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Ferritin Iron Regulators, PCBP1 and NCOA4, Respond to Cellular Iron Status in Developing Red Cells

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Abstract

Developing red blood cells exhibit multiple, redundant systems for regulating and coordinating the uptake of iron, the synthesis of heme, and the formation of hemoglobin during terminal differentiation. We recently described the roles of poly rC-binding protein (PCBP1) and nuclear coactivator 4 (NCOA4) in mediating the flux of iron through ferritin in developing erythroid cells, with PCBP1, an iron chaperone, delivering iron to ferritin and NCOA4, an autophagic cargo receptor, directing ferritin to the lysosome for degradation and iron release. Ferritin iron flux is critical, as mice lacking these factors develop microcytic anemia. Here we report that these processes are regulated by cellular iron levels in a murine model of *ex vivo* terminal differentiation. PCBP1 delivers iron to ferritin via a direct protein-protein interaction. This interaction is developmentally regulated, enhanced by iron deprivation, and inhibited by iron excess, both in developing cells and *in vitro*. NCOA4 activity also exhibited developmental regulation and regulation by cellular iron levels. Excess iron uptake during differentiation triggered lysosomal degradation of NCOA4, which was dependent on the E3 ubiquitin ligase HERC2. Thus, developing red blood cells express a series of proteins that both mediate and regulate the flux of iron to the mitochondria.

Keywords

Iron; Erythropoiesis; Ferritin; Iron chaperone; Ferritinophagy

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Author Contributions

M-S.R. conceived and designed the research studies, conducted experiments, acquired and analyzed data, prepared figures, and wrote the manuscript. K.A.D. conducted experiments. C.C.P. conceived and designed the research studies and wrote the manuscript.

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Introduction

The terminal phase of red blood cell development relies on an ordered series of events to ensure the coordinated accumulation of iron, heme, and globin, as insufficient or excess levels of any of these components are toxic to the developing erythrocyte. Accumulation of each of these components is regulated at multiple levels; back-up systems designed to relieve excess accumulation are in place as well [1]. For example, terminal differentiation begins with the activation of a transcriptional program that includes high levels of mRNA for transferrin receptor 1, which permits the influx of iron, and of aminolevulinate synthase 2 (ALAS2), which catalyzes the initial, rate-limiting, step of heme synthesis. However, iron must accumulate before heme synthesis is activated. Iron regulatory proteins (IRPs) 1 and 2 block the translation of *Alas2* mRNA until sufficient iron accumulates to relieve this repression and allow ALAS2 translation and heme synthesis to proceed [2]. Similarly, heme must accumulate before globin synthesis is fully activated, as the transcriptional repressor BACH1 must undergo heme-mediated degradation [3] and the inhibitory eIF2 α kinase must undergo heme-mediated inactivation for globin transcription and translation to proceed [4]. Even with these redundant regulatory systems in place, erythroid precursors express efflux pumps for iron and heme to ensure that toxic components do not accumulate and that iron, heme, and globin stay in balance during differentiation [5, 6].

Erythropoiesis proceeds in the face of a fairly wide range of systemic transferrin-iron levels and erythrocyte iron availability. Thus, red cells must also have the capacity to tolerate changes in the rate of iron accumulation and to regulate the flow of intracellular iron to the mitochondria, where the enzyme ferrochelatase completes heme synthesis with the insertion of iron into protoporphyrin IX. As red cells begin terminal differentiation, freshly-acquired iron first accumulates in ferritin [7]. Ferritin is the sole iron storage protein located in the cytosolic compartment of erythroid and other cell types. It is composed of 24 H- and L-subunits that form a hollow sphere into which iron is deposited [8]. Because terminal erythroid differentiation begins with a large, progressive increase in transferrin-iron uptake, synthesis of ferritin is upregulated by development [7, 9], which allows for the storage of iron within the ferritin nanocage.

