BACKGROUND & AIMS: The liver has one of the highest rates of heme synthesis of any organ. More than 50% of the heme synthesized in the liver is used for synthesis of P450 enzymes, which metabolize exogenous and endogenous compounds that include natural products, hormones, drugs, and carcinogens. Feline leukemia virus subgroup C cellular receptor 1a (FLVCR1a) is plasma membrane heme exporter that is ubiquitously expressed and controls intracellular heme content in hematopoietic lineages. We investigated the role of Flvcr1a in liver function in mice. METHODS: We created mice with conditional disruption of Mfsd7b, which encodes Flvcr1a, in hepatocytes (Flvcr1a fl/fl, alb-cre mice). Mice were analyzed under basal conditions, after phenylhydrazine-induced hemolysis, and after induction of cytochromes P450 synthesis. Livers were collected and analyzed by histologic, quantitative real-time polymerase chain reaction, and immunoblot analyses. Hepatic P450 enzymatic activities were measured. RESULTS: Flvcr1a fl/fl, alb-cre mice accumulated heme and iron in liver despite up-regulation of heme oxygenase 1, ferroportin, and ferritins. Hepatic heme export activity of Flvcr1a was closely associated with heme biosynthesis, which is required to sustain cytochrome induction. Upon cytochromes P450 stimulation, Flvcr1a fl/fl, alb-cre mice had reduced cytochrome activity, associated with accumulation of heme in hepatocytes. The expansion of the cytosolic heme pool in these mice was likely responsible for the early inhibition of heme synthesis and increased degradation of heme, which reduced expression and activity of cytochromes P450. CONCLUSIONS: In livers of mice, Flvcr1a maintains a free heme pool that regulates heme synthesis and degradation as well as cytochromes P450 expression and activity. These findings have important implications for drug metabolism.

Keywords: ALAS1; CYP; Flvcr; HO-1; Flvcr1.

Aerobic cells require heme as the prosthetic moiety of several hemoproteins, including hemoglobin, cytochromes, myoglobin, catalases, and peroxidases. In addition, heme plays important regulatory roles in cell signaling and in control of gene expression. Heme biosynthesis occurs partially in the mitochondria and partially in the cytoplasm by a multistep pathway involving 8 enzymatic reactions. 5-Aminolevulinic acid synthase (ALAS), which catalyzes the condensation of glycine and succinyl-CoA to form ALA (5-aminolevulinic acid) in the mitochondrion, is the first and rate-controlling enzyme of heme biosynthesis. The rate of heme synthesis is balanced by the rate of its degradation through heme oxygenases (HO-1 and HO-2) to ensure that heme supply is adequate to physiological needs, without a significant accumulation in excess. The tight control of heme synthesis vs heme degradation is essential because free heme is a pro-oxidant and toxic molecule. Both heme synthesis and heme degradation are finely regulated by heme itself. Heme controls Alas1 transcription, the stability of Alas1 messenger RNA (mRNA) and the accumulation of the mature protein in the mitochondrion.

On the opposite side, heme controls Ho-1 gene expression by removing the transcriptional repressor BACH1 from its promoter. The pool of heme that exerts this control, the so-called “free” or “regulatory” heme pool, is determined by a balance between heme synthesis and degradation and because of its small size, dynamic properties, and ability to readily exchange with heme-containing proteins, reflects the overall status of cellular heme content.

