**Supplementary Methods**

**Animal studies**

Animal experiments were undertaken under an approved UK Home Office Project License 40/3636 with ethics approval from the University of Oxford Animal Welfare and Ethical Review Body. Wild-type male C57BL/6 mice were purchased from Harlan Laboratories, UK. Embryos from Fam132b+/− mice on a mixed Sv129/C57BL/6 background were obtained from the Mutant Mouse Regional Resource Center (MMRRC) at UC Davis (strain B6;129S5-Fam132btm1Lex/Mmucd, ID MMRRC:032289-UCD) and backcrossed onto C57BL/6 background using marker-assisted accelerated backcrossing. Heterozygote pairs were mated to generate homozygous animals from which knockout and wild-type colonies were maintained. Animals were housed in individually ventilated cages in the Department of Biomedical Services, University of Oxford, and provided access to normal chow (163 ppm of iron, Special Diets Services 801700) and water *ad libitum*. All experiments were performed in 9-13 weeks old male mice. For EPO treatments, mice were injected intraperitoneally with 200 IU recombinant human EPO (Bio-Rad) in water or vehicle (water) daily for three consecutive days and culled 24h after the last EPO injection. For ERFE treatments, mice were injected intravenously with 200µg of recombinant mouse Erfe or a clipped (inactive) version of the protein as a control, and culled 3h after treatment. Mice were euthanized in increasing $CO_2$ concentrations.

**Protein production**

Expi293F™ Cells (Gibco A14527) cells were propagated in Expi293 expression medium at 36°C and transfected with intact expression vector DNA (human or mouse ERFE) according to manufacturer's recommended protocols. Following transfection, the cell culture was returned to 8% CO2 shaking incubator at 36°C. No supplementary tryptone feed was given to these cultures. Conditioned media (CM) was collected 120 hours post-transfection. CM was transferred to sterile 1L Nalgene bottles and centrifuged for 6min at 1800 RPM, 4°C in Sorvall H-6000A rotor (940x g). Clarified CM was collected and filtered using Sartopore 2XL 0.8/0.2 um filtration. ERFE proteins were linked to monoFc[21] using a G4S linker at the N-terminus. The recombinant monoFc-huERFE (human) and monoFc-muErfe (murine) were purified in the following manner. All chromatography steps were performed at 4°C. The proteins were captured from CM using MabSelect SuRe (GE Healthcare), washed extensively with Calcium-Magnesium-free PBS (PBS-CMF) pH 7.2, and eluted with a decreasing pH gradient. Fractions containing ERFE were exchanged into low sodium chloride pH 8.0 buffer, loaded onto a Q Sepharose HP column (GE Healthcare), and an increasing sodium chloride gradient elution was performed. A Hiload Superdex 200 column (GE Healthcare) was used as a final

purification step with mobile phase containing Arginine pH 7.0. Fractions with high purity of ERFE were pooled and buffer exchanged into PBS-CMF pH7.2.

