SUPPLEMENTAL MATERIALS AND METHODS

Animals

BMP6 deficient mouse study protocol (Figure S1A)

BMP6 deficient mice (*BMP6*$^{-/-}$) and their respective wildtype-counterparts (*BMP6*-Wt) were kept on an iron adequate diet (30mg/kg iron Ssniff, Germany) throughout their whole life. For experiments, 10-30 week old male and female mice were used and either received i.p. on "Day 0" a human IgG4 isotype control antibody (3mg/kg) or KY1070 (3mg/kg). Effects were evaluated after 72h (Day 3).

Healthy mouse study protocol (Figure S1B)

8 – 10 week old male C57BL/6N mice were fed with a standard rodent diet (160mg Fe/kg, Ssniff, Germany) and i.p. injected at "Day 0" with either a human IgG4 isotype antibody (3mg/kg), KY1070 (3mg/kg), Darbepoetin alfa (EPO; 10µg/kg) or combinations of EPO, KY1070 (3mg/kg) and EPO (10µg/kg). After 72h (Day 3), all mice were euthanized, blood and tissues were harvested for further analysis.

Healthy rats study protocol (Figure S1C)

8 – 10 week old female Lewis rats were purchased from Charles River (Sulzfeld, Germany) and fed with a standard rodent diet (160mg Fe/kg, Ssniff, Germany) throughout the whole experiment. On "Day 0" blood was withdrawn for baseline determination of hematological, iron homeostasis parameters and treatment was started. Rats received either a single s.c. dose of a human IgG4 isotype control antibody (3mg/kg), KY1070 (3mg/kg), Darbepoetin alfa (10µg/kg) or KY1070 (3mg/kg) and Darbepoetin alfa (10µg/kg) in combination. For evaluation of the short-term effects of treatment, blood was taken after 72h. Darbepoetin alfa treatment was repeated on Day 7 and Day 14. 7 days after the last dose of Darbepoetin alfa (Day 21) all rats were euthanized and blood and tissues were harvested for further analysis.

ACD rats study protocol (Figure S3A and S4G)

6-8 week old female Lewis rats were purchased from Charles River (Sulzfeld, Germany).
At "Week -2" rats received a single i.p. injection of 15µg/kg Group A Streptococcal Peptidoglycan-Polysaccharide (PG-APS, Lee Laboratories, Grayson, GA). After 2 weeks ("Week 0"), blood was withdrawn for complete blood count (CBC) analysis. Responders (ACD rats) were defined as animals having an increased granulocyte count (>60% of white blood cells) and arthritis (defined as an

increased diameter of the ankle joint of the hind feet). ACD rats were randomly divided into experimental groups and treatment started (i.e. 2 weeks after PG-APS administration). Rats were either injected s.c. with a human IgG4 isotype control antibody (3mg/kg), KY1070 (3mg/kg), Darbepoetin alfa (EPO; 10µg/kg) or KY1070 (3mg/kg) and EPO (EPO; 10µg/kg) and analyzed at the given time points.

CKD mouse study protocol (Figure S5A)

3 week-old male C57BL/6N mice were purchased from Charles River (Sulzfeld, Germany).
Kidney damage was induced by a diet, containing 0.2% adenine, 0.9% phosphate and 30mg/kg iron (referred to as adenine diet). This diet was fed throughout the whole experimental period. Untreated control mice were fed with a diet containing 30mg/kg iron without adenine. After 8 weeks of feeding ("Week 0") CKD animals were randomly allocated into treatment groups receiving either a human IgG4 isotype control antibody (3mg/kg), KY1070 (3mg/kg), Darbepoetin alfa (1µg/kg or 10µg/kg) or different doses of KY1070 (varying from 0.1 – 10 mg/kg) and Darbepoetin alfa (varying from 0.01µg/kg - 10µg/kg) in combination (all given i.p.). Darbepoetin alfa treatment was repeated weekly, isotype control antibody and KY1070 were given every second week, i.e antibodies were administered two times and Darbepoetin alfa four times in the treatment period.
7 days after the last dose of Darbepoetin alfa all mice were euthanized, blood and tissues were harvested for further analysis. The experimental setup is shown in Figure S5A.

ACD rat EPO-sparing protocol (Figure S7)

For this study a protocol was designed, which follows human ESA treatment regimen, where EPO is administered only when Hgb levels fall under a predefined level.
ACD induction and screening for ACD development was performed as described above ('ACD rat study protocol'). Inclusion of ACD rats was based on the same criteria as that at 2 weeks after PG-APS administration. From this time point on, Hgb values were determined weekly in all ACD animals. For Hgb < 13.5g/dl, the therapy-naïve rat was randomly allocated into one of the treatment groups: ACD (3mg/kg isotype control IgG4 s.c), ACD | EPO (10µg/kg, Darbepoetin alfa s.c) or ACD | KY1070 + EPO (3mg/kg KY1070 and 10µg/kg Darbepoetin alfa s.c) (Figure S7B).

For all treated animals, Hgb values were determined weekly. Animals in the ACD and ACD | KY1070 + EPO groups received the isotype antibody or KY1070, respectively, every third week. Darbepoetin alfa administration in the ACD | EPO and ACD | KY1070 + EPO groups was performed weekly depending on the current Hgb value (Hgb < mean Hgb in therapy-naïve non-ACD rats) (Figure S7C).

Iron overload mouse study protocol (Figure S10A)

3 week-old male C57BL/6N mice were purchased from Charles River (Sulzfeld, Germany) and fed with a diet containing 30mg/kg iron throughout the whole experiment. After 8 weeks, mice were i.p. injected at "Day 0" with 20mg iron dextran (Sigma) in combination with either a human IgG4 isotype antibody (3mg/kg), KY1070 (3mg/kg), Darbepoetin alfa (10µg/kg), or KY1070 (3mg/kg) and Darbepoetin alfa (10µg/kg) in combination. Iron was applied every day, the other treatments were repeated on Day 6. After one week (Day 7), all mice were euthanized, blood and tissues harvested for further analysis.

Antibody generation

KY1070 and respective human IgG4 isotype antibody was provided by Kymab Ltd. (Cambridge, UK). Kymouse™ HK BMP6 $^{-/-}$ mice were immunized with recombinant human BMP6 (Peprotech) using a prime/boost regime[1]. Six to seven days after the final boost spleens and lymph nodes were removed. Tissues were disaggregated into single cell suspensions for antigen-driven selection of B-cells using B-cell FACS selection technology. Antibody V-regions of selected B-cell clones were recovered by standard molecular biology techniques, sequenced and recombinantly expressed in suspension CHO cells as fully human IgG4 IgG (carrying mutations S228P and L235E; EU index to prevent human IgG4 half molecule formation and reduce residual Fc mediated effector functions) antibodies for screening and further analysis.

Isotype control antibodies were generated in house (human IgG4 isotype) or were commercially available (mouse IgG2b R&D Systems MAB004).

Assays for KY1070 *in vitro* testing

Surface Plasmon Resonance (SPR)

The binding kinetics for KY1070 to human BMP6 were determined by surface plasmon resonance (SPR) at 37°C at pH 7.6 using Fab fragments and a ProteOn™ XPR36 instrument (BioRad). Briefly, biotinylated recombinant human BMP6 (Peprotech 120-06) was immobilised on a Neutravidin Biosensor NLC chip (Biorad) and binding examined using a single cycle kinetic method run at 5 different Fab concentrations (0.25, 1, 4, 16 and 64 nM) in HBS-EP at pH7.6 (GE Healthcare). Injection containing no Fab was used to double reference the binding sensorgrams. In this experimental set-up the use of monomeric Fab fragments avoids any bivalent interactions with the dimeric BMP6 and therefore avidity has very limited or no contribution to the binding kinetics. The values generated are therefore true KD values.

