## Figure legends

**Figure 1.** **Manhattan plot of the gene-based recessive diplotype association results.** The association *P*-value testing hemochromatosis association for each gene (*p* plotted on the ordinate) on different chromosomes is shown in alternating navy blue and yellow along the abscissa, with the experiment-wise significance level for the gene-based analyses across the exome (experiment-wise  = 3.14 × 10−6) depicted in red.

**Figure 2.** **Protein sequence alignment for FGF-4, FGF-5 and FGF-6 with heparin and FGFR binding domains.** Protein domains summarized from a previous FGF-6 functional study52. Alignment and Heparin and FGFR-binding sites/regions (HBS and FGFR-BR, respectively) are shown for FGF-4, FGF-5 and FGF-6 proteins.

**Figure 3.** **FGF-6 Active protein dosage effect on intracellular iron concentration.** A ferrozine assay was applied for the evaluation of total cell iron content in HepG2 (Human liver hepatocellular carcinoma cell line), 786-O (Human kidney adenocarcinoma cell line), HCT-8 (Human ileocecal colorectal adenocarcinoma cell line), HCT116 (Human colon carcinoma cell line) and HFF-1 (Human skin fibroblast cell line) with 10μM FAC and 500μM ascorbate in the cell culture media, respectively, with different concentrations of FGF-6 active protein (0ng/ml, 2.5ng/ml, 25ng/ml, and 250ng/ml). Control group was treated with ascorbate alone. After 48h incubation, cells were lysed and iron contents were determined with the ferrozine assay. **(A)** Total iron content in HepG2 cells with increasing FGF-6 protein concentration. **(B)** Total iron content in 786-O cells with increasing FGF-6 protein concentration. **(C)** Total iron content in HCT-8 cells with increasing FGF-6 protein concentration. **(D)** Total iron content in HCT-116 cells with increasing FGF-6 protein concentration.

**Figure 4.** **The effect of *FGF6* nonsynonymous variants on hepcidin expression and intracellular iron concentration. (A)** The effect ofFGF-6 active protein treatment on mRNA expression of several iron metabolism genes in HepG2 liver hepatocellular carcinoma cell culture media compared to control. Protein concentration was 250ng/ml and the incubation time was 24h with 10μM FAC and 500μM ascorbate in the cell culture media. *HAMP* encodes for hepcidin. *HDAC2* encodes for histone deacetylase 2. *HMOX1* encodes for heme oxygenase 1. *TFRC* encodes for transferrin receptor 1 and HEPH encodes hephaestin. mRNA expression was quantified relative to *GAPDH* expression. Treatment with PBS served as control. A t-test was used test for pairwise differences between sets of observations. \**P*＜0.05; \*\**P*＜0.01. Results are the mean±SD of 3 observations in a single experiment. **(B)** Iron metabolism gene expression changes with *FGF6* mRNA transfection in the HepG2 cell culture media after 24h.Vector without*FGF6* served as control. A t-test was used to test for pairwise differences between sets of observations. \**P*＜0.05; \*\**P*＜0.01. Results are the mean±SD of 3 observations in a single experiment. **(C, D)** Iron metabolism gene expression changes after the transfection by *FGF6* mRNA into various cell types with wildtype (WT) and the identified variants E172X (M1), D174V (M2) and R188Q (M3). Cell lines: HepG2 are liver hepatocellular carcinoma cells, HCT116 are ileocecal colorectal adenocarcinoma cells and HFF-1 are human normal skin fibroblasts. A t-test was used to test for pairwise differences between sets of observations. \**P*＜0.05; \*\**P*＜0.01. Results are the mean±SD of 3 observations in a single experiment. **(E, F)** Total intracellular iron concentration changes after the transfection with *FGF6* mRNA into three cell types with WT and the identified M1, M2, and M3 variants in the presence of FAC for 48h. A t-test was used to test for pairwise differences between sets of observations. \**P*＜0.05; \*\**P*＜0.01. Results are the mean±SD of 3 observations in a single experiment. **(G, H)** Ferritin protein level changes after the transfection by *FGF6* mRNA into the three cell types with WT and the identified M1, M2, and M3 variants in the presence of FAC for 48h. A t-test was used to test for pairwise differences between sets of observations. \**P*＜0.05; \*\**P*＜0.01. Results are the mean±SD of 3 observations in a single experiment.

**Figure 5. Perls’ stain and Ferritin expression. (A)** FGF-6 protein level was evaluated by immunohistochemistry assay (IHC) in skin tissues from systemic sclerosis (SSc) patients and healthy controls (Normal).Staining was visualized by Nikon microscopy. A t-test was used to test for pairwise differences between average optical density (AOD) values between SSc and Normal observations. The ratio of positive stain areas to the total area was used to evaluate protein levels. AOD were quantified by Image J software. \*\**P*＜0.01. **(B)** IHC with Perls’ Prussian Blue stain for Ferritin protein was applied to evaluate the iron deposition in SSc skin tissues and Normal skin tissue. AOD values were quantified by Image J software.Staining was visualized by Nikon microscopy. \*\**P*＜0.01. **(C)** IHC of FGF-6 protein in liver cancer tissue and control tissue. AOD were quantified by Image J Software. Staining was visualized by Nikon microscopy. \**P*＜0.05. **(D)** IHC of Ferritin protein using Perls’ Prussian Blue stain in liver cancer tissue and control tissue. AOD were quantified by Image J Software. Staining was visualized by Nikon microscopy. \**P*＜0.05.

