Supplementary information

Materials and Methods

Mice

Female C57BL/6N wild-type mice (7-8 weeks of age) were treated with SLN124 to establish time courses and dose responses. The Hfe-ko mice used in this study were on a pure congenic C57BL/6J genetic background. Age and gender-matched wild type C57BL/6J mice were used to obtain reference values. Mice were housed under a constant light-dark cycle and maintained on a standard mouse diet containing 200 ppm iron with ad libitum access to food and water. Mouse breeding and animal experiments were approved by and conducted in compliance with the guidelines of the EMBL Institutional Animal Care and Use Committee and by the Regierungspräsidium Freiburg (G-16/117).

Tissue and serum iron quantification

Tissue non-heme iron content was measured using the bathophenanthroline method[1]. Fresh blood was collected by cardiac puncture from mice euthanized by CO_2 inhalation. Hematological parameters were measured on 50µl of heparinized blood using the ABC ScilVet blood analyzer (ABX Diagnostics, Montpellier, France). Serum iron concentration and unsaturated iron binding capacity were assessed using the SFBC and UIBC kits (Biolabo, Maizy, France). Transferrin saturation was calculated using the formula SFBC/(SFBC + UIBC) × 100. Serum hepcidin was quantified in duplicate in 12µl of sample using the "Hepcidin Murine-Compete ELISA Kit" (Intrinsic Lifescience, United States) according to manufacturer's instructions. Serum ferritin has been measured using an Olympus AU400 analyzer at the Claude Bernard Institute (Paris, France)

Gene expression analysis

Total RNA was isolated from SNAP-frozen tissues using the Trizol reagent (Invitrogen) according to the manufacturer's instruction. One microgram of total RNA was reverse transcribed in a 25µl reaction using M-MLV reverse transcriptase (Fermentas) and random oligomers as primers. SYBR green Real-Time PCR was performed with the ABI StepONE Plus Real Time PCR System (Applied Biosystems) using the following primers:

	Forward (5'→3')	Reverse (5'→3')
RPL19	AGGCATATGGGCATAGGGAAGAG	TTGACCTTCAGGTACAGGCTGTG
BMP2	GCGCAGCTTCCATCACGA	CCCACTCATCTCTGGAAGTTCCT
BMP6	ATGGCAGGACTGGATCATTGC	CCATCACAGTAGTTGGCAGCG
SMAD6	GTTGCAACCCCTACCACTTC	GGAGGAGACAGCCGAGAATA
SMAD7	GCAGGCTGTCCAGATGCTGT	GATCCCCAGGCTCCAGAAGA
A2M	CATCACCCAAGCCCTCAC	CAAAGTGACTTCATCTTCTACTCCTC

Relative target mRNA expression levels were normalized to the RPL19 (Ribosomal Protein L19) housekeeping mRNA Results were calculated using the Pfaffl method.[2]

For the gene expression quantification of TMPRSS6 and Hepcidin (Hamp), total RNA was isolated from SNAP-frozen tissues using InviTrap ® Spin Tissue RNA Mini Kit (Stratec) according to the manufacturer's instruction. TaqMan Real-Time-qPCR was performed with QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems) in a 384-well format using 100ng total RNA per 20µl reaction, the Takyon™ One-Step Low Rox Probe 5X MasterMix dTTP (Eurogentec) and the following probe sets:

TMPRSS6	Forward (5'→3')	CCGCCAAAGCCCAGAAG
	Reverse (5'→3')	GGTCCCTCCCCAAAGGAATAG
	Probe (5'→3')	6-FAM-CAGCACCCGCCTGGGAACTTACTACAAC-BHQ1
ApoB	Forward (5'→3')	AAAGAGGCCAGTCAAGCTGTTC
	Reverse (5'→3')	GGTGGGATCACTTCTGTTTTGG
	Probe (5'→3')	6-FAM-CAGCAACACACTGCATCTGGTCTCTACCA-BHQ1
HAMP	Forward (5'→3')	CCTGTCTCCTGCTTCTCCTCCT
	Reverse (5'→3')	AATGTCTGCCCTGCTTTCTTCC
	Probe (5'→3')	6-FAM-TGAGCAGCACCACCTATCTCCATCAACA-BHQ1

Results were calculated using the comparative C_T method also known as the $2^{-\Delta\Delta Ct}$ method.[3,4] Relative TMPRSS6 and Hamp mRNA expression were normalized to ApoB housekeeping gene specific for hepatocytes.

