Physiological Roles and Regulation of NCOA4 in Macrophages

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Cole Guggisberg

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Faculty Advisor: Dr. Moon-Suhn Ryu

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ABSTRACT

Iron is essential due to its vital role as a metalloprotein cofactor in numerous metabolic and physiological functions. Iron homeostasis is highly regulated at both the cellular and systemic levels, which is required for overall organismal well-being. Phagocytosing macrophages are critical to systemic iron homeostasis owing to their capability to recycle iron from senescent red blood cells, as well as storing iron under systemic distress. Ferritin, the cytosolic iron storage protein, and its regulation is imperative to the macrophages ability to perform these functions. Nuclear receptor co-activator 4 (NCOA4) has recently been identified as a key regulator of ferritin, mediating its degradation via ferritinophagy. Yet, its function in macrophages remains unclear. The present studies employed a cell culture model, J774 murine macrophages, to examine the role and regulation of macrophage NCOA4 by iron status, red cell iron recycling, and inflammation. By iron supplementation or iron chelation we found that macrophage NCOA4 is post-transcriptionally regulated by iron status, which is inversely related to ferritin abundance. The loss of NCOA4 impaired ferritin turnover which led to a reduction in viable cells in iron deprived conditions. To recapitulate erythrophagocytosis (EP) and red cell iron recycling macrophages were treated with opsonized erythrocytes. By EP, ferritin iron storage acts as a transition between heme iron and recycling of elemental iron by ferroportin. Ferritin abundance peaks at 12 h with a subsequent decrease at 24 h which is NCOA4-dependent. To simulate iron overload and inflammatory conditions, macrophages were treated with a minihepcidin analog, PR73. Hepcidin activity repressed NCOA4 protein leading to an accumulation of ferritin in basal macrophages, while preventing the turnover of ferritin between 12 and 24 h in erythrocyte laden macrophages. As a model of endotoxemia, macrophages were treated with lipopolysaccharide (LPS), which decreased both NCOA4 transcript and protein

abundance. This effect on the transcript level was confirmed *in vivo*, with *Ncoa4* mRNA abundance repressed in the spleens of mice treated with LPS, a tissue rich with phagocytosing macrophages. Altogether our studies demonstrate an active role of NCOA4-mediated ferritinophagy in macrophage iron homeostasis. The response of NCOA4 to hepcidin and LPS suggests systemic implications and thus may become a potential therapeutic target for treatments of iron disorders and anemia of chronic disease.

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Background

Iron is essential to nearly all living organisms due to the array of biological functions it is involved in, such as DNA replication, electron transfer, and heme biosynthesis. However, due to iron's redox reactivity, free iron can cause oxidative stress through the production of reactive oxygen species (ROS) via the Fenton reaction. Thus, cells possess sophisticated and complex regulatory systems to maintain cellular iron demands while also limiting accumulation and oxidative damage.

Iron functions in the cells and the body primarily in three forms – elemental (ionic) iron, heme, and iron-sulfur clusters. The cytosolic labile iron pool provides metabolically available iron atoms that become available to metalloproteins or transported to cellular compartments where the metal is in need. A class of iron chaperone proteins, poly-r(C) binding proteins (PCBPs), mediates the distribution of exchangeable cytosolic iron to iron-dependent enzymes and into ferritin, a protein of cellular iron storage.[1,2] Heme, a protoporphyrin ring with an iron atom coordinated at its center, is a multi-functional metalloprotein owing to its capability to participate in oxygenation and redox reactions. In mammals, the majority of heme in the body functions as an oxygen carrier as a component of hemoglobin in red blood cells and myoglobin in muscle tissue. Heme also serves as a prosthetic group of hemoproteins, such as cytochrome *c*, and thus is critical in energy metabolism.[3] Similar to heme, iron-sulfur clusters act as prosthetic groups in numerous metalloproteins to serve as electron donors-acceptors in oxidation-reduction reactions.

Elemental iron is required for optimal metabolic functioning, yet too much can be detrimental. Free, unbound iron is redox active, which leads to the production of hydroxy

radicals (·OH) by the Fenton reaction. The hydroxy radicals can then react with DNA, proteins, phospholipids, and other biomolecules causing cellular damage. Therefore, cellular iron homeostasis is tightly regulated.

Cellular Iron Homeostasis

To maintain cellular iron requirements cells take up transferrin bound iron through a transferrin receptor-mediated mechanism.[4,5] Transferrin (Tf) functions as an iron courier in the blood circulatory system, transporting and distributing redox inactive ferric (Fe^{3+}) iron throughout the body for utilization.[6,7] Tf-bound iron is taken up by the cell through the interaction of holo-Tf (diferric) with transferrin receptor 1 (TfR1) on the plasma membrane. This interaction initiates the internalization of the Tf-TfR1 complex by clathrin-mediated endocytosis.[8] Within the endosome a drop in the pH creates an acidic environment, resulting in the dissociation of ferric iron from Tf.[9,10] Metalloreductases, such as six transmembrane epithelial antigen of the prostate 3 (STEAP3), reduce ferric iron to ferrous iron, which is then transported to the cytosol by divalent metal transporter 1 (DMT1) and incorporated into the labile iron pool.[11,12] The remaining endosome containing the apo-Tf/TfR1 complex is transported back to the plasma membrane where apo-Tf dissociates, back into circulation.[13]

Cells can also obtain iron through methods separate from transferrin receptor-mediated endocytosis. Recent studies have identified the uptake of non-transferrin bound iron (NTBI) by several Zrt/Irt-like protein (ZIP) members, ZIP14 and ZIP8. Both ZIP14 and ZIP8 import ferrous iron, which can be immediately incorporated into the labile iron pool.[14–16]

Due to its potential cytotoxicity, labile iron is designated for utilization, storage, or export based on the iron requirements of the cell. In the cytosol iron can be utilized by

iron-dependent proteins or trafficked directly to organelles such as the mitochondria to be incorporated into heme or iron-sulfur clusters.[17] Assisting the trafficking of iron is the previously mentioned family of PCBPs, which have demonstrated iron chaperone activity within the cytosol, facilitating the distribution of ferrous iron.[18,19] For example, both PCBP1 and 2 have been shown to be required for the metalation of several iron-dependent enzymes regulating hypoxia-inducible factor (HIF) and cell proliferation.[20,21]

Iron that is not immediately utilized for metabolic purposes can be stored within the sole cytosolic iron storage protein, ferritin. Elemental iron is sequestered within ferritin in its nontoxic ferric state with each ferritin core accommodating up to 4,500 iron atoms. Expression of ferritin increases with rising intracellular iron levels.[22,23] Thus, ferritin provides two roles for the cell; during iron excess it protects the cell from iron toxicity, and serves as a reservoir when iron is required. The ferritin complex is a 24-subunit multimer consisting of two isoforms – ferritin heavy (FTH1) and light (FTL) chains. The expression and ratio of FTH1 and FTL that make up the protein shell varies by tissue type and physiological status.[24,25] Importantly, FTH1 possesses ferroxidase activity (Fe^{2+} to Fe^{3+}) which enables iron to be incorporated into its iron core.[26–28] Additionally, the metalation of ferritin has been shown to require the iron chaperone protein, PCBP1. *In vivo* and *in vitro* experiments have demonstrated a protein-protein interaction between PCBP1 and ferritin, facilitating metalation of the ferritin complex.[1,29] Furthermore, PCBP1 has been shown to regulate iron storage in developing erythroid cells, with microcytic anemia developing in *Pcbp1*-deficient mice.[30] The iron stored within ferritin is metabolically unavailable until the ferritin complex is degraded, which will be discussed in more detail in subsequent sections. Ultimately, the cells ability to store iron within ferritin is vital to the maintenance of cellular iron homeostasis.

Lastly, to maintain cellular iron homeostasis cells can export elemental iron through the only known mammalian cellular iron exporter, ferroportin (FPN1).[31–33] FPN1 activity can aid in the prevention of iron toxicity when intracellular iron levels rise beyond what the cell can handle. Additionally, flux of iron by FPN1 from duodenal enterocytes, hepatocytes, and macrophages is necessary to maintain systemic iron homeostasis.[34–36] Therefore, FPN1 possesses multi-level regulation owing to its vital role in cellular and systemic iron homeostasis, which will be described in more detail in the following sections. The structure of FPN1 and the mechanism by which it exports iron remains an area of intense research. Studies hypothesize that FPN1 consists of two transmembrane lobes that form a cavity for Fe^{2+} binding. It is believed that the Fe^{2+} binding causes a conformational shift of the two lobes from an inward (intracellular facing) to outward (extracellular facing) conformation, leading to the export of iron.[37–40] Ferroxidases in the extracellular space oxidize Fe^{2+} to Fe^{3+} which can then be bound and transported by Tf.[41,42] One study has reported that the iron chaperone PCBP2 interacts with FPN1, facilitating the export of iron. However, further studies are needed to confirm this finding.[43]

Mechanisms Regulating the Cellular Iron Pool

Cellular iron homeostasis is a tightly regulated and coordinated process between uptake, storage, export, and utilization. Central to the balance of these processes is the iron-responsive element/iron regulatory protein (IRE/IRP) system, which post-transcriptionally regulates the expression of multiple iron genes. The iron-dependent process is regulated by the binding of IRP1 (also functions as the cytosolic isoform of aconitase) or IRP2 to stem-loop structures, termed IREs, in the untranslated regions

(UTR) of messenger RNA (mRNA) transcripts. The binding activity of IRPs to IREs is high when cells are iron-deficient and low in iron-replete conditions.[44]

IREs can be present in the 5'- or 3'-UTR of the mRNA transcript. Transcripts with IREs located in the 3'-UTR, such as in *TfR1*, are stabilized by the binding of IRPs when the cell is iron-deficient, promoting translation of the protein and iron uptake by the cell. However, when iron levels increase IRPs dissociate from the IRE and the mRNA transcript is vulnerable to endonucleases, leading to translational repression.[45–47] Ferritin (*FTH1* and *FTL*) and *FPN1* mRNA contain an IRE in the 5'-UTR. Cellular iron deficiency and IRP binding in the 5'-UTR leads to a translational block, reducing ferritin and FPN1 expression and thus limiting iron storage and export. When iron levels are replete IRPs dissociate from the IRE and permit translation of the proteins promoting storage and export (Figure 1.1).[31,48–50]

The IRE/IRP system is not the only form of regulation for genes such as *TfR1*, ferritins, and *FPN1*, however the post-transcriptional nature of the mechanism is what makes it physiologically essential. As mentioned, excess intracellular iron can be cytotoxic due to ROS production by the Fenton reaction. Therefore, the IRE/IRP system provides a quick response to adapt to changes in intracellular iron to maintain homeostasis.

Another mechanism regulating intracellular iron homeostasis is the selective degradation of ferritin via a process termed ferritinophagy. When cells become iron deficient nuclear receptor coactivator 4 (NCOA4) acts as a selective cargo carrier receptor, mediating the delivery of ferritin to the autophagosome. This involves physical interaction and complex formation between NCOA4 and ferritin, particularly with its ferritin H units.[51,52] Fusion of the autophagosome with the lysosome leads to the

formation of the autophagolysosome and digestion of the contents by lysosomal enzymes. This results in the release of the ferritin iron core and an increase of bioavailable iron that can be utilized by the cell.[51–53]

The flux of iron through ferritinophagy is mediated by NCOA4 levels. NCOA4 protein abundance and activity is regulated by intracellular iron levels. Unlike other iron-regulatory genes such as FPN1, TfR1, and ferritin, the *Ncoa4* transcript does not contain an IRE in either the 5'- or 3'-UTRs. Instead, NCOA4 is post-translationally regulated by the activities of HERC2, an E3 ubiquitin ligase. When cellular iron is sufficient, NCOA4-HERC2 binding leads to ubiquitination and proteasomal degradation of NCOA4. This would limit ferritinophagy and promote iron storage within ferritin. Conversely, when cellular iron is deficient, NCOA4-HERC2 binding is reduced, stabilizing NCOA4 for enhanced NCOA4-mediated ferritinophagy (Figure 1.2).[51]

Systemic Iron Homeostasis at the Organismal Level

Iron is essential for every cell and tissue in the body; however, the bulk of our daily iron requirement is destined for the bone marrow for the development of new red blood cells (erythropoiesis). Each day nearly 200 billion red blood cells are produced, requiring roughly 20 to 25 mg of iron.[54] Systemic iron levels are maintained by dietary absorption, tissue iron stores, and recycling of senescent red blood cells by macrophages of the reticuloendothelial (RE) system. Systemic iron homeostasis requires the same level of intricacy and precision as cellular iron metabolism to sustain optimal tissue and organismal functioning.

Dietary iron is absorbed by duodenal enterocytes. Forms of dietary iron include heme and non-heme sources. Heme iron is generally considered more readily absorbed, however the mechanism of how this occurs remains unknown. Conversely, details

regarding non-heme iron absorption are more understood. Within the lumen of the intestine duodenal cytochrome B reduces ferric iron to ferrous iron, allowing it to be transported across the apical membrane by DMT1.[55–57] Enterocyte iron can be utilized, stored, or exported, depending on the iron requirements of the body. FPN1 on the basolateral membrane mediates the export of iron from the cell, where hephaestin oxidizes Fe^{2+} to Fe^{3+} which can then bind and be transported by Tf.[32,33,58]

Iron transport through absorptive enterocytes has been shown to be controlled at both the apical and basolateral membranes.[59] DMT1 expression at the apical surface of enterocytes is reduced by iron overload conditions, limiting the amount of non-heme iron being absorbed from intake.[60] On the basolateral membrane, FPN1 activity and expression is repressed by hepcidin, which in turn limits the amount of iron exported from the enterocyte into circulation.[61,62] These responses of enterocyte DMT1 and FPN1 mediates adaptation to changes in dietary or body iron status.

There is no known mechanism for regulated iron excretion. Small amounts of iron are lost by exfoliation of epithelial and intestinal cells; however, this is generally replenished by dietary iron absorption.[63]

The body's capability to store iron is a crucial component of systemic iron homeostasis. Central to iron storage is the liver, which performs three major functions to maintain systemic iron balance: sequestering and storing excess iron from the plasma, mobilization of iron stores to meet physiological demands, and synthesizing proteins integral to systemic iron homeostasis. Hepatocytes function as the major storage site of excess iron. When plasma iron levels increase Tf-bound iron is taken up by TfR1-mediated endocytosis.[64] Additionally, in iron overload conditions ZIP14 and DMT1 have been shown to be involved in the uptake of non-Tf-bound iron (NTBI) by the liver.[15,65–67]

Conversely, when iron is deficient or demands are increased, iron is mobilized and exported by FPN1 into circulation.[68]

The liver also functions as the primary location for the synthesis of Tf and hepcidin.[69,70] Hepcidin is the master regulator of systemic iron, controlling the amount of iron being exported into the plasma by FPN1 expressing cells. Hepcidin expression is tightly controlled by numerous factors such as inflammation, iron status, and hypoxia (discussed in more detail in the subsequent section).[71] Tf, in the blood serum, serves not only as the iron carrier of the body, but also as a buffering system to limit the amount of toxic, free iron in circulation. In normal conditions, roughly 30% of transferrin is saturated with iron, offering a sizable buffering capacity for handling increases in plasma iron levels.[72]

Macrophages of the RE system also contribute to the iron storage capacity of the body, however their main function and contribution to systemic iron homeostasis is the recycling of iron from effete red blood cells (RBCs). RE macrophages are a group of specialized phagocytic cells that originate as monocytes in the bone marrow. This includes central nurse macrophages in the bone marrow, Kupffer cells in the liver, and red pulp macrophages in the spleen. The spleen is regarded as the main site of iron recycling under normal conditions, however the liver has been shown to be important in RBC clearance during conditions of increased RBC turnover.[73–75] Together, iron recycled by RE macrophages provides over 90% of the 20 to 25 mg of iron required for new RBC production (adult human).[54] The ability to recycle iron is of the utmost importance due to the insufficient supply of iron coming from the diet.

RE macrophages recognize senescent or damaged RBCs by markers present on the red cells which promotes their uptake via phagocytosis. Lysosomal fusion with the

RBC-phagosome forms the phagolysosome, resulting red cell degradation into various components and heme.[76] Heme is then transported from the phagolysosome to the cytosol by heme-responsive gene 1 (HRG1) and degraded by heme-oxygenase 1 (HO1) into carbon monoxide, biliverdin, and iron.[77–79] Iron liberated from heme is incorporated into the labile iron pool where it can be utilized by the cell, stored within ferritin, or exported by FPN1. How iron transiently stored within ferritin is released or regulated in this process remains unknown.

