A crosstalk between hepcidin and IRE/IRP pathways controls ferroportin expression and determines serum iron levels in mice

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Abstract

The iron hormone hepcidin is transcriptionally activated by iron or inflammation via distinct, partially overlapping pathways. We addressed how iron affects inflammatory hepcidin levels and the ensuing hypoferremic response. Dietary iron overload did not mitigate hepcidin induction in LPS-treated wt mice but prevented effective inflammatory hypoferremia. Likewise, LPS modestly decreased serum iron in hepcidin-deficient Hjv-/- mice, model of hemochromatosis. Synthetic hepcidin triggered hypoferremia in control but not iron-loaded wt animals. Furthermore, it dramatically decreased hepatic and splenic ferroportin in Hjv-/- mice on standard or iron-deficient diet, but only triggered hypoferremia in the latter. Mechanistically, iron antagonized hepcidin responsiveness by inactivating IRPs in the liver and spleen, to stimulate ferroportin mRNA translation. Prolonged LPS treatment eliminating ferroportin mRNA permitted hepcidin-mediated hypoferremia in iron-loaded mice. Thus, *de novo* ferroportin synthesis is critical determinant of serum iron and finetunes hepcidin-dependent functional outcomes. Our data uncover a crosstalk between hepcidin and IRE/IRP systems that controls tissue ferroportin expression and determines serum iron levels. Moreover, they suggest that hepcidin supplementation therapy is more efficient combined with iron depletion.

Introduction

Systemic iron balance is controlled by hepcidin a peptide hormone that is produced by hepatocytes in the liver and operates in target cells by binding to the iron exporter ferroportin (1, 2). This results in ferroportin internalization and lysosomal degradation but also directly inhibits ferroportin function by occluding its iron export channel (3, 4). Ferroportin is highly expressed in duodenal enterocytes and tissue macrophages, which are instrumental for dietary iron absorption and iron recycling from senescent erythrocytes, respectively. Ferroportin is also expressed in hepatocytes, where excess iron is stored and can be mobilized on demand. Hepcidin-mediated ferroportin inactivation inhibits iron entry into plasma. This is a critical homeostatic response against iron overload, but also an innate immune response against infection (5). Thus, hepcidin expression is induced when systemic iron levels are high to prevent dietary iron absorption, or under inflammatory conditions to promote iron retention within ferroportin-expressing cells and render the metal unavailable to extracellular pathogens.

The hepcidin-encoding *HAMP* gene is transcriptionally induced by iron or inflammatory stimuli via BMP/SMAD (6) or IL-6/STAT3 (7) signaling, respectively. These pathways crosstalk at different levels. For instance, the BMP co-receptor hemojuvelin (HJV), a potent enhancer of iron-dependent BMP/SMAD signaling, is also essential for inflammatory induction of hepcidin. Thus, Hjv-/- mice, a model of juvenile hemochromatosis characterized by severe iron overload and hepcidin deficiency (8), exhibit blunted inflammatory induction of hepcidin and fail to mount a hypoferremic response following LPS treatment or infection with *E. coli* (9). Excess iron inhibits hepcidin induction via the BMP/SMAD and IL-6/STAT3 signaling pathways in cultured cells (10, 11), but the *in vivo* relevance of these findings is not known.

Hepcidin-dependent inhibition of ferroportin activity and expression is a major but not the sole contributor to inflammatory hypoferremia (12, 13). This is related to the fact that ferroportin expression is regulated by additional transcriptional and post-transcriptional mechanisms (14). Thus, ferroportin transcription is induced by iron (15) and suppressed by inflammatory signals (16), while translation of *Fpn(+IRE)* mRNA, a ferroportin transcript harboring an "iron responsive element" (IRE) within its 5' untranslated regions (5' UTR) is controlled by "iron regulatory proteins" (IRPs), IRP1 and IRP2. The IRE/IRP system accounts for coordinate post-transcriptional regulation of iron metabolism proteins in cells (17, 18). In a homeostatic response to iron deficiency, IRPs bind to the (IRE) within the *Fpn(+IRE)* and ferritin (*Fth1* and *Ftl1*) mRNAs, inhibiting their translation. IRE/IRP interactions do not take place in iron-loaded cells, allowing *de novo* ferroportin and ferritin synthesis to promote iron efflux and storage, respectively. The impact of the IRE/IRP system on regulation of tissue ferroportin and serum iron is not well understood.

The aim of this work was to elucidate mechanisms by which systemic iron overload affects hepcidin expression and downstream responses, especially under inflammatory conditions. Utilizing wild type and Hjv-/- mice, we demonstrate that serum iron levels reflect regulation of ferroportin in the liver and spleen by multiple signals. We further show that effective hepcidin-mediated hypoferremia is antagonized by compensatory mechanisms aiming to prevent cellular iron overload. Our data uncover a crosstalk between hepcidin and the IRE/IRP system that controls ferroportin expression in hepatocytes, as well as liver and splenic macrophages, and thereby determines serum iron levels.

Results

Dietary iron overload does not prevent further inflammatory Hamp mRNA induction in LPS-treated wt mice, but mitigates hepcidin responsiveness. In an exploratory experiment, wt mice were subjected to dietary iron loading by feeding a high-iron diet (HID) for short (1 day), intermediate (1 week) or long (5 weeks) time intervals; control animals remained on standard diet (SD). As expected, mice on HID for 1 day manifested maximal increases in serum iron (Fig. S1A) and transferrin saturation (Fig. S1B). They retained physiological liver iron content (LIC; Fig. S1E) and serum ferritin (Fig. S1C), a reflection of LIC. Serum iron and transferrin saturation plateaued after longer HID intake, while LIC and serum ferritin gradually increased to peak at 5 weeks. The dietary iron loading promoted gradual upregulation of serum hepcidin (Fig. S1D) and liver *Hamp* mRNA (Fig. S1F), with highest values at 5 weeks. This could not prevent chronic dietary iron overload, in agreement with earlier findings (19, 20).

LPS triggered appropriate hepcidin induction and a robust hypoferremic response in control mice. Interestingly, LPS-induced inflammation resulted in further proportional increase in hepcidin and *Hamp* mRNA in dietary iron-loaded mice (Fig. S1D and S1F). This was accompanied by significant drops in serum iron and transferrin saturation. However, values did not reach the nadir of LPS-treated control animals and were increasing in mice on HID for longer periods, despite significant hepcidin accumulation. These data suggest that hepatic iron overload does not prevent inflammatory induction of hepcidin; however, it impairs its capacity to decrease serum iron.

Uncoupling inflammatory hepcidin induction from hypoferremic response in wt and Hjv-/- mice following dietary iron manipulations. To further explore the potential of hepcidin to

107 promote hypoferremia under iron overload, wt and Hjv-/- mice, a model of hemochromatosis,
108 were subjected to dietary iron manipulations. Wt mice were fed SD or HID, and Hjv-/- mice
109 were fed SD or an iron-deficient diet (IDD) for 5 weeks, to achieve a broad spectrum of hepcidin
110 regulation. Wt mice on HID and Hjv-/- mice on SD or IDD manifested similarly high serum iron
111 and transferrin saturation (Fig. 1A-B). Serum non-transferrin bound iron (NTBI) levels appeared
112 modestly elevated in the dietary and genetic iron overload models and seemed to decrease in
113 Hjv-/- mice on IDD (Fig. 1C). LIC was substantially reduced in Hjv-/- mice in response to IDD,
114 but also compared to wt mice on HID (Fig. 1E). The quantitative LIC data were corroborated
115 histologically by Perls staining (Fig. 1F). Dietary iron loading increased splenic iron in wt mice
116 and confirmed that Hjv-/- mice fail to retain iron in splenic macrophages (Fig. S2). As expected,
117 serum hepcidin (Fig. 1D) and liver *Hamp* mRNA (Fig. 1G) were maximally induced in HID-fed
118 wt mice and were low in Hjv-/- mice on SD, and further suppressed to undetectable levels
119 following IDD intake.

120 LPS reduced serum iron and transferrin saturation in hyperferremic wt mice on HID and
121 Hjv-/- mice on SD or IDD, but not below baseline of control wt mice on SD, the only animals
122 that developed a robust hypoferremic response (Fig. 1A-B). The LPS treatment was associated
123 with significant accumulation of hepcidin (Fig. 1D) and induction of *Hamp* mRNA (Fig. 1G) in
124 all experimental groups, while NTBI (Fig. 1C) and LIC (Fig. 1E) were unaffected. Notably,
125 LPS-treated wt mice on HID and Hjv-/- mice on IDD exhibited dramatic differences in *Hamp*
126 mRNA but similar blunted hypoferremic response to the acute inflammatory stimulus. Thus, the
127 profound hepcidin induction in iron-loaded wt mice cannot decrease serum iron below that of
128 iron-depleted Hjv-/- mice with negligible hepcidin, which indicates reduced hepcidin
129 responsiveness. In support to this interpretation, *Id1* and *Socs3* mRNAs (Fig. 1H-I), which are

markers of BMP/SMAD and IL-6/STAT3 signaling, respectively, were appropriately induced by dietary iron loading or LPS treatment in wt mice. Thus, the major hepcidin signaling pathways were intact under these experimental conditions.

Fpn(+IRE) mRNA levels were significantly increased in the liver of iron-loaded wt mice on HID and Hjv-/- mice on SD (Fig. 1J), which is consistent with transcriptional induction (15). The LPS treatment strongly suppressed liver *Fpn(+IRE)* mRNA (Fig. 1J) and induced *Dmt1*, *Zip14* and *Lcn2* mRNAs in all animals (Fig. 1K-M). These encode the divalent metal transporter DMT1, the NTBI transporter Zip14 and the siderophore-binding protein Lcn2, respectively; *Lcn2* mRNA induction was dramatic. The transferrin receptor 1-encoding *Tfrc* mRNA was largely unaffected by LPS, except for a reduction in Hjv-/- mice on IDD (Fig. 1N).

To assess the downstream function of hepcidin, we first analyzed ferroportin in the liver, an organ that contributes to iron sequestration during inflammation. Immunohistochemical staining of liver sections revealed strong ferroportin expression in Kupffer cells, predominantly in periportal areas, under all experimental conditions (Figs. 2A and S3). Hepatocellular ferroportin staining is also evident in the iron overload models, mostly in periportal hepatocytes (Fig. S3), and in line with recent data (21). LPS triggered redistribution and decreased expression of ferroportin in Kupffer cells from wt but not Hjv-/- mice (Fig. S3), as reported (9).

We further analyzed ferroportin in liver homogenates by Western blotting. Levels of biochemically detectable liver ferroportin differed substantially between wt and Hjv-/- mice. Thus, they were relatively low in the former and highly induced in the latter (Fig. 2B), independently of iron load. The differences were more dramatic compared to those observed by immunohistochemistry (Figs. 2A and S3). Conceivably, the strong ferroportin signal in Western

152 blots from Hjv-/- liver homogenates reflects high ferroportin expression in hepatocytes, which
153 are the predominant cell population and make up ~80% of the liver cell mass (22). Yet,
154 hepatocellular ferroportin is less visible by immunohistochemistry because the signal is
155 substantially weaker compared to that in Kupffer cells (see also Fig. 5E). Interestingly, the LPS
156 treatment visibly suppressed total liver ferroportin in Hjv-/- mice on SD but not IDD, while it
157 appeared to modestly affect it in wt mice (Fig. 2B). These data are consistent with negative
158 regulation of ferroportin by residual LPS-induced hepcidin in Hjv-/- mice on SD, which could
159 explain the small drop in serum iron and transferrin saturation under these acute inflammatory
160 conditions, as reported (9). However, liver ferroportin remained detectable and apparently
161 functional, as it did not allow significant iron sequestration and dramatic drop in serum iron.
162 Notably, persistence of relatively high serum iron is also evident in LPS-treated wt mice on HID,
163 despite maximal hepcidin and minimal liver ferroportin levels.

164 Next, we analyzed ferroportin in the spleen, an organ with erythrophagocytic
165 macrophages that plays an important role in body iron traffic (23). Immunohistochemical
166 analysis shows that LPS reduced ferroportin in red pulp splenic macrophages from wt mice on
167 SD, but this effect was less evident in wt mice on HID and in Hjv-/- mice on SD or IDD (Figs.
168 2C and S4). Western blot analysis shows a stronger ferroportin signal in splenic extracts from
169 Hjv-/- animals (Fig. 2D), consistent with immunohistochemistry. However, in this assay LPS
170 appeared to suppress splenic ferroportin in wt animals (though statistical significance was not
171 reached) and in Hjv-/- mice on SD, but not IDD. This could be a result of residual hepcidin
172 upregulation (Figs. 1D and 1G), while the lack of significant splenic ferroportin suppression in
173 Hjv-/- mice on IDD may indicate hepcidin insufficiency. In any case, the relatively high

circulating iron levels in dietary iron-loaded and LPS-treated wt mice indicates continuous iron efflux to plasma despite hepcidin excess.

Insufficient hepcidin leads to blunted hypoferremic response in iron overload. We used human synthetic hepcidin to address whether the failure of mouse models of iron overload to mount an appropriate hypoferremic response to acute inflammation is caused by endogenous hepcidin insufficiency or other mechanisms. Wt and Hjv-/- mice subjected to dietary iron manipulations received 2.5 μg/g synthetic hepcidin every two hours for a total of four intraperitoneal injections. Each dose corresponds to ~200-fold excess over endogenous circulating hepcidin in control wt animals. The treatment caused hypoferremia in wt mice on SD but not HID, where the decrease in serum iron was significant but well above baseline of untreated wt controls (Fig. 3A-B). Likewise, synthetic hepcidin significantly decreased serum iron but failed to cause dramatic hypoferremia in hepcidin-deficient Hjv-/- mice on SD. Strikingly, hepcidin administration was much more effective in relatively iron-depleted Hjv-/- mice on IDD, and lowered serum iron and transferrin saturation below baseline. The treatments significantly reduced NTBI in Hjv-/- mice on SD, with a trend in mice on IDD (Fig. 3C) but did not affect LIC or splenic iron content (SIC) under any experimental conditions (Figs. 3D-E and S5). Serum iron represents <2% of total tissue iron and therefore its acute fluctuations are not expected to dramatically alter LIC or SIC.

Notably, synthetic hepcidin led to significant reduction of endogenous *Hamp* mRNA in wt mice on SD (Fig. 3F), as earlier reported (24). Conceivably, this is related to destabilization of the Hamp inducer Tfr2 in the liver (Fig. 3G), a known response to hypoferremia (25). Synthetic hepcidin did not promote inflammation, iron perturbations or alterations in BMP/SMAD signaling in the liver, as judged by the unaltered expression of hepatic *Fpn*, *Socs3*, *Bmp6* and *Id1*

mRNAs (Fig. S6A-D). Moreover, synthetic hepcidin did not affect *Dmt1*, *Zip14*, *Lcn2* or *Tfrc* mRNAs (Fig. S6E-H), which encode iron transporters; *Zip14* and *Lcn2* are also inflammatory markers.

Next, we analyzed liver ferroportin by immunohistochemistry. Figs. 4A and S7 show that exogenous hepcidin decreased ferroportin signal intensity in all animal groups to varying degrees. The hepcidin effect was particularly noticeable in hepatocytes from Hjv-/- mice (see low magnification images in Fig. S7). Kupffer cells seemed to retain some ferroportin in all groups except Hjv-/- mice on IDD. Interestingly, while synthetic hepcidin decreased ferroportin signal intensity in Kupffer cells, it did not alter intracellular ferroportin distribution as would be expected based on the data in LPS-treated wt mice (Fig. 4A).

