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BASIC AND TRANSLATIONAL—LIVER

Heme Exporter FLVCR1a Regulates Heme Synthesis and Degradation and Controls Activity of Cytochromes P450

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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.[1] In addition, heme plays important regulatory roles in cell signaling and in control of gene expression.[2] 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.[1]

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.[3] The tight control of heme synthesis vs heme degradation is essential because free heme is a pro-oxidant and toxic molecule.[4,5] Both heme synthesis and heme degradation are tunely 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.[6–8] On the opposite side, heme controls *Ho-1* gene expression by removing the transcriptional repressor BACH1 from its promoter.[9] 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.[10]

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.[11,12] *Flvcr1* gene is essential for erythropoiesis and systemic iron homeostasis.[12] 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.[12,13] FLVCR1b is a shorter protein with only 6 transmembrane domains, supposed to homodimerize to form a functional transporter.[13] We recently demonstrated a crucial role for FLVCR1b in the last step of heme biosynthetic pathway, ie, heme export from mitochondria.[13] 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

Abbreviations used in this paper: ALA, 5-aminolevulinic acid; ALAS, 5-aminolevulinic acid synthase; Be(a)P, benzo(a)pyrene; CYP, cytochrome P450; FLVCR1a, feline leukemia virus subgroup C cellular receptor 1a; Fpn, ferroportin; HO, heme oxygenase; mRNA, messenger RNA.

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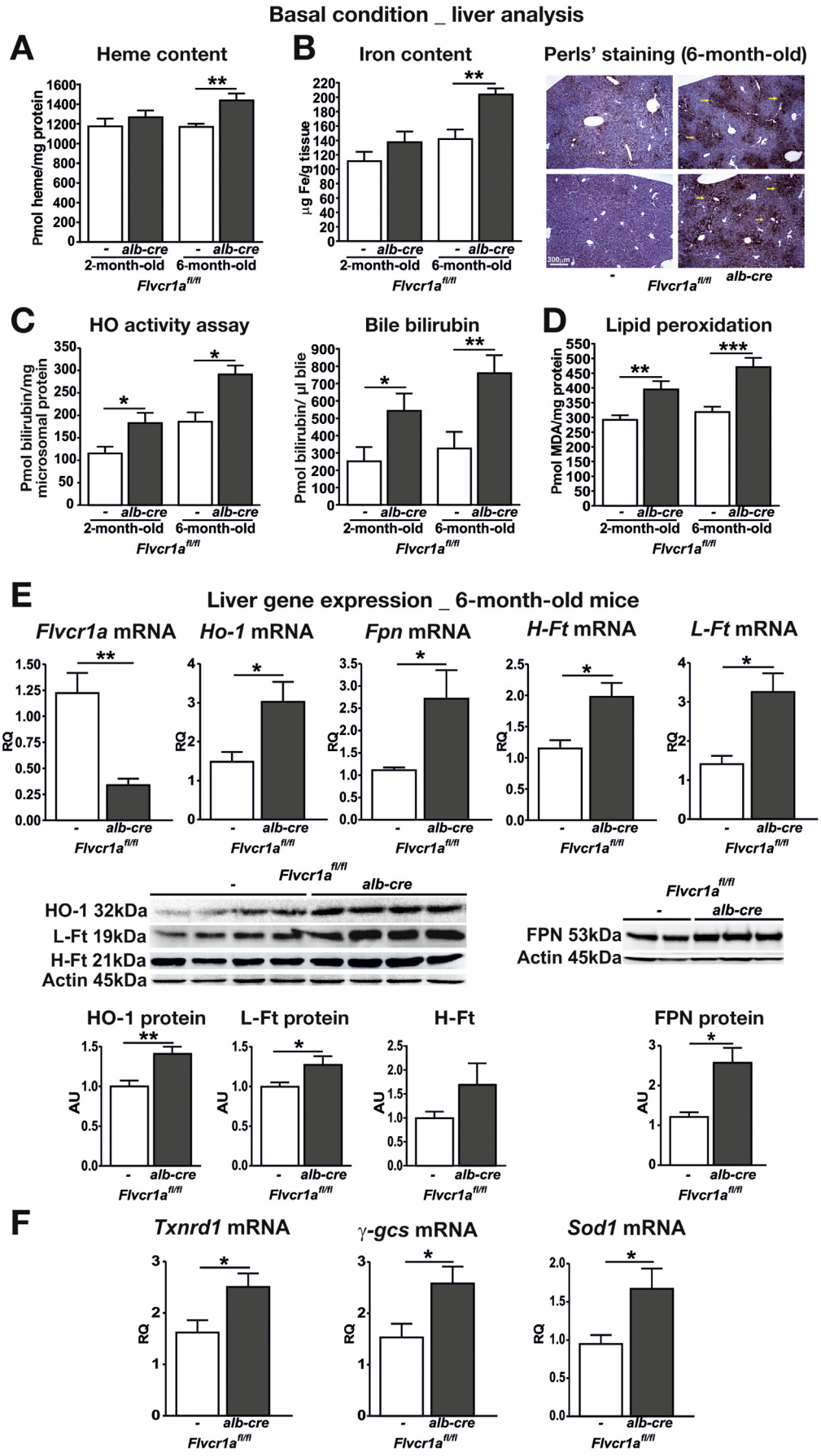


Figure 1. *Flvcr1a* deletion in the liver alters hepatic heme/iron homeostasis. Data on the livers of 2- (*A–D*) and 6-month-old (*A–F*) *Flvcr1a*$^{fl/fl}$ and *Flvcr1a*$^{fl/fl}$; *alb-cre* mice are shown. Heme (*A*) and iron (*B*) content. (*A*) n = 10. (*B*) n = 10. Liver sections of 6-month-old 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.

