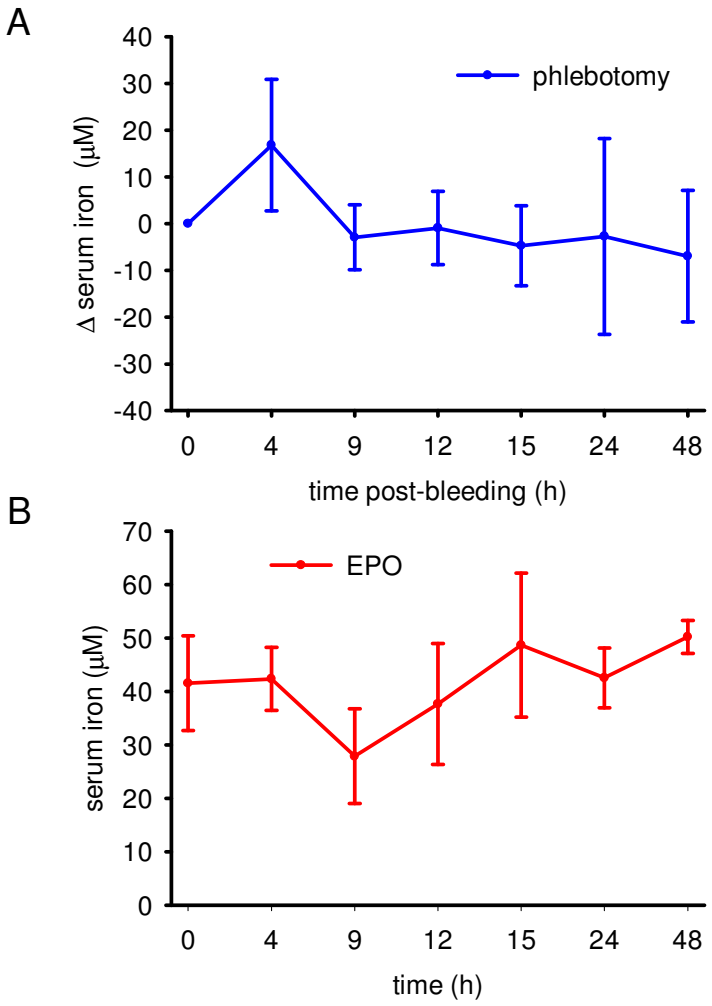
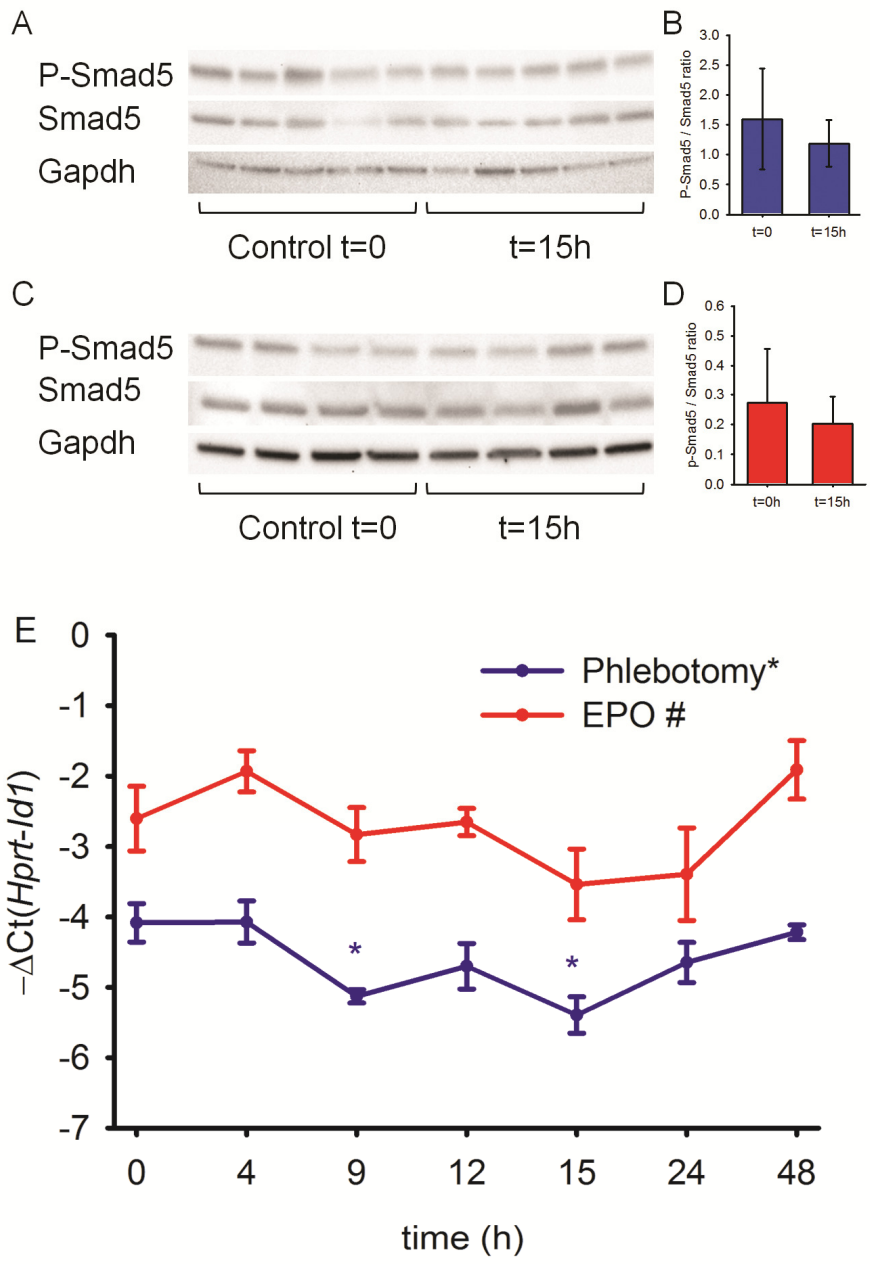
Supplementary Table 1 : Murine *Fam132b* Promoter Analysis 3k (Link to Supplementary Table 1 Murine Fam132b Erythroferrone Promoter Analysis 3k.xlsx Excel spreadsheet)

