Mice are poor heme absorbers and do not require intestinal *Hmox1* for dietary heme iron assimilation

Carine Fillebeen,[1] Konstantinos Gkouvatsos,[1] Gabriela Fragoso,[2] Annie Calvé,[2] Daniel Garcia-Santos,[3] Marzell Buffler,[4] Christiane Becker,[4] Klaus Schümann,[4] Prem Ponka,[3] Manuela M. Santos,[2] and Kostas Pantopoulos[1]

[1]Department of Medicine, McGill University, and Lady Davis Institute for Medical Research, Jewish General Hospital, Montreal, Quebec, Canada; [2]Département de Médecine, Université de Montréal, and Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM), Montreal, Quebec, Canada; [3]Department of Physiology, McGill University, and Lady Davis Institute for Medical Research, Jewish General Hospital, Montreal, Quebec, Canada; and [4]Science Center Weihenstephan, Technical University Munich, Am Forum 5, Freising, Germany

Correspondence: kostas.pantopoulos@mcgill.ca
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Online Supplementary Appendix

Methods

Animals

Hmox1$^{fl/fl}$ mice (1) were kindly provided by Dr. G. Kollias (BSRC Al. Fleming, Greece). Vil-Cre transgenic mice were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA). Hmox1$^{Vil\text{-}Cre}$ mice were generated by intercrossing Hmox1$^{fl/fl}$ and Vil-Cre animals. C57BL/6 wild type mice were purchased from Charles River Laboratories, Inc. (Wilmington, MA, USA). Gene-specific primers used for genotyping of mutant mice are listed in Table S1. All mice were housed in macrolone cages (up to 5 mice per cage, 12:12 hr light-dark cycle: 7 am - 7 pm; 22±1°C, 60±5% humidity), according to institutional guidelines. They were given free access to water and a standard rodent diet. Custom-made diets with variable iron content were used for dietary iron manipulations (Harlan Laboratories). The iron-deficient diet (IDD) contained 2-6 ppm iron (TD.80396 Teklad). The high-hemin diet (HHD) was the IDD supplemented with 30 mmol/kg hemin (TD.120342). The iron-sufficient diets were the IDD supplemented with either 50 ppm $FeSO_4$ (TD.120515) or 50 ppm hemin (TD.120516), respectively. At the endpoint, the animals were sacrificed by CO_2 inhalation. Experimental procedures were performed in accordance with the Canadian Council on Animal Care guidelines after approval by the Animal Care Committees of McGill University (protocol 4966), CRCHUM (protocol N09065) and the Government of Upper Bavaria (AZ 55.2-1-54-2531-88-09).

Hematology and serum biochemistry

Blood was collected via cardiac puncture. Hb, hematocrit (HCT) and mean corpuscular volume (MCV) were analyzed by using the Scil Vet-ABC hematology analyzer.

For serum preparation, the blood was clotted at room temperature for 1 h. Serum was separated by centrifugation (2000 g for 10 min) before being snap frozen in liquid nitrogen and stored at -80°C. Serum iron and total iron binding capacity (TIBC) were measured at the Biochemistry Department of the Jewish General Hospital using a Roche Hitachi 917 Chemistry Analyzer. Transferrin saturation was calculated from the ratio of serum iron and TIBC.

Quantification of tissue iron

Tissue non-heme iron content was quantified by the ferrozine assay (2). Tissue total (heme and non-heme) iron content was measured by atomic absorption spectroscopy (3, 4). Results are expressed as micrograms of iron per gram of dry tissue weight.

Histology

Duodenal specimens were fixed in 10% buffered formalin and embedded in paraffin. De-paraffinized tissue sections were stained with hematoxylin and eosin (H&E), to assess duodenal architecture.

