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Divergent roles of haptoglobin and hemopexin deficiency for disease progression of Shiga-toxin-induced hemolytic-uremic syndrome in mice

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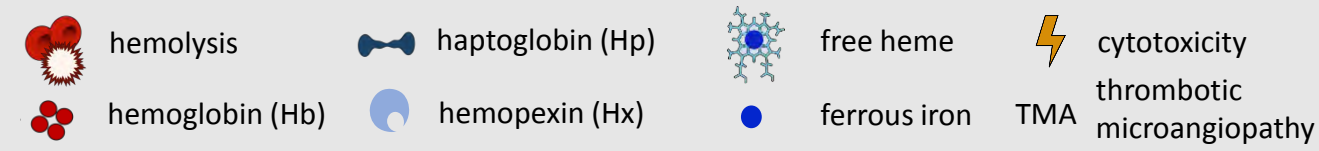
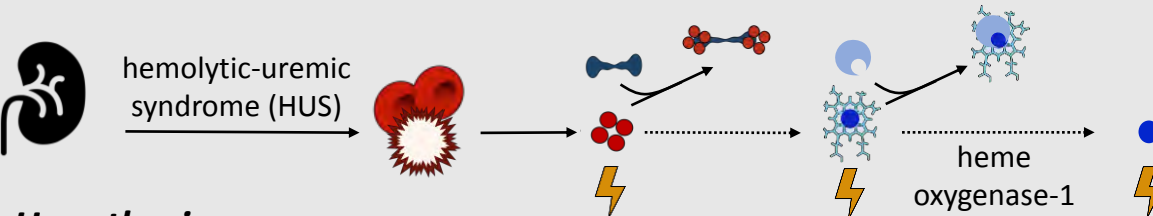
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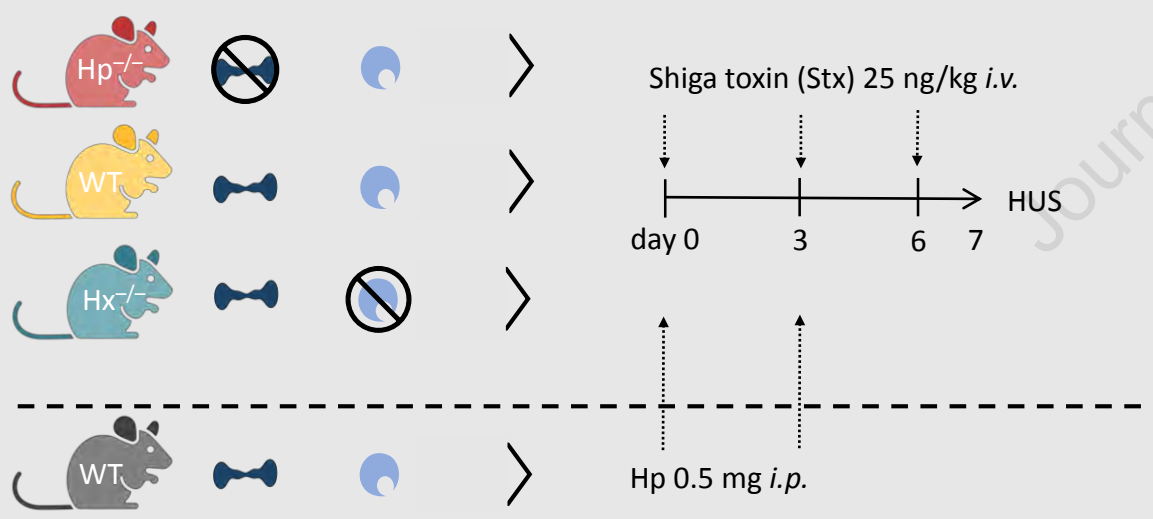
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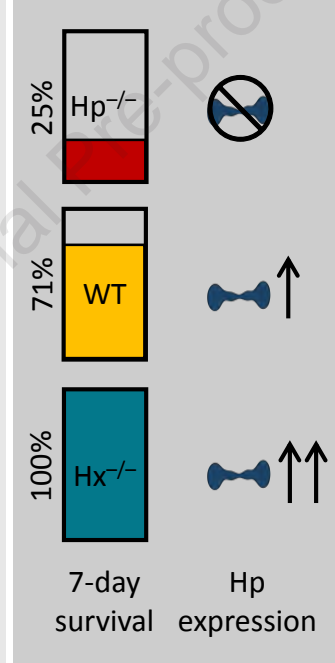


Hypothesis
The presence of Hp and Hx impacts outcome and renal pathology in HUS

Study design



Primary outcome



Pathological and molecular findings (day 5)

	fibrin (TMA)	platelets (TMA)	systemic hemolysis	neutrophil recruitment	tubular iron
Hp ^{-/-}	↑	↑	↑	↑	↑
WT	↔	↑	↑	↑	⊘
Hx ^{-/-}	↔	↔	↔	↔	⊘
WT + Hp	n. a.	↔	n. a.	↔	n. a.

Pirschel and Mestekemper, 2021

In mice with HUS, Hp deficiency aggravates disease progression associated with tubular iron deposition, while Hx deficiency conveys protection associated with supranormal plasma Hp, attenuated TMA and renal inflammation. Low dose Hp treatment of WT mice with HUS attenuated renal platelet deposition and neutrophil recruitment.

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2 **Divergent roles of haptoglobin and hemopexin deficiency for disease progression of Shiga-**
3 **toxin-induced hemolytic-uremic syndrome in mice**

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37 **Abstract**

38 Thrombotic microangiopathy, hemolysis and acute kidney injury are typical clinical
39 characteristics of hemolytic-uremic syndrome (HUS), which is predominantly caused by
40 Shiga-toxin-producing *Escherichia coli*. Free heme aggravates organ damage in life-
41 threatening infections, even with a low degree of systemic hemolysis. Therefore, we
42 hypothesized that the presence of the hemoglobin- and the heme-scavenging proteins,
43 haptoglobin and hemopexin, respectively impacts outcome and kidney pathology in HUS.
44 Here, we investigated the effect of haptoglobin and hemopexin deficiency (haptoglobin^{-/-},
45 hemopexin^{-/-}) and haptoglobin treatment in a murine model of HUS-like disease. Seven-day
46 survival was decreased in haptoglobin^{-/-} (25%) compared to wild type mice (71.4%), whereas
47 all hemopexin^{-/-} mice survived. Shiga-toxin-challenged hemopexin^{-/-} mice showed decreased
48 kidney inflammation and attenuated thrombotic microangiopathy, indicated by reduced
49 neutrophil recruitment and platelet deposition. These observations were associated with
50 supranormal haptoglobin plasma levels in hemopexin^{-/-} mice. Low dose haptoglobin
51 administration to Shiga-toxin-challenged wild type mice attenuated kidney platelet deposition
52 and neutrophil recruitment, suggesting that haptoglobin at least partially contributes to the
53 beneficial effects. Surrogate parameters of hemolysis were elevated in Shiga-toxin-challenged
54 wild type and haptoglobin^{-/-} mice, while signs for hepatic hemoglobin degradation like heme
55 oxygenase-1, ferritin and CD163 expression were only increased in Shiga-toxin-challenged
56 wild type mice. In line with this observation, haptoglobin^{-/-} mice displayed tubular iron
57 deposition as an indicator for kidney hemoglobin degradation. Thus, haptoglobin and
58 hemopexin deficiency play divergent roles in Shiga-toxin-mediated HUS, suggesting
59 haptoglobin is involved, and hemopexin is redundant for the resolution of HUS pathology.
60

61 **Key words**

62 hemolytic-uremic syndrome, Shiga toxin, haptoglobin, hemopexin, iron overload, acute renal failure

63 64 **Translational Statement**

65 Hemolytic-uremic syndrome (HUS) is a life-threatening complication of infection with enterohemorrhagic
66 *Escherichia coli* and characterized by microangiopathic hemolytic anemia and renal impairment.
67 Evidence suggests that free heme contributes to disease progression in systemic inflammation. We
68 show that the hemoglobin and heme scavenger proteins haptoglobin and hemopexin play divergent
69 roles in HUS pathogenesis: Our data indicate that hemopexin is redundant for the resolution of HUS
70 pathology, while haptoglobin deficiency aggravates disease progression in mice with HUS and higher
71 endogenous haptoglobin levels as well as haptoglobin administration are associated with an attenuation
72 of surrogate parameters of thrombotic microangiopathy and inflammation. (98/100)

73

74 **Introduction**

75 The hemolytic-uremic syndrome (HUS) is a rare but severe systemic complication upon infection with
76 Shiga-toxin (Stx)-producing enterohemorrhagic *Escherichia coli* (STEC). STEC-HUS, a thrombotic
77 microangiopathy (TMA) primarily affecting the kidneys, is clinically characterized by hemolytic anemia,
78 thrombocytopenia and end-organ damage caused by thrombosis in small blood vessels.¹ It is the most
79 frequent reason for acute kidney injury (AKI) in childhood,² but severe HUS courses have also been
80 described in adults.^{3, 4} Although the pathogenesis is still under investigation,⁵ it is evident that Stx,
81 comprising Stx1 and Stx2, is the major virulence factor of STEC.⁶ By binding to globotriaosylceramide
82 (Gb3) receptor with high affinity and interfering with protein synthesis, Stx leads to epithelial and
83 endothelial cell damage thereby initiating the occurrence of renal TMA.⁶ Clot deposition in the
84 microvasculature leads to subsequent tissue ischemia, organ injury, and hemolysis.^{1, 6} Therapeutic
85 options are currently supportive and dialysis is often required. Since there is no specific therapy, further
86 studies are needed to evaluate potential targets for therapeutic approaches. Free heme is a known
87 relevant factor in the maintenance of pathological processes in life-threatening infections by leading to
88 inflammation,^{7, 8} complement activation^{9, 10} and reactive oxygen species (ROS).¹¹ Recently, elevated
89 free heme could be detected in plasma of STEC-HUS patients.¹² However, the impact of heme and
90 heme degradation products on disease progression has not yet been investigated. In mammals,
91 clearance of cell-free hemoglobin (Hb) and heme-bound iron is mainly regulated by the scavenging
92 systems haptoglobin (Hp) and hemopexin (Hx). Hp is the plasma protein with the highest binding affinity
93 to Hb. As an acute-phase protein it is upregulated under inflammatory conditions and predominantly
94 produced in hepatocytes.¹³ Key functions of Hp are preventing glomerular filtration of Hb and enabling
95 Hb degradation by the reticuloendothelial system, especially in spleen and liver,^{14, 15} thereby protecting
96 the kidney from Hb-mediated cytotoxicity.¹⁶ CD163, a membrane receptor on macrophages, binds to
97 the Hp-Hb complex with high affinity and leads to its endocytosis.¹⁵ In the absence of Hp, glomerular
98 filtered Hb binds to the multiligand receptors megalin and cubilin mediating its tubular uptake.¹⁷ When
99 Hb becomes oxidized to methemoglobin, its heme groups dissociate and potentially exert cytotoxicity
100 via the centrally bound iron.¹⁸ Various plasma proteins, such as albumin, α 1-microglobulin (α 1M) and
101 Hx prevent iron-mediated damage by binding free heme.¹⁸ Hx is the scavenging protein with the highest
102 affinity to heme and a murine but not human acute-phase protein mainly produced in the liver.^{19, 20} The
103 Hx-heme complex is removed from plasma by low-density lipoprotein(LDL)-receptor related protein

