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► **To cite this version:**

Celine Besson, Alexandra Willemetz, Chloe Latour, Lorenne Robert, Helene Coppin, et al.. NEW INSIGHTS INTO THE HEPATIC IRON PHENOTYPE OF BMP6 KNOCKOUT MICE. 2024. hal-04666022

HAL Id: hal-04666022

<https://hal.science/hal-04666022v1>

Preprint submitted on 5 Aug 2024

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TITLE PAGE

Full title: NEW INSIGHTS INTO THE HEPATIC IRON PHENOTYPE OF BMP6
KNOCKOUT MICE

Running title: Deciphering hepatic iron homeostasis and Fpn expression pattern
in *bmp6* knockout mice

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Key words: iron overload; hepcidin; ferroportin; liver; *Bmp6* Knockout, macrophages,
lipogranuloma, lipid raft, zonal distribution,

1 **ABSTRACT**

2 **Objective:** *Bmp6* knockout (KO) mice progressively accumulate a significant amount of
3 iron in their liver as they age due to a defect in hepcidin (*Hamp*) expression and an upregulation
4 of the iron exporter ferroportin (*Fpn*). In this study, we conducted a comprehensive investigation
5 of the hepatic iron overload phenotype, with specific emphasis on the cellular and subcellular
6 localization of *Fpn* in *Bmp6* KO mice.

7 **Materials and Methods:** Livers obtained from *Bmp6* knockout (KO) mice at different
8 developmental stages were utilized for the quantification of iron content, investigation of iron
9 distribution, histological analysis, histoimmunofluorescence assays performed on paraffin-
10 embedded sections, confocal microscopy examinations, subcellular membrane fractionation, and
11 western blot analysis.

12 **Results:** In *Bmp6* KO livers, iron overload increased with age and was not homogeneous,
13 with certain hepatic lobes and specific areas in liver sections showing more pronounced iron
14 accumulation. In young mice, iron accumulated mostly in the centrilobular zone where low *Fpn*
15 expression was observed. *Fpn* was strongly detected in periportal Kupffer cells and at the apical
16 membrane of periportal hepatocytes lining the sinusoidal capillaries. The zonal distribution of
17 iron tended to disappear with age in strongly iron-overloaded areas, with the appearance of large
18 cellular aggregates strongly positive for *Fpn*, iron, and ceroid/lipofuscin. At the subcellular level,
19 hepatic *Fpn* seemed to concentrate in specific cell surface compartments and was enriched in a
20 lipid raft fraction.

21 **Conclusions:** Unregulated expression of *Fpn* on the cell surface of periportal
22 macrophages and hepatocytes results in centrilobular iron overload within hepatocytes. In areas

1 of pronounced iron overload, Fpn expression is present in lipogranulomas, identified as
2 aggregations of macrophages accumulating hemosiderin and ceroid/lipofuscin pigments. These
3 lesions likely form due to the phagocytosis of sideronecrotic/ferroptotic hepatocytes by
4 macrophages. In contrast to the duodenal form of Fpn, both splenic and hepatic Fpn demonstrated
5 robust enrichment within lipid rafts. The observed variations in the subcellular localization of
6 Fpn could play a significant role in influencing the transporter's iron transport activity and/or its
7 regulation by hepcidin.

8 **INTRODUCTION**

9 *Bmp6* knockout (KO) mice progressively accumulate a significant amount of iron in their
10 liver as they age due to a defect in hepcidin (*Hamp*) expression and a substantial upregulation of
11 the iron exporter ferroportin (Fpn, also referred to as *Slc40a1*, solute carrier family 40 member
12 1) ^{1,2}. Iron deposits in the *Bmp6* KO liver have been shown to be more pronounced in the
13 centrilobular areas ². However, the liver, being a complex organ, possesses a distinctive anatomy
14 characterized by distinct lobes that exhibit morphological and functional variations ³. In fact,
15 limited information is available concerning the precise distribution of iron in histologic sections
16 throughout the hepatic lobes of these mice. As a result, we undertook a meticulous examination
17 of iron overload in each hepatic lobe of *Bmp6* KO mice and tracked its changes with advancing
18 age. Furthermore, we investigated the expression and localization of Fpn in the liver, in
19 conjunction with the distribution of iron deposits.

20 In *Bmp6* KO mice, Fpn was described to be overexpressed in enterocytes of the
21 duodenum, in splenic macrophages and cells within the liver ². A similar phenotype was
22 observed in *Hamp* KO mice ⁴. However, the hepatic cellular localization of Fpn was only

1 partially characterized in both *Hamp* and *Bmp6* KO mice, using immunohistochemistry
2 techniques ^{2,4,5}. Interestingly, in *Hamp* KO mice, Fpn was found to be more expressed in
3 periportal areas ⁵. Such zonal tissue distribution has yet to be explored in *Bmp6* KO. Previously,
4 in both *Bmp6* and *Hamp* KO liver, Fpn was detected in Kupffer cells and in the perisinusoidal
5 space (aka space of Disse) lining the apical membranes of hepatocytes ^{4,5,5}. While hepatocytes
6 were also demonstrated to express Fpn ⁵, the exact subcellular localization of the iron exporter
7 within these cells remains unknown. Furthermore, beyond parenchymal hepatocytes, other cell
8 types such as sinusoidal endothelial cells (SEC) and hepatic stellate cells (HSC) coexist within
9 the space of Disse. Notably, mRNA studies have indicated substantial Fpn expression in HSC
10 within the rat liver ⁶, suggesting a plausible presence of the iron transporter within these cells.

11 Consequently, we conducted a meticulous analysis of the expression and the localization
12 of Fpn at the tissue, cellular, and subcellular levels in *Bmp6* KO mice. This investigation involved
13 immunofluorescence studies, colocalization labeling, and subcellular fractionation techniques.

14 **MATERIALS AND METHODS**

15 **Animals**

16
17 Animal experiments were carried out following approved conditions and protocols by the
18 local ethics committee (UMS006 CEEA-122 Toulouse) and in accordance with the European
19 Union directive 2010/63/EU. The generation of *Bmp6*^{tm1Rob} mice (*Bmp6*^{-/-}: *Bmp6* KO) on an
20 outbred CD1 background was previously documented ⁷. Mice were genotyped using high-
21 resolution amplicon melting via the LightCycler 480 System (Roche Diagnostics). Wildtype and
22 *Bmp6* KO mice (only males) were euthanized at various ages, as specified, and tissues were
23 collected for the different analyses.

24 **Antibodies sources**

1 To detect Fpn, experiments were conducted using either with an antibody purchased from
2 Alphadiagnostic (MTP11-A; immunofluorescence) or with a homemade produced and affinity-
3 purified polyclonal rabbit antisera against Fpn (immunofluorescence and western blotting) ⁸.
4 Polyclonal rabbit antisera against Dmt1 (Slc11a2 solute carrier family 11 member 2, aka as
5 Nramp2) were generated and affinity purified as described previously ⁹. The specificity of
6 homemade Fpn and Dmt1 sera was validated by immunoblotting, immunofluorescence and
7 histochemistry ^{2,8,9}. The anti-human caveolin-1 (Cav1) antibody was purchased from Tebu-bio
8 (N-20). The rabbit anti-heme-oxygenase-1 (Hmox1) polyclonal antibody (SPA-896) was
9 purchased from Stressgene Biotechnology. The mouse monoclonal anti-vinculin antibody
10 (VINC) was purchased from Sigma–Aldrich (CLONE hVIN-1). The rat monoclonal anti-mouse
11 dipeptidyl peptidase-4 (Dpp4 also known as CD26) and the rat monoclonal anti-mouse F4/80
12 antibodies were purchased from Abcam (ab42899) and AbD serotec (CL:A3-1), respectively.
13 The goat polyclonal anti-Desmin antibody (Y-20) was purchased from Santa Cruz (sc-7559).
14 Secondary peroxidase antibodies against mouse, rat and rabbit were obtained from DAKO. The
15 secondary antibodies used for immunofluorescence, including Alexa Fluor® 488 goat anti-rabbit
16 Ig (GAR-alexa488), Alexa Fluor® 568 Rat anti-mouse Ig (RAM-alexa568), and Alexa Fluor®
17 563 goat anti-rat Ig (GARa-alexa563), were obtained from Molecular Probes.

18

19 **Tissue iron staining and quantitative iron measurement.**

20 Left lateral lobes (LLL), right lateral lobes (RLL), medium lobes (ML) and caudate lobes
21 (CL) of the liver from three mice per genotype were fixed in 4% buffered formalin and
22 subsequently embedded in paraffin. Following deparaffinization, tissue sections were subjected
23 to staining using Perls Prussian blue stain to visualize nonheme iron, followed by counterstaining
24 with nuclear fast red. The slides were observed under a light microscope and images were

1 captured or scanned using a Panoramic 250 Flash II (3DHISTECH). Subsequent analysis was
2 performed utilizing a Panoramic Viewer software. Due to the size of certain lobes, quantitative
3 assessments of nonheme iron in hepatic lobes were conducted by pooling each specific lobes
4 from three mice per genotype (pooled lobes: 3xLLL, 3xRLL, 3xML or 3xCL). Iron
5 measurements of the pooled lobes were performed following the methodology recommended by
6 Torrance and Bothwell ¹⁰. Briefly, 100 mg of tissues were homogenized in 150 µl water using a
7 FastPrep-24 Instrument (MP Biomedicals) for 60 seconds at 6 m/second. The homogenates were
8 then mixed with a solution containing HCl 30%/trichloroacetic acid 10% to achieve a final
9 volume of 1.5 ml. These samples were allowed to incubate overnight at 65°C. The iron content
10 was subsequently complexed with the bathophenanthroline sulfonate chromogen, and the
11 absorbance was measured at a wavelength of 535 nm.

