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Developing erythrocytes take up exceptionally large amounts of iron, which must be transferred to mitochondria for incorporation into heme. This massive iron flux must be precisely controlled to permit the coordinated synthesis of heme and hemoglobin while avoiding the toxic effects of chemically reactive iron. In cultured animal cells, iron chaperones poly rC-binding protein 1 (PCBP1) and PCBP2 deliver iron to ferritin, the sole cytosolic iron storage protein, and nuclear receptor coactivator 4 (NCOA4) mediates the autophagic turnover of ferritin. The roles of PCBP, ferritin, and NCOA4 in erythroid development remain unclear. Here, we show that PCBP1, NCOA4, and ferritin are critical for murine red cell development. Using a cultured cell model of erythroid differentiation, depletion of PCBP1 or NCOA4 impaired iron trafficking through ferritin, which resulted in reduced heme synthesis, reduced hemoglobin formation, and perturbation of erythroid regulatory systems. Mice lacking Pcbp1 exhibited microcytic anemia and activation of compensatory erythropoiesis via the regulators erythropoietin and erythroferrone. Ex vivo differentiation of erythroid precursors from Pcbp1-deficient mice confirmed defects in ferritin iron flux and heme synthesis. These studies demonstrate the importance of ferritin for the vectorial transfer of imported iron to mitochondria in developing red cells and of PCBP1 and NCOA4 in mediating iron flux through ferritin.

Introduction
Iron deficiency is the most common nutritional deficiency in the world, affecting more than 2 billion people (1). Globally, iron deficiency accounts for 841,000 deaths per year, and the loss of disability-adjusted life years has been estimated to be above 35 million, primarily due to maternal and perinatal deaths from severe anemia (2). In the adult human, nearly 70% of total body iron is present in circulating rbc. These cells are produced in the bone marrow, which releases into the bloodstream more than 2 million reticulocytes every second (3). To accomplish this remarkable rate of production, 25 mg of iron per day must be routed to the erythron, taken up into erythroid precursors, delivered to mitochondria, and converted into heme for incorporation into hemoglobin (Hb).

The mechanisms of iron acquisition and utilization by developing rbc have been characterized in considerable detail (4). Upon initiation of development, erythroid progenitors dramatically expand the uptake of transferrin-bound iron (Fe3+,Tf) by elevating the expression of transferrin receptors on their cell surface. Iron that is internalized via transferrin receptor–mediated endocytosis is ultimately delivered into mitochondria for heme synthesis as the maturation process progresses. There, iron is incorporated into protoporphyrin IX to form heme, which is eventually delivered to globin proteins to form Hb. Yet the mechanisms by which internalized Fe3+,Tf is delivered to mitochondria are poorly understood.

Cytosolic free iron can harm cells due to its chemical reactivity when present in excess or mistrafficked. Thus, the acute expansion of cellular iron during the late stages of erythropoiesis necessitates a system that can either rapidly deliver the ions into the site of utilization or promptly sequester toxic iron molecules into a storage compartment. Ferritin is the exclusive storage site of elemental iron in the cytosol of mammalian cells. The biologically functional form of ferritin is a 24-subunit, hetero-oligomer composed of 2 peptides, ferritin-H and ferritin-L, featuring a hollow, spherical cage that can accommodate up to 4,500 iron atoms (5). Ferritin expression rises early in the terminal erythroid differentiation phase and reaches very high levels in parallel with the large amount of iron import in erythroid cells (6, 7). However, whether ferritin merely serves to sequester excess iron or plays a more integral role in cellular iron trafficking during maturation is still debated.

Currently, studies suggest 2 routes for the flux of iron from the uptake system to mitochondria: the ferritin-independent and ferritin-dependent paths. Iron-labeling experiments with immature rbc in circulation suggest that the transfer of iron to mitochondria does not require ferritin (8–10). Instead, these studies propose a direct transfer mechanism in which the iron-laden endosomes directly contact and transfer iron to mitochondria. In support of this hypothesis, a mouse model of ferritin deficiency exhibits relatively normal rbc production, although other iron parameters are highly altered and may have compensatory effects (11). Conversely, ex vivo studies using erythroid progenitor cells demonstrate a transient deposition of exogenous iron into ferritin, which is followed by iron translocation to the heme moiety of Hb (12, 13). Chemical inhibition of lysosome function in these cells leads to a retention of iron in ferritin and impairs incorporation of iron into Hb (13). These studies indicate that iron must first enter the cytosol and be stored in ferritin and thereafter released from ferritin prior to heme iron synthesis.
Accumulation of 55Fe in ferritin precedes incorporation into Hb in developing G1E-ER4 cells. Cells were analyzed by immunoblotting. X indicates nonspecific protein. (B) Quantitation of 55Fe accumulation in cells, heme extracts, ferritin, and Hb. Iron levels at 8 hours and 24 hours of development are expressed as percentage of final levels (48 hours). (C) Temporal protein expression patterns of PCBP1 and NCOA4 during erythroid maturation. Cells treated as in A were analyzed by immunoblotting. X indicates nonspecific protein. (D) Developmental regulation of physical interactions between PCBP1 or NCOA4 and ferritin in differentiating erythroid progenitors. Cells treated as in A were analyzed by quantitative IP of ferritin and immunoblotting. PCBP2 was not detectable in any ferritin precipitates. Ferritin-bound PCBP1 and NCOA4 were quantified and normalized to α-tubulin in post-IP supernatant. Bulk rabbit IgG included as negative control for nonspecific interactions.

