

Transient decrease of serum iron after acute erythropoietin treatment contributes to hepcidin inhibition by ERFE in mice

Irene Artuso,^{1,#} Mariateresa Pettinato,^{1,2} Antonella Nai,^{1,2} Alessia Pagani,¹ Ugo Sardo,³ Benjamin Billoré,³ Maria Rosa Lidonnici,⁴ Cavan Bennett,⁵ Giacomo Mandelli,⁴ Sant-Rayn Pasricha,^{5,6} Giuliana Ferrari,⁴ Clara Camaschella,¹ Léon Kautz³ and Laura Silvestri^{1,2}

¹Regulation of Iron Metabolism Unit, Division of Genetics and Cell Biology, IRCCS San Raffaele Scientific Institute, Milan, Italy; ²Vita-Salute San Raffaele University, Milan, Italy; ³IRSD, Université de Toulouse, INSERM U1220, INRA U1416, ENVT, UPS, France; ⁴SR-Tiget Unit, IRCCS San Raffaele Scientific Institute, Milan, Italy; ⁵The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia and ⁶Department of Medical Biology, The University of Melbourne, VC, Australia

IA and MP contributed equally to this work

[#]Present address: Cellular and Molecular Immunology, Department of Biotechnology and Biosciences, University of Milano-Bicocca, Milano, Italy

Correspondence: LAURA SILVESTRI - silvestri.laura@hsr.it
doi:10.3324/haematol.2018.199810

Supplemental Data

Methods

Mice treatment

Wild type (WT) C57BL/6N male mice 8-9 weeks old were from Charles River. *Erfe*^{-/-} o C57BL/6J background were bred and housed in a specific-pathogen-free barrier facility in the animal facilities of INSERM US006. Control WT C57BL/6J mice were obtained from Janvier Laboratories at 5 wk of age and housed with the *Erfe*^{-/-} mice until the age of 8-9 weeks. Mice were housed under a standard 12-hour light/dark cycle with water and standard laboratory mouse chow diet (250 mg iron/kg) ad libitum in a pathogen-free animal facility of San Raffaele Scientific Institute or in the animal facilities of INSERM US006, in accordance with the European Union guidelines. The study was approved by the San Raffaele Institutional Animal Care and Use and the Midi-Pyrénées Animal Ethics Committee. A single dose of 200 U/mouse EPO (Epoetin alpha, Eprex, Janssen) or saline was administered by intraperitoneal injections. Mice were sacrificed at 3, 6, 9 and 15 hrs post-injection, anesthetized and sacrificed by cervical dislocation. For human Holo-transferrin (hHoloTF) treatment, C57BL/6N male mice, at 8-9 weeks of age, were injected with 200 U/mouse EPO and one hour later 300 mg/kg hHoloTF (kindly provided by Marieke von Lindern, Sanquin, The Netherlands), or saline, was administered by intravenous injection. Mice were sacrificed after 6, 9 and 15 hrs post-EPO. All efforts were made to minimize suffering. Blood was collected by intracardiac puncture. Spleen was dissected and immediately snap-frozen for RNA or erythropoiesis analysis. Bone marrow cells were isolated by flushing of femurs and snap-frozen for RNA isolation or erythropoiesis characterization. Liver was dissected and immediately snap-frozen for RNA isolation. Samples of liver and spleen were dried for iron quantification.

Analysis of iron parameters

Blood was collected at sacrifice and processed for serum preparation. Serum iron, transferrin saturation, liver and spleen iron concentration were measured as previously described¹².

