Brief Report

RED CELLS, IRON, AND ERYTHROPOIESIS

Targeted disruption of hepcidin in the liver recapitulates the hemochromatotic phenotype

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Key Points

- Liver-specific hepcidin KO mice fully recapitulate the severe iron overload phenotype observed in the total KO mice.
- The hepcidin produced by hepatocytes is the main regulator of body iron homeostasis.

Hepcidin is a 25-amino-acid peptide demonstrated to be the iron regulatory hormone capable of blocking iron absorption from the duodenum and iron release from macrophages. Mutations affecting hepcidin regulators or the hepcidin gene itself cause hemochromatosis, a common genetic disorder. Hepcidin is produced mainly by the liver, but many cells and tissues express low levels of the hormone. To determine the contribution of these hepcidin-producing tissues in body iron homeostasis, we have developed a new mouse model in which the hepcidin gene can be conditionally inactivated. Here we compare a liver-specific knockout (KO) mouse model with total KO mice. We show that the liver-specific KO mice fully recapitulate the severe iron overload phenotype observed in the total KO mice, with increased plasma iron and massive parenchymal iron accumulation. This result demonstrates that the hepatocyte constitutes the predominant reservoir for systemic hepcidin and that the other tissues are unable to compensate. (*Blood*. 2014;123(23):3646-3650)

Introduction

Hepcidin expression is induced by iron accumulation and diminished in situations of iron needs (increased erythropoiesis and hypoxia).[1] Hepcidin controls serum iron levels by binding to ferroportin (FPN), the only known iron exporter, and inducing its degradation.[2] Even if the liver is the main site of hepcidin production, many tissues or cells (macrophages,[3,4] brain,[5] heart,[6] retina,[7] kidney,[8,9] adipocytes,[10] pancreas,[11] etc) have been shown to express hepcidin at low levels. However, the contribution of these tissues to circulating hepcidin levels and therefore to body iron homeostasis is unknown. To address this question, we generated mice with floxed *hamp1* alleles (with locus of crossover in P1 [loxP] sequences flanking exons 2 and 3 of the gene) allowing tissue-specific gene deletion with the classical Cre-loxP strategy. Using the ubiquitous Cre recombinase–deleter mice (E2a-Cre mice), we have validated our targeting strategy and reproduced the iron overload observed in the classical hepcidin knockout (KO) mice.[12] Whether non-hepatocyte sources of hepcidin could affect iron homeostasis has not been documented yet. We therefore studied the effect of hepcidin deficiency in the liver by crossing the floxed hepcidin mice ($hamp1^{lox/lox}$ mice) with transgenic mice expressing the hepatocyte-specific albumin Cre recombinase (Alb-Cre).[13] Liver-specific KO mice were compared with wild-type (WT) and total KO mice (E2a-Cre). The iron status of the animals was monitored.

Study design

Gene targeting and generation of conditional *hamp1* KO mice

The *hamp1* targeting construct was generated from polymerase chain reaction (PCR) products amplified from the DNA of 129/SV ES cells. The 5′ (4 kb) and 3′ (1.8 kb) homology arms (bold line) were inserted on either side of a phosphoglycerate kinase promoter-driven hygromycin selection cassette flanked by FLP recombinase target (FRT) sites, in the pL3-FRT-Hygro vector (Figure 1Aii). The generation of the *hamp1*-targeted clones is described in the supplemental Methods (see the *Blood* Web site). The hygromycin resistance cassette flanked by FRT sites was excised by crossing $hamp1^{lox/+}$ mice with flippase-expressing mice (Figure 1Aiii). The resulting heterozygous offspring were bred with deleter E2a-Cre transgenic mice[14] or hepatocyte-specific Alb-Cre mice[13] (Figure 1Aiv). These mice were crossed to generate WT, $hamp1^{-/-}$ ($Hepc^{\Delta total}$), or liver-specific KO ($Hepc^{\Delta liver}$) mice. Only males were used in the study. The animal studies described here were reviewed and approved (Agreement no. CEEA34.CP.003.13) by the "Président du Comité d'Ethique pour l'Expérimentation Animale Paris Descartes" and are in accordance with the principles and guidelines established by the European Convention for the Protection of Laboratory Animals.

