

1 **Coordinate regulation of liver ferroportin degradation and *de novo* synthesis**
2 **determines serum iron levels in mice**

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22

23 **Abstract**

24 The iron hormone hepcidin is transcriptionally activated by iron or inflammation via distinct,
25 partially overlapping pathways. We addressed how iron affects inflammatory hepcidin levels and
26 the ensuing hypoferremic response. Dietary iron overload did not mitigate hepcidin induction in
27 LPS-treated wt mice but prevented effective inflammatory hypoferremia. Likewise, LPS
28 modestly decreased serum iron in hepcidin-deficient *Hjv*^{-/-} mice, model of hemochromatosis.
29 Synthetic hepcidin triggered hypoferremia only in control but not iron-loaded wt animals.
30 Furthermore, it dramatically decreased hepatic and splenic ferroportin in *Hjv*^{-/-} mice on standard
31 or iron-deficient diet, but only triggered hypoferremia in the latter. Mechanistically, iron induced
32 liver ferroportin mRNA translation, thereby antagonizing hepcidin-mediated hypoferremia.
33 Conversely, iron depletion suppressed *de novo* ferroportin synthesis in *Hjv*^{-/-} livers, allowing
34 exogenous hepcidin to cause hypoferremia. Consequently, prolonged LPS treatment eliminating
35 ferroportin mRNA permitted hepcidin-mediated hypoferremia in iron-loaded mice. Thus, liver
36 ferroportin mRNA translation is critical determinant of serum iron and finetunes hepcidin-
37 dependent functional outcomes. Our data indicate a crosstalk between hepcidin/ferroportin and
38 IRE/IRP systems. Moreover, they suggest that hepcidin supplementation therapy is more
39 efficient combined with iron depletion.

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42 **Introduction**

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

55 The hepcidin-encoding *HAMP* gene is transcriptionally induced by iron or inflammatory
56 stimuli via BMP/SMAD (6) or JAK/STAT3 (7) signaling, respectively. These pathways
57 crosstalk at different levels. For instance, the BMP co-receptor hemojuvelin (HJV), a potent
58 enhancer of iron-dependent BMP/SMAD signaling, is also essential for inflammatory induction
59 of hepcidin. Thus, *Hjv*^{-/-} mice, a model of juvenile hemochromatosis characterized by severe
60 iron overload and hepcidin deficiency (8), exhibit blunted inflammatory induction of hepcidin
61 and fail to mount a hypoferremic response following LPS treatment or infection with *E. coli* (9).

62 Herein, we utilized wild type and *Hjv*^{-/-} mice to elucidate mechanisms by which systemic
63 iron overload affects downstream hepcidin responses, and particularly, the development of
64 inflammatory hypoferremia.

65

66 **Results**

67 *Dietary iron overload does not prevent further inflammatory *Hamp* mRNA induction in*
68 *LPS-treated wt mice, but mitigates hepcidin responsiveness.* In an exploratory experiment, wt
69 mice were subjected to dietary iron loading by feeding a high-iron diet (HID) for short (1 day),
70 intermediate (1 week) or long (5 weeks) time intervals; control animals remained on standard
71 diet (SD). As expected, mice on HID for 1 day manifested maximal increases in serum iron (Fig.
72 1A) and transferrin saturation (Fig. 1B) without changes in total iron-binding capacity (TIBC;
73 Fig. 1C). They retained physiological liver iron content (LIC; Fig. 1E) and serum ferritin (Fig.
74 1D), a reflection of LIC. Serum iron and transferrin saturation plateaued after longer HID intake,
75 while LIC and serum ferritin gradually increased to peak at 5 weeks. The dietary iron loading
76 promoted gradual upregulation of serum hepcidin and liver *Hamp* mRNA, with highest values at
77 5 weeks (Fig. 1F-G). This could not prevent chronic dietary iron overload, in agreement with
78 earlier findings (10, 11).

79 LPS triggered appropriate hepcidin induction and a robust hypoferremic response in
80 control mice. Interestingly, LPS-induced inflammation resulted in further proportional increase
81 in hepcidin and *Hamp* mRNA in dietary iron-loaded mice (Fig. 1F-G). This was accompanied by
82 significant drops in serum iron and transferrin saturation. However, values did not reach the
83 nadir of LPS-treated control animals and were increasing in mice on HID for longer periods,

84 despite significant hepcidin accumulation. These data suggest that hepatic iron overload does not
85 prevent inflammatory induction of hepcidin; however, it impairs its capacity to mount a
86 hypoferremic response.

87 *Uncoupling inflammatory hepcidin induction from hypoferremic response in dietary iron*

88 *manipulated wt and HJV^{-/-} mice.* To further explore the potential of hepcidin to promote
89 hypoferremia under iron overload, wt and HJV^{-/-} mice, a model of hemochromatosis, were
90 subjected to dietary iron manipulations. Wt mice were fed SD or HID, and HJV^{-/-} mice were fed
91 SD or an iron-deficient diet (IDD) for 5 weeks. Wt mice on HID and HJV^{-/-} mice on SD or IDD
92 manifested similarly high serum iron and transferrin saturation without changes in TIBC (Fig.
93 2A-C). Serum non-transferrin bound iron (NTBI) levels were slightly elevated in the dietary and
94 genetic iron overload models and appeared to decrease in HJV^{-/-} mice on IDD (Fig. 2D). LIC was
95 substantially reduced in HJV^{-/-} mice in response to IDD, but also compared to wt mice on HID
96 (Fig. 2E). The quantitative LIC data are corroborated histologically by Perls staining (Fig. 2M).
97 Dietary iron loading increased splenic iron in wt mice and confirmed that HJV^{-/-} mice fail to
98 retain iron in splenic macrophages (Fig. 2N). As expected, hepcidin and *Hamp* mRNA were
99 induced in HID-fed wt mice and were low in HJV^{-/-} mice on SD, and further suppressed to
100 undetectable levels following IDD intake (Fig. 2F-G).

101 LPS reduced serum iron and transferrin saturation in hyperferremic wt mice on HID and
102 HJV^{-/-} mice on SD or IDD, but not below baseline of control wt mice on SD, the only animals
103 that developed a robust hypoferremic response (Fig. 2A-B). The LPS treatment was associated
104 with significant accumulation of hepcidin and induction of *Hamp* mRNA in all experimental
105 groups where this was detectable (Fig. 2F-G), while TIBC, NTBI and LIC were unaffected (Fig.
106 2C-E). Notably, LPS-treated wt mice on HID and HJV^{-/-} mice on IDD exhibited dramatic

107 differences in *Hamp* mRNA but similar blunted hypoferremic response to the acute
108 inflammatory stimulus. Thus, the profound hepcidin induction in iron-loaded wt mice cannot
109 reduce serum iron below that of iron-depleted *Hjv*^{-/-} mice with negligible hepcidin.

110 The LPS treatment strongly suppressed liver *Fpn(+IRE)* (ferroportin IRE isoform)
111 mRNA (Fig. 2H) and induced *Dmt1*, *Zip14* and *Lcn2* mRNAs (Figs 2I-K). These encode the
112 divalent metal transporter DMT1, the NTBI transporter *Zip14* and the siderophore-binding
113 protein *Lcn2*, respectively; *Lcn2* mRNA induction was dramatic. The transferrin receptor 1-
114 encoding *Tfrc* mRNA was largely unaffected by LPS, except for a reduction in *Hjv*^{-/-} mice on
115 IDD (Fig. 2L).

116 To assess the potential role of hepcidin, we first analyzed ferroportin in the liver, an
117 organ that contributes to iron sequestration during inflammation. Immunohistochemical staining
118 of liver sections revealed strong ferroportin expression in Kupffer cells, predominantly in
119 periportal areas, under all experimental conditions (Figs. 3A and S1). Hepatocellular staining is
120 also evident in the iron overload models, mostly in periportal hepatocytes (Fig. S1), and in line
121 with recent data (12). LPS triggered a shift of ferroportin in Kupffer cells from elongated
122 dendritic branches to round intracellular compartments. LPS did not affect the intensity or
123 distribution of Kupffer cell ferroportin in *Hjv*^{-/-} mice (Fig. S1), in agreement with previous
124 findings (9).

125 We further analyzed ferroportin in liver homogenates by Western blotting. Levels of
126 biochemically detectable liver ferroportin differed substantially between wt and *Hjv*^{-/-} mice.
127 Thus, they were relatively low in the former and highly induced in the latter (Fig. 3B),
128 independently of iron load. The differences were more dramatic compared to those observed by

129 immunohistochemistry (Figs. 3A and S1). Conceivably, the strong ferroportin signal in Western
130 blots from *Hjv*^{-/-} liver homogenates reflects high ferroportin expression in hepatocytes, the
131 predominant cell population. Yet, hepatocellular ferroportin is less visible by
132 immunohistochemistry because the signal is substantially weaker compared to that in Kupffer
133 cells. Interestingly, the LPS treatment profoundly suppressed total liver ferroportin in *Hjv*^{-/-}
134 mice on SD but not IDD, while it modestly affected it in wt mice (Fig. 3B). These data are
135 consistent with negative regulation of ferroportin by residual LPS-induced hepcidin in *Hjv*^{-/-}
136 mice on SD, which could explain the small drop in serum iron and transferrin saturation under
137 these acute inflammatory conditions, as reported (9). However, liver ferroportin remained
138 detectable and apparently functional, as it did not allow significant iron sequestration and
139 hypoferremia. Notably, a lack of robust hypoferremic response is also evident in LPS-treated wt
140 mice on HID, despite maximal hepcidin and minimal liver ferroportin levels.

141 Next, we analyzed ferroportin in the spleen, an organ with erythrophagocytic
142 macrophages that plays an important role in body iron traffic. Immunohistochemical analysis
143 shows that LPS reduced ferroportin in red pulp splenic macrophages from wt mice on SD, but
144 this effect was less evident in wt mice on HID and in *Hjv*^{-/-} mice on SD or IDD (Figs. 3C and
145 S2). Western blot analysis shows a stronger ferroportin signal in splenic extracts from *Hjv*^{-/-}
146 animals (Fig. 3D), consistent with immunohistochemistry. However, in this assay LPS appeared
147 to suppress splenic ferroportin in all animals except for *Hjv*^{-/-} mice on IDD. This could be a
148 result of residual hepcidin upregulation (Fig. 2F-G), while the lack of significant splenic
149 ferroportin suppression in *Hjv*^{-/-} mice on IDD may indicate hepcidin insufficiency. In any case,
150 the lack of hypoferremic response in dietary iron-loaded wt mice indicates continuous iron efflux
151 to plasma despite hepcidin excess.

152 *Is the compromised hypoferremic response to inflammation under iron overload caused*
153 *by hepcidin insufficiency?* We used human synthetic hepcidin to address whether the failure of
154 mouse models of iron overload to mount an appropriate hypoferremic response to acute
155 inflammation is caused by endogenous hepcidin insufficiency or other mechanisms. Wt and *Hjv*-
156 *-* mice subjected to dietary iron manipulations received every two hours 2.5 µg/g synthetic
157 hepcidin for a total of four intraperitoneal injections. Each dose corresponds to ~200-fold excess
158 over endogenous circulating hepcidin in control wt animals. The treatment caused hypoferremia
159 in wt mice on SD but not HID, where the decrease in serum iron was significant but well above
160 baseline of untreated wt controls (Fig. 4A-C). Likewise, synthetic hepcidin significantly
161 decreased serum iron but failed to cause hypoferremia in hepcidin-deficient *Hjv*-*-* mice on SD.
162 Strikingly, hepcidin administration was much more effective in relatively iron-depleted *Hjv*-*-*
163 mice on IDD, and reduced serum iron and transferrin saturation below baseline. The treatments
164 significantly reduced NTBI in *Hjv*-*-* mice on SD, with a trend in mice on IDD (Fig. 4D) but did
165 not affect LIC or splenic iron content (SIC) under any experimental conditions (Figs. 4E-F and
166 S3). Serum iron represents <2% of total tissue iron and therefore its acute fluctuations are not
167 expected to dramatically alter LIC or SIC.

168 Notably, synthetic hepcidin led to significant reduction of endogenous *Hamp* mRNA in
169 wt mice on SD (Fig. 4G), as earlier reported (13). Conceivably, this is related to destabilization
170 of the *Hamp* inducer *Tfr2* in the liver (Fig. 4H), a known response to hypoferremia (14).
171 Synthetic hepcidin did not promote inflammation, iron perturbations or alterations in
172 BMP/SMAD signaling in the liver, as judged by the unaltered expression of hepatic *Fpn*, *Bmp6*
173 and *Id1* mRNAs (Fig. 4I-K). Moreover, synthetic hepcidin did not affect *Tfrc*, *Dmt1*, *Zip14* or

174 *Lcn2* mRNAs (Fig. S4), which encode iron transporters; *Zip14* and *Lcn2* are also inflammatory
175 markers.

