ATP-Binding Cassette B10 Regulates Early Steps of Heme Synthesis

Marina Bayeva,* Arineh Khechaduri,* Rongxue Wu, Michael A. Burke, J. Andrew Wasserstrom, Neha Singh, Marc Liesa, Orian S. Shirihai, Nathaniel B. Langer, Barry H. Paw, Hossein Ardehali

Rationale: Heme plays a critical role in gas exchange, mitochondrial energy production, and antioxidant defense in cardiovascular system. The mitochondrial transporter ATP-binding cassette (ABC) B10 has been suggested to export heme out of the mitochondria and is required for normal hemoglobinization of erythropoietic cells and protection against ischemia–reperfusion injury in the heart; however, its primary function has not been established.

Objective: The aim of this study was to identify the function of ABCB10 in heme synthesis in cardiac cells.

Methods and Results: Knockdown of ABCB10 in cardiac myoblasts significantly reduced heme levels and the activities of heme-containing proteins, whereas supplementation with δ-aminolevulinic acid reversed these defects. Overexpression of mitochondrial δ-aminolevulinic acid synthase 2, the rate-limiting enzyme upstream of δ-aminolevulinic acid export, failed to restore heme levels in cells with ABCB10 downregulation. ABCB10 and heme levels were increased by hypoxia, and reversal of ABCB10 upregulation caused oxidative stress and cell death. Furthermore, ABCB10 knockdown in neonatal rat cardiomyocytes resulted in a significant delay of calcium removal from the cytoplasm, suggesting a relaxation defect. Finally, ABCB10 expression and heme levels were altered in failing human hearts and mice with ischemic cardiomyopathy.

Conclusions: ABCB10 plays a critical role in heme synthesis pathway by facilitating δ-aminolevulinic acid production or export from the mitochondria. In contrast to previous reports, we show that ABCB10 is not a heme exporter and instead is required for the early mitochondrial steps of heme biosynthesis. (Circ Res 2013;113:279-287.)

Key Words: δ-aminolevulinic acid ■ ATP-binding cassette transporters ■ cardiomyopathies ■ heme ■ mitochondria

Heme synthesis has been extensively studied in the context of red blood cell development and hemoglobinization, and the defects of this pathway often cause sideroblastic anemia and neurological deficits in humans. Overexpression of mitochondrial δ-aminolevulinic acid synthase 2, the rate-limiting enzyme upstream of δ-aminolevulinic acid export, failed to restore heme levels in cells with ABCB10 downregulation. ABCB10 and heme levels were increased by hypoxia, and reversal of ABCB10 upregulation caused oxidative stress and cell death. Furthermore, ABCB10 knockdown in neonatal rat cardiomyocytes resulted in a significant delay of calcium removal from the cytoplasm, suggesting a relaxation defect. Finally, ABCB10 expression and heme levels were altered in failing human hearts and mice with ischemic cardiomyopathy.

ATP-binding cassette (ABC) transporters compose one of the largest protein families known and are found in all

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From the Feinberg Cardiovascular Research Institute, Northwestern University School of Medicine, Chicago, IL (M.B., A.K., R.W., M.A.B., J.A.W., N.S., H.A.); Department of Medicine, Obesity and Nutrition Section, Mitochondria Affinity Research Collaborative, Evans Biomedical Research Center, Boston University School of Medicine, Boston, MA (M.L., O.S.S.); and Hematology Division, Brigham & Women’s Hospital Harvard Medical School, Boston, MA (N.B.L., B.H.P.).

*These authors contributed equally to this work.

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Correspondence to Hossein Ardehali, MD, PhD, Feinberg Cardiovascular Research Institute, Northwestern University School of Medicine, Tarry 14-733, 303 E Chicago Ave, Chicago, IL 60611. E-mail h-ardehali@northwestern.edu

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organisms from single-cell bacteria to humans. They use the energy of ATP hydrolysis to transport diverse substrates across cellular membranes. Although >50 ABC proteins have been identified in the human genome, only 4 of them localize to the mitochondria, and their functions in the heart remain understudied. ABCB6 is an outer membrane protein and has been suggested to import coproporphyrinogen III into the mitochondria for the synthesis of heme, although a controversy about its mitochondrial localization and role in heme synthesis exists. ABCB7 regulates mitochondrial and cellular iron homeostasis and was shown to interact with ferrochelatase. A mutation of this protein was found in patients with X-linked sideroblastic anemia with ataxia, suggesting that ABCB7 may participate in heme biosynthesis. ABCB8 (also known as mABC1) was recently shown to protect against oxidative stress, facilitate iron export out of the mitochondria, and play a role in the maturation of cytosolic iron–sulfur cluster proteins. However, heme levels were not altered in the hearts of ABCB8 knockout mice, suggesting that this transporter may not be involved in the regulation of heme homeostasis. Finally, several reports suggested that ABCB10 (also known as ABC-me and mABC2) plays a role in hemoglobinization of erythropoietic cells and could function to export heme out of the mitochondria, although the biological substrate for this protein has not been identified yet. ABCB10 is a mitochondrial inner membrane half-protein that homodimerizes to form a functional transporter. Tissues with the highest levels of ABCB10 expression include hematopoietic lineages, heart, liver, and kidney. ABCB10 was shown to be required for normal hematopoiesis possibly via heme export from the mitochondria. To test this hypothesis, we measured heme levels and total mitochondrial iron content with ABCB10 modulation in H9c2 cardiac myoblasts. We effectively downregulated ABCB10 by lentiviral transduction of short hairpin RNA (shRNA) and overexpressed this protein using adenovirus, as evidenced by Western blot (Online Figure I). Furthermore, our isolation protocol yielded highly enriched mitochondrial and cytosolic fractions (Online Figure II). Overexpression of ABCB10 had no effect on mitochondrial or total cellular heme levels in H9c2 cells (Figure 1A and 1B). However, cellular and mitochondrial heme content was significantly reduced by ABCB10 knockdown in H9c2 cells (Figure 1C and 1D) and in NRCM (Online Figure IIB–IID). The reduction in heme was not caused by a decrease in nonheme mitochondrial iron levels, which were unaffected by ABCB10 downregulation (Online Figure IIE). It is important to note that mitochondrial heme levels were reduced, not increased, by ABCB10 knockdown, in contrast to the commonly stated hypothesis that ABCB10 functions in heme export.