Regulation of the Systemic Iron Pool

Central to systemic iron homeostasis is the peptide hormone hepcidin, which is encoded by the *HAMP* gene. Hepcidin is predominantly synthesized and released by hepatocytes of the liver.[70,80] It exerts its function on the only known mammalian cellular iron exporter, FPN1 (*SLC40A1*), regulating the amount of iron circulating in the plasma. This is accomplished through posttranslational regulation of FPN1.[81] Systemic iron homeostasis is maintained by FPN1 expression on three main cell types that export iron to the blood circulatory system – hepatocytes (body iron stores), duodenal enterocytes (dietary iron), and macrophages of the reticuloendothelial system (iron recycled from effete red cells).[31–33] Circulating hepcidin binds to FPN1 resulting in the ubiquitination of the transmembrane protein, causing its internalization via endocytosis and subsequent lysosomal degradation.[81,82] More recent studies have shown that hepcidin binding could also obstruct the export of iron from FPN1 without causing its internalization and degradation.[83]

Hepcidin expression is tightly regulated through transcription, responding to stimuli such as iron status (iron stores and plasma iron), inflammation, and erythropoiesis.[84] Each has its own unique molecular mechanism(s) to regulate hepcidin

transcription. Nutritional immunity (discussed more in the subsequent section) is the result of inflammatory cytokines stimulating hepcidin production, reducing dietary uptake of iron from duodenal enterocytes and sequestering iron within iron recycling macrophages and hepatocytes, consequently causing hypoferremia.[85,86] Under this condition hepcidin is stimulated predominantly by the cytokine interleukin 6 (IL-6), which is produced in response to inflammation or infection. The loss of IL-6 in mice treated with an inflammatory stimulus did not exhibit an increase in hepcidin expression or decrease in serum iron compared to stimulated controls. Additionally, this was demonstrated in human subjects injected with IL-6, exhibiting increased urinary hepcidin concentrations and reduced serum iron.[85] Studies have also demonstrated hepcidin induction by other cytokines such as IL-1β and IL-22, but their roles in vivo require further investigation.[87,88]

Hepcidin induction also occurs through an iron-sensing pathway. This requires bone morphogenic protein (BMP) signaling, which phosphorylates SMAD proteins, forming a complex that will be translocated into the nucleus where it can bind one of two response elements on the promoter of the *HAMP* gene, thus increasing transcription and expression of hepcidin.[89–91]

Consistently elevated levels of hepcidin, seen in iron overload and chronic inflammatory conditions, will severely limit the amount of iron entering the bloodstream to supply erythropoietic needs.[92,93] Conversely, elevated erythropoietic needs, e.g., by anemia, represses hepcidin expression via activation of the erythropoietin (EPO)-erythroferrone (ERFE) regulatory axis. Hypoxic signals produced by anemia induces the expression of erythropoietin (EPO) in the kidneys and liver.[94–96] EPO targets developing erythroblasts where ERFE becomes activated. ERFE is a hormone that directly acts on hepatocytes, suppressing hepcidin expression.[97] *Erfe*-deficient mice treated with an

inflammatory stimulus develop a more pronounced anemia with elevated hepcidin mRNA expression, lower serum iron, and delayed tissue iron mobilization compared to wild-type controls.[98] A decrease in hepcidin permits the mobilization of iron from liver stores and iron recycling macrophages, as well as increases the absorption of dietary iron. The efflux of iron increases plasma iron levels and provides the iron necessary for heme and hemoglobin synthesis in developing red blood cells.[98] The molecular mechanisms demonstrating how ERFE suppresses hepcidin expression are still being studied, but recent in vitro studies have shown that ERFE disrupts several of the BMPs that induce hepcidin expression through the BMP-SMAD pathway.[99] Altogether, these findings highlight the complexity of hepcidin regulation and its vital role in iron metabolism.

Disorders Resulting from Iron Dyshomeostasis

Organismal iron dyshomeostasis in humans is associated with a number of diseases and disorders. The most prevalent iron-related disorder is iron deficiency anemia, which affects billions of people worldwide.[100] Insufficient iron supply to developing red cells impairs hemoglobinization which leads to the formation of microcytic hypochromic red blood cells. Anemia affects individuals of all ages but pregnant women and preschool-age children have the highest prevalence in the population.[100] Anemia can negatively affect a child's cognitive and physical development, and in pregnant women anemia has been associated with reduced size and birth weight and increased risk of maternal and child mortality.[101,102] Across populations, anemia is linked to fatigue and poor productivity leading to economic losses.[103]

Another cause of anemia is due to chronic disease or inflammation, which is estimated to be the second leading cause of anemia behind iron deficiency.[104] During an

infection or inflammatory state, the innate immune system triggers an acute phase response, sequestering iron within tissues and limiting absorption from the diet resulting in hypoferremia. This intricate system of nutrient maneuverability is referred to as nutritional immunity, which is designed to protect the host by starving the pathogen and limiting virulence.[105,106] However, in certain chronic conditions such as cancer, chronic kidney disease, and obesity, this response is prolonged due to consistently elevated levels of inflammation leading to chronic hypoferremia, severely disrupting systemic iron homeostasis. Over time this can lead to anemia of chronic disease (ACD), which is also referred to as anemia of inflammation. Clinically, ACD is diagnosed as a normochromic normocytic anemia, with reduced serum iron, normal or increased serum ferritin, and elevated cytokine levels.[86,104] Commonly, due to the more severe underlying cause of chronic inflammation, ACD often goes unrecognized and untreated which can negatively affect the quality of life, recovery, or even survival of patients. The most effective treatment of ACD is to treat the underlying disease, however, in some scenarios this is not feasible.

Iron overload conditions are generally due to genetic mutations causing a disruption in iron homeostasis. The most common genetic iron overload disorder is hereditary hemochromatosis (HH), which can be caused by several different mutations involved in the regulation or synthesis of hepcidin or its target protein, FPN1. The most prevalent form of HH is type 1 hemochromatosis associated with a mutation in the *HFE* gene, which disrupts the iron sensing pathway involved in regulating hepcidin synthesis. This results in decreased hepcidin expression and increased absorption of iron from the diet causing iron overload. Type 1 HH affects about 1 million individuals in the United states and is most common among those with norther European ancestry (~1/250

people).[107,108] The mutation(s) in type 2 and 3 HH are in different genes (*HJV*, *HAMP*, and *TFR2*), however they result in the same outcome of impaired hepcidin expression causing iron accumulation. Type 2 HH is often called juvenile HH due to the earlier onset of symptoms compared to type 1, whereas type 3 is classified as an intermediate form. Type 2 and type 3 HH are considered rare genetic disorders and the actual incidence is unknown.[109,110] The classic treatment for HH is phlebotomy to remove excess iron from the body. However, recently researchers have developed minihepcidin compounds that have shown to be successful in preventing iron overload in hemochromatosis mouse models.[111] Therefore, minihepcidin treatment could be a supplemental therapy to help control HH iron overload disorders. Type 4 HH, commonly called ferroportin disease, is extremely rare and is the result of a mutation in FPN1 causing tissue iron accumulation. The severity varies between patients, with treatments targeted to the symptoms of the individual.[112,113]

Physiological Roles of NCOA4-mediated Ferritinophagy

NCOA4 was originally identified as an androgen receptor coactivator and has been shown to interact with additional nuclear receptors as well such as glucocorticoid, vitamin D, thyroid hormone.[114] Recently, a new role of NCOA4 in iron metabolism was discovered through quantitative proteomics, identifying it as an autophagic cargo carrier receptor for the autophagolysosome-mediated turnover of ferritin, i.e., ferritinophagy.[52,53] NCOA4 was shown to be required for ferritin turnover by iron chelation and that NCOA4 abundance was responsive to iron treatment, increasing with chelation and decreasing with supplementation.[53] The responsiveness of NCOA4 to iron status was supported in developing erythroid cells, demonstrating an inverse relationship with ferritin abundance.[29] Combined, these studies indicate that NCOA4-meditaed ferritinophagy is

upregulated during iron scarcity to increase bioavailable iron, and repressed when iron is replete to promote storage and limit free cytosolic iron levels.

Given the role of NCOA4 in cellular iron balance, researchers addressed the contributions of NCOA4-mediated ferritinophagy during erythropoiesis, a physiological process involving an exceptionally high level of iron accumulation and consumption by cells. In the early stages of development erythroid progenitor cells accumulate iron, storing the majority within ferritin, which is subsequently utilized during heme iron synthesis.[115,116] Two separate groups demonstrated that NCOA4 depletion in human and mouse erythroid cell lines results in impaired ferritin iron utilization and hemoglobinization.[51,117] These findings were confirmed in an erythroid-specific *Ncoa4* knockout murine model, with adult knockout mice exhibiting a mild hypochromic microcytic anemia with elevated ferritin protein abundance and iron contents in the bone marrow.[118]

The systemic knockout of *Ncoa4* in mice produces microcytic hypochromic anemia despite signs of systemic iron overload.[118,119] Recent comparisons between the hematological profiles of systemic *Ncoa4* knockout and erythroid-specific *Ncoa4* knockout mice revealed a more profound anemia phenotype in the systemic knockout model.[118] This supports a role of *Ncoa4* in a non-erythroid cell type or tissue contributing to erythroid iron homeostasis and red cell development. Notably, accumulation of iron in the red pulp of the spleen, a major site of RE macrophages and red cell iron recycling, by NCOA4 deficiency has been reported.[52] Thus, macrophage NCOA4 may play an integral role in the recycling process, which provides the majority of iron needed for maintaining adequate red cell production.

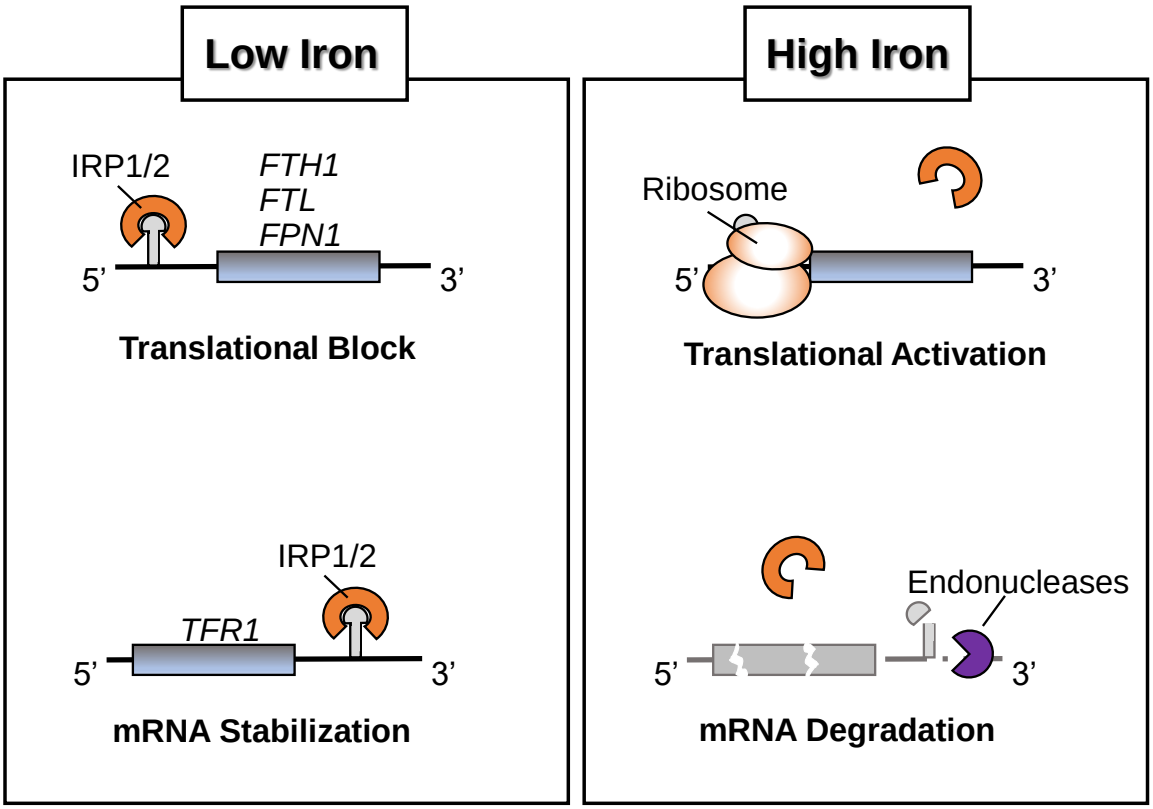

Figure 1.1: Iron-dependent IRE/IRP interactions post-transcriptionally regulate genes involved in cellular iron homeostasis.
Low iron conditions (left) promote the binding of IRPs to IREs in the 5'- and 3'-UTR of mRNA transcripts with the aim to increase cellular iron levels. Binding in the 5'-UTR results in a translational block, reducing expression of ferritin (storage) and ferroportin (export). Conversely, IRP binding in the 3'-UTR stabilizes the transferrin receptor (TFR1) transcript, promoting iron uptake by the cell. In high iron conditions (right) IRPs dissociate from the IREs in the 5' UTR, allowing the ribosome access to translate the protein, increasing expression of ferritin and ferroportin. IRP dissociation from the 3'-UTR IRE exposes the TFR1 transcript to endonucleases leading to mRNA degradation. IRP – iron responsive protein; IRE – iron response element; FTH1 – ferritin H subunit; FTL – ferritin L subunit; FPN1 – ferroportin 1; TFR1 – transferrin receptor 1. Image adapted from "Mammalian iron metabolism and its control by iron regulatory proteins". *Biochimica et Biophysica Acta*. **2012**.

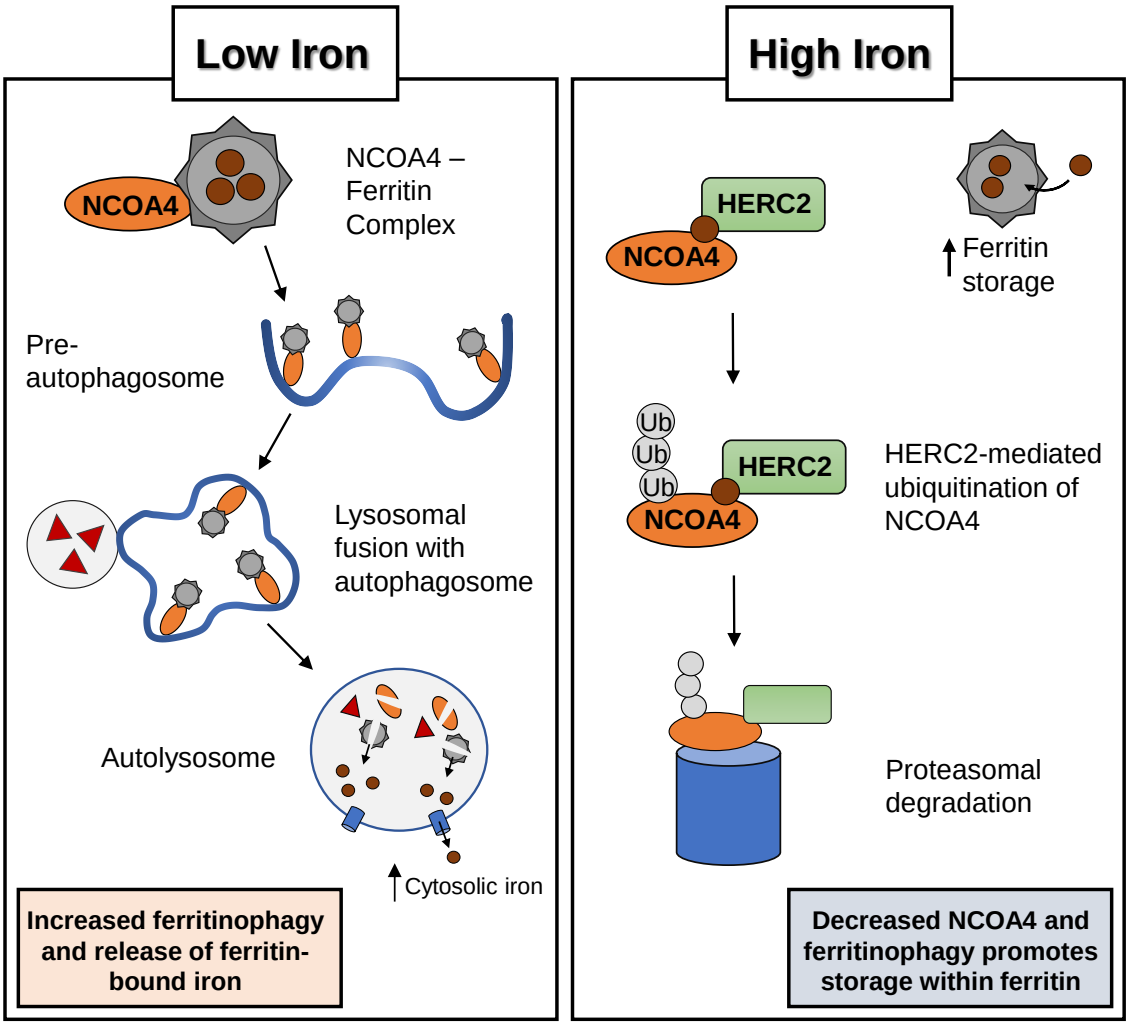

Figure 1.2: NCOA4-mediated ferritinophagy and regulation of NCOA4 by HERC2. Iron is stored with the ferritin complex. During iron deficient conditions (left) cells promote NCOA4-mediated ferritinophagy to meet cellular demands. NCOA4 binds ferritin and delivers it to a developing autophagosome. Fusion of the autophagosome with the lysosome results in the degradation of the ferritin complex and release of the iron core, which is subsequently transported to the cytosol for utilization or export. NCOA4 levels are regulated by HERC2 in an iron-dependent manner (right). During iron replete conditions, NCOA4 interacts with HERC2, targeting it for ubiquitination and proteasomal degradation. Decreased NCOA4 expression stabilizes ferritin and promotes iron storage. Image adapted from "The Role of NCOA4-Mediated Ferritinophagy in Health and Disease". *Pharmaceuticals*. **2018**.

