Biology of Mitochondrial ABCs and Their Contribution to Pathology

Yu Fukuda, John A. Lynch and John D. Schuetz

Abstract The mitochondria are key determinants in the life and death of a cell, and abnormalities in mitochondrial function are associated with a number of pathological conditions. Given that many crucial co-factors are generated in mitochondria, including heme and iron–sulfur clusters, it is a reasonable expectation that energy-dependent transporters might facilitate the efficient movement of these molecules through membrane barriers to their next enzymatic steps. The four mitochondrial ABC transporters are ABCB6, ABCB7, ABCB8, and ABCB10. Of these, ABCB6, ABCB7, and ABCB10 have been implicated in processes important to erythroid cells. Mitochondria are also the site of energy production, during which cytotoxic reactive oxygen species are generated. Through modulation of iron and heme biosynthesis, all four mitochondrial ABC transporters have important roles in protecting cells against excessive ROS. In humans, loss-of-function mutations in ABCB7 cause X-linked sideroblastic anemia with ataxia, and several variants that result in loss of ABCB6 at the surface of red cells have been used to identify a new blood group, *Lan*. ABCB8 has an important role in ischemia. Two of the mammalian mitochondrial transporters, ABCB6 and ABCB7, are closely related to homologs which serve to protect lower organisms from heavy metal toxicity, and mammalian ABCB6 has been demonstrated to provide resistance against some toxic metals, though the mechanism remains unclear. The physiology of mitochondrial transporters and how they contribute to pathological states will be discussed.

Keywords Mitochondria · Heme · Iron · Heavy metal · Reactive oxygen species · ABC transporter

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Abbreviations

ABC	ATP-Binding cassette
ALA	δ-aminolevulinic acid
ANT	Adenine nucleotide translocator
ATPase	decompose ATP
Fe–S	iron–sulfur
HMT1	Heavy metal tolerance protein
mitoK$_{ATP}$	Mitochondrial ATP-sensitive K^+
MDS	Myelodysplastic syndrome
NBD	Nucleotide-binding domain
PIC	Phosphate carrier
PPIX	Protoporphyrin IX
RARS	Refractory anemia with ring sideroblasts
ROS	Reactive oxygen species
SDH	Succinate dehydrogenase
SUR	Sulfonylurea receptor
XLSA/A	X-linked sideroblastic anemia with cerebellar ataxia

Introduction

The mitochondria are key regulators in cellular processes from the execution of apoptosis to generation of energy. The electron transport chain generates energy necessary for the cell, but an inherent by-product is the production of reactive oxygen species (ROS), which can damage proteins, lipids, and DNA. Many steps in the biosynthesis of essential molecules, such as heme, iron–sulfur (Fe–S) clusters, urea, steroid, and neurotransmitters, take place in the mitochondria. In addition, mitochondria are involved in the storage and homeostasis of iron. Mitochondrial iron is essential for the formation of heme and Fe–S clusters, which are co-factors to many enzymes with crucial roles in cellular events including oxidative phosphorylation.

As the major site of cellular metabolism, defects in mitochondrial proteins result in a variety of pathological conditions. For example, porphyrias reflect aberrant heme biosynthesis, whereby elevated levels of intermediates in heme metabolism (porphyrins) cause intracellular damage (Balwani and Desnick 2012). As hemoproteins are involved in protection against oxidative stress, disrupted heme biosynthesis produces oxidative damage. Some intermediates of defective heme synthesis are capable of forming reactive superoxides which account for their cytotoxicity. Efficient mitochondrial heme production also needs coordination with an iron delivery process, as excess iron results in oxidative stress. Impaired assembly of Fe–S clusters is inextricably linked to increased free iron in mitochondria. Thus, perturbations in iron homeostasis, heme biosynthesis, and Fe–S cluster formation are likely to produce oxidative stress.

Table 1 Summary of human mitochondrial ABC transporters

	Ortholog	Chr. location	Mitochondrial targeting sequence	Mito. location	Role	Substrates	Disease or human phenotype	Knockout	Protection against oxidative stress	Expression during erythroid differentiation
ABCB6	Hmt1	2q36	None N-terminal MSD_0 contains *N*-glycosylation and disulfide bond	OM	Heme biosynthesis	Porphyrins Binding and transport tested	Lan	Viable-elevated porphyrin biosynthesis Increased sensitivity to Phz toxicity	Increases hemoproteins such as catalase	Increase
ABCB7	Atm1	Xq12–q13	1–53	IM	Fe–S biogenesis	Sulfur-containing molecules GSH-conjugated and Fe–S-structure and ATPase in yeast	XLSA/A RARS	Embryonic lethal Mx1-Cre shows sideroblasts	Iron, Fe/S homeostatis	Increase
ABCB8	(Mdl1?)	7q35–36	1–55	IM[a]	Iron export (indirect)?	ND		Cardiac-specific knockout mild cardiomyopathy	Through reduction in mito iron	
ABCB10	Mdl1	I1q32	1–105	IM	Heme biosynthesis	ND ALA?		Embryonic lethal Mx1-Cre-anemia and iron accumulation	MitoROS, hemoproteins	Increase

[a]Not formally tested

There are four ATP-binding cassette (ABC) 'half-transporters' (one membrane-spanning domain and nucleotide-binding domain (NBD) in the gene) from the B subfamily of the ABC protein superfamily that have been shown to localize in mitochondria (Table 1). ABCB7, B8, and B10 localize to the mitochondrial inner membrane (Ardehali et al. 2004; Csere et al. 1998; Graf et al. 2004; Hogue et al. 1999; Taketani et al. 2003), whereas ABCB6 localizes to the outer membrane and most recently at the plasma membrane (Helias et al. 2012; Krishnamurthy et al. 2006; Mitsuhashi et al. 2000; Paterson et al. 2007). However, in this review, we will focus on the role of ABCB6 in mitochondria as little is known about its function at the plasma membrane. In the past few years, knockout animal models were generated for each of these mitochondrial ABC transporters, providing insights into their role in mitochondrial function. However, endogenous ligands have yet to be identified for ABCB8 and ABCB10. Nonetheless, available data (discussed below) suggest that all four mitochondrial ABC transporters at least contribute to iron and heme homeostasis (Fig. 1). This might account for why oxidative stress occurs in their absence. Interestingly, ABCB6, ABCB7, and ABCB10 all are highly expressed in erythroid progenitors, suggesting a prominent role in erythroid biology (Fig. 2), and their expression levels increase during erythroid differentiation (Taketani et al. 2003).

