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Régulation de l'hepcidine et le rôle de la lipocaline 2 dans l'homéostasie du fer / Novel insights into the regulation of hepcidin and the role of lipocalin 2 in iron homeostasis

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Régulation de l'hepcidine et le rôle de la lipocaline 2 dans l'homéostasie du fer / Novel insights into the regulation of hepcidin and the role of lipocalin 2 in iron homeostasis

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Résumé

Le fer, un métal de transition, est requis pour la survie de presque tout les organismes vivant à cause de son habilité à accepter ou donner un électron et donc à catalyser plusieurs réactions biochimique fondamentales. Cependant, la même propriété permet aussi au fer ionique d'accélérer la formation de radicaux libres et donc le fer peut potentiellement avoir des effets néfastes. Conséquemment, l'homéostasie du fer doit être étroitement régulé, tant au niveau cellulaire que systémique. Notre étude met l'emphase sur deux molécules importante pour régulation du métabolisme du fer : la lipocaline 2 (Lcn2) et l'hepcidine.

Lcn2, une protéine de phase aiguë, est impliquée dans le transport du fer par les sidérophores. Lcn2 est un candidat potentiel comme transporteur du fer qui pourrait être responsable de l'accumulation excessive du fer non lié à la transferrine dans le foie des patients atteints d'hémochromatose héréditaire (HH). Nous avons généré des souris double-déficiente *HfeLcn2* pour évaluer l'importance de Lcn2 dans la pathogenèse de surcharge en fer hépatique dans les souris knock-out *Hfe* (*Hfe* $^{-/-}$). Notre étude révèle que la délétion de Lcn2 dans les souris *Hfe*$^{-/-}$ n'influence pas leur accumulation de fer hépatique ou leur réponse à une surcharge en fer. Le phénotype des souries *HfeLcn2*$^{-/-}$ demeure indiscernable de celui des souris *Hfe*$^{-/-}$. Nos données impliquent que Lcn2 n'est pas essentiel pour la livraison du fer aux hépatocytes dans l'HH.

L'hepcidine, un régulateur clé du métabolisme du fer, est un petit peptide antimicrobien produit par le foie et qui régule l'absorption intestinale du fer et son recyclage par les macrophages. L'expression de l'hepcidine est induite par la surcharge en fer et l'inflammation, tandis que, à l'inverse, elle est inhibée par l'anémie et l'hypoxie. Dans certaine situations pathologique, l'hepcidine est régulée dans des directions opposées par plus d'un régulateur. Nous avons, en outre, analysé comment les différents facteurs influencent l'expression de l'hepcidine *in vivo* en utilisant un modèle de souris avec un métabolisme du fer altéré. Nous

avons examiné la régulation de l'hepcidine en présence de stimuli opposés, ainsi que la contribution des médiateurs et des voix de signalisation en aval de l'expression de l'hepcidine. Nous avons démontré que l'érythropoïèse, lorsque stimulé par l'érythropoïétine, mais pas par l'hypoxie, diminue l'expression de l'hepcidine d'une façon dépendante de la dose, même en présence de lipopolysaccharides ou de surcharge de fer alimentaire, qui peuvent agir de manière additive. De plus, l'entraînement érythropoïétique inhibe tant la voix inflammatoire que celle de détection du fer, du moins en partie, par la suppression du signal IL-6/STAT3 et BMP/SMAD4 *in vivo*. Au total, nos données suggèrent que le niveau d'expression de l'hepcidine en présence de signaux opposés est déterminé par la force du stimulus individuel plutôt que par une hiérarchie absolue. Ces découvertes sont pertinentes pour le traitement de l'anémie des maladies chronique et les désordres de surcharge en fer.

Mots-clés : Fer, système immunitaire, hepcidine, lipocaline 2, BMP, SMAD, STAT3

Abstract

Iron, a transition metal, is required for survival by almost all living organisms due to its ability to accept or donate electrons and thus to catalyze many fundamental biochemical reactions. However, the same properties also allow ionic iron to accelerate the formation of free radicals and as such iron has the potential for deleterious effects. Consequently, iron homeostasis must be tightly regulated at both cellular and systemic levels. Our studies focused on two important molecules in the regulation of iron metabolism, namely, lipocalin 2 (Lcn2) and hepcidin.