Consistent with a reduction in heme content, the activities of several heme-containing enzymes were decreased with ABCB10 knockdown (Figure 2A–2C), whereas their mRNA and protein levels remained unchanged (Online Figure IIIA and IIIB). However, we observed no change in the activities or mRNA levels of heme-containing enzymes with ABCB10 overexpression (Figure 2D–2F and Online Figure III), which is in agreement with unaltered heme content in these cells. Thus, our results suggest that ABCB10 regulates the heme biosynthetic pathway, but does not seem to be involved in heme export out of the mitochondria.

### Methods

**Methods for H9c2 cell culturing, lentiviral and adenoviral transduction, heme and total iron content determination, enzyme activity analyses, microscopy and ROS quantification, mRNA and protein level measurements, 14C-glycine incorporation, Ca2+ transient analysis, cell death quantification, neonatal rat cardiomyocyte (NRCM) isolation, as well as myocardial infarction (MI) surgery and tissue harvesting in mice, are described in the detailed methods provided in the online supplement. Data are expressed as mean±SEM. Statistical significance was assessed with the unpaired Student t test; *P*<0.05 was considered statistically significant.**

### Results

**ABCB10 Regulates Heme Levels in Cardiac Cells**

Studies have shown that ABCB10 is required for normal hematopoiesis possibly via heme export from the mitochondria. To test this hypothesis, we measured heme levels and total mitochondrial iron content with ABCB10 modulation in H9c2 cardiac myoblasts. We effectively downregulated ABCB10 by lentiviral transduction of short hairpin RNA (shRNA) and overexpressed this protein using adenovirus, as evidenced by Western blot (Online Figure I). Furthermore, our isolation protocol yielded highly enriched mitochondrial and cytosolic fractions (Online Figure II). Overexpression of ABCB10 had no effect on mitochondrial or total cellular heme levels in H9c2 cells (Figure 1A and 1B). However, cellular and mitochondrial heme content was significantly reduced by ABCB10 knockdown in H9c2 cells (Figure 1C and 1D) and in NRCM (Online Figure IIB–IID). The reduction in heme was not caused by a decrease in nonheme mitochondrial iron levels, which were unaffected by ABCB10 downregulation (Online Figure IIE). It is important to note that mitochondrial heme levels were reduced, not increased, by ABCB10 knockdown, in contrast to the commonly stated hypothesis that ABCB10 functions in heme export.

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ABCB10 Does Not Alter Levels of Heme Synthetic or Degrading Enzymes

To determine how knockdown of ABCB10 reduces heme content, we measured the levels of enzymes catalyzing each of the 8 steps in the heme biosynthesis pathway. The mRNA and protein levels of ALAS1, the rate-limiting enzyme in heme synthesis, were not altered with ABCB10 knockdown (Figure 3A and 3B; Online Figure IVA). In addition, there was a small increase in mRNA levels of uroporphyrinogen decarboxylase and protoporphyrinogen oxidase and no difference in mRNA levels of other heme synthetic enzymes (Online Figure IVA). Similarly, there was no change in the expression of ALAS1 and most of the other heme synthetic enzymes with ABCB10 overexpression (Online Figure IVB). Finally, we detected no difference in the levels of heme oxygenase 1 and 2 with ABCB10 expression (Online Figure IVC). Finally, we detected no difference in the levels of other heme synthetic enzymes (Online Figure IV A).

We next hypothesized that ABCB10 may play a role in the early mitochondrial steps of heme biosynthetic pathway, namely the production of ALA by ALAS1 or export of ALA from the mitochondria. This hypothesis predicts that addition of exogenous ALA would rescue the heme defect observed with ABCB10 knockdown. Consistently, the reduction in total cellular and mitochondrial heme levels with ABCB10 knockdown (Figure 1C) was completely reversed this defect (Figure 3D). However, incorporation of Fe into the PPIX ring was unaffected by ABCB10 knockdown (Figure 3E), arguing against the hypothesis that ABCB10 is involved in the last steps of heme synthesis.

ABCB10 Does Not Alter Levels of Heme Synthetic or Degrading Enzymes

To test this, we assessed the effects of ABCB10 knockdown on incorporation of Fe into PPIX by supplementing cells with ALA to enhance heme production and measuring radioactivity in the organic fraction containing heme, as described. Consistent with our earlier observations, ABCB10 downregulation reduced total cellular porphyrin levels (Figure 3C), and addition of ALA completely reversed this defect (Figure 3D). However, incorporation of Fe into the PPIX ring was unaffected by ABCB10 knockdown (Figure 3E), arguing against the hypothesis that ABCB10 is involved in the last steps of heme synthesis.

ABCB10 Facilitates ALA Production or Export From the Mitochondria

ABCB10 Modulates ALA Export

A recent study showed that ABCB10 transiently interacts with ferrochelatase, the last enzyme in the heme biosynthetic pathway. Thus, we hypothesized that ABCB10 may play a role in the incorporation of iron into the PPIX ring to form heme. To test this, we assessed the effects of ABCB10 knockdown on incorporation of Fe into PPIX by supplementing cells with ALA to enhance heme production and measuring radioactivity in the organic fraction containing heme, as described. Consistent with our earlier observations, ABCB10 downregulation reduced total cellular porphyrin levels (Figure 3C), and addition of ALA completely reversed this defect (Figure 3D). However, incorporation of Fe into the PPIX ring was unaffected by ABCB10 knockdown (Figure 3E), arguing against the hypothesis that ABCB10 is involved in the last steps of heme synthesis.

Figure 1. ATP-binding cassette (ABC) B10 regulates heme levels in cardiac myoblasts.

Cellular (A) and mitochondrial (B) heme content with ABCB10 overexpression in H9c2 cells (n=6). Cellular (C) and mitochondrial (D) heme levels with ABCB10 knockdown in H9c2 cells (n=6). Data are presented as mean±SEM. *P<0.05 vs control. Ad-ABC10 indicates adenovirus encoding ABCB10 protein; Ad-GFP, adenovirus encoding green fluorescent protein; and shRNA, short hairpin RNA.

Figure 2. Heme-containing enzyme activities are reduced by ATP-binding cassette (ABC) B10 knockdown.

Catalase (A), peroxidase (B), and mitochondrial complex IV (C) activities in H9c2 with ABCB10 knockdown (n=3–6). Data are presented as mean±SEM. *P<0.05 vs control. Ad-ABC10 indicates adenovirus encoding ABCB10 protein; Ad-GFP, adenovirus encoding GFP; and shRNA, short hairpin RNA.

