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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) <sup>1,2</sup>. Iron deposits in the *Bmp6* KO liver have been shown to be more pronounced in the  
13 centrilobular areas <sup>2</sup>. However, the liver, being a complex organ, possesses a distinctive anatomy  
14 characterized by distinct lobes that exhibit morphological and functional variations <sup>3</sup>. 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 <sup>2</sup>. A similar phenotype was  
22 observed in *Hamp* KO mice <sup>4</sup>. However, the hepatic cellular localization of Fpn was only

1 partially characterized in both *Hamp* and *Bmp6* KO mice, using immunohistochemistry  
2 techniques <sup>2,4,5</sup>. Interestingly, in *Hamp* KO mice, Fpn was found to be more expressed in  
3 periportal areas <sup>5</sup>. 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 <sup>4,5,5</sup>. While hepatocytes  
6 were also demonstrated to express Fpn <sup>5</sup>, 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 <sup>6</sup>, 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*<sup>tm1Rob</sup> mice (*Bmp6*<sup>-/-</sup>: *Bmp6* KO) on an  
20 outbred CD1 background was previously documented <sup>7</sup>. 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) <sup>8</sup>.  
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 <sup>9</sup>. The specificity of  
6 homemade Fpn and Dmt1 sera was validated by immunoblotting, immunofluorescence and  
7 histochemistry <sup>2,8,9</sup>. 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 <sup>10</sup>. 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<sup>8</sup>. 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 phenylmethanesulfonyl 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, <sup>11</sup>]. Grounded frozen tissues (liver, spleen and duodenum) were used to  
17 prepare crude membrane extracts as described before<sup>8</sup>. 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  $\beta$ -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 <sup>2</sup>, 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<sup>12,13</sup>. Previous observations in cultured  
3 macrophages have clearly demonstrated that Fpn is mostly present in lipid rafts<sup>11</sup>. 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 described<sup>2</sup>, 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 <sup>14</sup>. 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 <sup>5</sup>. This pattern of Fpn expression was  
8 similarly noted in rats at the mRNA level via *in situ* hybridization <sup>6</sup>, 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 <sup>15</sup>. 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 <sup>16</sup>. 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 <sup>17</sup>. Notably, Hjv was found to be  
17 selectively expressed by periportal hepatocytes in mouse livers <sup>17</sup>. 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. <sup>18</sup> 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 <sup>19,20</sup>. Despite expressing hepcidin, albeit at inappropriately low levels relative to their body iron  
18 stores <sup>21–24</sup>, 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 <sup>25</sup>, 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) <sup>25</sup>. 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 H<sub>2</sub>O<sub>2</sub>, 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 macrophages<sup>26</sup>. 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 hepatocytes<sup>27</sup>. Intriguingly, periportal macrophages have  
20 been found to be more prone to phagocytosis in comparison to their centrilobular counterparts  
21<sup>28</sup>. 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) <sup>29</sup>. 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) <sup>6</sup>, 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 <sup>12</sup>. Furthermore, in the hematopoietic environment, Dpp4 activity is primarily located  
16 with lipid rafts <sup>13</sup>. Lipid rafts correspond to specific molecular platforms, floating at the cell  
17 surface and involved in numerous biological functions such as cell signaling <sup>30-32</sup>. 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 <sup>11</sup>. 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 modifications<sup>8</sup>. 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 effects<sup>33-35</sup>. Intriguingly, during the suckling period, enterocyte ferroportin  
9 seems less responsive to the degradative impact of circulating hepcidin<sup>36</sup>. 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.