Reverse transcription and real-time quantitative PCR

RNA extraction, quantitative PCR, and sequences of the primers used have been previously described.[15]

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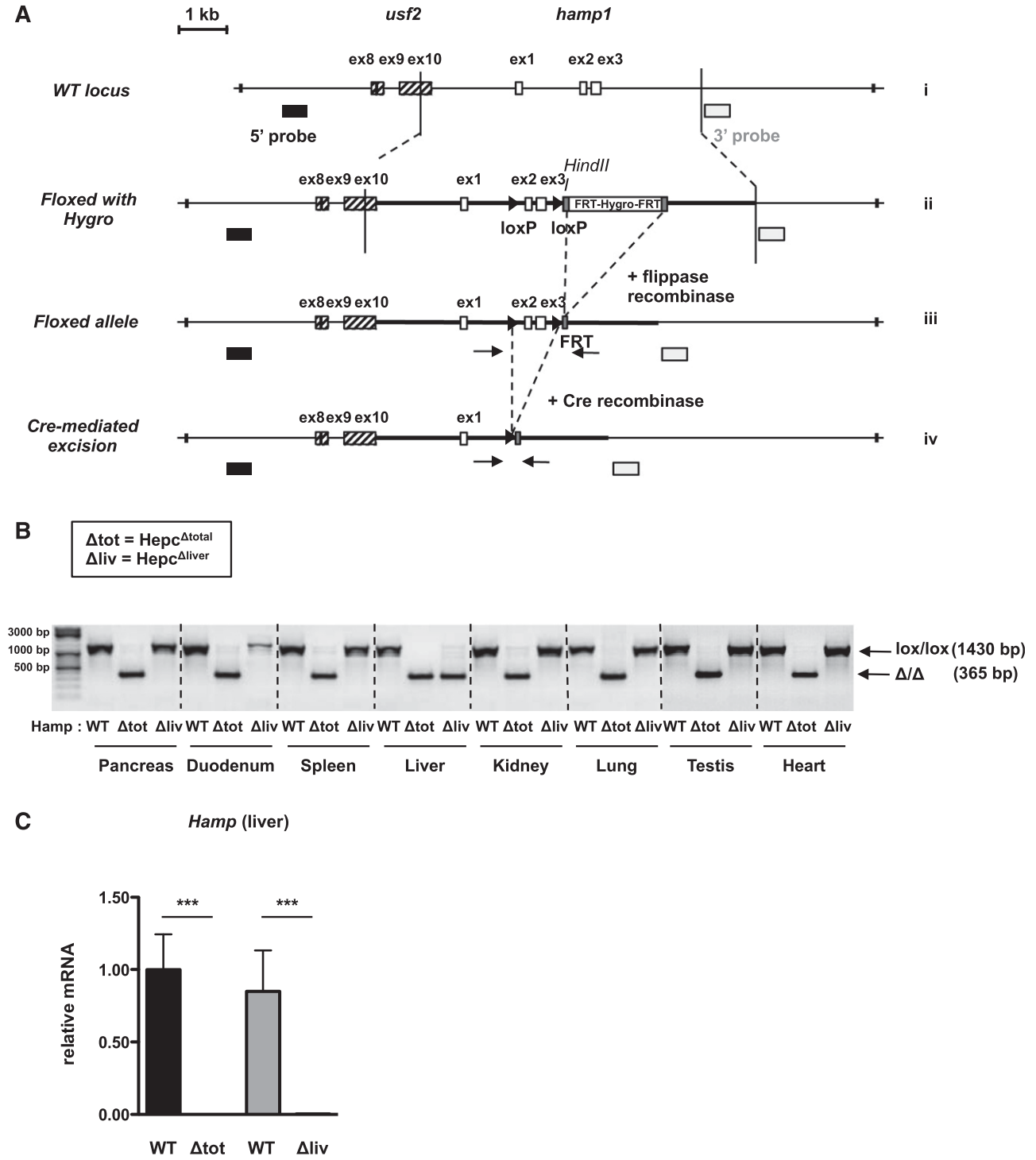