Exposure to xenobiotic agents is an ever-present threat to life. Heavy metals are an important subclass of these and organisms have adopted a wide range of strategies to cope with their presence in the environment. In plants, these mechanisms range from chelation by small molecules to incorporation into vacuoles (Hall 2002). In yeast and mammals, some heavy metal detoxification occurs through the excretion of glutathione-bound conjugates by plasma membrane ABC transporters

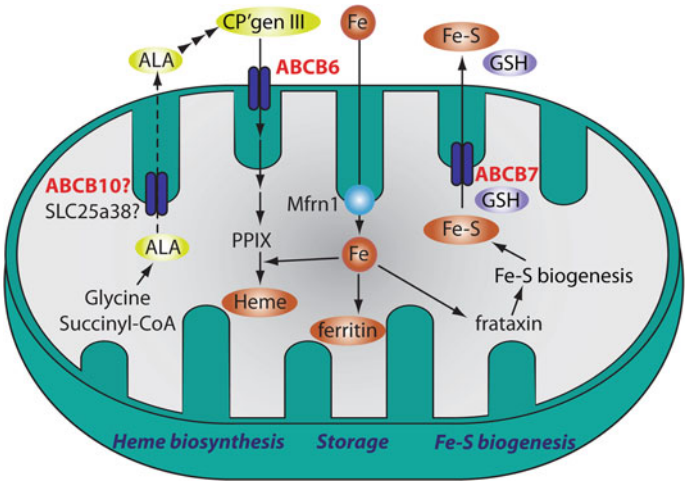

Fig. 1 ABC transporters and iron utilization in mitochondria. *ALA* δ-aminolevulinic acid; *CP'gen III* coproporphyrinogen III; *PPIX* protoporphyrin IX; *GSH* glutathione; *Mfrn1* mitoferrin

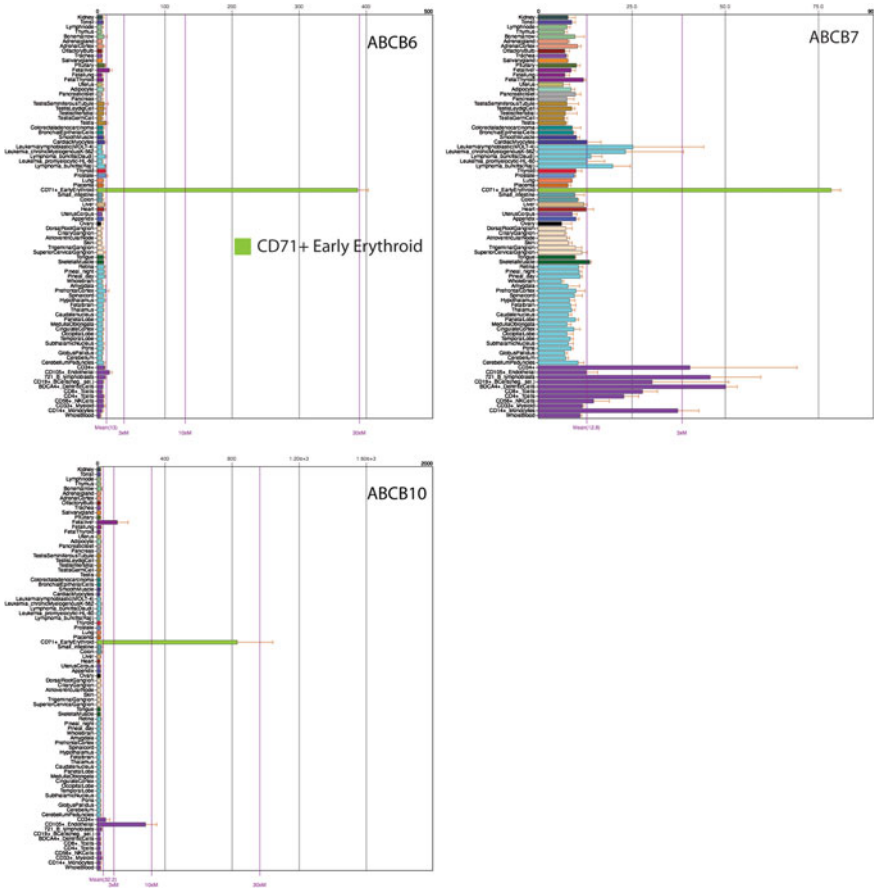

Fig. 2 *ABCB6, B7*, and *B10* are expressed at high levels in CD71+ early erythroid progenitors. Data obtained from biogps.org

(e.g., ABCC1, ABCC4) (Leslie et al. 2004). In yeast, ABC transporters ycf1p and Bpt1b reside in vacuolar membranes where they import and sequester heavy metals. Recent evidence demonstrates that some ABCB mitochondrial transporters are capable of providing resistance to heavy metals (Preveral et al. 2009).

Heavy Metal Toxicity and HMT1 Homologs

While many heavy metals, such as zinc, are necessary for metabolic processes such as reduction of alcohols by alcohol dehydrogenase, dissipation of free radicals by superoxide dismutase (Gautam et al. 2012), and the synthesis of methionine

(Jing et al. 2014), others, such as arsenic and cadmium, are harmful, and environmental exposure disrupts metabolic processes. Among the mechanisms organisms have developed for defense against cadmium toxicity is the sequestration of the chelated ion, a process involving the heavy metal tolerance proteins (HMT1) (Preveral et al. 2009). One protective mechanism that was characterized in the yeast strain, *Schizosaccharomyces pombe*, showed that the ABC half-transporter, *Sp*HMT1, was necessary for cadmium detoxification (Ortiz et al. 1992). Specifically, *Sp*HMT1 imported cadmium complexed with metal-binding peptides, the phytochelatins, into vacuoles. In higher animals, *Sp*HMT1 is homologous to the mammalian mitochondrial transporters, ABCB6 and ABCB7, and *Ce*HMT in *Caenorhabditis elegans*. *Sp*HMT1, for example, reduces cadmium toxicity in *Saccharomyces pombe* and *Caenorhabditis elegans*, *Ce*HMT1 performs likewise, indicating a conserved function (Schwartz et al. 2010). One homolog of *Sp*HMT1, *Na*Atm1, from *Novosphingobium aromaticivorans*, has been crystallized with the tripeptide glutathione, which provides the structural basis for detoxification related to glutathione binding and transport (Lee et al. 2014). A binding site for glutathione and its derivatives in *Na*Atm1 was demonstrated, as well as a capacity to confer heavy metal resistance, a function which is lost when a critical residue in the Walker B motif in the NBD is mutated.

