger sequencing. pLEXSYsat2-ABCB6 (K629M) expression plasmid encoding a catalytically inactive version of the human ABCB6 protein (with the inactivating K629M mutation within the Walker A motif [4]) was constructed by site-directed mutagenesis of pLEXSYsat2-ABCB6 construct with QuickChange Lightning Kit (Agilent, Santa Clara, CA, USA) and mutagenic primers:

ABCB6-A1886T-F 5′-CGCAAAATTGTGCTCATCCCTGCCCCAGATG-3′

ABCB6-A1886T-R 5′-CATCTGGGGCAGGGATGAGCACAATTTTGCG-3′

Expression sequence in pLEXSYsat2-ABCB6 (K629M) plasmid was verified by Sanger sequencing to be identical to that in pLEXSYsat2-ABCB6 construct apart from the expected change of AAG codon (629K) to ATG codon (629M; A to T in position 1886 of CDS of hABCB6; Fig. 1B).

L. tarentolae cells were transfected by electroporation. pLEXSY-sat2-ABCB6, pLEXSYsat2-ABCB6 (K629M) and empty pLEXSY-sat2 plasmids were first digested with SwaI restriction enzyme (Fig. 1A) and linear expression cassettes (without *E. coli*-relevant ori and antibiotic selection marker) of ~7.1 kbp (ABCB6) and ~4.6 kbp (control) were purified by gel extraction. Parasite cells in logarithmic growth phase were centrifuged (2500 ×g, 5 min), resuspended in Eppendorf Hypoosmolar Buffer (Eppendorf, Hamburg, Germany) at final concentration of 1×10^8 cells/ml and kept on ice for 10 min prior to the addition of 2.5 μg of linearized expression cassette. Following electroporation (Eppendorf Multiporator, 2 mm cuvette, 1000 V, 160 μs) and 10 min incubation on ice, cells were resuspended in fresh BHI culture medium and grown in suspension for the next 24 h. Single colony-derived, NTC resistant strains were then selected after 9 days of growth on solid media (BHI-agar containing 100 μg/ml NTC) and maintained in suspension culture with constant antibiotic concentration. Genomic integration of the expression cassette in control and ABCB6 transfected strains was verified by diagnostic PCR reaction with 3 sets of primers (Fig. 1):

1) ABCB6 specific primers (annealing temperature of 60 °C):
   ABCB6-F 5′-CTGGTCTCCTGCTCCTCTG-3′
   ABCB6-R 5′-GCGCATACTTTGTCACTGAGC-3′
2) *ssu* locus integration primers (annealing temperature of 60 °C):
   SSU-F 5′-GATCTGGTTGATTCTGCCAGTAG-3′
   APRT-R 5′-TATTCGTTGTCAGATGGCGCAC-3′
3) Nourseothricin resistance cassette integration primers (annealing temperature of 53 °C):
   SAT-F 5′-CCTAGTATGAAGATTTCGGTGATC-3′
   SSU-R 5′-CTGCAGGTTCACCTACAGCTAC-3′

2.3. Preparation of microsomal and plasma membrane fractions

Lysis and membrane fractioning of transgenic *L. tarentolae* strains (empty vector control and ABCB6) was made by a modification of the method of Chen et al. [25]. All preparations were made at 4 °C. Briefly, cultures containing approximately 5×10^{10} cells were harvested (2500 ×g, 5 min), washed twice with buffer A (10 mM Tris-HCl pH = 7.4, 0.3 M sucrose, 0.2 mM EDTA, 0.2% BSA) and lysed in hypoosmolar buffer B (10 mM Tris-HCl, pH = 7.4) with glass homogenizer (approximately 30 full strokes). Unbroken cells and cell debris were removed by centrifugation at 10,000 ×g for 10 min. The supernatant was centrifuged at 17,000 ×g for 20 min to spin down organelles and microsomal membranes. Finally, the supernatant was centrifuged again at 61,000 ×g for 1 h to obtain the pellet highly enriched in plasma membranes. Both microsomal and plasma membrane pellets were resuspended in 0.5 ml of buffer C (20 mM Tris HCl pH = 7.4, 0.25 M sucrose, 0.2 mM EDTA) and gently passed several times through a 25-gauge needle. Protein concentration in final membrane preparations was measured with 660 nm Protein Assay Reagent (ThermoFisher Scientific) and samples were rapidly frozen at −80 °C in small aliquots for further use.