176 Next, we analyzed liver ferroportin by immunohistochemistry. Figs. 5A and S5 show that
177 exogenous hepcidin decreased ferroportin signal intensity in all animal groups to varying
178 degrees. The hepcidin effect was particularly noticeable in hepatocytes from *Hjv*^{-/-} mice (see
179 low magnification images in Fig. S5). Kupffer cells seemed to retain some ferroportin in all
180 groups except *Hjv*^{-/-} mice on IDD. Interestingly, while synthetic hepcidin decreased ferroportin
181 signal intensity in Kupffer cells, it did not alter intracellular ferroportin distribution as would be
182 expected based on the data in LPS-treated wt mice (Fig. 5A).

183 Western blotting confirmed that total liver ferroportin is highly induced in *Hjv*^{-/-} mice
184 (Fig. 5B). Again, the signal intensity can be attributed to protein expressed in hepatocytes. The
185 treatment with synthetic hepcidin did not significantly affect liver ferroportin in wt mice (either
186 on SD or HID), but substantially reduced it in *Hjv*^{-/-} mice, to almost control levels. The effect
187 appeared more pronounced in *Hjv*^{-/-} mice on IDD; nevertheless, ferroportin remained detectable.

188 Splenic ferroportin was reduced in all animal groups following hepcidin treatment, with
189 stronger effects visualized by immunohistochemistry in wt mice on SD and *Hjv*^{-/-} mice on IDD
190 (Figs. 5C and S6). At the biochemical level, ferroportin expression was again much stronger in
191 the spleen of *Hjv*^{-/-} mice (Fig. 5D). Synthetic hepcidin did not significantly affect splenic
192 ferroportin in wt mice, but dramatically reduced it in all *Hjv*^{-/-} mice.

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

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

197 *Iron-dependent regulation of ferroportin mRNA translation in the liver.* We hypothesized
198 that the functional outcomes of exogenous hepcidin may not merely depend on its capacity to
199 degrade tissue ferroportin, but also on iron-dependent ferroportin regeneration via *de novo*
200 synthesis. We addressed this in the liver, which can export iron to plasma from ferroportin-
201 expressing parenchymal cells under iron overload. Thus, we assessed the effects of iron on whole
202 liver *Fpn(+IRE)* mRNA translation by polysome profile analysis. Liver extracts from wt mice on
203 SD or HID, and *Hjv*^{-/-} mice on SD or IDD were fractionated on sucrose gradients to separate
204 translationally inactive light monosomes from translating heavy polysomes. The relative
205 distribution of *Fpn(+IRE)*, *Fth1* (positive control for iron regulation) and *Actb* (negative control)
206 mRNAs within the different fractions was quantified by qPCR. Fig. 6A shows that dietary iron
207 loading stimulates *Fpn(+IRE)* (and *Fth1*) mRNA translation in wt mice (note the shifts from
208 monosomes to polysomes). Conversely, dietary iron depletion inhibits *Fpn(+IRE)* (and *Fth1*)
209 mRNA translation in *Hjv*^{-/-} mice. We also attempted to obtain polysome profiles of *Fpn(-IRE)*
210 mRNA but it was undetectable after fractionation. These data indicate that in mouse models of
211 iron overload, iron-stimulated ferroportin synthesis in the liver antagonizes hepcidin-mediated
212 ferroportin degradation and prevents an appropriate hypoferremic response.

213 Quantification of liver iron by ICP-MS (Fig. 6B) validated iron loading of wt mice by
214 HID, and relative iron depletion of *Hjv*^{-/-} mice by IDD intake, respectively (see also Fig. 2E).
215 Livers of wt mice on HID had significantly ($p < 0.01$) higher iron content compared to *Hjv*^{-/-} mice
216 on IDD. Iron redox speciation analysis by CE-ICP-MS revealed a profound increase in $\text{Fe}^{2+}/\text{Fe}^{3+}$
217 ratios in livers of *Hjv*^{-/-} mice on SD, which was corrected by dietary iron depletion (Fig. 6C).

218 Nevertheless, there was no difference in $\text{Fe}^{2+}/\text{Fe}^{3+}$ ratios among livers of wt mice on SD or HID,
219 and *Hjv*^{-/-} mice on IDD. We conclude that an increase in total iron content, rather than excessive
220 accumulation of redox active Fe^{2+} drives *Fpn(+IRE)* (and *Fth1*) mRNA translation in the liver.

221 *Effective hypoferremic response to hepcidin in mouse models of iron overload following*
222 *prolonged LPS treatment that eliminates ferroportin mRNA.* We reasoned that complete
223 inactivation of ferroportin mRNA would restore hepcidin-induced hypoferremia despite iron
224 overload. An 8-hour treatment of mice with LPS suppresses liver *Fpn(+IRE)* mRNA below
225 detection levels (Fig. 7A), as reported (9). The same holds true for the *Fpn(-IRE)* isoform (Fig.
226 7B), which is 290 times less abundant in control mouse livers compared to *Fpn(+IRE)*
227 ($\Delta\text{Ct}=8.18$), in agreement with published data (15). We went on to examine the effects of
228 synthetic hepcidin on serum iron under these conditions of maximal *Fpn* mRNA suppression.
229 Importantly, the prolonged LPS treatment decreased serum iron in wt mice on HID below the
230 control baseline (Fig. 7C). Furthermore, when combined with synthetic hepcidin, it promoted an
231 effective hypoferremic response in wt mice on HID and *Hjv*^{-/-} mice on SD (or IDD) (Figs. 7C-
232 E), and tended to decrease NTBI (Fig. 7F). These data strongly suggest that the expression of
233 actively translating *Fpn* mRNA in iron-exporting tissues under systemic iron overload mitigates
234 hepcidin-induced drop in serum iron.

235

236 **Discussion**

237 We sought to analyze how iron overload affects hepcidin-mediated inflammatory
238 responses. We reported that excess iron inhibits the major hepcidin signaling pathways
239 (BMP/SMAD and IL-6/STAT3) in cultured cells (16). To explore the physiological relevance of

240 these findings, wt mice were subjected to variable degrees of dietary iron loading and then
241 treated with LPS. All iron-loaded mice could further upregulate hepcidin in response to LPS-
242 induced acute inflammation (Fig. 1). This is consistent with other relevant findings (17) and
243 apparently contradicts the *in vitro* data. While experimental iron loading of cultured cells is
244 rapid, dietary iron loading of mice is gradual (11) and most of excess iron is effectively
245 detoxified within ferritin, which is highly induced (18). By contrast, the suppression of hepcidin
246 preceded ferritin induction in cultured cells (16), which may explain the discrepancy with the *in*
247 *vivo* data.

248 We noted that the unimpaired inflammatory induction of hepcidin in iron-loaded wt mice
249 correlated with significant drops in serum iron, but these appeared inversely proportional to the
250 degree of systemic iron loading (Fig. 1). Thus, LPS-treated mice on 5 weeks HID failed to
251 reduce serum iron below a baseline of untreated control mice on SD. This indicates a defect in
252 mounting a hypoferremic response to inflammation, which can be attributed to mechanisms
253 antagonizing hepcidin action. To explore how iron modulates the capacity of hepcidin to trigger
254 inflammatory hypoferremia, we established conditions of iron overload using wt and *Hjv*^{-/-} mice
255 with extreme differences in hepcidin expression. Figs. 2 and 3 demonstrate that iron overload
256 prevents effective inflammatory hypoferremia independently of hepcidin and tissue ferroportin
257 levels.

258 We used a ~200-fold excess of synthetic hepcidin to directly assess its capacity to divert
259 iron traffic in iron-loaded mice. Hepcidin injection caused hypoferremia in control wt mice on
260 SD and significantly reduced serum iron in wt mice on HID and *Hjv*^{-/-} mice on SD, but not
261 below baseline (Fig. 4). Thus, synthetic hepcidin failed to cause hypoferremia in iron overload
262 models with either high or low endogenous hepcidin. Importantly, synthetic hepcidin promoted

263 robust hypoferremia in relatively iron-depleted *Hjv*^{-/-} mice on IDD, with undetectable
264 endogenous hepcidin. It should be noted that synthetic hepcidin had similar effects on tissue
265 ferroportin among wt or *Hjv*^{-/-} mice, regardless of iron diet (Fig. 5). It reduced intensity of the
266 ferroportin signal in Kupffer cells and splenic macrophages of wt mice without significantly
267 affecting biochemically detectable total protein levels. In addition, it dramatically reduced total
268 ferroportin in the liver and spleen of *Hjv*^{-/-} mice. However, in all experimental settings there was
269 residual tissue ferroportin, which appears to be functionally significant.

270 We reasoned that at steady-state, tissue ferroportin may consist of fractions of newly
271 synthesized protein, and protein that is *en route* to hepcidin-mediated degradation. Conceivably,
272 the former may exhibit more robust iron export activity, at least before its iron channel gets
273 occluded by hepcidin. Increased *de novo* synthesis of active ferroportin could explain why
274 synthetic hepcidin cannot promote hypoferremia in iron overload. In fact, Fig. 6 demonstrates
275 that dietary iron overload augments *Fpn(+IRE)* mRNA translation in the liver of wt mice.
276 Conversely, relative dietary iron depletion inhibits *Fpn(+IRE)* mRNA translation in the liver of
277 *Hjv*^{-/-} mice, in line with the restoration of hepcidin-mediated hypoferremic response (Fig. 4).

278 Considering that *Fpn(+IRE)* is the predominant *Fpn* transcript in the liver (15) and
279 harbors an “iron responsive element” (IRE), our data are consistent with translational control of
280 liver ferroportin expression via the IRE/IRP system. This accounts for coordinate post-
281 transcriptional regulation of iron metabolism proteins in cells (19, 20). In a homeostatic response
282 to iron deficiency, “iron regulatory proteins” IRP1 and IRP2 prevent translation of *Fpn(+IRE)*
283 and *Fth1* mRNAs encoding the iron efflux and storage proteins ferroportin and H-ferritin,
284 respectively, by binding to the IRE within their 5' untranslated region. IRE/IRP interactions do
285 not take place in iron-loaded cells, allowing *Fpn(+IRE)* and *Fth1* mRNA translation. While

286 translational control of ferritin is well established (19, 20), regulation of ferroportin by the
287 IRE/IRP system has hitherto only been documented in cell models (21, 22) and the mouse
288 duodenum (23), but not directly validated in the mouse liver. Moreover, the physiological
289 relevance of this mechanism remained speculative.

290 The critical role of *de novo* ferroportin synthesis in fine-tuning hepcidin-dependent
291 functional outcomes is also highlighted in Fig. 7. Thus, synthetic hepcidin was highly effective
292 as promoter of hypoferremia in dietary iron-loaded wt mice when administered together with
293 LPS. LPS is known to suppress *Fpn* mRNA in cell lines (24) and mouse tissues, with a nadir in
294 the liver reached at 8 h (9). The recovery of hepcidin effectiveness in mouse models of iron
295 overload was only possible when *Fpn* mRNA was essentially eliminated. Under these conditions,
296 LPS treatment alone was sufficient to decrease serum iron in dietary iron-loaded wt mice below
297 baseline.

298 Tissue iron uptake may be another important determinant of the hypoferremic response to
299 inflammation. LPS did not affect *Tfrc* mRNA levels in the liver (Fig. 2L), which argues against
300 increased uptake of transferrin-bound iron via TfR1. On the other hand, LPS induced *Zip14*,
301 *Dmt1* and *Lcn2* mRNAs (Figs 2J-K). *Zip14* is the NTBI transporter accounting for hepatocellular
302 iron overload in hemochromatosis (25) and is upregulated by inflammatory cues in hepatocytes
303 (26). DMT1 is dispensable for NTBI uptake by hepatocytes (27), and its inflammatory induction
304 might promote iron acquisition by macrophages (24, 28). Nevertheless, considering that the
305 fraction of NTBI represents <2% of total serum iron even in the iron overload models (Figs. 2A
306 and D), it is implausible that NTBI uptake by *Zip14* and/or DMT1 substantially contributes to
307 inflammatory hypoferremia. *Lcn2* is an acute phase protein that can sequester intracellular iron
308 bound to catecholate siderophores (29), and is more likely to transport iron to tissues during

309 infection. In any case, synthetic hepcidin did not affect expression of iron transporters (Fig. S4).
310 This excludes the possibility for a synergistic effect on LPS-induced tissue iron uptake that could
311 promote effective hypoferremia in the iron overload models.