Arsenic remains one of the most common toxic heavy metals humans are exposed to and recent reports implicate ABCB6 as a protector against arsenic toxicity. Given its evolutionary relationship with *C. elegans* and *S. pombe* orthologs, this is not unexpected. Reports indicate arsenate exposure increases ABCB6 expression in vitro and in vivo (Chavan et al. 2011). These authors further demonstrated that ABCB6 knockdown in human liver cancer cell lines, HepG2 and Hep3B cells, was detrimental to survival in media containing arsenic. Together, the findings of heavy metal resistance by HMT1-related proteins suggest a conserved function.

The mechanism by which ABCB6 provides resistance to arsenic, or other heavy metals, in higher organisms is not immediately obvious. In fact, the localization of ABCB6 to the mitochondria provides a challenge in explaining the mechanism of resistance, as its outer mitochondrial membrane localization and orientation would lead to direct import of arsenic into the mitochondria. As mitochondria are key targets for heavy metal toxicity (Belyaeva et al. 2008), a new mechanism is required to account for this. Clearly, in contrast with the yeast vacuoles that use HMT1 proteins to sequester heavy metals, import of heavy metals by ABCB6 into the mitochondria is unlikely to produce any salutary effect. In fact, one would suspect heavy metals in mitochondria might be deleterious. One proposition is that ABCB6 is a functional paralog of *Na*ATM and co-transports glutathione. This might counteract the ROS produced by heavy metal disruption of the electron transport chain (Fig. 3). However, it is difficult to imagine the survival advantage of mitochondrial import of glutathione co-transport with a heavy metal.

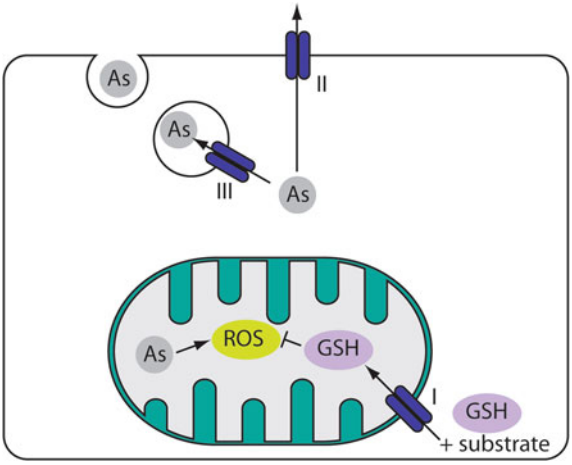

Fig. 3 Detoxification of heavy metals by ABC transporters. ATM1 analogs, such as ABCB6, could mitigate cellular damage (I) by importing GSH with other substrates, which could reduce the damage inflicted by heavy metal ROS generation within the mitochondria, (II) by directly exporting heavy metals out of the cell, or (III) by compartmentalizing metals into an unknown structure where they might be secreted from the cell. *As* arsenic/heavy metal; *ROS* reactive oxygen species; *GSH* glutathione

Artesunate

Mitochondrial transporters have also been implicated in resistance to chemotherapeutic agents that affect heme. For example, ABCB6 expression is upregulated by exposure to artesunate (Kelter et al. 2007). Artesunate is one of the modern classes of drugs used in the treatment of malaria, caused by the microbe *Plasmodium falciparum* (Dondorp et al. 2005). A member of the artemisinin class of compounds, artesunate's mechanism of action involves the generation of free radicals secondary to its reaction with the iron of heme (Pandey et al. 1999). Recently, artesunate has shown promise as a cancer chemotherapeutic, where its effectiveness for some tumors is also dependent upon the tumor's iron or heme concentration (Hamacher-Brady et al. 2011). Again, this mechanism also relies upon levels of sufficient heme production by the target tissue. This suggests that mitochondrial ABC transporters involved in heme and iron homeostasis might have a significant role in cellular response to this drug. Given ABCB6 role in heme generation in vitro and in vivo, one might suspect ABCB6 deficiency could impact responses to artesunate (Ulrich et al. 2012).

ABCB6

Background

ABCB6 was initially cloned and named MTABC3. It localized to chromosome 2q36 and was homologous to ATM1P, a *Saccaromyces cerevisiae* protein that regulates iron–sulfur clusters egress from the mitochondria to the cytosol (Mitsuhashi et al. 2000). The functional ortholog of ATM1P was eventually determined to be ABCB7. Nonetheless, the overexpression of ABCB6 was capable of partially overcoming the inherent iron accumulation defect in ATM1P deficient yeast, suggesting its mitochondrial localization contributed to mitochondrial iron homeostasis. Further characterization of ABCB6 indicated that it formed a homodimer at the outer mitochondrial membrane and it accelerated heme biosynthesis (Krishnamurthy et al. 2006). While both its regulation by heme and its high degree of expression, in early erythroid and liver cells, suggested an important role in heme regulation within the mitochondria, the lack of any known mitochondrial targeting sequence presents difficulties in explaining this localization (Tsuchida et al. 2008). Later, subcellular fractionation indicated that ABCB6 was localized to two locations within the cell, one mitochondrial and the other the plasma membrane (Paterson et al. 2007). Other locations have since been suggested, including lysosomes (Kiss et al. 2012) and Golgi (Tsuchida et al. 2008), but one could argue these locations are secondary and are related to either processes involved in ABCB6 biosynthesis and/or degradation. Recent studies have identified motifs in ABCB6 that are important for trafficking, such as the requirement of a pair of conserved cysteines that are required for ABCB6 stability and to exit from the ER (Fukuda et al. 2011). Most recently, a transmembrane domain has been identified as important for the movement of ABCB6 from plasma membranes into rab5-containing vesicles, which suggests it undergoes endosomal retrieval (Kiss et al. 2015). Undoubtedly, future studies will determine how ABCB6 traffics to the mitochondria and the plasma membrane.