2.4. Immunostaining of ABCB6

Membrane protein samples were directly denatured with SDS-PAGE sample buffer diluted from 5× Lane Marker Reducing Sample Buffer (ThermoFisher Scientific). Immunoblotting was performed with standard protocols and involved SDS-PAGE on 4%–20% pre-cast polyacrylamide gels (BioRad; Hercules, CA, USA), protein transfer onto polyvinylidene fluoride membrane and probing with anti-ABCB6 primary antibody (1:2000) and HRP-conjugated secondary antibody (1:1000). Immunolabelling was detected by chemiluminescence with Super Signal West Femto substrate (ThermoFisher Scientific) and visualized on Multispectral FX Pro In-Vivo Imager (Carestream; Rochester, NY, USA).

For immunofluorescence detection of ABCB6, aliquots of LEXSY-control and LEXSY-ABCB6 cultures were centrifuged (2500 ×g, 5 min, 4 °C), washed once with ice cold PBS and stained for 10 min at 4 °C with 0.5 μM membrane dye mCLING-ATTO488 (Synaptics Systems,

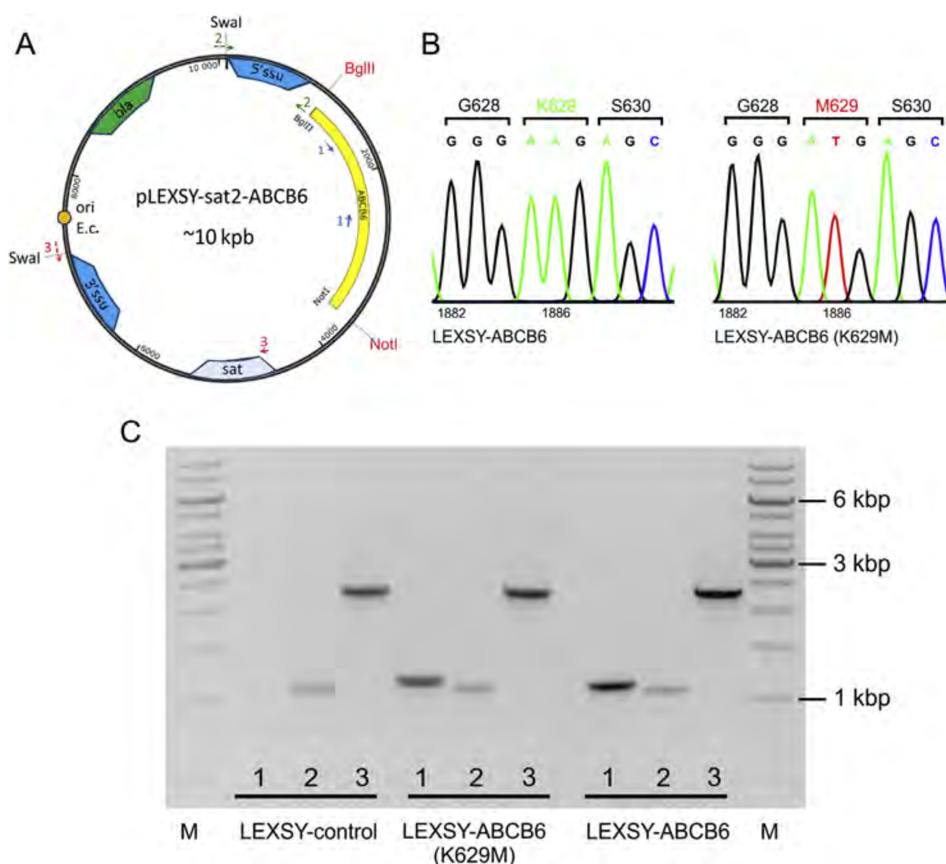

Fig. 1. Engineering of stable *L. tarentolae* control and hABCB6 (wild-type and K629M) expressing strains. A. Map of constructed pLEXSY expression vector containing human ABCB6 cDNA cloned into the commercial pLEXSY-sat2 plasmid using BglII/NotI restriction sites. Respective pairs of diagnostic primers used for genetic evaluation of NTC resistant cells are depicted and numbered indicating approximated localization within the construct or within parasite genomic *ssu* integration sites (dashed lines). B. Relevant fragments of Sanger sequencing chromatograms visualizing the vicinity of nucleotide position in CDS of human *ABCB6* gene resulting in K629M amino acid mutation. C. Diagnostic PCR products derived from genomic DNA of transgenic strains used as template. Both LEXSY-ABCB6 strains, having the human ABCB6 cDNA integrated in their genome, give a ~1.1 kbp product with ABCB6-specific primers (pair 1). The empty vector control strain contains an empty expression cassette integrated into the same *ssu* locus (pair 2) but has only the NTC resistance gene (*sat*, pair 3) and a noncoding 1 kbp stuffer DNA fragment in place of the ABCB6 sequence. M: 1 kbp DNA ladder marker.

