NCOA4 maintains murine erythropoiesis via cell autonomous and non-autonomous mechanisms

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ABSTRACT

Ncoa4 mediates autophagic degradation of ferritin, the cytosolic iron storage complex, to maintain intracellular iron homeostasis. Recent evidence also supports a role for Ncoa4 in systemic iron homeostasis and erythropoiesis. However, the specific contribution and temporal importance of Ncoa4-mediated ferritinophagy in regulating systemic iron homeostasis and erythropoiesis is unclear. Here, we show that Ncoa4 has a critical role in basal systemic iron homeostasis and both cell autonomous and non-autonomous roles in murine erythropoiesis. Using an inducible murine model of Ncoa4 knockout, acute systemic disruption of Ncoa4 impaired systemic iron homeostasis leading to tissue ferritin and iron accumulation, a decrease in serum iron, and anemia. Mice acutely depleted of Ncoa4 engaged the Hif2α-erythropoietin system to compensate for anemia. Mice with targeted deletion of Ncoa4 specifically in the erythroid compartment developed a pronounced anemia in the immediate postnatal stage, a mild hypochromic microcytic anemia at adult stages, and were more sensitive to hemolysis with higher requirements for the Hif2α-erythropoietin axis and extramedullary erythropoiesis during recovery. These studies demonstrate the importance of Ncoa4-mediated ferritinophagy as a regulator of systemic iron homeostasis and define the relative cell autonomous and non-autonomous contributions of Ncoa4 in supporting erythropoiesis in vivo.

Introduction

Iron is an essential element for life, required in a diverse set of processes including oxygen binding and transport,1 electron transport,2 and DNA synthesis and repair.3,4 The majority of cellular iron is stored in ferritin, the cellular iron storage complex composed of 24 subunits of ferritin light and heavy chain subunits (FTL, FTH1) that can chelate up to 4,500 iron atoms in a non-toxic ferrihydrite mineral core.5 Iron can be released from ferritin via a process termed ‘ferritinophagy’.6,7 FTH1 is bound by NCOA4 (Nuclear Receptor Coactivator 4) and transported to an autophagosome that fuses with a lysosome for ferritin degradation and iron release.8 NCOA4-mediated ferritinophagy is responsive to alterations in cellular iron levels to promote ferritinophagy under iron-depleted conditions or decrease ferritinophagy when iron is abundant in the cell.9 Given the established importance of NCOA4 in intracellular iron homeostasis, the role of NCOA4 in the response to systemic iron deficiency, and, particularly, its role in the pathophysiology of iron deficiency in anemia, has been of interest. Recent data from in vitro model systems of human, mouse, and zebrafish erythropoiesis support
a role for NCOA4-mediated ferritinophagy as an important step for iron release prior to mitochondrial iron import and heme biosynthesis. Specifically, perturbation of NCOA4 expression impairs hemoglobinization in the human K562 erythroleukemia and the murine erythropoietic G1E-ER4 cell line models, suggesting that NCOA4 has a cell autonomous role in erythropoiesis. Constitutive deletion of Ncoa4 in a murine model led to tissue ferritin and iron accumulation as well as a mild hypochromic microcytic anemia, supporting a role for Ncoa4 in both systemic iron homeostasis and erythropoiesis. The defect in erythropoiesis observed in this model was recently independently confirmed and extended to include a more dramatic anemia in the immediate postnatal period.

Several critical questions regarding in vivo Ncoa4 function remain. First, it is unclear whether a cell autonomous effect accounts for the entirety of the observed anemia phenotype in germline Ncoa4 knockout (KO) mice, or whether the non-erythroid functions of Ncoa4 also contribute to this phenotype. Secondly, Ncoa4 KO animals likely recruit adaptive mechanisms to compensate for decreased access to ferritin iron stores in the long-term and limit the severity of anemia. To evaluate the cell autonomous role of Ncoa4 in erythropoiesis, we generated a conditional Ncoa4fl/fl;EpoR-Cre mouse model with erythroid lineage ablation of Ncoa4. These mice develop a pronounced anemia at the immediate postnatal stage which resolves to a mild hypochromic microcytic anemia in adulthood. However, the anemia, whether in the neonate or adult, is less severe than the anemia that develops in a germline Ncoa4 KO. To examine the adaptive response to Ncoa4 depletion, we generated a tamoxifen-inducible Ncoa4fl/fl;UBC-cre/Ert2 model to control temporally the somatic deletion of Ncoa4. We found that Ncoa4 deletion in the adult mouse leads to acute anemia followed by a compensatory recovery. Furthermore, this phenotype is more pronounced under conditions of chemically induced stress erythropoiesis. In toto, these data support a model for both cell autonomous and non-autonomous functions of Ncoa4 in regulating basal and stress erythropoiesis.

Methods

Details of the experimental procedures are available in the Online Supplement. Primer and short hairpin (sh) RNA sequences are listed in Online Supplementary Table S11.

Mouse models and analysis

Ncoa4fl/fl mice were generated by insertion of loxp sites flanking exons 2 through 6. The NCOA4fl/fl mouse strain is deposited and available at the Jackson Laboratory (JAX#032959 Ncoa4fl/fl). Ncoa4fl/fl mice on a C57BL/6 (B6) background were crossed to B6.Cg-Aleu(Tcra-TCRb)1J/W and B6.EpoRcre/Ert2 mice alleles. Mice were maintained on the Prolab Isopro RMH diet (380 ppm iron). Ncoa4 recombination was induced in Ncoa4fl/fl;UBC-cre/Ert2 mice with five doses of 200 mg/kg tamoxifen. Hemolysis was induced with 40 mg/kg phenylhydrazine (PHZ). All experiments were done in adult mice (more than 8 weeks of age, age-matched within the colony) except those on postnatal bleeding (day 10).