Figure 1. PCBP1 and NCOA4 activities are differentially regulated during rbc maturation in G1E-ER4 cells. Cells were treated with β-estradiol (β-Est), EPO, and 55Fe- Tf for 48 hours. (A) Accumulation of 55Fe in ferritin precedes incorporation into Hb in developing G1E-ER4 cells. Cells harvested at the indicated times were imaged as cell pellets (top) and analyzed by native gel electrophoresis and phosphorimaging. (B) Quantitation of 55Fe accumulation in cells, heme extracts, ferritin, and Hb. Iron levels at 8 hours and 24 hours of development are expressed as percentage of final levels (48 hours). (C) Temporal protein expression patterns of PCBP1 and NCOA4 during erythroid maturation. Cells treated as in A were analyzed by immunoblotting. X indicates nonspecific protein. (D) Developmental regulation of physical interactions between PCBP1 or NCOA4 and ferritin in differentiating erythroid progenitors. Cells treated as in A were analyzed by quantitative IP of ferritin and immunoblotting. PCBP2 was not detectable in any ferritin precipitates. Ferritin-bound PCBP1 and NCOA4 were quantified and normalized to α-tubulin in post-IP supernatant. Bulk rabbit IgG included as negative control for nonspecific interactions. n = 3 independent experiments. *P < 0.05; **P < 0.01, repeated-measures ANOVA with Bonferroni’s post-test.

Two proteins, poly C–binding protein 1 (PCBP1) and nuclear receptor coactivator 4 (NCOA4) have recently been implicated in controlling the flow of iron into and out of ferritin, respectively, in mammalian cells. PCBP1 is a multifunctional adaptor protein that can bind cellular RNA, DNA, and proteins, altering the fate of its binding partners (14). In erythroid cells, PCBP1 and its close paralog, PCBP2, were found in a nucleoprotein complex that interacts with C-rich sequences in the 3′ UTR of globin transcripts, where they were thought to stabilize the mRNA transcripts prior to translation (15). Subsequent studies identified PCBP1 as an iron-binding protein that delivers iron to ferritin (16) and other iron-dependent proteins, including a mononuclear iron enzyme, deoxyhypusine hydroxylase (17), and 2 dinuclear iron enzymes, prolyl hydroxylase 2 and asparagyl hydroxylase, that regulate the α-subunit of hypoxia-inducible factors (18). This interprotein metal transfer is achieved via direct protein-protein interactions, defining PCBP1 as a cytosolic iron chaperone. Although this metal delivery function is apparent in general cell culture models, especially when cells are iron deficient, whether it is required for or regulated by physiological events in cells specialized for iron trafficking remains unknown.

Recently, 2 independent groups identified NCOA4 as a receptor for autophagic cargo that specifically binds to ferritin and directs it to autophagosomes (19, 20). Ferritin-containing autophagosomes are targeted to lysosomes through autophagosome-lysosome fusion, which results in the degradation of ferritin and the release of its iron core. Cells initiate the turnover of ferritin under iron-deprived conditions, i.e., when the cellular demand for iron is high, by increasing the expression of NCOA4 (19, 21). Enhanced gene expression of NCOA4 in cells of erythroid lineage (22) and its dependence on GATA1 (23) have been identified by high-throughput transcriptome analyses, suggesting a role in erythroid maturation. Hb production is impaired by systemic losses of NCOA4 in zebra fish and mice (21, 24) and by NCOA4 RNAi in human cell culture models of erythroid development (21). Yet whether these phenotypes can be attributed to a dysregulation of ferritin iron release during erythroid maturation remains unclear, as NCOA4 has other functions as a transcriptional coactivator involved in various ligand-induced gene activations (25, 26) or a repressor of DNA replication (27).

Because the erythron is the major consumer of iron in mammals, we questioned whether PCBP1 and NCOA4 were functioning as ferritin iron regulators in the erythroid system. Here we show that genetic depletion of these proteins leads to erythropoietic defects by inhibiting the cellular flux of iron through ferritin. Our results identify PCBP1 and NCOA4 as indispensable mediators of the vectorial transfer of exogenous iron through ferritin and demonstrate that ferritin is an intermediate source of iron for heme biosynthesis in developing erythroid progenitor cells.

**Results**

**PCBP1 and NCOA4 differentially interact with ferritin during erythroid maturation.** We used G1E-ER4 cells for our in vitro cell culture studies. These cells are an extensively validated murine cell line that recapitulates the early stages of terminal rbc development. G1E-ER4 cells can be induced to differentiate from progenitors identical when cells are iron deficient, whether it is required for or regulated by physiological events in cells specialized for iron trafficking remains unknown.

Recently, 2 independent groups identified NCOA4 as a receptor for autophagic cargo that specifically binds to ferritin and
Figure 2. Depletion of PCBP1 or NCOA4 impairs iron flux through ferritin and heme biosynthesis in maturing erythroid progenitors. G1E-ER4 cells were subjected to 2 sequential treatments with a nontargeting control siRNA or with siRNA against PCBP1 or NCOA4, then treated with β-estradiol and 55Fe2-Tf for 24 hours. (A) Reduced hemoglobinization of PCBP1- and NCOA4-deficient cells. Representative cell pellets after treatment are shown. Con, control. (B) Altered levels of 55Fe in ferritin and Hb in PCBP1- and NCOA4-depleted cells. Levels of 55Fe in heme extracts and whole cells were determined by scintillation counting (n = 3). (C) Reduced heme synthesis in PCBP1- and NCOA4-depleted cells. Levels of 55Fe in heme extracts and whole cells were determined by scintillation counting (n = 3). (D) Effects of PCBP1 and NCOA4 depletion on indicators of cellular iron balance. Cells treated as in A were analyzed by immunoblotting for ferritin and IRP2. X indicates nonspecific protein. IRP2 quantitation shown at right (n = 6). (E) Effects of PCBP1 and NCOA4 depletion on indicators of heme levels. Cells were analyzed by immunoblotting for BACH1 and β-globin; quantitation at right (n = 6). (F) Repression of Hba-a1/2 (α-globin) transcripts in PCBP1- and NCOA4-depleted cells. Cells treated as in A were analyzed by real-time PCR (RT-PCR) (n = 5). Protein and transcript abundance were normalized to β-actin and are shown as relative to control siRNA-treated cells. *P < 0.05; **P < 0.01; ***P < 0.001, repeated-measures ANOVA with Dunnett’s test for multiple comparisons with control.