12

13 **Histology and immunohistofluorescence analysis**

14 Following fixation and embedding in paraffin, each lobe (LLL, RLL, ML, and CL) was
15 prepared for tissue sectioning. Following blocking with BSA 1% and 10% heat inactivated goat
16 serum for 30 min at room temperature, deparaffinized liver sections were incubated with primary
17 antibodies for 1hr. After three washes with PBS containing 0.5% BSA, the sections were
18 incubated with Alexa conjugated secondary antibodies for an additional one hour at room
19 temperature. Once mounted, sections were visualized using an epifluorescence microscope
20 LEICA DM-IRM, a Zeiss confocal fluorescent microscope or a Panoramic 250 Flash II
21 (3DHISTECH) scanner. Image acquisition was performed using either ARCHIMED-PRO
22 (Microvision Instruments), Zeiss LSM Image Browser softwares or the Panoramic Viewer
23 software.

1 **Preparation of crude membrane and cytosolic protein extracts**

2 Cytosolic and membrane fractions were obtained following a previously established and
3 validated protocol ⁸. In brief, tissue samples were homogenized using an Ultraturax in lysis buffer
4 (10 mL/g of tissue in 0.25 mol/L sucrose/0.03 mol/L histidine, pH 7.2) which was supplemented
5 with protease inhibitors (PI), and phenylmethylsulfonyl fluoride (PMSF). The resulting lysates
6 were centrifuged at 6000 g for 15 min to remove nuclei and unbroken cells. The resulting
7 supernatants, termed post-nuclear supernatants (PNS) were subsequently subjected to
8 ultracentrifugation (Beckman) at 80,000 g for 30 minutes to separate the crude membrane
9 fractions from the cytosolic proteins. Supernatants corresponding to cytosolic extracts were
10 collected and the membrane pellets were resuspended in a lysis buffer (0.25 mol/L sucrose/0.03
11 mol/L histidine, pH 7.2), supplemented with PI's, and PMSF. The protein concentrations of both
12 the membrane and cytosolic fractions were determined via the Bradford assay (Bio-RAD) and
13 the fractions were stored at -80°C until utilization.

14 **Isolation of lipid rafts/detergent resistant membranes**

15 Lipid rafts from mouse tissues were isolated as detergent (Triton X-100) resistant
16 membranes [DRM, ¹¹]. Grounded frozen tissues (liver, spleen and duodenum) were used to
17 prepare crude membrane extracts as described before ⁸. Membrane pellets were resuspended in
18 lysis buffer (1ml/pellet from 100mg tissues; 150 mM NaCl, 25 mM MES, 5 mM EDTA, pH 6.5,
19 1% Triton X-100) supplemented with protease inhibitor cocktail (PI's; Sigma Aldrich) and
20 phenylmethanesulfonyl fluoride (PMSF; Sigma Aldrich). Samples were then homogenized using
21 a mini plastic potter and tissue lysates were incubated at 4°C for 60 min with gentle agitation.
22 Samples were then homogenized by 20 passages through a 25-gauge needle (5/8-inch) and
23 adjusted to a final concentration of 40% (w/v) Iodixanol (OptiPrep®, sigma Aldrich). The

1 mixture was then layered under a 20–40% discontinuous iodixanol gradient and centrifuged at
2 260,000 g for 15h at 4°C using an SW 41 Ti Rotor (Beckman Coulter). After spinning, twelve
3 fractions of 1 ml were collected from the top to the bottom of the gradient tube and the protein
4 concentration of each fraction was determined with a Bradford assay before immunoblotting.

5 **Immunoblotting**

6 Proteins samples (crude membrane extracts and DRM) were prepared in Laemmli buffer
7 (50mM Tris, 55mM SDS, 1M Glycerol, 570mM β -mercaptoethanol and bromophenol blue) and
8 incubated for 30 min at RT. Samples were separated in a 10% SDS-PAGE and transferred onto
9 PVDF membrane. To control protein loading and transfer, membranes were stained with 0,1%
10 Red Ponceau S. Membranes were then destained in 0,1% Tween-PBS (T-PBS) and blocked O/N
11 with 5% skim milk in T-PBS at 4°C or for 2h at RT. Each membrane was incubated with primary
12 antibodies diluted in blocking solution for 2h at RT or O/N at 4°C. After washing in T-PBS, blots
13 were incubated for 1h at RT with the secondary antibodies conjugated with horseradish
14 peroxidase (HRP) diluted in blocking solution. Membranes were washed in T-PBS and followed
15 by development with either Novex ECL (Invitrogen) or Immobilon Western (Millipore)
16 Chemiluminescent HRP substrate reagents.

17 **RNA studies**

18 Total RNA from the liver was extracted using Isol-RN lysis reagent (5 PRIME).
19 Complementary DNA was synthesized using MMLV-RT (Promega). The sequence of the
20 primers for the *Hamp*, *Fpn*, and the reference gene *Hprt* are listed in Supporting Table S1.
21 Quantitative polymerase chain reactions (PCRs) were prepared with LightCycler 480 DNA
22 SYBR Green I Master reaction mix and run on a LightCycler 480 System (both from Roche
23 Diagnostics). Cycle threshold difference (DCt) values were obtained by subtracting the reference

1 gene Ct value to the target gene Ct. GraphPad Prism (version 6) was used for statistical analyses
2 (GraphPad Software, La Jolla California USA) and DCt values in WT and *Bmp6* KO mice were
3 compared by Student's t-Tests.

4 5 **RESULTS**

6 7 **Ferroportin and *Hamp1* expression in *Bmp6* KO liver**

8 We assessed the hepatic expression of Fpn and Hamp mRNA through RT-qPCR in both
9 WT and *Bmp6* KO mice (Fig.1A). Consistent with prior findings ², the Hamp mRNA levels
10 exhibited a substantial decrease in *Bmp6* KO mice compared to their WT counterparts. On the
11 other hand, *Fpn* mRNA expression did not change significantly. In contrast to these observations,
12 the protein level of Fpn was notably elevated in *Bmp6* KO mice, as demonstrated by western blot
13 analysis (Fig.1B) and immunohistofluorescence of Fpn within liver sections (Fig.1C).

14 15 **Hepatic iron localisation in *Bmp6* KO liver**

16 Subsequently, we proceeded to track the iron accumulation pattern across distinct liver
17 lobes in both wildtype mice (WT) and *Bmp6* KO mice (Fig.1D). The left lateral lobe (LLL), right
18 lateral lobe (RLL), medium lobe (ML), and caudate lobe (CL) were subjected to Perls staining
19 and analyzed via microscopy. Among 12-week-old WT mice (CD1), there were no prominent
20 indicators of iron accumulation within their liver lobes. A meticulous examination revealed slight
21 iron loading in specific periportal regions (see Fig.2A). Importantly, no significant or marked
22 differences in iron loading were observable in WT mice from 4 weeks to 12 weeks (not shown).
23 In contrast to WT mice, *Bmp6* KO mice displayed a significant iron overload even at the age of
24 4 weeks (Fig.1D). In these mice, iron overload increased as they aged in each of the liver lobes

1 However, the distribution of iron deposits was not homogenous between hepatic lobes, with the
2 ML exhibiting the highest iron accumulation over time. Such observation was corroborated by
3 the quantitative measurements of iron in each lobe of *Bmp6* KO mice (Fig.S1). Interestingly, this
4 variation in iron accumulation among lobes was not accompanied by changes in *Hamp* and *Fpn*
5 mRNA expression (not shown). Importantly, within each lobe, except for the caudate lobe (CL),
6 certain specific areas [Fig.1D (*)] exhibited more pronounced iron accumulation compared to
7 other regions [Fig.1D (§)].

8 **Histological analysis of iron-rich hepatic regions in young and aged *Bmp6* KO mice**

9 Concurrently, we examined the pattern of *Fpn* expression alongside the distribution of
10 cellular iron overload in the livers of young *Bmp6* KO mice (Fig.2). As discussed earlier, under
11 high magnification, a subtle iron accumulation was discernible in specific hepatocytes within the
12 periportal area of WT (CD1) mice (Fig.2A, PP). In contrast, young *Bmp6* KO mice displayed
13 predominant iron accumulation in hepatocytes localized within the centrilobular zones (Fig.2A,
14 B, & C; CL), where a lower *Fpn* expression was observed (Fig.2B and C). Specifically, the
15 expression of *Fpn* was largely confined to cells localized in the periportal area (Fig.2C & D).
16 Histograms depicting the fluorescence of *Fpn* and F4/80 (a macrophage marker) (% of total
17 fluorescence dots, Fig.2F) from the images presented in panels D and E demonstrated that both
18 *Fpn* and F4/80 exhibited enrichment in the same regions corresponding to periportal zones
19 associated with lower iron levels.