Polymerase chain reaction genotyping

Genomic DNA was extracted from tissues or sorted Ter119+ red blood cells (RBC) and targeting was determined by polymerase chain reaction (PCR).

Hematologic and iron parameters

Blood was drawn by retro-orbital or submandibular bleeding (serial bleed, 35 μL) for determination of complete blood counts. The method of collection was kept consistent within the same time-course experiment. Serum was obtained from blood drawn by intracardiac bleeding. Serum erythropoietin (R&D MPE00B) and Fth (Abcam, ab157713) levels were determined by enzyme-linked immnosorbent assays. Tissue non-heme iron concentrations were determined as described previously. Serum iron levels were determined using a kit (Fisher:23666320).

Flow cytometry

Bone marrow or spleen cells were stained with anti-Ter119 anti-Cd44 antibodies. Stages were determined based on published methods.

Reactive oxygen species measurements

Quantitative real-time PCR was performed on isolated mRNA using SYBR Green, Actb was used as an internal control.

Western blot analysis

Cells or tissues were lysed in RIPA buffer. Protein was separated on sodium dodecylsulfate polyacrylamide gel electrophoresis gels and transferred to a membrane. Antibodies to the following compounds (and their sources) were used: Ncoa4 (Santa Cruz Biotechnology sc-373739, 1:100), Fth1 (Cell Signaling:4393), Fpn (Alpha Diagnostic International MTP11-A, 1:1000), Actb (Sigma A5441, 1:3000), Hif-2α (Novus NB100-122, 1:100), Bach1 (Santa Cruz Biotechnology sc-27121, 1:100), Hr (Millipore Sigma 07-728, 1:1000), Eif2α (Cell Signaling #9722, 1:1000), Eif2α-P (Cell Signaling #9722, 1:1000), anti-rabbit IgG (H+L) horse radish peroxidase conjugate (Promega W3456, 1:3000), and anti-mouse IgG(H+L) horse radish peroxidase conjugate (Promega W4021, 1:7000).

Histology

Tissues were processed as previously described, fixed in formalin and embedded in paraffin. After deparaffinization, the primary antibody was incubated followed by the secondary antibody and then developed with 3,3′-diaminobenzidine. Antibodies: Fth1 (Cell Signaling #4393) and Cd68 (Abcam:ab51680). For quantification of the number of erythroblastic islands, more than five fields (magnification: 10x) per mouse were analyzed and fields were averaged per mouse. Significance was determined by comparing the average of the control group versus that of the KO group. Islands were identified by the presence of groups of nucleated RBC shown by hematoxylin staining. For quantification of immunohistochemical staining, CD68 was quantified using five fields (magnification: 10x) from each mouse. The percentage of the positive area was calculated by highlighting positively stained regions and averaging them for each mouse.

Lentivirus-mediated short hairpin RNA

Lentiviral-mediated shRNA knockdown was performed as previously described.

Quantitative proteomics

Quantitative mass spectrometry-based proteomic analyses were performed as previously described. Briefly, 50 μg of digested peptides from each sample were labeled with tandem mass tag reagents and combined at a 1:1:1:1:1:1:1:1:1:1:1:1 ratio. Data
were collected using an Orbitrap Fusion Lumos mass spectrometer. Mass spectra were processed using a Sequest-based in-house software pipeline.

**Bioinformatic analysis**

Gene set enrichment analyses (GSEA) were performed as described elsewhere. A dataset from Gautier et al. provided proteomic data for seven stages of erythropoiesis.

**Statistics**

No statistical methods were used to predetermine sample size. For comparisons between two groups, a Student t-test (unpaired, 2-tailed) was performed for all experiments except for the Student t-tests (unpaired, 1-tailed) in Figures 3C, 4G and Figure 5G (hematocrit, day 2). Results from groups were considered statistically different when \( P<0.05 \).

**Study approval**

All animal studies were conducted under an approved Dana-Farber Cancer Institute Institutional Animal Care and Use Committee (IACUC) protocol (15-020).

**Results**

**Temporally induced systemic Ncoa4 loss in the adult leads to defective iron mobilization and anemia**

To assess the effects of deletion of Ncoa4 in the adult animal, we generated a conditional Ncoa4 KO model (Ncoa4\textsuperscript{fl/fl};UBC-cre/ERT2). We crossed these animals with the UBC-cre/ERT2 allele to generate a temporally inducible Ncoa4\textsuperscript{fl/fl};UBC-cre/ERT2 systemic KO model (hereafter Ncoa4\textsuperscript{rec}) with tamoxifen administration. One week after tamoxifen administration, there was efficient recombination of the Ncoa4 locus and corresponding depletion of Ncoa4 protein (Figure 1A, Online Supplementary Figure S1A). Acute depletion of Ncoa4 impaired ferritinophagy resulting in varying degrees of tissue accumulation of Fth1 (Figure 1B, Online Supplementary Figure S1D). Fth1 accumulation correlated with increased iron retention in the spleen and liver of Ncoa4\textsuperscript{rec} mice (Figure 1C,D) although no differences were observed in the bone marrow (Figure 1E). There was no significant difference in serum ferritin levels in control versus Ncoa4\textsuperscript{rec} mice (Online Supplementary Figure S1E). Consistent with a decrease in tissue ferritin turnover and decreased cellular iron release, serum iron was decreased after Ncoa4 deletion (Figure 1F). Ferroportin (Fpn) was increased in the spleen and liver and to a lesser extent in the duodenum (Figure 1G) of Ncoa4\textsuperscript{rec} mice, suggesting a compensatory response to low serum iron to increase iron export from tissues to support erythropoiesis. Despite the acute decrease in serum iron levels, no differences were observed in liver hepcidin expression (Online Supplementary Figure S1F). Taken together, these results suggest that ferritinophagy is important for maintaining serum iron levels. However, owing to systemic KO, the tissue source(s) of iron mobilized into the serum as a consequence of ferritinophagy is unclear.