312 In conclusion, our data reveal a crosstalk between the hepcidin pathway and the IRE/IRP
313 system in the liver for the control of tissue ferroportin and serum iron levels. Furthermore, they
314 suggest that application of hepcidin therapeutics for treatment of iron overload disorders should
315 be combined with iron depletion strategies to mitigate *Fpn* mRNA translation and increase
316 hepcidin efficacy.

317

318 **Materials and Methods**

319 Animals. Wild type C57BL/6J and isogenic *Hjv*^{-/-} mice (30) were housed in macrolone
320 cages (up to 5 mice/cage, 12:12 h light-dark cycle: 7 am - 7 pm; 22 ± 1°C, 60 ± 5% humidity).
321 The mice were fed either a standard diet (200 ppm iron), an iron-deficient diet (2-6 ppm iron) or
322 a high-iron diet (2% carbonyl iron) (31). Where indicated, mice were injected intraperitoneally
323 with 1 µg/g LPS (serotype 055:B5; Sigma-Aldrich) or subcutaneously with 2.5 µg/g synthetic
324 hepcidin; control mice were injected with phosphate-buffered saline. At the endpoints, animals
325 were sacrificed by CO₂ inhalation and cervical dislocation. Experimental procedures were
326 approved by the Animal Care Committee of McGill University (protocol 4966).

327 Serum biochemistry. Blood was collected via cardiac puncture. Serum was prepared by
328 using micro Z-gel tubes with clotting activator (Sarstedt) and was kept frozen at -20°C until
329 analysis. Serum iron, total iron binding capacity (TIBC) and, where indicated serum ferritin,

330 were determined at the Biochemistry Department of the Montreal Jewish General Hospital using
331 a Roche Hitachi 917 Chemistry Analyzer. Transferrin saturation was calculated from the ratio of
332 serum iron and TIBC. Serum hepcidin was measured by using an ELISA kit (HMC-001;
333 Intrinsic LifeSciences).

334 Quantification of serum non-transferrin bound iron (NTBI). NTBI was measured by
335 adapting the method developed by Esposito *et al* (32). Briefly, iron samples of known
336 concentration were created by mixing 70 mM nitrilotriacetate (NTA) (pH = 7.0) with 20 mM
337 ferrous ammonium sulfate. Fe^{2+} was allowed to oxidize to Fe^{3+} in ambient air for at least 30 min
338 and then the solution was diluted to 0.2 mM before further serial dilutions to create a ladder. 5 μl
339 of ladder was loaded in a 96-well plate containing 195 μl plasma-like medium with or without
340 100 μM deferiprone. The composition of the plasma-like medium was: 40 mg/ml bovine serum
341 albumin, 1.2 mM sodium phosphate dibasic, 120 μM sodium citrate, 10 mM sodium bicarbonate
342 in iron-free HEPES-buffered saline (HEPES 20 mM, NaCl 150 mM, treated with Chelex-100
343 chelating resin [Bio-Rad, Hercules, CA], 0.5 mM NTA, 40 μM ascorbic acid, 50 μM
344 dihydrorhodamine, pH=7.4). 5 μl of sample was loaded in a 96-well plate containing 195 μl of
345 iron-free HEPES-buffered saline with or without 100 μM deferiprone. Microplates were read
346 every 2 minutes at 37°C over 40 min at 485/520 nm (ex/em). Final NTBI was calculated by
347 comparing the oxidation rate of DHR in the presence or absence of the strong chelator
348 deferiprone.

349 Hepcidin synthesis. Human hepcidin (DTHFPICIFCCGCCHRSKCGMCKKT) was
350 synthesized at Ferring Research Institute, San Diego, CA. The linear peptide was assembled on
351 Rink amide resin using Tribute peptide synthesizer and the peptide was cleaved from the resin
352 with the TFA/TIS/EDT/H₂O 91:3:3:3 (v/v/v/v) cocktail. The solvents were evaporated, and the

353 crude peptide was precipitated with diethyl ether, reconstituted in 50% aqueous acetonitrile and
354 lyophilized. The lyophilizate was dissolved in 30% aqueous acetonitrile at the concentration of
355 0.05 mM and the pH of the solution was adjusted to 7.8 with 6 M ammonium hydroxide. Folding
356 was achieved within 4 hours using the cysteine/cystine redox (peptide/Cys/Cys₂ 1:6:6 molar
357 ratio). The reaction mixture was acidified to pH 3, loaded onto HPLC prep column and purified
358 in a TFA based gradient. The identity of the peptide was confirmed by mass spectrometry and by
359 coelution with a commercially available sample (Peptide International, #PLP-3771-PI).

360 Quantitative real-time PCR (qPCR). RNA was extracted from livers by using the RNeasy
361 kit (Qiagen). cDNA was synthesized from 1 µg RNA by using the OneScript® Plus cDNA
362 Synthesis Kit (Applied Biological Materials Inc.). Gene-specific primers pairs (Table 1) were
363 validated by dissociation curve analysis and demonstrated amplification efficiency between 90-
364 110 %. SYBR Green (Bioline) and primers were used to amplify products under following
365 cycling conditions: initial denaturation 95°C 10 min, 40 cycles of 95°C 5 s, 58°C 30 s, 72°C 10
366 s, and final cycle melt analysis between 58°-95°C. Relative mRNA expression was calculated by
367 the $2^{-\Delta\Delta C_t}$ method (33). Data were normalized to murine ribosomal protein L19 (*Rpl19*). Data are
368 reported as fold increases compared to samples from wild type mice on standard diet (SD).

369 Polysome fractionation. RNA was freshly prepared from frozen livers. Linear sucrose
370 gradients were prepared the day before the experiment by using 5% (w/v) and 50% (w/v) sucrose
371 solutions with 10x gradient buffer (200 mM HEPES pH=7.6, 1 M KCl, 50 mM MgCl₂, 0.1
372 mg/ml Cycloheximide, 1 tablet cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail
373 (Roche), 200 U/mL Recombinant RNasin® Ribonuclease Inhibitor (Promega), 2 mM DTT).
374 Linear gradients were prepared in Polyallomer Centrifuge Tubes (Beckman Coulter). Tubes were
375 marked using a gradient cylinder (BioComp), and 5% sucrose solution was added using a syringe

376 with a layering needle (BioComp) until solution level reached the mark. Then, 50% sucrose
377 solution was layered underneath the 5% solution until the interface between the two solutions
378 reached the mark. Tubes were capped with rate zonal caps (BioComp) and linearized using a
379 Gradient Master 108 (Biocomp). All reagents were nuclease-free and all solutions were kept on
380 ice or at 4°C. Sample preparation was adapted from Liang *et al.* (34). Briefly, livers were flash
381 frozen upon collection. Roughly 30-80 mg of tissue was crushed using a mortar and pestle in the
382 presence of liquid nitrogen to prevent thawing. Tissues were lysed in up to 1 ml of hypotonic
383 lysis buffer (5 mM Tris-Hcl pH=7.5, 1.5 mM KCl, 2.5 mM MgCl₂, 2 mM DTT, 1 mg/ml
384 Cycloheximide, 200 U/ml Recombinant RNasin® Ribonuclease Inhibitor (Promega), 1 tablet
385 cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (Roche), 0.5% (v/v) Triton X-100,
386 0.5% (v/v) Sodium Deoxycholate) and homogenized using Dounce homogenizers (60
387 movements with both loose and tight pestles) on ice. Samples were centrifuged at 4°C, 16,060g
388 for 4 minutes and supernatants were collected. Sample optical density was measured at 260 nm
389 and samples were normalized to either the lowest value or 30 ODs. 450 µl of sucrose gradient
390 was removed from the top and replaced with normalized sample. Tube weights were balanced by
391 weight before centrifugation at 200,000g for 2 h at 4°C in a SW 41 Ti rotor and a Beckman
392 Optima L-60 Ultracentrifuge. Samples were fractionated using a BR-188 Density Gradient
393 Fractionation System (Brandel). Immediately upon collection, 800 µl of samples were mixed
394 with 1 ml of TRIzol™ and kept on ice before storage at -80°C. Polysomal RNA was processed
395 according to the manufacturer's protocol. mRNA distribution was analyzed as previously
396 described (35).

397 Western blotting. Livers were washed with ice-cold PBS and dissected into pieces.
398 Aliquots were snap frozen at liquid nitrogen and stored at -80°C. Protein lysates were obtained

399 as described (12). Lysates containing 40 µg of proteins were analyzed by SDS-PAGE on 9-13%
400 gels and proteins were transferred onto nitrocellulose membranes (BioRad). The blots were
401 blocked in non-fat milk diluted in tris-buffered saline (TBS) containing 0.1% (v/v) Tween-20
402 (TBS-T), and probed overnight with antibodies against ferroportin (36) (1:1000 diluted
403 monoclonal rat anti-mouse 1C7, kindly provided by Amgen Inc), β-actin (1:2000 diluted;
404 Sigma), or Tfr2 (1:1000 diluted rabbit polyclonal; Alpha Diagnostics). Following a 3x wash with
405 TBS-T, the membranes were incubated with peroxidase-coupled secondary antibodies for 1 h.
406 Immunoreactive bands were detected by enhanced chemiluminescence with the Western
407 Lightning ECL Kit (Perkin Elmer).

408 Immunohistochemistry. Tissue specimens were fixed in 10% buffered formalin and
409 embedded in paraffin. Samples were cut at 4-µm, placed on SuperFrost/Plus slides (Fisher) and
410 dried overnight at 37°C. The slides were then loaded onto the Discovery XT Autostainer
411 (Ventana Medical System) for automated immunohistochemistry. Slides underwent de-
412 paraffinization and heat-induced epitope retrieval. Immunostaining was performed by using
413 1:500 diluted rabbit polyclonal antibodies against ferroportin (37) and an appropriate detection
414 kit (Omnimap rabbit polyclonal HRP, #760-4311 and ChromoMap-DAB #760-159; Roche).
415 Negative controls were performed by the omission of the primary antibody. Slides were
416 counterstained with hematoxylin for four minutes, blued with Bluing Reagent for four minutes,
417 removed from the autostainer, washed in warm soapy water, dehydrated through graded
418 alcohols, cleared in xylene, and mounted with Permount (Fisher). Sections were analyzed by
419 conventional light microscopy and quantified by using the Aperio ImageScope software (Leica
420 Biosystems) (9).

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

423 Quantification of liver iron content (LIC). Total liver iron was quantified by using the
424 ferrozine assay (11) or inductively coupled plasma mass spectrometry (ICP-MS) (38).

425 Iron speciation analysis. Iron redox speciation analysis in the liver was performed by
426 capillary electrophoresis (CE) coupled to ICP-MS (CE-ICP-MS). Dynamic reaction cell (DRC)
427 technology (ICP-DRC-MS) with NH₃ as DRC-gas was applied for non-interfered monitoring of
428 the iron isotopes. A "PrinCe 706" CE system (PrinCe Technologies B.V., Emmen, Netherlands)
429 was employed for separation of iron species at +20 kV. Temperature settings for sample/buffer
430 tray and capillary were set to 20°C. An uncoated capillary (100 cm x 50 µm ID; CS-
431 Chromatographie Service GmbH, Langerwehe, Germany) was used for separation and
432 hyphenation to the ICP-DRC-MS. A CE-ICP-MS interface (38, 39) was installed which
433 provided the electrical connection between CE capillary end and outlet electrode. The self-
434 aspiration mode allowed for best flow rate adjustment and avoided suction flow. Electrolytes for
435 sample stacking and electrophoretic separation were 10% HCl = leading electrolyte, 0.05 mM
436 HCl= terminating electrolyte and 50 mM HCl = background electrolyte. The Fe²⁺/Fe³⁺ ratio was
437 calculated from quantitative determined concentrations of Fe-species.

438 Statistics. Statistical analysis was performed by using the Prism GraphPad software
439 (version 9.1.0). Lognormally distributed data including qPCR and ELISA results were first log
440 transformed before analysis with ordinary two-way ANOVA (Tukey's multiple comparisons
441 test) for comparisons within same treatment groups (denoted by a or b on figures) or with
442 multiple unpaired t tests using the Holm-Sidak method to compare effects between treatments.

443 Normally distributed data was analyzed by two-way ANOVA using either Sidak's method for
444 comparisons between treatment groups or Tukey's multiple comparisons test within treatments
445 groups. Probability value $p < 0.05$ was considered statistically significant.