20 In areas of pronounced iron overload [Fig.1D and Fig.S2 (*)] which became more
21 prominent with advancing age, particularly within the medium lobe (ML), the characteristic
22 zonal distribution of iron disappeared with the appearance of large aggregates strongly enriched
23 in iron (Fig.3A, arrows; Fig.S2 A and A', arrowheads). Such aggregates were shown to be

1 positive for both Fpn (Fig.3B and C; Fig.S2 B and B’; green fluorescence) and Soudan black
2 (Fig.3B and C; Fig.S2 C and C’, brown coloration). Furthermore, these aggregates exhibited
3 autofluorescence (Fig.3D, in red) and were also positive for F4/80 (Fig3E). In comparison to
4 other liver lobes, the CL seemed to exhibit a greater resistant to strong iron accumulation (Fig.1
5 & S1) and did not display cellular aggregates (Fig.S2D).

6 Collectively, these observations suggest that within liver regions characterized by
7 substantial iron accumulation, the emergence of iron-positive cellular aggregates primarily
8 consists of macrophages. These macrophages exhibit strong expression of Fpn and contain
9 ceroid/lipofuscin (identified by Soudan black staining, as further discussed).

10 **Cellular and subcellular localisation of ferroportin in *Bmp6* KO liver**

11 To precisely ascertain the subcellular localization of Fpn, we conducted a detailed confocal
12 analysis within liver sections from 8-week-old *Bmp6* KO mice, involving co-immunofluorescence
13 staining of the iron exporter Fpn alongside markers such as F4/80 (macrophage marker), CD26
14 (also known as dipeptidyl peptidase-4, Dpp-4, expressed both in the apical membrane of
15 hepatocytes and in hepatocyte plasma membrane forming the biliary canaliculi), and desmin (a
16 stellate cell marker) (Fig. 4). Fpn was strongly detected in periportal Kupffer cells (Fig.4A and A’,
17 arrowhead) as well as at the apical membrane of hepatocytes lining the sinusoidal capillaries
18 (arrows in Fig.4A, A’ and 4B, B’). However, Fpn was not detected in the Dpp4 positive membrane
19 of the biliary canaliculi (arrowhead in Fig.4B, B’). The absence of colocalization between Fpn and
20 desmin (Fig.3C, C’) suggested that stellate cells did not express Fpn. In addition, there was no
21 detection of Fpn in endothelial CD34-positive cells (not shown).

22 Interestingly, the hepatic Fpn detected in *Bmp6* KO mice appeared to concentrate within
23 specific membrane compartments at the cell surface of both hepatocytes (arrows) and Kupffer cells

1 (arrowheads in Fig.5A). Notably, Dpp4, which colocalised with Fpn in apical membrane of
2 hepatocytes (Fig.5B), is known to be enriched in lipid rafts^{12,13}. Previous observations in cultured
3 macrophages have clearly demonstrated that Fpn is mostly present in lipid rafts¹¹. Therefore, we
4 decided to investigate the subcellular localisation of Fpn within lipid rafts in *Bmp6* KO tissues,
5 namely the liver, spleen, and duodenum, using the detergent-rich membrane (DRM) analysis (see
6 M&M and Fig.S4). As previously described², Fpn was also strongly increased in spleen and
7 duodenum of *Bmp6* KO mice (Fig.S3). Co-staining of F4/80 and Fpn in the spleen indicated that
8 Fpn was in macrophages of the red pulp (Fig.5C). In the duodenum, Fpn was strongly expressed at
9 the basolateral membrane of enterocytes, in sharp contrast to the brush border localization of the
10 iron transporter Dmt1 (Fig.5C). Using iodixanol density gradient centrifugation and western blot
11 analysis (Fig.S4), we compared the subcellular fractionation of hepatic Fpn with those obtained
12 from splenic and duodenal Fpn of 8 week-old *Bmp6* KO mice (Fig.5D and E). Similar volumes
13 (Fig.5D) or similar amounts of protein (Fig.5E) from pooled fractions F1, F2, F3 and F4 (see M&M
14 and Fig.S4) were analyzed by western blotting. Interestingly, hepatic Fpn as well as splenic Fpn
15 were strongly enriched in the fraction containing the lipid raft marker caveolin 1 (Cav1). As a
16 control and in contrast to Fpn, Hmox1 (Heme oxygenase 1) was mainly detected in Non Detergent
17 resistant membrane (F4). On the other hand, duodenal Fpn as well as Dmt1 were mainly detected
18 in non-raft (Cav1 negative) containing fractions F3 and F4, respectively.

19 **DISCUSSION**

20
21 In this investigation, we examined the hepatic iron overload phenotype of *Bmp6* KO mice
22 while concurrently exploring the expression pattern of the iron exporter Fpn at tissue, cellular,
23 and subcellular levels. This approach has provided novel insights into the intricacies of liver iron
24 homeostasis.

1 Initially, we identified some heterogeneity in the iron overload phenotype across distinct
2 hepatic lobes. These findings underscore the necessity of ensuring uniformity in iron dosage or
3 Perls staining during investigations of liver iron overload in *Bmp6* KO models. It is advisable to
4 maintain consistency either by focusing solely on the same lobe or by utilizing homogenates of
5 the entire liver for normalization purposes. Furthermore, we observed no discernible correlation
6 between iron levels in each lobe at different ages and the mRNA expression levels of *Hamp* or
7 *Fpn* which remains constant between liver lobes (not shown). The variations in iron
8 accumulation between lobes in *Bmp6* KO mice can likely be attributed to a strong iron overload
9 and anatomical considerations pertaining to the distribution of iron-enriched blood from the
10 intestine. This blood enters the branches of the portal veins and hepatic artery, thereby likely
11 contributing to the observed interlobe disparities.

12 Importantly, in 24-weeks aged *Hfe* KO mice (a model of hemochromatosis), no such
13 difference in iron loading between lobes was observed ¹⁴. The varying levels of iron
14 accumulation observed between *Bmp6* and *Hfe* KO mouse models may reflect differences in the
15 severity or mechanisms of iron overload in response to genetic mutations. Indeed, the specificity
16 of each iron overload model should be carefully considered when designing and interpreting
17 experimental investigations.

18 Within *Bmp6* KO mice, the phenomenon of iron overload also displayed heterogeneity
19 within the same hepatic lobe. In young mice and in specific areas of the liver section within each
20 lobe (except for CL, harboring the lowest iron content), two distinct iron overload patterns
21 emerged: areas of low iron overload (LI) exhibiting concentrated iron levels in the centrilobular
22 zone, and high iron overload regions (HI) marked by discrete iron aggregates and lacking a
23 defined zonation. Once more, this underscores the importance of exercising caution when

1 conducting liver lobe analyses in this model and emphasizes the need to avoid sampling a portion
2 of the lobe but rather examining the lobe in its entirety. As these mice aged, iron overload
3 increased across all hepatic lobes, resulting in a loss of iron zonation. Critically, the liver iron
4 zonation in LI corresponded to an opposing zonation of Fpn expression. Specifically, in LI, Fpn
5 protein is predominantly detected in periportal hepatocytes and Kupffer cells, indicating distinct
6 regulatory mechanisms governing Fpn expression in periportal versus centrilobular hepatocytes.
7 Parallel observations were made in *Hamp* KO mice ⁵. This pattern of Fpn expression was
8 similarly noted in rats at the mRNA level via *in situ* hybridization ⁶, suggesting contributions of
9 transcriptional and post-transcriptional (mRNA stability) regulations to periportal zonal Fpn
10 expression. However, the possibility of other regulatory mechanisms cannot be excluded.
11 Remarkably, *Hamp* mRNA expression was localized within the same periportal zone where Fpn
12 is expressed ¹⁵. Regulation of *Hamp1* expression in response to body iron levels in the liver
13 involves BMP ligand binding to their receptors, particularly BMP6, which triggers SMAD
14 phosphorylation and gene expression regulation ¹⁶. Hemojuvelin (Hjv), a BMP co-receptor,
15 enhances the SMAD phosphorylation pathway by binding to BMP ligands and receptors. *Hamp*
16 expression is significantly reduced in the absence of Hjv ¹⁷. Notably, Hjv was found to be
17 selectively expressed by periportal hepatocytes in mouse livers ¹⁷. Collectively, these findings
18 suggest that the localized expression and function of *Hamp* in the periportal zone contribute to
19 sustaining Fpn expression in this area (Fig.S5). Worth noting, our observations revealed that
20 periportal zones contain a higher abundance of hepatic macrophages compared to centrilobular
21 regions.