Histology

Tissues were fixed for 24h in 10% neutral buffered formalin (Sigma), dehydrated, paraffin embedded and sectioned at 3 µm on polylysine slides (Thermo scientific). For iron staining, slides were treated for 10 minutes with 3% H_2O_2 to block the endogenous peroxidases and incubated for 20minutes in Prussian Blue (Sigma) followed by a 20minutes incubation with DAB (Sigma). Meyer's hematoxylin was used as counterstain.

Statistical analyses.

Data are shown as mean±SEM. Statistical analyses were performed using Prism v8.1 (GraphPad Software, La Jolla, CA). The p-values were calculated using Welch-ANOVA with Dunnett T3 multiple comparisons tests. This approach takes into account both unequal group sizes and unequal SDs (SEMs) and returns the adjusted p-values when testing multiple hypotheses on the same data set. p-values <0.05 (*), <0.01 (**), <0.001 (***) and <0.0001 (****) are indicated.

Supplementary references

1. Torrance JD, Bothwell TH. A simple technique for measuring storage iron concentrations in formalinised liver samples. *S Afr J Med Sci.* 1968;33(1):9-11.
2. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 2001;29(9):2002-2007.
3. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2-[Delta][Delta]CT Method. *Methods.* 2001;25(4):402-408.
4. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc.* 2008;3(6):1101-1108.

Supplementary Figure Legends

Figure S1: Gene expression analysis of hepcidin regulators in C57BL/6N wild type mice treated with SLN124, **A-B:** mRNA expression analysis of the SMAD6 (A) and SMAD7 (B) BMP/SMAD target genes. **C-D:** mRNA expression analysis of the bone morphogenetic proteins BMP2 (C) and BMP6 (D). **E:** mRNA expression of the acute phase gene alpha-2-macroglobulin (A2M). Data are reported as mean±S.E.M. Welch-ANOVA with Dunnett T3 multiple comparisons test against PBS control group. p-values <0.05 (*), <0.01 (**), <0.001 (***) and <0.0001 (****) are indicated.

Figure S2: SLN124 reduces transferrin saturation and increases the iron content in the red pulp of the spleen of Hfe-ko mice. A: Transferrin saturation of Hfe-ko mice treated with PBS, GalNAc-Luciferase siRNA (CTRL) and two different doses of SLN124, as indicated. **B:** DAB-enhanced Perls' iron stain of spleen sections of Hfe-ko mice treated as indicated. Data are reported as mean±S.E.M. Welch-ANOVA with Dunnett T3 multiple comparisons test against PBS control group. p-value <0.01 (**).The dashed line indicates the average value of the parameter measured on age, gender and genetic background (C57BL/6J) matched wild type mice.

Figure S3: Analysis of iron content and distribution of Hfe-ko mice treated with SLN124. A: Non-heme hepatic iron measurement of Hfe-ko mice treated with PBS, GalNAc-Luciferase siRNA (CTRL) or two different doses of SLN124, as indicated. The dashed line indicates the average value of the parameter measured on age, gender and genetic background (C57BL/6J) matched wild type animals. **B:** DAB-enhanced Perls' iron stain of hepatic sections of Hfe-ko mice treated as indicated. Data are reported as mean±S.E.M.