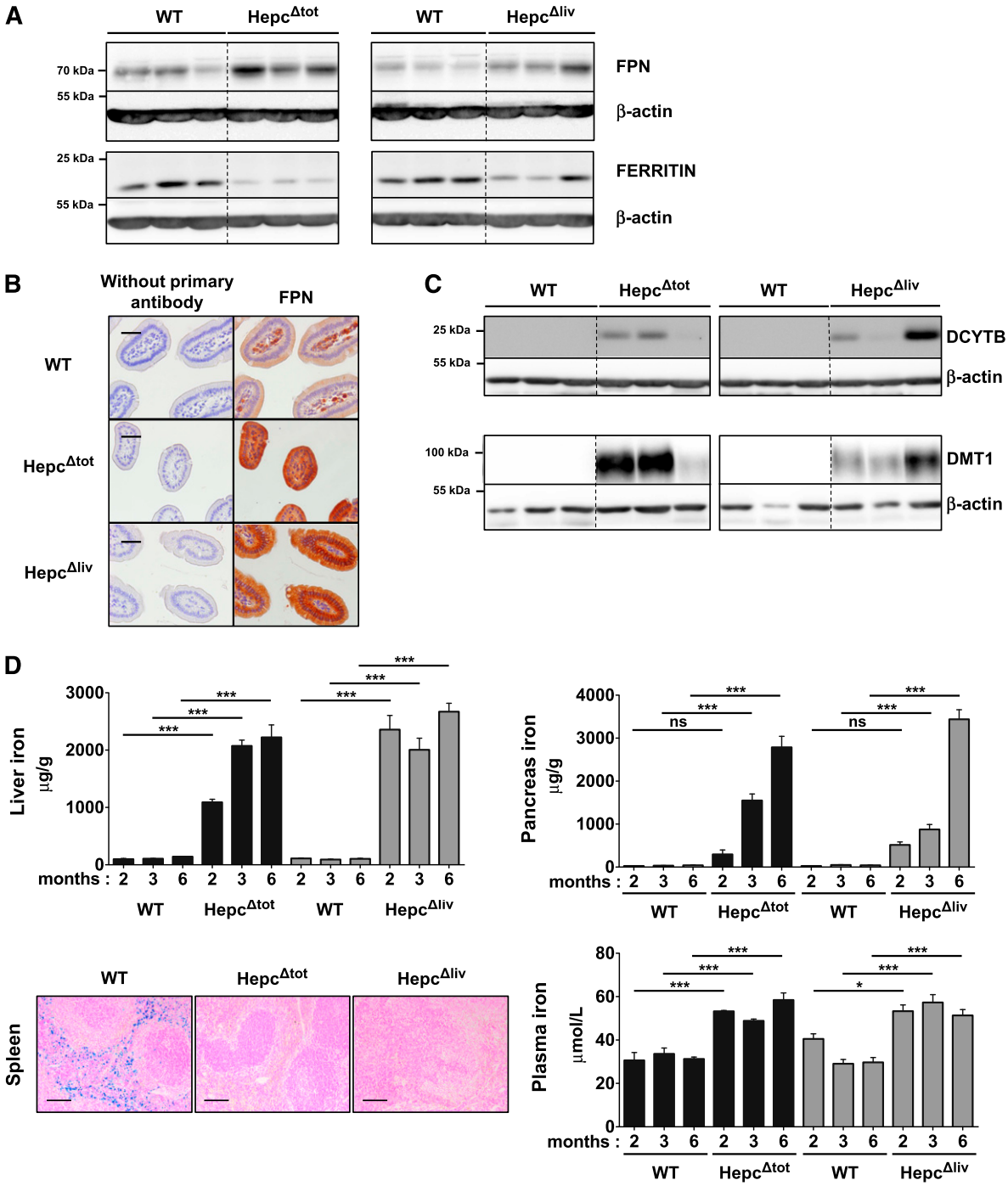
Figure 1. Generation of conditional KO mice for hepcidin. (A) Scheme of the mouse WT *hamp1* genomic sequence (i), preceded by the *usf2* gene loxP-flanked allele with FRT-flanked Hygro sequence (ii), floxed allele after flippase recombination (iii), and conditional KO allele after Cre-mediated excision (iv). Arrows: P1 and P2 primers used for deletion verification. (B) Recombination of the floxed *hamp1* allele by genomic PCR in the pancreas, duodenum, spleen, liver, kidney, lung, testis, and heart of the $Hepc^{\Delta total}$, $Hepc^{\Delta liver}$ and WT mice. (C) Hepcidin messenger RNA (mRNA) expression in the liver of $Hepc^{\Delta total}$ and $Hepc^{\Delta liver}$ mice and control littermates. $n > 5$. Values of WT mice were set up at 1. Samples were normalized to the threshold cycle value for cyclophilin. $^{***}P < .001$.