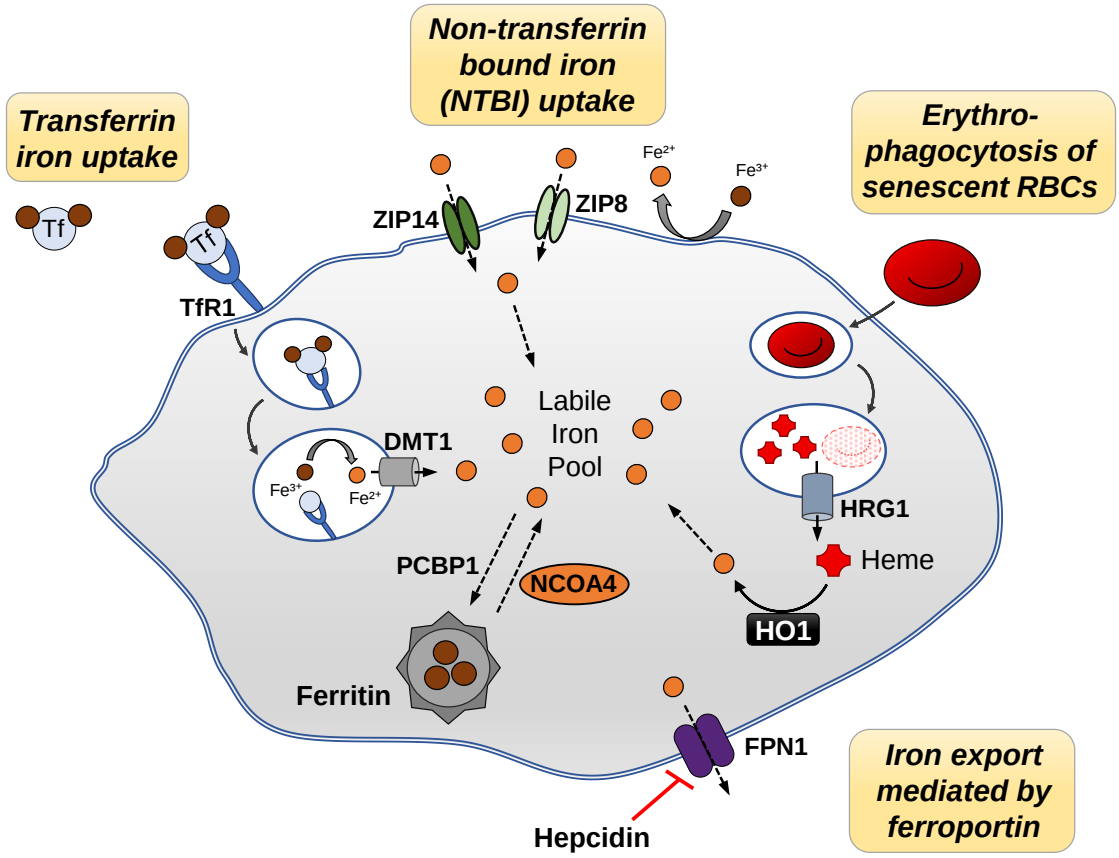

Figure 1.3: General methods of macrophage iron metabolism.
Macrophages can acquire iron through several different routes and sources. Transferrin-bound iron (left) is taken up by TfR1-mediated endocytosis. Within the endosome ferric iron (Fe^{3+}) is reduced to its ferrous form (Fe^{2+}) by metalloreductases and transported to the cytosol by DMT1. NTBI can be imported directly by ZIP8 and ZIP14 (middle). Erythrophagocytosis of senescent red blood cells is the major form of iron acquisition by RE macrophages (right, top). Red blood cell digestion results in the release of heme from hemoglobin which is translocated to the cytosol by HRG1. HO-1 liberates iron from heme which can then be incorporated into the labile iron pool (LIP). When the LIP is in excess, the iron chaperone PCBP1 delivers iron to ferritin for storage. Conversely, NCOA4 mediates ferritin degradation in a process called ferritinophagy, increasing cytosolic iron. Iron can be exported from the cell by FPN1 (right, bottom). FPN1 is post-translationally regulated by hepcidin. Abbreviations: Tf – transferrin; TfR1 – transferrin receptor 1; DMT1 – divalent metal transporter 1; ZIP8/14 – Zrt/Irt-like protein 8/14; HRG1 – heme-responsive gene 1; HO1 – heme oxygenase-1; PCBP1 – poly(rC)-binding protein 1; NCOA4 – nuclear receptor coactivator 4; FPN1 – ferroportin 1. Image adapted from “Iron Regulation: Macrophages in Control”. *Pharmaceuticals*. **2018**

INTRODUCTION

At the cellular level, iron is required for the formation and function of numerous metalloproteins. Yet, free iron is redox-active and thus cytotoxic if not properly handled.[120] A key function of iron is oxygen transport as a component of hemoglobin in red blood cells, which comprises approximately two-thirds of the total iron found in the body. In humans, erythrocytes turn over every 120 days and thus need to be replaced through constant production of new red blood cells.[121,122] The large demand for iron by erythropoietic cells is primarily met by the recycling of iron from senescent and effete red blood cells. Under normal steady-state conditions, the RE macrophages contribute up to ~90% of the daily iron requirement for red blood cell replenishment.[54]

Macrophage-mediated iron recycling is initiated by the engulfment of erythrocytes, termed, erythrophagocytosis. Digestive processes begin in the lysosome where red cell hemoglobin (Hb) is reduced to heme and subsequently transported to the cytosol, where heme oxygenase-1 extracts iron molecules, destined for export by ferroportin.[78,79,123,124] An individual human erythrocyte contains about 270 million molecules of Hb, with each Hb composed of up to four heme iron molecules.[125] Thus, macrophages must be equipped to handle the large expansion in its iron load after erythrophagocytosis. Ferritin, the sole cytosolic iron storage protein in mammals, provides cells the capability to handle excess iron by storing the metal in a stable and nontoxic form. An induction in ferritin expression by erythrophagocytosis has been reported,[124] however, how the stored iron is released from ferritin for ferroportin-mediated export remains poorly understood.

Ferritin functions as a 24-subunit heteropolymer of ferritin L (FTL) and ferritin H (FTH) subunits, which can accommodate up to 4,500 iron atoms. Ferritin expression is

post-transcriptionally regulated by the presence of an IRE in the 5'-UTR of its H and L transcripts. With elevated cellular iron levels, IRP-IRE binding decreases, enabling the translation of ferritin. Conversely, when iron levels are replete, IRP binding creates a translational block to prevent the synthesis of new proteins.[22] The regulated release of iron from this highly stable form of iron storage is through either proteasomal[126] or lysosomal degradation.[127,128] More recently, the involvement of autophagy in the latter process has been revealed, giving rise to the term, ferritinophagy. The process of ferritinophagy is initiated by the binding of a selective cargo receptor, NCOA4, to iron-laden ferritin, which leads to the selective autophagy and subsequent lysosomal degradation of the NCOA4-ferritin complex.[52,53] Notably, NCOA4 only binds to ferritin H to initiate ferritinophagy,[51] which is also the subunit conferring ferroxidase activity, promoting the retention of iron within ferritin in its non-toxic ferric state.[129]

In developing erythroid cells, ferritin iron storage has been proposed as an intermediate form of iron, between enhanced import, and delivery to the mitochondria for heme biosynthesis.[115,116] Recently, NCOA4 has been identified to be integral to the regulation of ferritin iron storage and heme production during terminal erythroid differentiation.[51,117] In agreement with these *in vitro* studies, microcytic hypochromic anemia has been observed in systemic and erythroid-specific *Ncoa4* knockout mice.[118,119] A role of NCOA4 in a non-erythroid blood lineage in the regulation of red blood cell production has been recently proposed,[130] which supports the more profound anemia seen by systemic versus erythroid-specific *Ncoa4* knockout.[118] Notably, systemic knockout of *Ncoa4* causes iron accumulation in the red pulp of the spleen, a major site of RE macrophages.[52,119] These suggest a key role of macrophage NCOA4 in red cell metabolism and, presumably, organismal iron recycling.

A primary innate response to inflammation or infection is hypoferremia, which involves elevated hepcidin activity.[85,131] This acute-phase response, referred to as nutritional immunity, is intended to limit the availability of iron to invasive pathogens by sequestration of systemic iron within iron-supplying cells, including hepatocytes, enterocytes, and RE macrophages. However, elevated cellular iron may rather promote the virulence of intracellular pathogens, such as *Salmonella*.[132,133] Additionally, cellular iron accumulation can impose cytotoxic oxidative stress to cells by producing reactive oxygen species.[134] Notably, macrophages coordinately regulated the genes of iron homeostasis towards iron sequestration in response to classical activation by inflammation. This includes elevated ferritin[135] and repressed ferroportin[136] expressions, collectively promoting cellular iron retention. Whether NCOA4 expression and ferritinophagy serves as a target of nutritional immunity or inflammatory responses remains unknown.

Macrophages contribute to organismal iron homeostasis and produce innate immunity through actively regulating its intracellular iron contents. The studies, herein, employ J774 murine macrophages, and examine the roles of NCOA4 and ferritin regulation during the adaptation of macrophages to changes in cellular iron availability, and the processes of iron recycling after erythrophagocytosis. Moreover, the responses of macrophage NCOA4 to hepcidin activity and an inflammatory stimulus were recapitulated and characterized *in vitro*. These cell culture experiments revealed regulatory roles of macrophage NCOA4 and ferritinophagy, which are integral to both cellular and systemic iron homeostasis during (patho)physiological conditions of iron recycling, systemic iron overload, infection, and chronic inflammation.

MATERIALS AND METHODS

Cell Culture and Iron Treatments

J774, clone E, murine macrophages were maintained at sub-confluency and cultured in alpha-minimum essential medium (AMEM; HyClone, GE Life Sciences) containing 10% fetal bovine serum (FBS; Sigma), 100 U/mL penicillin and 100 µg/mL streptomycin (Corning) at 37°C in 5% CO_2.

Cellular iron deficiency was produced by treatment of an iron chelator, deferoxamine (DFO; Sigma-Aldrich), to the culture medium at a final concentration of 100 µM for 18 hours. Iron loading was achieved by supplementing the medium with 100 µg/mL of ferric ammonium citrate (FAC; Sigma-Aldrich).

Erythrocyte Treatment for Erythrophagocytosis by Macrophages

I adopted a protocol to recapitulate the process of erythrophagocytosis and red cell iron recycling using J774 cells, *in vitro*. Sheep erythrocytes (HemoStat Laboratories) were opsonized by incubation with rabbit anti-sheep IgG (MP Biomedicals) at 550 µg of IgG per 250 x 10^6 erythrocytes at 37°C for 20 minutes. Opsonized erythrocytes were added to the J774 monolayer (~60% confluency) at an erythrocyte:macrophage ratio of 10:1, which was considered 0-hour. After 1.5 hours of incubation, cells were washed with water to remove noningested erythrocytes by hypotonic lysis. Following two additional washes with 1X PBS, cells were incubated in fresh growth medium until harvest at the designated times to assess red cell digestion and iron recycling.

Hepcidin Mimic (PR73) and LPS Treatments

PR73, a synthetic minihepcidn analog, from Drs. Ganz and Nemeth (UCLA) was used to assess the impact of hepcidin activity on our study model. PR73 mimics the biological effects of hepcidin by blocking the flux of iron through ferroportin.[137] However,

it is considerably more stable compared to natural hepcidin, making it suitable for cell culture experiments.[138] The stock was prepared in DMSO, thus DMSO was added as a carrier control. Macrophages not undergoing red cell digestion were treated with 1 μM PR73 for 24 hours. Considering the elevated ferroportin expression after erythrophagocytosis,[124] erythrocyte-laden macrophages were treated with a higher dose of 4 μM PR73. For experiments involving erythrophagocytosis, PR73 was added to cell culture 4 hours after red cell treatments.

To study classically activated macrophages *in vitro*, J774 cells were treated with lipopolysaccharide (LPS) as a model of endotoxemia. LPS (Sigma-Aldrich) was added to the growth medium at a concentration of 1.0 μg/mL for a duration of 24 hours prior to harvest.

Gene Silencing by siRNA Transfection

Gene silencing was achieved by liposome mediated delivery of siRNA using HiPerFect transfection reagent (Qiagen). J774 cells were seeded and treated with mixtures of siRNA and transfection reagent, 24 hours prior to any cell treatment. Cells were seeded at a density to maintain sub-confluency throughout experiments. Control or NCOA4 siRNA (Silencer Select; Ambion) diluted in Opti-MEM (Invitrogen) were mixed with HiPerFect (Qiagen) and added to cells. Following 6 hours of incubation, fresh growth medium was added to the transfected cells for subsequent incubation. Final siRNA concentrations were at 50 nM per well or culture dish.

RNA Isolation, Reverse Transcription, and qPCR

Relative abundance of mRNA transcripts was assessed by qPCR. Cells were harvested and treated with TRI Reagent (Sigma-Aldrich) for RNA isolation. Total RNA was extracted using the TRI Reagent manufacturer's protocol. Equal amounts of RNA

were reversed transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qPCR was performed using PowerUp SYBR Green Master Mix (Applied Biosystems) with amplifications detected using the CFX Connect Real-Time System (Bio-Rad). CFX Connect Software was used to assess the melt curve analysis to confirm reaction specificity and set the Cq threshold. qPCR data was processed by the ΔΔCq method with *Actb* or *Tbp* as housekeeping genes for normalization.

Protein Extraction and Western Blot Analysis

Cell protein extract was prepared using an NP-40 based lysis buffer [100 mM Tris-HCl (pH 7.5), 50 mM KCl, 0.1% NP40, 5.0% glycerol, water, and protease inhibitor cocktail (Roche)]. Following centrifugation, the supernatant was collected, and protein concentrations were determined using the Pierce BCA Protein Assay kit (Thermo Scientific).

For western blot analyses, equal amounts of protein were mixed with Laemmli buffer (Bio-Rad) and a reducing agent, 2-mercaptoethanol (Sigma), and boiled for 10 minutes. Proteins were separated by electrophoresis on a Mini-PROTEAN TGX 4-20% polyacrylamide precast gel (Bio-Rad). Proteins were then transferred to a nitrocellulose membrane using the Trans-Blot Turbo Transfer System (Bio-Rad) and RTA Transfer kit (Bio-Rad). Protein transfer was confirmed by Ponceau staining. Membranes were blocked with a 5% nonfat milk-PBS solution. Primary antibodies were prepared in 5% nonfat milk-PBS-T and the following dilutions were used to detect each protein of interest: rabbit anti-ferritin at 1:1000 (Sigma-Aldrich, F5012); anti-GAPDH 1:2000 (Bio-Rad, Cat# 12004167); rabbit anti-NCOA4 at 1:1000 (Bethyl Laboratories, Cat# A302-272A); and rabbit anti-IRP2 at 1:1000 (Dr. Betty Liebold, University of Utah). Following

primary incubation, proteins were visualized and quantified with IRDye anti-rabbit secondary antibodies (1:10,000; LI-COR) using a LI-COR Odyssey FC imager system.

Cell Viability Assay (CCK-8)

The Cell Counting Kit-8 (CCK-8; Sigma) assay is a colorimetric method to assess the number of viable cells based on cellular dehydrogenase activity. J774 cells were plated in a 96-well plate at a density of 2000 cells/well and transfected as described above in a total well volume of 100 µL. Following 24 hours of incubation, cells had reached a sub-confluent monolayer and DFO (100 µM) was added to the medium and allowed to incubate for a total of 18 hours. Two hours prior to measuring the absorbance, 10 µL of CCK-8 solution was added to each well. Control wells were prepared without cells as a background correction for cell culture medium. The absorbance was measured at 450 nm and cell viability was determined as percentage of control.

Measures of Total Cellular and Heme Iron Contents

Total cellular mineral contents were measured using inductively coupled plasma mass spectrometry (ICP-MS) by Dr. Jaekwon Lee at the University of Nebraska. Cells were washed twice with PBS supplemented 10 mM EDTA to remove metals non-specifically bound to cell membranes. Acid-washed tubes were used to prevent contamination by external metal sources. Cells were pelleted at 130 x *g* at 4°C for 10 minutes and submitted for ICP-MS. Cellular metal contents were normalized to total protein contents determined by BCA assays (Thermo Scientific).

