ZIP14 and DMT1 in the liver, pancreas, and heart are differentially regulated by iron deficiency and overload: implications for tissue iron uptake in iron-related disorders

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Online Supplementary Design and Methods

Sample preparation and western blot analysis

Samples of liver and pancreas were homogenized by Dounce homogenization in ice-cold RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% IGEPAL CA-630 (Sigma), 0.5% Na deoxycholate, 0.1% SDS) containing 1× Complete, Mini Protease Inhibitor Cocktail (Roche). Homogenates were clarified by centrifugation at 10,000 x g for 10 min at 4 °C. Heart samples were homogenized by Dounce homogenization in ice-cold phosphate-buffered saline containing 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 1 mM ethylenediaminetetraacetic acid and 1× Complete, Mini Protease Inhibitor Cocktail (Roche). Heart homogenates were incubated on ice for 30 min and then clarified by centrifugation at 13,000 x g for 10 min at 4 °C. In validation studies of the anti-ZIP14 antibody, membranes from rat liver were isolated as described previously[1] and HEK 293T cell lysates were prepared in RIPA buffer. For measurement of ZIP14 and ferritin levels in HepG2 cells, SDS lysis buffer was used.[2] Protein concentrations were determined by using the *RC DC* Protein Assay (Bio-Rad).

Proteins were mixed with Laemmli buffer and incubated for 15 min at 37 °C. Proteins were separated electrophoretically on an SDS 7.5% polyacrylamide gel, transferred to nitrocellulose, and incubated for 1 h in blocking buffer [5% non-fat dry milk in Tris-buffered saline (TBS)-Tween 20 (TBS-T)]. Blots were incubated overnight at 4 °C in blocking buffer containing 2.5 μg/mL affinity-purified rabbit anti-ZIP14 antibody or rabbit anti-DMT1 (+/- IRE isoforms) antiserum (1:4000), supplied by Dr. Philippe Gross (McGill University, Montreal, Canada). After washing in TBS-T, blots were incubated for 40 min with a 1:4,000 dilution of horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG secondary antibody (Amersham Biosciences). Blots were then washed with TBS-T and TBS, and immunoreactivity was visualized by using enhanced chemiluminescence (SuperSignal West Pico, Pierce) and X-ray film or the FluorChem E digital darkroom (Cell Biosciences). Transferrin receptor 1 (TfR1) levels were detected as described above using mouse anti-TfR1 (1:1000, Invitrogen) antibody, followed by goat HRP-conjugated anti-mouse (1:5000; Invitrogen) secondary antibody. ZIP14 in HepG2 cells was detected by using rabbit anti-human SLC39A14 Prestige Antibody (1:200; Sigma-Aldrich) and donkey anti-rabbit IgG secondary antibody (1:5000; Amersham). Ferritin was detected by using goat anti-ferritin light chain (1:1000; Novus Biologicals) and rabbit anti-goat secondary antibody (1:5000; Invitrogen). To indicate lane loading, blots were stripped and reprobed with rabbit anti-scavenger receptor class B type I, SR-B1 (Novus Biologicals), rabbit pan-cadherin antibody (Santa Cruz), or mouse anti-tubulin (Sigma-Aldrich) followed by HRP-conjugated secondary antibodies. Immunoreactive band intensities on X-ray film were quantified by densitometry using GeneTools software (SynGene).

RNA isolation and quantitative reverse transcriptase polymerase chain reaction analysis

Total RNA was isolated from liver and heart by using RNA-Bee (TelTest). The RNeasy Mini Kit (Qiagen) was used to isolate RNA from pancreas. The integrity of the isolated total RNA was evaluated by using denaturing gel electrophoresis. Residual DNA was removed by the Turbo-DNA-free kit (Ambion). The purified total RNA was used for cDNA synthesis employing the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). The cDNA was then used as the template for quantitative reverse transcription polymerase chain reaction (qRT-PCR) with gene-specific primers. For PCR, Power SYBR Green PCR Master Mix (Applied Biosystems) was used with five $\log_{10}$ standard curves on an Applied Biosystems 7300 Real-Time PCR System. The gene-specific forward (F) and reverse (R) primers used were: ZIP14: 5'- GATGCT GCCTGCTG-GTTGTA-3' (F), 5'- ACAGTGGCTCACCTAGGTGAT G-3' (R); DMT1 + IRE (iron-responsive element): 5'-TGTGGCCTG-GCGTTACG -3' (F), 5'-CGCAGAAGAA-CGAGGACCAA -3' (R); DMT1 – IRE: 5'-TTTGAACCAAGGCGAAGAAGA -3' (F), 5'-ACCCATTCACAGCCG-TTAGC-3' (R); Hepcidin: 5'-GGCAGAAAGCAAGACTGATGAC-3' (F), 5'-ACAGGAATAAATAATGGGGCG -3' (R); Cyclophilin: 5'-CGCACAGCCGGGACAA -3' (F), 5'-TTCGATCTTGC-CACAGTCTAC-3' (R).