Supplementary Table 2: qRT-PCR primers

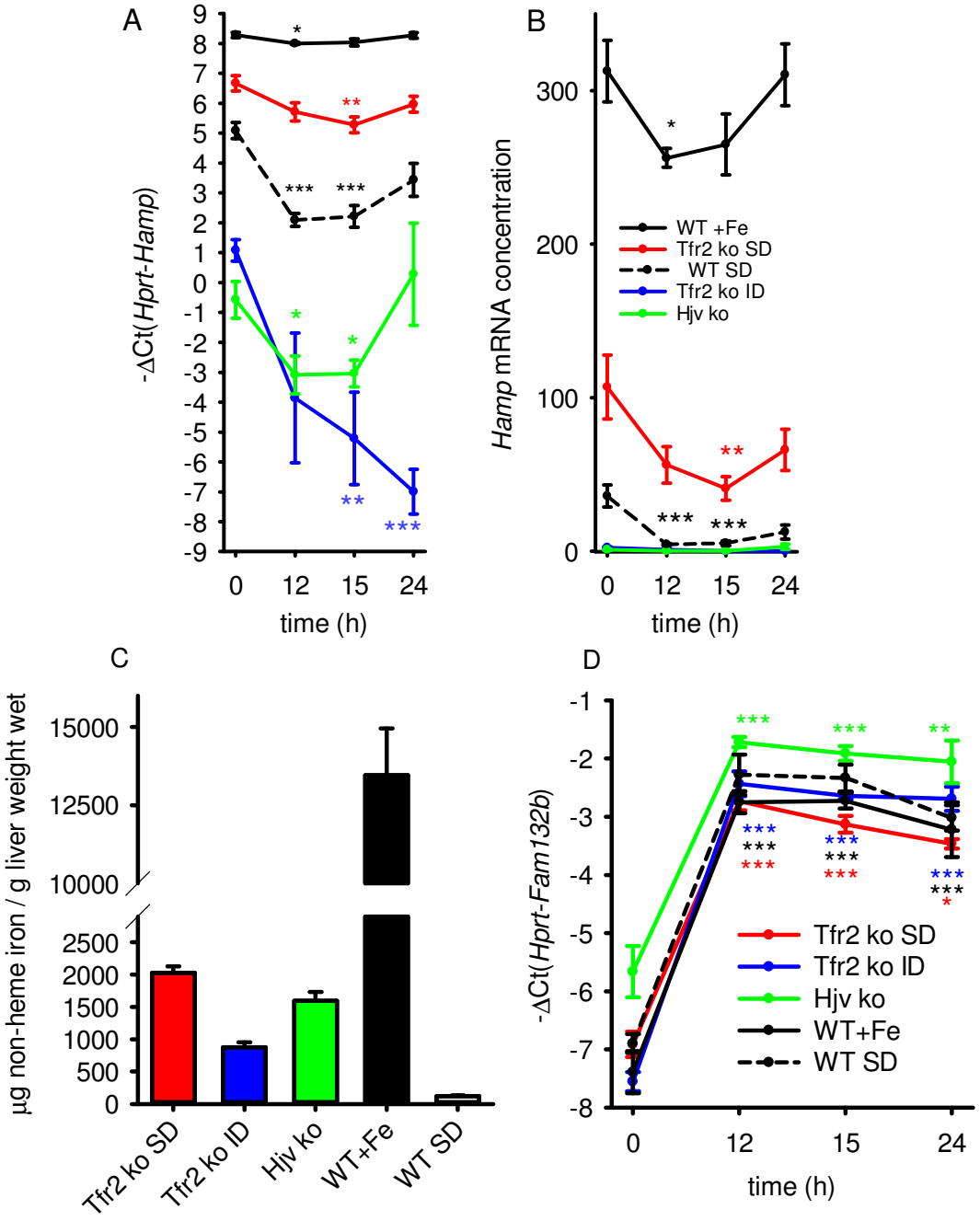
Transcript	Forward primer	Reverse primer
Mouse *Hprt*	CTG-GTT-AAG-CAG-TAC-AGC-CCC-AA	CAG-GAG-GTC-CTT-TTC-ACC-AGC
Mouse *Rpl4*	TGA-AAA-GCC-CAG-AAA-TCC-AA	AGT-CTT-GGC-GTA-AGG-GTT-CA
Mouse *Hamp*	TTG-CGA-TAC-CAA-TGC-AGA-AGA	GAT-GTG-GCT-CTA-GGC-TAT-GTT
Mouse *Fam132b*	ATG-GGG-CTG-GAG-AAC-AGC	TGG-CAT-TGT-CCA-AGA-AGA-CA
Mouse *Id1*	ACC-CTG-AAC-GGC-GAG-ATC-A	TCG-TCG-GCT-GGA-ACA-CAT-G
Mouse *Saa1*	AGT-CTG-GGG-TGC-TGA-GAA-AA	ATG-TCT-GTT-GGC-TTC-CTG-GT
Mouse *Gypa*	ATG-GCA-GGG-ATT-ATC-GGA-AC	CAC-CCT-CAG-GAG-ATT-GGA-TG
Human *HPRT*	CAG-CAG-TGA-GCT-CTT-CAC-CA	CAA-GAA-CAC-GGA-GGT-CCA-CT
Human *FAM132B*	GCC-CTG-GCG-TCG-TGA-TTA-GT	AGC-AAG-ACG-TTC-AGT-CCT-GTC



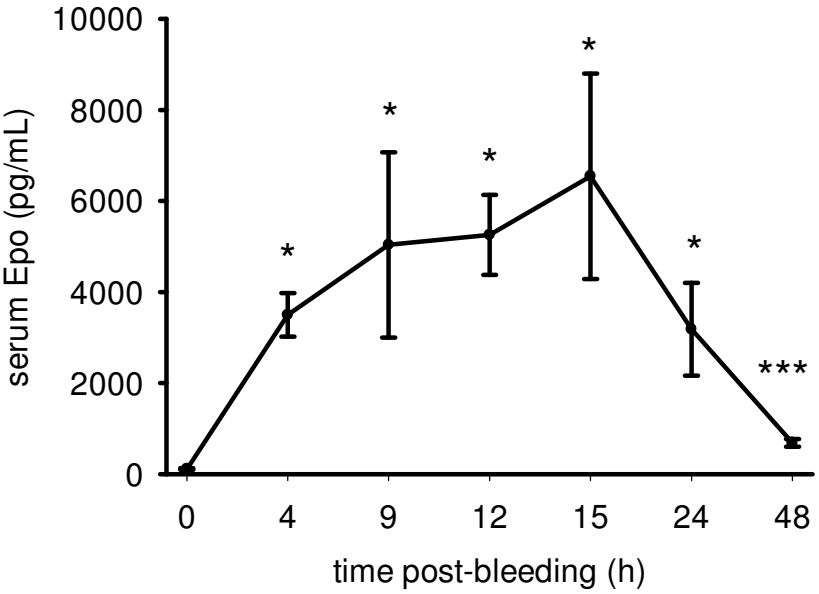
Supplementary Figure 1: Serum iron levels after erythropoietic stimulation. Changes in serum iron levels between paired samples from the initial phlebotomy and terminal analysis (blue line) **(A)** and serum iron concentrations after EPO injection (red line) (**B**) in 6 week-old C57BL/6 mice. No significant changes in serum iron concentrations were observed. Values shown are means ± standard deviation. For each time-point, means of serum iron changes were compared to the initial value (zero) (A) or serum iron concentrations were compared to untreated t=0 mice (B) by two-tailed Student t-test (n=4 per group).