104 1-mediated endocytosis.²¹ After its uptake, the intracellular degradation of heme into equimolar amounts
105 of ferrous iron (Fe²⁺), carbon monoxide (CO), and biliverdin is mediated via the two heme oxygenase
106 isoforms (HO-1, HO-2).²² HO-1 is ubiquitously expressed, inducible, and gains cytoprotective properties
107 by modulating the tissue response in the presence of various stress factors.²² First evidence from cell-
108 culture experiments suggest that Stx augments heme-mediated toxicity in renal epithelial cells which
109 can be attenuated by HO-1 induction.²³ Heme degradation by HO-1 increases the availability of free
110 iron.²⁴ While biliverdin is converted to bilirubin by biliverdin reductase,²⁵ labile iron is rapidly bound by
111 the intracellular iron-storage protein ferritin to prevent ROS formation.²⁶ Ferritin consists of a heavy
112 (Fth1) and a light (Ftl1) chain, the former has ferroxidase activity being crucial for iron storage.²⁷ The
113 transmembrane protein ferroportin (SCL40A1) mediates iron transport into the circulation where it is
114 bound by transferrin.²⁸ Ferroportin expression is locally regulated by iron-regulatory proteins and
115 systemically by the acute-phase protein hepcidin.²⁸

116 Hitherto, the role of the Hb- and heme-scavenging proteins Hp and Hx in HUS pathology has not been
117 addressed. We hypothesized, that Hp and Hx impact disease progression of STEC-HUS by ameliorating
118 Hb- and heme-mediated cytotoxicity and kidney injury. Thus, we analyzed the effect of Hp and Hx
119 deficiency as well as Hp treatment in a murine model of HUS-like disease. Elucidating the role of these
120 proteins in STEC-HUS provides a deeper understanding of the pathogenesis and offers the potential to
121 develop novel therapeutic strategies.

122

123 **Methods**

124 Information on commercially available kits, buffers, antibodies employed in the study and other
125 methodical details including methods relevant to supplementary results are provided in the supplement.

126 *Animal experiments*

127 Generation of the Hp^{-/-} and Hx^{-/-} mice was described in ²⁹ and ¹⁶, respectively. HUS was induced in 10-15
128 weeks old male C57BL/6J wild-type (WT), Hp^{-/-} and Hx^{-/-} mice.³⁰ Mice were subjected to 25 ng/kg
129 bodyweight (BW) Stx2 (WT Stx, Hp^{-/-} Stx, Hx^{-/-} Stx) or 0.9% NaCl (WT sham, Hp^{-/-} sham, Hx^{-/-} sham)
130 intravenously (*i.v.*) on days 0, 3 and 6 accompanied by volume resuscitation with 800 µl Ringer's lactate
131 subcutaneously (*s.c.*) three times daily. BW and HUS score (supplementary Table S1) were determined
132 as described previously.³⁰ Survival was assessed up to day 7 or mice were sacrificed when an HUS
133 score of 4 (high-grade disease state) was reached to comply with ethical regulations. All further analyses
134 were performed in samples obtained at day 5 after HUS induction. For Hp treatment WT mice received

135 0.5 mg Hp (ABIN491578, antibodies-online GmbH) in 200 µl PBS intraperitoneally (*i.p.*) on day 0 and 3.
136 All procedures were approved by the regional animal welfare committee (Thuringian State Office for
137 Consumer Protection, Bad Langensalza, Germany; registration number 02-040/16) and performed in
138 accordance with the German legislation.

139 *Plasma analysis*

140 Blood withdrawal, plasma preparation and analysis of hemolysis were performed as described
141 previously.³⁰ Plasma α1M, albumin, Hp, Hx, urea, neutrophil gelatinase-associated lipocalin (NGAL),
142 bilirubin and hepcidin were analyzed with commercial kits according to manufactures instructions
143 (supplementary Table S2).

144 *Histological and immunohistochemical analysis*

145 Kidneys were histopathologically and immunohistochemically evaluated using periodic acid Schiff
146 (PAS), kidney injury molecule-1 (KIM-1), CD31, F4-80, complement component 3 (C3c), cleaved
147 caspase-3 (CC-3) staining as described previously,³⁰ as well as ferroportin, lymphocyte antigen 6
148 complex, locus G (Ly6G), glycoprotein 1b (GP1b) and iron staining (antibodies in supplementary
149 Table S3, 4).

150 *Gene expression analysis*

151 Isolation of RNA, performance of real-time PCR (supplementary Table S5) and data analysis were
152 described previously.^{31, 32}

153 *Protein expression analysis*

154 Immunoblot analysis was performed as described previously.³¹ For blotting of renal HO-1 100 µg and
155 for Fth1, hepatic HO-1 and CD163 25 µg of total protein were used (antibodies in supplementary
156 Table S6). Proteins of interest were normalized to total protein load using the stain-free technology (Bio-
157 Rad Laboratories, Inc.). Bands with normalization factors less than 0.7 and more than 1.3 were excluded
158 from analysis.³³ Samples from 6 animals per group were pooled to equal protein amounts for the
159 representative blots of renal HO-1 and Fth1. Individual blots (1 animal/group) are shown in
160 supplementary Figure S1. Data are presented relative to the mean of sham animals.

161 *Statistics*

162 Data were analyzed with GraphPad Prism 7.03 and are depicted as median ± interquartile range (IQR)
163 for n observations. Survival was analyzed generating Kaplan-Meier curves and evaluated by Mantel-Cox
164 test. Mann-Whitney *U*-test was used to compare the Stx groups of each strain with the corresponding

165 sham group, each knockout sham group to the WT sham group and each knockout Stx group to the WT
166 Stx group. A *P*-value < 0.05 was considered significant.

167

168 **Data sharing statement**

169 For original data, please contact sina.coldewey@med.uni-jena.de

170

171 **Results**

172 *SEVEN-DAY SURVIVAL IS WORSE IN HP^{-/-} AND IMPROVED IN HX^{-/-} MICE*

173 Survival rate of Stx-challenged WT mice (71.4%) was decreased but not significantly altered compared
174 to WT sham mice (100%) (Figure 1a). Seven-day survival of Stx-challenged Hp^{-/-} mice (25%) was lower
175 compared to Hp^{-/-} sham mice (100%). Most notably, all Stx-challenged Hx^{-/-} mice survived (100%). Both,
176 Stx-challenged WT and Hx^{-/-} mice, showed significantly higher survival rates compared to
177 Stx-challenged Hp^{-/-} mice.

178 *THE COURSE OF DISEASE IS MORE SEVERE IN HP^{-/-} AND WT MICE THAN IN HX^{-/-} MICE*

179 Disease progression, indicated by increased HUS scores, was apparent in all Stx-challenged mice
180 (Figure 1b). However, while HUS scores of Stx-challenged Hp^{-/-} and WT mice were comparable on
181 day 5, Stx-challenged Hx^{-/-} mice showed less disease progression (Figure 1c). All Stx-challenged mice
182 lost weight during the course of disease (Figure 1d). Five days after HUS induction, weight loss of
183 Hp^{-/-} mice was higher compared to WT mice, while weight loss of Hx^{-/-} mice was comparable to WT mice
184 (Figure 1e).

185 *EXPRESSION OF THE HB AND HEME SCAVENGER PROTEINS HX, α1M, ALBUMIN AND HP IN WT, HP^{-/-} AND 186 HX^{-/-} MICE*

187 A compensatory upregulation of α1M in Hx^{-/-} mice with sickle cell disease³⁴ as well as Hp in Hx^{-/-} mice
188 and Hx in Hp^{-/-} mice with artificial hemolysis has been described.³⁵ Thus, we investigated plasma levels
189 of Hb- and heme-binding proteins.

190 Hepatic *Hx* gene expression was increased in Stx-challenged WT and Hp^{-/-} mice compared to their
191 corresponding sham group (Figure 2a). A similar pattern was found for Hx plasma levels, they were
192 higher in Hp^{-/-} sham mice compared to WT sham mice (Figure 2b).

193 Plasma α1M was decreased in Stx-challenged WT but unchanged in Hp^{-/-} and Hx^{-/-} mice compared to
194 their corresponding sham group (Figure 2c).

195 Heme-binding properties have been described for albumin.³⁶ However, plasma albumin was unchanged
196 in Stx-challenged WT and knockout mice compared to their corresponding sham group (Figure 2d).

197 Hepatic *Hp* gene expression was increased in Stx-challenged WT and *Hx*^{-/-} mice compared to their
198 corresponding sham group (Figure 2e). A similar pattern was found for plasma *Hp* (Figure 2f). Notably,
199 plasma *Hp* was higher in *Hx*^{-/-} compared to WT mice irrespective of Stx challenge.

200 *RENAL IMPAIRMENT IN WT, HP*^{-/-} *AND HX*^{-/-} *MICE*

201 Liver, lung, colon and kidneys of WT, *Hp*^{-/-}, and *Hx*^{-/-} mice were assessed for morphological alterations.
202 While no relevant morphological changes appeared in lung and colon, diffuse granulomatous changes
203 were detected in liver sections of Stx-challenged mice and knockout sham animals (supplementary
204 Figure S2, 3), accompanied by unchanged liver enzymes (supplementary Figure S4).