For heme assays, cell protein extract was prepared by solubilizing macrophages in Triton X-100 based lysis buffer [20 mM Tris-HCl (pH 7.5), 40 mM KCl, 0.5% Triton X-100, and water]. Heme contents were colorimetrically measured using the QuantiChrom

Heme Assay Kit (BioAssay Systems) following the manufacturers protocol. Absorbance at 400 nm was measured using a plate reader and normalized to total protein contents.

Preparation of Splenic Tissue

Frozen spleen samples from LPS treated and control (PBS) mice were from Dr. Xiaoli Chen's lab (University of Minnesota). Sixteen-week old, male C57/BL6 mice were intraperitoneally injected with 0.3 mg/kg PBS or LPS. The spleen was harvested 6 hours after LPS injection, and snap-frozen until processing. For RNA isolation, spleen tissues were homogenized in TRI Reagent. After centrifugation to remove residual tissue debris, the supernatant was collected for RNA isolation as described above.

Statistical Analyses

Cell culture experimental values are presented as mean ± SD. Splenic transcript abundance from *in vivo* experiments are presented as mean ± SEM. *n* numbers are detailed in the figure legends. Comparisons between two groups were performed using Student's *t*-test. When comparing more than two groups of the same variable, one-way ANOVA with a Tukey's post-hoc test was used. Experiments with multiple independent variables were compared by two-way ANOVA with a Tukey's post-hoc test. For all analyses statistical significance was set at $*P < 0.05$, 2-sided. Statistical analyses were completed using Microsoft Excel (Student's *t*-test) and JMP Pro 14 (1-way and 2-way ANOVA).

Table 1.1: qPCR Primer Sequences.

Transcript	Primer	Sequence
Ncoa4	*Ncoa4* forward	5' – AGCTAAGGCACCCAAGGCTA – 3'
	Ncoa4 reverse	5' – CTTAGGGCCTCCTTTGCACG – 3'
Tfrc	*Tfrc* forward	5' – TCACTTCCTGTCGCCCTATGT – 3'
	Tfrc reverse	5' – AGAGTGTGAGAGCCAGAGCC – 3'
Fth1	*Fth1* forward	5' – CCACGTGACCAACTTACGCA – 3'
	Fth1 reverse	5' – TCTCATCACCGTGTCCCAGG – 3'
Ftl1	*Ftl1* forward	5' – GGAGCGTCTCCTCGAGTTTC – 3'
	Ftl1 reverse	5' – GAGATGGCTTCTGCACATCCT – 3'
Fpn1	*Fpn1* forward	5' – CCCATAGTCTCTGTCAGCCTGC – 3'
	Fpn1 reverse	5' – CCGTCAAATCAAAG GACCAAA – 3'
Hmox1	*Hmox1* forward	5' – TGGTGCAAGAT ACTGCCCCT – 3'
	Hmox1 reverse	5' – GTCTGGGATGAGGTAGTGCTGAT – 3'
IL6	*IL6* forward	5' – CTCGGCAAACCTACTGCGTT – 3'
	IL6 reverse	5' – TGACCACAGTGAGGAATGTCCA – 3'
Actb	*Actb* forward	5' – AGGAGTACGATGAGTCCGGC – 3'
	Actb reverse	5' – AGCTCAGTAACAGTCCGCCT – 3'
Tbp	*Tbp* forward	5' – AGTTGTGCAGAAGTTGGGCT – 3'
	Tbp reverse	5' – TACTGAACTGCTGGTGGGTCA – 3'

RESULTS

Post-transcriptional regulation of NCOA4 in J774 macrophages.

In prototypic mammalian and erythroid cell culture models, NCOA4 is highly responsive to cellular iron status at the protein level.[51,117] However, whether NCOA4 is regulated by iron in macrophages remain unknown. Thus, I first assessed if NCOA4 is responsive to cellular iron status in J774 murine macrophages. Cellular iron overload by FAC (100 µg/mL) resulted in markedly increased ferritin protein abundance while repressing NCOA4 (Figure 2.1). Conversely, iron deficiency by DFO (100 µM), increased NCOA4 abundance with a concomitant decrease in ferritin protein abundance. These findings demonstrate an inverse relationship between NCOA4 and ferritin expression in J774 macrophages. Notably, the transcript abundance of *Ncoa4* was not affected by either iron treatment, indicating a post-translational mechanism for regulation (Figure 2.2). *Tfrc* abundance confirmed the effectiveness of each iron treatment and demonstrate an active IRP/IRE system in macrophages (Figure 2.2). These findings indicate that NCOA4 is actively regulated by iron status in macrophages and that regulated ferritinophagy may serve as a potential mechanism for controlling macrophagic iron storage and homeostasis.

NCOA4-dependent ferritin regulation in J774 macrophages.

To determine the role of NCOA4 in ferritin regulation and macrophage iron homeostasis, NCOA4 was knocked down by siRNA transfection. The siRNA-mediated gene silencing of NCOA4 was confirmed by repression in both protein and transcript abundance versus control levels (Figure 2.3A,B). The loss of NCOA4 produced an increase in ferritin protein abundance that was on average 2.5-folds greater than control (Figure 2.4). A decrease in IRP/IRE complexes can lead to enhanced translation of

ferritin.[48,50] NCOA4 deficiency had a minimal impact on IPR2 protein abundance and *Tfrc* mRNA expression, a known target of IRP-mediated regulation (Figure 2.5A,B). These identify NCOA4 as a key regulator of ferritin turnover and iron storage in macrophages.

NCOA4-dependent ferritin turnover and survival of iron-deficient macrophages.

The marked increase in NCOA4 expression by iron deprivation and its role in ferritin degradation suggests its involvement in the adaptation of macrophages to cellular iron restriction. To test this, J774 cells were treated with NCOA4 siRNA and DFO to restrict ferritin turnover and cellular iron import, respectively. By NCOA4 depletion, ferritin accumulation occurred in both iron-adequate and DFO-treated cells (Figure 2.6). The repression in ferritin by DFO was present in both control and NCOA4 siRNA-treated cells (Figure 2.6), likely mediated by increased IRP activity (Figure 2.7A,B). Yet, the ferritin levels of DFO-treated NCOA4-deficient cells were comparable to iron-adequate control cells, indicating less supply of iron from both storage and import (Figure 2.6).

Iron is essential for cell survival and growth.[139] Thus, the impact of limiting iron from the extracellular (import) and intracellular (ferritin) sources was assessed by the CCK-8 cell viability assay. The number of viable cells was significantly reduced by DFO-induced iron chelation only when J774 cells were NCOA4-deficient (Figure 2.8). Notably, DFO treatment or NCOA4 deficiency alone did not significantly influence cell viability or growth. These identify NCOA4-dependent turnover of ferritin as an essential mechanism for macrophages to adapt to cellular iron deficiency, and ferritin as an alternative source of iron when its import becomes restricted.

Cellular heme and non-heme iron contents in macrophages after erythrophagocytosis.

A primary role of macrophages in systemic iron homeostasis is in the recycling of iron from effete and senescent red blood cells. This process requires the conversion of heme iron from ingested red cells to elemental iron by macrophages.[54] To assess the potential role of NCOA4 in iron handling during this process, J774 cells were treated with opsonized erythrocytes to induce erythrophagocytosis.[124]

After 3 hours of red cell treatment, J774 cells featured a red coloration, indicative of the cellular ingestion of erythrocytes (Figure 2.9). This was confirmed by an acute increase in macrophage heme and total iron contents after 4 hours of red cell treatments (Figures 2.10A and 2.11). Heme oxygenase-1 (Hmox1) mediates heme digestion, with gene expression upregulated by elevated cellular heme contents.[79,124] *Hmox1* mRNA levels peaked 3 hours after erythrophagocytosis (Figure 2.10B), which preceded heme digestion evidenced by the disappearance of the red coloration of erythrocyte-treated macrophages (Figure 2.9). Cellular heme contents returned to the basal levels after 12 hours (Figure 2.10A). Despite the disappearance of heme, macrophage iron content remained elevated at 12 hours, indicating the cellular retention of digested iron in a non-heme iron form (Figure 2.11).

Ferritin accumulation and NCOA4-mediated ferritin turnover after erythrophagocytosis.

Accumulation of non-heme iron in cells is expected to result in increased iron storage within ferritin. Thus, I measured the temporal pattern of ferritin expression over 24 hours after erythrophagocytosis. Ferritin protein abundance gradually increased until 12 hours of erythrophagocytosis, and subsequently decreased at 24 hours (Figure 2.12).

No change was seen in ferritin transcript abundance (*Fth1*, *Ftl1*; Figure 2.13). Thus, the changes in ferritin protein levels after erythrophagocytosis are due to post-transcriptional regulation. IRP2 protein and Tfrc mRNA featured a transient decline 3-4 hours after red cell ingestion, indicating an expansion in the cytosolic labile iron pool (Figure 2.14). By 12 hours IRP2 and *Tfrc* returned to near-basal levels. These indicate that macrophages transiently increase ferritin expression to handle the expansion in the cytosolic non-heme iron contents prior to export by ferroportin activity.[124]

Notably, between 12 and 24 hours after erythrophagocytosis, when both macrophage iron and ferritin levels declined, a moderate increase in NCOA4 abundance was identified (Figures 2.11, 2.15). To test whether NCOA4 plays a role in the regulation of macrophage ferritin at the later stages of iron handling after erythrophagocytosis, J774 cells were treated with NCOA4 siRNA and then with opsonized erythrocytes. The loss of NCOA4 by siRNA transfection was confirmed by a repression in protein abundance (Figure 2.16). Both control and NCOA4 siRNA-treated cells demonstrated an increase in ferritin abundance at 12 hours after erythrophagocytosis. However, the decrease in ferritin at 24 hours was lost when cells were lacking adequate NCOA4 (Figure 2.16). These demonstrate that NCOA4 mediates the turnover of ferritin in the late stages of red cell iron recycling by macrophages.

Repression of NCOA4 by hepcidin activity in macrophages.

Cellular iron export by macrophages is a target of hepcidin due to their role in systemic iron metabolism and innate immunity.[124,140] Hepcidin limits the flux of macrophage iron into the bloodstream by blocking ferroportin activity and causing retention of iron within macrophages.[81,141] To assess the potential role of NCOA4 in

macrophage iron regulation by hepcidin, J774 cells were treated with a synthetic minihepcidin analog, PR73.[137]

Hepcidin activity repressed NCOA4 abundance with a concomitant increase in ferritin protein abundance (Figure 2.17A). Ncoa4 transcript abundance was unaffected by hepcidin activity, indicating a post-transcriptional regulatory mechanism (Figure 2.17B). Notably, IRP2 protein and Tfrc mRNA abundance were not affected by PR73 (Figure 2.18A,B). These suggest that less NCOA4 expression and ferritinophagy may facilitate the adaptation of macrophages to cellular iron accumulation by hepcidin activity. To test this, I artificially repressed NCOA4 by siRNA transfection in J774 cells and assessed if ferritin is further enhanced by PR73. The loss of NCOA4 produced an increase in ferritin abundance compared to control levels (Figure 2.19, −Hepcidin). Interestingly, the increase in ferritin abundance produced by PR73 was lost in NCOA4-depleted cells (Figure 2.19), indicating that the expansion in ferritin expression by NCOA4 depletion was sufficient for handling the increase in cellular iron by hepcidin activity. Additionally, no change in IRP2 abundance was observed by hepcidin activity in either siRNA treatment group (Figure 2.19).

To test the hepcidin effect in macrophages undergoing red cell iron recycling, J774 cells were first treated with opsonized erythrocytes and then PR73. Hepcidin activity by PR73 repressed NCOA4 abundance 24 hours after erythrophagocytosis and prevented the drop in ferritin abundance between 12 and 24 hours (Figure 2.20). Hepcidin activity had no effect on IRP2 abundance after 24 hours of erythrophagocytosis (Figure 2.21). To further confirm whether the repression of NCOA4 by PR73 is mediating the ferritin responses, the effects of PR73 on ferritin in erythrocyte-treated control and NCOA4-deficient J774 cells were compared. After 24 hours of erythrophagocytosis,

NCOA4-deficient macrophages produced more ferritin when PR73 was not present (Figure 2.22, −Hepcidin). However, the effect of NCOA4 deficiency was absent when cells were treated with PR73 (Figure 2.22, +Hepcidin). In other words, NCOA4 repression by siRNA was sufficient to reproduce the effects of PR73 on ferritin expression. These identify the accumulation of ferritin by a repression in NCOA4 as a mechanism to sequester excess cytosolic iron by hepcidin activity in macrophages.

NCOA4 repression in macrophages activated by LPS.

Macrophages are intimately tied to innate immunity.[106] Classical activation towards inflammatory M1 macrophages involve coordinated regulation of iron genes leading to cellular iron sequestration.[135,136,142] Thus, to test whether NCOA4 is involved in this response, J774 cells were activated by LPS and NCOA4 expression was assessed. LPS repressed NCOA4 on the transcript and protein levels (Figure 2.23A,B). Additionally, ferritin protein abundance increased by LPS, with the lower band of the ferritin doublet producing a particularly pronounced response (Figure 2.24A). An increase in ferritin expression by LPS was measurable at the transcript level, with a 2.5-fold upregulation of ferritin H (*Fth1*) mRNA. However, LPS had no effect on ferritin L (*Ftl1*) transcript levels (Figure 2.24B). LPS-mediated activation of J774 cells was confirmed by a dramatic increase in *IL6* mRNA[143,144] (Figure 2.25). This was further supported by a repression in *Fpn1* mRNA, which has been previously characterized as a response of activated macrophages (Figure 2.23B).[136,145] These identify a repression in NCOA4 as a part of the coordinated regulation of iron genes by inflammation or infection to sequester iron within macrophages. Notably, the response of Ncoa4 was measurable at the mRNA level, which is distinct from the post-translational mechanism for NCOA4 regulation by iron status.[29,51]

Downregulation of splenic NCOA4 by LPS-induced endotoxemia in mice.

The spleen is the primary tissue of RE macrophage-mediated iron recycling.[54] To determine if inflammation produces a similar repression of NCOA4 *in vivo*, *Ncoa4* mRNA in splenic tissue of mice with LPS-induced endotoxemia were measured. Splenic *Ncoa4* mRNA was repressed by nearly 40% in LPS-treated mice compared to PBS-injected controls (Figure 2.26). Similar to the expression pattern of J774 cells, ferritin H (*Fth1*) abundance increased by 2.5-fold, whereas ferritin L (*Ftl1*) abundance slightly decreased by LPS (Figure 2.26). As expected, *Fpn1* mRNA was repressed, along with a marked increase in *IL6* mRNA, indicating the effective stimulation of an inflammatory response by LPS *in vivo* (Figure 2.26). These findings reinforce the immunological implications of the *in vitro* LPS studies, and further support the role of NCOA4 in regulating macrophagic iron metabolism and distribution during inflammatory conditions.

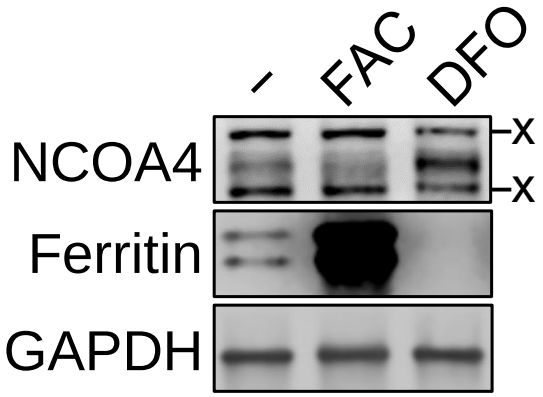

Figure 2.1: Macrophage NCOA4 responds to iron status demonstrating an inverse expression pattern with ferritin abundance.
Iron overload by FAC (100 µg/mL; 18 hours) repressed NCOA4 and increased ferritin abundance in J774 cells. Conversely, by DFO-mediated iron chelation (100 µM; 18 hours) NCOA4 increased with a reduction in ferritin abundance.

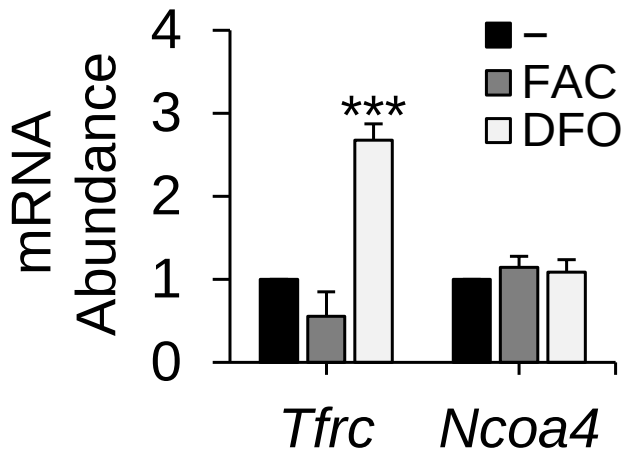

Figure 2.2: Transferrin receptor (*Tfrc*) mRNA responds to iron treatment, whereas *Ncoa4* mRNA is unchanged.
J774 cells were treated as described in Figure 2.1. Transcript abundance was measured by qPCR and normalized by *Tbp*; $n = 3$ independent experiments. ***$P < 0.001$ vs control by 1-way ANOVA with Tukey's post-test.