HepG2 *Hamp* luciferase reporter gene assay

A human hepatic cell line (HepG2) that expresses a luciferase reporter gene under control of the *Hamp* regulatory was generated by amplifying the hamp gene regulatory region (Casanovas et al., 2014) from HepG2 cells and cloning this into a luciferase expression vector (pMCS-Red Firefly Luc; ThermoFisher) by standard molecular biology methods. The entire regulatory region-luciferase cassette was then re-amplified by PCR and subcloned into a PiggyBac expression vector containing a puromycin selection cassette constructed in house[2]. HepG2 cells (ATCC) were transfected with the above construct using Freestyle max transfection agents (ThermoScientific). For stimulating with various BMPs, solutions with human BMPs were prepared at 40nM (10nM final assay concentration) in MEM containing 1% FBS. Control inhibition curves were generated by titrating commercial anti-BMP antibodies starting at 600nM final concentration. KY1070 titrations were generated for the same concentration range. Human isotype control was also included in each assay. HepG2 reporter cells were detached from flasks, pelleted and re-suspended in MEM supplemented with 1% FBS at $3.3x10^5$ cells/ml and 30µl of this suspension added at 10,000 cells/well. Plates were incubated overnight at 37°C, 5% CO_2 and the next day, 30µL of luciferase substrate (Pierce Firefly Luc one step glow assay kit) was added. Plates were incubated for 10 minutes at room temperature in the dark and read using an Envision plate reader (Perkin Elmer).

For HepG2 *Hamp* luciferase reporter assay the control antibodies used were (anti-BMP2/4 R&D Systems MAB3552; anti-BMP5 R&D Systems MAB7151, anti-BMP7 R&D Systems MAB3541, anti-BMP9 R&D Systems 3209). Human BMPs used were BMP2 (R&D Systems 355-BM), BMP4 (R&D Systems 314-BP), BMP5 (R&D Systems 615-BMC), BMP6 (Peprotech 120-06), BMP7 (R&D Systems 354-BP) and BMP9 (R&D Systems 3209-BP).

Receptor dimerization assay

PathHunter® eXpress BMPR1A - BMPR2 transfected U2OS cells (DiscoverX #93-1053C3) were resuspended and $1x10^4$ cells added to each well of a white-walled clear bottom 96-well tissue culture plate (DiscoverX#15-073) and incubated for 24h at 37°C. For inducing dimerization, human BMP6 (Peprotech 120-06) was used at a fixed final concentration of 200ng/mL. The effect of anti-BMP6 antibody was tested by pre-incubating a serial dilution of anti-BMP6 antibodies with 200ng/ml human BMP6 for one hour at room temperature. Following this, 10µl of this pre-incubated mixture was then added to U2OS cells and incubated at 37°C for a further 16h. Detection reagent was prepared as recommended by the manufacturer and 110µl of this prepared Detection Mix was added to each well and incubated for 1h at room temperature in the dark. Plates were read and analysed using an Envision (PerkinElmer) plate reader.

HepG2 cell culture for hepcidin RNA analysis

HepG2 cells were cultured in RPMI 1640 (PAN Biotech) + 10% FCS + 1% Penicillin/Streptomycin.

For hepcidin mRNA measurements 200.000 HepG2 cells/well were plated in a 12-well plate. Cells were left to rest for 24 hours before medium was removed and replaced with serum-free RPMI. In addition, HepG2 cells were treated with 1nM human BMP6 (R&D) alone or in combination with murine ERFE (1nM, 5nM or 10nM) and/or KY1070 (1nM, 5nM or 10nM) as indicated. After 6 hours of incubation, cells were harvested and analyzed accordingly.

Recombinant ERFE production and purification

Mouse ERFE cDNA sequence was cloned into pFUSEN-hG2Fc plasmid (Invivogen). Recombinant proteins were produced in suspension culture in Freestyle 293F cells (Life Technologies) transiently transfected using FectoPRO transfection reagent (Polyplus). Supernatants from cells overexpressing Fc-tagged ERFE protein were collected after 5 days and supplemented with protease inhibitor cocktail (Roche). Recombinant ERFE was purified using an Hitrap protein A HP column on an ÄKTA pure chromatography system (GE healthcare) and eluted with 0.1M Glycine pH3.5. The eluted fractions were concentrated using centrifugal concentrators Spin-X UF 20 (Corning), and recombinant ERFE protein was resuspended in saline (0.9% NaCl). Protein concentration was determined using Coomassie Imperial Protein Stain and Pierce bicinchoninic acid protein assay (Thermo Fisher Scientific).

Protein extraction for Western blot analysis

For the examination of pSMAD1/5/8 nuclear extracts were prepared from freshly isolated tissue using a commercially available kit (NE-PER, Thermo Scientific, Rockford, IL). All other proteins were prepared as whole cell lysates.

Tissue iron measurement

Briefly, the determination of tissue iron content was carried out with acid-hydrolysed tissue homogenates with a colorimetric method employing bathophenanthroline disulfonic acid and L-ascorbic acid in a sodium acetate assay buffer. The calculated iron quantity was normalized to the wet tissue weight for each sample.

Plasma biochemistry

Plasma iron was determined in mice and rats as described previously (QuantiChrom Iron Assay kit and BioAssay Systems, Hayward, CA). ELISAs were used to measure transferrin (Tf) (Rat Transferrin ELISA Kit; Mouse Tf ELISA Kit both form Abcam, Austria), erythropoetin (Mouse Erythropoietin

Quantikine ELISA Kit, human Erythropoietin Quantikine ELISA Kit both from R&D, Austria), hepcidin (hepcidin-25 (rat) enzyme immunoassay kit extraction free, Peninsula Laboratories International, Inc., San Carlos, CA, HMC-001 Hepcidin Murine-Compete ELISA Kit Intrinsic LifeSciences (ILS), and erythroferrone (ERF-200, Intrinsic Mouse Erythroferron ELISA Kit Intrinsic LifeSciences (ILS)). Analysis was always performed according to the manufacturer's protocol. Blood Urea Nitrogen (BUN) was measured using a colorimetric method (Urea Nitrogen (BUN) Colorimetric Detection Kit, Invitrogen, Austria) Optical densities were measured at indicated wavelengths in a microplate reader (Infinite M200pro, Tecan, Männedorf, Switzerland).

Tf saturation (Tf-Sat) was calculated based on iron and Tf measurement using the following formula: Tf-Sat [%] = (Fe [µg/dL]/Tf [mg/dL]) × 70.9

Antibodies

Western blot antibodies used

Rabbit anti-human FPN1 (1:400; Eurogentec, Liège, Belgium), Rabbit anti-human Ft (1:400; Sigma, Schnelldorf, Germany) and Rabbit anti-human pSMAD1/5/9 (CST #13820) Rabbit anti-human β-actin (1:500; Sigma, Schnelldorf, Germany) and anti-GAPDH antibody ([6C5] - Loading Control (#ab8245)) were used.

Antibodies and staining reagents used for flow cytometry

FITC-anti-Ter119 (Ter119, Biolegend), PE-anti-CD71 (C2, BD), APC-anti-CD44 (IM7), eFluor 450-anti-CD49b (DX5, eBioscience), eFluor450-anti-CD3 (17A2, eBioscience), eFluor450-anti-CD19 (eBio1D3, eBioscience), eFluor450-anti-CD11b (RB6-8C5, eBioscience), eFluor 450- anti-CD11c (N418, eBioscience). eFluor450-anti-Gr1 (M1I70 eBioscience), FITC-anti-CD45 (30-F11, eBiosciences), PerCP-anti-Gr1 (RB6-8C5, eBioscience), APC-Cy7-anti-CD11b (M1I70, Biolegend).

Calcein staining protocol for determination of intracellular free iron levels

Isolated bone marrow cells were resuspended in PBS containing Calcein at a concentration of 1µg/mL according to the recommendation by the manufacturer (Thermo Fisher). Bone marrow cell pellets were re-suspended in 50µl of Calcein-AM containing PBS and incubated for 5 minutes at 37°C protected from light. Afterwards cells were washed with PBS and the staining procedure with fluorophore-conjugated antibodies was continued.