To evaluate the effects of a temporally induced block in ferritinophagy on erythropoiesis, we analyzed complete blood counts in Ncoa4\textsuperscript{fl/fl} and Ncoa4\textsuperscript{rec} mice (Figure 1H, Online Supplementary Table S1) 7 days after the last tamoxifen administration. Ncoa4 deletion resulted in a drop in RBC number, hematocrit and hemoglobin (Figure 1H). No significant differences were observed in mean corpuscular volume or red cell distribution width, likely due to the acuteness of the model (Figure 1H). The phenotype was independent of gender and not solely due to the administration of tamoxifen or the UBC-cre/ERT2 allele (Online Supplementary Tables S1-3).

As Ncoa4 is highly expressed at the orthochromatic erythroblast stage, we evaluated the effects of Ncoa4 depletion on erythroid differentiation using flow cytometric analysis of Ter119 and Cd44 in bone marrow precursors. Ncoa4 depletion had no impact on erythroid differentiation through the reticulocyte stage (Online Supplementary Figure S1G), similarly to a previously described model of germline Ncoa4 deletion. Given the acute anemia, we next analyzed mice for phenotypic and molecular markers of a recovery response to anemia. Ncoa4\textsuperscript{rec} mice showed increased erythropoietin (Epo) levels in serum (Figure 1I) and increased hypoxia-induced factor-2α (Hif-2α) expression in the kidney (Figure 1J) indicating an appropriate compensatory response to anemia. However, spleen size, a marker of stress erythropoiesis, was not different (Figure 1K). On the other hand, Bach1, Hri and Eif2a-P were upregulated in Ncoa4\textsuperscript{rec} mice (Figure 1L). This is consistent with activation of a transcriptional program in erythroblasts of Ncoa4 KO mice which would limit globin synthesis when heme levels are low. Collectively, our data demonstrate a dynamic role for Ncoa4 in systemic iron homeostasis with constant flux through the ferritinophagy pathway under basal conditions which supports erythropoiesis.

**Anemia induced by loss of Ncoa4 in the adult leads to a compensatory response over time**

To evaluate the effects of acute Ncoa4 deletion over the lifespan of a murine RBC, we induced Ncoa4 recombination and performed serial complete blood counts. We observed the expected anemia immediately following depletion (day 11) (Figure 2A). However, over a period of 5 weeks, the hematocrit and hemoglobin values recovered to levels comparable to those of control animals (Figure 2A). Conversely, reticulocyte hemoglobin content and mean corpuscular volume were both significantly decreased, consistent with a compensated microcytosis (Figure 2A). The normal hematocrit and hemoglobin levels are in contrast to the constitutive model of Ncoa4 deletion in which hematocrit and hemoglobin were decreased, suggesting differences in the outcome of an adaptive response to Ncoa4 deletion in adult animals versus life-long Ncoa4 loss. Nevertheless, similarly to constitutive knock-out mouse models, we found no differences in erythrocyte precursor differentiation in the adult animal (Online Supplementary Figure S2A).

At the end point of the experiment, Ncoa4\textsuperscript{rec} mice showed Fth1 accumulation in tissues, including those not affected by acute Ncoa4 depletion (pancreas, Figure 2B,C). In contrast to acute deletion of Ncoa4, serum iron levels were increased in long-term Ncoa4\textsuperscript{rec} mice (Figure 2D), which is in line with results from a constitutive model of Ncoa4 deletion. This may be a consequence of defective ferritinophagy leading to tissue iron overload and shunting of intracellular iron to export pathways or consequent to sustained mobilization of iron induced by anemia. To evaluate the former, we analyzed Fpn expression in several tissues. After long-term Ncoa4 ablation, we observed normalization of Fpn levels in spleen and liver compared
to the levels after acute depletion (Figure 2E). This normalization is consistent with an increase in hepcidin levels (Figure 2F), increase in serum iron and resolution of anemia. However, Fpn levels were sustainably increased in the duodenum as part of a compensatory response to iron deficiency and, in part, explaining the high iron in serum. It is to be expected that duodenal Fpn expression would decrease with a longer period of time as demonstrated in