ABCB6 and Porphyrin Transport

ABCB6 has a bona fide role in porphyrin transport. The movement of porphyrins, such as heme and protoporphyrin IX (PPIX), through membrane barriers via transport, was thought to occur by passive diffusion. We now know of a wide range of carriers and transporters for porphyrins, including those present in mammals with a specificity for heme, such as HCP1 (SLC46A1) (Le Blanc et al. 2012), FLVCR (slc49A1/2) (Keel et al. 2008), and ABCG2 (Gnana-Prakasam et al. 2011; Khan and Quigley 2011), which reportedly also localizes to mitochondria, although we do not know exactly where it localizes (e.g., inner membrane, outer membrane)

(Kobuchi et al. 2012). In erythroid cells, diffusion would be an inefficient mechanism to control porphyrin movement into the mitochondria and would hamper hemoglobin production during erythroid differentiation. An active transporter, which could aid in this process, could allow for high rates of heme required to synthesize hemoglobin. Available evidence indicates ABCB6 is the only ATP-dependent porphyrin transporter capable of importing porphyrins into the mitochondria (Chavan et al. 2011; Lynch et al. 2009; Ulrich et al. 2012). The ATP-dependent transport of porphyrins into the mitochondria, particularly the heme precursor copropophyrinogen III, has long been established (Ponka 1999; Rebeiz et al. 1996), but the molecular entity that performed this was unknown. ABCB6, as an outer mitochondrial transporter, with its nucleotide-binding domains facing the cytosol and levels regulated by porphyrin concentration, was a strong candidate. Further, binding studies demonstrated that ABCB6 has a preference for binding tetrapyrroles like heme and not monopyrroles. Transport studies using ^{55}Fe-labeled hemin showed that it could be imported into mitochondria in an ATP- and ABCB6-dependent manner (Krishnamurthy et al. 2006). It is not known if the cytosolic coproporphyrinogen III is actively transported by ABCB6 (Ulrich et al. 2012). This is because the rapid oxidation of copropophyrinogen III to coproporphyrin III precludes its ready use as a transport substrate in vitro. However, coproporphyin III retains the primary tetrapyrrole feature of coproporphyrinogen III (differing only by two double bonds), and was shown to be readily transported by ABCB6 in mouse liver mitochondria (Ulrich et al. 2012). Interestingly, a passive coproporphyrin uptake transport component existed regardless of ABCB6 presence.

Porphyrin and Physiological Significance

As would be expected as a consequence of porphyrin transport, ABCB6 has been proposed as a regulator of heme synthesis. The overexpression of ABCB6 was shown to enhance the cellular levels of the penultimate heme precursor, PPIX, in cultured K562 human erythroblasts (Krishnamurthy et al. 2006). Studies using either a non-functional ABCB6 (harboring a mutation in the Walker A domain) or a mutation in a crucial cysteine (required for stability) exhibited reduced formation of PPIX (Fukuda et al. 2011). This increased porphyrin generation by ABCB6 was further enhanced by the co-addition of δ-aminolevulinic acid (ALA), an early porphyrin precursor, whose presence enhances the concentration of porphyrin biosynthetic intermediates. Curiously, the lack of ABCB6, demonstrated in *Abcb6*$^{-/-}$ mice, was associated with an increase in PPIX in reticulocytes (Ulrich et al. 2012). This unexpected result is related to compensatory adaptations where deficiency of Abcb6 drives changes in the expression of genes involved in both heme biosynthesis and degradation.

ABCB6 and Disease

Evidence suggests that the function of ABCB6 may be an important prognostic factor in some cancers. This is demonstrated by its capacity to enhance the accumulation of PPIX in human gliomas (Zhao et al. 2013). These authors found a strong correlation between ABCB6 expression and prognostic characteristics of the tumor, such as higher amounts of ABCB6 in those with both a higher grade and more aggressive glioma. In addition, the amount of PPIX generated by treatment with a porphyrin biosynthetic precursor, ALA, was greater in those expressing more ABCB6. These findings are in agreement with ABCB6 as a positive regulator of porphyrin biosynthesis. However, it is unknown exactly how an increase in porphyrin biosynthesis might contribute to a more aggressive tumor phenotype.

ABCB6 role in synchronizing the coupling between heme biosynthesis and hemoglobin production was demonstrated in *Abcb6*-null animals (Ulrich et al. 2012). Absence of Abcb6 in mice and humans does not produce any obvious pathological condition under normal unperturbed circumstances (Helias et al. 2012). However, in an experimental condition mimicking profound anemia, Abcb6 was essential for normal stress erythropoiesis. In this work, mice exhibited adaptive changes which, coupled with the stress of enhanced porphyrin generation but decreased heme formation, produced a profound defect in *Abcb6* knockout mice survival (Ulrich et al. 2012).

Clearly, ABCB6 role in porphyrin homeostasis is important in pathological conditions. Some roles are clear, like the coupling accelerated heme biosynthesis to hemoglobin production while others are less so. For example, how does Abcb6 promote resistance to heavy metals? Does ABCB6 expression change the biology of gliomas? Based upon ABCB6 impact on some enzymes (e.g., the mitochondrial electron transport chain (Lynch et al. 2009), it is likely that ABCB6 importance extends to other areas as well. Many unanswered questions remain to be addressed. For example, without a canonical targeting sequence, how does ABCB6 localize to the mitochondria? What controls its differential localization between cellular compartments like mitochondria and plasma membrane? Does the existence of a loop formed by conserved cysteine signify any importance of redox in its trafficking? Presumably, all of these questions will be soon answerable given the current interest in ABCB6.

ABCB7

Background

ABCB7 was first identified as the causative gene for a form of X-linked sideroblastic anemia with cerebellar ataxia (XLSA/A). Further, ABCB7 has been implicated in another form of anemia, refractory anemia with ring sideroblasts (RARS).

The human *ABCB7* sequence was identified in the expressed sequence tag (EST) database based upon its sequence homology with yeast *Atm1* (discussed below) (Shimada et al. 1998), and mapped to chromosome Xq12–13 (Savary et al. 1997). Human *ABCB7* shares ~50 % sequence identity with yeast *Atm1* and is highly conserved in other species. Most of the functional studies on ABCB7 have come from the use of an *Atm1*-insufficient yeast strain, in which ABCB7 expression rescues both the aberrant mitochondrial iron accumulation and lack of Fe–S cluster proteins, suggesting a conserved function (Csere et al. 1998). ABCB7 is predicted to have six membrane-spanning helices with a C-terminal NBD. Mitochondrial localization of ABCB7 was confirmed by subcellular fractionation as well as immunofluorescence microscopy (Csere et al. 1998; Taketani et al. 2003). Unlike ABCB6, ABCB7 harbors an N-terminal mitochondrial targeting sequence in the first 135 amino acid residues that is capable of targeting a cytosolic protein, dihydrofolate reductase, to the mitochondria (Csere et al. 1998).