Flow cytometry

BM and spleen cells isolated from C57BL/6N animals were resuspended in MACS Buffer (Milteny Biotech), pre-treated with rat-anti mouse CD16/CD32 (BD Pharmingen) to block unspecific Ig binding, and subsequently stained with BV421 Rat Anti-Mouse TER-119 (BD Biosciences), PE Rat Anti-Mouse CD71/TFR1 (BD Pharmingen) and APC rat anti-mouse CD44 (BD Biosciences) for 30 min in the dark at 4°C, as described in Wang et al., PNAS 2009. Samples were acquired at FACS CantoTMII and analyzed with FCS express. For experiments involving *Erfe*^{-/-} mice, BM and spleen cells from C57BL/6J wild type and KO mice were resuspended in PBS 5% FBS, pre-treated with rat-anti mouse CD16/CD32 (BD Pharmingen), and stained with FITC Rat Anti-Mouse TER-119 (BD Biosciences), PE Rat Anti-Mouse CD71/TFR1 (BD Pharmingen) and APC rat anti-mouse CD44 (BD Biosciences) for 30 min in the dark at 4°C. Samples were acquired on a MACS-Quant 10 (Milteny Biotech) and analyzed with FCS express.

Quantitative Real Time PCR

Total RNA was isolated using the UPzol reagent (Biotechrabbit, Düsseldorf, Germany) or the RNeasy kit (Qiagen), following manufacturer's instructions. cDNA was synthesized with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Massachusetts, USA) or with the MMLV-RT (Promega) for *Erfe*^{-/-} mice. Gene expression levels were measured by quantitative real-time PCR using the SybrGreen PCR Master Mix (Applied Biosystems) or the TaqMan Gene Expression Master Mix (Applied Biosystems). *Hprt1* or *Gapdh* were used as housekeeping genes. Changes in the expression levels were represented as $-\Delta\Delta Ct$ (saline-EPO). Quantitative PCR (qPCR) reactions involving *Erfe*^{-/-} mice were prepared with LightCycler[®] 480 DNA SYBR Green I Master reaction mix (Roche Diagnostics) and run in duplicate on a LightCycler[®] 480 System (Roche Diagnostics). *HAMP* mRNA transcript abundance was normalized to the reference gene *HPRT*. Primers used for qRT-PCR are listed in **Supplemental Table 1** and **2**.

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation was performed on snap frozen liver samples as reported⁵. Briefly, frozen samples were minced and fixed in 1% formaldehyde followed by quenching with glycine, washing, douncing (10 strokes), lysing in lysis buffer (1% SDS, 10mM EDTA, 50mM Tris) and subsequently sonicated. Aliquots of sonicated cells were diluted in dilution buffer (0.01% SDS, 1.1% TritonX-100, 1.2mM EDTA, 16.7mM Tris-HCl, 165mM NaCl) and pre-cleared using mixed Protein A/G beads (ThermoFisher Scientific). After 16 hrs incubation at 4°C with relevant antibodies against H3k9ac and H3k4me3 samples were incubated 5 hours with Protein A/G beads at 4°C and then washed 5 times with standard RIPA buffer. Chromatin elution was achieved by heating at 65°C in an elution buffer (50mM Tris-HCl, 10mM EDTA, 1% SDS) for 30 minutes, after which DNA was decrosslinked overnight. Quantitative PCR (qPCR) was performed by using specific primers to amplify sections of genomic DNA of interest (**Figure 3A**) and SensiFAST SYBR-Green Mastermix (Bioline) to measure relative enrichment of sample relative to input (% input). A genomic region known to be enriched for the relevant mark was run as a positive control (the promoter of *Hprt* for H3K9ac and H3K4me3) and a negative control primer in a gene desert was also assessed to exclude non-specific binding. Results in regions of interest were normalized to positive controls to improve comparability between replicates (%input/% *Hprt* input). Primer sequences are in **Supplemental Table 3**.

Statistical analysis

One-way ANOVA, combined with the non-parametric analysis (Kruskal-Wallis test), or two-way ANOVA were used for statistical significance calculation. $P < 0.05$ in a 2-tailed test was considered statistically significant. For Chromatin immunoprecipitation assays, three-way ANOVA with Tukey's correction for multiple comparisons was used for significance calculation. Statistical analysis was performed using GraphPad Prism 5.0 or 7.0 (GraphPad).