**Anti-ERFE antibody production**

Ten-to-twelve week old male ErfeKO mice were injected i.p. with 50µg/dose of monoFc-huERFE on days 0, 14, 28 and 42. Mice were culled on day 45 and the spleen harvested for hybridoma production using the ClonaCell-HY Hybridoma kit (StemCell technologies) following the manufacturer's instructions. Briefly, spleen cells were fused with SP2/O myeloma cells for 16-24h. Hybridomas were selected using the HAT (hypoxanthine-aminopterin-thymidine) method, plated and incubated for 13 days. Colonies (1000-2000) were picked into 96-well plates containing 200 µL of ClonaCell-HY Growth Medium E and incubated for 3 days. Supernatants were screened by ELISA in a 96-well plate coated with monoFc-huERFE or control (at 1ug protein/ml in PBS), and colonies with a positive signal for monoFc-huERFE but negative for the control were selected. The six (sister) clones with the highest signal were selected, expanded in DMEM, 10% ultra low IgG FBS, 2mM L-glutamine, 100 U/ml penicillin and 0.1mg/ml streptomycin. Cell culture supernatants were loaded onto Protein G Agarose Pierce columns (2ml per column). IgGs were eluted with 10ml 100mM glycine, pH 2.7; buffered immediately with 350µl 1M Trizma hydrochloride solution pH 9.0 (Sigma) and buffer exchanged into PBS using an Amicon Ultra-15 Ultracel 30K centrifugal filter (Merck). Purified IgGs were sequenced and cloned in to mammalian expression vectors as described previously[22]. Transiently transfected HEK293 cells expressing anti-ERFE were cultured in FreeStyle™ 293 medium or Expi293™ medium (ThermoFisher). These cells were pre-seeded in a wave bioreactor at a cell density of $1.25 \times 10^6$ cells/ml and transfected with polyethylenimine (Polysciences). The wave bioreactors were incubated at 37 °C with a rocking rate of 20 rpm for 120 h before harvest. The conditioned media was centrifuged using a Sorvall BIOS 16 Bioprocessing Centrifuge (Thermo Fisher Scientific) and filtered with a 0.22 µm filter device prior to purification. The clarified conditioned media was loaded onto a 5 ml MAbSelect SuRe column (GE Life Sciences) equilibrated with PBS, pH 7.2. The column was washed with 10 column volumes of PBS, pH 7.2 before the protein was step eluted using a low pH buffer. The protein was immediately loaded onto a 320 ml Superdex 200 size exclusion column (GE Life Sciences) equilibrated in PBS, pH 7.2. Peak fractions were pooled and filtered through a 0.2um PES filter. A monoclonal antibody that bound both mouse and human Erfe proteins, and neutralized the ability of both mouse and human ERFE proteins to suppress hepcidin *in vitro*, was then selected for further use.

**Cell treatments**

Huh7 and HepG2 were cultured in Dulbecco's Modified Eagle's Medium – High Glucose (Sigma), supplemented with 10% Fetal Bovine Serum (Sigma), 1% Penicillin-Streptomycin (Sigma) and 1% L-Glutamine (Sigma), unless otherwise indicated. Cells were plated 24h before treatments in 24-well (gene expression analysis) or 12-well (protein analysis) cell culture plates. At the time of treatment, cells were washed with PBS and fresh media was added. Cells were treated with recombinant human or mouse ERFE, BMP2, 4, 5, 6, 7 or 9 (R&D systems), Activin B (R&D systems), anti-Erfe antibody LDN- 193189 (MedChem Express) or IL-6 (R&D systems), for 30 minutes (Western Blot), 6 or 24 hours (gene expression). siRNA-mediated gene knockdown of *TMPRSS6* in HepG2 cells was performed in antibiotic-free medium for 24h using Lipofectamine RNAiMAX reagent (Invitrogen) with 20nM of siGENOME SMARTpool siRNA (Dharmacon) targeting mouse *Tmprss6* (M-006052-02-0005) following the manufacturer's instructions. Non-targeting siRNA (D-001210-02-05) was used as control. Silencing efficiency was assessed by qPCR.

**Gene expression microarray**

RNA from Huh7 cells was isolated using RNeasy Plus kit (Qiagen), followed by RNA quantification and quality assessment using a 2100 Bioanalyzer (Agilent). RNA was converted into biotin labelled cRNA for hybridization and gene expression analysed using the Human HT12v4.0 Expression Beadchip (Illumina) and the Illuminas's iScan Scanner. Raw data was normalised using the lumi package (Bioconductor) and compared using LIMMA (Bioconductor). Statistical significance was set at $p<0.05$.

**RNA sequencing**

mRNA sequencing libraries were constructed from 1µg of total RNA with Illumina truSeq Stranded mRNA sample prep protocol. Sequencing was performed on the Illumina NextSeq 500 platform at single-End 75bp.

**Western Blot**

Cells were lysed at 4 °C using RIPA buffer (Thermo Scientific) containing protease/phosphatase inhibitor (Cell Signalling). Lysates were denatured at 95 °C and separated on a 10% SDS polyacrylamide gel (Bio-Rad), following the manufacturer's instruction. Protein sizes were estimated by using the Novex Sharp Pre-Stained Protein Ladder (life technologies). Protein was transferred to a nitrocellulose membrane, then blocked with milk/TBS for 1 h. Antibodies used were anti-P-SMAD 1/5 (S463s/465)/ 9(S465/467) (Cell signalling 13820S 1:500), anti-Smad1 (Cell Signalling 6944S

1:500), anti-β-actin-peroxidase (Sigma A3854 1:10 000), and Anti-rabbit IgG HRP conjugated (RnD systems HAP008 1:5000).