**Figure 1.** Acute systemic knockout of Ncoa4 impairs ferritinophagy and erythropoiesis. A murine model of Ncoa4 deficiency was generated using a tamoxifen-inducible Cre recombinase system (Ncoa4fl/fl;UBC-cre/ERT2). Ncoa4fl/fl and Ncoa4fl/fl;UBC-cre/ERT2 mice were administered tamoxifen to generate Ncoa4-deficient (Ncoa4rec) and control animals (Ncoa4fl/fl). (A) Genomic DNA isolated from spleens of Ncoa4fl/fl and Ncoa4fl/fl;UBC-cre/ERT2 yielded a polymerase chain reaction (PCR) product at 470 bp when using primers directed at the floxed allele (left panel, see Methods). This band was not detected in Ncoa4rec mice. Using primers directed at the recombined allele, a PCR product of 540 bp was detected for Ncoa4rec but not Ncoa4fl/fl (right panel). (B) Increased Fth1 protein levels in liver, spleen, kidney, brain but not pancreas or bone marrow from Ncoa4rec mice 11 days after initiation of tamoxifen administration. β-actin (Actb) served as a loading control. (C-E) Tissue iron levels in spleen (C), liver (D) and bone marrow (E) from Ncoa4rec in comparison to Ncoa4fl/fl mice [3-5 mice/group, error bars represent the standard error of mean (s.e.m.)] (F) Lower serum iron levels in Ncoa4rec mice (3 mice/group, error bars represent the s.e.m.). (G) Increased Fpn protein levels in spleen, liver, and duodenum from Ncoa4rec mice 11 days after initiation of tamoxifen administration. Actb served as a loading control. (H) Whole blood from adult (>12 weeks) Ncoa4fl/fl and Ncoa4rec mice 7 days after the last administration of tamoxifen was acquired by retro-orbital bleeding for complete blood count profiling (Ncoa4fl/fl, n = 12; Ncoa4rec, n = 11, error bars represent the s.e.m.). (I) Elevated erythropoietin levels in serum of Ncoa4rec mice (8 mice/group, error bars represent the s.e.m.). (J) Increased Hif-2α protein levels in kidney from Ncoa4rec mice 11 days after initiation of tamoxifen administration. Actb served as a loading control. (K) No significant change in spleen size of Ncoa4fl/fl and Ncoa4rec male and female mice 11 days after the initiation of tamoxifen administration (4 males and 7 females/group). (L) Bach1, Hri, Eif2ak1 and Eif2ak1-P protein levels in red blood cells of Ncoa4fl/fl and Ncoa4rec. Actb served as a loading control. For all panels, statistical comparison was performed using a two-tailed Student t-test: *P<0.05, **P<0.01, ***P<0.001, TAM: tamoxifen; Fth1: ferritin heavy chain 1; Fpn: ferroportin; RBC: red blood cell count; HCT: hematocrit; HGB: hemoglobin; MCH: mean corpuscular hemoglobin; CHr: reticulocyte hemoglobin content; MOV: mean corpuscular volume; RDW: red blood cell distribution width; RETIC: reticulocyte count; PLT: platelet count; Epo: erythropoietin; b.w.: body weight.
a constitutive Ncoa4 KO model, in which duodenal Fpn expression was elevated in 2-month old mice but decreased at 6 months.

Alternatively, or in addition, serum iron overload may be a secondary consequence of ineffective erythroid utilization of iron. To examine this, we evaluated the Hif-2α-Epo pathway. As shown in Figure 1J, this compensatory mechanism is activated early after induction. Five weeks later, we observed a trend towards an increase in Epo levels in Ncoa4−/− mice (Figure 2G) which correlated with sustained overexpression of Hif-2α in the kidneys (Figure 2H). As in the early response, longer-term Ncoa4 deletion had no effect on spleen size (Online Supplementary Figure S2B). Taken together, these data show that Ncoa4 depletion induces an acute, uncompensated anemia that normalizes over time and achieves a new steady state in which the Hif-2α-Epo axis response induces erythropoiesis sufficient to overcome the hemoglobin deficit by virtue of producing more RBC that are poorly hemoglobinized. In the setting of adequate serum iron, which is achieved by increased iron export from tissues and iron import from the diet, the persistent cellular hemoglobin deficit suggests a defect intrinsic to the RBC itself, specifically that the erythroid precursor is unable to mobilize sufficient iron for heme synthesis.

The stress-erythropoietic response to hemolysis is less effective in maintaining erythrocyte production in Ncoa4-deficient mice

To evaluate the role of Ncoa4 under conditions of stress erythropoiesis, we induced hemolysis with PHZ (Online Supplementary Figure S2C). As shown in Figure 2I, the stress response in Ncoa4−/− mice was less effective than in Ncoa4+/+ mice, as indicated by the lower levels of Epo and the persistent increase in serum iron levels (Figure 2J). These data suggest that Ncoa4 plays a crucial role in the regulation of erythropoiesis under conditions of stress hemolysis.
Supplementary Figure 2SC). Ncoa4<sup>−/−</sup> mice reached lower RBC numbers, hematocrit and hemoglobin nadirs which required a longer time to recover than those of Ncoa4<sup>fl/fl</sup> mice (Figure 3A, Online Supplementary Figure S2D) although they did reach the same levels as those of controls at a later time-point (Online Supplementary Table S4). Reticulocyte percentage was lower in KO mice at initial time points after PHZ suggesting an initial sluggish reticulocytosis response to PHZ-induced anemia (Figure 3B). However, KO animals demonstrated a higher reticulocyte peak at day 10 and percentage reticulocyte level remained significantly elevated compared to that of controls at the endpoint (day 12). The trend observed in absolute reticulocyte number (Online Supplementary Figure S2D) was the same as the reticulocyte percentage, indicating that the increase in reticulocytosis observed is not a consequence of decreased RBC number due to increased fragility of the cells.

Both Epo (Figure 3C) and Hif-2a (Figure 3D) levels were elevated in mutant animals, which also had enlarged
spleens and an increase in erythrocyte precursors when euthanized prior to complete recovery (Figure 3E,F). However, unlike a previously reported germline mutant maintained on a low iron diet, there was no block in differentiation at the orthochromatid erythroblast stage, indicating that different erythropoietic stresses affect the Ncoa4 phenotype differently.

