ATP-dependent Mitochondrial Porphyrin Importer ABCB6 Protects against Phenylhydrazine Toxicity*^S

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Background: The role of the mitochondrial ABC transporter, Abcb6, in vivo is unknown.

Results: Abcb6-null mice are incapable of ATP-dependent import of mitochondrial porphyrins. Despite compensatory changes in the porphyrin pathway, Abcb6-null mice are less viable after a porphyrin-inducing stress.

Conclusion: Abcb6 absence abolished ATP-dependent mitochondrial porphyrin uptake and deregulated porphyrin pathway genes.

Significance: Disrupted Abcb6 function may produce porphyria after certain stresses.

Abcb6 is a mammalian mitochondrial ATP-binding cassette (ABC) transporter that regulates *de novo* porphyrin synthesis. In previous studies, haploinsufficient $(Abcb6^{+/-})$ embryonic stem cells showed impaired porphyrin synthesis. Unexpectedly, Abcb6^{-/-} mice derived from these stem cells appeared phenotypically normal. We hypothesized that other ATP-dependent and/or -independent mechanisms conserve porphyrins. Here, we demonstrate that $Abcb6^{-/-}$ mice lack mitochondrial ATPdriven import of coproporphyrin III. Gene expression analysis revealed that loss of Abcb6 results in up-regulation of compensatory porphyrin and iron pathways, associated with elevated protoporphyrin IX (PPIX). Phenylhydrazine-induced stress caused higher mortality in $Abcb6^{-/-}$ mice, possibly because of sustained elevation of PPIX and an inability to convert PPIX to heme despite elevated ferrochelatase levels. Therefore, Abcb6 is the sole ATP-dependent porphyrin importer, and loss of Abcb6 produces up-regulation of heme and iron pathways necessary for normal development. However, under extreme demand for porphyrins (e.g. phenylhydrazine stress), these adaptations appear inadequate, which suggests that under these conditions Abcb6 is important for optimal survival.

ATP-binding cassette $(ABC)^2$ transporters move structurally diverse compounds across membranes. We recently demonstrated that the mitochondrial ABC transporter ABCB6 activates *de novo* heme and porphyrin biosynthesis and elevates intracellular protoporphyrin IX (PPIX), whereas the loss of one



Abcb6 allele in embryonic stem (ES) cells impairs porphyrin synthesis (1). Porphyrin synthesis requires the coordination of cellular and mitochondrial processes, as the initial steps require precursors from the Krebs cycle and the terminal step requires that ferrous iron (Fe²⁺) be delivered to the mitochondria and added via ferrochelatase (FECH) catalysis to the PPIX ring to complete heme formation. It is currently unknown whether Abcb6 is required for normal basal porphyrin synthesis or instead has a more selective role during high demand for porphyrin precursors (*e.g.* during stress erythropoiesis).

Here, we present the first evidence that Abcb6 is the only ATP-dependent mitochondrial importer of porphyrins. For example, ATP-driven import of such porphyrins such as coproporphyrin III (CP), is completely absent in the mitochondria of Abcb6^{-/-} mice, whereas non-ATP-dependent mitochondrial CP uptake is identical in $Abcb6^{-/-}$ and wild-type mice. Moreover, there is no evident functional up-regulation of other potential mitochondrial porphyrin importers, such as peripheral benzodiazepine receptor (also known as TSPO), 2-oxoglutarate carrier (also known as SLC25a11), the adenine nucleotide translocator (ANT1), and voltage-dependent anion channel, in the absence of Abcb6 (2-6). In addition, constitutive absence of Abcb6 results in increased expression of genes important for porphyrin biosynthesis (e.g. SLC48a) (7) and iron homeostasis (e.g. SLC25a37) (8) in erythroid cells. During phenylhydrazine toxicity, genes important for porphyrin and iron conservation are up-regulated in wild-type mice but not in Abcb6-null mice. Taken together, our findings suggest that although Abcb6 is not essential for basal porphyrin synthesis, it has an important role in coordinating heme and iron homeostasis during times when high porphyrin demand occurs such as during phenylhydrazine stress.

EXPERIMENTAL PROCEDURES

Generation of ES Cells Expressing Nonfunctional Abcb6—An Abcb6-targeting vector was constructed in the pKO vector by standard molecular procedures. Briefly, the 5' arm consisted of a 3.2-kb HindIII/XhoI fragment 5' of *Abcb6* exon 6 ligated into the pKONTKV1901 vector (Stratagene). The 3' arm was a

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This article contains supplemental Figs. S1–S6, Tables S1–S4, "Materials and Methods," and additional references.

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² The abbreviations used are: ABC, ATP-binding cassette; FECH, ferrochelatase; HMBA, hexamethylenebisacetamide; ZnPP, zinc-protoporphyrin; Phz, phenylhydrazine; ISC, iron-sulfur cluster; AMP-PNP, adenosine 5'-(β,γ-imino)triphosphate.

5.4-kb EcoRI fragment of *Abcb6* containing exons 16–19. The fragments were verified by DNA sequence analysis. The targeting vector was linearized with NotI and electroporated into 129/SVJ-derived ES cells. Genomic DNA from 798 ES clones that survived 2 weeks of G418 selection was screened first by PCR analysis and subsequently by Southern blot analysis.

Animals—All procedures involving animals were approved by the St. Jude IAUCAC committee. All mice were born and housed in the St. Jude Children's Research Hospital animal care facility. Mice were maintained on a standard rodent diet. In these experiments, littermates were used as controls.

Coproporphyrin III Uptake Assay-Mitochondria were isolated from livers of female mice, prepared as described (9, 10), and used immediately. Mitochondria (50–100 μ g) were resuspended in Tris-sucrose (TS) buffer (50 mM Tris-HCl, 250 mM sucrose, pH 7.4) (final volume, 50 µl). Equal volumes of reaction mix and control mix were freshly prepared for each assay. Reactions (in triplicate) were started by adding 50 μ l of reaction or control mix to each sample and incubated the time interval indicated at 37 °C. Reactions were stopped by dilution with 1 ml of ice-cold TS buffer, and samples were stored on ice. Blanks were prepared by adding mitochondria and reaction/control mix directly to 1 ml of ice-cold TS buffer and incubating on ice. Samples were centrifuged at 16,000 \times g in a refrigerated tabletop centrifuge for 5 min, and the pellet was washed twice with ice-cold TS buffer. Pellets were lysed by incubation for 10 min in 1% Nonidet P-40 solution with agitation, and fluorescence was measured at excitation wavelength 405 nm and emission wavelength 630 nm. Concentration of CP was determined from a standard curve of CP in 1% Nonidet P-40. The rate of active transport (pmol·min⁻¹ mg⁻¹) was calculated as the difference between ATP-dependent and -independent uptake.

Determination of Kinetic Constants— K_m and V_{max} values were determined for coproporphyrin transport using a combination of two methods. First, the transport rates *versus* CP concentration graphs were transposed to generate Lineweaver-Burke graphs and values of K_m and V_{max} estimated by linear regression analysis. To confirm these estimates, nonlinear regression was conducted using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego). The values obtained by these two methods varied by less than 10%.

Analysis of Expression Screening—Gene expression screening was based on the methods of Nilsson et al. (11). Freely available mammalian microarray data were used to search for genes consistently coexpressed with a query dataset. 15 GEO datasets (314 chips) were obtained from the NCBI Gene Expression Omnibus between September and December 2009. A prior p_0 value of 15% (versus 5% in the Nilsson method) was used to calculate the integrated p value, as we started from a more narrowly defined list of genes (those up-regulated in Abcb6⁻ erythroblasts). We used the following eight heme biosynthetic genes as controls: alad, alas2, cpox, fech, hmbs, ppox, urod, and uros. The gene expression value in each data set was calculated by using the Affymetrix MAS5 method. We mapped Affymetrix probe sets in the Affymetrix Mouse 430_2 array to other Affymetrix array platforms, using either the best matched probe or probe ortholog files available from the Affymetrix web site. When multiple probe sets were applicable to genes, those

with the maximum expression values were used. The probability of coexpression of each gene with the eight known heme genes was calculated within each dataset, and the genes were ranked in order of their likelihood of coexpression with the heme genes. The dataset weight (w_d) was defined as the mean coexpression probability across the eight heme genes.

For the heat map showing *Abcb6* expression throughout erythroid differentiation, the dataset GSE4655 (erythroid differentiation *in vitro*, *Homo sapiens*) was used. Heat maps were generated on the basis of Z-scores, calculated as Z = (x - m)/s, where *x* is the raw gene expression value to be standardized; *m* is the mean gene expression across all samples, and *s* is the corresponding standard deviation.

HPLC Measurement of PPIX, ZnPP, and Heme-Samples of peripheral blood mixed with EDTA were lysed in the HPLC mobile phase (described below) for 30 min at room temperature and then centrifuged (50,000 \times g) for 10 min at 4 °C. Twenty microliters of supernatant was injected into the HPLC system (Beckman System Gold Programmable Solvent Module 126). ZnPP and PPIX were separated on a Hibar 125 \times 4-mm LiChrospher 100 RP-18 column (5 μ m; Merck) with a mobile phase containing 10% 1 M ammonium acetate buffer, pH 5.0, 30% methanol, and 60% (v/v) acetonitrile at a flow rate of 1 ml/min. ZnPP and PPIX were detected by fluorescence (ZnPP, excitation 415 nm and emission 590 nm; PPIX, excitation 405 nm and emission 630 nm). Under these conditions, ZnPP and PPIX were eluted at 2.5 and 6.1 min, respectively. Results were quantified by comparison to known quantities of external standards.

For heme and porphyrin analysis, porphyrins were extracted from whole-blood samples with acidified acetone and centrifuged at $16,000 \times g$ for 10 min. Heme was separated from other porphyrins on a Shimadzu system, using a mobile phase of acetonitrile in water containing 0.05% trichloroacetic acid at 1 ml/min on a reverse-phase C18 column (MC Medical), applying a 30-66% linear gradient over 5 min followed by a 66-90% linear gradient over 20 min. Heme absorbance was read at 400 nm, whereas porphyrin fluorescence was measured at 395 nm (excitation) and 630 nm (emission). The heme/ZnPP ratio of each sample was determined by dividing the heme absorbance by the PPIX fluorescence. The heme absorbance value of each sample was normalized to the absolute reticulocyte count (as determined by both Ter119 and thiazole-orange double-positive cells).

Erythroid Krüppel-like Transcription Factor (EKLF Also Known as KLF1) Binding Site Analysis—EKLF binds specifically to the sequence CCACACCCT (12) and loosely to the sequence CCNCNCCCN (13) (core binding sequence, CACCC) (14). For each of the 71 submitted query genes, 3 kb of upstream sequence plus 5'-UTR were derived from the UCSC genome browser. Three EKLF binding patterns were mapped by using DNA pattern analysis and visualized by using Feature map in the RSA suite of tools (15).

RESULTS

Abcb6 Knock-out and Expression—Abcb6 comprises 19 exons and is highly conserved in humans and mice (16). *Abcb6* was disrupted in embryonic stem (ES) cells by using homolo-





FIGURE 1. Generation of *Abcb6^{-/-}* mice. *A*, construct used. *B*, PCR analysis of genomic DNA from wild-type, *Abcb6^{+/-}*, and *Abcb6^{-/-}* mice. The wild-type allele amplifies a 0.6-kb fragment and the null allele a 1.2-kb fragment. *C*, Western blot (*upper panel*) and quantitative graph (*lower panel*) of Abcb6 in mitochondria isolated from various tissues of adult *Abcb6^{-/-}* mice. A separate gel was used for bone marrow lysates. *VDAC*, voltage-dependent anion channel.

gous recombination to replace exons 13 and 14 (encoding the Walker A ATP-binding motif essential for ABC transporter function) (17) with the neomycin resistance cassette (Fig. 1*A*), as verified by PCR (Fig. 1*B*). Immunoblot analysis of mitochondrial lysates from various tissues revealed a gene dose effect, *i.e.* loss of one allele reduced Abcb6 protein by half (supplemental Fig. S1). After normalization with the mitochondrial protein voltage-dependent anion channel, Abcb6 was highly expressed in the mitochondria of multiple tissues, most highly in the liver and spleen (Fig. 1*C*). In several tissues (bone marrow and heart), substantial proteolytic cleavage of Abcb6 resulted in a reduced size immunoreactive band.