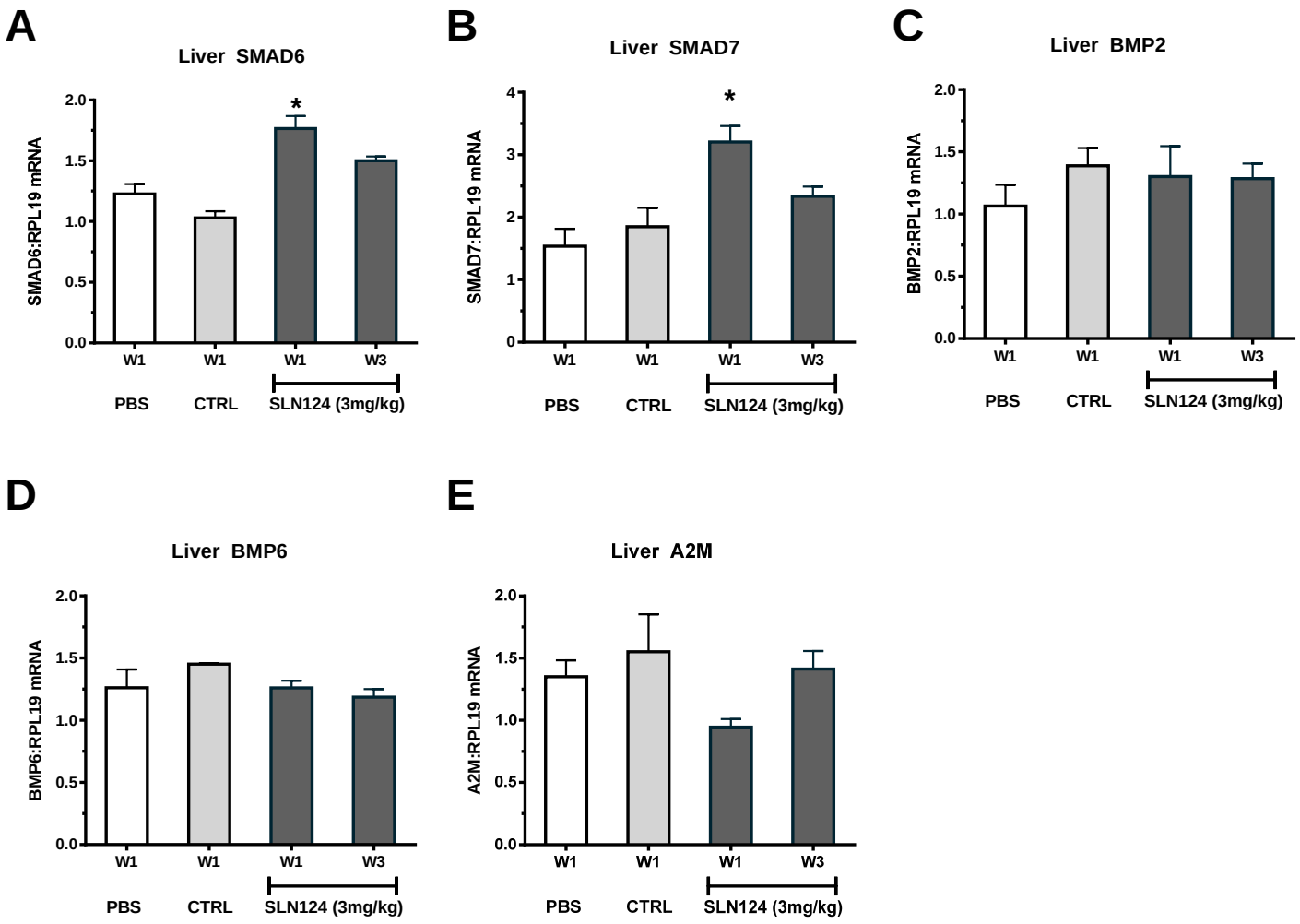
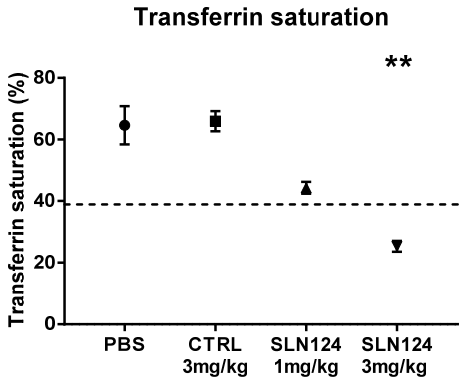

A
Liver SMAD6
SMAD6:RPL19 mRNA
2.0
1.5
1.0
0.5
0.0
*
W1
W1
W1
W3
PBS
CTRL
SLN124 (3mg/kg)
B
Liver SMAD7
SMAD7:RPL19 mRNA
4
3
2
1
0
*
W1
W1
W1
W3
PBS
CTRL
SLN124 (3mg/kg)
C
Liver BMP2
BMP2:RPL19 mRNA
2.0
1.5
1.0
0.5
0.0
W1
W1
W1
W3
PBS
CTRL
SLN124 (3mg/kg)
D
Liver BMP6
BMP6:RPL19 mRNA
2.0
1.5
1.0
0.5
0.0
W1
W1
W1
W3
PBS
CTRL
SLN124 (3mg/kg)
E
Liver A2M
A2M:RPL19 mRNA
2.0
1.5
1.0
0.5
0.0
W1
W1
W1
W3
PBS
CTRL
SLN124 (3mg/kg)

Figure S1

Figure S2

A


Transferrin saturation
Transferrin saturation (%)
80
60
40
20
0
**
PBS
CTRL 3mg/kg
SLN124 1mg/kg
SLN124 3mg/kg