446

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455

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558

559

560 **Figures legends**

561 **Fig. 1. Dietary iron loading does not disrupt inflammatory hepcidin induction in LPS-**
562 **treated wild type mice but prevents hepcidin-mediated hypoferremia.** Nine-week-old male
563 mice (n=12-14 per group) were fed SD or HID for one day, one week, or five weeks prior to
564 sacrifice. Half of the mice were injected intraperitoneally with saline and the other half with 1
565 $\mu\text{g/g}$ LPS 4 hours before sacrifice. Sera were collected by cardiac puncture and analyzed for: (A)
566 iron, (B) transferrin saturation, (C) TIBC, (D) ferritin, and (F) hepcidin. Livers were dissected
567 and processed for biochemical analysis of: (E) liver iron content (LIC) by the ferrozine assay and
568 (G) *Hamp* mRNA by qPCR. The dotted line in (A) and (B) indicates baseline serum iron and
569 transferrin saturation, respectively, of control mice on SD. Data (A-E) are presented as the
570 mean \pm SEM, while (F-G) are presented as geometric mean \pm SD. Statistically significant
571 differences ($p < 0.05$) over time compared to values from saline- or LPS-treated control mice are
572 indicated by a or b, respectively.

573

574 **Fig. 2. Iron overload prevents hypoferremic response to LPS-induced inflammation in**
575 **dietary iron-manipulated wt and *Hjv*^{-/-} mice with dramatic differences in hepcidin**
576 **expression.** Four-week-old male wt mice (n=12-14 per group) were placed on HID for five
577 weeks. Conversely, age- and sex-matched isogenic *Hjv*^{-/-} mice (n=12-14 per group) were placed
578 on IDD for five weeks to prevent excessive iron overload. Controls from both genotypes were
579 kept on SD. Half of the mice were injected with saline and the other half with 1 $\mu\text{g/g}$ LPS; all
580 animals were sacrificed 4 hours later. Sera were collected by cardiac puncture and analyzed for:
581 (A) iron, (B) transferrin saturation, (C) TIBC, (D) NTBI, and (F) hepcidin. Livers were dissected

582 and processed for LIC quantification by the ferrozine assay (E), and for qPCR analysis of *Hamp*
583 (*G*), *Fpn(+IRE)* (*H*), *Dmt1* (*I*), *Zip14* (*J*), *Lcn2* (*K*) and *Tfrc* (*L*) mRNAs. Sections of liver (*M*)
584 and spleen (*N*) were analyzed for iron deposits by Perls' staining (magnification: 10x). The
585 dotted line in (*A*) and (*B*) indicates baseline serum iron and transferrin saturation, respectively,
586 of control wt mice on SD. Data in (*A-F*) are presented as the mean±SEM while in (*G-L*) are
587 presented as geometric mean±SD. Statistically significant differences ($p<0.05$) compared to
588 values from saline- or LPS-treated wt control mice are indicated by a or b, respectively.

589

590 **Fig. 3. Effects of LPS on hepatic and splenic ferroportin of iron-manipulated wt and *Hjv*^{-/-}**
591 **mice.** Livers and spleens from mice described in Fig. 2 were dissected and processed for
592 immunohistochemical and biochemical analysis of ferroportin. Immunohistochemical staining of
593 ferroportin in liver (*A*) and spleen (*C*) sections (magnification for liver is 20x and for spleen 5x).
594 Western blot for ferroportin and β -actin in liver (*B*) and spleen (*D*) extracts from four
595 representative mice in each condition. Blots were quantified by densitometry and ferroportin/ β -
596 actin ratios are shown on the right. Densitometric data are presented as the mean±SEM.
597 Statistically significant differences ($p<0.05$) compared to values from saline- or LPS-treated wt
598 control mice are indicated by a or b, respectively.

599

600 **Fig. 4. Iron depletion of *Hjv*^{-/-} mice improves the efficacy of synthetic hepcidin to promote**
601 **hypoferremia.** Four-week-old wt male mice (n=12-14 per group) were placed on HID for five
602 weeks. Conversely, age- and sex-matched isogenic *Hjv*^{-/-} mice (n=12-14 per group) were placed
603 on IDD for five weeks to prevent excessive iron overload. Controls from both genotypes were

604 kept on SD. Half of the mice were injected every 2 hours for a total of 4 injections with saline,
605 and the other half with 2.5 $\mu\text{g/g}$ synthetic hepcidin. Sera were collected by cardiac puncture and
606 analyzed for: (A) iron, (B) transferrin saturation, (C) TIBC, and (D) NTBI. Livers and spleens
607 were dissected and processed for analysis of LIC (E) and SIC (F) by the ferrozine assay. Livers
608 were also used for qPCR analysis of *Hamp* (G), *Fpn(+IRE)* (I), *Bmp6* (J) and *Id1* (K) mRNAs.
609 Finally, livers were used for Western blot analysis of Tfrc, Tfr2, and β -actin; a representative
610 image (out of n=3 samples) is shown in (H). The dotted line in (A) and (B) indicates baseline
611 serum iron and transferrin saturation, respectively, of control wt mice on SD. Data in (A-F) are
612 presented as the mean \pm SEM while data in (G and I-K) are presented as geometric mean \pm SD.
613 Statistically significant differences ($p<0.05$) compared to values from saline- or LPS-treated wt
614 control mice are indicated by a or b, respectively.

615

616 **Fig. 5. Effects of synthetic hepcidin on hepatic and splenic ferroportin of iron-manipulated**
617 **wt and *Hjv*^{-/-} mice.** Livers and spleens from mice described in Fig. 4 were dissected and
618 processed for immunohistochemical and biochemical analysis of ferroportin.
619 Immunohistochemical staining of ferroportin in liver (A) and spleen (C) sections (magnification
620 for liver is 20x and for spleen 10x). Western blot for ferroportin and β -actin in liver (B) and
621 spleen (D) extracts from four representative mice in each condition. Blots were quantified by
622 densitometry and ferroportin/ β -actin ratios are shown on the right. Densitometric data are
623 presented as the mean \pm SEM. Statistically significant differences ($p<0.05$) compared to values
624 from saline- or hepcidin-treated wt control mice are indicated by a or b, respectively.

625

626 **Fig. 6. Iron regulation of Fpn(+IRE) mRNA translation in the mouse liver.** Four-week-old
627 wt male mice (n=10-14 per group) were placed on HID for five weeks. Conversely, age- and sex-
628 matched isogenic H₂v^{-/-} mice (n=10-14 per group) were placed on IDD for five weeks to prevent
629 excessive iron overload. Controls from both genotypes were kept on SD. At the endpoint, the
630 mice were sacrificed, and livers were used for polysome profile analysis and iron assays. (A)
631 Liver polysome profiles from n=3 mice in each experimental group. Top: Recording of
632 absorbance at 254 nm of representative samples. Fraction numbers and direction of the gradient
633 are indicated. Bottom left: Distribution of *Fpn(+IRE)*, *Fth1* and *Actb* mRNAs among light
634 monosomal and heavy polysomal fractions (separated by dashed line) was analyzed by qPCR.
635 Bottom right: Bar graph comparisons of pooled fractions. Numbers indicate the fold change
636 compared to wt mice on SD. (B and C) Analysis of total iron (B), and redox iron speciation (C)
637 in the liver by CE-ICP-MS. Data are presented as the mean±SEM. Statistical analysis in (A) was
638 performed by two-way ANOVA and in (B, C) by one-way ANOVA or unpaired t-test.
639 Statistically significant differences (p<0.05) compared to values from wt control mice on SD are
640 indicated by *.

641

642 **Fig. 7. Elimination of ferroportin mRNA by prolonged LPS treatment potentiates hepcidin-**
643 **induced hypoferremia in mouse models of iron overload.** Four-week-old wt male mice (n=10-
644 14 per group) were placed on HID for five weeks. Conversely, age- and sex-matched isogenic
645 H₂v^{-/-} mice (n=10-14 per group) were placed on IDD for five weeks to prevent excessive iron
646 overload. Controls from both genotypes were kept on SD. (A and B) Half of the mice were
647 injected with saline and the other half with 1 µg/g LPS and sacrificed after 8 h. Livers were
648 dissected and processed for qPCR analysis of *Fpn(+IRE)* (A) and *Fpn(-IRE)* (B) mRNAs. (C-F)

649 All mice were injected with 1 $\mu\text{g/g}$ LPS. Half of the animals were subsequently injected with
650 saline, and the other half with 2.5 $\mu\text{g/g}$ synthetic hepcidin every two hours for a total of 4
651 injections. At the endpoint the mice were sacrificed. Sera were collected by cardiac puncture and
652 analyzed for: (C) iron, (D) transferrin saturation, (E) TIBC, and (F) NTBI. The dotted line in (C)
653 and (D) indicates baseline serum iron and transferrin saturation, respectively, of control wt mice
654 on SD. Data are presented as (A-B) geometric mean \pm SD or (C-F) mean \pm SEM. Statistically
655 significant differences ($p < 0.05$) compared to values from saline-treated (A and B) or
656 LPS/hepcidin-treated (C and F) wt control mice on SD are indicated by a, b or c, respectively.

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Table 1. List of primers used for qPCR.

Gene	GenBank accession	Forward primer sequence	Reverse primer sequence
<i>Hamp1</i>	NM_032541.1	AAGCAGGGCAGACATTGCGAT	CAGGATGTGGCTCTAGGCTATGT
<i>Fpn(+IRE)</i>	NM_016917.2	GGCATAAGGCTGTTGTGCTT	TCATGACACCAGGCGTTCTC
<i>Fpn(-IRE)</i>	XM_006496137.4	GCCGGTTGGAGTTTCAATGT	TCATGACACCAGGCGTTCTC
<i>Dmt1</i>	NM_001146161.1	CTTGGGATACTGACGGTGAC	GATTTGCAGTCTGGAGCAGT
<i>Zip14</i>	NM_001135151.1	TGGATAGTGAGGCTGCGTGG	ATGGTGAGGCCAAGGCTAAT
<i>Lcn2</i>	NM_008491	GGCCAGTTCACCTCTGGGAAA	TGGCGAACTGGTTGTAGTCC
<i>Tfrc</i>	NM_011638.4	AGCCAGATCAGCATTCTCTAACT	GCCTTCATGTTATTGTCCGGCAT
<i>Bmp6</i>	NM_007556.2	ACTCGGGATGGACTCCACGTCA	CACCATGAAGGGCTGCTTGTCTG
<i>Id1</i>	NM_010495.2	GGTACTTGGTCTGTCCGGAGC	GCAGGTCCCTGATGTAGTCTG
<i>Fth1</i>	NM_010239.2	AAGTGCGCCAGAACTACCAC	AGCCACATCATCTCCGGTCAA
<i>Rpl19</i>	NM_009078.2	AGGCATATGGGCATAGGGAAGAG	TTGACCTTCAGGTACAGGCTGTG
<i>Actb</i>	NM_007393.3	GACGACATGGAGAAGATCTG	GTGAAGCTGTAGCCACGCTC

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664 **Supplemental Figure legends**

665 **Fig. S1. Low magnification immunohistochemical images of ferroportin in liver sections of**
666 **dietary iron-manipulated wild type and *Hjv*^{-/-} mice following LPS treatment.** Liver sections
667 from mice described in Fig. 2 were used for immunohistochemical analysis of ferroportin
668 (magnifications: 10x and 5x).

669

670 **Fig. S2. Low magnification immunohistochemical images of ferroportin in spleen sections**
671 **of dietary iron-manipulated wild type and *Hjv*^{-/-} mice following LPS treatment.** Spleen
672 sections from mice described in Fig. 2 were used for immunohistochemical analysis of
673 ferroportin (magnification: 2x).

674 **Fig. S3. Perls staining for iron deposits in liver and spleen sections of dietary iron-**
675 **manipulated wild type and *Hjv*^{-/-} mice following treatment with synthetic hepcidin.** Liver
676 and spleen sections from mice described in Fig. 4 were stained with Perls Prussian blue
677 (magnification: 10x).

678

679 **Fig. S4. Effects of LPS treatment on expression of mRNAs encoding iron transport proteins**
680 **in the liver of dietary iron-manipulated wild type and *Hjv*^{-/-} mice.** Livers from mice
681 described in Fig. 2 were dissected and processed for qPCR analysis of mRNAs encoding iron
682 transport proteins. (A) *Tfrc* mRNA, (B) *Dmt1* mRNA, (C) *Zip14* mRNA and (D) *Lcn2* mRNA.
683 All data are presented as the geometric mean \pm SD. Statistically significant differences ($p < 0.05$)

684 compared to values from saline- or LPS-treated control mice are indicated by a or b,
685 respectively.