Western blotting confirmed that total liver ferroportin is highly induced in Hjv-/- mice (Fig. 4B). Again, the signal intensity can be attributed to protein expressed in hepatocytes. The treatment with synthetic hepcidin did not significantly affect liver ferroportin in wt mice (either on SD or HID), but substantially reduced it in Hjv-/- mice, to almost control levels. The effect appeared more pronounced in Hjv-/- mice on IDD; nevertheless, ferroportin remained detectable.

Splenic ferroportin was reduced in all animal groups following hepcidin treatment, with stronger effects visualized by immunohistochemistry in wt mice on SD and Hjv-/- mice on IDD (Figs. 4C and S8). At the biochemical level, ferroportin expression was again much stronger in the spleen of Hjv-/- mice (Fig. 4D). Synthetic hepcidin did not significantly affect splenic ferroportin in wt mice, but dramatically reduced it in all Hjv-/- mice.

Taken together, our data suggest that synthetic hepcidin overcomes endogenous hepcidin deficiency in Hjv-/- mice. However, it only triggers hypoferremia in these animals following

relative iron depletion. On the other hand, in iron-loaded wt mice with already high endogenous hepcidin, excess synthetic hepcidin fails to promote hypoferremia.

Dietary iron manipulations are sensed by IRPs in the liver and spleen of wt and Hjv-/- mice. The IRE/IRP system orchestrates homeostatic adaptation to cellular iron supply (17, 18). To evaluate the responses of IRPs in the whole liver and spleen to the above-described dietary iron manipulations, we analyzed tissue extracts from wt and Hjv-/- mice by an electrophoretic mobility shift assay (EMSA) using a ^{32}P-labelled IRE probe. The data in Fig. 5A-B show that HID intake decreases the IRE-binding activities of IRP1 and IRP2 in both liver and spleen of wt mice. Conversely, IDD intake stimulates the IRE-binding activities of IRP1 and IRP2 in the liver and spleen of Hjv-/- mice. IRE/IRP2 interactions are better visible in longer exposures (left panels). EMSAs with tissue extracts previously treated with 2-mercaptoethanol (2-ME) were performed as loading controls (26) and are shown in the bottom panels.

To clarify which cell types of the liver account for the responses of IRPs to dietary iron, separate EMSAs were performed using extracts from isolated hepatocytes or non-parenchymal liver cells. The data in Fig. 5C-D uncover that IRP1 and IRP2 in both liver cell populations from wt and Hjv-/- mice are sensitive to dietary iron loading or restriction, respectively. The EMSA analysis of non-parenchymal liver cells, which contains Kupffer cells, showed a high experimental variability (Fig. 5D). Nevertheless, the overall results are consistent with those obtained with splenic extracts, which contain red pulp macrophages (Fig. 5B).

Relative expression of ferroportin in hepatocytes and non-parenchymal liver cells from wt and Hjv-/- mice. We determined the relative abundance of ferroportin in hepatocytes and non-parenchymal liver cells from wt and Hjv-/- mice on SD by Western blotting. As expected,

241 ferroportin expression (normalized to β-actin) was ~1.5-2-fold higher in the non-parenchymal
242 cell fraction as compared to hepatocytes in both wt and Hjv-/- mice (Fig. 5E). In comparison
243 across genotypes, ferroportin expression was ~2-fold higher in hepatocytes and ~50% higher in
244 non-parenchymal cells from Hjv-/- vs wt mice.

245 *Iron-dependent regulation of ferroportin mRNA translation in the liver*. Having
246 established that dietary iron manipulations trigger IRP responses in the liver and spleen, we
247 hypothesized that the functional outcomes of exogenous hepcidin may not merely depend on its
248 capacity to degrade tissue ferroportin, but also on iron-dependent ferroportin regeneration via *de*
249 *novo* synthesis. Ferroportin is encoded by two alternatively spliced transcripts, *Fpn(+IRE)* and
250 *Fpn(-IRE)*, which exhibit different tissue distribution (27). *Fpn(+IRE)* mRNA is predominant in
251 the mouse liver and spleen (27) and is considered as a target of IRPs.

252 Thus, we assessed the effects of dietary iron on whole liver *Fpn(+IRE)* mRNA
253 translation by polysome profile analysis. We focused on the liver because this organ contains the
254 highest number of iron-recycling macrophages (28) and can also export iron to plasma from
255 ferroportin-expressing parenchymal cells. Liver extracts from wt mice on SD or HID, and Hjv-/-
256 mice on SD or IDD were fractionated on sucrose gradients to separate translationally inactive
257 light monosomes from translating heavy polysomes. The relative distribution of *Fpn(+IRE)*,
258 *Fth1* (positive control for iron regulation) and *Actb* (negative control) mRNAs within the
259 different fractions was quantified by qPCR. Fig. 6A shows that dietary iron loading stimulates
260 *Fpn(+IRE)* (and *Fth1*) mRNA translation in wt mice (note the shifts from monosomes to
261 polysomes). Conversely, dietary iron depletion inhibits *Fpn(+IRE)* (and *Fth1*) mRNA translation
262 in Hjv-/- mice. We also attempted to obtain polysome profiles of *Fpn(-IRE)* mRNA but it was
263 undetectable after fractionation. These data indicate that in mice subjected to iron overload, iron-

264 stimulated ferroportin synthesis in the liver antagonizes hepcidin-mediated ferroportin
265 degradation and prevents an appropriate hypoferremic response. Considering that levels of
266 *Fpn(+IRE)* mRNA are elevated in iron-loaded wt and Hjv-/- mice (Figs. 1J and S6A), it is
267 possible that increased *de novo* ferroportin synthesis is further enhanced by transcriptional
268 induction.

269 Quantification of liver iron by ICP-MS (Fig. 6B) validated iron loading of wt mice by
270 HID, and relative iron depletion of Hjv-/- mice by IDD intake, respectively (see also Fig. 1E).
271 Iron redox speciation analysis by CE-ICP-MS revealed a profound increase in Fe^{2+}/Fe^{3+} ratios in
272 livers of Hjv-/- mice on SD, which was corrected by dietary iron depletion (Fig. 6C).
273 Nevertheless, there was no difference in Fe^{2+}/Fe^{3+} ratios among livers of wt mice on SD or HID,
274 and Hjv-/- mice on IDD. We conclude that an increase in total iron content, rather than excessive
275 accumulation of redox active Fe^{2+} drives *Fpn(+IRE)* (and *Fth1*) mRNA translation in the liver.

276 *Restoration of effective hypoferremic response under iron overload following maximal*
277 *Fpn mRNA suppression*. We reasoned that complete inactivation of ferroportin mRNA would
278 restore hepcidin-induced hypoferremia despite iron overload. An 8-hour treatment of mice with
279 LPS suppresses liver *Fpn(+IRE)* mRNA below detection levels (Fig. 7A), as reported (9). The
280 same holds true for the *Fpn(-IRE)* isoform (Fig. 7B), which is 290 times less abundant in control
281 mouse livers compared to *Fpn(+IRE)* (ΔCt=8.18), in agreement with published data (27). We
282 went on to examine the effects of synthetic hepcidin on serum iron under these conditions of
283 maximal *Fpn* mRNA suppression. Importantly, the prolonged LPS treatment decreased serum
284 iron in wt mice on HID below the control baseline (Fig. 7C). Furthermore, when combined with
285 synthetic hepcidin, it promoted an effective hypoferremic response in wt mice on HID and Hjv-/-
286 mice on SD (or IDD) (Figs. 7C-D) and tended to decrease NTBI (Fig. 7E). These data strongly

suggest that the expression of actively translating *Fpn* mRNA in iron-exporting tissues under systemic iron overload mitigates hepcidin-induced drop in serum iron.

Discussion

We sought to analyze how iron overload affects hepcidin-mediated inflammatory responses. We and others reported that excess iron inhibits the major hepcidin signaling pathways (BMP/SMAD and IL-6/STAT3) in cultured cells (10, 11). To explore the physiological relevance of these findings, wt mice were subjected to variable degrees of dietary iron loading and then treated with LPS. All iron-loaded mice could further upregulate hepcidin in response to LPS-induced acute inflammation (Fig. S1). This is consistent with other relevant findings (29) and apparently contradicts the *in vitro* data. While experimental iron loading of cultured cells is rapid, dietary iron loading of mice is gradual (20) and most of excess iron is effectively detoxified within ferritin, which is highly induced (30). By contrast, the suppression of hepcidin preceded ferritin induction in cultured cells (10), which may explain the discrepancy with the *in vivo* data.

The unimpaired inflammatory induction of hepcidin in iron-loaded wt mice correlated with significant drops in serum iron, but these appeared inversely proportional to the degree of systemic iron loading (Fig. S1). Thus, LPS-treated mice on 5 weeks HID failed to reduce serum iron below a baseline of untreated control mice on SD. This indicates a defect in mounting a hypoferremic response to inflammation, which can be attributed to mechanisms antagonizing hepcidin action. To explore how iron modulates the capacity of hepcidin to trigger inflammatory hypoferremia, we established conditions of iron overload using wt and Hjv-/- mice with extreme

differences in hepcidin expression. Figs. 1 and 2 demonstrate that iron overload prevents effective inflammatory hypoferremia independently of hepcidin and tissue ferroportin levels.

We used a ~200-fold excess of synthetic hepcidin to directly assess its capacity to divert iron traffic in iron-loaded mice. Hepcidin injection caused hypoferremia in control wt mice on SD and significantly reduced serum iron in wt mice on HID and Hjv-/- mice on SD, but not below baseline (Fig. 3). Thus, synthetic hepcidin failed to drastically drop serum iron levels in iron overload models with either high or low endogenous hepcidin. Importantly, synthetic hepcidin promoted robust hypoferremia in relatively iron-depleted Hjv-/- mice on IDD, with undetectable endogenous hepcidin. It should be noted that synthetic hepcidin had similar effects on tissue ferroportin among wt or Hjv-/- mice, regardless of iron diet (Fig. 4). It reduced intensity of the ferroportin signal in Kupffer cells and splenic macrophages of wt mice without significantly affecting biochemically detectable total protein levels. In addition, it dramatically reduced total ferroportin in the liver and spleen of Hjv-/- mice. However, in all experimental settings there was residual tissue ferroportin, which appears to be functionally significant.

We reasoned that at steady-state, tissue ferroportin may consist of fractions of newly synthesized protein, and protein that is *en route* to hepcidin-mediated degradation. Conceivably, the former may exhibit more robust iron export activity, at least before its iron channel gets occluded by hepcidin. Increased *de novo* synthesis of active ferroportin could explain why synthetic hepcidin cannot drastically drop serum iron levels under iron overload. In fact, Fig. 6 demonstrates that dietary iron overload augments *Fpn(+IRE)* mRNA translation in the liver of wt mice. Conversely, relative dietary iron depletion inhibits *Fpn(+IRE)* mRNA translation in the liver of Hjv-/- mice, in line with the restoration of hepcidin-mediated hypoferremic response (Fig. 3).

Our data are consistent with translational control of liver ferroportin expression via the IRE/IRP system and do not exclude the possibility for an additional contribution of iron-dependent transcriptional regulation of *Fpn(+IRE)* mRNA. Direct evidence for activation of IRP responses in the liver and spleen to dietary iron manipulations is provided in Fig. 5. While translational control of ferritin in tissues is established (31), regulation of ferroportin by the IRE/IRP system is less well characterized and has hitherto only been documented in cell models (32, 33), the mouse duodenum (34), and the rat liver (35). Moreover, the physiological relevance of this mechanism remained speculative. The data in Figs. 5 and 6 show that the IRE/IRP system is operational and controls *Fpn(+IRE)* mRNA translation in both hepatocytes and non-parenchymal liver cells. Presumably, this offers a compensatory mechanism to protect these cells from iron overload and iron-induced toxicity. On the other hand, this mechanism attenuates hepcidin responsiveness and promotes a state of hepcidin resistance, as higher amounts of hepcidin are required to achieve effective hypoferremia. Because hepcidin has a short plasma half-life, it is reasonable to predict that the use of more potent hepcidin analogs (36) will overcome the antagonistic effects of increased ferroportin mRNA translation under iron overload.

The critical role of *de novo* ferroportin synthesis in fine-tuning hepcidin-dependent functional outcomes is also highlighted in Fig. 7. Thus, synthetic hepcidin was highly effective as promoter of hypoferremia in dietary iron-loaded wt mice when administered together with LPS. LPS is known to suppress *Fpn* mRNA in cell lines (16) and mouse tissues, with a nadir in the liver reached at 8 h (9). The recovery of hepcidin effectiveness in mouse models of iron overload was only possible when *Fpn* mRNA was essentially eliminated. Under these conditions,

LPS treatment alone was sufficient to decrease serum iron in dietary iron-loaded wt mice below baseline.

Tissue iron uptake may be another important determinant of the hypoferremic response to inflammation. LPS did not affect *Tfrc* mRNA levels in the liver (Fig. 1N), which argues against increased uptake of transferrin-bound iron via TfR1. On the other hand, LPS induced *Zip14*, *Dmt1* and *Lcn2* mRNAs (Figs 1K-M). Zip14 is the NTBI transporter accounting for hepatocellular iron overload in hemochromatosis (37) and is upregulated by inflammatory cues in hepatocytes (38). DMT1 is dispensable for NTBI uptake by hepatocytes (39), and its inflammatory induction might promote iron acquisition by macrophages (16, 40). Nevertheless, considering that the fraction of NTBI represents <2% of total serum iron even in the iron overload models (Figs. 1A and C), it is implausible that NTBI uptake by Zip14 and/or DMT1 substantially contributes to inflammatory hypoferremia. Lcn2 is an acute phase protein that can sequester intracellular iron bound to catecholate siderophores (41), and is more likely to transport iron to tissues during infection. In any case, synthetic hepcidin did not affect expression of iron transporters (Fig. S6E-H). This excludes the possibility for a synergistic effect on LPS-induced tissue iron uptake that could promote effective hypoferremia in the iron overload models.

In conclusion, our data reveal a crosstalk between the hepcidin pathway and the IRE/IRP system in the liver and spleen for the control of tissue ferroportin and serum iron levels. Furthermore, they suggest that application of hepcidin therapeutics for treatment of iron overload disorders should be combined with iron depletion strategies to mitigate *Fpn* mRNA translation and increase hepcidin efficacy. Future work is expected to clarify whether optimizing the hypoferremic response to inflammation under systemic iron overload decreases susceptibility to pathogens.

Materials and Methods

Animals. Wild type C57BL/6J and isogenic Hjv-/- mice (42) were housed in macrolone cages (up to 5 mice/cage, 12:12 h light-dark cycle: 7 am - 7 pm; 22 ± 1°C, 60 ± 5% humidity). The mice were fed either a standard diet (200 ppm iron), an iron-deficient diet (2-6 ppm iron) or a high-iron diet (2% carbonyl iron) (43). Where indicated, mice were injected intraperitoneally with 1 μg/g LPS (serotype 055:B5; Sigma-Aldrich) or subcutaneously with 2.5 μg/g synthetic hepcidin; control mice were injected with phosphate-buffered saline. At the endpoints, animals were sacrificed by CO_2 inhalation and cervical dislocation. Experimental procedures were approved by the Animal Care Committee of McGill University (protocol 4966).

