SUPPLEMENTARY APPENDIX

Hemolytic anemia repressed hepcidin level without hepatocyte iron overload: lesson from Günther disease model

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Supplementary informations

I/ Supplementary Materials/ Methods

Special reagents and mice treatments

For acute hemolysis and heme-arginate injections, BALB/c strain males were provided by Janvier laboratory. All experimental procedures involving animals were performed in compliance with the French and European regulations on Animal Welfare and Public Health Service recommendations.

Phenylhydrazine: Phenylhydrazine (PHZ, Sigma Aldrich, France) was freshly dissolved in water and pH-adjusted (pH: 7.4), as a 10mg/mL stock solution, before use. Intra-peritoneal (i.p.) injections of 50µL (500µg) to 100µL (1mg) of PHZ were performed per day, during 2 weeks. Hemolysis was verified and only anemic mice were retained for further analysis.

Heme-arginate: Heme arginate (Normosang®; Orphan Europe), was i.p. injected to mice at 8mg/kg per day, during 3 weeks. Control mice were injected with a solution corresponding to the excipients. Following treatment, mice were maintained for 48 hours in metabolic cages to collect urine.

Hematological and iron status:

Mouse erythrocyte and reticulocyte cellular indices were analyzed with the Advia 120 hematology analyzer (Siemens-Healthcare Diagnostics, Saint Denis, France)

Serum and/or urinary non-heme iron, bilirubin, LDH, ferritin, and Tf levels, creatinin, total protein, albumin were measured using AU400 automate (Beckman Coulter Paris Nord 2, France). Total urinary iron levels were determined at the maximal follow-up by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) on a JY 24 spectrometer (Horiba Jobin Yvon, Longjumeau, France).

Serum haptoglobin (Hp) was measured using direct sandwich ELISA for mouse serum Hp, as previously described.(20) Urinary free heme was measured using hemin assay kit (BioVision, Inc. Headquarters, CliniSciences, Nanterre, France), according to the manufacturer's instructions. Determination of serum concentrations of Epo was carried out using ELISA kits (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. Serum hepcidin was performed with LCMSMS as described (ref).

Tissue iron content was determined by acid digestion of tissue samples, followed by iron quantification (IL test; Instrumentation Laboratory, Lexington, MA) on an AU400 automate (Beckman Coulter). Blood smears were fixed and stained with hematoxylin-eosin.

Immunohistochemical Methods

Tissues were isolated and fixed in 3.5% formaldehyde for 3 to 5 hours. Sections were then performed for immunostaining and histological examinations. Images were acquired using a ScanScope digital scanner (Aperio, TRIBVN, France).

RBC turnover an osmotic fragility assay

For RBC lifespan, mice received sulfo-NHS-LC-biotin [*N*-succinimidyl-6-(biotinamido) hexanoate; Pierce Biotechnology, Rockford, USA] by intravenous retro-orbital injection (40 µg/g body weight) on three consecutive days. Aliquots of blood drawn in PBS-G (PBS supplemented with 0.1% glucose) were then collected every two or three days. Washed RBCs ($3x10^6$) were incubated with 2 µL of FITC-conjugated streptavidin solution (BD Biosciences, Le Pont de Claix, France) for 1 hour at 25°C in the dark. FITC-labeled RBCs were then detected by flow cytometry analysis (FACS Canto, BD Biosciences). The percentage of biotinylated cells was calculated as a ratio of positive cells to all RBCs. For osmotic fragility assay, and to account for differences in hematocrit levels, 10 µL (for *WT*) and 15µL (for *CEP*) of blood was dissolved in 3 mL distilled water containing from 10 to 0 g/L NaCl, adjusted to pH 7.4. Ten minutes later, tubes were centrifuged 5 minutes at 2000g,

1mL of the supernatant was collected and the optical density of the supernatant was measured at 546 nm. The percentage of hemolysis was calculated as the ratio of the optical density for a given NaCl concentration to the optical density of the 100 % hemolysis, and plotted against the NaCl concentration. The NaCl concentration at 50% hemolysis was determined by regression analysis.

