

Erythroferrone inhibits the induction of hepcidin by BMP6

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Key points:

- Erythroferrone suppresses BMP/SMAD signalling in vitro and in vivo
- Erythroferrone inhibits hepcidin induction by BMP5, BMP6 and BMP7

Abstract

Decreased hepcidin mobilizes iron, which facilitates erythropoiesis, but excess iron is pathogenic in beta-thalassemia. Erythropoietin (EPO) enhances erythroferrone (ERFE) synthesis by erythroblasts, and ERFE suppresses hepatic hepcidin production, through an unknown mechanism. The BMP/SMAD pathway in the liver is critical for control of hepcidin, and we show that EPO suppressed hepcidin and other BMP target genes *in vivo* in a partially ERFE-dependent manner. Furthermore, recombinant ERFE suppressed the hepatic BMP/SMAD pathway independently of changes in serum and liver iron, and *in vitro*, ERFE decreased SMAD 1/5/8 phosphorylation and inhibited expression of BMP target genes. ERFE specifically abrogated the induction of hepcidin by BMP5, BMP6 and BMP7, but had no or little effect on hepcidin induction by BMP2, 4, 9 or Activin B. A neutralising anti-ERFE antibody prevented the ability of ERFE to inhibit hepcidin induction by BMP5, BMP6 and BMP7. Cell-free HTRF assays showed that BMP5, BMP6 and BMP7 competed with anti-ERFE for binding to ERFE. We conclude that ERFE suppresses hepcidin by inhibiting hepatic BMP/SMAD signalling via preferentially impairing an evolutionarily closely related BMP sub-group of BMP5, BMP6 and BMP7. ERFE can act as a natural ligand trap generated by stimulated erythropoiesis in order to regulate availability of iron.

Introduction

Iron absorption is tightly regulated by erythropoietic demand via control of hepcidin expression¹. Hepcidin inhibits the cellular iron exporter ferroportin^{2,3}, reducing iron recycling through splenic macrophages and uptake of dietary iron through enterocytes. When iron is required, following acute blood loss or due to hypoxia, hepcidin is suppressed to allow iron mobilization for increased erythropoiesis⁴. Erythropoietin (EPO) causes hepcidin suppression⁵⁻⁷, at least in part by increasing synthesis of the hormone erythroferrone (ERFE)⁸. ERFE is produced by erythroblasts after bleeding or EPO treatment, and acts on hepatocytes to suppress hepcidin expression. ErfeKO mice fail to

suppress hepcidin after phlebotomy and show delayed recovery from blood loss⁸. Furthermore, serum ERFE concentrations are increased in humans after blood loss and EPO administration, and in β -thalassemia patients⁹. Hepcidin expression is modulated via the BMP/SMAD signalling pathway¹⁰⁻¹²: BMPs bind to BMP receptors on hepatocyte cell membranes, which phosphorylate cytosolic SMADs (SMAD1/5/8) that translocate to the nucleus complexed with SMAD4 to activate the transcription of target genes, including hepcidin (*HAMP*)¹¹. Here we demonstrate that ERFE directly inhibits the induction of hepcidin expression by BMP5, BMP6 and BMP7.

Methods

(Supplementary Information contains detailed methods).

Animal studies Experiments were performed in 9-13 week old Wild-type and ErfeKO males. EPO: intraperitoneal injection of 200IU recombinant human EPO or vehicle daily for three consecutive days, analysis 24h after the last injections. ERFE: intravenous injection of 200 μ g of recombinant mouse ERFE or vehicle, analysis 3h after treatment. Blood parameters were quantified using a hematology analyser. Serum and non-heme liver iron were quantified as previously described¹³.

Cell treatments Huh7 and HepG2 were cultured in DMEM–High Glucose, 10% FBS, 1% Penicillin-Streptomycin and 1% L-Glutamine, unless otherwise indicated. Cells were plated 24h before treatments and treated for 30 minutes, 6 or 24 hours.

RNA isolation, cDNA synthesis and qRT-PCR RNA was isolated using Qiagen's RNeasy Plus kit. cDNA was synthesized using the High Capacity RNA-to-cDNA kit. qRT-PCR was undertaken using Taqman Gene Expression Master Mix and Expression Assays (Supplementary Table 1).

Microarray RNA was converted into biotin labelled cRNA for hybridization and analysed using the Human HT12v4.0 Expression Beadchip (Illumina) and the Illumina's iScan Scanner. Raw data were normalised using the lumi package and compared using LIMMA (Bioconductor).

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

Western Blot Western Blot was performed as described¹⁴. Antibodies used: anti-P-SMAD1/5/9, anti-SMAD1, anti- β -actin-peroxidase and anti-rabbit-IgG HRP-conjugated.

Luciferase assay C2C12 BRE-Luc cells were obtained and cultured as described¹⁵. Cells were treated with BMP (2nM) alone or in combination with mouse ERFE (7.5pM-0.5μM) in 1% FBS. Luminescence was measured 24h after treatment using the britelite Plus Reporter Gene Assay System.

Homogeneous Time Resolved Fluorescence Reaction mix contained 15 nM biotinylated murine ERFE, SA-XL665, europium cryptate-labeled antibody, and BMPs (0.1-200nM), incubated for 3h at room temperature and read on the EnVision Multilabel Plate Reader (excitation: 340 nm; emission 615 nm and 665 nm).

Statistical analysis

Statistical significance was assessed by Student's t-test or one-way ANOVA with Tukey test using Prism 6 GraphPad.

Results and discussion

Mice challenged with erythropoietin or Erfe downregulate hepatic BMP-target genes

First we tested the effect of EPO treatment in wild-type (WT) and ErfeKO mice. WT mice displayed enhanced erythropoiesis, with a blunted or no effect in ErfeKO mice (Figure S1). WT and ErfeKO mice had similar hepcidin expression at baseline as previously reported⁸. EPO strongly suppressed hepatic *Hamp* and other Bmp-target genes - *Id1*, *Id2*, *Atoh8* and *Smad7* – indicating decreased BMP-signalling activity (Figure 1A). In ErfeKO mice, suppression of *Hamp*, *Id2* and *Atoh8* was blunted or prevented, suggesting that the effect of EPO on Bmp signalling is partially Erfe-dependent. The Erfe-independent decrease in hepatic Bmp-signalling may be explained by increased iron consumption by EPO-stimulated erythroblasts, leading to decreased transferrin saturation (Figure 1B). To dissociate between the effects of Erfe and iron on hepatic BMP signalling we injected recombinant murine Erfe protein into WT mice and performed analysis 3h later, at which timepoint serum and liver iron were unchanged (Figure 1C). Erfe suppressed hepatic *Hamp* and BMP-target genes, without altering *Bmp6* and *Bmp2* mRNA levels (Figure 1D). A previous study showed that peak Erfe expression was observed 4h after phlebotomy or EPO treatment, followed by a decrease in *Id1* between 4h and 9h⁸, consistent with our data.