205 All Stx-challenged genotypes showed severe renal injury, indicated by increased plasma urea
206 (Figure 3a) and NGAL (Figure 3b), altered morphology in PAS-stained sections (Figure 3c,
207 supplementary Figure S5A) and elevated KIM-1 expression (Figure 3d), suggesting that the kidney is
208 the primarily affected organ in this murine model. Plasma creatinine was elevated in all Stx-challenged
209 genotypes compared to their corresponding sham group, and slightly increased in Stx-challenged *Hp*^{-/-}
210 compared to WT mice (supplementary Figure S6A). Potassium plasma levels were elevated in Stx-
211 challenged WT and *Hp*^{-/-} but not in *Hx*^{-/-} mice compared to their corresponding sham group
212 (supplementary Figure S6B). Furthermore, enhanced potassium levels were observed in Stx-challenged
213 *Hp*^{-/-} compared to WT mice.

214 In human STEC-HUS, glomerular damage is predominant, but tubular damage also contributes to the
215 pathology.³⁷ Ultrastructural analysis revealed severe tubular injury in all Stx-challenged mice but no
216 alterations of podocytes (supplementary Figure S7). Murine Stx models do not completely reconstruct
217 human HUS. Several models have been developed to highlight certain aspects of HUS, comprising
218 genetic modifications to study the lectin pathway³⁸ or enhance thrombotic processes³⁹ and co-injection
219 of LPS to provoke broader HUS symptoms like glomerular changes and thrombocytopenia.⁴⁰ This study
220 focuses on Stx-mediated pathomechanisms.

221 Renal endothelial cells are the main target of Stx by binding Gb3-receptors⁶ and apoptotic cells are
222 increased in kidneys of STEC-HUS patients.³⁷ A comparable loss of endothelial cells in all Stx-
223 challenged genotypes indicated by CD31 expression (Figure 3e, supplementary Figure S5B), and
224 raised apoptosis indicated by CC-3 expression (Figure 3f, supplementary Figure S5C) compared to their

225 corresponding sham group was observed. Compared to Stx-challenged WT mice, Hx^{-/-} mice expressed
226 less CC-3.

227 Renal HO-1 expression was increased in Stx-challenged strains compared to their corresponding sham
228 group (Figure 3g, supplementary Figure S1A). Interestingly, HO-1 levels were the highest in Stx-
229 challenged Hp^{-/-} (15-fold), followed by WT mice (10-fold) whereas Hx^{-/-} mice (6-fold) had the lowest
230 levels.

231 Renal microthrombi formation is a hallmark of HUS pathology. Fibrin deposition was detected by SFOG
232 staining in all Stx-challenged Hp^{-/-} mice but only in some Stx-challenged WT and Hx^{-/-} mice
233 (supplementary Figure S8).

234 Increased numbers of renal GP1b-positive thrombocytes were observed in Stx-challenged WT and
235 Hp^{-/-} but not in Hx^{-/-} mice compared to their corresponding sham group (Figure 3h).

236 *ELEVATED HEMOLYSIS IN WT AND HP^{-/-} MICE*

237 Increased hemolysis and plasma bilirubin were detected in Stx-challenged WT and Hp^{-/-} but not in
238 Hx^{-/-} mice compared to their corresponding sham group (Figure 4a-b). Hepatic and renal gene
239 expression of proteins taking part in heme and iron homeostasis displayed varying regulations
240 (supplementary Figure S9, 10). Hepatic *Hmox1* expression (Figure 4c) as well as levels of hepatic HO-1,
241 Fth1, and CD163 were elevated in Stx-challenged WT but not in Hp^{-/-} and Hx^{-/-} mice compared to their
242 corresponding sham group (Figure 4d-l).

243 *RENAL INFLAMMATION IS ATTENUATED IN HX^{-/-} MICE*

244 Macrophage³⁷ and neutrophil⁴¹ recruitment to kidneys of STEC-HUS patients has been described and
245 neutrophilia was shown to be associated with poor prognosis.^{42, 43} F4-80-positive macrophages were
246 increased in kidneys of Stx-challenged WT and Hp^{-/-} but not in Hx^{-/-} mice compared to their
247 corresponding sham group (Figure 5a). F4-80 expression was elevated in Hp^{-/-} sham compared to WT
248 sham mice. Macrophages were decreased in Stx-challenged Hp^{-/-} and Hx^{-/-} compared to WT mice.

249 Ly6G expression, indicating neutrophil granulocyte recruitment, was elevated in kidneys of
250 Stx-challenged WT and Hp^{-/-} but not in Hx^{-/-} mice compared to their corresponding sham group
251 (Figure 5b).

252 C3c deposition, indicating complement activation, was increased in all Stx-challenged mice compared
253 to their corresponding sham group. C3c expression was elevated in Stx-challenged Hp^{-/-} compared to
254 WT mice (Figure 5c).

255 *HP INTERVENTION IN WT MICE*

256 Stx-challenged WT mice received a low dose Hp, which has been reported to be beneficial in septic
257 mice,⁴⁴ to evaluate its protective function in HUS-like disease (Figure 6a). Stx groups showed enhanced
258 plasma NGAL, altered renal morphology, increased expression of KIM-1, CC-3 and F4-80-positive
259 macrophages, C3c in the kidneys compared to their corresponding sham groups (Figure 6b-g). Renal
260 GP1b and Ly6g expression was elevated in the Stx-challenged vehicle group but not in Hp-treated mice
261 with HUS compared to the corresponding control group (Figure 6h-i). Hp-treated mice with HUS showed
262 decreased Ly6g expression compared to the corresponding vehicle group (Figure 6i).

263 *TUBULAR IRON DEPOSITION IS INCREASED IN HP^{-/-} MICE BUT NOT IN WT AND HX^{-/-} MICE*

264 Hp and Hx take part in iron homeostasis by their scavenging function regarding Hb and heme-bound
265 iron. Pronounced iron deposition was detected in tubules of the Hp^{-/-} but not in the WT and Hx^{-/-} strain,
266 irrespective of treatment (Figure 7a). Iron-positive tubules were increased in Stx-challenged Hp^{-/-} mice
267 compared to their corresponding sham group. In accordance, highest renal Fth1 expression was found
268 in Hp^{-/-}, followed by Hx^{-/-} mice, whereas WT mice had the lowest levels (Figure 7b). Fth1 expression was
269 slightly elevated in all Stx-challenged mice compared to their corresponding sham group.

270 We analyzed DMT1, megalin and cubilin which are responsible for cellular uptake of iron⁴⁵ and Hb
271 respectively.⁴⁶ DMT1 expression was reduced in Stx-challenged Hx^{-/-} but not in WT and Hp^{-/-} mice
272 compared to their corresponding sham group (supplementary Figure S11A). Megalin and cubilin
273 expression was high in all genotypes independent of Stx challenge (supplementary Figure S11B-C).

274 Plasma Heparin was increased in all Stx-challenged mice compared to their corresponding sham group
275 (Figure 7c). In Stx-challenged Hp^{-/-} mice, heparin levels were elevated compared to WT Stx mice.
276 Ferroportin-positive tubules were decreased in all Stx-challenged genotypes compared to their
277 corresponding sham group (Figure 7d), in Stx-challenged Hp^{-/-} compared to WT mice and in Hp^{-/-} sham
278 compared to WT sham mice.

279 Renal MDA, nitrotyrosine and NOX-1 were investigated as markers for oxidative stress. MDA was
280 enhanced in Hp^{-/-} compared to WT mice, independent of Stx challenge. Nitrotyrosine and NOX-1
281 expression were increased in Stx-challenged Hp^{-/-} compared to WT mice (supplementary Figure S12).

282

283 **Discussion**

284 We showed that Hp and Hx play divergent roles for disease progression in HUS, indicated by a survival
285 advantage of Hx^{-/-} mice and a higher mortality rate in Hp^{-/-} mice compared to WT mice. Albeit the role of
286 Hx in infectious diseases is discussed controversially, we hypothesized that both scavenger proteins

287 are required for the resolution of HUS pathology, accompanied by hemolysis. Thus, the survival benefit
288 of $Hx^{-/-}$ mice appeared unexpected to us, in particular, as Hx administration has been described to be
289 protective in a murine model of sepsis, with a moderate degree of hemolysis⁸ as well as during severe
290 hemolysis.⁷ However, in line with our results, Spiller *et al.* showed that Hx deficiency was protective in
291 septic mice.⁴⁷ The high mortality rate of $Hp^{-/-}$ mice with HUS was consistent with previously reported
292 aggravated vulnerability under hemolytic²⁹, inflammatory⁴⁸, and septic⁴⁴ conditions and emphasizes the
293 physiological relevance of Hp in diseases accompanied by hemolysis.

294 To evaluate possible mechanisms underlying the observed outcome of mice with HUS, we assessed
295 various organ systems, such as kidneys, liver, lung and colon for pathological changes. We only found
296 obvious morphological alterations in kidneys of Stx-challenged mice.

297 Acute TMA-derived hemolysis is a disease-defining feature in patients with STEC-HUS.⁴⁹ Renal fibrin
298 deposition was present in some $Hx^{-/-}$ and WT mice with HUS. However, unlike in Stx-challenged WT
299 mice, renal platelet deposition as surrogate parameter of TMA was not significantly increased in Stx-
300 challenged $Hx^{-/-}$ mice compared to the corresponding sham group. These findings indicate attenuated
301 TMA in Stx-challenged $Hx^{-/-}$ mice. Furthermore, renal apoptosis as well as HO-1 expression, as
302 surrogate parameter for inflammation,⁵⁰ hypoxia,⁵¹ and accumulation of heme⁵², were less pronounced
303 in $Hx^{-/-}$ compared to WT mice with HUS. In line with this, we found a moderate hemolysis, increased
304 bilirubin levels in WT but not in $Hx^{-/-}$ mice with HUS. Consequently, we observed an induction of hepatic
305 HO-1, Fth1, and CD163 in Stx-challenged WT but not in $Hx^{-/-}$ mice, most likely indicating the clearance
306 of Hp-Hb complexes by liver macrophages via CD163.^{15, 53} Of note, in patients with HUS, high plasma
307 heme has been reported to be associated with high plasma HO-1 levels.¹²

308 It has been reported that Stx- and heme-mediated cytotoxicity is sensitized by inflammation.^{54, 55}
309 Furthermore, renal macrophage³⁷ and neutrophil recruitment⁴¹ are observed in renal biopsies of STEC-
310 HUS patients. In Stx-challenged $Hx^{-/-}$ mice, renal inflammation was less pronounced. This was indicated
311 by a reduced macrophage expression compared to Stx-challenged WT mice and by an attenuated
312 neutrophil expression. Considering our results, we conclude that Hx deficiency improves the survival of
313 mice with HUS by ameliorating renal pathology and consequently reducing fatal events resulting from
314 end stage kidney disease.