Elevated cellular iron availability also leads to an increase in ferritin protein expression. This increase is largely mediated through enhanced translation, which occurs as intracellular iron relieves the translational block imposed by IRP 1 and IRP2 [8]. Both H- and L-ferritin transcripts contain an iron-regulatory element (IRE) in the 5' UTR that can be bound by IRP1 and IRP2, blocking the assembly of the ribosome on the mRNA. As iron increases, the incorporation of an iron-sulfur cluster into IRP1 prevents its binding to IRE structures in ferritin mRNA, allowing ferritin protein synthesis to occur. Rising cytosolic iron levels also trigger the degradation of IRP2, thereby also relieving the translational block on ferritin mRNA.

While the amount of iron deposited into ferritin is generally proportional to the amount of ferritin protein synthesized, ferritin iron storage is controlled by multiple mechanisms. The actual delivery of iron to ferritin is dependent on the iron chaperone, poly rC-binding protein 1 (PCBP1), which binds iron in the cytosol and transfers it to ferritin through a direct

protein-protein interaction [10]. Iron leaves ferritin primarily through its degradation in a process termed ferritinophagy [11]. The transfer of ferritin iron to the mitochondria is initiated by the recruitment of cytosolic ferritin into autophagosomes, which are then delivered to the lysosome for degradation and iron release [11–13]. The targeting of ferritin to the autophagosome is mediated by nuclear coactivator 4 (NCOA4), an autophagic cargo receptor that specifically recognizes ferritin [11, 13]. While both PCBP1 and NCOA4 were initially identified as members of RNA- and DNA-binding complexes affecting gene expression, respectively [14, 15], subsequent studies revealed both as adaptor proteins with critical roles in cellular iron trafficking. Both have been found to control iron flux in developing red cells [16]. Here, we examine whether the iron flux through ferritin is a locus of regulation in developing red cells and find that the activities of both PCBP1 and NCOA4 are regulated directly by iron. This regulation is achieved through distinct mechanisms that permit the fine tuning of iron trafficking in the developing red cell.

Materials and Methods

Cell culture

G1E-ER4 cells were maintained in IMDM supplemented with 15% FBS, 100 U/mL penicillin-streptomycin, 2 U/mL erythropoietin (Epogen, Amgen), 0.1% monothioglycerol, and 0.5% conditioned medium from Kit ligand-producing CHO cells (CHO-KL cells). Committed erythroid differentiation of G1E-ER4 cells was induced by treating cells at 2×10^5 cells/mL with 100 nM of β -estradiol. Dr. Mitchell Weiss at the St. Jude Children's Hospital, Tennessee, USA generously provided both G1E-ER4 and CHO-KL cell-lines.

Cellular iron treatments and RNAi

Ferric ammonium citrate (FAC) was added to cell culture at a final concentration of 100 μ g/mL for cellular iron supplementation. Cellular iron availability was limited by desferrioxamine treatment (Dfo; 50 μ M). For the gene knockdown experiments, G1E-ER4 cells were transfected with siRNA twice, with an interval of 24 h. At each transfection, six million cells were mixed with 480 pmoles of siRNA in 100 μ L of Nucleofector Solution R (Lonza), and electroporated using a Nucleofector II device (Lonza). Silencer Select siRNA s77517 and s67460 (Ambion) were used for NCOA4 and HERC2 RNAi, respectively. Control transfections were done with non-targeting siRNA (Ambion 4390847). Differentiation of transfected cells were induced by β -estradiol treatment immediately after the second transfection.

Protein isolation and western analyses

G1E-ER4 cells were incubated on ice for 30 min in basal lysis buffer: 100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Igepal CA-630, 5% glycerol, and an EDTA-free protease inhibitor cocktail (Roche). Insoluble debris was removed by centrifugation. For western analyses, proteins were denatured by boiling in $1\times$ LDS containing 2.5% 2-mercaptoethanol (BME), separated by 4%–12% Bis-Tris PAGE in MES buffer (Invitrogen), and then transferred to nitrocellulose membrane using the iBlot System (Invitrogen). HERC2 proteins were labeled using a commercial antibody from Bethyl Laboratories, Inc (A301-905A). Primary antibodies for detection of other proteins as listed in reference [16]. All western images were

visualized using IRDye-conjugated secondary antibodies and the Odyssey CLx system (Li-Cor).

