ARTICLE

NCOA4-mediated ferritinophagy in macrophages is crucial to sustain erythropoiesis in mice

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ABSTRACT

Nuclear receptor coactivator 4 (NCOA4) promotes ferritin degradation and *Ncoa4-ko* mice in a C57BL/6 background show microcytosis and mild anemia, aggravated by iron deficiency. To understand tissue-specific contributions of NCOA4-mediated ferritinophagy we explored the effect of *Ncoa4* genetic ablation in the iron-rich Sv129/J strain. Increased body iron content protects these mice from anemia and, in basal conditions, Sv129/J *Ncoa4-ko* mice show only microcytosis; nevertheless, when fed a low-iron diet they develop a more severe anemia compared to that of wild-type animals. Reciprocal bone marrow (BM) transplantation from wild-type donors into *Ncoa4-ko* and from *Ncoa4-ko* into wild-type mice revealed that microcytosis and susceptibility to iron deficiency anemia depend on BM-derived cells. Reconstitution of erythropoiesis with normalization of red blood count and hemoglobin concentration occurred at the same rate in transplanted animals independently of the genotype. Importantly, NCOA4 loss did not affect terminal erythropoiesis in iron deficiency, both in total and specific BM *Ncoa4-ko* animals compared to controls. On the contrary, upon a low iron diet, spleen from wild-type animals with *Ncoa4-ko* BM displayed marked iron retention compared to (wild-type BM) controls, indicating defective macrophage iron release in the former. Thus, erythropoietin administration failed to mobilize iron from stores in *Ncoa4-ko* animals. Furthermore, *Ncoa4* inactivation in thalassemic mice did not worsen the hematologic phenotype. Overall our data reveal a major role for NCOA4-mediated ferritinophagy in macrophages to favor iron release for erythropoiesis, especially in iron deficiency.

Introduction

Nuclear receptor coactivator 4 (NCOA4), originally identified as an androgen receptor interactor,[1] is a novel player in iron metabolism contributing to the regulation of cell and systemic iron homeostasis. NCOA4 acts as a cargo receptor that promotes "ferritinophagy", the selective autophagy-mediated degradation of the iron storage protein ferritin.[2,3] This process is induced in conditions of iron deficiency to facilitate iron recovery from intracellular stores.[4,5]

As expected, inactivation of *Ncoa4* increases cell ferritin aggregates[2-4] and C57BL/6 *Ncoa4*-knockout (*ko*) mice show ferritin and iron accumulation in several organs, in particular splenic macrophages.[3,6] In addition, they display a mild microcytic anemia and develop a more severe anemia than wild-type (wt) mice when fed

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an iron-deficient diet.[6] Whether this phenotype is due to an intrinsic defect of erythroid cells or to retention of stored iron because of impaired ferritin degradation due to *Ncoa4* inactivation is a matter of investigation.

Some evidence argues in favor of an intrinsic erythroid function for NCOA4. First, *NCOA4* is expressed at high levels in maturing orthochromatic erythroblasts;[7] second, *in vitro*[4,8] and *ex vivo*[9] data suggest that NCOA4 is required for the differentiation and hemoglobinizationof erythroid cells, modulating iron incorporation into heme. An erythropoietic role for NCOA4 was also suggested *in vivo* in zebrafish embryos treated with morpholinos to *Ncoa4*.[4] A moderate-to-severe anemia was observed in *Ncoa4-ko* mice at birth, which was mostly rescued in adult animals.[9,10] Although all these findings suggest a role for NCOA4 in erythropoiesis, formal proof that anemia of adult *Ncoa4-ko* mice is due to loss of protein activity in erythroid cells is still lacking. Recent data[10] point toward both an autonomous and non-autonomous effect of NCOA4 in erythropoiesis. A condional tamoxifen-induced total *Ncoa4-ko* and a tissue-specific one (through the expression of CRE-recombinase under the control of the erythropoietin receptor promoter in *Ncoa4-floxed* transgenic animals) were generated by Santana Codina *et al.* However, both tamoxifen-induced toxic effects on red blood cells[11] and non-erythroid-restricted expression of erythropoietin receptor[12] still leave the question open.

In order to clarify the *in vivo* function of NCOA4, its role in erythropoiesis and to identify the cell type mostly affected by *Ncoa4* deficiency *in vivo* we used different approaches. First we generated *Ncoa4-ko* mice on the iron-rich Sv129/J strain and analyzed the animals in basal conditions and after different challenges. We then performed reciprocal transplantation of bone marrow from wt and *Ncoa4-ko* mice into *Ncoa4-ko* and wt mice. Finally, we crossed *Ncoa4-ko* with $Hbb^{th3/+}$ mice, a model of transfusion-independent β-thalassemia. We proved that reduced iron release by macrophages is the principal driver of anemia in *Ncoa4-ko* animals and excluded a relevant role for NCOA4 in erythroid cells *in vivo*.

Methods

Mouse models and bone marrow transplantation

Ncoa4-ko mice on a Sv129/J background were generated as described by Bellelli *et al.*[6] and in the *Online Supplementary Materials*. Wild-type littermates were used as controls in all the experiments. When not specified otherwise, mice were fed a standard diet containing 280 mg/kg of carbonyl iron.

Blood was collected by tail vein puncture for complete blood count (CBC) at 3, 5, 8 and 9 months of age. Mice were sacrificed when they were 3 or 9 months old and blood was collected for determination of the transferrin saturation. Liver, spleen and kidneys were dissected, weighed and snap-frozen for RNA and protein analysis or dried for iron quantification or processed for FACS analysis. BM cells were harvested and processed for methylcellulose assay, flow cytometry or RNA analysis. Duodenum was washed and formalin-fixed for Perls staining.

BM transplantation was performed as described by Nai *et al.*[13] and in the *Online Supplementary Material*. The CBC was evaluated monthly. At sacrifice animals were analyzed as above.

A subset of *Ncoa4-ko* mice was crossed to C57BL/6N $Hbbt^{th3/+}$ animals[14] (Jackson Laboratories, Bar Harbor, ME, USA) obtaining $Ncoa4^{+/-}$ and $Ncoa4^{+/-}/Hbb^{th3/+}$ progenies on a mixed C57/129 background; these animals were back-crossed generating $Ncoa4^{-/-}/Hbb^{th3/+}$, $Ncoa4^{+/-}/Hbb^{th3/+}$ and $Hbb^{th3/+}$ mice. Blood was collected for CBC evaluation from 1, 2 and 4-month old animals of both genders.

All mice were maintained in the San Raffaele Institute animal facility in accordance with European Union guidelines. The study was approved by the Institutional Animal Care and Use Committee of San Raffaele Institute.

Treatments

For the induction of iron deficiency, *Ncoa4-ko* mice of both genders were fed an iron-deficient diet containing less than 3 mg/kg of carbonyl iron, (SAFE, Augy, France) for 6 months starting when they were 3 months old. Transplanted mice were fed a standard diet for 2 months and then the iron-deficient diet until sacrificed 5 months after BM transplantation.

For the evaluation of duodenal iron absorption, 9-month old wt and *Ncoa4-ko* mice (of both genders) were administered 100 μL of a solution containing 228.5 mg/L of the stable iron isotope ^{57}Fe (Sigma-Aldrich) by oral gavage. The animals were fasted for 16 h before ^{57}Fe administration and 1 h after gavage they were anesthetized by intraperitoneal administration of Avertin (2,2,2-tribromoethanol, 250 mg/kg; Sigma-Aldrich). Blood for preparation of serum was withdrawn from retro-orbital vessels and mice were subsequently perfused transcardially with phosphate-buffered saline. Duodenum and liver were recovered, washed with phosphate-buffered saline, weighed and immediately snap-frozen.

For the induction of acute erythropoietic expansion, wt and *Ncoa4-ko* mice (of both genders) were treated with a single injection of erythropoietin (0.8 IU/g) or saline as a control and sacrificed 15 h later.

At sacrifice all animals were analyzed as described above.

Phenotypic characterization

Determination of the CBC, transferrin saturation and tissue iron content, flow cytometry analysis, colony-forming unit assay, Perls blue staining, inductively coupled plasma mass spectrometry, western-blot analysis and quantitative real-time polymerase chain reaction were performed by standard methods. Details are provided in the *Online Supplementary Material.*

Statistics

Data are presented as the mean ± standard error (SE). An unpaired two-tailed Student *t*-test (for variables with a normal distribution) or Mann-Whitney test (for variables with a non-Gaussian distributions) was performed using GraphPad Prism 5.0 (GraphPad). *P* values <0.05 are considered statistically significant.