Measurement of ZIP14 and DMT1 messenger RNA copy number

Calibration curves were obtained by using plasmids containing cDNA for rat ZIP14 and DMT1 as templates in qRT-PCR. Serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} μg/PCR reaction) for each plasmid were analyzed to plot the linear cal-

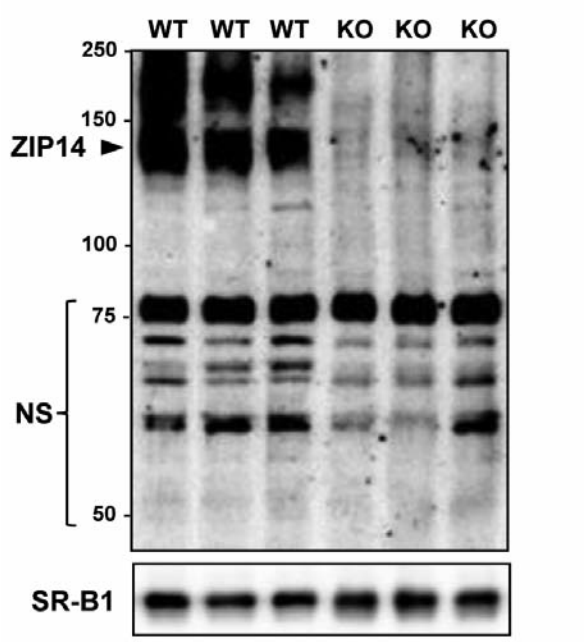
ibration curves [log10 (mass) versus ΔC_t]. Total RNA from iron-adequate rats (n=6) was analyzed in parallel, and mRNA copy numbers of ZIP14 and DMT1 were calculated by comparison to calibration curves. Primers used for DMT1 targeted both the + and - IRE isoforms: 5'-GCTTTGCCCGCGTGATC -3' (F), 5'-TCTTGGAAGACAGCAACAAGCA -3' (R).

Immunofluorescence staining of ZIP14 in liver and pancreas

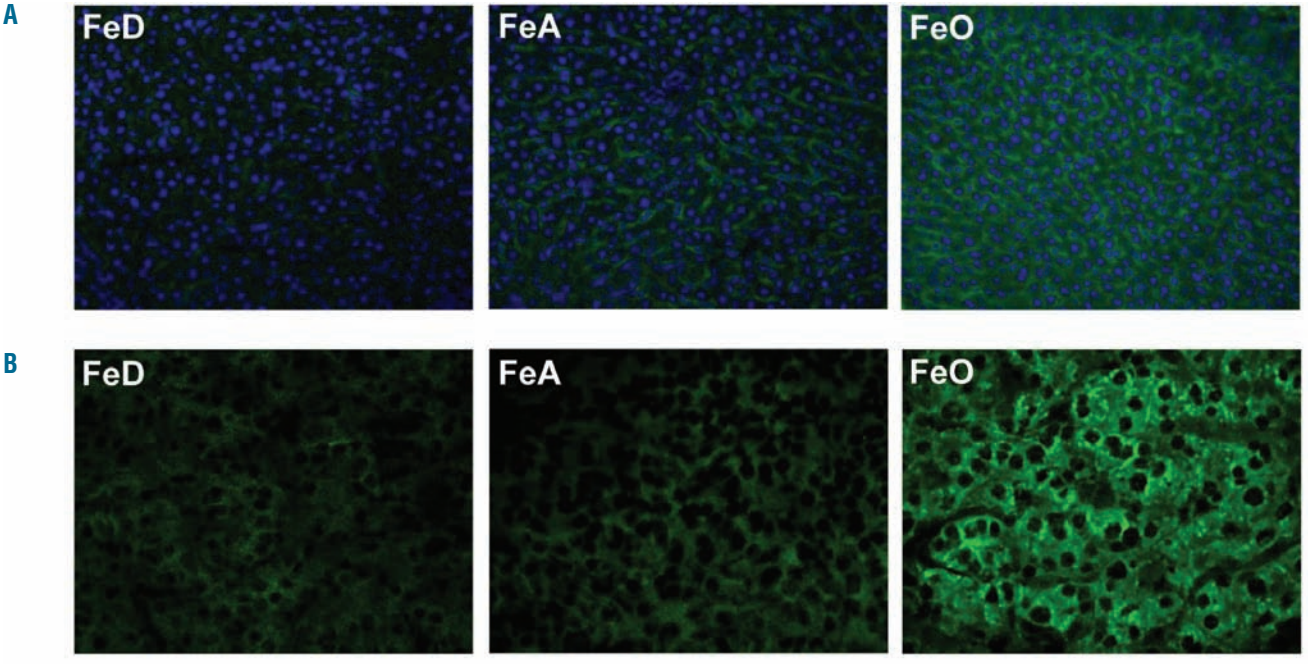
After sacrificing rats, tissues were harvested, immediately embedded in Tissue-Tech OCT compound (Sakura), and frozen on dry ice. Seven micrometer-thick serial frozen sections were prepared by using a cryostat, dried overnight at room temperature, and incubated in acetone at -20 °C for 5 min. Non-specific immunoglobulin binding was blocked with 2% normal goat serum (Vector lab) for 30 min, and endogenous avidin and biotin were blocked with avidin/biotin blocking solution (Vector lab). Tissue section slides were incubated with rabbit anti-ZIP14 antibody (2 μg/mL) at 4 °C overnight in a humidified chamber. Slides were subsequently incubated with biotinylated goat anti-rabbit IgG (1:500; Vector lab) for 40 min, followed by Alexa Fluor 488 streptavidin (1:500; Invitrogen) for 30 min. Slides were washed in TBS between all antibody incubations. After fixing in 10% formalin in buffered saline, cover slips were mounted on slides using VECTASHIELD mounting medium with 4',6-diamino-2-phenylindole (DAPI) (Vector lab). Immunofluorescence staining for insulin in pancreas sections was performed as described above but with guinea pig anti-insulin primary antibody (1:200; Abcam) and Alexa Fluor 594 goat anti-guinea pig IgG (1:200; Invitrogen). Confocal microscopy was performed with an Olympus IX2-DSU spinning disk confocal fluorescent microscope equipped with a Hamamatsu ORCA-AG camera and 3i SlideBook v4.2 software.