Supplemental Table 1. Oligonucleotide primers used for qRT-PCR by TaqMan

Gene	Transcript Assay Id
<i>Hamp</i>	Mm00519025_m1
<i>Id1</i>	Mm00775963_m1
<i>Smad7</i>	Mm03023958_m1
<i>Atoh8</i>	Mm00464055_m1
<i>Bmp6</i>	Mm01332882_m1
<i>Tfr2</i>	Mm00443703_m1
<i>Hprt1</i>	Mm01318743_m1

Supplemental Table 2. Oligonucleotide primers used for qRT-PCR by SybrGreen

Gene	Forward primer	Reverse primer
<i>Erfe</i>	atggggctggagaacagc	tggcattgtccaagaagaca
<i>Tfr1</i>	ccaagtattctcagatatgattca	cagtcagctggcaaagattat
<i>Gypa</i>	ttatcacacggcccctactg	gttgaattggtgacggcatt
<i>Hamp</i>	aagcagggcagacattgcat	caggatgtggctctaggctatgt
<i>Id1</i>	acctgaacggcgagatca	tcgtcggctggaacacatg
<i>Smad7</i>	gcaggctgtccagatgctgt	gatccccaggctccagaaga
<i>Atoh8</i>	cagaagggcgagccaagaaacgg	ctggtggctcccagctttctctca
<i>Tfr2</i>	tacaacgtgcgcatcatgagg	gagtcggctggcgacaca
<i>Hprt1</i>	ctggttaagcagtacagcccaa	caggaggtcctttcaccagc
<i>Gapdh</i>	tccactcacggcaaattcaa	tttgatgtagtggggctctcg

Supplemental Table 3. Oligonucleotide primers used for chromatin immunoprecipitation

Oligos name	Forward primer	Reverse primer
<i>Hamp 600 bp</i>	gcattggctctgcctatgat	cgtggagaccactgtgaaga
<i>Hamp exon 1</i>	accacctatctccatcaacagg	ccatcactcctgagccattc
<i>Hprt</i>	tctagaaggagcttcggtcc	tggatctgcagaattaggg
<i>negative</i>	ataaaggcttgactcgtc	cagttccctttgcttgatcc

Legend to Supplemental Figures

Supplemental Figure 1. Time course analysis of the effect of a single EPO injection on *Erfe*, *hepcidin*, BMP-SMAD target genes and circulating iron in C57BL/6N wild type mice. C57BL6/N wild type male mice were treated with EPO (200 U/mouse) or saline (8-18 mice for each time point) and sacrificed at different time points. *Erfe* was measured by qRT-PCR in bone marrow (A) and spleen (B). Liver expression of hepcidin (*Hamp*) (C) and the BMP-SMAD target genes *Id1* (D) and *Smad7* (E) was analyzed by qRT-PCR. *Gapdh* (A, B) or *Hprt1* (C, D, E) were used as housekeeping genes. Changes in the expression levels were represented as $-\Delta\Delta Ct$ (saline-EPO). Data are shown as means \pm s.e.m. Serum iron (F) and transferrin saturation (TS; G) were analyzed in C57BL/6N wild type mice (7-16 mice for each time point) treated with a single EPO injection (200 U/mouse) and expressed as a difference between the EPO-treated and the saline treated values. Data are shown as means \pm s.e.m. The one-way ANOVA combined with a non-parametric analysis (Kruskal-Wallis test) was used for significance calculation (**: $P < .01$; ***: $P < .001$). Two-tailed Student t-test was used to calculate statistically significant differences of EPO treatment at different time points compared to $t=0$; ns: non significant; #: $P < .05$; ##: $P < .01$; ###: $P < .001$; ####: $P < .0001$. BM: bone marrow; SP: spleen.