22 The study conducted by Latour et al. ¹⁸ presents a comprehensive analysis using a range
23 of mouse models, including single and double knockouts of iron regulators. These models exhibit

1 diverse iron overload patterns—periportal versus centrilobular—coupled with varying
2 reductions in hepcidin expression. Upon examining the collective dataset, a clear correlation
3 emerges between the detected level of hepcidin expression (mRNA) in the mouse models and
4 the spatial distribution of Perls staining (indicative of iron levels) within the liver (Fig.S5). In
5 instances where mice displayed extremely low hepcidin levels [Group D: *Bmp6* KO (male),
6 *Hamp* KO, *Hjv* KO (male), *Bmp6* KO / *B2m* KO, *Bmp6* KO / *Tfr2* KO, and *Bmp6* KO / *B2m* KO
7 / *Trf2* KO], iron deposition predominantly occurred in the centrilobular regions, aligning with
8 the observations in this study (particularly evident in young *Bmp6* KO mice). In these mice, the
9 notably diminished hepcidin levels exert dual positive impacts on both systemic (macrophages,
10 duodenum) and local (hepatic) *Fpn* expression, consequently contributing to the redistribution
11 of iron from periportal hepatocytes to hepatocytes encircling the central veins (Fig.6C and
12 Fig.S5).

13 Conversely, in *Bmp6* KO females (Fig.S5; Group C), who exhibit slightly elevated
14 hepcidin levels compared to *Bmp6* KO males, iron distribution predominantly occurs within the
15 periportal regions (Fig 6B) (18). Similarly, zonal patterns of injury, characterized by periportal
16 hemosiderin deposition, are observed in *Hfe* KO mice and individuals with hemochromatosis
17 ^{19,20}. Despite expressing hepcidin, albeit at inappropriately low levels relative to their body iron
18 stores ^{21–24}, both *Hfe* KO mice and hemochromatosis patients experience such periportal iron
19 overload. This phenomenon extends to mice with low or inadequate hepatic *Hamp* expression,
20 encompassing various genotypes [*Bmp6* KO (female), *Hfe* KO, *B2m* KO, *TFR2* KO, *Hamp* +/-,
21 *Hjv* KO (females), *Neogenin* KO], and promote intestinal iron absorption and heme iron
22 recycling from the spleen while preserving a local regulation of hepatic *Fpn* (Fig.6B; Fig.S5). In
23 such scenarios, a continuous influx of iron-rich blood from the intestines via the portal vein,

1 coupled with minimal fluctuations in periportal Fpn repression, facilitates iron accumulation
2 within hepatocytes surrounding the periportal veins. Moreover, we demonstrate that CD1 WT
3 mice exhibit modest iron accumulation in periportal hepatocytes, a phenomenon not observed in
4 other wildtype strains like C57BL/6 (no discernible iron accumulation, data not shown). Once
5 again, CD1 mice were shown to express less Hamp than other WT mice (personal
6 communication). Consequently, contingent on the threshold of hepcidin levels, iron
7 accumulation occurs either within the periportal or centrilobular zones (Fig 6 & Fig.S5). As time
8 progresses, this distinctive zonal distribution diminishes, resulting in a more diffuse distribution
9 of iron across the entire liver (Fig.6D).

10 The liver, an intricate organ, encompasses a diverse array of metabolic pathways. To
11 ensure its optimal functionality, metabolic zonation is imperative ²⁵, and for this purpose, the
12 liver parenchyma exhibits notable heterogeneity and functional adaptability. This demand for
13 spatial organization appears to extend to hepatic iron metabolism. The redistribution of iron from
14 periportal to centrilobular zones, facilitated by the gradient of Fpn expression, could constitute a
15 protective mechanism aimed at limiting hepatic injury. Indeed, the centrilobular area is
16 acknowledged as a detoxification zone characterized by reduced oxygenation, potentially
17 rendering centrilobular hepatocytes less prone to iron and hemosiderin accumulation.
18 Pathological iron accumulation within tissues exacerbates the generation of reactive oxygen
19 species (ROS) and triggers detrimental effects primarily associated with oxidative stress.
20 Compared to centrilobular cells, periportal hepatocytes contain a substantial number of
21 peroxisomes and mitochondria, significant sources of reactive oxygen species (ROS) ²⁵. In order
22 to mitigate the impact of ROS, these cells also exhibit elevated levels of superoxide dismutase
23 2, an enzyme responsible for generating H₂O₂, a potent catalyst for dangerous free radicals

1 formed in conjunction with ferrous iron through the Fenton reaction. Consequently, the
2 accumulation of iron within cells situated in regions characterized by lower oxygenation, such
3 as the centrilobular area, might potentially confer reduced harm to the overall organism.

4 Interestingly, as time progressed, the prominence of hepatic lesions, characterized as
5 lipogranulomas, exhibited a more marked manifestation in periportal zones in *Bmp6* KO mice.
6 These lesions were discerned as substantial clusters of macrophages displaying elevated levels
7 of Fpn and containing significant quantities of both hemosiderin and ceroid/lipofuscin. Ceroid,
8 akin to lipofuscin, constitutes a yellow-brown pigment originating from an insoluble polymer of
9 oxidized lipids and proteins. It is recognized as a pathological pigment formed through the
10 phagocytosis of lipids from deceased hepatocytes (exogenous lipids/heterophagy), subsequently
11 accumulating in hypertrophied macrophages following liver injury. Interestingly, in old mice
12 exhibiting increased erythrophagocytosis and iron overload in the spleen, similar large iron-rich
13 aggregates were observed through tissue staining inside some morphologically damaged
14 macrophages²⁶. In addition to these intracellular aggregates, some large amorphous extracellular
15 aggregates were also detected, likely emerging from damaged red pulp macrophages.

16 As previously discussed, elevated iron deposition in periportal hepatocytes could trigger
17 a potent oxidative stress reaction culminating in cell death. In certain murine models of
18 hemochromatosis, ferroptosis — a specific form of cell death instigated by iron-dependent lipid
19 peroxidation — has been observed in hepatocytes²⁷. Intriguingly, periportal macrophages have
20 been found to be more prone to phagocytosis in comparison to their centrilobular counterparts
21²⁸. In addition, we observed the presence of more macrophages in periportal zone than in the
22 centrolobular one. Moreover, it is plausible that the composition of lipogranulomas in *Bmp6* KO
23 involves distinct macrophage populations. In healthy liver, Kupffer cells do not solely constitute

1 the recognized tissue macrophages, and during liver diseases, additional macrophage subsets are
2 recruited, such as the lipid-associated macrophages (LAM) ²⁹. Consequently, it can be inferred
3 that lipogranulomas in *Bmp6* KO likely encompass resident and/or recruited macrophages laden
4 with iron and lipids, acquired through the phagocytosis of sideronecrotic and ferroptotic
5 hepatocytes.

6 The iron exporter Fpn was found to be strongly present at the membranes of Kupffer cells
7 and on the surface of hepatocytes. However, Fpn was not detected on the membrane that lines
8 the biliary duct, suggesting that the transporter does not directly transport iron for biliary
9 secretion. Despite earlier evidence of Fpn mRNA expression in hepatic stellate cells (HSC) ⁶, we
10 did not observe Fpn protein expression in these cells.

11 At the subcellular level, in both Kupffer cells and hepatocytes, the iron exporter was
12 present in discrete domains in the plasma membrane. In hepatocytes, Fpn was shown to strongly
13 colocalize with Dpp4 (dipeptidyl peptidase-4 aka CD26), a cell surface glycoprotein.
14 Interestingly, Dpp4 interacts with Caveolin-1 which facilitates its recruitment into lipids rafts in
15 T cells ¹². Furthermore, in the hematopoietic environment, Dpp4 activity is primarily located
16 with lipid rafts ¹³. Lipid rafts correspond to specific molecular platforms, floating at the cell
17 surface and involved in numerous biological functions such as cell signaling ³⁰⁻³². In cultured
18 macrophages, Fpn has been found to be highly concentrated within lipid rafts, a crucial
19 localization for the regulation of the iron exporter by Hamp ¹¹. For the first time, our study
20 demonstrates that both hepatic cells (hepatocytes and Kupffer cells) as well as splenic
21 macrophages exhibit Fpn enrichment within lipid rafts in an *in vivo* context. These findings
22 suggest that Fpn resides within specific subdomains of the plasma membrane in both hepatocytes
23 and tissue macrophages (Kupffer cells and splenic macrophages). In contrast, the form of Fpn

1 found in the duodenum, expressed at the basolateral membrane of enterocytes, is predominantly
2 detected in a non-raft fraction. These observations emphasize the existence of differences
3 between duodenal, hepatic, and macrophage Fpn, a phenomenon previously noted in relation to
4 post-transcriptional modifications⁸. It is plausible to speculate that these observed differences,
5 including lipid raft localization and glycosylation, could play a significant role in the iron
6 exporter's transport activity and/or its regulation. Notably, the degradation of Fpn induced by
7 Hamp appears to be more efficient in macrophages compared to enterocytes, which are more
8 resilient to Hamp's effects³³⁻³⁵. Intriguingly, during the suckling period, enterocyte ferroportin
9 seems less responsive to the degradative impact of circulating hepcidin³⁶. This finding correlates
10 with a change in the electrophoretic mobility of Fpn between suckling and adult mice. In suckling
11 mice, the ferroportin protein is smaller, which could be indicative of an alternatively spliced form
12 of the transporter or different post-translational modifications, such as glycosylation. Subsequent
13 investigations are necessary to elucidate the biological implications underlying the disparities
14 observed among the various cellular forms of Fpn.