315 $Hx^{-/-}$ mice with or without artificial hemolysis have been described to display higher endogenous Hp
316 levels.³⁵ We could reproduce this finding in $Hx^{-/-}$ mice with or without HUS. Unlike STEC-HUS patients,
317 who often display depleted Hp levels¹² most likely as a sign of plasma Hp consumption, the acute-phase

318 reaction with high Hp expression seems to predominate in mice with HUS. A variety of anti-inflammatory
319 and immunomodulatory functions of Hp have been reported, such as inhibiting calcium influx and
320 subsequent oxidative burst by binding to activated neutrophils⁵⁶ and suppressing LPS-induced TNF- α
321 production of macrophages.⁴⁸ Therefore, we hypothesized that increased Hp plasma levels in Hx^{-/-}
322 compared to WT mice might contribute to the protective effects of the constitutional Hx knockout.
323 Treatment of Stx-challenged WT mice with low dose Hp attenuated renal platelet deposition and
324 neutrophil recruitment. Interestingly, it has been shown recently that reduction of neutrophil recruitment
325 to kidneys of WT mice by inhibition of CXC chemokine receptor 2 conveys renal protection.⁵⁷ However,
326 as low dose Hp administration did not attenuate renal injury and CC-3 expression, our results indicate
327 that the elevated endogenous Hp expression in Hx^{-/-} mice alone does not explain all beneficial effects
328 observed in these mice.

329 We further investigated the impact of Hp deficiency on renal pathology. We identified similar patterns of
330 tubular damage and renal thrombocyte depositions in Hp^{-/-} and WT mice with HUS. This is consistent
331 with findings of Fagoonee *et al.* showing no differences in renal injury between Hp^{-/-} and WT mice
332 subjected to ischemia reperfusion injury (IRI).⁴⁶ But renal fibrin deposition indicating microthrombi
333 formation, a surrogate parameter for TMA, was increased in Stx-challenged Hp^{-/-} compared to WT mice.
334 Interestingly, Hx plasma levels were higher in Hp^{-/-} sham compared to WT sham mice, suggesting a
335 compensatory adaptation of the Hp deficient genotype. After Stx challenge, plasma Hx increased in WT
336 and even further in Hp^{-/-} mice, suggesting that, similar to Hp, rather the acute-phase reaction than the
337 Hx consumption prevails in mice with HUS. However, in STEC-HUS patient with hemolysis Hx depletion
338 has been reported.¹² There is first evidence that Hx can cause a nephrin-dependent remodeling of the
339 actin cytoskeleton in podocytes⁵⁸, which is supported by the observation that unilateral renal infusion of
340 rats with Hx leads to glomerular alterations with concomitant proteinuria.^{59,60} In our studies, we detected
341 no ultrastructural changes of podocytes independent of genotype or intervention. Assumably, the
342 increase of Hx reflects a compensatory mechanism to detoxify heme in the absence of Hp and/or in Stx-
343 induced HUS-like disease with a moderate degree of hemolysis.

344 We observed a disturbed iron homeostasis, elevated markers of oxidative stress and increased renal
345 complement activation in kidneys of Stx-challenged Hp^{-/-} compared to WT mice, which might explain the
346 detrimental survival of Hp^{-/-} mice with HUS.

347 Specifically, we found not only elevated plasma hepcidin and decreased renal ferroportin levels in Stx-
348 challenged Hp^{-/-} compared to WT mice, but also tubular iron deposition in Hp^{-/-} sham mice, that further

349 increased following Stx challenge. In line with this observation, a strong enhancement of Hb-derived
350 iron in tubules of adult Hp^{-/-} sham mice has been described to accumulate with age and after IRI.⁴⁶ Other
351 studies showed nephrotoxic effects in experimental hemochromatosis⁶¹ or chronic hemosiderosis in
352 rats.⁶² Thus, the observed iron deposition is likely to contribute to the detrimental outcome of Stx-
353 challenged Hp^{-/-} mice. To date, there are no studies examining iron homeostasis in STEC-HUS patients.
354 However, a study analyzing genetic polymorphisms in STEC-HUS patients suggests that genes
355 encoding for proteins involved in iron transport might influence the host susceptibility to develop HUS.⁶³
356 There is increasing evidence that in the absence of Hp, Hb is glomerular filtrated and that the tubular
357 uptake through megalin and cubilin prevents urinary iron loss.^{17, 64} We observed elevated renal HO-1
358 expression and acute tubular iron deposition in Stx-challenged Hp^{-/-} mice, indicating alterations in renal
359 heme and iron homeostasis. Unlike in WT mice, we found no induction of hepatic HO-1, Fth1, and
360 CD163 in Stx-challenged Hp^{-/-} mice, suggesting that Hb cannot be cleared by liver macrophages via
361 CD163 due to the Hp deficiency. In hemolytic disease, it has been shown that liver macrophages can
362 switch to a proinflammatory phenotype in the presence of heme and iron.⁷ In this study, we did not
363 characterize the macrophage phenotype. However, quantitatively, renal macrophage recruitment was
364 surprisingly attenuated in Stx-challenged Hp^{-/-} compared to WT mice.
365 Furthermore, markers of oxidative stress were elevated in Stx-challenged Hp^{-/-} compared to WT mice.
366 This finding might result from the observed tubular iron increase, as it has been described that heme-
367 bound iron is a potent mediator for ROS generation which can lead to ferroptosis⁶⁵ and has been
368 associated to thrombocyte activation *in vitro*.⁶⁶ In patients with HUS, enhanced lipid oxidation as marker
369 for oxidative stress has been shown to be increased and linked to hemolysis.⁶⁷
370 There is evidence, that complement activation occurs in the presence of heme in models of artificial
371 hemolysis¹⁰ and sickle cell disease.⁹ Increased complement activation in the plasma of STEC-HUS
372 patients has been described,⁶⁸ and preclinical studies suggest that this activation might lead to an
373 aggravation of HUS pathology.⁶⁹⁻⁷¹ In accordance, we found elevated renal C3c deposition in Hp^{-/-}
374 compared to WT mice with HUS.
375 We conclude, that Hp and Hx deficiency play divergent roles for HUS disease progression in mice. While
376 Stx-challenged Hx^{-/-} mice were characterized by less disease severity and an attenuated renal
377 pathology, Hp^{-/-} mice displayed a higher mortality rate, accompanied by renal iron and complement
378 deposition. Low dose Hp treatment of Stx-challenged WT mice attenuated surrogate parameters of renal

379 TMA and inflammation, but not kidney injury. Thus, we suggest, that Hp-dependent mechanisms convey
380 – at least in part – protection and that Hp is important for the resolution of STEC-HUS pathology.

381

382 **Disclosures**

383 The authors have no conflict of interest to declare.

384

385 **Authorship Contribution**

386 SMC designed, planned and supervised the study. SMC, WP, ANM wrote the manuscript and the
387 revisions. WP, BW performed animal experiments with WT, Hp^{-/-}, Hx^{-/-} mice, including data analysis. SK,
388 BW, NK performed animal experiments with Hp administration including data analysis. WP, ANM
389 analyzed ELISA data. WP, SK, ANM performed histology and immunohistochemistry including data
390 analysis. ANM performed gene expression, western blot analyses and hemolysis assay including data
391 analysis. FG provided Shiga toxin. CD, KA planned and supervised histology for liver, lung and colon,
392 immunohistochemistry for GP1b, electron microscopy and analyzed corresponding data. SMC, WP,
393 ANM, BW, NK, SK, CD, FG, ET, MB, KA and SHH provided important intellectual content and revised
394 the manuscript. All authors carefully reviewed and approved the manuscript.

395

396 **Supplementary Material**

397 Supplementary File (PDF)

398 Supplementary Methods.

399 Table S1. HUS score

400 Table S2. Commercial Kits

401 Table S3. Primary antibodies used for immunohistochemistry

402 Table S4. Secondary antibodies used for immunohistochemistry

403 Table S5. Primer used for quantitative real-time PCR

404 Table S6. Primary and secondary antibodies used for western blot analyses

405 Supplementary Results.

406 Supplementary Figures.

407 Figure S1.

408 **Supplementary Figure S1. Renal protein expression of HO-1 and Fth1 in WT, Hp^{-/-} and Hx^{-/-} mice**
409 **with experimental HUS.** Protein expression on day 5 of (A) HO-1 (28 kDa) and (B) Fth1 (21 kDa) in

410 kidneys of sham mice and mice subjected to Stx. Each line represents a single blot of indicated strains
411 and groups. Fth1, ferritin heavy chain; Hp, haptoglobin; HO-1, heme oxygenase-1; Hx, hemopexin; Stx,
412 Shiga toxin; WT, wild type. Representative blots of pooled samples are shown in Figure 3g (HO-1) and
413 Figure 7b (Fth1).

414

415 Figure S2.

416 **Supplemental Figure S2. Granulomatous alterations in the liver of WT, Hp^{-/-} and Hx^{-/-} mice with**
417 **experimental HUS.** Quantification and representative pictures of H&E staining in liver sections on day 5
418 of sham mice and mice subjected to Stx (n = 6 per group). Bars = 500 µm. Data are expressed as scatter
419 dot plot with median ± IQR for n observations. **P* < 0.05 vs. corresponding sham group, #*P* < 0.05 vs.
420 WT sham group (Mann-Whitney *U*-test). Hp, haptoglobin; H&E, hematoxylin and eosin; Hx, hemopexin;
421 IQR, interquartile range; Stx, Shiga toxin, WT, wild type.