Abcb6^{-/-} Mice Lack ATP-driven Coproporphyrin III Mitochondrial Import-Among the porphyrins that interact with Abcb6, CP was a prime candidate substrate because it potently disrupted the hemin-agarose interaction with Abcb6 and inhibited heme import into mitochondria (17). Although CP differs electronically from the endogenous substrate coproporphyrinogen III, the main feature distinguishing the porphyrins interacting with Abcb6 is the tetratrapyrrole structure (17). Furthermore, unlike coproporphyrinogen III, which is highly susceptibility to oxidation and not suitable for isolated mitochondrial transport assays, CP is a stable substrate that is very similar to the endogenous coproporphyrinogen III. Mitochondrial CP uptake assays have been described previously (18, 19). However, these studies did not provide unequivocal evidence for CP transport because they either did not include ATP or the concentrations were exceedingly high. To determine the kinetics of CP binding and transport, we developed a CP uptake assay using hepatic mitochondria from adult wild-type and $Abcb6^{-/-}$ mice. We utilized the nonhydrolyzable ATP analog AMP-PNP to distinguish between ATP-dependent (requiring ATP hydrolysis) and nondependent import (Fig. 2A). The wild-type and $Abcb6^{-/-}$ mice showed nearly identical non-ATP-dependent CP uptake (4.5 ± 0.9 and 5.4 ± 1.8 pmol/mg, respectively). In contrast, ATP-dependent transport (~4 pmol/min/mg) was measurable only in the wild-type mice. In the wild-type mice, ATP-dependent CP transport was saturable and displayed Michaelis-Menten kinetics with a V_{max} of 9.8 pmol·min⁻¹ mg⁻¹ and a K_m of 12.6 μ M (Fig. 2B).

We hypothesized that Abcb6 up-regulation is required to meet the increased demand for porphyrins during erythropoiesis. We tested this hypothesis in an immortalized erythroid cell line, the murine MEL cells, which can be induced by hexamethylenebisacetamide (HMBA) to activate heme/porphyrin synthesis. MEL cells were untreated or treated with HMBA for 120 h before mitochondrial isolation. The initial rate of ATPdependent CP transport was four times as great in HMBAtreated cells (18.24 pmol·min⁻¹ mg⁻¹) as in untreated cells $(4.67 \text{ pmol}\cdot\text{min}^{-1} \text{ mg}^{-1})$ (Fig. 2C, upper panel). In contrast, non-ATP-dependent CP uptake was almost identical in treated and untreated MEL cells (Fig. 2C, lower panel). Quantitative analysis indicated that mitochondrial expression of Abcb6 was increased by a factor of \sim 5 by HMBA treatment. Although the expression of peripheral benzodiazepine receptor (an energyindependent porphyrin importer) also increased in HMBAtreated MEL cells (Fig. 2D), it did not appear to substantially contribute to the ATP-independent import of CP, consistent





FIGURE 2. **Abcb6 is the sole ATP-dependent mitochondrial transporter of coporphyrin III.** *A*, comparison of CP import in wild-type and $Abcb6^{-/-}$ mice, showing ATP-dependent transport (*ATP*), non-ATP-dependent transport (*A-N-P*), and total transport (ATP transport with non-ATP transport subtracted). *B*, import kinetics of CP in mitochondria isolated from mouse liver. *C*, comparison of rate of CP import into untreated and HMBA-treated MEL cells. *D*, immunoblot of mitochondrial lysates shows that non-ATP-dependent carriers are not up-regulated in $Abcb6^{-/-}$ mitochondria and that Abcb6 is maximally up-regulated in HMBA-treated MEL cells. *E*, time course of heme concentration in untreated and HMBA-treated MEL cells, as measured by HPLC and normalized by cell number. *F*, HPLC analysis of intracellular porphyrins in untreated and HMBA-treated MEL cells. *VDAC*, voltage-dependent anion channel; *PBR*, peripheral benzodiazepine receptor; *OGC*, 2-oxoglutarate carrier; *ANT*, adenine nucleotide translocator; *Cpx III*, coproporphyrin III.

with the weak affinity of peripheral benzodiazepine receptor for CP compared with hemin or PPIX (Fig. 2*C*). Moreover, immunoblot analysis showed no greater mitochondrial expression of other non-ATP-dependent porphyrin importers, voltage-dependent anion channel, ANT1, or 2-oxoglutarate carrier, in $Abcb6^{-/-}$ mice than in wild-type mice (Fig. 2*D*). The increased CP transport we observed in HMBA-treated MEL cells corresponded to a 20-fold increase in heme (measured by HPLC) during the same time course of differentiation (Fig. 2*E* and supplemental Fig. S2). Although small amounts of heme accumulate in the uninduced MEL cells, it is most notable that endogenous coproporphyrinogen III only accumulates in the

noninduced cells and not in the MEL cells in which Abcb6 was strongly induced. Together, these data indicate that Abcb6 is required for ATP-dependent mitochondrial import of CP and that in MEL cells, Abcb6 expression levels parallel increased ATP-dependent CP import. Given the parallel increase in heme and Abcb6 function and expression, these findings suggest that Abcb6 is crucial to coordinating porphyrin and iron pathways to synthesize heme.

Non-Mendelian Inheritance and Increased Erythroid PPIX in $Abcb6^{-/-}$ Mice—Although the $Abcb6^{-/-}$ mice appeared phenotypically normal, multiple independent intercrosses among $Abcb6^{+/-}$ mice (153 viable offspring) yielded 10% fewer mice of



TABLE 1	
Non-Mendelian inheritance of the Abcb6 gene	

	Abcb6 ^{+/+}	Abcb6 ^{+/-}	Abcb6 ^{-/-}	
Expected % $(n)^a$	25% (38.25) ^b	50% (76.5)	25% (38.25)	
Observed % (n)	35% (53)	42% (64)	23% (36)	

^a Expected inheritance in a population of 153 is given in parentheses.

 $^{b}p = 0.02$, Pearson's χ^{2} test with 2 degrees of freedom.

TABLE 2

Mean (±S.E.) peripheral blood hematological values in Abcb6 $^{+/+}$ and Abcb6 $^{-/-}$ mice

	Abcb6 ^{+/+}	Abcb6 ^{-/-}	<i>p</i> value
Hematocrit	$34.8 \pm 1.2\%$	$36.5 \pm 0.9\%$	0.2465
Hemoglobin	12.0 ± 0.5 g/dl	13.0 ± 0.2 g/dl	0.042
Red cell distribution width	$14.3 \pm 0.3\%$	$14.7 \pm 0.2\%$	0.2842
Mean corpuscular volume	$45.7 \pm 0.3 \text{fl}$	$44.7 \pm 0.2 \text{ fl}$	0.0083
PPIX (mean fluorescence)	1498 ± 112.6	2122 ± 179.7	0.0064
Reticulocytes	$9.5\pm0.9\%$	$10.5\pm0.7\%$	0.3704

the $Abcb6^{-/-}$ genotype and of the $Abcb6^{+/-}$ genotype than expected (Table 1). Because of the role of Abcb6 in mitochondrial porphyrin transport and regulation of intracellular porphyrins, we examined the peripheral blood parameters of $Abcb6^{-/-}$ mice (Table 2). Surprisingly, the mean erythrocyte PPIX concentration was 41% greater in $Abcb6^{-/-}$ mice than in their wild-type littermates (p = 0.006), although the mean corpuscular volume and hemoglobin differed only slightly. All other hematologic parameters measured were comparable in $Abcb6^{+/+}$ and $Abcb6^{-/-}$ animals.

Compensatory Changes in Gene Expression in Abcb6^{-/-} Mice-The increased erythrocyte PPIX and reduced inheritance of the Abcb6-null allele suggested that mice survived by compensation. Other knock-out animals with severe defects in heme synthesis have been observed to survive (20-22), although the mechanisms of compensation have not been identified. In particular, the elevated erythrocyte PPIX in *Abcb6*^{-/-} mice suggested that porphyrin biosynthesis may be altered, possibly leading to alteration of iron usage and other interrelated pathways. To determine whether gene expression is altered in the absence of Abcb6, we compared transcript expression in $Abcb6^{-/-}$ and wild-type erythroblasts (MACSpurified Ter119-positive cells) by using an Affymetrix microarray (Fig. 3). Pathway analysis (GENEGO, KEGG, and Gseap) indicated the highest ranked pathways were in the porphyrin metabolism and oxidative phosphorylation pathways (p = 2.18×10^{-12} and 2.04×10^{-9} , respectively), consistent with the known role of Abcb6 in activating heme synthesis and with the up-regulation of Abcb6 during erythroid development (10, 17).

To determine the relationship of these genes to heme biosynthesis, we adapted an expression screening approach described by Nilsson *et al.* (11). Analysis of public gene expression datasets available on GEO (23) revealed that during erythroid differentiation *Abcb6* is up-regulated in parallel with many of the eight heme pathway (*alas2, alad, hmbs, uros, urod, cpox, ppox,* and *fech*) genes (Fig. 3*A*). We focused on the 69 mitochondrial and known erythroid-associated genes (of 175) that were upregulated by a factor of 2 or more in *Abcb6^{-/-}* erythroblasts, plus eight other heme pathway genes (supplemental Table S1), of the total of more than 3500 genes, whose expression was altered by a factor of 2 or more (Fig. 3*B* and supplemental Table S2). Nearly all of the 69 genes were independently verified by

ABCB6 Protects against Phenylhydrazine Toxicity

ABI gene card analysis (supplemental Fig. S3). Thus, a total of 72 candidate genes were examined within a collection of 314 microarray datasets (supplemental Table S3) representing a wide range of erythropoiesis-relevant conditions. The dataset weight and individual rankings were used to select candidate genes whose expression was highly related to heme gene expression across a range of cell types and experimental conditions as illustrated in Fig. 3C. Many of the highest ranking genes in our final list (the top 20 candidates) were potential EKLF targets (24), as supported by putative EKLF-binding sites within 1 kb upstream (supplemental Table S4). Furthermore, heme biosynthetic pathway genes such as Alas2 and Hmbs are known to have EKLF-binding sites, and there is evidence of endogenous binding to EKLF (25, 26). Importantly, Abcb6 has bona fide EKLF-binding sites (supplemental Table 4 and supplemental Fig. S4A) and is activated by EKLF (supplemental Fig. S4B).

Elevation of EKLF and FECH in $Abcb6^{-/-}$ Erythroid Cells— Several transcription factors with known roles in erythropoiesis (EKLF, Nfe2, and MyD116) were expressed more highly in $Abcb6^{-/-}$ than in $Abcb6^{+/+}$ erythroid cells (supplemental Table S1). However, EKLF was the transcription factor with the highest integrated *p* value in the expression screen of those that play a role in regulating heme biosynthetic genes (Table 3). Furthermore, EKLF expression was strongly up-regulated, and FECH protein was increased in $Abcb6^{-/-}$ mice (Fig. 4A), consistent with the observed elevation of FECH mRNA and the fact that FECH is a known EKLF target (27).

Impaired Stress Erythropoiesis in Abcb6^{-/-} Mice—The elevated PPIX levels and up-regulation of heme biosynthetic genes in the $Abcb6^{-/-}$ mice suggested erythroid cells might not respond to an increased demand in porphyrin synthesis. To determine this, we induced porphyrin synthesis in vivo, and mice were injected subcutaneously with phenylhydrazine (Phz, 50 mg/kg) on days 0, 1, and 3 and monitored from day 0 to 8-10. Unexpectedly, $Abcb6^{-/-}$ mice showed greater mortality during days 3–7 post-Phz (25/58 or 43% versus 11/40 or \sim 27% in wild-type mice) (p = 0.043, two-tailed χ^2 test) (Fig. 4B). The $Abcb6^{-/-}$ mice that died during days 3–7 had an overall lower mean hematocrit (12.8 versus 16%) at day 3 and a greater rate of hematocrit decline than did survivors. Surviving Abcb6^{-/-} mice had hematocrits almost identical to those of $Abcb6^{+/+}$ mice through day 3, when they reached a nadir of $17.4 \pm 1.2\%$ $(Abcb6^{+/+})$ and $18.5 \pm 0.7\%$ $(Abcb6^{-/-})$. However, the time to maximal recovery was significantly delayed in the surviving *Abcb6*^{-/-} mice (day 10 *versus* day 7, p = 0.004) (Fig. 4*C*), and the reticulocyte count remained elevated (Fig. 4D), indicating that Abcb6 is required for a normal stress response in erythroid cells. The greater red cell distribution width in $Abcb6^{-/-}$ mice after day 7 reflected their greater number of reticulocytes, which are larger than erythrocytes (Fig. 4*E*). These findings suggest that $Abcb6^{-/-}$ mice remain anemic longer than $Abcb6^{+/+}$ mice, consistent with their lower hemoglobin levels during days 5-10 (Fig. 4F). Mean corpuscular volume values, which were slightly lower in the $Abcb6^{-/-}$ mice before treatment, remained lower during days 3–10, consistent with hemoglobin levels (Fig. 4G). These results demonstrate that loss of Abcb6 impairs the normal erythroid response to phenylhydrazine.