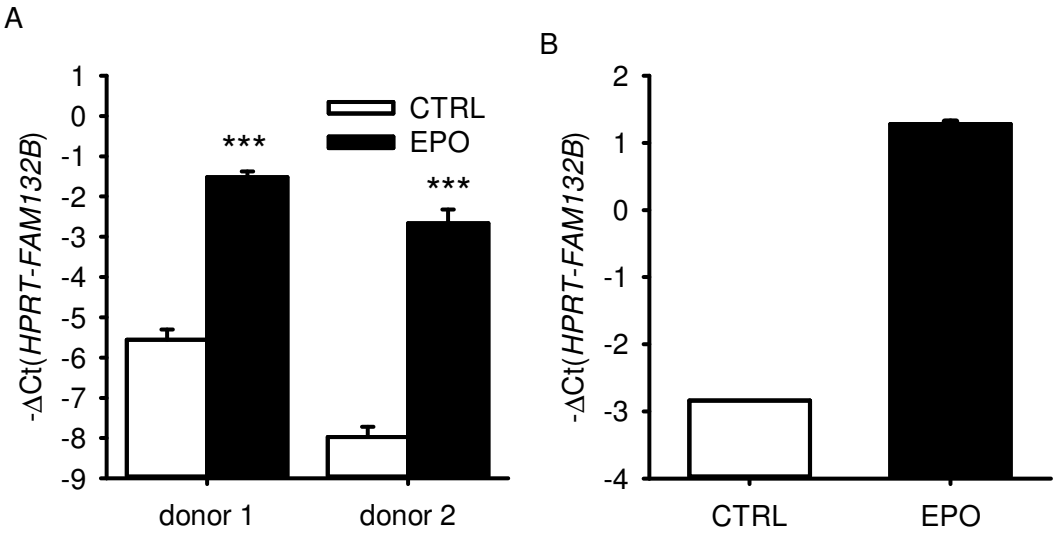
Supplementary Figure 2: Smad5 phosphorylation and *Id1* mRNA expression in response to erythropoietic stimulation. (A) Smad5 phosphorylation was compared by western blot in the livers of wild-type Sv129/C57BL/6 mice at 0 and 15 hours after phlebotomy. **(B)** Densitometric ratio of phosphorylated Smad5 to total Smad5 for phlebotomized mice. Means ± SEM are shown. **(C)** Smad5 phosphorylation was compared by western blot in the livers of wild-type C57BL/6 mice at 0 and 15 hours after EPO injection. **(D)** Densitometric ratio of phosphorylated Smad5 to total Smad5 for the EPO-treated mice. Means ± SEM are shown. **(E)** Inhibitor of DNA binding 1 (*Id1*) mRNA was measured in 6 week-old C57BL/6 wild-type males after phlebotomy (blue line) or EPO injection (red line) and was only slightly decreased within 9 hours after phlebotomy but not significantly after EPO injection. *Id1* mRNA levels were measured by qRT-PCR. Values shown are means ± SEM of -ΔCt (i.e., Ct *Hprt* – Ct *Id1*) and were compared for each time-point to the control mice by two-tailed Student t-test (n=4). *p<0.05.



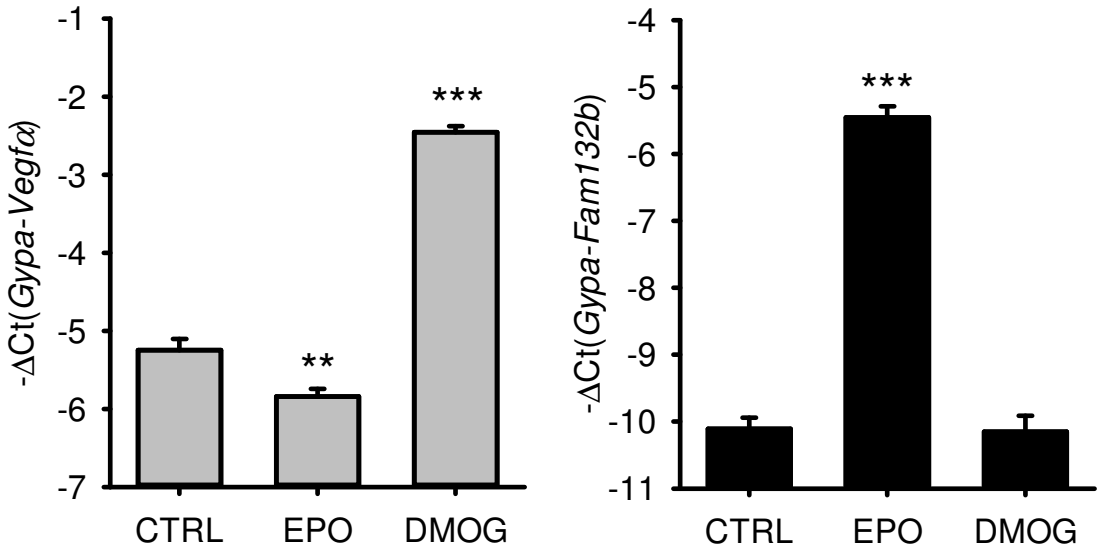
Supplementary Figure 3: Hepcidin suppression by increased erythropoietic activity is independent of the effect of the BMP pathway. (**A and B**) Hepcidin (*Hamp*) mRNA response to bleeding in 6 week-old males *Tfr2*^{Y245X} on standard diet 336 ppm Fe (Tfr2 ko SD, red line) or iron-deficient diet containing 4 ppm Fe for 2 weeks after weaning (Tfr2 ko ID, blue line), *Hjv*-deficient mice (Hjv ko, green line), 7 week-old C57BL/6 WT mice treated with 50 mg iron-dextran (WT+Fe, black line) and 6 week-old C57BL/6 WT mice (also shown in Figure 1) as a reference (dashed black line). (**A**) *Hamp* mRNA level is shown on a logarithmic scale as –ΔCt (i.e., Ct *Hprt* – Ct *Hamp*) measured by qRT-PCR. (**B**) Relative *Hamp* transcript abundance was calculated as $2^{-\Delta Ct(Hprt\ -\ Hamp)}$ and represented on a linear scale. (**C**) Liver iron content and (**D**) *Fam132b* mRNA levels in the bone marrow were measured in the same groups of mice. Values shown are means ± SEM and were compared for each time-point to the control t=0 mice by two-tailed Student t-test (n=4 mice per group and time point). ***p<0.001, **p<0.01, *p<0.05.