422

423 Figure S3.

424 **Supplemental Figure S3. Inflammatory alterations in lung and colon of WT, Hp^{-/-} and Hx^{-/-} mice**
425 **with experimental HUS.** Representative pictures of (A) PAS reaction in lungs and (B) H&E staining in
426 colon sections on day 5 of sham mice and mice subjected to Stx (n = 6 per group). (A) Bars = 200 µm
427 (B) Bars = 500 µm. Since no morphological changes were observed in the intestine and lung, only the
428 presence of inflammatory cell aggregates was determined for these two organs (0 = absent;
429 1 = present). Few inflammatory cell aggregates were observed in the lung of WT sham (1/6), WT Stx
430 (3/6), Hp^{-/-} sham (2/6), Hp^{-/-} Stx (2/6), Hx^{-/-} sham (3/6), and Hx^{-/-} Stx (3/6) mice. Few inflammatory cell
431 aggregates were observed in the colon of WT sham (2/6), WT Stx (1/6), Hp^{-/-} sham (4/6), Hp^{-/-} Stx (3/6),
432 Hx^{-/-} sham (0/6), and Hx^{-/-} Stx (2/6) mice. Hp, haptoglobin; H&E, hematoxylin and eosin; Hx, hemopexin;
433 IQR, interquartile range; PAS, periodic acid Schiff; Stx, Shiga toxin, WT, wild type.

434

435 Figure S4.

436 **Supplementary Figure S4. Plasma values of ALAT and ASAT in WT, Hp^{-/-} and Hx^{-/-} mice with**
437 **experimental HUS.** Determination of plasma (A) ALAT (WT sham: n = 12; WT Stx, Hp^{-/-} sham and Stx,
438 Hx^{-/-} Stx: n = 6, sham; Hx^{-/-}: n = 5) and (B) ASAT (n = 12 per group) in sham mice and mice subjected
439 to Stx on day 5. (A-B) Data are expressed as scatter dot plot with median ± IQR for n observations.

440 * $P < 0.05$, vs. corresponding sham group, # $P < 0.05$ vs. WT sham group (Mann-Whitney U -test). ALAT,
441 alanine aminotransferase; ASAT, aspartate aminotransferase; Hp, haptoglobin; Hx, hemopexin; IQR,
442 interquartile range; Stx, Shiga toxin; WT, wild type.

443

444 Figure S5.

445 **Supplementary Figure S5. Renal PAS reaction, CD31 and CC-3 staining in WT, Hp^{-/-} and Hx^{-/-} mice**
446 **with experimental HUS.** Representative pictures on day 5 of (A) PAS reaction, immunohistochemical
447 (B) CD31 and (C) CC-3 staining in renal sections of sham mice and mice subjected to Stx (n = 8 per
448 group). Bars = 100 μ m. (A-C) Quantifications are shown in Figures 3c (PAS), 3e (CD31) and 3f (CC-3).
449 CC-3, cleaved caspase-3; Hp, haptoglobin; Hx, hemopexin; PAS, periodic acid Schiff; Stx, Shiga toxin,
450 WT, wild type.

451

452 Figure S6.

453 **Supplementary Figure S6. Kidney dysfunction in WT, Hp^{-/-} and Hx^{-/-} mice with experimental HUS.**
454 Determination of (A) creatinine and (B) potassium in plasma of sham mice and mice subjected to Stx
455 (n = 8 per group). (A-B) Data are expressed as scatter dot plot with median \pm IQR for n observations.
456 * $P < 0.05$, vs. corresponding sham group, § $P < 0.05$ vs. WT Stx group (Mann-Whitney U -test). Hp,
457 haptoglobin; Hx, hemopexin; Stx, Shiga toxin, WT, wild type.

458

459 Figure S7.

460 **Supplementary Figure S7. Electron microscopic analysis of kidney tissue from WT, Hp^{-/-} and**
461 **Hx^{-/-} mice with experimental HUS.** Representative ultrastructural images on day 5 of sham mice and
462 mice subjected to Stx. After HUS induction, only occasional widening of the podocyte foot processes
463 (FP) and slightly swollen endothelium were observed in all genotypes. The fenestration of the
464 endothelium (EC) was not noticeably altered due to Stx challenge. The glomerular basement
465 membranes were neither widened nor injured and mesangial cells appeared normal (N = nucleus;
466 P = podocyte; RBC = red blood cell). Scale bar = 1 μ m. Hp, haptoglobin; Hx, hemopexin; Stx, Shiga
467 toxin; WT, wild type.

468

469 Figure S8.

470 **Supplementary Figure S8. Renal fibrin depositions in WT, Hp^{-/-} and Hx^{-/-} mice with experimental**
471 **HUS.** Quantifications and representative pictures of SGOF staining on day 5 in renal sections of sham
472 mice and mice subjected to Stx (n = 8 per group). Data are expressed as scatter dot plot with
473 median ± IQR for n observations. **P* < 0.05, vs. corresponding sham group, §*P* < 0.05 vs. WT Stx group
474 (Mann-Whitney *U*-test). Hp, haptoglobin; Hx, hemopexin; IQR, interquartile range; SFOG; acid fuchsin
475 orange G; Stx, Shiga toxin, WT, wild type.

476

477 Figure S9.

478 **Supplementary Figure S9. Hepatic heme and iron metabolism in WT, Hp^{-/-} and Hx^{-/-} mice with**
479 **experimental HUS.** mRNA expression of (A) *CD163*, (B) *Trf*, (C) *Lrp1*, (D) *Fth1*, (E) *Ftl1*, (F) *Alb* and
480 (G) *SCL40A1* in livers of sham mice and mice subjected to Stx (n = 6 per group). (A-H) Data are
481 expressed as scatter dot plot with median ± IQR for n observations. **P* < 0.05 vs. corresponding sham
482 group, #*P* < 0.05 vs. WT sham group, §*P* < 0.05 vs. WT Stx group (Mann-Whitney *U*-test). *Alb*, albumin;
483 *Fth1*, ferritin heavy chain; *Ftl1*, ferritin light chain; Hp, haptoglobin; *Hmox1*, heme oxygenase-1; Hx,
484 hemopexin; IQR, interquartile range; *Lrp1*, LDL-receptor related protein 1; *Trf*, transferrin; *SCL40A1*,
485 ferroportin; Stx, Shiga toxin; WT, wild type.

486

487 Figure S10.

488 **Supplementary Figure S10. Renal heme and iron metabolism in WT, Hp^{-/-} and Hx^{-/-} mice with**
489 **experimental HUS.** mRNA expression of (A) *Alb*, (B) *Trf*, (C) *SCL40A1* (D) *Lrp1*, (E) *Ftl1*, (F) *Fth1*,
490 (G) *Lrp*, (H) *Cubn* and (I) *Hmox1* on day 5 in kidneys of sham mice and mice subjected to Stx (n = 6 per
491 group). (A-I) Data are expressed as scatter dot plot with median ± IQR for n observations. **P* < 0.05 vs.
492 corresponding sham group, #*P* < 0.05 vs. WT sham group, §*P* < 0.05 vs. WT Stx group (Mann-Whitney
493 *U*-test). *Alb*, albumin; *Cubn*, cubilin; *Fth1*, ferritin heavy chain; *Ftl1*, ferritin light chain; Hp, haptoglobin;
494 *Hmox1*, heme oxygenase-1; Hx, hemopexin; IQR, interquartile range; *Lrp1*, LDL-receptor related protein
495 1; *Lrp2*, LDL-receptor related protein 2; *Trf*, transferrin; Stx, Shiga toxin; WT, wild type.

496

497 Figure S11.

498 **Supplementary Figure S11. Renal expression of DMT1, megalin and cubilin in WT, Hp^{-/-} and Hx^{-/-}**
499 **mice with experimental HUS.** Quantification and representative pictures of immunohistochemical
500 (A) DMT1, (B) megalin and (C) cubilin staining on day 5 in renal sections of sham mice and mice

501 subjected to Stx (n = 8 per group). Bars = 100 μ m. (A-B) Data are expressed as scatter dot plot with
502 median \pm IQR for n observations. * P < 0.05 vs. corresponding sham group, $^{\S}P$ < 0.05 vs. WT Stx group
503 (Mann-Whitney U -test). DMT1, divalent metal transporter 1; Hp, haptoglobin; Hx, hemopexin; IQR,
504 interquartile range; Stx, Shiga toxin; ROI, region of interest; WT, wild type.

505

506 Figure S12.

507 **Supplementary Figure S12. Oxidative stress in the kidney of WT, Hp^{-/-} and Hx^{-/-} mice with**
508 **experimental HUS.** (A) MDA levels on day 5 in kidneys of sham mice and mice subjected to Stx (n = 6
509 per group). Quantification of immunohistochemical (B) nitrotyrosine and (C) NOX-1 staining on day 5 in
510 renal sections of sham mice and mice subjected to Stx (n = 8 per group). Bars = 100 μ m. (A-C) Data
511 are expressed as scatter dot plot with median \pm IQR for n observations. * P < 0.05 vs. corresponding
512 sham group, $^{\#}P$ < 0.05 vs. WT sham group, $^{\S}P$ < 0.05 vs. WT Stx group (Mann-Whitney U -test). Hp,
513 haptoglobin; Hx, hemopexin; IQR, interquartile range; MDA, malondialdehyde; NOX-1, NADPH oxidase
514 1; Stx, Shiga toxin; WT, wild type.

515

516 Supplementary References

517

518 Supplementary information is available on Kidney International's website.

519

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706

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718 **Figure Legends**

719

720 **Figure 1. Clinical presentation of WT, $Hp^{-/-}$, and $Hx^{-/-}$ mice with experimental HUS.** (a) Kaplan-Meier
 721 survival analysis of sham mice and mice subjected to Stx (WT sham: n = 9, WT Stx: n = 14, $Hp^{-/-}$ sham:
 722 n = 8, $Hp^{-/-}$ Stx: n = 8, $Hx^{-/-}$ sham: n = 8, $Hx^{-/-}$ Stx: n = 8) in experimental HUS followed up for 7 days.
 723 * $P < 0.05$ vs. corresponding sham group, $^{\S}P < 0.05$ vs. indicated Stx group (Log-rank Mantel-Cox test).
 724 (b-e) Experimental HUS followed up for 5 days in sham mice and mice subjected to Stx (WT sham:
 725 n = 19, WT Stx: n = 14, $Hp^{-/-}$ sham: n = 13, $Hp^{-/-}$ Stx: n = 13, $Hx^{-/-}$ sham: n = 12, $Hx^{-/-}$ Stx: n = 12).
 726 (b) Evaluation of disease progression by HUS score (ranging from 1 = very active to 5 = dead) over 5
 727 days. (c) Significant changes of HUS score on day 5 of sham mice and mice subjected to Stx.
 728 (d) Progression of weight loss on day 1 to 5 in sham mice and mice subjected to Stx. (e) Significant
 729 changes of weight loss on day 5 in sham mice and mice subjected to Stx. (b-e) Data are expressed as
 730 (b, d) dot plot, (c) bar graph, (e) scatter dot plot with median \pm IQR. * $P < 0.05$ vs. corresponding sham
 731 group, $^{\S}P < 0.05$ vs. WT Stx group (Mann-Whitney *U*-test). Hp, haptoglobin; Hx, hemopexin; IQR,
 732 interquartile range; Stx, Shiga toxin; WT, wild type.