**RNA isolation, cDNA synthesis and qRT-PCR**

Liver (preserved in RNA later prior to lysis using a TissueRuptor(Qiagen)) or cells were lysed and RNA was isolated using the RNeasy Plus kit (Qiagen), followed by RNA quantification and quality assessment using Nanodrop (Thermo Fisher). cDNA was synthesized using the High Capacity RNA-to-cDNA kit (Applied Biosystems). Gene expression was assessed using quantitative real-time PCR with Taqman Gene Expression Master Mix and inventoried Taqman Gene expression assays (Applied Biosystems) specific for the genes of interest (supplementary table 1). *GAPDH* or *Hprt1* were used as endogenous control genes for human cells and mouse tissues respectively, and qPCR was performed using the QuantStudio 7 Flex Real-Time PCR system.

**Tissue non-heme iron measurement**

Liver tissues were dried for 4 hours at 100 °C, weighed and digested in 10% tricholoroacetic acid (Sigma)/ 30% hydrochloric acid (Sigma) for 20 hours at 65 °C. A standard curve was generated using a dilution series of ferric ammonium citrate (Sigma) in the 10% (w/v) trichloroacetic acid/ 30% hydrochloric acid mixture. Non-heme iron content was determined colorimetrically by measuring absorbance at 535nm following reaction with chromogen reagent containing 0.1% (w/v) bathophenoldisulphonic acid (Sigma) / 0.8% thioglycolic acid (Sigma).

**Blood parameters and serum iron analysis**

Blood was taken by cardiac puncture immediately after euthanising mice and collected in BD EDTA or SST (serum) Microtainer tubes. Whole EDTA-blood was immediately used for quantification of blood parameters using a hematology analyser (Sysmex KX-21-N). Serum was prepared by centrifugation of clotted blood at 8000 x g for 3 minutes in BD Microtainer SST tubes (Beckton Dickinson) and used for serum iron quantification using a Abbott Architect c16000 automated analyzer (Abbott Laboratories).

**Luciferase assay**

C2C12 mouse myoblast cells (ATCC CRL-1772) were transfected with a pTal-Luc reporter plasmid in which a synthetic BMP-response element (BRE) was inserted into the NheI site as previously described[23]. Cells were cultured in Low Bicarbonate DMEM supplemented with 4 mM L-glutamine; 4.5 g/L glucose, 100ug/ml of Pen/Strep; 10% fetal bovine serum (FBS). Cells remained undifferentiated mononucleated cells with no morphological changes throughout the course of the

culture and experiments. Cells were plated in a 96 well plate (10 000 cells per well) and treated 24h later in 1% FBS media containing BMP (2nM) alone or in combination with a gradient of mouse ERFE concentrations (4-fold dilutions from 0.5µM) with two replicates per condition. Luminescence was measured 24h after treatment using the britelite Plus Reporter Gene Assey System (PerkinElmer) and an EnVision Plate Reader (PerkinElmer) following the manufacturer's instructions.

**Homogeneous Time Resolved Fluorescence** A competition HTRF assay[24,25] was established in order to assess whether BMPs could compete with neutralising anti-ERFE antibodies for binding to the same/overlapping epitope on recombinant Erfe protein. The anti-ERFE antibody was labelled with europium cryptate using a cryptate labelling kit (CisBio) according to the manufacturer's instructions. The final reaction mix contained 15 nM biotinylated monoFc-murine Erfe, 1:1000 dilution of Streptavidin-XL665 (CisBio), 1:300 dilution of the europium cryptate-labeled anti-ERFE antibody, and BMPs or unlabelled anti-ERFE mAb at differing concentrations, in a total reaction volume of 20 µl in 1× assay buffer [50 mM sodium phosphate, pH 7.5, 400 mM potassium fluoride, and 0.1% BSA (w/v)]. Reagents were added sequentially into 384-well low-volume black plates (Nunc). Reactions were allowed to proceed for 3 h at room temperature, and plates were subsequently read on the EnVision Multilabel Plate Reader (Perkin-Elmer) with excitation at 340 nm and two emission readings at 615 nm (measuring input donor fluorescence from antiERFE antibody-europium cryptate) and 665 nm (measuring output acceptor fluorescence from Streptavidin-XL665). All readings were expressed as the percentage of change in fluorescence, %ΔF, where: %ΔF=[(F665 Sample/F615 Sample)−(F665 Control/F615 Control)(F665 Control/F615 Control)]×100 . "Control" represents the background fluorescence energy transfer in wells containing 1:1000 labelled antibody, in assay buffer, alone.