Recently, heme export through the cell-surface transporter feline leukemia virus subgroup C cellular receptor 1a (Flvcr1a) was proposed as an additional control step to prevent the intracellular accumulation of heme. Flvcr1a gene is essential for erythropoiesis and systemic iron homeostasis. It encodes for 2 proteins, FLVCR1a and FLVCR1b, expressed at the plasma membrane and on the mitochondrion, respectively. FLVCR1a belongs to the SLC49 family of the major facilitator superfamily of transporters with 12 hydrophobic transmembrane domains. FLVCR1b is a shorter protein with only 6 transmembrane domains, supposed to homodimerize to form a functional transporter. We recently demonstrated a crucial role for FLVCR1b in the last step of heme biosynthetic pathway, ie, heme export from mitochondria. On the other hand, FLVCR1a exerts its heme export activity at the plasma membrane and avoids intracellular heme loading. Previous studies showed that FLVCR1a-mediated heme export in macrophages prevents heme-derived iron accumulation after...
Figure 1. Flvcr1a deletion in the liver alters hepatic heme/iron homeostasis. Data on the livers of 2- (A–D) and 6-month-old (A–F) Flvcr1a<sup>fl/fl</sup> and Flvcr1a<sup>fl/fl; alb-cre</sup> mice are shown. Heme (A) and iron (B) content, (A) n = 10. Liver sections of 6-month-old mice stained with Perl’s reaction are shown on the right. Scale bar = 300 μm. (C) HO activity and bile bilirubin content, n = 5. (D) malondialdehyde (MDA) content, n = 14. (E) Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of Flvcr1a, Ho-1, Fpn, H- and L-ferritin (Ft) mRNA level (n = 6) and representative Western blots of HO-1, L- and H-Ft and FPN protein, n = 10. (F) qRT-PCR analysis of Txnrd1, γ-gcs, and Sod1 mRNA level (n = 5). Unpaired T test analysis with Welch’s correction was performed. AU, arbitrary units; RQ, relative quantity. *P < .05; **P < .01; ***P < .001.
Figure 2. FLVCR1a is required to export heme on heme accumulation inside hepatocytes. Data on the liver of hemin-treated Flvcr1a<sup>fl/fl</sup> and Flvcr1a<sup>fl/fl</sup>:alb-cre mice are shown. Heme content in the liver (A, C) and bilirubin content in the bile (B) at different time points after 30 μmol/kg heme injection. In (B, C) mice were injected with 15 μmol/kg Tin-Protoporphrin IX (SnPP) 30 minutes before heme treatment (n = 5). (D) SnPP content in the liver 4 hours after SnPP injection (n = 4). (E) Quantitative real-time polymerase chain reaction analysis (qRT-PCR) of Flvcr1a, Ho-1, Fpn, H- and L-ferritin (Ft), Txnrd1, and γ-gcs mRNA level (n = 6). (F) Representative Western blots of HO-1, L- and H-Ft protein (n = 4). Two-way analysis of variance with Bonferroni post-test analysis and unpaired T test analysis with Welch’s were performed on data in (A), (B), (C), (F), and in (D), respectively. *P < .05; **P < .01; ***P < .001; ****P < .0001.
erythrophagocytosis.\textsuperscript{14} Consistently, silencing of \textit{Flvcr1a} in HeLa cells results in cytosolic heme loading, HO-1 induction, and oxidative stress. Finally, \textit{Flvcr1a} deletion in mice causes embryo lethality due to extended hemorrhages.\textsuperscript{13}

The liver is one of the body compartments with the highest heme rate synthesis. More than 50% of the heme synthesized in the liver is committed to the synthesis of cytochromes P450 (CYPs),\textsuperscript{15} the major enzymes involved in drug metabolism.\textsuperscript{16} As the prosthetic moiety of all CYPs, heme is responsible for the catalytic activity of these enzymes. In addition, the free heme pool also regulates CYP protein synthesis and disposal.\textsuperscript{10}

Here we show that Flvcr1a function in hepatocytes is critical for the maintenance of a heme pool that controls CYP expression and activity.

\section*{Methods}

\subsection*{Mice and Treatment}

Mice used in these studies were 2/3-month-old and 6-month-old littermates, maintained on a standard chow diet and kept with free access to food and water. All experiments were approved by the animal ethical committee of the University of Torino (Italy).

\subsection*{Heme and Iron Content}

Heme content in tissues and bile was quantified by the oxalic acid method. Tissue nonheme iron content was determined by a colorimetric method using 4,7-diphenyl-1,10-phenanthroline disulfonic acid (Sigma, St Louis, MO) as chromogen.

\subsection*{HO Activity}

HO activity was measured by spectrophotometric determination of bilirubin produced from hemin added as substrate.