733

734 **Figure 2. Heme and Hb scavengers in WT, $Hp^{-/-}$, and $Hx^{-/-}$ mice with experimental HUS.** (a) mRNA
 735 expression of *Hx* on day 5 in livers of sham mice and mice subjected to Stx (n = 6 per group, except:
 736 n = 5 for $Hp^{-/-}$ Stx). (b) Plasma Hx levels on day 5 of sham mice and mice subjected to Stx (n = 12 per
 737 group). Determination of plasma (c) $\alpha 1M$ and (d) albumin on day 5 in sham mice and mice subjected to
 738 Stx (n = 12 per group). (e) mRNA expression of *Hp* on day 5 in livers of sham mice and mice subjected
 739 to Stx (n = 6 per group). (f) Plasma Hp levels on day 5 of sham mice and mice subjected to Stx (n = 12
 740 per group). (a-e) Data are expressed as scatter dot plot with median \pm IQR for n observations. * $P < 0.05$
 741 vs. corresponding sham group, $^{\#}P < 0.05$ vs. WT sham group, $^{\S}P < 0.05$ vs. WT Stx group
 742 (Mann-Whitney *U*-test). $\alpha 1M$, alpha-1-microglobulin; *Hp*/*Hp*, haptoglobin; *Hx*/*Hx*, hemopexin; IQR,
 743 interquartile range; Stx, Shiga toxin; WT, wild type.

744

745 **Figure 3. Kidney injury and renal stress burden in WT, $Hp^{-/-}$, and $Hx^{-/-}$ mice with experimental**
 746 **HUS.** Determination of plasma (a) urea and (b) NGAL on day 5 in sham mice and mice subjected to Stx
 747 (n = 12 per group). Quantification of (c) PAS reaction on day 5 in renal sections of sham mice and mice
 748 subjected to Stx (n = 8 per group). Quantification and representative pictures of immunohistochemical

749 (d) KIM-1 staining on day 5 in renal sections of sham mice and mice subjected to Stx (n = 8 per group).
 750 Quantification of immunohistochemical (e) CD31 and (f) CC-3 staining on day 5 in renal sections of
 751 sham mice and mice subjected to Stx (n = 8 per group). (g) Protein expression of HO-1 on day 5 in
 752 kidneys of sham mice and mice subjected to Stx. Samples from 6 animals per group were pooled to
 753 equal protein amounts for this representative blot (n = 6 per group). Individual blots (1 animal/group) are
 754 shown in supplementary Figure S1A. (h) Quantification and representative pictures of
 755 immunohistochemical GP1b staining on day 5 in renal sections of sham mice and mice subjected to Stx
 756 (n = 8 per group). Bars = 100 μ m. Data are expressed as (a-f, h) scatter dot plot, (g) bar graph with
 757 median \pm IQR for n observations. * P < 0.05 vs. corresponding sham group, # P < 0.05 vs. WT sham
 758 group (Mann-Whitney U -test). CC-3, cleaved caspase-3; GP1b; glycoprotein 1b; Hp, haptoglobin; HO-1,
 759 heme oxygenase-1; Hx, hemopexin; IQR, interquartile range; KIM-1, kidney injury molecule-1; NGAL,
 760 neutrophil gelatinase-associated lipocalin; PAS, periodic acid Schiff; Stx, Shiga toxin, WT, wild type.

761

762 **Figure 4. Hemolysis in WT, Hp^{-/-}, and Hx^{-/-} mice with experimental HUS.** Determination of
 763 (a) hemolysis and (b) plasma bilirubin on day 5 in sham mice and mice subjected to Stx (hemolysis: WT
 764 sham: n = 10, WT Stx n = 10, Hp^{-/-} sham n = 8, Hp^{-/-} n = 7, Hx^{-/-} sham n = 8, Hx^{-/-} Stx n = 9; bilirubin:
 765 n = 12 per group). (c) mRNA expression of *Hmox1* on day 5 in the liver of sham mice and mice subjected
 766 to Stx (n = 6 per group). Protein expression of HO-1 on day 5 in the liver of (d) WT, (e) Hp^{-/-}, and (f) Hx^{-/-}
 767 sham mice and mice subjected to Stx (n = 5 per group). Protein expression of Fth1 on day 5 in the
 768 liver of (g) WT, (h) Hp^{-/-}, and (i) Hx^{-/-} sham mice and mice subjected to Stx (n = 5 per group). Protein
 769 expression of CD163 on day 5 in the liver of (j) WT, (k) Hp^{-/-}, and (l) Hx^{-/-} sham mice and mice subjected
 770 to Stx (n = 5 per group). (a-l) Data are expressed as scatter dot plot with median \pm IQR for n
 771 observations. * P < 0.05 vs. corresponding sham group. Fth1, ferritin heavy chain; Hp, haptoglobin;
 772 *Hmox1*/HO-1; heme oxygenase-1; Hx, hemopexin; Stx, Shiga toxin; WT, wild type.

773

774 **Figure 5. Immune response in WT, Hp^{-/-}, and Hx^{-/-} mice with experimental HUS.** Quantification and
 775 representative pictures of immunohistochemical (a) F4-80, (b) Ly6G, and (c) C3c staining on day 5 in
 776 renal sections of sham mice and mice subjected to Stx (n = 8 per group). Bars = 100 μ m. (a-c) Data are
 777 expressed as scatter dot plot with median \pm IQR for n observations. * P < 0.05 vs. corresponding sham
 778 group, # P < 0.05 vs. WT sham group, § P < 0.05 vs. WT Stx group (Mann-Whitney U -test). Hp,

779 haptoglobin; Hx, hemopexin; IQR, interquartile range; Ly6G, lymphocyte antigen 6 complex, locus G;
 780 Stx, Shiga toxin; WT, wild type.

781

782 **Figure 6. Effect of Hp treatment on kidney injury and inflammation in WT mice with experimental**

783 **HUS. (a)** Application regime for low dose Hp treatment of sham mice and mice subjected to Stx.

784 **(b)** Determination of plasma NGAL on day 5 in sham mice and mice subjected to Stx, which were treated

785 with Hp or vehicle (n = 6 per treatment group). Quantification of **(c)** PAS reaction, immunohistochemical

786 **(d)** KIM-1, **(e)** CC-3, **(f)** F4-80, **(g)** C3c, **(h)** GP1b and **(i)** Ly6G staining on day 5 in renal sections of

787 sham mice and mice subjected to Stx, which were treated with Hp or vehicle (sham + vehicle,

788 sham + Hp: n = 4 per group; Stx + vehicle, Stx + Hp: n = 6 per group; GP1b: Stx + Hp: n = 5 per group).

789 **(c-h)** Data are expressed as scatter dot plot with median \pm IQR for n observations. * P < 0.05 vs.

790 corresponding sham group (Mann-Whitney U -test). CC-3, cleaved caspase-3; GP1b; glycoprotein 1b;

791 Hp, haptoglobin; i.p., intraperitoneal; IQR, interquartile range; KIM-1, kidney injury molecule-1; Ly6G,

792 lymphocyte antigen 6 complex, locus G; NGAL, neutrophil gelatinase-associated lipocalin; PAS, periodic

793 acid Schiff, s.c., subcutaneous; Stx, Shiga toxin, WT, wild type.

794

795 **Figure 7. Renal iron homeostasis in WT, Hp^{-/-}, and Hx^{-/-} mice with experimental HUS.**

796 **(a)** Quantification and representative pictures of iron staining on day 5 in renal sections of sham mice

797 and mice subjected to Stx (n = 8 per group). **(b)** Protein expression of Fth1 on day 5 in kidneys of sham

798 mice and mice subjected to Stx. Samples from 6 animals per group were pooled to equal protein

799 amounts for this representative blot (n = 6 per group). Individual blots (1 animal/group) are shown in

800 supplementary Figure S1B. **(c)** Plasma hepcidin levels on day 5 of sham mice and mice subjected to

801 Stx (n = 6 per group). Quantification and representative pictures of immunohistochemical **(d)** ferroportin

802 staining on day 5 in renal sections of sham mice and mice subjected to Stx (n = 8 per group).

803 Bars = 100 μ m. Data are expressed as **(a, c-d)** scatter dot plot, **(b)** bar graph with median \pm IQR for n

804 observations. * P < 0.05 vs. corresponding sham group, # P < 0.05 vs. WT sham group, § P < 0.05 vs. WT

805 Stx group (Mann-Whitney U -test). Fth1, ferritin heavy chain; Hp, haptoglobin; Hx, hemopexin; IQR,

806 interquartile range; Stx, Shiga toxin; ROI, region of interest; WT, wild type.

