Liver cell isolation:  Hepatocytes were isolated from normal male Wistar rats by the Cell Culture Core of the NIDDK-funded USC Research Center for Liver Diseases. In brief, the livers were perfused and digested in situ with collagenase and dissociated hepatocytes were separated by sedimentation and purified by Percoll density centrifugation. Kupffer cells, sinusoidal endothelial cells (SEC), and hepatic stellate cells (HSC) were provided by the NIAAA-funded Non-Parenchymal Liver Cell Core of the USC Research Center for Alcoholic Liver and Pancreatic Diseases. Kupffer cells and HSC were isolated by sequential digestion with pronase and collagenase followed by arabinogalactan gradient ultracentrifugation. Kupffer cells were further purified by the adherence method. SEC were isolated by collagenase perfusion, metrizamide gradient centrifugation and elutriation as previously described.

The purity of hepatocytes, HSC and SEC were examined by phase-contrast microscopy, UV-excited autofluorescence (HSC), and uptake of diacetylated LDL (SEC). The purity of Kupffer cells was demonstrated by the functional analysis by means of phagocytosis of 1-µm latex beads. The purity of each cell type was: hepatocytes (>92%), Kupffer cells (>96%), HSC (>98%) and SEC (95%). Cell viability was tested by trypan blue exclusion right after isolation and always exceeded 96% except for hepatocytes (>93%). All cell types were cultured in DMEM containing 5% FCS for 16 hr after isolation before total RNA was extracted. Viability of the cells after the 16 hr culture exceeded 98% for all cell types. This short culture period was chosen to allow the cells to recuperate from the isolation stress but to minimize the loss of their in vivo gene expression.

Generation of rats with acute iron deprivation and acute iron loading. Weanling male Sprague-Dawley rats were purchased from Harlan Sprague Dawley (Indianapolis, IN). Upon arrival, animals were first housed at the animal facility in the Department of Nutritional Sciences of University of Wisconsin for three days to allow the adaptation to control diet (50 mg Fe/kg diet, control diet) fed ad lib before feeding experiments were initiated. Day 0 is referred to the time point after the adaptation period and the start of the dietary treatment. The weanling rats used in this study were about 25 days old and weighed 50 to 52 grams on day 0.

The generation of rats with iron deprivation was performed as previously described. Briefly, weanling rats after adaptation were randomly assigned to two different categories with free access to a control diet (50 mg Fe/kg diet, group control) or were pair-fed an iron deficient diet (less than 2 mg Fe/kg diet, group ID). Pair-feeding involves providing the control group with the same amount of diet consumed by the animals fed the iron deficient diet on the previous day with the two groups on a staggered schedule differing by one day. Pair-feeding is a necessary control because animals fed the iron-deficient diet exhibit reduced food intake. Pair-feeding ensures the two groups of animals have equivalent energy intake but different iron intake. All animals had free access to water. Animals fed the diets for 1, 2 or 3 days were anesthetized with isoflurane and blood was collected by heart puncture for serum preparation. The animals were euthanized while under anesthesia by incising the diaphragm. Skeletal muscle (gastrocnemius) and liver were rapidly removed and snap-frozen in liquid nitrogen and then stored at -80°C for qRT-PCR and western blot analysis. There are either 4 or 5 animals per each group as indicated in the text.

The procedure for generation of rats with acute iron loading was similar to that described above. Briefly, rats after adaptation were randomly assigned to two different categories with free access to a control diet (50 mg Fe/kg diet, group control) or were pair-fed an ID diet (less than 2 mg Fe/kg diet, group ID) for 3 days. Animals in each category were then divided into three groups. One group of animals was euthanized while under anesthesia by incising the diaphragm. Blood was collected by cardiac puncture for the analysis of serum iron, total iron binding capacity (TIBC) and transferrin saturation at Animal Health Diagnostic Center of Cornell University. Liver was rapidly removed and
snap-frozen in liquid nitrogen and then stored at -80°C for qRT-PCR and western blot analysis. The other two groups were first injected intraperitoneally with ferric ammonium citrate (FAC) in PBS at approximately 2.5 mg iron per 100 gram body weight, or pair-injected with an equal amount of ammonium citrate (AC). At 3 hr post-injection, these two groups of animals were euthanized as described above. In this set of experiment, we also included the analysis of animals at day 0. There were 5 animals per group for the 0 day timepoint and 4 animals per group for 3 day control and 3 day ID with or without the injection of FAC or AC. All procedures for animal use met the requirements of the University of Wisconsin Research Animal Resource Center.

**TMPRSS6 cDNA cloning and transfection.** The coding sequences of human and rat TMPRSS6 cDNA were amplified from a human liver cDNA preparation and a rat liver cDNA preparation by PCR using the Expand High Fidelity PCR system (Roche Applied Science), respectively. The amplicons were first cloned into pGEM-T vector (Promega). Sequences were verified by DNA sequencing, and cDNAs were then subcloned into pcDNA3.1 vector (Invitrogen). The primers used for amplifications are listed in Table 2. The human TMPRSS6 construct with an addition of C-terminal myc tag (pcDNA3- myc TMPRSS6) was generated by PCR using TMPRSS6 cDNA as a template.

Transient transfection of human TMPRSS6 cDNA (pcDNA3.1-TMPRSS6), rat TMPRSS6 cDNA (pcDNA3-TMPRSS6), or pcDNA3.1 empty vector into HEK293 cells, was performed using Lipofectamine 2000 (Invitrogen). Cell lysates were prepared at 48 hr post-transfection and used as controls for matriptase-2 in immunodetection. HepG2 cells stably expressing human matriptase-2 and HEK293 cells stably expressing HJV were generated using the Nucleofector kit V (Amaxa Biosystems) and Lipofectamine 2000, respectively.

