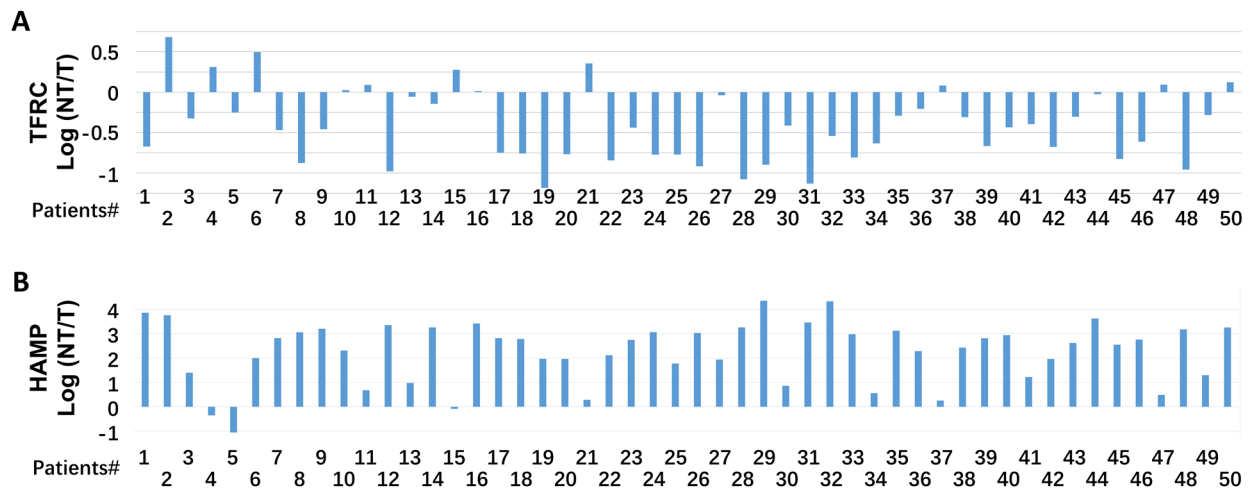**Supplemental Information**

Sphingosine-1-phosphate transporter spinster

homolog 2 is essential for iron-regulated

metastasis of hepatocellular carcinoma

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SUPPLEMENTARY FIGURES AND FIGURE LEGENDS

Fig. S1. Most HCC patients showed low iron statues in the HCC samples compared to their non-tumor adjacent samples. Data was retrieved from the public TCGA database (https://portal.gdc.cancer.gov/). The mRNA expression levels of **(A)** TFRC and **(B)** HAMP in the non-tumor (NT) samples were divided by those in the solid tumor (T) samples from the same patient. The log value is presented. HCC: hepatocellular carcinoma; TFRC: transferrin receptor; HAMP: hepcidin.

A

IA Diet

Day -7　0　10　　　　　　50

HCC Inoculation　　　　Sacrifice

B
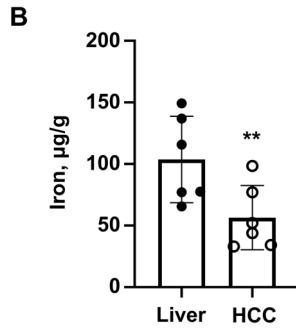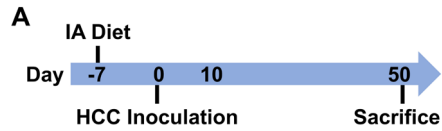
Fig. S2. HCC tumor had less iron than normal liver in an orthotopic mouse model.

(A) C57BL/6 mice were fed with IA diet at Day -7. Mouse HCC H22 cells were

orthotopically administrated at Day 0. All mice were sacrificed at Day 50. N=5 **(B)** Iron

content in the normal liver and growing HCC was determined.
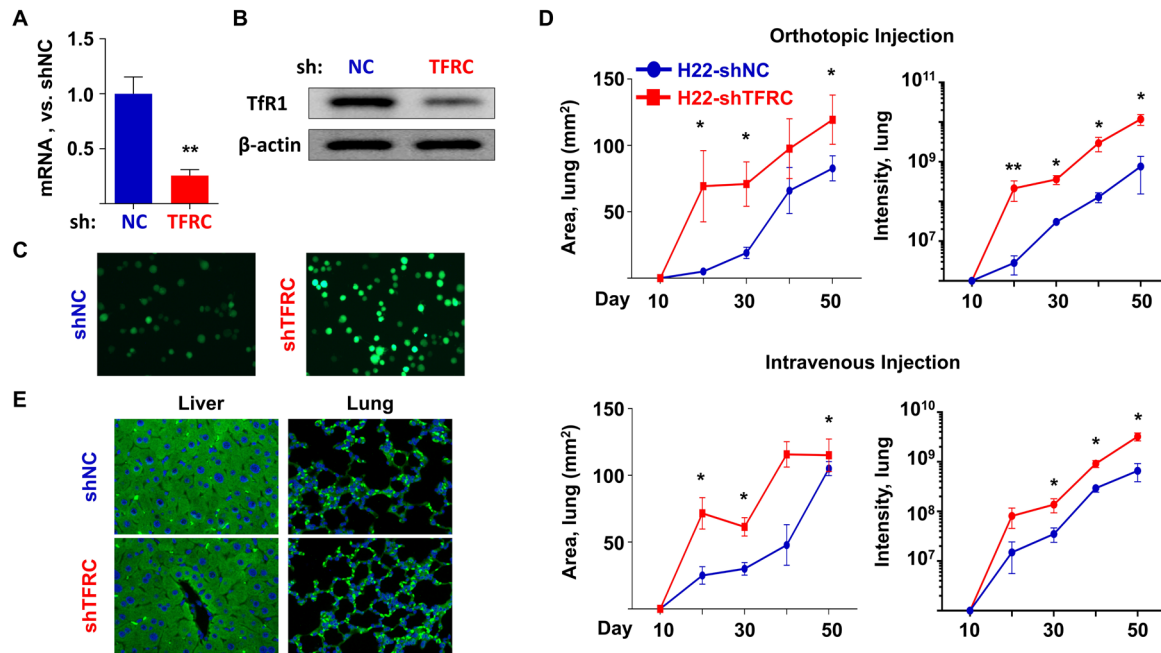
Fig. S3. TFRC-knockdown enhanced H22 cell metastasis *in vivo*.