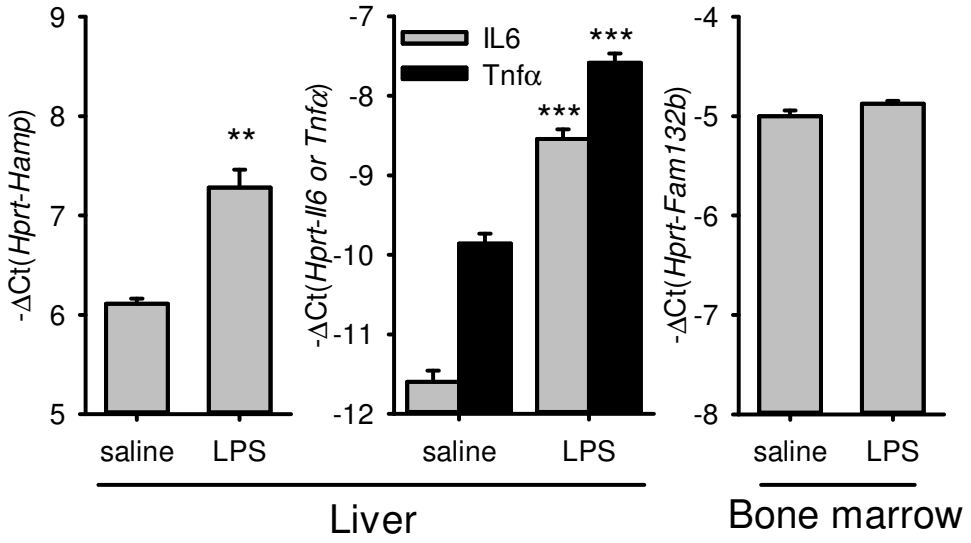
Supplementary Figure 4: Serum erythropoietin levels after erythropoietic stimulation. Serum Epo levels were measured in 6 week-old C57BL/6 wild-type males after phlebotomy and in control mice. Epo levels are rapidly increased within 4 hours and start to decrease after 15 hours. Values shown are means ± SEM. Means of serum Epo values were compared for each time-point to the t=0 control mice by two-tailed Student t-test (n=4 mice per group and time point). ***$p<0.001$, *$p<0.05$.



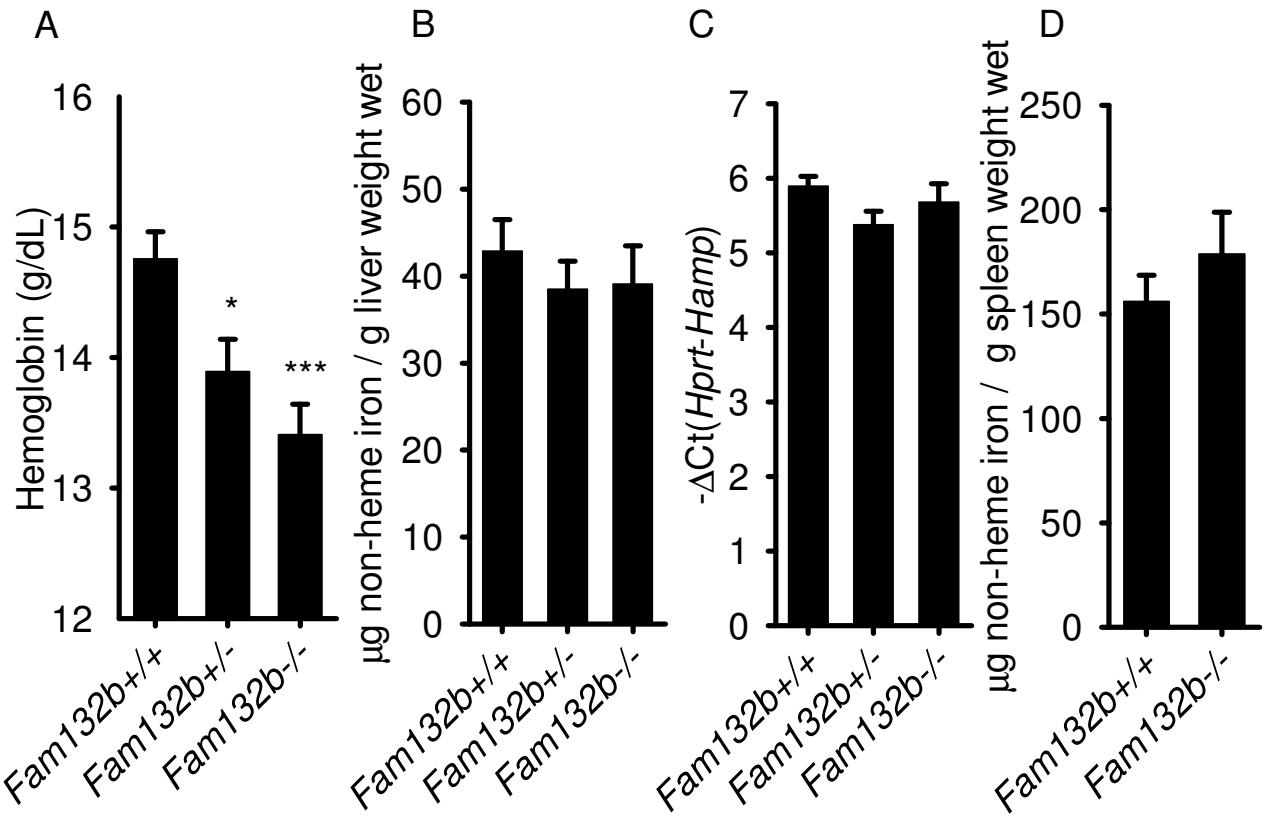
Supplementary Figure 5: Erythroferrone is induced by EPO in human erythroblasts. *FAM132B* mRNA expression was induced between 16 to 40-fold in human fetal erythroblasts (**A**) and 16-fold in erythroblasts from human adult bone marrow (**B**) 15 hours after treatment with erythropoietin (10 U/mL). Cells from three different donors were analyzed individually. Mean values ± SEM of 6 (donor 1), 3 (donor 2) or 2 (bone marrow) replicates where –ΔCt (i.e., Ct *HPRT*- Ct *FAM132B*) were compared between untreated and treated cells by two-tailed Student t-test. ***p<0.001