References

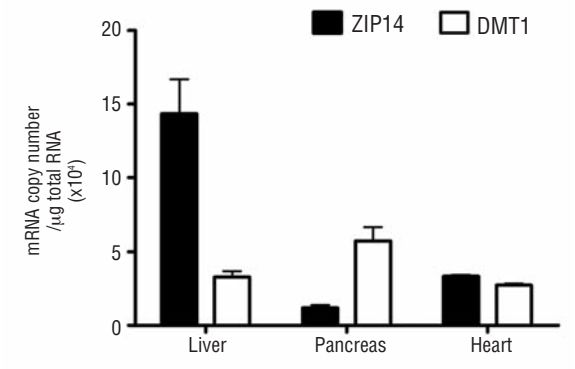
1. Jenkitkasemwong S, Broderius M, Nam H, Prohaska JR, Knutson MD. Anemic copper-deficient rats, but not mice, display low hepcidin expression and high ferroportin levels. J Nutr. 2010;140(4):723-30.
2. Knutson MD, Vafa MR, Haile DJ, Wessling-Resnick M. Iron loading and erythrophagocytosis increase ferroportin 1 (FPN1) expression in J774 macrophages. Blood. 2003;102(12):4191-7.



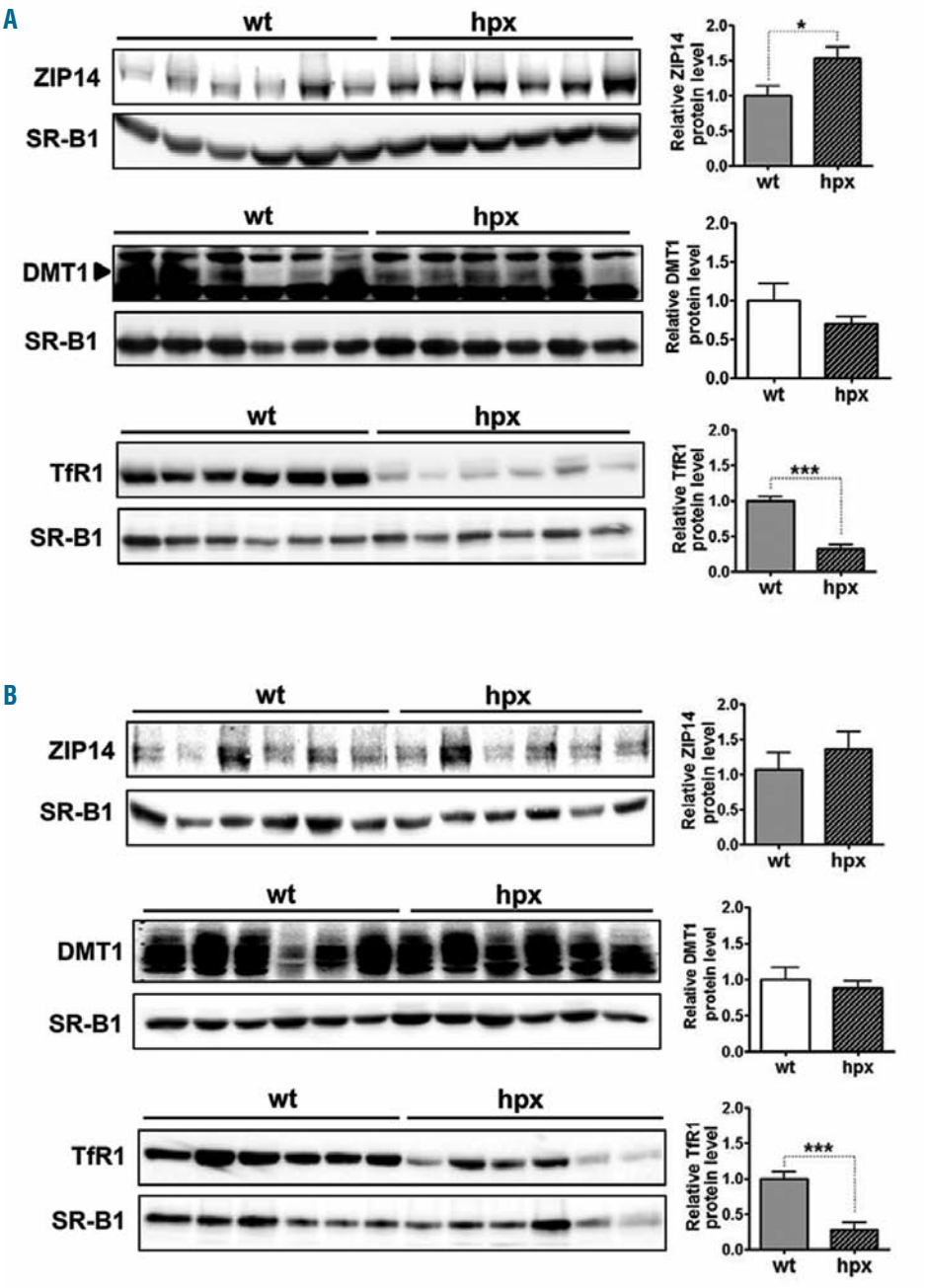
Online Supplementary Figure S1. Western blot analysis of wild-type *vs. Zip14* knockout mouse liver. Western blot analysis of wild-type (WT) and *Zip14* knockout (KO) mouse liver (n=3). The ZIP14-specific (arrowhead) and non-specific (NS) immunoreactive bands are indicated. ZIP14 immunoreactivity ≥130 kDa in liver represents glycosylated ZIP14, likely as dimers/oligomers. To indicate lane loading, the blot was stripped and reprobed with the integral membrane protein SR-B1.



Online Supplementary Figure S2. Effect of iron deficiency and overload on Zip14 immunofluorescence in sections of rat liver and pancreas. Zip14 immunofluorescence (Alexa Fluor 488, green) in sections of (A) liver and (B) pancreas from FeD, FeA, and FeO rats, original magnification × 20. Each square is representative of four animals per group. Images were obtained by using a spinning disk confocal fluorescent microscope system.



Online Supplementary Figure S3. ZIP14 and DMT1 mRNA copy numbers in liver, pancreas, and heart. Total RNA was isolated from iron-adequate rat tissues, and mRNA copy numbers were determined by using quantitative RT-PCR. Values are means ± SE, n=6.



Online Supplementary Figure S4. Effect of genetic iron overload on ZIP14 and DMT1 levels in liver and pancreas. (A) Immunoblot analysis of ZIP14, DMT1, and TfR1 in livers of wild-type (wt) and hpx mice. To indicate lane loading, blots were stripped and reprobed with SR-B1. For DMT1, the arrow indicates the position of the DMT1-specific band, as determined by using Dmt-1 knockout mouse liver (data not shown). (B) Immunoblot analysis of ZIP14, DMT1, and TfR1 in pancreas of wild-type (wt) and hpx mice. Band intensities were quantified by densitometry and relative protein levels were normalized to the levels of SR-B1. Values are means ± SE, n=6.