15 **CONCLUSION**

16 In summary, our exploration of hepatic iron regulation in *Bmp6* KO has unveiled intricate
17 patterns of ferroportin (Fpn) expression and its impact on zonal iron distribution in the liver. It
18 reveals the detailed aspects of Fpn localization, from its specific presence in Kupffer cells and
19 hepatocyte apical membranes within periportal zones to its association with macrophages in iron-
20 rich regions and hepatic lesions. Finally, our study underscores the importance of understanding
21 Fpn's subcellular localization for comprehending its transport activity and its modulation by
22 hepcidin.

1 REFERENCES

- 2 1. Donovan, A. *et al.* Positional cloning of zebrafish ferroportin1 identifies a conserved
3 vertebrate iron exporter. *Nature* **403**, 776–781 (2000).
- 4 2. Meynard, D. *et al.* Lack of the bone morphogenetic protein BMP6 induces massive iron
5 overload. *Nat. Genet.* **41**, 478–481 (2009).
- 6 3. Malarkey, D. E., Johnson, K., Ryan, L., Boorman, G. & Maronpot, R. R. New insights into
7 functional aspects of liver morphology. *Toxicol. Pathol.* **33**, 27–34 (2005).
- 8 4. Viatte, L. *et al.* Deregulation of proteins involved in iron metabolism in hepcidin-deficient
9 mice. *Blood* **105**, 4861–4864 (2005).
- 10 5. Ramey, G. *et al.* Hepcidin targets ferroportin for degradation in hepatocytes. *Haematologica*
11 **95**, 501–504 (2010).
- 12 6. Zhang, A.-S., Xiong, S., Tsukamoto, H. & Enns, C. A. Localization of iron metabolism-
13 related mRNAs in rat liver indicate that HFE is expressed predominantly in hepatocytes.
14 *Blood* **103**, 1509–1514 (2004).
- 15 7. Solloway, M. J. *et al.* Mice lacking Bmp6 function. *Dev. Genet.* **22**, 321–339 (1998).
- 16 8. Canonne-Hergaux, F., Donovan, A., Delaby, C., Wang, H. & Gros, P. Comparative studies
17 of duodenal and macrophage ferroportin proteins. *Am. J. Physiol. Gastrointest. Liver Physiol.*
18 **290**, G156-163 (2006).
- 19 9. Canonne-Hergaux, F., Gruenheid, S., Ponka, P. & Gros, P. Cellular and subcellular
20 localization of the Nramp2 iron transporter in the intestinal brush border and regulation by
21 dietary iron. *Blood* **93**, 4406–4417 (1999).
- 22 10. Torrance, J. D. & Bothwell, T. H. A simple technique for measuring storage iron
23 concentrations in formalinised liver samples. *S. Afr. J. Med. Sci.* **33**, 9–11 (1968).
- 24 11. Auriac, A., Willemetz, A. & Canonne-Hergaux, F. Lipid raft-dependent endocytosis: a new
25 route for hepcidin-mediated regulation of ferroportin in macrophages. *Haematologica* **95**,
26 1269–1277 (2010).
- 27 12. Ohnuma, K. *et al.* Caveolin-1 triggers T-cell activation via CD26 in association with
28 CARMA1. *J. Biol. Chem.* **282**, 10117–10131 (2007).
- 29 13. Pereira, D. A., Gomes, L., El-Cheikh, M. C. & Borojevic, R. Dipeptidyl peptidase IV (CD26)
30 activity in the hematopoietic system: differences between the membrane-anchored and the
31 released enzyme activity. *Braz. J. Med. Biol. Res. Rev. Bras. Pesqui. Medicas E Biol.* **36**, 567–
32 578 (2003).
- 33 14. Colucci, S., Carvalho Oliveira, T., Muckenthaler, M. U. & Marques, O. Iron homeostasis in
34 mice: does liver lobe matter? *Am. J. Physiol. Gastrointest. Liver Physiol.* **325**, G453–G457
35 (2023).

- 1 15. Lee, D.-H. *et al.* Neogenin inhibits HJV secretion and regulates BMP-induced hepcidin
2 expression and iron homeostasis. *Blood* **115**, 3136–3145 (2010).
- 3 16. Roth, M.-P., Meynard, D. & Coppin, H. Regulators of hepcidin expression. *Vitam. Horm.*
4 **110**, 101–129 (2019).
- 5 17. Niederkofler, V., Salie, R. & Arber, S. Hemojuvelin is essential for dietary iron sensing, and
6 its mutation leads to severe iron overload. *J. Clin. Invest.* **115**, 2180–2186 (2005).
- 7 18. Latour, C. *et al.* Differing impact of the deletion of hemochromatosis-associated molecules
8 HFE and transferrin receptor-2 on the iron phenotype of mice lacking bone morphogenetic
9 protein 6 or hemojuvelin. *Hepatology* **63**, 126–137 (2016).
- 10 19. Zhou, X. Y. *et al.* HFE gene knockout produces mouse model of hereditary
11 hemochromatosis. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 2492–2497 (1998).
- 12 20. Iancu, T. C., Deugnier, Y., Halliday, J. W., Powell, L. W. & Brissot, P. Ultrastructural
13 sequences during liver iron overload in genetic hemochromatosis. *J. Hepatology* **27**, 628–638
14 (1997).
- 15 21. Ahmad, K. A. *et al.* Decreased liver hepcidin expression in the Hfe knockout mouse. *Blood*
16 *Cells. Mol. Dis.* **29**, 361–366 (2002).
- 17 22. Muckenthaler, M. *et al.* Regulatory defects in liver and intestine implicate abnormal hepcidin
18 and Cybrd1 expression in mouse hemochromatosis. *Nat. Genet.* **34**, 102–107 (2003).
- 19 23. Nicolas, G. *et al.* Constitutive hepcidin expression prevents iron overload in a mouse model
20 of hemochromatosis. *Nat. Genet.* **34**, 97–101 (2003).
- 21 24. Piperno, A. *et al.* Blunted hepcidin response to oral iron challenge in HFE-related
22 hemochromatosis. *Blood* **110**, 4096–4100 (2007).
- 23 25. Gebhardt, R. & Matz-Soja, M. Liver zonation: Novel aspects of its regulation and its impact
24 on homeostasis. *World J. Gastroenterol.* **20**, 8491–8504 (2014).
- 25 26. Slusarczyk, P. *et al.* Impaired iron recycling from erythrocytes is an early hallmark of aging.
26 *eLife* **12**, e79196 (2023).
- 27 27. Chen, J., Li, X., Ge, C., Min, J. & Wang, F. The multifaceted role of ferroptosis in liver
28 disease. *Cell Death Differ.* **29**, 467–480 (2022).
- 29 28. Sleyster, E. C. & Knook, D. L. Relation between localization and function of rat liver Kupffer
30 cells. *Lab. Investig. J. Tech. Methods Pathol.* **47**, 484–490 (1982).
- 31 29. Guilliams, M. & Scott, C. L. Liver macrophages in health and disease. *Immunity* **55**, 1515–
32 1529 (2022).
- 33 30. Lajoie, P. & Nabi, I. R. Lipid rafts, caveolae, and their endocytosis. *Int. Rev. Cell Mol. Biol.*
34 **282**, 135–163 (2010).

- 1 31. Le Roy, C. & Wrana, J. L. Clathrin- and non-clathrin-mediated endocytic regulation of cell
2 signalling. *Nat. Rev. Mol. Cell Biol.* **6**, 112–126 (2005).
- 3 32. Lingwood, D. & Simons, K. Lipid rafts as a membrane-organizing principle. *Science* **327**,
4 46–50 (2010).
- 5 33. Brasse-Lagnel, C. *et al.* Intestinal DMT1 cotransporter is down-regulated by hepcidin via
6 proteasome internalization and degradation. *Gastroenterology* **140**, 1261-1271.e1 (2011).
- 7 34. Chaston, T. *et al.* Evidence for differential effects of hepcidin in macrophages and intestinal
8 epithelial cells. *Gut* **57**, 374–382 (2008).
- 9 35. Chung, B., Chaston, T., Marks, J., Srai, S. K. & Sharp, P. A. Hepcidin decreases iron
10 transporter expression in vivo in mouse duodenum and spleen and in vitro in THP-1
11 macrophages and intestinal Caco-2 cells. *J. Nutr.* **139**, 1457–1462 (2009).
- 12 36. Frazer, D. M. *et al.* Ferroportin Is Essential for Iron Absorption During Suckling, But Is
13 Hyporesponsive to the Regulatory Hormone Hepcidin. *Cell. Mol. Gastroenterol. Hepatol.* **3**,
14 410–421 (2017).

15

16

17 **AUTHORSHIP**

18 **Contribution:** C.B., A.W., and L.R. designed protocols, conducted experiments, and analyzed data
19 (CB: Perls staining, RT-PCR & immunofluorescence, A.W: western blot and immunofluorescence;
20 L.R.: lipid raft studies). C.L. carried out experiments (mice genotyping and RT-PCR) and analyzed
21 data. H.C. and M-P.R. participated in data discussions and manuscript review. F.C-H. formulated
22 protocols, conducted experiments (western blot and IF), critically evaluated the data, and wrote the
23 paper. The correction and improvement of the English language in this article were assisted by
24 ChatGPT, a language model developed by OpenAI.