(A) Quantitative reverse-transcription PCR and **(B)** Western blot assays to validate the knockdown efficiency of lentiviral mediated shTFRC in H22 cells.

(C) Phen Green-FL fluorescence signal quenching assay showed significantly decreased iron accumulation in the TFRC-knockdown H22 cells.

(D to E) C57BL/6 mice were fed with IA diet at Day -7. TFRC-knockdown (shTFRC) mouse HCC H22 cells or their control counterparts (shNC) were orthotopically or intravenously administrated at Day 0. All mice were sacrificed at Day 50. N=3 **(D)** Growth of tumor cells over time in the lung of C57BL/6 mice. **(E)** Fluorescent immunostaining showed ferritin expression in the liver and lung of the tumor-bearing C57BL/6 mice at sacrifice. Data is related to Figure. 2G.

Data was presented as mean $\pm$ SEM. *$p < 0.05$, **$p < 0.01$ vs. shNC. HCC: hepatocellular carcinoma; TFRC: transferrin receptor.
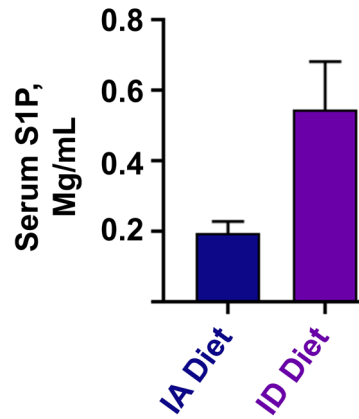
Fig. S4. Iron deficiency induced serum S1P level. C57BL/6 mice were fed with either

IA or ID diet for 2 months. The S1P content in serum was determined by liquid

chromatography – mass spectrometry (LC-MS).
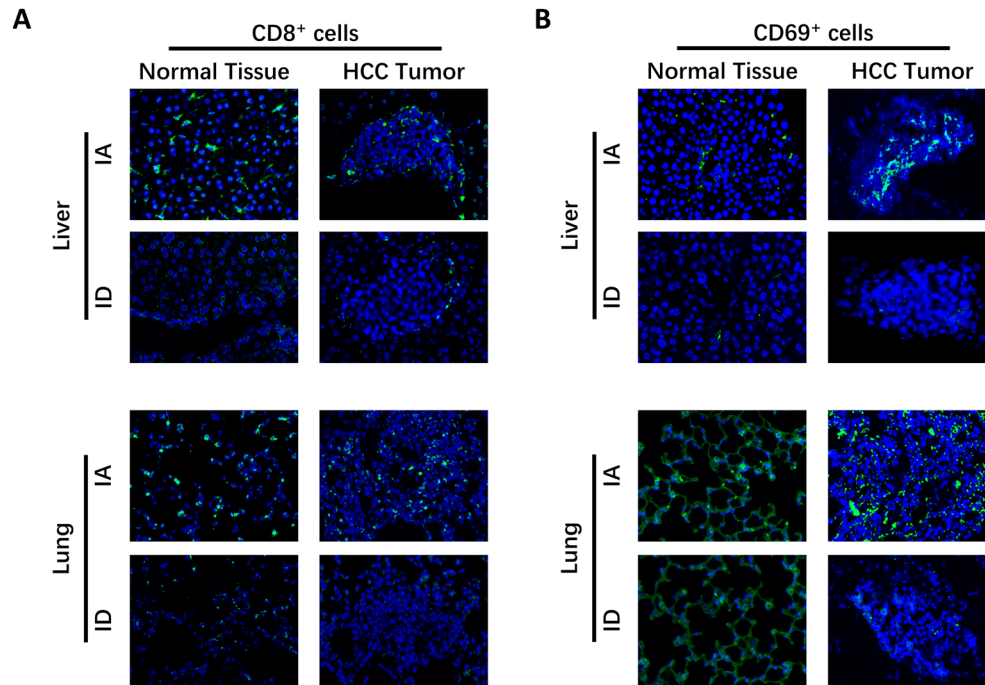
Fig. S5. Iron deficiency reduced CD8+ and CD69+ cells in the tumor-bearing mouse liver and lung. C57BL/6 mice were fed with either IA or ID diet at Day -7. Mouse HCC H22 cells were orthotopically administrated at Day 0. All mice were sacrificed at Day 50. The liver and lung tissues were obtained, followed by immune fluorescent staining against **(A)** CD8 and **(B)** CD69. Data is related to Fig. 3C. N=3.