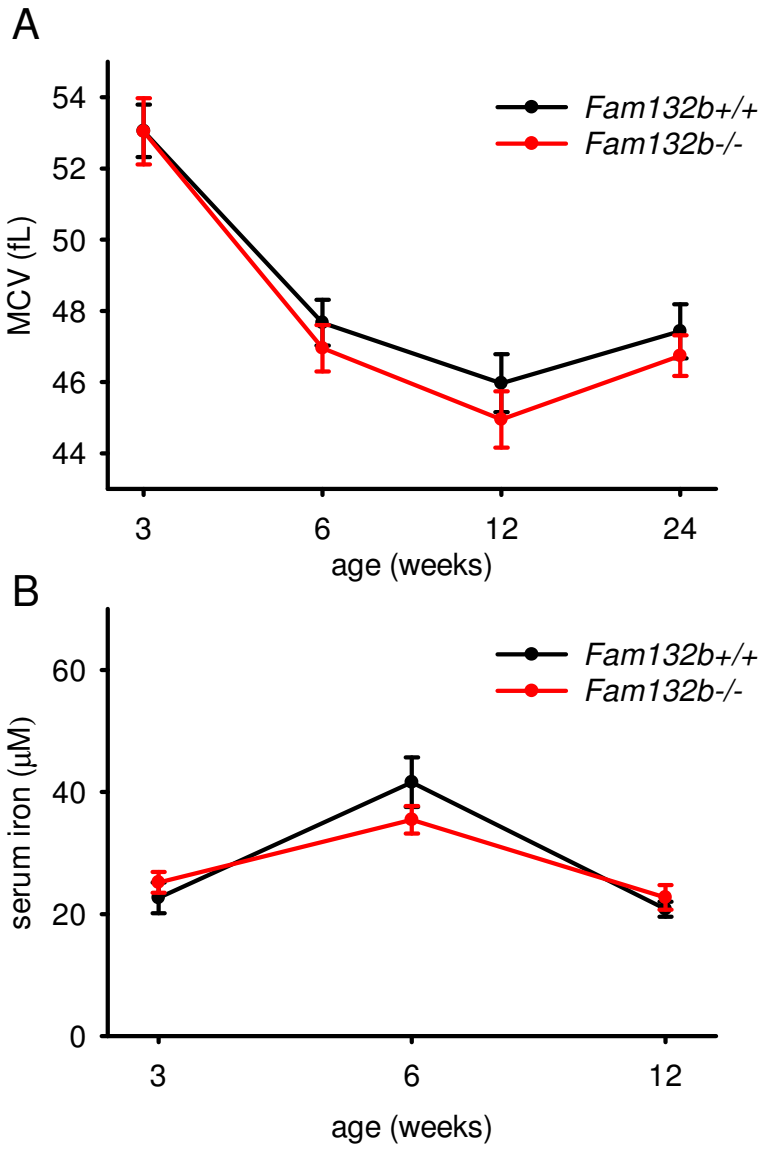
Supplementary Figure 6: Erythroferrone *Fam132b* mRNA expression is not influenced by the hypoxia pathway. Vascular endothelial growth factor α (*Vegfα*) and *Fam132b* mRNA expression were compared in mouse bone marrow 15 hours after treatment with 10 U/mL EPO or 1 mM DMOG (Dimethyloxalylglycine, N-(Methoxyoxoacetyl)-glycine methyl ester). As *Vegfα* mRNA expression (a marker of hypoxia) was induced 15 hours after treatment with DMOG, *Fam132b* mRNA levels remained unchanged. EPO treatment is shown as a positive control to show the cells responsiveness. Mean values ± SEM of –ΔCt (i.e., Ct *Gypa* - Ct *Vegfα or Fam132b*) were compared between control and EPO or DMOG treated cells by two-tailed Student t-test. Results are shown from three independent experiments, each done in triplicate. ***p<0.001, **p<0.01.



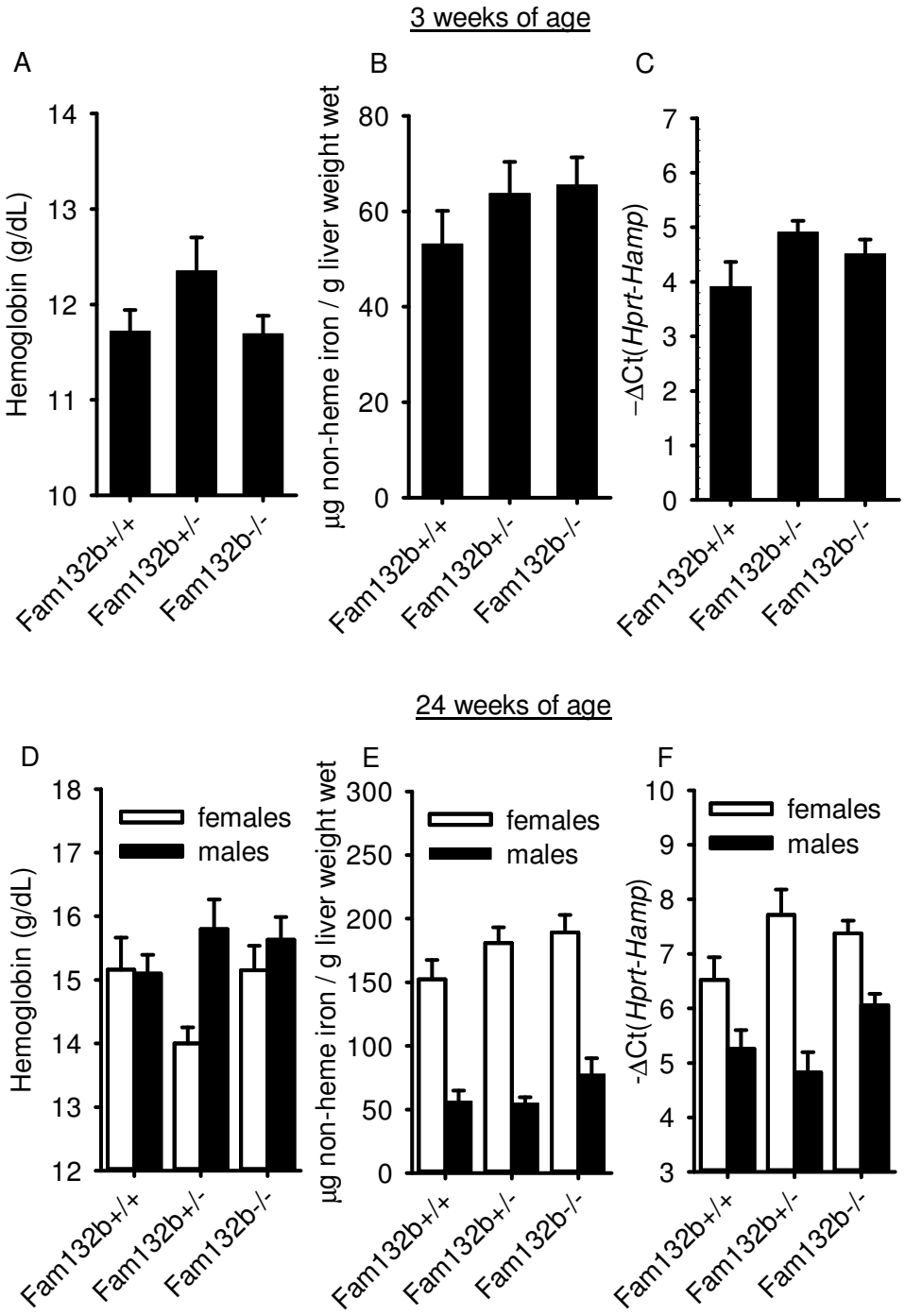
Supplementary Figure 7: Erythroferrone *FAM132B* mRNA expression is not influenced by systemic inflammation. Liver *Hamp*, *Il-6* and *Tnfα,* and bone marrow *Fam132b* mRNA levels in 6 week-old C57BL/6 mice (n=3 mice per group) were compared between mice injected with saline or with LPS (Sigma 055:B5, 1µg/g) 4 hours after treatment. As hepcidin, *Il-6* and *Tnfα* mRNA expression was induced 4 hours after LPS injection, bone marrow *Fam132b* levels remained unchanged. Mean values ± SEM of –ΔCt (i.e., Ct *Hprt* - Ct *Hamp, Il-6, Tnfα or Fam132b*) were compared between saline and LPS treated mice by Student t-test. ***p<0.001, **p<0.01.