Results

Ncoa4-ko mice on a Sv129/J background have microcytic red cells but not anemia

Given the significant impact of mouse strain on iron metabolism,[15] we investigated the phenotype of *Ncoa4-ko* mice on a Sv129/J background, a strain more iron rich than C57BL/6 (AN and LS, *unpublished data* and Levy *et al.*[16]). For comparison with *Ncoa4-ko* C57BL/6 age-matched animals,[6] the CBC was periodically determined until 9 months of age when mice were sacrificed. Differently from C57BL/6 animals, red blood cell (RBC) count, hematocrit (not shown) and hemoglobin levels were similar in Sv129/J *Ncoa4-ko* mice and wt littermates. Only the erythrocyte indices, mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH), were slightly

lower (decreases of about 4.4% and 6%, respectively) in mutant mice than in controls, at all ages analyzed, as occurred in the C57BL/6 strain[6] (Figure 1A). Bone marrow (BM) and spleen erythroid differentiation (Figure 1B) was similar in wt and *Ncoa4-ko* mice at 3 and 9 months of age (Figure 1C and *data not shown*). *Ncoa4-ko* BM cells generated the same number of burst-forming units-erythroid (BFU-E) and colony-forming units-granulocyte/monocyte (CFU-GM) as wt BM cells in methylcellulose assays (Figure 1D). Consistent with normal hemoglobin levels, erythropoietin (*Epo*) expression in the kidney was comparable in wt and *Ncoa4-ko* animals (*Online Supplementary Figure S1A*).

Overall these results show that the lack of *Ncoa4* in an iron-rich background causes only mild microcytosis without anemia, preserves normal maturation and does not impair the clonogenic capcity of erythroid cells, thus excluding important differentiation defects *in vivo*.

Ncoa4-ko mice have normal iron parameters but tissue iron retention in ferritin

At difference from C57BL/6 mice,[6] 9-month old Sv129/J *Ncoa4-ko* mice had transferrin saturation, serum iron levels and liver, spleen and kidney non-heme iron content comparable to those in wt mice (Table 1). However, iron retention was evident in sections of duodenum from *Ncoa4-ko* animals (Figure 2A), as observed in C57BL/6 mice.[6]

Duodenal iron accumulation was not due to increased expression of the iron-regulatory hormone hepcidin (*Hamp*), which was instead reduced in mutant mice (Figure 2B). The inhibition of *Hamp* was likely mediated by downregulation of the BMP-SMAD pathway, as suggested by the low expression of the BMP-SMAD target gene, inhibitor of differentiation 1 (*Id1*) (Figure 2C). This occurred in the presence of normal levels of expression of the hepcidin activators *Bmp6* and *Bmp2* and of the hepcidin inhibitor erythroferrone (*Erfe*) both in BM and spleen (*Online Supplementary Figure S1B-E*). Despite normal liver iron content, transferrin receptor 1 (*Tfr1*) expression, which is inversely correlated to intracellular iron, was increased in the livers of *Ncoa4-ko* mice (Figure 2D), in line with results obtained in *NCOA4*-depleted cells.[2] *Tfr1* expression was also increased in the kidneys of mutant mice (*Online Supplementary Figure S1F*), despite normal kidney iron content. On the other hand, liver ferritin H levels were increased, as expected because of the impaired ferritinophagy (Figure 2E). Collectively, these data suggest that, irrespective of normal iron concentration, *Ncoa4-ko* tissues sense a signal of iron deficiency, likely due to a reduced "free" iron pool secondary to ferritin iron retention.

Ncoa4-ko mice are susceptible to iron deficiency

To characterize their capacity to release iron from the stores in chronic iron deficiency, a cohort of wt and *Ncoa4-ko* mice was challenged with an iron-poor diet for 6 months, starting at 3 months of age. Surprisingly this diet did not significantly affect the hematologic parameters of wt mice, likely because of the high body iron content of the Sv129/J strain. Like C57BL/6 mice, Sv129/J *Ncoa4-ko* mice fed with a low-iron diet developed severe anemia, especially in the last month of the diet (Figure 3A). BM and spleen erythropoiesis in *Ncoa4-ko* mice remained substantially comparable to that in wt animals, with similar percentages of Ter119$^+$ cells and without evident maturation differences (Figure 3B). However, *Ncoa4-ko* cells isolated from iron-deficient mice generated significantly fewer BFU-E and CFU-GM than did wt cells (Figure 3C), suggesting that severe iron deficiency impairs the clonogenic capacity of early progenitors cells lacking *Ncoa4*.

Iron deficiency reduced the levels of circulating and tissue iron in both genotypes. After 6 months *Ncoa4-ko* mice had dramatically lower transferrin saturation and serum iron levels than had wt littermates, while liver, speen and kidney content (Table 1) and Perls staining on duodenal sections (*Online Supplementary Figure S2A*) were comparably decreased. These results suggest that in circumstances of prolonged, chronic iron deficiency, *Ncoa4-ko* mice likely mobilize stored iron through NCOA4-independent mechanisms. Nevertheless, liver ferritin levels remained higher in *Ncoa4-ko* mice than in wt ones, consistent with reduced ferritin degradation (*Online Supplementary Figure S2B*). Levels of liver *Hamp*, *Id1* and *Bmp6* expression were similarly decreased in *Ncoa4-ko* and wt mice (*Online Supplementary Figure S2C-E*). *Ncoa4-ko* mice showed a trend towards increased liver *Tfr1* expression (*Online Supplementary Figure S2F*) and a significant upregulation of kidney *Tfr1* (*Online Supplementary Figure S2G*), despite iron content being comparable to that of wt mice. This is consistent with a functional iron deficiency, as observed under a standard diet. As expected, kidney *Epo* expression was higher in anemic *Ncoa4-ko* mice than in wt ones (*Online Supplementary Figure S2H*).

Overall these results suggest that in chronic iron deficiency impaired ferritinophagy decreases iron availability for erythropoiesis in *Ncoa4-ko* mice, thus causing severe anemia.

Because of the relevance of iron absorption in iron deficiency and hypoxia,[17,18] we wondered whether the lack of *Ncoa4* could also affect duodenal iron uptake. To investigate this point, we treated 9-month old *Ncoa4-ko* and wt controls with a single dose of ^{57}Fe *via* oral gavage (100 μL/mouse of a solution containing 228 mg/L ^{57}Fe) and sacrificed them 1 h later.[19] The amount of ^{57}Fe determined *via* inductively coupled plasma mass spectrometry in the duodenum, serum and liver (*Online Supplementary Figure S3*) was similar in wt and *Ncoa4-ko* animals, excluding that the lack of *Ncoa4* significantly impairs dietary iron uptake.

Ncoa4-ko bone marrow reconstitutes normal erythropoiesis *in vivo*

To investigate erythropoiesis of *Ncoa4-ko* mice in more depth, we used BM transplantation to assess the capacity of *Ncoa4*-deficient hematopoietic stem cells to reconstitute erythropoiesis. BM transplantation replaces all recipient hematopoietic lineages, spleen macrophages[20] and some liver macrophages[21] with donor cells. Lethally irradiated *Ncoa4-ko* mice were transplanted with BM cells from wt littermates (and from *Ncoa4-ko* as controls) (Figure 4A). Wild-type mice were transplanted with BM cells from *Ncoa4-ko* donors (and from wt littermates as controls) (*Online Supplementary Figure S4A*).

Two months after the transplant, erythropoiesis was fully recovered in animals transplanted with both *Ncoa4-ko* and wt BM, with comparable RBC counts and hemoglobin levels (Figure 4B and *Online Supplementary Figure S4B-E*). This result disputes the impaired differentiation capacity of *Ncoa4-ko* erythroid progenitors/precursors *in vivo*.