IA: iron-adequate; ID: iron-deficient; HCC: hepatocellular carcinoma.
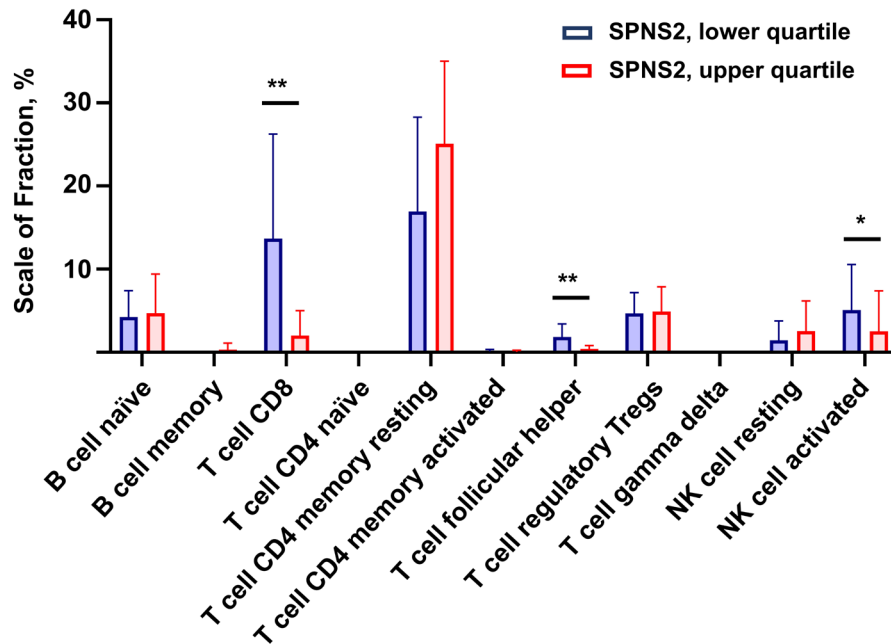
Fig. S6. Comparison of CIBERSORT immune cell fraction between HCC tissues with high and low SPNS2 expression. Gene-expression profiles of HCC tissues were obtained from the TCGA database and patients were divided into two groups based on the lower and upper quartile of transcripts per million values of SPNS2. The data were uploaded to the Cell-type Identification by Estimating Relative Subsets of RNA Transcripts (CIBERSORT) web portal (http://cibersort.stanford.edu/) and were analyzed by the algorithm using the LM22 signature and 1,000 permutations (Nat Methods. 2015;12(5):453-7.). *, P<0.05; **, P<0.05 vs. lower quartile.
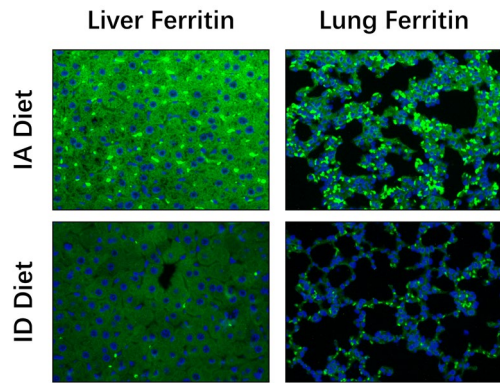
Fig. S7. Characterization of iron deficiency in the tumor-bearing nude mice. Nude mice were fed with either IA or ID diet at Day -7. Human HCC Huh7 cells were orthotopically or intravenously administrated at Day 0. All mice were sacrificed at Day 50. Ferritin expression in the liver and lung were determined by immunofluorescence staining at sacrifice. Data is related to Fig. 4A. N=6.

IA: iron-adequate; ID: iron-deficient; HCC: hepatocellular carcinoma;
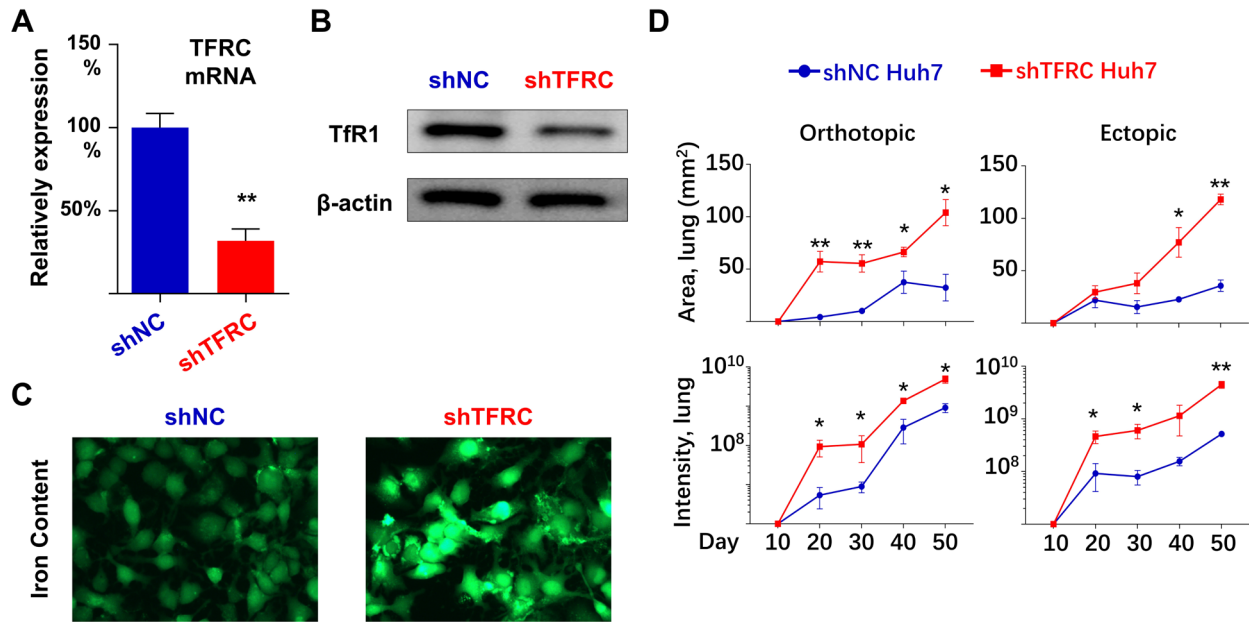
Fig. S8. Characterization of TFRC knockdown in the Huh7 cells. (A) RT-qPCR, **(B)** Western blot and **(C)** Phen Green-FL fluorescence signal quenching assays indicated the reduced TFRC mRNA, TfR1 protein and iron accumulation, respectively. **(D)** Growth of tumor cells over time in the lung of nude mice. Nude mice were fed with IA diet at Day -7. TFRC-knockdown (shTFRC) human HCC Huh7 cells or their negative control counterparts (shNC) were orthotopically or intravenously administrated at Day 0. All mice were sacrificed at Day 50. N=3.