Figure 3. ABCB10 does not alter heme synthetase expression.

Cellular (A) and mitochondrial (B) heme content with ABCB10 knockdown in H9c2 (n=6). Data are presented as mean±SEM. *P<0.05 vs control. Ad-ABC10 indicates adenovirus encoding ABCB10 protein; Ad-GFP, adenovirus encoding green fluorescent protein; and shRNA, short hairpin RNA.
and 1D) was completely abolished by ALA supplementation (Figure 4A and 4B), and the difference in heme content between ABCB10 shRNA and control shRNA groups was no longer significant. Similar results were obtained in ABCB10 shRNA-transfected cells. Accordingly, ABCB10 knockdown resulted in a reduced

in ALA-treated cells transduced with ABCB10 shRNA lentivirus (Figure 4C–4E). To confirm these findings, we measured incorporation of 55Fe into protoporphyrin IX (PPIX), with ABCB10 knockdown in H9c2 cells. Cells are treated with ALA to promote porphyrin synthesis and incubated with 55Fe, followed by quantification of 55Fe saturation of PPIX in the organic fraction containing porphyrins (n=6). Data are presented as means±SEM. *P<0.05 vs control. shRNA indicates short hairpin RNA.

Figure 3. ATP-binding cassette (ABC) B10 does not regulate heme synthesis. A, Protein levels of δ-aminolevulinic acid synthase 1 (ALAS1), the rate-limiting enzyme in heme synthesis. B, Densitometry analysis of ALAS1 Western blots (n=6). Total porphyrin levels with ABCB10 knockdown alone (C) or with ABCB10 knockdown and ALA supplementation (D: n=6). E, Incorporation of 55Fe into protoporphyrin IX (PPIX), with ABCB10 knockdown in H9c2 cells. Cells are treated with ALA to promote porphyrin synthesis and incubated with 55Fe, followed by quantification of 55Fe saturation of PPIX in the organic fraction containing porphyrins (n=6). Data are presented as means±SEM. *P<0.05 vs control. shRNA indicates short hairpin RNA.

Figure 4. ATP-binding cassette (ABC) B10 promotes mitochondrial δ-aminolevulinic acid (ALA) export. Cellular (A) and mitochondrial (B) heme levels in ABCB10 knockdown cells incubated with 1.2 mmol/L ALA for 6 hours (n=6–18). ALA treatment reversed the decrease in heme levels associated with ABCB10 knockdown (as shown in Figure 1A and 1B). C–E, Activities of heme-containing enzymes in cells with ABCB10 knockdown after supplementation with ALA (n=6–6). ALA supplementation restored the activities of heme-containing enzymes to control levels in ABCB10 knockdown cells. F, Cellular heme levels with ABCB10 knockdown and supplementation with 500 μmol/L methyl succinate (m-succinate) or vehicle (n=6). G, Cellular heme levels with overexpression of zebrafish ALAS2 (zALAS2) and knockdown of ABCB10 (n=6). Data are presented as means±SEM. *P<0.05 vs control, #P<0.05 vs zALAS2 overexpressing control short hairpin RNA (shRNA)-transfected cells.
incorporation of radioactive glycine into heme that was measured in the organic phase (Online Figure IVE).

One potential explanation for the defect in heme synthesis with ABCB10 shRNA and its reversal by the addition of exogenous ALA is a reduction in mitochondrial bioenergetic function as a result of increased ROS levels and subsequent deficiency in succinyl CoA, a necessary substrate for the synthesis of ALA and heme. To address this possibility, we supplemented cells with membrane-permeable methyl succinate and measured the effects of ABCB10 knockdown on heme production. The addition of methyl succinate led to a small but significant increase in heme levels in the control cells, but failed to restore heme content in ABCB10 shRNA cells (Figure 4F), suggesting that the defect observed with ABCB10 knockdown is not because of succinyl CoA deficiency.

As ABCB10 knockdown is associated with a defect in hemo-globinization and differentiation of erythropoietic lineage both in vitro and in vivo, we assessed the effects of ALA supplementation on these parameters in MEL cells with ABCB10 knockdown. We used small interfering RNA (siRNA) approach to knock down ABCB10 in MEL cells and achieved a 50% reduction in its mRNA and protein levels (Online Figure VA and VB). Consistent with our hypothesis, ABCB10 knockdown reduced both heme content and differentiation of MEL cells (Online Figure VC and VE), whereas addition of exogenous ALA reversed these defects (Online Figure VD and VE). These findings suggest that ABCB10 also facilitates mitochondrial ALA synthesis or export in erythroid progenitor cells, and this function of ABCB10 is necessary for erythrocytic differentiation.

ALAS2 Overexpression Does Not Restore Heme Levels With ABCB10 Knockdown

Finally, to show that ABCB10 functions downstream of ALA synthesis step, we measured heme levels with simultaneous knockdown of ABCB10 and overexpression of ALAS2, the mitochondrial rate-limiting enzyme that combines glycine and succinyl-CoA to form ALA. We successfully overexpressed zebrafish ALAS2 using a lentiviral vector (Online Figure VIA and VIB), which resulted in a significant increase in total cellular heme content (Online Figure VIC). However, we observed no increase in heme levels with zebrafish ALAS2 overexpression in cells treated with ABCB10 siRNA (Figure 4G). Overexpression of zebrafish ALAS2 in NRCM similarly increased cellular heme levels in control shRNA group, but failed to rescue the heme defect in ABCB10 shRNA group (Online Figure VID–VIF). These data, together with our finding that ABCB10 knockdown or overexpression does not alter the mRNA and protein levels of ALAS1, suggest that ABCB10 functions to facilitate ALA export, rather than to regulate production of ALA by ALAS1 enzyme.