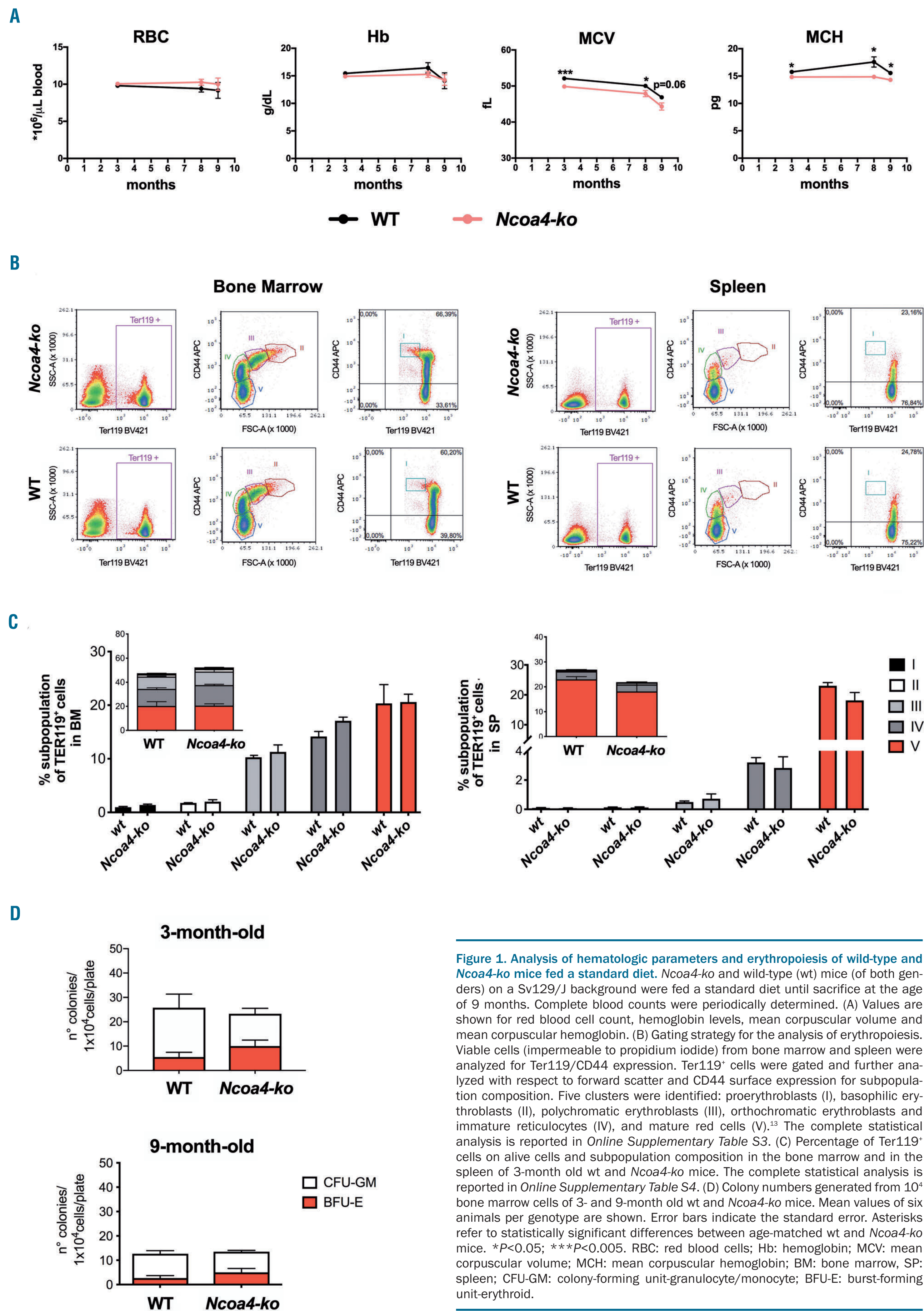

Figure 1. Analysis of hematologic parameters and erythropoiesis of wild-type and *Ncoa4-ko* mice fed a standard diet. *Ncoa4-ko* and wild-type (wt) mice (of both genders) on a Sv129/J background were fed a standard diet until sacrifice at the age of 9 months. Complete blood counts were periodically determined. (A) Values are shown for red blood cell count, hemoglobin levels, mean corpuscular volume and mean corpuscular hemoglobin. (B) Gating strategy for the analysis of erythropoiesis. Viable cells (impermeable to propidium iodide) from bone marrow and spleen were analyzed for Ter119/CD44 expression. $Ter119^{+}$ cells were gated and further analyzed with respect to forward scatter and CD44 surface expression for subpopulation composition. Five clusters were identified: proerythroblasts (I), basophilic erythroblasts (II), polychromatic erythroblasts (III), orthochromatic erythroblasts and immature reticulocytes (IV), and mature red cells (V).[13] The complete statistical analysis is reported in *Online Supplementary Table S3*. (C) Percentage of $Ter119^{+}$ cells on alive cells and subpopulation composition in the bone marrow and in the spleen of 3-month old wt and *Ncoa4-ko* mice. The complete statistical analysis is reported in *Online Supplementary Table S4*. (D) Colony numbers generated from 10^4 bone marrow cells of 3- and 9-month old wt and *Ncoa4-ko* mice. Mean values of six animals per genotype are shown. Error bars indicate the standard error. Asterisks refer to statistically significant differences between age-matched wt and *Ncoa4-ko* mice. **P*<0.05; ****P*<0.005. RBC: red blood cells; Hb: hemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; BM: bone marrow, SP: spleen; CFU-GM: colony-forming unit-granulocyte/monocyte; BFU-E: burst-forming unit-erythroid.

Table 1. Iron parameters of 9-month old wild-type and *Ncoa4*-knockout mice fed a standard and an iron-poor diet.

	Standard diet		Iron-poor diet	
	WT (n=5-10)	*Ncoa4-ko* (n=6-13)	WT (n=4)	*Ncoa4-ko* (n=5)
TS(%)	57.21±2.58	57.60±1.80	36.70±5.03	14.10±2.88**
SI(μg/dL)	296.1±34.5	331.9±55.6	176.9±24.5	74.5±13.4*
LIC (μg iron/g dry tissue)	522.1±37.4	596.6.5±37.5	136.6±10.9	165.9±5.0
SIC (μg iron/g dry tissue)	4897±713	3518±417	1161±215	632±155
KIC (μg iron/g dry tissue)	1382±168	1276±122	748±59	772±53

TS: transferrin daturation; SI: serum iron; LIC: liver iron content; SIC: spleen iron content; KIC: kidney iron content. The values reported are the mean ± standard error for each group. The number of animals analyzed in each group is indicated in the header. Asterisks refer to statistically significant differences between *Ncoa4*-knockout (*ko*) and wild-type (wt) mice fed the same diet. *P<0.05; **P<0.01.