FIGURE 3. **Gene expression screening of** $Abcb6^{-/-}$ **mice.** *A*, during *in vitro* erythroid differentiation, *Abcb6* is up-regulated simultaneously with heme biosynthesis genes. *B*, transcripts were compared in erythroblasts from 1-month-old female wild-type and $Abcb6^{-/-}$ mice. Genes whose expression changed by a factor of 2 or more included more than 110 mitochondrial genes and 66 known erythroid-associated genes (candidate genes). *C*, more than 314 arrays were searched to identify genes consistently coexpressed with the candidate genes.

Erythropoietin and Red Blood Cell Turnover—The reticulocytosis observed in *Abcb6^{-/-}* mice might be caused by an overproduction of erythropoietin or by altered differentiation and maturation of erythroid progenitors. *Abcb6^{+/+}* and *Abcb6^{-/-}* mice had similarly elevated serum erythropoietin concentration at day 8, followed by a return to normal levels by day 10 (Fig. 5*A*), ruling out sustained erythropoietin elevation as the primary cause of the prolonged reticulocytosis.

To determine whether reduced cell survival caused the prolonged reticulocytosis and delayed hematocrit recovery, we measured red cell turnover by injecting mice with *N*-hydroxysuccinimide-biotin at day 7 (peak reticulocyte production; see Fig. 4*C*) and monitoring the proportion of *N*-hydroxysuccinimide- and Ter119-positive cells. The mean half-life of Ter119positive cells did not differ substantially in wild-type and $Abcb6^{-/-}$ mice (10.46 *versus* 10.39 days) (Fig. 5*B*). We also found no significant difference between the genotypes in apoptosis of Ter119⁺ cells from spleen and bone marrow during recovery from Phz treatment (Fig. 5*C*).

Because the spleen becomes the primary erythroid organ during Phz-induced stress erythropoiesis in mice (28), we also examined splenic reticulocyte levels during recovery. At day 8, the percentage of reticulocytes in $Abcb6^{-/-}$ spleens was more than eight times that in wild-type spleens (Fig. 5*D*). We also examined erythroid colony-forming units (CFU-E), the rapidly divided progenitors that differentiate into orthochromic erythroblasts and then into reticulocytes (29). Untreated wild-type

and $Abcb6^{-/-}$ mice showed comparable capacity to form CFU-E (Fig. 5*E*), consistent with equal distribution of bone marrow and spleen progenitors expressing the maturation markers CD71 and Ter119 (supplemental Fig. S5). However, splenocytes from Phz-treated $Abcb6^{-/-}$ mice showed a significant increase in CFU-E during recovery (Fig. 5*E*), consistent with the observed elevation of reticulocytes and suggesting an intrinsic dysregulation of porphyrin synthesis in $Abcb6^{-/-}$ mice is associated with a defect in erythroid maturation.

Elevated PPIX and Deficient Heme Production in $Abcb6^{-/-}$ Mice—Because erythroid-cell PPIX was elevated in untreated $Abcb6^{-/-}$ mice, we measured PPIX concentration in peripheral and splenic erythroid cells during and after Phz treatment (Fig. 6). The erythrocyte PPIX concentration was approximately twice as high in $Abcb6^{-/-}$ mice as in wild-type mice during days 7–10 after Phz treatment (Fig. 6A). PPIX concentration was also elevated in $Abcb6^{-/-}$ splenic erythroid cells (Fig. 6B), particularly in splenic reticulocytes (1.9 times that in wild-type splenic reticulocytes on day 10).

If the $Abcb6^{-/-}$ mouse erythrocyte heme-to-PPIX ratio is altered it might suggest a defect in conversion of PPIX to heme. The $Abcb6^{-/-}$ heme-to-PPIX ratio was less than 25% that in wild-type mice at days 8 and 10, as was the reticulocyte heme concentration at day 10 (Fig. 6, *C* and *D*). Although FECH is elevated in $Abcb6^{-/-}$ mice, it might be nonfunctional. FECH uses Zn^{2+} as a cofactor when iron is insufficient. The ratio of ZnPP to heme provides an *in vivo* measure of the iron available