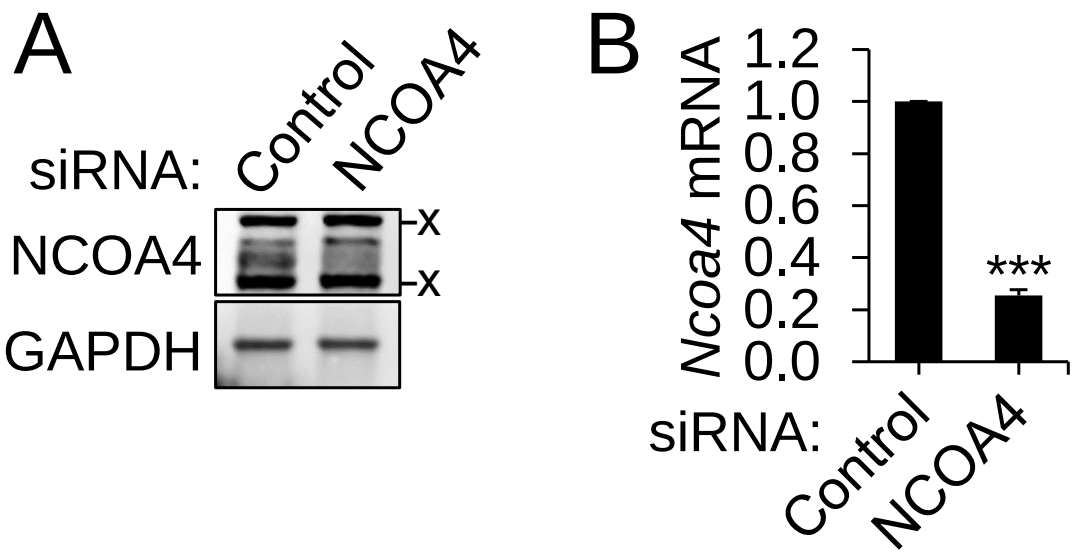

Figure 2.3: Macrophage NCOA4 is effectively knocked down by siRNA transfection.
(**A**) Western analysis demonstrates repressed NCOA4 abundance by siRNA transfection. (**B**) A significant repression in *Ncoa4* mRNA confirms an effective knockdown. Total siRNA treatment time was 42 hours. qPCR samples are normalized to *Tbp*; $n = 4$ independent experiments. ***$P < 0.001$ by 2-tailed t test.

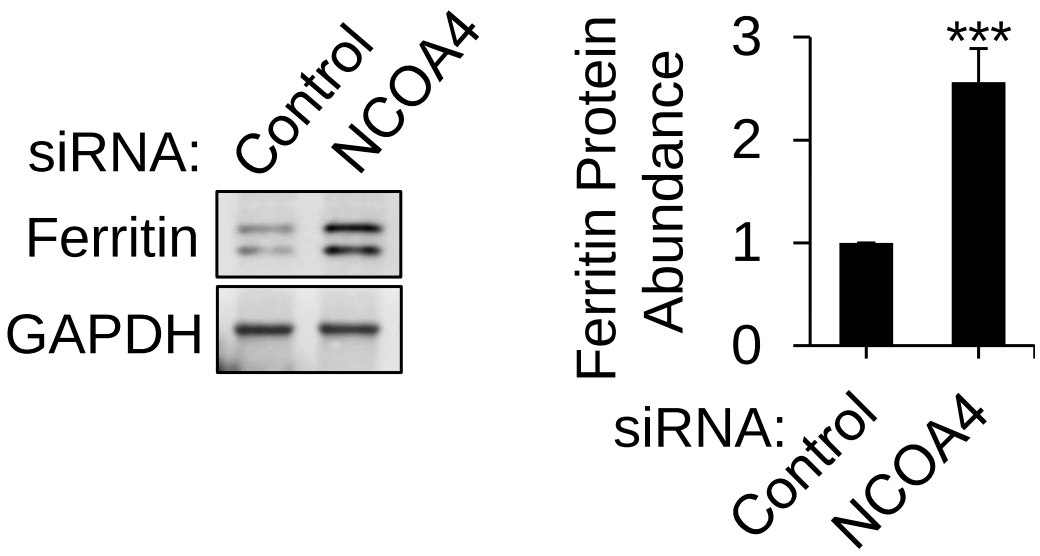

Figure 2.4: The loss of NCOA4 results in an accumulation of ferritin.
Ferritin protein abundance significantly increases by the loss of NCOA4. Ferritin abundance is normalized to GAPDH; $n = 5$ from 4 independent experiments. ***$P < 0.001$ by 2-tailed t test.

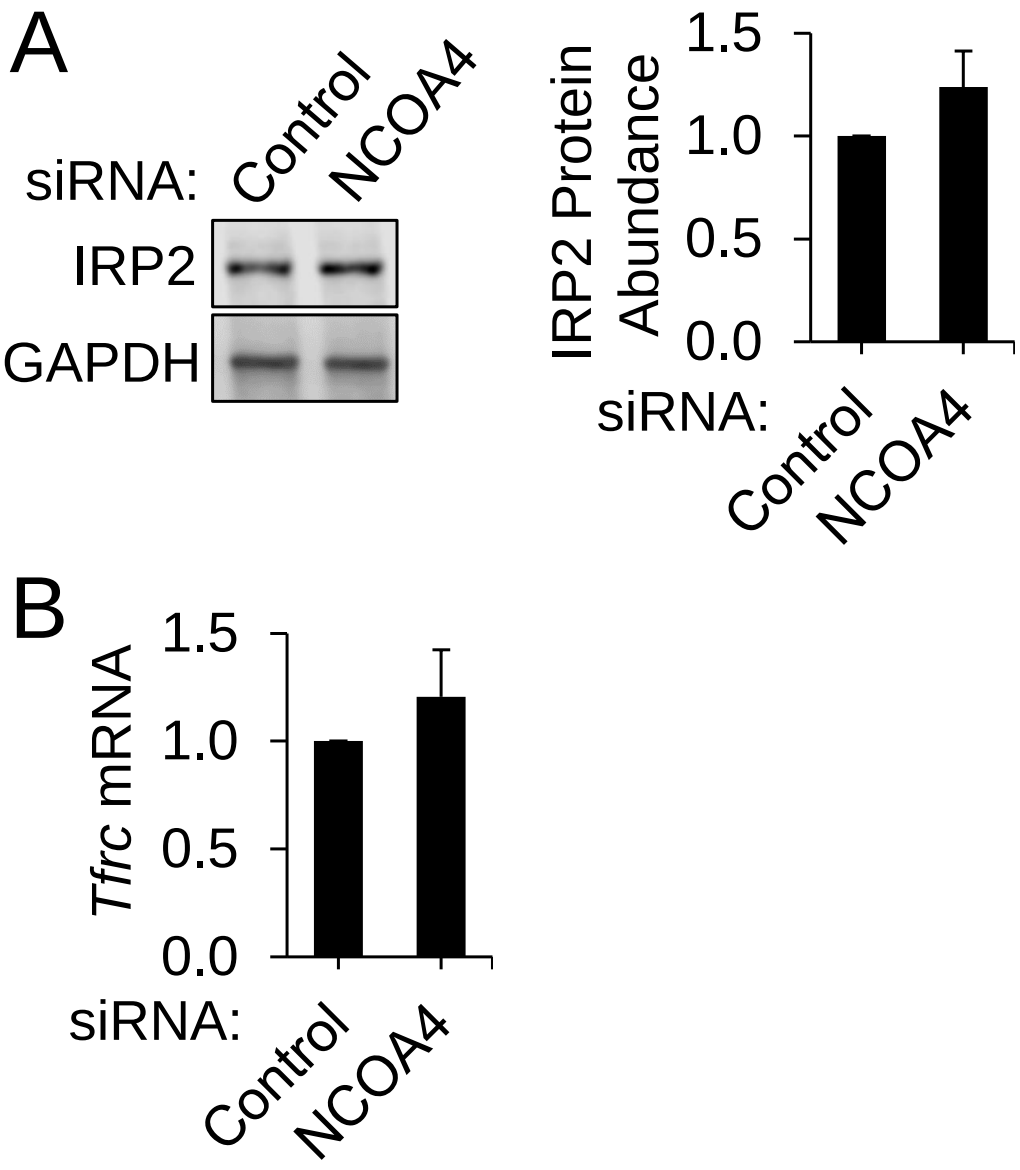

Figure 2.5: NCOA4 depletion has no effect on cellular iron status markers. IRP2 (**A**) and *Tfrc* (**B**) abundance are minimally affected by the loss of NCOA4. IRP2 abundance is normalized to GAPDH; *n* = 3 from 3 independent experiments. *Tfrc* is normalized to *Tbp*; *n* = 4 from 4 independent experiments. *P* value measured by 2-tailed *t* test.

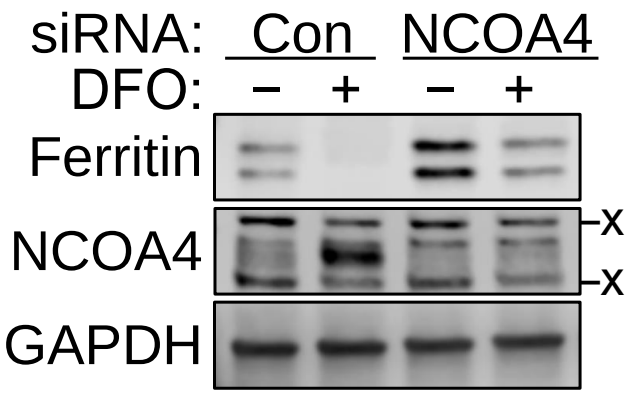

Figure 2.6: The depletion of NCOA4 impairs ferritin turnover during iron restriction.
Ferritin degradation is impaired in NCOA4-depleted cells under iron adequate and iron restricted conditions. Cells were transfected with siRNA 24 hours prior to DFO (100 μM, 18 h) treatment.

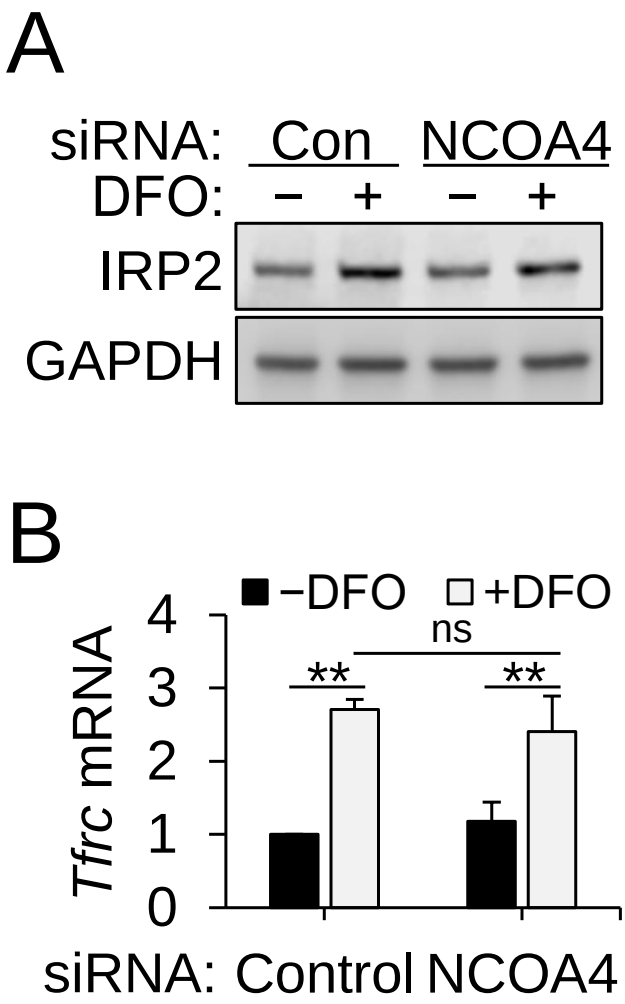

Figure 2.7: Cellular iron markers are unaffected by the loss of NCOA4 in iron deficient conditions.
(**A**) Iron restriction by DFO increases IRP2 abundance (**A**) and *Tfrc* mRNA (**B**) in both control and NCOA4-deficient cells. Cells were treated as described in Figure 2.6. *Tfrc* is normalized to *Tbp*; $n = 3$ from 3 independent experiments. $^{**}P < 0.01$ by 2-way ANOVA with Tukey's post-test. 'ns' indicates not significant.

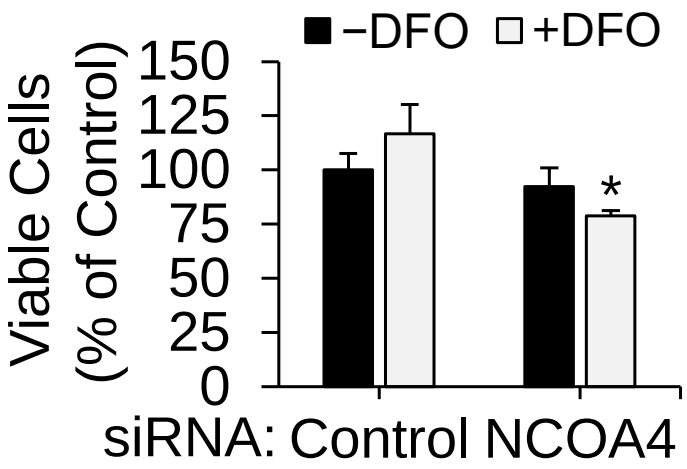

Figure 2.8: The loss of NCOA4 combined with iron restriction reduces the number of viable cells compared to controls, measured by CCK-8 assay.
Restricting iron from both extracellular (DFO) and intracellular (NCOA4 siRNA) iron sources reduces the number of viable cells compared to control conditions (control siRNA). NCOA4 depletion or DFO treatment alone did not affect cell viability. Cells were treated as described in Figure 2.6. $n = 4$ biological replicates. $^*P < 0.05$ vs Control siRNA by 2-way ANOVA with Tukey's post-test.

Figure 2.9: Changes in cell pellet color after erythrophagocytosis.
Red cell treatment (+RBC) produces a pronounced red cell pellet color that gradually disappears by 12 h. Macrophages were fed opsonized red blood cells (10:1 erythrocyte:macrophage) for 1.5 hours and harvested at the indicated time points. Control cells (−RBC) are used for color comparison at each time point.

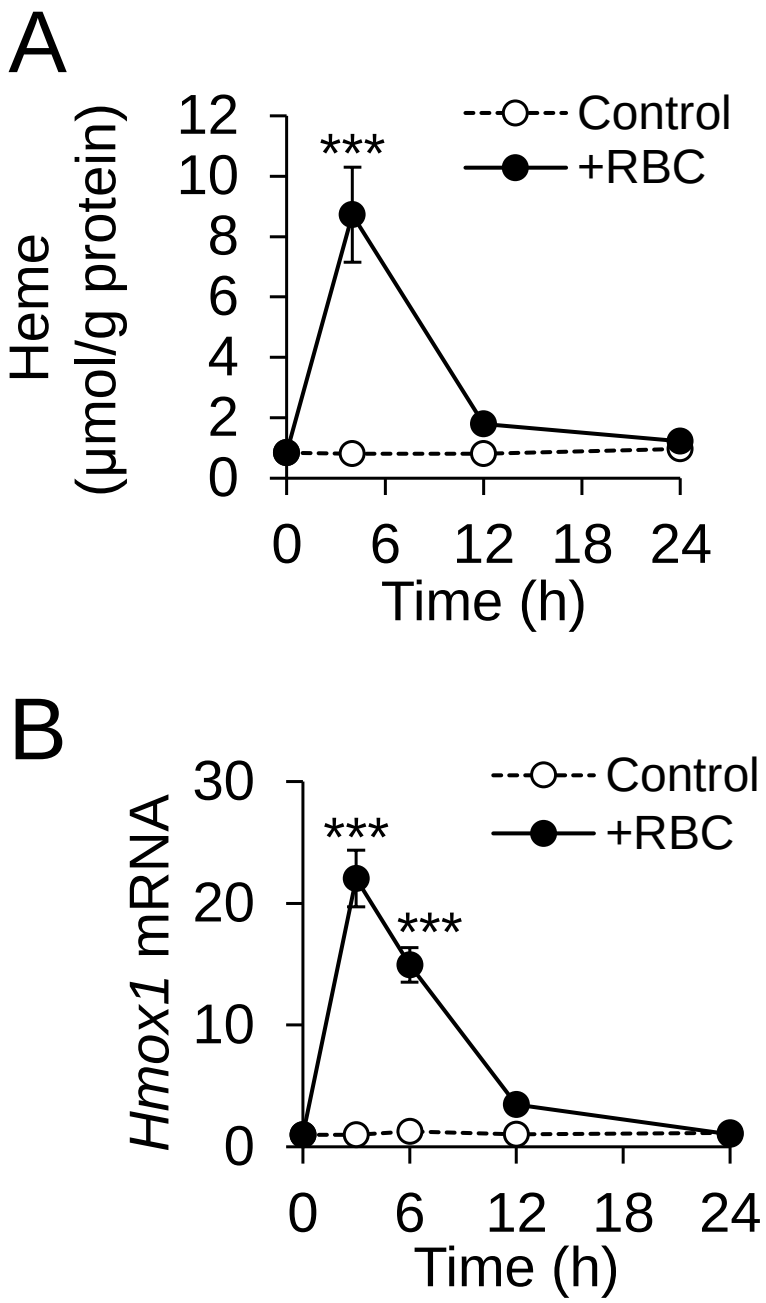

Figure 2.10: Erythrophagocytosis acutely increases macrophage heme contents and *Hmox1* expression.