\subsection*{Lipid Peroxidation}

Lipid peroxidation from tissue extracts was measured using the colorimetric assay kit Brixotech LPO-586 from Oxis International (Portland, OR).

\subsection*{Quantitative Real-Time Polymerase Chain Reaction}

Total RNA was extracted using Pure Link RNA Mini Kit (Ambion, Life Technologies Italia, Torino, Italy). One microgram total RNA was reverse transcribed using M-MLV reverse transcriptase and random primers (Life Technologies Italia). Quantitative real-time polymerase chain reaction was performed on a 7300 Real Time PCR System (Applied Biosystems, Life Technologies Italia, Torino, Italy). Primers and probes were designed using the ProbeFinder software (www.roche-applied-science.com).

\section*{Protein Extraction and Western Blotting}

Tissue and cell proteins were extracted as reported previously\textsuperscript{17} and concentration was determined using the Bio-Rad protein assay system (Bio-Rad, Munich, Germany). Fifty micrograms total protein extracts were separated on 8\%–12\% sodium dodecyl sulfate polyacrylamide gel electrophoresis and analyzed by Western blotting using antibodies against HO-1 (Stressgen, Victoria, Canada), L- and H-ferritin (kindly provided by Sonia Levi), ferroportin (Fpn; Alpha Diagnostic Intl. Inc, San Antonio, TX), CYP1A1, CYP3A, CYP2E1, and actin (Santa Cruz Biotechnology, Inc, Dallas, TX).

\section*{Histology}

Tissues were fixed in 10\% formalin overnight at room temperature and embedded in paraffin. Microtome sections, 5-\textmu m thick, were stained with Perl’s reaction followed by methanol 3,3-diaminobenzidine (Boehringer Mannheim, Germany) development.

\section*{ALAS Activity}

ALAS activity was assayed by measuring ALA formation in liver homogenates after glycine addition.

\section*{CYP Activity}

CYP1A1 activity was assessed by measuring ethoxyresorufin-0-deethylase activity in liver microsomes using 7-ethoxyresorufin as a substrate. CYP3A activity was assessed by measuring conversion of the substrate proluciferin-PFBE to luciferin (V8901 P450-Glo CYP3A4 Assay; Promega, Madison, WI). CYP2E1 activity was determined by assaying the hydroxylation of p-nitrophenol to 4-nitrocatechol in the liver microsomal fraction.

\section*{Statistical Analysis}

Results were expressed as mean ± SEM. Comparisons between 2 groups were performed with 2-sided Welch $t$ tests and among more than 2 groups with 1- or 2-way analysis of variance followed by Bonferroni post test. $P$ values <0.05 were regarded as significant ($P$ < 0.05; **$P$ < 0.01; ***$P$ < 0.001; ****$P$ < 0.0001).

See also Supplementary Material.

\section*{Results}

\subsection*{Generation of Liver-Specific Flvcr1a Knockout Mice}

To study the function of the heme exporter FLVCR1a in the liver, we generated a liver-specific \textit{Flvcr1a} knockout mouse (Supplementary Figure 1A). Liver-specific \textit{Flvcr1a}
knockout (Flvcr1a<sup>fl/fl</sup>;alb-cre) mice were born at the expected Mendelian ratio and were viable and fertile. Flvcr1a<sup>fl/fl</sup>;alb-cre mice showed the recombinant allele only in the liver (Supplementary Figure 1B) and a strong reduction of hepatic Flvcr1a expression (Supplementary Figure 1C and D). As expected, Flvcr1a mRNA could not be detected in primary hepatocytes isolated from Flvcr1a<sup>0/0</sup>;alb-cre mice (Supplementary Figure 1E), demonstrating that this mouse is a liver-specific knockout model for Flvcr1a.

Flvcr1a<sup>0/0</sup>;alb-cre mice showed no gross liver abnormalities (Supplementary Figure 1F). Blood analysis did not reveal any difference between Flvcr1a<sup>fl/fl</sup>;alb-cre and Flvcr1a<sup>0/0</sup> mice (Supplementary Table 1).