TABLE 3

The final 72 candidates identified by gene expression screening

CPCN Comparphysingen online 999 X X X X X ERG1 Errochelissen ultage 0.99 X X X ERG1 Errochelissen ultage 0.99 X X X ERG2 Unoperphysingen dicatelosythes 0.99 X X X PPOX Protopiphyringen onlane 0.999 X X X REG23B10 Penticatel gene, EG023B1 HMBS 0.998 X X X REG32B17 Penticatel gene, EG023B1 HMBS 0.998 X X X REG32B17 Penticatel gene, EG023B1 HMBS 0.998 X X X REG4 Intercentricatel depre, EG023B1 HMBS 0.998 X X X REG4 Intercentricatel depre, EG023B1 HMBS 0.998 X X X REG4 Intercentricatel depre, EG023B 0.998 X X X REG4 Intercentricatel depre, EG023B 0.998 X X X <th>Gene</th> <th>Description</th> <th>Integrated probability</th> <th>EKLF target^a</th> <th>Unique heme biosynthetic genes^b</th> <th>Mitochondrial</th>	Gene	Description	Integrated probability	EKLF target ^a	Unique heme biosynthetic genes ^b	Mitochondrial
FFC/I Ferror behavior 0.999 X X X HABS Holdson the bound of the total interphone on the set of the total interphone of the set of the total interphone of the set of the	CPOX	Coproporphyrinogen oxidase	0.999	Х	х	Х
HHBS Hydroxyneitybilane synthase 0.999 X X X LRD Looporphytingen (Sectional Sectional Secti	FECH	Ferrochelatase	0.999	X	x	X
EKLF Kingpel-lake factor 1 (crythoud) 0.99 X URON Unoperphythologe flog registre 0.99 X X URON Unoperphythologe flog registre 0.99 X X URON Unoperphythologe flog registre 0.99 X X URON Under factor, erythoud-dartowed 2 0.98 X X URON Annobec inter depression (direct a construct a	HMBS	Hydroxymethylbilane synthase	0.999	Х	Х	Х
IBODUnspenpiptingen declared0.99XXIPACAProduced propriorNumber factor, erythenid-learned 20.99XXINFE2Number factor, erythenid-learned 20.998XXIACLDAnnobeu linit is dely drates0.998XXIACLDAnnobeu linit is dely drates0.998XXIACLDAnnobeu linit is dely drates0.998XXIEFZAKIElasorotic Healesian nolecula 40.996XXIEFZAKIElasorotic Translation initiation factor 2 a kinase 10.993XXIEFZAKIElasorotic Translation initiation factor 2 a kinase 10.993XXIEFZAKIElasorotic Translation initiation factor 2 a kinase 10.996XXXIEFZAKIElasorotic Translation initiation factor 2 a kinase 10.996XXXIEFZAKIElasorotic Translation initiation factor 2 a kinase 10.996XXXIEFZAKIElasorotic Translation initiation factor 2 a kinase 10.997XXXIEFRALElasorotic Translation initiation factor 2 a kinase 10.996XXXIFRALItself inton sublicit and the dup drogenase 20.977XXXIFRALItself inton sublicit and the dup drogenase 20.977XXXIFRALItself inton sublicit and the dup drogenase 20.977XXXIFRALItself inton sublicit and the dup drogenase 20.977 <td>EKLF</td> <td>Krüppel-like factor 1 (erythroid)</td> <td>0.999</td> <td>Х</td> <td></td> <td></td>	EKLF	Krüppel-like factor 1 (erythroid)	0.999	Х		
PPOX Protopophyringen stake 0.999 X X URDS Upopophyringen II syntase 1 0.998 X X URDS Prediced gene, EGG338 IS HARS 0.998 X X ALAD Annonevillant, 6-syntase 2 0.997 X X ALAS Annonevillant, 6-syntase 2 0.997 X X CREXS Glazardonin Shomolog (Saccharowyces cervisiae) 0.992 X X CREXS Glazardonin Shomolog (Saccharowyces cervisiae) 0.994 X X CREXS Glazardonin schutzer 6 0.984 X X CREXS Glazardonin schutzer 6 0.984 X X CNCX Pyrovale carboxylase 0.981 X X CREXA Pyrovale carboxylase 0.976 X X MTHED2 Hels ion-sultir cluster ox-chaptene honolog (Exherichia cal) 9.977 X X MTCRD2 Hels ion-sultir cluster ox-chaptene honolog (Exherichia cal) 9.976 X X MTHED2 Hels	UROD	Uroporphyrinogen decarboxylase	0.999	Х	Х	
UBOS Upperphytringen III synthas 0.999 X ARL2 Nuclear fairs reprinted deliver 2 0.986 X ALAD Anniolocultance deliver 2 0.987 X ALAD Anniolocultance deliver 2 0.987 X ILCAM Introcellular adhesion molecule 4 0.966 X ILCAM Introcellular adhesion molecule 4 0.967 X X ILCAM Introcellular adhesion molecule 4 0.988 X X ILCAM Billwedin recleature 8 0.988 X X ILCAM Epsthi Epsthi X X ILCAM Provide cataboxias 0.981 X X ILCAM Provide cataboxias 0.976 X X ILCAM Epsthi Epsthi 0.976 X X ILCAM Subtac carie framing 16, member 3 0.971 X X ILCAM Subtac carie framing 1 0.975 X X ILCAM Subtac carie framing 1 0.971<	PPOX	Protoporphyrinogen oxidase	0.999			Х
ME2 Nuclear factor, erythenel derived 2 0.98 X ALAD Predicted purch (Ed2X) 0.996 X ALAD Aninolevalinate, 6-synthase 2 0.997 X CLMAH Intercellular abcissin molecule 4 0.996 X X EGG27S57 Dedication protein compote peripheral-like 1 0.986 X X EZRFLI Beconstructure protein band 41 0.981 X X TRCC Transferrin receptor 0.977 X X MICTORD Interferring functions at 7 0.968 X X SCAS138 Solute carrie family 25, member 38 0.977 X X SCAS140 Microsonal glutations 5: transferrin sceptor X X X SCAS143 Solute carrie family 26, member 38 0.968	UROS	Uroporphyrinogen III synthase	0.999		Х	
LLC282/8 Predicted gam, EuG2818 HMMS 0.986 ALLO Anniholowing, Edynamics (2) 0.977 X LCMAH Intercellular adhesion molecule 4 0.986 X LCMAK Editaryoit translotion initiation factor 2a kinase 1 0.993 X X GLRXS Glutareloxin 5 horning (Saccharomyce corresidue) 0.988 X X BCRAR Therakering park (Editar) 0.988 X X BCRAR Thiorefoodin reductase 2 0.984 X X PCA Pruvate carboxylase 0.981 X X Discover reductase protein hard 11 0.976 X X TRNDB2 Trainslocator protein hard 21 0.976 X X TRNDB2 Trainslocator protein hard 20 0.976 X X TRNDB2<	NFE2	Nuclear factor, erythroid-derived 2	0.998	Х		
ALAD Annolecularitie delyratises 1989 V V ICAM4 Intercellular albesion molecule 4 0.966 X V EIPZAK1 Eukaryote translation initiation factor Zn kinase 1 0.993 X X EIPZAK1 Eukaryote translation initiation factor Zn kinase 1 0.992 X X EIRZS Glutzelosito, showing (Schwarz) 0.993 X X EIRZN Headrong (Schwarz) 0.993 X X EIRZN Headrong (Schwarz) 0.993 X X EIRZN Headrong (Schwarz) 0.994 X X EIRZN Headrong (Schwarz) 0.991 X X EIRZN Headrong (Schwarz) 0.977 X X EIRZN Medition-schwarz 0.976 X X MITHPD2 Methylenetertalydrong (Schwarz) 0.976 X X MITHPD2 Methylenetertalydrong (Schwarz) 0.968 X X MITHPD2 Methylenetertalydrong (Schwarz) 0.968 X X MITHPD2 Methylenetertalydrong (Schwarz) 0.968 X X RD24A ATTBee inhibitor factor 0.964 X X RD40H Hy	EG623818	Predicted gene, EG623818 HMBS	0.998			
ALAGA Minimizer symmatics 0.396 X X CH2AKI Eakspott cranslation initiation factor 2a kinase 1 0.993 X X GLRNS Glutterdoxin 5 homolog (Saccharomyce cerevisiae) 0.992 X X DEWB Billwrrdin reductase 8 0.989 X X DEWB Diversion reductase 8 0.988 X X TANRD2 Thioredoxin reductase 7 0.984 X X TANRD2 Thioredoxin reductase 7 0.984 X X TANRD2 Thioredoxin reductase 7 0.984 X X TANRD2 Thioredoxin reductase 7 0.977 X X TANRD2 Methylometical hydrogenase 2 0.975 X X GCD24A CD25a antigen 0.975 X X X SICC538 Solutic carrie family 3, member 38 0.986 X X X SICC538 Solutic carrie family 3, member 38 0.986 X X X SICC538 <t< td=""><td>ALAD</td><td>Aminolevulinate dehydratase</td><td>0.998</td><td></td><td></td><td>V</td></t<>	ALAD	Aminolevulinate dehydratase	0.998			V
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	ALASZ ICAMA	Aminolevulinate, o-synthase 2	0.997	v		λ
cf 25.3 control of the interval on the biomage (state interval on the biomage) 0.992 X X EGG275.57 Predicted period (state interval on the biomage) 0.989 X X BLVBB Billwerdin reductase B 0.988 X X BLVBB Berroduze period (state 2) 0.984 X X BLVDD Theredoxin reductase 2 0.986 X X BLVBL Berroduze protein in a dt 1 0.981 X X TRRC Transferin receptor 0.977 X X TRRC CD24a CD24a CD24a CD24a X X SC23A38 Soluto carrier finally 25, member 38 0.966 X X SRPO Transform carrier finally 25, member 38 0.966 X X ACC2A art P binding casetic sub-finally (WITLE), member 2 0.917 X X ABVERI BLT2/adenovirus E1B interacting protein 3-like 0.966 X X ADCC27 AT Profit binding casetic sub-finally (WITLE), member 2 0.916 X <td>EIEDAVI4</td> <td>Eukamotic translation initiation factor 2 a kinasa 1</td> <td>0.996</td> <td>л</td> <td></td> <td></td>	EIEDAVI4	Eukamotic translation initiation factor 2 a kinasa 1	0.996	л		
LGG757ST Predicted gene, ESG757ST 0.989 X X BLVBB Billwordin reductase B 0.988 X X BZRPL1 Bernzollazepine receptor, peripheral-like 1 0.986 X X PCX Pyrruate carboxylase 0.981 X X PCX Pyrruate carboxylase 0.984 X X PCX Pyrruate carboxylase 0.984 X X PCX Pyrruate carboxylase 0.981 X X PCX Pyrruate carboxylase 0.976 X X PCX Hack inon-sulfur cluster co-chaperone honnolog (Eacherichia call) 0.977 X X CD24A CD24a antigen 9.975 X X X SIGC738 Solute carrie family 25, member 38 0.966 X X X APCG27 ATP-binding cassets, sub-family 6 (WHTF), member 2 0.951 X X APCG47 Hoosehood carrie family 35, member 39 0.945 X X ACLART Mioc	GLRX5	Glutaredoxin 5 homolog (Saccharowyces cerevisiae)	0.993		x	x
BL/VBB Bibliverdin reductase B 0.088 X N BZRPL1 Benzolizappine receptor, peripheral-like 1 0.086 X X DTXNRD2 Thioredoxin reductase 2 0.084 X X DTXC Private carboxylase 0.081 X X DTXC Private carboxylase 0.081 X X DTXC Private carboxylase 0.0977 X X MTRPD2 Methylencetralydrololate dehydrogenase 2 0.976 X X MC373 Microsomal glurathione 5-transferase 3 0.977 X X MC573 Microsomal glurathione 5-transferase 3 0.976 X X MC573 Recours protein	EG627557	Predicted gene, EG627557	0.989		X	X
Beznodlizerpine receptor, perpheral-like 1 0.096	BLVRB	Biliverdin reductase B	0.988	х		
TXND22 Thioredoxin reducts 2 - 1 0984 X X PPCX Pryrute carboxylas 0981 X X PBFAL Erythrocyte protein band 4.1 0.981 X X ITREC Transferrin receptor 0.977 X X IFTRED Inderborn suffic clastofic dehydrogenase 2 0.975 X X OCD24A CD2bia and the dehydrogenase 2 0.975 X X MGST3 Microsonal glutathione 5-transferase 3 0.971 X X SIC25A38 Solute carrier family 25, member 38 0.966 X X SIC25A37 Microsonal glutathione 5-transferase 3 0.971 X X SIC25A38 Solute carrier family 25, member 38 0.966 X X MRG1 BC12/addenorins E1B infrarcting protein 3-like 0.965 X X MCG2 ATT 1 Mitochonfail carrier triple repet 1 0.945 X X GLUL Glutanate-amnonia ligase (glutanine synthetase) 0.92 X X <t< td=""><td>BZRPL1</td><td>Benzodiazepine receptor, peripheral-like 1</td><td>0.986</td><td></td><td></td><td></td></t<>	BZRPL1	Benzodiazepine receptor, peripheral-like 1	0.986			
PCX Pyrvate carboxylase 0.981 X X TPRC Transferrin receptor 0.977 X X TRC Transferrin receptor 0.977 X X MTHD Missilitor colling constraints of the programs 2 0.977 X X MTTRC Transferrin receptor X X X MGST3 Microsom glutalitions 5*/ransferase 3 0.971 X X SLC25A38 Solute carrier family 25, member 38 0.966 X X BMP3L BC12/ademovirus 118 interacting protein 3-like 0.96 X X ABCC22 ATPbr16 Ading cossette, sub-family G (WHTE), member 2 0.951 X X ABCC32 Hold Hydrogenase (bicquinone) 1 b 9 0.916 X X X IBGLH Hemoglobin Z, Bilke embryonic chain 0.94 X X X IBDL2 Peroxiredoxia 2 0.909 X X X IBDL2 Peroxiredoxia 2 0.909 X X X	TXNRD2	Thioredoxin reductase 2	0.984		Х	Х