Goettingen, Germany) which stains only membranes accessible from the outside. Following 20 min fixation (4% PFA, 0.2% glutaraldehyde, PBS) at 4 °C, cells were incubated overnight with blocking buffer (1% BSA, 0.1% Triton X-100, PBS). ABCB6 was then immunodetected by subsequent 1 h incubations in blocking buffer with anti-ABCB6 antibody (1:100) and secondary, Alexa Fluor 555-conjugated antibody (1:1000). Cells were imaged using 780 LSM confocal microscope (Zeiss; Oberkochen, Germany) with Plan-Apochromat 63×/1.4 Oil DICM27 objective. Zeiss Microscope Software ZEN2012 was used to calculate co-localization between ABCB6 signal and plasma membrane signal.

2.5. ATPase and transport activity measurement

Basal ATPase activity of crude membrane fractions was measured by kinetics of inorganic phosphate release. Reaction was performed at 37 °C in buffer P (100 mM Tris–HCl buffer pH = 7.4, 85 mM NaCl, 10 mM $MgCl_2$, 10 mM KCl, 0.1 mM EGTA and 2.5 mM ATP) and 20 μg of membrane preparation in 360 μl was used for each reaction. At 0, 5, 10 and 15 min time points, 50 μl aliquots were transferred to ice-cold 0.6 M TCA and centrifuged at 14,000 ×*g* for 10 min. Concentration of inorganic phosphate was determined according to the improved malachite green assay [26], adapted for use in 96-well plates. Supernatant samples of 40 μL were placed in 96-well plates and 160 μL of water was added, followed by the addition of 40 μL of malachite green solution (0.9 mM malachite green oxalate in 0.35% PVA solution). After a 10 min incubation at room temperature, 40 μL of ammonium heptamolybdate (14 mM in 3.15 M H_2SO_4) was added and absorbance at 610 nm was read after further 30 min of incubation. Phosphate concentration was taken from a calibration curve made using 0–10 μM of KH_2PO_4 as a standard. The enzyme activity was expressed in nmol of phosphate released per mg of protein per minute.

In chlorin e6 accumulation assay, 5 μl of 10 mM chlorin e6 in DMSO was added to 1 ml of transgenic *L. tarentolae* cells (final concentration 50 μM) in their logarithmic growth phase (~8×10^7 cells) and cells were incubated at 26 °C or 37 °C for 1 h. After that time, samples were put on ice, washed two times with ice cold PBS and lysed (small aliquots were withdrawn for confocal imaging before lysis) with 0.5% Triton X-100 in PBS. Cell debris were removed with centrifugation (14,000 ×*g*, 10 min) and concentration of chlorin e6 in supernatant was quantified by its fluorescence (Ex = 405 nm, Em = 665 nm) using a calibration curve of chlorin e6 in 0.5% Triton X-100/PBS at concentrations 0-10 μM. Assay was repeated three times and chlorin e6 concentration in each lysate was normalized using protein concentration measured with 660 nm Protein Assay Reagent.

2.6. Statistical analysis

All pairwise comparisons of arithmetic means were performed using Student's two-tailed *t*-test. Data were presented as mean ± S.E.M. and asterisks were used to denote statistical significance of differences, with n and *p* values given in figure legends.

3. Results

3.1. Overexpression of human ABCB6 in *L. tarentolae*

We used the commercial pLEXSY-sat2 vector (containing a Ssu integration sequence for integration into ribosomal DNA and transcription from the rDNA promoter as well as a nourseothricin resistance selection marker) to generate human ABCB6-expressing *L. tarentolae* strains. We constructed the pLEXSY-sat2-ABCB6 expression vector (Fig. 1A) and the pLEXSY-sat2-ABCB6 (K629M) expression vector (Fig. 1B), verified their integrity by full Sanger sequencing of the inserted cDNA and its flanking sequences, and introduced them by electroporation into the P10 *L. tarentolae* basal strain which is optimal for heterologous protein expression. After generation of nourseothricin-resistant *L. tarentolae* strains, we confirmed the successful integration of the pLEXSY-sat2-ABCB6 expression cassette by diagnostic PCR of genomic DNA in comparison to a