B

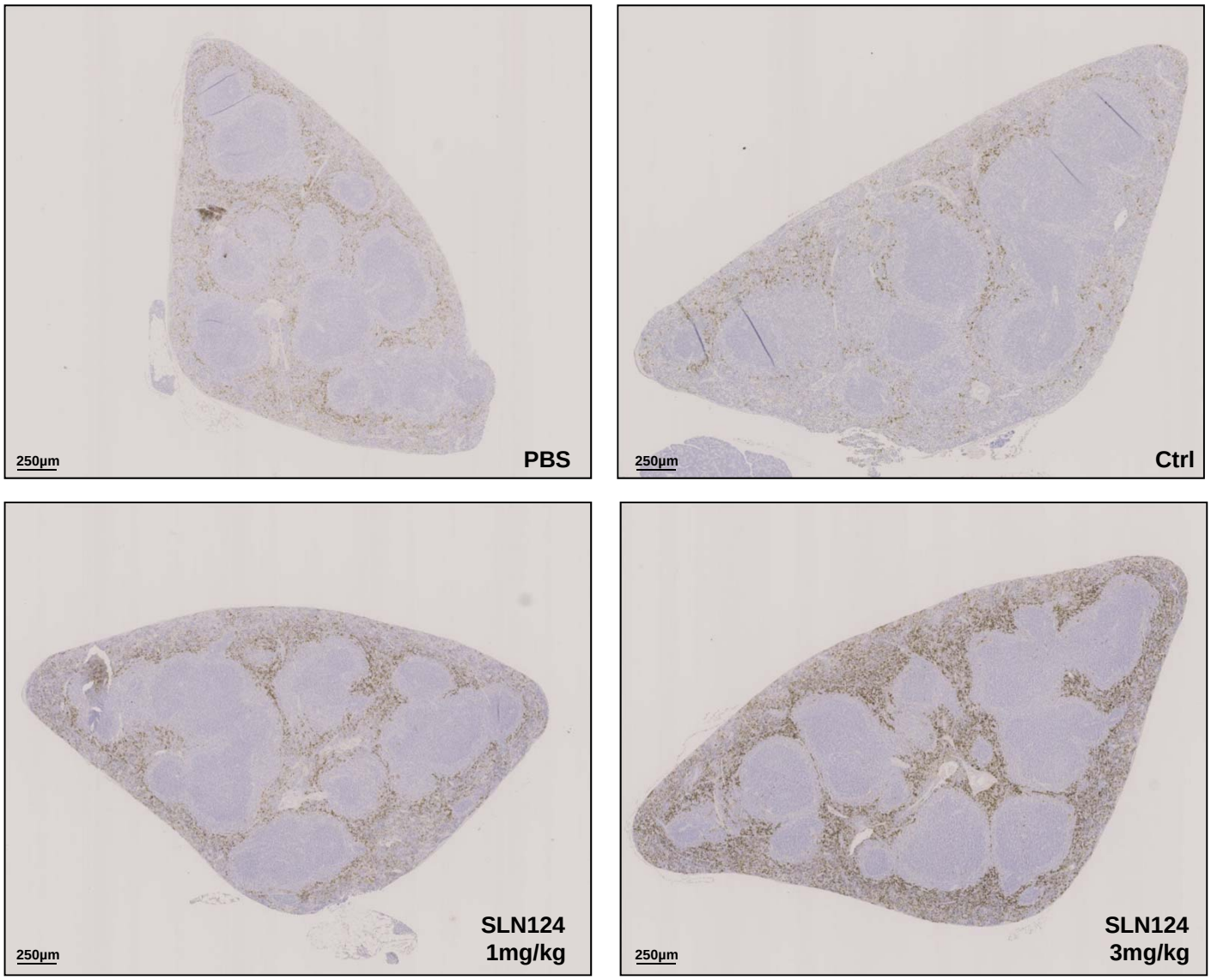
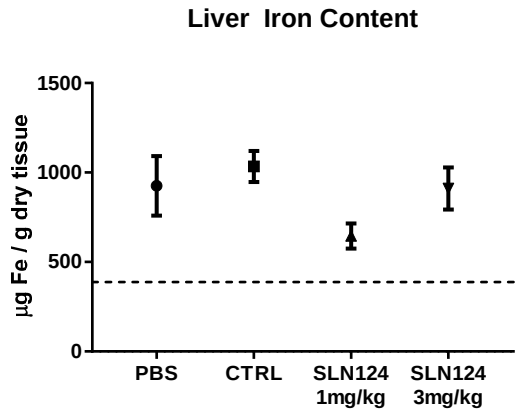
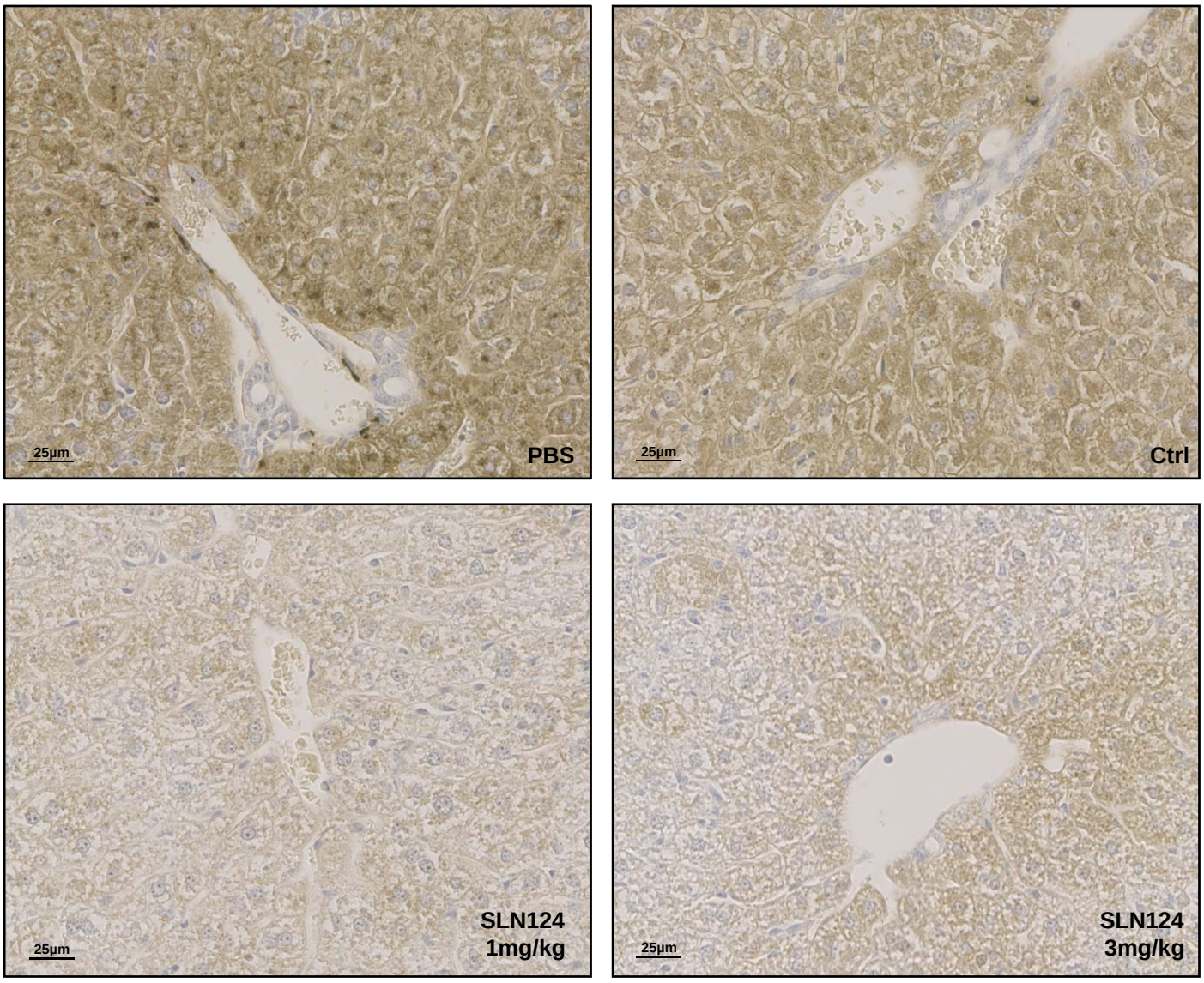

250µm
PBS
250µm
Ctrl
250µm
SLN124 1mg/kg
250µm
SLN124 3mg/kg

Figure S3

A


Liver Iron Content
µg Fe / g dry tissue
1500
1000
500
0
PBS
CTRL
SLN124 1mg/kg
SLN124 3mg/kg

B


25µm
PBS
25µm
Ctrl
25µm
SLN124 1mg/kg
25µm
SLN124 3mg/kg