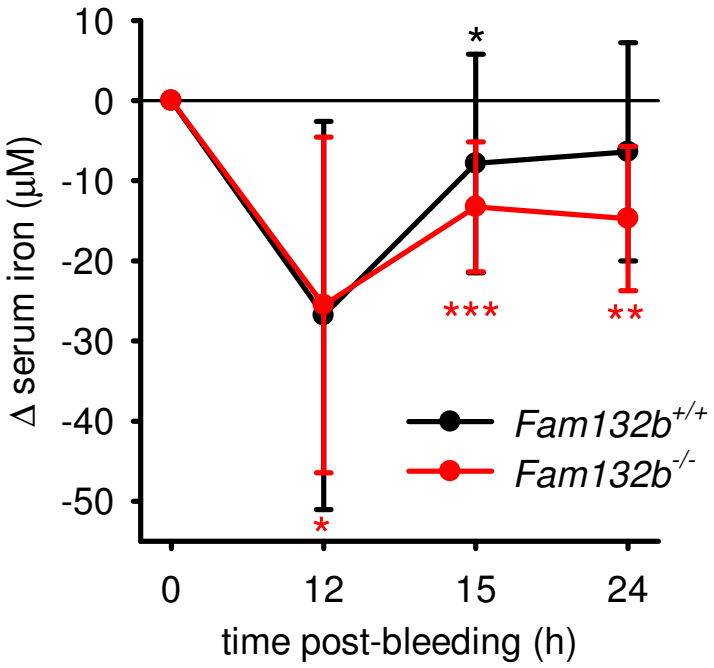
Supplementary Figure 8: 6-week old ERFE-deficient mice have normal iron stores and hepcidin expression but have reduced hemoglobin levels compared to wild-type mice. Hemoglobin (**A**), liver iron (**B**) and liver hepcidin (*Hamp*) mRNA levels (**C**) were measured in 6 week-old *Fam132b*$^{+/+}$, *Fam132b*$^{+/-}$ and *Fam132b*$^{-/-}$ littermates. Spleen iron levels (**D**) were compared between *Fam132b*$^{+/+}$ and *Fam132b*$^{-/-}$ mice. The graphs show means ± SEM. Because no gender differences were observed, the genders were combined for each parameter. All comparisons were done by two-tailed Student t-test (n=11 to 14). ***p<0.001, *p<0.05



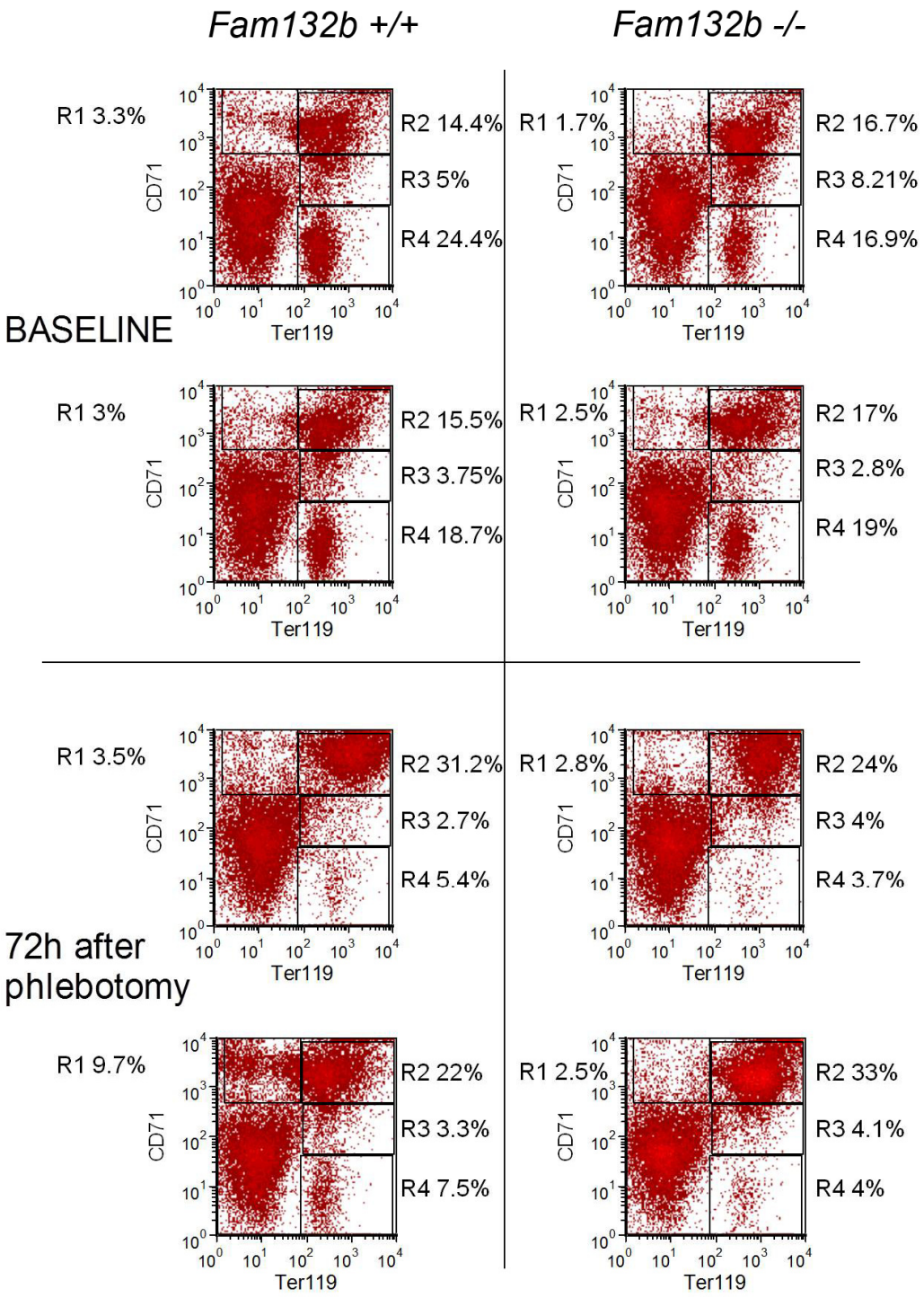
Supplementary Figure 9: ERFE-deficient mice have normal mean corpuscular volume (MCV) and serum iron concentrations. MCV (**A**) and serum iron concentrations (**B**) were compared at various ages in *Fam132b*$^{-/-}$ mice (red line) and wild-type mice (black line). The graphs show mean values ± SEM, with n= 8-16 mice per time point and genotype. No statistically significant difference was observed between WT and KO by Student t-test.