Western blot

Extraction of membrane proteins was performed as previously described.[15] The following antibodies were used: divalent metal transporter 1 (DMT1)[16] (courtesy of François Cannone-Hergaux), duodenal cytochrome B (DCYTB) (DCYTB11-A; Alpha Diagnostic), FPN (MTP11-A; Alpha Diagnostic), and ferritin (SAB2500431; Sigma-Aldrich).

Iron measurements and immunostaining

Plasma and tissue iron levels were quantified colorimetrically by a previously described method.[15] For histology, tissues were fixed in 4% formaldehyde and embedded in paraffin. Five micrometer slides were either stained with Perls's Prussian blue and nuclear fast red counterstain or stained by immunohistochemistry using FPN antibody (MTP11-A; Alpha Diagnostic).



E

	WT	Hepc$^{\Delta tot}$	Hepc$^{\Delta liv}$
Serum hepcidin (ng/mL)	291,4 (± 39,6)	nd	nd

Figure 2. The liver-specific KO mice recapitulate the phenotype of the total KO mice. (A) FPN and ferritin expression in the spleen of 6-month-old Hepc$^{\Delta total}$ and Hepc$^{\Delta liver}$ mice and their control littermates by western blotting. (B) Immunohistochemistry for FPN in the duodenum of Hepc$^{\Delta total}$ and Hepc$^{\Delta liver}$ mice and WT mice. Bars represent 50 μm. (40/0.60, Leica DMI3000B microscope, Leica DFC310FX camera [Leica LAS Core software]). (C) Western blotting in duodenum scrapings of 6-month-old Hepc$^{\Delta total}$, Hepc$^{\Delta liver}$, and control mice. (D) Liver, pancreas, and plasma iron in 2-, 3-, and 6-month-old Hepc$^{\Delta total}$ and Hepc$^{\Delta liver}$ mice and their control littermates. n > 5. *$P < .05$, ***$P < .001$. ns indicates not significant. Perls's blue staining of the spleen of 6-month-old Hepc$^{\Delta total}$ and Hepc$^{\Delta liver}$ mice and WT mice. Bars represent 100 μm. (20/0.4, Leica DMI3000B microscope, Leica DFC310FX camera [Leica LAS Core software]). (E) Plasma hepcidin in 6-month-old control, Hepc$^{\Delta total}$, and Hepc$^{\Delta liver}$ mice. n = 4.

Hepcidin enzyme-linked immunosorbent assay

Plasma hepcidin was measured following the manufacturer's instructions (Bachem).