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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

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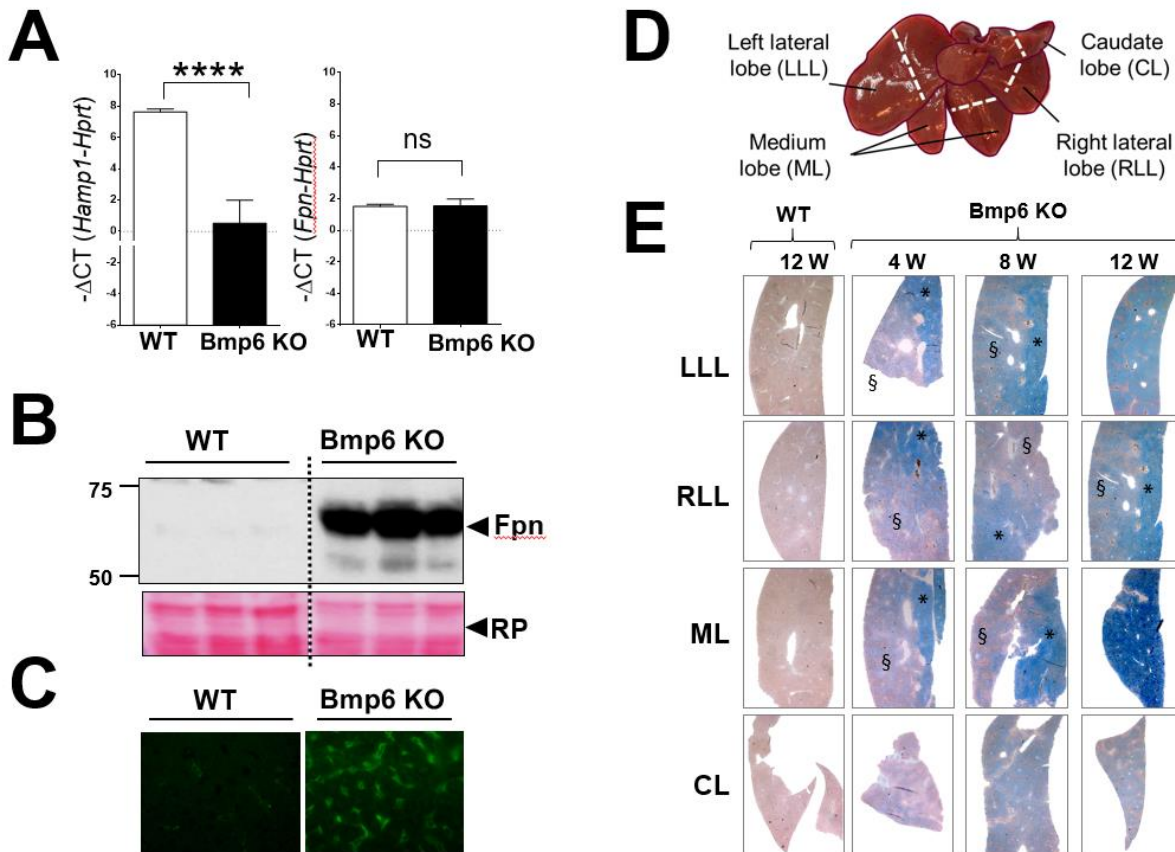
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## 1 FIGURES & LEGENDS