**Flvcr1a Deletion in the Liver Results in Altered Hepatic Heme/Iron Homeostasis**

To evaluate if the deletion of Flvcr1a alters hepatic heme homeostasis, we analyzed the livers of 2- and 6-month-old Flvcr1a<sup>0/0</sup>;alb-cre compared with those of an Flvcr1a<sup>0/0</sup> counterpart. Hepatic heme and iron content were comparable at 2 months of age, but were significantly higher in 6-month-old Flvcr1a<sup>0/0</sup>;alb-cre than in Flvcr1a<sup>0/0</sup> mice (Figure 1A and B). Iron accumulation in 6-month-old Flvcr1a<sup>0/0</sup>;alb-cre mice was further confirmed by Perl’s staining on liver sections (Figure 1B). Consistently, Flvcr1a<sup>0/0</sup>;alb-cre mice showed an enhanced HO activity as well as an increased bilirubin excretion in the bile.

**Figure 4.** Flvcr1a deficiency affects liver homeostasis after dexamethasone-induced cytochrome synthesis. Data on the liver of dexamethasone-treated Flvcr1a<sup>0/0</sup> and Flvcr1a<sup>0/0</sup>;alb-cre mice are shown. Heme (A) and malondialdehyde (MDA) (B) content. (A) <i>n</i> = 5. (B) <i>n</i> = 5. (C, E, F) Quantitative real-time polymerase chain reaction analysis of Flvcr1a and Ho-1, Txnrd1, Alas1, Flvcr1b, and TfR1 mRNA level (<i>n</i> = 5). (D) Representative Western blot of L-Ft protein (<i>n</i> = 4). Two-way analysis of variance with Bonferroni post-test analysis was performed. *<i>P</i> < .05; **<i>P</i> < .01; ***<i>P</i> < .001.
compared with Flvcr1a\textsuperscript{fl/fl} mice (Figure 1C). In addition, Flvcr1a\textsuperscript{fl/fl};alb-cre mice showed increased lipid peroxidation in the liver (Figure 1D). The analysis of hepatic gene expression revealed that Flvcr1a\textsuperscript{fl/fl};alb-cre mice up-regulated genes that encode for proteins involved in heme metabolism (Ho-1),\textsuperscript{18,19} iron export (Fpn),\textsuperscript{20,21} and storage (H- and L-Ferritin),\textsuperscript{22} and antioxidant response (Txnrd1, γ-gcs, Sod1),\textsuperscript{23} compared with Flvcr1a\textsuperscript{fl/fl} mice (Figure 1E and F; Supplementary Figure 2 for gene expression analysis of 2-month-old mice). On the other hand, expression of the other known heme exporter Abcg2 was not increased in the liver of Flvcr1a\textsuperscript{fl/fl};alb-cre mice (Supplementary Figure 3), indicating that no other heme exporter was able to compensate for the lack of Flvcr1a.

**FLVCR1a Is Required to Export Heme on Heme Accumulation Inside Hepatocytes**

The phenotype of liver-specific Flvcr1a knockout mice suggests that FLVCR1a-mediated heme export prevents hepatic heme accumulation. To further address this point, mice were injected with hemin, the substrate of FLVCR1a. One hour after heme injection, heme accumulated in the liver of both Flvcr1a\textsuperscript{fl/fl};alb-cre and Flvcr1a\textsuperscript{fl/fl} mice at the same extent, but bilirubin production was significantly higher in Flvcr1a\textsuperscript{fl/fl};alb-cre mice than in Flvcr1a\textsuperscript{fl/fl} mice, likely because of the enhanced HO activity (Figure 2A and B). Consistently, if animals were pretreated with the heme analog Tin-Protoporphyrin IX that inhibits HO, before heme injection, Flvcr1a\textsuperscript{fl/fl};alb-cre mice showed a significantly higher hepatic heme content 1 hour after heme infusion compared with control mice (Figure 2C). When we injected mice with Tin-Protoporphyrin IX alone, we found it accumulated more in the liver of Flvcr1a\textsuperscript{fl/fl};alb-cre mice than in that of Flvcr1a\textsuperscript{fl/fl} controls (Figure 2D), strengthening the FLVCR1a export function. Measurement of heme content in the bile at 1 hour after heme injection demonstrated that it was excreted at the same extent in both Flvcr1a\textsuperscript{fl/fl};alb-cre and Flvcr1a\textsuperscript{fl/fl} mice (Supplementary Figure 4A), suggesting that Flvcr1a did not export heme in the bile but likely vs the bloodstream. Accordingly, the analysis of a human hepatocarcinoma cell line, HepG2, overexpressing Flvcr1a-myc,
showed that FLVCR1a localized at the plasma cell membrane, along the sinusoidal surface (Supplementary Figure 4B).