Inhibition of hepcidin by Erfe requires BMP signalling but not TMPRSS6

Microarray and RNAseq analysis of human hepatoma cells treated with Erfe for 24h revealed suppression of several BMP/SMAD target genes (Figure 2A and S2), four of which were detected in both assays - *ID1*, *ID2*, *SMAD6* and *HAMP* (confirmed by qRT-PCR) (Figure 2B). The decreased

expression of SMAD6 *in vitro* and SMAD7 *in vivo* (Figure 1A) indicate that suppression of BMP target genes by Erfe is not caused via upregulation of inhibitory SMADs. A recent study reported that conditional ablation of *Smad1* and *Smad5* in hepatocytes does not further suppress *Hamp* after EPO injection, suggesting that BMP/SMAD signalling is required for ERFE activity¹⁶. We found that Erfe in combination with LDN-193189 (a potent inhibitor of BMP signalling¹⁷) did not further suppress *HAMP* or *ID1* compared to LDN-193189 alone, at baseline or upon *HAMP* stimulation by IL-6 (Figure S3A), consistent with a requirement for intact BMP signalling for Erfe function. There are conflicting data regarding the involvement of Matriptase-2 in Erfe-mediated *HAMP* suppression^{18,19}. We found that after silencing TMPRSS6 in Huh7 cells, Erfe still suppressed *HAMP* to the same levels as wild-type, suggesting Matriptase-2 is not required for *HAMP* suppression by Erfe (Figure S3B).

Erfe inhibits hepcidin induction by BMP5, BMP6 and BMP7

Erfe caused a decrease in SMAD 1/5/8 phosphorylation relative to non-Erfe-treated cells (Figure 2C), both at baseline and after BMP6 stimulation. Because *HAMP* can be stimulated by a variety of ligands¹¹, we tested the effect of Erfe on cells treated with various BMPs using C2C12 Bre-Luc cells. Increased concentrations of Erfe dose-dependently decreased activation of BMP signalling by BMP5, BMP6 and BMP7 (IC50: 15nM, 25nM and 22nM, respectively), but had no effect on Bre-Luc activity stimulated by BMP2, BMP4 and BMP9 (Figure 2D). Similarly, using hepatoma cells, Erfe strongly suppressed endogenous *HAMP* and *ID1* induction by BMP5, BMP6 and BMP7, with no or minor effects on BMP2, BMP4, BMP9 or Activin B (Figures 2E and S4). Next, by immunizing ErfeKO mice with recombinant ERFE, we generated an anti-ERFE monoclonal antibody that neutralised the ability of Erfe to inhibit hepcidin induction by BMP5, BMP6 and BMP7, but did not influence the hepcidin-stimulating activity of the BMPs in the absence of Erfe (Figure S5). Using this antibody and recombinant BMPs, we then performed Homogeneous Time Resolved Fluorescence (HTRF) competition assays (Figure S6). Unlabelled anti-ERFE efficiently competed with labelled anti-ERFE to bind Erfe as expected. BMP5, BMP6 and BMP7 (but not BMP4) dose-dependently prevented the labelled anti-ERFE from binding Erfe, suggesting that the neutralising antibody functions by disrupting Erfe's ability to bind these BMPs (Figure 2F). Taken together, these data demonstrate that Erfe preferentially binds and inhibits members of the BMP5/6/7 subgroup of BMPs, leading to decreased hepcidin expression. In mice, BMP2 and BMP6 are both required for appropriate hepcidin expression^{12,20}. The relative inability to inhibit BMP2 indicates that ERFE may not fully suppress hepcidin. Our findings have important consequences for understanding hepcidin regulation by erythropoiesis, and for potential therapies aimed at manipulating hepcidin expression.

Acknowledgements

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Author contributions

JA, NF, KM, AEA, SRP, OC, ML, SJD, RJ and HD designed research; JA, NF, KM, AS, DQ, VT, AB, MT, EL, AEA and SRP performed research; JA, NF, KM, AS, VT, AS, AEA and SRP collected data; JA, NF, KM, AS, VT, ST, AEA, SRP, OC, ML, SJD, RJ and HD analyzed and interpreted data; JA, AS and ST, performed statistical analysis; JA, NF, KM, SJD, RJ and HD wrote the manuscript

Conflict of Interest Disclosure

This work was supported in part by funding from Pfizer to JA, KM, DQ, SJD and HD. NF, AS, VT, AB, MT, EL, AS, OC, ML and RJ are employed by Pfizer. NF, OC, RJ, JA, KM, SJD and HD are named inventors on a patent application under evaluation. AEA, ST and SRP declare no conflict of interest.