Preparation of a radiolabeled ^{59}Fe-Hb lysate

Female mice (11-week old) were injected intraperitoneally once per day for 3 consecutive days with 50 mg/kg of phenylhydrazine, to induce anemia. During the last phenylhydrazine dose the mice were co-injected with 200 µl of radiolabeled ^{59}Fe-citrate (0.03 µM, ~12 million cpm). Following a 3-day rest, the mice were sacrificed by avertin overdose (1 ml of 2.5% avertin). The blood was isolated by cardiac puncture and lysed using bi-distilled water. The crude ^{59}Fe-Hb lysate was acidified to pH 3 by using 10 mM HCl and digested with pepsin (P7012-1G, Sigma-Aldrich, Taufkirchen, Germany), to facilitate

intestinal heme absorption. The pepsin/Hb ratio was 6% w/w (5). Pepsin hydrolysis was carried out at 40°C for 18 h and the reaction was terminated by addition of NaOH to reach pH 8. Subsequently, cysteine (30089, Sigma-Aldrich) was added in a molar ratio (Fe/cysteine) of 1:10 to ensure solubility of the ^{59}Fe-Hb lysate. Prior to use, the hydrolyzed ^{59}Fe-Hb solution was diluted 1:10.

Determination of intestinal ^{59}Fe-heme absorption

Intestinal heme absorption was measured using a slightly modified version of the previously described ligated duodenal loops method (6). Briefly, after overnight fasting, mice were anaesthetized using ketamine (CP-Pharma, Burgdorf, Germany) plus medetomidin (Eurovet Animal Health B.V. Bladel, The Netherlands) in a dosage of 100 and 25 mg/kg, respectively. A tied-off intestinal segment of approximately 15 cm beginning directly after the pylorus encompassing duodenum, jejunum and ileum was flushed with saline and filled with the previously hydrolyzed ^{59}Fe-Hb solution (see above). Following an absorption period of 2 h, the mesenteric blood supply was ligated and the intestinal segment was removed. After determining its length, the segment was extensively flushed with stop medium (10 mmol/l cold hemin in phosphate buffered saline) and blotted. ^{59}Fe radioactivity remained in the intestine or transferred to the carcass were measured in a whole body counter for small animals (type AW3, Mab Solutions).

Quantitative real-time PCR (qPCR)

Total RNA was extracted from frozen tissues by using the RNeasy kit (Qiagen). Purity was assessed by 260/280 nm absorbance ratios and quality was monitored by agarose gel electrophoresis. cDNA was synthesized from 1 µg of RNA with the QuantiTect Reverse Transcription kit (Qiagen) as per the manufacturer's protocol. SYBR Green (Qiagen) and

gene-specific primers (Table S2) were used to amplify products with the following cycling conditions: initial denaturation 95°C 10 min, 40 cycles of 95°C 30 sec, 58°C 1 min, 72°C 1 min, and final cycle melt analysis from 58°C to 95°C (7). Data were normalized to two reference genes (β-actin and ribosomal protein S18) according to the MIQE guidelines (8), and reported as fold increases compared to samples from $Hmox1^{fl/fl}$ or wild type mice fed the IDD.

Western blotting

Tissue lysates containing 30 μg of protein were analyzed by SDS-PAGE on 10% or 15% gels. Following transfer of the proteins onto nitrocellulose membranes (BioRad), the blots were saturated with 5% non-fat milk in Tris-buffered saline (TBS) containing 0.1% (v/v) Tween-20 (TBS-T) and probed with primary antibodies against HO-1 (1:500 diluted; StressGen), HO-2 (1:1000 diluted; StressGen), ferritin 1:500 diluted; Novus), villin (1:1000 diluted; Cell Signaling) or β-actin (1:2000 diluted; Sigma). After three washes with TBS-T, the blots were incubated with appropriate peroxidase-coupled secondary antibodies, and then washed again. The peroxidase signal was detected by enhanced chemiluminescence with the Western Lightning ECL kit (Perkin Elmer).

Statistics

Quantitative data were expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed by using the Prism GraphPad software (version 5.0d). Analysis of multiple groups was done by one- or two-way ANOVA, and for only two groups by the Student's t-test. A probability value $p<0.05$ was considered to be statistically significant.

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Table S1. List of primers used for genotyping.