Co-immunoprecipitation (co-IP) and post-lysis iron treatments

Ferritin and ferritin-bound proteins in G1E-ER4 lysates were magnetically captured using Dynabead-conjugated anti-ferritin rabbit IgG (Sigma-Aldrich F5012). The bead-free supernatant were saved as post-IP samples. Control IP was conducted with nonspecific rabbit IgG (Santa Cruz Biotechnology Inc., sc-2027). Tris-2-chloroethyl phosphate (5 mM), and different levels of ferrous iron sulfate (FAS) and bathophenanthrolinedisulfonic acid (BPS) were supplemented to the basal lysis buffer for IP experiments involving post-lysis iron treatments. Immunocomplexes and post-IP lysates were boiled in 2X LDS with 5% BME, and subjected to western analyses as describe above.

Real-time quantitative PCR (qPCR)

G1E-ER4 cells treated with TRI reagent, and processed for RNA extraction by the RiboPure kit (Ambion). cDNA was generated using the High-Capacity cDNA Reverse Transcription kit (ABI), and amplified with QuantiTect primers (Qiagen) and Power SYBR Green Master Mix (ABI). Relative transcript abundance was determined by the 2^{-C_t} method.

Statistical Analysis

One-way ANOVA or repeated measures ANOVA followed by Bonferroni's post-test was conducted for comparisons. A p -value < 0.05 was considered statistically significant.

Results

Elevated cellular iron suppresses the interaction of PCBP1 with ferritin in developing erythroid progenitors

We recently identified new regulatory roles for PCBP1 and NCOA4 in the flux of imported iron through ferritin during erythroid development [16]. This study also demonstrated that loss of ferritin iron management by PCBP1 or NCOA4 depletion produces functional iron deficiency, causing impaired heme synthesis and hemoglobinization of differentiating red blood cells. These suggest that PCBP1 and NCOA4 potentially are mediators for the cellular response to fluctuating levels of iron, and thus may be functionally regulated by iron in the erythroid system. G1E-ER4 cells are murine erythroid progenitors that, when treated with β -estradiol *in vitro*, undergo differentiation that recapitulates the committed erythroid differentiation of the mammalian system *in vivo* [17]. Our previous studies identified substantial expression levels and activities of both PCBP1 and NCOA4 developing G1E-ER4 cells. We also noted previously that both PCBP1 and NCOA4 activities changed over the course of differentiation. PCBP1 protein levels are relatively constant in differentiating G1E-ER4 cells while ferritin protein levels increase substantially over 48 h. Yet the binding activity of PCBP1 for ferritin, as measured by quantitative co-immunoprecipitation, is maximal at early time points (8 h), then progressively falls (24–48 h) despite the rise in ferritin levels ([16] and shown again in Fig. 1A and B). We considered whether fluctuating cellular iron levels in these cells could account for this alteration in PCBP1-ferritin binding activity.

We first assessed the effects of cellular iron availability on the expression levels of PCBP1 and ferritin. Because much of the expression and activity of both PCBP1 and NCOA4 occurs within the first 24 h of differentiation, we focused on this time-point for our studies. G1E-ER4 cells were induced to differentiate and were treated with ferric iron and Dfo, a ferric iron chelator, to produce cellular iron repletion and deprivation, respectively. Total protein abundance of PCBP1 was not affected by either treatment in G1E-ER4 cells that are both proliferating and differentiating (Fig. 1C). Ferritin and β -globin protein levels were measured to confirm the effects of iron treatment and β -estradiol-induced differentiation, respectively. Notably, ferritin expression was highly upregulated by supplemental iron while β -globin levels remained relatively unaffected. Iron depletion by Dfo resulted in clear losses of both ferritin and β -globin proteins.