**Statistical analysis**

Statistical analyses were performed using Prism 6 (GraphPad Software). Statistical significance was assessed using Student's t-test or one-way ANOVA followed by Tukey test for multiple comparisons.

**Supplementary table 1:** List of TaqMan Gene Expression assays (Applied Biosystems)

| **Protein** | **Species** | **Gene** | **Assay code** |
|---|---|---|---|
| Glyceraldehyde 3-phosphate dehydrogenase | Human | *GAPDH* | Hs99999905_m1 |
| Hepcidin | Human | *HAMP* | Hs00221783_m1 |
| Inhibitor of DNA-binding protein 1 | Human | *ID1* | Hs03676575_s1 |
| Inhibitor of DNA-binding protein 2 | Human | *ID2* | Hs00171409_m1 |
| Sons of mothers against decapentaplegic 6 | Human | *SMAD6* | Hs00178579_m1 |
| Matriptase-2 | Human | *TMPRSS6* | Hs00542184_m1 |
| Hypoxanthine-guanine phosphoribosyltransferase | Mouse | *Hprt1* | Mm01545399_m1 |
| Hepcidin | Mouse | *Hamp1* | Mm04231240_s1 |
| Inhibitor of DNA-binding protein 1 | Mouse | *Id1* | Mm00775963_g1 |
| Inhibitor of DNA-binding protein 2 | Mouse | *Id2* | Mm00711781_m1 |
| Atonal BHLH Transcription Factor 8 | Mouse | *Atoh8* | Mm00464055_m1 |
| Sons of mothers against decapentaplegic 7 | Mouse | *Smad7* | Mm00484742_m1 |
| Bone morphogenetic protein 2 | Mouse | *Bmp2* | Mm01340178_m1 |
| Bone morphogenetic protein 6 | Mouse | *Bmp6* | Mm01332882_m1 |

**Supplementary Bibliography**


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22. Babrak L, McGarvey JA, Stanker LH, Hnasko R. Identification and verification of hybridoma-derived monoclonal antibody variable region sequences using recombinant DNA technology and mass spectrometry. *Mol Immunol*. 2017;90:287-294.

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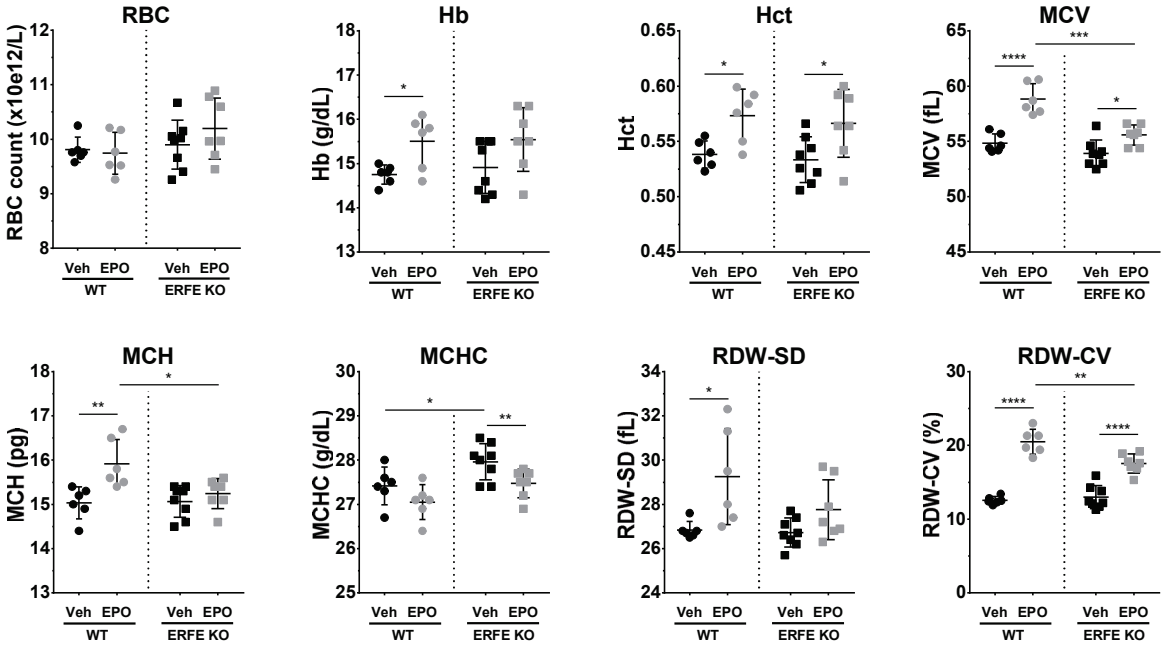