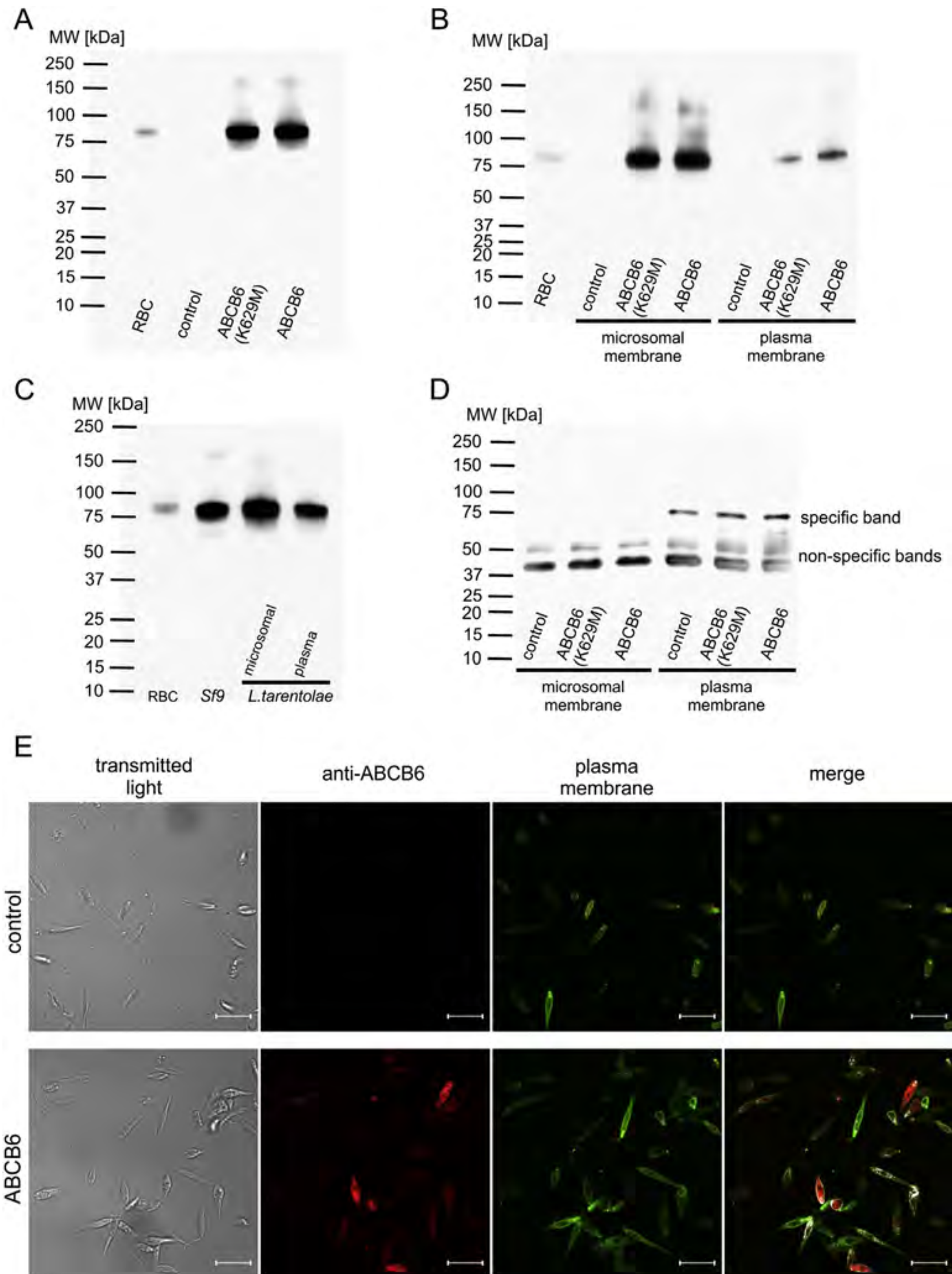

Fig. 2. Heterologous expression of human ABCB6 in *L. tarentolae*. A, B, C. Western blotting of ABCB6-expressing samples with anti-ABCB6 antibody. 5 μg of normal red blood cell (RBC) membranes lysate was used as a positive control. A. Western blotting of total cell lysates (10 μg of protein per lane) of all transgenic strains. B. LEXSY-control, LEXSY-ABCB6 (K629M) and LEXSY-ABCB6 cells were lysed and proteins were fractionated (see Materials and methods) resulting in cytoplasmic as well as microsomal and plasma membrane fractions. 2 μg of the membrane fraction samples were analysed by Western blotting. C. LEXSY-ABCB6 membrane fraction samples and Sf9-ABCB6 membrane samples (2 μg per lane) were analysed by Western blotting. D. Western blotting of membrane samples (2 μg of protein per lane) of all transgenic strains with anti-Gp63 antibody, only the upper band of ca. 75 kDa (labelled as specific) corresponds to *L. tarentolae* plasma membrane metalloproteinase. E. Immunofluorescence staining of human ABCB6 protein in transgenic *L. tarentolae* strains (red channel). Cellular membranes accessible from the outside were stained with mCLING-ATTO488 dye and are shown in green. In merged images, white pixels represent colocalizing regions (positive/above threshold in both red and green channels). Scale bars: 10 μm.

control (empty pLEXSY-sat2 vector-transfected) strain generated in parallel (designated LEXSY-control; Fig. 1C). For further experiments, the fastest-growing ABCB6 coding sequence-containing strains were used, designated LEXSY-ABCB6 and LEXSY-ABCB6 (K629M).