Lcn2, an acute phase protein, is involved in iron trafficking via siderophores. Lcn2 has emerged as a candidate iron-transporter that may be responsible for excessive non-transferrin-bound iron (NTBI) accumulation in the liver of hereditary hemochromatosis (HH) patients. We generated *HfeLcn2* double-deficient mice to evaluate the importance of Lcn2 in the pathogenesis of hepatic iron loading in *Hfe* knockout mice. Our studies revealed that deletion of Lcn2 in *Hfe*-knockout mice does not influence hepatic iron accumulation in $Hfe^{-/-}$ mice, or their response to iron loading, as the phenotype of $HfeLcn2^{-/-}$ mice remained indistinguishable from that of $Hfe^{-/-}$ mice. Our data imply that Lcn2 is not essential for iron delivery to hepatocytes in HH.

Hepcidin, a key regulator of iron metabolism, is a small antimicrobial peptide produced by the liver that regulates intestinal iron absorption and iron recycling by macrophages. Hepcidin expression is induced by iron-loading and inflammation while, conversely, being inhibited by anemia and hypoxia. Under certain pathologic situations, hepcidin is regulated in opposite directions by more than one regulator. We further investigated how different factors influence hepcidin expression *in vivo* using mouse models of altered iron metabolism. We examined hepcidin regulation in the presence of opposing stimuli as well as the contributions of

mediators and downstream signaling pathways of hepcidin expression. We show that erythropoiesis drive, when stimulated by erythropoietin but not by hypoxia, down-regulates hepcidin in a dose-dependent manner, even in the presence of lipopolysaccharide or dietary iron-loading, which may act additively. Moreover, erythropoietic drive inhibited both the inflammatory and iron-sensing pathways, at least in part, via the suppression of IL-6/STAT3 and BMP/SMAD4 signaling *in vivo*. Altogether, our data suggest that hepcidin expression levels in the presence of opposing signaling are determined by the strength of the individual stimuli rather than by an absolute hierarchy. These findings are pertinent for the treatment of the anemia of chronic disease and iron-loading disorders.

Keywords: iron, immune system, hepcidin, lipocalin 2, BMP, SMAD, STAT3,

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List of acronyms and abbreviations

ACD, anemia of chronic disease

β2m, β2-microglobulin .