RNA preparation from tissue, reverse transcription and TaqMan real-time PCR

Total RNA was prepared from liquid nitrogen-frozen rat and mouse tissues using acid guanidinium thiocyanate-phenol-chloroform extraction with peqGOLD Tri-Fast™ (Peqlab, Germany). For reverse transcription 4µg RNA was used. TaqMan real-time PCR was performed at least in duplicates on a CFX96 light cycler (Bio-Rad). Ssofast Probes Supermix and Ssofast EvaGreen Supermix (Bio-Rad Laboratories GmbH, Vienna, Austria) were used according to the manufacturer's instructions. Real-time PCR reactions were performed on a CFX Cycler and analyzed with CFX software (BioRad). Gene expression was normalized using ΔΔct calculations.

Primer sequences

The following TaqMan PCR primers and probes were used:

Rat hepcidin: 5'-TGAGCAGCGGTGCCTATCT-3', 5'-CCATGCCAAGGCTGCAG-3', FAM-CGGCAACAGACGAGACAGACTACGGC-BHQ1

Rat Gusb (beta-glucoronidase): 5'-ATTACTCGAACAATCGGTTGCA-3', 5'-GACCGGCATGTCCAAGGTT-3', FAM-CGTAGCGGCTGCCGGTACCACT-BHQ1

Mouse hepcidin 5`-GGC AGA CAT TGC GAT ACC AAT-3`, 5`-TGCAACAGATACCAC ACTGGGAA-3`, probe FAM-CCAACTTCCCCATCTGCATCTGC-BHQ1

Mouse IL-6: 5′- TGTTCTCTGGGAAATCGTGGA-3′, 5′-AAGTGCATCATCGTTGTTCATACA-3′, FAM-ATGAGAAAAGAGTTGTGCAATGGCAATTCTG- BHQ1

Mouse FoxP3: 5'-AGGAGAAGCTGGGAGCTATGC-3', 5′- TGGCTACGATGCAGCAAG AG -3′, FAM - AGCGCCATCTTCCCAGCCAGG - BHQ1

Mouse Gata3: 5'- CTACCGGGTTCGGATGTAAGTC-3', 5′-GTTCACACACTCCCTGCCTTCT -3′, FAM -AGGCCCAAGGCACGATCCAGC- BHQ1

Mouse TNF-α: 5′-TTCTATGGCCCAGACCCTA-3′, 5′- TTGCTACGACGTGGGCTACA-3′, FAM-CTCAGATCATCTTCTCAAAATTCGAGTGACAAGC-BHQ1

Human hepcidin: 5′- TTTCCCACAACAGACGGGAC-3′, 5′- AGCTGGCCCTGGCTCC-3′, probe FAM−CAGAGCTGCAACCCCAGGACAGAGC−BHQ1

Human tubulin: 5′- TTCAACACCTTCTTCAGT-3′, 5′-GGTGCCAGTGCGAACTTC-3′

Statistics

Significant differences between groups was determined using a 2-tailed unpaired t-test (two groups) or two way ANOVA (more than 2 groups, two factors) with Tukey-corrected post-hoc t-tests for multiple comparison as indicated in the respective figure legends. Significance for differences in number of consumed EPO doses (Figure 4C) was determined with Fisher's exact test. Differences in hematological parameters from the EPO-sparing CKD experiment (Figure 4D and S9) were fed into a first order linear model considering effects of EPO, KY1070 and their interaction. Model estimates

and 95% confidence intervals were calculated with the least square method. Significance for the estimates (estimate $\neq 0$) was calculated with two-sided t-test.

SUPPLEMENTAL FIGURES

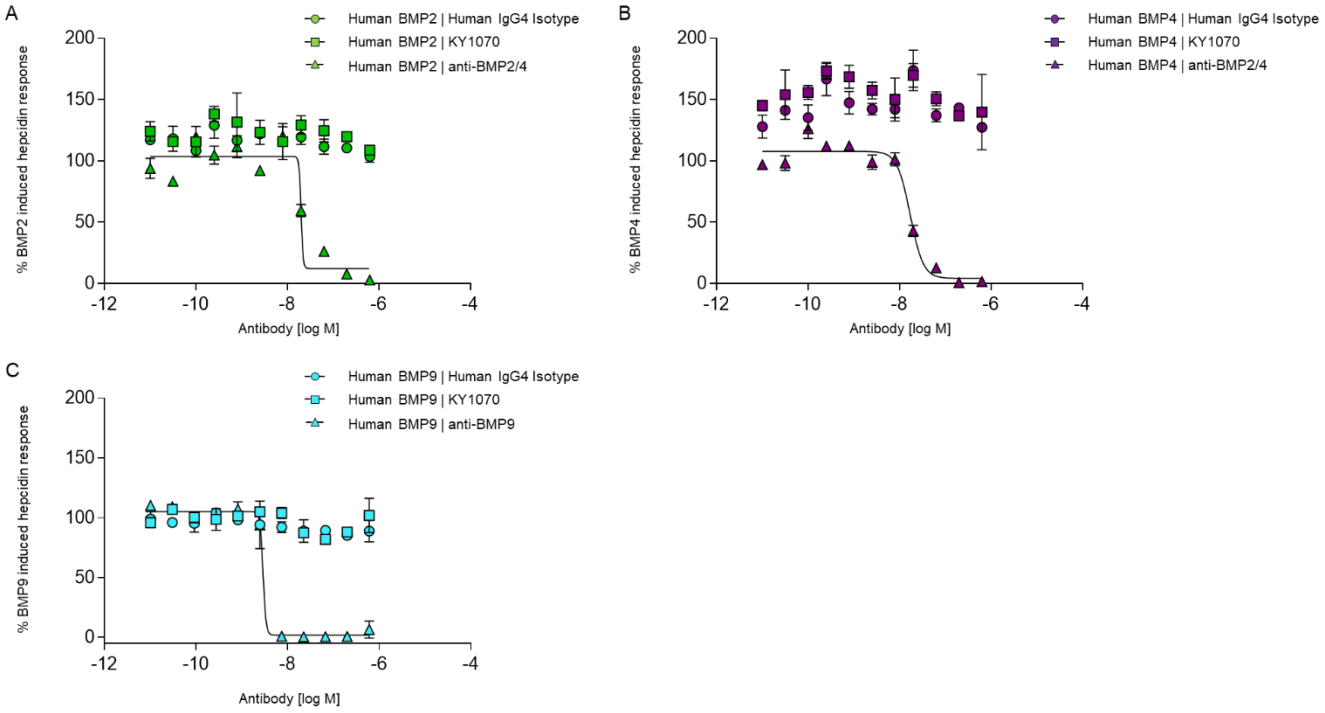

Figure S1. (B) KY1070 specificity was tested against human BMP2, BMP4 and BMP9. *Hamp* luciferase reporter gene cells were stimulated with a fixed concentration of recombinant human BMP2, BMP4 or BMP9 of 10nM. Inhibition curves for KY1070 were generated by titration starting at a final concentration of 600nM. A titration of commercially available murine anti-BMP2/4 and anti-BMP9 antibodies as well as a human IgG4 isotype control was included.