We confirmed the efficient transcription of both variants of human ABCB6-coding sequence by detecting cognate ABCB6 mRNA in LEXSY-ABCB6 *L. tarentolae* cells by real-time PCR (data not shown). Furthermore, we set out to confirm the efficient expression of ABCB6 in these strains at the protein level, since successful expression of mammalian membrane proteins in lower eukaryotes is sometimes difficult to achieve. In total cell lysates, a band corresponding to the expected molecular weight of ca. 90 kDa was detected by Western blotting with anti-ABCB6 antibodies in LEXSY-ABCB6 and LEXSY-ABCB6 (K629M) strains, but not in LEXSY-control (Fig. 2A). The detected molecular weight corresponded very well to a band detected in the positive control — human erythrocyte membranes known to contain ABCB6. To confirm and further pinpoint the intracellular localization of ABCB6 as a membrane protein, we performed fractionation of *L. tarentolae* cells with subsequent immunodetection by Western blotting. The resulting signal was strong in microsomal membranes and weaker in plasma membrane fraction (Fig. 2B). In order to provide a direct quantitative comparison to other, more conventional expression systems, we used a total membrane preparation from insect cells (Sf9 cell line) overexpressing human ABCB6 from a baculoviral vector. We compared the expressed protein yield by Western blotting of samples with equal total protein content (Fig. 2C) and subsequent quantification of band intensity using densitometric analysis. In comparison to the Sf9-ABCB6 membranes, LEXSY-ABCB6 plasma membranes expressed ca. 22% less ABCB6, while LEXSY-ABCB6 microsomal membranes expressed ca. 27% more.

The subcellular localization of overexpressed ABCB6 to both types of membrane fractions was confirmed by proving the efficient separation of plasma from microsomal membranes during fractionation. We used Western blotting against Gp63, a plasma membrane metalloproteinase, to confirm that our microsomal membrane preparation did not contain plasma membrane impurities (Fig. 2D). We further confirmed this subcellular localization by immunofluorescence: while no staining at all was seen in LEXSY-control, LEXSY-ABCB6 cells demonstrated diffuse staining, with highly variable intensity between individuals (Fig. 2E). When using a fixable dye specific for biological membranes accessible from the outside (in the case of *Leishmania*, plasma membrane and flagellar pocket), we were able to show significant colocalization between ABCB6 signal and external membrane signal, calculated on the basis of several fields of view to be 20.8% $\pm$ 0.6% (white pixels in Fig. 2E). The remaining ABCB6 signal was located intracellularly in perinuclear locations consistent with intracellular organelle membranes.

3.2. Functional analysis of human ABCB6 overexpressed in L. tarentolae

Since the physiological function and substrates of human ABCB6 is a matter of substantial controversy in the literature, we selected two potential activities linked to functional ABCB6 that we considered most relevant for biochemical validation: ATPase activity and porphyrin transporter activity. Whereas we demonstrated membrane localization for the heterologously expressed hABCB6 in *L. tarentolae*, we performed ATPase assays on crude membrane preparations isolated according to the same protocols used for immunodetection. As expected, in both types of membrane preparation (microsomal/intracellular membranes and plasma membrane), the basal magnesium-dependent ATPase activity (corresponding to endogenous membrane ATPases as well as heterologous ABCB6 activity that is substrate-independent or stimulated by endogenous substrates) was significantly increased in *Leishmania* cells expressing hABCB6 in comparison to control cells (Fig. 3). Crucially, this increase in ATPase activity was absent from both membrane fractions isolated from the LEXSY-ABCB6 (K629M) strain, i.e. the mutant designed to be catalytically inactive does indeed show no ATPase activity. This increase was smaller in plasma membranes, but larger in the