686

687 **Fig. S5. Low magnification immunohistochemical images of ferroportin in liver sections of**
688 **dietary iron-manipulated wild type and HJV^{-/-} mice following treatment with synthetic**
689 **hepcidin.** Liver sections from mice described in Fig. 4 were used for immunohistochemical
690 analysis of ferroportin (magnifications: 10x and 5x).

691

692 **Fig. S6. Low magnification immunohistochemical images of ferroportin in spleen sections**
693 **of dietary iron-manipulated wild type and HJV^{-/-} mice following treatment with synthetic**
694 **hepcidin.** Spleen sections from mice described in Fig. 4 were used for immunohistochemical
695 analysis of ferroportin (magnification: 2x).

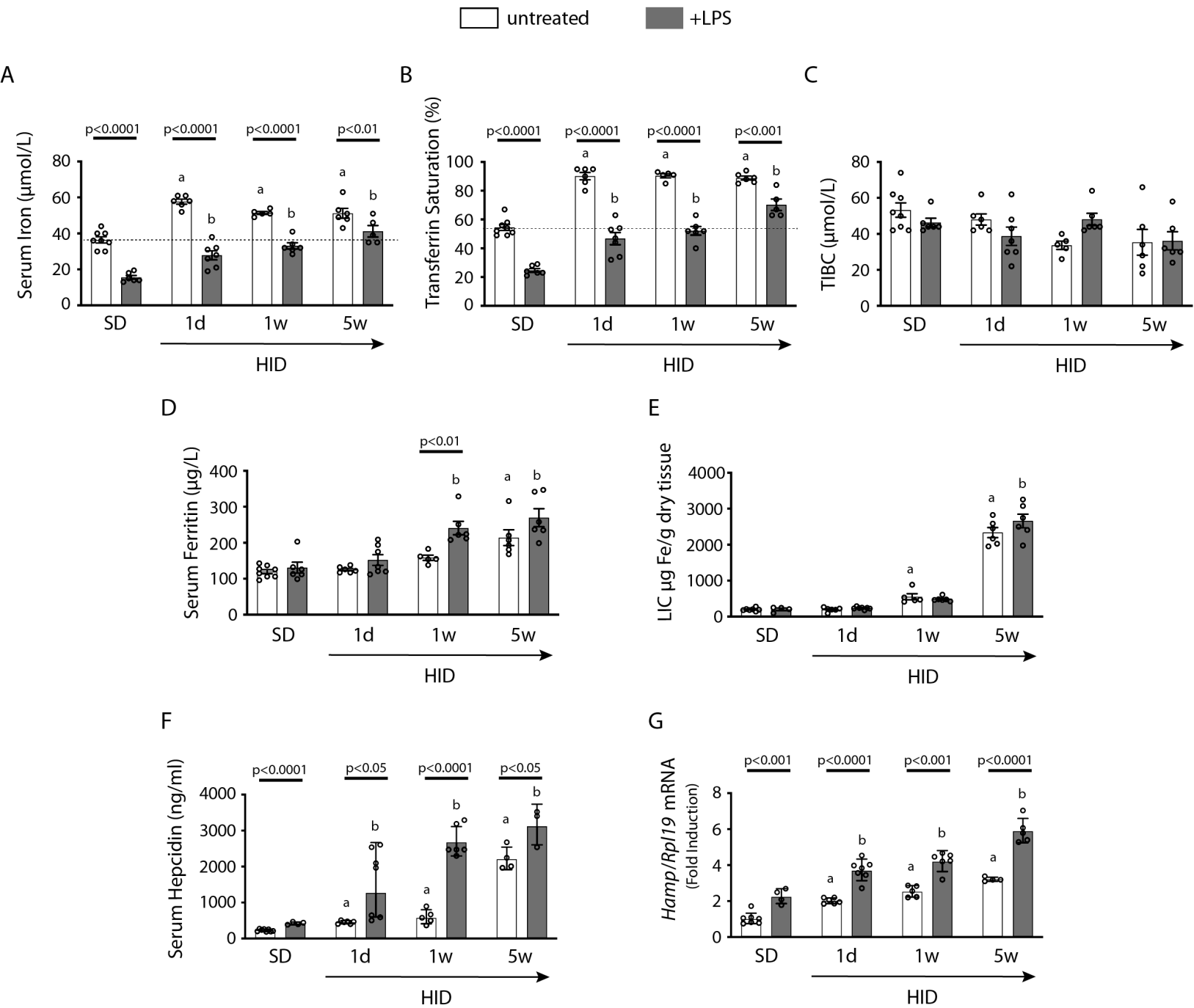
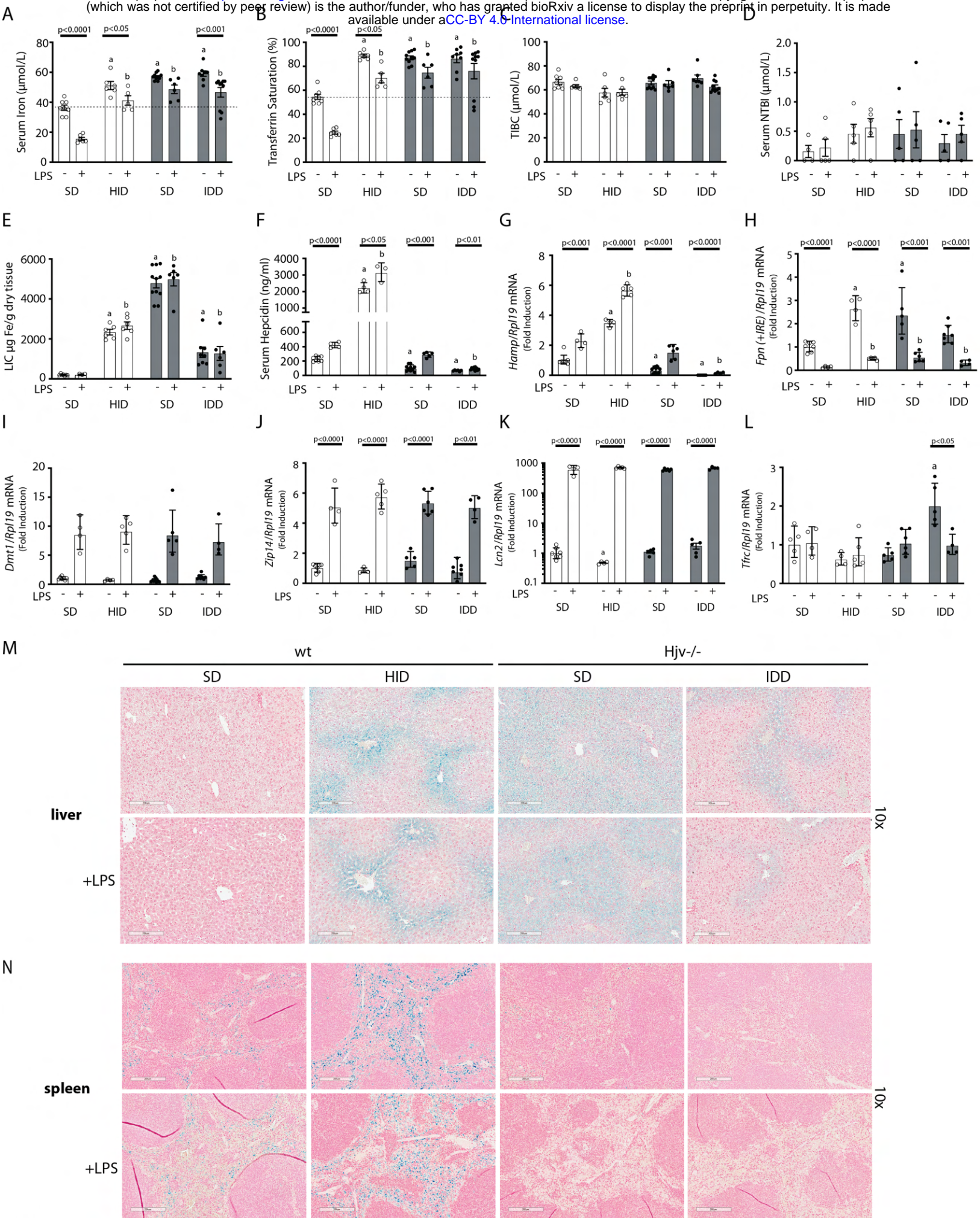