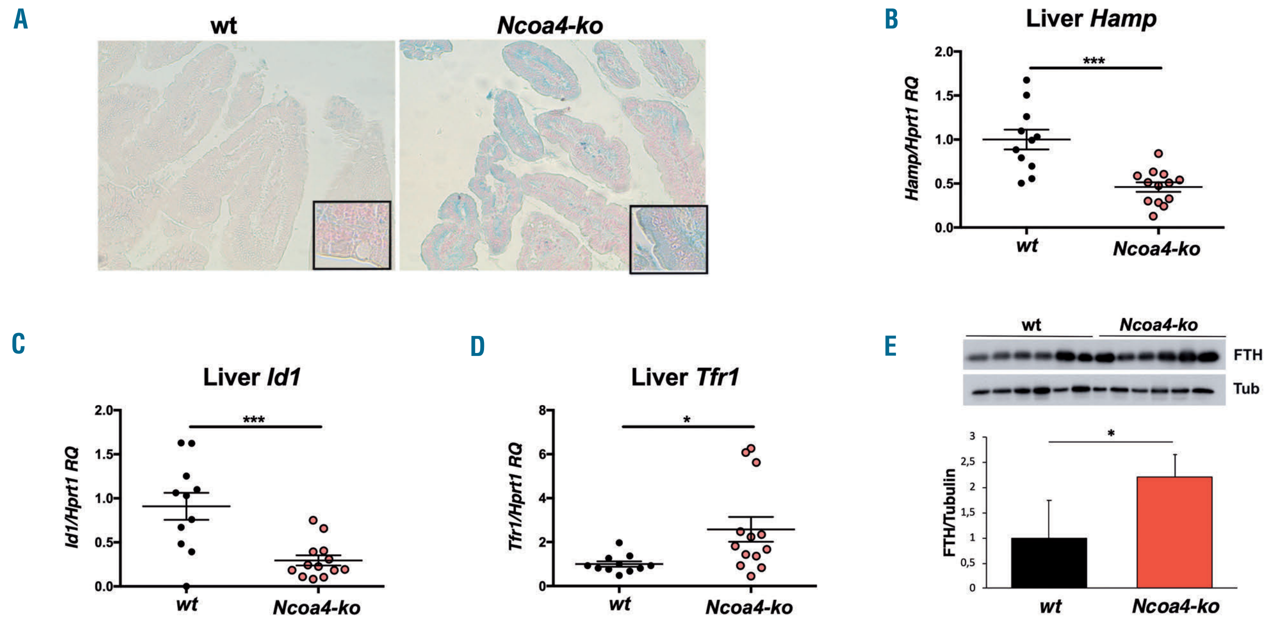

Figure 2. Analysis of the iron phenotype of 9-month old wild-type and *Ncoa4-ko* mice fed a standard diet. *Ncoa4-ko* and wild-type (wt) mice (of both genders) on a Sv129/J background were fed a standard diet until sacrifice at the age of 9 months. (A) Representative pictures of Perls staining performed on duodenal sections (thickness of section 5 μm; magnification 20X, 40X in the inset). (B-D) Quantitative real-time polymerase chain reaction analysis of hepcidin (*Hamp*) (B), inhibitor of differentiation 1 (*Id1*) (C) and transferrin receptor 1 (*Tfr1*) (D) to measure mRNA levels in the liver relative to those of hypoxanthine phosphoribosyltransferase 1 (*Hprt1*). Data were normalized on a wild-type mean value of 1. (E) Western blot and relative densitometric analysis of ferritin H protein levels in the liver. Tubulin was used as a loading control; Mean values of 6-13 animals per genotype are shown. Error bars indicate the standard error. Asterisks refer to statistically significant differences between age-matched wt and *Ncoa4-ko* mice. *P<0.05; **P<0.01; ***P<0.005. RQ: relative quantification; FTH: ferritin H; Tub: tubulin.

The microcytosis of *Ncoa4-ko* mice transplanted with wt BM was fully corrected with their MCV reaching levels of untransplanted wt mice (Figure 4B). Conversely, wt mice transplanted with *Ncoa4-ko* BM developed a mild microcytosis, comparable to that of germ-line *Ncoa4-ko* animals (*Online Supplementary Figure S4B-E*).

These experiments clearly demonstrate that the microcytosis of *Ncoa4-ko* mice is due to the loss of NCOA4 exclusively in BM-derived cells, either erythroid cells or macrophages or both. However, considering not only the normal erythropoiesis documented in *Ncoa4-ko* mice but also the ability of ko BM cells to completely reconstitute erythropoiesis in lethally irradiated animals, our conclusion is that a major defect in erythroid precursors lacking *Ncoa4* is unlikely.

Ncoa4-ko macrophages display impaired ferritinophagy *in vivo*

To better characterize ferritinophagy impairment in *Ncoa4-ko* macrophages, *Ncoa4-ko* mice reconstituted with *Ncoa4-ko* (*Ncoa4-ko$^{ko\ BM}$*) or wt BM cells (*Ncoa4-ko$^{wt\ BM}$*) were fed a low iron diet for 3 months starting 2 months after BM transplantation. The duration of this iron-deficient diet was shorter than the one used in germ-line animals to avoid the activation of the NCOA4-independent compensatory mechanisms of iron release observed in total *Ncoa4-ko* mice. The iron-deficient diet reduced hemoglobin levels, MCV and MCH without affecting RBC count in both genotypes, with a trend toward a more severe effect in *Ncoa4-ko$^{ko\ BM}$* transplanted mice (Figure 4B), confirming that the susceptibility to iron-deficiency of *Ncoa4-ko* mice is due to a defect in BM-derived cells. The comparable BM and spleen erythroid differentiation in the two genotypes (Figure 4C) argues against an intrinsic defect of *Ncoa4-ko* erythroid cells, rather suggesting that microcytosis and increased susceptibility to iron deficiency result from defective iron release by *Ncoa4-ko* macrophages.

In support of this interpretation, after 3 months of diet, the spleen iron content in *Ncoa4-ko$^{ko\ BM}$* mice was higher than that in controls (Figure 4D), consistent with a defect

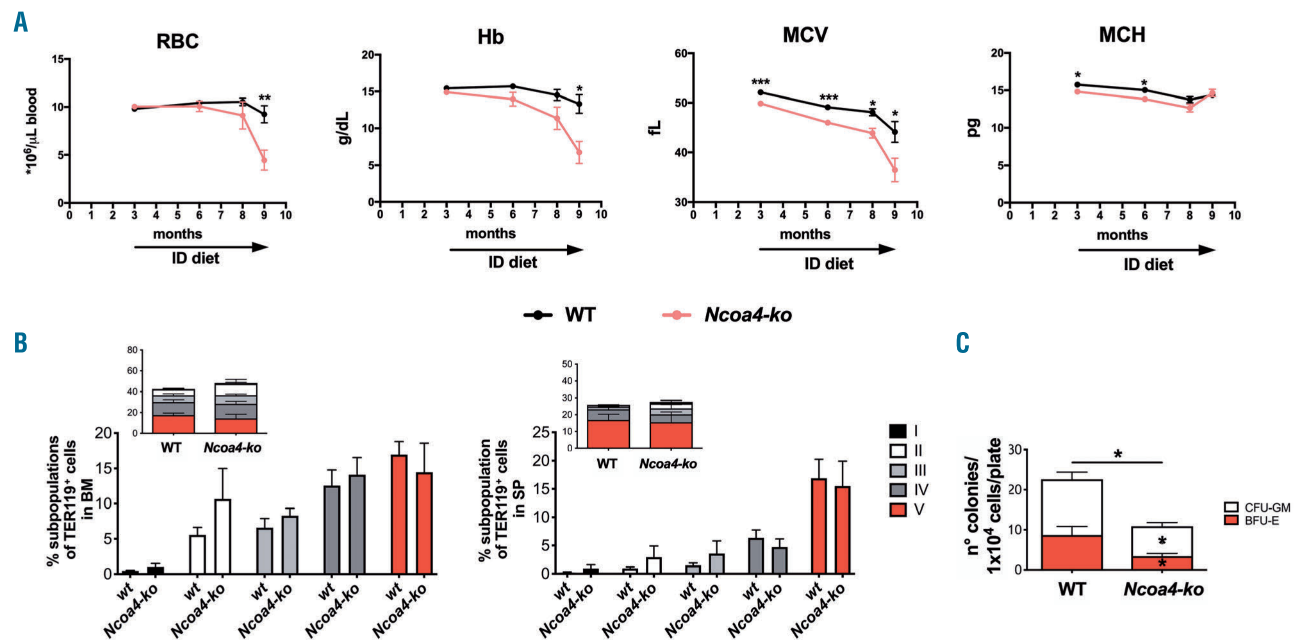

Figure 3. Hematologic parameters and analysis of erythropoiesis in wild-type and *Ncoa4-ko* mice fed an iron-deficient diet. *Ncoa4-ko* and wild-type (wt) mice (of both genders) on a Sv129/J background were fed an iron-deficient diet for 6 months starting from the age of 3 months. Complete blood counts were determined periodically. (A) Values are shown for red blood cell count, hemoglobin levels, mean corpuscular volume and mean corpuscular hemoglobin. (B) Percentage of $Ter119^+$ cells on alive cells and subpopulation composition (determined as described in Figure 1B) both in the bone marrow and in the spleen of 9-month old iron-deficient wt and *Ncoa4-ko* mice. The complete statistical analysis is reported in *Online Supplementary Table S5*. (D) Colony numbers generated from 10^4 bone marrow cells of 9-month old iron-deficient wt and *Ncoa4-ko* mice. Mean values of four or five animals per genotype are shown. Error bars indicate the standard error. Asterisks refer to statistically significant differences between age-matched wt and *Ncoa4-ko* mice. *P<0.05; **P<0.01; ***P<0.005. ID: iron-deficient; RBC: red blood cells; Hb: hemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; BM: bone marrow, SP: spleen; CFU-GM: colony-forming unit-granulocyte/monocyte; BFU-E: burst-forming unit-erythroid.

of *Ncoa4-ko* macrophages in releasing iron *in vivo*, despite comparable transferrin saturation (*Online Supplementary Figure S5A*), serum iron levels (*Online Supplementary Figure S5B*) and liver iron content (Figure 4E) in the two genotypes. Splenic iron accumulation did not result from increased hepcidin levels (*Online Supplementary Figure S5C*), but from impaired degradation of ferritin. Indeed ferritin levels in the spleen of *Ncoa4-ko*$^{ko\ BM}$ animals remained higher than in mice transplanted with wt cells (Figure 4F).