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 (Hamp1) and ferroportin

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

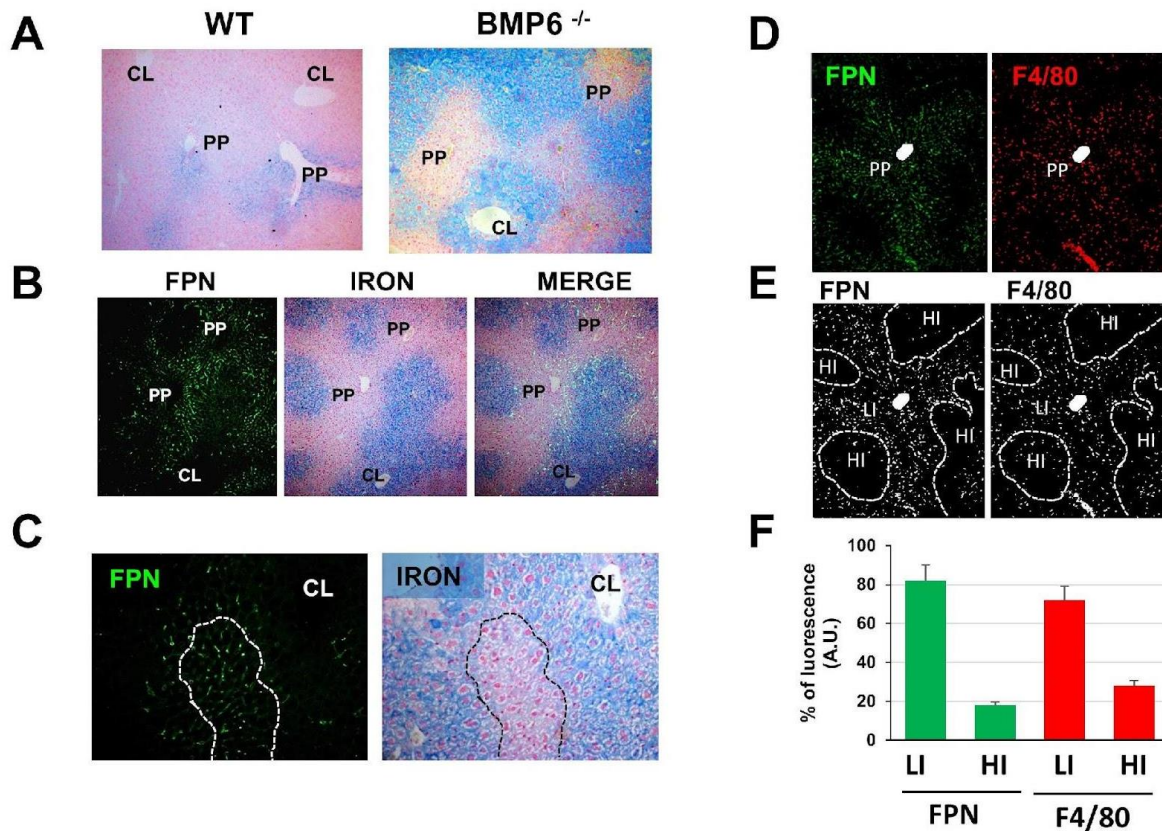
9 *Bmp6* KO livers (three mice per genotype) and subjected to Western blot analysis. Protein

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)

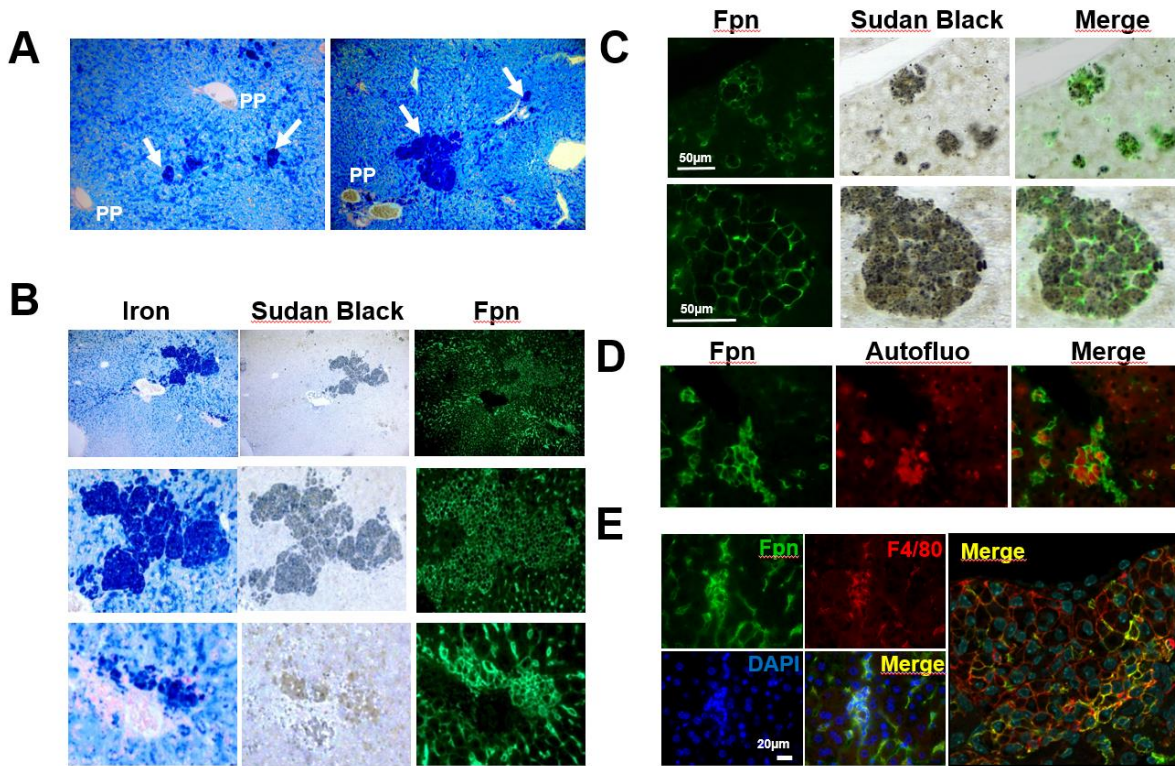
1 Immunofluorescence validation of the substantial upregulation of Fpn protein in liver sections.  
2 (D) Illustration of the different parts of the distinct lobes [left lateral lobe (LLL), right lateral lobe  
3 (RLL), medium lobe (ML), and caudate lobe (CL)] used for tissue sectioning and other analyses. (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)

