Shifting the Paradigm: The Putative Mitochondrial Protein ABCB6 Resides in the Lysosomes of Cells and in the Plasma Membrane of Erythrocytes

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Abstract

ABCB6, a member of the adenosine triphosphate–binding cassette (ABC) transporter family, has been proposed to be responsible for the mitochondrial uptake of porphyrins. Here we show that ABCB6 is a glycoprotein present in the membrane of mature erythrocytes and in exosomes released from reticulocytes during the final steps of erythroid maturation. Consistent with its presence in exosomes, endogenous ABCB6 is localized to the endo/lysosomal compartment, and is absent from the mitochondria of cells. Knock-down studies demonstrate that ABCB6 function is not required for de novo heme biosynthesis in differentiating K562 cells, excluding this ABC transporter as a key regulator of porphyrin synthesis. We confirm the mitochondrial localization of ABCB7, ABCB8 and ABCB10, suggesting that only three ABC transporters should be classified as mitochondrial proteins. Taken together, our results challenge the current paradigm linking the expression and function of ABCB6 to mitochondria.

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Introduction

ATP-binding cassette (ABC) transporters comprise a superfamily of membrane spanning multidomain proteins. ABC proteins are located in the membrane compartment of the cells, where they mediate translocation of various molecules across these barriers. The functional significance of ABC transporters is suggested by the evolutionary conservation of this protein superfamily. Based on sequence homology, 48 different ABC proteins (grouped into seven subfamilies ranging from A to G) have been defined in the human genome. Most human ABC proteins function as efflux pumps, translocating their substrates into the extracellular space or intracellular organelles. Human ABC transporters may be expressed in the plasma membranes of the cells, and also in intracellular membrane compartments including the endoplasmic reticulum (ER), lysosomes, peroxisomes and mitochondria. ABCB6 belongs to the B (MDR/TAP) sub-family of ATP-binding cassette transporters. The B subfamily consists of 11 members, with functions ranging from the translocation of phosphatidylcholine (ABCB4-MDR3) to secretion of bile acids (ABCB11) or the protection of cells against xenobiotics (ABCB1-MDR1/Pgp). The ABCB subfamily contains seven half transporters, of which four (ABCB6, ABCB7, ABCB8 and ABCB10) are believed to reside in the mitochondria. ABCB2 and ABCB3 (TAP1–2) act as heterodimers mediating the translocation of immunogenic peptides from the cytosol into the endoplasmic reticulum, ABCB9 has been localized to the lysosomes [1–2], and the cellular localization of ABCB5 remains to be clarified.

The exact physiological function of mitochondrial ABC transporters is not known. Presumably, they play a role in the transport of solutes across mitochondrial membranes (thus regulating the intramitochondrial milieu), or in the communication of signals transmitted from the mitochondria to the cell. The inner mitochondrial membrane ABC transporters most likely act as exporters: mutations of ABCB7 result in mitochondrial iron overload [3] (although the exact nature of the transported substrate remains elusive), and ABCB8 is also believed to be involved in mitochondrial iron export [4]. The high expression of ABCB10 in tissues related to hematopoiesis suggests a role in erythroid differentiation, with a function presumably contributing to the tight regulation of mitochondrial iron acquisition and heme synthesis [5].

The first report describing ABCB6 function was based on the investigation of a Saccharomyces cerevisiae mutant strain lacking the mitochondrial ABC transporter Atm1p [6]. Atm1p knockout cells accumulate high mitochondrial iron levels and are more sensitive to oxidative stress [7,8], iron-starvation [9] and heavy metal toxicity [10]. Based on the analysis of this complex phenotype, Atm1p is believed to transport a yet unknown substrate that has an important role in the biogenesis of cytosolic/nuclear iron-sulfur proteins as well as in regulating the overall mitochondrial iron homeostasis.
ABC6 is Expressed as a Full Length Glycoprotein in RBCs

ABC6 is highly expressed in fetal liver, and ABC6 protein levels were reported to increase coordinately with globin levels during the induced differentiation of proerythroblast cell lines [14]. To expand the scope of our analysis from cancer cell lines to models of higher physiological relevance, we analyzed terminally differentiated red blood cells. As shown in Figure 3A, Western blot analysis indicates that ABC6 is expressed in mature erythrocytes; immunofluorescence analysis of fresh human blood using two different anti-ABC6 antibodies confirmed this result (Figure S3). Following treatment of erythrocyte ghosts with peptide N-glycosidase F (PNGaseF), a clear shift of the apparent molecular weight is observed, indicating that ABC6 is expressed as an N-glycosylated protein in the plasma membrane of mature erythrocytes (Figure 3B). According to the conventional wisdom, mature red blood cells lack intracellular organelles. In fact, red blood cells are completely devoid of mitochondria; HEK cells expressing endogenous ABC6 gave identical results (Figure S1). To investigate whether the reported mitochondrial localization of ABC6 may be explained by an artefact linked to overexpression systems used in those studies [14,16,17,21], we transfected HeLa cells with a construct encoding a Flag-tagged ABC6 variant. This experimental approach also resulted in an endolysosomal expression pattern that was clearly distinct from the mitochondrial expression of ABCB7, ABCB9 or ABCB10 (Figure 2B and Table S1). Interestingly, at very high expression levels, ABC6 was also found in the plasma membrane, whereas under the same experimental conditions the canonical mitochondrial ABC transporters remained confined to the mitochondria (Figure S2).