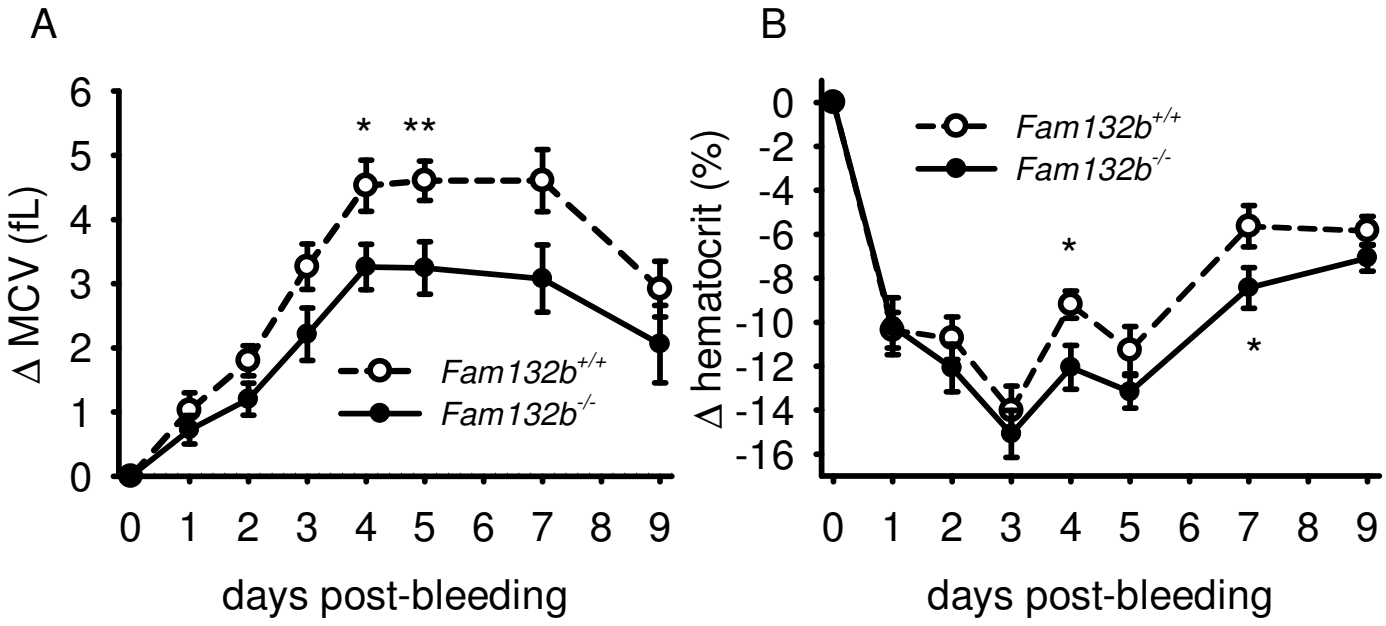
Supplementary Figure 10: ERFE-deficient mice have normal hemoglobin, iron stores and hepcidin at 3 and 24 weeks of age. Hemoglobin (**A, D**), liver iron (**B, E**) and liver hepcidin (*Hamp*) mRNA levels (**C, F**) were measured in 3 week-old (**A, B, C**) and 6 month-old (**D, E, F**) *Fam132b*$^{+/+}$, *Fam132b*$^{+/-}$ and *Fam132b*$^{-/-}$ littermates. No significant differences were observed between genotypes. Values shown for hepcidin expression are means ± SEM of -ΔCt (i.e., Ct *Hprt* – Ct *Hamp*). Mean values in hemoglobin, liver iron and hepcidin levels were compared between each genotype by two-tailed Student t-test. In the absence of gender differences at 3 weeks of age, the genders were combined for each parameter (n=9 to 12). Gender-separated study was performed at 6 months of age as females have higher liver stores than males (n=3 to 11).



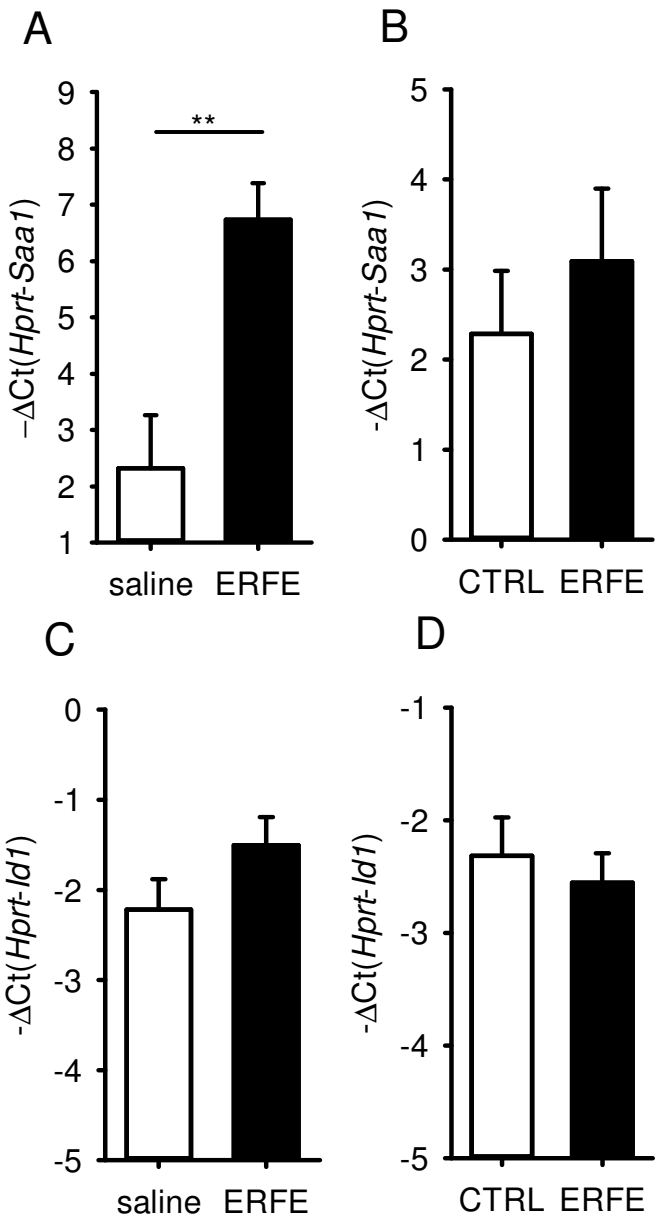
Supplementary Figure 11: Serum iron concentrations in ERFE-deficient mice in response to phlebotomy. Changes of serum iron from the initial phlebotomy levels to the end time point were compared for each mouse in *Fam132b*$^{+/+}$ (black line) and *Fam132b*$^{-/-}$ (red line) littermates (n=8 to 14 per genotype and time-point). Serum iron levels were significantly decreased at all time points in *Fam132b*$^{-/-}$ mice but not in *Fam132b*$^{+/+}$ confirming that the lack of hepcidin suppression after phlebotomy leads to impaired iron regulation. Values shown are means ± standard deviation. Means of serum iron values were compared for each time-point to the control mice by two tailed Student t-test. ***p<0.001, **p<0.01, *p<0.05