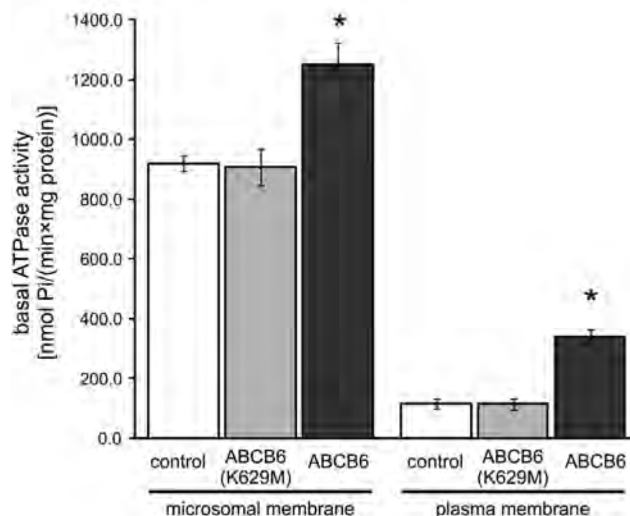

Fig. 3. Basal ATPase activity of two membrane fractions of transgenic *L. tarentolae* strains. ATPase activity of membrane fractions isolated from cellular lysates of LEXSY-control, LEXSY-ABCB6 (K629M) and LEXSY-ABCB6 strains was measured using the malachite green method, and the activity was expressed as amount of inorganic phosphate liberated by 1 mg of membrane protein per minute. Asterisks denote statistically significant difference from the respective control, $n = 7$, $p < 0.001$.

microsomal fraction, in line with results concerning subcellular localization of the heterologous protein. While this ATPase activity corresponds to substrate-independent activity of hABCB6 and/or to its activity stimulated by an endogenous substrate, we were unable to identify the potential substrate, the transport of which is driving this activity, or to further stimulate it with potential ABCB6 substrates known from literature (data not shown).

The least controversial putative physiological activity of ABCB6 in mammalian cells relates to transport of endogenous porphyrins and porphyrin derivative xenobiotics. Thus, we tested the impact of heterologous expression of hABCB6 in *L. tarentolae* cells on the cellular content of exogenously added chlorin e6, a porphyrin-like tetrapyrrole compound of plant origin. This compound has been identified as a potential ABCB6 substrate, and the rationale for our experiment was that presence of an active transporter in cellular membranes will alter the equilibrium distribution of chlorin (which is hydrophobic and can diffuse through biological membranes passively) in the cytoplasm and in intracellular compartments. Indeed, while at 26 °C, when human ABCB6 should be catalytically inactive for thermodynamic reasons, we observed no difference between the control an ABCB6-expressing strains in total chlorin content after 1 h of incubation, the same incubation at 37 °C (physiological temperature for ABCB6) leads to the appearance of a significant difference between strains: the ABCB6 strain accumulates significantly more chlorin than the control one (Fig. 4A). The difference of basal chlorin e6 accumulation in two studied temperatures is most probably a direct result of increased membrane fluidity at 37 °C (since membrane fluidity strongly influences the passive diffusion rate). Again, as for ATPase measurements, the LEXSY-ABCB6 (K629M) strain overexpressing the catalytically inactive form of the transporter protein is indistinguishable from the control strain. Microscopic observation showed that this accumulation in LEXSY-ABCB6 cells is intracellular rather than linked with plasma membranes (Fig. 4B), suggesting that it corresponds to chlorin transport (by ABCB6 expressed in intracellular compartments) into the lumen of these compartments.

4. Discussion

Human ABCB6 is an interesting member of the ABC superfamily: first identified as a member of group of “mitochondrial half-transporters” that included ABCB6, 7, 8 and 10 [27]. It subsequently became the