Splenic macrophages play a key role in making iron available for erythropoiesis by phagocytosing senescent RBC and recycling iron from heme. Consistent with enlarged spleen size and activation of the Epo pathway, Ncoa4 deletion increased spleen erythropoiesis in PHZ-treated Ncoa4 mice compared to controls (Online Supplementary Figure S2E). In addition, specific populations of transient macrophages in the liver have been implicated in RBC phagocytosis and erythroid island formation under conditions of stress erythropoiesis. Indeed, macrophages were detected by Cd68 staining in the livers of Ncoa4 and Ncoa4 mice (Figure 3G) and there was a significant increase in the number of erythroblastic islands of macrophages were detected by Cd68 staining in the livers of Ncoa4 mice (Figure 3J). Likewise, iron concentration and Fth1 staining were increased in these tissues (Figure 3K, L, bottom panel, Online Supplementary Figure S2F). This suggests an enhanced reliance on both spleen- and liver-based erythropoiesis for recovery. Interestingly, we observed decreased hepcidin levels in livers of Ncoa4 null mice suggesting an active requirement for mobilization of cytosolic iron to support recovery from anemia (Figure 3I). Of note, erthroferrone (Erfe) levels in bone marrow were unchanged (Figure 3J).

According to the kinetics of activation of the Erfe-Hamp pathway we would expect an increase in Erfe after hemolytic insult coincident with the high Epo/low Hamp levels observed; therefore, these results point towards normalization of Erfe expression during the recovery period. Lower RBC numbers, hematocrit and hemoglobin nadirs in acute Ncoa4 mice treated with PHZ could suggest either delayed engagement of the reticulocytosis machinery and/or increased turnover of RBC or RBC precursors. Data described above appear to support an initial sluggish reticulocytosis followed by robust engagement of compensatory reticulocytosis. One marker of RBC turnover in response to PHZ is accumulation of hemosiderin deposits in spleen and liver macrophages, likely representing iron from phagocytosed RBC. Perls Prussian blue staining of hemosiderin deposits confirmed higher iron retention in spleen and liver (Figure 3K, L) from Ncoa4 mice. Likewise, iron concentration and Fth1 staining were increased in these tissues (Figure 3K, L, bottom panel, Online Supplementary Figure S2G). Another marker of RBC turnover is accumulation of filtered iron in renal proximal tubules. Ncoa4 mice accumulated higher levels of iron in the kidney in response to PHZ (Figure 3M). While these accumulations of iron could suggest a higher turnover of RBC, these markers are complicated to interpret in mice with systemic Ncoa4 depletion as a block in ferroinophagy flux will lead to accumulation of tissue iron deposits even in the absence of an increase in RBC turnover. We also measured haptoglobin and hemopexin levels in serum as measures of RBC lysis. Haptoglobin and hemopexin levels were similarly downregulated in Ncoa4 and control animals, likely due to massive PHZ-induced RBC lysis (Online Supplementary Figure S2H). Given the complexity of the model, the relative contribution of sluggish reticulocytosis or increased RBC turnover is unclear and supports investigation of the phenotype in model systems with targeted Ncoa4 deletion.

Ncoa4 has a cell autonomous role in erythropoiesis

To evaluate the cell autonomous role of Ncoa4 in erythropoiesis in vivo, we crossed Ncoa4 mice with the erythropoietin receptor EpoR-Cre allele which results in erythropoietin-specific deletion in mid-gestation. Ter119–specific efficient Ncoa4 recombination was demonstrated in Ncoa4;EpoR-Cre mice (Online Supplementary Figure S3A,B). Adult Ncoa4;EpoR-Cre animals had a mild hypochromic microcytic anemia compared to EpoR-Cre mice, with decreased hemoglobin, hematocrit, mean corpuscular hemoglobin, reticulocyte hemoglobin content, and mean corpuscular volume (Figure 4A, Online Supplementary Table S5). Epo levels were downregulated in KO mice (Figure 4B); however, discordant with the Epo levels, Hif-2α kidney protein levels were slightly elevated (Figure 4C). Correlating with Ncoa4 depletion in the erythroid compartment, Fth1 (Figure 4D) and iron levels (Figure 4E) were elevated in bone marrow from Ncoa4;EpoR-Cre mice. This increase was in contrast to the lack of Fth1 accumulation in the acute depletion model (Figure 1B, E) likely due to long-term Ncoa4 depletion. This is consistent with Fth1 accumulation in the bone marrow of mice with constitutive systemic deletion of Ncoa4. Consistent with an anemic phenotype, Bach1 and Eif2α-P levels were elevated in RBC from KO mice (Figure 4F). Similarly to the findings in adult systemic KO mice, there were no differences in differentiation of bone marrow progenitors at any stage (Online Supplementary Figure S3D). Germline Ncoa4 null mice are severely anemic in the immediate postnatal period. We found that erythropoietin-specific deletion of Ncoa4 similarly leads to a more dramatic phenotype when we analyzed complete blood counts from mice at postnatal day 10 (P10). P10 Ncoa4;EpoR-Cre mice were anemic in comparison to control mice with significant decreases in RBC numbers, hematocrit and hemoglobin (Figure 4G, Online Supplementary Table S6). However, the Ncoa4;EpoR-Cre P10 anemia was less severe than the postnatal anemia observed in mice with germline Ncoa4 loss. Overall, these findings support a cell autonomous role for Ncoa4 in erythropoiesis which is more severe in the postnatal period, but this phenotype is milder than induced systemic (Figure 1H) or germline Ncoa4 deletion, suggesting a concurrent, non-cell autonomous role for Ncoa4 in supporting erythropoiesis.