Serum biochemistry. Blood was collected via cardiac puncture. Serum was prepared by using micro Z-gel tubes with clotting activator (Sarstedt) and was kept frozen at −20°C until analysis. Serum iron, total iron binding capacity (TIBC) and, where indicated serum ferritin, were determined at the Biochemistry Department of the Montreal Jewish General Hospital using a Roche Hitachi 917 Chemistry Analyzer. Transferrin saturation was calculated from the ratio of serum iron and TIBC. Serum hepcidin was measured by using an ELISA kit (HMC-001; Intrinsic LifeSciences).

Quantification of serum non-transferrin bound iron (NTBI). NTBI was measured by adapting the method developed by Esposito *et al* (44). Briefly, iron samples of known concentration were created by mixing 70 mM nitrilotriacetate (NTA) (pH = 7.0) with 20 mM ferrous ammonium sulfate. Fe^{2+} was allowed to oxidize to Fe^{3+} in ambient air for at least 30 min and then the solution was diluted to 0.2 mM before further serial dilutions to create a ladder. 5 μl

399 of ladder was loaded in a 96-well plate containing 195 µl plasma-like medium with or without
400 100 µM deferiprone. The composition of the plasma-like medium was: 40 mg/ml bovine serum
401 albumin, 1.2 mM sodium phosphate dibasic, 120 µM sodium citrate, 10 mM sodium bicarbonate
402 in iron-free HEPES-buffered saline (HEPES 20 mM, NaCl 150 mM, treated with Chelex-100
403 chelating resin [Bio-Rad, Hercules, CA], 0.5 mM NTA, 40 µM ascorbic acid, 50 µM
404 dihydrorhodamine, pH=7.4). 5 µl of sample was loaded in a 96-well plate containing 195 µl of
405 iron-free HEPES-buffered saline with or without 100 µM deferiprone. Microplates were read
406 every 2 minutes at 37°C over 40 min at 485/520 nm (ex/em). Final NTBI was calculated by
407 comparing the oxidation rate of DHR in the presence or absence of the strong chelator
408 deferiprone.

409 *Hepcidin synthesis*. Human hepcidin (DTHFPICIFCCGCCHRSKCGMCCKT) was
410 synthesized at Ferring Research Institute, San Diego, CA. The linear peptide was assembled on
411 Rink amide resin using Tribute peptide synthesizer and the peptide was cleaved from the resin
412 with the TFA/TIS/EDT/H_2O 91:3:3:3 (v/v/v/v) cocktail. The solvents were evaporated, and the
413 crude peptide was precipitated with diethyl ether, reconstituted in 50% aqueous acetonitrile and
414 lyophilized. The lyophilizate was dissolved in 30% aqueous acetonitrile at the concentration of
415 0.05 mM and the pH of the solution was adjusted to 7.8 with 6 M ammonium hydroxide. Folding
416 was achieved within 4 hours using the cysteine/cystine redox (peptide/Cys/Cys_2 1:6:6 molar
417 ratio). The reaction mixture was acidified to pH 3, loaded onto HPLC prep column and purified
418 in a TFA based gradient. The identity of the peptide was confirmed by mass spectrometry and by
419 coelution with a commercially available sample (Peptide International, #PLP-3771-PI).

420 *Quantitative real-time PCR (qPCR)*. RNA was extracted from livers by using the RNeasy
421 kit (Qiagen). cDNA was synthesized from 1 µg RNA by using the OneScript® Plus cDNA

422 Synthesis Kit (Applied Biological Materials Inc.). Gene-specific primers pairs (Table S1) were
423 validated by dissociation curve analysis and demonstrated amplification efficiency between 90-
424 110 %. SYBR Green (Bioline) and primers were used to amplify products under following
425 cycling conditions: initial denaturation 95°C 10 min, 40 cycles of 95°C 5 s, 58°C 30 s, 72°C 10
426 s, and final cycle melt analysis between 58°-95°C. Relative mRNA expression was calculated by
427 the $2^{-\Delta\Delta Ct}$ method (45). Data were normalized to murine ribosomal protein L19 (*Rpl19*). Data are
428 reported as fold increases compared to samples from wild type mice on standard diet (SD).

429 *Polysome fractionation*. RNA was freshly prepared from frozen livers. Linear sucrose
430 gradients were prepared the day before the experiment by using 5% (w/v) and 50% (w/v) sucrose
431 solutions with 10x gradient buffer (200 mM HEPES pH=7.6, 1 M KCl, 50 mM $MgCl_2$, 0.1
432 mg/ml Cycloheximide, 1 tablet cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail
433 (Roche), 200 U/mL Recombinant RNasin® Ribonuclease Inhibitor (Promega), 2 mM DTT).
434 Linear gradients were prepared in Polyallomer Centrifuge Tubes (Beckman Coulter). Tubes were
435 marked using a gradient cylinder (BioComp), and 5% sucrose solution was added using a syringe
436 with a layering needle (BioComp) until solution level reached the mark. Then, 50% sucrose
437 solution was layered underneath the 5% solution until the interface between the two solutions
438 reached the mark. Tubes were capped with rate zonal caps (BioComp) and linearized using a
439 Gradient Master 108 (Biocomp). All reagents were nuclease-free and all solutions were kept on
440 ice or at 4°C. Sample preparation was adapted from Liang *et al.* (46). Briefly, livers were flash
441 frozen upon collection. Roughly 30-80 mg of tissue was crushed using a mortar and pestle in the
442 presence of liquid nitrogen to prevent thawing. Tissues were lysed in up to 1 ml of hypotonic
443 lysis buffer (5 mM Tris-Hcl pH=7.5, 1.5 mM KCl, 2.5 mM $MgCl_2$, 2 mM DTT, 1 mg/ml
444 Cycloheximide, 200 U/ml Recombinant RNasin® Ribonuclease Inhibitor (Promega), 1 tablet

445 cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (Roche), 0.5% (v/v) Triton X-100,
446 0.5% (v/v) Sodium Deoxycholate) and homogenized using Dounce homogenizers (60
447 movements with both loose and tight pestles) on ice. Samples were centrifuged at 4°C, 16,060g
448 for 4 minutes and supernatants were collected. Sample optical density was measured at 260 nM
449 and samples were normalized to either the lowest value or 30 ODs. 450 µl of sucrose gradient
450 was removed from the top and replaced with normalized sample. Tube weights were balanced by
451 weight before centrifugation at 200,000g for 2 h at 4°C in a SW 41 Ti rotor and a Beckman
452 Optima L-60 Ultracentrifuge. Samples were fractionated using a BR-188 Density Gradient
453 Fractionation System (Brandel). Immediately upon collection, 800 µl of samples were mixed
454 with 1 ml of TRIzol™ and kept on ice before storage at -80°C. Polysomal RNA was processed
455 according to the manufacturer's protocol. mRNA distribution was analyzed as previously
456 described (47).

457 *Electrophoretic mobility shift assay (EMSA)*. IRE-binding activities from liver and spleen
458 were analyzed by EMSA using a radioactive ^{32}P-labelled IRE probe, according to established
459 procedures (26). EMSAs were also performed in extracts from hepatocytes and non-parenchymal
460 cells, which were separated by using a 2-step collagenase perfusion technique, as previously
461 described (9).

462 *Western blotting*. Livers were washed with ice-cold PBS and dissected into pieces.
463 Aliquots were snap frozen at liquid nitrogen and stored at −80°C. Protein lysates were obtained
464 as described (21). Lysates containing 40 µg of proteins were analyzed by SDS-PAGE on 9-13%
465 gels and proteins were transferred onto nitrocellulose membranes (BioRad). The blots were
466 blocked in non-fat milk diluted in tris-buffered saline (TBS) containing 0.1% (v/v) Tween-20
467 (TBS-T), and probed overnight with antibodies against ferroportin (48) (1:1000 diluted

468 monoclonal rat anti-mouse 1C7, kindly provided by Amgen Inc), β-actin (1:2000 diluted;
469 Sigma), or Tfr2 (1:1000 diluted rabbit polyclonal; Alpha Diagnostics). Following a 3x wash with
470 TBS-T, the membranes were incubated with peroxidase-coupled secondary antibodies for 1 h.
471 Immunoreactive bands were detected by enhanced chemiluminescence with the Western
472 Lightning ECL Kit (Perkin Elmer).

473 *Immunohistochemistry*. Tissue specimens were fixed in 10% buffered formalin and
474 embedded in paraffin. Samples from 3 different mice for each experimental condition were cut at
475 4-µm, placed on SuperFrost/Plus slides (Fisher) and dried overnight at 37°C. The slides were
476 then loaded onto the Discovery XT Autostainer (Ventana Medical System) for automated
477 immunohistochemistry. Slides underwent de-paraffinization and heat-induced epitope retrieval.
478 Immunostaining was performed by using 1:500 diluted rabbit polyclonal antibodies against
479 ferroportin (49) and an appropriate detection kit (Omnimap rabbit polyclonal HRP, #760-4311
480 and ChromoMap-DAB #760-159; Roche). Negative controls were performed by the omission of
481 the primary antibody. Slides were counterstained with hematoxylin for four minutes, blued with
482 Bluing Reagent for four minutes, removed from the autostainer, washed in warm soapy water,
483 dehydrated through graded alcohols, cleared in xylene, and mounted with Permount (Fisher).
484 Sections were analyzed by conventional light microscopy and quantified by using the Aperio
485 ImageScope software (Leica Biosystems) (9).

486 *Perls Prussian blue staining*. To visualize non-heme iron deposits, deparaffinized tissue
487 sections were stained with Perls' Prussian blue using the Accustain Iron Stain kit (Sigma).

488 *Quantification of liver iron content (LIC)*. Total liver iron was quantified by using the
489 ferrozine assay (20) or inductively coupled plasma mass spectrometry (ICP-MS) (50).

490 *Iron speciation analysis*. Iron redox speciation analysis in the liver was performed by
491 capillary electrophoresis (CE) coupled to ICP-MS (CE-ICP-MS). Dynamic reaction cell (DRC)
492 technology (ICP-DRC-MS) with NH_3 as DRC-gas was applied for non-interfered monitoring of
493 the iron isotopes. A "PrinCe 706" CE system (PrinCe Technologies B.V., Emmen, Netherlands)
494 was employed for separation of iron species at +20 kV. Temperature settings for sample/buffer
495 tray and capillary were set to 20°C. An uncoated capillary (100 cm x 50 µm ID; CS-
496 Chromatographie Service GmbH, Langerwehe, Germany) was used for separation and
497 hyphenation to the ICP–DRC-MS. A CE-ICP-MS interface (50, 51) was installed which
498 provided the electrical connection between CE capillary end and outlet electrode. The self-
499 aspiration mode allowed for best flow rate adjustment and avoided suction flow. Electrolytes for
500 sample stacking and electrophoretic separation were 10% HCl = leading electrolyte, 0.05 mM
501 HCl= terminating electrolyte and 50 mM HCl = background electrolyte. The Fe^{2+}/Fe^{3+} ratio was
502 calculated from quantitative determined concentrations of Fe-species.

503 *Statistics*. Statistical analysis was performed by using the Prism GraphPad software
504 (version 9.1.0). Lognormally distributed data including qPCR and ELISA results were first log
505 transformed before analysis with ordinary two-way ANOVA (Tukey's multiple comparisons
506 test) for comparisons within same treatment groups (denoted by a or b on figures) or with
507 multiple unpaired t tests using the Holm-Sidak method to compare effects between treatments.
508 Normally distributed data was analyzed by two-way ANOVA using either Sidak's method for
509 comparisons between treatment groups or Tukey's multiple comparisons test within treatments
510 groups. Probability value $p<0.05$ was considered statistically significant.

511

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Competing interests

The authors declare no competing financial interests.

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Figures legends

Fig. 1. Iron overload blunts hepcidin responsiveness to LPS-induced inflammation. Four-week-old male wt mice (n=12-14 per group) were placed on HID for five weeks. Conversely, age- and sex-matched isogenic Hjv-/- mice (n=12-14 per group) were placed on IDD for five weeks to prevent excessive iron overload. Controls from both genotypes were kept on SD. Half of the mice were injected with saline and the other half with 1 μg/g LPS; all animals were sacrificed 4 hours later. Sera were collected by cardiac puncture and analyzed for: (A) iron, (B) transferrin saturation, (C) NTBI, and (D) hepcidin. Livers were dissected and processed for LIC quantification by the ferrozine assay (E) and for histological detection of iron deposits by Perls' staining (magnification: 10x). Livers were also used for qPCR analysis of following mRNAs: (G) *Hamp*, (H) Id1, (I) Socs3, (J) *Fpn(+IRE)*, (K) *Dmt1*, (L) *Zip14*, (M) *Lcn2* and (N) *Tfrc*. The

663 dotted line in (A) and (B) indicates baseline serum iron and transferrin saturation, respectively,
664 of control wt mice on SD. Data in (A-E) are presented as the mean±SEM while in (G-N) are
665 presented as geometric mean±SD. Statistically significant differences ($p<0.05$) compared to
666 values from saline- or LPS-treated wt control mice are indicated by a or b, respectively.

667

668 **Fig. 2. Effects of LPS on hepatic and splenic ferroportin of iron-manipulated wt and Hjv-/-**
669 **mice.** Livers and spleens from mice described in Fig. 1 were dissected and processed for
670 immunohistochemical and biochemical analysis of ferroportin. Immunohistochemical staining of
671 ferroportin in liver (A) and spleen (C) sections (magnification for liver is 20x and for spleen 5x).
672 Western blot for ferroportin and β-actin in liver (B) and spleen (D) extracts from four
673 representative mice in each condition. Blots were quantified by densitometry and ferroportin/β-
674 actin ratios are shown on the right. Densitometric data are presented as the mean±SEM.
675 Statistically significant differences ($p<0.05$) compared to values from saline- or LPS-treated wt
676 control mice are indicated by a or b, respectively.

677

678 **Fig. 3. Iron depletion of Hjv-/- mice improves the efficacy of synthetic hepcidin to promote**
679 **hypoferremia.** Four-week-old wt male mice (n=12-14 per group) were placed on HID for five
680 weeks. Conversely, age- and sex-matched isogenic Hjv-/- mice (n=12-14 per group) were placed
681 on IDD for five weeks to prevent excessive iron overload. Controls from both genotypes were
682 kept on SD. Half of the mice were injected every 2 hours for a total of 4 injections with saline,
683 and the other half with 2.5 μg/g synthetic hepcidin. Sera were collected by cardiac puncture and
684 analyzed for: (A) iron, (B) transferrin saturation, and (C) NTBI. Livers and spleens were

685 dissected and processed for analysis of: (D) LIC and (E) SIC by the ferrozine assay. (F) qPCR
686 analysis of liver *Hamp* mRNA (F). (G) Western blot analysis of liver Tfrc, Tfr2, and β-actin; a
687 representative image (out of n=2 samples) is shown on the left; the blots were quantified by
688 densitometry and Tfrc/β-actin or Tfr2/β-actin ratios are shown on the right. The dotted line in
689 (A) and (B) indicates baseline serum iron and transferrin saturation, respectively, of control wt
690 mice on SD. Data in (A-E) are presented as the mean±SEM and in (F) as geometric mean±SD.
691 Statistically significant differences ($p<0.05$) compared to values from saline- or hepcidin-treated
692 wt control mice are indicated by a or b, respectively.