25 **Conflict-of-interest statement:** The authors declare no competing financial interests.

26

27 **ACKNOWLEDGEMENTS:**

28 We wish to acknowledge Ophélie Gourbeyre and Benjamin BILLORE for technical assistance.

29

1 **FUNDING:** This work was supported by INSERM and the "Agence Nationale de la Recherche"
2 in France, awarded to F.C-H (ANR ANR-08-GENO-014-01 / GENOPAT).

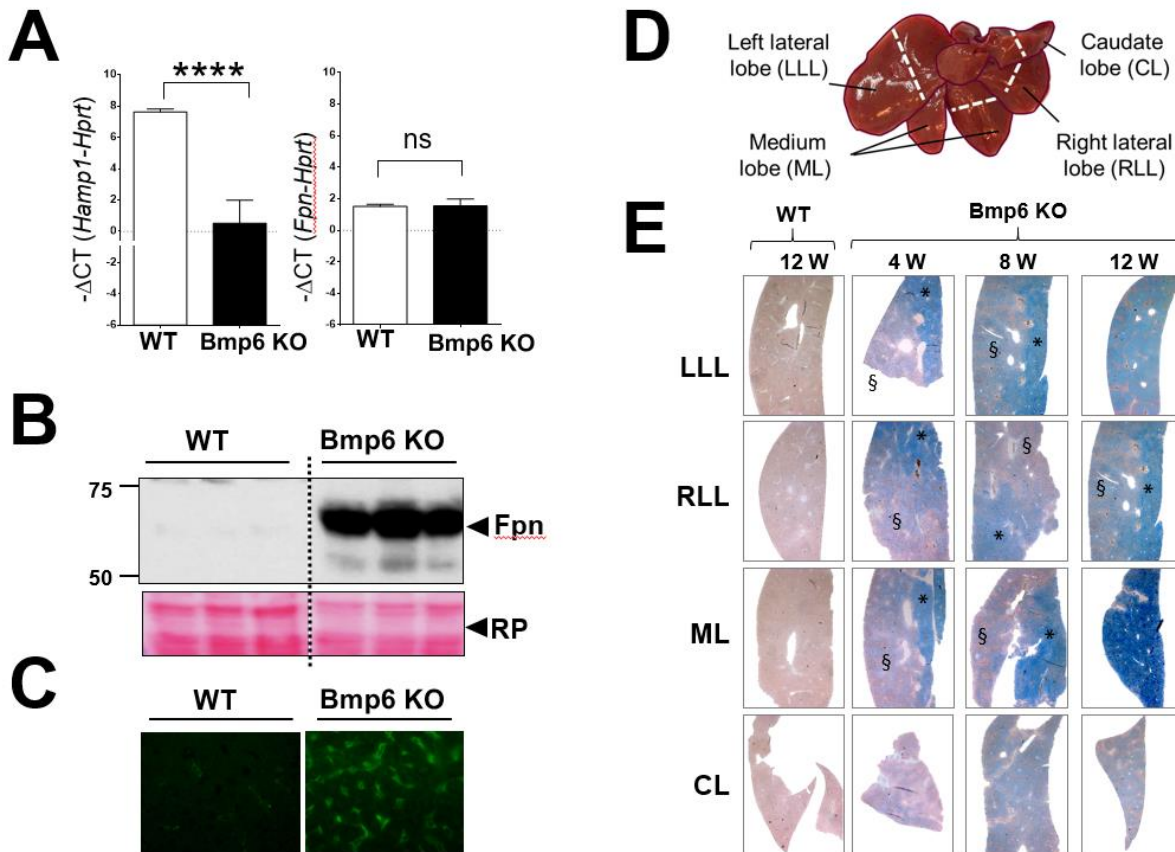
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1 FIGURES & LEGENDS

2



3

4 **Fig.1: Expression of ferroportin (Fpn) and hepcidin 1 (Hamp1), and iron overload**

5 **in *Bmp6* KO liver.** (A) Messenger RNA expression levels of hepcidin (Hamp)

6 (Fpn) in total liver samples from wildtype (WT) or *Bmp6* KO mice (n=6). The relative gene

7 expression is presented as $-\Delta\text{CT}$ (CT gene of interest - CT Hprt). Statistical significance:

8 $P < 0.0001$ (****), ns: not significant. (B) Total membrane proteins were extracted from WT or

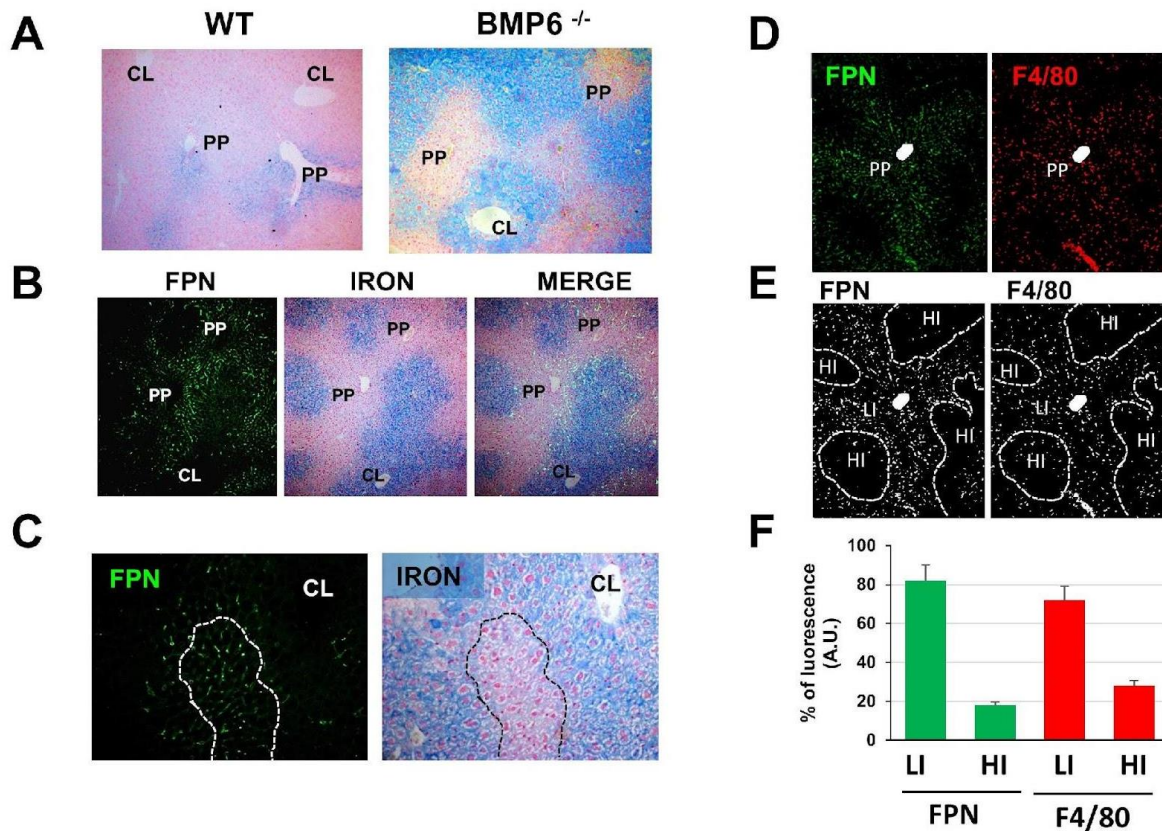
9 *Bmp6* KO livers (three mice per genotype) and subjected to Western blot analysis. Protein

10 visualization was assessed both quantitatively and qualitatively using Ponceau S staining (PS).