References

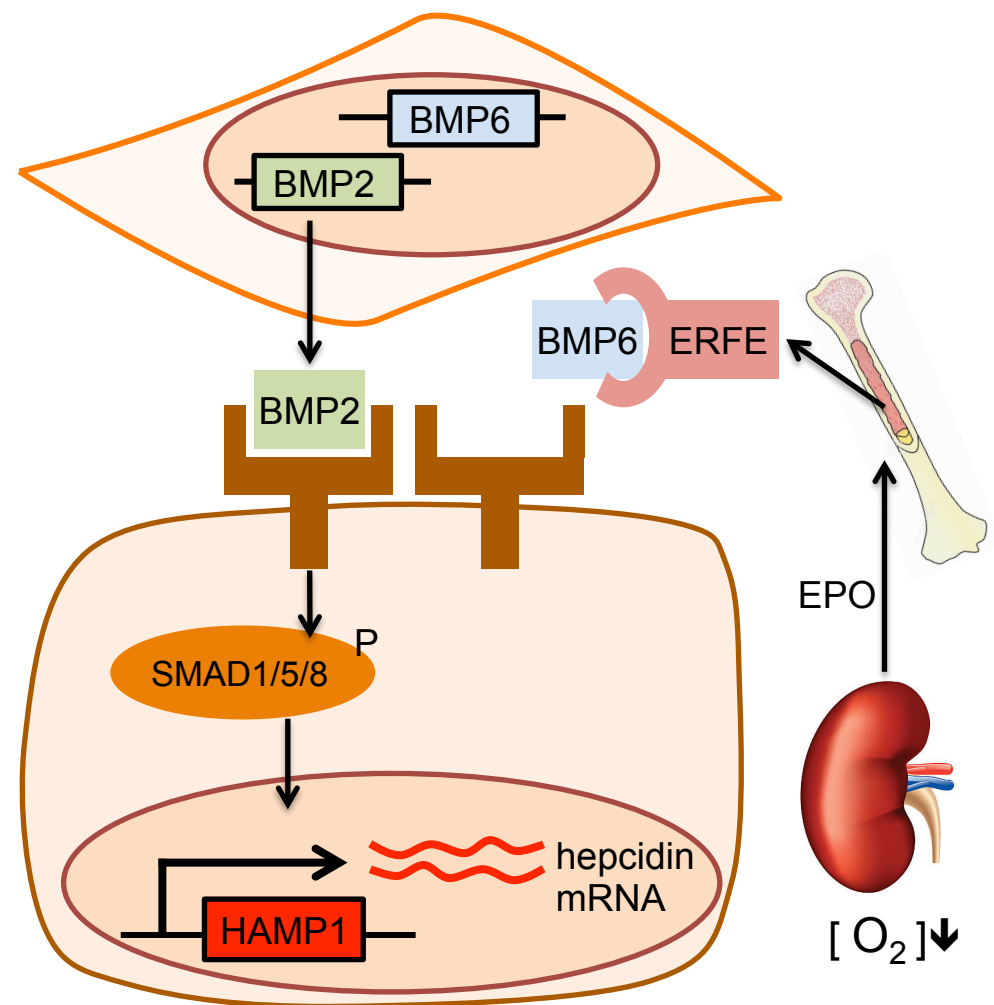
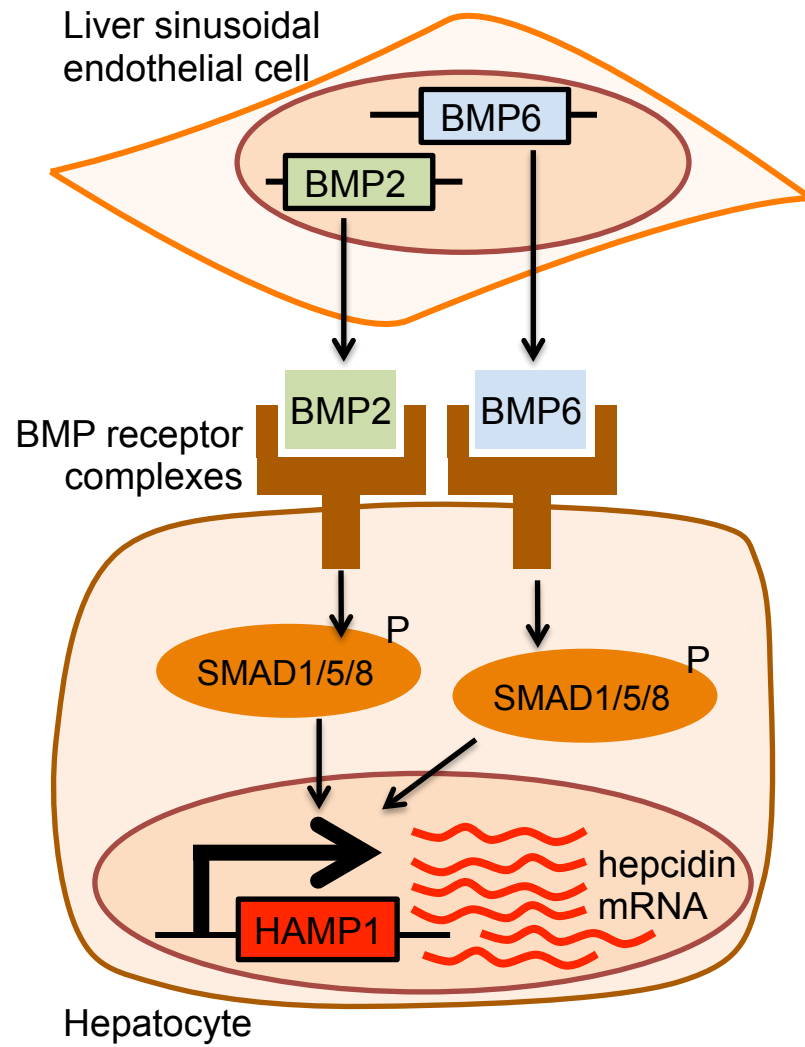
1. Ganz T. Hepcidin and iron regulation, 10 years later. *Blood*. 2011;117(17):4425-4433.
2. Nemeth E, Tuttle MS, Powelson J, et al. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science*. 2004;306(5704):2090-2093.
3. Aschemeyer S, Qiao B, Stefanova D, et al. Structure-function analysis of ferroportin defines the binding site and an alternative mechanism of action of hepcidin. *Blood*. 2017.
4. Gardenghi S, Grady RW, Rivella S. Anemia, ineffective erythropoiesis, and hepcidin: interacting factors in abnormal iron metabolism leading to iron overload in beta-thalassemia. *Hematol Oncol Clin North Am*. 2010;24(6):1089-1107.
5. Pak M, Lopez MA, Gabayan V, Ganz T, Rivera S. Suppression of hepcidin during anemia requires erythropoietic activity. *Blood*. 2006;108(12):3730-3735.
6. Vokurka M, Krijt J, Sulc K, Necas E. Hepcidin mRNA levels in mouse liver respond to inhibition of erythropoiesis. *Physiol Res*. 2006;55(6):667-674.
7. Ashby DR, Gale DP, Busbridge M, et al. Erythropoietin administration in humans causes a marked and prolonged reduction in circulating hepcidin. *Haematologica*. 2010;95(3):505-508.
8. Kautz L, Jung G, Valore EV, Rivella S, Nemeth E, Ganz T. Identification of erythroferrone as an erythroid regulator of iron metabolism. *Nat Genet*. 2014;46(7):678-684.
9. Ganz T, Jung G, Naeim A, et al. Immunoassay for human serum erythroferrone. *Blood*. 2017;130(10):1243-1246.
10. Yadin D, Knaus P, Mueller TD. Structural insights into BMP receptors: Specificity, activation and inhibition. *Cytokine Growth Factor Rev*. 2016;27:13-34.

