Western blot analysis

Mouse livers were homogenized using the Omni TissueMaster-125 (USA Scientific) in modified RIPA buffer [50 mM Tris [tris(hydroxymethyl)aminomethane] base, pH 7.5, 150 mM NaCl, 1 mM EDTA (ethylenediaminetetraacetic acid), 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulfate)] containing protease inhibitor cocktail tablets (Complete Mini, Roche) and phosphatase inhibitors cocktail tablets (PhosSTOP, Roche), per the manufacturer's instructions. Lysates were cleared by centrifugation at 10000g for 10 minutes at 4°C (two times). Proteins were quantified using the DC Protein Assay kit (Bio-Rad). After dilution in 1X Laemmli Sample buffer (Bio-Rad) and incubation at 95°C for 5 minutes, 75 µg of protein extracts were resolved by SDS-polyacrylamide gel electrophoresis, using a pre-cast 10% Tris-HCl Criterion gel (BioRad). Proteins were transferred to an Immun-Blot PDVF membrane (BioRad), which was then blocked in 5% bovine serum albumin (BSA) in TBST buffer (1X Tris buffered saline, 0.1% Tween-20) for 1 hour. The membrane was probed overnight at 4°C with rabbit polyclonal antibody to phosphorylated Smad1/5/8 (1/2000, Cell Signaling Technology) in 5% BSA in TBST and washed with TBST. Following incubation with a peroxidase-coupled donkey anti-rabbit IgG antibody (1/5000, GE Healthcare), enzyme

activity was detected with the Amersham ECL Western Blotting kit (GE Healthcare). The blot was then stripped with Re-blot Plus Strong solution (Millipore) and reprobed with rabbit polyclonal antibody to Smad1 (1/1000, Cell Signaling Technology). β-actin expression was measured using a mouse monoclonal antibody (1/20000, Sigma) and a peroxidase-coupled sheep anti-mouse IgG secondary antibody (1/10000, GE Healthcare).

Histological analysis

Mouse spleens were fixed in 10% buffered formalin for 24 hours. Following transfer to 70% ethanol, spleens were embedded in paraffin, and 8-micrometer sections were prepared by the Duke University Medical Center Research Histology Laboratory. Deparaffinized tissue sections were stained by the diaminobenzidine-enhanced Perls reaction. Brightfield images were acquired at room temperature using the 10X/0.30 NA objective lens of an Eclipse E600 microscope (Nikon) with attached Spot RT Digital Camera with Spot 4.5 software (Diagnostic Instruments). The whole image was adjusted for brightness and labeled using Adobe Photoshop CS3 with no further modifications.