ABCB10 Is Important for Cardiomyocyte’s Response to Hypoxia

We recently showed that hypoxia increases heme production in the heart via upregulation of the rate-limiting enzyme, ALAS2, catalyzing mitochondrial synthesis of ALA. The increased rate of ALA synthesis would require higher export capacity to efficiently deliver this intermediate into the cytosol. Consistently, mRNA and protein levels of ABCB10 were higher in H9c2 cells subjected to 48 hours of 1% O₂ than in cells grown in normoxia (Figure 5A). Treatment of cells with ABCB10 siRNA completely reversed hypoxic induction of this

Figure 5. Hypoxic upregulation of ATP-binding cassette (ABC) B10 is required for cell survival. A. Western blot analysis showing that ABCB10 is upregulated by hypoxia in H9c2 cells, and this increase is prevented by treatment of hypoxic cells with ABCB10 small interfering RNA (siRNA). B. Heme levels in H9c2 subjected to 48 hours of hypoxia (1% O₂) or normoxia, with or without suppression of hypoxic ABCB10 upregulation by siRNA (n=6). C. Mitosox staining (red) of H9c2 cells with and without reversal of ABCB10 upregulation by siRNA subjected to 48 hours of hypoxia followed by 30 minutes of reoxygenation. Nuclei are counterstained with Hoescht (blue). Representative images are shown on the left, and quantification is presented on the right (n=8, 4 fields per sample). Cell death as assessed by propidium iodine (PI) and annexin V staining and quantified by flow cytometry in H9c2 cells with and without ABCB10 siRNA treatment subjected to hypoxia–reoxygenation injury as in C (n=8); D) or with additional incubation with 200 μmol/L hydrogen peroxide for 12 hours in hypoxia before reoxygenation (n=6); E) Data are presented as mean±SEM. *P<0.05 vs control.
protein (Figure 5A) and abolished the hypoxia-mediated increase in heme (Figure 5B). These findings suggest that upregulation of ABCB10 is needed to support the increase in heme synthesis in hypoxia, and inhibition of ABCB10 upregulation is sufficient to halt heme production under hypoxic conditions.

To determine whether hypoxia-driven upregulation of ABCB10 bears physiological consequences, we assessed ROS levels and cell death in cardiac cells subjected to 48 hours of hypoxia, followed by 30 minutes of reoxygenation with and without knockdown of ABCB10. Failure to upregulate ABCB10 in hypoxia as a result of siRNA treatment was associated with increased oxidative stress, as determined by the superoxide-sensitive dye MitoSox (Figure 5C). The accumulation of MitoSox in the mitochondria was not caused by changes in mitochondrial membrane potential, as tetramethylrhodamine, ethyl ester staining did not differ between the groups (Online Figure VIG). Furthermore, cell death was significantly increased in hypoxic cells in which ABCB10 upregulation was suppressed (Figure 5D) and further augmented by addition of hydrogen peroxide as a source of oxidative stress (Figure 5E). Thus, in contrast to forced overexpression of ABCB10 in normoxia which does not alter heme synthesis, physiological induction of ABCB10 in hypoxia is necessary to support increased rates of heme synthesis, and failure to maintain adequate ABCB10 levels compromises cell survival.

**ABCB10 Levels Are Increased in Cardiomyopathy**

To determine whether ABCB10 expression is altered in ischemic heart disease, we measured the levels of this protein in the hearts of mice subjected to MI. We observed a progressive increase in ABCB10 expression in the heart after MI compared with sham-operated controls, with >2-fold elevation in ABCB10 protein levels 1 month after MI (Figure 6A). The increase in ABCB10 protein was associated with higher cellular and mitochondrial heme levels in mouse hearts 1 month after MI (Figure 6B and 6C). Furthermore, there was also an increase in ABCB10 content in the explanted hearts from human patients with ischemic cardiomyopathy compared with the nonmyopathic human hearts (Figure 6D), which was paralleled by increased heme levels in myopathic hearts (Figure 6E).

Finally, to determine whether ABCB10 has an effect on cardiomyocyte physiology, we assessed Ca\(^{2+}\) handling in NRCM with ABCB10 knockdown. In the absence of electric pacing, fewer cells in the ABCB10 siRNA group were beating spontaneously compared with the control group (17/27 versus 9/24 spontaneously beating total cell count for control and ABCB10 siRNA, respectively). Analysis of Ca\(^{2+}\) transients revealed no difference in the magnitude of transient (\(F/F_0\)) and rise time, but a significant prolongation of the decay time (Figure 6F–6H), indicative of impaired Ca\(^{2+}\) removal from the cytoplasm after systolic influx. Consistent with a calcium removal defect, we

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**Figure 6. ATP-binding cassette (ABC) B10 is upregulated in the ischemic heart and modulates Ca\(^{2+}\) transients.** A, ABCB10 protein levels in sham-operated mice and mice subjected to myocardial infarction (MI) injury 1 week and 1 month after the surgery. Densitometric analysis is presented below the Western blot (n=3–4). Total cellular (B) and mitochondrial (C) heme levels in mouse hearts 1 month after MI or sham operation (n=3). D, ABCB10 protein levels in explanted human hearts with ischemic cardiomyopathy or in noncardiomyopathic control human hearts. Densitometric analysis is shown on the right. E, Heme levels in myopathic and nonmyopathic human hearts (n=10). Analysis of Ca\(^{2+}\) transients in neonatal rat cardiomyocytes with ABCB10 and control small interfering RNA (siRNA); representative images (F), \(F/F_0\) as a measure of transient magnitude (n=10–11 cells from 3 coverslips; G), and rise and decay times (n=15 cells from 4 coverslips; H). Data are presented as mean±SEM. *P<0.05 vs control. ICM indicates ischemic cardiomyopathy; and NM, nonmyopathic.
observed a significant reduction in protein levels of sodium–calcium exchanger in NRCM treated with ABCB10 siRNA, compared with control (Online Figure VII).

In summary, we showed that ABCB10 facilitates ALA export from the mitochondria into the cytosol and is required for heme synthesis. ABCB10 is positively regulated by hypoxia, and failure to increase the levels of this protein results in increased oxidative stress and loss of cell viability after hypoxia–reoxegenation injury. Finally, low ABCB10 levels are associated with slower Ca\(^{2+}\) removal from the cell, and upregulation of ABCB10 in ischemic myocardium may help to protect cardiac function.

**Discussion**

The production of heme requires efficient shuttling of synthetic intermediates between mitochondrial and cytosolic compartments, but the transporters mediating these events are not known.\(^6,31\) In this report, we show for the first time that ABCB10 plays a role in early mitochondrial steps of heme synthesis, likely by facilitating ALA export out of the mitochondria, and is important for maintenance of cellular heme homeostasis in cardiac cells. Knockdown of ABCB10 in cardiac myoblasts reduces heme levels and the activities of heme-containing proteins, whereas addition of exogenous ALA completely reverses these defects. However, increased production of ALA in the mitochondria by overexpression of the rate-limiting enzyme ALAS2 or supplementation with methyl succinate, a precursor of succinyl-CoA, fails to restore heme levels. These results suggest that ABCB10 may be involved in ALA export out of the mitochondria (Figure 7).