ABC6 Is Dispensable for Erythropoiesis

ABCB6 is highly expressed in fetal liver, and ABC6 protein levels were reported to increase coordinately with globin levels during the induced differentiation of proerythroblast cell lines [14]. To expand the scope of our analysis from cancer cell lines to models of higher physiological relevance, we analyzed terminally differentiated red blood cells. As shown in Figure 3A, Western blot analysis indicates that ABC6 is expressed in mature erythrocytes; immunofluorescence analysis of fresh human blood using two different anti-ABC6 antibodies confirmed this result (Figure S3). Following treatment of erythrocyte ghosts with peptide N-glycosidase F (PNGaseF), a clear shift of the apparent molecular weight is observed, indicating that ABC6 is expressed as an N-glycosylated protein in the plasma membrane of mature erythrocytes (Figure 3B). According to the conventional wisdom, mature red blood cells lack intracellular organelles. In fact, red blood cells are completely devoid of mitochondria; since mitochondria are removed in the final stages of erythroid differentiation [31]. Thus, the presence of a glycosylated ABC6 in mature erythrocytes is inconsistent with the presumed mitochondrial localization of this protein in erythrocyte precursors.
ABC6 is Released in Exosomes from the Reticulocytes

As erythrocytes differentiate from reticulocytes, we assessed the presence and fate of ABC6 in immature red blood cells. Mice were phlebotomized to increase their reticulocyte count in blood (up to 20%). Consistently, an increase in transferrin receptor (TfR) expression was displayed by circulating red blood cells without noticeable change in spectrin content (Figure S4A). Interestingly, RBCs from anemic mice presented much higher amounts of ABC6 compared to those of healthy animals, suggesting a loss of the ABC transporter during reticulocyte maturation into erythrocyte. Such loss of specific proteins during reticulocyte maturation has been shown to occur through their association with exosomes. Exosomes correspond to intraluminal vesicles of internal compartments called multivesicular endosomes (MVE), secreted in the extracellular medium upon fusion of MVE with the plasma membrane [32]. Thus, reticulocytes from mice treated with phenylhydrazine were differentiated in vitro for 48 h and the secreted exosomes were collected from the maturation medium. The TfR and proteins classically taken as exosomal markers (Alix, Tsg101, flo18) were found in the released vesicles. Remarkably, ABC6 was also detected in the secreted exosomes and consistently the amount of cell-associated transporter decreased during in vitro maturation period, as in the case of the TfR (Figure S4B). Conversely, B-spectrin, which is not lost during reticulocyte maturation, was not detected in exosomes [33]. Moreover, the absence of porin in the exosomal fraction rules out a contamination by mitochondria exocytosed during reticulocyte differentiation [31]. Accordingly, after reticulocyte fractionation, ABC6 was recovered in plasma membrane, endosome and exosome subfractions (not shown).

Human RBCs from reticulocyte-enriched blood was similarly used for in vitro maturation (Figure 4A). Correspondingly, the vesicles containing the exosomal markers (and not spectrin) were enriched with TfR and ABC6. Note that the exosomal enrichment of the two cargo proteins is especially noticeable due to the low reticulocyte count of human RBCs, accentuating the final TfR and ABC6 concentration in exosomal membrane compared to RBCs plasma membrane. To confirm ABC6 association with the vesicles, exosomes collected after reticulocyte maturation were fractionated by flotation on sucrose gradient and analyzed for the presence of TfR, Tsg101 and ABC6 by Western blot (Figure S5). TfR and Tsg101 were mainly detected in fractions 5 to 8, corresponding to densities between 1.11 and 1.20 g/mL, in agreement with exosome characteristics [34]. Outprisingly, ABC6 was distributed in the same fractions, attesting its exosome association. Immunofluorescence study was carried out on human RBCs and confirmed the higher expression of ABC6 on TfR-positive reticulocytes (Figure 4B).

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**Discussion**

Of the ~1500 proteins identified in the erythrocytes, proteomic studies have confirmed the presence of several ABC transporters,
ABCB6 Is Dispensable for Erythropoiesis

A

B

Flag

CoxIV

merged

ABCB7

ABCB8

ABCB10

ABCB6
ABC6 Is Dispensable for Erythropoiesis

suggested that the mature erythrocyte membrane is a major repository of ABC proteins [36]. Here we show that ABCB6, a protein currently assigned to mitochondria in the major protein databases [37], is abundantly expressed in the erythrocyte membrane. Our findings expand red blood cell proteomic data that include ABCB6 fragments [38–40]. We show that ABCB6 is expressed in its full length, glycosylated form, suggesting that the plasma membrane localization in red blood cells does not rely on erythrocyte-specific posttranslational modifications. The presence of ABCB6 in red blood cells may seem logical in view of the increased expression of ABCB6 in erythrocyte differentiation models (Figure 5A and [14]). However, since mature erythrocytes do not contain intracellular membrane compartments, plasma membrane localization of ABCB6 is inconsistent with the presumed mitochondrial localization of this protein in erythrocyte precursors. Intracellular organelles such as mitochondria and endosomes are lost during the final stage of differentiation through autophagy and secretion [41]. In fact, the red blood cell ghosts are negative for mitochondrial markers such as porin (Figure 3). We show that ABCB6 is at least partly evacuated through exosome secretion. Since exosomal cargo proteins must originate from the plasma membrane-endosomal continuum, this result provides further arguments against the presumed mitochondrial localization of ABCB6. By filtering components to be secreted vs. components to be recycled to the plasma membrane, exosome biogenesis also contributes to RBC plasma membrane remodeling. Similarly to the lysosomal protein LAMP2 [42], ABCB6 could be partly redistributed to the plasma membrane of mature erythrocytes during reticulocyte maturation by fusion of lysosomes with the plasma membrane [43]. The functional relevance of such “neolocalized” cell surface proteins in RBCs awaits further investigation.