Flow cytometry assay

All tissue samples were used at a final concentration of $3x10^6$ cells/mL. Cells were then immunostained for 20 min at 4°C with the following antibodies: FITC-conjugated anti-Ter119 (BD Pharmingen, Le Pont de Claix, France), phycoerythrin conjugated anti-CD71 (AbD Serotec, Oxford, UK). Propidium iodide was used to exclude dead cells and apoptosis was assessed using the AnnexinV-FITC apoptosis detection kit (BD Pharmingen, Le Pont de Claix, France). Flow cytometry analysis was carried out on BD Biosciences FACScalibur.

Sequences primers

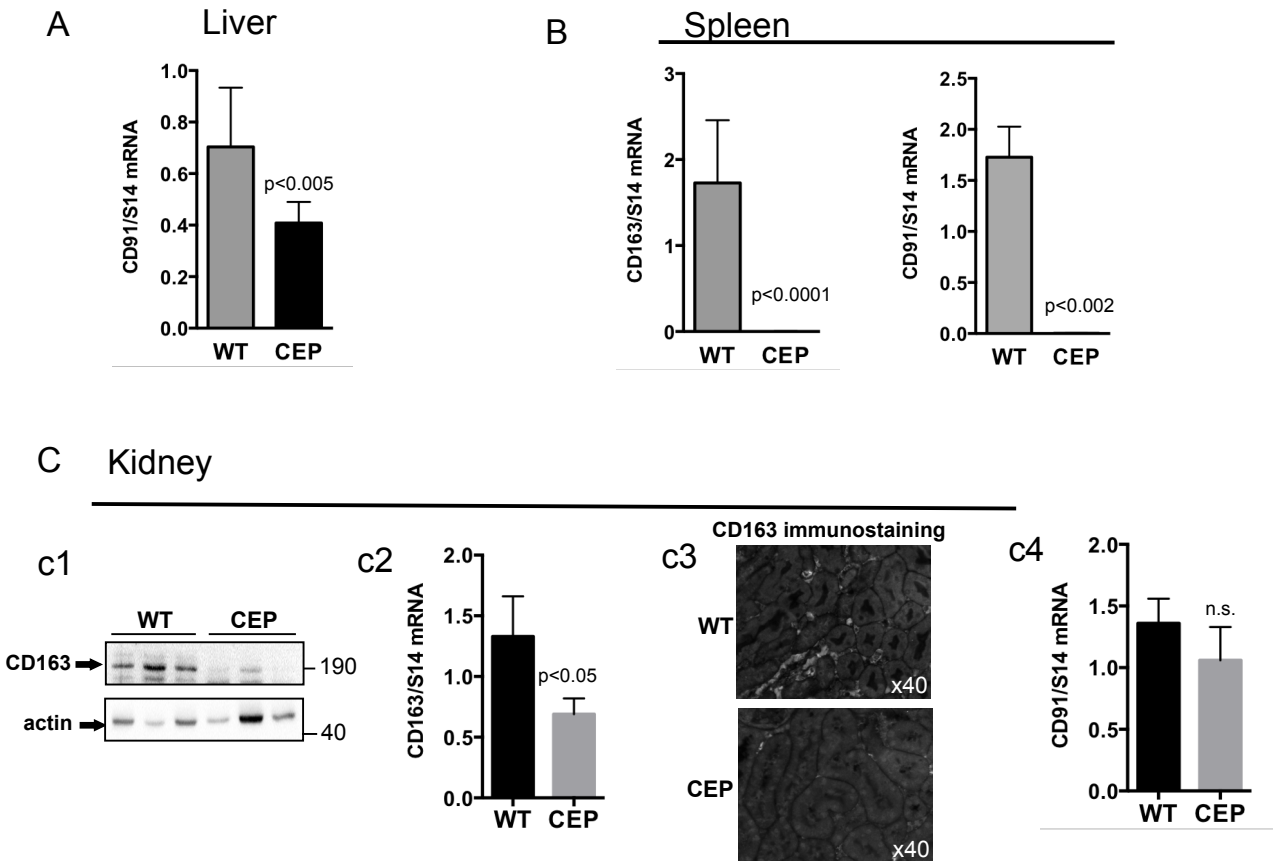
mRNA	Forward primer	Reverse primer
Actin	5'-gctgtgctgtccctgtatgcctct	5'-cttctcagctgtggtggtgaagc-3'
CD91	5'-gtgccgagaccaggtgt-3'	5'-ttggcacaggcaaactcg-3'
CD163	5'-catgtgggtagatcgtgtgc-3'	5'-tgtatgcccttcctggagtc-3'
DMT1	5'-ggctttcttatgagcattgccta- 3'	5'-ggagcacccagagcagctta-3',
FAM 132	5'- cgagctcttcaccatctcagta-3'	5'- tgagagccactgcgtaccg-3'
Ferroportin	5'-cccatagtctctgtcagcctgc-3'	5'-ccgtcaaatcaaaggaccaaa-3'
GAPDH	5'-tgaagcaggcatctgaggg-'	5'-cgaaggtggaagagtgggag-3'
HAMP1	5'- cgagctcttcaccatctcagta-3'	5'-cagataccacactgggaatt-3'
H-ferritin	5'-gcctcctacgtctatctgtctatgtcttg-3'	5'-gagaaagtatttggcaaagttcttcagagc-3'
HO-1	5'-gatttgtctgaggccttgaaggag-3'	5'-catagactgggttctgcttgttgc-3'
S14	5'-caggaccaagacccctggacctgga-3'	5'-atcttcatcccagagcgagcaagagctc-3'
TfR1	5'-gaggaaccagaccgttatgttgt-3'	5'-cttcgccgcaacaccag-3'