693

694 **Fig. 4. Effects of synthetic hepcidin on hepatic and splenic ferroportin of iron-manipulated**
695 **wt and Hjv-/- mice.** Livers and spleens from mice described in Fig. 3 were dissected and
696 processed for immunohistochemical and biochemical analysis of ferroportin.
697 Immunohistochemical staining of ferroportin in liver (A) and spleen (C) sections (magnification
698 for liver is 20x and for spleen 10x). Western blot for ferroportin and β-actin in liver (B) and
699 spleen (D) extracts from four representative mice in each condition. Blots were quantified by
700 densitometry and ferroportin/β-actin ratios are shown on the right. Densitometric data are
701 presented as the mean±SEM. Statistically significant differences ($p<0.05$) compared to values
702 from saline- or hepcidin-treated wt control mice are indicated by a or b, respectively.

703

704 **Fig. 5. Dietary iron manipulations trigger IRP responses in the liver and spleen, as well as**
705 **in primary hepatocytes and non-parenchymal liver cells of wt and Hjv-/- mice.** Whole liver
706 (A), whole spleen (B), isolated hepatocytes (C) or isolated non-parenchymal liver cells (D) from

707 the mice described in Fig. 3 were analyzed for IRE-binding activity by EMSA with a ^{32}P-labelled
708 IRE probe in the absence (top) or presence (bottom) of 2% mercaptoethanol (2-ME). Two or
709 three representative samples from each condition are shown. The positions of IRE/IRP1 and
710 IRE/IRP2 complexes are indicated by arrows. Shorter and longer exposures of the
711 autoradiograms are shown in the left and right panels, respectively. (E) Isolated hepatocytes and
712 isolated non-parenchymal liver cells were analyzed by Western blotting for expression of
713 ferroportin and β-actin. Blots were quantified by densitometry and ferroportin/β-actin ratios are
714 shown on the right. Densitometric data are presented as the mean±SEM.

715

716 **Fig. 6. Iron regulation of Fpn(+IRE) mRNA translation in the mouse liver.** Four-week-old
717 wt male mice (n=10-14 per group) were placed on HID for five weeks. Conversely, age- and sex-
718 matched isogenic Hjv-/- mice (n=10-14 per group) were placed on IDD for five weeks to prevent
719 excessive iron overload. Controls from both genotypes were kept on SD. At the endpoint, the
720 mice were sacrificed, and livers were used for polysome profile analysis and iron assays. (A)
721 Liver polysome profiles from n=3 mice in each experimental group. Top: Recording of
722 absorbance at 254 nm of representative samples. Fraction numbers and direction of the gradient
723 are indicated. Bottom left: Distribution of *Fpn(+IRE)*, *Fth1* and *Actb* mRNAs among light
724 monosomal and heavy polysomal fractions (separated by dashed line) was analyzed by qPCR.
725 Bottom right: Bar graph comparisons of pooled fractions. Numbers indicate the fold change
726 compared to wt mice on SD. (B and C) Analysis of total iron (B), and redox iron speciation (C)
727 in the liver by CE-ICP-MS. Data are presented as the mean±SEM. Statistical analysis in (A) was
728 performed by two-way ANOVA and in (B, C) by one-way ANOVA or unpaired t-test.

729 Statistically significant differences ($p<0.05$) compared to values from wt control mice on SD are
730 indicated by a.

731

732 **Fig. 7. Elimination of ferroportin mRNA by prolonged LPS treatment potentiates hepcidin-**
733 **induced hypoferremia in mouse models of iron overload.** Four-week-old wt male mice (n=10-
734 14 per group) were placed on HID for five weeks. Conversely, age- and sex-matched isogenic
735 Hjv-/- mice (n=10-14 per group) were placed on IDD for five weeks to prevent excessive iron
736 overload. Controls from both genotypes were kept on SD. (A and B) Half of the mice were
737 injected with saline and the other half with 1 μg/g LPS and sacrificed after 8 h. Livers were
738 dissected and processed for qPCR analysis of *Fpn(+IRE)* (A) and *Fpn(-IRE)* (B) mRNAs. (C-E)
739 All mice were injected with 1 μg/g LPS. Half of the animals were subsequently injected with
740 saline, and the other half with 2.5 μg/g synthetic hepcidin every two hours for a total of 4
741 injections. At the endpoint the mice were sacrificed. Sera were collected by cardiac puncture and
742 analyzed for: (C) iron, (D) transferrin saturation, and (E) NTBI. The dotted line in (C) and (D)
743 indicates baseline serum iron and transferrin saturation, respectively, of control wt mice on SD.
744 Data are presented as (A-B) geometric mean±SD or (C-E) mean±SEM. Statistically significant
745 differences ($p<0.05$) compared to values from saline-, LPS- or hepcidin-treated wt control mice
746 on SD are indicated by a, b or c, respectively.

747

748

Table S1. List of primers used for qPCR.

Gene	GenBank accession	Forward primer sequence	Reverse primer sequence
Hamp1	NM_032541.1	*AAGCAGGGCAGACATTGCGAT*	*CAGGATGTGGCTCTAGGCTATGT*
Fpn(+IRE)	NM_016917.2	*GGCATAAGGCTGTTGTGCTT*	*TCATGACACCAGGCGTTCTC*
Fpn(-IRE)	XM_006496137.4	*GCCGGTTGGAGTTTCAATGT*	*TCATGACACCAGGCGTTCTC*
Dmt1	NM_001146161.1	*CTTGGGATACTGACGGTGAC*	*GATTTGCAGTCTGGAGCAGT*
Zip14	NM_001135151.1	*TGGATAGTGAGGCTGCGTGG*	*ATGGTGAGGCCAAGGCTAAT*
Lcn2	NM_008491	*GGCCAGTTCACTCTGGGAAA*	*TGGCGAACTGGTTGTAGTCC*
Tfrc	NM_011638.4	*AGCCAGATCAGCATTCTCTAACT*	*GCCTTCATGTTATTGTCGGCAT*
Bmp6	NM_007556.2	*ACTCGGGATGGACTCCACGTCA*	*CACCATGAAGGGCTGCTTGTCG*
Id1	NM_010495.2	*GGTACTTGGTCTGTCGGAGC*	*GCAGGTCCCTGATGTAGTCG*
Socs3	NM_007707.3	*TGCGCCTCAAGACCTTCAG*	*GCTCCAGTAGAATCCGCTCTC*
Fth1	NM_010239.2	*AAGTGCGCCAGAACTACCAC*	*AGCCACATCATCTCGGTCAA*
Rpl19	NM_009078.2	*AGGCATATGGGCATAGGGAAGAG*	*TTGACCTTCAGGTACAGGCTGTG*
Actb	NM_007393.3	*GACGACATGGAGAAGATCTG*	*GTGAAGCTGTAGCCACGCTC*

754 **Supplemental Figure legends**

755

756 **Fig. S1. Dietary iron loading does not disrupt inflammatory hepcidin induction in LPS-**
757 **treated wild type mice but blunts hepcidin-mediated hypoferremia.** Nine-week-old male
758 mice (n=12-14 per group) were fed SD or HID for one day, one week, or five weeks prior to
759 sacrifice. Half of the mice were injected intraperitoneally with saline and the other half with 1
760 μg/g LPS 4 hours before sacrifice. Sera were collected by cardiac puncture and analyzed for: (A)
761 iron, (B) transferrin saturation, (C) ferritin, and (D) hepcidin. Livers were dissected and
762 processed for biochemical analysis of: (E) liver iron content (LIC) by the ferrozine assay and (F)
763 *Hamp* mRNA by qPCR. The dotted line in (A) and (B) indicates baseline serum iron and
764 transferrin saturation, respectively, of control mice on SD. Data (A-E) are presented as the
765 mean±SEM and in (F) as geometric mean±SD. Statistically significant differences ($p<0.05$) over
766 time compared to values from saline- or LPS-treated control mice are indicated by a or b,
767 respectively.

768

769 **Fig. S2. Effects of dietary iron manipulations in splenic iron of wt and Hjv-/- mice.** Spleen
770 sections from mice described in Fig. 1 were analyzed histologically for iron deposits by Perls
771 staining (magnification: 10x).

772

773 **Fig. S3. Low magnification immunohistochemical images of ferroportin in liver sections of**
774 **dietary iron-manipulated wild type and Hjv-/- mice following LPS treatment.** Liver sections

from mice described in Fig. 1 were used for immunohistochemical analysis of ferroportin (magnifications: 10x and 5x).

Fig. S4. Low magnification immunohistochemical images of ferroportin in spleen sections of dietary iron-manipulated wild type and Hjv-/- mice following LPS treatment. Spleen sections from mice described in Fig. 1 were used for immunohistochemical analysis of ferroportin (magnification: 2x).

Fig. S5. Perls staining for iron deposits in liver and spleen sections of dietary iron-manipulated wild type and Hjv-/- mice following treatment with synthetic hepcidin. Liver and spleen sections from mice described in Fig. 3 were stained with Perls Prussian blue (magnification: 10x).

Fig. S6. Effects of LPS treatment on expression of mRNAs encoding iron transport proteins and signaling endpoints in the liver of dietary iron-manipulated wild type and Hjv-/- mice. Livers from mice described in Fig. 3 were dissected and processed for qPCR analysis of mRNAs encoding iron transport proteins and signaling endpoints. (A) *Fpn(+IRE)*, (B) *Socs3*, (C) *Id1*, (D) *Bmp6*, (E) *Dmt1*, (F) *Zip14*, (G) *Lcn2* and (H) *Tfrc*. All data are presented as the geometric mean ± SD. Statistically significant differences ($p<0.05$) compared to values from saline- or hepcidin-treated control mice are indicated by a or b, respectively.

795

796 **Fig. S7. Low magnification immunohistochemical images of ferroportin in liver sections of**
797 **dietary iron-manipulated wild type and Hjv-/- mice following treatment with synthetic**
798 **hepcidin.** Liver sections from mice described in Fig. 3 were used for immunohistochemical
799 analysis of ferroportin (magnifications: 10x and 5x).

800

801 **Fig. S8. Low magnification immunohistochemical images of ferroportin in spleen sections**
802 **of dietary iron-manipulated wild type and Hjv-/- mice following treatment with synthetic**
803 **hepcidin.** Spleen sections from mice described in Fig. 3 were used for immunohistochemical
804 analysis of ferroportin (magnification: 2x).

