Fetal liver hepcidin supports acquisition of iron stores in utero

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Running title- Fetal hepcidin regulates iron stores

Abstract

The liver-derived hormone hepcidin (HAMP) controls systemic iron homeostasis by blocking the iron export protein ferroportin (FPN) in the gut and the spleen, the sites of iron absorption and recycling respectively. In the adult, disruption of HAMP expression or of FPN responsiveness to HAMP leads to increased liver iron stores. In the newborn, liver iron stores are important for supporting postnatal growth. However, it is not clear if fetal liver HAMP plays a role in regulating fetal iron stores. To address this question, we generated fetuses harbouring a ubiquitous or liver-specific knock-in the HAMP-resistant *fpn*C326Y allele, or liver-specific loss of the *hamp* gene. This was achieved using paternal inheritance in order to safeguard against the confounding effects of altered iron control in the mothers and in the maternal part of the placenta. We found that these fetuses had reduced liver iron stores compared with littermate controls. This was associated with marked increase in FPN in the fetal liver but not in the placenta. These results demonstrate that fetal liver HAMP operates in a cell-autonomous manner to increase fetal liver iron stores in utero. They also suggest that the function of FPN in the placenta is permissive of iron transport rather than regulatory.

Keywords

Hepcidin

Ferroportin

Iron stores

Fetal liver

Placenta

Introduction

Iron is essential for neonatal growth and development. However, the neonatal gut is not fully competent in regulating iron absorption in response to iron needs until 6-9 months of age (1). During this time, the neonate is dependent on its liver iron stores .Suboptimal iron stores at birth are associated with irreversible cognitive, behavioural and motor skill deficits (2,3,4). In humans, umbilical cord ferritin concentrations <76 mg/L are associated with impaired language ability, tractability, and fine motor skills in children subsequently studied at 5 years of age (5). In some human follow-up studies, it was also observed that these developmental deficits were not reversed by iron therapy in neonatal or adult life (6). These studies highlight the importance of acquiring adequate liver iron stores in utero.

In the adult, the liver-derived hormone hepcidin HAMP controls systemic iron homeostasis, and consequently liver iron stores, by blocking the iron export protein ferroportin (FPN) in the gut and the spleen, the sites of iron absorption and recycling respectively (7, 8). Disruption of the hepcidin/ferroportin axis in a manner that reduces hepcidin expression or responsiveness leads to the iron overload disease hemochromatosis. This disease is characterised by increased serum iron concentration, iron deposition in the liver and depletion of iron from splenic macrophages (9). Previous work from this laboratory has shown that these effects are recapitulated in mice harbouring a ubiquitous knock-in of the *fpn*C326Y allele, which encodes a non-hepcidin responsive FPN (10). The fetal liver also expresses hepcidin, and the widely-accepted consensus is that it regulates iron transfer across the placenta, by blocking FPN in the fetally-derived SynTII layer that lines the fetal capillaries of the syncytiotrophoblast (STB) (11). There is some evidence supporting a role for hepcidin in fetal iron homeostasis. One study examining the effects of the hemochromatosis gene *hfe* (HFE is a protein that is necessary for hepcidin expression), found that *hfe* knockout (*hfe*-ko) fetuses accumulated higher liver stores than wild type fetuses, but only when mothers were provided an iron-rich diet (12). Another found that transgenic mice over-expressing hepcidin develop iron deficiency in utero (13). A third study found that fetuses lacking the *matriptase-2* gene (Matriptase-2 is a negative regulator of hepcidin expression) contained less iron than wild type controls (14). One common feature of these studies is that the genetic variant (*hfe*-ko allele, hamp-transgene or *matriptase-2*-ko) is also present in the mothers, with the potential to affect not only maternal iron status but also homeostatic iron control in the maternal part of the placenta.

We sought to establish formally the role of fetal hepcidin in utero. To safeguard against the confounding effects of altered homeostatic iron control in the mothers and in maternally-derived part of the placenta, we used paternally-inherited alleles to disrupt hepcidin or hepcidin responsiveness in the fetal tissues, against a background of normal maternal iron control.