Data was presented as mean $\pm$ SEM. *p < 0.05, **p < 0.01 vs. shNC. HCC: hepatocellular carcinoma; TFRC: transferrin receptor.
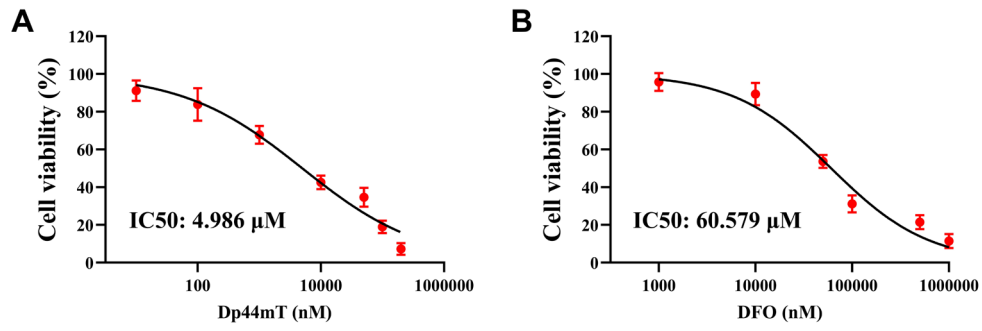
Fig. S9. The IC50s of Dp44mT and DFO in the Huh7 cells.
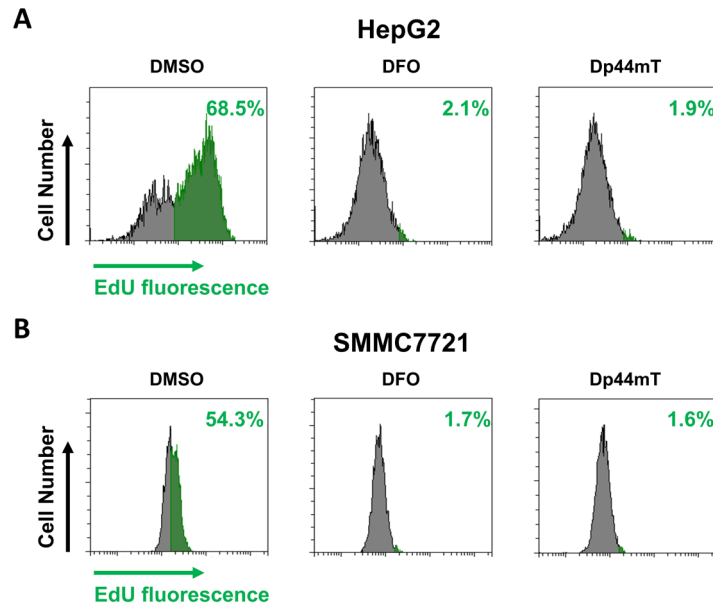
Fig. S10. Iron depletion led to cell growth inhibition of human liver cancer cell lines *in vitro*. Human HCC cell lines **(A)** HepG2 and **(B)** SMMC7721 were treated with either DMSO or iron chelators (DFO and Dp44mT) for 24h. The number of dividing cells were determined by EdU fluorescence levels.

DMSO: dimethyl sulfoxide; DFO: deferoxamine; Edu: 5-Ethynyl-2´-deoxyuridine.
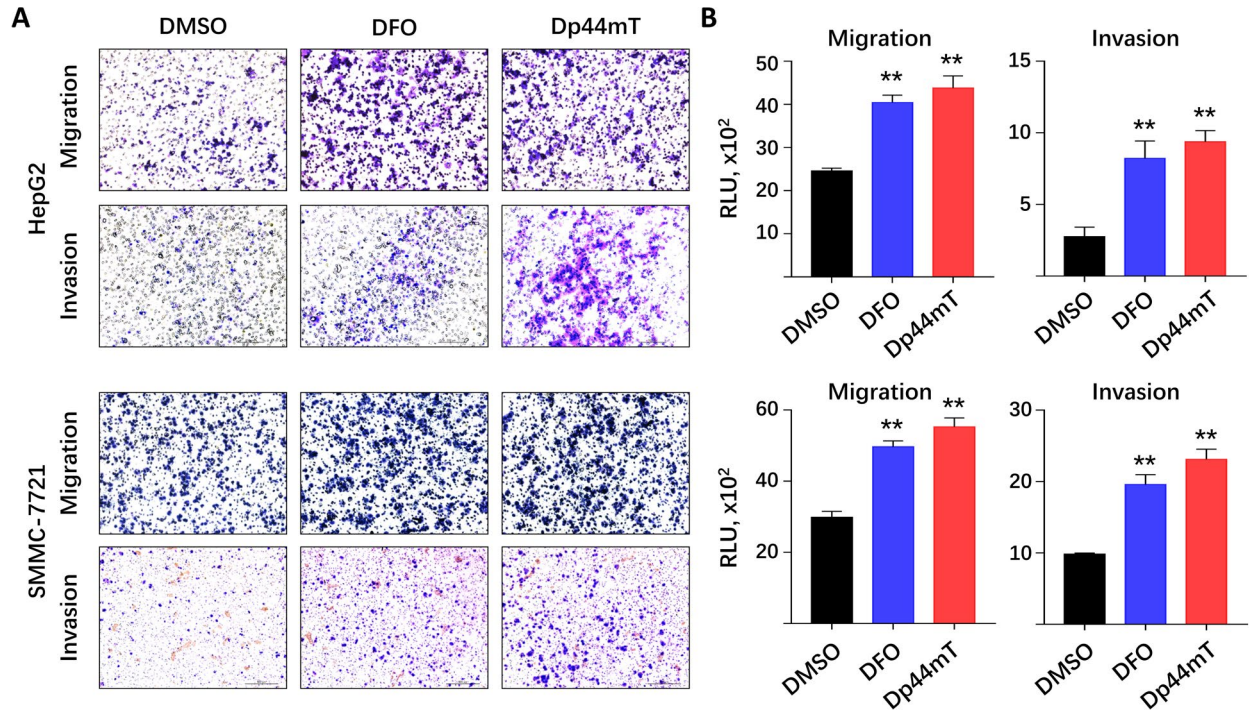
Fig. S11. Iron depletion increased migration and invasion abilities of various human liver cancer cell lines *in vitro*. Human HCC cell lines HepG2 and SMMC7721 were treated with either DMSO or iron chelators (DFO and Dp44mT) for 24h. Both migration and invasion abilities were determined. **(A)** Representative figures and **(B)** quantitative data were showed.