We further evaluated the erythroid cell autonomous effects of Ncoa4 deletion during PHZ-induced hemolysis. Adult mice were dosed with PHZ on days 0, 1 and 3 and analyzed at day 7. At day 7, RBC numbers, hematocrit, hemoglobin and mean corpuscular hemoglobin were decreased equally in EpoR-Cre and Ncoa4;EpoR-Cre mice (Figure 5A, Online Supplementary Table S7). Despite similar complete blood count parameters 7 days after administration of PHZ, Ncoa4;EpoR-Cre mice had elevated serum Epo levels (Figure 5B) and kidney Hif-2α protein and mRNA expression (Figure 5C, D) compared to EpoR-Cre mice, pointing to differential engagement of the Epo system for proportionate erythrocyte recovery. Despite differential increases in Epo levels, PHZ induced an equally potent increase in spleen size (Online Supplementary Figure S3E) and upregulation of erythocyte precursors in bone marrow and spleen (Online Supplementary Figure S3F). There was an increase in erythroid islands in the livers of Ncoa4;EpoR-Cre mice (Figure 5E) as well as an
increase in splenic erythropoiesis (Online Supplementary Figure S3G), confirming a differential activation of stress erythropoiesis in these mice. We also noted an increase in Cd68+ macrophages in Ncoa4fl/fl;EpoR-Cre mice (Figure 5F), suggesting liver macrophages are present in these mice for recycling damaged RBC but also to contribute to stress erythropoiesis. However, there were no apparent differences in liver hepcidin expression (Online Supplementary Figure S3H). To evaluate RBC lysis, we analyzed haptoglobin and hemopexin levels. Here, we observed a trend towards decreased hemopexin in Ncoa4fl/fl;EpoR-Cre mice which could suggest higher RBC fragility after PHZ administration (Online Supplementary Figure S3I).

Next, we performed a time-course experiment to understand the kinetics of recovery from PHZ-induced anemia (Figure 5G). Similarly to the Ncoa4fl/fl;UBC-cre/ERT2 model, Ncoa4fl/fl;EpoR-Cre mice reached a lower hematocrit nadir (Figure 5G). However, within the time course of the experiment, Ncoa4fl/fl;EpoR-Cre mice recovered the same hematocrit levels as EpoR-Cre mice (Figure 5G), suggesting reticulocytosis is not impaired in this model. This is in contrast to the systemic KO mice in which recovery was incomplete in the same time period suggesting that non-erythroid loss of Ncoa4 impairs recovery, likely through disrupting systemic iron metabolism. At the endpoint of the time-course experiment (day 11 after PHZ), spleen size
Figure 5. Stress erythropoiesis increases iron retention and macrophages in Ncoa4^{fl/fl};EpoR-Cre mice. EpoR-Cre and Ncoa4^{fl/fl};EpoR-Cre animals were treated with phenylhydrazine (PHZ) to induce red blood cell (RBC) lysis. (A) RBC count, hematocrit, and hemoglobin in Ncoa4^{fl/fl};EpoR-Cre and EpoR-Cre mice after PHZ treatment (4 mice/group, error bars represent the standard error of mean (s.e.m.). Data are also presented in Online Supplementary Table S7. (B) Erythropoietin levels in serum of EpoR-Cre and Ncoa4^{fl/fl};EpoR-Cre mice (n=7/group, error bars represent the s.e.m.). (C) Hif-2α protein levels from Ncoa4^{fl/fl};EpoR-Cre mice. β-actin (Actb) served as a loading control. (D) Hif-2α mRNA fold-change for EpoR-Cre versus Ncoa4^{fl/fl};EpoR-Cre mice. (n=3/group, error bars represent the s.e.m.). (E) Erythroblastic islands associated with Cd68 staining in liver of EpoR-Cre and Ncoa4^{fl/fl};EpoR-Cre mice. (Top) Representative field (40x, scale=50 μm) of five quantified fields per animal (3 mice per group). (Bottom) Quantification of the number of erythroblastic islands (3 mice/group, error bars represent the s.e.m.). (F) Elevated Cd68 staining in liver of EpoR-Cre and Ncoa4^{fl/fl};EpoR-Cre mice. Representative field (20x) of 3 mice/group (scale bar=200 μm). Bottom: Relative Cd68 expression of CD68 EpoR-Cre and Ncoa4^{fl/fl};EpoR-Cre mice (n=3/group, error bars represent the s.e.m.). (G) Serial complete blood count profiling of PHZ-administered EpoR-Cre and Ncoa4^{fl/fl};EpoR-Cre mice. (EpoR-Cre, 5 mice/group, Ncoa4^{fl/fl};EpoR-Cre, 4 mice/group, error bars represent the s.e.m.). (H-J) Increase in tissue iron staining in Ncoa4^{fl/fl};EpoR-Cre mice as determined by Prussian blue staining of spleen (H), liver (I) and kidney (J) (representative fields of 3 mice/group, 10x, scale bar=200 μm). (Bottom) Tissue iron levels in spleen, liver, and kidney (3 mice/group, error bars represent the s.e.m.). For all panels, statistical comparison was performed using a two-tailed Student t-test: *P<0.05, **P<0.01, ***P<0.001, except for panel G (hematocrit, day 2), where a one-tailed Student t-test was performed: *P<0.05. RBC: red blood cell count; HCT: hematocrit; HGB: hemoglobin; EPO: erythropoietin; Hif-2α: heat-inducible factor-2α; MCH: mean corpuscular hemoglobin; CHr: reticulocyte hemoglobin content; MCV: mean corpuscular volume; RDW: red blood cell distribution width; PLT: platelet count; WBC: white blood cell count.
Mechanisms of NCOA4-maintained murine erythropoiesis

(Online Supplementary Figure S4A) and Epo levels (Online Supplementary Figure S4B) normalized suggesting complete recovery. No differences were observed in the differentiation profile and number of precursors in bone marrow (Online Supplementary Figure S4C).