METHODS

Mice

All animal procedures were compliant with the UK Home Office Animals (Scientific Procedures) Act 1986 and approved by the University of Oxford Medical Sciences Division Ethical Review Committee. The conditional fpnC326Y^{fl} allele was generated as described previously (10). Females were mated between 9 and 12 weeks of age and foetuses were harvested from first pregnancies.

Immunostaining

Formalin-fixed paraffin-embedded FFPE tissue sections were with rabbit polyclonal anti-mouse FPN antibody (NBP1-21502, Novus biologicals) at 1/200 dilution. Alexa488-conugated anti-rabbit antibody (ab150073, Abcam) was then used 1/500 as a secondary antibody.

Iron indices
Serum iron and ferritin levels were determined using the ABX-Pentra system (Horiba Medical, CA). Determination of total elemental iron in tissues from PBS-perfused animals was carried out by inductively coupled plasma mass spectrometry (ICP-MS) as described previously (10). Haemoglobin was recorded from fresh blood using the HemoCue Hb 201+ system. DAB-enhanced Perls' iron stain was carried out in FFPE sections as described previously (10).

Diet provision during pregnancy
Unless otherwise stated, mothers were fed a standard chow diet containing 200ppm iron. In iron-loading studies, mothers were fed a diet containing 5000ppm iron (Teklad TD.140464) as soon as mated.

Gene expression
Gene expression was measured by quantitative real-time PCR, using Applied Biosystems Taqman gene expression assay probes for fpn, tfr1 and hamp and house-keeping gene β-Actin (Life Technologies*, Carlsbad, CA*). The CT value for the gene of interest was first normalised by deducting CT value for β-Actin to obtain a delta CT value. Delta CT values of test samples were further normalised to the average of the delta CT values for control samples to obtain delta delta CT values. Relative gene expression levels were then calculated as $2^{-\text{delta deltaCT}}$.

Statistics
Values are shown as mean± standard error of the mean (S.E.M). Paired comparisons were performed using Student's T test.

RESULTS AND DISCUSSION

Fetal liver iron concentration and hepcidin expression increase in the third trimester

In the mouse, the fetal liver forms in the second half of gestation. We found that the total concentration of elemental iron in the fetal liver, measured by inductively coupled plasma mass spectrometry ICP-MS as described previously (10), increased by 2.75 fold between e13.5 and e17.5, to levels well above those in the maternal liver, consistent with increased liver iron stores (Figure 1A). Over the same period of time, fetal liver hamp mRNA expression increased by 24 fold (Figure 1B). Nevertheless, at 17.5, fetal liver hepcidin expression was ~ 18 fold lower than that of maternal liver hepcidin (Figure 1B). As expected, and consistent with a role of iron stores in supporting growth, there was a positive correlation between the concentration of iron in the fetal liver and fetal weight at e.17.5 (Figure 1C). Additionally, there was a positive correlation between the concentration of iron and the expression of hamp mRNA in the fetal liver at e.17.5 (Figure 1D).

Ubiquitous loss of FPN responsiveness to hepcidin reduces fetal liver iron stores and haemoglobin at birth

In the adult, liver HAMP regulates liver iron stores (7,8,9). To probe the role of fetal HAMP in regulating fetal liver iron stores, we used mice harbouring a ubiquitous knock-in of a HAMP-resistant FPN. To guard against a confounding effect of maternal iron overload, we set up matings using wild type $fpn^{wt/wt}$ mothers and $fpn^{wt/C326Y}$ fathers heterozygous for the knock-in mutation of *fpn*C326Y, an isoform of FPN with intact iron export function but which is resistant to HAMP. Adult mice harbouring this mutation develop the iron overload disease hemochromatosis, characterised by increased liver iron stores and depletion of iron from the spleen, by 12 weeks of age (10). In contrast to the adult mice, $fpn^{wt/C326Y}$ fetuses at e.17.5, had ~30% lower fetal liver iron concentration compared with $fpn^{wt/wt}$ littermate controls (Figure 2A). This was further confirmed histologically by DAB-enhanced Perls' iron stain