Our results demonstrate that ABCB10 knockdown reduces heme synthesis in 3 distinct cell lines, nonerythroleukemic H9c2, NRCM, and erythropoietic MEL cells, providing first evidence that hemoglobinization defect observed in ABCB10 knockout mice may be because of reduced cytosolic ALA availability and impaired heme synthesis. However, the exact function of ABCB10 in hematopoiesis and the ability of ALA supplementation to reverse embryonic lethality of ABCB10 knockout mice warrant in-depth investigation. In addition, the finding of elevated heme content in mouse MI hearts indicates a novel role for ABCB10 and heme in cardiac pathology. Given that heme synthesis pathway is stimulated by hypoxia, inability to upregulate ABCB10 and provide adequate export capacity for ALA would result in ALA trapping inside the mitochondria. Because ALA is a prooxidant molecule,\(^32\) increased cell death after hypoxia–reoxegenation injury may be secondary to the oxidative stress caused by ALA accumulation inside the mitochondria.

Our studies show that ABCB10 is needed for the cytosolic steps of heme synthesis, localizing ABCB10 function to the first 2 mitochondrial steps of the pathway: ALA production or export. The first possibility is less likely, given that ALAS1 mRNA and protein levels are unchanged with ABCB10 knockdown. Furthermore, overexpression of zebrafish ALAS2, whose sequence and regulation are distinct from that of cardiac-specific ALAS1 isoform, fails to rescue the defect in heme observed with ABCB10 knockdown. However, we cannot exclude the possibility of a primary defect in mitochondrial function, mediated by oxidative stress or other factors, which would impede ALA synthesis by inhibiting the activity of ALAS1/2 enzymes as reported previously.\(^33\) Furthermore, although our results suggest that ABCB10 may facilitate ALA export out of the mitochondria, they do not establish ABCB10 as the actual ALA exporter. Although Graf et al.\(^22\) concluded that ABCB10 exists exclusively in the form of homodimers and higher-order homo-oligomeric complexes, the possibility still exists that ABCB10 transiently/noncovalently interacts with yet another transporter that functions to export ALA. Recent reports of interactions among ABCB10, Mfrn1, and ferrochelatase,\(^21,25\) as well as ferrochelatase and ABCB7,\(^16\) highlight the complexity of protein–protein interactions occurring at the inner mitochondrial membrane. Finally, the direct measurement of ALA export out of the isolated mitochondria could not be performed using an earlier-established method for measurement of mitochondrial export activity\(^20\) because radioactive labeling of ALA precursors (succinyl CoA and glycine) will result in their incorporation into a variety of other cellular molecules.

ABCB10 knockout mice die in utero and ABCB10\(^{-/-}\) erythroid cells fail to differentiate as a result of a defect in hemoglobinization and ROS-mediated increase in apoptosis.\(^36\) Furthermore, ABCB10 heterozygous mice display increased susceptibility to ischemia–reperfusion injury of the heart because of an acute increase in oxidative stress triggered by ischemia–reperfusion,\(^27\) although the source of ROS has not been identified yet. We also show that knockdown of ABCB10 in cardiac myoblasts leads to increased ROS production and is associated with reduced activities of heme-containing antioxidant enzymes, peroxidases and catalases. Thus, it is tempting to speculate that the potential mechanism for the increase in ROS observed with ABCB10 deletion/knockdown may be through a decrease in the antioxidant capacity of the cells caused by reduced availability of heme, although mitochondrial accumulation of prooxidant ALA may contribute to and further exacerbate the damage. In addition to increases in oxidative stress and cell death, we show that ABCB10 knockdown impairs Ca\(^{2+}\) handling by prolonging transient decay time. Although the exact mechanism for this finding is yet to be elucidated, we show that sodium–calcium exchanger protein levels are reduced by ABCB10 knockdown, consistent
with previous findings of sodium–calcium exchanger being sensitive to oxidative stress.34,35 Furthermore, sarcoplasmic reticulum Ca2+ ATPase 2 is thought to play an important role in Ca2+ removal during the diastolic phase. Sarcoplasmic reticulum Ca2+ ATPase 2 is exquisitely sensitive to oxidative damage, undergoing multiple posttranslational modifications, including oxidation and sulfenylation of cystines, which impair its activity and cause prolongation of decay phase of the transient.36,37 Interestingly, Liesa et al7 found sarcoplasmic reticulum Ca2+ ATPase 2 activity to be significantly reduced in the hearts of ABCB10 heterozygous mice after ischemia–reperfusion injury, which is supportive of our data. Disruption of Ca2+ transients by ABCB10 knockdown suggests that changes in the levels of this protein may indirectly influence cardiac contractility.

Overexpression of ABCB10 in cardiac myoblasts did not increase heme content, consistent with previous reports and with the rate-limiting role of ALAS1 in heme synthesis,7 whereas knockdown of ABCB10 significantly reduced heme levels. This raises a question about physiological significance of ABCB10 upregulation in the mouse MI model and in human hearts with ischemic cardiomyopathy. Important to note, however, that in the former set of experiments ABCB10 levels were increased artificially through forced adenoviral overexpression. As shown in Online Figure IVB, ABCB10 overexpression did not alter the levels of heme synthetic enzymes. Although ALA export capacity was increased in this setting, the levels of ALAS1, the enzyme required for ALA production in the mitochondria, remained unchanged. Thus, one would not expect heme levels to be altered by forced overexpression of ABCB10. However, upregulation of ABCB10 in hypoxia and ischemic hearts parallels overall induction of heme synthetic pathway and heightened requirements for ALA export. We reported earlier that heme synthesis is increased in ischemic human failing hearts and in cardiac cells subjected to hypoxia through upregulation of ALAS2.19 In this setting, upregulation of ABCB10 is required to support increased demand for ALA transport. Consistently, we show that suppression of ABCB10 upregulation by hypoxia is sufficient to repress heme production and leads to increased cell death. Our conclusions are supported by Shirihai et al18 who also showed that forced overexpression of ABCB10 does not increase hemoglobinization of undifferentiated MEL cells in which heme synthesis pathway is inactive. However, ABCB10 overexpression, together with induction of differentiation (which is a strong stimulus for increased heme production), promotes hemoglobinization.20 Thus, overexpression of ABCB10 facilitates heme synthesis when other steps in heme synthesis (particularly those needed for ALA production) are also active.