BMP, bone morphogenetic protein

BMT, Bone marrow transplantation

GPx, catalase, glutathione peroxidase

C/EBPα, CCAAT/enhancer-binding protein alpha

GVHD, chronic graft-versus-host disease

CDA, congenital dyserythropoietic anemia

CI, carbonyl-iron

DFX, deferasirox

DFP, deferiprone

DFO, deferoxamine

holoTf, diferric-Tf

2,5-DHBA , 2,5-dihydroxybenzoic acid

DMT1, divalent metal transporter 1

DcytB , duodenal cytochrome B

EPO, erythropoietin

Erk, extracellular signal-regulated kinase

ESAs , erythropoiesis stimulating agents

Fe-S, iron-sulfur

FtH, ferritin heavy chains

FtL, ferritin light chains

FtMt, mitochondrial ferritin

FPN, ferroportin

GDF15, growth differentiation factor 15

GPx, glutathione peroxidase

HCT, hematocrit

Hb , hemoglobin

HJV, hemojuvelin

Hepc, hepcidin

HH, hereditary hemochromatosis

HIF, hypoxia-inducible factor

H_2O_2, hydrogen peroxide

$^{\bullet}$HO, hydroxyl radical

holo, iron loaded

ISC, iron-sulfur cluster

IL-6, interleukin-6

IFN-γ, interferon-gamma

IREs, iron-responsive elements

IRF-8, interferon regulatory factor 8

IRIDA, iron-refractory iron deficiency anemia

IRPs, iron regulatory proteins

IDA, iron deficiency anemia

JAKs, Janus kinases

JH, juvenile hemochromatosis

Lcn2, lipocalin 2

LPS, lipopolysaccharide

LEAP-1, liver-expressed antimicrobial peptide

LVDCC, L-type voltage-dependent calcium channels

MCHC, mean corpuscular hemoglobin concentration

MCH, mean corpuscular hemoglobin

MCV, mean corpuscular volume

m-HJV, membrane-bound hemojuvelin

Nramp1, natural resistance-associated macrophage protein-1

Nramp2, natural resistance-associated macrophage protein-2

NGAL, neutrophil gelatinase associated lipocalin

NTBI , non-transferrin bound iron

NMR, nuclear magnetic resonance spectroscopy

OsM, oncostatin M

$O_2^{\bullet-}$, superoxide anion

PBS, phosphate-buffered saline

PHD, prolyl hydroxylase domain

PNS, peripheral nervous system

ROS, reactive oxygen species

rhEPO , recombinant human EPO

RBC, red blood cells

RGMC, repulsive guidance molecule C

RNAi, RNA interference

Scara5, scavenger receptor class A, member 5

s-HJV, soluble hemojuvelin

shRNA, short hairpin RNA

SMAD4, SMA- and mothers against decapentaplegic-related protein

siRNA, small interfering RNA

SOD, superoxide dismutase

STAT-3, signal transducer and activator of transcription 3

TIM-2, T cell immunoglobulin and mucin domain containing 2

TNF-α, tumor necrosis factors-α

TLR-4, toll-like receptor 4

Tf, transferrin

TfR, transferrin receptor

TMPRSS6,Transmembrane protease, serine 6

TWSG1, twisted gastrulation protein

USF2, upstream stimulatory factor 2

UTRs, untranslated regions

VHL, von-Hippel–Lindau

ZIP14, Zrt/Irt-like protein 14

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CHAPTER 1 INTRODUCTION

Iron, a transition metal, is required for survival by almost all living organisms due to its ability to switch between two different redox states and as such catalyze many fundamental biochemical reactions. However, ionic iron can accelerate the formation of free radicals and as such has a potential for deleterious effects. Therefore, iron levels need to be tightly regulated such as to meet body iron needs, while at the same time avoiding toxicity. The importance of iron in biological systems is best illustrated by the ever-increasing number of diseases associated with deregulated iron metabolism. Thus, understanding the mechanisms for the regulation of iron homeostasis is essential for human health.

1. The importance of iron for biological systems

Iron is the fourth most abundant element on the surface of our planet [1]. Despite this relative abundance in the rocks constituting the earth's crust, iron is present in rather small quantities in living matter. However, iron remains an essential part in many metabolic processes essential for life.

The metabolic importance of this element comes from its chemical properties, as iron can form up to six coordination bonds by accepting an electron pair on each of its six atomic orbitals. Electronegative elements such as oxygen, nitrogen and sulfur thus have the ability to bind iron. This fundamental property allows iron to associate with many biologically-relevant proteins. Furthermore, as a transition metal, iron can exist in two forms, the reduced (Fe^{2+}, ferrous iron) and oxidized forms (Fe^{3+}, ferric iron). This ability to alternatively accept and give electrons makes iron the cofactor of choice for many redox enzymatic reactions (**Figure 1**). Therefore, the widespread use of iron can be accounted for by its ability to shuttle electrons, flexibility for binding ligands in diverse orientations, and its high bioavailability during the early stages of evolution under the reducing conditions of a sulphur-rich atmosphere.

1.1 Iron-containing proteins

Many proteins that have crucial roles in cellular physiology require iron to function. Interestingly, most iron-binding proteins are highly conserved across prokaryotes and eukaryotes, and the central position of iron in cellular metabolism is maintained in almost all forms of life. Depending on how the metal is bound to the protein, iron-containing proteins can be classified into three classes: hemoproteins, iron-sulfur proteins and iron-binding proteins.