Data shown in Figure 2C indicate that the enhanced HO activity was able to compensate for the lack of FLVCR1a to maintain heme content in the normal range on transient heme accumulation. This was further demonstrated by the analysis of gene expression. On heme treatment, Flvcr1ala/b mice showed a strong induction of Flvcr1a in the liver, as well as an up-regulation of Ho-1, Fpn, H- and L-ferritin. Flvcr1ala/b;alb-cre mice that were unable to induce Flvcr1a, showed a stronger induction of the heme degradation and iron storage/export pathways, as an attempt to compensate for the lack of heme export (Figure 2E and F). This was not sufficient to control oxidative stress, as demonstrated by the significantly higher induction of the antioxidant genes in the liver of Flvcr1a-deleted mice after heme injection (Figure 2E).

These data demonstrate that FLVCR1a is a heme exporter in hepatocytes that works in close association with the heme degradation pathway to maintain heme/iron homeostasis.

**FLVCR1a-Mediated Heme Export Function Is Strictly Associated With Heme Biosynthesis**

The liver is, at the same time, one of the organs with the highest rate of heme synthesis and the main body site devoted to the detoxification of heme coming from the bloodstream. We asked in which of these processes is FLVCR1a mainly involved. To address this point, we treated mice with the heme precursor ALA or with the hemolytic agent phenylhydrazine, to promote heme synthesis or heme accumulation, respectively.

Although we did not observe any difference after phenylhydrazine treatment (Supplementary Results, Supplementary Figure 5), increased heme content was found in the liver of Flvcr1ala/b;alb-cre mice compared with Flvcr1ala/b mice after ALA treatment, suggesting that de novo synthesis, heme accumulated in the liver when FLVCR1a was absent (Figure 3A). This resulted in a marked increase in the hepatic lipid peroxidation index (Figure 3B). Interestingly, Flvcr1a was strongly induced by ALA treatment in the liver of Flvcr1ala/b mice (Figure 3C). On the other hand, the genes involved in heme and iron metabolism, such as Ho-1 and Fpn, were up-regulated to a higher extent in the liver of Flvcr1ala/b;alb-cre mice than in that of Flvcr1ala/b mice, and this was associated with a higher induction of the genes of the antioxidant response (Figure 3C). Conversely, we observed a reduced expression of Alas1, Flvcr1b, and Tf1r1 in the liver of Flvcr1ala/b;alb-cre compared with Flvcr1ala/b mice (Figure 3C), suggesting an attenuation of the heme biosynthetic pathway in these animals. These results were confirmed in vitro on primary hepatocytes treated with ALA (Figure 3D–F; Supplementary Material).

These data indicate that FLVCR1a-mediated heme export function is strictly associated with heme synthesis.

**FLVCR1a-Mediated Heme Export Function Is Associated With CYP Induction**

In the liver, most of the newly synthesized heme is committed to CYP synthesis. To test whether FLVCR1a function is linked to heme synthesis stimulation on cytochromes induction, we treated our mice with inducers of 3 distinct classes of CYPs.