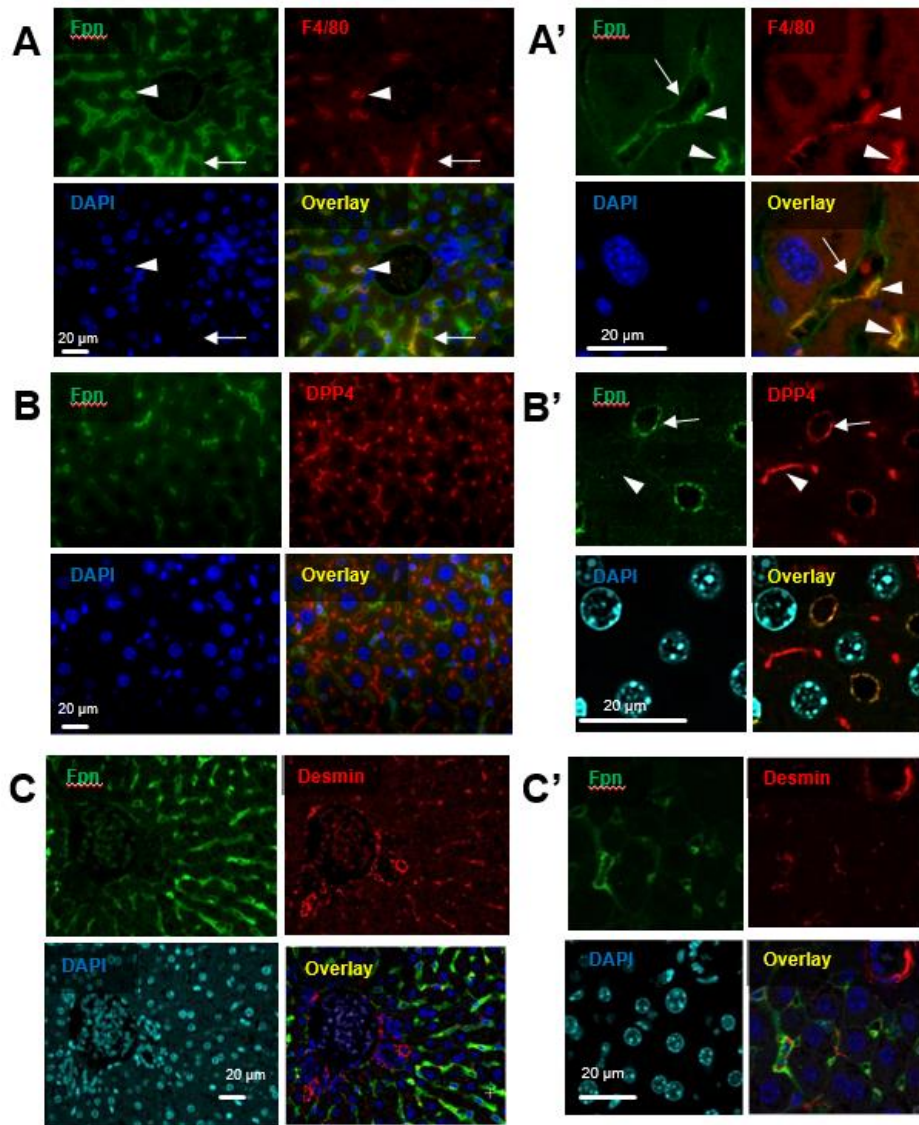
9 Ferroportin staining strongly correlates with ceroid/lipofuscin accumulation. (D) Ferroportin