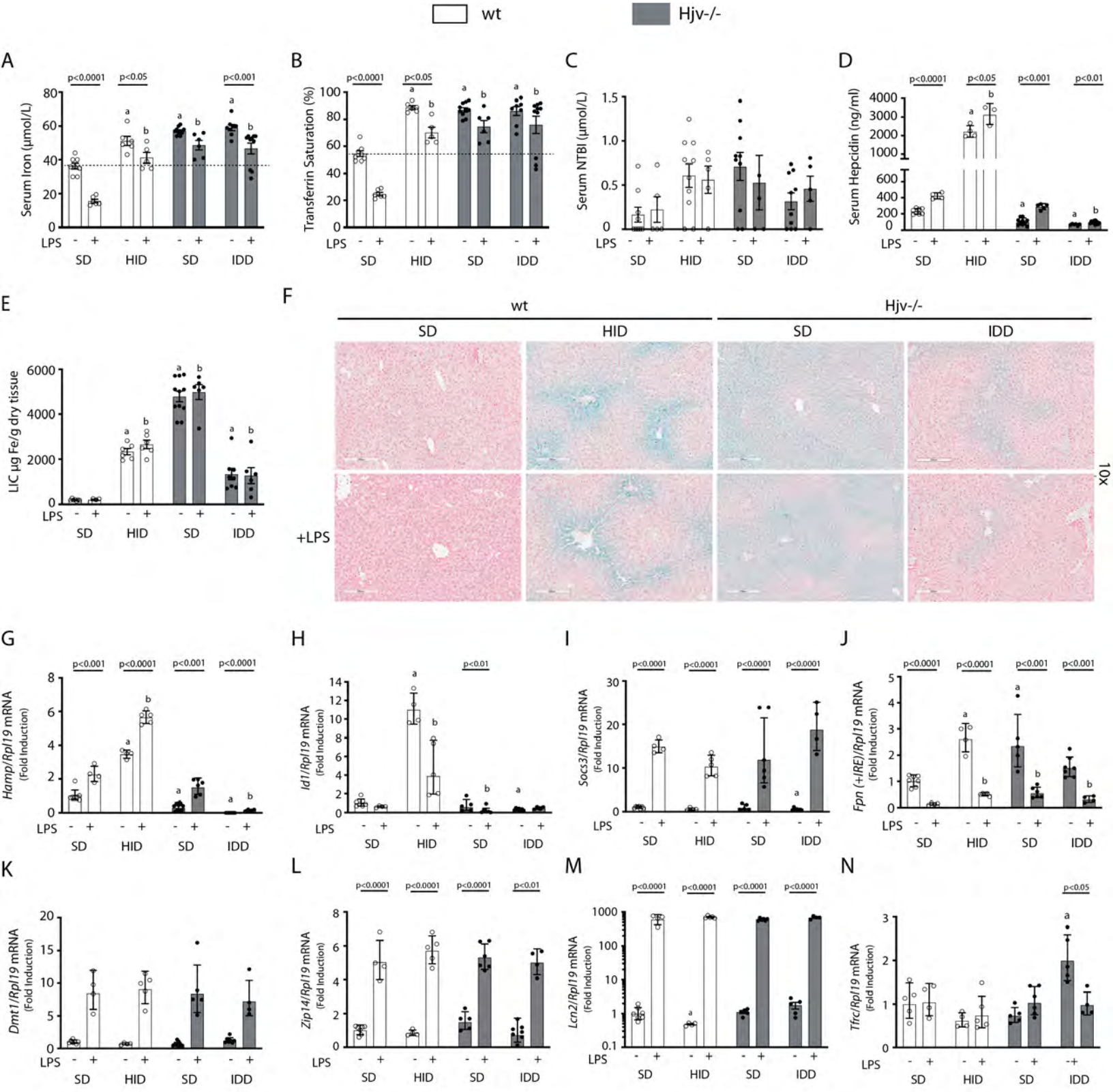
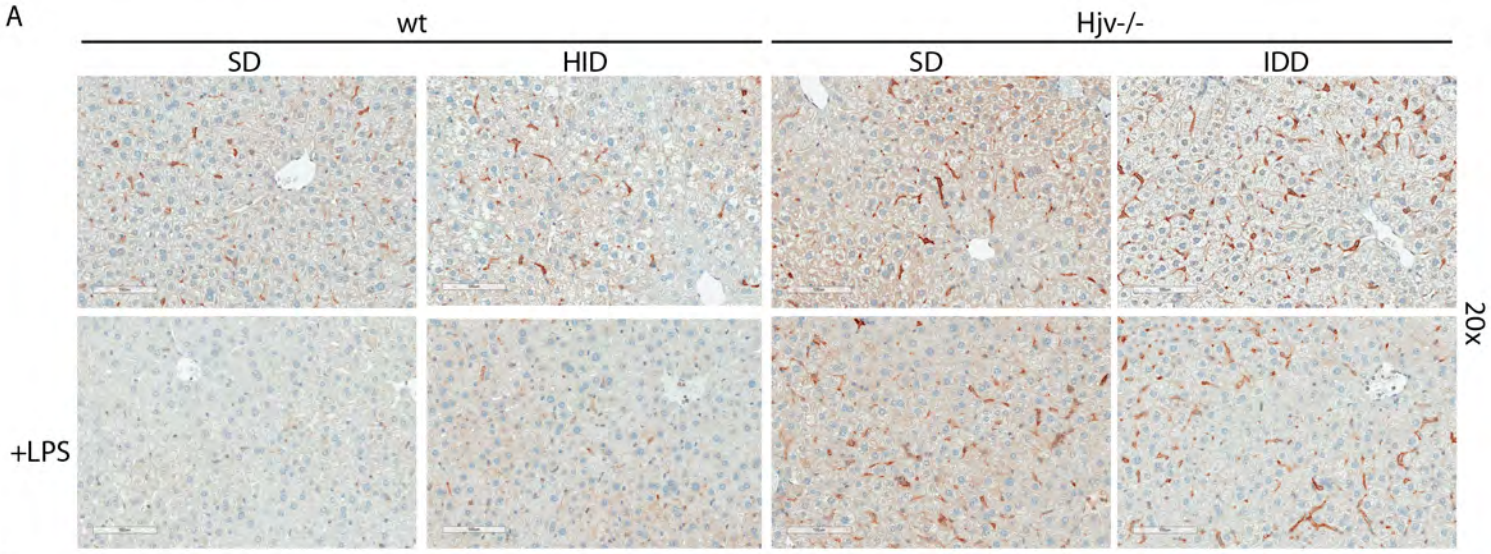
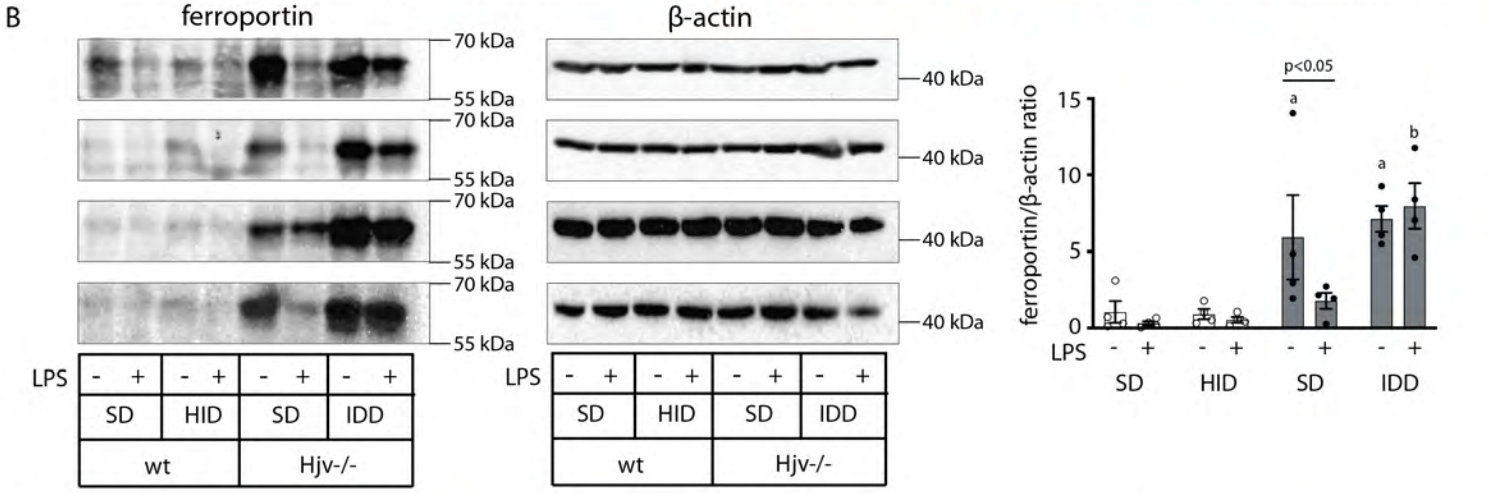
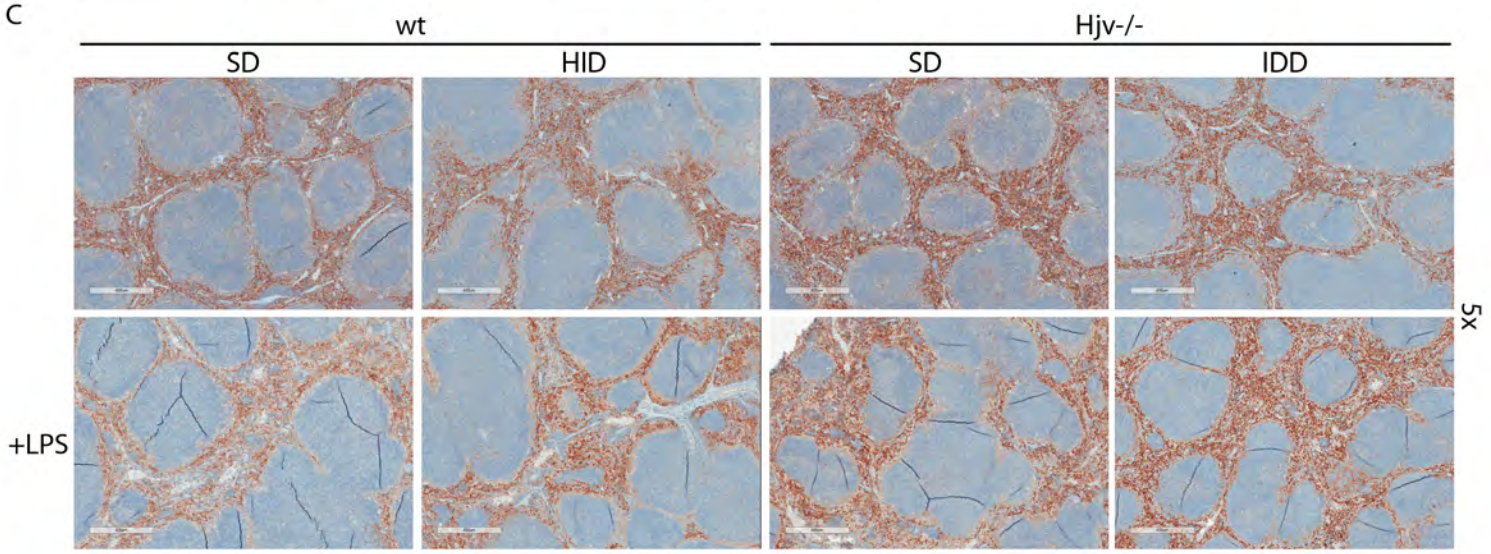

wt
Hjv-/-
A
Serum Iron (µmol/L)
B
Transferrin Saturation (%)
C
Serum NTBI (µmol/L)
D
Serum Hepcidin (ng/ml)
E
LIC µg Fe/g dry tissue
F
+LPS
10x
G
Hamp/Rpl19 mRNA (Fold Induction)
H
Id1/Rpl19 mRNA (Fold Induction)
I
Socs3/Rpl19 mRNA (Fold Induction)
J
Fpn (+IRE)/Rpl19 mRNA (Fold Induction)
K
Dmt1/Rpl19 mRNA (Fold Induction)
L
Zip14/Rpl19 mRNA (Fold Induction)
M
Lcn2/Rpl19 mRNA (Fold Induction)
N
Tfrc/Rpl19 mRNA (Fold Induction)
LPS
SD
HID
IDD

Fig. 1


A
wt
Hjv-/-
SD
HID
SD
IDD
20x
+LPS


B
ferroportin
β-actin
70 kDa
55 kDa
40 kDa
LPS
SD
HID
SD
IDD
wt
Hjv-/-
p<0.05
ferroportin/β-actin ratio
15
10
5
0
a
b


C
wt
Hjv-/-
SD
HID
SD
IDD
5x
+LPS

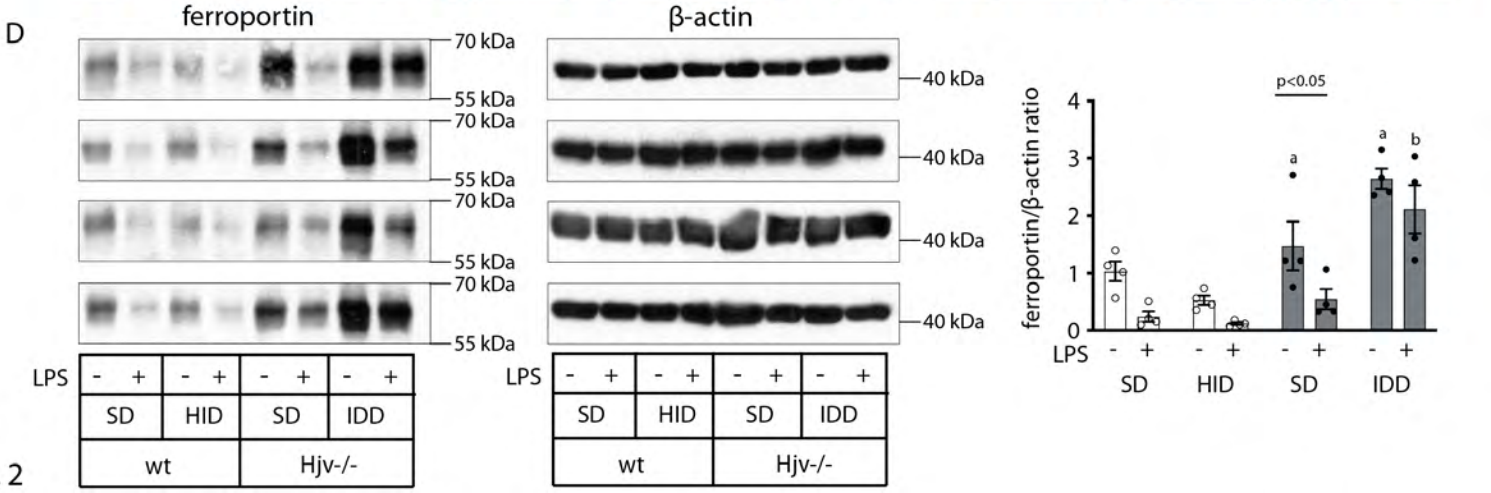

D
ferroportin
β-actin
70 kDa
55 kDa
40 kDa
LPS
SD
HID
SD
IDD
wt
Hjv-/-
p<0.05
ferroportin/β-actin ratio
4
3
2
1
0
a
b

Fig. 2

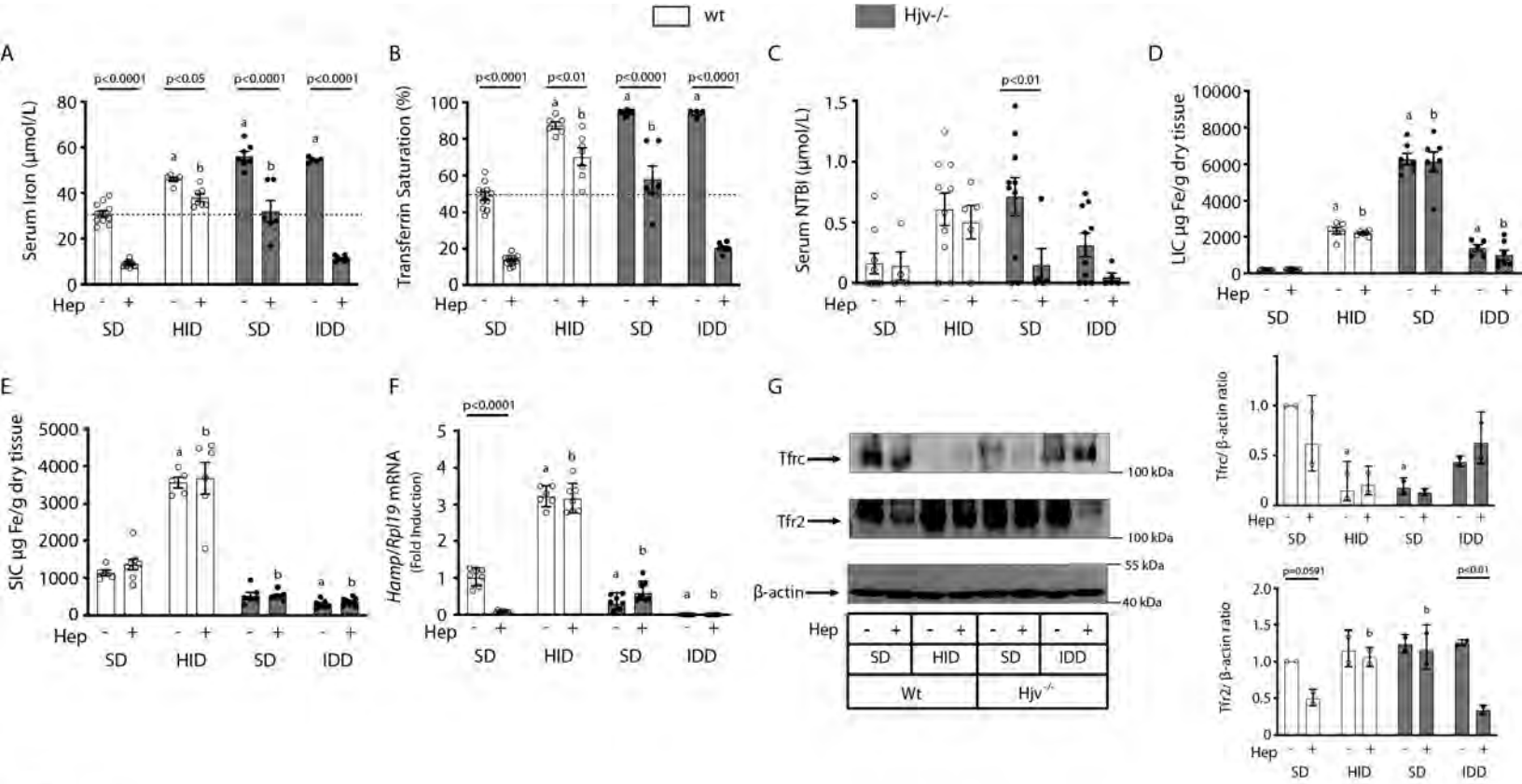

wt
Hjv-/-
A
Serum Iron (µmol/L)
p<0.0001
p<0.05
p<0.0001
p<0.0001
B
Transferrin Saturation (%)
p<0.0001
p<0.01
p<0.0001
p<0.0001
C
Serum NTBI (µmol/L)
p<0.01
D
LIC µg Fe/g dry tissue
E
SIC µg Fe/g dry tissue
F
Hamp/Rpl19 mRNA
(Fold Induction)
p<0.0001
G
Tfrc
Tfr2
β-actin
100 kDa
100 kDa
55 kDa
40 kDa
Wt
Hjv-/-
Tfrc/ β-actin ratio
Tfr2/ β-actin ratio
p=0.0591
p<0.01
Hep
SD
HID
SD
IDD