All *in vitro* experiments were performed as 3 replications. Data was presented as mean $\pm$ SEM. *p < 0.05, **p < 0.01 vs. DMSO. HCC: hepatocellular carcinoma; DMSO: dimethyl sulfoxide; DFO: deferoxamine.
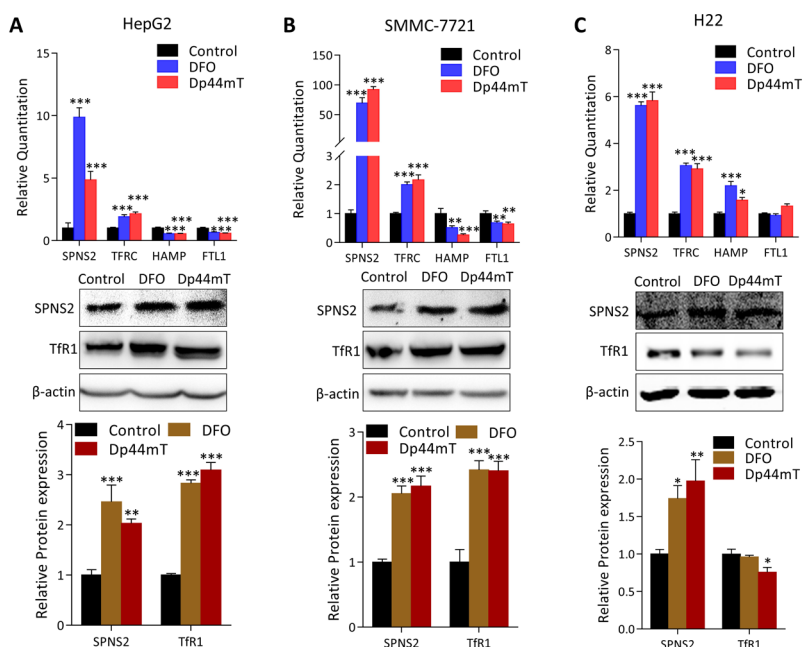
Fig. S12. The mRNA and protein expression of SPNS2 and iron-related genes in various HCC cells *in vitro*, under the treatment of iron depletion. (A) HepG2, **(B)** SMMC-7721, and **(C)** H22 cells were treated with either DMSO or iron chelators (DFO or Dp44mT) for 24h. The mRNA (upper) and protein (lower) expression of SPNS2 and iron-related genes were determined.