A recent study showed that ABCB10 binds to and stabilizes Mfrn1 in MEL cells,25 although this interaction has not yet been confirmed in the heart. Thus, ABCB10 knockdown is expected to reduce mitochondrial iron levels through destabilization of Mfrn1, which we failed to observe in our studies. Mfrn1 is expressed in low levels in the heart, whereas Mfrn2 is the predominant isofrom responsible for the majority of iron imported into the mitochondria.7,9 Given that ABCB10 does not regulate expression or stability of Mfrn2,25 reduction in Mfrn1 with ABCB10 loss is unlikely to alter mitochondrial iron uptake in cardiac myoblasts. Finally, a weak interaction between ABCB10 and ferrochelatase has been reported in hematopoietic cells21 but has not been studied in other cell types. We show that ABCB10 knockdown in cardiac cells does not affect ferrochelatase levels or activity, as we observed no change in iron incorporation into PPIX. Consistent with our findings, Chen et al22 also report no difference in iron incorporation into PPIX, with coexpression of Mfrn1 and ABCB10 in MEL cells. Thus, the relevance of ferrochelatase–ABCBO10 interaction remains to be determined.

In summary, we have shown that knockdown of ABCB10 causes a reduction in cytosolic and mitochondrial heme levels, possibly as a result of reduced cytosolic ALA levels. Importantly, our findings are contrary to the commonly held hypothesis that ABCB10 functions in mitochondrial heme export, which would predict increased mitochondrial and reduced cytosolic heme levels because of mitochondrial trapping of heme with ABCB10 knockdown.

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Disclosures

None.

References

Novelty and Significance

What Is Known?

- ATP-binding cassette protein (ABC) B10 is a mitochondrial ABC protein with no identified function.
- A portion of heme synthesized in the mitochondria is exported into the cytoplasm for incorporation into cytosolic heme-containing proteins.
- ABCB10 was suggested to be involved in heme synthesis, possibly by exporting heme out of mitochondria.

What New Information Does This Article Contribute?

- ABCB10 is not a mitochondrial heme exporter.
- ABCB10 promotes early steps of heme synthesis in the mitochondrion.
- ABCB10 facilitates the export of δ-aminolevulinic acid from the mitochondria to the cytosol for subsequent synthetic steps.

Heme is an essential cofactor of proteins involved in gas transport, energy production, and antioxidant defense. However, our understanding of the heme biosynthetic pathway remains incomplete. Production of heme requires coordinated movement of synthetic intermediates between mitochondria and cytosol. ABCB10 is a mitochondrial transporter that was previously suggested to participate in heme export out of this organelle. In contrast to this view, our data indicate that knockdown of ABCB10 is required for efficient red blood cell development. Proc Natl Acad Sci U S A. 2012;109:4152–4157.


ATP-Binding Cassette B10 Regulates Early Steps of Heme Synthesis
Marina Bayeva, Arineh Khechaduri, Rongxue Wu, Michael A. Burke, J. Andrew Wasserstrom, Neha Singh, Marc Liesa, Orian S. Shirihai, Nathaniel B. Langer, Barry H. Paw and Hossein Ardehali

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SUPPLEMENTAL METHODS

Cell culture and reagents

H9c2 cardiac myoblasts were purchased from ATCC and kept in complete DMEM medium (ATCC, VA) supplemented with 10% FBS (Invitrogen, CA) and 1% penicillin-streptomycin (P/S). MEL cells were grown in suspension in complete RPMI-1640 (Cellgro, VA) medium supplemented with L-glutamine, 10% FBS and 1% P/S. Isolation and culture of neonatal rat cardiomyocytes (NRCM) was performed as described previously.1

Adenoviral and Lentiviral Transduction of Cells

A recombinant adenoviral vector encoding GFP and human ABCB10 cDNA, separated by the polio virus internal ribosome entry site (IRES) element and under the transcriptional control of CMV promoter, was constructed. Briefly, ABCB10 cDNA was cloned into the adenovirus shuttle vector pAdCGI to make the vector designed pAdCGI-ABCB10. A 25 cm² flask (T25; Sarstedt, Newton, NC) of CRE8 cells was cotransfected with 2.1 μg of y5 viral packaging plasmid and 2.1 μg of shuttle vector plasmid using Lipofectamine Plus (Invitrogen). Cells were incubated 5–9 days until cytopathic effects were observed. Cells and supernatant were collected and subjected to three cycles of freeze-thaw. Following centrifugation, 2 ml of the vector-containing supernatant was added to a 90% confluent T25 and returned to the incubator until cytopathic effects were observed. This procedure was repeated three to four times after which the vector was plaque purified and expanded. Large-scale vector preparations were purified on a cesium chloride gradient, as described.3 Virus titers were determined by plaque assays. The insertion of ABCB10 cDNA was confirmed by sequencing the vector genome, and the vector was functionally validated by the expression of GFP and ABCB10. NRCMs were transduced on day 6 after isolation, and were imaged 2 days following transduction by a laser scanning confocal microscope (UltraVIEW; PerkinElmer), using a 403 water-immersion lens and
X40 optical zoom. Western blot analysis was also performed to assess gene expression. Lentivirus for shRNA-mediated knockdown of ABCB10 was purchased from Open Biosystems (cat #: V2LMM_5302) and non-silencing shRNA was used for control experiments. For ALAS2 overexpression experiments, zebrafish ALAS2 cDNA (GenBank #NM_131682) were cloned into pMMPA-HA retroviral vectors as described.\(^4\) Viral transduction of H9c2 cells was carried out in complete medium for 48 hours.

**MEL Cell Differentiation and siRNA treatment**

On the day of differentiation induction 1 x 10\(^5\) to 1 x 10\(^6\) cells/mL were collected by centrifugation and transfected with siGENOME SMARTpool siRNA against mouse ABCB10 (Dharmacon, CO) using DharmaFECT 1 siRNA transfection reagent (Dharmacon, CO) according to the manufacturer’s protocol. Cells were incubated with the transfection mix for 6 hours and differentiation was initiated by addition of complete medium containing 2% DMSO and 5mM hexamethylene bisacetamide (HMBA, Sigma-Aldrich, USA). To maintain efficient ABCB10 knockdown, the transfection was repeated on Day 3 of differentiation, and the differentiation medium was replaced. Cells were collected five days after induction of differentiation and knockdown of ABCB10 was confirmed by qRT-PCR and Western blot analyses. Differentiation was assessed by staining cells with benzidine and counting blue (differentiated) and unstained (undifferentiated) cells.