The iron chaperone activity of PCBP1 for ferritin is mediated by direct interaction between the two proteins [10] and the ferritin-binding activity of PCBP1 is post-translationally regulated by the developmental status of erythroid progenitor cells ([16] and Fig. 1A and B). Accordingly, we next tested whether the complex formation between PCBP1 and ferritin could be affected by cellular iron supply in a similar fashion. Ferritin and its bound proteins were isolated via IP from G1E-ER4 cells differentiated with or without iron treatments. In spite of higher ferritin protein levels, iron-supplemented cells contained much less PCBP1 protein in their ferritin immunocomplexes (Fig. 1D and E). Iron deprivation produced a level of ferritin-bound PCBP1 similar to that of controls, despite the lower ferritin protein levels, indicating an elevated PCBP1-to-ferritin ratio within ferritin precipitates and suggesting an increased affinity of PCBP1 for ferritin. For all IP experiments, complete isolation of ferritin-containing complexes was confirmed by assessing the depletion of ferritin in post-IP lysates (Fig. 1D and F).

We questioned whether this iron-induced, post-translational change in PCBP1 affinity occurred rapidly or after a delay (which could suggest an indirect mechanism). Thus, G1E-ER4 cells were induced to differentiate for 24 h, and supplemented with Fe for 1 to 4 h prior to harvesting. We observed that a drastic reduction in the amount of PCBP1 bound to ferritin occurred as early as one hour after cellular iron treatment (Fig. 1F). Notably, the iron effects on PCBP1 activity began prior to any dramatic changes in ferritin protein abundance, suggesting that the differential binding of PCBP1 to ferritin was not merely due to the changes in total PCBP1-to-ferritin ratio.

Excess ferrous iron dissociates PCBP1 from erythroid ferritin

The rapid loss of ferritin-bound PCBP1 after cellular iron supplementation led us to test if the interaction between PCBP1 and ferritin could directly respond to ferrous iron, the primary oxidation state of cytosolic labile iron *per se*. Accordingly, G1E-ER4 cells after 24 h of differentiation were lysed with buffers containing different levels of supplemental ferrous iron and BPS, a ferrous iron chelator. Addition of ferrous iron (FAS) led to a decrease in ferritin-bound PCBP1 levels in a concentration-dependent manner. These iron effects were blocked by addition of the ferrous iron chelator, again, in a fashion proportional to the concentration of BPS (Fig. 2). Notably, BPS alone led to a pronounced increase in PCBP1 complexed with ferritin versus untreated control lysates, suggesting the presence of

chelatable, labile iron that can suppress the ferritin-binding capability of PCBP1 in developing G1E-ER4 cells.

Impaired ferritinophagy caused by RNAi-mediated NCOA4 depletion leads to very high levels of iron loading into ferritin in developing G1E-ER4 cells ([16] and reproduced in Fig. 3A and B). We used this genetic approach to produce iron saturation in ferritin, and measured whether this metallation of ferritin could affect its levels of bound PCBP1. While NCOA4 depletion had no effect on total PCBP1 expression (Fig. 3A), NCOA4 depletion caused a reduction in ferritin-bound PCBP1 when compared to control siRNA-treated cells (Fig. 3C). Levels of intact ferritin protein (slower migrating ferritin band) in IP were not diminished by NCOA4 depletion, indicating that changes in total ferritin could not account for loss of PCBP1 interaction. The faster-migrating ferritin band, indicative of NCOA4-mediated ferritinophagy [13], is absent in NCOA4-depleted cells. Collectively, the experimental conditions used here produced increases in the cytosolic labile iron pool (Fig. 1), the *in vitro* ferrous iron pool (Fig. 2), and ferritin iron pool (Fig. 3); each of these conditions led to decreases in the amount of PCBP1 bound to ferritin, which mechanistically indicates that iron directly controls the affinity of PCBP1 for ferritin.