Despite comparable iron content, liver ferritin levels were higher in the *Ncoa4-ko*$^{ko\ BM}$ mice than in *Ncoa4-ko*$^{wt\ BM}$ controls (Figure 4G), suggesting impaired ferritin degradation also in this tissue. Since macrophages are the only hepatic cells derived, at least in part, from donor BM,[21] these results further confirm the reduced ability of *Ncoa4-ko* macrophages to degrade ferritin. Limited macrophage ferritinophagy, which restricts iron recycling, explains the severity of anemia in iron deficiency and supports a prevalent non-autonomous role of NCOA4 in erythropoiesis.

The circulating iron levels in *Ncoa4-ko* mice fail to rise upon acute erythropoietic expansion

Iron recycling is essential not only to compensate for chronic anemia but also in response to the acute expansion of erythropoiesis, such as after bleeding or stimulation with erythropoietin. To verify the role played by NCOA4 in response to an acute increase of iron demand *in vivo*, we exploited a published protocol,[22,23] treating wt and *Ncoa4-ko* mice with a single injection of erythropoietin (8 IU/g body weight) to induce erythropoietic expansion, increased erythroferrone release[24] and inhibition of hepcidin. In normal mice, iron uptake from the diet and release from stores are enhanced to supply erythropoietic needs, resulting in a transient increase in the levels of serum iron 15 h after the administration of erythropoietin[22] (Figure 5A). At this time point no significant changes of BM erythropoiesis were observed, while the percentage of early erythroid precursors was increased in the spleen in both wt and *Ncoa4-ko* mice (Figure 5B and *Online Supplementary Figure S6A*). The induction of splenic *Erfe* was comparable in both genotypes (Figure 5C) and hepcidin inhibition was even stronger in mutant mice than in wt ones (Figure 5D). Despite low hepcidin levels and differently from the situation in wt mice, transferrin saturation and serum iron levels were not increased in erythropoietin-treated *Ncoa4-ko* mice (Figure 5E, F). These findings indicate that the latter mice fail to mobilize iron stores in response to an acute increase of iron demand. The hypoferremia observed in the latter animals likely contributes to decrease hepcidin, suppressing the BMP-SMAD pathway, as suggested by the concomitant reduction of *Id1* mRNA, in the absence of changes in *Bmp6* and *Bmp2* (*Online Supplementary Figure S6B-D*).

Ncoa4 deletion does not further worsen anemia in thalassemic mice

To further prove the non-autonomous role of NCOA4 in erythropoiesis, we investigated the effect of *Ncoa4* heterozygous or homozygous deletion in *Hbb*$^{th3/+}$ animals, a model of transfusion-independent thalassemia, characterized by deficiency of β-globin chains, ineffective erythropoiesis and anemia.[14] We reasoned that *Ncoa4* genetic

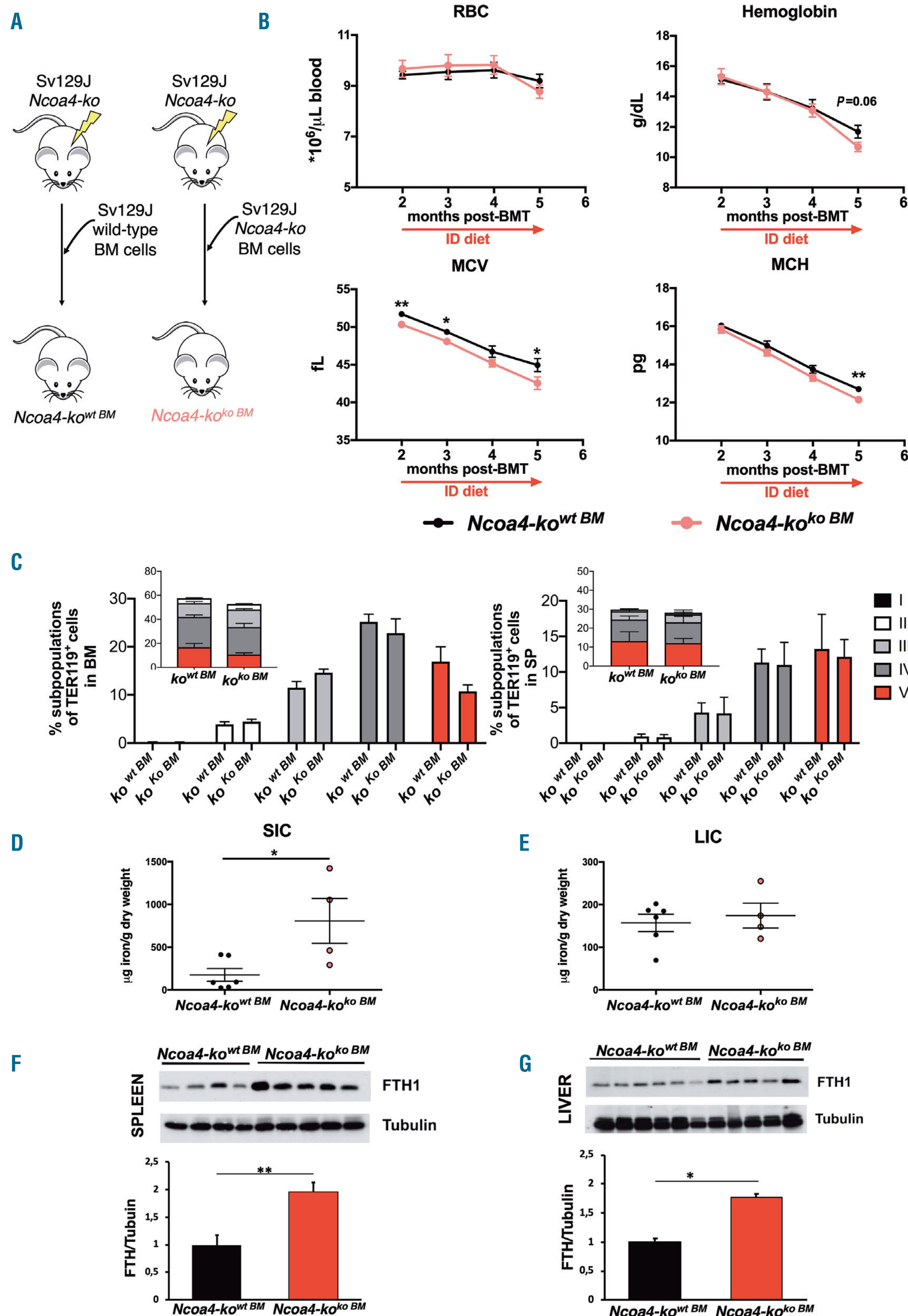

Figure 4. Hematologic parameters, erythropoiesis and iron phenotype of ***Ncoa4-ko*** $^{wt\ BM}$ **and** ***Ncoa4-ko*** $^{ko\ BM}$ **mice fed an iron-poor diet.** *Ncoa4-ko* mice on a Sv129/J background were transplanted with Sv129J wt (*Ncoa4-ko*$^{wt\ BM}$) or *Ncoa4-ko* (*Ncoa4-ko*$^{ko\ BM}$) bone marrow (BM). Animals were fed an iron-deficient diet for 3 months starting 2 months after BM transplantation (BMT) and the complete blood count was determined periodically. (A) Scheme of the BMT procedure. (B) Red blood cell count, hemoglobin levels, mean corpuscular volume and mean corpuscular hemoglobin. (C) Percentage of Ter119^{+} cells on alive cells and subpopulation composition (determined as described in Figure 1B) both in the bone marrow and in the spleen of *Ncoa4-ko*$^{wt\ BM}$ and *Ncoa4-ko*$^{ko\ BM}$ mice 5 months after BMT. The complete statistical analysis is reported in *Online Supplementary Table S6*. (D) Spleen iron content. (E) Liver iron content. (F, G) Ferritin H protein levels in the spleen (F) and liver (G) (representative western blot, upper panels and densitometric analysis, lower panels; tubulin used as a loading control) in *Ncoa4-ko*$^{wt\ BM}$ and *Ncoa4-ko*$^{ko\ BM}$ mice 5 months after BMT. Mean values of four or five per genotype are shown. Error bars indicate the standard error. Asterisks refer to statistically significant differences between age-matched *Ncoa4-ko*$^{wt\ BM}$ and *Ncoa4-ko*$^{ko\ BM}$ mice. *P<0.05; **P<0.01. RBC: red blood cells; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; SP: spleen; FTH: ferritin H.