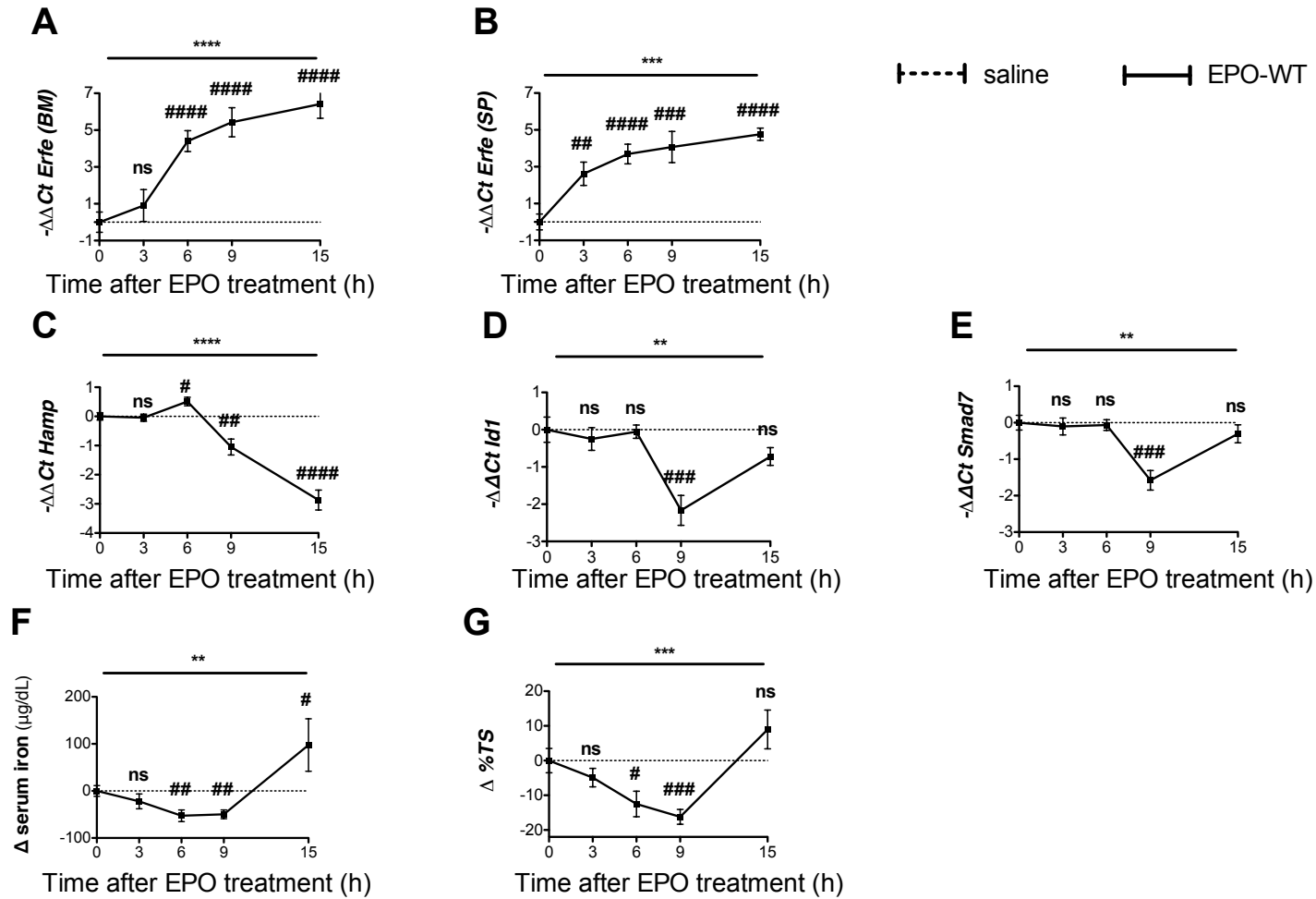
Supplemental Figure 2. Chromatin modifications at the hepcidin promoter locus. Diagram of the hepcidin genetic locus [downloaded from ENCODE (<https://www.encodeproject.org/>)] demonstrating the position of the amplified regions (-600 bp and exon1) used for subsequent qPCR analysis of ChIP experiments (Figure 1).