Elevated cellular iron supply decreases the expression and activity of NCOA4 in developing erythroid cells

In general cell culture models, ferritinophagy, the autophagic sorting of ferritin into the lysosome for degradation, appears to be a regulated process that is augmented in response to cellular iron or nutrient depletion [11, 18]. Our recent study highlighted the critical role that ferritin plays in the trafficking of iron to mitochondria in the developing erythrocyte [16]. NCOA4, the autophagic receptor for ferritin, is transcriptionally upregulated during terminal erythroid differentiation, particularly during stages of elevated iron demand, i.e., hemoglobin synthesis. Yet, whether iron affects NCOA4 activity in erythroid cells remains unknown. Therefore, we measured NCOA4 protein abundance under conditions of iron excess or deficiency in both undifferentiated and differentiating G1E-ER4 cells (Fig. 4A). NCOA4 protein levels were low in undifferentiated cells and did not significantly change with iron or Dfo treatment. Differentiation of G1E-ER4 cells triggered a significant increase in NCOA4 protein levels under control and iron-deprived conditions. NCOA4 levels were repressed, however, in developing G1E-ER4 cells treated with supplemental iron. Iron and Dfo treatments effectively changed ferritin protein abundance in both undifferentiated and differentiating cells, confirming the effects of iron treatment on the IRP regulatory system and suggesting a different mechanism for NCOA4 regulation. We measured the amount of NCOA4 bound to ferritin in iron-treated cells and found that the amount of NCOA4 protein bound to ferritin was proportionate to the total NCOA4 protein abundance (Fig. 4B). Under all iron conditions approximately 50% of the total NCOA4 pool was captured by ferritin immunoprecipitation and thus was in ferritin-bound form (Fig. 4B and C). This implies that regulation of NCOA4 activity by iron status primarily occurs through alteration of protein abundance.

As previously reported, formation of the lower molecular weight ferritin band is NCOA4-dependent, and reflects proteolytic cleavage of ferritin chains within the lysosome after

ferritinophagy [13]. The ratio of the full-length ferritin to the cleaved ferritin thus could serve as a semi-quantitative measure of ferritinophagy. Examination of the ratio of full-length to cleaved ferritin suggests that the sorting of ferritin into the lysosome occurred at a lower rate in the iron-treated cell, consistent with the loss of the NCOA4 receptor under these conditions (Fig. 4D). Ratios of full-length to cleaved ferritin were unchanged from control in Dfo-treated cells, consistent with the similar cellular levels of NCOA4 under those conditions. Thus, the reduction in NCOA4 expression and activity by high iron contributed to the uncoupling of ferritin from the ferritinophagy pathway.

Iron-induced turnover of erythroid NCOA4 requires HERC2 and intact lysosomal activity

The above studies demonstrate that NCOA4 is regulated by excess iron at the protein expression level in G1E-ER4 cells only during development. To identify the molecular mechanism by which NCOA4 protein responds to supplemental iron, we first assessed the effects of iron on *Ncoa4* transcript abundance in β -estradiol-treated G1E-ER4 cells. Unlike the protein, iron treatments had no effect on the transcript level (Fig. 5A). Recently, both lysosomes and proteasomes were shown to partially contribute to the constitutive turnover of NCOA4 [18]. We tested whether inhibitors of proteasomal or lysosomal proteolysis could prevent the loss of NCOA4 protein by excess iron. Control cells treated with vehicle (DMSO) exhibited the expected loss of NCOA4 protein after iron supplementation (Fig. 5B). In contrast, cells treated with the lysosomal inhibitor Bafilomycin A1 maintained NCOA4 expression levels in the presence of iron. Treatment with the proteasome inhibitor Bortezomib had no effect on the iron-mediated loss of NCOA4. These observations indicated that iron accelerates the turnover of NCOA4, a process that is dependent on the lysosome rather than the proteasome. Bortezomib treatment led to a mild accumulation of ferritin in both control and iron-treated cells, presumably by inhibiting a constitutive proteasome-mediated ferritin turnover [19]. The effectiveness of cellular iron supplementation was confirmed by measurement of elevated ferritin protein under both inhibitor treatments.