Fig. 1



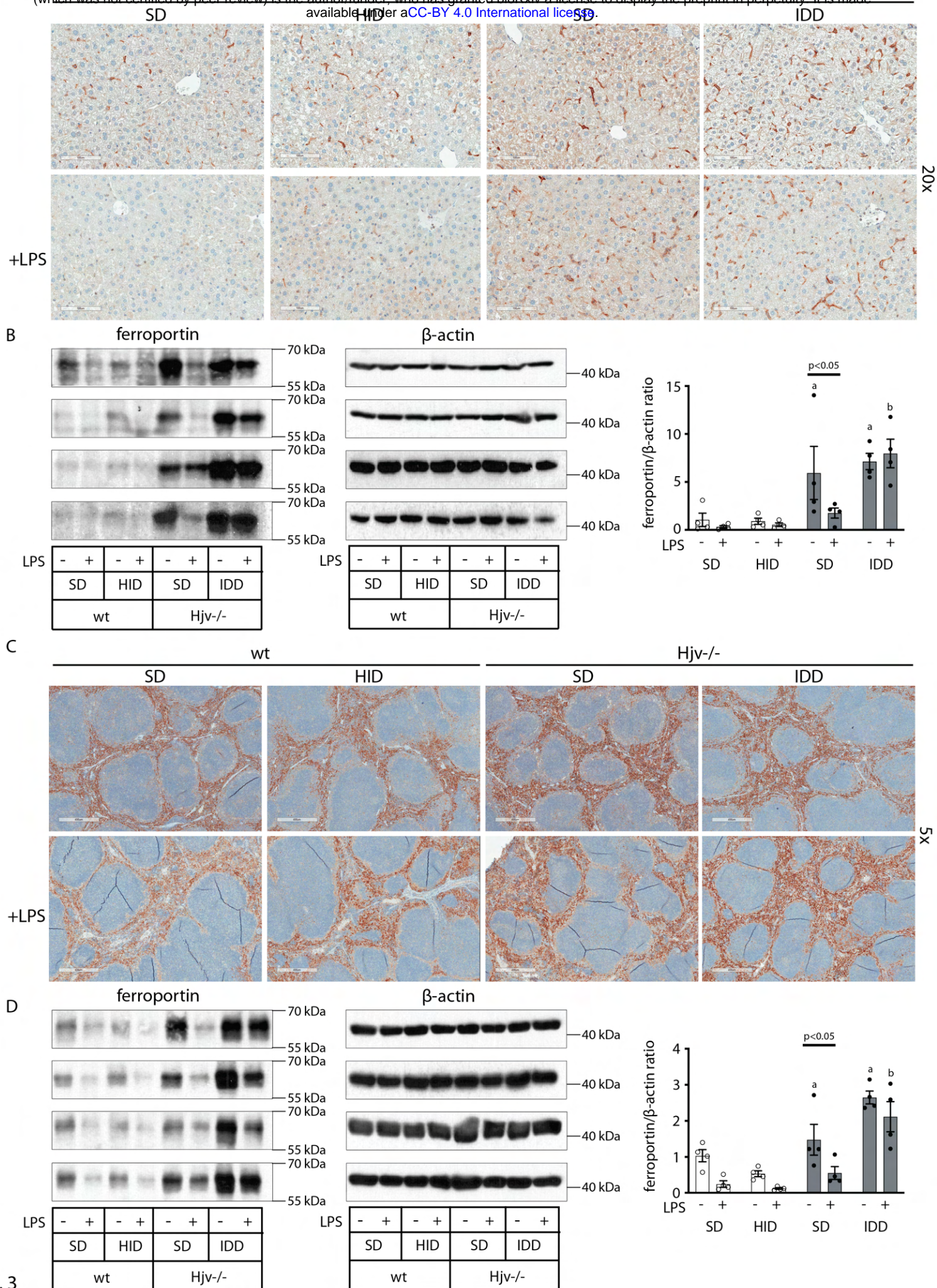


Fig. 3

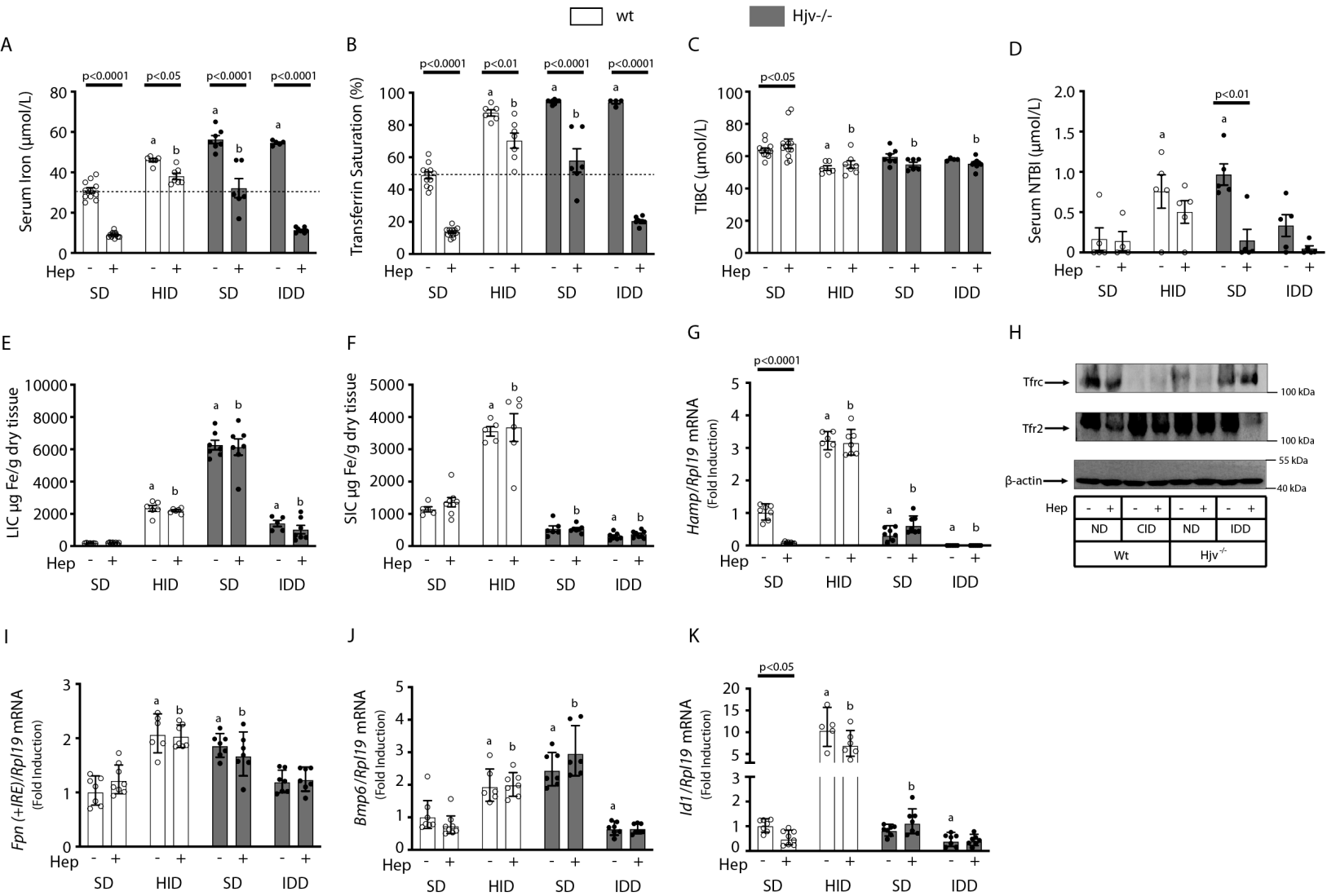


Fig. 4

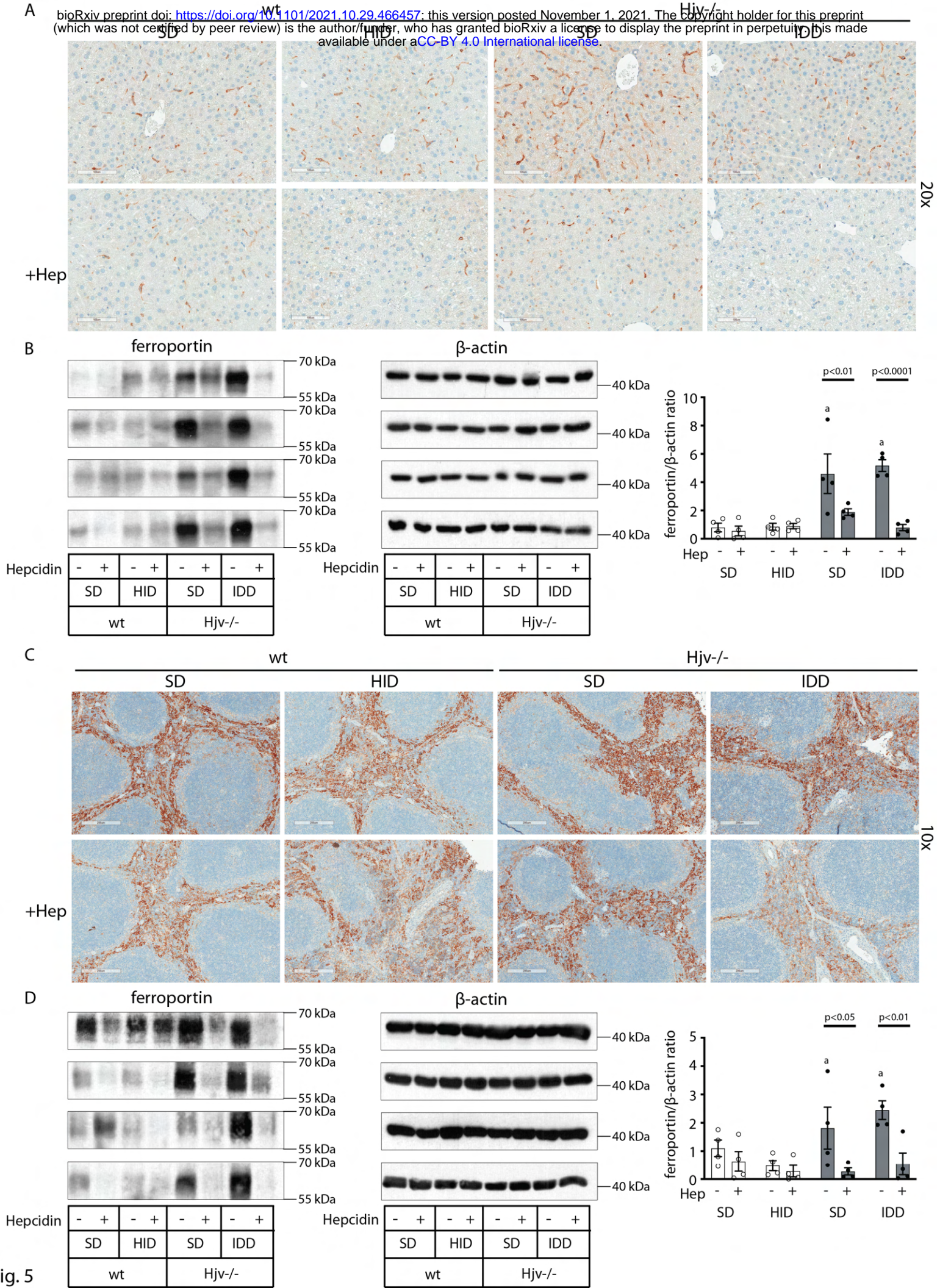
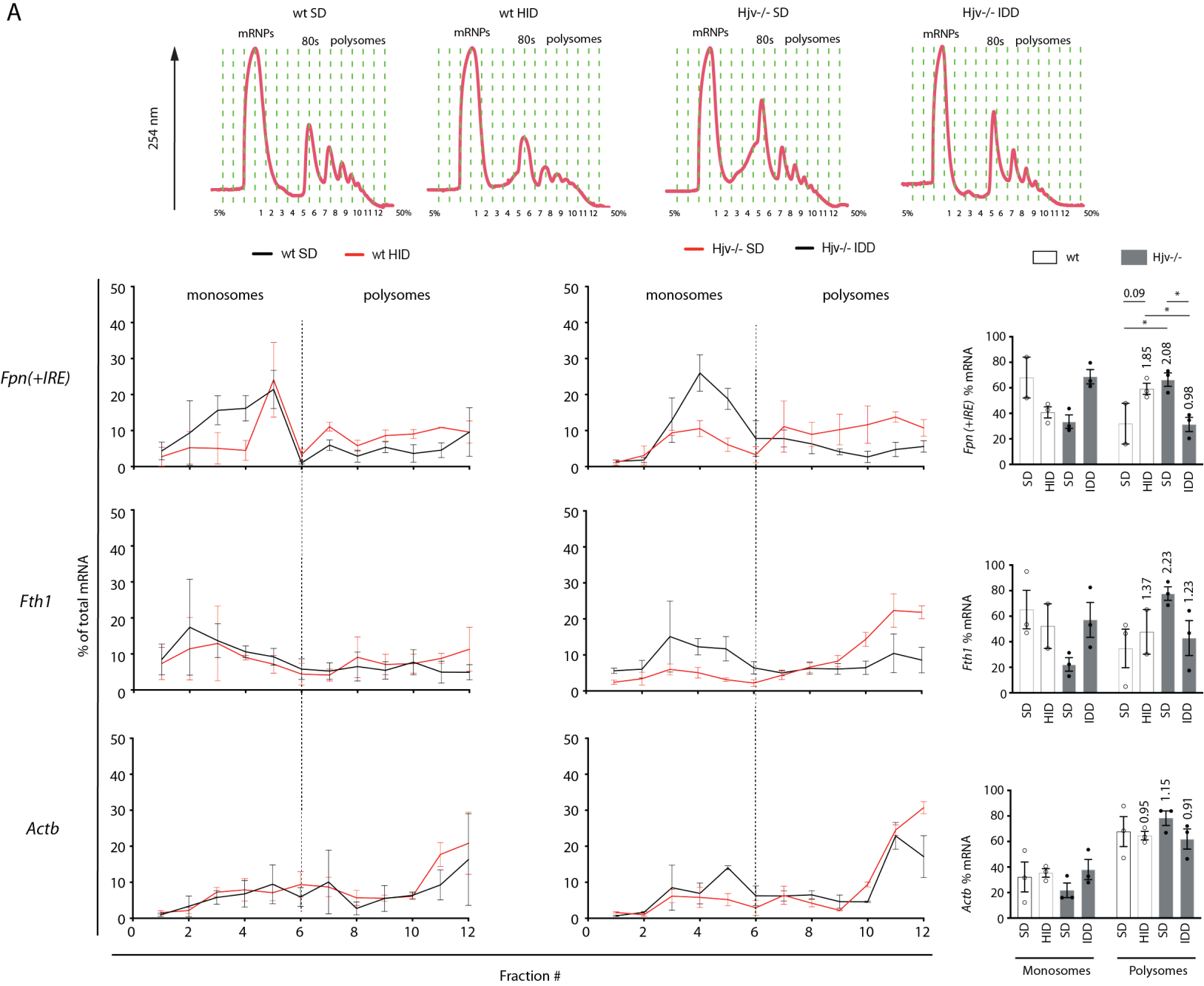
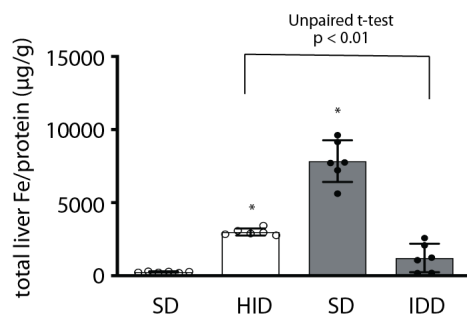