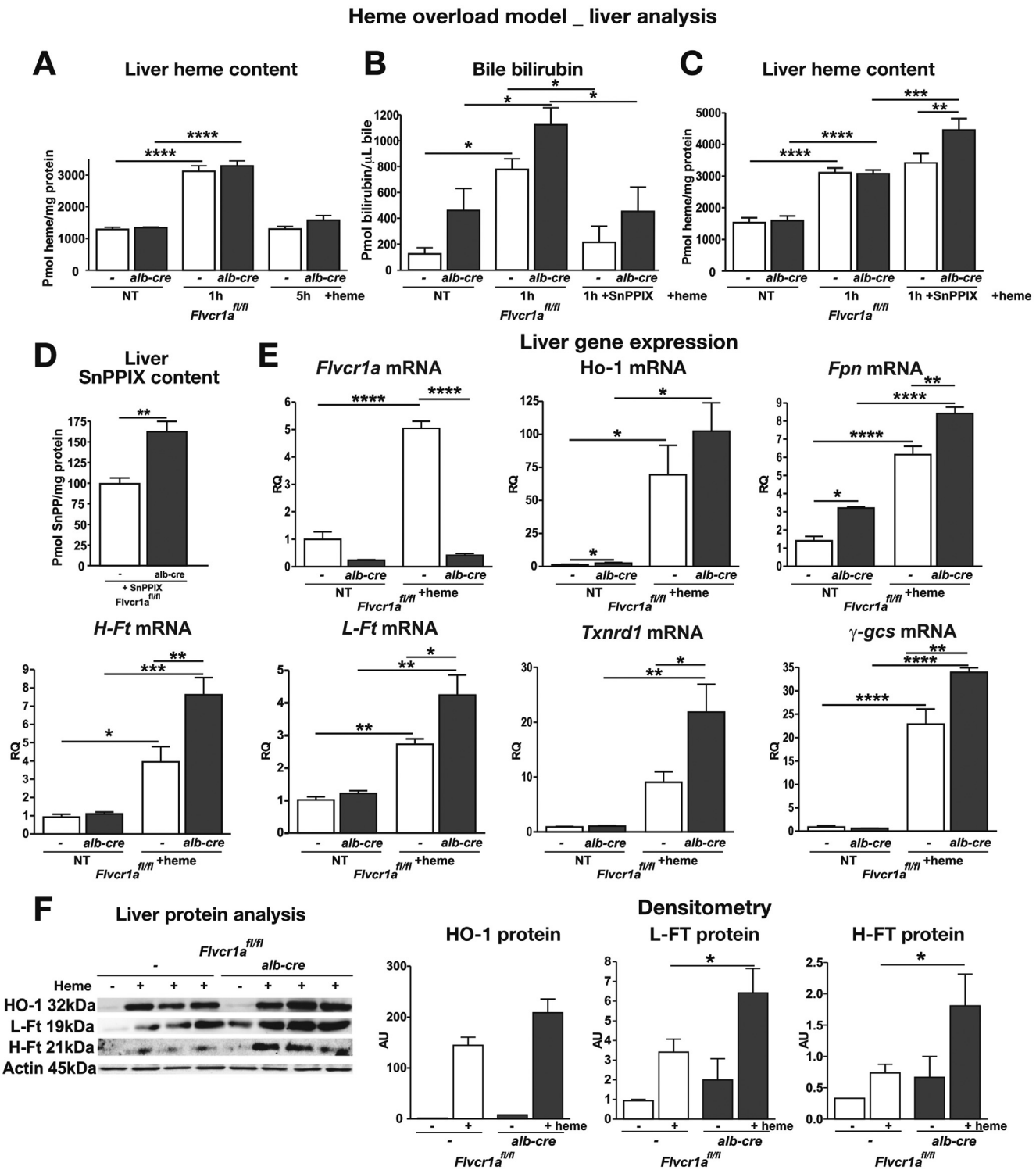
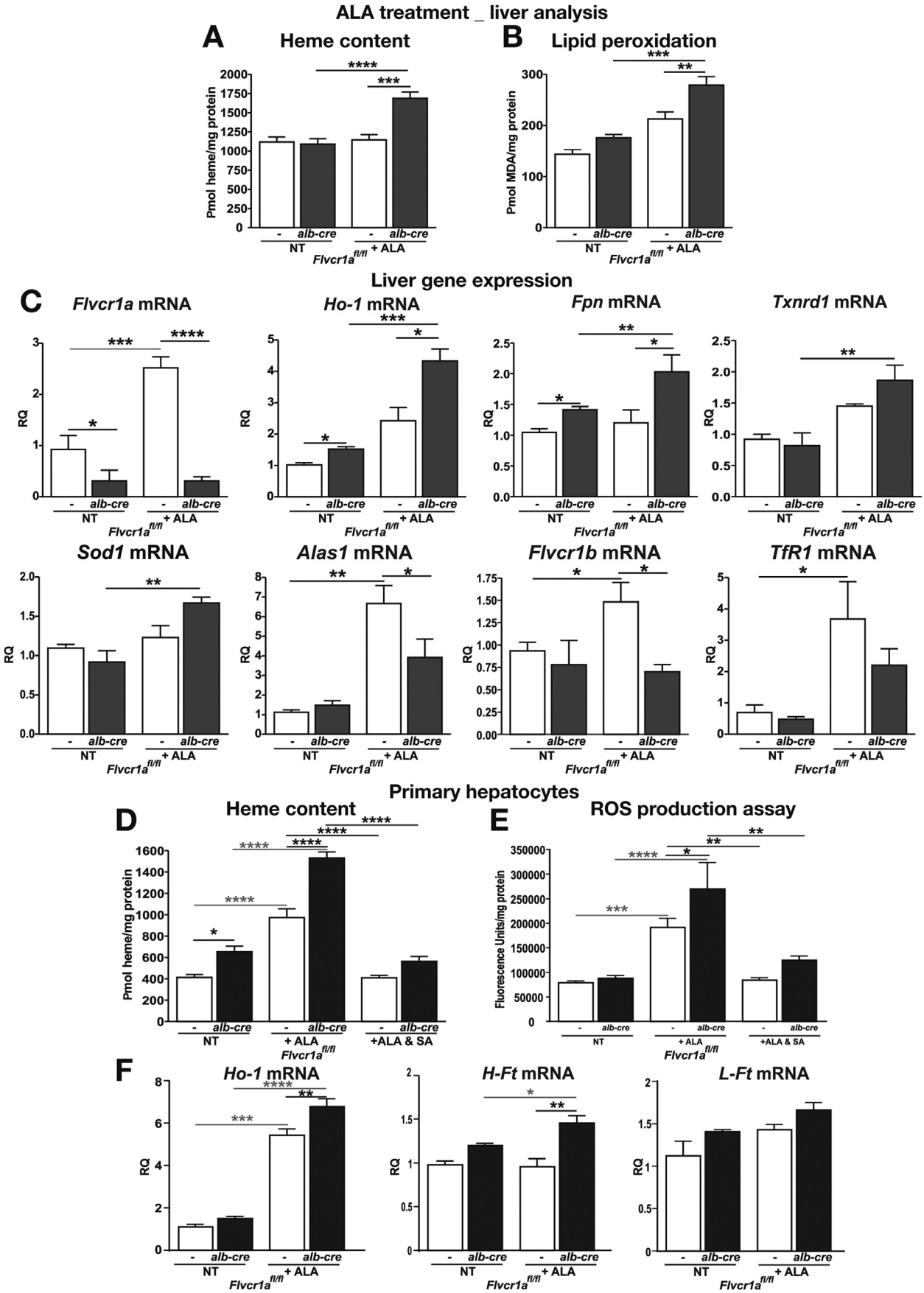


Figure 2. FLVCR1a is required to export heme on heme accumulation inside hepatocytes. Data on the liver of hemin-treated *Flvcr1a*$^{fl/fl}$ and *Flvcr1a*$^{fl/fl}$*;alb-cre* mice are shown. Heme content in the liver (*A, C*) and bilirubin content in the bile (*B*) at different time points after 30 μmol/kg heme injection. In (*B, C*) mice were injected with 15 μmol/kg Tin-Protoporphyrin 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. *$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$.


ALA treatment _ liver analysis
A
Heme content
Pmol heme/mg protein
B
Lipid peroxidation
Pmol MDA/mg protein
- alb-cre - alb-cre
NT + ALA
Flvcr1a fl/fl
Liver gene expression
C
Flvcr1a mRNA
Ho-1 mRNA
Fpn mRNA
Txnrd1 mRNA
Sod1 mRNA
Alas1 mRNA
Flvcr1b mRNA
TfR1 mRNA
RQ
Primary hepatocytes
D
Heme content
E
ROS production assay
Fluorescence Units/mg protein
NT
+ ALA
+ALA & SA
F
Ho-1 mRNA
H-Ft mRNA
L-Ft mRNA

erythrophagocytosis.[14] Consistently, silencing of *Flvcr1a* in HeLa cells results in cytosolic heme loading, HO-1 induction, and oxidative stress. Finally, *Flvcr1a* deletion in mice causes embryo lethality due to extended hemorrhages.[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),[15] the major enzymes involved in drug metabolism.[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.[10]

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

Methods

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).

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-phenantroline disulfonic acid (Sigma, St Louis, MO) as chromogen.

HO Activity

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

Lipid Peroxidation

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

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). Primers and probes were designed using the ProbeFinder software (www.roche-applied-science.com).

Protein Extraction and Western Blotting

Tissue and cell proteins were extracted as reported previously[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).