and labeled with 55Fe bound to transferrin (55Fe2-Tf), the physiologic iron source for erythroid cells (Figure 1A). Visual inspection of cell pellets demonstrated accumulation of red color, which indicated efficient hemoglobinization over this period. Cell lysates were analyzed by native gel electrophoresis and phosphorimaging to measure the 55Fe incorporated into ferritin and 55Fe-heme into Hb. We measured total cellular 55Fe and 55Fe-heme by extraction and scintillation counting. Each measurement at 8 and 24 hours was expressed as a percentage of the final level at 48 hours (Figure 1B). Earlier studies suggested that the formation of ferritin iron precedes heme biosynthesis during erythroid maturation (6, 12, 13). We confirmed that after 8 hours of differentiation, ferritin iron accumulation was readily apparent, while very little iron had been incorporated into heme or Hb. In contrast, after 24 hours of differentiation, iron was substantially present in heme and Hb as well as in ferritin. These quantitative approaches allowed us to determine that erythroid progenitors rapidly increased iron uptake and ferritin iron storage during differentiation and that iron incorporation into heme and Hb lagged behind iron uptake and ferritin storage, as previously described (6, 12, 13).

Proteins involved in heme and Hb synthesis are upregulated during terminal erythroid differentiation. We measured the abundance of our proteins of interest in the developing G1E-ER4 cells (Figure 1C). While there was no apparent increase in either PCBP1 or PCBP2 levels (PCBP2 levels actually fell), we found a substantial increase in NCOA4, ferritin, and β-globin expression at laterpoints of maturation. Notably, the increase in NCOA4 production occurred in the stages in which iron incorporation into heme and Hb was maximal and β-globin expression was present.

In general cell culture models, both PCBP1 and NCOA4 regulate cellular iron storage through direct protein–protein interaction with ferritin. Thus, the amount of protein bound to ferritin is indicative of their levels of activity. To test whether PCBP1, PCBP2, and NCOA4 bind to ferritin or whether their activities are regulated during erythroid maturation, we quantitatively isolated ferritin from G1E-ER4 cells at different stages of differentiation via IP and measured the amounts of coprecipitated PCBP1, PCBP2, and NCOA4 (Figure 1D). Although levels of PCBP1 in whole cell extracts changed little during differentiation, the amount of PCBP1 in the ferritin immunocomplexes fluctuated, with a peak early in differentiation (8 hours) that fell at later time points. This decrease in coprecipitated PCBP1 occurred despite an increase in total ferritin at later time points. In contrast, PCBP2 was not detectable in any ferritin isolates (data not shown). These data suggested that PCBP1, but not PCBP2, could function as an iron chaperone for ferritin mineralization in erythroid cells undergoing maturation. Ferritin-bound NCOA4 levels correlated well with NCOA4’s total cellular expression, featuring higher levels of ferritin-NCOA4 complexes at the later stages of differentiation. These data are consistent with a role for NCOA4 in the mobilization of iron from ferritin when heme synthesis occurs.

PCBP1 and NCOA4 are required for iron storage and hemoglobinization in developing erythroid progenitors. G1E-ER4 cells recapitulated the iron flux previously reported for developing erythroid cells. To characterize the roles of PCBP1 and NCOA4 in this process, we carried out a series of loss-of-function experiments using RNAi in G1E-ER4 cells (Supplemental Figure 2A). Again, we labeled cells with 55Fe2-Tf during differentiation. PCBP1- or NCOA4-deficient cells exhibited diminished red coloration after 24 hours of differentiation when compared with control-transfected cells, indicating impaired hemoglobinization (Figure 2A). We hypothesized that this was due to a dysregulation in cellular iron distribution. Phosphorimaging showed a 65% decrease in 55Fe incorporation into ferritin in cells depleted of PCBP1. Con-
versely, depletion of NCOA4 resulted in a 4-fold increase of iron in ferritin (Figure 2B). These data indicate that, in developing rbc, PCBP1 functions as an iron chaperone, facilitating iron accumulation into ferritin, and that NCOA4 is required for iron mobilization from ferritin, consistent with its role as an autophagic cargo receptor. We confirmed the depletion of PCBP1 and NCOA4 to 55% and 70%, respectively, Figure 2C), as suggested by the loss of red pigment and erythropoiesis in a manner opposite from that of PCBP1 depletion. NCOA4 protein abundance is posttranscriptionally upregulated by PCBP2 depletion (n = 4). Transcript and protein abundance were normalized to β-actin and α-tubulin, respectively. (F) PCBP2-depleted cells exhibit increased binding of ferritin to PCBP1 by co-IP. *P < 0.05; **P < 0.01; ***P < 0.001, paired 2-tailed t tests.

Figure 3. PCBP2 deficiency enhances the flux of exogenous iron through ferritin in developing erythroid progenitors. G1E-ER4 cells were depleted of PCBP2 by siRNA, then treated and analyzed as in Figure 2. (A) Red coloration is more prominent in PCBP2-deficient G1E-ER4 cells. (B) Confirmation of PCBP2 depletion by Western blotting. (C) PCBP2 deficiency increases 55Fe levels in ferritin and Hb during erythroid development (n = 3). (D) PCBP2 depletion affects protein indicators of cellular iron and erythropoiesis in a manner opposite from that of PCBP1 depletion. (E) NCOA4 protein abundance is posttranscriptionally upregulated by PCBP2 depletion (n = 4). Transcript and protein abundance were normalized to β-actin and α-tubulin, respectively. (F) PCBP2-depleted cells exhibit increased binding of ferritin to PCBP1 by co-IP. *P < 0.05; **P < 0.01; ***P < 0.001, paired 2-tailed t tests.