Fig. 5

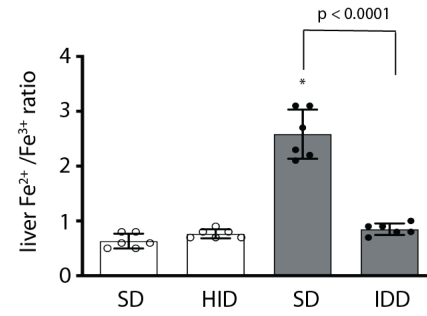
A



B



C



□ wt ■ Hvj^{-/-}

Fig. 6

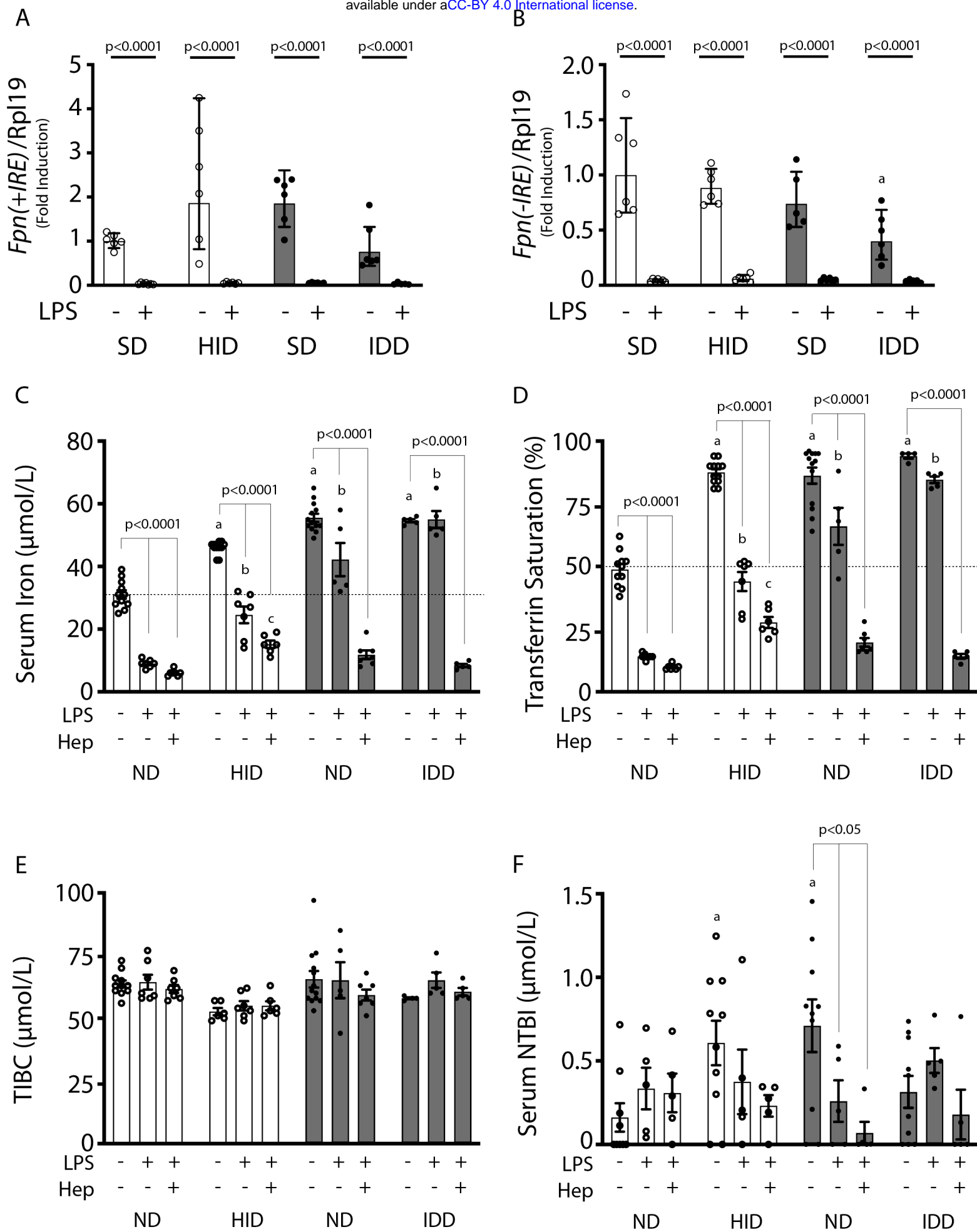


Fig. 7

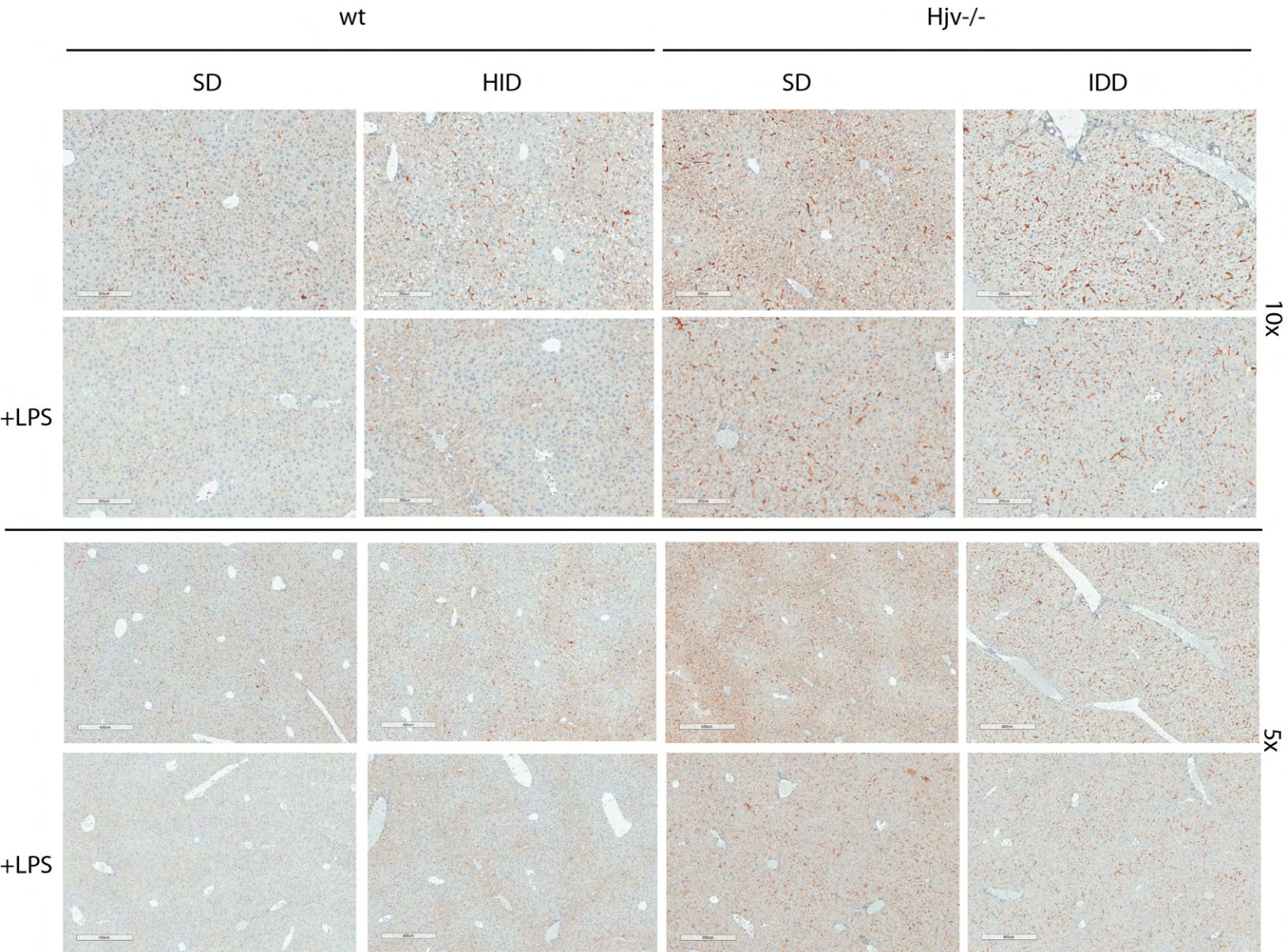


Fig. S1

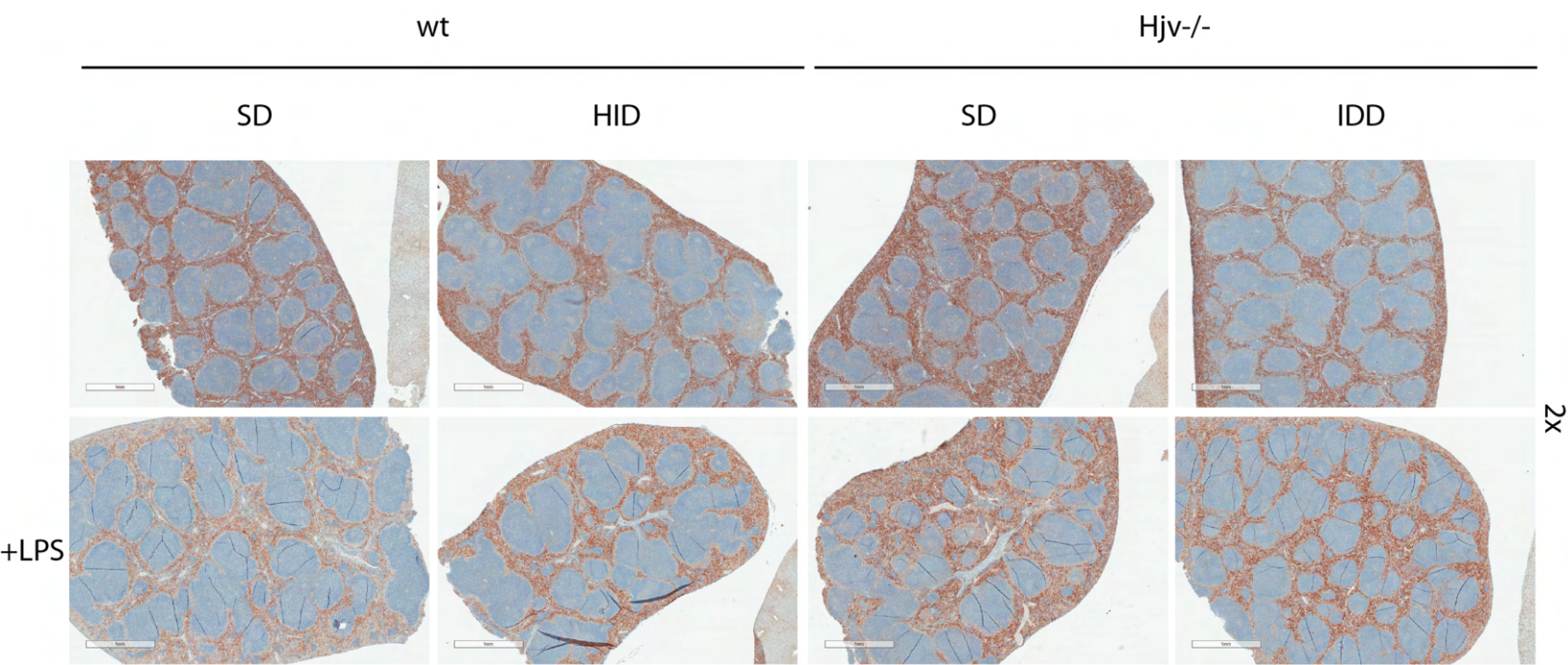