Macrophage heme content (**A**) and heme oxygenase-1 (*Hmox1*) expression (**B**) significantly increase after erythrophagocytosis, with both returning to near basal levels by 12 h. Cells were treated as described in Figure 2.9. Heme content is normalized to total cellular protein; $n = 4$ biological replicates. *Hmox1* is normalized to *Actb*; $n = 3$ biological replicates. ***$P < 0.001$ by 2-way ANOVA with Tukey's post-test.

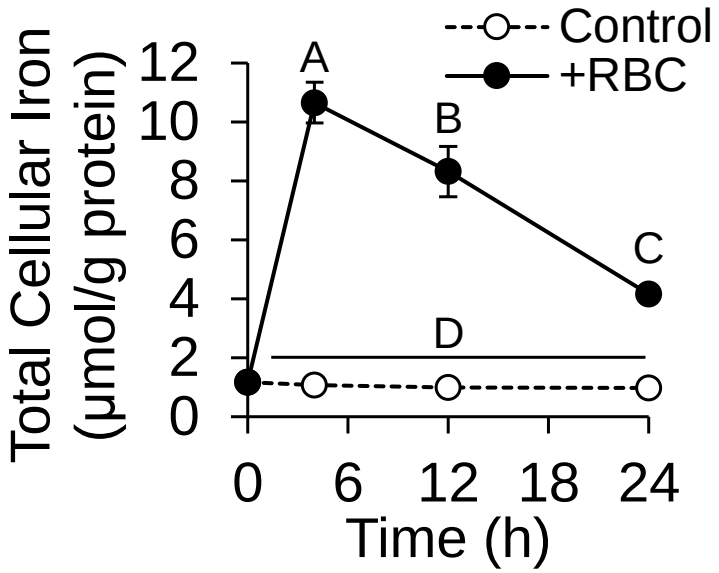

Figure 2.11: Macrophage iron content dramatically changes throughout red cell iron recycling.
Erythrophagocytosis significantly increases cellular iron content with a steady decline thereafter. Cells were treated as described in Figure 2.9. Cellular iron content was measured by ICP-MS and normalized to total cellular protein; $n = 4$ biological replicates. Different letters represent significant differences at $P < 0.001$ by 2-way ANOVA with Tukey's post-test.

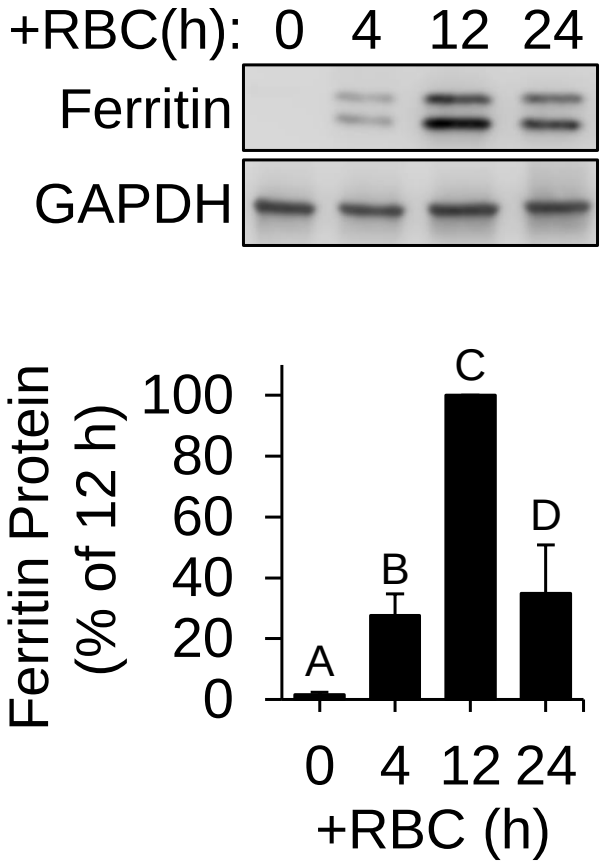

Figure 2.12: Ferritin protein abundance peaks 12 h after erythrophagocytosis. Ferritin protein increases following red cell ingestion, peaking at 12 h with a subsequent decrease by 24 h. Cells were treated with red cells as described in Figure 2.9. Ferritin abundance is normalized to GAPDH; $n = 6$ from 3 independent experiments. Different letters represent significant differences with $P < 0.001$ by 1-way ANOVA with Tukey's post-test.

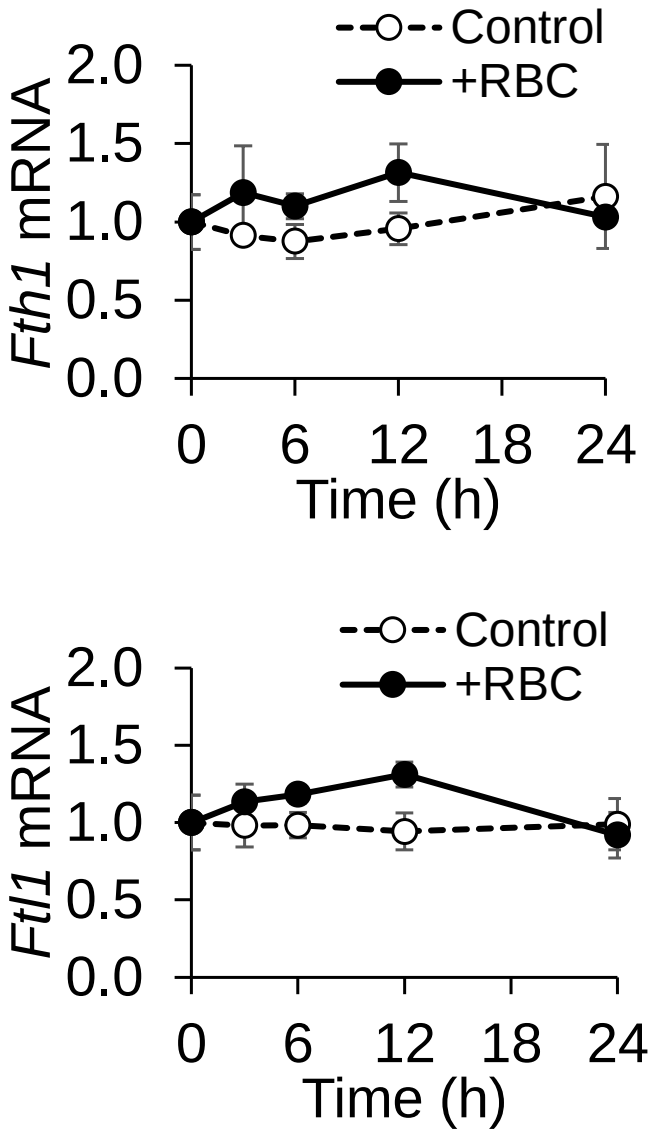

Figure 2.13: Erythrophagocytosis and iron recycling has no effect on ferritin transcript abundance.
Ferritin H (*Fth1*) and L (*Ftl1*) mRNA abundance is consistent throughout red cell iron recycling. Cells were treated with opsonized red cells as described in Figure 2.9. *Fth1* and *Ftl1* are normalized to *Actb*; *n* = 3 biological replicates. Statistical significance was determined by 2-way ANOVA with Tukey's post-test.

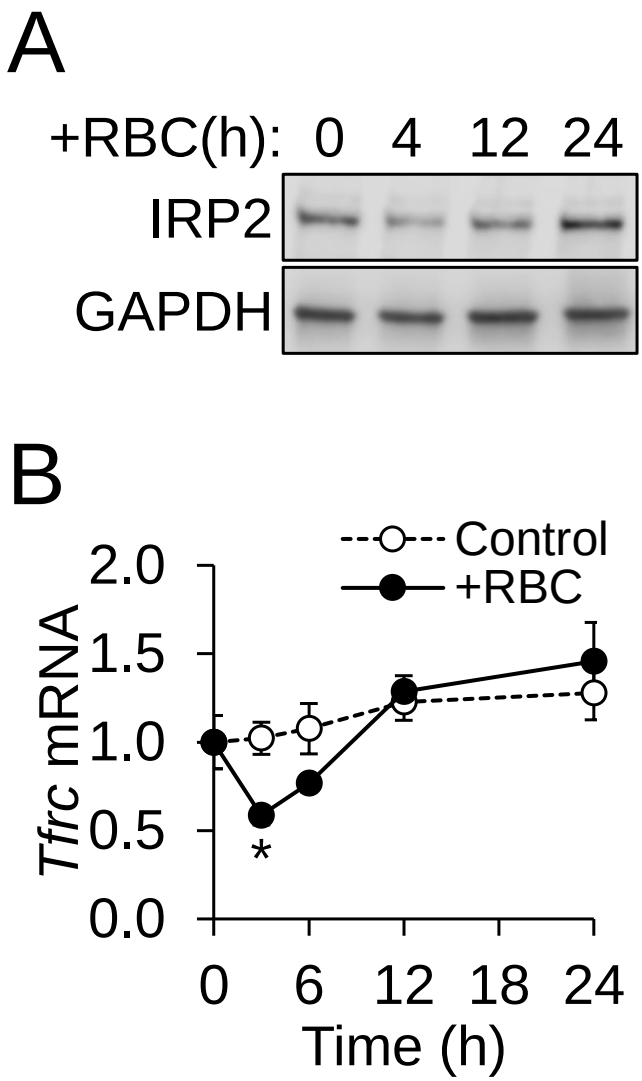

Figure 2.14: Erythrophagocytosis decreases IRP2 protein and *Tfrc* mRNA abundance in macrophages.
IRP2 abundance (**A**) and *Tfrc* mRNA (**B**) are repressed following red cell ingestion. Both markers return to basal levels by 12 h. Cells were treated with opsonized red cells as described in Figure 2.9.*Tfrc* is normalized to *Actb*; $n = 3$ biological replicates. $^*P < 0.05$ vs control by 2-way ANOVA with Tukey's post-test.

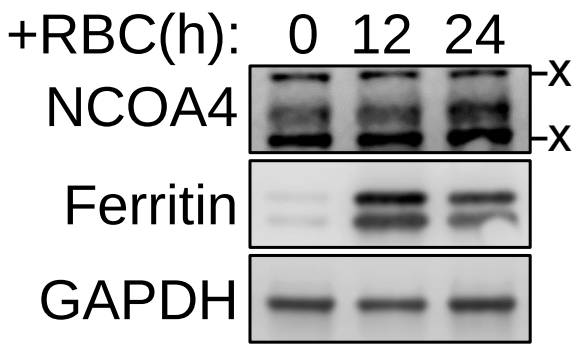

Figure 2.15: NCOA4 increases in the late stages of iron recycling by macrophages. The decrease in ferritin between 12 and 24 h is seen with a concomitant increase in NCOA4 abundance. Macrophages were treated with opsonized red cells as described in Figure 2.9.

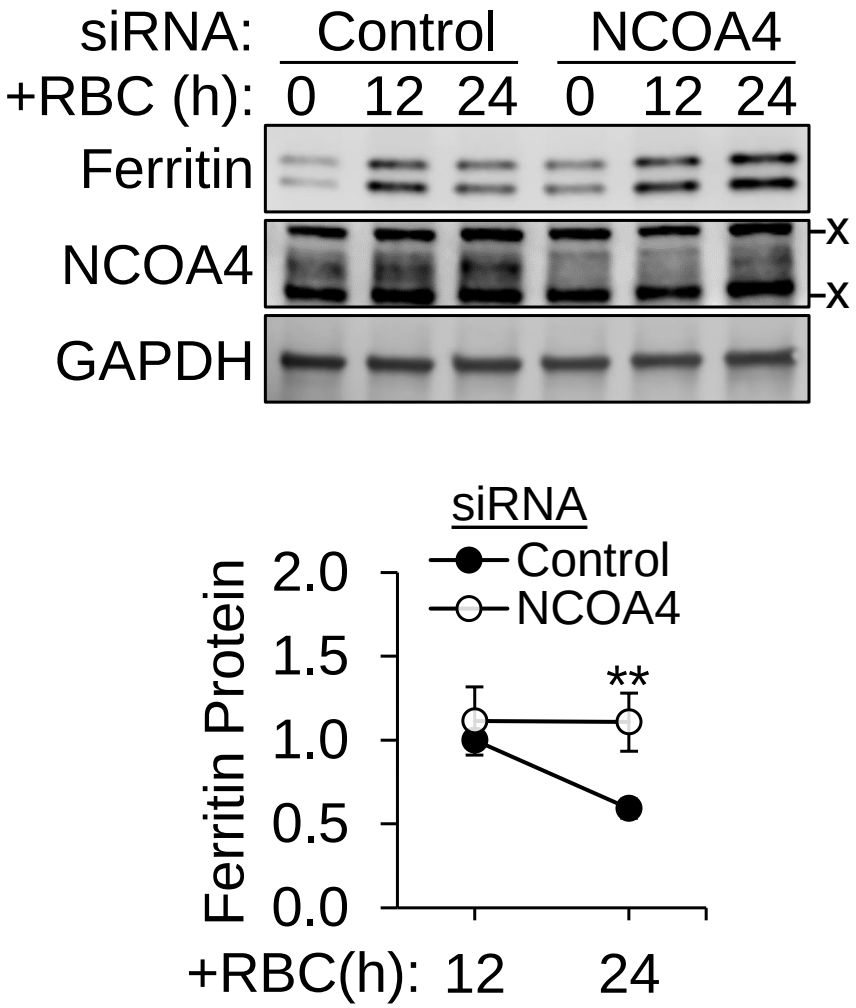

Figure 2.16: The loss of NCOA4 prevents the turnover of ferritin between 12 and 24 hours.

The decrease in ferritin abundance between 12 and 24 h (Control siRNA) is lost by NCOA4 depletion. Cells were transfected 24 hours prior to erythrophagocytosis. Ferritin is normalized to GAPDH; $n = 3$ from 3 independent experiments. $^{**}P < 0.01$ vs Control 24h by 2-way ANOVA with Tukey's post-test.