**Western Blot**

Fifteen-30 μg of protein were resolved on SDS-PAGE gels and transferred to nitrocellulose membranes (Invitrogen, CA). The membranes were probed with antibodies against ABCB10, ALAS1/2 (Abcam, MA), ferrochelatase (Proteintech,IL), GAPDH (Santa Cruz, CA), NCX (Swant, Switzerland) and tubulin (Abcam, MA). HRP-conjugated donkey anti-rabbit and donkey anti-
mouse were used as secondary antibodies (Santa Cruz, CA) and visualized by Pierce SuperSignal Chemiluminescent Substrates.

**Quantitative RT-PCR**

RNA was isolated with RNA STAT-60 (TEL-TEST, Inc, TX), reverse-transcribed with a Random Hexamer (Applied Biosystems, CA), and amplified on a 7500 Fast Real-Time PCR system with SYBR Green PCR Master Mix (Applied Biosystems, CA). Primers were designed using Primer3 (v. 0.4.0) software to target sequences spanning an exon-intron-exon boundary and their specificity was confirmed by running a dissociation curve. mRNA levels were calculated by the comparative threshold cycle method and normalized to β-actin gene.

**Heme Content Determination**

For determination of cellular heme levels, cells were lysed in 1% Triton-X100 in TBS, followed by centrifugation at 5,000 x g for 10 minutes to remove debris. For heme content in mouse hearts, ~5 mg of frozen tissue was homogenized in 1% Triton-X100 in TBS and centrifuged at 5,000 x g for 10 minutes to remove debris. For determination of mitochondrial heme levels, mitochondrial fraction was isolated using Mitochondrial Isolation Kit for Cultured Cells or Tissue (Pierce) according to the manufacturer’s protocol. Protein concentration of cellular or mitochondriallysate was quantified by BCA assay (Pierce, IL) and heme was quantified as described. Briefly, equal amounts of protein were mixed with 2M oxalic acid, heated to 95˚C for 30 minutes to release iron from heme and generate protoporphyrin IX. Samples were then centrifuged for 10 min at 1,000 x g at 4˚C to remove debris, the fluorescence of the supernatant was assessed at 405nm / 600nm on Spectra Max Gemini fluorescence microplate reader and normalized to protein concentration of each sample.

**Mitochondrial ⁵⁵Fe Analysis**
$^{55}$Fe (Perkin-Elmer, MA) was conjugated to nitriloacetic acid (NTA, Sigma-Aldrich, USA) and dissolved to the final concentration of 150-250 nM in complete, serum-containing medium. H9c2 cells were grown until 80% confluent, followed by modulation of ABCB10 levels and incubation in $^{55}$Fe containing-medium for 48 hours. Cells were then washed once with cold PBS and twice with 500 μM bathophenanthrolinedisulfonate (BPS, Sigma, USA) in PBS to remove membrane-associated radioactivity. Mitochondrial fraction was isolated using the Mitochondrial Isolation Kit for Cultured Cells (Pierce), washed once with 500 μM BPS in PBS to remove residual $^{55}$Fe, and resuspended in 1% Triton-X100 in TBS. The radioactivity of mitochondrial fraction was analyzed on Beckman scintillation counter and normalized to the protein concentration of each sample determined by BCA assay.

**Total Porphyrin Content Quantification**

The assay was modified from Sorte et al. Briefly, cells were resuspended in 1% Triton X-100 in TBS and centrifuged for 5 min at 5,000 x g to remove debris. Supernatant was collected, mixed with one part of Ehrlich’s reagent and two parts of saturated sodium acetate solution. The fluorescence was measured at 405 nm on Spectra Max Gemini fluorescence microplate reader and normalized to protein concentration of each sample. Porphobilinogen (Sigma-Aldrich, USA) was used to generate the standard curve.

**Assessment of $^{55}$Fe and $^{14}$C-glycine Incorporation into Heme**

Following lentiviral treatment, cells were incubated with 1.2mM δ-aminolevulinic acid (ALA) and 200 nM $^{55}$Fe-NTA for 8 hours, washed once with PBS and twice with 500μM BPS in PBS to remove membrane-associated $^{55}$Fe, and lysed. For $^{14}$C-glycine incorporation into heme, complete growth medium was supplemented with 10 miCi/mL $^{14}$C-glycine followed by 3-hour incubation, three washes with PBS to remove radioactivity, and cell lysis. Protein content of each sample was determined by BCA assay. Next, one part of 0.1M HCl was added to 5 parts
of cell lysate, followed by mixing with an equal volume of ethylacetate: acetic acid (3:1) and vortexing. Fractions were separated by centrifugation at 15,000 x g for 5 min and the radioactivity in the organic fraction containing heme was determined by scintillation counting and normalized to protein concentration of each sample.

**Enzyme Activities**

Complex IV activity was measured using the Sandwich ELISA Kits – Dipsticks assay (MitoSciences) according to the manufacturer’s protocol. Peroxidase activity was assessed with the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, CA) as absorbance at 560nm and normalized to protein concentration of each sample. Catalase activity was determined using the Catalase Assay Kit (Abcam) in accordance with manufacturer’s guidelines as absorbance at 570nm, and normalized to protein content.

**Hypoxia-Reoxygenation Treatment**

H9c2 cells were transfected with ABCB10 or control siRNA as described above, followed by incubation in a humidified hypoxic chamber set at 1%O₂, 5%CO₂, at 37°C for 48 hours, and 30 minutes of reoxygenation at 21%O₂ and 5%CO₂ at 37°C. To induce additional oxidative stress, cells were treated with 200μM H₂O₂ for 12 hours prior to reoxygenation, where indicated.

**Mitochondrial ROS and Membrane Potential Quantification**

MitoSox Red (Invitrogen) was used to assess mitochondrial O₂⁻ production and TMRE staining was employed as a marker of mitochondrial membrane potential. Cells were visualized by confocal microscopy and ROS levels were quantified by ImageJ software. Four fields per each sample were obtained and averaged. Nuclei were counterstained with Hoescht and subtracted from the total Mitosox fluorescence to exclude the dye which localized to the nucleus.
Assessment of Cell Death

Following hypoxia-reoxygenation, cells were collected by trypsinization and labeled with propidium iodine (Sigma-Aldrich, USA) and Alexa Fluor® 350-conjugated Annexin V (Molecular Probes, NY). Cell death was analyzed by flow cytometry in a FacsCanto flow cytometer (BD Bioscience).