(Figure 2B). At birth (d0), liver iron concentration remained lower in *fpn*$^{wt/C326Y}$ fetuses than in *fpn*$^{wt/wt}$ littermate controls (Figure 2A). This difference disappeared within the first week of life, with mice of both genotypes having comparable liver iron stores at 1 and 4 weeks of age (Figure 2A). By 12 weeks of age, *fpn*$^{wt/C326Y}$ mice had liver iron concentration that is 115% higher than that of *fpn*$^{wt/wt}$ littermate controls (Figure 2A), consistent with the hemochromatosis phenotype. Reduced liver iron stores in utero and at birth could not be attributed to re-distribution of iron into extra-hepatic tissues, as iron concentration in the remaining carcass (fetus-liver) of *fpn*$^{wt/C326Y}$ fetuses was still lower than in *fpn*$^{wt/wt}$ littermates at e17.5 (Figure 2C). Splenic iron concentration was comparable between mice of the two genotypes at birth (d0), and at 1 and 4 weeks of age, but lower in *fpn*$^{wt/C326Y}$ mice than in *fpn*$^{wt/wt}$ littermate controls at 12 weeks of age (Figure 2C), consistent with the hemochromatosis phenotype (10). Limited iron availability affects haemoglobin synthesis, and consistent with this, hemoglobin levels at birth (d0) were 16% lower in *fpn*$^{wt/C326Y}$ mice than in *fpn*$^{wt/wt}$ littermate controls (Figure 2D). By one week of age, hemoglobin levels were comparable between animals of the two genotypes (Figure 2D). Thus, ubiquitous loss of FPN responsiveness to hepcidin in fetal tissues results in reduced fetal liver iron stores and lower haemoglobin at birth.

Next, we sought to determine whether the reduction in fetal iron stores in *fpn*$^{wt/C326Y}$ animals could be attributed to changes in placental iron handling. However, we found that placental iron concentration, and the expression of iron-regulated transcripts *Fpn*, *Transferrin receptor 1* (TfR1) and *Hamp* were all comparable between placentae of *fpn*$^{wt/C326Y}$ mice and placentae of *fpn*$^{wt/wt}$ littermates (Figure 2E-F), suggesting that placental iron handling is not different between animals of the two genotypes. When we examined the STB, we found that FPN levels were not visibly different between placentae of *fpn*$^{wt/C326Y}$ mice and placentae of *fpn*$^{wt/wt}$ littermates (Figure 2G), indicating FPN at this site is not actively regulated by hepcidin. Consistent with this, we found that provision of an iron-loaded diet to *fpn*$^{wt/wt}$ mothers carrying *fpn*$^{wt/wt}$ litters failed to alter FPN in the placental STB (Figure 2H), despite concomitantly increasing the expression of hepcidin in the fetal and maternal livers and in the placenta itself (Figure 2I).Thus altered placental iron handling does not explain reduced liver iron stores seen in *fpn*$^{wt/C326Y}$ fetuses. Apart from the placenta, the other site of FPN expression is the fetal liver itself. Therefore, we examined whether FPN expression in the livers of *fpn*$^{wt/C326Y}$ fetuses was altered, and found that it was markedly increased compared to livers of *fpn*$^{wt/wt}$ littermates at e17.5 (figure 2J). Therefore, we hypothesised that this marked increase in FPN expression is the cause of decreased fetal liver iron stores seen in *fpn*$^{wt/C326Y}$ fetuses.

Liver-specific loss of FPN responsiveness to hepcidin reduces fetal liver iron stores.

To test the above hypothesis, we generated foetuses harbouring a liver-specific knock-in of the *fpn*C326Y allele, by mating *fpn*C326Y$^{fl/fl}$ mothers with *fpn*C326Y$^{fl/fl}$ fathers transgenic for Cre recombinase driven by the hepatocyte-specific albumin promoter (*fpn*C326Y$^{fl/fl}$, Alb.Cre+). We then harvested *fpn*C326Y$^{fl/fl}$,Alb.Cre+ fetuses and *fpn*C326Y$^{fl/fl}$ littermates at e17.5, and found that *fpn*C326Y$^{fl/fl}$,Alb.Cre+ fetuses had a 26% reduction in liver iron concentration relative to *fpn*C326Y$^{fl/fl}$ littermates (Figure 3A).This was confirmed histologically by DAB-enhanced Perls' iron stain (Figure 3B). *fpn*C326Y$^{fl/fl}$,Alb.Cre+ fetuses also had increased hepatocyte FPN expression compared with *fpn*C326Y$^{fl/fl}$ littermates at e17.5 (Figure 3C). These results confirm that FPN in fetal hepatocytes is subject to regulation by HAMP and that this regulation is important for the control of fetal liver iron stores.