**Figure S1. Blood parameters in EPO-treated mice.** WT and ERFE KO male mice (10-13 weeks old) were injected with 3 doses of 200 iu of EPO or vehicle (Veh), one dose every 24h, and analysed 24h after the last injection. Blood parameters were measured using a Sysmex analyser (one-way ANOVA followed by Tukey test for multiple comparisons *p <0.05, **p <0.01, ***p<0.001, ****p <0.0001). RBC: Red Blood Cells; Hb: Hemoglobin; Hct: Hematocrit; MCV: Mean Corpuscular Volume; MCH: Mean Corpuscular Hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration; RDW-SD: Red blood cell Distribution Width – Standard Deviation; RDW-CV: Red blood cell Distribution Width – Coefficient of Variation.

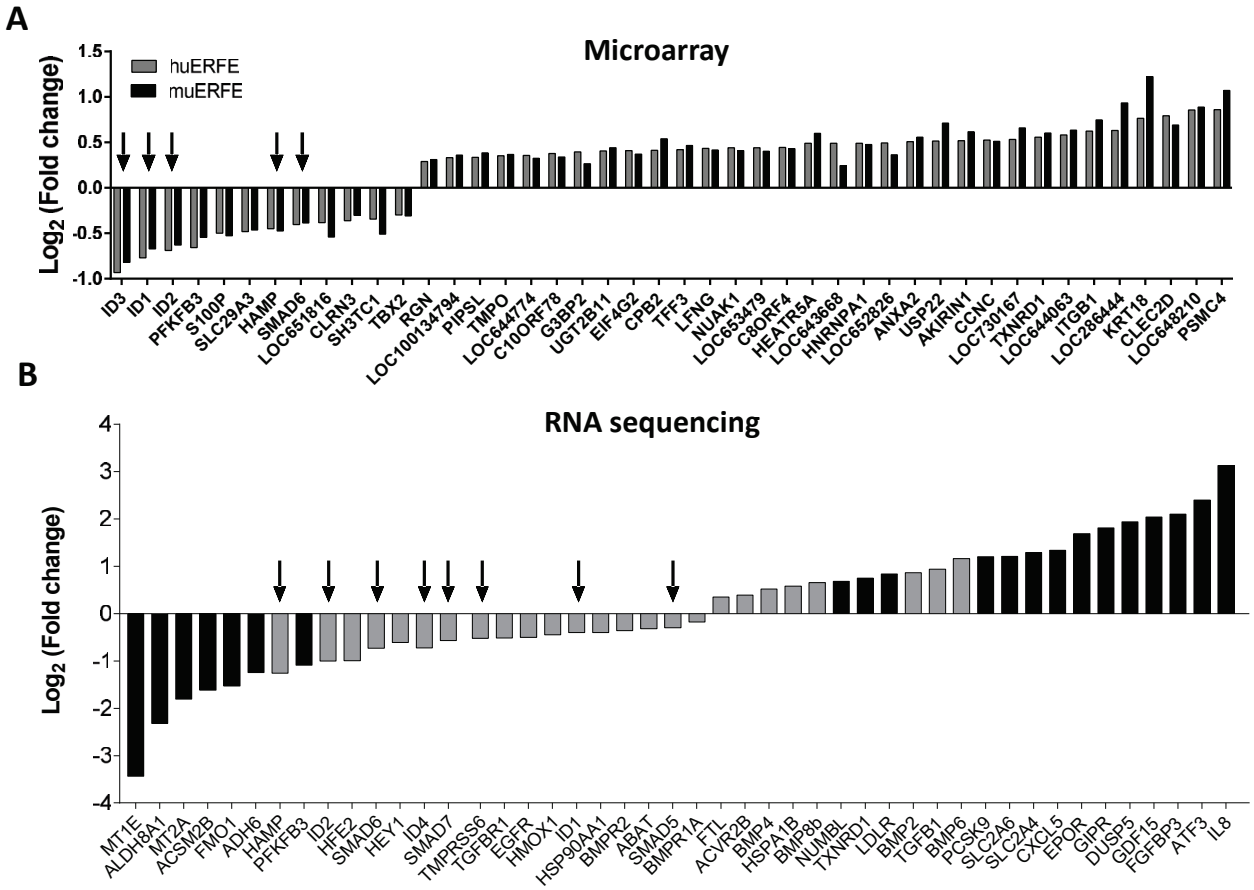


**Figure S2. BMP/SMAD target genes are suppressed by ERFE. (A)** Gene expression analysis (Illumina) of Huh7 cells treated with human (gray) or mouse (black) ERFE (10µg/ml) for 24h. Values represent Log(fold change) of genes differentially expressed in cells treated with human or mouse ERFE. **(B)** RNA sequencing of Huh7 cells treated with human or mouse ERFE (10µg/ml) for 24h. Represented are a list of 46 differentially expressed genes (of a total of 691) selected for the their involvement in BMP/SMAD signalling or iron metabolism (grey), and genes differentially expressed with a high significance ($p<10^{-20}$, black). Values represent Log(fold change). Arrows in both panels indicate BMP/SMAD-target genes.