1.1.1 Hemoproteins

Hemoproteins have a tetrapyrrole core at the center of which is encased an iron atom. When the iron atom is in the reduced form, the prosthetic group is called heme, whereas it is called hemin when the iron atom is in the ferric form [2]. The most representative hemoproteins include hemoglobin (Hb), myoglobin [3], cytochromes [4], catalases and peroxidases. Hemoglobin and myoglobin are responsible for stabilizing, transporting and storing oxygen. Oxygen transport through hemoglobin is one of the most important biological functions of iron. Cytochromes constitute major components of the mitochondrial electron transport chains (cytochromes a, b, c) that participate in electron transport and energy metabolism [5], while catalases and peroxidases are hemoproteins that play a fundamental role in the elimination of hazardous reactive oxygen species (ROS) and as such have antioxidant functions.

1.1.2 Iron-sulfur proteins

This type of protein contains what is called an iron-sulfur center (or cluster) consisting of combinations between iron and sulfur atoms of variable stoichiometry. Iron atoms bridge with inorganic sulfides and are bound to proteins through cysteine residues to form the iron-sulfur (Fe-S) proteins [6]. [2Fe-2S], [4Fe-4S] and [3Fe-4S] are the most common cluster variants in eukaryotes [7]. Iron-sulfur proteins include ferredoxins, NADH dehydrogenases, hydrogenases, cytochrome c reductase and nitrogenases. They play a critical role in a wide range of cellular activities as components of the respiratory electron transport complexes, and of tricarboxylic acid cycle enzymes, aconitase and succinate dehydrogenase [7, 8]. In addition, Fe-S clusters are components of DNA repair enzymes fundamental for the recognition of DNA damage and repair.

1.1.3 Iron-binding proteins

This class of proteins is characterized by the presence of iron directly associated to the protein.

Examples of iron-binding proteins include ribonucleotide reductase necessary during the S phase of DNA synthesis, and lipoxygenases that catalyze the oxidation of fatty acids. Other iron-binding proteins are fundamental for the transport and storage of iron, namely transferrin and ferritin.

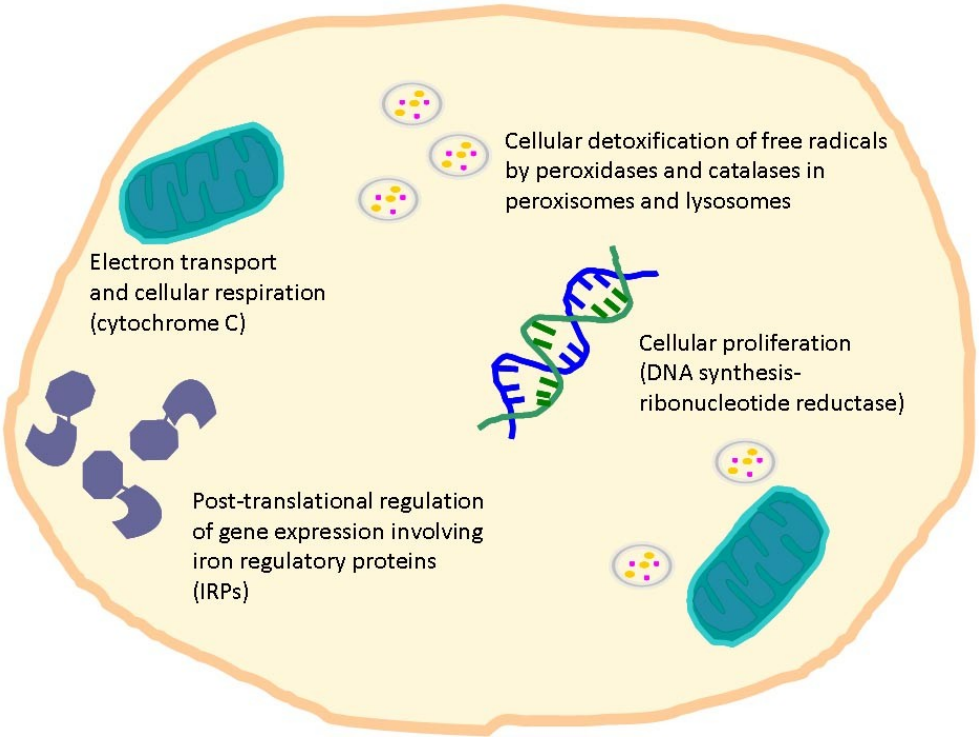

Figure 1 The importance of iron for biological systems

Iron is vital for almost all living organisms due to participation in a wide variety of cellular processes such as cellular respiration, proliferation, detoxification and differentiation [9-13].