807

808

Figure 1

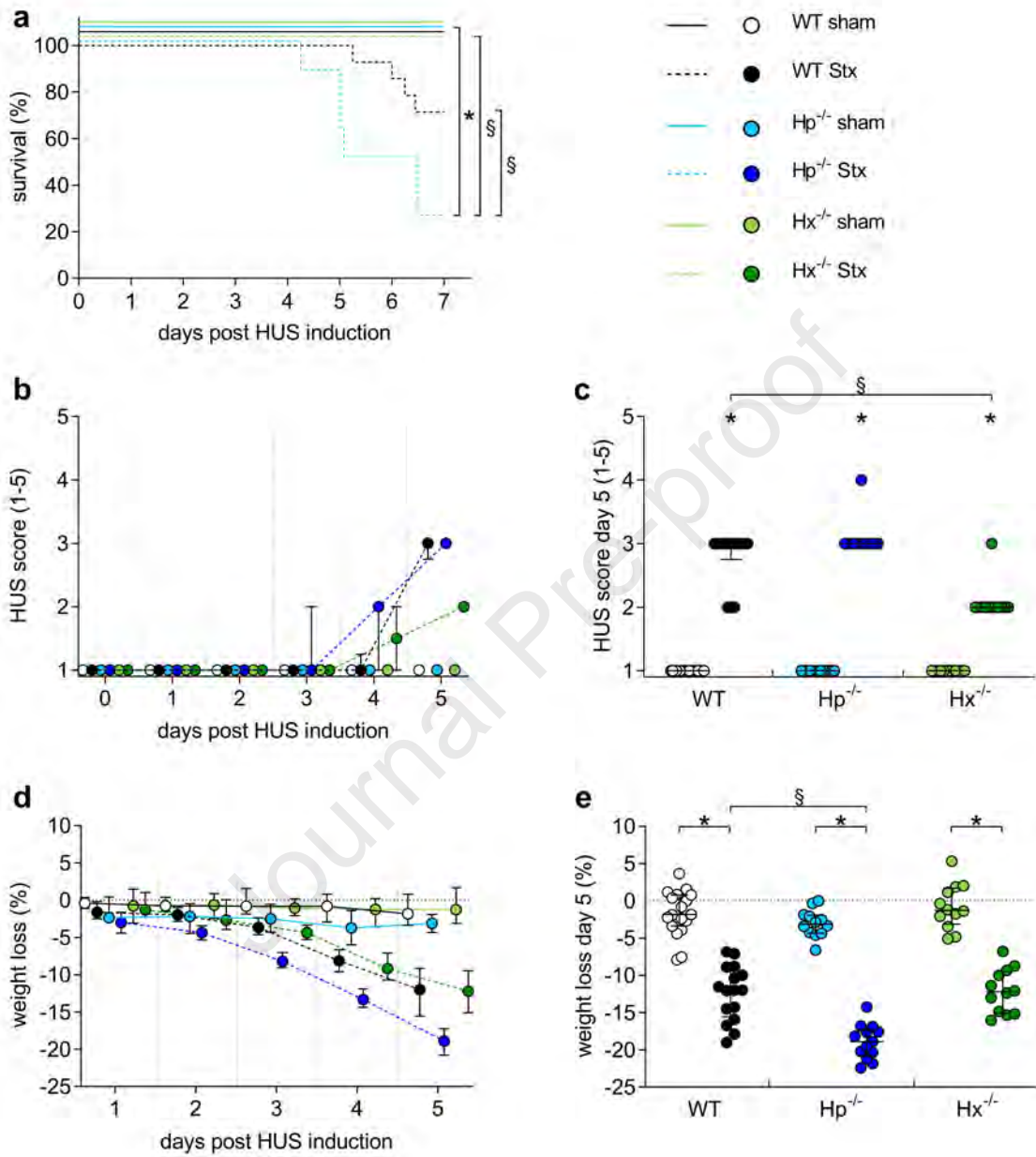


Figure 2

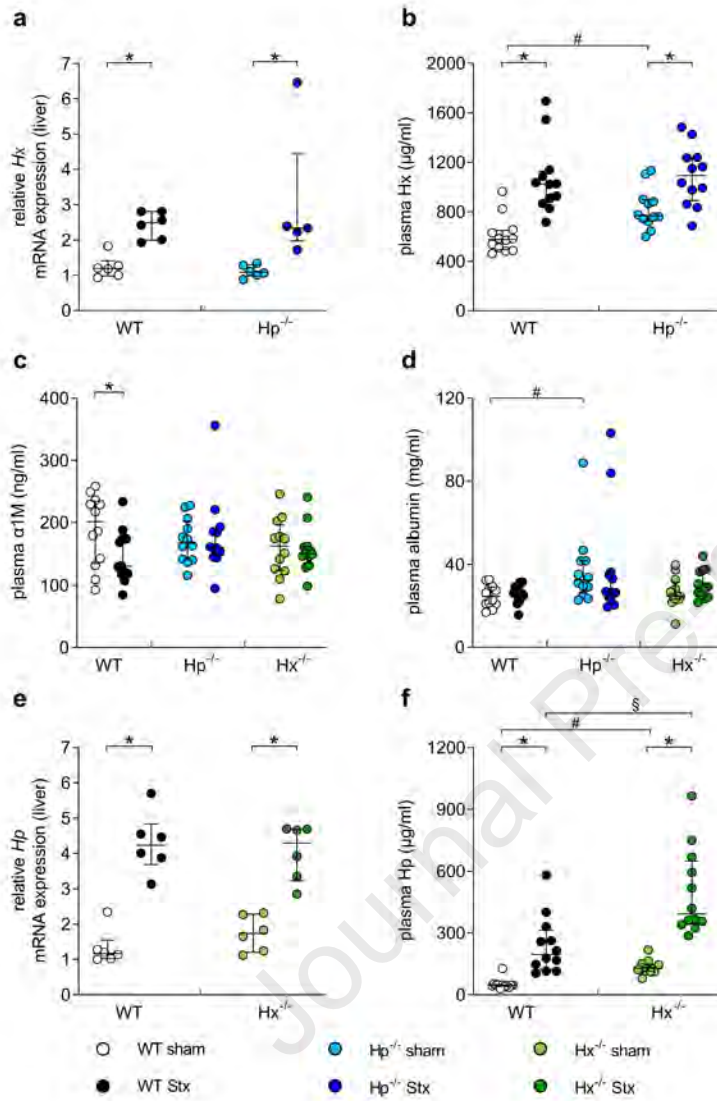


Figure 3

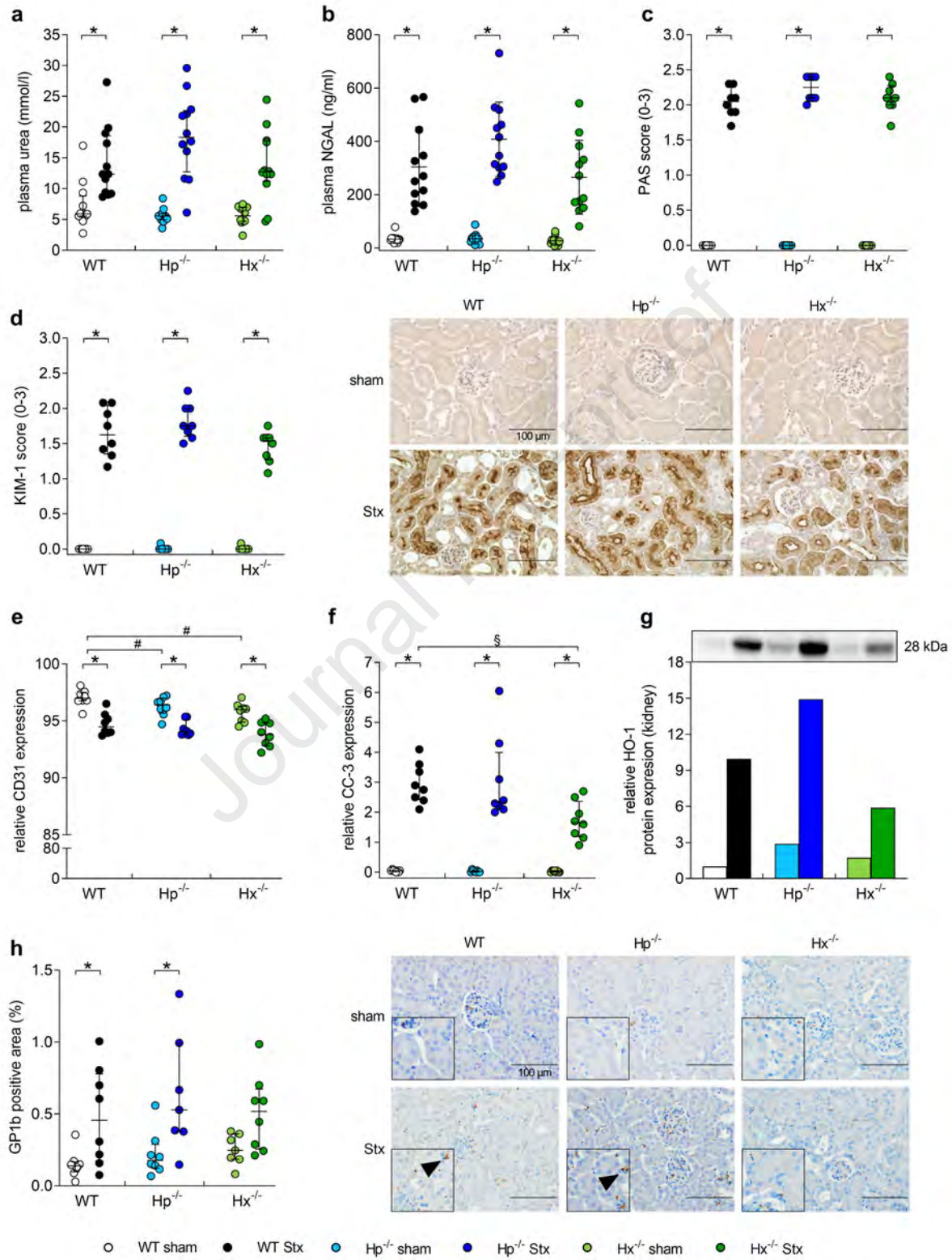