All *in vitro* experiments were performed as 3 replications. Data was presented as mean $\pm$ SEM. *$p < 0.05$, **$p < 0.01$ vs. DMSO. HCC: hepatocellular carcinoma; DMSO: dimethyl sulfoxide; DFO: deferoxamine; SPNS2: transporter spinster homologue 2; TFRC: transferrin receptor; HAMP: hepcidin; FTL: ferritin light chain.
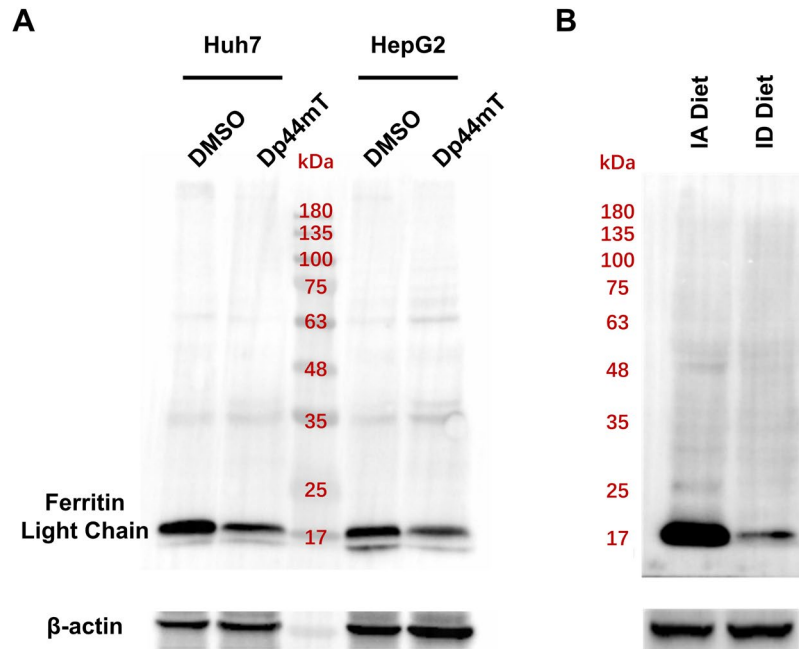
Fig. S13. The protein expression of FTL and validation of the anti-FTL antibody. (A) Huh7 and HepG2 cells were treated with either DMSO control, or Dp44mT for 24 hours . (B) C57BL/6 mice were fed with IA or ID diet for 2 months. Total protein was extracted and Western blot assay was performed against for Ferritin light chain.
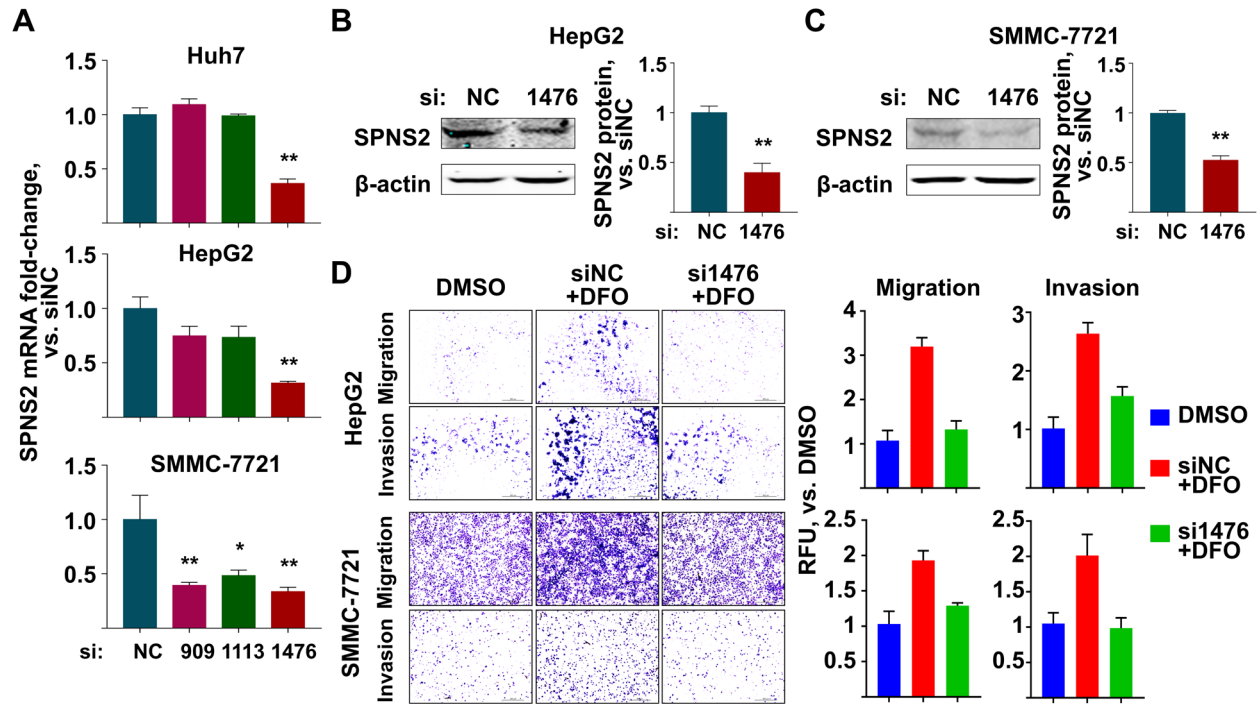
Fig. S14. Inhibition of SPNS2 expression rescued the enhancement effect of iron-depletion on the migration and invasion abilities of human HCC cell lines *in vitro*.

(A) Screening the inhibition efficiency of various siRNAs against SPNS2. mRNA expression was determined at 24 hours post-siRNA treatment.

(B and C) Western blot assays indicated the inhibition of SPNS2 expression post-siRNA treatment.

(D) Migration and invasion abilities of HepG2 (upper) and SMMC-7721 cells (lower) after the treatment of siRNA against SPNS2.

All *in vitro* experiments were performed as 3 replications. Data was presented as mean $\pm$ SEM. *p < 0.05, **p < 0.01 vs. siNC. HCC: hepatocellular carcinoma; DMSO: dimethyl sulfoxide; DFO: deferoxamine; SPNS2: transporter spinster homologue 2.
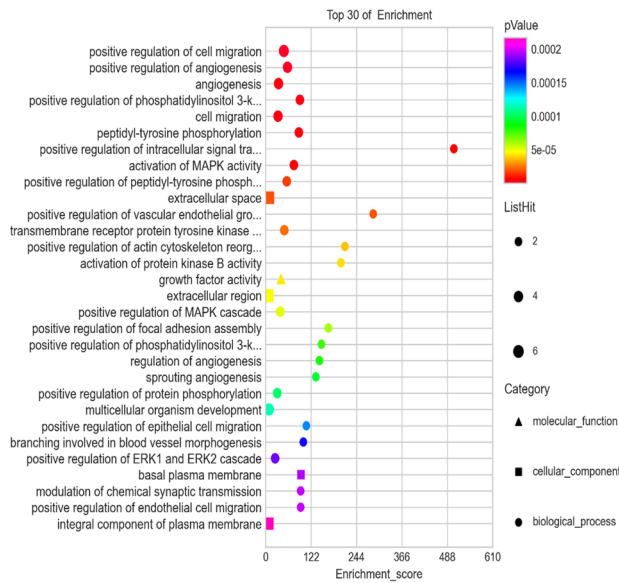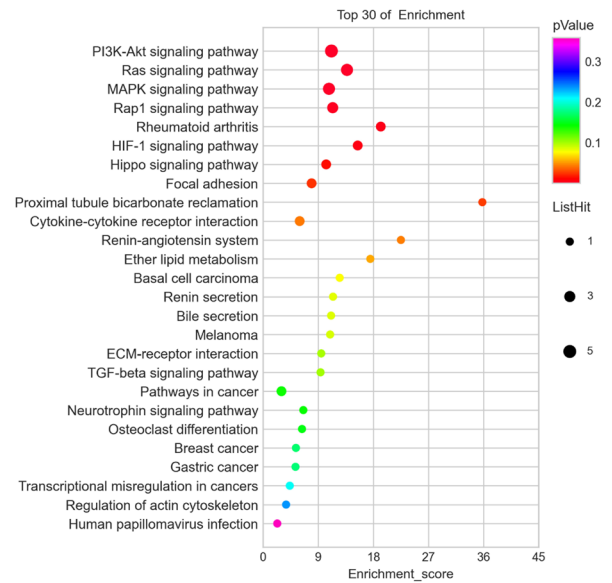
Fig. S15. The GO and KEGG enrichment analysis for metastasis-related genes.