1.2 Iron toxicity

Although iron is indispensable for life, it may also become toxic. The toxicity of iron is mainly associated with its potential participation in the excessive production of ROS, a byproduct of oxygen metabolism that describes a variety of molecules and free radicals (chemical species with one unpaired electron).

Under normal physiological conditions, superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) are continuously produced in tissue cells as byproducts of aerobic metabolism [14, 15]. More than 500 liters of oxygen is utilized daily by tissue cells of a normal human subject, and 1-5% of the oxygen consumed by the respiratory chain is incompletely reduced to $O_2^{\bullet-}$ and H_2O_2 [14, 16]. If not efficiently removed, hydroxyl radical ($^{\bullet}HO$) may be generated from H_2O_2 *via* the Fenton reaction in the presence of Fe^{2+} and cause oxidative damage to cellular components, including nucleic acids [17], proteins [18] and lipids [19] .

To cope with the oxidative stress elicited by aerobic metabolism, mammalian cells have developed an ubiquitous antioxidant defense system, which consists of superoxide dismutases (SODs), catalase, glutathione peroxidases (GPxs) and glutathione reductase together with a number of low molecular weight antioxidants such as ascorbate, α-tocopherol and glutathione [20, 21] (**Figure 2**). However, this antioxidant defense system may be overwhelmed by various pathological or environmental factors so that a fraction of ROS may escape destruction and form the far more reactive hydroxyl radical ($^{\bullet}HO$) [20, 21]. An increase in ROS-elicited oxidative damage to DNA and other biomolecules may impair normal functions of tissue cells and lead to human aging and disease [17, 20].

Because of its destructive potential, iron is suspected to play a role in many pathological conditions, including carcinogenesis, atherosclerosis, and a number of neurodegenerative disorders, such as Parkinson's or Alzheimer's disease [22-27]. To minimize these potential toxic effects, highly sophisticated mechanisms and specialized molecules for the acquisition, transport, and storage of iron in soluble, non-toxic forms have evolved to meet cellular iron requirements and to systemically-regulate iron homeostasis.