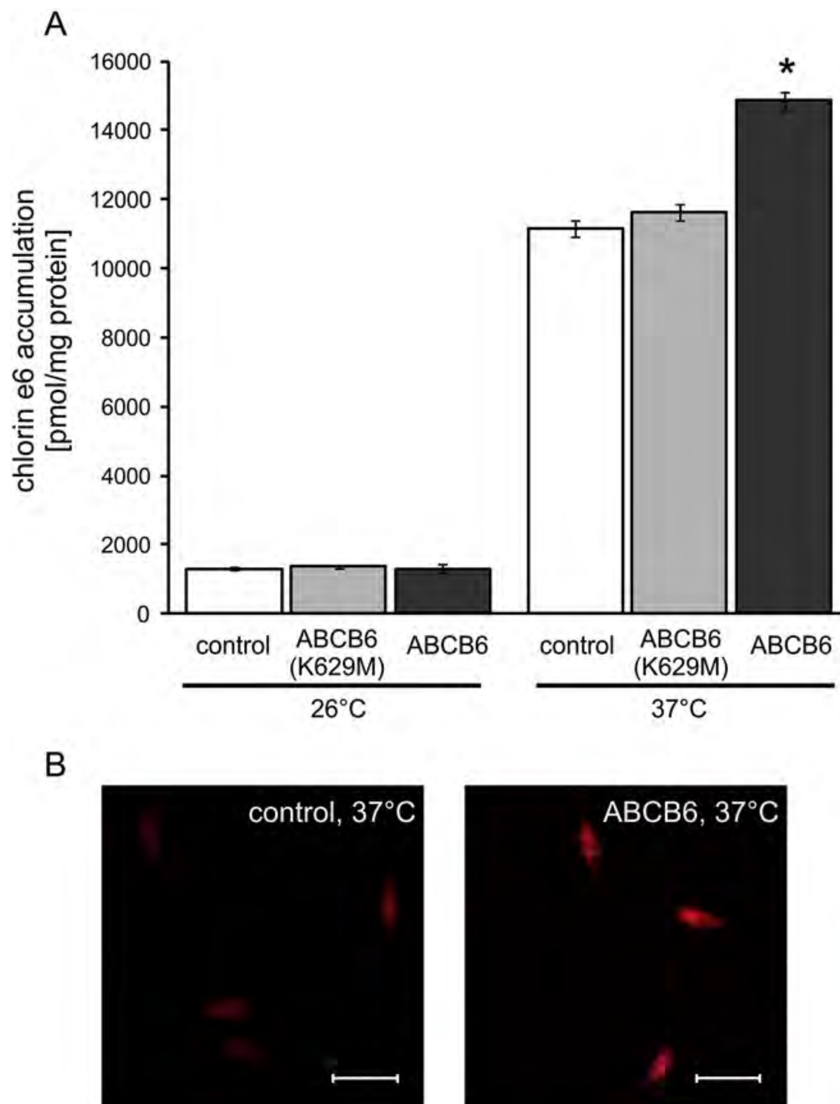

Fig. 4. Transport activity of ABCB6 using chlorin e6 as a substrate. A. Concentration of chlorin e6 in lysates of transgenic *L. tarentolae* after 1 h of chlorin e6 treatment in two temperature conditions: optimal for parasite growth (26 °C) and physiological for human ABCB6 transport activity (37 °C). Asterisks denote statistically significant difference from the respective control, $n = 6$, $p < 0.001$. B. Representative confocal images of LEXSY-control and LEXSY-ABCB6 cells after 1 h incubation with chlorin e6 at 37 °C. Scale bars: 20 μm.

subject of controversy concerning its actual subcellular localization, with extramitochondrial locations (including the plasma membrane) postulated as most relevant to its physiological function [4,7,28]. Moreover, the identity of its physiological substrate, although accepted by most researchers to involve porphyrins or porphyrin-like molecules [5,29], is not entirely clear and is difficult to study due to the hydrophobic character of candidate compounds. Nevertheless, it is clear that human ABCB6 participates in and influences many physiological and pharmacological phenomena on cellular and tissue level: mutations in the ABCB6 gene have been implicated in hereditary forms of coloboma [11], dyschromatosis universalis [12] and pseudohyperkalemia [13]. It has also been suggested that ABCB6 activity towards porphyrins impacts heme metabolism [29], hematopoiesis [30], photodynamic therapy [31] and parasite killing in macrophages [32]. Therefore, a heterologous expression system with correctly folded human ABCB6 expressed in a eukaryotic host with glycosylation patterns and membrane lipid composition similar to mammalian ones would greatly help the complicated task of unraveling the molecular questions of ABCB6 biochemistry. Hitherto, the controversy over site of expression in mammalian cells of various origin and the difficulty in direct biochemical studies has hindered research on its structure, kinetics and substrate specificity. While it is known that human ABCB6 is glycosylated in vivo and this modification has a potential role in protein trafficking and stability [33], studies on the potential role of glycosylation in functional activity have been hindered by the lack of suitable models: e.g. Sf9 cells produce only core-glycosylated ABCB6 [20].

We have succeeded in expressing human ABCB6 in *L. tarentolae*, an attractive heterologous expression host with many mammalian-like features [14]. Our studies show that ABCB6 is expressed in host cell membranes, with the majority retained in intracellular organelle membranes and a significant minority expressed in the plasma membrane. While membrane protein trafficking mechanisms in *Leishmania* are insufficiently studied and their relation to mammalian systems is tentative, it is tempting to speculate that this subcellular distribution is to an extent analogous to that observed in some human cell types. Still, we do not postulate that *L. tarentolae* cells with human ABCB6 may directly model the physiological function of ABCB6 in human cells — a deeper understanding of this function would be necessary to draw such inferences. We have rather generated and characterized this strain as a cheap, abundant source of active protein for biochemical studies.