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.

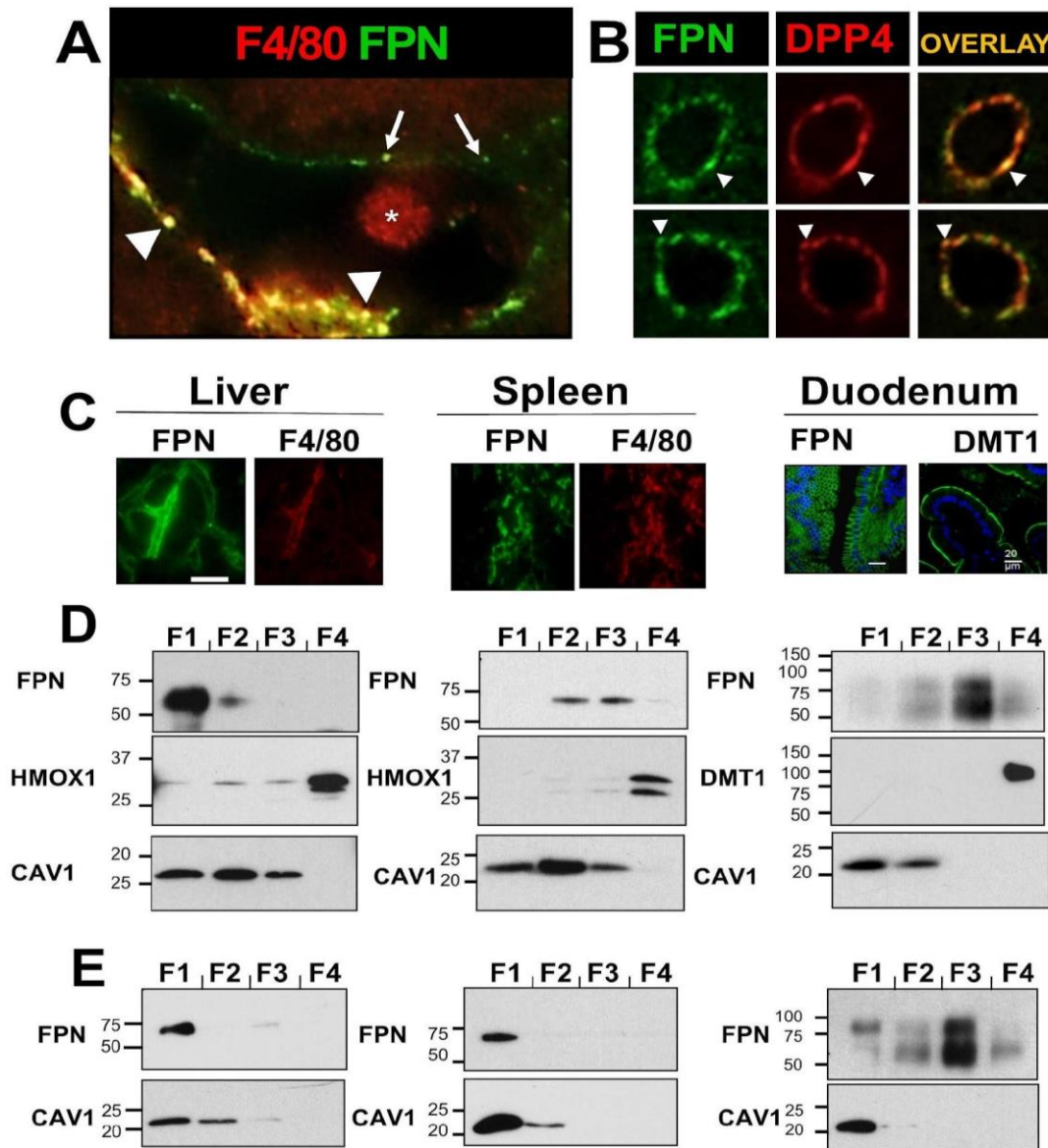
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3 **Fig.4: Confocal analysis of co-immunofluorescence staining of ferroportin (Fpn)**  
4 with F4/80 (A & A'), Dipeptidyl peptidase-4 (DPP-4, B & B') or Desmin (C & C') in liver  
5 sections of 8 weeks-old *Bmp6* KO mice. Orange/yellow color in the 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