Fig. S2

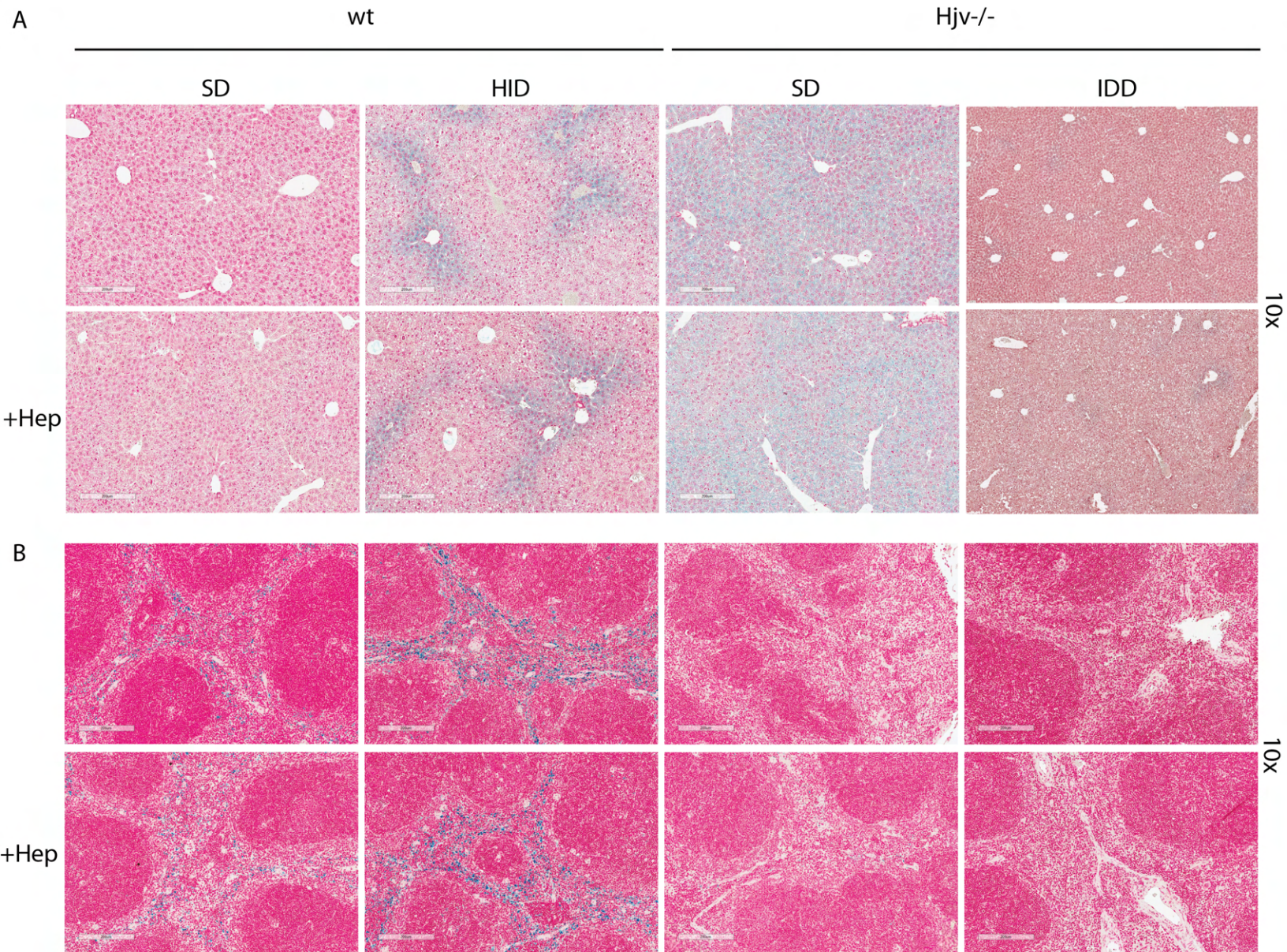


Fig. S3

□ wt ■ H₂v^{-/-}

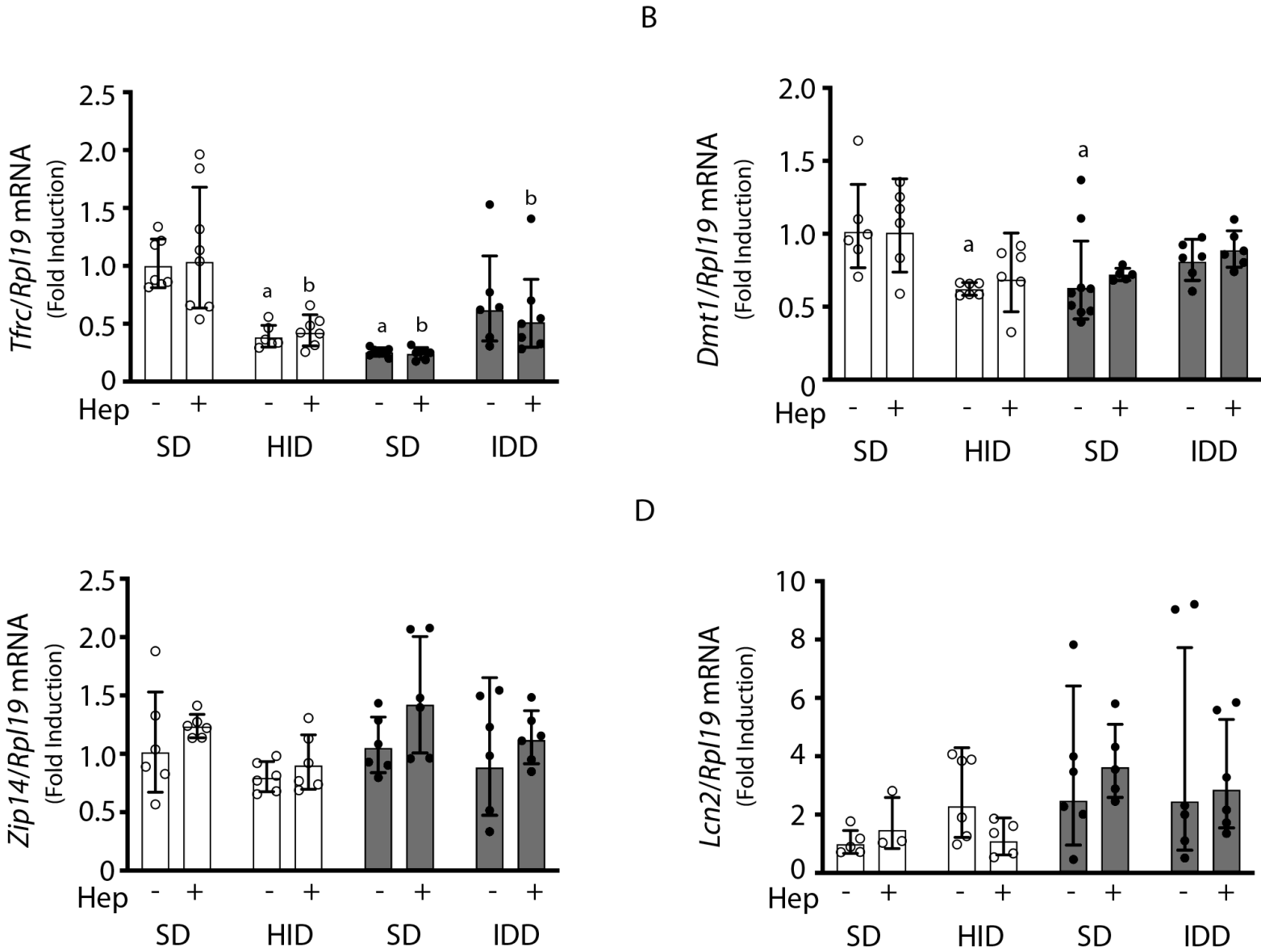


Fig. S4

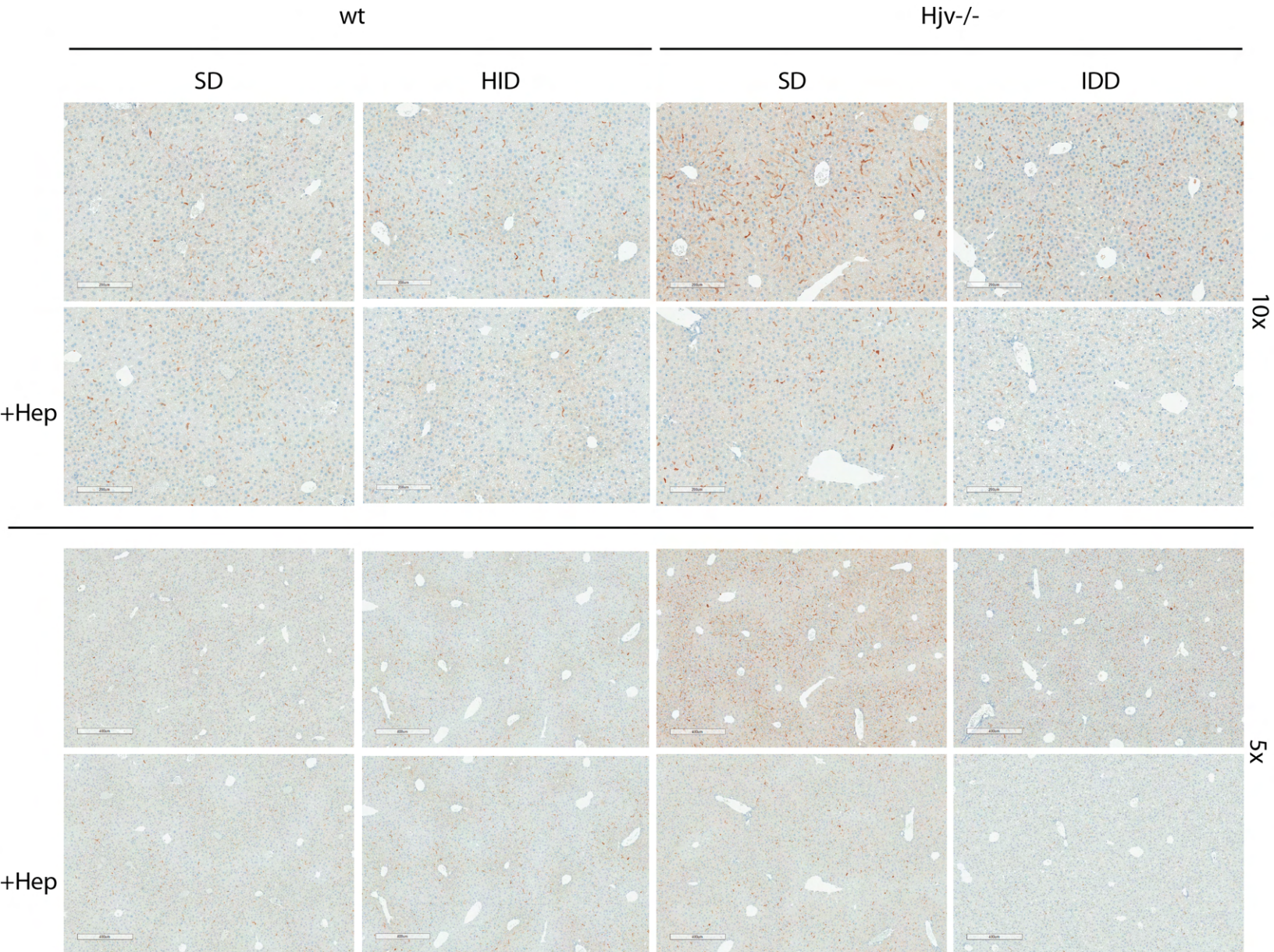


Fig. S5

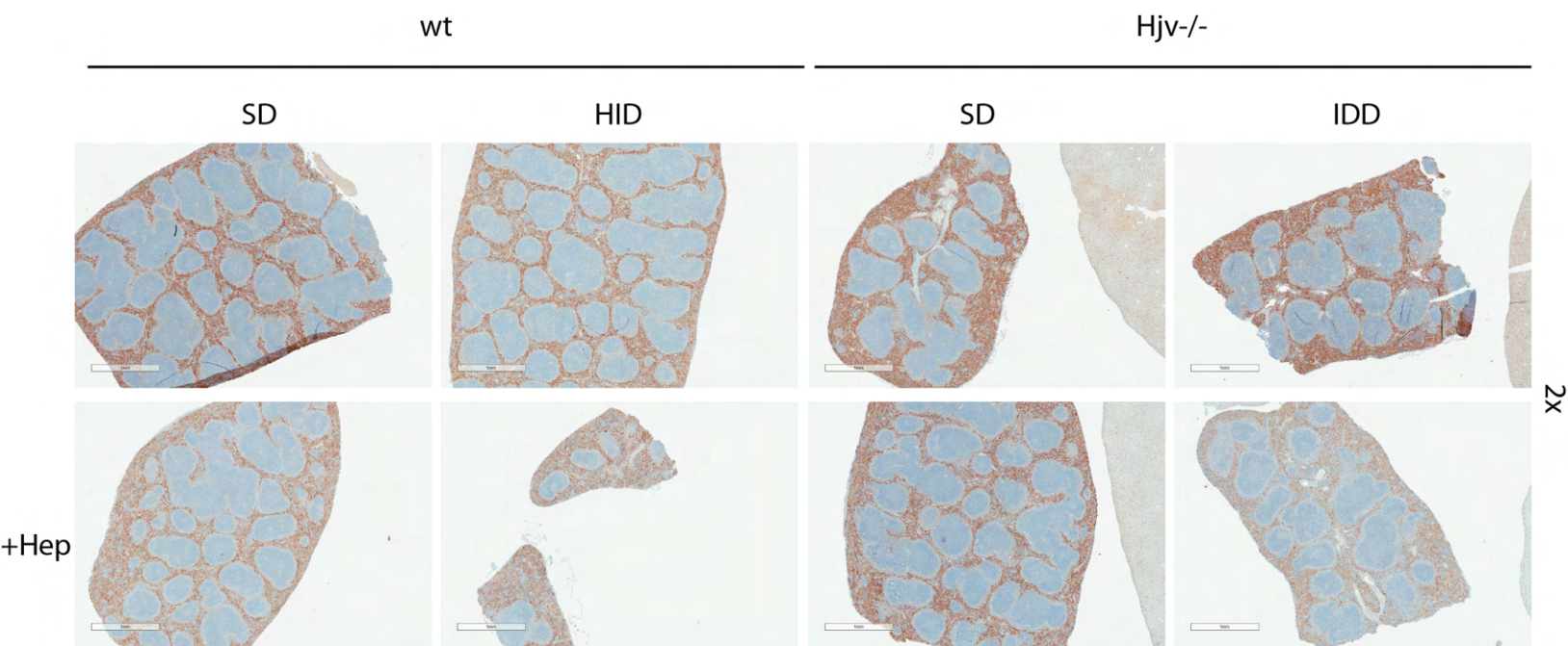


Fig. S6