Fig. 3

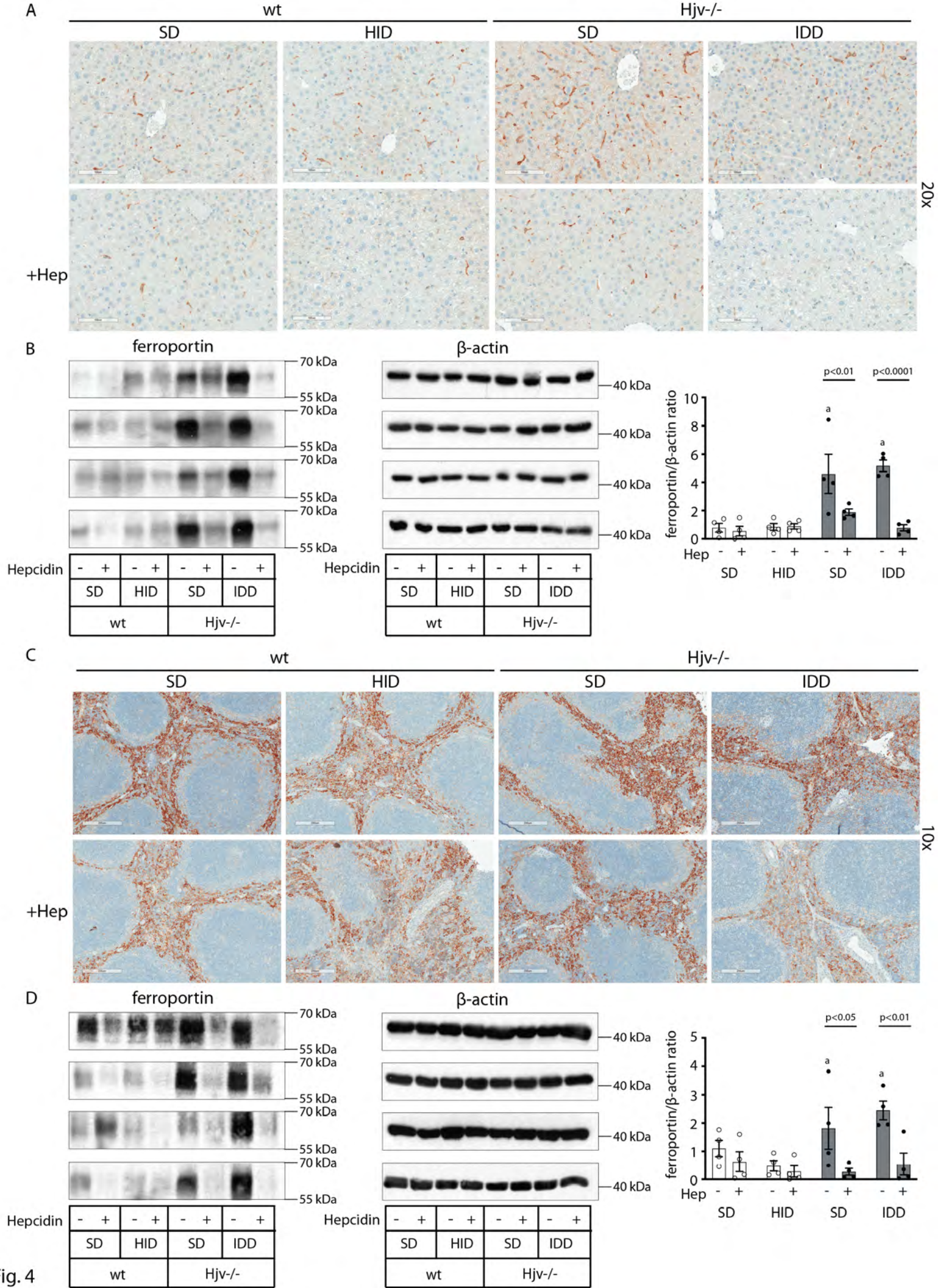

A
wt
Hjv-/-
SD
HID
SD
IDD
20x
+Hep
B
ferroportin
β-actin
70 kDa
55 kDa
40 kDa
Hepcidin
- +
SD
HID
SD
IDD
wt
Hjv-/-
ferroportin/β-actin ratio
p<0.01
p<0.0001
Hep
C
10x
D
p<0.05
p<0.01

Fig. 4

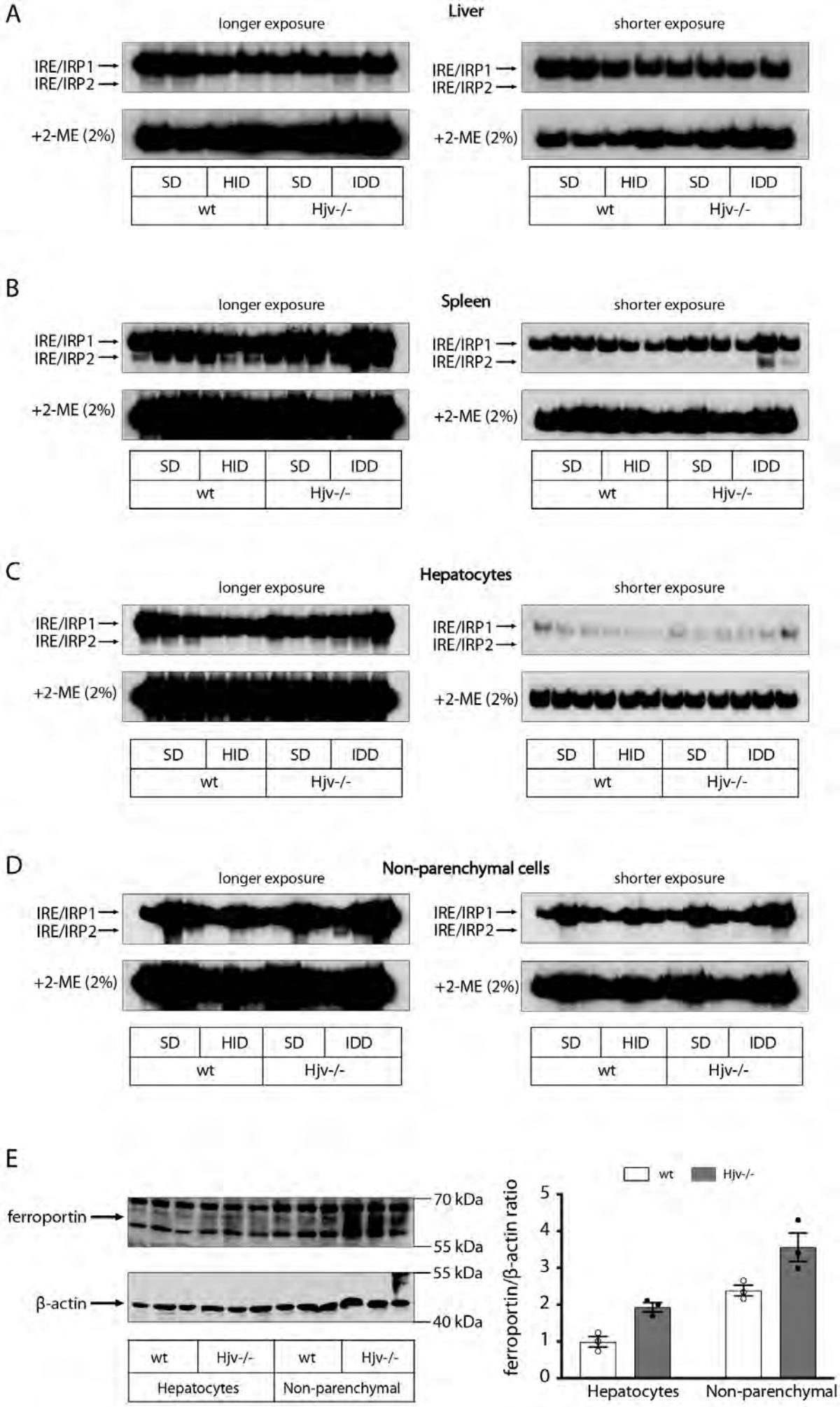

A
Liver
longer exposure
shorter exposure
IRE/IRP1
IRE/IRP2
+2-ME (2%)
SD
HID
SD
IDD
wt
Hjv-/-
B
Spleen
C
Hepatocytes
D
Non-parenchymal cells
E
ferroportin
β-actin
70 kDa
55 kDa
55 kDa
40 kDa
Hepatocytes
Non-parenchymal
ferroportin/β-actin ratio
0
1
2
3
4
5

Fig. 5

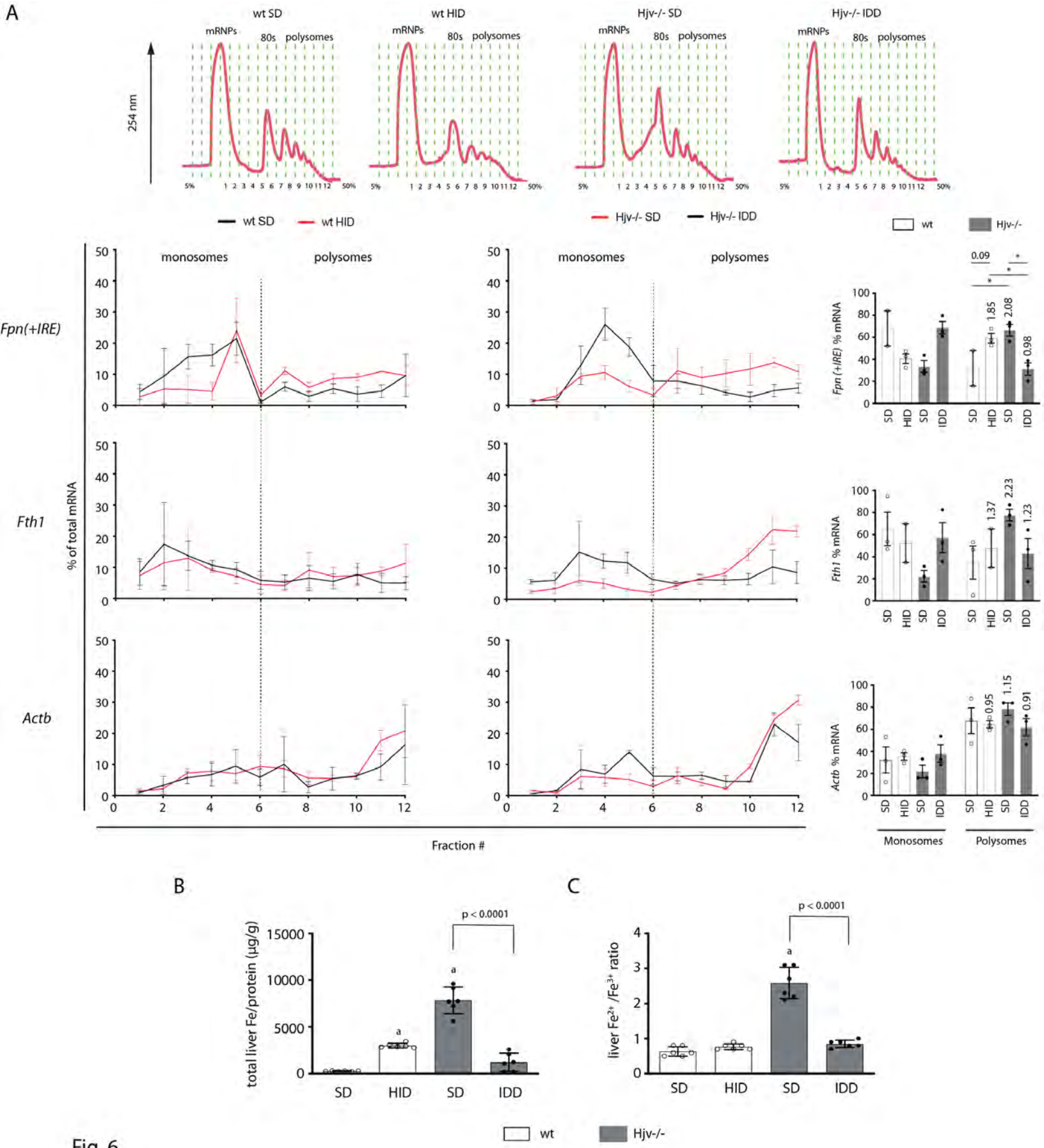

A
wt SD
wt HID
Hjv-/- SD
Hjv-/- IDD
mRNPs
80s
polysomes
254 nm
5%
50%
wt SD
wt HID
Hjv-/- SD
Hjv-/- IDD
wt
Hjv-/-
monosomes
polysomes
Fpn(+IRE)
Fth1
Actb
% of total mRNA
Fraction #
Fpn (+IRE) % mRNA
Fth1 % mRNA
Actb % mRNA
0.09
1.85
2.08
0.98
1.37
2.23
1.23
0.95
1.15
0.91
SD
HID
SD
IDD
Monosomes
Polysomes
B
total liver Fe/protein (µg/g)
p < 0.0001
C
liver Fe2+ /Fe3+ ratio
p < 0.0001
wt
Hjv-/-

Fig. 6

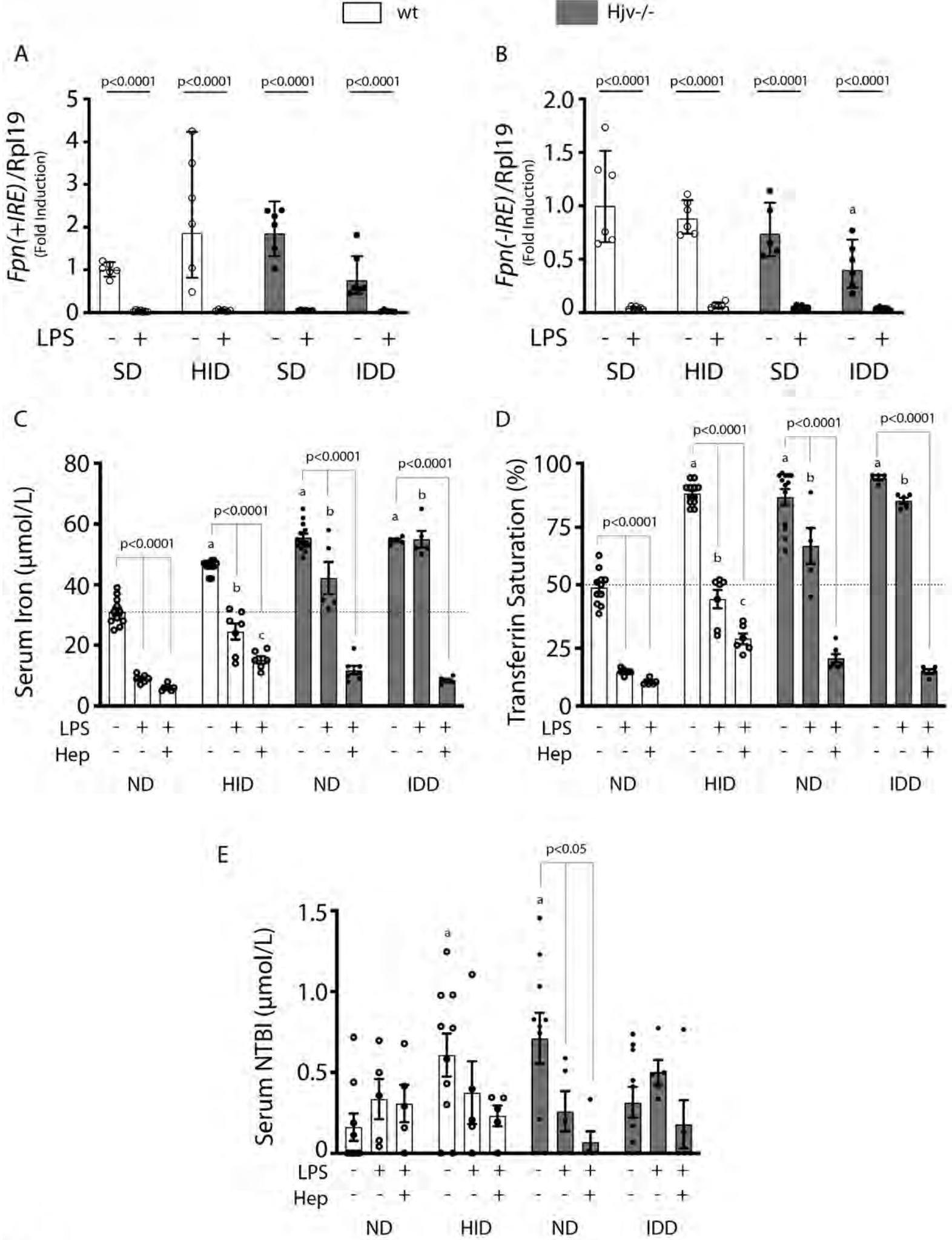

wt
Hjv-/-
A
p<0.0001
Fpn(+IRE)/Rpl19
(Fold Induction)
LPS
SD
HID
SD
IDD
B
Fpn(-IRE)/Rpl19
(Fold Induction)
C
Serum Iron (µmol/L)
Hep
ND
HID
ND
IDD
D
Transferrin Saturation (%)
E
p<0.05
Serum NTBI (µmol/L)