At present, it is uncertain whether erythrocytic ABCB6 is actually a bioactive molecule or a vestigial heritage from an erythroid precursor. Similarly to ABCB7 and ABCB10, expression of ABCB6 was shown to increase upon DMSO-induced differentiation of mouse erythroleukemia (MEL) cells [44], suggesting that these proteins may all share an important role in the regulation of the heme synthetic pathway. While our data do not exclude the possibility that ABCB6 may play a role in heme metabolism under certain conditions that remain to be defined, the results presented in this paper suggest that it does not mediate direct mitochondrial uptake of porphyrins.

First, our morphological data show that ABCB6 is not identified in purified mitochondria. Second, we show that the endogenous and cDNA-derived human ABCB6 (NM_005689) is targeted to the endolysosomal compartment, unlike ABCB7, ABCB8 or ABCB10, which were found in the mitochondria. Third, ABCB6 is glycosylated, and to date only one mammalian glycoprotein has been described in mitochondria, although this number could be underestimated [45]. Finally, we show that differential expression of ABCB6 does not influence baseline or induced porphyrin levels in K562 cells.

It is well known that overexpression takes a toll on the processing of proteins; tags may also derail intracellular targeting. Whereas most localization data in the literature are based on the

Figure 2. Determination of the subcellular localization of ABCB6 by double immunofluorescence labeling and laser-scanning confocal microscopy. A. Expression of the endogenous ABCB6 protein in K562 cells was visualized by the monoclonal ABCB6-567 antibody (green), lysosomes and mitochondria were labeled with LAMP1 (yellow on the overlay, r = 0.79), but not with CoxIV (r = 0.14). B. Flag-tagged ABC proteins were expressed following transient transfection of HeLa cells. The cDNA-derived ABC transporters were visualized with an anti-FLAG tag antibody (green, left panel); mitochondria were labeled with CoxIV (red, middle panel). Whereas the canonical mitochondrial ABC transporters are confined to the mitochondria (merged image, right panel, r = 0.87, r = 0.7, r = 0.84 for ABCB7, ABCB8 and ABCB10, respectively), ABCB6 does not show colocalisation with the mitochondrial marker (merged image, right panel, r = 0.13).

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Figure 3. ABCB6 is expressed as a full length glycoprotein in mature erythrocytes. A. Red blood cell ghosts (60 ug total protein, lane 1) were solubilized and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After transfer to a PVDF membrane, Western blot analysis was performed using a panel of custom-made and commercially available anti-ABCB6 antibodies as well as anti-porin to exclude contamination by mitochondria. Membranes of K562 cells engineered to overexpress HA-tagged ABCB6 were loaded as a control (10 ug total protein, lane 2). B. Red blood cell ghosts and ABCB6 overexpressing K562 cell lysates were treated with PNGase F enzyme to remove N-glycans. 20 ug total protein was analyzed by SDS-PAGE, ABCB6 was visualized by the ABCB6-567 antibody.

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Figure 4. Human reticulocytes release ABCB6 in association with exosomes. A. 250 μL packed cells volume (PCV) of human RBCs from reticulocyte-enriched (4.5%) blood was cultured for 48 h and exosomes were collected from the medium as previously described in [42]. 0.5 μL PCV of RBCs before (t0) or after (48 h) maturation, as well as the completeness of exosomes were loaded on 10% SDS-PAGE for immunoblot analysis of the indicated proteins. The molecular mass (kDa) standards are indicated on the left. Note that the Tfr is not detected in RBCs due to the low reticulocyte count but revealed in exosomes due to its concentration in the vesicles. B. Human RBCs from reticulocyte-enriched (10%) blood were adsorbed on cover slips pretreated with poly-L-lysine, fixed 20 min by 1% PFA and immunostained for ABCB6 (antibody Ab 7474 at; dilution 1/50/Alexa Fluor 594; A21207) or and Tfr (MoAb H68.4 at; dilution 1/200/Alexa Fluor 488; A11029) after permeabilization, as described in the Materials and Methods. Coverslips were observed using a Leica confocal SPE and a Leica 63× ACS APO 1.33 objective. Note that none of the cells shown are positive to DAPI although contained in the mounting reagent. Arrows point to possible colocalization of ABCB6 and Tfr in MVE.