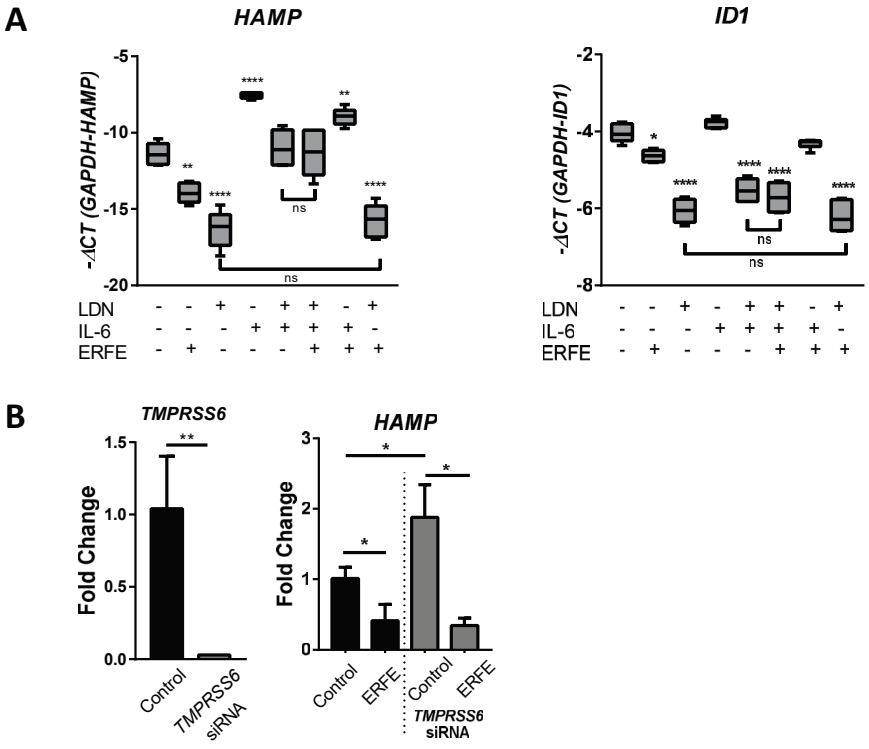


**Figure S3. ERFE-mediated hepcidin suppression is independent of IL-6/JAK-STAT pathway and TMPRSS6.** **(A)** Huh7 cells treated with mouse ERFE (10µg/ml), IL-6 (100ng/ml) and LDN (18nM) in serum-free media, alone or in combination, for 6h, for analysis of *HAMP* and *ID1* expression by qRT-PCR. Stars represent statistical significance relative to non-treated cells. **(B)** HepG2 cells were transfected with 20nM of scramble (control) or TMPRSS6 siRNA. After 24h cells were treated with vehicle or mouse ERFE (10µg/ml) and gene expression of *Tmprss6* and *Hamp* was measured by qRT-PCR 24h after treatment. Values represent mean +/- standard deviation. (*p <0.05, **p <0.01, ***p<0.001, ****p <0.0001, Student's t test, n=3 independent experiments).