Although PCBP1 and NCOA4 depletion produced opposing effects on ferritin iron accumulation, both depletions led to lower levels of 55Fe-Hb (55% and 70% reductions, respectively, Figure 2D). Ferritin protein levels tend to increase when cytosolic iron levels rise due to enhanced translation, which is controlled by the iron regulatory protein–iron responsive element (IRP-IRE) regulatory system (30). Iron regulatory protein 2 (IRP2) is targeted for degradation when iron levels rise, which relieves the IRP2-mediated block on ferritin translation. PCBP1 deficiency produced a 63% decrease in IRP2 protein abundance, suggesting an expansion in the cytosolic iron pool and enhanced degradation of IRP2. In contrast, NCOA4 depletion produced a 180% increase in IRP2, consistent with a decrease in cytosolic iron levels and IRP2 degradation, likely due to ferritin sequestration of iron. These changes in IRP2 indicate that the cytosolic labile iron pool was affected by impaired loading and release of iron from ferritin caused by depletion of PCBP1 and NCOA4, respectively. Ferritin protein levels are also regulated via autophagic turnover, mediated by NCOA4 (19, 20). Autolysosomal degradation of ferritin is associated with the appearance of a lower molecular weight, truncated form of ferritin (20). This form was apparent in control cells and increased in PCBP1-depleted cells, but not present in NCOA4-depleted cells (Figure 2D). The changes in total ferritin protein levels were small, likely because changes in ferritin translation were offset by concomitant changes in ferritin turnover.

Erythroid cells express multiple regulatory systems to avoid excess globin production under conditions of diminished heme production. We questioned whether the heme deficiency produced by PCBP1 and NCOA4 depletion resulted in altered regulation of globin production. BTB domain and CNC homolog 1 (BACH1) is an erythroid transcriptional repressor that is rapidly degraded under conditions of heme sufficiency (31). We observed that BACH1 levels were elevated and that transcripts of Hba-a1/2 (α-globin), which are targets of BACH1 (32), were repressed in PCBP1- and NCOA4-depleted cells (Figure 2, E and F). PCBP1 and NCOA4 depletion also resulted in lower β-globin protein levels, although changes in Hbb-b1 mRNA were much smaller (Supplemental Figure 5A). In contrast, transcript levels of Alas2, the rate-limiting enzyme for heme biosynthesis, were increased in cells depleted of PCBP1 or NCOA4. These data indicate that the heme deficiency associated with PCBP1 or NCOA4 depletion altered the regulatory systems designed to coordinate heme and globin synthesis.

Iron supplementation fails to restore Hb levels in PCBP1- and NCOA4-depleted cells. We questioned whether the requirement for iron flux through ferritin was critical for erythroid maturation or whether the requirement could be bypassed by iron supplementation. Therefore, we depleted G1E-ER4 cells of PCBP1 or NCOA4 and allowed them to differentiate for 24 or 48 hours in the presence or absence of supplemental iron. Treatment of cells with iron...
for 4 or 24 hours produced a time-dependent increase in ferritin protein levels in control and PCBP1-depleted cells, consistent with an iron-mediated increase in ferritin translation, confirming the uptake of supplemental iron (Supplemental Figure 5, B and C). The increased iron did not restore β-globin levels in PCBP1-depleted cells, however, suggesting that the defect in heme synthesis persisted. Similarly, supplemental iron did not correct the β-globin deficit of NCOA4-depleted cells. As the translational upregulation of ferritin was also blocked in the NCOA4-deficient cells, these data suggest the supplemental iron was sequestered in ferritin. Taking these data together, we demonstrate that the iron flux through ferritin is an obligate component of mitochondrial heme synthesis in early to intermediate stages of terminal differentiation regardless of cellular iron availability.

Loss of PCBP2 enhances the flux of iron through ferritin. The above loss-of-function experiments demonstrated the requirement of PCBP1 for the entrance of iron into ferritin in developing erythroid cells. The PCBP protein family is composed of 4 members, all of which have been implicated in controlling cytosolic iron distribution (16, 33). Although PCBP2 was not detectable in ferritin immunoprecipitates, it was highly expressed in developing GIE-ER4 cells (Figure 1C). This led us to test whether PCBP2 affects cellular iron during rbc maturation. Again, we used siRNA to deplete cells of PCBP2 and 55Fe2-Tf labeling to track iron flux in GIE-ER4 cells. Surprisingly, cells exhibited enhanced hemoglobinization and E). These data demonstrate that PCBP2 suppressed the iron chaperone activity of its homolog, PCBP1, in ferritin mineralization. Notably, Hb iron contents were slightly higher in cells with the simultaneous loss of PCBP1 and PCBP2 when compared with those deficient in PCBP1 only. This implies that iron may reach mitochondria for heme synthesis independently of PCBP1, possibly through enhanced transfer of ferritin iron to the lysosome via NCOA4-mediated ferritinophagy.

Conditional PCBP1 deficiency in mice leads to microcytic anemia. Consistent with our cell-based studies, mouse models of NCOA4 deficiency are associated with defects in rbc hemoglobinization (21, 24). The role of PCBP1 in erythropoiesis has not been studied in a live animal model; therefore, we tested to determine whether loss of PCBP1 activity in mice could lead to erythropoietic defects. Pcbp1 is indispensable for murine embryonic development (34). To circumvent the lethality of constitutive germline Pcbp1 deletion, we generated a conditional deletion model in which the Pcbp1 locus is flanked by loxP sites (Pcbp1fl/fl). By breeding the Pcbp1fl/fl mice into a tamoxifen-inducible Cre recombinase background (Cre-ER), we were able to produce Pcbp1fl/fl and Pcbp1fl/fl Cre-ER mice (Supplemental Figure 7). Recombination was induced by weaning mice onto a tamoxifen diet for 15, 30, and 45 days prior to phenotypic analyses.