Results and discussion

We introduced, in the mouse genome, loxP sites flanking exons 2 and 3 of *hamp1* gene, which encodes the hepcidin protein ("Study design" and Figure 1A). Mice for floxed *hamp1* alleles (*hamp1*$^{lox/lox}$) were bred to a transgenic line (E2a-Cre) expressing a Cre recombinase under the control of the ubiquitous E2a promoter[17] or to transgenic mice expressing the hepatocyte-specific Alb-Cre recombinase,[13] to generate total (Hepc$^{\Delta total}$) and liver-specific (Hepc$^{\Delta liver}$) homozygote KO mice, respectively. We observed an efficient truncation of the floxed *hamp1* allele in all the organs of the Hepc$^{\Delta total}$ mice we tested. As expected, deletion of the floxed *hamp1* allele was observed only in the liver of the Hepc$^{\Delta liver}$ mice and not in the other organs (Figure 1B). Hepcidin expression, measured by quantitative PCR, was completely absent in the liver of the Hepc$^{\Delta total}$ and Hepc$^{\Delta liver}$ mice compared with their WT littermates (Figure 1C).

Plasma hepcidin inhibits iron efflux into plasma by directly binding to and inducing the degradation of FPN, which is present on macrophages and enterocytes. We therefore measured FPN expression in spleen and duodenum extracts of Hepc$^{\Delta total}$ and Hepc$^{\Delta liver}$ mice. FPN was similarly increased in the spleen of 6-month-old Hepc$^{\Delta total}$ and Hepc$^{\Delta liver}$ mice compared with control littermates, as assessed by western-blot analysis (with no alteration of FPN mRNA level; not shown). As a consequence, ferritin levels were decreased in both Hepc$^{\Delta total}$ and Hepc$^{\Delta liver}$ mice (Figure 2A), corroborated by the absence of iron staining in the spleen compared with WT animals (Figure 2D). In the duodenum, we showed by immunohistochemistry (Figure 2B) that FPN expression was also similarly increased in both mutant mice (with a modest twofold increase of FPN mRNA level; not shown). The increased rate of the duodenal iron absorption proteins (DCYTB and DMT1) was identical in the Hepc$^{\Delta total}$ and Hepc$^{\Delta liver}$ mice compared with their control littermates (Figure 2C).

Finally, we examined the age-dependent variation (2, 3, and 6 months) of the iron parameters in the Hepc$^{\Delta total}$, Hepc$^{\Delta liver}$, and control mice (Figure 2D). Even though liver iron content was higher in the liver-specific KO compared with the total KO mice at 2 months (with, however, a similar Perls's blue staining; supplemental Figure 1), both mutant mice had a massive iron accumulation at 3 and 6 months. Iron gradually accumulated in the pancreas, in an age-dependent manner in both Hepc$^{\Delta total}$ and Hepc$^{\Delta liver}$ mice. Plasma iron increased at 2 months and remained constant during the following months. Hepcidin was undetectable in the plasma of Hepc$^{\Delta total}$ but also in Hepc$^{\Delta liver}$ mice (Figure 2E), confirming the lack of contribution of extrahepatic organs in the overall circulating hepcidin.

In conclusion, hepatic hepcidin is fundamental for the maintenance of systemic iron homeostasis in basal conditions. The liver-specific KO mice fully recapitulate the severe iron overload phenotype observed in the total KO mice demonstrating that the hepatocyte constitutes the predominant reservoir for systemic hepcidin, the other tissues being unable to compensate for the severe iron overload. Our results correlate with the liver-specific KOs of hepcidin regulators HFE,[18] hemojuvelin,[19] or transferrin receptor 2,[20] which all display a severe iron overload, similar to that of the respective total KO mice.

This new mouse model, in which the hepcidin gene can be spatiotemporally inactivated, will be important for future research to determine the impact of hepcidin deficiency in different tissues in pathophysiological conditions. For example, hypoxia induces a strong increase of hepcidin expression in the heart,[6] suggesting its implication in cardiac diseases. Inflammatory stimuli have been shown to strongly upregulate hepcidin in myeloid cells, such as macrophages and neutrophils,[3,4] or in the brain.[21] Therefore, nonhepatic hepcidin may be essential at the site of infections and/or in poorly perfused tissues, inaccessible by systemic hepcidin from the circulation.

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Authorship

Contribution: S.Z., J.R.R.M., S.D., M.H., L.V., and C.P. performed experiments; S.Z., J.R.R.M., S.D., M.H., L.V., S.V., and C.P. conceived, analyzed, and interpreted the experiments; and C.P. wrote the manuscript.

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