Histology

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

ALAS Activity

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

CYP Activity

CYP1A1 activity was assessed by measuring ethoxyresorufin-O-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.

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 <.05 were regarded as significant ($^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$; $^{****}P < .0001$).

See also Supplementary Material.

Results

Generation of Liver-Specific Flvcr1a Knockout Mice

To study the function of the heme exporter FLVCR1a in the liver, we generated a liver-specific *Flvcr1a* knockout mouse (Supplementary Figure 1*A*). Liver-specific *Flvcr1a*

Figure 3. FLVCR1a-mediated heme export function is strictly associated with heme biosynthesis. Data on ALA-treated *Flvcr1a*$^{fl/fl}$ and *Flvcr1a*$^{fl/fl}$*;alb-cre* mice are shown. Heme (*A*) and malondialdehyde (MDA) (*B*) content in the liver (n = 4). (*C*) Quantitative real-time polymerase chain reaction analysis of *Flvcr1a*, *Ho-1*, *Fpn*, *Txnrd1*, *Sod1*, *Alas1*, *Flvcr1b*, and *TfR1* mRNA level in the liver (n = 4). (*D*, *E*) Heme uptake and reactive oxygen species (ROS) production on primary hepatocytes isolated from the liver of *Flvcr1a*$^{fl/fl}$ and *Flvcr1a*$^{fl/fl}$*;alb-cre* mice, untreated or treated with 5 mM ALA or 5mM ALA and 0.5 mM succinylacetone (SA) (n = 4). (*F*) qRT-PCR analysis of *Ho-1*, *H-*, and *L-Ft* mRNA level in ALA-treated primary hepatocytes (n = 6). Two-way analysis of variance with Bonferroni post-test analysis was performed. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$; $^{****}P < .0001$.

knockout (*Flvcr1a*$^{fl/fl}$;*alb-cre*) mice were born at the expected Mendelian ratio and were viable and fertile.

Flvcr1a$^{fl/fl}$;*alb-cre* mice showed the recombinant allele only in the liver (Supplementary Figure 1*B*) and a strong reduction of hepatic *Flvcr1a* expression (Supplementary Figure 1*C* and *D*). As expected, *Flvcr1a* mRNA could not be detected in primary hepatocytes isolated from *Flvcr1a*$^{fl/fl}$;*alb-cre* mice (Supplementary Figure 1*E*), demonstrating that this mouse is a liver-specific knockout model for *Flvcr1a*.

Flvcr1a$^{fl/fl}$;*alb-cre* mice showed no gross liver abnormalities (Supplementary Figure 1*F*). Blood analysis did not reveal any difference between Flvcr1afl$^{/fl}$;*alb-cre* and *Flvcr1a*$^{fl/fl}$ 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*$^{fl/fl}$;*alb-cre* compared with those of an *Flvcr1a*$^{fl/fl}$ counterpart. Hepatic heme and iron content were comparable at 2 months of age, but were significantly higher in 6-month-old *Flvcr1a*$^{fl/fl}$;*alb-cre* than in *Flvcr1a*$^{fl/fl}$ mice (Figure 1*A* and *B*). Iron accumulation in 6-month-old *Flvcr1a*$^{fl/fl}$;*alb-cre* mice was further confirmed by Perl's staining on liver sections (Figure 1*B*). Consistently, *Flvcr1a*$^{fl/fl}$;*alb-cre* mice showed an enhanced HO activity as well as an increased bilirubin excretion in the bile

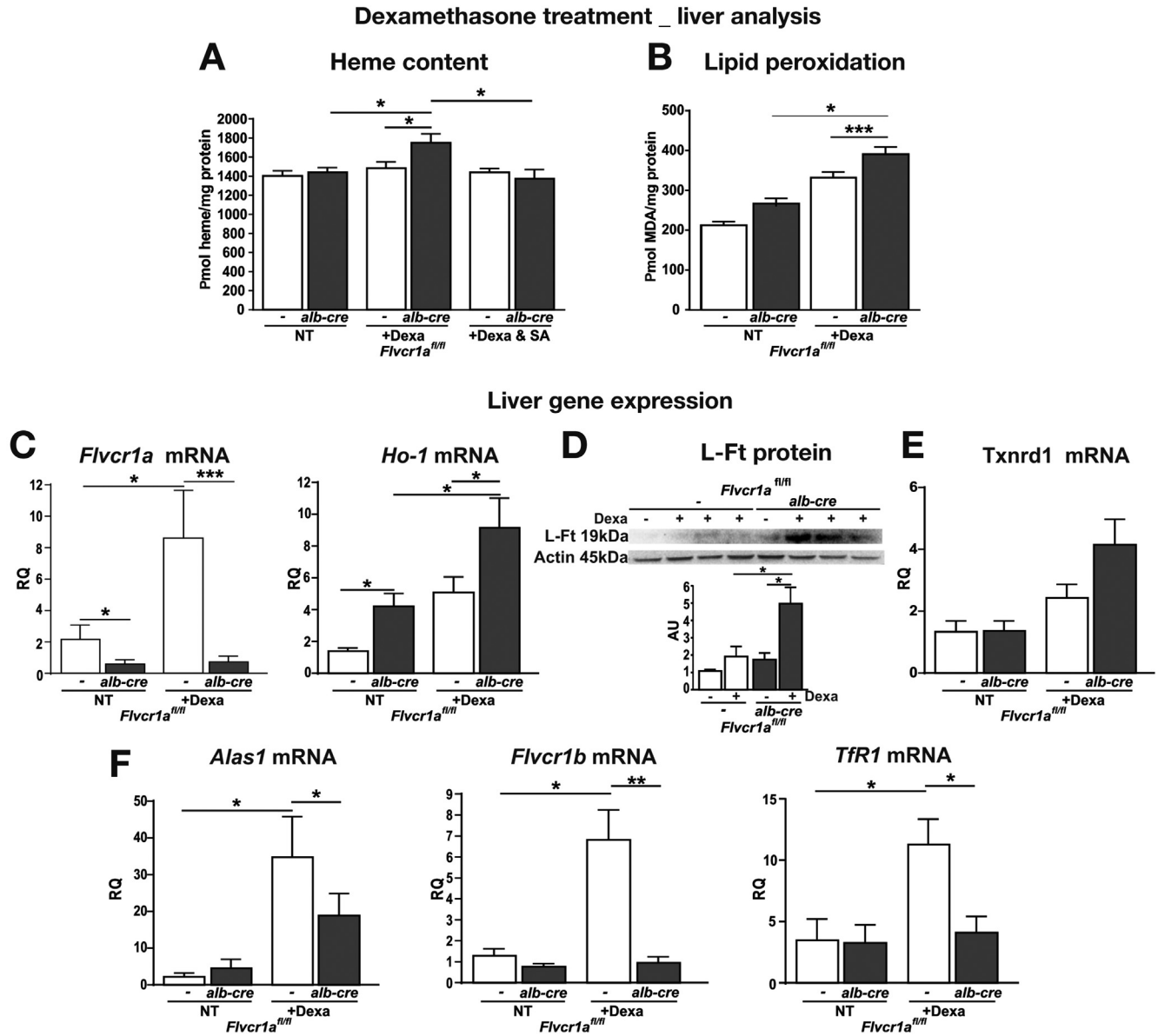


Figure 4. *Flvcr1a* deficiency affects liver homeostasis after dexamethasone-induced cytochrome synthesis. Data on the liver of dexamethasone-treated *Flvcr1a*$^{fl/fl}$ and *Flvcr1a*$^{fl/fl}$;*alb-cre* mice are shown. Heme (*A*) and malondialdehyde (MDA) (*B*) content. (*A*) n = 5. (*B*) n = 5. (*C*, *E*, *F*) Quantitative real-time polymerase chain reaction analysis of *Flvcr1a* and *Ho-1, Txnrd1, Alas1*, *Flvcr1b,* and *TfR1* mRNA level (n = 5). (*D*) Representative Western blot of L-Ft protein (n = 4). Two-way analysis of variance with Bonferroni post-test analysis was performed. *$P < .05$; **$P < .01$; ***$P < .001$.