**Figure 6. The proposed mechanism of FGF-6 in the regulation of hepcidin expression and iron concentrations.** Paracrine FGF-6 interacts with FGFR with heparin or heparin sulphate proteoglycan (HPSG) as the cofactor to initial FGF pathway.59 Activated FGFRs have the ability to phosphorylate specific tyrosine residues and activate STAT3 pathway.60 Iron overload, and inflammation could positively regulate hepcidin by BMP/Smad pathway61 and inflammatory IL-6/STAT3 pathways62,63. However, loss-of-function *FGF6* variants will silence the FGF6-FGFR pathway, increase free heparin and reduce expression of hepcidin, thereby decreasing the inhibition of ferroportin-mediated iron transfer from the intracellular compartment to the blood (i.e. increasing plasma levels of iron). In the SSc patients, IL-6 is increased so that hepcidin will be positively regulated which suppresses iron release to the plasma generating higher iron levels in skin cells.

## Supplementary Figure Legends

**Fig. S1. Two-Site Power Calculations.** Power calculations for a two-site disease model comparing the Armitage trend test of disease association at each site to a log-likelihood ratio test explicitly evaluating recessive diplotype effects. Baseline haplotype frequencies, case and control diploid sample sizes, and relative risk of disease-predisposing diplotypes parameters are shown. The initial haplotype frequencies (A1B1, A1B2, A2B1, A2B2) are presented. Different combinations of haplotypes are generated by generating recombination between the two sites and the results are presented in a collapsed manner through a single linkage disequilibrium metric. Hardy-Weinberg equilibrium of haplotypes/diplotypes in the general population is assumed. R is the relative risk of disease for recessive diplotypes compared to the remaining diplotypes. ncs and nct are the number of cases and controls, respectively. The type I error rate, adjusted for an exome-wide scan, was set to 2.5x10-6 for all calculations.

**Fig. S2. Minor Allele Frequency Distribution to PMRP dataset.** Displayed is the histogram of the minor allele frequency (MAF) at each variant within the 10,000 PMRP subjects following removal of variants from the QC procedures.

**Fig. S3.** **Quantile-Quantile Plot.** Q-Q plot for the exome-wide, gene-based recessive diplotype scanning in hemochromatosis is shown. Numerous genes had no recessive diplotypes with putative functional alleles and therefore yielded P-values of 1. The two data points exceeding the confidence interval represented *HFE* and *FGF6*.

**Fig. S4. Comparative genomic analysis and protein-protein interaction (PPI).** The comparative genomic analyses revealed that *FGF6* evolved synchronously with other iron metabolism genes. **(A)** Main iron metabolism genes were collected and alignment was conducted to make the comparative genomic analysis together with *FGF6*. The earliest gene appearance over time was inferred by comparing species and corresponding evolution and appearance time was labelled. **(B)** Protein-protein interaction network was estimated by String (version 10.0)51 using the highest confidence setting (confidence score>0.9).

**Fig. S5. Total iron content in HFF-1 cells with increasing FGF-6 protein concentration**. \* P＜.05; \*\* P＜0.01. Results are the mean±SD of 3 observations in each experiment.

**Fig. S6. FGF6 mutation Plasmid Structures in the study. M1 (GAG->TAG)** E172X, M2 (GAC->GTC) D174V and M3 (CGG -> CAG) R188Q

**Fig. S7. Relative transcription of iron metabolism genes with wildtype FGF6 and three mutations.** mRNA levels of FGF6 and four iron metabolism genes are measured relative to GAPDH following transfection of vector, wildtype FGF6 (WT), E172X FGF6 (M1), D174V FGF6 (M2), and R188Q FGF6 (M3). Measurements were taken in three cell lines: 786-O, A498, and HCT-8.

**Fig. S8. FGF6 loss-of-function nonsynonymous variants cause hepcidin downregulation and iron deposition in HFF-1. (A)** Iron metabolism gene expression changes after the transfection by *FGF6* mRNA into HFF-1 with wildtype and the identified variants R188Q, D174V and E172X. **(B)** Total iron contents changes after the transfection by *FGF6* mRNA into HFF-1 with wildtype and the identified variants R188Q, D174V and E172X. **(C)** Ferritin protein level changes after the transfection by *FGF6* mRNA into HFF-1 with wildtype and the identified variants R188Q, D174V and E172X. **(D)** The densitometry data of Western blot for Ferritin protein were shown in the column chart. \**P*＜.05; \*\**P*＜0.01. Results are the mean±SD of three observations in one experiment.

**Fig. S9. Perls’ stain reveals that FGF6 loss-of-function nonsynonymous variants cause iron deposition.** Perls’ stain in HepG2 **(A)**, HCT-116 **(B)**, HCT-8 **(C)**, 786-O **(D)** and HFF-1 **(E)** in the presence of FAC differs among transfection by *FGF6* mRNA with wildtype and the identified variants R188Q, D174V and E172X.

**Fig. S10. Perls’ stain in SSc and liver cancer. (A)** Perls’ stain was applied to evaluate the iron deposition in SSc skin tissues.Perls’ stain was visualized by Nikon microscopy. The ratio of iron-positive stain areas to the total area was used to evaluate the iron deposition levels by Image J software. Arrows indicated positive stain area. **(B)** Perls’ stain in liver cancer tissues.Perls’ stain was visualized by Nikon microscopy. The ratio of iron-positive stain areas to the total area was used to evaluate the iron deposition levels by Image J software.

**Fig. S11. FGF6 protein levels were different among normal, cancer and metastatic cells. (A)** IHC of FGF-6 in normal hepatocytes and metastatic cells. The blue circle indicated normal liver tissue and the arrows indicated metastatic cells. (B) IHC of FGF-6 in non-metastatic liver cancer cells.

**Supplemental Reference**

SR1. Szklarczyk, D. et al. STRING v10: protein-protein interaction networks, integrated over the tree of life. Nucleic Acids Res 43, D447-52 (2015).