11. Andriopoulos B, Jr., Corradini E, Xia Y, et al. BMP6 is a key endogenous regulator of hepcidin expression and iron metabolism. *Nat Genet.* 2009;41(4):482-487.
12. Meynard D, Kautz L, Darnaud V, Canonne-Hergaux F, Coppin H, Roth MP. Lack of the bone morphogenetic protein BMP6 induces massive iron overload. *Nat Genet.* 2009;41(4):478-481.
13. Armitage AE, Lim PJ, Frost JN, et al. Induced Disruption of the Iron-Regulatory Hormone Hepcidin Inhibits Acute Inflammatory Hypoferraemia. *J Innate Immun.* 2016;8(5):517-528.
14. Pasricha SR, Lim PJ, Duarte TL, et al. Hepcidin is regulated by promoter-associated histone acetylation and HDAC3. *Nat Commun.* 2017;8(1):403.
15. Korchynskyi O, ten Dijke P. Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter. *J Biol Chem.* 2002;277(7):4883-4891.
16. Wang CY, Core AB, Canali S, et al. Smad1/5 is required for erythropoietin-mediated suppression of hepcidin in mice. *Blood.* 2017;130(1):73-83.
17. Cuny GD, Yu PB, Laha JK, et al. Structure-activity relationship study of bone morphogenetic protein (BMP) signaling inhibitors. *Bioorg Med Chem Lett.* 2008;18(15):4388-4392.
18. Nai A, Rubio A, Campanella A, et al. Limiting hepatic Bmp-Smad signaling by matriptase-2 is required for erythropoietin-mediated hepcidin suppression in mice. *Blood.* 2016;127(19):2327-2336.
19. Aschemeyer S, Gabayan V, Ganz T, Nemeth E, Kautz L. Erythroferrone and matriptase-2 independently regulate hepcidin expression. *Am J Hematol.* 2017.
20. Canali S, Wang CY, Zumbrennen-Bullough KB, Bayer A, Babitt JL. Bone morphogenetic protein 2 controls iron homeostasis in mice independent of Bmp6. *Am J Hematol.* 2017;92(11):1204-1213.

Figure legends

Figure 1. EPO suppresses BMP-target genes in an ERFE-dependent manner. (A,B) WT and ErfeKO 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 to measure expression of BMP-target genes in the liver (A), serum and liver iron (B). **(C,D)** Nine weeks old WT male mice were injected i.v. with 200µg of murine Erfe or a clipped (inactive) version of the protein as a control (Ctrl). Mice were analysed 3h after the injections to measure serum and liver iron (C) and expression of BMP-target genes, *Bmp2* and *Bmp6* in the liver (D). Bars represent mean +/- standard deviation (*p <0.05, **p <0.01, ***p<0.001, ****p <0.0001, using one-way ANOVA followed by Tukey test for multiple comparisons (A, B) or Student's t test (C, D), n=6-8 mice per group).

Figure 2. ERFE suppresses BMP/SMAD signalling by inhibiting BMP5, BMP6 and BMP7. (A) Venn diagram representing the number of genes differentially expressed genes by Huh7 cells treated with murine Erfe (10µg/ml) compared to vehicle-treated cells, and analysed by Illumina microarray or RNA sequencing. **(B)** Gene expression measured by qRT-PCR of the common four differentially expressed genes in Huh7 cells treated with vehicle or murine Erfe (10µg/ml). **(C)** Huh7 cells treated with mouse ERFE (10µg/ml), BMP6 (6nM or 18nM) and LDN (100nM), alone or in combination, for 30min. pSMAD/SMAD ratios values were calculated by densitometry. **(D)** C2C12 Bre-Luc cells were treated with 2nM of BMP in combination with a gradient of mouse ERFE concentrations (7.5pM to 0.5 µM) for 24h, and luminescence measured in each well. Data was normalized to percentage of maximum luminescence (no ERFE). **(E)** Huh7 cells 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 for HAMP gene expression by qRT-PCR. **(F)** Homogeneous Time Resolved Fluorescence (HTRF) assay for detection of binding between ERFE and BMP, using cryptate-labelled antiERFE antibody and BMPs (0.1 – 200nM), and unlabelled antibody as positive control. Values calculated as $\% \Delta F = [(F665 \text{ Sample}/F615 \text{ Sample}) - (F665 \text{ Control}/F615 \text{ Control})] / (F665 \text{ Control}/F615 \text{ Control}) \times 100$, in which control is the background fluorescence energy transfer in wells containing labelled antibody alone. Results B-F represented as mean +/- standard deviation from three independent experiments (*p <0.05, **p <0.01, ***p<0.001, ****p <0.0001, Student's t test).