compared with *Flvcr1a*$^{fl/fl}$ mice (Figure 1*C*). In addition, *Flvcr1a*$^{fl/fl}$*;alb-cre* mice showed increased lipid peroxidation in the liver (Figure 1*D*). The analysis of hepatic gene expression revealed that *Flvcr1a*$^{fl/fl}$*;alb-cre* mice up-regulated genes that encode for proteins involved in heme metabolism (*Ho-1*),[18,19] iron export (*Fpn*)[20,21] and storage (*H-* and *L-Ferritin*),[22] and antioxidant response (*Txnrd1, γ-gcs, Sod1*),[23] compared with *Flvcr1a*$^{fl/fl}$ mice (Figure 1*E* 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*$^{fl/fl}$*;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*$^{fl/fl}$*;alb-cre* and *Flvcr1a*$^{fl/fl}$ mice at the same extent, but bilirubin production was significantly higher in *Flvcr1a*$^{fl/fl}$*;alb-cre* mice than in *Flvcr1a*$^{fl/fl}$ mice, likely because of the enhanced HO activity (Figure 2*A* and *B*). Consistently, if animals were pretreated with the heme analog Tin-Protoporphyrin IX that inhibits HO, before heme injection, *Flvcr1a*$^{fl/fl}$*;alb-cre* mice showed a significantly higher hepatic heme content 1 hour after heme infusion compared with control mice (Figure 2*C*). When we injected mice with Tin-Protoporphyrin IX alone, we found it accumulated more in the liver of *Flvcr1a*$^{fl/fl}$*;alb-cre* mice than in that of *Flvcr1a*$^{fl/fl}$ controls (Figure 2*D*), 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*$^{fl/fl}$*;alb-cre* and *Flvcr1a*$^{fl/fl}$ mice (Supplementary Figure 4*A*), 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,

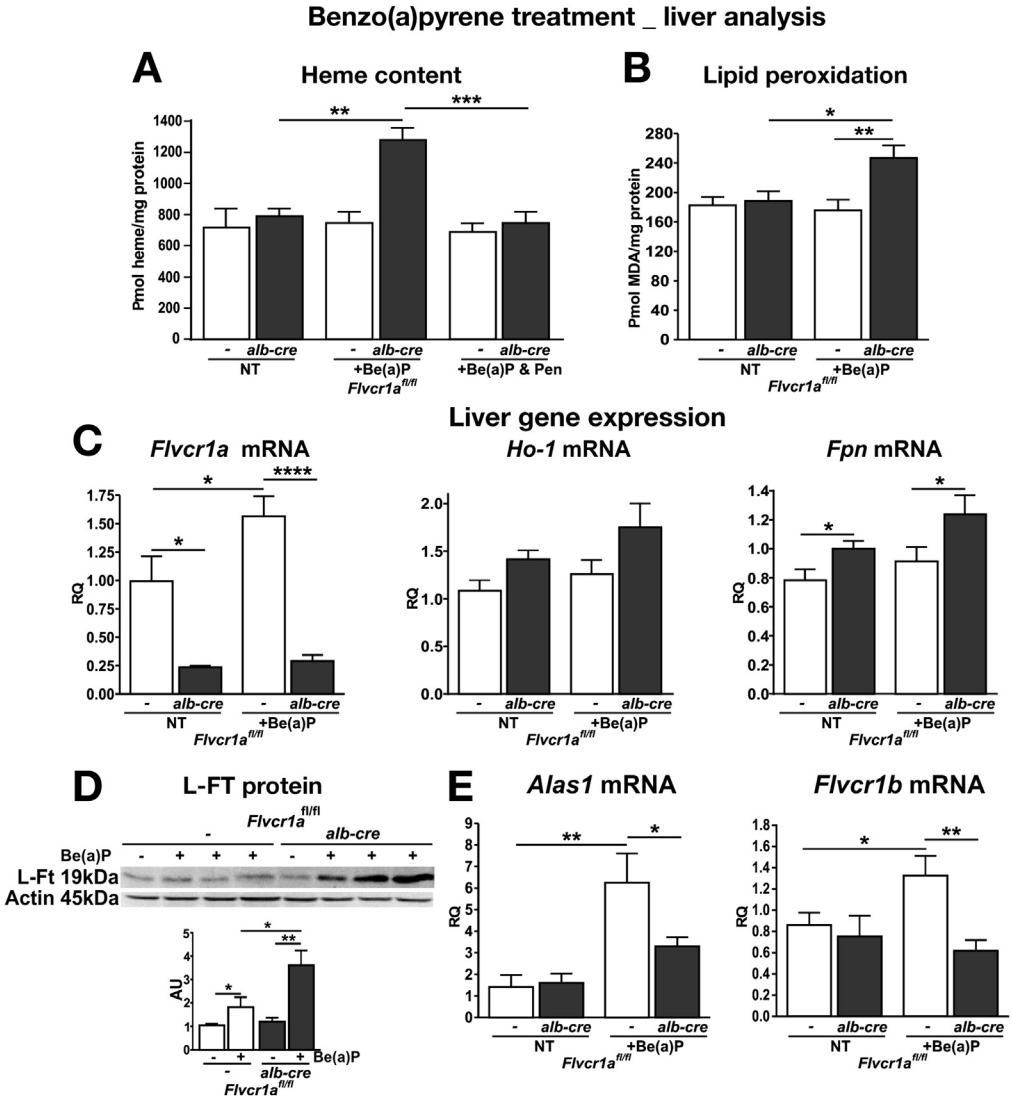
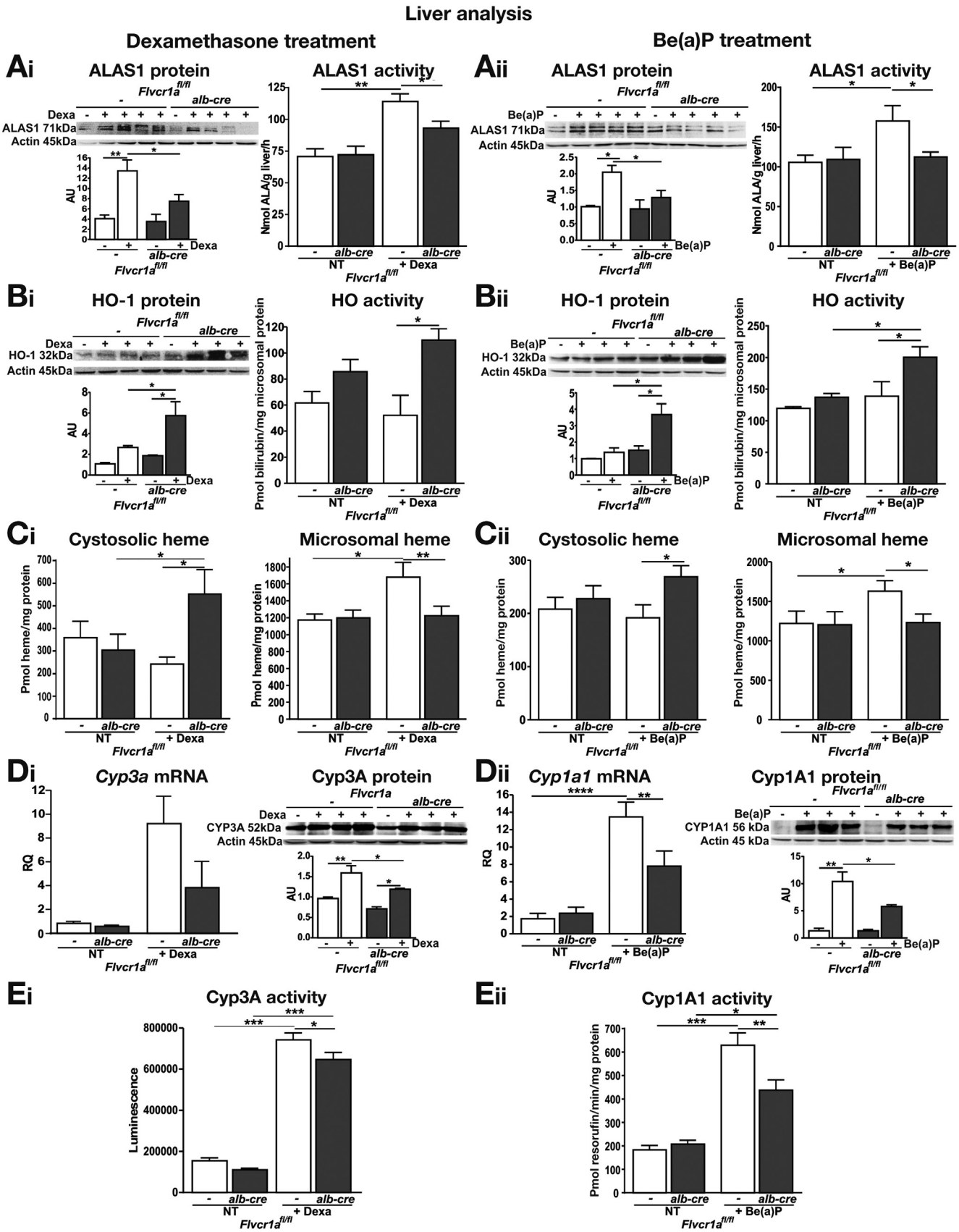