Fetal liver hepcidin regulates fetal liver FPN and iron stores.

Next, we set out to identify the source of HAMP that regulates fetal liver FPN and consequently iron stores. The previous observations that fetal liver hamp mRNA increases 24 fold between e13.5 and e17.5 and correlates positively with fetal liver iron stores (Figues 1B, D) suggest that the fetal liver is the source of HAMP that regulates fetal liver FPN and iron concentration. To test this hypothesis, we deleted the *hamp* gene specifically in the fetal liver by mating *hamp*$^{fl/fl}$ mothers with *hamp*$^{fl/fl}$, Alb.Cre+ fathers. We found that at e17.5, hepcidin mRNA expression was reduced by 80% in the livers of *hamp*$^{fl/fl}$, Alb.Cre+ fetuses relative to *hamp*$^{fl/fl}$ littermates, confirming the activity of the Cre allele (Figure 4A). Loss of fetal liver HAMP resulted in a 47% decrease in fetal liver iron concentration compared with *hamp*$^{fl/fl}$ littermates (Figure 4B), and this was further confirmed histologically by DAB-enhanced Perls' iron stain (Figure 4C). In contrast, adult *hamp*$^{fl/fl}$, Alb.Cre+ mice had 19 fold increase in liver iron concentration compared with *hamp* $^{fl/fl}$ controls, consistent with the well-recognised role for hepatic HAMP in controlling systemic iron availability in the adult (Figure 4D). When we compared FPN expression in *hamp*$^{fl/fl}$ and *hamp*$^{fl/fl}$, Alb.Cre+ fetuses at e17.5, we found that FPN expression was markedly increased in livers of *hamp*$^{fl/fl}$, Alb.Cre+ fetuses (Figure 4E), but comparable between placentas of the two genotypes (Figure 4F).

The widely accepted consensus is that fetal liver HAMP regulates iron availability to the foetus by inhibiting placental FPN (11). This consensus is based on animal studies in which the iron homeostatic machinery is altered in the foetus, the mother and in the placenta itself (12,13,14). By using paternally inherited alleles, our studies eliminate the confounding effects of altered maternal iron status, and altered homeostatic iron control in the maternal side of the placenta. Furthermore, the additional use of paternally-inherited liver-specific Cre recombinase to disrupt the hepcidin/ferroportin axis in the fetal liver eliminates the confounding factors of altered homeostatic iron control in the fetal part of the placenta. One important finding from our studies is that fetal liver HAMP operates in a cell-autonomous manner to increase fetal liver iron concentration. The finding that fetal liver HAMP expression is significantly lower than that of maternal hepcidin is also consistent with an autocrine rather than endocrine mode of action. Liver iron endowment at birth supports neonatal growth, and indeed these stores are rapidly depleted in early life. Therefore, there is a need for a rapid build-up of iron levels in fetal hepatocytes during the third trimester. In most cells, rapid iron build up of intracellular iron levels would normally be prevented by the action of iron regulatory proteins IRPs, through in part, stabilisation of the *fpn* transcript (15). The cell autonomous blockade of FPN by fetal HAMP may well serve to allow this rapid build-up by counter-acting the action of IRPs. Another important finding from our studies is that FPN in the placenta is not subject to regulation by HAMP, suggesting that its role in this setting is permissive rather than regulatory. In the future, it would be important to establish, formally, the roles of placental FPN and HAMP, using genetic tools that can target the placenta specifically.