Consistent with the UBC-Cre/ERT2 model, we observed a lower hematocrit nadir in the Ncoa4fl/fl;EpoR-Cre model in comparison to that of the control. Consistent with previous reports,14 hematocrit levels recovered 3 days after nadir, which is in contrast with the systemic KO model in which the hematocrit level decreased even further in the same period of time, coincident with a delay in reticulocytosis. These data suggest that reticulocytosis is not impaired in Ncoa4fl/fl;EpoR-Cre mice, a model in which tissue Ncoa4 expression and systemic iron metabolism are intact. On the other hand, in the Ncoa4fl/fl;EpoR-Cre model Prussian blue staining (Figure 5H-J, top) and tissue iron levels (Figure 5 H-J, bottom) were increased after PHZ induction. Non-heme iron in RBC from Ncoa4fl/fl;EpoR-Cre animals was not elevated (Online Supplementary Figure S4D), suggesting that elevated tissue iron is not a consequence of elevated iron in RBC but more likely due to increased hemolysis. Overall these data suggest that RBC from Ncoa4fl/fl;EpoR-Cre mice are more sensitive to PHZ-induced lysis.

NCOA4 depletion impairs hemoglobinization and redox balance in K562 cells

In order to develop a deeper understanding of the cell autonomous role of NCOA4, we depleted NCOA4 in a K562 cellular model of erythropoiesis and analyzed the proteomic changes under basal and differentiation conditions. Using mass spectrometry-based quantitative proteomics, we identified and quantified 7,869 proteins (Online Supplementary Table S8). NCOA4 was depleted in both basal and hemin-differentiated conditions (Online Supplementary Table S9). Hemoglobin subunits (HBD, HBE1, HBZ) were significantly decreased in NCOA4-depleted cells (Figure 6A, Online Supplementary Table S9) as were the associated GSEA pathways (hemoglobin complex and AHSP pathway: hemoglobin chaperone system) (Figure 6B, Online Supplementary Figure S5A). Several heme synthesis pathway proteins were decreased under basal conditions, including FECH and UROS (Figure 6A, Online Supplementary Table S9). Likewise, the heme metabolism GSEA pathway was downregulated (Figure 6B, Online Supplementary Figure S5A). These results are consistent with a phenotypic defect in hemoglobinization of NCOA4-depleted K562 cells and in vivo in erythroid cells and aligns with a defect in iron mobilization to mitochondria to support heme synthesis.9

We next used erythroid differentiation stage-specific proteomic maps to analyze the effects of NCOA4 depletion during differentiation.25 Control cells treated with hemin (shGFP+HM vs. shGFP) showed the strongest correlation with advanced stages of erythropoiesis whereas NCOA4-depleted cells (shNCOA4 vs. shGFP) were associated with early-stage progenitor proteomes (Figure 6C, Online Supplementary Table S10). In comparison to basal conditions, NCOA4-depleted cells treated with hemin (shNCOA4+HM vs. shGFP+HM) were closer to the later-stage proteomes showing that there was some ability of NCOA4-depleted cells to differentiate (Figure 6C, Online Supplementary Figure S5B, Online Supplementary Table S10).

We further analyzed proteomic changes for potential molecular explanations of the increased oxidant sensitivity of Ncoa4-deficient erythrocytes. Catalase, important for mitigating oxidative stress in erythroid cells, was significantly decreased in NCOA4-depleted cells (Figure 6A, Online Supplementary Table S9). The decrease in catalase is likely due to reduced ferritinophagy which decreases bioavailable iron and thereby decreases the basal accumulation of reactive oxygen species by the Fenton reaction of the Haber-Weiss cycle.42,43 We next determined basal and induced levels of reactive oxygen species in RBC from Ncoa4fl/fl;EpoR-Cre and EpoR-Cre mice. While a decrease in basal levels of catalase would portend increased sensitivity to redox stress, there was no significant increase in baseline or induced reactive oxygen species levels. On the contrary, consistent with prior results in NCOA4-depleted cancer cell lines, RBC from Ncoa4fl/fl;EpoR-Cre mice appeared resistant to direct challenge by reactive oxygen species (Figure 6D). However, there were no differences in reactive oxygen species levels (Figure 6E) in response to ex vivo PHZ challenge. Further investigation is warranted to determine the cause of the increased sensitivity of Ncoa4-depleted RBC to PHZ.