C57BL/6 mice were fed a purified diet with no added iron and separated into ID, IA, and IO groups. Two weeks after iron injection, total hepatic RNA was subjected to Affymetrix GeneChip Mouse Gene 1.0 ST Array analysis. The magnitude of gene counts compared to all the background genes is represented by the horizontal bar length. The significance levels are represented by the legend's color saturation.
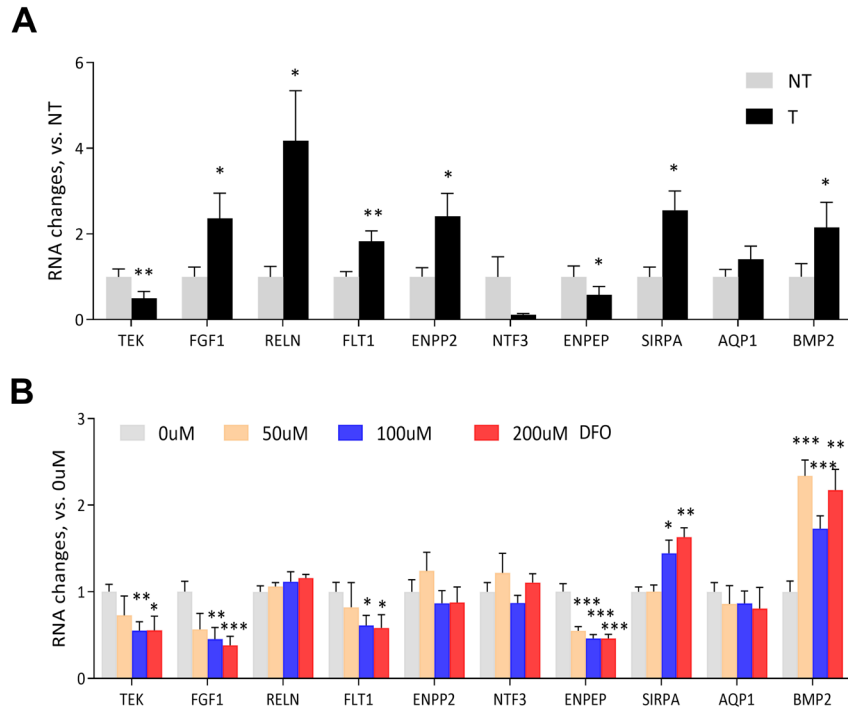
**Figure S16. Expressions of metastasis-related genes in iron-deficient cells.** Total RNAs were isolated from (A) human liver cancer samples and (B) iron chelator DFO-treated Huh7 cells, followed by qRT-PCR to determine the expression levels of metastasis-related genes. NT, adjacent non-tumor tissue. T, tumor tissue.

Fig. S17. The strategy to generate and identify SPNS2 knockout mice. The knockout of SPNS2 gene was performed by CRISPR /Cas9 system and guided by two gRNAs, as shown in the following figure. This strategy made KO mice loss exon 3, 4, and 5 in the SPNS2 gene. The pairs of PCR primers, F1&R1 and F2&R1, were used to amplify DNA bands for KO and WT mice. The WT band amplified by R1 and F2 equals to 409bp, while the KO band amplified by R1 and F1 equals to 613bp.
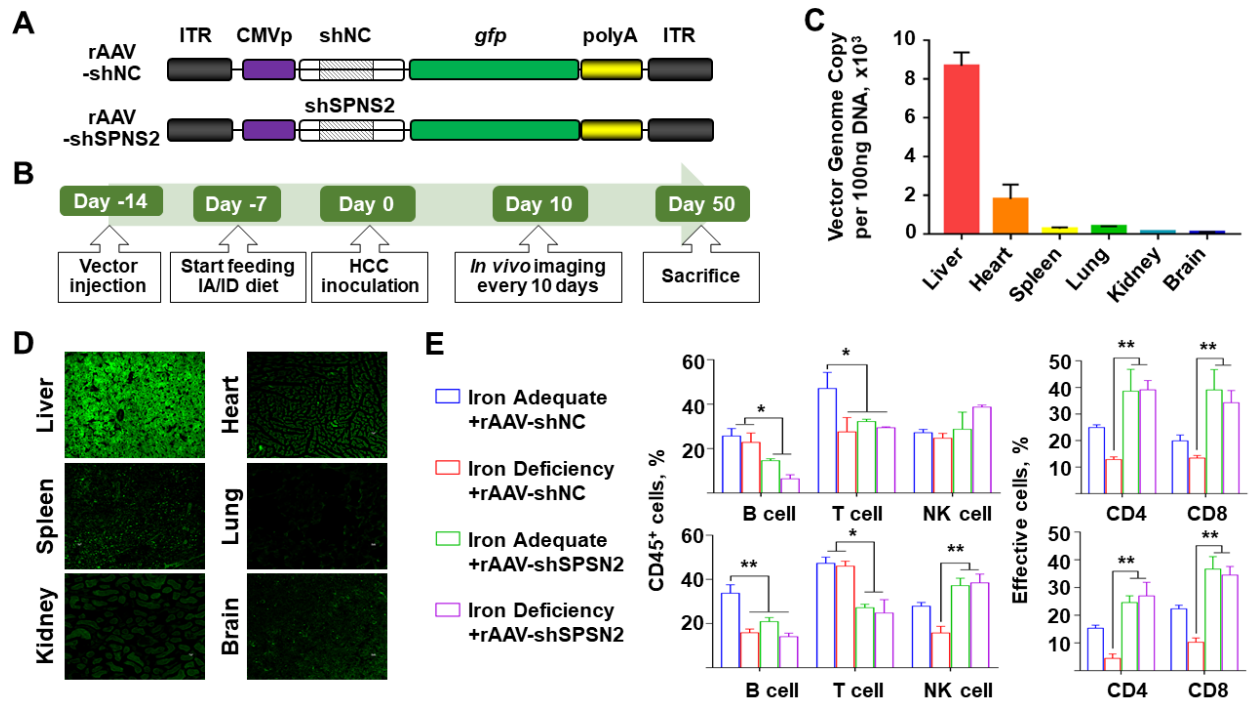
Fig. S18. Validation of recombinant adeno-associated virus (rAAV) serotype 8 vectors *in vivo*.