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Figure 5. Porphyrin synthesis and ABCB6 expression in K562 cells. A. ABCB6 protein expression is upregulated in parental K562 cells upon chemically induced erythroid differentiation. K562 cells were cultured for 72 h with 150 nM Gleevec or 50 μM hemin, the number of viable cells was determined by trypan-blue staining. Hemoglobin levels were quantified by benzidine staining. Error bars represent SD of at least 5 experiments. ABCB6 expression is revealed by the ABCB6-567 monoclonal antibody (60 μg total protein, 72 hours post-induction). Actin is shown as loading control. B. Baseline PPIX-fluorescence of K562 cells is not influenced by ABCB6 levels. PPIX fluorescence was measured by flow cytometry as explained in the Methods section, ABCB6 expression of the cells was revealed by the anti human ABCB6-567 monoclonal antibody (actin is shown as loading control). Error bars represent SD of at least 3 experiments. Parental K562 cells (lane 1); K562 cells stably transfected with a non-target control shRNA (lane 2) or ABCB6 shRNA construct (lane 3); K562 cells stably overexpressing wild-type ABCB6 (lane 4) or a non-functional mutant variant of ABCB6 (ABCB6KM, lane 5). C. Downregulation of ABCB6 does not impede induced heme synthesis of K562 cells. K562 cells stably transfected with an shRNA construct targeting ABCB6 or a scrambled control were cultured for 72 h with 150 nM imatinib or 50 μM hemin, hemoglobin levels were quantified by benzidine staining. Error bars represent SD of at least 5 experiments. doi:10.1371/journal.pone.0037378.g005
overexpression of tagged ABCB6 protein forms [6,14,16,17,21], we characterized the intracellular distribution of the endogenous protein, in three human cell lines of different origin, using complementary methods. We used a panel of custom-made and commercially available antibodies. The specificity of the antibodies was confirmed by immunoblotting and confocal microscopy studies of cell lines expressing tagged variants of ABCB6 (Figure S2, [17]). The major advantage of using antibodies to visualize the subcellular localization of proteins is that genetic constructs are not needed and that the possible artificial effects of protein fusions are avoided. However, while fusion proteins may enable monitoring of dynamic changes, antibodies detect only a snapshot of the dynamic process of protein distribution. Immunocytochemical labeling of the ABCB6 protein with the currently available antibodies requires fixed and permeabilized cells, limiting the study to dynamically fixed end points. Under these conditions, the distribution of the native ABCB6 primarily shows colocalisation with lysosomes, and less so with the Golgi/ER markers (Table S2), presumably because of the short half-life of ABCB6 in the latter organelles (glycosylation proves that ABCB6 transits through the ER-Golgi system [15,17]). Conversely, lack of mitochondrial localization, especially in the context of the observed expression pattern of ABCB7, ABCB8 and ABCB10 in the same experimental system indicates that ABCB6 does not reside in mitochondria.

K562 is a Philadelphia-positive erythroleukemia cell line that has been widely used to study induced hemoglobin production, even if this model does not fully recapitulate the complex physiology of terminal erythropoiesis [29]. In K562 cells, targeted inhibition of the oncogenic kinase initiates the entire differentiation program leading to enhanced de novo heme biosynthesis and ultimately to increased cellular hemoglobin levels [35]. In our hands, treatment with hemin or the Bcr-Abl kinase inhibitor Gleevec induced hemoglobin production and differentiation of K562 cells toward the erythroid lineage, which was accompanied by a significant increase of ABCB6 protein levels. However, overproduction or downregulation of ABCB6 did not result in a shift of baseline PPIX levels, and stable silencing by inducible shRNA did not prevent differentiation-induced hemoglobin upregulation of K562 cells. Notably, in a similar experimental setup, silencing of ABCB10 interfered with the ability of K562 cells to undergo induced differentiation [46].

Careful analysis of the literature provides additional support to our conclusions. A recent publication suggests that ABCB6 can be silenced without a consequent decrease of cellular PPIX levels [47]. Interestingly, abcb6-KO mice are viable and appear to be hematologically normal, suggesting that ABCB6 is dispensable [Jill Paterson, Gergely Szakacs, Michael M. Gottesman, unpublished data]. While this manuscript was in preparation, ABCB6 was also shown to be dispensable for erythropoiesis in humans [48]. Heterologous expression of rat Abcb6 in a human adenocarcinoma cell line resulted in a subcellular localization confined to the endo/lysosomal compartment [21]. ABCB6 was also found to be expressed in the plasma membrane [17]. While we don’t see evidence of the plasma membrane expression of ABCB6 in the cell lines analyzed in this study, we note that at high expression levels, cDNA-derived ABCB6 appears in the plasma membrane of HeLa cells. The continuum of the endolysosomal and plasma membrane compartments, as well as the abundance of ABCB6 in red cells adds further relevance of this finding. Data assembled by recent proteomic efforts contain reference to lysosomal localization [20]. Lysosomes are organelles of eukaryotic cells that are critically involved in the degradation of macromolecules mainly delivered by endocytosis and autophagy. The lysosomal membrane facilitates interaction and fusion with other compartments and harbors transport proteins catalyzing the export of catabolites, thereby allowing their recycling [49]. Given the ATP-dependence of ABC transporters, the ATP-binding domain of ABCB6 is expected to face the cytoplasm, predicting ABCB6 to be a lysosomal importer. In addition to Atm1p, ABCB6 is also homologous to the heavy metal tolerance factor 1 (HMT1) of Caenorhabditis elegans and Schizosaccharomyces pombe. HMT1 is a half transporter that confers tolerance to heavy metal toxicity in Schizosaccharomyces pombe [50] and tolerance to cadmium in Caenorhabditis elegans [51]. Notably, HMT1 is a vacuolar protein, which protects cells against metal toxicity by promoting the sequestration of metal adducts into the vacuolar interior. Interestingly, a possible role of ABCB6 in metal tolerance has been suggested in the literature. Direct correlation between arsinite resistance and ABCB6 expression was observed in various human and mouse cell lines [17,52]. Knockdown of ABCB6 expression sensitized HepG2 and Hep3B cells to arsinite toxicity, and stable overexpression of ABCB6 conferred a strong survival advantage towards arsenite-induced oxidative stress [53]. These results are consistent with the prediction that ABCB6, as a functional orthologue of HMT1, pumps transition metal complexes into the interior of acidic vesicles.