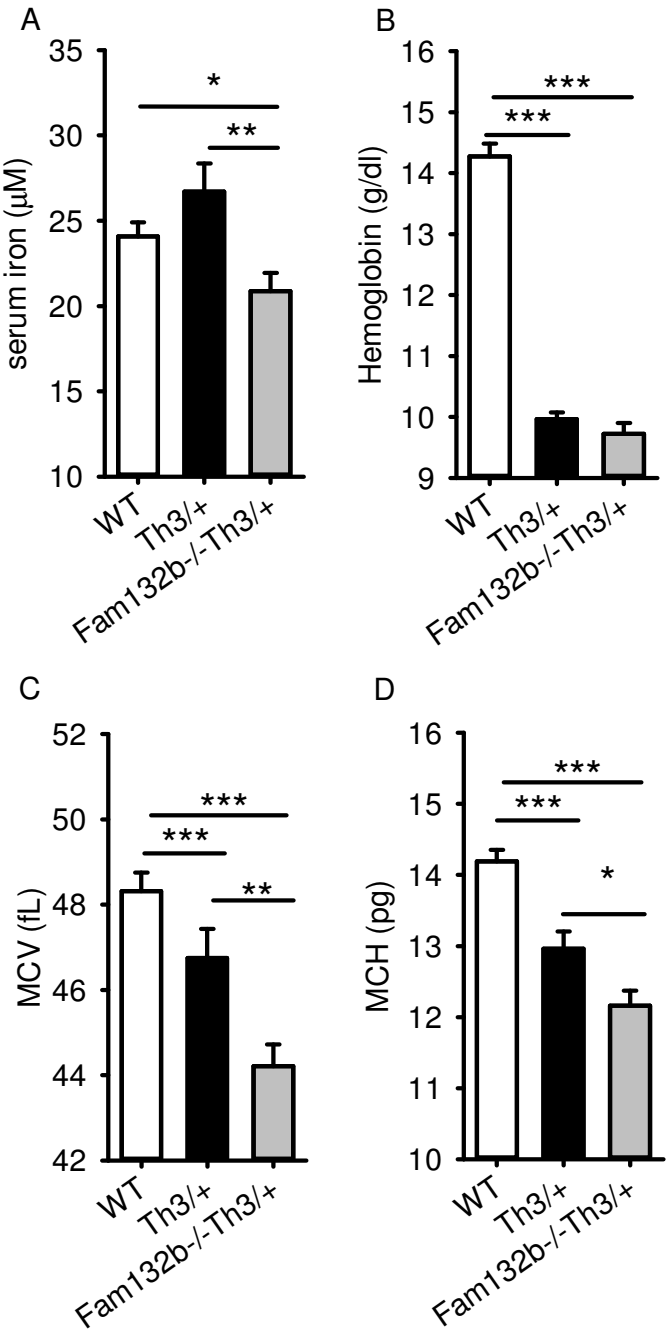
Supplementary Figure 12: ERFE is not required for erythroblast differentiation during stress erythropoiesis. Mouse bone marrow erythroblasts of *Fam132b*$^{+/+}$ and *Fam132b*$^{-/-}$ mice were analyzed by flow cytometry in control mice and mice 72h after phlebotomy (2 mice per group are shown). No significant difference in erythroblasts differentiation was observed between *Fam132b*$^{+/+}$ and *Fam132b*$^{-/-}$ mice. Flow cytometry data were analyzed using FCS express software (DeNovo software).



Supplementary Figure 13: ERFE-deficient mice show lower MCV and hematocrit during recovery from anemia. Eight week-old phlebotomized *Fam132b*$^{-/-}$ mice (solid line) had lower mean corpuscular volume (MCV) (**A**) and hematocrit (**B**) compared to wild-type mice (dashed line). Hematological parameters (**A, B**) were compared for each measurement between WT and KO by two tailed Student t-test (n=15 to 17). The graphs show means ± SEM. In the absence of gender differences, the genders were combined for each parameter. **p<0.01, *p<0.05. See also Figure 4 in the manuscript.



Supplementary Figure 14: *Saa1* and *Id1* expression in ERFE-treated mice. Liver *Saa1* and *Id1* mRNA (**A, C**) expression was measured in mice treated with recombinant ERFE (n=6) or saline (n=6) for 15 hours. Liver *Saa1* and *Id1* mRNA (**B, D**) was measured in mice expressing ERFE-lentivirus (n=8) or control lentivirus (n=7) for 3 weeks. Mean values ± SEM of –ΔCt (i.e., Ct *Hprt* - Ct *Id1 or Saa1*) were compared between control and ERFE-treated mice by two-tailed Student t-test.



Supplementary Figure 15: Ablation of ERFE in thalassemic mice decreases serum iron, erythrocyte mean corpuscular volume and mean corpuscular hemoglobin. Serum iron (**A**), hemoglobin (**B**), MCV (**C**) and MCH (**D**) levels were determined in littermate wild-type (n=12), *Hbb*$^{Th3/+}$ (n=12) and *Fam132b*$^{-/-}$, *Hbb*$^{Th3/+}$ (n=18) mice at 6 weeks of age. Consistent with decreased iron overload, lower serum iron, MCV and MCH levels were observed in *Fam132b*$^{-/-}$, *Hbb*$^{Th3/+}$ mice compared to their *Hbb*$^{Th3/+}$ counterparts. Hemoglobin concentration was similar between *Fam132b*$^{-/-}$, *Hbb*$^{Th3/+}$ and *Hbb*$^{Th3/+}$ mice. Mean values ± SEM in serum iron, hemoglobin, MCV and MCH levels were compared between each genotype by two-tailed Student t-test.