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Figure 1

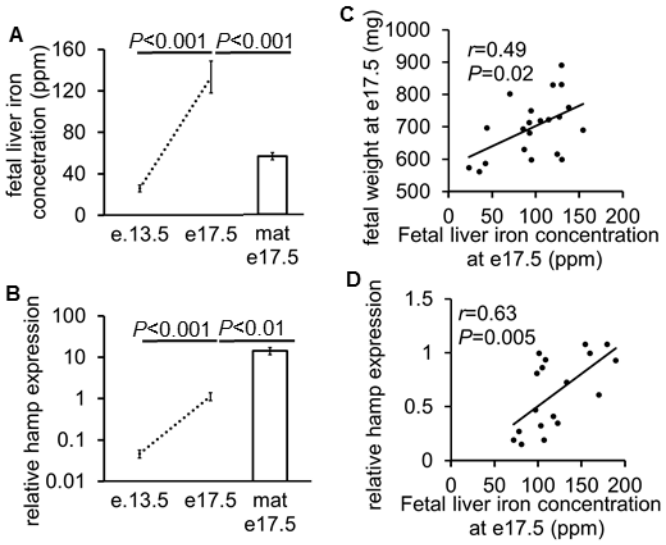

Figure 1: Fetal liver iron concentration and hepcidin expression increase in the third trimester. **A.** Total iron concentration in fetal livers harvested at e13.5 (n=27) and e17.5 (n=28) and in maternal livers at e17.5 (n=5). **B.** Relative hamp mRNA expression in fetal livers harvested at e13.5 (n=6) and e17.5 (n=6) and in maternal livers at e17.5 (n=6). **C.** Correlation between fetal weight and fetal liver iron concentration at e17.5 (n=21). **D.** Correlation between relative hamp expression and total iron concentration in fetal livers at e17.5 (n=18). Values are shown as mean±S.E.M. P values are calculated using Student's t test. R is Pearson's correlation coefficient.