Further work is needed to elucidate the functional relevance of ABCB6 in the pathophysiology of erythroid cells. It is possible that ABCB6 offers protection of the erythrocyte by excreting heme degradation products. In addition to the erythrocytic membrane, the snapshots of our morphological studies locate ABCB6 in the endo-lysosomal compartment of cells. This compartment includes different membrane organelles that interact together through specific targeting mechanisms and fusion processes in order to degrade, sort and recycle material of different origin. Interestingly, ABCB6 and TIR are expressed in the same endosomal compartment in HeLa cells (not shown), which is consistent with a colocalisation in an intracellular compartment of the erythroid precursors such as the multivesicular endosome, bringing about exosome production. In the complex dynamics of a living cell, endosomes may transiently contact mitochondria as part of the organelle-mediated delivery of iron observed in reticulocytes [54,55,56]. Could this transient interaction account for the reported mitochondrial localization and presumed function of ABCB6 in cell lines? The findings presented in this paper do not support this notion. While we do not exclude the contribution of ABCB6 to porphyrin metabolism, our results clearly show that ABCB6 is unlikely to be directly responsible for the mitochondrial uptake of porphyrins. We also show that ABCB6 function is not required for de novo heme biosynthesis in differentiating K562 cells, excluding this ABC transporter as a key regulator of porphyrin synthesis. At the same time we confirm the mitochondrial localization of ABCB7, ABCB8 and ABCB10, suggesting that only three ABC transporters should be classified as mitochondrial proteins. Taken together, our results challenge the current paradigm linking the expression and function of ABCB6 to mitochondria. The identification of the subcellular localization of ABCB6 should pave the way for studies aimed at the elucidation of its true physiological function.

Materials and Methods

Ethics Statement

Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University Montpellier II. Reticulocyte-enriched blood (always >4%) was
obtained from patients with pathological states, such as hemolytic anemia, during routine follow-up visits to the “Hôpital St Eloi” (Montpellier, France), as approved by the Montpellier University Hospital’s ethics committee. Control blood was obtained from healthy volunteers, as approved by the Montpellier University Hospital’s ethics committee. All subjects gave their written informed consent to participate in the study, which was approved by the Montpellier University Hospital’s ethics committee.

**DNA Constructs**

cDNA encoding wild-type human ABCB6 (NM_005689) bearing an HA-tag at its C-terminus [17] was mutated by mutagenic PCR to generate a variant harboring mutation of a universally conserved lysine residue in the Walker A sequence (K629M). The HA-tagged ABCB6 and the non-functional ABCB6-variant (ABCB6HA and ABCB6HA-KM, respectively) were cloned into pBabe retroviral vectors (AddGene plasmid 1764). An untagged form of both the wild-type and the non-functional mutant variant of ABCB6 (K629M, ABCB6-KM) were cloned into pSEW lentiviral vectors. The pLKO.1-puro-3×HAeclectococcus indolcipoides shRNA vectors targeting human ABCB6 or expressing a nonsense hairpin were purchased from Sigma. For transient transfection, a C-terminally Flag-tagged ABCB6 construct was created by PCR mutagenesis and subcloning into a 3×FLAG-CMV-14 vector (Sigma-Aldrich). pCMV-ABCB7, pCMV-ABCB8 and pCMV-ABCB10, containing C-terminally Flag-tagged constructs were kind gifts from Jill Paterson.

**Cell Lines, Culture Conditions**

The K562 cell line (American Type Culture Collection) was kindly provided by Bela Papp, Inserm, Paris. Cells were grown in RPMI medium without nucleosides, supplemented with 10% (v/v) fetal bovine serum (Invitrogen Cat.No. 10106-169) and with 2 mM glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin (Invitrogen-Gibco, Carlsbad, CA, USA) at 37°C in humidified air/CO2 (19:1) atmosphere. ABCB6-HA and its non-functional KM mutant variant (ABCB6-HA KM) were expressed in K562 cells using 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 PG13 cells. The Phoenix-eco cell line [57] was a gift from G. Nolan (Department of Pharmacology, Stanford University, Stanford, CA); PG13 retrovirus producing cells were obtained from ATCC (Manassas, VA). For the generation of ABCB6 knock-down cell lines, lentiviral particles were produced by following the manufacturer’s instructions. To induce the expression of the shRNA constructs, IPTG (1 mM) was added to the cells for 48 hours before additional treatments. Untagged variants of ABCB6 were stably expressed in K562 cells by lentiviral transduction as described above, and transiently expressed in HeLa cells (Sigma) following the instructions of the FuGENE HD Transfection kit (Promega). Since we found that Mycoplasma infection significantly influences the baseline fluorescence of cells (thus interfering with measurements of protoporphyrin IX (PPIX) levels), cell lines were regularly screened with the Mycoplasma Detection Kit (Lonza), and the assays were carried out in mycoplasma-negative cells.