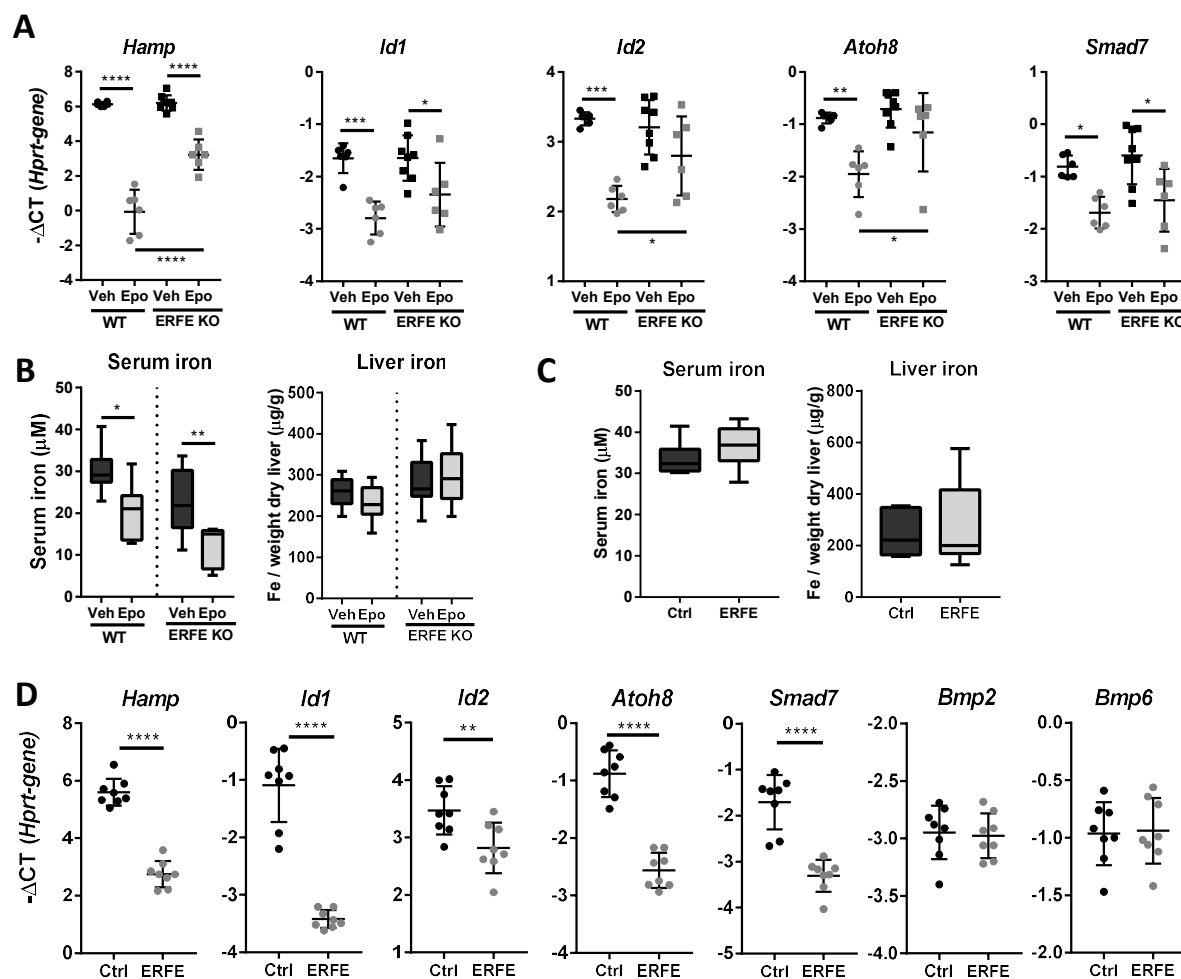


Figure 1. EPO suppresses BMP-target genes in an ERFE-dependent manner. (A,B) WT and ERFE KO male mice (10-13 weeks old) were injected with 3 doses of 200 iu of EPO or vehicle (Veh), one dose every 24h, and analysed 24h after the last injection to measure expression of BMP-target genes in the liver (A), serum and liver iron (B). (C,D) Nine weeks old WT male mice were injected i.v. with 200 μg of murine ERFE or a clipped version of the protein as a control (Ctrl). Mice were analysed 3h after the injections to measure serum and liver iron (C) and expression of BMP-target genes, *Bmp2* and *Bmp6* in the liver (D). Bars represent mean \pm standard deviation (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, using one-way ANOVA followed by Tukey test for multiple comparisons (A, B) or Student's t test (C, D), $n = 6-8$ mice per group).