EPBAl Erpthrocyte protein band 4.1 0.981 TTRC Tansler (laster co-chaperone homolog (Escherichia coll) 0.977 X HSCB HesB iron-sallar cluster co-chaperone homolog (Escherichia coll) 0.977 X MGSTH Microsonnal glutathione S-transferase 3 0.971 X MGSTH Microsonnal glutathione S-transferase 3 0.966 X X SLC25A3B Solute control Fill Intracting protein 3-like 0.966 X X MISTH Microsonnal glutathione S-transferase 3 0.956 X X ATTPO Translocator protein 0.966 X X ATTPI All Pase inhibitory factor 1 0.955 X X MIGH Hydrogynatic kontrol (QIWIITE), member 2 0.916 X X MIGH Honologica Z, Silice empryonic chain 0.945 X X X GLUI Glutanate-ammonia ligase (glutanine synthetase) 0.921 X X X MGL1 Monolyceride lipase 0.916 X X X X	PCX	Pyruvate carboxylase	0.981	Х		Х
$\begin{array}{c c c c c c c c c c c c c c c c c c c $	EPB4.1	Erythrocyte protein band 4.1	0.981			
HSCB HeeB iron-suffar cluster co-chaperone homolog (Exderichia coli) 0.977 X MTHFD2 Methylemettahydrofialae (edyhorgenase 2 0.975 X CD24A CD24a antigen 0.975 X CD25A Microsoma glatahilers Stransferse 3 0.966 X X CD25A Microsoma glatahilers Stransferse 3 0.966 X X CD25A Microsoma glatahilers Stransferse 3 0.967 X X SUP20 Translocator protein 0.967 X X ARCG2 ATP-binding cassette, sub-family G (WHITE), member 2 0.955 X X HACH Hydroxyacyl glatathione bydrolase 0.955 X X X HACH Mydroxyacyl glatathione synthetase) 0.916 X X X Glata Guaranse annonia lagae (glatannie synthetase) 0.916 X X X MG1L Monoglyceride lipse 0.916 X X X X MG1L Monoglyceride lipse 0.916 X X	TFRC	Transferrin receptor	0.977	Х		
MTHPD2 Methyleneteriarydrofolate dehydrogenase 2 0.976 X CD24A CD24A antigon 0.975 X MGST3 Solute carrier family 5, member 38 0.964 X SVD3A3A Solute carrier family 5, member 38 0.964 X SVD3D1 BCl2/adenovirus E18 interacting protein 3-like 0.96 X X ARDER RECL2/adenovirus E18 interacting protein 3-like 0.95 X X ARDER ACRATT Mitochondrial carrier triple repeat 1 0.945 X X ARCARTT Mitochondrial carrier triple repeat 1 0.945 X X X GULU Glutanate-ammonia ligase (glutanine synthetase) 0.92 X X X MGLL Monoglyceride lipase 0.916 X X X HRX Heroxinetann 0.940 X X X MGLI Monoglyceride lipase 0.901 X X X HRX Heroxinetann 0.901 X X X	HSCB	HscB iron-sulfur cluster co-chaperone homolog (Escherichia coli)	0.977			Х
$ \begin{array}{cccc} CD24A & CD24a antigon & 0.975 & X & \\ MGST3 & Microsconal glutatione S-transferase 3 & 0.976 & X & X & X \\ SLGSA38 & Solute carrier family 15, member 38 & 0.946 & X & X & X \\ MSTB1, BC Cl2/denovins E18 interacting protein 3-like & 0.96 & X & X & X \\ ATPEH & ATPase inhibitory factor 1 & 0.957 & X & X & X \\ ARGC3 & ATP-binding casetse, uso family 6 (WHTE), member 2 & 0.951 & X & X \\ MCART & Mitochondrial carrier training is represented as 0.955 & X & X & X \\ MCART & Mitochondrial carrier training is represented as 0.945 & X & X \\ HBB-BHI & Henoglobin Z, Pike embryonic chain & 0.945 & X & X \\ HBB-BHI & Henoglobin Z, Pike embryonic chain & 0.94 & X & X \\ MCMID & NADH dehydrogenase (lubiquinoe) 1 b 9 & 0.916 & X & X \\ MTD10 & NADH dehydrogenase (lubiquinoe) 1 b 9 & 0.916 & X & X \\ EPBA3 & Erythrocyte protein band 4.9 & 0.900 & X & X \\ EPBA4 & Solute carrier family 48 (Hrg1) & 0.877 & X & X \\ MTD116 & Myeloid differentiation primary response gene 116 & 0.877 & X & X \\ MTD116 & Myeloid differentiation primary response gene 116 & 0.877 & X & X \\ MTSC3 & 5'-Nucleotids A -1 & 0.874 & X \\ MTSC3 & 5'-Nucleotids A -1 & 0.874 & X \\ MTSC3 & 5'-Nucleotids -1 & 0.876 & X & X \\ MTSC3 & 5'-Nucleotids -1 & 0.876 & X & X \\ MTSC3 & 5'-Nucleotidse, cytosolic III & 0.808 & X & X \\ MTSC4 & Intervised M1 + Monogles (S correstizae) & 0.836 & X & X \\ MTSC3 & 5'-Nucleotidse, cytosolic III & 0.767 & X \\ SLC25A37 & Solute carrier family 45, member 37 & 0.75 & X & X \\ MTSC4 & Trep MTB4 & Henoglobin N, Pike embryonic chain & 0.736 & X \\ MTSC4 & Magnesium homestasis factor homolog (S correstizae) & 0.861 & X \\ MTSC3 & Structeride distributes 0 & 0.974 & X \\ SLC25A37 & Solute carrier family 45, member 37 & 0.75 & X & X \\ MTSC4 & Magnesium homestasis factor homolog (S correstizae) & 0.663 & X \\ MTSC4 & Magnesium homestasis factor homolog (S correstizae) & 0.648 & X \\ QTVA4 & Clubatione proxidae & 0.659 & X \\ MTSC4 & Magnesium homestasis factor homolog (S correstizae) & 0.648 & X \\ QTM4 & Clubatione proxidae & 4 & 0.629 & X \\ SLC25A39 &$	MTHFD2	Methylenetetrahydrofolate dehydrogenase 2	0.976			Х
MICrosomal guitatione 3-transferate 3 0.9/1 X SUC2X.13 Solute carrier family 32, member 3 0.96 X X TDVD Translocator protein 0.96 X X TDVD Translocator protein 0.96 X X TDVD ATTREFI ATTREFI 0.95 X X ATTREFI Attract inplication protein 0.94 X X ARGC2 ATP-binding cassetts.sub-family 63 (WHTTE), member 2 0.95 X X HACH Hydroxya gliutathione hydrolase 0.95 X X X GLULI Glutarate-ammonia ligase (gliutarine synthetase) 0.918 X X X GLULI Honoglyceride lipase 0.916 X X X X PRDX2 Peroxincetoxin 2 0.901 X X X MICH hydroglase (differentiation primary response gene 116 0.874 X X SUCASAL Solute carrier family 48 (Hrg1) 0.874 X X	CD24A	CD24a antigen	0.975	X		
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	MGST3	Microsomal glutathione S-transferase 3	0.971	Х		X
IARDOITRIBUCTOR0.79XXBNR21BCC12/aderovirus IE interacting protein 3-like0.96XXATTUTAATE-ase inhibitory factor0.95XXATTUTAATE-ase inhibitory factor0.95XXARGEArgeneric interacting protein 3-like0.95XXARGEArgeneric interacting protein 3-like0.945XXARGEArgeneric interacting protein 3-like0.945XXARGEClubar and state ase mayonic chain0.94XXGLILGlutarate ase mayonic ingase (glutaraine synthetase)0.92XXMDLFB9NADH (delydrogenars (ubiquionon) b 90.916XXMGLLMonoglyceride lipase0.916XXMRD1Heroscheristica0.909XXXMDLFB9NADH (delydrogenars (kl frg1)0.875HK1Heroscheristica0.875HK1Heroscheristica (X-1)0.875HAX1HCLS1 associated X-10.874XXBPCM2.3-Bisphosphogreerate mutase0.824XXGMPRGiuanosine monophosphate reductase0.841XXCOX245'-Nucleatidase, crisocial III0.808XXCOX245'-Nucleatidase, crisocial III0.808XXGMPRGiuanise monophosphate reductase0.824XXGMPASoluce traiter aning 3.6 <td>SLC25A38</td> <td>Solute carrier family 25, member 38</td> <td>0.968</td> <td>v</td> <td></td> <td>X</td>	SLC25A38	Solute carrier family 25, member 38	0.968	v		X
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	1 SPO	PCL2/adaptivity F1P interacting protoin 2 like	0.96	Λ		A V
Alt ProcessArt Pase miniting in Kui LNoAAAlt GHHydroxyard glutathiner hydrolase0.95XXAlt AGHHittochondrial carrier triple repeat.10.945XXAlt AGHHittochondrial carrier triple repeat.10.945XXGULUGlutamate-ammonia ligase (glutamine synthesase)0.92XXGULUGlutamate-ammonia ligase (glutamine synthesase)0.918XXMDUEBPN.DH dehydrogensac (ublquinone) 1.b 90.918XXMGLLMonoglyceride lipase0.916XXXMRDLPeroxiredoxin 20.909XXXMRDLHydrocyte protein band 4.90.901XXXMVD1/6Myeloid differentiation primary response gene 1160.875XXHX1HCL31 associated X-10.875XXHAX1HCL31 associated X-10.875XXHX1Conclorables, criteriantia0.826XXSIC48A1Solut carier family 48 (Hrg1)0.826XXISCA1Iron-sulfur cluster assembly 1 homolog (S. cerevisiae)0.828XXGCN74Cytochrome coxidase, subanit Vila 20.747XXGVA74Cytochrome coxidase, subanit Vila 20.747XXGURPHemoglobin X, β-like embryonic chain0.733XXGCN74Cytochrome coxidase, subanit Vila 20.631XXGURPGlu	DINIPSL ATDIE1	A TDese inhibitory factor 1	0.96		v	A V
IACGTHydroxyacy [guitathione hydrolase0.95XXMCARTWitchondrial carrier triple report0.945XXHBB-BH1Henoglobin 2, B-like embryonic chain0.94XXGLULGuitanter-amnonic ligase (guitamine synthetase)0.92XNDUFB9NADH dehydrogenase (ubiquinone) 1 b 90.916XMGLLMongoyeende [ipase]0.916XPRDX2Peroxiredoxin 20.9008XPRDX2Peroxiredoxin 10.9008XVIND1/6Myeloid differentiation primary response gene 1160.877XMYD1/6Myeloid differentiation primary response gene 1160.877XSLC48A1Solute carrier family 48 (Hg1)0.874XBPGM2,3-Bisphosphoglycerate mutase0.8841XRESSDRieske (fe-S) domain containing0.824XXNTSC35'-Nucleotidase, cytosolic 1110.8754XXSLC25A37Solute carrier family 25, member 370.754XXSOD2Superoxide dismutase 2, mitchondrial0.733XXCOX5A2Cytochrome coxidase, subunit Vb0.731XXSDD2Superase factor formaly 25, member 370.754XXSUC25A37Solute carrier family 25, member 370.754XXCOX5A2Cytochrome coxidase, subunit Vb0.731XXSDD2Superase factor formaly 25, member 370.754XXCOX5A2Cytochrome	ABCG2	ATP-binding cassette sub-family G (WHITE) member 2	0.951		Λ	А
$\begin{array}{c c c c c c c c c c c c c c c c c c c $	HAGH	Hydroxyacyl glutathione bydrolase	0.95		х	х
$\begin{array}{c c c c c c c c c c c c c c c c c c c $	MCART1	Mitochondrial carrier triple repeat 1	0.945		X	X
GLIL GLIL INDUFB9RADH delydrogenes (lubranine synthetase) NDUFB90.918XMGLL Monoglyceride lipase0.916 0.918XXMGLL PRDX2 Peroxiredoxin 20.909 0.909XXXPRDX2 PEPB49Erythrocyte protein band 4.90.909 0.901XXXMTD116 Myeloid differentiation primary response gene 1160.877 0.877XXXMYD116 Myeloid differentiation primary response gene 1160.877 0.877XXHAX1 HCLS1 associated X-1 HAX10.876XXHAX1 HCLS1 associated X-1 HCL 	HBB-BH1	Hemoglobin Z. <i>B</i> -like embryonic chain	0.94	х		
	GLUL	Glutamate-ammonia ligase (glutamine synthetase)	0.92			Х
	NDUFB9	NADH dehydrogenase (ubiquinone) 1 b 9	0.918			Х
PRDX2Peroxiredoxin 20.909XXXXEPB4.9Erythrocyte protein band 4.90.908XXHK1Hexokinase 10.901XXHK1Hexokinase 10.901XXSLC48A1Solute carrier family 48 (Hrg1)0.875XBPGM2,3-Bisphosphoghycerate mutase0.867XBPGM2,3-Bisphosphoghycerate mutase0.867XBCA1Kronsonie momophosphate reductase0.841XRFEDRieske (Fe-5) domain containing0.826XXSCA1Kron-suffur cluster assembly 1 homolog (S. cerevisiae)0.826XXSCA2Cytochrome c oxidase, subunit VIIa 20.754XXCOX7A2Cytochrome c oxidase, subunit VIIa 20.755XXXCOX5A37Solute carrier family 25, member 370.75XXXFTH1Ferritin heavy chain 10.731XXXCOX5BCytochrome c oxidase, subunit Vb0.731XXXATP51ATP synthase mitochondrial \mathcal{P}_0 complex0.709XXARS2Phenylalanine-tRNA synthetase 2 (mitochondrial)0.653XXATP54ATP synthase, rif, complex, subunit 40.591XXATP54ATP synthase, rif, complex, subunit 40.591XXATP54ATP synthase, rif, complex, subunit 40.591XXATP54ATP synthase, rif, complex, subunit 4 <t< td=""><td>MGLL</td><td>Monoglyceride lipase</td><td>0.916</td><td>Х</td><td></td><td></td></t<>	MGLL	Monoglyceride lipase	0.916	Х		