HERC2, an E3 ubiquitin ligase protein, has been implicated in the iron-induced turnover of NCOA4 in non-erythroid cells [18]. Thus, we tested whether this protein could carry out a similar regulatory role in erythroid cells, particularly in the developing ones. As described earlier, the iron responsiveness of NCOA4 was only present in G1E-ER4 cells undergoing differentiation (Fig. 4A). Thus, we first assessed temporal patterns of HERC2 expression during β -estradiol-induced maturation. Western analysis identified a strong induction of HERC2 expression during differentiation, which peaked after 24 h (Fig. 5C) and corresponded to the time when the effect of iron on NCOA4 was detectable. The developmental changes in *Herc2* mRNA level mirrored the protein abundance (Fig. 5D). Thus, HERC2 is expressed in G1E-ER4 cells in a developmental pattern that could affect NCOA4. Finally, we determined whether the iron-responsiveness of NCOA4 is in fact dependent on HERC2 activity via a genetic loss-of-function approach. Control and HERC2-depleted G1E-ER4 cells were induced to differentiate for 24 h with iron added for 0, 4, or 24 h. As expected, NCOA4 protein abundance was diminished by excess iron in control cells, but not in cells where HERC2 was depleted (Fig. 5E). Notably, HERC2 expression in control siRNA-treated cells did not change by iron treatments, indicating that HERC2

controls NCOA4 turnover without changes in its own expression. Similar to cells treated with Bafilomycin A1, HERC2-depleted cells still exhibited elevated ferritin protein when cultured with high iron despite the high levels of NCOA4 expression, consistent with multiple mechanisms operating to control ferritin protein levels. Overall, our data identify the HERC2- and lysosome-mediated turnover of NCOA4 as the primary mechanism for repressing NCOA4 activity in erythroid cells developing under excess iron.

4. Discussion

Red blood cell differentiation is remarkable for the speed in which the contents of the cell are replaced with a concentrated solution of hemoglobin and for the multiple layers of regulation that allow for the smooth coordination of iron accumulation, heme synthesis, and hemoglobin formation [1]. Herein, we have described additional control mechanisms that permit the fine-tuning of iron flux through ferritin as red cells develop (Figure 6).

Under conditions of reduced iron availability, PCBP1 demonstrated a high ferritin-binding activity, which would lead to more efficient transfer of limited cytosolic iron to ferritin. NCOA4 is stabilized under these iron-limited conditions, thereby more efficiently transferring ferritin to the lysosome for iron liberation and transfer to mitochondria. Under conditions of high iron uptake, PCBP1 exhibited a loss of binding activity for ferritin that was directly mediated by excess free ferrous iron. In this condition, iron-binding sites on both PCBP1 and ferritin may become saturated with iron, and the capacity of PCBP1 to utilize a bridging iron ligand to stabilize interaction with ferritin and mediate iron transfer may be impaired. HERC2 targeted NCOA4 for degradation in high iron, likely because the iron-bound form of NCOA4 is recognized and ubiquitinated by HERC2 [18]. Thus, when iron is in excess, ferritin loading becomes less efficient and turnover is inhibited, blocking the excess delivery of iron to mitochondria. The excess iron that is not loaded into ferritin can be directly detoxified by export via ferroportin, which is expressed and active in developing erythrocytes [6]. This iron is not “lost” *per se*, as it can be reacquired by transferrin and re-presented to the red cell for uptake.

The regulatory mechanisms described here can operate very rapidly, within minutes, and allow the developing red cell to ensure a consistent flow of iron to the mitochondria. Why does the cell need a regulatory system that operates on such a short time scale? Because transferrin-iron uptake and the amount of iron presented to the red cell cytosol can vary significantly over minutes to hours under normal physiologic conditions [20]. Levels of iron and transferrin saturation can vary by 100% in a normal healthy individual over the course of a day, but similar changes in iron availability within the mitochondria would be detrimental to efficient heme and hemoglobin synthesis. Thus, the obligatory accumulation of iron in ferritin [7, 16, 21] and the regulation of iron flux through ferritin ensures that variations in iron availability are minimized.