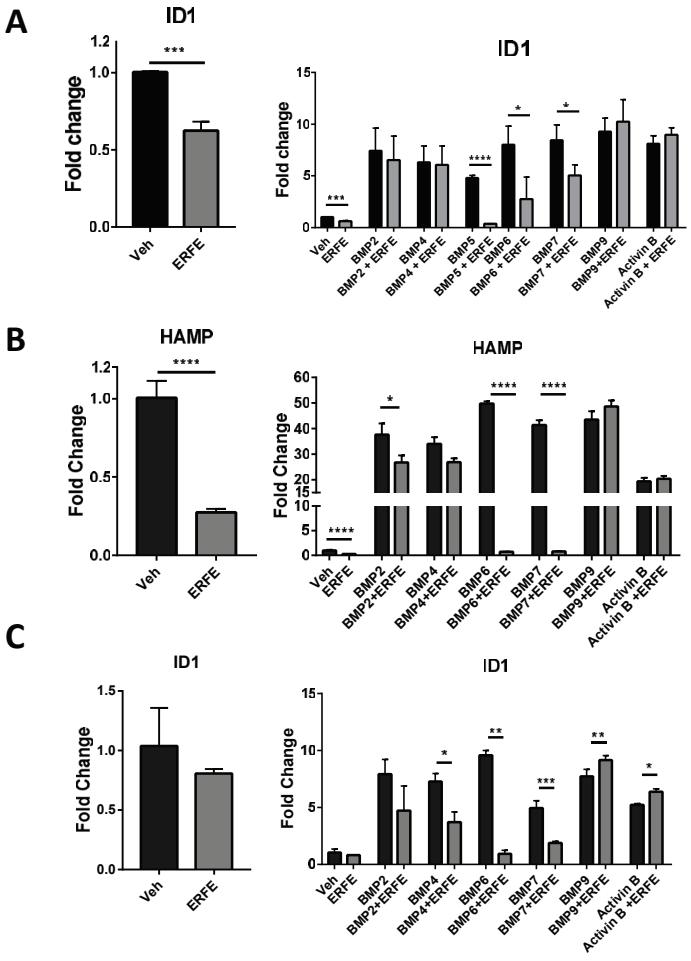


**Figure S4. ERFE suppresses BMP/SMAD signalling by inhibiting BMP5, BMP6 and BMP7.** Huh7 **(A)** and HepG2 **(B-C)** cells were treated with 2nM of BMPs, alone or in combination with 10 µg/ml of mouse ERFE, in serum-free media, and analysed 6h after treatment. Gene expression of *HAMP* and *ID1* was measured by qRT-PCR. Results expressed as fold change relative to non-treated cells from 3 independent experiments. Statistical significance was analysed for each pair of BMP treatments (*p <0.05, **p <0.01, ***p<0.001, ****p <0.0001, Student's t test).