Firstly, we injected mice with dexamethasone, an inducer of CYP3A. Dexamethasone treatment caused an increase in heme content in the liver of Flvcr1ala/b;alb-cre mice, that was almost negligible in Flvcr1ala/b counterpart (Figure 4A). This effect was abrogated by co-treatment with the inhibitor of heme biosynthesis, succinylacetone (Figure 4A). As a consequence of heme accumulation, a higher amount of lipid peroxides was generated on dexamethasone treatment in the liver of Flvcr1ala/b;alb-cre mice compared with Flvcr1ala/b mice (Figure 4B). The analysis of gene expression demonstrated that Flvcr1a was induced in the liver of Flvcr1ala/b mice after dexamethasone treatment, as occurred on ALA treatment (Figure 4C). On the other hand, the heme-, iron-, and stress-related genes were induced to a higher extent in the liver of dexamethasone-treated Flvcr1ala/b;alb-cre mice compared with the Flvcr1ala/b counterpart (Figure 4C–E), suggesting that the higher induction of these genes compensated for the lack of Flvcr1a. In addition, genes involved in heme biosynthesis, such as Alas1, Flvcr1b, and Tf1r1, were found to be significantly less expressed in Flvcr1ala/b;alb-cre mice compared with Flvcr1ala/b mice after dexamethasone treatment (Figure 4F).

Similar results were obtained when mice were treated with benzo(a)pyrene (Be[a]P), an inducer of CYP1A1 and CYP1A2 (Figure 5, Supplementary Figure 6), and imidazole, an inducer of CYP2E1 (Supplementary Results; Supplementary Figure 7). Because the induction of Alas1 8h after Be(a)P injection was comparable in Flvcr1ala/b;alb-cre and Flvcr1ala/b (Supplementary Figure 8), the difference found at 16 hours post injection likely indicates that the heme biosynthetic pathway was switched off earlier in Flvcr1a-deleted mice than in its wild-type counterparts, as an attempt to compensate for the excess of heme accumulated in the liver.

Collectively, these data indicate that FLVCR1a-mediated heme export is associated with CYP induction.
FLVCR1a Controls CYP Activity by Regulating Heme Synthesis and Degradation

In the previous section, we showed that Ho-1 and Alas1 mRNA levels were higher and lower, respectively, in the liver of dexamethasone-, Be(a)P-, or imidazole- treated Flvcr1floxed/; alb-cre compared with Flvcr1flox/flox mice, suggesting that heme degradation is increased and heme synthesis is inhibited when FLVCR1a-mediated heme export is blocked. Consistently, Ho-1 was found up-regulated and Alas1, as well as Flvcrr1b, down-regulated in the liver of sickle cell anemia and β-thalassemia mice, in which Flvcr1a levels were strongly decreased (Supplementary Figure 9). This observation strengthens the idea that Flvcr1a deletion/down-regulation leads to the coordinated induction of heme degradation and down-regulation of the heme biosynthetic pathway. To evaluate this point, we analyzed Ho-1 as well as Alas1 protein and activity in the liver of Flvcr1floxed/; alb-cre and Flvcr1flox/flox mice, treated with dexamethasone or Be(a)P. After the stimulation of CYP synthesis, Ho-1 and ALAS1 expression were induced in the liver of both Flvcr1floxed/; alb-cre and Flvcr1flox/flox mice (Figure 6A and B). Ho-1 induction was significantly higher and ALAS1 expression was markedly reduced in the liver of Flvcr1floxed/; alb-cre mice compared with Flvcr1flox/flox counterparts. This correlated with the enzymatic activities of Ho-1 and ALAS1, which were respectively higher and lower in the liver of Flvcr1floxed/; alb-cre mice than in that of Flvcr1flox/flox animals (Figure 6A and B). Ho-1 induction as well as ALAS1 inhibition were likely mediated by heme overload occurring in Flvcr1floxed/; alb-cre mice. Consistently, after the stimulation of CYP synthesis, heme accumulated to a higher extent in the cytosolic fraction of Flvcr1floxed/; alb-cre mice compared with Flvcr1flox/flox controls. On the other hand, heme content was significantly lower in the microsomal fraction of Flvcr1floxed/; alb-cre mice than in that of Flvcr1flox/flox animals (Figure 6C). As microsomal heme reflects the heme fraction contained in CYPs, we measured mRNA and protein expression and enzymatic activity of CYP3A and CYP1A1 in the livers of our mice. In agreement with heme levels, CYP3A and CYP1A1 mRNA, protein levels and activities were significantly lower in the livers of dexamethasone- and Be(a)P-treated Flvcr1floxed/; alb-cre mice than in those of treated-Flvcr1flox/flox animals (Figure 6D and E). Similar results were obtained when mice were treated with imidazole (Supplementary Results; Supplementary Figure 10).