Function Inferred from ABCB7 Homolog Atm1p

The first mitochondrial ABC transporter identified, appropriately named Atm1 for ABC transporter of mitochondria, was essential for normal growth, iron homeostasis, and formation of Fe–S clusters in yeast (Kispal et al. 1997, 1999; Leighton and Schatz 1995; Senbongi et al. 1999). Immunofluorescence microscopy and subcellular fractionation demonstrated mitochondrial localization, consistent with its 53 amino acid targeting sequence being sufficient to target an unrelated protein to the mitochondria. Atm1p orientation and localization in the mitochondria was determined by the proteinase K insensitivity of a C-terminally-tagged Atm1p both in purified mitochondria and mitoplasts. These studies indicated Atm1p resides in the inner mitochondrial membrane with the C-terminal NBD facing the matrix. From this orientation, Atm1p was proposed to transport its substrates from the matrix into the intermembrane space. The reduction in Fe–S cluster-containing proteins and holo-forms of hemoproteins in a yeast strain lacking *Atm1* suggested its substrates were Fe–S clusters (Kispal et al. 1997; Leighton and Schatz 1995). Coupled with these deficiencies was enhanced mitochondrial iron accumulation which, undoubtedly, accounted for the increased oxidative stress observed in Δ*atm1* cells. As might be expected, Δ*atm1* cells were hypersensitive to oxidative stress and exhibited impaired growth in the presence of H_2O_2 (Kispal et al. 1997; Senbongi et al. 1999). Further evidence of functional conservation was suggested by *C. elegans* lacking the homolog of *ABCB7*, *abtm-1*; free iron and oxidative stress increased in this strain (Gonzalez-Cabo et al. 2011).

While Atm1 appears integral to providing cytosolic Fe–S clusters to certain apo-proteins, its direct role in Fe–S cluster transport has not been confirmed. In eukaryotes, the multi-step Fe–S cluster assembly occurs in the mitochondrial matrix where the cysteine desulfurase, Nfs1p, provides elemental sulfur and frataxin acts as an iron chaperone. Lack of *Nsf1* (in yeast) results in a complete deficiency in

both mitochondrial and cytosolic Fe–S-containing proteins. In contrast, *Atm1* absence only affects cytosolic Fe–S-containing proteins (Kispal et al. 1999). From this, one infers that Atm1 is primarily involved in Fe–S cluster transport, but not in their biogenesis.

Fe–S-containing proteins are involved in multiple cellular processes such as electron transport and heme biosynthesis. Fe–S cluster proteins such as iron regulatory protein 1 (IRP1, holo-protein acts as c-aconitase) use Fe–S cluster to sense cellular iron levels and ultimately regulate the expression of proteins involved in iron homeostasis. In mammals, ferrochelatase, the enzyme responsible for the insertion of iron into PPIX to make heme, requires an Fe–S cluster inserted into its C-terminus (Crooks et al. 2010). In total, mitochondrial iron, Fe–S cluster, and heme biosynthesis share intimate regulatory overlap.

The homodimeric crystal structure of Atm1 revealed a binding site for glutathione (GSH), suggesting thiol-containing compounds are endogenous substrates (Lee et al. 2014; Srinivasan et al. 2014). Both *Saccharomyces cerevisiae* Atm1 and bacterial *Na*Atm1 ($\sim$45 % sequence identity with yeast Atm1 and human ABCB7) were crystallized in a nucleotide-free state, with structures solved in either the GSH- or GSSG-bound (oxidized GSH) states, respectively (Lee et al. 2014; Srinivasan et al. 2014). In the absence of nucleotides, Atm1 adopted an inward-facing open conformation with GSH binding at positively charged residues in an internal cavity. Because the structure was similar to the GSH-free form, it suggested some substrates bind without producing conformational changes. Nucleotide binding to the NBDs on a substrate-bound ABC transporter induced a conformational change to an outward-facing conformation with substrates released to the other side. Overall, these Atm1 structures showed good agreement with previously reported ABC transporters, i.e., where transmembrane helices from each monomer intertwined with the helices from the other monomer. Notably, the hydrophilic amino acid residues where GSH binds are conserved in human ABCB7.

Several GSH and related compounds stimulated ATPase activity of *Na*Atm1, which suggests that these compounds are ligands for this transporter, with the highest activity observed for metallated, aromatic hydrocarbon-conjugated, and oxidized GSH-related compounds (Lee et al. 2014). For example, S–Ag GSH and S–Hg GSH strongly activated *Na*Atm1 ATPase activity. Furthermore, overexpression of *Na*Atm1 in metal-sensitive *E. coli* strains protected against toxic amounts of heavy metals. *Na*Atm1 also mediated ATP-dependent transport of GSSG into the vesicles prepared from *E. coli* membranes. These results suggested that Atm1 alleviates heavy metal toxicity through export of metallated GSH. Because GSH is involved in extra-mitochondrial Fe–S cluster assembly, GSH may be a part of the substrate (e.g. GSH-coordinated Fe–S clusters).

A further benefit of the crystal structure of Atm1 was the insight into a mutation in ABCB7 that causes the disease, XLSA/A (E433 in human, see below). This residue corresponds to D398 in yeast Atm1, which was identified as one of the key residues required for the interaction with GSH. From the ATPase studies, one might speculate that the GSH-binding site is part of the substrate-binding pocket. Interestingly, key residues identified to interact with GSSG are highly conserved

among atm1, hmt1, Abcb7, and Abcb6 from different species. Other residues found to be mutated in XLSA/A patients were also found in the membrane-spanning domain, E208 in the matrix side (E173 in yeast), whereas I400 and V411 are on the intermembrane side (V365 and V376 in yeast, respectively). The impact of these residues on protein function remains to be tested.

ABCB7 in Human Diseases

ABCB7 loss of function is linked to two human diseases that exhibit ring sideroblasts (erythroblasts with perinuclear iron-loaded mitochondria). To date, four mutations have been identified in the *ABCB7* gene from patients with X-linked sideroblastic anemia with cerebellar ataxia (XLSA/A). However, the reduced levels of *ABCB7* mRNA seen in refractory anemia with ring sideroblasts (RARS) are not due to mutation, suggesting other defects.

X-linked Sideroblastic Anemia with Cerebellar Ataxia (XLSA/A)

XLSA/A is a rare form of congenital sideroblastic anemia, which is characterized by a microcytic anemia with mitochondrial iron accumulation in erythroid precursors of affected males and attributed to defects in ABCB7. While XLSA/A is characterized by neurological defects (e.g., cerebellar ataxia), the anemia and alterations in erythropoiesis resemble those found in patients harboring a defect in the porphyrin biosynthetic enzyme, ALAS2. Despite the neurological defects associated with this disease, *ABCB7* transcripts were not detectable in the adult brain. The four non-synonymous mutations of *ABCB7*, among affected individuals, resulted in amino acid changes of E209D, I400M, V411L, or E433K (Allikmets et al. 1999; Bekri et al. 2000; D'Hooghe et al. 2012; Maguire et al. 2001), which, according to predictions, produce a partial loss of ABCB7 function. Because absence of *Abcb7* in mice is embryonically lethal (suggesting this gene is critical to early development), one infers from this that only mutations with mild effects on ABCB7 function will be tolerated in humans. Consistent with this, yeast harboring the corresponding ABCB7 I400M mutation in Atm1p (analogous to V365M) grew slower (Allikmets et al. 1999). Moreover, yeast expressing mutant Atm1p (D398K) with the corresponding E433K mutation in ABCB7 only produced ~50 % of the normal cytosolic Fe–S cluster proteins (Bekri et al. 2000). The structural studies of Atm1p showed that D398 participates in GSH binding further suggesting the importance of this residue in ABCB7 substrate recognition (Srinivasan et al. 2014). Interestingly, transgenic mice carrying the E433 K allele resulted in male infertility (Pondarre et al. 2007).