**Antibodies**

The following primary antibodies were used in Western blotting experiments: β-actin (A1978, Sigma-Aldrich); ABCB6-367, ABCB6-788 and ABCB6-7474 [17], ABCB6 (Sc98685, Santa Cruz); porin (A31855, Invitrogen); LAMP1 (611043, BD Biosciences); NaK-ATPase (BML-SA247-0100, Biomarker); CoxIV (A21348, Invitrogen); Lac-I (AP09404PU-S, Acris antibodies, Germany); BXP21; TRIF (H60.4, Invitrogen); Follistatin-1 (610820, BD Biosciences); Tsg 101 (4A10, Abcam); Alix (R. Sadoul, INSERM U836, Grenoble) and B-spectrin (N. Mohandas, NY Blood Center). HRP-conjugated secondary antibodies were purchased from Jackson Immuno Research Laboratories. The following primary antibodies were used for confocal microscopy: anti-Flag (F3165, Sigma); panCadherin (Ab16505, Abcam); giantin (Ab24586, Abcam); Lamp1 (L1418, Sigma); LAMP1 (611043, BD Bioscience); calnexin and CoxIV (Cellular Localization Antibody Kit, Cell Signaling, #4753). Fluorescently labeled secondary antibodies (goat anti-mouse IgGs conjugated with Alexa Fluor 488 and Alexa Fluor 594) were purchased from Invitrogen.

**Differentiation of K562 Cells, Measurement of Baseline PPIX Fluorescence**

K562 cells (2×10^5/mL) were treated with various drugs for 3 days. Cells were grown beyond the logarithmic growth phase (i.e. over 10^5 cells/mL) diluted to 2×10^5 cells/mL and treated with Gleevec immediately. For hemin treatments, the inducer was added to the diluted cultures after a four-hour-long lag period; hemin was solubilized as described [58]. Special attention was given not to disturb air/CO2 (19:1) atmosphere throughout the differentiation process. Viability was determined by trypan blue exclusion. Hemoglobin content of K562 cells was determined by the benzidine technique of Luftig [59] et al. with minor modifications: cells were lysed in a lysis buffer (50 mM Tris pH = 7.4; 250 mM NaCl; 2% Nonidet-P 40) at a density of 5×10^5/mL. The hemoglobin content of clear supernatants was determined as described previously [60]. PPIX fluorescence was measured using an Attune acoustic focusing cytometer (Applied Biosystems, Life Technologies Corp. USA) equipped with a 405 nm (violet) laser and a 640 nm longpass filter.

**Confocal Microscopy**

K562 cells were gently washed with Dulbecco’s modified phosphate-buffered saline (DPBS), fixed with 4% paraformaldehyde (PFA) in DPBS for 10 minutes at room temperature, dried on histopathological slides in drops, washed with DPBS, and permeabilized in methanol for 90 seconds at room temperature. The samples were blocked for 1 h at room temperature in DPBS containing 2 mg/mL BSA, 1% fish gelatin, 0.1% Triton-X 100 and 5% goat serum. The cells were then incubated for 1 h at room temperature with the primary antibody diluted in blocking buffer. After washing with DPBS, the cells were incubated for 1 hour at room temperature 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 minutes at room temperature. Wheat Germ Agglutinin was added to living cells before the immunostaining procedure and incubated for 10 min at 37°C. To label cellular organelles, the following dyes were used: MitoTracker Red CMXros dfixable dye (Invitrogen, M7512), LysoTracker Red DND-99 (Invitrogen, L7528), DAPI (4',6-diamidino-2-phenylindole, dithacamidolindole, D1306) and Alexa Fluor 633 conjugate of WGA (W21404). Samples were studied with an Olympus IX-81/FV500 laser scanning confocal microscope, using an Olympus PLAPO 60× (1.4 NA) oil immersion objective (Olympus Europa GmbH). Dual channel colocalization analysis was performed by the ImageJ software with
the Colocalization Threshold and Colocalization Test plugins. Dual channel colocalization analysis was performed by the ImageJ software with the Colocalization Threshold and Colocalization Test plugins. Averaged Manders’ and Pearson’s coefficients are reported for each subcellular marker from 5 random pictures manders [61–62].

Isolation of Erythrocytes and Reticulocytes

Human blood was freshly drawn into heparin from healthy volunteers. Cells were spun at 10000 rpm at 4°C for 10 min. The plasma and the buffy coat were removed by suction, and the red blood cells were washed three times with PBS. Reticulocyte-enriched blood (always >4%) was obtained from patients with pathological states, such as hemolytic anemia, during routine follow-up visits to the "Hôpital St Eloi" (Montpellier, France), as approved by the relevant institutional review boards. After centrifugation, the plasma and the buffy coat were removed, and the red blood cells were washed twice with Ringer solution. Anemia was induced in swiss-OF1 mice via two injections of an aqueous solution of phenylhydrazine hydrochloride (PHZ) (6 mg/mL) at the dose 60 mg/kg of on days 1 and 2. Mice were then sacrificed on day 6 by cardiac puncture under anesthesia. Alternatively, reticulocytosis was induced by phlebotomy. Blood (0.5 mL) was removed from retroorbital vein on days 0 and 2. On day 4, animals were sacrificed and blood was collected by cardiac puncture. After removing the buffy coat, red blood cells were washed twice with Ringer solution, and used for fractionation or in vivo maturation. For control experiments, erythrocytes were obtained from the blood of untreated mice.