On the enhancement of heme demand, Flvcr1a deletion resulted in an expansion of the cytosolic heme pool that stimulates heme degradation and inhibits heme and CYP synthesis. To test whether the main determinant for CYP expression/function was the size of heme pool or the rate of heme synthesis, both impaired in Flvcr1a-deleted liver, we treated wild-type mice with dexamethasone or Be(a)P alone or together with hemin, to mimic heme overload occurring in Flvcr1floxed/; alb-cre mice, or with succinylacetone or DL-penicillamine, 2 inhibitors of heme biosynthesis. As expected, dexamethasone and Be(a)P treatment caused a marked increase in ALAS1 activity as well as in CYP expression/activity, and Ho-1 expression/activity was only slightly induced (Figure 7A and B). Hemin co-treatment significantly reduced hepatic ALAS1 activity, while increasing Ho-1 expression/activity, compared with mice treated with dexamethasone or Be(a)P only (Figure 7A and B). This correlated nicely with a reduced expression and activity of CYPs (Figure 7B). Similarly, we observed that co-treatment with Be(a)P and the ALAS-inhibitor DL-penicillamine decreased ALAS activity as well as the expression and activity of CYP1A1 (Figure 7A and B, right). Administration of succinylacetone, a heme synthesis inhibitor acting on 5-aminovaleric acid dehydratase downstream of ALAS1, caused a feedback up-regulation of ALAS1 activity, as expected, but a decrease in CYP3A activity, as a consequence of reduced heme availability (Figure 7A and B, left). We can conclude that the effect of heme overload on cytochrome function parallels that of heme synthesis inhibition, fostering the concept that cytochrome function is strictly associated to de novo heme production rather than to heme pool size itself.

As further confirmation, we observed that 6-month-old Flvcr1floxed/; alb-cre mice showed a reduction in ALAS1 activity as well as an increase in HO activity (Figure 7C). This misbalance in heme synthesis/degradation resulted in a reduced CYP expression at both mRNA and protein level (CYP1A1 and CYP3A, Figure 7D; CYP2E1, Supplementary Figure 11) and reduced CYP activity (Figure 7E).

These data indicate that FLVCR1a-mediated heme export in hepatocytes controls the expansion of the heme pool, which in turns determines the balance between heme synthesis and degradation and CYP activity.

Discussion

Here we showed that FLVCR1a is essential for the maintenance of heme and iron homeostasis in the liver and that its function is strictly associated with the heme biosynthetic process that is crucial for the control of CYP activity. Previous studies demonstrated that FLVCR1a exerts a detoxifying function in macrophages and erythroid cells, by
exporting heme excess. Our results indicate that FLVCR1a is similarly important in the liver, as its deletion leads to progressive heme and iron loading and to the compensatory up-regulation of the genes responsible for heme degradation and iron storage. Consistently with our finding in mice, Flvcr1 was found mutated in human subjects with mild hepatic iron overload.

Our data show that FLVCR1a export function is associated with heme biosynthesis in agreement with data showing that ALA treatment causes heme accumulation in Flvcr1a-silenced HeLa cells. In addition, we observed a concerted up-regulation of Flvcr1a and Flvcr1b, Alas1, and TfR1 in the liver of ALA-treated wild-type mice that strengthens the link between FLVCR1a function and heme biosynthesis.

More than half of the hepatic production of heme is used for the formation of CYPs, which are engaged in steroid metabolism and in the oxidative metabolism of foreign compounds, including pharmaceutical drugs. Our data showed that Flvcr1a is up-regulated after CYP induction, suggesting that its function is strictly associated with enhanced heme demand to support cytochrome induction. Similarly, Alas1 as well as Flvcr1b and TfR1 are up-regulated to sustain newly heme synthesis in such condition.