Patients affected with XLSA/A exhibit Zn–PPIX accumulation, a rare occurrence, but usually an indication of iron deficiency. The production of Zn–PPIX, instead of heme in iron excess, suggests this iron pool is inaccessible for heme synthesis. In HeLa

cells, knockdown of ABCB7 using siRNA recapitulated some of the phenotypes, including altered iron homeostasis and reduced cytosolic Fe–S cluster proteins (Cavadini et al. 2007). While transferrin receptor expression was upregulated, ferritin, the iron storage molecule, was reduced and ferritin-unbound iron accumulated in mitochondria. The mitochondrial iron was not available for heme formation as evidenced by an increase in PPIX. Unlike yeast ferrochelatase, the mammalian ferrochelatase is an Fe–S cluster-containing protein, and an essential co-factor required for its enzyme activity. ABCB7 and ferrochelatase physically interact in in vitro binding assays, and overexpression of ABCB7 in cells increased both ferrochelatase expression and activity (Taketani et al. 2003). The importance of the interaction between ABCB7 and ferrochelatase requires further investigation because formation of Zn–PPIX in XLSA/A patients implies that ferrochelatase activity is intact, but iron is unexpectedly unavailable when ABCB7 function is impaired.

Refractory Anemia with Ring Sideroblasts

Refractory anemia with ring sideroblasts (RARS) is a type of myelodysplastic syndrome (MDS) characterized by excessive mitochondrial iron accumulation in erythroblasts much like what is seen with XLSA/A patients. Like other forms of MDS, which are heterogeneous but display aberrant hematopoietic cell proliferation and differentiation and loss of circulating blood cells; 10–20 % of RARS progress to acute myeloid leukemia. Although the ring sideroblasts are phenotypically similar to those from XLSA/A patients, no mutations in the *ABCB7* gene, including its promoter, have been identified to date. Nonetheless, *ABCB7* mRNA levels are lower in individuals with RARS compared to healthy controls, or individuals with refractory anemia or refractory anemia with excess blasts (Boultwood et al. 2008). In RARS patients, a splicing factor SFB1 is mutated at a higher frequency (64–90 %), which might explain the reduced *ABCB7* mRNA. Knockdown of ABCB7, using shRNA, in hematopoietic progenitor-enriched bone marrow cells ($CD34^+$), resulted in reduced erythroid progenitors and impaired growth in liquid culture after erythropoietin stimulation, and as expected, impaired erythroid maturation (Nikpour et al. 2013). Conversely, overexpression of ABCB7 gene in $CD34^+$ bone marrow cells from RARS patients enhanced erythroid progenitor formation. While the reduced ABCB7 transcripts appear secondary to SFB1 mutation in RARS, these results further support the role for ABCB7 in erythroid biology.

Abcb7 Knockout Mouse Models Highlight the Importance of This Gene

Mouse models show that constitutive absence of *Abcb7* is embryonically lethal. To overcome this, mice harboring *Abcb7* alleles that can be conditionally deleted were bred to mice harboring *Cre* transgenes that enabled *Abcb7* gene deletion in a

tissue-specific manner (Pondarre et al. 2006). This approach revealed that general deletion of *Abcb7* in embryos prevented them from surviving past day 7.5. Histological analysis of these embryos showed some with growth retardation and hemorrhage; however, there was no evidence for excess iron accumulation. Because of the preferential activation of the female-derived X chromosome in extra-embryonic tissues in mammals, it was speculated that Abcb7 was important for the development of extra-embryonic tissues. This hypothesis was confirmed by crossing *Abcb7* homozygous floxed female mice to males hemizygous for either *Sox-Cre* or *Villin-Cre* transgene, which drives *Cre* expression in embryonic epiblast (sparing much of the extra-embryonic tissues) or in ciliated cells of the extra-embryonic visceral endoderm. Live-born females were obtained from the *Sox-Cre* line but not from the *Villin-Cre* line demonstrating that *Abcb7* is essential in the extra-embryonic tissues during early development.

Abcb7 floxed mice were crossed to inducible- or tissue-specific *Cre* lines to further investigate whether loss of *Abcb7* hemoglobinization during recapitulates human disease. Post-natal deletion of *Abcb7* in the hematopoietic compartment resulted in bone marrow failure and subsequent death within ~20 days (Pondarre et al. 2007). Coupled with this was an early transient siderocytosis, occurring shortly after the deletion of *Abcb7*. These findings support the idea that only mutations resulting in a mild defect in ABCB7 function are tolerated. To determine if an *ABCB7* mutation that was associated with sideroblastic anemia produced a similar phenotype in mice, mice harboring the disease-causing E433 K mutation in *Abcb7* were generated. Notably, red blood cells in these animals were iron-laden (siderocytes), and consistent with anemia as reticulocytes had increased Zn–PPIX compared to Fe–PPIX (heme).

ABCB8

Background

ABCB8 was the first mammalian mitochondrial ABC transporter to be identified and was appropriately named human mitochondrial ABC1 (M-ABC1) (Hogue et al. 1999 JMB). ABCB8, isolated after a cDNA library from a T-cell line, was screened using an EST clone that had nucleotide sequence similarities to the NBD from P-glycoprotein (*ABCB1*). The resulting cDNA encoded a protein with an open reading frame of 718 amino acids that mapped to chromosome 7q35–36. ABCB8 is ubiquitously expressed in human tissues and, like Atm1, contains an N-terminal mitochondrial targeting sequence that mapped to its first 55 amino acids. A cleavable signal sequence accounted for a molecular mass (~60 kDa) that was smaller than predicted. Because inner mitochondrial membrane proteins typically contain cleavable signal sequences, ABCB8 was predicted, like ABCB7 and ABCB10, to reside in the inner mitochondrial membrane. The inner membrane ABC transporters, Atm1p (ABCB7 ortholog) and ABCB10, form homodimers;

therefore, without any obvious potential partners, ABCB8 appears to form a homodimer as a protein complex of an apparent molecular mass of ~120–130 kDa is detected after cross-linking. Unlike ABCB6 and Atm1p, the conserved Walker A motif is critical for the stability of ABCB8, as point mutations in key residues in this motif rendered ABCB8 undetectable after transient expression in COS-7 cells.