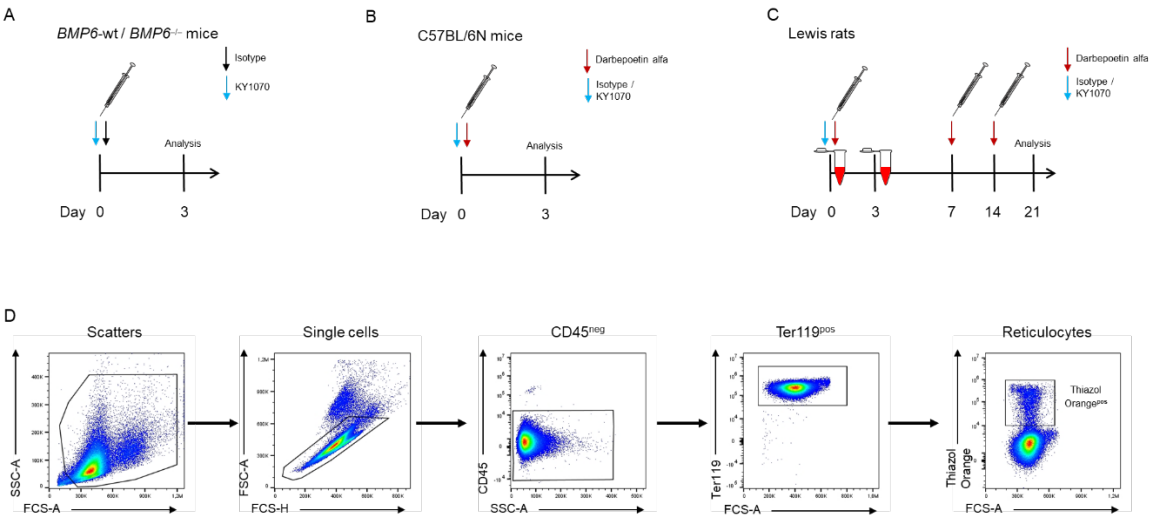

Figure S2. Experimental setup performed in *BMP6* Wt/ *BMP6*$^{-/-}$ mice (A), healthy C57BL/6N mice (B) and Lewis rats (C) is shown. (D) Gating strategy and analysis procedure of peripheral blood for analysis of reticulocytes**.** Whole blood was stained with antibodies against CD45, Ter119 and Thiazol Orange for the detection of reticulocytes.

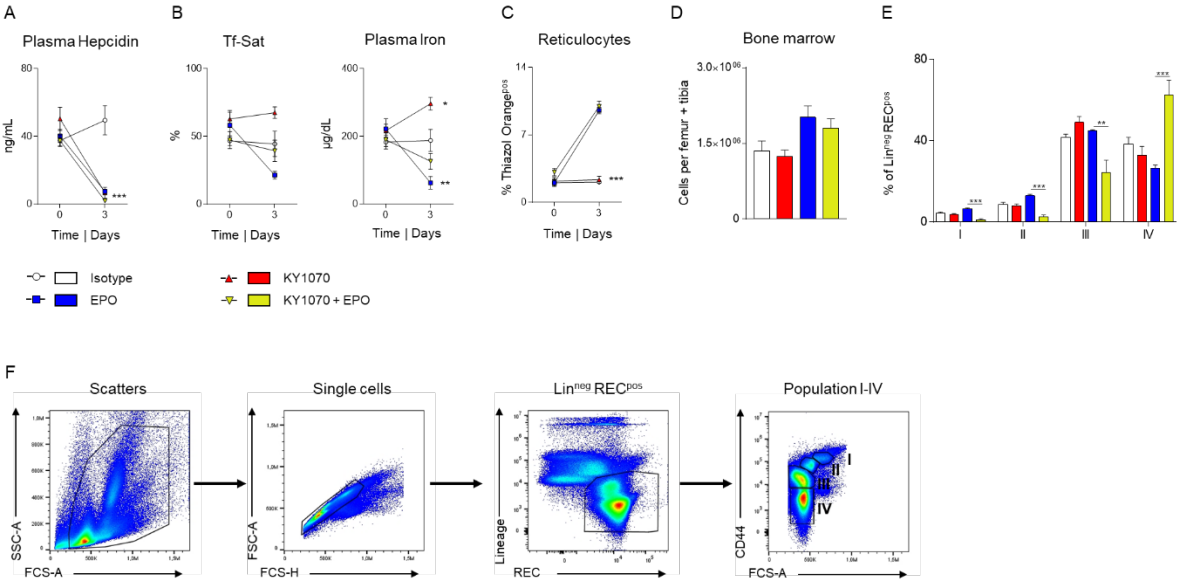

Figure S3. (A-C) Plasma hepcidin levels, Tf-Sat, plasma iron levels and reticulocyte counts determined in healthy Lewis rats at baseline (= Day 0) and 3 days after a single injection of IgG4 isotype control (3mg/kg; n=5), KY1070 (3mg/kg; n=5), EPO (10µg/kg; n=6) or KY1070 (3mg/kg) and EPO (10µg/kg; n=7). (D) Total bone marrow cell count per leg (isolated from femur and tibia) at the end of the experiment (Day 21). (E) Percentages of the different erythropoietic populations (Population I-IV) per femur expressed as percent of Lineage^{neg} REC^{pos} cells. (F) Gating strategy and analysis procedure of rat bone marrow cells for erythroid precursor populations. Bone marrow cells were stained with antibodies against CD44, REC and CD45 (Lineage cells). Identification of late erythroblasts: Basophilic (population I), polychromatic (population II), orthochromatic erythroblasts and reticulocytes (population III) and mature red blood cells (population IV) among Lineage^{neg} single cells was accomplished with CD44 and size discrimination. Two-way ANOVA with Tukey corrected post hoc t-test for multiple comparisons was applied for A-E. Results are shown as mean ± SEM. Significant levels are indicated for Isotype treated control group against all treatment groups and EPO vs. KY1070 + EPO treated rats. *p <0.05, ***p<0.001; Tf-Sat (transferrin saturation), Hgb (hemoglobin levels), EPO (Darbepoetin alfa).

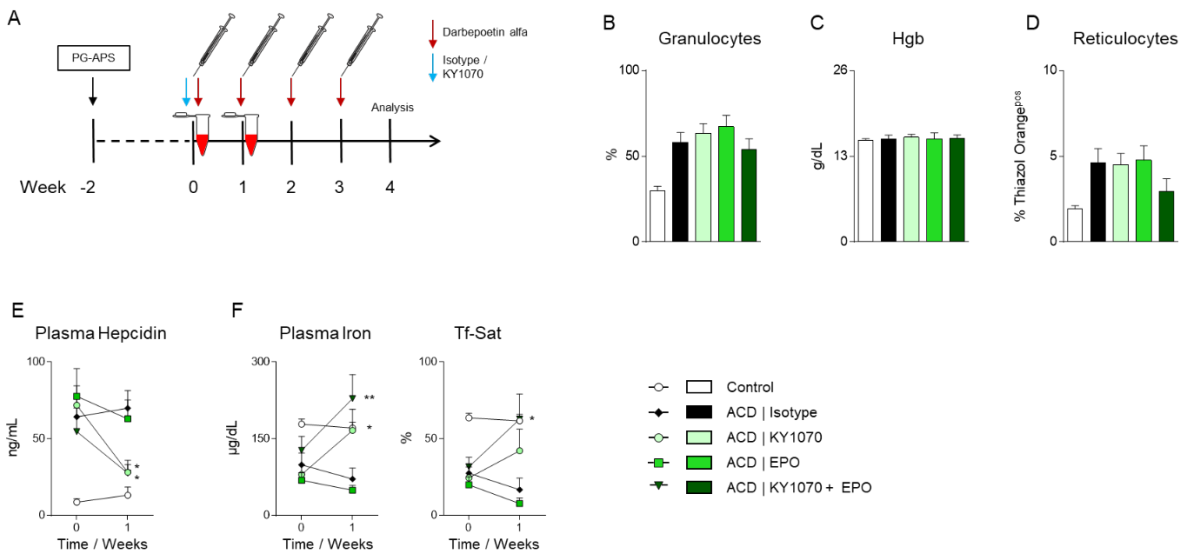

Figure S4. (A) The experimental timeline for experiments performed in ACD rats is shown. Two weeks after PG-APS injection ("Week 0") rats were bled, screened for ACD development, allocated to the treatment groups (ACD; n=8; ACD | KY1070 n=9; ACD | EPO, n=8; ACD | KY1070 + EPO n=7) and treatment started. (B, C, D) Baseline parameters of ACD rats at timepoint "Week 0" (B) granulocytes as percent of white blood cells and (C) Hgb levels (D) reticulocytes as determined by flow cytometric analysis (shown as % Thiazol Orange^{pos} of CD45^{neg} Ter119^{pos}). (E, F) Kinetic analysis of plasma hepcidin, plasma iron and Tf-Sat one week after isotype antibody (ACD), KY1070 (3mg/kg), Darbepoetin alfa (10µg/kg) or combination treatment in ACD rats. Two-way ANOVA with Tukey corrected post hoc t-test for multiple comparisons was applied for E and F at Week 1. Results are shown as mean ± SEM. Significant levels are indicated for Isotype treated control group against all treatment groups and EPO vs. KY1070 + EPO treated rats. *p <0.05, **p<0.01; ACD (anemia of chronic disease), PG-APS (Group A Streptococcal Peptidoglycan-Polysaccharide), Hgb (hemoglobin levels), Tf-Sat (transferrin saturation).