Discussion

Our data support a model of both cell autonomous and non-autonomous roles for Ncoa4 in erythropoiesis and establish the importance of Ncoa4 in regulating basal systemic iron homeostasis. Prior data from cell culture model systems revealed an important role for constitutive activity of Ncoa4-mediated ferritinophagy in supporting basal intracellular iron homeostasis. Using a tamoxifen-inducible murine model of Ncoa4 depletion, we now demonstrate that there is a similar constitutive flux through the Ncoa4-mediated ferritinophagy pathway in vivo to support systemic iron homeostasis. We further demonstrate that there appears to be variable flux through this pathway depending on the organ. After acute Ncoa4 ablation, Fth1 accumulates in liver, spleen, and kidney but not in the pancreas or bone marrow and to a varying degree in the brain. This suggests a higher basal flux through the ferritinophagy pathway in specific tissues involved in systemic iron homeostasis. Further exploration of additional tissues and specific cell types under basal and stimulated conditions will be instructive regarding the tissues with the highest basal and stimulated requirements for ferritinophagy for maintaining intracellular iron homeostasis. Furthermore, our work supports additional study of the role of Ncoa4 under pathological conditions in which iron homeostasis is disrupted, such as hemochromatosis and iron deficiency anemia.44 Impaired whole body Ncoa4 function induces a hypochromatic microcytic anemia consistent with an iron deficiency anemia despite tissue iron overload, highlighting the importance of Ncoa4-mediated ferritinophagy in supporting erythropoiesis.12 Our data are the first to show that Ncoa4 has both cell autonomous and non-autonomous roles in erythropoiesis. Constitutive targeted ablation of Ncoa4 in the erythroid compartment leads to a pronounced anemia in postnatal stages and a mild hypochromic microcytic anemia in adult mice. The impaired hemoglobinization of RBC (low mean corpuscular hemoglobin) and reticulocytes (reticulocyte hemoglobin content) shows that Ncoa4-mediated fer-
ritinophagy is an important step in vivo in the intra-erythrocyte itinerary and utilization of iron for heme synthesis. This aligns with in vitro data showing that ferritin is an important intermediate step in iron destined for heme synthesis. In comparison to the anemia in a constitutive systemic Ncoa4 KO mouse model, the anemia in our erythroid targeted model is less severe, also suggesting a non-cell autonomous role of Ncoa4 in supporting erythropoiesis.

Given the constitutive KO nature of prior model systems, it was initially unclear whether the observed microcytic anemia in adult Ncoa4-deficient mice was an early or late consequence of Ncoa4 depletion. Gao et al. recently showed that the most severe defect in erythropoiesis is in the immediate postnatal period, highlighting the importance of accounting for temporal variations in the requirement for Ncoa4 function. Similarly, targeted deletion of Ncoa4 in the erythroid compartment provokes a more significant anemia in postnatal mice, albeit less severe than that of the systemic knockout model. The severity of the phenotype in the postnatal period is likely a consequence of the significant demand for iron mobilization from ferritin in all tissues, in particular from the liver where iron stores are high in neonatal mice, to rapidly expand the erythroid compartment. In this prior study, Gao et al. proposed a new role for Ncoa4 in mediating a thyroid hor-
mone-dependent transcriptional program that supports erythropoiesis. While our results do not exclude this possibility, it is clear that the erythroid-specific function of Ncoa4 does not account for the entirety of the anemia effect in systemic Ncoa4 KO mice. These results will allow us to reconcile with the clear role of Ncoa4 in mediating ferritinophagy as well as reports that Ncoa4 participates in DNA replication origin activation.

Contrasting these results from the postnatal stage with those from adult mice, Ncoa4-deficient mice recover from the profound postnatal anemia either because they are no longer dependent on cell autonomous or non-autonomous Ncoa4 function or because compensatory mechanisms replace Ncoa4 function. For the first time, we are now able to temporally control Ncoa4 ablation. Our results show that Ncoa4 is important in the adult mouse given that an acute ablation of Ncoa4 leads to anemia. While we have not determined the cause of the acute anemia it is interesting to speculate based on results from Gao et al. that show a block in terminal differentiation of primary human erythroblasts upon acute NCOA4 knockdown, which may render a subset of newly synthesized reticulocytes particularly sensitive to turnover.

The role of Ncoa4 in erythroid differentiation has been studied intensively with mixed results. Our results with targeted constitutive ablation of Ncoa4 in the erythroid compartment showed no appreciable defect in differentiation of erythrocyte precursors derived from an adult mouse. Likewise, we saw no effect on erythroid differentiation after acute systemic Ncoa4 depletion. Our results could suggest a number of possibilities with respect to the role of Ncoa4 in erythroid differentiation: (i) erythroid intrinsic Ncoa4 function may not be important for in vivo differentiation; (ii) erythroid intrinsic Ncoa4 function in differentiation may be dispensable and compensated for by other intrinsic or extrinsic mechanisms; and/or (iii) erythroid differentiation is dependent on Ncoa4 in postnatal mice but not in adult mice. Erythroid compartment-specific Ncoa4 rescue in the setting of a Ncoa4 null mouse may answer the question of extrinsic effects on differentiation.

Overall, we have shown that Ncoa4 is important for basal and stimulated erythropoiesis by regulating both systemic and RBC iron metabolism. Loss of Ncoa4 triggers significant compensatory mechanisms to mitigate baseline and induced anemias. In response to Ncoa4 depletion, the Hif-2α-Epo axis is upregulated to compensate for anemia. We note that with a concurrent iron deficiency, activation of Irp1 may repress Hif-2α translation; however, the net result in our system is induction of Hif-2α protein expression. Activation of the Hif-2α-Epo pathway mediates a compensatory increase in Fpn expression, which increases iron availability from tissues and iron import from the diet. Furthermore, both the Epo system and extramedullary erythropoiesis are required for recovery from chemically-induced anemias in Ncoa4-null mice. As Ncoa4-depleted RBC maintain their ability to synthesize heme and hemoglobin, there must exist additional baseline or compensatory pathways to ensure continued iron delivery to the mitochondria. Our erythroid compartment-specific mouse model shows a cell autonomous role of Ncoa4 but also highlights the non-autonomous role of Ncoa4 in maintaining erythropoiesis. Future work will be directed at determining the non-autonomous contribution of Ncoa4 to erythropoiesis, with the liver (hepatocytes) and macrophages being two likely cell types given their established roles in systemic iron homeostasis.

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