ABCB8 as a Part of MitoK_{ATP} Channel

The physiological substrate of ABCB8 has not been determined, yet. However, ABCB8 protects against oxidative stress. This unanticipated finding was revealed after the discovery of ABCB8 in a macromolecular complex encompassed in the mitochondrial ATP-sensitive K^+ channel (mitoK_{ATP}) (Ardehali et al. 2004). Activation of mitoK_{ATP} protected cardiac and neuronal cells against oxidant-induced apoptosis during brief ischemic episodes. However, ABCB8 role is unclear as the mitochondrial enzyme, succinate dehydrogenase (SDH), is also a component of this complex. Further, immunoprecipitation of SDH from inner mitochondrial membranes revealed additional interaction between SDH, ABCB8, and three more mitochondrial proteins, phosphate carrier (PIC), adenine nucleotide translocator (ANT), and ATP synthase (ATPase). PIC and ANT have been suggested to exhibit ion channel activity, thus it is conceivable that ABCB8 regulates the mitoKATP. This would be analogous to the sulfonylurea receptors' (SUR, ABCC8/9) regulation of the plasma membrane K_{ATP} channel. At this point, how ABCB8 regulates mitoK_{ATP} is unclear, but warrants further investigation.

ABCB8 and Iron Modulation

As discussed above, mitoK_{ATP} activity protects against oxidative stress (e.g., ischemia). ABCB8 role under such stress was directly tested using a ROS-generating system. In this system, knockdown of ABCB8 in ROS-exposed neonatal rat cardiomyocytes resulted in a loss of mitochondrial membrane potential and cell viability (Ardehali et al. 2005). In contrast, ABCB8 overexpression protected the cells against ROS-mediated mitochondrial injury. This protection appears unrelated to the mitoK_{ATP} channel as its inhibition prior to ROS formation did not alter the mitochondrial membrane potential. Therefore, ABCB8 protects against ROS independent of mitoK_{ATP}. Modulation of mitochondrial iron levels might be how ABCB8 protects against oxidative stress (see below).

The function of ABCB8 in cardiomyocytes was further investigated by generating a cardiac-specific *Abcb8*-null mouse model (Ichikawa et al. 2012). Hearts deficient in *Abcb8* exhibited multiple defects, including reduced heart contractility and cardiac output, the latter being consistent with defects in systolic and diastolic function. While no gross alteration in heart morphology was observed, electron

microscopic examination of *Abcb8*-null hearts revealed aberrant mitochondrial structures. The mitochondria were smaller and cristae morphology was abnormal. Furthermore, mitochondria contained electron-dense material similar to those of animals exposed to iron loading. Indeed, mitochondrial iron levels modestly increased in *Abcb8*-null hearts. These in vivo findings were supported by knockdown of *Abcb8* in cultured cardiomyocytes. Abcb8 deficient cardiomyocytes had enhanced mitochondrial iron accumulation, whereas Abcb8 overexpression reduced mitochondrial iron levels. Subsequent studies, using radiolabeled iron, suggested iron export was impaired after knockdown of ABCB8, although further experiments are needed to determine what form of iron is exported (i.e., is it heme or an Fe–S cluster?). Notably, mitochondrial membrane potential was unaffected in these Abcb8 siRNA-treated cells, suggesting that the mitochondrial injury seen in the *Abcb8*-null mouse heart is likely due to chronic iron accumulation in the mitochondria. Surprisingly, the activity of cytosolic Fe–S cluster proteins, such as xanthine oxidase and aconitase, was reduced in either the *Abcb8*-null mouse heart or siRNA-treated cardiomyocytes. So it is conceivable that ABCB8 not only regulates iron export, but Fe–S cluster assembly, too. Whether ABCB8 is directly involved in iron transport or regulates another transporter of mitochondrial iron remains to be seen. It is of interest that both *Abcb7* and *Abcb8* knockout mice have some overlapping characteristics (i.e., mitochondrial iron accumulation and reduced cytosolic Fe–S cluster proteins), suggesting they share redundant function in modulating mitochondrial iron levels. However, how ABCB8 regulates iron homeostasis remains a mystery. Moreover, without direct determination of heme (there was no change in PPIX levels), it is difficult to gain insight into how *Abcb8* deficiency affects mitochondrial iron homeostasis.

ABCB8 Protects Against Doxorubicin-Induced Oxidative Stress

Doxorubicin is a widely used cancer chemotherapeutic with cardiomyopathy as an unfortunate side effect. This is independent of its primary mechanism of inhibiting topoisomerase. One hypothesized mechanism of doxorubicin-induced cardiomyopathy is the “ROS and iron” hypothesis. This was supported by the finding that the iron chelator dexrazoxane (DXZ) protected against doxorubicin-induced cardiomyopathy. In further support, an animal model shows doxorubicin-mediated heart injury is related to greater systemic iron absorption. Notably, ABCB8 knockdown sensitized cardiomyocytes to doxorubicin toxicity, accompanied by an increased cellular ROS. It is possible, by regulating mitochondrial iron, ABCB8 may indirectly protect against doxorubicin toxicity, an idea supported by knockdown of ABCB8, which enhances doxorubicin cytotoxicity in normally resistant melanoma cells.

ABCB10

Background

ABCB10, first identified as a target of GATA-1, (a transcription factor essential for differentiation of erythroid cells), was initially named ABC-me for mitochondrial erythroid transporter (Shirihai et al. 2000). The gene (*ABCB10, ABC-me, M-ABC2, MTABC2*) was localized to chromosome 1q32 in man and encodes a protein of 716 amino acids. ABCB10 is highly expressed at sites of definitive hematopoiesis such as fetal liver erythroid cells (i.e., $Ter119^{+}$) and adult bone marrow (Fig. 2). In adult mice, *Abcb10* is also highly expressed in other tissues including liver, kidney, and heart, and, to a lower extent, in brain and spleen.

Biochemical Characteristics of ABCB10

The biochemical properties of ABCB10 have been extensively studied, including a well-characterized mitochondrial targeting signal that is crucial to ABCB10 localization to the mitochondrial inner membrane with its C-terminal NBD facing the matrix (Graf et al. 2004). The mitochondrial targeting sequence comprises the first 105 amino acids, which, upon cleavage, produce a protein with an apparent molecular mass of 65 kDa instead of a predicted 78 kDa. The deletion of this N-terminal signal sequence abolished its mitochondrial localization.