Analysis of Calcium Transients

NRCM were cultured on glass cover slips followed by transfection with ABCB10 or control siRNA. Cells were loaded with 10μM fluo-4AM for 20 minutes. Intracellular Ca^{2+} transients were measured at 36°C via linescan imaging using a Zeiss LSM510 confocal microscope. Fluorescence emission was measured through a 40x water immersion objective (Apochromat, NA 1.2) at wavelengths >520nm during excitation at 488nm with a 25mW argon laser (<10% laser power). Linescan imaging was recorded in spontaneously contracting cells and in cells paced at BCL=2000msec at equilibrium. Calcium transients were recorded from 10-15 cells on 4 separate coverslips in each group and F/F0, transient rise and decay times were analyzed using Zeiss LSM Examiner and ClampFit (Molecular Devices) software.

Human Samples

Tissue samples were obtained from the tissue bank at Feinberg Cardiovascular Research Institute (Northwestern University) and consisted of samples from non-failing (n=10) and failing ischemic (n=10) human hearts. Failing ischemic tissues were obtained from the explanted hearts of cardiac transplant recipients. Non-failing heart tissue samples were obtained from unmatched organ donors whose hearts were unsuitable for transplantation but had no known cardiac disease. Explanted hearts were immediately placed in cold cardioplegic solution and subsequently frozen in liquid nitrogen. Protocols for tissue procurement were approved by the Institutional Review Board of the Northwestern University. Informed consent
was obtained from all transplant patients and from the families of organ donors before tissue collection.

**Myocardial Infarction and Tissue Harvesting**

Myocardial infarction without reperfusion was induced in 10-12 week-old wild type C57 black mice by permanent ligation of the left coronary descending artery with a silk suture as previously described. Anesthesia was induced with 2% isoflurane and maintained with 1.5% isoflurane throughout the procedure. Disruption of blood flow was confirmed by the pallor of left ventricle, as well as ST segment elevation and QRS complex widening on ECG, followed by chest closure in layers. In sham-operated animals the chest wall was surgically opened and closed without coronary ligation. Mice were allowed to recover for one week or one month for the MI group, and one month for the sham group. After that, mouse hearts were excised under anesthesia, washed in cold PBS and flash-frozen in liquid nitrogen. All animal studies were performed in accordance with the guidelines established by Northwestern University.
REFERENCES


Online Figure I. Overexpression and Downregulation of ABCB10 in H9c2 cells

A, ABCB10 protein in H9c2 cells transduced with control non-silencing shRNA or ABCB10 shRNA lentivirus. Densitometry analysis of the Western blot is presented on the right. B, Adenoviral overexpression of GFP and ABCB10 using various multiplicities of infection (MOI). Densitometry analysis of the Western blot is presented on the right. 5MOI dose of each virus was used in all subsequent overexpression studies. Data are presented as mean ± SEM. * p<0.05 vs. control.
Online Figure II. ABCB10 knockdown reduces heme levels in NRCM

A, Enrichment of mitochondrial and cytosolic fractions isolated from H9c2 cells was evaluated by Western blot analysis of mitochondria- and cytosol-specific proteins, cytochrome c and actin, respectively. B, Western blot analysis of ABCB10 knockdown in NRCM with using shRNA-encoding lentivirus (n=3). C,D, Cellular (C) and mitochondrial (D) heme levels in NRCM with shRNA-mediated knockdown of ABCB10 (n=6). E, Mitochondrial $^{55}$Fe content in H9c2 with ABCB10 knockdown (n=6). Mito, mitochondrial. Data are presented as mean ± SEM. * p≤0.05 vs. control.
Online Figure III. Levels of heme containing enzymes are unaffected by ABCB10 modulation

A,B qRT-PCR analysis of mRNA levels (n=6) (A) and Western blot analysis of protein levels (n=3) (B) of heme-containing enzymes with ABCB10 knockdown. C, mRNA levels of heme-containing enzymes with ABCB10 overexpression (n=6). Data are presented as mean ± SEM.
Online Figure IV. ABCB10 does not regulate heme synthesis or degradation

A,B, qRT-PCR analysis of mRNA levels of enzymes involved in heme synthesis in H9c2 with ABCB10 downregulation (A) and ABCB10 overexpression (B) (n=5-6). C, mRNA levels of heme oxygenases (HOX), enzymes involved in heme degradation, with ABCB10 shRNA (n=6). D, Cellular heme levels in NRCM supplemented with ALA with and without ABCB10 shRNA (n=6). E, Incorporation of C14-glycine into heme in H9c2 cells with and without ABCB10 knockdown (n=6). Data are presented as mean ± SEM. * p<0.05 vs. control.
Online Figure V. ABCB10 knockdown and ALA supplementation in MEL cells

A,B, mRNA (A) and protein (B) levels of ABCB10 in MEL cells following ABCB10 knockdown at day 5 of differentiation. Densitometry analysis is shown on the right. C, Cellular heme levels with ABCB10 knockdown in MEL cells (n=6). D, Cellular heme levels in MEL cells with ABCB10 knockdown following ALA supplementation (n=6). E, Effects of ABCB10 knockdown with and without ALA supplementation of MEL cell differentiation (n=6). Data are presented as mean ± SEM. * p<0.05 vs. control.
Online Figure VI. zALAS2 overexpression increases cellular heme levels at baseline, but not with ABCB10 knockdown

A, B, mRNA (A) and protein (B) levels of zALAS2 in H9c2 cells transduced with zALAS2 or GFP lentivirus. Densitometry analysis is shown below the Western blot. C, Cellular heme levels with zALAS2 overexpression (n=6). D, E, mRNA (D) and protein (E) levels of zALAS2 in NRCM (n=3-6). F, Cellular heme levels with zALAS2 overexpression in NRCM with and without ABCB10 knockdown (n=6). G, Flow cytometry analysis of TMRE intensity as an indicator of mitochondrial membrane potential in H9c2 with ABCB10 knockdown (n=6). Data are presented as mean ± SEM. * p<0.05 vs. control.
Online Figure VII. ABCB10 knockdown alters expression of a cellular calcium transporter

A, Western blot analysis of NCX expression in NRCM treated with ABCB10 or control siRNA. B, Densitometry analysis of the blot in A (n=3). Data are presented as mean ± SEM. * p<0.05 vs. control.