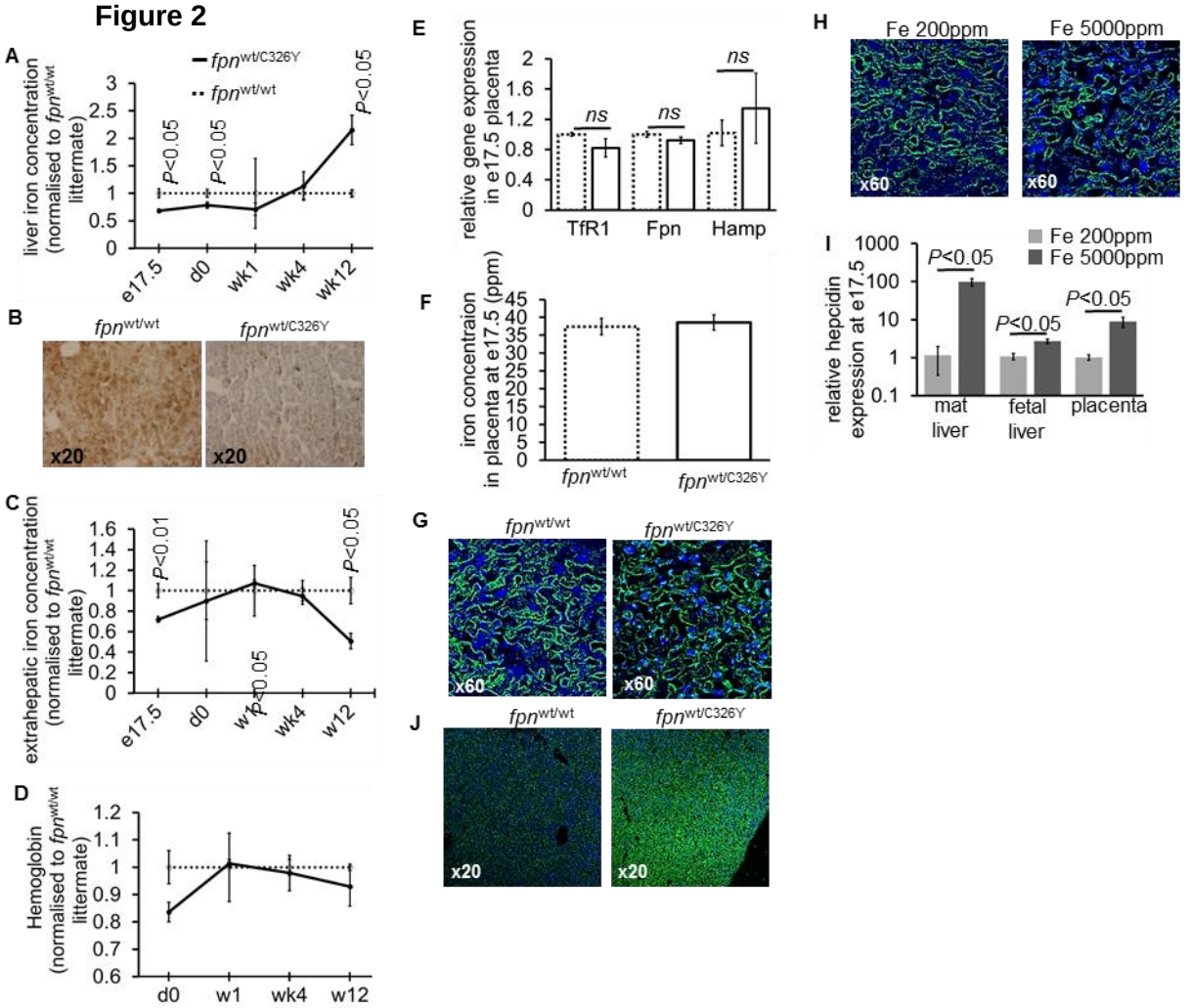

Figure 2: Ubiquitous loss of FPN responsiveness to hepcidin in the foetus reduces fetal liver iron stores and haemoglobin at birth. A. Liver iron concentration in *fpn*$^{wt/C326Y}$ animals relative to *fpn*$^{wt/wt}$ littermate controls at e17.5 (n=4, 8 respectively), day 0 (n=14,17), week1 (n=4, 6), week 4 (n=3, 3) and week 12 (n=3, 3) of age. *P* values are shown relative to *fpn*$^{wt/wt}$ littermate controls at the respective timepoint. **B.** Representative images of DAB-enhanced Perls' iron stain in the liver of *fpn*$^{wt/C326Y}$ animals and *fpn*$^{wt/wt}$ littermate controls at e17.5. **C.** Extra-hepatic iron concentration in *fpn*$^{wt/C326Y}$ animals relative to *fpn*$^{wt/wt}$ littermate controls at e17.5 (carcass-liver) (n=6, 11) and in the spleens of mice at day 0 (n=3,4), week 1(n=3, 4), week 4 (n=3 per group) and week 12 (n=3 per group) of age. *P* values are shown relative to *fpn*$^{wt/wt}$ littermate controls at the respective timepoint. **D**. Hemoglobin concentration in *fpn*$^{wt/C326Y}$ animals relative to *fpn*$^{wt/wt}$ littermate controls at day 0 (n=6, 11), week1 (n=3, 4), week 4 (n=3 per group) and week 12 (n=3 per group) of age. **E.** Representative images of FPN immunostaining in the STB from placentae of *fpn*$^{wt/C326Y}$ animals and *fpn*$^{wt/wt}$ littermate controls at e17.5. **F.** Iron concentration in placentae of *fpn*$^{wt/C326Y}$ fetuses (n=11) and *fpn*$^{wt/wt}$ littermates (n=6) at e17.5. **G.** Relative expression of TfR1, Fpn and Hamp mRNA transcripts in placentae of *fpn*$^{wt/C326Y}$ fetuses (n=5) and *fpn*$^{wt/wt}$ littermates (n=3) at e17.5. **H.** Representative images of FPN immunostaining in the STB of placentae harvested at e17.5 from *fpn*$^{wt/wt}$ mothers carrying *fpn*$^{wt/wt}$ litters and fed a normal iron diet (200ppm) or an iron-rich diet (5000ppm). **I.** Relative expression of Hamp mRNA transcript in maternal livers (n=3 per group), fetal livers (n=6 per group) and corresponding placenta (n=6 per group) harvested at e17.5 from mothers fed a normal iron diet (200ppm) or an iron-rich diet (5000ppm). **J.** Representative images of FPN immunostaining in the liver of *fpn*$^{wt/C326Y}$ animals and *fpn*$^{wt/wt}$ littermate controls at e17.5. Values are shown as mean±S.E.M. P values are calculated using Student's t test.