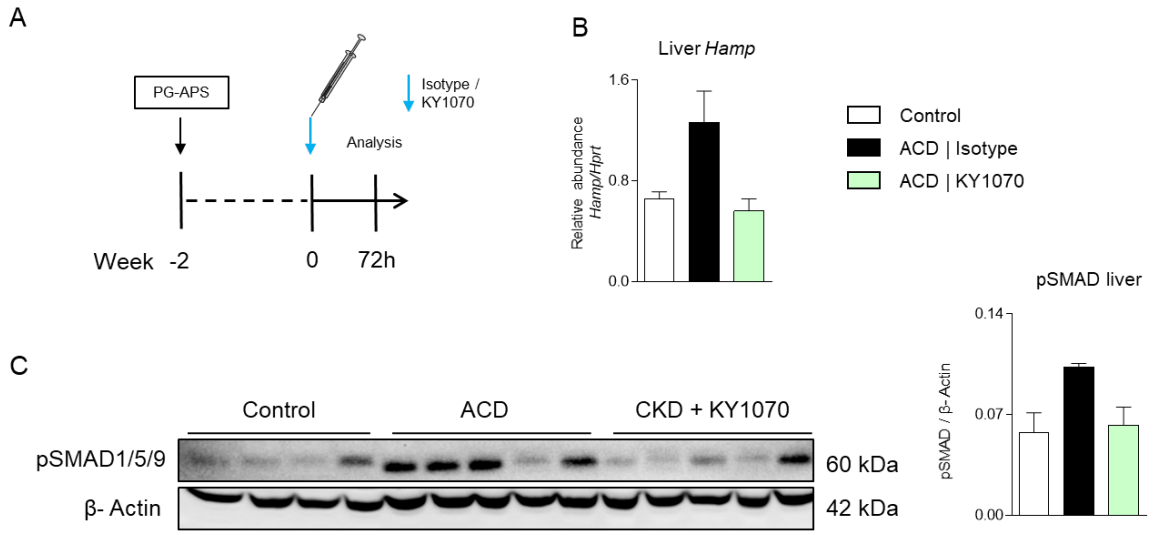

Figure S5. (A) Experimental set-up for analysis of short-term effects of KY1070 is shown. Two weeks after PG-APS injection ("Week 0") rats were allocated to the treatment groups (ACD; n=6; ACD | KY1070 n=6) and treatment started. (B) Hepatic *Hamp* levels 72h after treatment with isotype antibody (ACD) or KY1070 (3mg/kg). (C) Western blot and densitometric quantification of pSMAD protein expression (n=4-5 per group; each lane represents one individual animal; β-actin level was used for normalization). Results are shown as mean ± SEM. ACD (anemia of chronic disease), PG-APS (Group A Streptococcal Peptidoglycan-Polysaccharide), pSMAD (phospho-SMAD).

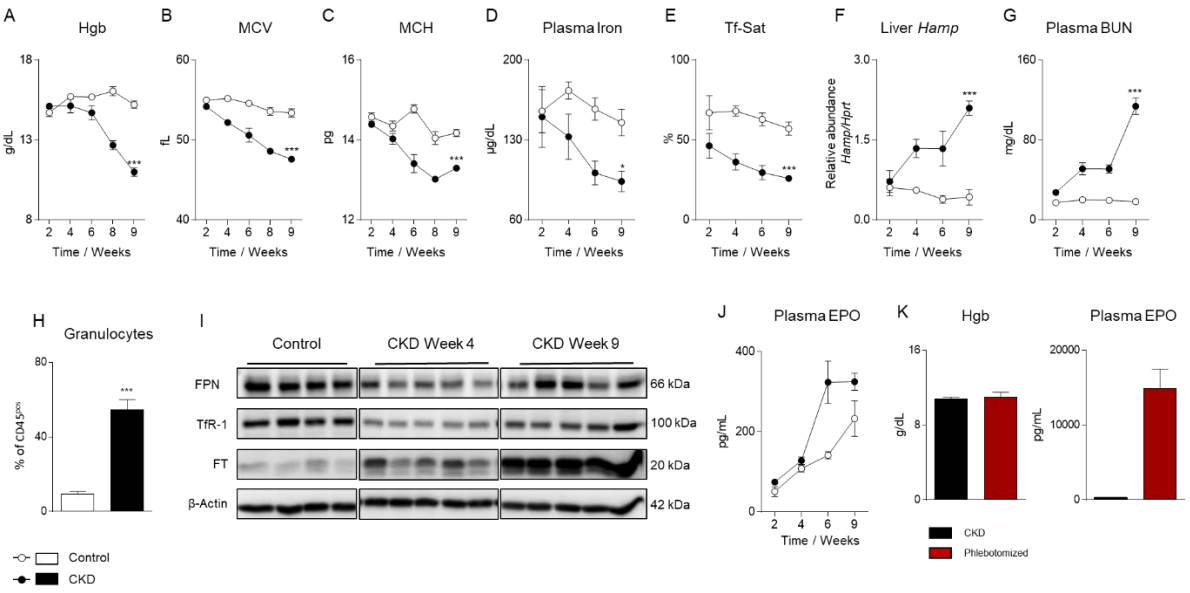

Figure S6. Analysis of hematological parameters, iron homeostasis and kidney function in the model of dietary adenine-induced CKD in male C57BL/6N wild type mice. 3-week old mice (n=5 for each experimental group at each endpoint (Week 2, 4, 6, and 9) were either fed with adenine diet or control

diet throughout the whole experimental period and sacrificed after 2, 4, 6 or 9 weeks. Mice terminated at week 9 were additionally bled at week 8 for determination of hematological parameters. (A) Hgb, (B) MCV, (C) MCH, (D) plasma iron levels, (E) Tf-Sat, (F) qPCR analysis of liver *Hamp* mRNA, and (G) plasma BUN levels were determined at all given timepoints. (H) Granulocytes determined at week 9 by flow cytometry, shown as percent of $CD45^{pos}$ cells. (I) Western blot analysis of FPN, TfR1 and FT in whole spleen lysates in mice fed the control diet (control) and in CKD mice 4 weeks and 9 weeks being fed with the adenine diet (n=4-5 per group; each lane represents one individual animal; β-actin level was used for normalization). (J) Kinetics of plasma EPO levels in control and CKD animals. (K) Hgb and plasma EPO levels of CKD mice and mice after phlebotomy. Unpaired student t-test was applied for comparison between control mice and CKD mice at week 9. Results are shown as mean ± SEM. ***p<0.001; CKD (Chronic kidney disease), Hgb (hemoglobin levels), MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), Tf-Sat (transferrin saturation), BUN (blood urea nitrogen), FPN (Ferroportin), TfR-1 (Transferrin Receptor-1), FT (Ferritin), EPO (Erythropoetin).