We measured PCBP1 transcript and protein abundance in bone marrow cells after tamoxifen induction and found that abundance was decreased in the Cre-ER–expressing mice (Pcbp1fl/fl) to 40%–50% of the levels in Pcbp1fl/fl mice, suggesting a moderate level of recombination efficiency (Figure 4, A and B). To determine the level of recombination in erythroid progenitors in the bone marrow, we isolated Ter119+ cells from Pcbp1fl/fl and Pcbp1fl/fl mice after tamoxifen treatment and measured a 70%–
75% decrease in PCBP1 transcript levels (Supplemental Figure 8A). The partial depletion of Pcbp1 was not enhanced by longer periods of dietary tamoxifen. Bone marrow cells from Pcbp1 mice featured 30%–35% lower β-globin protein levels and less intense red coloration, suggesting defects in hemoglobinization of the erythropoietic cells (Figure 4, B and C). Direct measurement of heme content indicated that Pcbp1 mice exhibited 25%–40% lower heme levels in the bone marrow compared with Pcbp1 animals (Figure 4D). These data suggested a defect in erythroid cell maturation in PCBP1-deficient bone marrow.

Although the erythropoietic defects in bone marrow cells of Pcbp1 mice occurred within 15 days of tamoxifen treatment, rbc parameters of circulating whole blood appeared relatively spared during this early stage of PCBP1 deficiency. After 30 days of tamoxifen, however, PCBP1-deficient animals exhibited hematological changes as measured by complete blood counts (CBC) (Figure 5 and Supplemental Table I). The mean corpuscular Hb (MCH) and mean corpuscular volume (MCV) were significantly lower in the Pcbp1-deficient mice when compared with wild-type controls (Figure 5A). By day 45, Pcbp1 mice additionally exhibited significant decreases in total Hb and hematocrit (Figure 5B) and in the total heme content of whole blood (Figure 5C). Red cell distribution width (RDW) was increased by 45 days in Pcbp1 (Supplemental Table I). These changes in erythrocyte indices reflected progressive development of microcytic anemia in PCBP1-deficient mice and resembled the prototypical manifestations of iron deficiency anemia. Consistent with iron-limited erythropoiesis, Pcbp1 mice at 30 days exhibited a precipitous loss of circulating reticulocytes (56% of Pcbp1, Figure 5D), which increased to control levels by 45 days. Wright-Giemsa stains of blood smears also demonstrated a loss of reticulocytes at day 30, followed by recovery of reticulocyte production by day 45. These changes suggested a potential compensatory response triggered by the anemia due to PCBP1 deficiency.

Compensatory erythropoiesis accompanies anemia in Pcbp1 deficiency. Reticulocyte numbers reflect the capacity of erythropoietic tissues to increase production of immature rbc to meet the demands of the animal. Histological analysis of bone marrow sections from both Pcbp1 and Pcbp1 mice at day 30 revealed a highly cellular marrow with nucleated cells of multiple lineages present. Assay for apoptotic cells revealed no differences between genotypes (Supplemental Figure 8B), indicating the absence of acute bone marrow failure and suggesting that the reduction in reticulocyte release from the erythropoietic tissue was not due to apoptotic loss of erythroid progenitor cells.

The increase in reticulocyte number detected at day 45 in the Pcbp1 mice suggested a compensatory response to the anemia. Anemia is sensed in the renal cortex as a relative hypoxia, which triggers the synthesis and release of EPO. We measured the Epo transcripts in the kidneys of Pcbp1 and Pcbp1 mice and found that, at 30 days of tamoxifen, levels were similar, but at 45 days of tamoxifen, Pcbp1 mice exhibited a 2-fold increase in Epo transcript levels (Figure 6A). PCBP1 deficiency was detected in kidney at both 30 and 45 days of tamoxifen, suggesting that the Epo response was not primarily due to PCBP1 deficiency in the kidney. Recent work has identified erythroferrone (ERFE, Fam132b) as an erythroid factor that responds to the EPO released under conditions of stimulated erythropoiesis (35). We measured Erfe transcripts in the bone marrow of Pcbp1 and Pcbp1 mice and again found that Erfe transcript levels were similar at days 15 and 30, but that levels were 3-fold higher in the Pcbp1 mice after 45 days (Figure 6B). ERFE is released from the bone marrow and sensed in the liver, where it functions to transcriptionally repress the expression of hepcidin (HAMP), the major regulator of dietary iron assimilation and macrophage iron release. We measured HAMP transcripts in the liver and found that, at day 45, their levels were reduced by 70% in the Pcbp1 mice (Figure 6C). These data all indicate that prolonged PCBP1 deficiency was associated with iron-limited anemia, which
activated the EPO-ERFE-hepcidin regulatory axis for compensatory iron uptake and rbc production.