While the mechanisms that direct iron through ferritin to the lysosome are becoming clear, the mechanisms that allow iron to move from the lysosome to the mitochondrial matrix for incorporation into heme are becoming clearer, as well. Although some studies have described localization of DMT1 to lysosomes and the mitochondrial membrane [22, 23],

other work suggests that mucolipin-1 (MCOLN1) functions as a lysosomal iron channel, facilitating the movement of iron ions from the lysosome to the cytosolic compartment [24]. In developing erythrocytes, lysosomal iron destined for mitochondria may not be freely released into the cytosol, however. Direct contact between lysosome-like compartments and mitochondria have been observed in both melanocytes [25] and developing erythroid precursors [26]. Mitofusin-2 (Mfn2), which is required for tethering endosomes to mitochondrial membranes, is also required for the formation of these lysosome-mitochondria contact sites [25, 26]. Furthermore, Khalil, et al. found that both Mfn2 and MCOLN1 were required for normal heme synthesis, hemoglobin formation, and red cell development [26]. Whether the expression or activity of these proteins are also tightly regulated by iron status remains elusive, and warrants further investigation.

Collectively, we demonstrate that the actions of PCBP1 and NCOA4 on ferritin allow erythroid cells to rapidly respond to changes in cellular iron availability, which could provide a strategy to maintain cellular iron storage and utilization at a constant rate despite fluctuation in the total cellular iron supply.

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Figure 1. Cellular iron supplementation acutely suppresses PCBP1 binding to ferritin in developing erythroid progenitors

A. Constant PCBP1 and increasing ferritin protein levels in developing G1E-ER4 cells. G1E-ER4 cells were treated with β -estradiol (β -Est) for 48 h to induce committed erythroid development. Proteins were analyzed by immunoblotting of whole cell extracts. B. Developmental changes in PCBP1 binding to ferritin. Cells treated as in A were subjected to immunoprecipitation (IP) using anti-ferritin antibody or control IgG and immune complexes were analyzed by immunoblotting. Supernatant after IP was analyzed with α -tubulin antibody. Experiments similar to A and B were previously reported [16] and are included for reference. C. Total cellular PCBP1 protein abundance is not affected by cellular iron status in G1E-ER4 cells. G1E-ER4 cells were treated with β -Est, supplemental iron (Fe) was provided as ferric ammonium citrate at a final concentration of 100 μ g/mL, and desferrioxamine (Dfo), a ferric iron chelator, was added at 50 μ M to produce cellular iron deprivation. β -Globin abundance was measured as a marker of hemoglobinization. D. Loss of ferritin-bound PCBP1 in G1E-ER4 cells differentiating under high iron (24 h). Physical interaction of PCBP1 with ferritin was measured by IP of ferritin and immuno-blotting of PCBP1. Control IgG indicate precipitates from nonspecific antibodies. E. Quantitation of ferritin-bound PCBP1 normalized to respective post-IP α -tubulin values shown in Fig. 1C. Data are shown as mean $\pm$ SEM of $n = 4$ independent experiments. ***, $p < 0.001$. F. Rapid inhibition of the ferritin-binding activity of PCBP1 by iron supplementation to developing

G1E-ER4 cells. Fe was added to medium at each indicated time before reaching 24 h of development. Cells were analyzed by IP and western blotting as in D.

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Figure 2. Excess ferrous iron inhibits the interaction of erythroid PCBP1 with ferritin
Differentiated G1E-ER4 cells (24 h) were lysed in buffer containing the indicated amounts of ferrous ammonium sulfate (FAS) and a ferrous iron chelator, batho-phenanthroline disulfonate (BPS). Ferritin and its complexed proteins were isolated by IP and analyzed by immunoblotting. $n = 3$, representative immunoblots are shown.

Figure 3. Excess iron deposition in ferritin inhibits PCBP1 binding

A. Cellular depletion of NCOA4 by RNAi confirmed by western analyses. G1E-ER4 cells were transfected with each siRNA and induced with β -estradiol to differentiate for 24 h. B. Excess accumulation of iron in cells lacking NCOA4. Control cells and cells depleted of NCOA4 as in A, *above*, were incubated with $^{55}\text{Fe}_2$ -Transferrin for 24 h to radiolabel intracellular iron. Whole cell lysates were analyzed by native gel electrophoresis and phosphorimaging. Signal corresponds to iron in ferritin. A similar experiment was previously reported [16] and is included here for reference. C. NCOA4 depletion decreases the amount of PCBP1 bound to ferritin in developing G1E-ER4 cells. Relative abundance of PCBP1 in ferritin IPs was assessed by western analyses.