inactivation would worsen the erythroid phenotype of $Hbb^{th3/+}$ animals if NCOA4 was playing an important role in hemoglobinization of erythroid precursors, as suggested.[5] To investigate this, we bred *Ncoa4-ko* mice with $Hbb^{th3/+}$ animals and evaluated the CBC at 1, 2 and 4 months of age in double mutants. *Ncoa4* haploinsufficiency had no effect on anemia of $Hbb^{th3/+}$ mice at any time point (Figure 6). Deletion of both *Ncoa4* alleles induced a modest, statistically significant increase of RBC count at 2 and 4 month of age (Figure 6A), which may result from the iron-restricted phenotype caused by reduced iron recycling, as suggested by further decreased MCV (Figure 6C) and MCH (Figure 6D) of double-mutant mice compared to $Hbb^{th3/+}$ controls.

In conclusion *Ncoa4* deletion did not aggravate anemia in this model, suggesting a minor, if any, effect of NCOA4

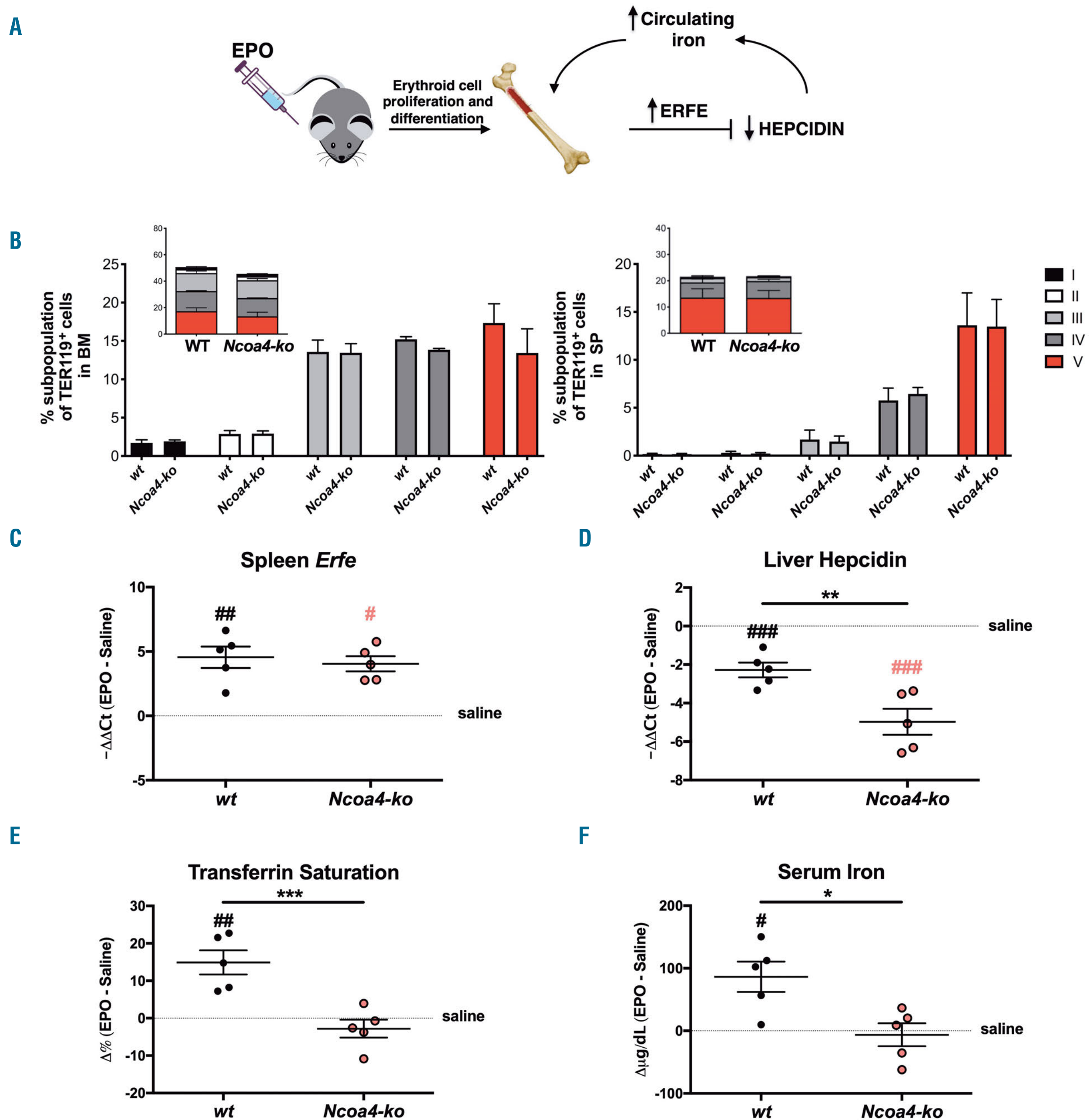

Figure 5. Erythropoiesis and iron phenotype of wild-type and *Ncoa4-ko* mice after an acute erythropoietin challenge. Three-month old *Ncoa4-ko* and wild-type (wt) mice on a Sv129/J background were treated with a single intraperitoneal injection of erythropoietin (EPO; 8 IU/g) or saline as a control and sacrificed 15 h later. (A) A scheme of the expected alterations of iron homeostasis induced by the EPO injection. (B) The percentage of Ter119+ alive cells and subpopulation composition (determined as described in Figure 1B) both in the bone marrow and in the spleen of EPO-treated wt and *Ncoa4-ko* mice. The complete statistical analysis is reported in *Online Supplementary Table S7*. (C) Real-time polymerase chain reaction (PCR) of splenic erythroferrone (*Erfe*) to measure mRNA levels relative to those of glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*). (D) Real-time PCR of hepatic hepcidin (*Hamp*) to measure mRNA levels relative to those of hypoxanthine phosphoribosyltransferase 1 (*Hprt1*). (E) Transferrin saturation. (F) Serum Iron levels. Data in (C-F) are expressed as the difference (Δ) between mice of the same genotype treated with EPO or saline (dotted gray line). Mean values of five animals per genotype are shown. Error bars indicate the standard error. Asterisks refer to statistically significant differences between age-matched EPO-treated wt and *Ncoa4-ko* mice. *P<0.05; **P<0.01; ***P<0.005. Hashtags refer to statistically significant differences between EPO- and saline-treated mice of the same genotype. #P<0.05; ##P<0.01; ###P<0.005. ERFE: erythroferrone; BM: bone marrow; SP: spleen.

in erythropoiesis. The stable or even slightly improved phenotype secondary to the loss of *Ncoa4* is in line with mild iron restriction, a condition described to ameliorate the thalassemic phenotype.[25-27]

Discussion

NCOA4-mediated ferritinophagy is essential for maintaining cell and systemic iron homeostasis.[2-6,9] Importantly, loss of NCOA4 function *in vivo* induces anemia, especially in conditions of low iron. Which cell type(s) depend most on NCOA4 and ferritinophagy in order to sustain iron-dependent processes, and especially erythropoiesis, remains uncertain.

Here, by combining different experimental approaches, we dissected the tissue-specific role of NCOA4 and ferritinophagy in supporting RBC production and showed that the major function of NCOA4 is in iron recycling macrophages.