EP84.9Erythrocyte protein band 4.90.908XHK1Hexokinase10.901XXMYD116Myeloid differentiation primary response gene 1160.877XSUC48A1Solute carrier family 48 (Hrg1)0.875XHAX1HCLS1 associated X-10.874XBPGM2.3-Bisphosphogycerate mutase0.877XGMPRGuanosine monophosphate reductase0.841XRFESDRicek (Fe-3) domain containing0.826XSCA1Iron-sulfur cluster assembly 1 homolog (S. cerevisiae)0.824XXNTSC35'-Nucleotidase, subunit VIIa 20.754XXCOX7A2Cytochrome c oxidase, subunit VIIa 20.754XXSUC2SA37Solute carrier family 25, member 370.75XXSOD2Superoxide dismutse 2, mitochondrial0.736XXGOXSBCytochrome c oxidase, subunit Vb0.731XXATPS:LATP synthase mitochondrial 7, complex0.663XXGPX4Glutathione peroxidase 40.629XXXGPX4Glutathione peroxidase 40.631XXXGPX4Glutathione peroxidase, subunit V1 a, p0/peptide 10.516XXGPX4Glutathione peroxidase, subunit V1 a, p0/peptide 10.516XXGPX4Glutathione peroxidase, subunit V1 a, p0/peptide 10.516XXGPX4Glutathione peroxidase, subunit V1 a, p0/peptide 10	PRDX2	Peroxiredoxin 2	0.909	Х	Х	Х
HKIHexokinase 10.901XXMYD116Myleloid differentiation primary response gene 1160.877XSLC48A1Solute carrier family 48 (Hrg1)0.875XHAX1HCLS1 associated X.10.875XBPGM2.3-Bisphosphoghycerate mutase0.857XGMPRGuansine monophosphate reductase0.841XRFESDRieske (Fe-S) domain containing0.824XXNTSC3S'-Nucleotidase, cytosolic III0.808XXCOX7A2Cytochrome c oxidase, subunit VIIa 20.754XXSLC25A37Solute carrier family 25, member 370.75XXXFTH1Ferritin heavy chain 10.736XXXCOX5ACytochrome c oxidase, subunit Vb0.733XXSOD2Superoxide dismutase 2, mitochondrial0.733XXGMRS2Magnesium homeostasis factor homolog (S. cerevisiae)0.648XXATPS1.ATP synthase mitochondrial F_0 complex0.653XXGPX4Glutathione peroxidase 40.629XXXQUOTEOHER/KRIKSN CDNA 2010107E04 gene0.516XXATPS1.ATP synthase, Fi-q complex, subunit V1 a polypeptide 10.516XXQUOTEOHER/KRIKSN CDNA 2010107E04 gene0.428XXQUAGA4Cytochrome c oxidase, subunit V1 a polypeptide 10.516XXQUAGA4Guatahione preceptor-interacting	EPB4.9	Erythrocyte protein band 4.9	0.908	Х		
$\begin{array}{c c c c c c c c c c c c c c c c c c c $	HK1	Hexokinase 1	0.901		X	Х
	MYD116	Myeloid differentiation primary response gene 116	0.877	Х		
$\begin{array}{c c c c c c c c c c c c c c c c c c c $	SLC48A1	Solute carrier family 48 (Hrg1)	0.875			V
Dr On2,5-Displosibility of the full data for the eductase0.841XRFESDRieske (Fe-S) domain containing0.826ISCA1Iron-sulfar cluster assembly 1 homolog (S. cerevisiae)0.824XXCX732Cytochrome c vidase, subunit VIIa 20.754XCOX7A2Cytochrome c vidase, subunit VIIa 20.754XSLC25A37Solute carrier family 25, member 370.75XXFTH1Ferritin heavy chain 10.747XXCOX5BCytochrome c vidase, subunit Vb0.733XXSOD2Superoxide dismutase 2, mitochondrial0.733XXCOX5BCytochrome c vidase, subunit Vb0.731XXATPS1ATP synthase mitochondrial F_0 complex0.709XXATPS1ATP synthase mitochondrial F_0 complex0.663XXMRS2Magnesium homeostasis factor homolog (S. cerevisiae)0.648XXSLC25A39Solute carrier family 25, member 390.591XXXATPS4ATP synthase, F ₁ F ₀ complex, subunit e0.53XXXOU1007C04RIKRIKEN cDNA 2010107E04 gene0.516XXXAIPArtly hydrocarbon receptor-interacting protein0.503XXXLARS2Leuryl-RINA synthetase, mitochondrial0.443XXZOU1007C04RIKRIKEN coldase, subunit VIc0.428XXZLARS2Leuryl-RINA synthetase, mitochondrial <td>PDCM</td> <td>HULSI associated X-1</td> <td>0.8/4</td> <td></td> <td></td> <td>Λ</td>	PDCM	HULSI associated X-1	0.8/4			Λ
On TAOutstate Minophysical relationship0.871XRFESDRieske (Fe-S) domain containing0.826XXISCA1Iron-suffur cluster assembly 1 homolog (S. cerevisiae)0.824XXNT5C35'-Nucleotidase, cytosolic III0.808XXCOX7A2Cytochrome c oxidase, subunit VIa 20.754XXSubte carrier family 25, member 370.75XXXFTH1Ferritin heavy chain 10.747XXHBP-YHemoglobin Y, β-like embryonic chain0.736XXSOD2Superoxide dismutase 2, mitochondrial0.733XXCOX5BCytochrome c oxidase, subunit Vb0.731XXATPSLATP synthase mitochondrial P_0 complex0.693XXMRS2Magnesium homeostasis factor homolog (S. cerevisiae)0.648XXGPX4Glutathione peroxidase 40.629XXX2010107E04RIKRIKEN cDNA 2010107E04 gene0.531XXATPSAryl hydrocarbon receptor-interacting protein0.503XXALPAryl hydrocarbon receptor-interacting protein0.503XXLARS2Leuxyl-tRNA synthetase, mitochondrial0.486XXCOX6ACCytochrome c oxidase, subunit VIc0.443XXLARS2Leuxyl-tRNA synthetase, mitochondrial0.496XXLARS2Leuxyl-tRNA synthetase, mitochondrial0.486XX <td>CMDP</td> <td>Cuanosino mononhosphata reductase</td> <td>0.837</td> <td>v</td> <td></td> <td></td>	CMDP	Cuanosino mononhosphata reductase	0.837	v		
ISCA1Iron-sulfur cluster assembly 1 homolog (S. cerevisiae)0.854XXNTSC35'-Nucleotidase, cytosolic III0.808XXCOX7A2Cytochrome c oxidase, subunit VIIa 20.754XSLC25A37Solute carrier family 25, member 370.75XXFTH1Ferritin heavy chain 10.774HBB-YHemoglobin Y, β -like embryonic chain0.736XXSOD2Superoxide dismutase 2, mitochondrial0.733XXCOX5BCytochrome c oxidase, subunit Vb0.731XXATP5LATP synthase mitochondrial β_1 complex0.709XXFARS2Phenylalanine-tRNA synthetase 2 (mitochondrial)0.653XXMRS2Magnesium homeostasis factor homolog (S. cerevisiae)0.648XXSL225A39Solute carrier family 25, member 390.591XXX201007E04RIKRIKEN cDNA 2010107E04 gene0.53XXXATP5KATP synthase, Fi_c complex, subunit e0.516XXXAIPAryl hydrocarbon receptor-interacting protein0.503XXXBE73Early B-cell factor 30.428XXXCOX6CCytochrome c oxidase, subunit VIc0.425XXLARS2Leucyl-tRNA synthetase, nitochondrial0.411XXADPAryl hydrocarbon receptor-interacting protein0.412XXDI007E04RIKBCL21Li <td>RFFSD</td> <td>Rieske (Fe-S) domain containing</td> <td>0.826</td> <td>А</td> <td></td> <td></td>	RFFSD	Rieske (Fe-S) domain containing	0.826	А		
NT5C35'-Nucleotidase, cytosolic III0.808XXCOX7A2Cytochrome c oxidase, subunit VIIa 20.754XCOX7A2Cytochrome c oxidase, subunit VIIa 20.754XSU225A37Solute carrier family 25, member 370.75XXFTH1Ferritin heavy chain 10.747	ISCA1	Iron-sulfur cluster assembly 1 homolog (S. cerevisiae)	0.824		Х	Х
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SLC25A37Solute carrier family 25, member 370.75XXFTH1Ferritin heavy chain 10.747HBB-YHemoglobin Y, B-like embryonic chain0.736SOD2Superoxide dismutase 2, mitochondrial0.733XCOX5BCytochrome c oxidase, subunit Vb0.731XCOX5EATP synthase mitochondrial Γ_{0} complex0.709XFARS2Phenylalanine-tRNA synthetase 2 (mitochondrial)0.653XMRS2Magnesium homeostasis factor homolog (S. cerevisiae)0.648XGPX4Glutathione peroxidase 40.629XSLC25A39Solute carrier family 25, member 390.591XX2010107E04RIKRIKEN cDNA 2010107E04 gene0.53XX2010107E04RIKRIKEN cDNA 2010107E04 gene0.503XXLARS2Leucyl-tRNA synthetase, mitochondrial0.448XXLARS2Leucyl-tRNA synthetase, mitochondrial0.443XXLARS2Leucyl-tRNA synthetase, mitochondrial0.443XXLARS2Leucyl-tRNA synthetase, mitochondrial0.428XXCOX6CCytochrome c reductase hinge protein0.415XXNDNNecdin (Ndn), mRNA0.415XXNDNNecdin (Ndn), mRNA0.415XXNDNNecdin (Ndn), mRNA0.364XXFSU1IseU iron-sulfur cluster scafold homolog (E. coli)0.364XNDNFisso 1 homolog (yeast) <t< td=""><td>COX7A2</td><td>Cytochrome <i>c</i> oxidase, subunit VIIa 2</td><td>0.754</td><td></td><td></td><td>Х</td></t<>	COX7A2	Cytochrome <i>c</i> oxidase, subunit VIIa 2	0.754			Х
FTH1Ferritin heavy chain 10.747HBB-YHemoglobin Y, β -like embryonic chain0.736SOD2Superoxide dismutase 2, mitochondrial0.731XCOX5BCytochrome c oxidase, subunit Vb0.731XATP5LATP synthase mitochondrial F_0 complex0.709XMRS2Phenylalanine-tRNA synthetase 2 (mitochondrial)0.653XMRS2Magnesium homeostasis factor homolog (S. cerevisiae)0.648XGPX4Glutathione peroxidase 40.629XSLC2SA39Solute carrier family 25, member 390.511XXATP5KATP synthase, F_1F_0 complex, subunit e0.533XX2010107E04RIKRIKEN cDNA 2010107E04 gene0.516XXAIPAryl hydrocarbon receptor-interacting protein0.503XXBCL2.11BCL2.11ke 10.443XXEBF3Early B-cell factor 30.428XCOX6CCytochrome c raidase, subunit VIc0.425XLQCRHUbiquinol-cytochrome c raidase, subunit VIc0.425XNDNNecdin (Mah), mRNA0.411XNDNNecdin (Mah), mRNA0.411NDNNecdin (Mah), mRNA0.411NDNNecdin (Mah), mRNA0.411NDNNecdin (Mah), mRNA0.411NDNKetin Pision 1 homolog (yeast)0.272X	SLC25A37	Solute carrier family 25, member 37	0.75		Х	Х
HB-YHemoglobin Y, β -like embryonic chain0.736SOD2Superoxide dismutase 2, mitochondrial0.733XSOD2Cytochrome c oxidase, subunit Vb0.731XATP5LATP synthase mitochondrial F_0 complex0.709XFARS2Phenylalanine-tRNA synthetase 2 (mitochondrial)0.653XMRS2Magnesium homeostasis factor homolog (S. cerevisiae)0.648XGPX4Glutathione peroxidase 40.629XSLC25A39Solute carrier family 25, member 390.591XX TP5KATP synthase, F, F_0 complex, subunit e0.543X2010107E04RIKRIKEN cDNA 2010107E04 gene0.533XCX6A1Cytochrome c oxidase, subunit VI a, polypeptide 10.516XAIPAryl hydrocarbon receptor-interacting protein0.443XBCL21.1BCL21.1ke 10.443XEBF3Early B-cell factor 30.428XCOX6CCytochrome c oxidase, subunit VIc0.425XUQCRHUbiquinol-cytochrome c reductase hinge protein0.415XNDNNecdin (Ndn), mRNA0.41XNDNNecdin (Ndn), mRNA0.41XFIS1Fision 1 homolog (yeast)0.272X	FTH1	Ferritin heavy chain 1	0.747			
SOD2Superoxide dismutase 2, mitochondrial0.733XCOX5BCytochrom c oxidase, subunit Vb0.731XATP5LATP synthase mitochondrial F_0 complex0.709XFARS2Phenylalanine-tRNA synthetase 2 (mitochondrial)0.653XMRS2Magnesium homeostasis factor homolog (S. cerevisiae)0.648XGPX4Glutathione peroxidase 40.629XSLC25A39Solute carrier family 25, member 390.591XX TP5KATP synthase, F ₁ F ₀ complex, subunit e0.543X2010107E04RIKRIKEN CDNA 2010107E04 gene0.516XCOX6A1Cytochrome c oxidase, subunit VI a, polypeptide 10.516XAIPAryl hydrocarbon receptor-interacting protein0.503XLARS2Leucyl-tRNA synthetase, mitochondrial0.448XBCL2L1BCL2-like 10.443XEBF3Early B-cell factor 30.428XCOX6CCytochrome c oxidase, subunit VIc0.425XUQCRHUbiquinol-cytochrome c reductase hinge protein0.419XACTBActin, β 0.415XNDNNeedin (Ndn), mRNA0.415XNDNNeedin (Ndn), mRA0.364XISCUIscU iron-sulfur cluster scaffold homolog (E. coli)0.364XFIS1Fision 1 homolog (yeast)0.272X	HBB-Y	Hemoglobin Y, β -like embryonic chain	0.736			