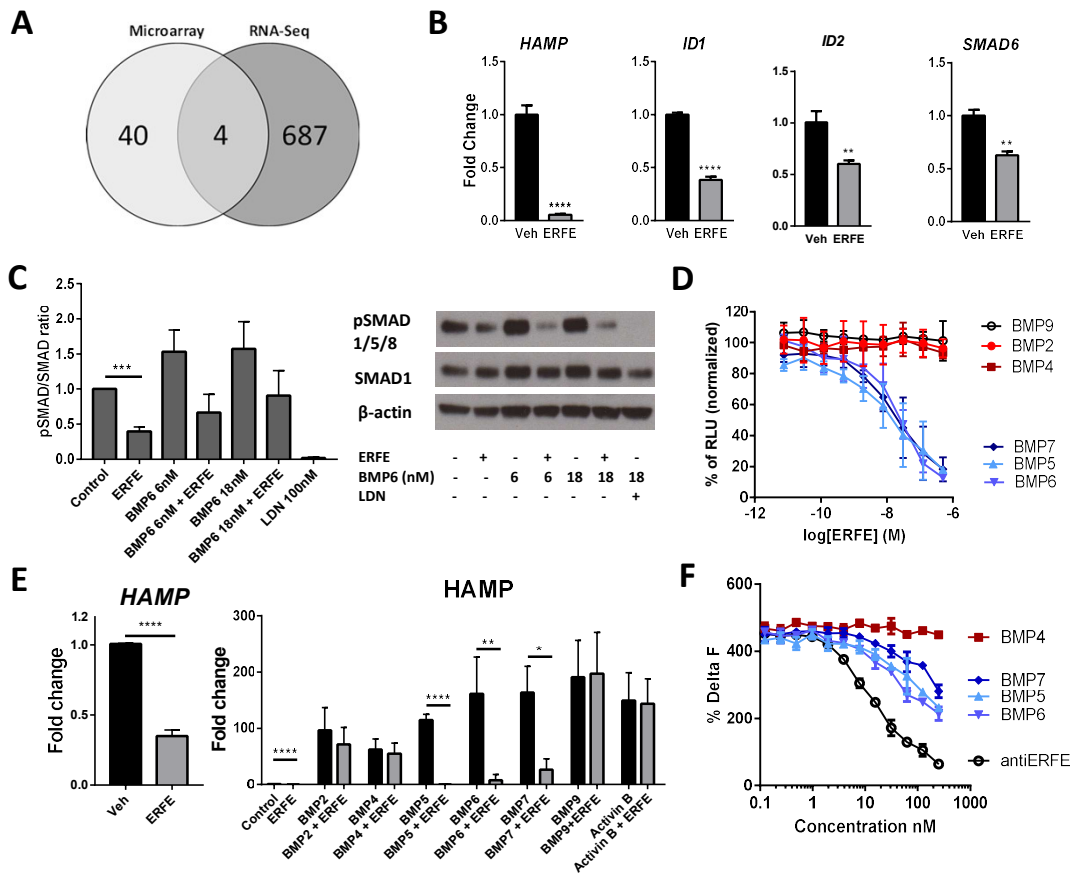


Figure 2. ERFE suppresses BMP/SMAD signalling by inhibiting BMP5, BMP6 and BMP7. (A) Venn diagram representing the number of differentially expressed genes in Huh7 cells treated with murine ERFE (10μg/ml) compared to vehicle-treated cells, and analysed by Illumina microarray or RNA sequencing. (B) Gene expression measured by qRT-PCR of the common four differentially expressed genes in Huh7 cells treated with vehicle or murine ERFE (10μg/ml). (C) Huh7 cells treated with mouse ERFE (10μg/ml), BMP6 (6nM or 18nM) and LDN (100nM), alone or in combination, for 30min. pSMAD/SMAD ratios values were calculated by densitometry. (D) C2C12 Bre-Luc cells were treated with 2nM of BMP in combination with a gradient of mouse ERFE concentrations (7.5pM to 0.5 μM) for 24h, and luminescence measured in each well. Data was normalized to percentage of maximum luminescence (no ERFE). (E) Huh7 cells 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 for *HAMP* gene expression by qRT-PCR. (F) Homogeneous Time Resolved Fluorescence (HTRF) assay for detection of binding between ERFE and BMP, using cryptate-labelled antiERFE antibody and BMPs (0.1 – 200nM), and unlabelled antibody as positive control. Values calculated as $\% \Delta F = \frac{(F665 \text{ Sample} / F615 \text{ Sample}) - (F665 \text{ Control} / F615 \text{ Control})}{(F665 \text{ Control} / F615 \text{ Control})} \times 100$, in which control is the background fluorescence energy transfer in wells containing labelled antibody alone. Results B-F represented as mean \pm standard deviation from three independent experiments (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, Student's t test).

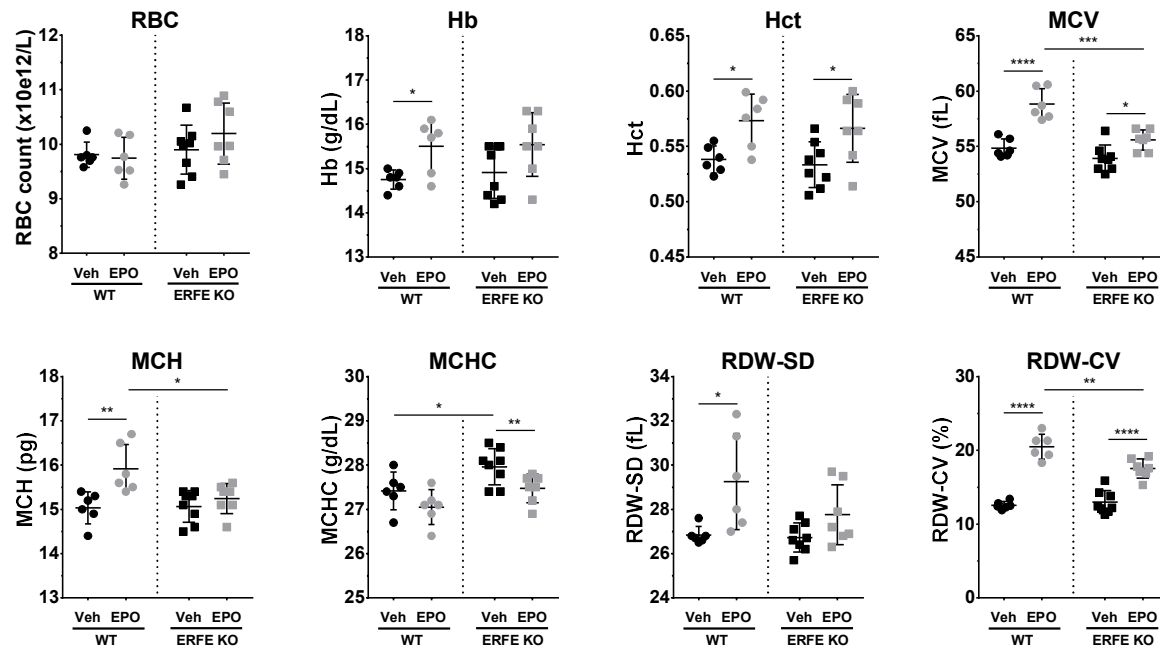


Figure S1. Blood parameters in EPO-treated mice. WT and ERFE KO male mice (10-13 weeks old) were injected with 3 doses of 200 iu of EPO or vehicle (Veh), one dose every 24h, and analysed 24h after the last injection. Blood parameters were measured using a Sysmex analyser (one-way ANOVA followed by Tukey 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.