**Generation of Rabbit anti-matriptase-2 antibody.** Rabbit anti-matriptase-2 antibody was generated by the injection of purified human matriptase-2 fragment into rabbits at Pocono Rabbit Farm & Laboratory Inc. (Canadensis, PA). Purified human matriptase-2 fragment was prepared using baculovirus. Briefly, the DNA encoding the human matriptase-2 residues K79-D567 (NM_153609) was PCR-amplified and cloned into pAcGP67A vector, a baculovirus transfer vector with gp67 secretion leader. 6x His-tag was introduced in front of the N-terminus. Supernatants from baculovirus-infected high five cells were harvested and buffer exchanged into 20 mM Tris, 300 mM NaCl and then loaded to a Ni-NTA column (Qiagen) with the addition of imidazole at a final concentration of 10 mM. Nickel column eluates were further purified over Superdex 200 10/30 size exclusion column (GE Healthcare) using 20 mM Tris, pH 7.4, 150 mM NaCl, and 5 mM EDTA as a running buffer. Protein purity was visualized using SDS/PAGE before protein was sent for antibody production. Rabbit anti-matriptase-2 IgG was purified with protein A-sepharose beads (Invitrogen).

**REFERENCES**

**Figure S1.** Western blot analysis of matriptase-2 (M2) in muscle and whole liver tissues. Gastrocnemius muscle and liver extract proteins (250 g) from rats fed either a control diet (control) or an iron deficient diet for 3 days were separated using 11% SDS-PAGE under reducing conditions, followed by transfer onto nitrocellulose membrane. Membranes were probed with rabbit anti-matriptase-2 (M2), followed by immunodetection using corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies. The chemiluminescent bands were exposed to X-ray film. Cell lysates (100 g protein per lane) from HEK293 cells transiently transfected with either empty vector (C), human TMPRSS6 cDNA (hM2) or rat TMPRSS6 cDNA (rM2) were included as the negative and positive controls, respectively. A and B illustrate the whole immunoblot images for Fig. 5A with different exposure time. n.s: non-specific band.
Figure S2. Suppression of hepcidin mRNA in response to acute iron deprivation does not result from the alteration of HAI-1 and HAI-2 expression in the liver. A. qRT-PCR analysis of HAI-1 and HAI-2 mRNA in the liver tissues from rats fed either a control diet (white bar) or an ID diet (striped bar) for 1 day (day 1) and 2 days (day 2). Results are expressed as the amounts relative to β-actin. There are 5 animals per group. The mean values and the standard deviation (SD) for each group are presented. The paired two-tailed Student’s T-Test was used to evaluate the statistical significance of the qRT-PCR results between the ID and the corresponding control groups. No significant difference (n.s.) was detected. B. Western blot analysis of HAI-1 in the liver extracts from rats fed either a control (Ctrl) or an ID diet for 1 day. Extract proteins (250 µg) were separated using 11% SDS-PAGE under reducing conditions, followed by immunodetection of HAI-1 and β-actin (actin) with rabbit anti-HAI-1 antibody (1:100; Santa Cruz), mouse anti-β-actin, and the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies. C. Western blot analysis of HAI-1 in the liver extracts from rats fed either a control (ctrl) or an ID diet for 2 day. Extract proteins (250 µg) were separated using 11% SDS-PAGE under reducing conditions, followed by immunodetection of HAI-1 and β-actin with rabbit anti-HAI-1 antibody (1:100; Santa Cruz), mouse anti-β-actin, and the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies. We also tried to immunodetect HAI-2 using goat anti-HAI-1 antibody (Santa Cruz) in these samples. No signal was obtained (data not shown).
Figure S3. Acute iron loading does not alter either TMPRSS6 mRNA or matriptase-2 protein levels in the liver. Liver tissues are collected from the same animals as described in the legend to Figure 7. Briefly, weanling male rats were first fed a control diet (50 mg Fe/kg diet, group control) or were pair-fed an ID diet (less than 2 mg Fe/kg diet, group ID) for 3 days. Animals in each category were then injected intraperitoneally with either ferric ammonium citrate (FAC) in PBS at approximately 2.5 mg iron per 100 gram body weight, or pair-injected with an equal amount of ammonium citrate (AC). Animals were euthanized for analysis after 3 hr. A. qRT-PCR analysis of hepatic TMPRSS6 mRNA. Results are expressed as the amounts relative to β-actin. There are 4 animals per group. The mean values and the standard deviation (SD) for each group are presented. The one-way ANOVA and Tukey’s post test were used to compare the difference between groups. No significant difference was detected. B. Western blot analysis of matriptase-2 (M2) and β-actin (actin) in liver tissues. Liver extract proteins (250 µg) from rats as described in A were separated using 11% SDS-PAGE under reducing conditions, followed by transfer onto nitrocellulose membrane. Membranes were probed with rabbit anti-matriptase-2 (M2) and mouse anti-β-actin, followed by immunodetection using corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies. The blot was exposed to X-ray film. Cell lysates (50 µg protein per lane) from HEK293 cells transiently transfected with human TMPRSS6 cDNA (hM2) was included as a positive control for matriptase-2. Representative images from 2 animals in control groups and 3 animals in ID groups are presented.