II Supplementary results

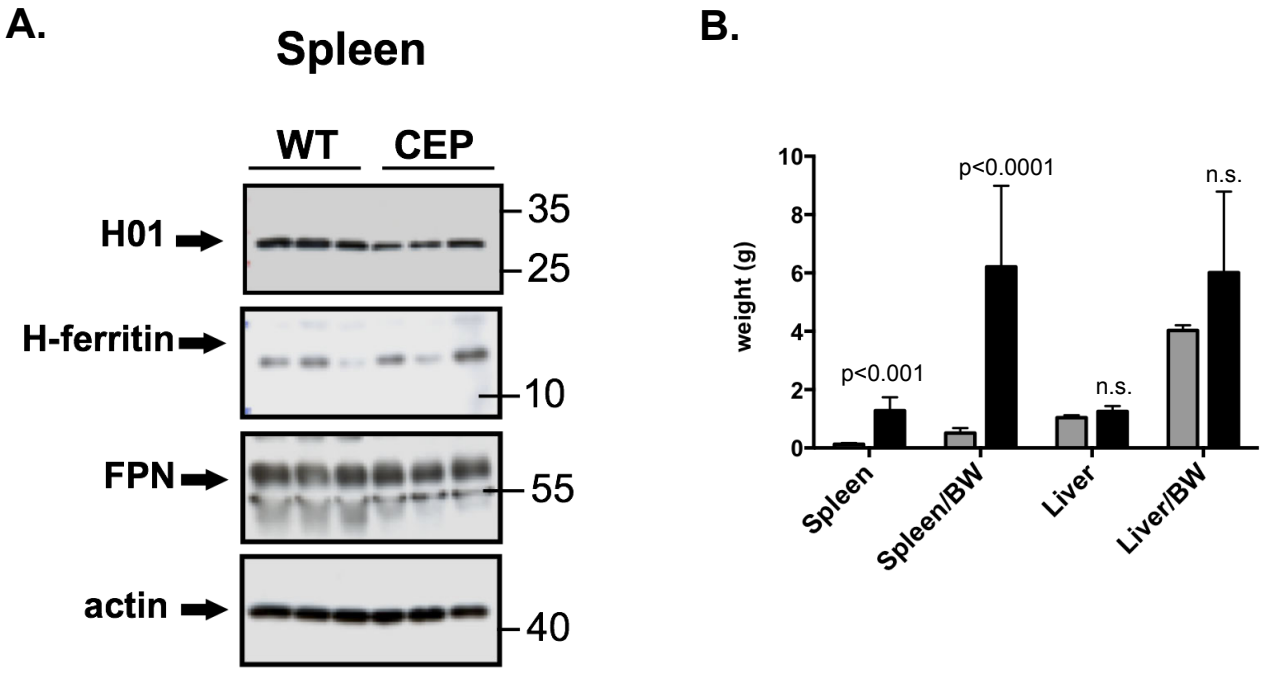
Supplementary table1:

gene	Control mice	PHZ-mice	Control mice	HA-mice
HO-1	0,45 ± 0,02	3,49 ± 0,90 *	0,86 ± 0,18	1,13 ± 0,14
Fpn	1,35 ± 0,29	3,76 ± 0,59 *	1,07 ± 0,12	1,10 ± 0,35

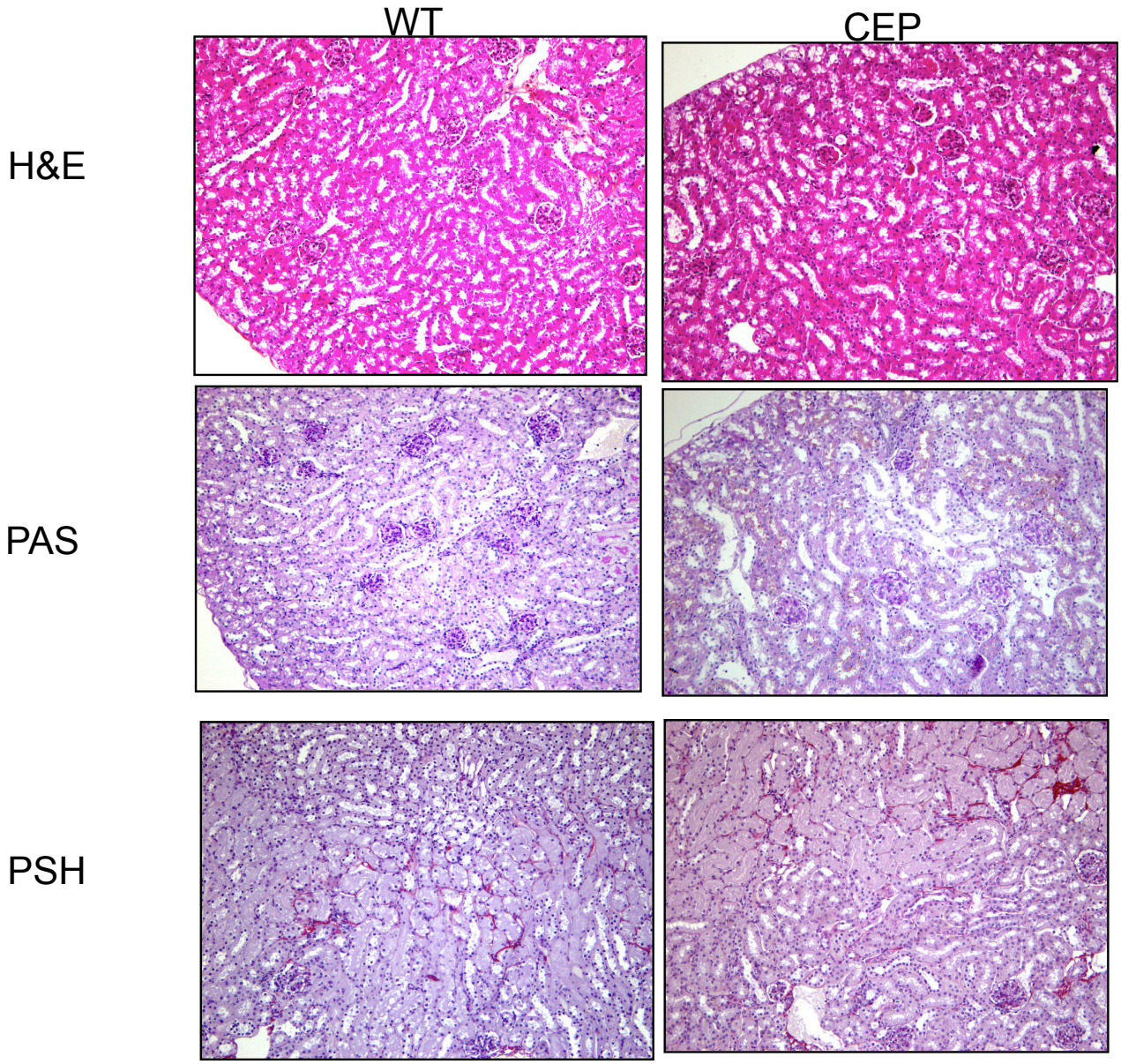