Perturbations in systemic iron homeostasis can also affect Hamp gene expression through regulation of bone morphogenetic protein 6 (BMP6) (36). We measured plasma iron levels in Pcbp1fl/fl and Pcbp1rec mice and observed a small but significant decrease in plasma iron levels in PCBP1-deficient mice on day 45 (Figure 6D). The decrease in plasma iron was not associated with a decrease in Bmp6 transcripts, however, but rather a small increase (Figure 6E). These data suggest that the repression in liver Hamp expression was not primarily due to the changes in circulating iron, as these would be expected to decrease, rather than increase, expression of Bmp6. Similarly, Pcbp1 recombination in the liver did not account for the repression in Hamp (Supplemental Figure 9). Thus, hepcidin repression was likely a consequence of elevated ERFE transcript abundance in bone marrow of Pcbp1rec mice at 45 days. (C) Repression of Hamp (hepcidin) expression at 45 days in Pcbp1rec mice. (D) Small decrease in plasma iron levels in Pcbp1rec mice. Plasma was collected and nonheme iron levels were measured. (E) Small increase in liver Bmp6 transcripts not accounting for change in hep- cidin. n = 5–7 Pcbp1fl/fl; n = 3–7 Pcbp1rec per time point. *P < 0.05; **P < 0.01; ***P < 0.001, 2-way ANOVA with Bonferroni’s post-test.}

Figure 6. Compensatory responses to anemia in Pcbp1-deficient animals. Tissues from Pcbp1fl/fl and Pcbp1rec mice treated with tamoxifen as in Figure 4 were harvested and analyzed by quantitative RT-PCR, nonheme iron measurement, or histochemistry. (A) Increased Epo mRNA levels in kidney of Pcbp1rec mice at 45 days. Depletion of PCBP1 was measured at 30 and 45 days. (B) Elevated Farn532b (ERFE) transcript abundance in bone marrow of Pcbp1rec mice at 45 days. Our animal model Erfe erythropoiesis via EPO-mediated production. Collectively, we identified microcytic anemia as a compensatory step in iron trafficking to mitochondria for heme biosynthesis in maturing rbc.

Discussion

In general cell culture systems, mammalian cells exhibit a requirement for iron chaperone activity, mainly under conditions of iron limitation (16–18, 33). Whether animals or tissues and cells that are specialized for iron transport and utilization also depend on iron chaperones was not known. Our studies, using data derived from cultured cell, whole animal, and ex vivo differentiation models, all indicate that the iron chaperone activity of PCBP1 is required for erythroid ferritin mineralization, which is an obligatory step in iron trafficking to mitochondria for heme biosynthesis in maturing rbc.
from lysosomes to mitochondria. Rates of heme synthesis and Hb formation are high. In the latest stage of differentiation, PCBP1-mediated iron delivery to ferritin is low, iron delivery to mitochondria may include direct transfer from endosomes, and the last fractions of heme and Hb are synthesized before organelles are ejected and the circulating reticulocyte takes its mature form.

Depletion of PCBP1 or NCOA4 in erythroid cells appears to uncouple ferritin iron accumulation from the IRP-IRE regulatory system. IRP1 and IRP2 are part of a cell-autonomous, posttranscriptional, iron regulatory system in which the cytosolic labile iron pool is sensed and the IRPs respond by altering the synthesis of proteins involved in iron homeostasis (30). IRP1 and IRP2 repress the translation of ferritin mRNA when cytosolic iron is low. As iron levels rise, IRP1 and IRP2 RNA-binding activity is progressively lost and ferritin protein is synthesized. Typically, iron accumulation in ferritin parallels ferritin protein synthesis. In PCBP1-depleted G1E-ER4 cells, IRP regulation appears intact; iron supplementation leads to increased ferritin protein synthesis. PCBP1 depletion also promoted a decrease in the levels of IRP2, the degradation of which is triggered by elevated cytosolic iron (30). Yet iron accumulation into ferritin is drastically impaired. Reciprocally, in NCOA4-depleted cells, ferritin protein levels were comparable to control levels, a somewhat paradoxical effect for a protein that mediates ferritin turnover. In this instance, it appears that iron accumulation in ferritin triggers cytosolic iron depletion and concomitant activation of RNA binding by IRP1 and IRP2. IRP binding leads to repression of ferritin synthesis, even under iron supplementation. However, ferritin continues unabated, leading to large increases in ferritin iron without increases in ferritin protein. Thus, despite appropriate iron sensing by the IRP-IRE system, ferritin iron accumulation becomes uncoupled from ferritin protein levels. In a sense, PCBP1 and NCOA4 are essential components of the cell-autonomous iron regulatory system.

Ferritin is the only cytosolic storage protein for elemental iron identified in mammalian cells. Because excess cytosolic iron is toxic, some investigators hypothesize that the primary function of erythroid ferritin is the removal and sequestration of surplus iron from the cytosol and the mitochondrial heme-producing machinery (8–10). Our work confirms an alternative hypothesis that identifies ferritin as an indispensable intermediate in the transfer of acquired iron between the transferrin-iron uptake machinery and the mitochondria (12, 13). These studies were carried out with G1E-ER4 cells at an intermediate stage of terminal development, from proerythroblasts to basophilic erythroblasts; the ex vivo studies were carried out with splenic erythroid precursors synchronized at the CFU-E stage. Both approaches reveal the early stages of committed erythroid differentiation as the developmental period requiring coordinated PCBP1 and NCOA4 activity and their common target, ferritin, for efficient iron utilization. Our whole animal studies indicate that disruption of efficient iron utilization by PCBP1 depletion ultimately leads to microcytic anemia, underscoring the importance of these early events in erythroid iron utilization.

The role of ferritin in early erythroid development does not negate evidence for ferritin-independent iron trafficking in late stages of development (8–10). Reticulocytes that are newly released from the bone marrow contain RNA, ribosomes, mitochondria, and lysosomes (38). After release into the circulation, these early reticulocytes further mature, ejecting their intracellular organelles, e.g., mitochondria and lysosomes. Early reticulocytes actively synthesize heme and Hb, but deposit very little iron in ferritin (6). Thus, the role of ferritin in erythrocyte iron flux may be limited to the earliest stages of rbc development. The interaction of PCBP1 with ferritin is greatly lessened in the late stages of development in G1E-ER4 cells, consistent with a diminishing role of ferritin iron transit as erythrocytes mature. The mechanism that accounts for this change in PCBP1-ferritin interaction remains to be investigated. Although mice with partial depletion of PCBP1 or systemic Ncoa4 knockout exhibit microcytic anemia (24), mice with conditional deletion of ferritin H do not (11). The reason for this difference is not yet identified and may be multifactorial. Mouse with ferritin H depletion exhibit increased transferrin saturation and serum iron; they may exhibit other alterations in iron balance, such as circulating non-transferrin-bound iron. These changes may serve to increase iron delivery to the erythron and compen-
sate for the loss of ferritin. Alternatively, PCBP1 may be involved in ferritin-independent iron trafficking that serves to directly or indirectly facilitate iron delivery to mitochondria. Further studies are needed to clarify these possible mechanisms in erythroid cells.