Gene	GenBank accession	Forward primer sequence	Reverse primer sequence
Hmox1	NM_010442.1	*AGGGGAAGAAGACTCATCGA*	*AGAAGGCTCGGAGGTTAAAT*
Cre	X03453.1	*GCGGTCTGGCAGTAAAAACTATC*	*GTGAAACAGCATTGCTGTCACTT*

Table S2. List of primers used for qPCR.

Gene	GenBank accession	Forward primer sequence	Reverse primer sequence
Actb	NM_007393.3	*GACGACATGGAGAAGATCTG*	*GTGAAGCTGTAGCCACGCTC*
Rn18s	NR_003278	*GAATAATGGAATAGGACCGCGG*	*GGAACTACGACGGTATCTGATC*
Hamp1	NM_032541	*AAGCAGGGCAGACATTGCGAT*	*CAGGATGTGGCTCTAGGCTATGT*
Hcp1	NM_026740.2	*TGCTGTGGCCTGTGTGAATA*	*GTCAGTGTGGGTCCATTGCT*
Hmox1	NM_010442.1	*TGCTCGAATGAACACTCTGG*	*TCCTCTGTCAGCATCACCTG*
Hmox2	NM_001042660	*TCGGACAGCTCAATTCGGAC*	*GGTAACTGCTGCGGTTGTAA*
Hpx	NM_017371.2	*TCCCAGATACCCTCTGGATG*	*GAATGCATAGGATGGGTGCT*
Flvcr1	NM_001081259	*CCGTCGCCTCGGTATGG*	*CACTAAAACAGGTGGCAACAAAAA*
Fth1	NM_010239.2	*AAGTGCGCCAGAACTACCAC*	*AGCCACATCATCTCGGTCAA*