11 Molecular weight markers and their corresponding sizes in kilodaltons (kDa) are indicated. (C)

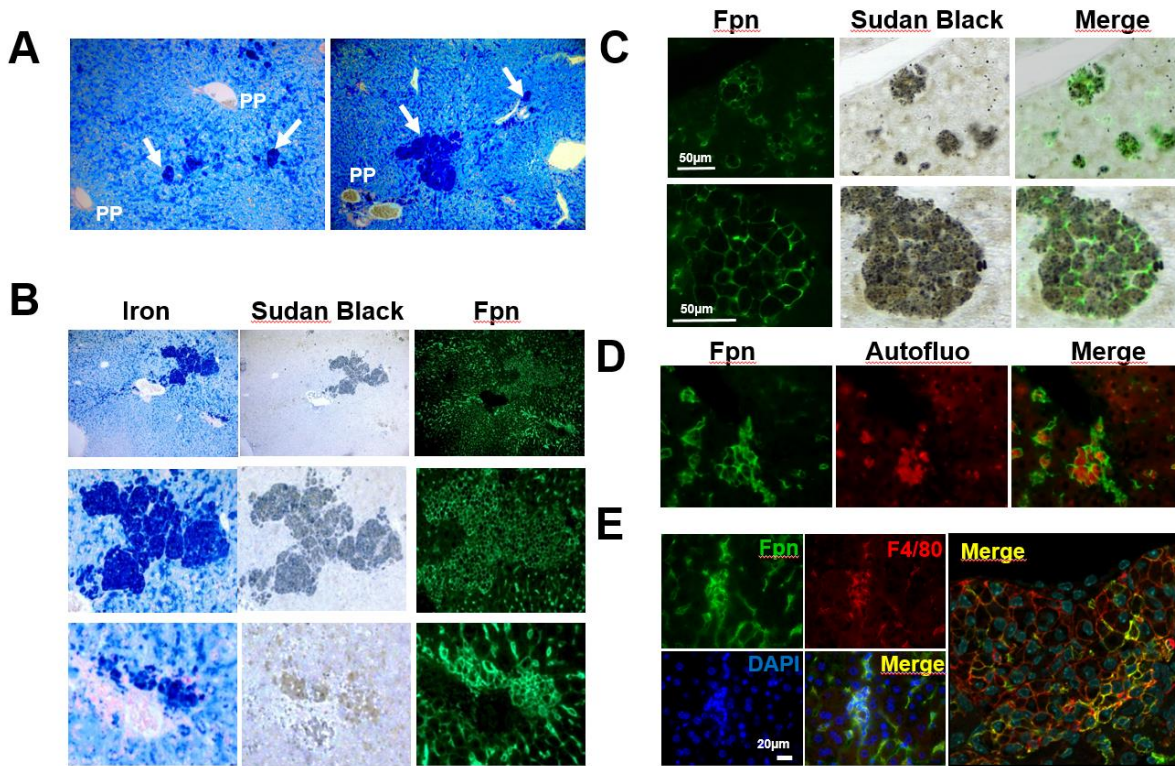
1 Immunofluorescence validation of the substantial upregulation of Fpn protein in liver sections.
2 (D) Illustration of the different parts of the distinct lobes [left lateral lobe (LLL), right lateral lobe
3 (RLL), medium lobe (ML), and caudate lobe (CL)] used for tissue sectioning and other analyses. (E)
4 Representative images (of three mice per genotype) illustrating iron distribution in liver lobes [
5 (LLL), (RLL), (ML), and (CL)] from wildtype (WT) and *Bmp6* KO mice at different ages. Perls
6 staining was performed for iron localization. In *Bmp6* KO mice (B), iron distribution was not
7 uniform among lobes or within the same lobe, with certain zones displaying lower (§) and higher
8 iron accumulation (*).

9



1
2 **Fig.2: Iron, ferroportin (Fpn), and macrophage distribution in *Bmp6* KO liver from**
3 **young mice. (A)** Perls staining of hepatic left lateral lobe (LLL) from 8-week-old wildtype (WT)
4 and *Bmp6* knockout (KO) mice. Images for *Bmp6* KO mice in (A) depict zones with mild iron
5 accumulation, marked as (§). In contrast to wildtype mice showing some iron in the periportal
6 zone (PP), *Bmp6* KO mice exhibited iron accumulation in the centrilobular zone (CL). **(B and**
7 **C)** Overlay of Perls staining images with Fpn immunofluorescence visualization, indicating the
8 concentration of Fpn staining in periportal low iron zones. **(D to F)** Immunofluorescence analysis
9 of Fpn and F4/80 (macrophage marker) staining. **(F)** Histogram representation of Fpn and F4/80
10 fluorescence (% of all fluorescence dots) from the images in panel E. Fpn and F4/80 were
11 enriched in the same zones corresponding to periportal low iron zones. LI: Low iron zones; HI:
12 High iron zones

1



2

3 **Fig.3: Histology and immunofluorescence analysis of high iron-containing zones**

4 **identified in *Bmp6* KO liver. (A)** Perl's staining of a medium lobe (ML) from *Bmp6* KO mice

5 at 12 weeks of age. The zonal iron distribution is disrupted in regions with significant iron

6 overload, displaying the emergence of prominent iron aggregates (arrow). **(B)** Sudan black

7 (ceroid/lipofuscin) and Fpn immunofluorescence staining in areas of intense iron overload (Perl's

8 staining) present in the left lateral lobe (LLL) from a 12-week-old KO *Bmp6* Mice. **(C)**

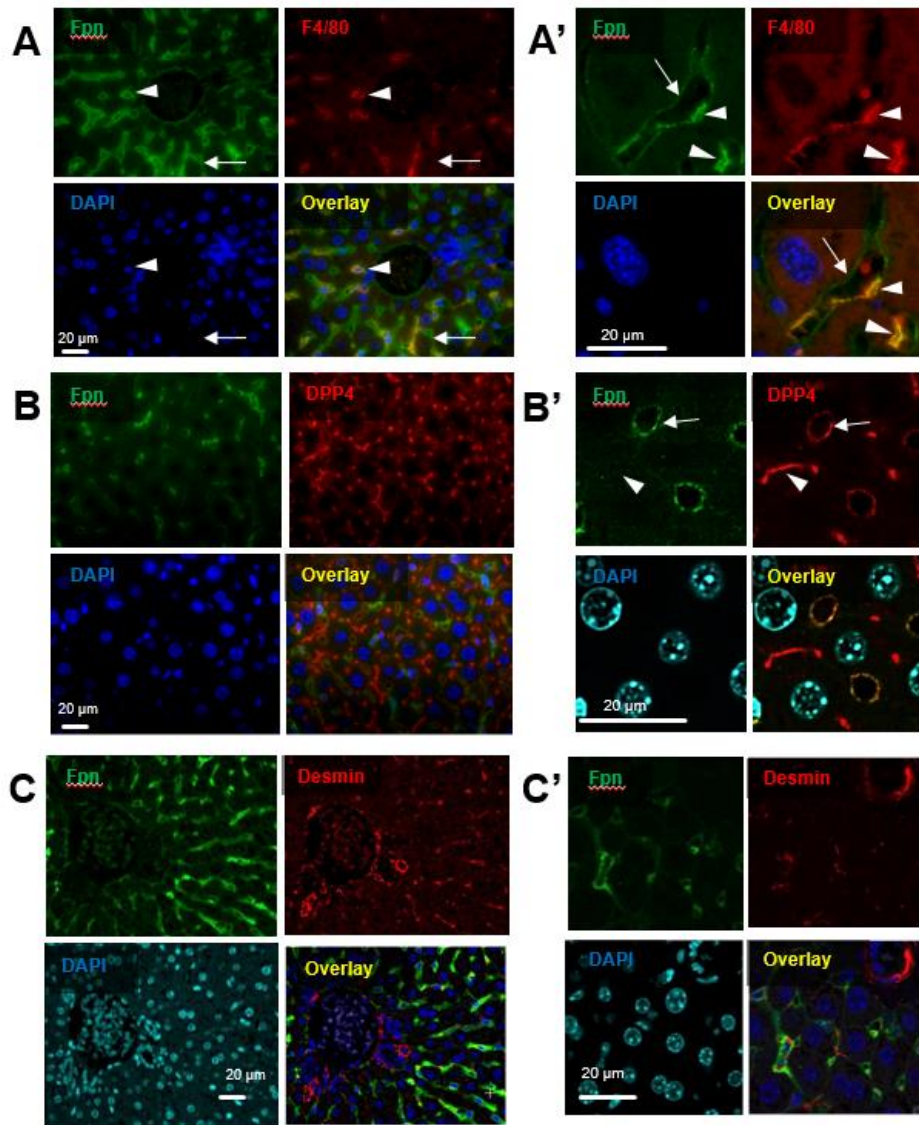
9 Ferroportin staining strongly correlates with ceroid/lipofuscin accumulation. **(D)** Ferroportin

10 staining is associated with autofluorescence within the aggregates. **(E)** Merged colabeling of Fpn

11 and F4/80 highlights the presence of numerous macrophages within these cellular aggregates.