Because either a deficiency or an excess of heme is toxic to the cell, hepatic heme production has to be tightly controlled. Previous works showed that in primary cultures of adult rat hepatocytes, 20% of newly formed heme is converted to bile pigments, and 80% is used for the formation of hemoproteins, mainly CYPs. Our data indicate that not only heme degradation, but also FLVCR1a-mediated heme export, is critical to ensure that the amount of available heme matches cell requirements. The alteration of one of these pathways, heme synthesis, degradation or export, in hepatocytes leads to an imbalance in heme homeostasis. In particular, FLVCR1a deletion causes an increase in the cytosolic heme fraction, when heme demand is increased to support CYP induction.

The cytosolic heme fraction contains a pool of newly synthesized heme that serves both precursor and regulatory functions. The free heme pool controls heme biosynthesis, through the regulation of ALAS1. If increased, the regulatory heme pool may repress ALAS1, and its depletion causes ALAS1 induction. Our results indicate that ALAS1 induction occurs in wild-type as well as in Flvcr1a-null mice shortly after cytochrome stimulation, to sustain heme synthesis for cytochrome formation. Then, Alas1 down-regulation occurs earlier in Flvcr1a-null mice than in wild-type animals because of the negative feedback exerted by the expanded cytosolic free heme pool. This is in agreement with many observations, according to which the addition of heme in hepatocyte cultures inhibits the drug-induced synthesis of ALAS. Although xenobiotics might have some primary inducing effect on hepatic ALAS1, many chemical inducers are believed to increase ALAS1 by depleting the free heme pool in hepatocytes. This is in agreement with our observation in wild-type mice in which ALAS1 expression, CYP activity, and microsomal heme are increased, and cytosolic heme levels are reduced after drug treatment. Conversely, liver-specific Flvcr1a-null mice showed an expansion of the cytosolic heme pool, suggesting that Flvcr1a deletion promotes intracellular heme accumulation, preventing the depletion of the free heme pool as a stimulus for ALAS1 induction and on the contrary, promoting its inhibition.

In liver-specific Flvcr1a-null mice, the decreased heme synthesis well correlates with a reduction of CYP expression and activity, in line with the previous observation that the enhancement in heme synthesis is required to sustain the induction/activity of CYPs. Conversely, when a bolus of hemin is administered to experimental animals, the induction/activity of CYPs is greatly suppressed and this effect is considered to be the result of inhibition of heme biosynthesis by ALAS1. Short-term hemin administration is known to both increase HO1 expression and interfere with the formation of CYP. Consistently, drug-treated Flvcr1a-null mice showed a significantly higher induction of HO1 and reduction in the expression and activity of ALAS1 and CYPs compared with wild-type animals, indicating that heme accumulation resulting from Flvcr1a deletion resembles what occurs after hemin administration.

In conclusion, the block of heme export due to Flvcr1a deletion promotes the expansion of the cytosolic heme pool, thus leading to ALAS inhibition and HO induction. We propose that the lack of FLVCR1a causes a reduction in the newly synthesized heme, impairing both CYP expression and activity (Figure 7F). It appears that in the hepatocytes, heme is formed in slight excess over its metabolic needs and its levels are maintained adequate by a combination of synthetic, degradative, and export mechanisms, suggesting that they are equipped with a “sensing” system to monitor changes in the size of “uncommitted” heme pool.

We can speculate that FLVCR1a is part of this sensing system and that, by sensing heme levels and exporting heme excess out of the cell, it controls the size of the cytosolic heme pool, playing a crucial regulatory role in cell metabolism and in the maintenance of a proper oxidative status. We expect that mutations in Flvcr1a and/or pathologic situations that affect its expression can result in a reduced CYP activity, altering drug metabolism, in particular in individuals that routinely assume drugs for therapeutic purposes.

Supplementary Material
Note: To access the supplementary material accompanying this article, visit the online version of Gastroenterology at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2014.01.053.

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Conflicts of interest
The authors disclose no conflicts.

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