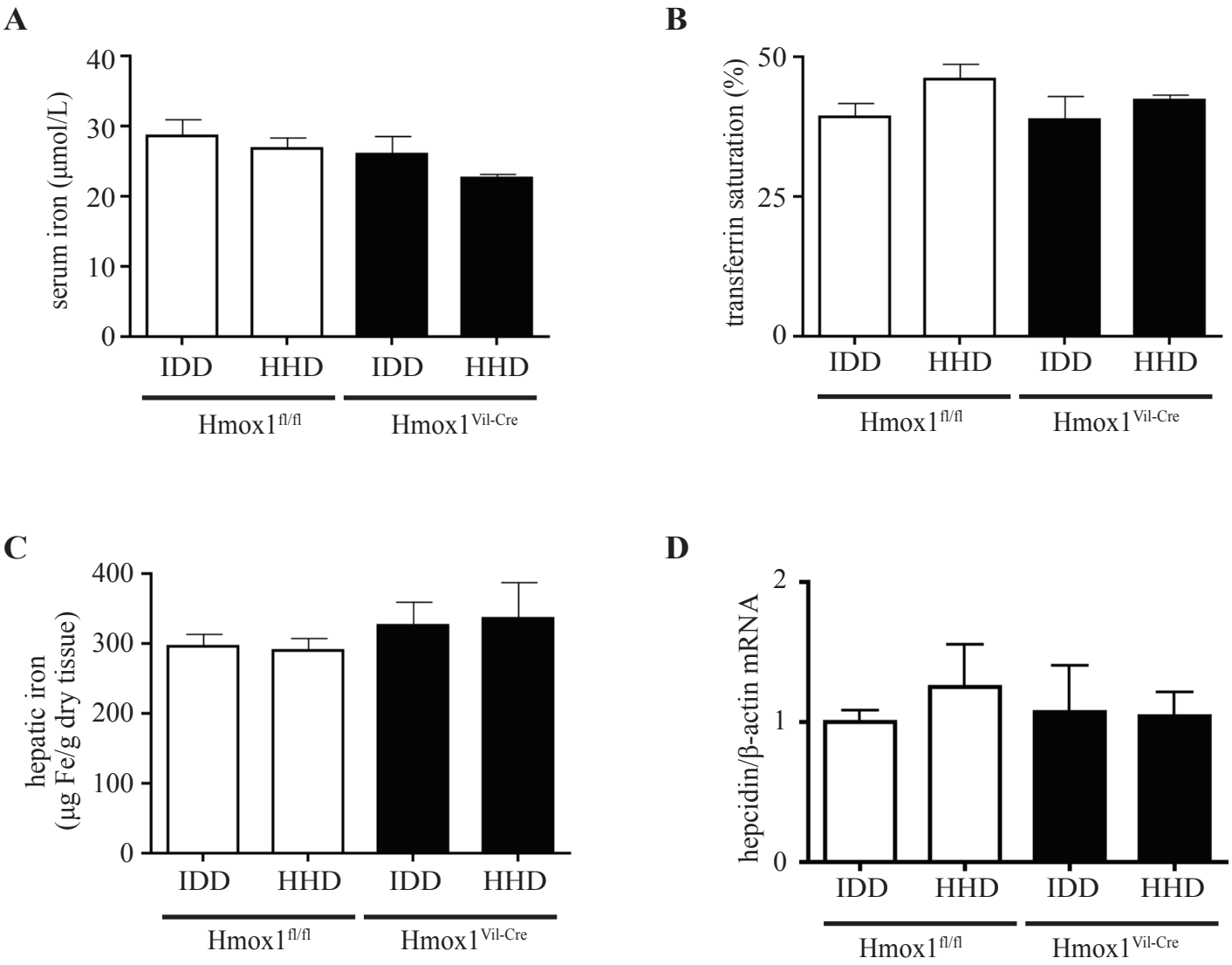

Fig. S1. Dietary heme excess did not increase systemic iron levels in control Hmox1$^{fl/fl}$ and in Hmox1$^{Vil\text{-}Cre}$ mice. The mice described in Fig. 1 were analyzed for serum iron (A), transferrin saturation (B), hepatic non-heme iron content (C) and hepatic hepcidin mRNA (D). Data are presented as the mean ± SEM. Statistical analysis was performed by one-way ANOVA.