$\begin{array}{ccccc} COXSB & Cytochrome c oxidase, subunit Vb & 0.731 & X \\ ATP5L & ATP synthase mitochondrial F_0 complex 0.709 & X \\ FARS2 & Phenylalanine-tRNA synthetase 2 (mitochondrial) 0.653 & X \\ MRS2 & Magnesium homeostasis factor homolog (S. cerevisiae) 0.648 & X \\ GPX4 & Glutathione peroxidase 4 & 0.629 & X \\ SLC25A39 & Solute carrier family 25, member 39 & 0.591 & X & X \\ 2010107E04RIK & RIKEN cDNA 2010107E04 gene & 0.53 & X \\ COX6A1 & Cytochrome c oxidase, subunit VI a, polypeptide 1 & 0.516 & X \\ LARS2 & Leucyl-tRNA synthetase, mitochondrial & 0.486 & X \\ BCL2L1 & BCL2-like 1 & 0.443 & X \\ EBF3 & Early B-cell factor 3 & 0.428 & COX6C & Cytochrome c reductase hinge protein & 0.419 & X \\ UQCRH & Ubiquinol-cytochrome c reductase hinge protein & 0.415 & X \\ ACTB & Actin, \beta & 0.411 & THSD7B & Thrombospondin, type I, domain containing 7B & 0.398 & X \\ ISCU & IscU iron-sulfur cluster scaffold homolog (E. coli) & 0.364 & X \\ IDATter of the Therm of the Total cluster scaffold homolog (E. coli) & 0.364 & X \\ IDATter of the Total cluster scaffold homolog (E. coli) & 0.364 & X \\ IDATter of the Total cluster scaffold homolog (E. coli) & 0.364 & X \\ IDATTER of the Total cluster scaffold homolog (E. coli) & 0.364 & X \\ IDATTER of the Total cluster scaffold homolog (E. coli) & 0.364 & X \\ IDATTER of the Total cluster scaffold homolog (E. coli) & 0.364 & X \\ IDATTER of the Total cluster scaffold homolog (E. coli) & 0.364 & X \\ IDATTER of the Total cluster scaffold homolog (E. coli) & 0.364 & X \\ IDATTER of the Total cluster scaffold homolog (E. coli) & 0.364 & X \\ IDATTER of the Total cluster scaffold homolog (E. coli) & 0.364 & X \\ IDATTER of the Total cluster scaffold homolog (E. coli) & 0.364 & X \\ IDATTER of the Total cluster scaffold homolog (E. coli) & 0.364 & X \\ IDATTER of the Total cluster scaffold homolog (E. coli) & 0.364 & X \\ IDATTER of the Total cluster scaffold homolog (E. coli) & 0.364 & X \\ IDATTER of the Total cluster scaffold homolog (E. coli) & 0.364 & X \\ IDATTER of th$	SOD2	Superoxide dismutase 2, mitochondrial	0.733			Х
ATPSLATP synthase mitochondrial Γ_0 complex0.709XFARS2Phenylalanine-tRNA synthetase 2 (mitochondrial)0.653XMRS2Magnesium homeostasis factor homolog (S. cerevisiae)0.648XGPX4Glutathione peroxidase 40.629XSLC25A39Solute carrier family 25, member 390.591XX2010107E04RIKRIKEN cDNA 2010107E04 gene0.53XX2010107E04RIKRIKEN cDNA 2010107E04 gene0.516XXCOX6A1Cytochrome c oxidase, subunit V1 a, polypeptide 10.516XXAIPAryl hydrocarbon receptor-interacting protein0.503XXBCL2L1BCL2-like 10.448XXBCL2L1BCL2-like 10.443XXEBF3Early B-cell factor 30.415XXVQCRHUbiquinol-cytochrome c reductase hinge protein0.415XXNDNNecdin (Ndn), mRNA0.41XXXISCUIscU iron-sulfur cluster scaffold homolog (E. coli)0.364XXFIS1Fision 1 homolog (yeast)0.272XX	COX5B	Cytochrome <i>c</i> oxidase, subunit Vb	0.731			Х
PARS2Phenylalanne-tKNA synthetase 2 (mitochondrial)0.653XMRS2Magnesium homeostasis factor homolog (S. cerevisiae)0.648XGPX4Glutathione peroxidase 40.629XSLC25A39Solute carrier family 25, member 390.591XXATP5KATP synthase, F_1F_0 complex, subunit e0.543X2010107E04RIKRIKEN cDNA 2010107E04 gene0.53XCOX6A1Cytochrome c oxidase, subunit V1 a, polypeptide 10.516XAIPAryl hydrocarbon receptor-interacting protein0.503XLARS2Leucyl-tRNA synthetase, mitochondrial0.486XBCL2L1BCL2-like 10.443XEBF3Early B-cell factor 30.428COX6CCytochrome c oxidase, subunit VIc0.415XUQCRHUbiquinol-cytochrome c reductase hinge protein0.415XNDNNecdin (Ndn), mRNA0.41TTHSD7BThrombospondin, type I, domain containing 7B0.398XISCUIscU iron-sulfur cluster scaffold homolog (<i>E. coli</i>)0.364XISCUIscU iron-sulfur cluster scaffold homolog (<i>E. coli</i>)0.364XISCUIscU iron-sulfur cluster scaffold homolog (<i>E. coli</i>)0.272X	ATP5L	ATP synthase mitochondrial F_0 complex	0.709			X
MRS2Magnesium homeostasis factor homolog (S. cereviside)0.648XGPX4Glutathione peroxidase 40.629XSILC25A39Solute carrier family 25, member 390.591XXATP5KATP synthase, F_1F_0 complex, subunit e0.543X2010107E04RIKRIKEN cDNA 2010107E04 gene0.53XCOX6A1Cytochrome c oxidase, subunit VI a, polypeptide 10.516XAIPAryl hydrocarbon receptor-interacting protein0.503XLARS2Leucyl-tRNA synthetase, mitochondrial0.4486XBCL2L1BCL2-like 10.443XEBF3Early B-cell factor 30.428XCOX6CCytochrome c oxidase, subunit VIc0.425XUQCRHUbiquinol-cytochrome c reductase hinge protein0.415XNDNNecdin (Ndn), mRNA0.41TTHSD7BThrombospondin, type I, domain containing 7B0.398XISCUIscU iron-sulfur cluster scaffold homolog (<i>E. coli</i>)0.364XFIS1Fision 1 homolog (yeast)0.272X	FARS2	Phenylalanine-tRNA synthetase 2 (mitochondrial)	0.653			X
GPA4Glutatione peroxidase 40.629XSILC25A39Solute carrier family 25, member 390.591XXATP5KATP synthase, F_1F_0 complex, subunit e0.543X2010107E04RIKRIKEN cDNA 2010107E04 gene0.53XCOX6A1Cytochrome c oxidase, subunit VI a, polypeptide 10.516XAIPAryl hydrocarbon receptor-interacting protein0.503XLARS2Leucyl-tRNA synthetase, mitochondrial0.486XBCL2L1BCL2-like 10.443XEBF3Early B-cell factor 30.425XCOX6CCytochrome c oxidase, subunit VIc0.415XUQCRHUbiquinol-cytochrome c reductase hinge protein0.415XNDNNecdin (Ndn), mRNA0.41THSD7BThrombospondin, type I, domain containing 7B0.398XISCUIscU iron-sulfur cluster scaffold homolog (E. coli)0.364XXFIS1Fision 1 homolog (yeast)0.272XX	MKS2 CDV4	Magnesium homeostasis factor homolog (S. cerevisiae)	0.648	v		X
SUC25A55Solute Calibration (Ndn), mRNAComplexityComplexityXXXATP5 XATP synthase, F,F_0 complex, subunit e0.543X2010107E04RIKRIKEN cDNA 2010107E04 gene0.53XCOX6A1Cytochrome c oxidase, subunit V1 a, polypeptide 10.516XAIPAryl hydrocarbon receptor-interacting protein0.503XLARS2Leucyl-tRNA synthetase, mitochondrial0.486XBCL2L1BCL2-like 10.443XEBF3Early B-cell factor 30.428COX6CCOX6CCytochrome c oxidase, subunit VIc0.425XUQCRHUbiquinol-cytochrome c reductase hinge protein0.415XNDNNecdin (Ndn), mRNA0.41THSD7BThrombospondin, type I, domain containing 7B0.398XISCU1IscU iron-sulfur cluster scaffold homolog (E. coli)0.364XXFIS1Fission 1 homolog (yeast)0.272X	GPA4 SLC25420	Giutatnione peroxidase 4	0.629	А	v	v
ATT SNATT SN thatse, 110 complex, suburit e0.545X2010107E04RIKRIKEN cDNA 201007E04 gene0.53XCOX6A1Cytochrome c oxidase, subunit VI a, polypeptide 10.516XAIPAryl hydrocarbon receptor-interacting protein0.503XLARS2Leucyl-tRNA synthetase, mitochondrial0.486XBCL2L1BCL2-like 10.443XEBF3Early B-cell factor 30.428XCOX6CCytochrome c oxidase, subunit VIc0.425XUQCRHUbiquinol-cytochrome c reductase hinge protein0.419XACTBActin, β 0.411THSD7BTHSD7BThrombospondin, type I, domain containing 7B0.398XISCUIscU iron-sulfur cluster scaffold homolog (E. coli)0.364XFIS1Fission 1 homolog (yeast)0.272X	ATD5K	ATD synthese E E complex subunit e	0.591		л	A Y
2010/07/1001 (Subtraction Loss of the Line of the Subtraction Constraints)10.516XCOX6A1Cytochrome c oxidase, subunit VI a, polypeptide 10.516XAIPAryl hydrocarbon receptor-interacting protein0.503XLARS2Leucyl-tRNA synthetase, mitochondrial0.486XBCL2L1BCL2-like 10.443XEBF3Early B-cell factor 30.428XCOX6CCytochrome c oxidase, subunit VIc0.425XUQCRHUbiquinol-cytochrome c reductase hinge protein0.419XACTBActin, β 0.415XNDNNecdin (Ndn), mRNA0.41THSD7BThrombospondin, type I, domain containing 7B0.398XISCU1IscU iron-sulfur cluster scaffold homolog (E. coli)0.364XFIS1Fission 1 homolog (yeast)0.272X	2010107F04RIK	RIKEN cDNA 2010107E04 gene	0.545			X
AIPAryl hydrocarbon receptor-interacting protein0.503XLARS2Leucyl-tRNA synthetase, mitochondrial0.486XBCL2L1BCL2-like 10.443XEBF3Early B-cell factor 30.428XCOX6CCytochrome c oxidase, subunit VIc0.425XUQCRHUbiquinol-cytochrome c reductase hinge protein0.415XNDNNecdin (Ndn), mRNA0.41THSD7BTHSD7BThrombospondin, type I, domain containing 7B0.398XISCUIscU iron-sulfur cluster scaffold homolog (E. coli)0.364XFIS1Fission 1 homolog (yeast)0.272X	COX6A1	Cytochrome c oxidase, subunit VI a, polypeptide 1	0.516			X
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	AIP	Arvl hydrocarbon receptor-interacting protein	0.503			X
BCL2L1BCL2-like 10.443XEBF3Early B-cell factor 30.428COX6CCytochrome c oxidase, subunit VIc0.425XUQCRHUbiquinol-cytochrome c reductase hinge protein0.419XACTBActin, β 0.415NDNNecdin (Ndn), mRNA0.41THSD7BThrombospondin, type I, domain containing 7B0.398XISCUIscU iron-sulfur cluster scaffold homolog (E. coli)0.364XFIS1Fission 1 homolog (yeast)0.272X	LARS2	Leucyl-tRNA synthetase, mitochondrial	0.486			Х
EBF3Early B-cell factor 30.428COX6CCytochrome c oxidase, subunit VIc0.425XUQCRHUbiquinol-cytochrome c reductase hinge protein0.419XACTBActin, β 0.4151NDNNecdin (Ndn), mRNA0.411THSD7BThrombospondin, type I, domain containing 7B0.398XISCUIscU iron-sulfur cluster scaffold homolog (E. coli)0.364XFIS1Fission 1 homolog (yeast)0.272X	BCL2L1	BCL2-like 1	0.443			Х
$\begin{array}{ccc} COX6C & Cytochrome c oxidase, subunit VIc & 0.425 & X \\ UQCRH & Ubiquinol-cytochrome c reductase hinge protein & 0.419 & X \\ ACTB & Actin, \beta & 0.415 & \\ NDN & Necdin (Ndn), mRNA & 0.41 & \\ THSD7B & Thrombospondin, type I, domain containing 7B & 0.398 & X & \\ ISCU & IscU iron-sulfur cluster scaffold homolog (E. coli) & 0.364 & X \\ FIS1 & Fission 1 homolog (yeast) & 0.272 & X & \\ \end{array}$	EBF3	Early B-cell factor 3	0.428			
$\begin{array}{ccc} UQCRH & Ubiquinol-cytochrome c reductase hinge protein & 0.419 & X \\ ACTB & Actin, \beta & 0.415 & \\ NDN & Necdin (Ndn), mRNA & 0.41 & \\ THSD7B & Thrombospondin, type I, domain containing 7B & 0.398 & X & \\ ISCU & IscU iron-sulfur cluster scaffold homolog (E. coli) & 0.364 & X \\ FIS1 & Fission 1 homolog (yeast) & 0.272 & X & \\ \end{array}$	COX6C	Cytochrome c oxidase, subunit VIc	0.425			Х
ACTBActin, β 0.415NDNNecdin (Ndn), mRNA0.41THSD7BThrombospondin, type I, domain containing 7B0.398XISCUIscU iron-sulfur cluster scaffold homolog (E. coli)0.364XFIS1Fission 1 homolog (yeast)0.272X	UQCRH	Ubiquinol-cytochrome <i>c</i> reductase hinge protein	0.419			Х
NDN Necdin (Ndn), mRNA 0.41 THSD7B Thrombospondin, type I, domain containing 7B 0.398 X ISCU IscU iron-sulfur cluster scaffold homolog (E. coli) 0.364 X FIS1 Fission 1 homolog (yeast) 0.272 X	ACTB	Actin, β	0.415			
1 FISD/B 1 nrombospondin, type I, domain containing / B 0.398 X ISCU IscU iron-sulfur cluster scaffold homolog (E. coli) 0.364 X FIS1 Fission 1 homolog (yeast) 0.272 X	NDN TUCD7D	Necdin (Ndn), mKNA	0.41	V		
Fisch Fisch Transform Driven test for the scale of th	THSD/B	I nrombospondin, type I, domain containing /B	0.398	х		v
A Date are from Date of A	FIS1	Fission 1 homolog (veset)	0.304			X
	4 Data and Di		0.272			24