Fig. 7

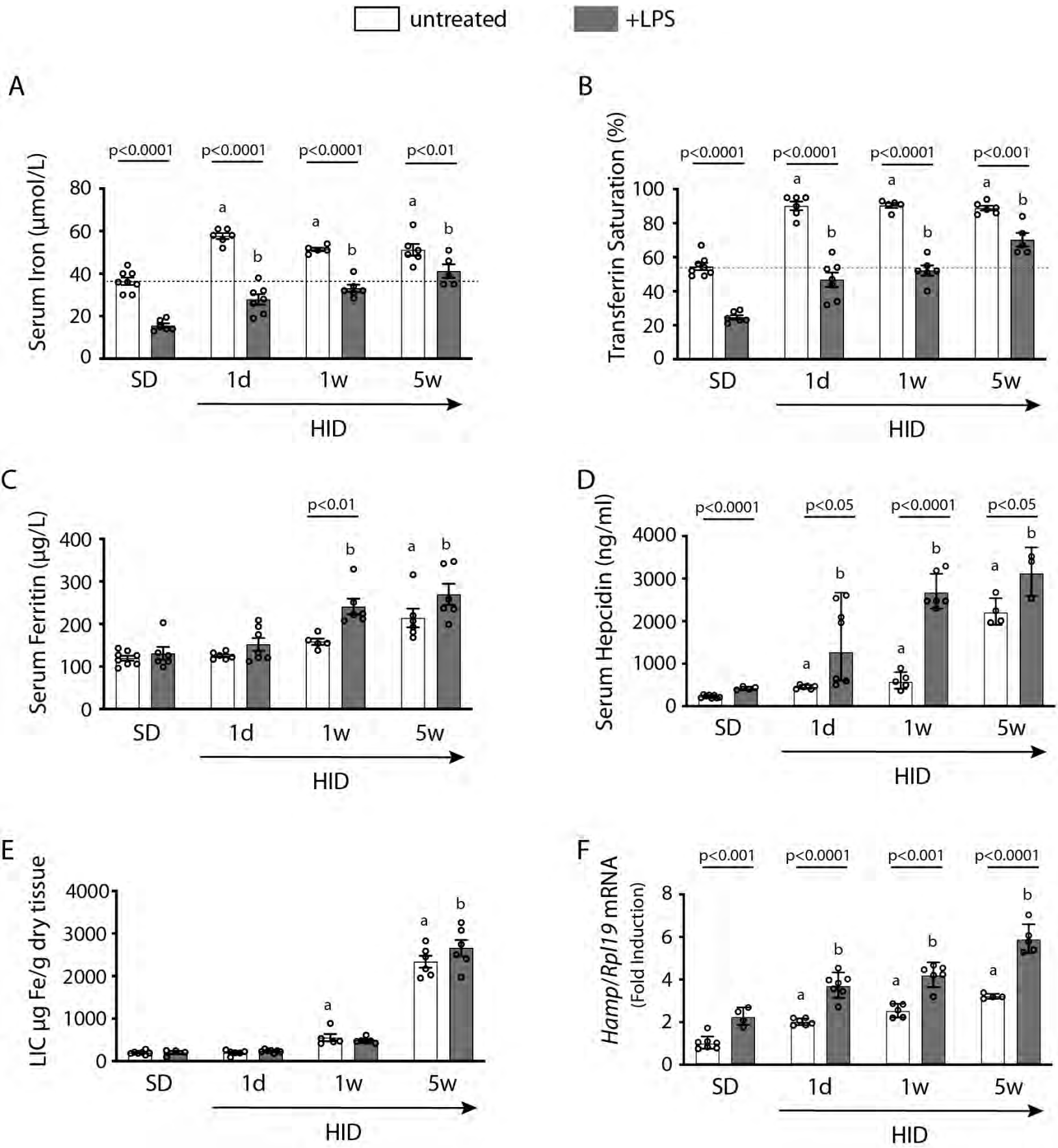

untreated
+LPS
A
Serum Iron (µmol/L)
p<0.0001
p<0.0001
p<0.0001
p<0.01
SD
1d
1w
5w
HID
B
Transferrin Saturation (%)
p<0.0001
p<0.0001
p<0.0001
p<0.001
C
Serum Ferritin (µg/L)
p<0.01
D
Serum Hepcidin (ng/ml)
p<0.0001
p<0.05
p<0.0001
p<0.05
E
LIC µg Fe/g dry tissue
F
Hamp/Rpl19 mRNA
(Fold Induction)
p<0.001
p<0.0001
p<0.001
p<0.0001