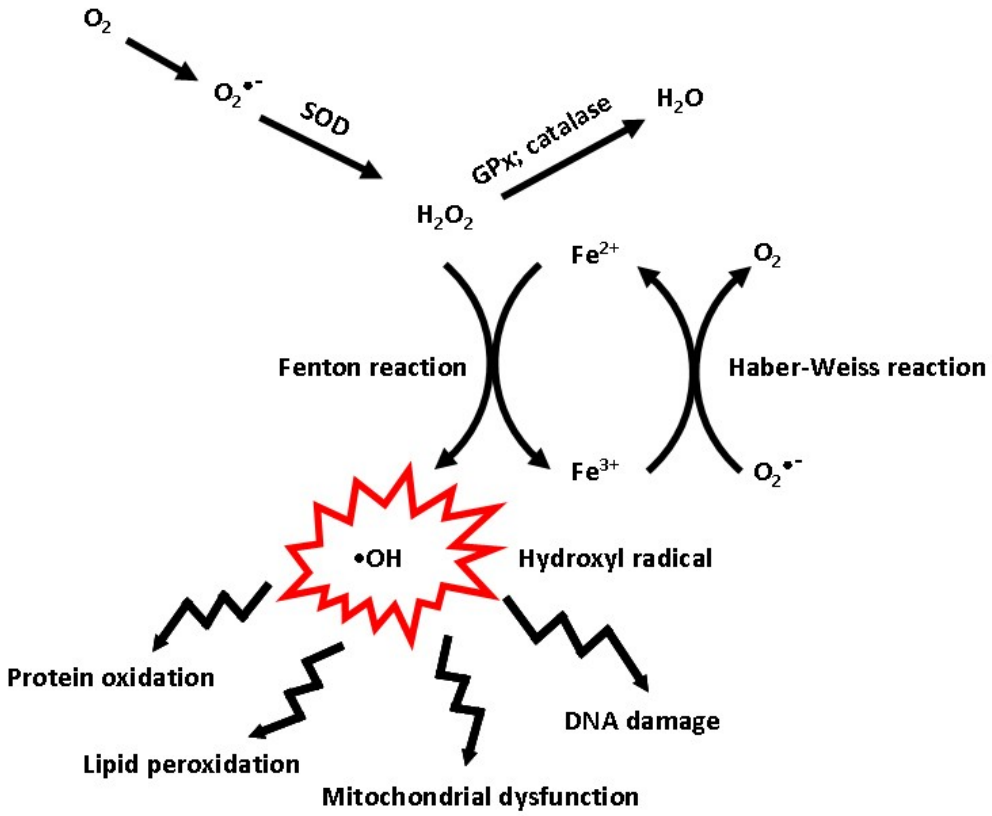

Figure 2 The basis for iron toxicity.

The Haber-Weiss reaction refers to the net reaction ($\bullet O_2^- + H_2O_2 \rightarrow \bullet OH + OH^- + O_2$).The reaction generates hydroxyl radical (•OH) from hydrogen peroxide (H_2O_2) and is catalyzed by iron. The first step involves reduction of ferric iron to ferrous ($Fe^{3+} + \bullet O_2^- \rightarrow Fe^{2+} + O_2$). The second step is the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \bullet OH$). The highly reactive hydroxyl radical (•OH) can lead to oxidative stress-induced protein oxidation, lipid peroxidation, mitochondrial dysfunction, and DNA damage. SOD - superoxide dismutase; GPx – glutathione peroxidase.

2. Iron homeostasis

The chemical properties of iron place two limitations on the biological behavior of this element. First, although iron is abundant, the metal is most commonly found in nature as the insoluble ferric hydroxide. Therefore, organisms have evolved complex mechanisms to obtain iron from their environment. Second, iron is potentially toxic by participating in the generation of toxic oxygen radicals. Consequently, while iron is accumulated in amounts sufficient for metabolism, organisms must ensure that their intracellular concentration of "free" iron does not reach toxic levels.

To avoid iron excess and its potential toxic consequences while at the same time providing enough of iron for growth and development, iron homeostasis must be tightly controlled at both the cellular and systemic levels. Iron needs to be transported and safely stored, processes that are mediated by a number of specialized proteins involved in maintaining iron homeostasis such as to ensure the acquisition of iron and its distribution to different organs and tissues, as well as intracellular organelles.

2.1 Cellular iron homeostasis

At the cellular level, iron homeostasis is achieved through the coordinated regulation of iron uptake, storage, export and management of intracellular iron distribution. Importantly, cellular iron homeostasis seems to be differentially regulated in iron-acceptor cells (primarily erythroid precursors) and in iron-donor cells (macrophages, hepatocytes and intestinal epithelial cells in the duodenum). **Figure 3** summarizes the general mechanisms that intervene in the uptake, intracellular distribution and transport of iron to cell populations.

2.1.1 Cellular iron uptake

Under normal physiological conditions, virtually all iron in circulation is bound to transferrin (Tf), an abundant plasma glycoprotein that can bind one or two ferric ions with high affinity [28]. Therefore, nearly all-cellular acquisition of iron from blood occurs via transferrin receptor 1 and 2 (TfR1- and TfR2-) mediated uptake.

TfR-mediated iron uptake

In circulation, iron-free Tf, or apoTf, captures extracellular ferric iron and forms the diferric-Tf (holoTf) complex that has high affinity for TfR1 expressed at the cell surface. The Tf/TfR1 complex is then internalized via endocytosis into endosomes. Acidification of the early endosomes through the entry of protons (pH=5.5) triggers conformational changes in both Tf and TfR1, facilitating the release of ferric iron from the Tf/TfR1 complex. Subsequently, the STEAP3 ferrireductase (six-transmembrane epithelial antigen of prostate 3) reduces ferric iron to ferrous iron (Fe^{2+}) [29] allowing its transportation across the endosomal membrane into the cytoplasm by the divalent metal transporter 1 (DMT1; also known as Natural Resistance Associated Macrophage Protein 2, or NRAMP2) [30]. The apoTf/TfR1 complex is then recycled back to the cell surface and apoTf is released into the bloodstream to recapture ferric iron, thereby completing the transferrin cycle (**Figure 3**).