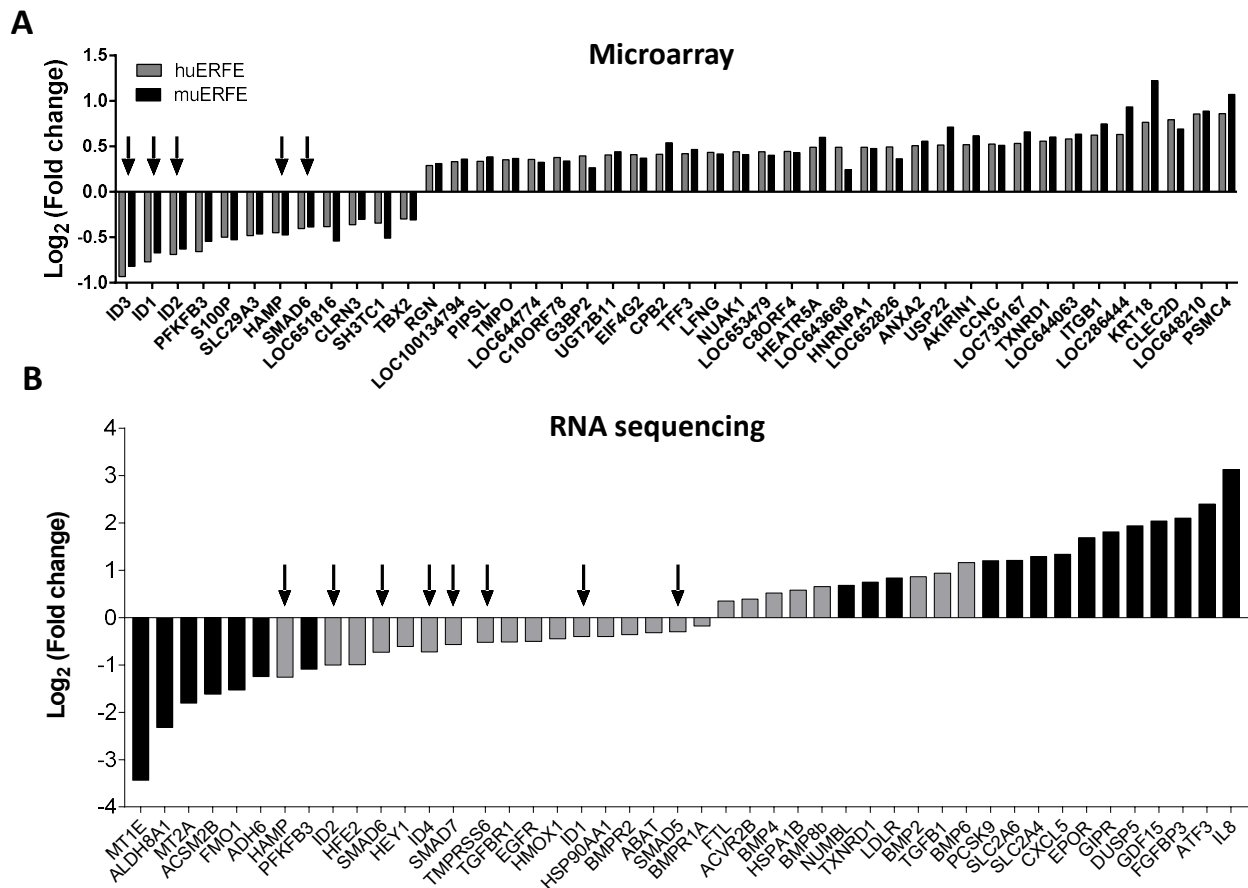


Figure S2. BMP/SMAD target genes are suppressed by ERFE. (A) Gene expression analysis (Illumina) of Huh7 cells treated with human (gray) or mouse (black) ERFE (10 μ g/ml) for 24h. Values represent Log(fold change) of genes differentially expressed in cells treated with human or mouse ERFE. **(B)** RNA sequencing of Huh7 cells treated with human or mouse ERFE (10 μ g/ml) for 24h. Represented are a list of 46 differentially expressed genes (of a total of 691) selected for 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.

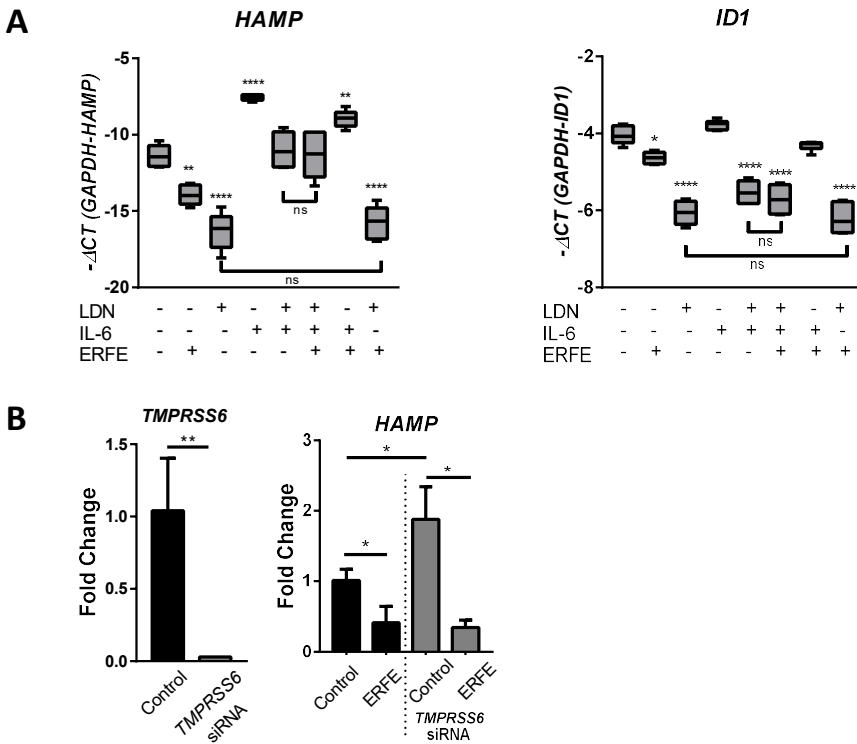


Figure S3. ERFE-mediated hepcidin suppression is independent of IL-6/JAK-STAT pathway and TMPRSS6. **(A)** Huh7 cells treated with mouse ERFE (10μg/ml), IL-6 (100ng/ml) and LDN (18nM) in serum-free media, alone or in combination, for 6h, for analysis of *HAMP* and *ID1* expression by qRT-PCR. Stars represent 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).

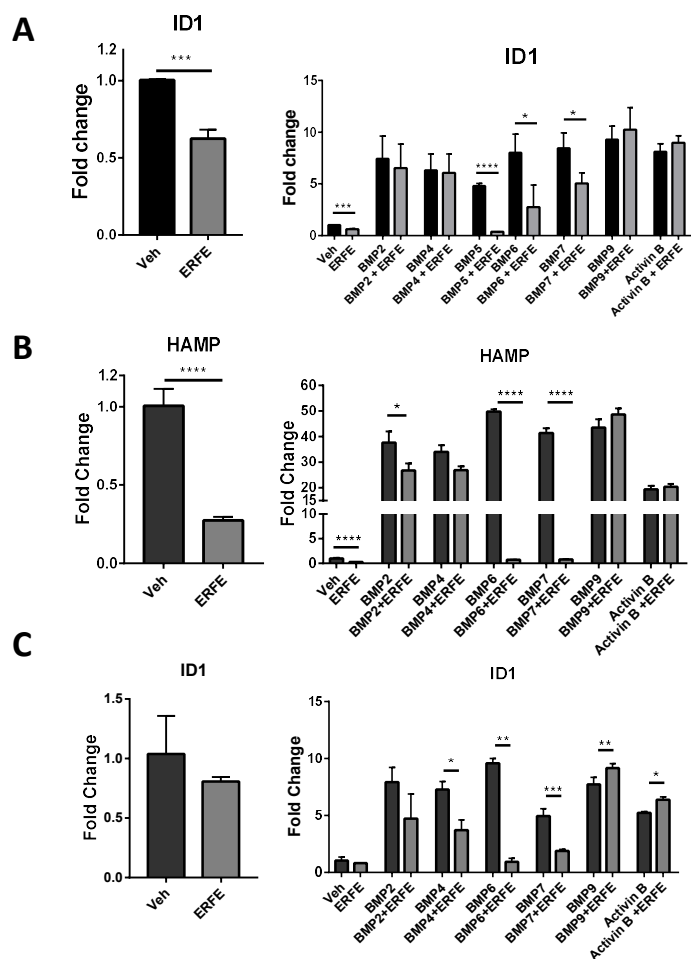


Figure S4. ERFE suppresses BMP/SMAD signalling by inhibiting BMP5, BMP6 and BMP7. Huh7 (A) and HepG2 (B-C) cells were treated with 2nM of BMPs, alone or in combination with 10 μ g/ml of mouse ERFE, in serum-free media, and analysed 6h after treatment. Gene expression of *HAMP* and *ID1* was measured 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).