Figure 5. *Flvcr1a* deficiency affects liver homeostasis after Be(a)P-induced cytochrome synthesis. Data on the liver of Be(a)P-treated *Flvcr1a*$^{fl/fl}$ and *Flvcr1a*$^{fl/fl}$*;alb-cre* mice are shown. Heme (*A*) and malondialdehyde (MDA) (*B*) content (n = 6). (*C*, *E*) Quantitative real-time polymerase chain reaction analysis of *Flvcr1a*, *Ho-1*, and *Fpn* and of *Alas1* and *Flvcr1b* mRNA level (n = 8). (*D*) Representative Western blot of L-Ft protein (n = 4). Two-way analysis of variance with Bonferroni post-test analysis was performed. **P* < .05; ***P* < .01; ****P* < .001; *****P* < .0001.


Liver analysis
Dexamethasone treatment
Be(a)P treatment
Ai
ALAS1 protein
ALAS1 activity
Aii
ALAS1 protein
ALAS1 activity
Bi
HO-1 protein
HO activity
Bii
HO-1 protein
HO activity
Ci
Cystosolic heme
Microsomal heme
Cii
Cystosolic heme
Microsomal heme
Di
Cyp3a mRNA
Cyp3A protein
Dii
Cyp1a1 mRNA
Cyp1A1 protein
Ei
Cyp3A activity
Eii
Cyp1A1 activity
Flvcr1a fl/fl
alb-cre
Dexa
Be(a)P
NT
+ Dexa
+ Be(a)P
AU
RQ
Luminescence
Nmol ALA/g liver/h
Pmol bilirubin/mg microsomal protein
Pmol heme/mg protein
Pmol resorufin/min/mg protein
ALAS1 71kDa
Actin 45kDa
HO-1 32kDa
CYP3A 52kDa
CYP1A1 56 kDa
Actin 45 kDa

showed that FLVCR1a localized at the plasma cell membrane, along the sinusoidal surface (Supplementary Figure 4*B*).

Data shown in Figure 2*C* 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, *Flvcr1a*$^{fl/fl}$ mice showed a strong induction of *Flvcr1a* in the liver, as well as an up-regulation of *Ho-1*, *Fpn*, *H-* and *L-ferritin. Flvcr1a*$^{fl/fl}$*;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 2*E* 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 2*E*).

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 deputed 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 recovery from the bloodstream, 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 *Flvcr1a*$^{fl/fl}$*;alb-cre* mice compared with *Flvcr1a*$^{fl/fl}$ mice after ALA treatment, suggesting that on de novo synthesis, heme accumulated in the liver when FLVCR1a was absent (Figure 3*A*). This resulted in a marked increase in the hepatic lipid peroxidation index (Figure 3*B*). Interestingly, *Flvcr1a* was strongly induced by ALA treatment in the liver of *Flvcr1a*$^{fl/fl}$ mice (Figure 3*C*). On the other hand, the genes involved in heme and iron metabolism, such as *Ho-1* and *Fpn*, were up-regulated to an higher extent in the liver of *Flvcr1a*$^{fl/fl}$*;alb-cre* mice than in that of *Flvcr1a*$^{fl/fl}$ mice, and this was associated with a higher induction of the genes of the antioxidant response (Figure 3*C*). Conversely, we observed a reduced expression of *Alas1*, *Flvcr1b*, and *Tfr1* in the liver of *Flvcr1a*$^{fl/fl}$*;alb-cre* compared with *Flvcr1a*$^{fl/fl}$ mice (Figure 3*C*), suggesting an attenuation of the heme biosynthetic pathway in these animals. These results were confirmed in vitro on primary hepatocytes treated with ALA (Figure 3*D*–*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

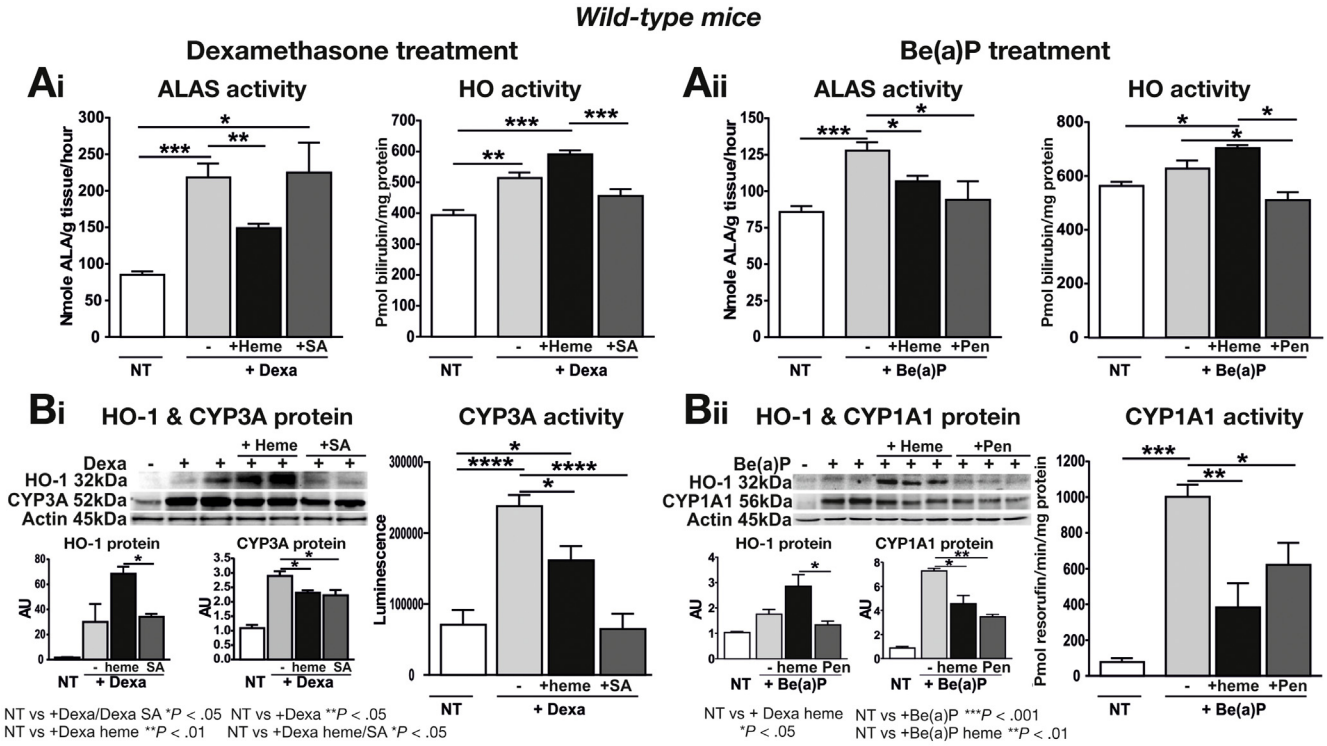
In the liver, most of the newly synthesized heme is committed to CYP synthesis. To test whether FLVCR1a function is linked to heme synthesis stimulation on cytochromes induction, we treated our mice with inducers of 3 distinct classes of CYPs.