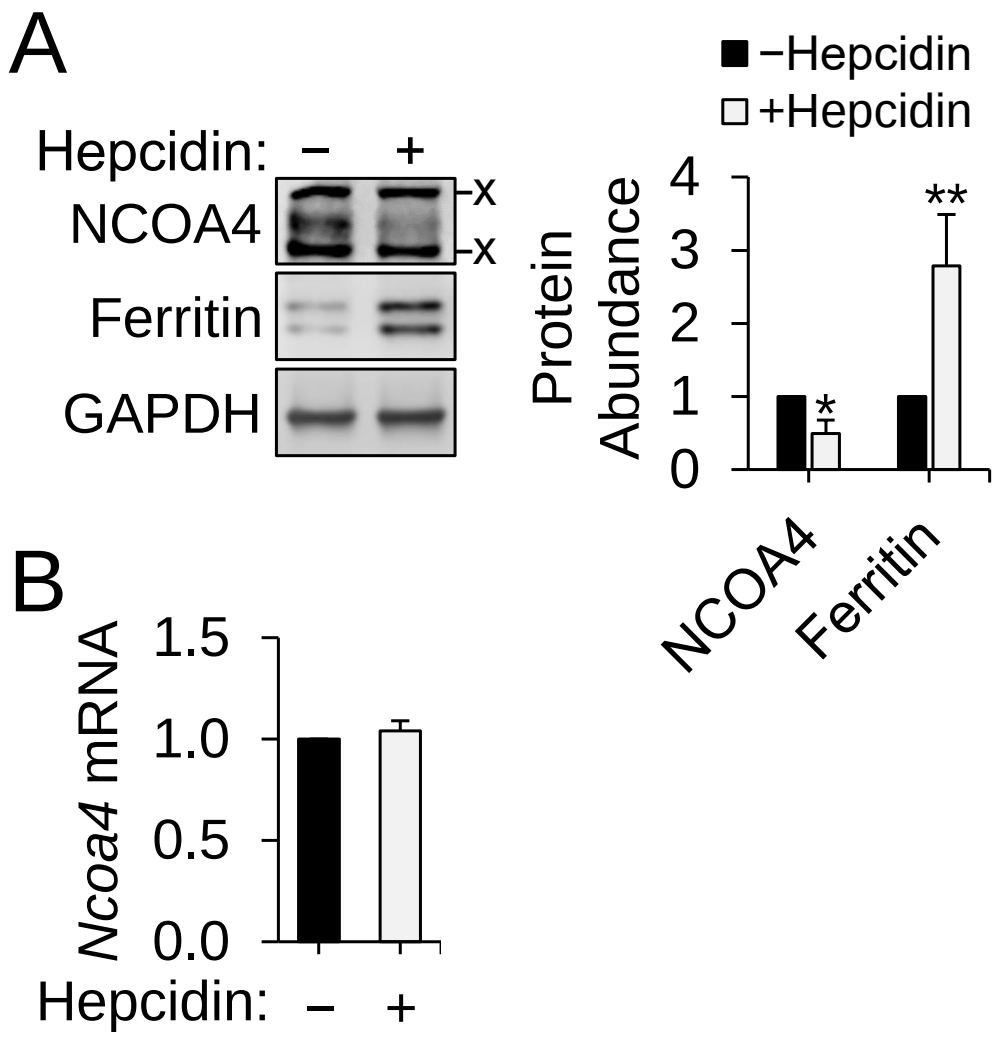

Figure 2.17: Hepcidin activity represses NCOA4and increases ferritin protein abundance in macrophages.
(**A**) Hepcidin activity (1 μM; 24 hours) significantly decreases NCOA4 abundance resulting in an accumulation of ferritin. (**B**) *Ncoa4* mRNA does not change by hepcidin activity. NCOA4 and ferritin are normalized to GAPDH; NCOA4 *n* = 3 from 3 independent experiments; ferritin *n* = 5 from 3 independent experiments. *Ncoa4* is normalized to *Tbp*; *n* = 3 from 3 independent experiments. Statistical significance was determined by 2-tailed *t* test. **P* < 0.05; ***P* < 0.01.

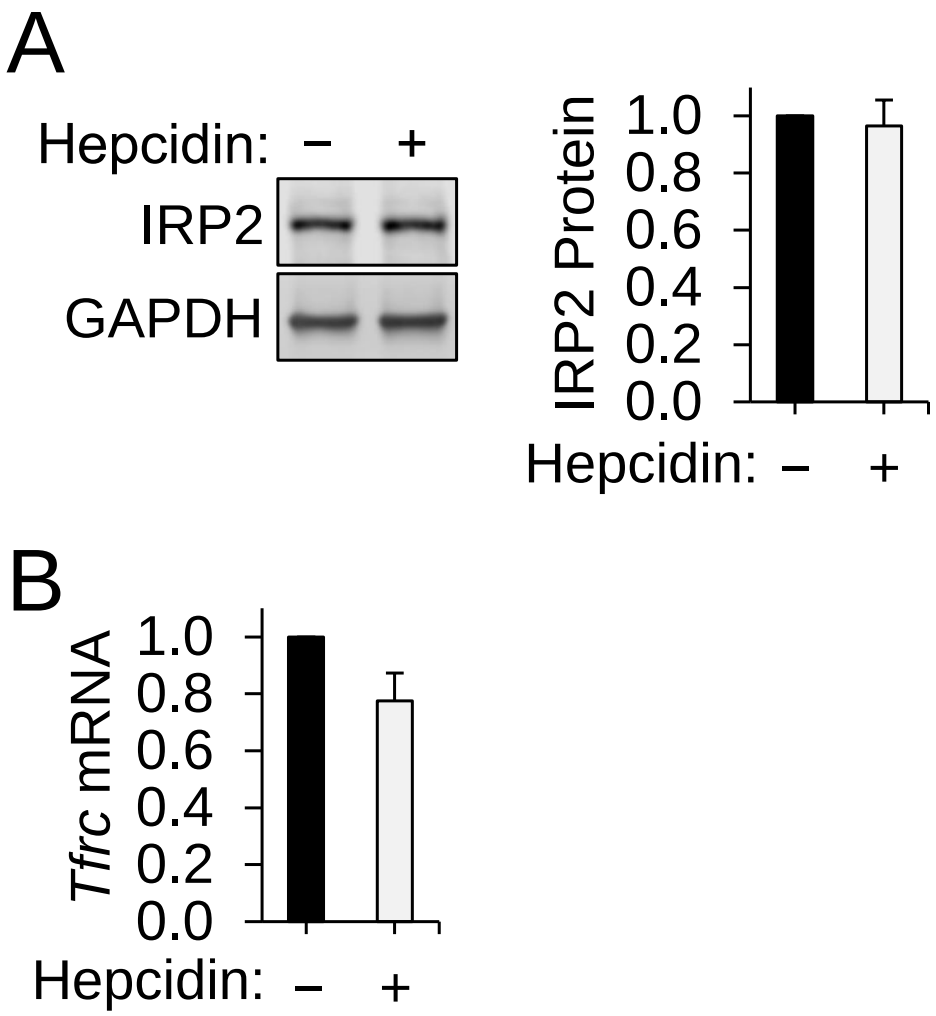

Figure 2.18: Markers of cellular iron status are unaffected by hepcidin activity. IRP2 protein (**A**) and *Tfrc* mRNA (**B**) abundance are unchanged by hepcidin activity (1 μM; 24 hours). IRP2 is normalized to GAPDH; *n* = 3 biological replicates. *Tfrc* is normalized to *Tbp*; *n* = 3 from 3 independent experiments. Statistical significance was determined by 2-tailed *t* test.

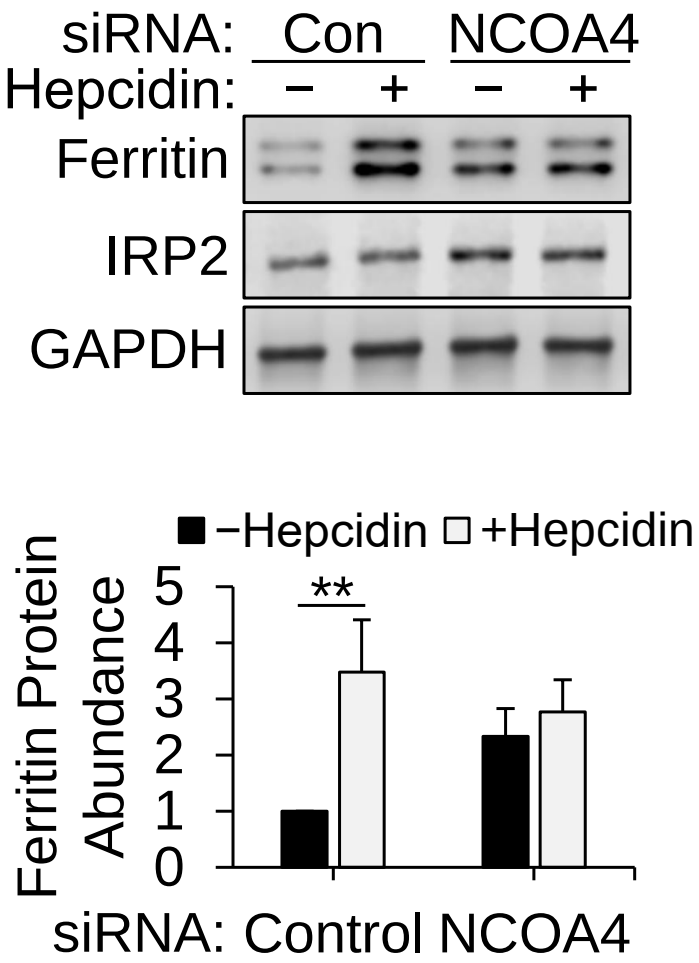

Figure 2.19: Artificially repressing NCOA4 negates the increase in ferritin by hepcidin.
No significant difference was seen in ferritin abundance by hepcidin in NCOA4-deficient macrophages. Additionally, hepcidin does not affect IRP2 expression in Control and NCOA4 siRNA treated macrophages. Macrophages were transfected with siRNA 24 hours prior to hepcidin treatment (1 μM; 24 hours). Ferritin is normalized to GAPDH; n = 5 from 3 independent experiments. $^{**}P < 0.01$ by 2-way ANOVA with Tukey's post-test.

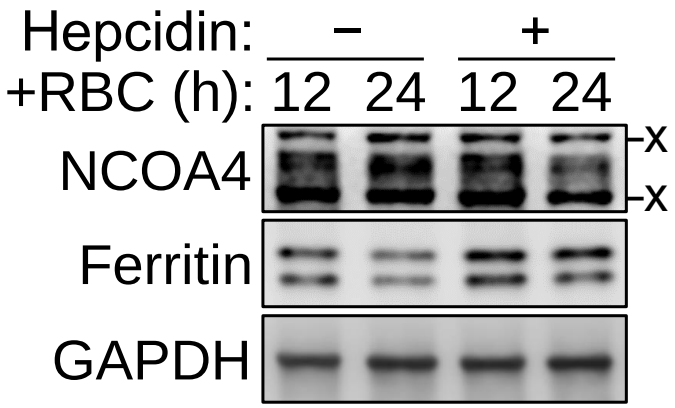

Figure 2.20: Hepcidin activity prevents the turnover of ferritin in the late stages of red cell iron recycling.
Hepcidin represses NCOA4 and abolishes the drop in ferritin between 12 and 24 h. J774 cells were fed opsonized red cells to induce erythrophagocytosis and subsequently treated with or without PR73.

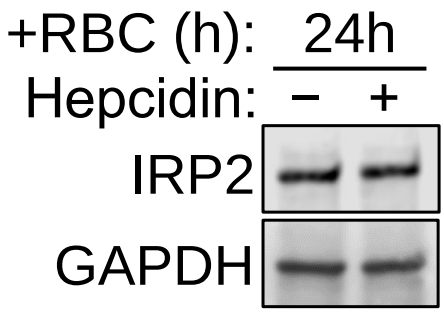

Figure 2.21: IRP2 abundance is unchanged by hepcidin activity at 24 hours of red cell iron recycling.
Cells were treated as described in Figure 2.20.

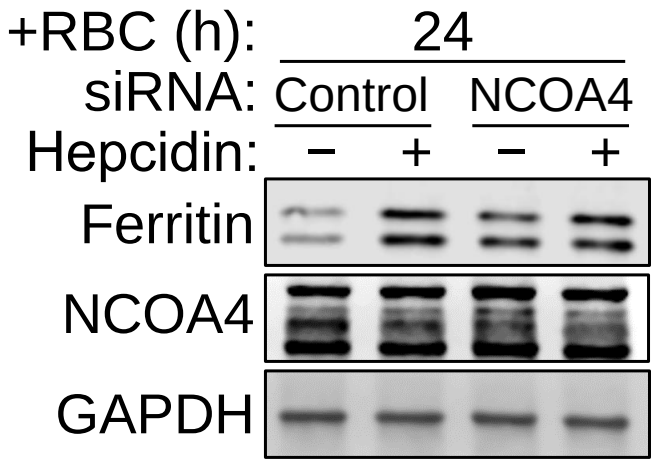

Figure 2.22: The loss of NCOA4 represses the magnitude of the ferritin response to hepcidin at 24 hours of red cell iron recycling.
Macrophages were transfected with siRNA 24 hours prior to erythrophagocytosis and PR73 (4 μM) treatment.

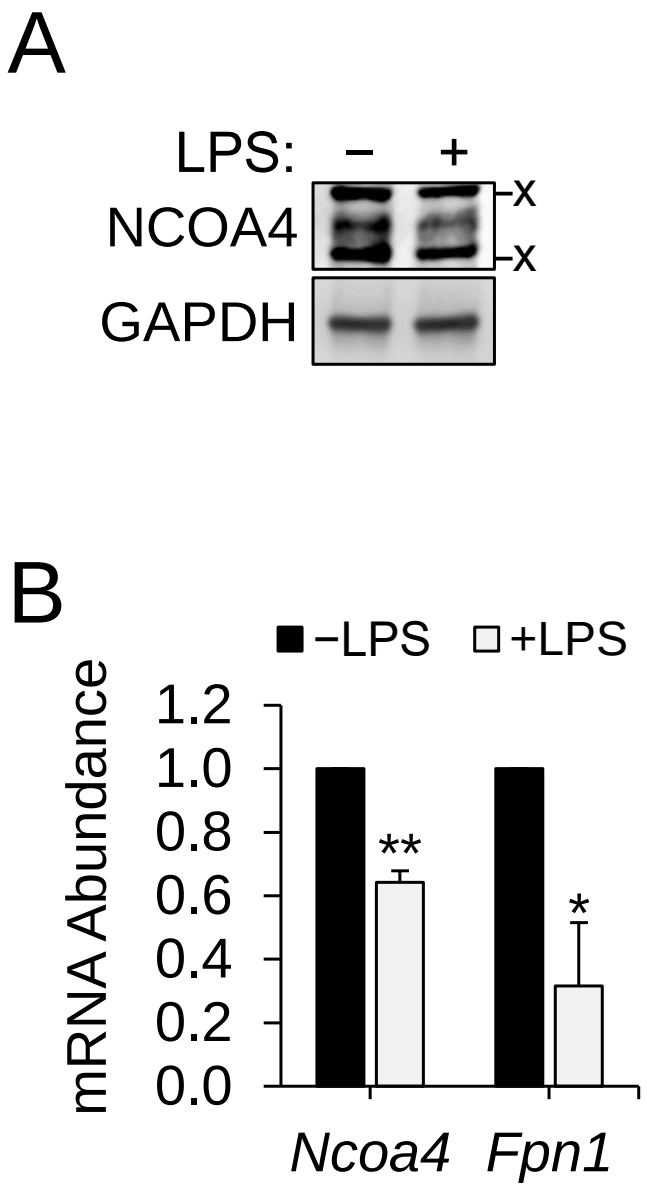

Figure 2.23: LPS represses NCOA4 and *Fpn1* in macrophages.
(**A**) NCOA4 protein abundance is decreased by LPS (1 μg/mL, 24 hours). (**B**) LPS treatment significantly represses *Ncoa4* and *Fpn1* transcript abundance. *Ncoa4 and Fpn1* are normalized to *Actb*; *n* = 3 independent experiments. **P* < 0.05; ***P* < 0.01, 2-tailed *t* test.

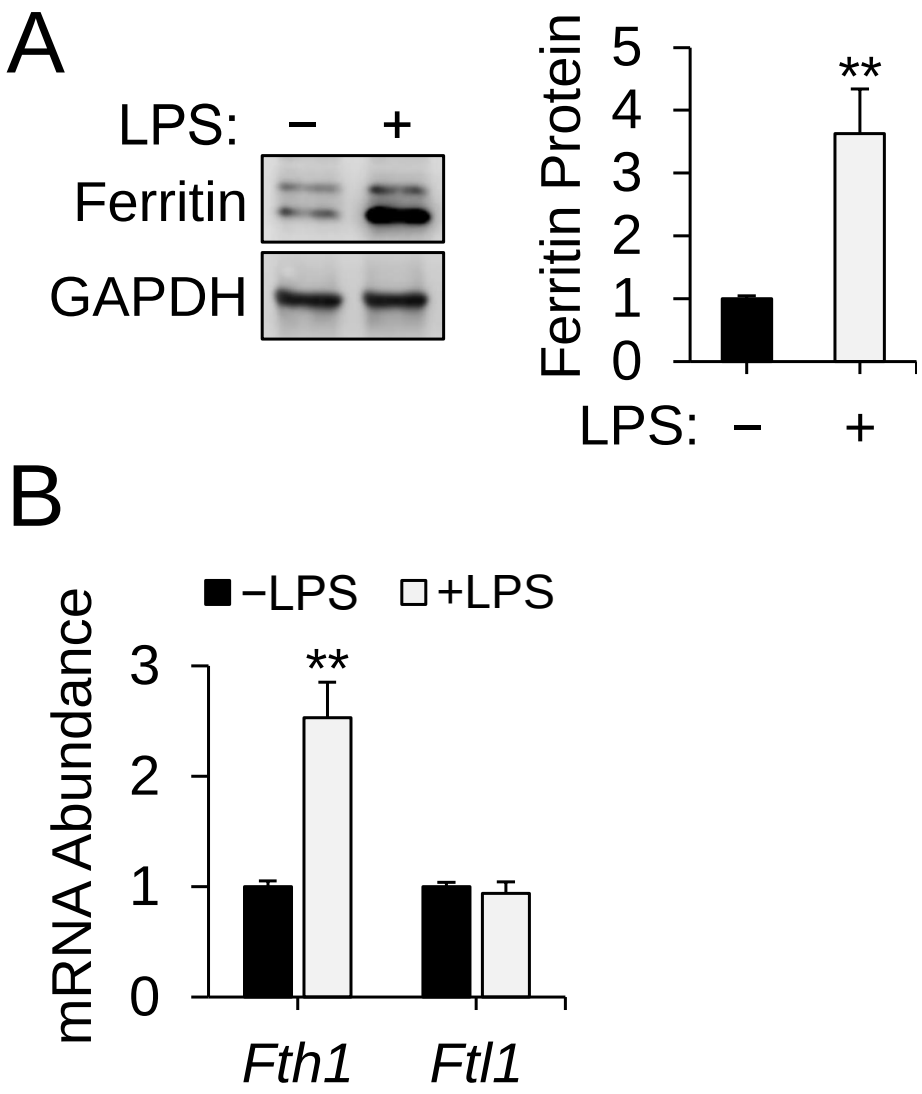

Figure 2.24: LPS activation promotes ferritin iron storage in macrophages. (**A**) Ferritin protein abundance increases by LPS treatment (1 μg/mL; 24 hours). (**B**) Ferritin H (*Fth1*) transcript increases by LPS, however ferritin L (*Ftl1*) does not change. Ferritin protein is normalized to GAPDH; *n* = 3 biological replicates. *Fth1* and *Ftl1* are normalized to *Actb*; *n* = 3 biological replicates. ***P* < 0.01 by 2-tailed *t* test.