In order to prove the suitability of *Leishmania*-expressed ABCB6 for further research, we focused on the least controversial aspects of its

function as an ABC transporter: ATPase activity and porphyrin transport. We were able to demonstrate that ABCB6 overexpression modulates both of these biochemical features in *L. tarentolae*, increasing unstimulated membrane ATPase activity and capability for chlorin (a porphyrin congener) accumulation. Even more convincingly, overexpression of the active site lysine mutant ABCB6 (Walker A mutation, previously shown to be catalytically inactive [4]) did not confer any of the phenotypical changes, even though it was expressed in analogous amount and intracellular locations, corroborating the requirement for expression of both correctly folded and active ABCB6. While we did not purify the protein further to demonstrate that both of these activities are directly mediated by ABCB6 molecules, we believe that the overall consistence of subcellular distribution of effects adds further credence to this postulate: not only do ABCB6-expressing cells differ in the expected manner from control ones, but within the overexpressing cells these activities are concentrated in intracellular compartments, where we have shown that human ABCB6 expression is stronger than in the plasma membrane. Thus, the increased chlorin content in the ABCB6-expressing strain is in line with expected direction of ABCB6-mediated transport: ABCB6, as most ABC transporters, functions as an exporter [20], so while plasma membrane ABCB6 potentially removes chlorin from the cytoplasm, the much more abundant microsomal ABCB6 transfers chlorin into the lumen of intracellular vesicular structures and concentrates it there.

The question of ABCB6 ATPase activity is complex and slightly controversial in the literature: while some researchers were able to show both basal and stimulated activity in an overexpression system [6], others have not been able to demonstrate either [20]. In our case, presence of basal activity and absence of stimulated activity may be explained e.g. by lack of putative necessary co-factors in the membrane preparation or by the "basal" activity being already saturated by an endogenous substrate. Indeed, Chavan et al. [6] have shown that purified ABCB6 ATPase activity can be stimulated by hemin, a compound that is present in our study in the *L. tarentolae* culture medium and may be insufficiently removed upon purification. To wit, the specific basal ATPase activity reported in that study for purified or reconstituted ABCB6 at the ATP concentration used by us (ca. 350–450 nmol Pi/min/mg protein at 2.5 mM ATP) is only slightly higher than that measured in the present study as difference between the basal activities of *L. tarentolae* membranes expressing and not expressing ABCB6 (ca. 300 nmol Pi/min/mg protein), suggesting a common source for both activities. Therefore, such high specific activity in an impure protein preparation hints at additional activation stimuli in our experimental setup.

Since ABCB6 is a relatively large membrane protein (with 11 transmembrane domains), its heterologous expression in a correctly folded and post-translationally modified, active conformation is non-trivial. Establishment of such models has been one of main directions of scientific activity in ABC transporter research for decades, with most activity concentrating on earlier-characterized proteins relevant to multidrug resistance (Pgp, the ABCC family). Less "mainstream" proteins such as ABCB6 have been relatively neglected. Within a broad study including many other ABC transporters, the establishment of a strain of *P. pastoris* expressing human ABCB6 was reported [19], but this work does not include any validation of its function, location or even immunoreactivity. In a study on ABCB6 biochemistry, a homologous expression model in HEK293 cells was established and tailored to cell biology functional studies in this particular cell background [6]. This model is reliable and physiologically relevant, but very non-cost-effective if it were to be used for direct biochemical assays. A more relevant heterologous expression system that was used in some biochemical studies [20] is the baculovirus-insect cell system. For this reason, we selected this system for a quantitative comparison: with regard to protein yield, our system performs marginally better.

Therefore, we consider our novel model to be a helpful, innovative tool for studying various aspects of ABCB6 biochemistry. *L. tarentolae* is a versatile host, with far cheaper and faster culture than mammalian or insect cells; its cells are large enough for direct microscopy in live or fixed cells, while the cultures grow to high densities, facilitating bulk biochemical studies or biochemical isolation procedures [14]. A relative drawback is the labor-intensive and lengthy process of establishing and validating a stable modified strain, but once such strain is established and characterized, as we describe here, it remains highly stable and cheap to propagate. An often-mentioned advantage of *Leishmania* is its propensity for mammalian-like post-translational modifications, especially glycosylation [34]. While it is not known whether such modifications are important for ABCB6 function, our results suggest that we were able to obtain a physiologically comparable level of necessary modifications: the molecular weight of *Leishmania*-expressed protein is indistinguishable from ABCB6 from human erythrocyte membranes, and functional assays yield predicted results for functional protein. A further significant advantage is the lack of a cell wall (present in most microbial expression hosts), making membrane isolation much easier and less time-consuming. In summary, we consider our establishment of a reliable, well-characterized heterologous expression model for functional human ABCB6 of significant interest to the membrane transport research community, as it is a proof of concept for other difficult-to-characterize membrane transporters.

Transparency document

The Transparency document associated with this article can be found, in online version.

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