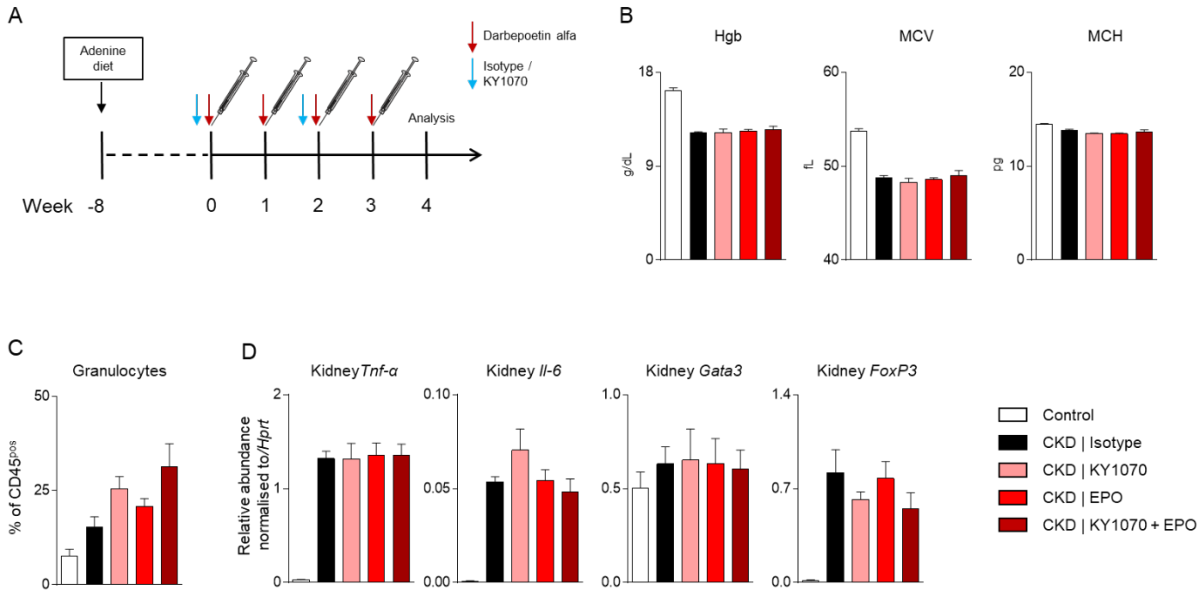

Figure S7. (A) Experimental setup for CKD mice treated with KY1070 and/or Darbepoetin alfa is shown. CKD in male C57BL/6N mice was induced by a diet containing 0.2% adenine. At "Week 0" CKD mice were either treated with human IgG4 Isotype (CKD; n=5), KY1070 (3mg/kg; n=6), Darbepoetin alfa (10µg/kg; n=6) or both (n=6). (B) Hematological parameters (Hgb, MCV, MCH) at baseline in CKD mice (Week 0, after 8 week feeding). (C) Granulocyte counts (shown as % of $CD45^{pos}$ cells) in the blood of treated and untreated CKD animals and healthy controls. (D) Levels of *Tnf-α, Gata3*, *Il-6* and *FoxP3* mRNA levels in the kidney of treated and untreated CKD mice and healthy controls determined by quantitative reverse transcription polymerase chain reaction analysis.

Results are shown as mean ± SEM. *p <0.05, **p<0.01, ***p<0.001; (A-F). CKD (Chronic kidney disease), Hgb (hemoglobin levels), EPO (Darbepoetin alfa).

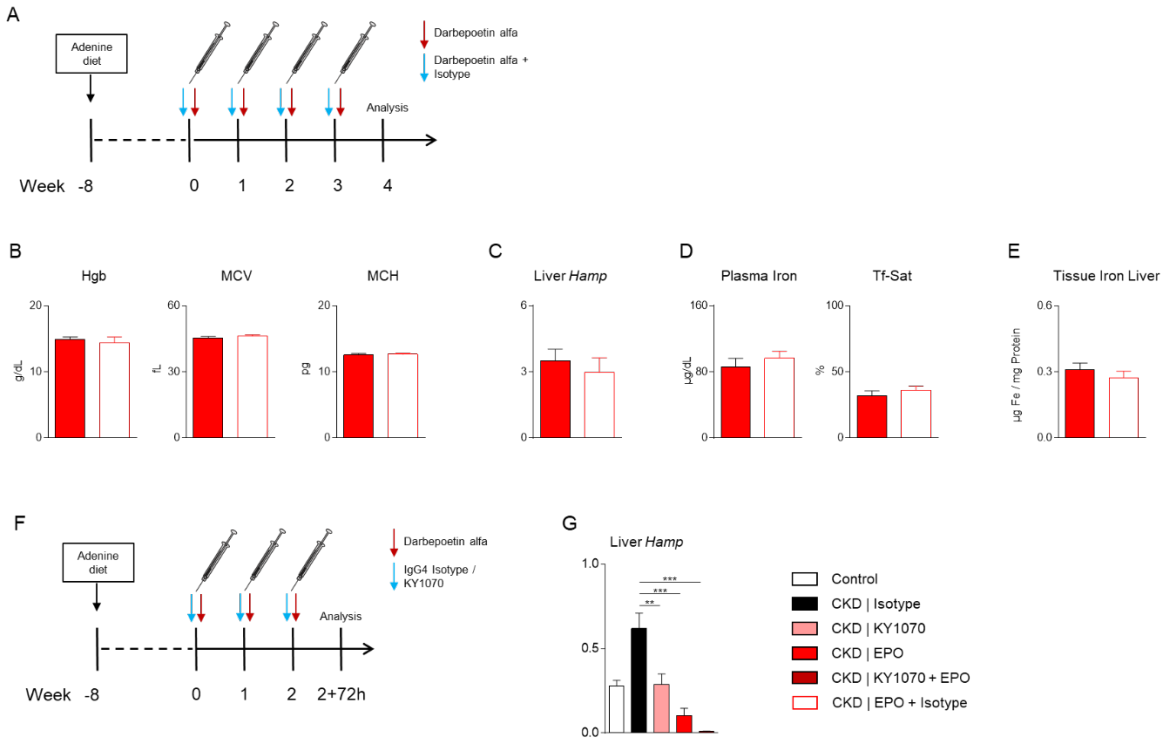

Figure S8. (A) Experimental setup for CKD mice treated with Darbepoetin alfa (10µg/kg; n= 5) or Darbepoetin alfa plus human IgG4 isotype (3mg/kg; n=6) is shown. Experimental setup resembles the protocol detailed in Figure S5. (B) Hematological parameters (Hgb, MCV, MCH), (C) liver *Hamp* mRNA levels, (D) Plasma iron, Tf-Sat and (E) hepatic tissue iron levels at timepoint of analysis (Week 4) is shown. (F, G) Experimental set-up for analysis of short-term effects of KY1070, EPO and combination therapy on hepatic *Hamp* levels after induction of CKD is shown. Results are shown as mean ± SEM. **p<0.01, ***p<0.001; (A-F). CKD (Chronic kidney disease), Hgb (hemoglobin levels), EPO (Darbepoetin alfa), MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin).

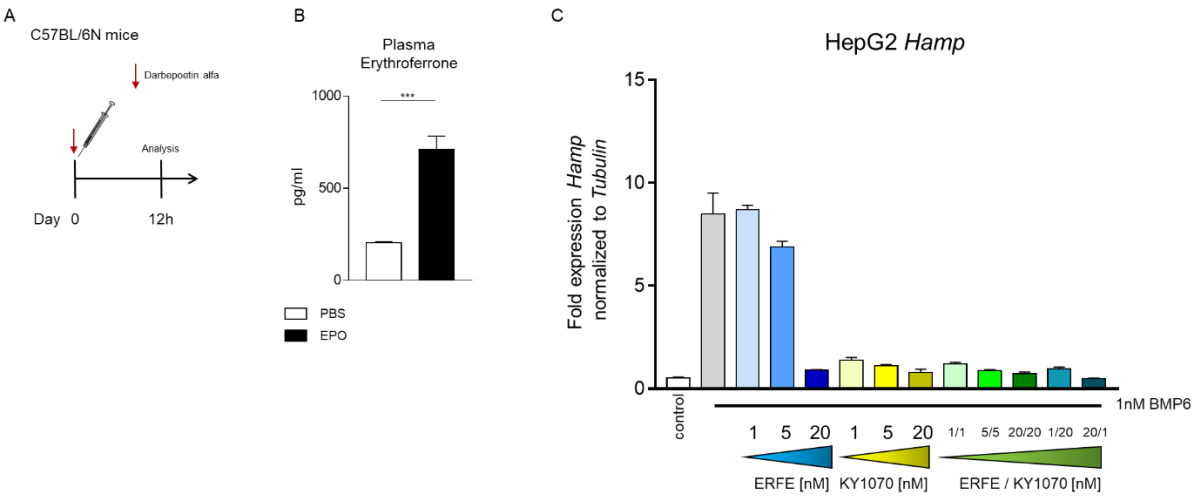

Figure S9. (A) Experimental setup for testing of EPO-mediated ERFE in C57BL/6N mice is shown. (B) ERFE plasma levels in wild-type mice 12h treated with a single dose of Darbepoetin alfa (10µg/kg; n= 4) or PBS. (C) qRT-PCR analysis of *Hamp* mRNA levels in HepG2 cells stimulated with 1nM BMP6 in combination with ERFE recombinant protein (1nM, 5nM, 20nM), KY1070 (1nM, 5nM 20nM) or combinations thereof for 6 hours. Results of one representative experiment are shown. Results are shown as mean ± SEM. ***p<0.001; (A). ERFE (Erythroferrone), BMP6 (Bone morphogenetic protein 6).