First, we demonstrated the relevance of total body iron as a modifier of the phenotype of *Ncoa4-ko* mice. Anemia is not a feature of *Ncoa4-ko* mice on Sv129/J, a strain with higher body iron than the C57BL/6 one (Levy *et al.*[16] and personal data). Terminal erythropoiesis proceeds normally in *Ncoa4-ko* mice and BM cells isolated from both 3- and 9-month old mice fed a standard diet generate the same number of BFU-E and CFU-GM as do wt cells. However, anemia is induced more easily in *Ncoa4-ko* mice than in controls by a prolonged iron-deficient diet. Lack of anemia in iron-replete conditions and increased susceptibility to anemia in iron deficiency suggest that the erythroid phenotype in *Ncoa4-ko* mice is strongly dependent on the efficacy of iron supply. Indeed, if NCOA4 is crucial for erythroid cell differentiation and hemoglobinization, anemia would develop in *Ncoa4-ko* animals irrespectively of their genetic background. Intriguingly, while NCOA4 is fundamental for providing iron in acute conditions, a prolonged (6 months) iron-deficient diet leads to substantial splenic and hepatic iron mobilization in *Ncoa4-ko* mice, suggesting that NCOA4-independent mechanisms are likely activated in chronic conditions, although these mechanisms are insufficient to prevent the development of anemia.

Second, after BM transplantations *Ncoa4-ko* BM cells reconstituted normal RBC counts and hemoglobin levels in both wt and *Ncoa4-ko* recipients, with microcytosis being the only RBC abnormality. The ability to reconstitute normal erythropoiesis and normal hemoglobin levels and RBC counts proves that loss of *Ncoa4 in vivo* has a limited, if any, effect on the erythroid lineage. In addition, haploinsufficiency and total *Ncoa4* ablation did not worsen anemia of a β-thalassemia mouse in the long term. If NCOA4 has an autonomous erythroid role, it would be expected that its deletion in a context of a constitutionally abnormal erythropoiesis would further decrease hemoglobin levels. Our results, instead, suggest that the iron-restricted phenotype of *Ncoa4-ko* mice protects erythroid precursors from oxidative stress and improves RBC, as observed with other iron restrictive approaches,[25-27] although enhancing microcytosis.

The autonomous erythroid function proposed for NCOA4 in a recent publication was based on data from tamoxifen-inducible total *Ncoa4-ko* mice, which develop

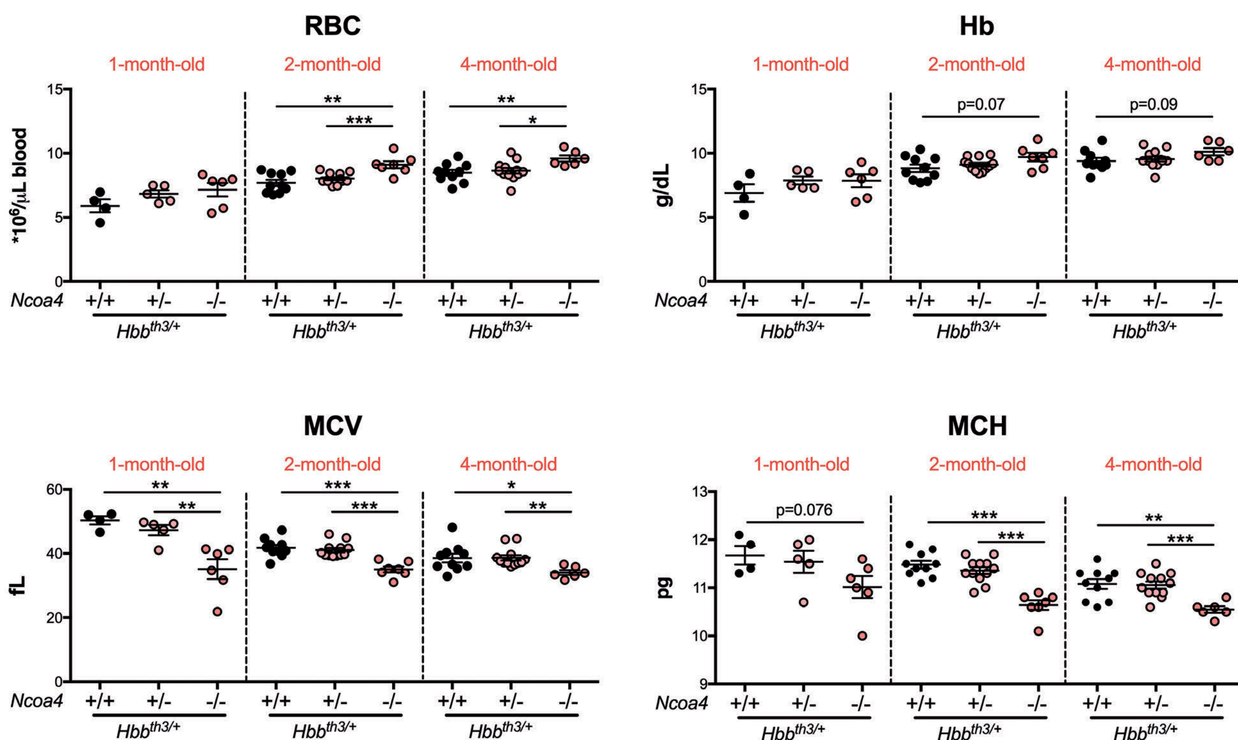

Figure 6. Hematologic parameters of *Hbb*$^{th3/+}$ mice with germ-line deletion of *Ncoa4*. Hematologic parameters of both male and female *Hbb*$^{th3/+}$ mice with wt *Ncoa4* (*Ncoa4*$^{+/+}$) or germ-line ablation of a single (*Ncoa4*$^{+/-}$) or both (*Ncoa4*$^{-/-}$) alleles were determined at 1, 2 and 4 months of age. The figure shows the values for red blood cell count, hemoglobin concentration, mean corpuscular volume and mean corpuscular hemoglobin. Asterisks refer to statistically significant differences. ***P*<0.01; ****P*<0.005. RBC: red blood cell count; Hb: hemoglobin concentration; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin.

acute anemia after tamoxifen treatment.[10] However, our recent finding that tamoxifen has a toxic effect on RBC production, an effect that is even stronger in mice lacking *Ncoa4*, weakens the authors' conclusion.[11] The same authors also generated an erythropoietin receptor (*Epor)-Cre Ncoa4*-floxed mice in which *Ncoa4* genetic ablation was induced by Cre recombinase expressed under the control of the *Epor* promoter.[10] The microcytic anemia of these animals was considered a proof of NCOA4 cell-autonomous function in the erythroid compartment. However, *Epor* is not exclusively expressed in the erythroid lineage but also in other cell types, including macrophages.[12] This raises concerns about the erythroid specificity of the model and makes the reported findings not conclusive regarding a NCOA4 self-autonomous erythroid role.

It is important to note that the modest microcytosis of *Ncoa4-ko* animals was reproduced by reconstituting lethally irradiated wt mice with *Ncoa4-ko* BM cells, while it was completely rescued by transplanting *Ncoa4-ko* animals with wt BM-derived cells. These findings indicate that the microcytosis resulted from the lack of NCOA4 in BM-derived cells, excluding the role of other cells such as iron absorptive enterocytes, which are knocked-out in *Ncoa4-ko* mice transplanted with wt donors. Concordantly, *Ncoa4-ko* mice show normal uptake and distribution of an orally administered iron isotope, ^{57}Fe. Although the technique is likely unable to detect subtle differences in absorption, we concluded that iron sequestration into ferritin caused by *Ncoa4* deficiency, despite inducing a condition of functional iron deficiency, does not substantially alter the HIF-2α-mediated orchestration of duodenal iron homeostasis[17,18] *in vivo*, as recently shown in an *in vitro* cellular model in which *Ncoa4* was silenced.[28]

While limiting the role of NCOA4 in erythroid precursors and enterocytes, our data point to a crucial function for ferritinophagy in macrophages. *Ncoa4-ko* macrophages have impaired iron recycling capacity *in vivo*, likely secondary to their reduced ability to degrade ferritin, a defect exacerbated by acute increases of iron demand. However, this does not translate into decreased saturation of transferrin, except in conditions of acute needs, such as after the administration of erythropoietin. Our observation is in line with results obtained in mice with selective inactivation of the iron exporter ferroportin which develop a more severe anemia than wt controls when fed an iron-poor diet[29] because of their impaired iron export capacity. We also speculate that impaired ferritinophagy in the central nurse macrophages of erythroblastic islands might affect the iron supply to maturing erythroblasts,[30] resulting in slightly microcytic RBC when circulating iron is normal and in anemia when transferrin-bound iron is limited in iron deficiency.