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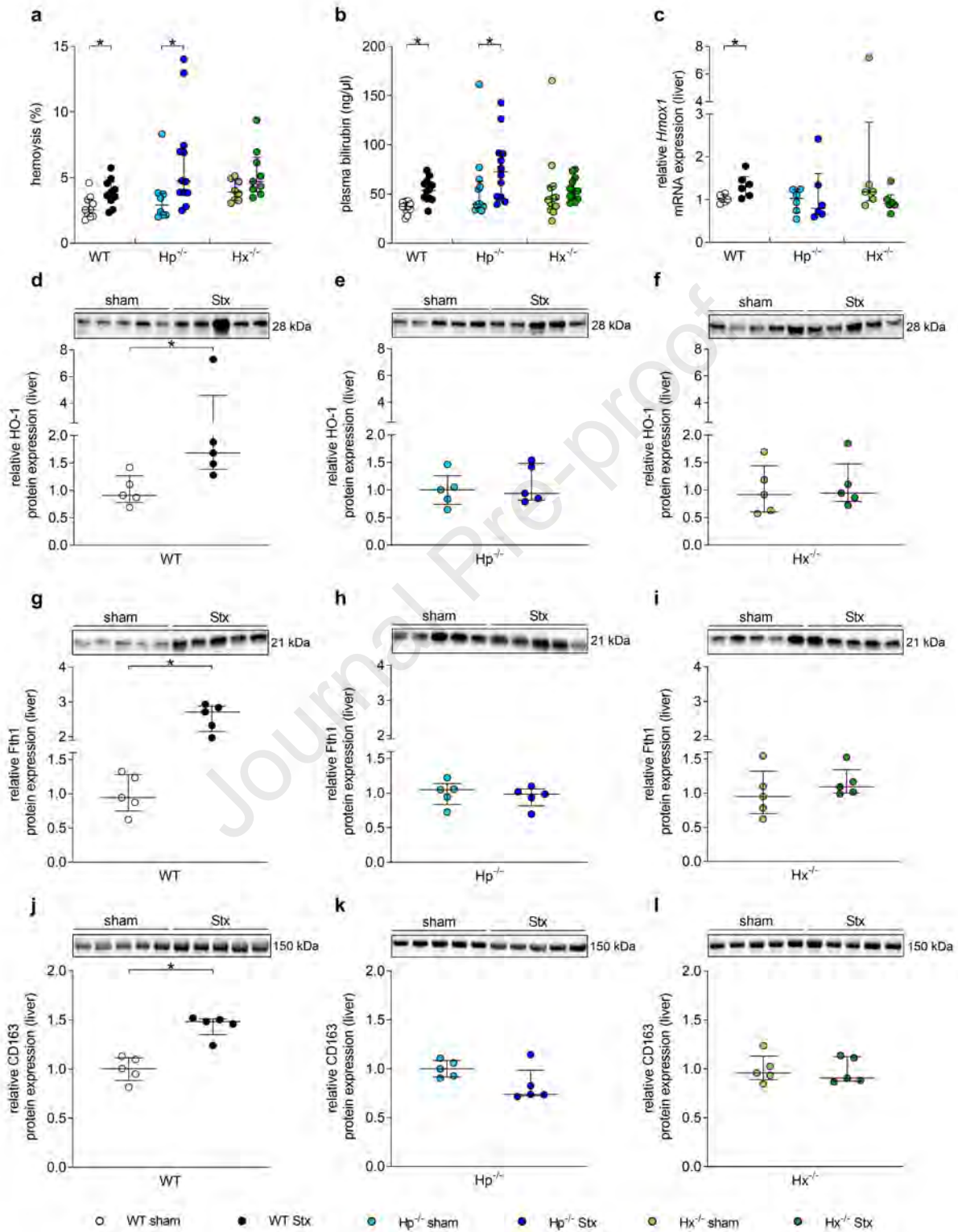


Figure 5

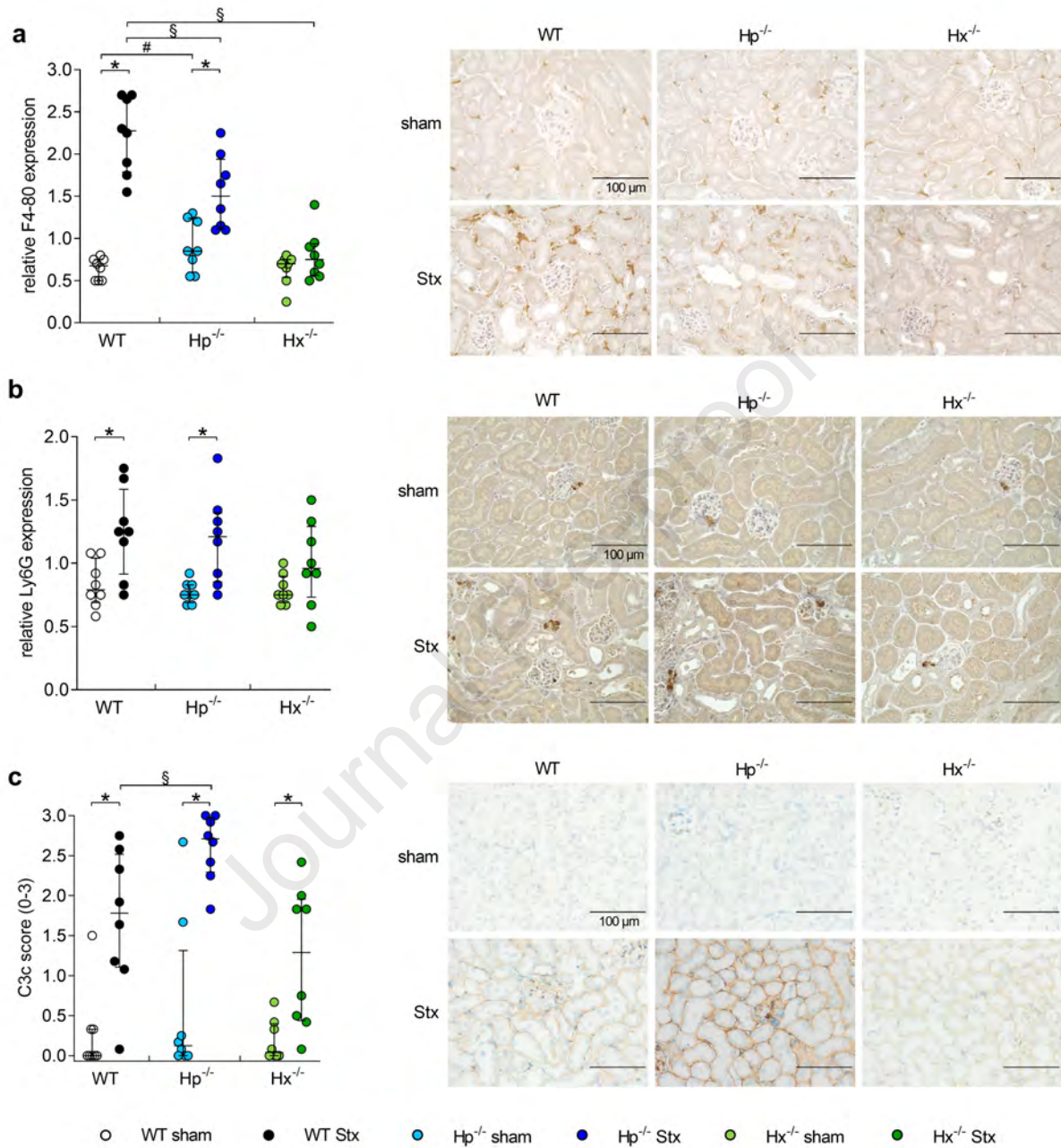


Figure 6

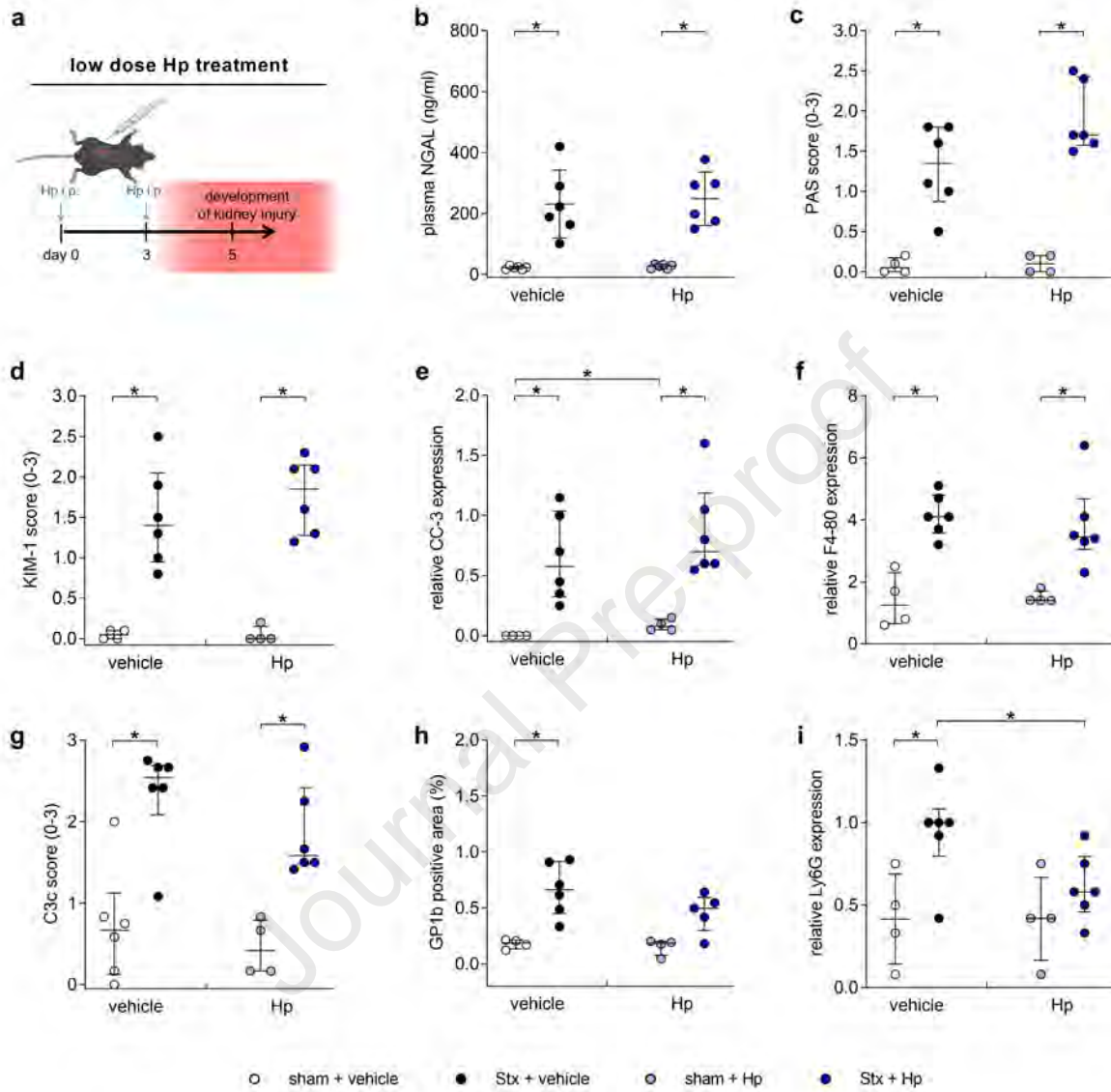


Figure 7