Figure 3

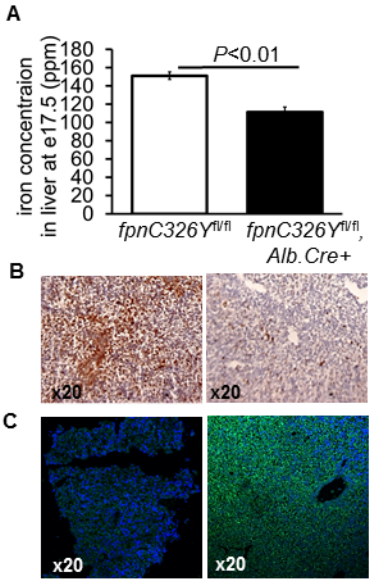

Figure 3: Liver-specific loss of FPN responsiveness to hepcidin reduces fetal liver iron stores. A. Total iron concentration in the liver of *fpnC326Y*$^{fl/fl}$,Alb.Cre+ animals and *fpnC326Y*$^{fl/fl}$ littermate controls at e17.5 (n=3 per group). **B.** Representative images of DAB-enhanced Perls' iron stain in the liver of *fpnC326Y*$^{fl/fl}$,Alb.Cre+ animals and *fpnC326Y*$^{fl/fl}$ littermate controls at e17.5. **C.** Representative images of FPN immunostaining in the liver of *fpnC326Y*$^{fl/fl}$,Alb.Cre+ animals and *fpnC326Y*$^{fl/fl}$ littermate controls at e17.5. Values are shown as mean±S.E.M. P values are calculated using Student's t test.

Figure 4

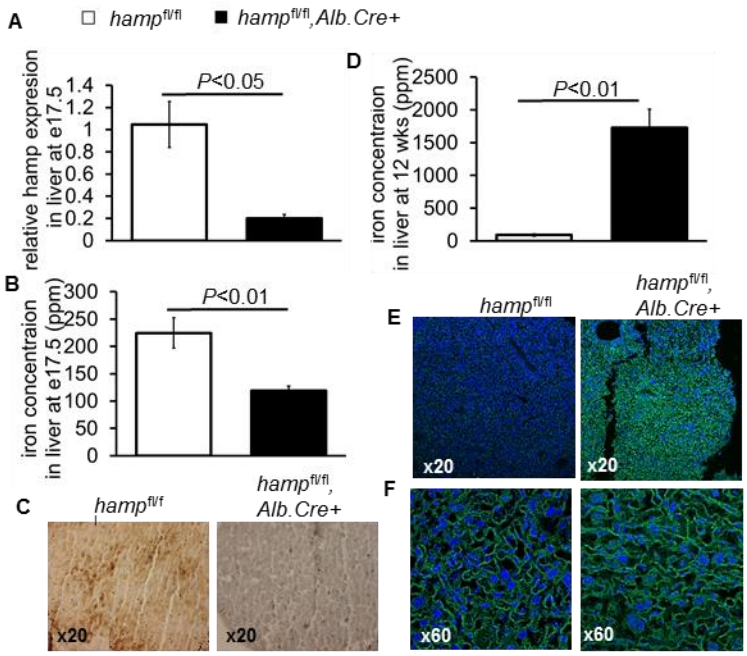

Figure 4: Liver-specific loss of hepcidin reduces fetal liver iron stores.
A. Relative hamp mRNA expression in liver of *hamp*$^{fl/fl}$,Alb.Cre+ animals (n=5) and *hamp*$^{fl/fl}$ littermate controls (n=4) at e17.5. **B**. Total iron concentration in the liver of *hamp*$^{fl/fl}$,Alb.Cre+ animals and *hamp*$^{fl/fl}$ littermate controls at e17.5 (n=8 per group). **C.** Representative images of DAB-enhanced Perls' iron stain in the liver of *hamp*$^{fl/fl}$,Alb.Cre+ animals and *hamp*$^{fl/fl}$ littermate controls at e17.5 . **D.** Total iron concentration in the liver of *hamp*$^{fl/fl}$,Alb.Cre+ animals and *hamp*$^{fl/fl}$ littermate controls at 12 weeks of age (n=3 per group). **E.** Representative images of FPN immunostaining in the liver of *hamp*$^{fl/fl}$,Alb.Cre+ animals and *hamp*$^{fl/fl}$ littermate controls at e17.5. **F.** Representative images of FPN immunostaining in the STB from placenta of *hamp*$^{fl/fl}$,Alb.Cre+ animals and *hamp*$^{fl/fl}$ littermate controls at e17.5.Values are shown as mean±S.E.M. P values are calculated using Student's t test.