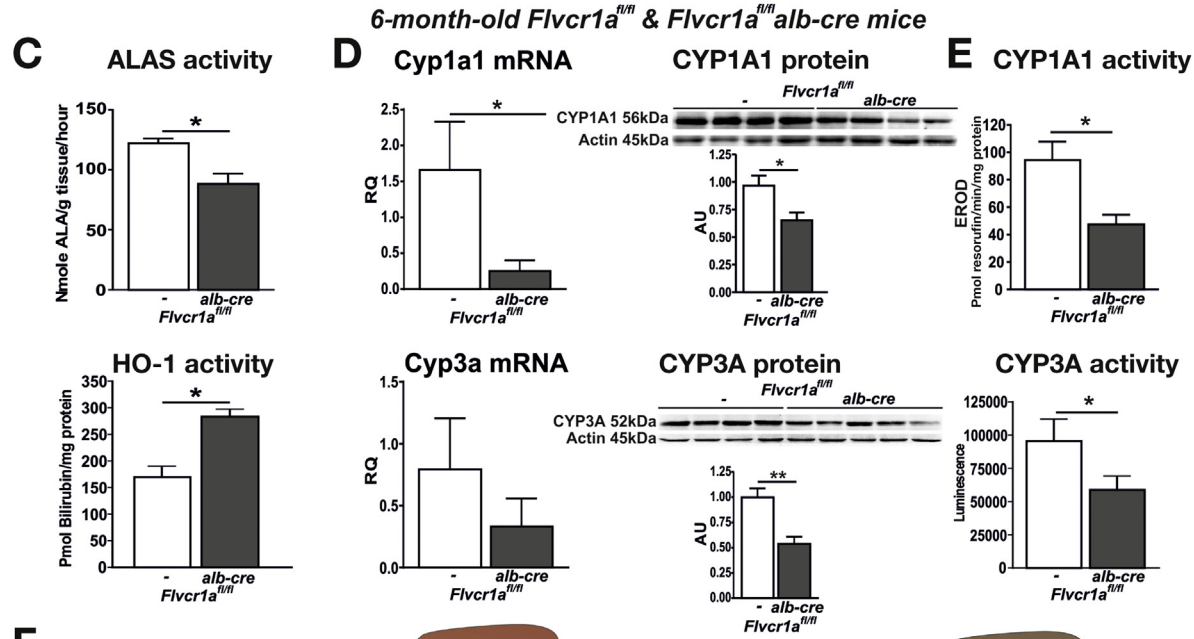
Firstly, we injected mice with dexamethasone, an inducer of CYP3A. Dexamethasone treatment caused an increase in heme content in the liver of *Flvcr1a*$^{fl/fl}$*;alb-cre* mice, that was almost negligible in *Flvcr1a*$^{fl/fl}$ counterpart (Figure 4*A*). This effect was abrogated by co-treatment with the inhibitor of heme biosynthesis, succinylacetone (Figure 4*A*). As a consequence of heme accumulation, a higher amount of lipid peroxides was generated on dexamethasone treatment in the liver of *Flvcr1a*$^{fl/fl}$*;alb-cre* mice compared with *Flvcr1a*$^{fl/fl}$ mice (Figure 4*B*). The analysis of gene expression demonstrated that *Flvcr1a* was induced in the liver of *Flvcr1a*$^{fl/fl}$ mice after dexamethasone treatment, as occurred on ALA treatment (Figure 4*C*). On the other hand, the heme-, iron-, and stress-related genes were induced to a higher extent in the liver of dexamethasone-treated *Flvcr1a*$^{fl/fl}$*;alb-cre* mice compared with the *Flvcr1a*$^{fl/fl}$ counterpart (Figure 4*C*–*E*), suggesting that the higher induction of these genes compensated for the lack of *Flvcr1a*. In addition, genes involved in heme biosynthesis, such as *Alas-1*, *Flvcr1b,* and *Tfr1,* were found to be significantly less expressed in *Flvcr1a*$^{fl/fl}$*;alb-cre* mice compared with *Flvcr1a*$^{fl/fl}$ mice after dexamethasone treatment (Figure 4*F*).

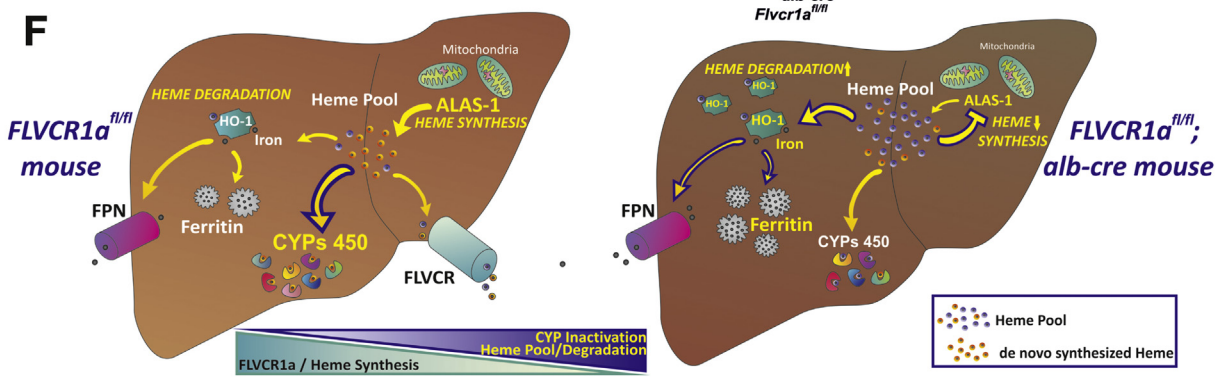
Similar results were obtained when mice were treated with benzo(a)pyrene (Be[a]P), an inducer of CYP1A1 and CYP1A2 (Figure 5, Supplementary Figure 6), and imidazole, an inducer of CYP2E1 (Supplementary Results; Supplementary Figure 7). Because the induction of *Alas1* 8h after Be(a)P injection was comparable in *Flvcr1a*$^{fl/fl}$*;alb-cre* and *Flvcr1a*$^{fl/fl}$ (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.