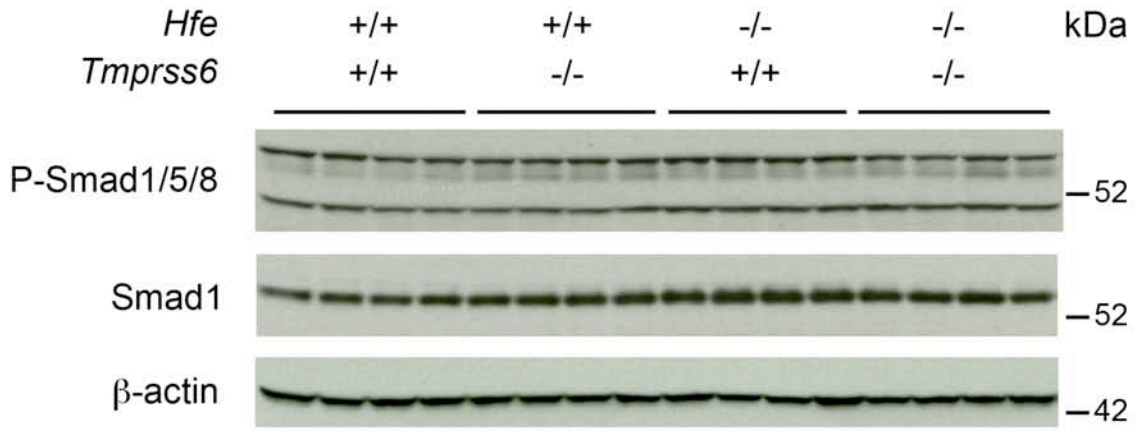

Figure S1. Genetic loss of Tmprss6 does not alter phosphorylation status of SMAD1/5/8 proteins in livers of *Hfe*$^{-/-}$ mice. Immunoloblot analysis of phosphorylated Smad1/5/8, total Smad1, and β-actin in liver lysates prepared from 8-week-old female *Hfe*$^{+/+}$*Tmprss6*$^{+/+}$, *Hfe*$^{+/+}$*Tmprss6*$^{-/-}$, *Hfe*$^{-/-}$*Tmprss6*$^{+/+}$, and *Hfe*$^{-/-}$*Tmprss6*$^{-/-}$ mice are shown. Four mice per genotype were analyzed.

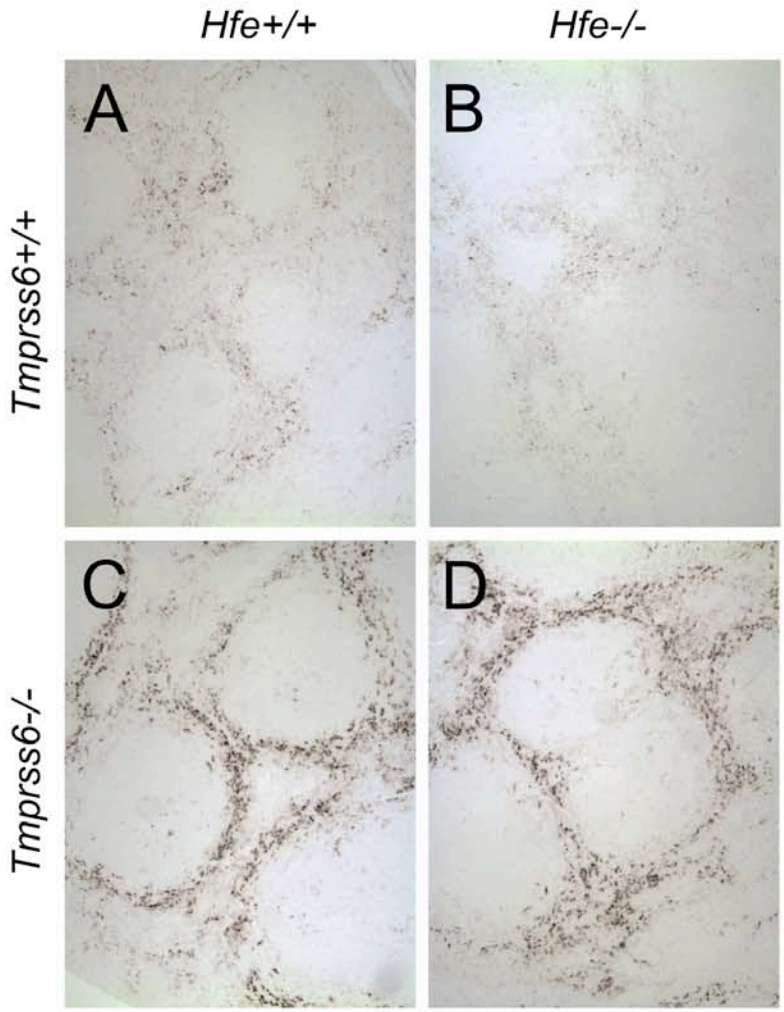

Figure S2. Genetic loss of Tmprss6 causes a qualitative increase in iron accumulation in splenic macrophages of *Hfe*$^{-/-}$ mice. DAB-enhanced Perls' Prussian blue staining of histological sections of spleens of 8-week-old female mice of selected *Hfe-Tmprss6* genotype combinations. Brown staining represents iron accumulation in cells. Splenic sections from *Hfe*$^{+/+}$*Tmprss6*$^{+/+}$ (A), *Hfe*$^{-/-}$*Tmprss6*$^{+/+}$ (B), *Hfe*$^{+/+}$*Tmprss6*$^{-/-}$ (C), and *Hfe*$^{-/-}$*Tmprss6*$^{-/-}$ (D) mice are shown. Representative results obtained from examination of three animals per genotype are presented. Original magnification x100.