NCOA4 is a multifunctional protein. We observed that BM cells isolated from iron-deficient *Ncoa4-ko* mice generate a lower number of BFU-E and CFU-GM-derived colonies compared to the numbers generated by wt animals, indicating a role for NCOA4 in early BM progenitors. We hypothesize that in mild iron deficiency, wt progenitors increase their rate of proliferation and DNA replication to sustain erythropoiesis and that this process likely requires NCOA4 in order to promote ferritinophagy and simultaneously control DNA replication origin activation to avoid replication stress, as suggested by Bellelli *et al.*[31] and Federico *et al.* (manuscript in preparation). In agreement, a defect in clonogenic capacity was found in cells isolated from neonatal *Ncoa4-ko* mice,[9] a condition characterized by high iron demands and functional iron deficiency. The clonogenic defect recovers in adulthood, when iron availability increases.

All together our results demonstrate the crucial role of NCOA4 in regulating iron homeostasis, in particular in response to increased iron requirements. Although a minor erythroid effect cannot be definitely excluded by all the available approaches, NCOA4 function in macrophages is the main driver of the hematologic alterations observed in *Ncoa4-ko* mice.

Disclosures

CC is an advisor for Vifor Iron Core and has received honoraria from Vifor Pharma. The other authors declare that they have no financial conflicts of interests.

Contributions

AN designed and performed experiments, analyzed data and wrote the manuscript; MRL, GFed, MP, VO, FCarr. and SGC performed research and contributed to the data analysis; GFer contributed to data analysis and manuscript writing; CC contributed to the experimental design and manuscript preparation; LS and FCarl conceived the experiments and critically reviewed the paper. All authors approved the final version of the manuscript.

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References

1. Yeh S, Chang C. Cloning and characterization of a specific coactivator, ARA70, for the androgen receptor in human prostate cells. Proc Natl Acad Sci U S A. 1996;93(11):5517-5521.
2. Mancias JD, Wang X, Gygi SP, Harper JW, Kimmelman AC. Quantitative proteomics identifies NCOA4 as the cargo receptor mediating ferritinophagy. Nature. 2014;509 (7498):105-109.
3. Dowdle WE, Nyfeler B, Nagel J, et al. Selective VPS34 inhibitor blocks autophagy and uncovers a role for NCOA4 in ferritin degradation and iron homeostasis in vivo. Nat Cell Biol. 2014;16(11):1069-1079.
4. Mancias JD, Pontano Vaites L, Nissim S, et al. Ferritinophagy via NCOA4 is required for erythropoiesis and is regulated by iron dependent HERC2-mediated proteolysis. Elife. 2015;4.
5. Ryu MS, Duck KA, Philpott CC. Ferritin iron regulators, PCBP1 and NCOA4, respond to cellular iron status in developing red cells. Blood Cells Mol Dis. 2018;69:75-81.
6. Bellelli R, Federico G, Matte A, et al. NCOA4 deficiency impairs systemic iron homeostasis. Cell Rep. 2016;14(3):411-421.
7. An X, Schulz VP, Li J, et al. Global transcriptome analyses of human and murine terminal erythroid differentiation. Blood. 2014;123(22):3466-3477.
8. Ryu MS, Zhang D, Protchenko O, Shakoury-Elizeh M, Philpott CC. PCBP1 and NCOA4 regulate erythroid iron storage and heme biosynthesis. J Clin Invest. 2017;127(5):1786-1797.
9. Gao X, Lee HY, Li W, et al. Thyroid hormone receptor beta and NCOA4 regulate terminal erythrocyte differentiation. Proc Natl Acad Sci U S A. 2017;114(38):10107-10112.
10. Santana-Codina N, Gableske S, Quiles Del Rey M, et al. NCOA4 maintains murine erythropoiesis via cell autonomous and non-autonomous mechanisms.

Haematologica. 2019;104(7):1342-1354.
11. Nai A, Pettinato M, Federico G, Olivari V, Carlomagno F, Silvestri L. Tamoxifen erythroid toxicity revealed by studying the role of nuclear receptor co-activator 4 in erythropoiesis. Haematologica. 2019;104(8):e383-e384.
12. Jelkmann W, Bohlius J, Hallek M, Sytkowski AJ. The erythropoietin receptor in normal and cancer tissues. Crit Rev Oncol Hematol. 2008;67(1):39-61.
13. Nai A, Lidonnici MR, Rausa M, et al. The second transferrin receptor regulates red blood cell production in mice. Blood. 2015;125(7):1170-1179.
14. Yang B, Kirby S, Lewis J, Detloff PJ, Maeda N, Smithies O. A mouse model for beta 0-thalassemia. Proc Natl Acad Sci U S A. 1995;92(25):11608-11612.
15. Fleming RE, Holden CC, Tomatsu S, et al. Mouse strain differences determine severity of iron accumulation in Hfe knockout model of hereditary hemochromatosis. Proc Natl Acad Sci U S A. 2001;98(5):2707-2711.
16. Levy JE, Montross LK, Cohen DE, Fleming MD, Andrews NC. The C282Y mutation causing hereditary hemochromatosis does not produce a null allele. Blood. 1999;94(1):9-11.
17. Mastrogiannaki M, Matak P, Keith B, Simon MC, Vaulont S, Peyssonnaux C. HIF-2alpha, but not HIF-1alpha, promotes iron absorption in mice. J Clin Invest. 2009;119(5):1159-1166.
18. Shah YM, Matsubara T, Ito S, Yim SH, Gonzalez FJ. Intestinal hypoxia-inducible transcription factors are essential for iron absorption following iron deficiency. Cell Metab. 2009;9(2):152-164.
19. Fiorito V, Geninatti Crich S, Silengo L, Altruda F, Aime S, Tolosano E. Assessment of iron absorption in mice by ICP-MS measurements of (57)Fe levels. Eur J Nutr. 2012;51(7):783-789.
20. Kaur S, Raggatt LJ, Millard SM, et al. Self-repopulating recipient bone marrow resident macrophages promote long-term hematopoietic stem cell engraftment. Blood. 2018;132(7):735-749.
21. Beattie L, Sawtell A, Mann J, et al. Bone marrow-derived and resident liver macrophages display unique transcriptomic signatures but similar biological functions. J Hepatol. 2016;65(4):758-768.
22. Artuso I, Pettinato M, Nai A, et al. Transient decrease of serum iron after acute erythropoietin treatment contributes to hepcidin inhibition by ERFE in mice. Haematologica. 2018;104(3):e87-e90.
23. Mirciov CSG, Wilkins SJ, Hung GCC, Helman SL, Anderson GJ, Frazer DM. Circulating iron levels influence the regulation of hepcidin following stimulated erythropoiesis. Haematologica. 2018;103(10):1616-1626.
24. 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.
25. Gardenghi S, Ramos P, Marongiu MF, et al. Hepcidin as a therapeutic tool to limit iron overload and improve anemia in beta-thalassemic mice. J Clin Invest. 2010;120(12):4466-4477.
26. Li H, Rybicki AC, Suzuka SM, et al. Transferrin therapy ameliorates disease in beta-thalassemic mice. Nat Med. 2010;16(2):177-182.
27. Nai A, Pagani A, Mandelli G, et al. Deletion of TMPRSS6 attenuates the phenotype in a mouse model of beta-thalassemia. Blood. 2012;119(21):5021-5029.
28. Schwartz AJ, Das NK, Ramakrishnan SK, et al. Hepatic hepcidin/intestinal HIF-2alpha axis maintains iron absorption during iron deficiency and overload. J Clin Invest. 2019;129(1):336-348.
29. Zhang Z, Zhang F, An P, et al. Ferroportin1 deficiency in mouse macrophages impairs iron homeostasis and inflammatory responses. Blood. 2011;118(7):1912-1922.
30. Nairz M, Theurl I, Swirski FK, Weiss G. "Pumping iron"-how macrophages handle iron at the systemic, microenvironmental, and cellular levels. Pflugers Arch. 2017;469(3-4):397-418.
31. Bellelli R, Castellone MD, Guida T, et al. NCOA4 transcriptional coactivator inhibits activation of DNA replication origins. Mol Cell. 2014;55(1):123-137.