In addition to PCBP1, PCBP2 can deliver iron to ferritin in yeast and cultured human cells (33). Our findings from erythroid cells, however, support a different role for PCBP2 in regulating cellular iron distribution. PCBP2 was not detected in complex with ferritin in erythroid progenitors, and the loss of PCBP2 increased iron incorporation into both ferritin and heme without compromising cellular accumulation of exogenous iron, suggesting that PCBP2 serves to limit ferritin iron accumulation in these cells. Codereplication of PCBP1 and PCBP2 resulted in a complete loss of PCBP2’s capacity to limit ferritin metatallation, meaning that PCBP2’s effects on ferritin are mediated through PCBP1. Furthermore, PCBP2 depletion enhanced the binding of PCBP1 to ferritin. Thus, PCBP2 functions to repress the PCBP1-mediated iron transfer through ferritin. How erythroid PCBP2 exerts this unique regulation on the iron pool would seem to be counterproductive and expose the cell to the toxic effects of iron. A direct transfer mechanism has been proposed to move iron from the endosomal compartment to mitochondria in developing reticulocytes (9, 10). It is tempting to speculate that a similar mechanism exists to transfer lysosomal iron to mitochondria, perhaps via direct contact between organelar membranes, as has been observed in yeast and melanocytes (41).

Methods

Cell lines, siRNA-mediated knockdown, and iron treatment. G1E-ER4 cells were maintained in Iscove’s modified Dulbecco’s medium (IMDM) with 15% FBS, 100 U/ml penicillin-streptomycin, 2 U/ml EPO (Amgen Epogen), monothioglycerol (1:10,000), and conditioned medium from Kit ligand–producing CHO cells (1:200). For differentiation, cells were plated and induced for differentiation at conditions above. All knockdown experiments were carried out using Silencer Select siRNA for mouse genes as identified: s76739 for PCBP1, s71297 for PCBP2, s77517 for NCOA4 (Ambion). Negative control siRNA (Ambion 4390847) was used for nontargeting transfecions. Ferric ammonium citrate was added to cell cultures as a supplement at a final concentration of 100 μg/ml.

Animals and tissue collection. Wild-type (C57BL/6), CMV-Cre (B6.C-Tg[CMV-Cre]1Cgn/J) and Cre-ER (B6.Cg-Tg[CAG-cre/Esr1*]5Amc/J) mice were purchased from The Jackson Laboratory. Targeting vectors and mice with a floxed allele of Pebp1 were generated by InGenious Targeting Laboratory. Targeted C57BL/6-129SvEv hybrid embryonic stem cells were microinjected into C57BL/6 blastocysts. Resulting chimeras with a high percentage agouti coat color were mated to C57BL/6 FLP mice to remove the FRT-flanked neo selection cassette. Mice carrying floxed alleles of Pebp1 were backcrossed for 4 to 6 generations with C57BL/6 mice. Pebp1/− males were crossed with...
Animals were euthanized by CO2, and blood was collected using heparin-pretreated syringes and tubes via cardiac puncture. The liver and kidney were excised and placed in RNAlater (Ambion) for preservation. Marrow cells were ejected from the femur and tibia with ice-cold PBS, pooled, and immediately processed for protein or RNA preparations. Ter119+ erythroid cells were isolated from bone marrow cells via MACS cell separation using anti-Ter-119 MicroBeads (Miltenyi Biotec 130-049-901). Genotyping was performed by PCR reactions of tail DNA using the MyTaq Extract-PCR Kit (Bioline). Primers for identifying wild-type, floxed, and null alleles of Pcbp1 are listed in Supplemental Table 2.

Primary erythroid cell preparation and culture. Primary erythroid progenitors were prepared from animals fed a tamoxifen diet for more than 4 weeks. Acute hemolytic anemia was produced by i.p. injections of PHZ in saline (60 mg/kg body weight) on days 0 and 1. Spleens were harvested on day 4 and gently mashed on and passed through a 100-μm cell strainer with chilled PHZ culture medium (alpha modification of Eagle’s medium [AMEM] containing 10% FBS and 100 U/ml penicillin-streptomycin). Mature rbc were removed using ammonium-chloride-potassium (ACK) lysis buffer. After washes with PHZ culture medium, recovery and viability of nucleated erythroid progenitors were assessed using an automated cell counter (Invitrogen) with trypan blue exclusion. For ex vivo erythroid maturation, the isolated spleen suspension was collected for total cell 55Fe measures and protein determinations. Radiolabeled cells were collected and washed with ice-cold PBS containing 10 mM EDTA and kept in chilled PBS. A part of cell samples, equal amounts of proteins were boiled in 1× LDS buffer containing 2.5% mercaptoethanol (BME) separated by 4%-12% Bis-Tris PAGE in 2-(N-morpholino)ethanesulfonic acid (MES) SDS running buffer (Invitrogen) and transferred to nitrocellulose membrane using the iBlot System (Invitrogen). Effective transfer was confirmed by Ponceau staining. Immoblotting was carried out using the following primary antibodies and dilutions: anti-ferritin at 1:2,000 (Sigma-Aldrich, catalog F5012); anti-PCBP1 at 1:5,000 (ref. 16); anti-PCBP2 at 1:500 (Abnova, M07, clone 5F12); anti-NCOA4 at 1:1,000 (Bethyl Laboratories, catalog A302-272A); anti-β-globin at 1:200 (Santa Cruz Biotechnology Inc., catalog sc-22718); anti-IRP2 at 1:1,000 (clone UT-30, gift from Elizabeth Leibold); anti-BACH1 at 1:1,000 (clone A1-6, gift from Mitsuyo Matsumoto); anti-α-tubulin at 1:10,000 (Abcam, catalog ab7291); and anti-β-actin, 1:5,000 (Abcam, catalog ab8224). Proteins were visualized and quantified using relevant IRDye-conjugated secondary antibodies and an ODYSSEY CLx infrared imager (LiCor). For isolation of ferritin and its bound protein, equal amounts of protein were incubated with the aforementioned ferritin antibodies precoupled to Dynabeads Protein A (Life Technologies). Immune complexes were captured by magnet, extracted from beads by boiling in 2× LDS buffer plus 5% BME, and processed for Western blot analyses as described above. Nonspecific rabbit IgG (Santa Cruz Biotechnology Inc., sc-2027) was used for control IP, and post-IP supernatants were analyzed to assure complete immunodepletion of ferritin.