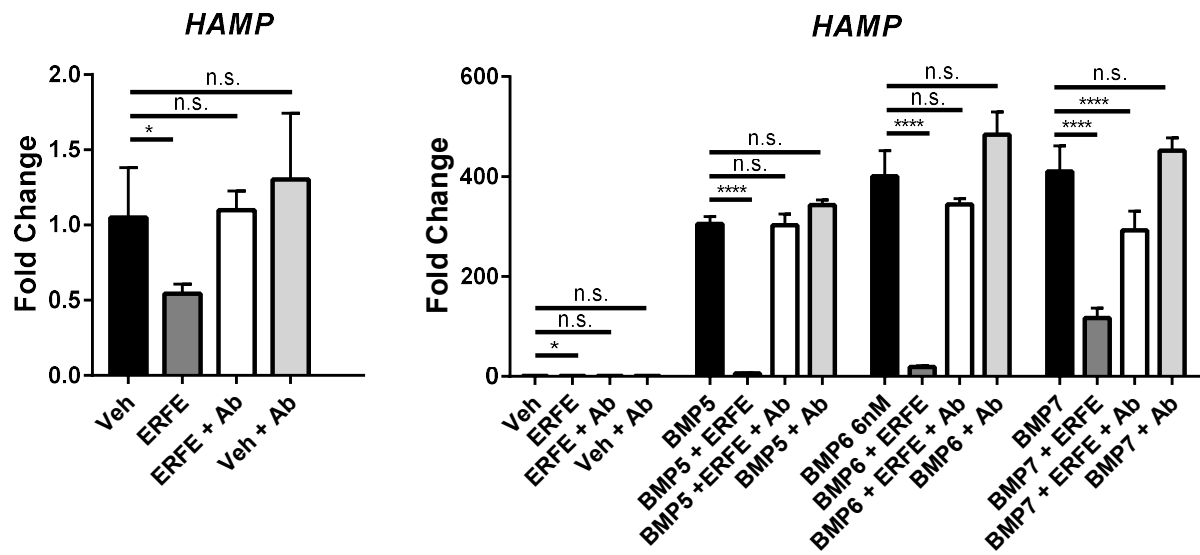


Figure S5. Anti-ERFE antibody neutralizes ERFE activity. Huh7 cells were treated with 10µg/ml of anti-ERFE antibody ER-0033 (Ab), 6nM of BMPs and 1 µg/ml of mouse ERFE, alone or in combination, in serum-free media, and analysed 6h after treatment. Gene expression of *HAMP* was measured by qRT-PCR. Results 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).

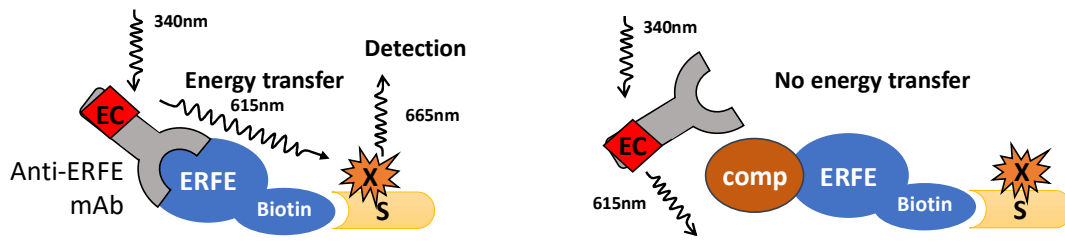


Figure S6: Homogeneous Time Resolved Fluorescence (HTRF) to test binding of BMPs to ERFE.

Left panel: monoclonal anti-ERFE antibodies labelled with Europium 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.

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 CO₂ 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 µm filtration. ERFE proteins were linked to monoFc²¹ 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 Hilo 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 1µg 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 previously²². 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 1.25×10^6 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 0.2µm 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 Illumina'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% trichloroacetic 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 described²³. 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 Assay 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: $\% \Delta F = [(F_{665} \text{ Sample} / F_{615} \text{ Sample}) - (F_{665} \text{ Control} / F_{615} \text{ Control})] \times 100$. "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	<i>GAPDH</i>	Hs99999905_m1
Hepcidin	Human	<i>HAMP</i>	Hs00221783_m1
Inhibitor of DNA-binding protein 1	Human	<i>ID1</i>	Hs03676575_s1
Inhibitor of DNA-binding protein 2	Human	<i>ID2</i>	Hs00171409_m1
Sons of mothers against decapentaplegic 6	Human	<i>SMAD6</i>	Hs00178579_m1
Matriptase-2	Human	<i>TMPRSS6</i>	Hs00542184_m1
Hypoxanthine-guanine phosphoribosyltransferase	Mouse	<i>Hprt1</i>	Mm01545399_m1
Hepcidin	Mouse	<i>Hamp1</i>	Mm04231240_s1
Inhibitor of DNA-binding protein 1	Mouse	<i>Id1</i>	Mm00775963_g1
Inhibitor of DNA-binding protein 2	Mouse	<i>Id2</i>	Mm00711781_m1
Atonal BHLH Transcription Factor 8	Mouse	<i>Atoh8</i>	Mm00464055_m1
Sons of mothers against decapentaplegic 7	Mouse	<i>Smad7</i>	Mm00484742_m1
Bone morphogenetic protein 2	Mouse	<i>Bmp2</i>	Mm01340178_m1
Bone morphogenetic protein 6	Mouse	<i>Bmp6</i>	Mm01332882_m1

Supplementary Bibliography

21. Ishino T, Wang M, Mosyak L, et al. Engineering a monomeric Fc domain modality by N-glycosylation for the half-life extension of biotherapeutics. *J Biol Chem*. 2013;288(23):16529-16537.
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.
23. Korchynskyi O, ten Dijke P. Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter. *J Biol Chem*. 2002;277(7):4883-4891.
24. Finlay WJ, Cunningham O, Lambert MA, et al. Affinity maturation of a humanized rat antibody for anti-RAGE therapy: comprehensive mutagenesis reveals a high level of mutational plasticity both inside and outside the complementarity-determining regions. *J Mol Biol*. 2009 May 8;388(3):541-58.
25. Degorce F, Card A, Soh S, et al. HTRF: A technology tailored for drug discovery - a review of theoretical aspects and recent applications. *Curr Chem Genomics*. 2009 May 28;3:22-32