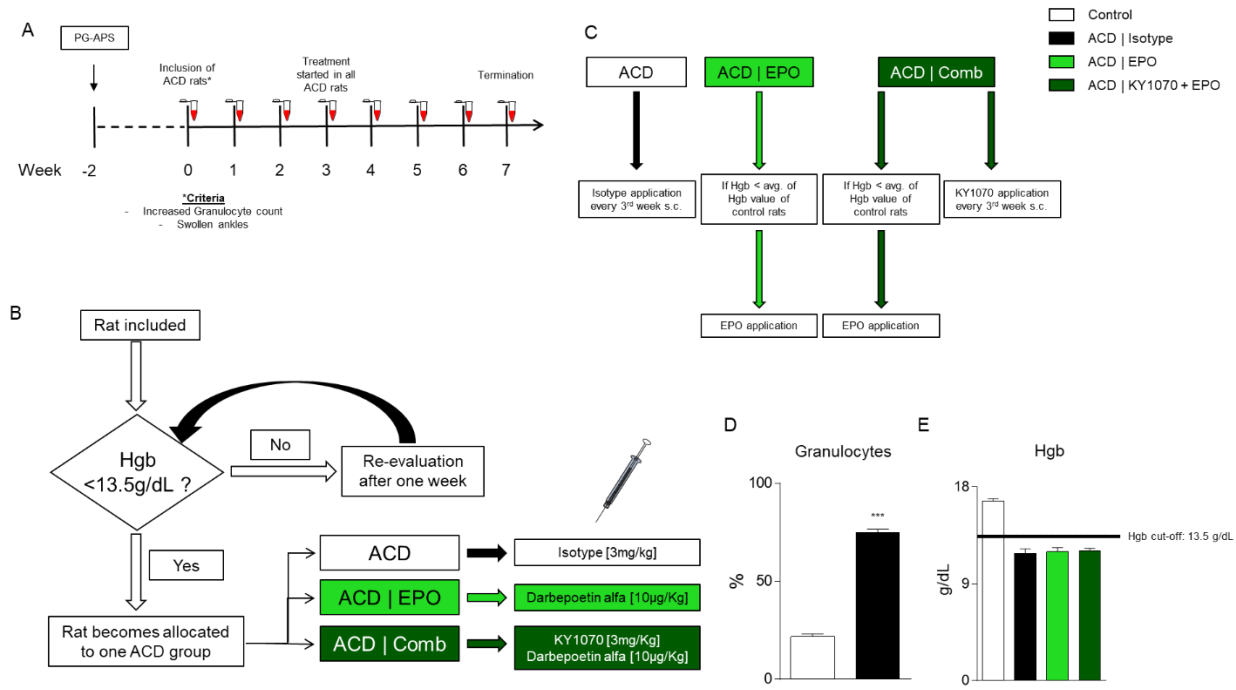

Figure S10. (A-C) Experimental setup for assessment of EPO-sparing in ACD rats is shown. (A) The experimental timeline for experiments performed in the ACD rats. (B,C) Study protocol in ACD rats. Once a rat has been included into the study, Hgb values were determined weekly in all ACD animals.

If the Hgb value of a therapy-naïve rat was < 13.5g/dL, the rat was randomly allocated into one of the treatment groups: ACD (3mg/kg isotype control antibody), ACD | EPO (10µg/kg) or ACD | KY1070 + EPO (3mg/kg KY1070 and 10µg/kg EPO). After treatment initialization, animals in the ACD and ACD | KY1070 + EPO groups were administered the isotype control antibody or KY1070, respectively, every third week. For decision of further EPO application, Hgb values were determined weekly. EPO administration in the ACD | EPO and ACD | KY1070 + EPO groups was performed depending on the current Hgb value (Hgb < mean Hgb in therapy-naïve control rats). All further information for the study protocol is given in the Supplemental File Section 'ACD rats EPO-sparing protocol'. (D) Percentage of granulocytes among white blood cells of control rats (n=5) and ACD rats (n=28) included at timepoint "Week 0". (E) Hgb values distribution among treatment groups at 1st treatment day (control n=5, ACD n=7, ACD | EPO n=10, ACD | KY1070 + EPO n=11). The Hgb cut-off value of 13.5g/dL is indicated. Unpaired student t-test was applied for comparison between groups for D. Results are shown as mean ± SEM. ***p<0.001. EPO (Darbepoetin alfa), ACD (anemia of chronic disease), Hgb (hemoglobin levels).

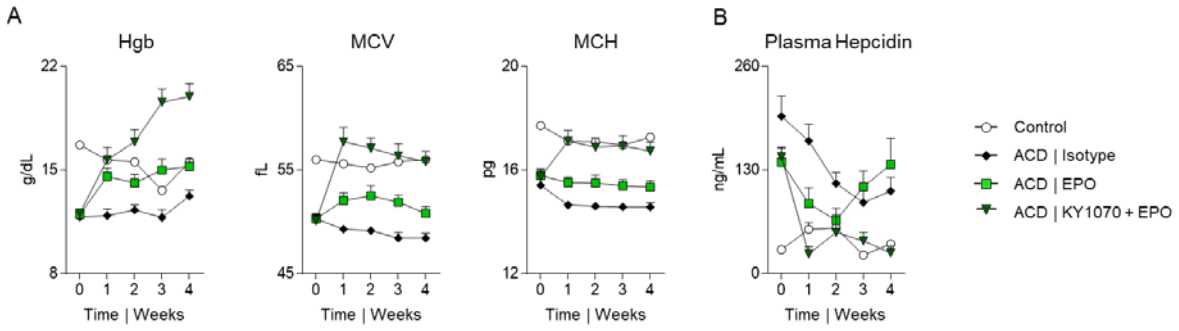

Figure S11. (A) Time course of weekly measured hematological parameters (Hgb, MCV, MCH) and (B) plasma hepcidin levels of anemic ACD rats with a human IgG4 isotype control (3mg/kg; n=7), KY1070 Darbepoetin alfa (10µg/kg; n=10) or both (n=11). Results are shown as mean ± SEM. ACD (Anemia of chronic disease), Hgb (hemoglobin levels), MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin).