Isolation of Organelle Membranes

Cellular fractions were isolated by differential centrifugation. Cells were washed with ice cold PBS and were resuspended in buffer A [10 mM NaCl, 1.5 mM MgCl2, 10 mM Tris (pH 7.4)] containing protease inhibitors (8 μg/mL aprotinine, 10 μg/mL leupeptine, 50 μg/mL PMSF) and 2 mM EDTA, swollen on ice, and disrupted with a Dounce homogenizer (150 strokes). Buffer B [525 mM mannitol, 175 mM sucrose, 12.5 mM Tris (pH 7.4), and 2.5 mM EDTA] was added in a ratio of 4:10 homogenate/buffer B (cell lysate: Figure 1A, lane 1). The nuclei and unbroken cells were removed by a low speed centrifugation (13000g) for 10 min at 4°C as described in [63] (Figure 1A, lane 2). This step was repeated until no pellet was visible. The clear supernatant was centrifuged at medium speed (8000g for 20 min) to remove cellular debris. The supernatant was further centrifuged at 12000g for 30 min. The resulting pellet was free of mitochondria (Figure 1A, lane 3) and the supernatant was further centrifuged at 12000g for 30 min. The resulting pellet was produced mitochondria (Figure 1A, lane 4). The supernatant was further centrifuged at 20000g for 45 min (Figure 1A, lane 5). The pellet of each separation step was dissolved in Laemmli buffer (0.05 M Tris-PO4 (pH 6.8), 2% SDS, 2% β-mercaptoethanol, 0.002 M EDTA (pH 6.8), 20% glycerol, 0.02% bromphenol blue in ultra pure water) and sonicated for 2 sec. Equal amounts (protein) were subjected to SDS PAGE analysis.

Mitochondria were isolated using Miltenyi’s Mitochondria Isolation Kit (cat.no: 130-094-532), with minor modifications of the protocol to increase the purity of the mitochondria. Briefly, 2 x 10^7 cells were washed with ice cold PBS and resuspended in 1 mL Lysis Buffer supplemented with 8 μg/ml aprotinine, 10 μg/mL leupeptine and 50 μg/mL PMSF (Sigma). Cells were broken in a Dounce homogenizer (150 strokes) and a 29G needle (10 strokes) (Figure 1B, lane 1). Nuclei and unbroken cells were removed with a low speed centrifugation step (5 min, 250×g, 4°C). 800 μL cell lysate was mixed with 6.3 mL of Separation Buffer and 30 μL anti-TOM22 MicroBeads was added to label mitochondria. The mixture was incubated for 60 min at 4°C in a tube rotator. The labeled cell lysate was transferred into a 4 mL polystyrene tube, which was placed into a magnetic separator column (EasySep, StemCell Technologies) for 10 minutes at 4°C. Following magnetic separation the supernatant was removed and the beads were washed with isolation buffer (3 mL). After 3 cycles of separation and washing, the solution containing labeled mitochondria was centrifuged at 12000g for 10 min, 4°C, the pellet was resuspended in Laemmli buffer (Figure 1B, lane 2). The supernatants from the washing steps were pooled and centrifuged at 10000g for 10 min; the resulting supernatant was centrifuged at 18000g for 30 min at 4°C, the pellet was subjected to SDS PAGE (Figure 1B, lane 3).

Reticulocyte Subcellular Fractionation

Mouse red blood cells were lysed by freezing/thawing as described previously [64]. The lysed cells were pelleted at 1500g for 5 min and the supernatant was centrifuged (30 000 rpm for 15 min in a Beckman TLA110 rotor) to pellet mitochondria and cell debris. An ultracentrifugation (70000 rpm for 2 h in a Beckman TLA110 rotor) finally allowed separating endosomes (pellet) from cytosol (supernatant). Endosomes were resuspended in PBS. Erythrocyte and reticulocyte plasma membranes were prepared according to Steck and Kant [65] with red blood cells from healthy and anemic mice, respectively. Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University Montpellier II.