Fig. S1

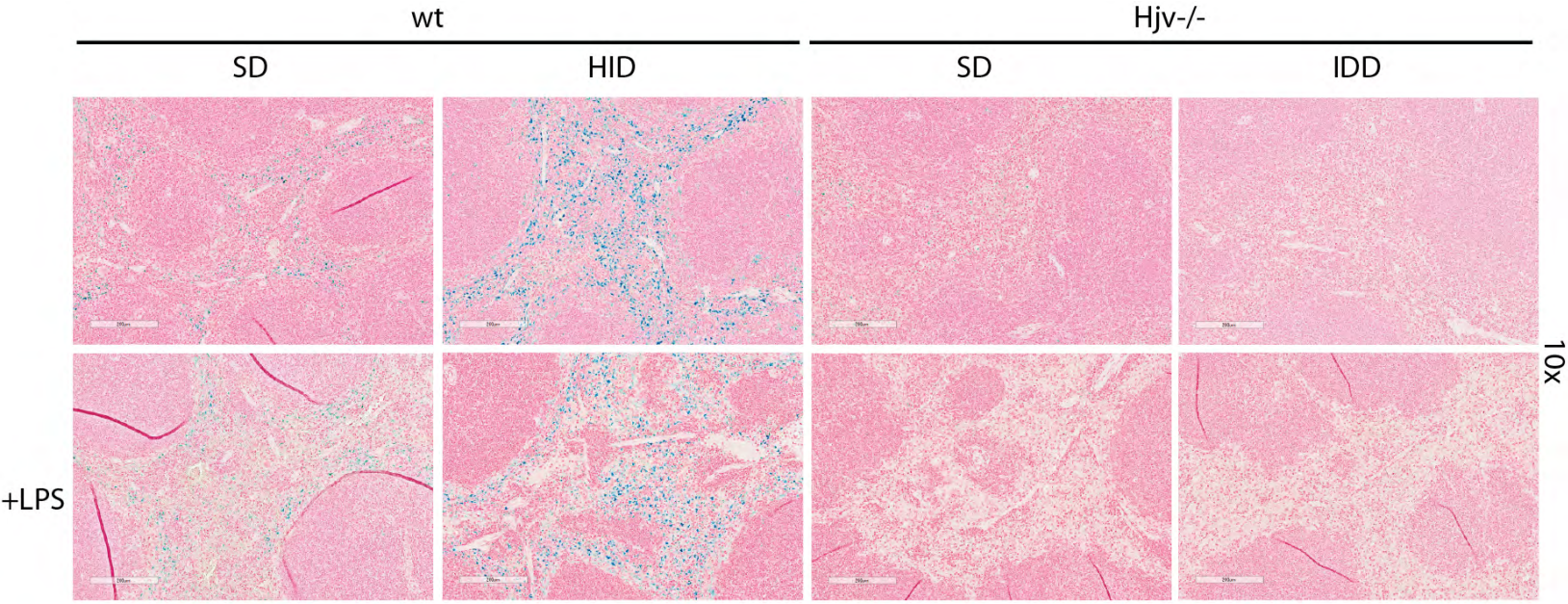


Fig. S2

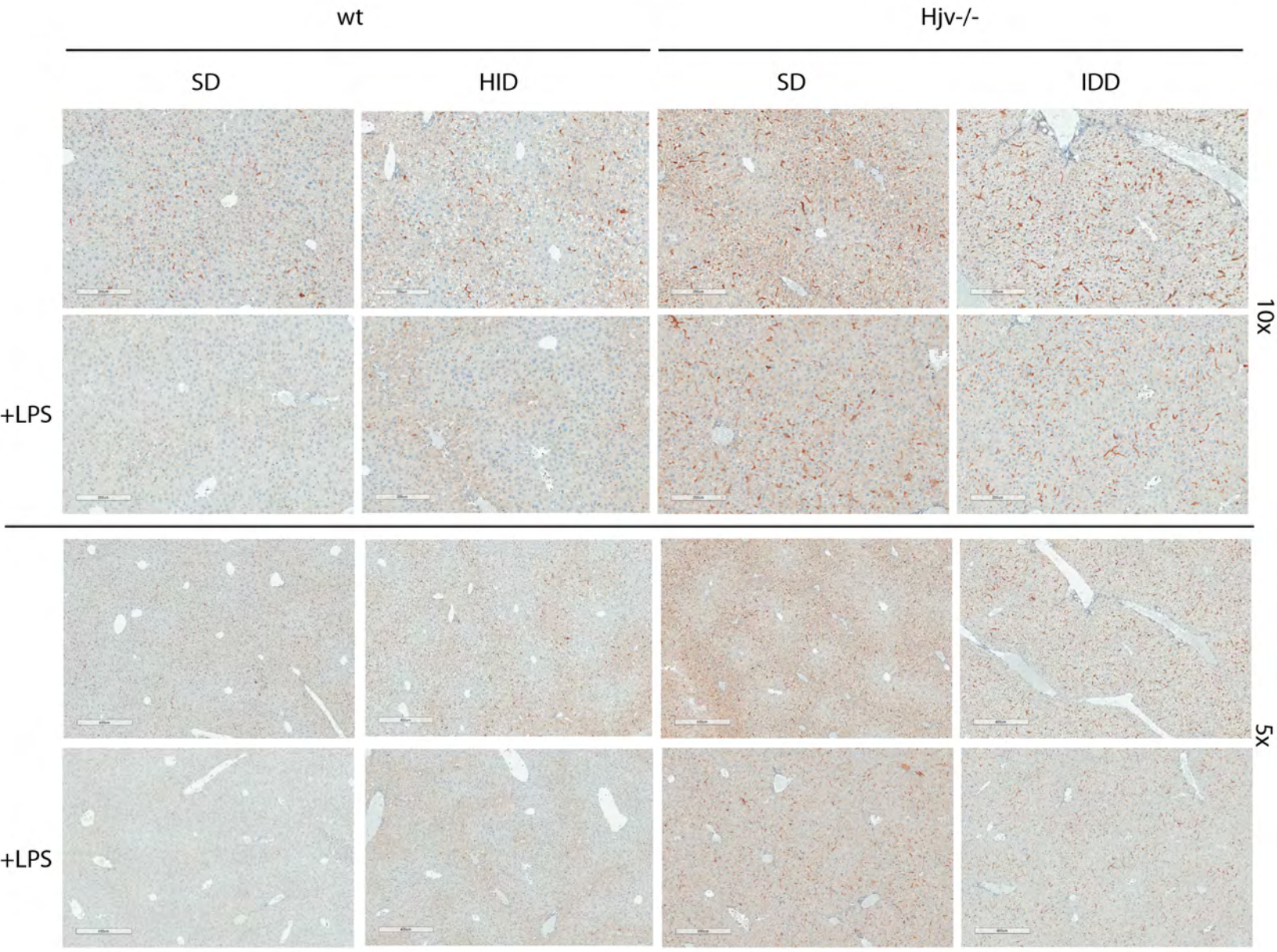


Fig. S3

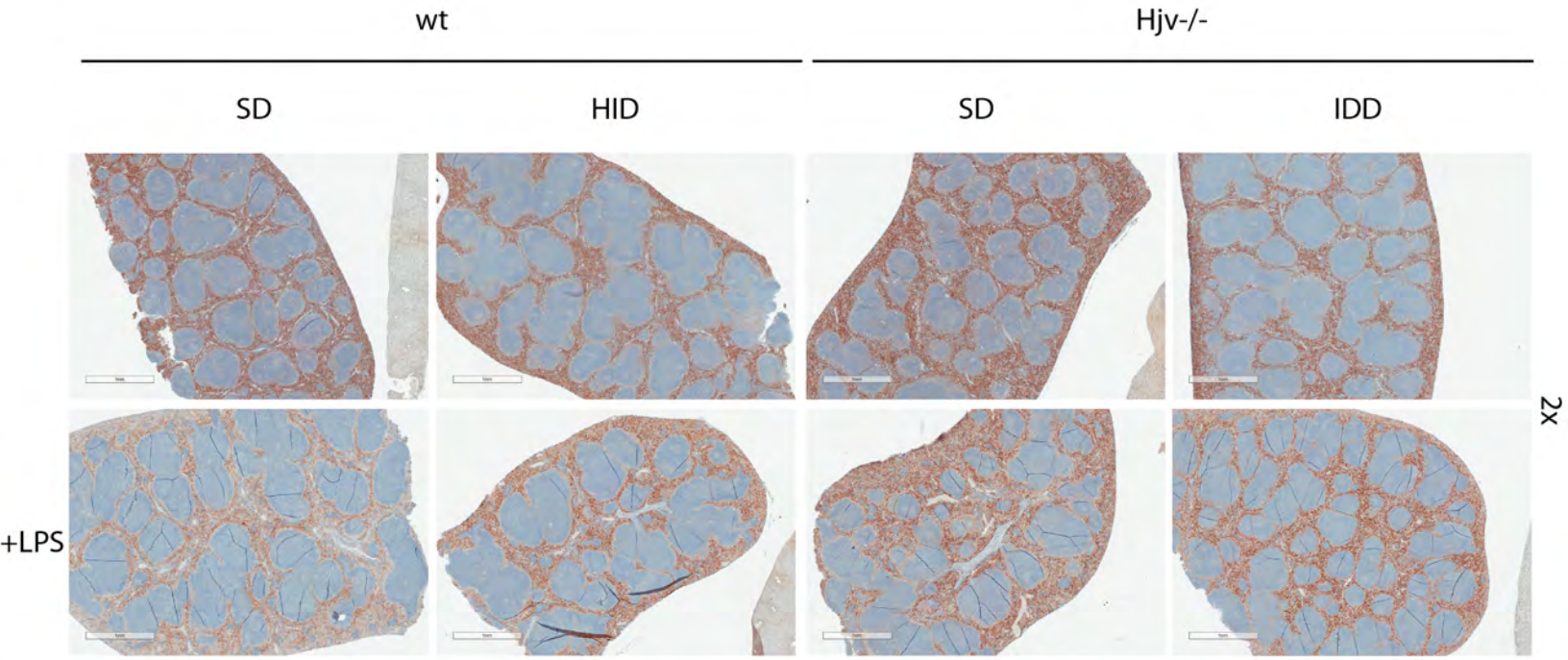


Fig. S4

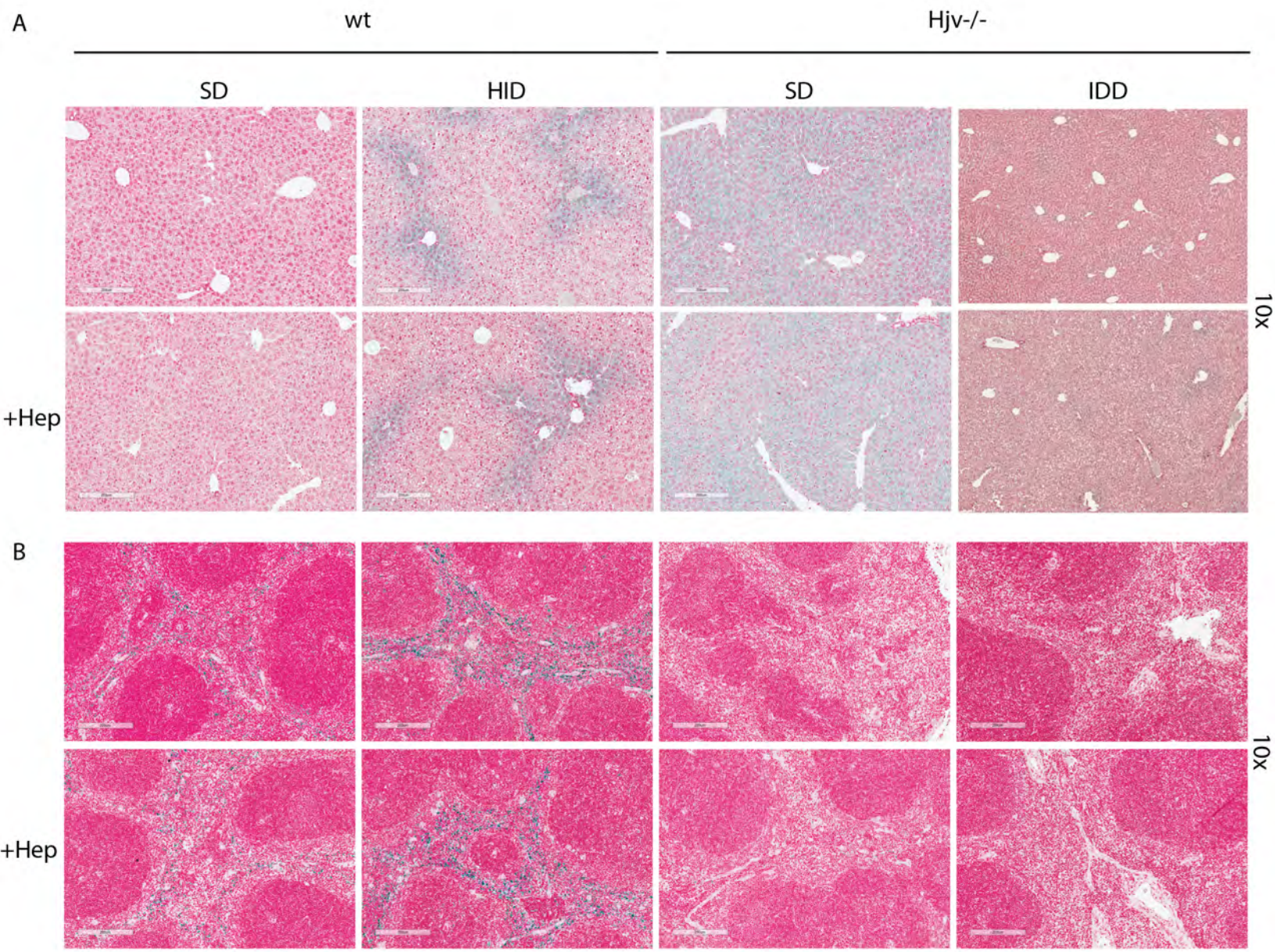


Fig. S5

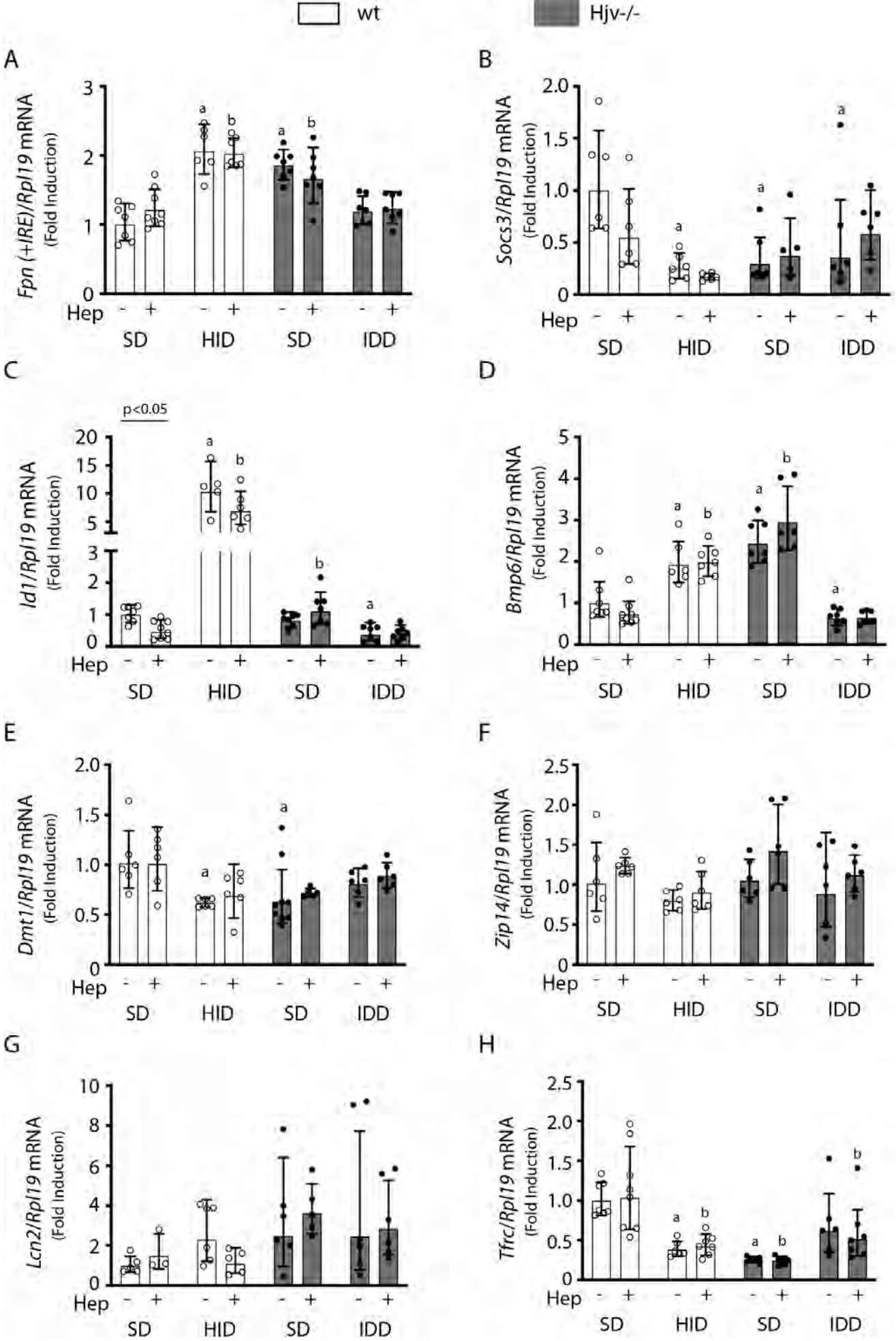

wt
Hjv-/-
A
Fpn (+IRE)/Rpl19 mRNA
(Fold Induction)
B
Socs3/Rpl19 mRNA
(Fold Induction)
C
p<0.05
Id1/Rpl19 mRNA
(Fold Induction)
D
Bmp6/Rpl19 mRNA
(Fold Induction)
E
Dmt1/Rpl19 mRNA
(Fold Induction)
F
Zip14/Rpl19 mRNA
(Fold Induction)
G
Lcn2/Rpl19 mRNA
(Fold Induction)
H
Tfrc/Rpl19 mRNA
(Fold Induction)
Hep
SD
HID
SD
IDD

Fig. S6

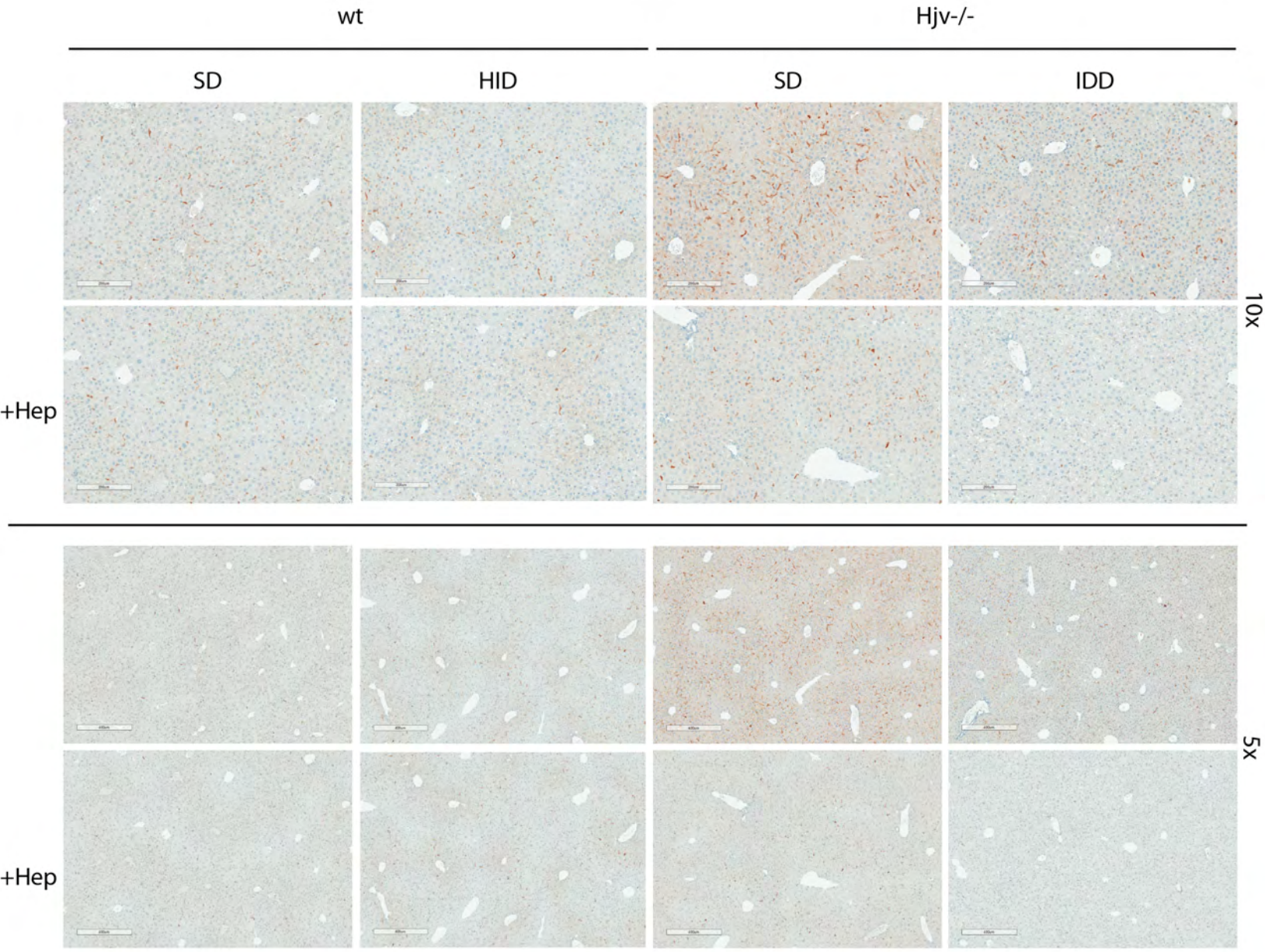


Fig. S7

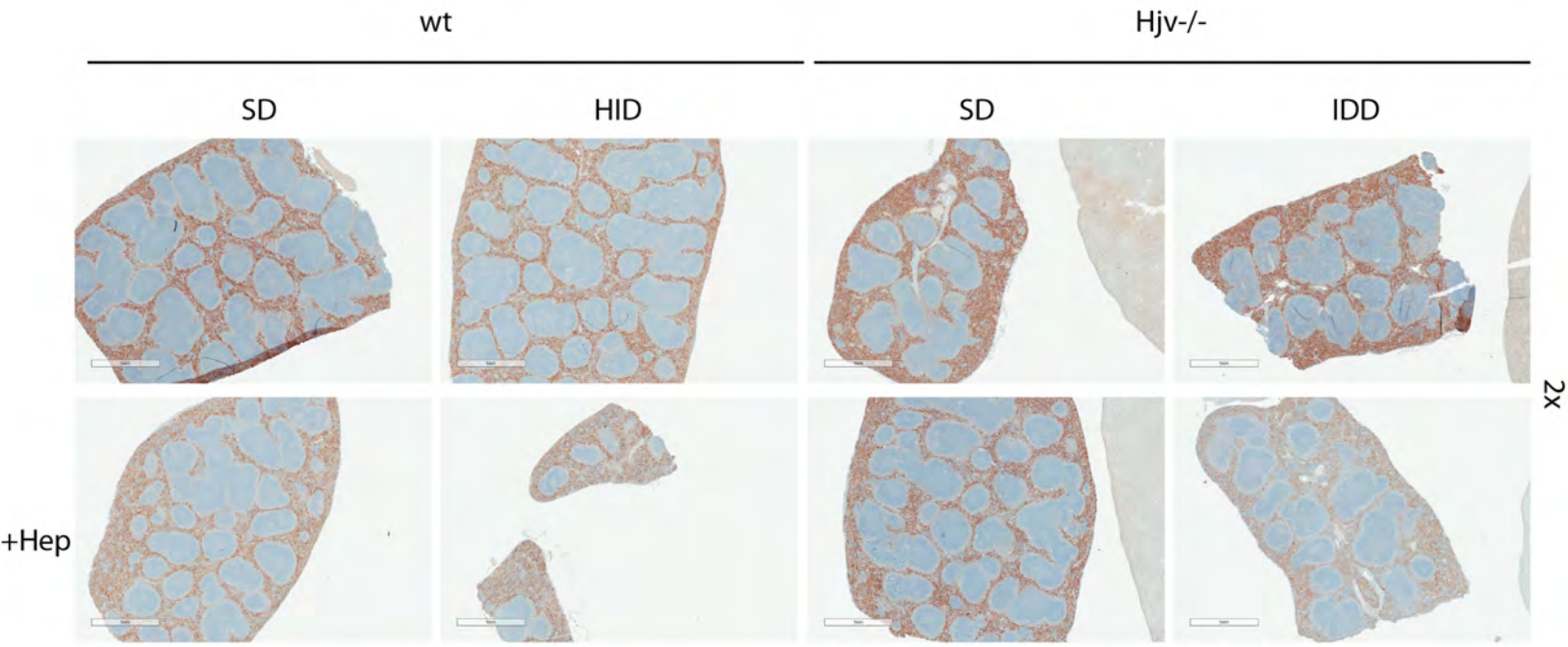


Fig. S8