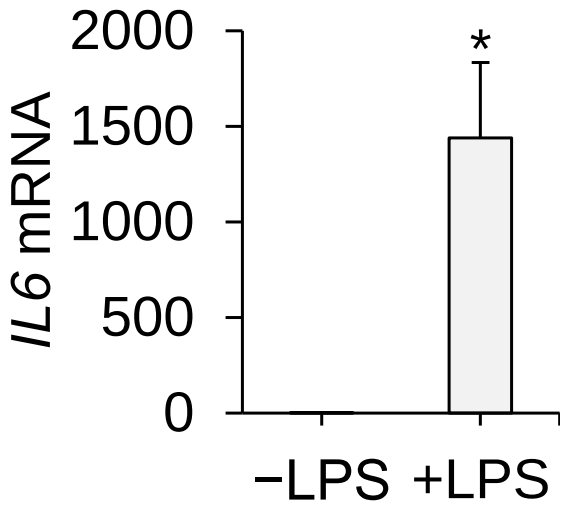

Figure 2.25: LPS treatment elicits an inflammatory response in J774 macrophages. *IL6* transcript dramatically increases in response to LPS in macrophages. Cells were treated with LPS (1 μg/mL) for 24 hours. *IL6* is normalized to *Actb*; $n = 3$ biological replicates that is representative of 3 independent experiments. $^{**}P < 0.01$ by 2-tailed *t* test.

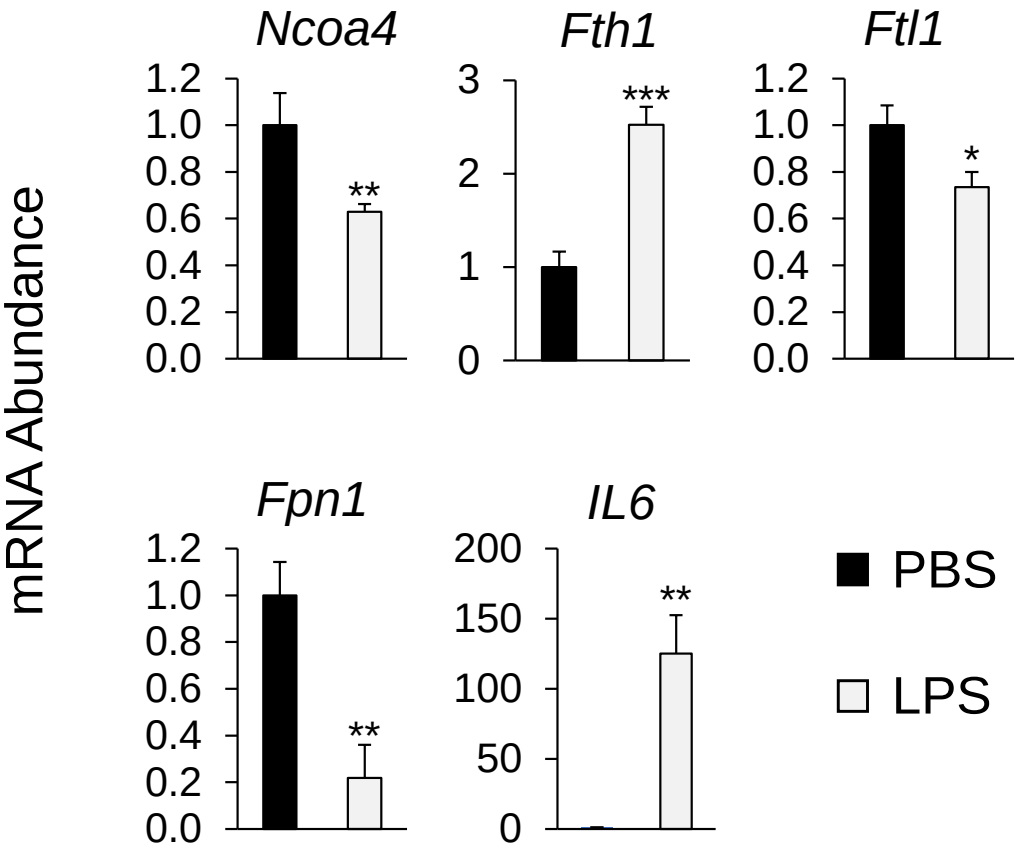

Figure 2.26: Endotoxemia by LPS represses splenic *Ncoa4*, *in vivo*.
Differential expression of splenic inflammatory and iron regulatory genes by LPS administration. Mice were treated with 0.3 mg/kg LPS or PBS (control) via intraperitoneal injection 6 hours prior to harvest. All genes are normalized to *Tbp*; $n = 5$ control; $n = 9$ LPS. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ by 2-tailed t test.

DISCUSSION

Iron is required for numerous metabolic processes. Yet, mishandled cellular iron can be cytotoxic.[120] Thus, coordinated cellular mechanisms adapt to iron fluctuations and cellular demands to maintain homeostasis and proper functioning. Cellular iron homeostasis is maintained by balancing iron import, utilization, storage, and export, which is largely mediated by the IRP/IRE regulatory system. The targets of this regulation include the iron importer genes TfR1 and DMT1, iron exporter ferroportin, and the cytosolic iron storage complex ferritin.[31,33,48,146,147] Iron storage within ferritin is a crucial component of the cells capacity to prevent cellular iron overload and toxicity.[148,149] Recently, NCOA4 has been characterized as the selective cargo receptor for autophagy-mediated release of iron from ferritin, termed, ferritinophagy. NCOA4 initiates ferritinophagy by binding to ferritin, which is subsequently degraded via the lysosome.[52,53] Notably, cellular iron restriction can enhance NCOA4 expression, which promotes the liberation of stored iron from ferritin.[29,53] Macrophages are multifunctional cells, which carry out central roles in systemic iron homeostasis and innate immunity.[150] The current studies, employing J774 macrophages, demonstrate an essential role of NCOA4-mediated ferritinophagy in macrophage iron homeostasis, and its regulation by cellular iron status. Moreover, the present studies determined the roles of NCOA4 in ferritin regulation after erythrophagocytosis and by systemic cues of iron overload and inflammation by hepcidin activity and LPS.

Reticuloendothelial macrophages are critical to systemic iron homeostasis due to their role in recycling iron from effete red cells, a process that provides over 90% of the daily iron requirement for new red cell production.[54] Red blood cells contain vast amounts of iron as a component of heme, therefore red cell digestion and iron recycling

would involve acute expansion in the cellular iron contents of erythrophagocytosing macrophages. The current studies identify a transitional storage of iron within ferritin between heme digestion and the export of elemental iron by ferroportin. This is remarkably similar to the role of ferritin during terminal erythroid development, where it functions as an intermediate form of iron between iron import and mitochondrial heme biosynthesis.[51,117] At the later stages of erythroid differentiation, NCOA4 is highly upregulated and mediates the release of stored iron towards the mitochondria.[51,117] The results from the present studies reveal a similar role of NCOA4 in macrophages, mediating a directional flux of intracellular iron, but rather from red cell heme digestion towards the exporter, ferroportin. Notably, recent *Ncoa4* knockout mouse models have demonstrated the contribution of non-erythroid NCOA4 to the maintenance of erythropoiesis.[118,130] Additionally, the accumulation of splenic iron has been associated with microcytic anemia by systemic and bone-marrow-specific losses of *Ncoa4* in mice,[118,119,130] proposing a role of NCOA4 in RE macrophage iron recycling. This would suggest that NCOA4 is essential to erythropoiesis not only during red cell development, but also during the recycling of iron back to the erythron by RE macrophages.

Similar to cellular iron balance, organismal iron distribution and homeostasis is regulated through an orchestrated control of iron utilization, acquisition, recycling, and storage. Central to the regulation of systemic iron homeostasis is the peptide hormone, hepcidin. Upregulated by systemic iron overload and inflammation, circulating hepcidin targets ferroportin on the plasma membrane of enterocytes, hepatocytes, and RE macrophages to decrease ferroportin activity and limit the flux of iron into the blood circulatory system.[122] However, the retention of cellular iron by hepcidin may impose greater oxidative stress due to the redox-active nature of iron. Therefore, due to the loss

of exporter activity by hepcidin, expanded iron storage capacity would become the primary mechanism to handle excess iron. This would include increased production, metalation, and/or fewer losses of ferritin complexes. The present studies demonstrate a repression of NCOA4 by hepcidin activity, which reveals repressed ferritinophagy as a mechanism to prevent cellular iron overload by hepcidin in macrophages. It was recently demonstrated that ferroportin overexpression in HEK293 cells increased ferritin turnover in an NCOA4-dependent manner,[151] supporting the interaction between ferroportin activity and NCOA4-mediated ferritinophagy. Together, these identify a role of NCOA4 in mediating the cross-talk between systemic and cellular regulatory mechanisms to remove excess iron, by hepcidin and ferritin, respectively. Of note, is the remarkably similar phenotypic characteristics of systemic *Ncoa4*-knockout mice and mice with macrophage-specific ferroportin deletion, both exhibiting mild anemia with tissue ferritin and iron accumulation, predominantly in the spleen and liver.[118,119,152] The similarities in presentation could be representative of the relationship between the two mechanisms.

The multifunctional capabilities of macrophages is due to their incredible plasticity, which allows them to adapt their physiology to a changing environment. In response to environmental cues, macrophages are activated to unique polarization states with distinct functions and genetic profiles. By inflammation or infection, macrophages are considered classically activated, or M1 macrophages.[153] This induces a coordinated shift in iron metabolism genes to sequester iron within the macrophage as a function of nutritional immunity.[106] Increased hepcidin activity is also an integral element of nutritional immunity. Hepcidin-induced hypoferremia is protective against extracellular pathogens, however it produces an iron-rich microenvironment within macrophages that is favored by intracellular pathogens, such as *Salmonella*.[106,132,133]

Therefore, sequestered iron must be properly handled to limit intracellular microbial burden and minimize oxidative stress. Separate from the post-translational regulation by hepcidin, previous studies have demonstrated a profound repression in ferroportin mRNA levels in response to inflammation.[136,145,154] Severely limiting cellular iron flux would necessitate an increase in ferritin iron storage, which has been demonstrated by increased ferritin H expression through enhanced transcription and protein synthesis. Interestingly, ferritin L expression is not affected under these conditions.[135,155–157] Unlike ferritin L, ferritin H possesses ferroxidase activity (converting Fe^{2+} to Fe^{3+}) which permits intracellular iron to be stored in its non-toxic form.[129] Moreover, a recent group demonstrated that ferritin H is required for iron sequestration by inflammation and contributes to macrophage activation.[157] The present studies identified repressed *Ncoa4* expression as a mechanism contributing to the iron retention by classically activated M1 macrophages. This further demonstrates a regulatory role of NCOA4 in altered macrophage iron metabolism by inflammation.

Notably, Ncoa4 has been shown to be necessary for the mobilization of iron during an acute increase in iron demand. Compared to wild-type controls, *Ncoa4* knockout mice failed to increase transferrin saturation and serum iron levels following administration of erythropoietin.[130] Thus, the differences in ferritin H and ferritin L expression by inflammation could serve another physiological purpose, which is that NCOA4 specifically binds ferritin H for initiating ferritinophagy.[51,52] Therefore, ferritin complexes predominantly composed of ferritin H subunits would be more efficient targets for NCOA4-mediated ferritinophagy once infection or inflammation subsides. Importantly, this would provide a more rapid recovery from hypoferremia to restore erythropoietic iron demands.

NCOA4 has been demonstrated to be post-translationally regulated in an iron-dependent manner. When cellular iron is replete, HERC2, an E3 ubiquitin ligase, mediates the proteasomal degradation of NCOA4 to limit ferritinophagy and promote ferritin iron storage. Conversely, when cellular iron is low, NCOA4 is stabilized, permitting ferritin binding for lysosomal degradation.[29,51] The present studies demonstrate macrophage Ncoa4 regulation is predominantly occurring on the protein level, supporting HERC2-mediated regulation. However, the LPS-induced repression in macrophage and splenic *Ncoa4* at the transcript level indicates a regulatory mechanism distinct from the HERC2-mediated post-translational mechanism. Of relevance is the transcriptional upregulation of *Ncoa4* by GATA1 in developing erythroid cells,[158] which permits promoted ferritinophagy despite elevated cellular iron contents. This novel mode of *Ncoa4* regulation will require future studies to determine the mechanisms leading to the repression in its mRNA.

Iron disorders and conditions leading to dysregulated iron affects millions of people, therefore the search for effective treatments to combat them is constant. The present studies have several clinical implications owing to the role of RE macrophages in systemic iron homeostasis. The most common genetic disorder leading to iron dysregulation is hereditary hemochromatosis (HH).[159] There are several types of HH, with the most prevalent types characterized by low hepcidin expression leading to hyperabsorption of dietary iron, resulting in tissue iron overload. If untreated, HH can lead to organ damage, cirrhosis, cardiovascular complications, diabetes mellitus and increased risk of infections.[159,160] To date, the most common treatment strategy for HH is phlebotomy.[160] However, more recently, synthetic minihepcidin analogs have begun clinical trial testing as a pharmacological strategy to combat HH.[161] Due to impaired

hepcidin activity by HH, iron flux from RE macrophages is unabated. The current studies have identified macrophage NCOA4 and ferritinophagy as an important contributor to systemic iron homeostasis. Thus, therapeutic treatments targeting macrophage NCOA4-mediated ferritinophagy, upstream of ferroportin-mediated export, to limit the cellular iron flux could be an effective treatment strategy to combat iron overload by HH.

Due to advancements in health care and effective treatments of certain diseases, survival from chronic health conditions and the presence of chronic inflammation has become more prevalent in the population.[162] Acute inflammation leads to hypoferremia as a host defense mechanism.[163] Repeated and prolonged stimulation of this pathway by chronic inflammation can lead to anemia of chronic disease (ACD) due to iron-restricted erythropoiesis.[86] ACD is the second most common form of anemia behind only iron deficiency anemia, affecting individuals with chronic infections, autoimmune disorders, cancer, chronic kidney disease, and many other chronic conditions.[164] It is characterized by normocytic or microcytic anemia and tissue iron accumulation. This is remarkably similar to the phenotypic presentation of *Ncoa4*-knockout mice.[118,119] The current studies demonstrate that inflammation and hepcidin represses NCOA4 and ferritinophagy, contributing to tissue iron accumulation. Therefore, potential therapeutic treatments could be developed to target ferritinophagy, or NCOA4 specifically, to promote the flux of iron from RE macrophages. Additionally, based on the cross-talk between ferritin regulation and hepcidin, targeting an increase in ferroportin activity would promote ferritinophagy and the recycling of iron into circulation to meet erythropoietic demands.

CONCLUSIONS

The current studies detail the roles and regulation of NCOA4 and ferritinophagy in macrophages, demonstrating significant importance in both cellular and systemic iron homeostasis. Specifically, NCOA4 is required for ferritin iron utilization in iron deprived conditions. Additionally, macrophage NCOA4 is imperative to the turnover of ferritin in the late stages of red cell iron recycling, a process that provides a majority of the iron necessary to sustain erythropoiesis. Treatment of macrophages with PR73, a minihepcidin analog possessing hepcidin activity, repressed NCOA4-mediated ferritinophagy, identifying a cross-talk between systemic and cellular iron homeostatic mechanisms. Lastly, macrophage *Ncoa4* mRNA was shown to be repressed by LPS-mediated inflammation likely as a mechanism to promote iron sequestration. This novel effect was confirmed *in vivo* by analyzing the splenic tissue of mice with LPS-induced endotoxemia. Further research employing animal models of macrophagic *Ncoa4* knockout would provide new insights on the impact of our findings at the organismal level, and thus is warranted.

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