Supplemental Figure 3. Analysis of bone marrow and spleen erythroid populations in EPO-treated C57BL/6N wild type mice. C57BL/6N wild type animals (3 mice for each time point) were treated with EPO (200 U/mouse) or saline, sacrificed at 3 and 6 hrs and analyzed for bone marrow (BM) (A) and spleen (SP) (B) erythroid differentiation. Cells were isolated and analyzed for Ter119/CD44 expression. Ter119 was gated and analyzed with respect to FSC

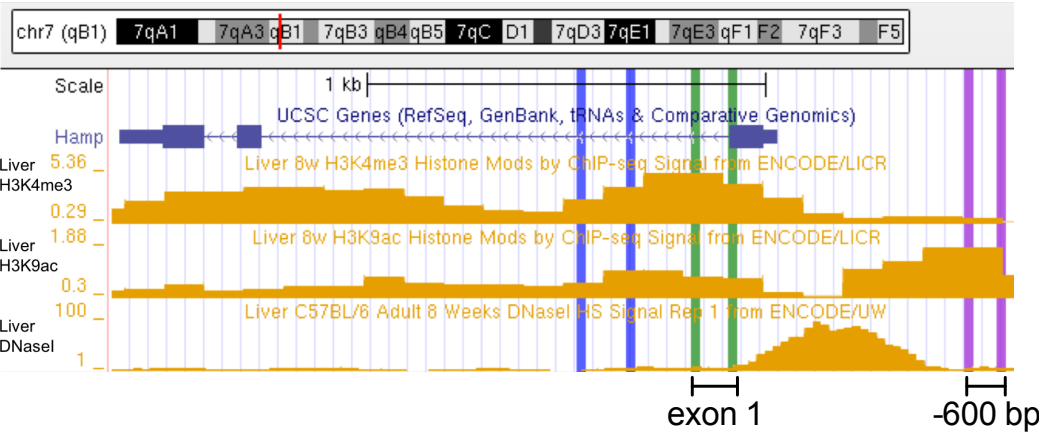
and CD44 surface expression for subpopulation composition (gated cluster I-V). The percentage of erythroid cell populations in the BM (A) and spleen (B) was shown.

Supplemental Figure 4. Analysis of bone marrow erythroid populations and TFR1 surface expression of EPO-treated *Erfe*^{-/-} and WT mice. A) Representative gating strategy for analysis of Ter119 subpopulations in C57BL/6J wild type and *Erfe*^{-/-} mice. Ter119⁺ cells were gated and further analyzed with respect to FSC and CD44 surface expression for subpopulation composition (gated cluster I-V). B) The percentage of bone marrow erythroid subpopulation in saline and EPO injected mice was shown. C) *Erfe*^{-/-} and C57BL/6J wild type mice (3 mice for each time point) were treated with EPO (200 U/mouse) or saline and sacrificed at 6 hrs post-injection. Surface CD71/TFR1 expression in the BM-derived erythroid populations was evaluated as mean fluorescent intensity (MFI). Unpaired two-tailed Student t-test was used for significance calculation. ns: non significant; *: p<0.05.