Mass spectrometry revealed that ABCB10 is a homodimer (Graf et al. 2004), and the crystal structure of ABCB10 in a nucleotide-free state showed an open inward-facing conformation in agreement with previously studied ABC transporters (Shintre et al. 2013). The ABCB10 protein fold is similar to those of other ABC transporters, where α-helices from each monomer are intertwined with each other. Unexpectedly, ABCB10 maintained an open inward-facing conformation when a non-hydrolyzable ATP analog is bound. This finding contrasted with the classic model of substrate transport whereby the binding of nucleotide induced a dynamic conformational change to yield an outward-facing conformation.

Role of ABCB10 in Erythroid Biology

ABCB10 has a crucial role in erythroid development as constitutive deletion produced anemic embryos that failed to thrive (embryos died by gestational day 12.5 accompanied by apoptotic erythroid precursors) (Hyde et al. 2012). To determine whether Abcb10 was required for erythrogenisis, red blood cell differentiation markers CD71 (aka transferrin receptor) and TER119 were analyzed in wild-type and *Abcb10*-null embryos. During erythrogenesis, CD71 expression increased,

followed by TER119 expression. Because CD71 is involved in iron acquisition, it is noteworthy that *Abcb10*-null embryos have dramatically reduced CD71 positive cells. Iron is crucial for hemoglobin biogenesis; therefore, it is not surprising that *Abcb10*-null erythroid differentiation is stalled with increased apoptosis at the peak of hemoglobin synthesis. ABCB10 role in hemoglobin synthesis appears conserved in humans and mice as knockdown of ABCB10 reduced hemoglobinization during differentiation in the human erythroleukemia cell line K562 (Tang et al. 2012). ABCB10 role in erythrogenesis is not only confined to the embryonic stage; deletion of *Abcb10* from the hematopoietic cells of adult mice harboring floxed *Abcb10* mice resulted in a progressive anemia characterized both by reduced red blood numbers and red cells exhibiting impaired hemoglobin and heme production (Yamamoto et al. 2014). The defect in heme biosynthesis was accompanied by an elevation in reticulocyte PPIX. These results indicate ABCB10 regulation of heme biosynthesis is crucial to erythroid differentiation.

Although ABCB10 was initially proposed as a mitochondria exporter of heme (Shirihai et al. 2000), more recent studies suggest that ABCB10 might regulate the terminal steps in heme biosynthesis through an interaction with other mitochondrial proteins (Chen et al. 2009, 2010). In differentiated mouse erythroleukemia (MEL) cells, ABCB10 co-immunoprecipitated with mitoferrin (Slc25a37), a mitochondrial iron importer (Chen et al. 2009). The stability and activity of mitoferrin are increased by the interaction with ABCB10. Subsequent studies using mass spectrometry identified ferrochelatase (the terminal enzyme in heme biosynthesis) as part of an oligomeric protein complex containing ABCB10, mitoferrin, and ferrochelatase (Chen et al. 2010).

Based on ABCB10 knockdown in cardiac myoblasts exhibiting reduced porphyrins including heme and hemoproteins (Liesa et al. 2011), an alternative role for ABCB10 was proposed. The authors suggested that ABCB10 increased the export of ALA out of the mitochondria because ALA augmentation normalized porphyrin levels in ABCB10 knockdown cells. A caveat to this interpretation is that these studies did not directly measure transport of ALA by ABCB10. Therefore, how ABCB10 modulates heme biosynthesis is not clear: is it at the terminal step or the beginning?

ABCB10 Role in Protection Against Oxidative Stress

ABCB10 expression protected against oxidative stress in yeast and mice, which is not surprising given aberrant accumulation of heme precursors is related to oxidative stress. In yeast, Mdl1p, an ABCB10 ortholog (Mdl1 shares sequence homology to ABCB8 to a lesser extent), conferred resistance to oxidative stress and heavy metal toxicity (Chloupkova et al. 2003). Accordingly, *Abcb10*-null embryos exhibit mitochondrial ROS and protein carbonylation in red blood cells (Hyde et al. 2012), which appears to be related to reduced erythroid differentiation capacity. Notably, quenching ROS with a superoxide dismutase 2 mimetic rescued erythroid

differentiation. In humans, differentiation of erythroid colonies was also impaired by *ABCB10* knockdown (Yamamoto et al. 2014), suggesting a conserved function among different species.

ABCB10 role in protection against oxidative stress extends beyond the erythroid compartment. For example, loss of one allele of *Abcb10* in mice resulted in reduced recovery of cardiac function after ischemic perfusion (Liesa et al. 2011). Mitochondrial defects consistent with increased oxidative stress accompanied the defect. Furthermore, attenuation of oxidative damage by the superoxide dismutase/catalase mimetic, EUK-203, restored cardiac function to wild-type levels after the ischemic insult.

Conclusion and Future Perspective

In the last decade, animal models lacking each of the four mitochondrial ABC transporters have been established, all of which have highlighted the importance of these proteins in the health and/or function of the mitochondria. Although the precise roles for some of them remain elusive, largely because substrate identification has lagged, the physiological roles of these transporters are emerging. Notably, there are several common threads that tie these transporters together: (a) role in erythroid biology through iron and heme homeostasis and (b) protection against oxidative stress.

ABCB6, ABCB7, and ABCB10 are upregulated during erythroid differentiation with each having some similar, but non-overlapping roles in erythroid biology. ABCB7 and ABCB10 are essential for normal erythropoiesis, whereas ABCB6 role was only evident under stress erythropoiesis. ABCB6 and ABCB10 are both involved in heme and hemoprotein homeostasis, where ABCB6 shuttles porphyrins into mitochondria. ABCB10 role in heme biosynthesis is unclear, with either a role in the terminal step in heme biosynthesis or export of ALA into the cytoplasm. Although exact substrates are yet to be identified, ABCB7 role in maintaining iron homeostasis through Fe–S cluster biogenesis is evident. Notably, loss of ABCB6, ABCB7, ABCB8, and ABCB10 all results in increased sensitivity to oxidative stress. Because their homologs, especially Atm1p and Hmt1p, have been shown to alleviate heavy metal toxicity and oxidative stress in different organisms, it is plausible that their mammalian counterparts also have a role in heavy metal detoxification. Currently, ABCB6 and ABCB8 have been implicated in modulating drug toxicity for artemisinin and doxorubicin, respectively. Future studies will undoubtedly address how these transporters affect and modify disease states.

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