A

$$\Delta Hb = \alpha + \beta_{anti-BMP6} C_{anti-BMP6} + \beta_{EPO} C_{EPO} + \beta_{anti-BMP6:EPO} C_{anti-BMP6} C_{EPO}$$

	Δ Hb		
	Value	95% CI	p
α	0.99	(0.67; 1.32)	< 0.0001
β(anti-BMP6)	0.14	(0.061; 0.23)	0.0011
β(EPO)	0.26	(0.17; 0.34)	< 0.0001
β(anti-BMP6:EPO)	0.055	(0.014; 0.096)	0.0086

B

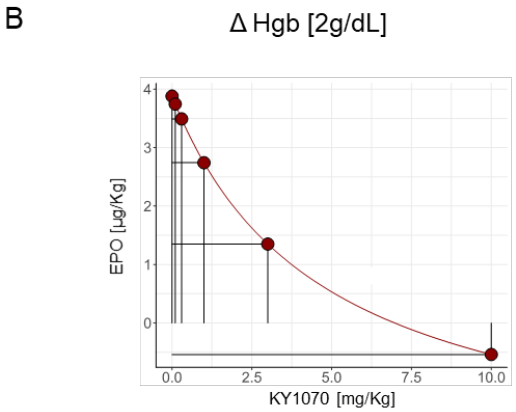

Figure S12. Data on Hgb presented in Figure 4 were fed into first order linear models, which model the given Δ parameter as a function of Darbepoetin alfa and KY1070 concentrations applied and interaction thereof. Model estimates and 95% confidence intervals were calculated with the least square method. (A) Model formula and value of model estimates with 95% confidence intervals and p value (p(estimate) ≠ 0). (B) Curve depicting concentrations of KY1070 and Darbepoetin alfa calculated from the model, which are required to reach the given increase in the ΔHgb [2g/dL]. Dots indicate the KY1070 and EPO dose used in our experiments. Δ (difference in increase), Hgb (hemoglobin levels).

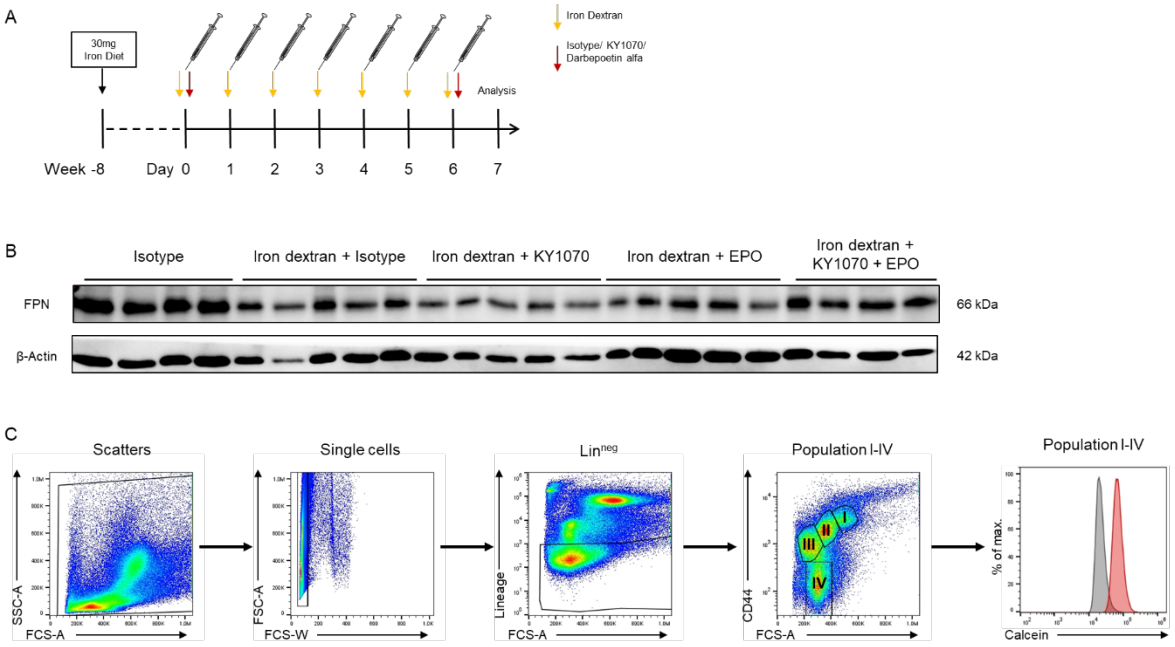

Figure S13. (A) Experimental timeline for C57BL/6N mice treated with iron dextran (20mg) in combination with human IgG4 isotype control (3mg/kg; n=7), KY1070 (3mg/kg; n=8) Darbepoetin alfa (10µg/kg; n=6) or both (n=6). (B) Western blot (n=4-5 per group; each lane represents one individual animal; β-actin level was used for normalization) of FPN of MACS-separated erythroid precursor cells of the bone marrow. (C) Gating strategy and analysis procedure of mouse bone marrow cells for determination of intracellular free iron levels by Calcein-AM. Bone marrow cells were stained with antibodies against CD44, Ter119 and lineage containing CD3, CD19, CD11b, CD11c, CD49b and Gr-1. Identification of basophilic (population I), polychromatic (population II), orthochromatic erythroblasts and reticulocytes (population III) and mature red blood cells (population IV) among Lineage^{neg} single cells was accomplished with CD44 and size discrimination. Finally, MFI of Calcein were analysed for each individual precursor population. FPN (Ferroportin).

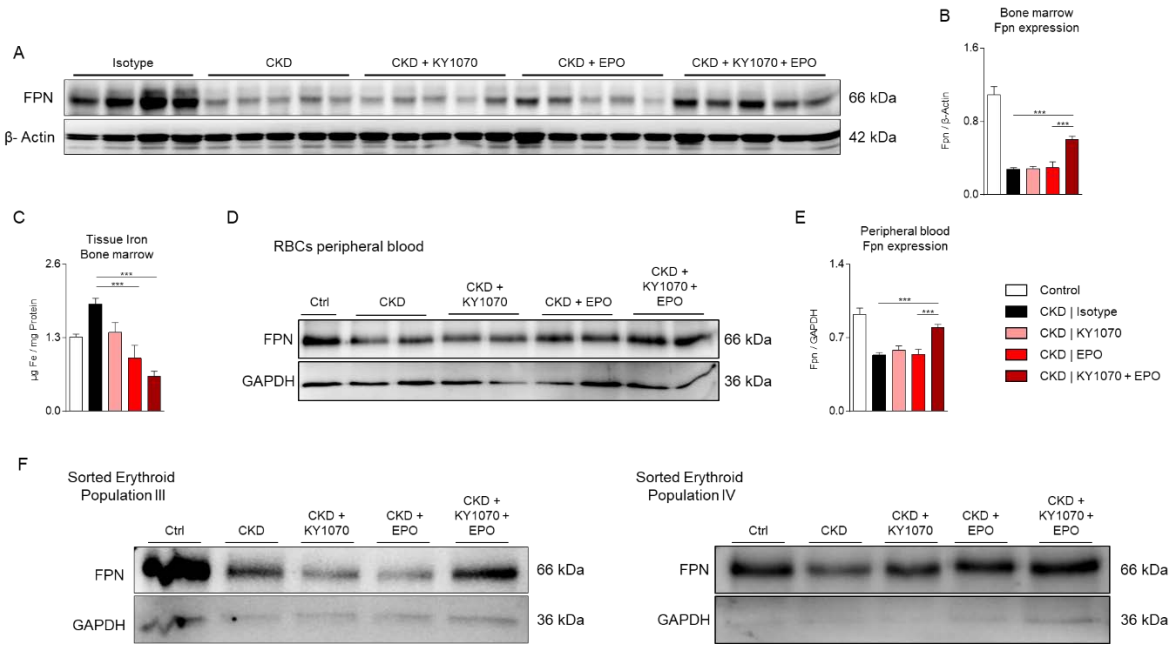

Figure S14. (A, B) Western blot and densitometric quantification of FPN protein expression (n=4-5 per group; each lane represents one individual animal; β-actin level was used for normalization) in the bone marrow. (C) Bone marrow tissue iron levels at timepoint of analysis is shown. (D, E) Densitometric quantification of FPN protein expression (n=4-5 per group) on mature red blood cells isolated from the peripheral blood (E). Representative Western blot is shown (each lane represents one individual animal; GAPDH was used for normalization). (F) Western blot of sorted erythroid progenitor cell populations for FPN (each lane represents bone marrow of 3-6 mice from the same treatment group; GAPDH was used for normalization). For details on experimental setup please see Figure S8F. Two-way ANOVA with Tukey corrected post hoc t-test for multiple comparisons between CKD animals was applied. Results are shown as mean ± SEM. ***p<0.001. CKD (Chronic kidney disease), EPO (Darbepoetin alfa), RBCs (Red Blood Cells), FPN (Ferroportin).

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