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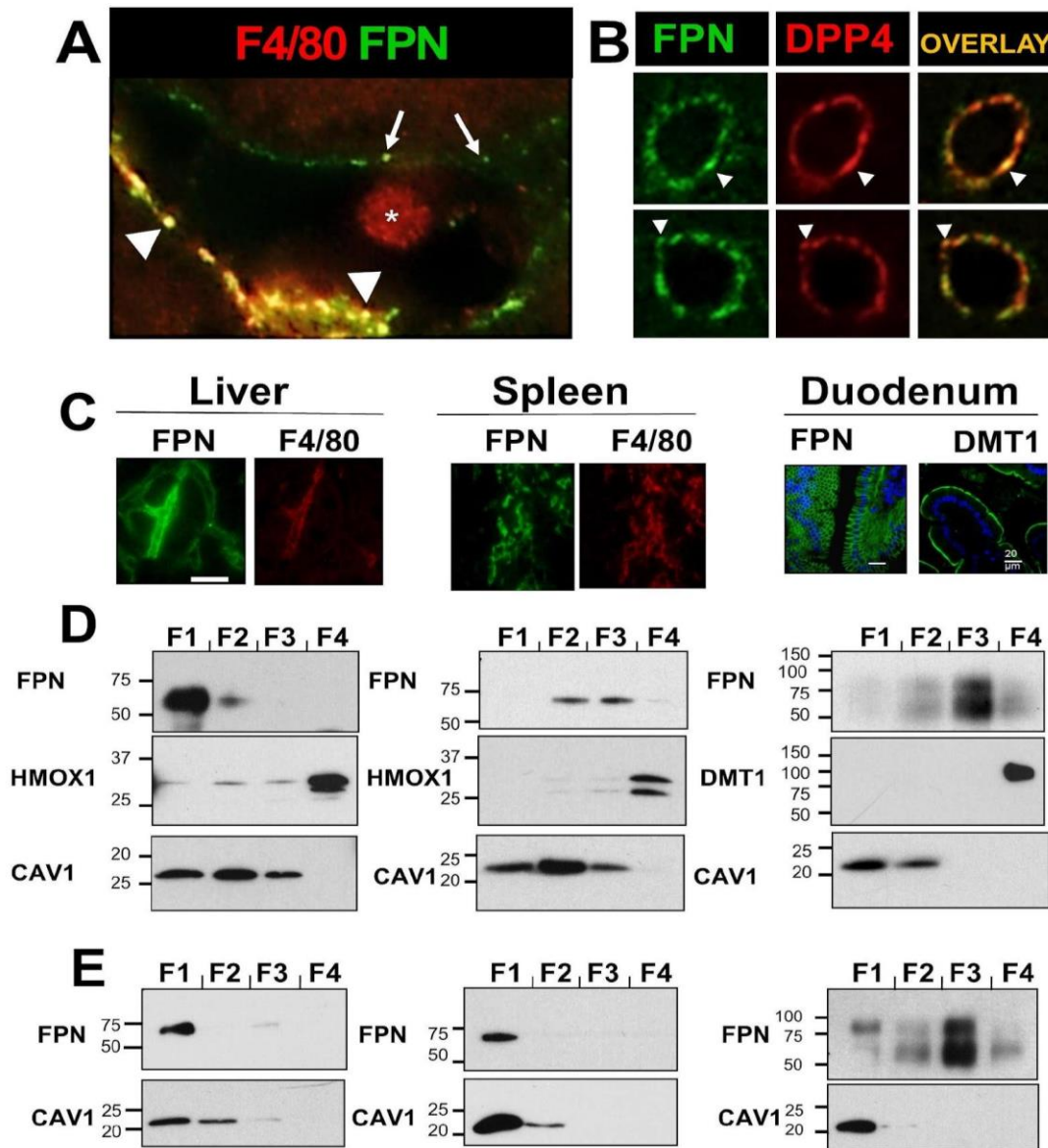
3 **Fig.4: Confocal analysis of co-immunofluorescence staining of ferroportin (Fpn)**

4 with F4/80 (A & A'), Dipeptidyl peptidase-4 (DPP-4, B & B') or Desmin (C & C') in liver

5 sections of 8 weeks-old *Bmp6* KO mice. Orange/yellow color in the overlays (A, A' and B, B')

6 indicates the colocalization of Fpn with F4/80 and DPP-4. No such colocalization of Fpn was

7 observed with Desmine. Nuclear DNA labeling was achieved using DAPI.



1

2 **Fig.5: Enrichment of *Bmp6* KO hepatic ferroportin (Fpn) in detergent resistant**

3 **membrane compartment.** (A) Confocal analysis of Fpn staining with F4/80, indicating the

4 enrichment of the iron exporter in membrane compartments at the cell surface of tissue

5 macrophages (arrowheads) and hepatocytes (arrows) in *Bmp6* KO mice. (B) Confocal analysis

6 of Fpn staining with Dipeptidyl peptidase-4 (Dpp4), showing the enrichment of Fpn in membrane

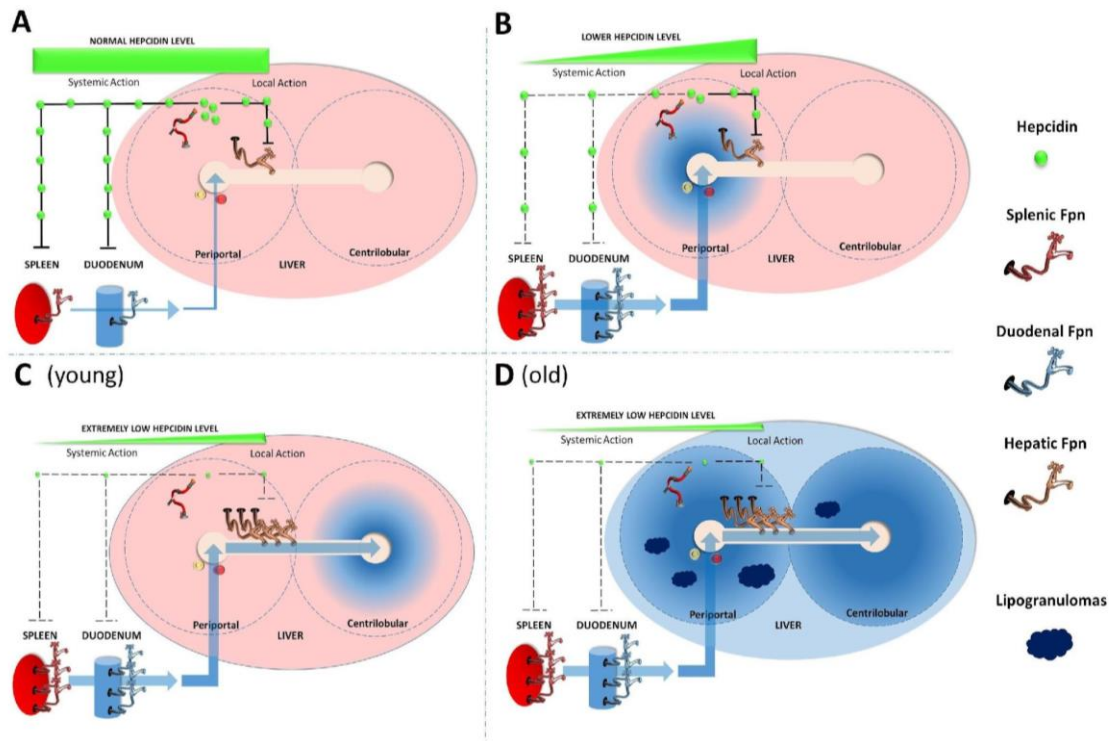
7 compartments at the cell surface of hepatocytes (arrows) in *Bmp6* KO mice. (C)

1 Immunohistofluorescence of Fpn in liver, spleen, and duodenum from *Bmp6* KO mice. F4/80
2 staining indicates Fpn expression in Kupffer cells and hepatocytes in the liver, and in splenic
3 macrophages in the red pulp. In the duodenum, Fpn is strongly expressed at the basolateral
4 membrane of enterocytes, opposite to the brush border localization of Dmt1. **(D & E)**
5 Immunoblotting detection of Fpn, Hmox1, Dmt1, and caveolin1 (Cav1; raft marker) in detergent
6 resistant membrane (DRM) and non-detergent resistant membrane (NDRM) fractions isolated
7 from liver, spleen, and duodenum of 8 weeks-old *Bmp6* KO mice. Similar volumes (D) or similar
8 amounts of protein (E) from pooled fractions A, B, C, and D were analyzed. Hepatic and splenic
9 Fpn are enriched in fractions containing Cav1, while intestinal Fpn is not. The molecular weight
10 marker's position and size in kilodaltons (kDa) are indicated on the left.

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3 **Fig.6: Influence of hepcidin and ferroportin centrilobular expression on hepatic**

4 **iron distribution.** (A) In most wildtype mice like C57BL6 with normal hepcidin levels, both

5 local (hepatic) and systemic (splenic and intestinal) effects of Hamp prevent liver iron overload.

6 (B) A modest decrease in Hamp levels, observed in CD1 wildtype mice, *Hfe*, *B2m*, and *Tfr2*

7 knockout mice, heterozygote Hamp +/- mice, *Bmp6* and *Hjv* knockout females, and Neogenin

8 mutants, leads to iron accumulation primarily in the periportal area. Reduced systemic Hamp

9 action results in increased iron recycling by macrophages and intestinal absorption, leading to

10 iron accumulation in periportal hepatocytes due to the maintained downregulation of hepatic

11 Fpn. (C) A dramatic decrease in Hamp levels impairs both systemic and local Hamp actions,

12 leading to strong Fpn expression in tissue macrophages, enterocytes, and periportal hepatocytes.

13 This results in increased iron recycling by macrophages and intestinal absorption, leading to iron

1 flux through the periportal vein. Due to high Fpn expression in periportal hepatocytes, iron is
2 mainly redistributed to the centrilobular area, where hepatocytes accumulate iron. This iron
3 zonation is observed in *Bmp6* and *Hjv* knockout males, *Hamp* knockout mice, and double or
4 triple knockouts (*Bmp6/B2m*, *Bmp6/Tfr2*, *Bmp6/B2m/TfR2*). (D) In contexts of extremely low
5 *Hamp* expression, iron zonation tends to disappear over time, leading to the formation of
6 lipogranulomas predominantly in the periportal zone.

7

8