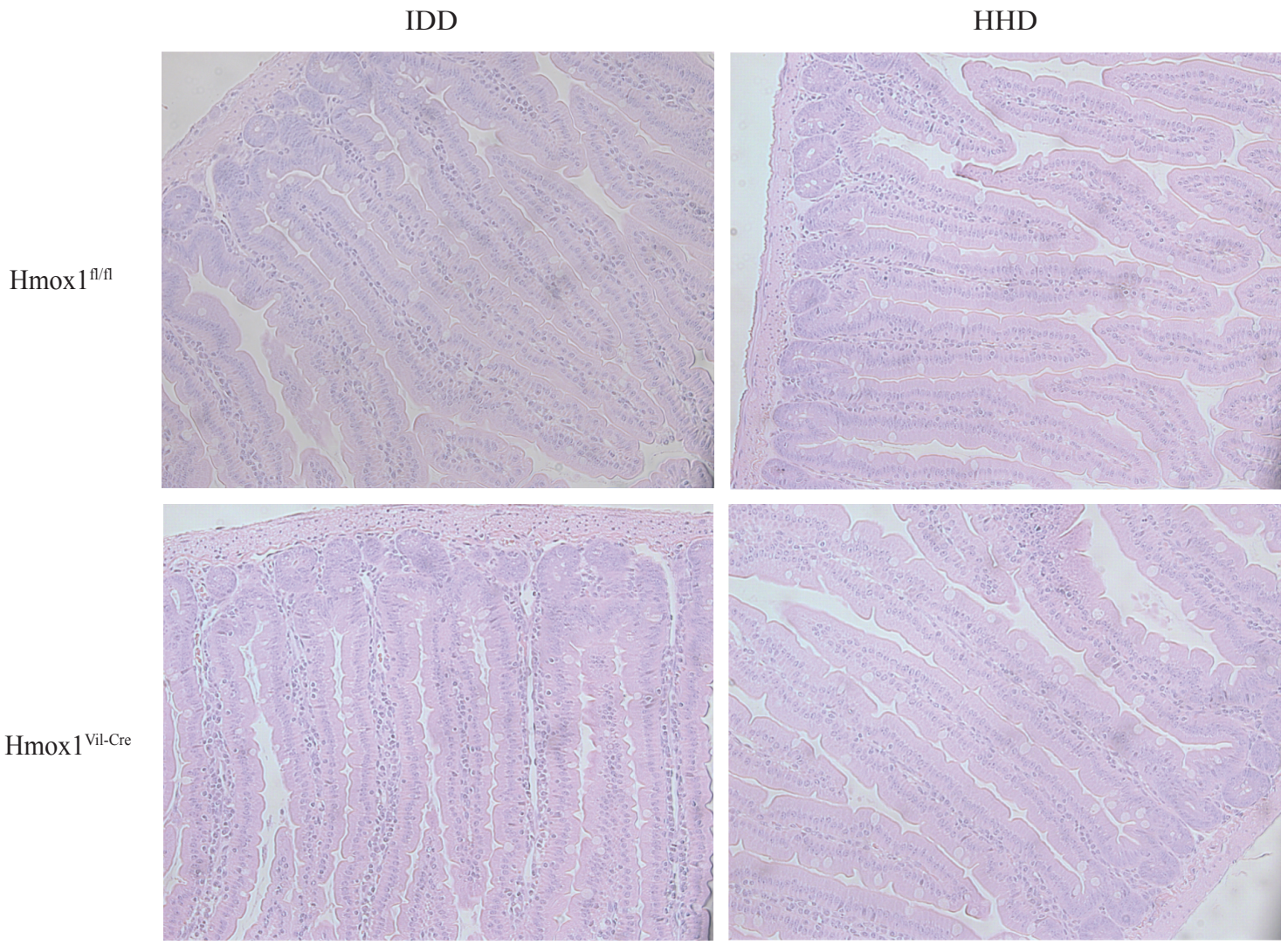

Fig. S2. Dietary heme excess did not alter architecture of intestinal epithelial cells. Hematoxylin and eosin (H&E) staining of intestinal sections.

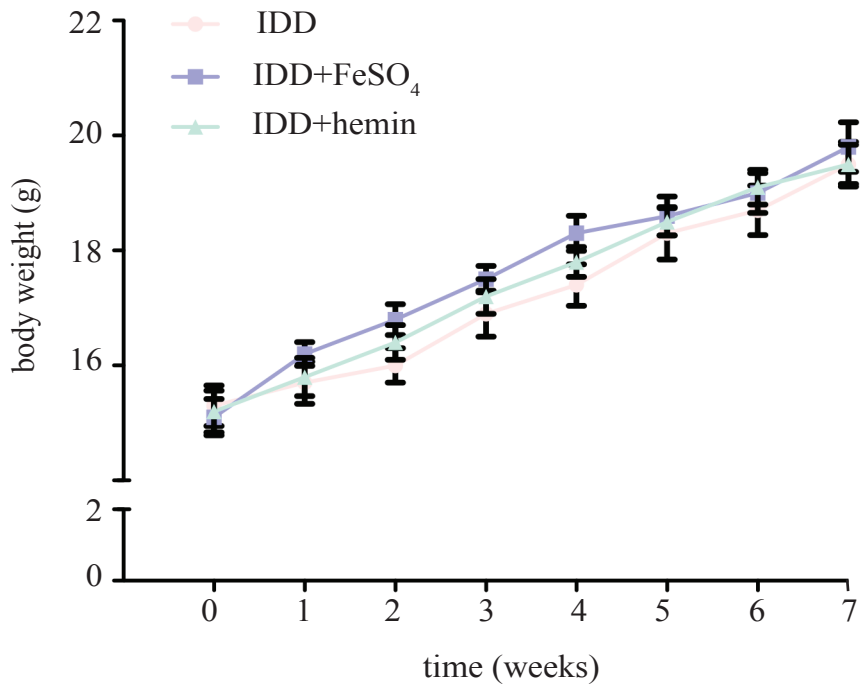

Fig. S3. Supplementation with $FeSO_4$ or hemin following dietary iron restriction did not significantly affect growth of C57BL/6 mice. Animal weight was measured at the indicated time intervals. Data are presented as the mean ± SEM. Statistical analysis was performed by two-way ANOVA.