^b Data are from Nilsson *et al.* (11).

Data are nom Misson et ut. (11).

for erythropoiesis rather than of general iron stores (30). This ratio increases when mitochondrial iron delivery is impaired and is an early indicator of iron-deficient erythropoiesis (30–32). The ZnPP-to-heme ratio in $Abcb6^{-/-}$ animals was more than twice that in wild-type animals post-Phz (Fig. 6*E*), suggesting an inability to meet the demand for mitochondrial iron. We

determined if this was a systemic defect in iron absorption by measuring serum iron and transferrin levels. The comparable levels in $Abcb6^{-/-}$ and $Abcb6^{+/+}$ mice (supplemental Fig. S6) indicate a systemic defect in iron absorption is unlikely. This deficiency is likely to be caused by defective mitochondrial iron regulation.





FIGURE 4. Increased mortality and impaired stress erythropoiesis in $Abcb6^{-/-}$ mice. *A*, EKLF and FECH protein expression is greater in $Abcb6^{-/-}$ than $Abcb6^{+/+}$ hematopoietic cells. *B*, mortality on days 3–7 of Phz treatment. *C*–*F*, mean (±S.E.) hematologic parameters of 1-month-old female mice injected with Phz on days 0, 1, and 3 (total dose, 50 mg/kg). *C*, hematocrit ($n = 11-30 \ Abcb6^{+/+}$, 13–46 $Abcb6^{-/-}$ mice). *D*, corrected reticulocyte count ($n = 9-25 \ Abcb6^{+/+}$, 12–37 $Abcb6^{-/-}$ mice). *E*, red cell distribution width (*RDW*) ($n = 10-26 \ Abcb6^{+/+}$, 13–44 $Abcb6^{-/-}$ mice). *F*, hemoglobin ($n = 10-26 \ Abcb6^{+/+}$, 13–44 $Abcb6^{-/-}$ mice). *F*, hemoglobin ($n = 10-26 \ Abcb6^{+/+}$, 13–44 $Abcb6^{-/-}$ mice). *C*, mean corpuscular volume (*MCV*) ($n = 8-27 \ Abcb6^{-/-}$ mice). *, p = 0.02-0.03; **, p = 0.01-0.02; ***, p = 0.003-0.009; ****, p = <0.002.

Gene Expression Changes after Phenylhydrazine Treatment— We hypothesized that the up-regulated genes in the $Abcb6^{-/-}$ mice might not be regulated appropriately under Phz stress. To test this proposition, we compared gene transcripts in erythroblasts from $Abcb6^{+/+}$ and $Abcb6^{-/-}$ mice before and 10 days after Phz treatment (Fig. 7 and supplemental Table S1). Many of the genes found to be up-regulated during stress erythropoiesis (by a factor of 1.5 or more) in $Abcb6^{+/+}$ erythroblasts were the same genes constitutively up-regulated in the untreated $Abcb6^{-/-}$ mice (Table 3). During Phz stress, the $Abcb6^{-/-}$



FIGURE 5. Erythroblast response to stress erythropoiesis is altered in Abcb6^{-/-} **mice.** A, serum erythropoietin assayed by ELISA in Abcb6⁺ mice that were untreated (n = 7), at day 8 of Phz treatment (n = 5), or at day 10 of Phz treatment (n = 5) and in Abcb6^{-/-} mice that were untreated (n = 7), at day 8 of Phz treatment (n = 4), or at day 10 of Phz treatment (n = 8). *B*, duration of erythrocyte survival in 2–4 Abcb6^{+/+} and 6–7 Abcb6^{-/-} Phz-treated Phz-treated mice. Day 0 of N-hydroxysuccinimide-biotin labeling = day 6 or 7 of the Phz regimen. C, apoptotic spleen and bone marrow Ter119-positive cells on day 8 of Phz treatment in 9 $Abcb6^{+/+}$ and 10 $Abcb6^{-/-}$ mice. D, percentage of splenic reticulocytes on days 8 and 10 in 3–4 $Abcb6^{+/+}$ and 5–6 $Abcb6^{-/-}$ mice. *E*, erythroid CFU in spleens of $Abcb6^{+/+}$ mice that were untreated (n =5), at day 8 of Phz treatment (n = 11), at day 10 of Phz treatment (n = 9), and mice that were untreated (n = 6), at day 8 of Phz treatment (n = 14), Abcb6⁻ or at day 10 of Phz treatment (n = 7) on days 8 and 10. Values are means (±S.E.).

erythroblasts showed either no change or reduced expression of these genes (including *fech, blvrb, cpox, slc48a1, EKLF, nfe2,* and *eif2ak1*), indicating that genes that are important in stress erythropoiesis are constitutively up-regulated in the absence of *Abcb6*.

DISCUSSION

We have presented the first evidence that Abcb6 is the sole mammalian ATP-dependent mitochondrial porphyrin importer and is required for normal porphyrin synthesis during phenylhydrazine stress. Non-ATP-dependent mitochondrial porphyrin transport was not enhanced in the absence of Abcb6, indicating that basal levels of such transport are sufficient for unstressed survival. We demonstrated that in erythroid cells, loss of Abcb6 is compensated for by up-regulation of multiple genes in the porphyrin biosynthetic pathway. Although these changes appear sufficient for survival under normal conditions, the increased mortality, elevated PPIX levels, and decreased

ABCB6 Protects against Phenylhydrazine Toxicity



FIGURE 6. PPIX and ZnPP are elevated in Phz-treated Abcb6mice. A, PPIX was assayed in blood samples from 7 to 23 Abcb6^{+/+} and 5-26 mice on the indicated days after treatment. B, PPIX in splenic Abcb6⁻ erythroid cells from $Abcb6^{+/+}$ mice that were untreated (n = 8), at day 8 of Phz treatment (n = 11), or at day 10 of Phz treatment (n = 3) and from mice that were untreated (n = 8), at day 8 of Phz treatment (n = 14), Ahch6 or at day 10 of Phz treatment (n = 5). C, heme/PPIX ratio in blood from 4 and 2–6 $Abcb6^{-/-}$ mice. D, reticulocyte heme on day 10 of Phz in $4Abcb6^{+/+}$ and $6Abcb6^{-/-}$ mice. E, ZnPP/heme ratio on day 10 Abcb6+/ treatment in 4 Abcb6^{+/} mice. E, ZnPP/heme ratio on day 10 in blood from 4 Abcb6+/+ and 4 Abcb6^{-/-} mice. Values are means ±S.E. **, p = 0.01 - 0.02; ***, p = 0.003 - 0.009; ****, p = 0.002. NS, not significant.

production of heme in $Abcb6^{-/-}$ mice suggest that Abcb6 is crucial to surviving some stresses.

In view of our previous finding that loss of one Abcb6 allele in embryonic stem cells was associated with reduced PPIX levels, it was surprising to find elevated PPIX in Abcb6^{-/-}erythroid cells. However, loss of Abcb6 is associated with constitutive changes in gene expression that are consistent with conservation of porphyrins. The elevated porphyrins and altered gene expression do not produce overt effects in viable Abcb6-null mice under normal conditions. However, the deleterious effects of these adaptive changes become apparent during chemically induced acute stress erythropoiesis, i.e. greater mortality and a more sustained anemia associated with reduced erythroid heme concentration despite elevated PPIX. Elevated PPIX can result from iron deficiency or from defective function of FECH (22), the final enzyme in the heme pathway. FECH incorporates iron into the porphyrin ring of PPIX in the presence of an ironsulfur cluster (ISC) (33). Under normal conditions, serum and tissue iron levels are similar in $Abcb6^{+/+}$ and $Abcb6^{-/-}$ mice, arguing against a general iron storage defect or malabsorption of dietary iron. Systemic iron usage appears to be unimpaired





FIGURE 7. *Abcb6^{-/-}* mouse erythroblasts show impaired up-regulation of heme biosynthetic, ISC, and iron homeostasis genes. Many genes identified in the expression screening analysis were up-regulated during stress erythropoiesis (day 10 after the start of Phz treatment) in wild-type mice but not in *Abcb6^{-/-}* mice. Each microarray contained RNA from 2 to 4 erythroblast samples.

after stress erythropoiesis, as serum iron and transferrin levels in $Abcb6^{-/-}$ and $Abcb6^{+/+}$ mice are similar at that time. Defective FECH activity could explain the increased PPIX, but the increase in ZnPP indicates that FECH is functional (23, 34), ruling out defective FECH.

The basal up-regulation of many heme and iron genes in the $Abcb6^{-/-}$ mice strongly resembled that in the wild-type mice after phenylhydrazine treatment. However, loss of Abcb6 is associated with up-regulation of many ISC biogenesis genes. In vertebrates, GIrx5 deficiency provided the first indication that ISC biogenesis and heme biosynthesis are related (35, 36). Of these, Glrx5, Isca1, Rfesd, and HSCB showed a strong association (integrated p values >0.82) with the heme biosynthetic genes in our expression screening. We propose that the impaired stress response to phenylhydrazine is partially compensated by up-regulation of *Glrx5* and *Isca1*. However, the inadequate response reflected in a lower survival of Abcb6^{-/-}mice suggests compensation is incomplete. Deficiencies in the mitochondrial ISC transporter Abcb7 produces anemia characterized by elevated erythrocyte PPIX and elevated ZnPP-to-heme ratio (37). The lack of change in *Abcb7* gene expression in the $Abcb6^{-/-}$ mice suggests this is a potential explanation for poor compensation.

Limitation of a factor required for mitochondrial iron acquisition might also explain the reduced heme in $Abcb6^{-/-}$ mice. One potential candidate is Abcb10, a mitochondrial ABC transporter that forms a complex with FECH and the mitochondrial inner membrane transporter Slc25a37 (mitoferrin) (8). Slc25a37 is responsible for importing iron for heme synthesis. Abcb10 interacts with and stabilizes Slc25a37, resulting in increased mitochondrial iron in differentiated MEL cells (38). Targeted deletion of *Abcb10* in mice results in embryonic lethality due to defective erythropoiesis (39). The constitutive level of Abcb10, unlike that of FECH and Slc25a37, is not increased in *Abcb6^{-/-}* erythroid cells. Thus, one possible explanation of impaired heme formation despite adequate PPIX and active FECH is insufficient availability of Abcb10 to form the necessary complexes with FECH and Slc25a37. Under these conditions, decreased mitochondrial iron might account for the reduced heme production.

Other associated gene expression changes suggest that $Abcb6^{-/-}$ erythroblasts preserve pre-existing heme within the cell. Our microarray analysis indicated that $Abcb6^{-/-}$ erythroblasts express lower levels of heme oxygenase 1 (Hmox 1), which catalyzes heme degradation. This finding is consistent with the observed reduction in heme, which positively regulates Hmox1 (40–42). Slc48a1 (Hrg-1), a heme-binding protein that associates with the endosomes and acts as a heme chaperone (7, 43), was also up-regulated in $Abcb6^{-/-}$ erythroblasts, suggesting additional possible mechanisms to conserve heme and maintain iron homeostasis.

Elevated intracellular PPIX is highly cytotoxic to red blood cells and can lead to damage from free radicals. In the disease protoporphyria, excess PPIX causes extensive tissue damage (44). The sustained PPIX levels observed in $Abcb6^{-/-}$ red blood cells after phenylhydrazine treatment are a possible explanation for the greater mortality of $Abcb6^{-/-}$ mice. The membrane transporter Abcg2 is known to modulate the effects of protoporphyria and may serve to export excess intracellular PPIX, reducing potential cytotoxic damage (1, 44). The increased expression of Abcg2 mRNA in the splenic erythroblasts of Abcb6^{-/-} mice may be a survival response to elevated PPIX. As an adaptive change, the increased Abcg2 expression may reduce the concentration of PPIX, under normal conditions. However it's reduction during phenylhydrazine treatment may be insufficient to protect against PPIX-induced cytotoxicity, consistent with the increased mortality observed.

We have shown that the absence of Abcb6 results in loss of ATP-dependent mitochondrial porphyrin import. One implication of these findings is that developmental absence of Abcb6 is survivable, provided rapid porphyrin import is not required. However, given the requirement of functional Abcb6 for an optimal porphyrin metabolism, it is possible that deficiency of Abcb6 (because of mutations or drug inhibitors) contributes to sporadic acute porphyrias.

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