Figure 6. The increase of cytosolic heme pool size due to *Flvcr1a* deficiency inhibits ALAS1 and induces HO-1, impairing CYP activity. Data on the liver of *Flvcr1a*$^{fl/fl}$ and *Flvcr1a*$^{fl/fl}$*;alb-cre* mice after dexamethasone or B(a)P treatment are shown on the left and right, respectively. (*A*) ALAS1 expression and ALAS activity (n = 8). (*B*) HO-1 expression and HO activity (n = 8). (*C*) Cytosolic and microsomal heme content (n = 5). (*D*) Quantitative real-time polymerase chain reaction analysis of Cyp3a11/Cyp1a1 mRNA level and representative Western blot of CYP3A/CYP1A1 protein. (*E*) CYP3A and CYP1A1 activity (n = 7). Two-way analysis of variance with Bonferroni post-test analysis was performed. **P* < .05; ***P* < .01; ****P* < .001; *****P* < .0001.


Wild-type mice
Dexamethasone treatment
Be(a)P treatment
Ai
ALAS activity
HO activity
Nmole ALA/g tissue/hour
Pmol bilirubin/mg protein
NT
+ Dexa
+ Be(a)P
+Heme
+SA
+Pen
Aii
Bi
HO-1 & CYP3A protein
CYP3A activity
HO-1 32kDa
CYP3A 52kDa
Actin 45kDa
HO-1 protein
CYP3A protein
Luminescence
AU
Bii
HO-1 & CYP1A1 protein
CYP1A1 activity
CYP1A1 56kDa
CYP1A1 protein
Pmol resorufin/min/mg protein
NT vs +Dexa/Dexa SA *P < .05 NT vs +Dexa **P < .05
NT vs +Dexa heme **P < .01 NT vs +Dexa heme/SA *P < .05
NT vs + Dexa heme *P < .05
NT vs +Be(a)P ***P < .001
NT vs +Be(a)P heme **P < .01


6-month-old Flvcr1a fl/fl & Flvcr1a fl/fl alb-cre mice
C
ALAS activity
D
Cyp1a1 mRNA
CYP1A1 protein
E
CYP1A1 activity
HO-1 activity
Cyp3a mRNA
CYP3A protein
CYP3A activity
Nmole ALA/g tissue/hour
Pmol Bilirubin/mg protein
RQ
AU
EROD
Pmol resorufin/min/mg protein
Luminescence
CYP3A 52kDa
Actin 45kDa
alb-cre
Flvcr1a fl/fl


F
FLVCR1a fl/fl mouse
FLVCR1a fl/fl; alb-cre mouse
HEME DEGRADATION
Heme Pool
ALAS-1
HEME SYNTHESIS
HO-1
Iron
Ferritin
FPN
CYPs 450
FLVCR
Mitochondria
CYP Inactivation
Heme Pool/Degradation
FLVCR1a / Heme Synthesis
Heme Pool
de novo synthesized Heme

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 *Flvcr1a*$^{fl/fl}$*;alb-cre* compared with *Flvcr1a*$^{fl/fl}$ 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 *Flvcr1b,* 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 *Flvcr1a*$^{fl/fl}$*;alb-cre* and *Flvcr1a*$^{fl/fl}$ 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 *Flvcr1a*$^{fl/fl}$*;alb-cre* and *Flvcr1a*$^{fl/fl}$ mice (Figure 6*A* and *B*). HO-1 induction was significantly higher and ALAS1 expression was markedly reduced in the liver of *Flvcr1a*$^{fl/fl}$*;alb-cre* mice compared with *Flvcr1a*$^{fl/fl}$ counterparts. This correlated with the enzymatic activities of HO-1 and ALAS1, which were respectively higher and lower in the liver of *Flvcr1a*$^{fl/fl}$*;alb-cre* mice than in that of *Flvcr1a*$^{fl/fl}$ animals (Figure 6*A* and *B*). HO-1 induction as well as ALAS1 inhibition were likely mediated by heme overload occurring in *Flvcr1a*$^{fl/fl}$*;alb-cre* mice. Consistently, after the stimulation of CYP synthesis, heme accumulated to a higher extent in the cytosolic fraction of *Flvcr1a*$^{fl/fl}$*;alb-cre* mice compared with *Flvcr1a*$^{fl/fl}$ controls. On the other hand, heme content was significantly lower in the microsomal fraction of *Flvcr1a*$^{fl/fl}$*;alb-cre* mice than in that of *Flvcr1a*$^{fl/fl}$ animals (Figure 6*C*). 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 *Flvcr1a*$^{fl/fl}$*;alb-cre* mice than in those of treated-*Flvcr1a*$^{fl/fl}$ animals (Figure 6*D* 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 *Flvcr1a*$^{fl/fl}$*;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 7*A* 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 7*A* and *B*). This correlated nicely with a reduced expression and activity of CYPs (Figure 7*B*). 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 7*A* and *B*, *right*). Administration of succinylacetone, a heme synthesis inhibitor acting on 5-aminolevulinic 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 7*A* 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 *Flvcr1a*$^{fl/fl}$*;alb-cre* mice showed a reduction in ALAS1 activity as well as an increase in HO activity (Figure 7*C*). This misbalance in heme synthesis/degradation resulted in a reduced CYP expression at both mRNA and protein level (CYP1A1 and CYP3A, Figure 7*D*; CYP2E1, Supplementary Figure 11) and reduced CYP activity (Figure 7*E*).

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

Figure 7. Heme overload as well as heme synthesis inhibition decrease CYP expression and activity. Data on the liver of wild-type mice after dexamethasone or B(a)P treatment are shown on the *upper left* and *upper right panels,* respectively. (*A*) ALAS activity and HO activity (n = 5). (*B*) Representative Western blots of HO-1 and CYP3A/CYP1A protein, and CYP3A/CYP1A1 activity (n = 6). Data on the liver of 6-month-old *Flvcr1a*$^{fl/fl}$ and *Flvcr1a*$^{fl/fl}$*;alb-cre* mice are shown on *bottom panel*. (*C*) ALAS activity and HO activity (n = 5). (*D*) Quantitative real-time polymerase chain reaction analysis of Cyp1a1/Cyp3a11 mRNA level and representative Western blot of CYP1A1/CYP3A protein (n = 5). (*E*) CYP1A1 and CYP3A activity (n = 5). One-way analysis of variance with Bonferroni post-test analysis and unpaired *T* test analysis with Welch's correction was performed on data in the *upper* and *bottom panel*, respectively. (*F*) Illustration showing FLVCR1a mood of action in the liver (see also Supplementary Material). *$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$.

exporting heme excess.[11,13,14] 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.[24]

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.[13] 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,[25,26] which are engaged in steroid metabolism and in the oxidative metabolism of foreign compounds, including pharmaceutical drugs.[10,15,27] 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.[28] 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.[10] The free heme pool controls heme biosynthesis, through the regulation of ALAS1. If increased, the regulatory heme pool may repress ALAS1,[7] and its depletion causes ALAS1 induction.[10] 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.[29–33] Although xenobiotics might have some primary inducing effect on hepatic ALAS1,[34,35] many chemical inducers are believed to increase ALAS1 by depleting the free heme pool in hepatocytes.[10] 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.[26,36–38] 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.[39,40] Short-term hemin administration is known to both increase HO1 expression[41] and interfere with the formation of CYP.[40] 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 7*F*). It appears that in the hepatocytes, heme is formed in slight excess over its metabolic needs[28] 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.

References

1. Ponka P. Cell biology of heme. Am J Med Sci 1999; 318:241–256.
2. Furuyama K, Kaneko K, Vargas PD. Heme as a magnificent molecule with multiple missions: heme determines its own fate and governs cellular homeostasis. Tohoku J Exp Med 2007;213:1–16.