Figure 4. Cellular iron supplementation during erythroid development suppresses NCOA4 expression and activity

A. NCOA4 protein expression is downregulated by added iron only in developing G1E-ER4 cells. Cells were induced to differentiate in the presence of iron treatments as indicated. Whole cell lysates were analyzed by immunoblotting. Ferritin abundance was measured as a marker of cellular iron status. B. NCOA4 levels in immunoprecipitates of ferritin from developing G1E-ER4 cells decrease by cellular iron supplementation. C. Amounts of NCOA4 immuno-depleted by IP of ferritin are proportionate to total NCOA4 abundance. NCOA4 abundance in post-IP lysates were quantified from Fig. 4B. Difference in NCOA4 abundance between the post-IP samples from control and ferritin IP experiments are expressed in percentage of control post-IP values. D. Iron inhibits turnover of ferritin via autophagy. Full length (upper) and cleaved (lower) forms of ferritin were quantified using western blot images from Fig. 4B. Ferritin ratio represents ratio of full-length to cleaved ferritin. All quantitative values were normalized to α -tubulin values, and are presented as mean $\pm$ SEM of $n = 4$ independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Figure 5. NCOA4 suppression by high iron during erythroid development requires HERC2 and intact lysosomal activity

A: *Ncoa4* transcript abundance in developing G1E-ER4 cells does not respond to cellular iron treatments. *Ncoa4* transcript levels were measured using qPCR, and normalized to *Actb* mRNA abundance. Data expressed as mean $\pm$ SEM of $n = 3$ biological replicates. **B:** Loss of NCOA4 protein by high iron is inhibited by a lysosome inhibitor bafilomycin A1 (Baf), but not a proteasome inhibitor bortezomib (Bort). DMSO was added as a carrier control for both inhibitors. Effectiveness of iron treatment was confirmed by elevated ferritin expression. **C:** Differential expression of HERC2 protein during G1E-ER4 maturation. Erythroid development is demonstrated through enhanced β -Globin expression. **D:** Upregulation of HERC by erythroid development is detected at the transcript level. *Herc2* mRNA abundance in equal amounts of total RNA was assessed using qPCR. Data expressed as mean $\pm$ SEM of $n = 3$ independent experiments. **E:** HERC2 is required for the iron-induced turnover of NCOA4 in developing erythroid cells. Control or HERC2 siRNA-treated G1E-ER4 cells differentiated for 24 h with iron added 0, 4, or 24 h prior to harvesting. Ferritin was measured to validate each iron treatment. NCOA4 abundance was normalized to α -tubulin values. Data presented as mean $\pm$ SEM of $n = 3$ independent experiments.

Figure 6. PCBP1- and NCOA4-dependent iron flux through ferritin is regulated by cytosolic iron levels via distinct mechanisms

A. Schematic model of the effects of excess iron on the physical interaction between PCBP1 and ferritin. In normal [Fe], ferrous iron atoms incorporated into PCBP1 function as a bridging ligand, stabilizing PCBP1 interaction with ferritin. When iron is in surfeit (High [Fe]), ferrous iron occupies the bridging ligand binding site on both PCBP1 and the surface of ferritin, which results in destabilization of PCBP1 interaction with ferritin. Small circles indicate ferrous iron atoms. B. Schematic model of the effects of excess iron on HERC2 interaction with ferritin. In normal [Fe], NCOA4 binds to ferritin and recruits it to the autophagosome (ferritinophagy). In high [Fe], NCOA4 binds iron, which is recognized by the E3 ubiquitin ligase HERC2 [18], and ubiquitinated NCOA4 is targeted for lysosomal degradation.