Supplementary Figure1



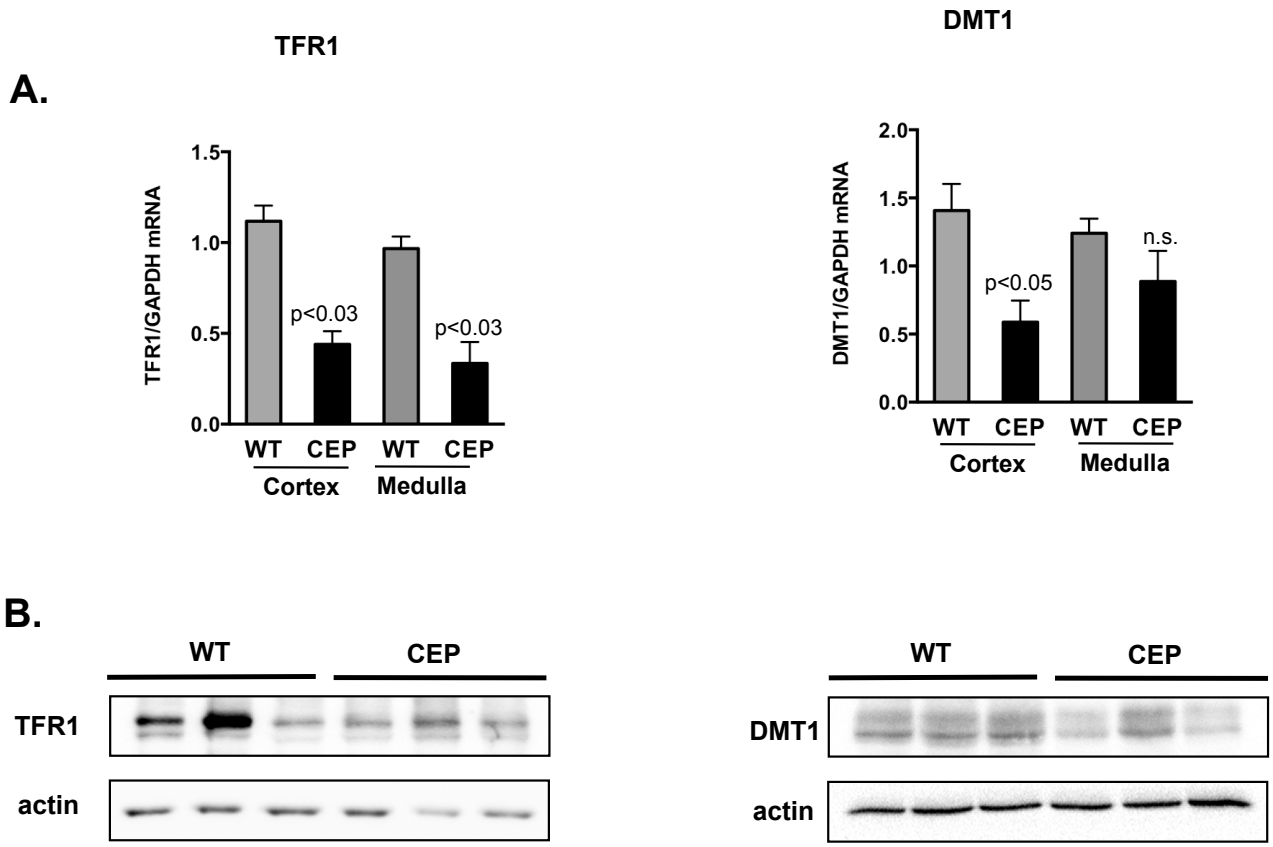
Supplementary Figure2



Supplementary Figure3



Supplementary Figure4



Supplementary Figure5