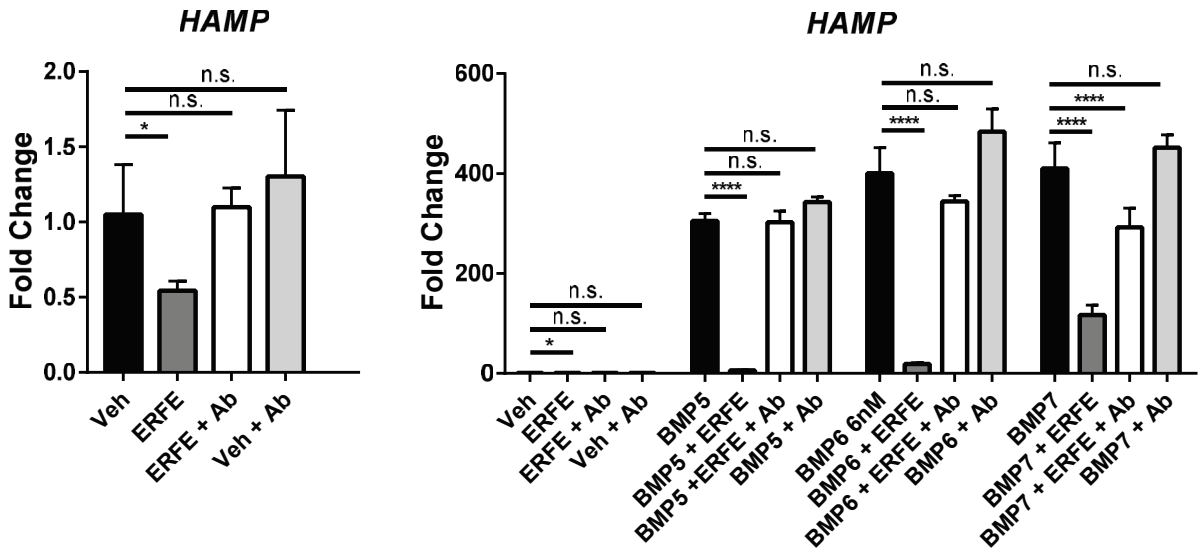


**Figure S5. Anti-ERFE antibody neutralizes ERFE activity.** Huh7 cells were treated with 10µg/ml of anti-ERFE antibody ER-0033 (Ab), 6nM of BMPs and 1 µg/ml of mouse ERFE, alone or in combination, in serum-free media, and analysed 6h after treatment. Gene expression of *HAMP* and was measured by qRT-PCR. Results expressed as mean +/- standard deviation fold change relative to non-treated cells. Statistical significance was analysed for the indicated columns (*p <0.05, ****p <0.0001, n.s.= non significant; Student's t test).

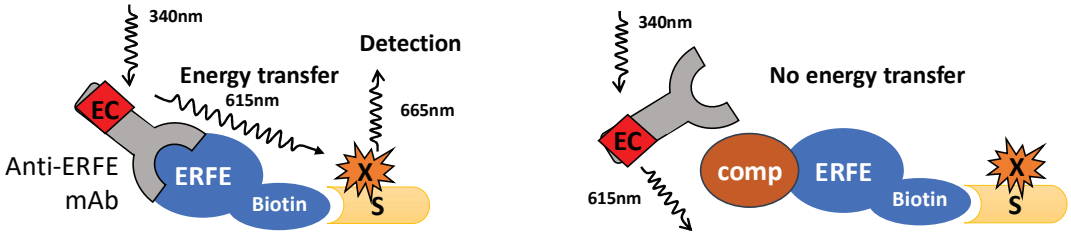


**Figure S6: Homogeneous Time Resolved Fluorescence (HTRF) to test binding of BMPs to ERFE.**
**Left panel:** monoclonal anti-ERFE antibodies labelled with Europeum Cryptate (EC) – excitation at 340nm, emission at 615nm - bind biotin–labelled ERFE, which reacts to streptavidin (S) labelled with XL665 (X) – excitation at 615nm, emission at 665nm. Binding of ERFE to antiERFE antibody promotes energy transfer from cryptate to XL665, increasing fluorescence signal at 665nm.
**Right panel:** Competition for ERFE binding by unlabelled proteins (comp: BMP4, 5, 6, 7 or unlabelled antiERFE antibody were tested) can disrupt binding of labelled-antibodies to ERFE and prevents energy transfer, thus decreasing detection of fluorescence at 665nm.