3. Abraham NG, Kappas A. Pharmacological and clinical aspects of heme oxygenase. Pharmacol Rev 2008; 60:79–127.
4. Tolosano E, Fagoonee S, Morello N, et al. Heme scavenging and the other facets of hemopexin. Antioxid Redox Signal 2010;12:305–320.
5. Vinchi F, Tolosano E. Therapeutic approaches to limit hemolysis-driven endothelial dysfunction: scavenging free heme to preserve vasculature homeostasis. Oxid Med Cell Longev 2013:396527.
6. Gotoh S, Nakamura T, Kataoka T, et al. Egr-1 regulates the transcriptional repression of mouse δ-aminolevulinic acid synthas8e 1 by heme. Gene 2011;472:28–36.
7. Kolluri S, Sadlon TJ, May BK, Bonkovsky HL. Haem repression of the housekeeping 5-aminolaevulinic acid synthase gene in the hepatoma cell line LMH. Biochem J 2005;392:173–180.
8. Zheng J, Shan Y, Lambrecht RW, et al. Differential regulation of human ALAS1 mRNA and protein levels by heme and cobalt protoporphyrin. Mol Cell Biochem 2008;319:153–161.
9. Sun J, Hoshino H, Takaku K, et al. Hemoprotein Bach1 regulates enhancer availability of heme oxygenase-1 gene. EMBO J 2002;21:5216–5224.
10. Correia MA, Sinclair PR, De Matteis F. Cytochrome P450 regulation: the interplay between its heme and apoprotein moieties in synthesis, assembly, repair, and disposal. Drug Metab Rev 2011;43:1–26.
11. **Quigley JG, Yang Z**, Worthington MT, et al. Identification of a human heme exporter that is essential for erythropoiesis. Cell 2004;118:757–766.
12. Khan AA, Quigley JG. Control of intracellular heme levels: heme transporters and heme oxygenases. Biochim Biophys Acta 2011;1813:668–682.
13. Chiabrando D, Marro S, Mercurio S, et al. The mitochondrial heme exporter FLVCR1b mediates erythroid differentiation. J Clin Invest 2012;122:4569–4579.
14. **Keel SB, Doty RT**, Yang Z, et al. A heme export protein is required for red blood cell differentiation and iron homeostasis. Science 2008;319:825–828.
15. Guengerich FP. Cytochrome P450s and other enzymes in drug metabolism and toxicity. AAPS J 2006;8: E101–E111.
16. Guengerich FP. Cytochrome p450 and chemical toxicology. Chem Res Toxicol 2008;21:70–83.
17. Vinchi F, Gastaldi S, Silengo L, et al. Hemopexin prevents endothelial damage and liver congestion in a mouse model of heme overload. Am J Pathol 2008;173:289–299.
18. Gozzelino R, Jeney V, Soares MP. Mechanisms of cell protection by heme oxygenase-1. Annu Rev Pharmacol Toxicol 2010;50:323–354.
19. Fraser ST, Midwinter RG, Berger BS, Stocker R. Heme oxygenase-1: a critical link between iron metabolism, erythropoiesis, and development. Adv Hematol 2011: 473709.
20. McKie AT, Marciani P, Rolfs A, et al. A novel duodenal iron-regulated transporter, IREG1, implicated in the basolateral transfer of iron to the circulation. Mol Cell 2000;5:299–309.
21. Marro S, Chiabrando D, Messana E, et al. Heme controls ferroportin1 (FPN1) transcription involving Bach1, Nrf2 and a MARE/ARE sequence motif at position -7007 of the FPN1 promoter. Haematologica 2010;95: 1261–1268.
22. Arosio P, Levi S. Ferritin, iron homeostasis, and oxidative damage. Free Radic Biol Med 2002;33:457–463.
23. Vinchi F, De Franceschi L, Ghigo A, et al. Hemopexin therapy improves cardiovascular function by preventing heme-induced endothelial toxicity in mouse models of hemolytic diseases. Circulation 2013;127:1317–1329.
24. Lee PL, Gaasterland T, Barton JC. Mild iron overload in an African American man with SLC40A1 D270V. Acta Haematol 2012;128:28–32.
25. Buzaleh AM, del Camen Martinez M, del Carmen Batlle AM. Relevance of cytochrome P450 levels in the actions of enflurane and isoflurane in mice: studies on the haem pathway. Clin Exp Pharmacol Physiol 2000; 27:796–800.
26. Baron J, Tephly TR. The role of heme synthesis during the induction of hepatic microsomal cytochrome P-450 and drug metabolism produced by benzpyrene. Biochem Biophys Res Commun 1969;36:526–532.
27. Wrighton SA, Stevens JC. The human hepatic cytochromes P450 involved in drug metabolism. Crit Rev Toxicol 1992;22:1–21.
28. Ponka P. Tissue-specific regulation of iron metabolism and heme synthesis: distinct control mechanisms in erythroid cells. Blood 1997;89:1–25.
29. Sassa S, Granick S. Induction of -aminolevulinic acid synthetase in chick embryo liver cells in cluture. Proc Natl Acad Sci U S A 1970;67:517–522.
30. Granick S, Kappas A. Steroid control of porphyrin and heme biosynthesis: a new biological function of steroid hormone metabolites. Proc Natl Acad Sci U S A 1967; 57:1463–1467.
31. Granick S. The induction in vitro of the synthesis of delta-aminolevulinic acid synthetase in chemical porphyria: a response to certain drugs, sex hormones, and foreign chemicals. J Biol Chem 1966;241:1359–1375.
32. Marver HS, Collins A, Tschudy DP, Rechcigl M. Delta-aminolevulinic acid synthetase. II. Induction in rat liver. J Biol Chem 1966;241:4323–4329.
33. Marver HS, Tschudy DP, Perlroth MG, Collins A. Delta-aminolevulinic acid synthetase. I. Studies in liver homogenates. J Biol Chem 1966;241:2803–2809.
34. Baron J, Tephly TR. Further studies on the relationship of the stimulatory effects of phenobarbital and 3,4-benzpyrene on hepatic heme synthesis to their effects on hepatic microsomal drug oxidations. Arch Biochem Biophys 1970;139:410–420.
35. Marver HS, Schmid R, Schützel H. Heme and methemoglobin: naturally occurring repressors of microsomal cytochrome. Biochem Biophys Res Commun 1968; 33:969–974.
36. Dwarki VJ, Francis VN, Bhat GJ, Padmanaban G. Regulation of cytochrome P-450 messenger RNA and apoprotein levels by heme. J Biol Chem 1987;262: 16958–16962.

37. Tephly TR, Hasegawa E, Baron J. Effect of drugs on heme synthesis in the liver. Metabolism 1971;20:200–214.
38. Baron J, Tephly TR. Effect of 3-amino-1,2,4-triazole on the stimulation of hepatic microsomal heme synthesis and induction of hepatic microsomal oxidases produced by phenobarbital. Mol Pharmacol 1969;5:10–20.
39. Schacter BA, Nelson EB, Marver HS, Masters BS. Immunochemical evidence for an association of heme oxygenase with the microsomal electron transport system. J Biol Chem 1972;247:3601–3607.
40. Marver HS. The role of heme in the synthesis and repression of microsomal protein. In: Gillette JR, Conney AH, Cosmides GJ, eds. Microsomes and drug oxidation. New York: Academic Press, 1969:495–511.
41. Maines MD, Kappas A. Metals as regulators of heme metabolism. Science 1977;198:1215–1221.

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Conflicts of interest
The authors disclose no conflicts.

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