Supplemental Figure 1

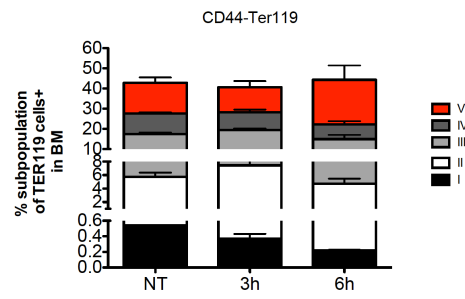


Supplemental Figure 2

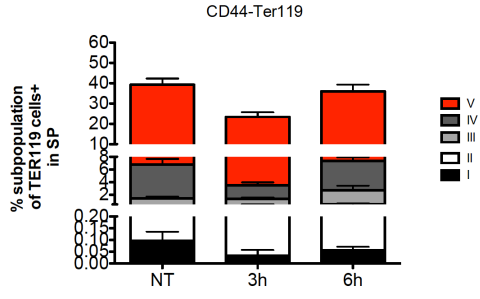


Supplemental Figure 3

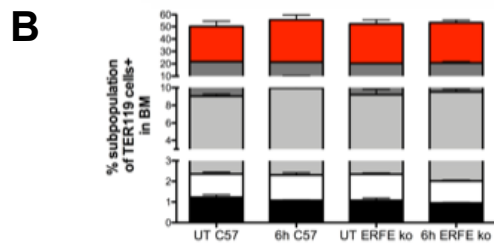
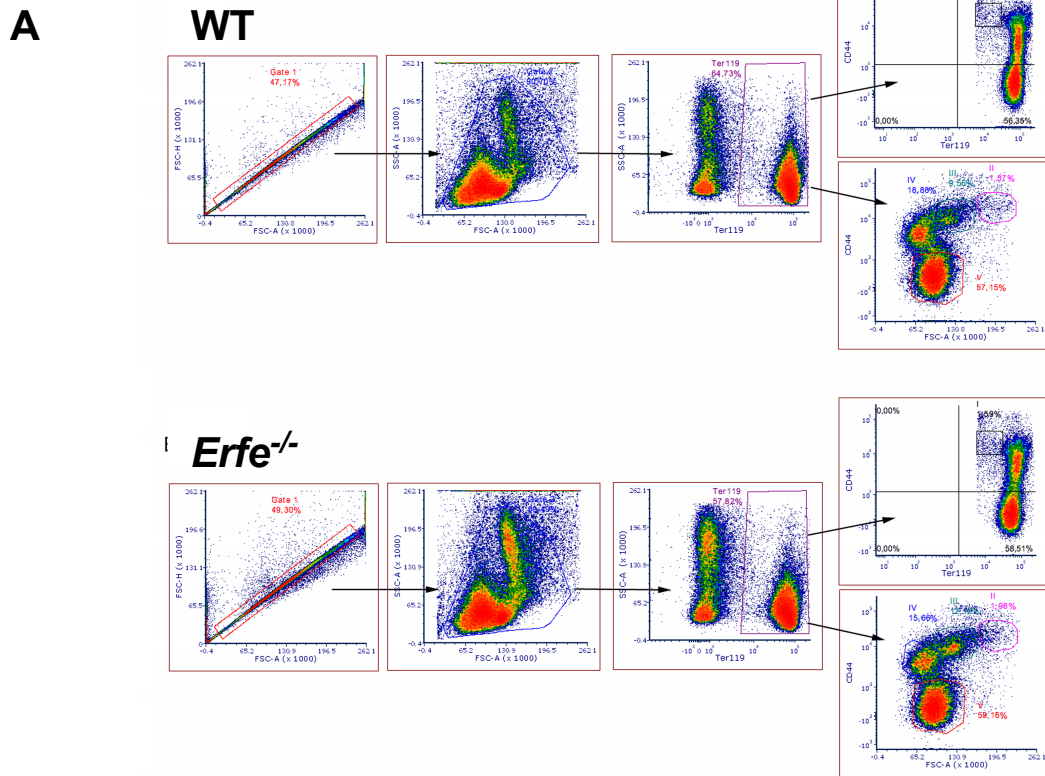
A



B



Supplemental Figure 4



C

	Subpopulations	UT	6hrs	6hrs vs UT
WT	I	15704 ± 2392	24650 ± 1749	*
	II	20618 ± 547.9	26831 ± 655.8	*
	III	23012 ± 752.3	30720 ± 3474	ns
	IV	10978 ± 953.8	15545 ± 2246	*
	V	7648 ± 368.6	7268 ± 545.7	ns
Erfe KO	I	12889 ± 1245	22589 ± 2753	*
	II	15698 ± 968	28650 ± 3408	*
	III	14646 ± 763.2	33355 ± 4686	*
	IV	8104 ± 518.1	21128 ± 2584	*
	V	6920 ± 124.7	7892 ± 468	ns