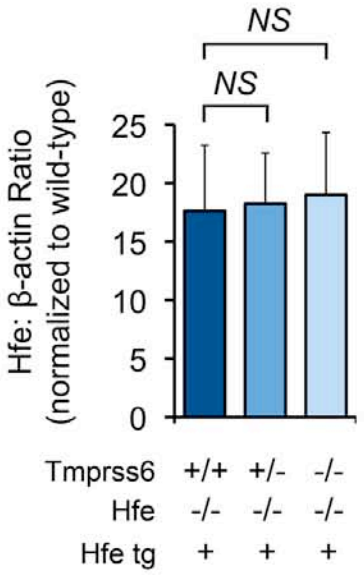

Figure S3. Hepatic expression of the *Hfe* transgene is similar in *Hfe*$^{-/-}$ mice with 0, 1, or 2 mutant *Tmprss6* alleles. Graphed are mean values obtained from analysis of *Hfe* mRNA expression relative to β-actin mRNA expression in 8-week-old *Hfe*$^{-/-}$ mice that are hemizygous for the liver-specific *Hfe* transgene and harbor 0, 1, or 2 mutant *Tmprss6* alleles. Results from 6 *Hfe*$^{-/-}$*Tmprss6*$^{+/+}$*Hfe tg*, 8 *Hfe*$^{-/-}$*Tmprss6*$^{+/-}$*Hfe tg*, and 8 *Hfe*$^{-/-}$*Tmprss6*$^{-/-}$*Hfe tg* mice are shown. mRNA expression ratios are normalized to an *Hfe*$^{+/+}$*Tmprss6*$^{+/+}$ mean value of 1. Error bars represent SD. *NS*, not significant.

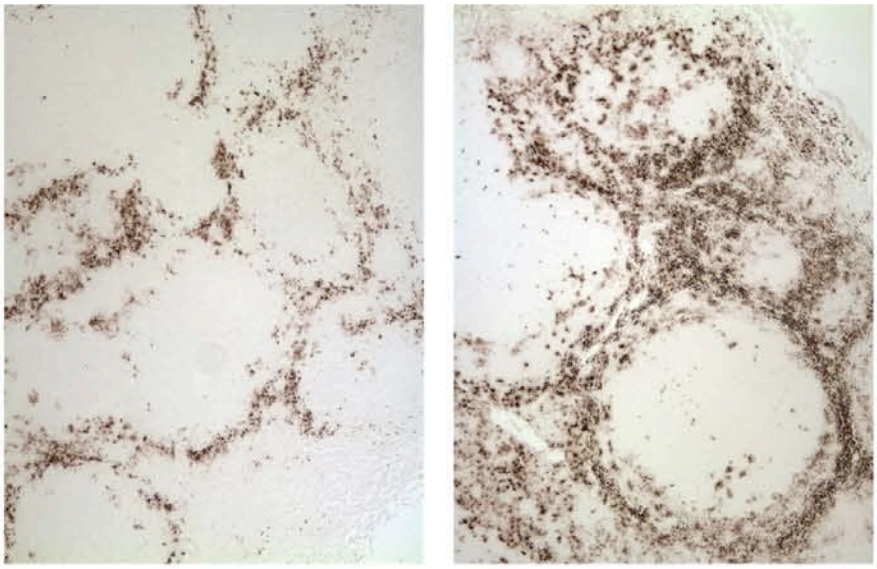

Figure S4. Hepatic over-expression of Hfe in 8-week-old *Hfe*$^{-/-}$ *Tmprss6*$^{-/-}$ mice causes a qualitative increase in iron accumulation in splenic macrophages. DAB-enhanced Perls' Prussian blue staining of histological sections of spleens. Brown staining represents iron accumulation in cells. Splenic sections from *Hfe*$^{-/-}$*Tmprss6*$^{-/-}$ mice (left) and *Hfe*$^{-/-}$*Tmprss6*$^{-/-}$ mice with the *Hfe* transgene (right) are shown. Representative results obtained from examination of three animals per genotype are presented. Original magnification x100 (B). Hfe tg, *Hfe* transgene.