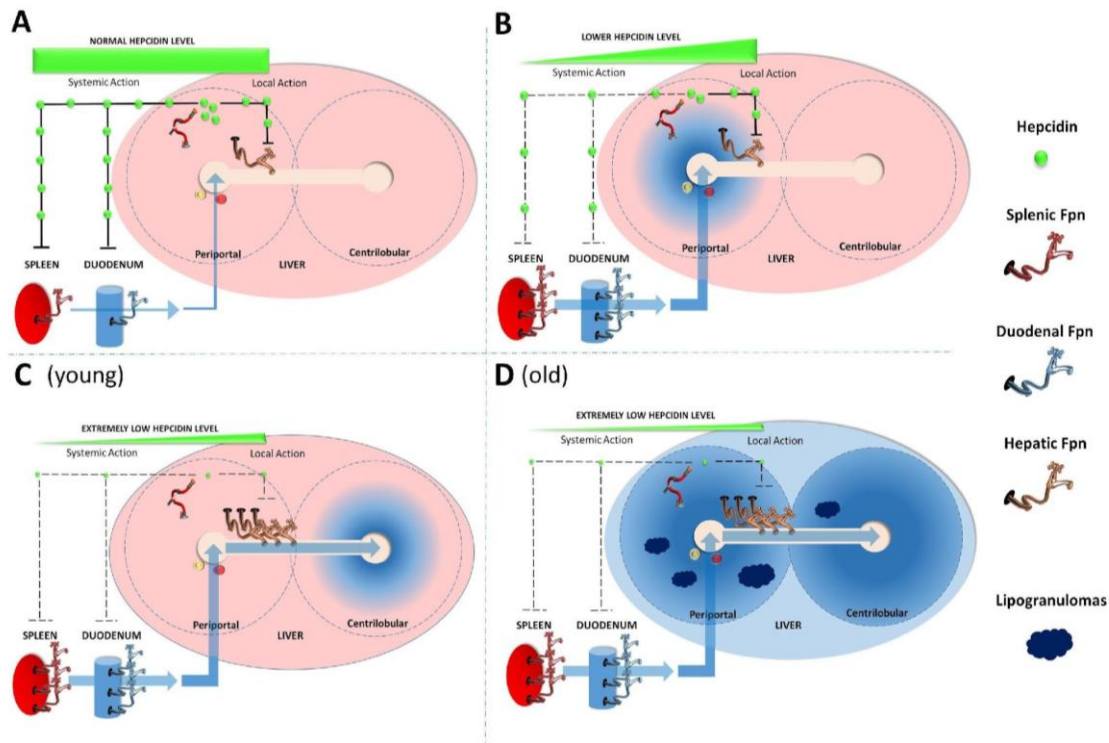
7 compartments at the cell surface of hepatocytes (arrows) in *Bmp6* KO mice. (C)

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