Exosome Isolation

Red blood cells were cultured for 48 h at 37°C in RPMI 1640 supplemented with 5 mM glutamine, 5 mM adenosine, 10 mM inosine, 3% FCS, depleted for endogenous exosomes by overnight ultracentrifugation (100 000×g), 50 U/mL penicillin and 50 μg/ml streptomycin. After pelleting the cells, the culture supernatant was centrifuged (20 000×g for 20 min) to remove cellular debris. Exosomes were separated from the supernatant by ultracentrifugation (100 000×g for 2 h) and resuspended in sucrose 250 mM, Heps 5 mM pH 7.4 or directly in Laemmli buffer, depending on the experiments. Exosomes collected from the 48 h maturation medium of human reticulocyte-enriched red blood cells (RBGs) were layered on top of a linear sucrose gradient (0.5-2.5 M sucrose) in a Beckman SW/S5 tube. Gradients were centrifuged at equilibrium for 16 h at 39 000 rpm, after which 350 μL fractions were collected from the top of the tube. Fraction densities were determined by refractometry. Proteins in collected fractions were precipitated by TCA, and samples were separated by SDS-PAGE using 10% polyacrylamide gels and the proteins were electrophoretically transferred to PVDF membrane (Immobilon-P; Millipore, Bedford, MA). Membranes were blocked for 1 hour in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 5% skim milk, followed by 1 h incubation with the indicated primary antibodies. Blots were washed and incubated for 1 h at room temperature with the secondary antibody (horseradish-peroxidase (HRP)-conjugated). Immuno reactive bands were visualized by the enhanced chemiluminescence method (ECL, Amersham Bioscience) according to standard procedures. The membranes were stripped by overnight incubation with 0.2 M glycine, 0.1% SDS, 1% Tween 20, pH 2.2 and re-probed after washing with TBST and blocking with 5% skim milk.
Supporting Information

Figure S1 Determination of the subcellular localization of endogenous ABCB6 by double immunofluorescence labeling and laser-scanning confocal microscopy. A. HeLa cells probed by the ABCB6-567 antibody to detect the endogenous ABCB6 protein (green) in the context of intracellular markers (red): CoxIV (mitochondria), panCadherin (plasma membrane), giantin (Golgi), calnexin (ER) and LysoTracker (lysosomes). ABCB6 colocalizes essentially with the lysosomal marker. B. HEK cells probed by the ABCB6-567 antibody to detect the endogenous ABCB6 protein (green) in the context of intracellular markers (red): WGA (plasma membrane), CoxIV (mitochondria) and Lamp1 (lysosomes). ABCB6 colocalizes essentially with the lysosomal marker.

(TIF)

Figure S2 Flag-tagged ABCB6 expressed in HeLa cells. Following transient transfection of HeLa cells with Flag-tagged ABCB6, cells were probed with anti-tag and ABCB6-567 antibodies. Proteins recognized by the antitag antibody are also labeled with ABCB6-567, suggesting that the monoclonal antibody ABCB6-567 specifically recognizes ABCB6. Note that at very high expression levels, ABCB6 can also be found in the plasma membrane in addition to its main intracellular localization (bottom panel).

(TIF)

Figure S3 Immunofluorescence analysis of fresh human blood using two different anti-ABCB6 antibodies: 74740 (A) and Santa Cruz (B), both revealed with an Alexa 594-coupled secondary antibody. A control with the secondary only is shown (C). DIC: differential interference contrast. Fresh human RBC (<48 h after sampling) group O+ were obtained from the French Blood center. The cells were washed with PBS and fixed in PBS with 4% of paraformaldehyde (EMS sciences) 4 hours at room temperature (RT). Cells were washed, treated with 0.1 M glycine in PBS for 15 minutes at (RT), and then permeabilized with 0.1% Triton X-100 in PBS for 10 minutes at RT. The cells were washed once and resuspended in 3% fetal calf serum (FCS). Antibodies were diluted in Washing Solution (PBS 1% FCS). The cells were incubated with the primary antibodies for 1 h at RT. After 3 washes, the cells were incubated with the secondary antibodies (anti-rabbit alexa 594, Molecular Probes) for 1 h and washed 3 times. A thin film was made on a glass slide and mounted with a coverslip and one drop of vectashield (Vector). Observations were made using a Zeiss AxioImager equipped with an apotome, with a 63× apochromat objective and Differential interference contrast. Luminosity and contrasts were adjusted using the Axiovision software.

(TIF)

Figure S4 ABCB6 expression and fate during reticulocyte maturation. A. Proteins from 0.5 μL packed cell volume (PCV) of RBCs from healthy or phlebotomized mice were separated on 10% SDS-PAGE, transferred on PVDF membrane and analyzed by Western blot for the presence of the indicated proteins. Following transient transfection of HeLa cells with Flag-tagged ABC proteins were expressed following transient transfection of HeLa cells. The cDNA-derived ABC transporters were visualized with an anti-FLAG tag antibody, mitochondria were labeled with CoxIV. Dual channel colocalization analysis was performed by the ImageJ software with the Colocalization Threshold and Colocalization Test plugins. Averaged Pearson’s coefficients were calculated for each subcellular marker from 5 random pictures.

(DOC)

Table S1 Statistics of colocalization analysis between the mitochondrial marker CoxIV, ABCB6 and the mitochondrial ABC proteins. Flag-tagged ABC proteins were expressed following transient transfection of HeLa cells. The cDNA-derived ABC transporters were visualized with an anti-FLAG tag antibody, mitochondria were labeled with CoxIV. Dual channel colocalization analysis was performed by the ImageJ software with the Colocalization Threshold and Colocalization Test plugins. Averaged Pearson’s coefficients were calculated for each subcellular marker from 5 random pictures.

Table S2 Colocalization of endogenous ABCB6 and different subcellular markers in HeLa cells. LysoTracker was used as lysosomal, pancadherin as plasma membrane, calnexin as ER, giantin as Golgi and CoxIV as mitochondrial marker, as described in Methods section.

(DOC)

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Author Contributions

Conceived and designed the experiments: MV GS. Performed the experiments: KK NK AT AB MG AV LB. Analyzed the data: KK NK AB HV LB MV GS. Wrote the paper: MV GS.
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