(A) Schematic genome structures of rAAV vectors containing shNC/shSPNS2 and a green fluorescent protein (*gfp*) gene. Both vectors were under the control of a ubiquitous CMV promoter (CMVp).

(B) Protocols for analysis of HCC metastasis post-viral injection.

(C and D) Non-tumor C57BL/6 mice were tail-vein injected with rAAV vectors. **(C)** The vector genome copy number and **(D)** GFP expression in various mouse tissues at Week 4 post-viral injection. N=3

(E) C57BL/6 mice were tail-vein injected with rAAV vectors at Day -14 and fed with either IA or ID diet at Day -7. Mouse HCC H22 cells were orthotopically administrated at Day 0. All mice were sacrificed at Day 50. The percentage of lymphocyte subsets and

that of effective T cells in the liver (upper) and lung (lower) were determined by flow

cytometry assay. Effective: CD 44^{high}, CD62^{Low}. Data is related to Fig 4E. N=3

Data was presented as mean $\pm$ SEM. *p < 0.05, **p < 0.01. IA: iron-adequate; ID: iron-

deficient; HCC: hepatocellular carcinoma; SPNS2: transporter spinster homologue 2;

ITR: inverted terminal repeat; CMVp: cytomegalovirus promoter.

SUPPLEMENTARY TABLE

Table S1. Sequences of primers for amplification of cDNA.

Name	sequence
hsa18s-F	TAACGAACGAGACTCTGGCAT
hsa18s-R	CGGACATCTAAGGGCATCACAG
hsaTFRC-F	GGGATACCTTTCGTCCCTGC
hsaTFRC-R	ACCGGATGCTTCACATTTTGC
hsaHAMP-F	CTGTTCCCTGTCGCTCTGTT
hsaHAMP-R	AGTTGTCCCGTCTGTTGTGG
hsaFerritin-F	GGACCCCCATCTCTGTGACT
hsaFerritin-R	AGTCGTGCTTGAGAGTGAGC
hsaSPNS2-F	AACGTGCTCAACTACCTGGAC
hsaSPNS2-R	GAAGCTACAGATGAACACTGACTG
mmu-18s-F	GTAACCCGTTGAACCCCATT
mmu-18s-R	CCATCCAATCGGTAGTAGCG
mmuHAMP-F	CAGGGCAGACATTGCGATAC
mmuHAMP-R	GCAACAGATACCACACTGGGA
mmuFerritin-F	GCTCCTTGCCCGGGACTTA
mmuFerritin-R	AAAAAGAAGCCCAGAGAGAGGT

mmuTFRC-F	GGCGCTTCCTAGTACTCCCT
mmuTFRC-R	ATAGCCCAGGTAGCCACTCA
mmuSPNS2-F	TGAAGGCCCTGATCCGAAAC
mmuSPNS2-R	ATGAGGCTGTCTTTGGCTCC

Table S2. Sequences of primers for amplification of genomic DNA.

TFRC-sg-5in-tF	TCTTTCTATATTGCCTCAGGTTGAC
TFRC-sg-3in-tR	GGTGTCAGCAAACTCTATGGAGTTC
SPNS2-F1	AGAATACATGAGACCCTGCGTTTG
SPNS2-F2	CAGAGACCAGGCTTTGACCTTC
SPNS2-R1	CACTTTGTCTTGAGCTTCCGC

Table S3. Sequences for siRNA, shRNA and Cas9 guide RNA.

SPNS2-gRNA-F	ATCCCCAGGGCCGCAGTCCAGGG
SPNS2-gRNA-R	TTACTTACTGCATCACCCCCAGG
Lenti-has-TFRC shRNA	CCGGTTGTATGTTGAAAATCAATTTCGCTCGA GCGAAATTGATTTTCAACATACAAATTTTT
Lenti-mmu-TFRC shRNA	CCGGTGCTAATTTTGGCACTAAAAAGGCTCGA GCCTTTTTAGTGCCAAAATTAGCATTTTT
siSPNS2-909-sense	CCGUCUUCUACUUCGCCAUTT

siSPNS2-909-antisense	AUGGCGAAGUAGAAGACGGTT
siSPNS2-1113-sense	CCUCAUGGCUCCGAGAUAUTT
siSPNS2-1113-antisense	AUAUCUCGGAGCCAUGAGGTT
siSPNS2-1476-sense	GCAUCGUAGGAGCCUAUAUTT
siSPNS2-1476-antisense	AUAUAGGCUCCUACGAUGCTT