Real-time PCR. RNA-later-treated (Ambion) organs were disrupted using a rotor-stator homogenizer (Omini International), while bone marrow, spleenocytes, and G1E-ER4 cells were lysed via repeated pipetting in TRI Reagent (Ambion). RNA was isolated using the Ribo-Pure Kit (Ambion) and reverse transcribed for 2-step SYBR green real-time PCR (ABI). Pcbp1, Pcbp2, Ncoa4, Tfrc, Hbb-b1, and Actb transcript abundance was assessed using QuantiTect primer assays (QiAGEN). A primer set for simultaneous detection of Hba-a1 and Hba-a2 mRNAs was designed from a sequence conserved between the 2 transcripts using NCBI’s Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Hba, forward, 5′-CGTGTGTCCTCCTCAAGTTACC-3′; Hba, reverse, 5′-GGTACAGTGCCAAGGGAGAG-3′; Alx2, Epo, HAMP, and ERF products were amplified via primers reported elsewhere (35, 42, 43). Specificity of each assay was confirmed by ampiclon melt curves, and data were analyzed using the double ACt method.

Radiolabeled iron assays. 59Fe-laden Tf was prepared by saturation of apo-Tf with 31FeCl2 complexed to nitritolriacetic acid as previously described (44). 59Fe-Tf was added to erythroid cell cultures at a physiological concentration, 2.6 μM, upon induction of development. Radiolabeled cells were collected and washed with ice-cold PBS containing 10 mM EDTA and kept in chilled PBS. A part of cell suspension was collected for total cell 59Fe measures and protein determination. Heme fractions were prepared by an acidified organic solvent extraction method previously reported (17). 59Fe contents were determined by scintillation counting and normalized to cellular protein levels from BCA assays. For assessment of 59Fe incorporation into ferritin cages and Hb, radiolabeled cells were lysed in 20 mM Tris-HCl (pH 7.5), 40 mM KCl, 0.5% Triton X-100, and an EDTA-free protease inhibitor cocktail (Roche). Equal amounts of protein were separated in 50 mM Tris-HCl (pH 7.5), 0.02% bromophenol blue, and 5% glycerol by 3%-8% Tris-Acetate PAGE with Tris-Glycine native running buffer (Invitrogen). Gels were dried under vacuum and exposed to a Storage Phosphor screen (GE Healthcare) for visualization by a Typhoon TRIO imager (Amersham Biosciences). Radio-iron signals were quantified via densitometric analyses using ImageQuant TL software (GE Healthcare).

CBC, total cellular heme, and plasma iron assays. CBC profiles of heparinized blood were assessed using a ProCyte Dx Hematology Analyzer (IDEXX). Heme contents in cell lysates were measured using the QuantiChrom Heme Assay Kit (BioAssay Systems) according to the manufacturer’s instructions. A ferrozine chromogenic method with iron standard solutions (Fisher Scientific) determined the total plasma iron levels as described elsewhere (45). Histology. Wright-Giemsa staining of blood smears and cytospins of G1E-ER4 cells were carried out at the Department of Laboratory Medicine of the NIH Clinical Center. Bone marrow slides were prepared from femurs fixed in 10% buffered formalin and decalcified by 5% EDTA. Tissue sectioning and H&E staining were performed at American Histo Labs Inc. Images of stained slides were produced using a NanoZoomer Digital Pathology system (Hamamatsu). Apoptotic cells within the marrow were identified using the In situ BrdU-Red DNA Fragmentation (TUNEL) Assay kit (Abcam).

Statistics. Data are presented as mean ± SEM of the indicated n of independent cell culture experiments or animals. Statistical significance was determined using paired or unpaired Student’s t tests,
repeated measures of ANOVA, or 2-way ANOVA with Bonferroni’s or Dunnett’s post hoc tests, depending on the study design and number of comparisons (Prism 5.0a, GraphPad Software). Significance was set at P < 0.05, 2-sided, for all analyses.

Study approval. All animal studies were reviewed and approved by the NIDDK Animal Care and Use Committee and performed in compliance with NIH guidelines for the humane care of animals.

Author contributions
MSR conceived and designed the research studies, conducted experiments, acquired and analyzed data, prepared figures, and wrote the manuscript. DZ designed and conducted experiments and acquired and analyzed data. OP designed and conducted experiments, acquired and analyzed data, prepared figures, and wrote the manuscript. MSE conducted experiments and acquired data. CCP conceived and designed the research studies and wrote the manuscript.

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