The TfR1 is ubiquitously expressed at lower levels in normal cells and at higher levels on proliferating cells and cells that require high amounts of iron, such as intestinal epithelial cells [31], placental trophoblasts and erythroid precursors [32]. A homologue of TfR1, termed TfR2, has additionally been identified [33]. Unlike TfR1, TfR2 is exclusively expressed in liver hepatocytes, duodenal crypt cells and early erythroid precursors [34] [35]. Similar to TfR1, TfR2 binds to Tf at the cell surface and mediates iron uptake by binding to Tf. However, TfR2 has a 25-fold lower affinity for Tf compared to TfR1 [33, 36] and seems to be pivotal for iron

uptake during embryonic development, particularly from day 13 to postnatal day 1, a period during which TfR1 expression is suppressed while TfR2 is activated in hepatocytes [35].

Non-transferrin bound iron (NTBI) uptake

Besides Tf iron, which represents the normal form of circulating iron, a second mechanism of iron uptake occurs through a transferrin-independent process. This NTBI transport process is considered to have a minor role in iron uptake under normal physiological conditions but becomes the primary uptake mechanism when serum iron is severely elevated and surpasses the iron-biding capacity of Tf, as seen in primary and secondary iron overload disorders [37]. NTBI has been directly connected with the production of harmful ROS and ensuing tissue damage, as most organs, including the liver, heart, pancreas and brain have a high capacity to rapidly uptake NTBI. The mechanism(s) responsible for NTBI uptake remain to be fully understood, however several molecules, including DMT1, ZIP14 (also known as Slc39a14), and L-type voltage-dependent calcium channels (L-type VDCC) have all been implicated in NTBI uptake.

DMT1. Divalent metals such as Mn and Zn are able to inhibit NTBI uptake by mouse hepatocytes indicating that NTBI may share the same transporter with other divalent metals. DMT1 has been proposed to be a putative transporter of NTBI in hepatic cells under iron overloading situations, because DMT1 expression is upregulated in the liver of iron-overloaded mice [38]. However, DMT1-deficient mice are able to develop hepatic iron overload, suggesting that another alternative pathway could play a major role in Fe^{3+} uptake and indicating that there is an alternative iron-transporter for hepatic NTBI uptake [39, 40].

ZIP14. The Zrt/Irt-like protein 14 is a zinc transporter that is also involved in NTBI uptake by hepatocytes [41]. When ZIP14 is overexpressed in the AML12 mouse hepatocyte cell line, NTBI uptake increases, while the opposite occurs when the expression of the endogenous

ZIP14 is suppressed [41].

LVDCC. The L-type voltage-dependent calcium channels, where “L" stands for long-lasting activation, represent a group of calcium channels that allow the influx of Ca^{2+} essential for normal excitability and excitation-contraction coupling in cardiomyocytes. Several findings have been shown to support the role of LVDCC in cardiac NTBI uptake [42]. For example, LVDCC agonists can augment myocyte iron uptake by perfused rat hearts, while conversely, LVDCC blockers have an inhibitory effect [43]. Moreover, treatment of iron-loaded mice with LVDCC blockers, such as amlodipine and verapamil, were shown to inhibit LVDCC current in cardiomyocytes, reduce myocardial iron accumulation and improve survival [44]. Conversely, overexpression of LVDCC in transgenic mice was shown to lead to increased myocardial iron accumulation and oxidative stress, resulting in impaired cardiac function in comparison with wild-type mice [44]. More recently, it has been demonstrated that, unlike wild-type mice, treatments of DMT1-deficient mice with the LVDCC blocker nifedipine had no effect in reducing iron accumulation in the liver, suggesting that this effect of nifedipine-mediated modulation of iron transport occurs trough DMT1 [45]. However, McKenzie et al reported that photodegraded nifedipine is an iron-specific ionophore, and the ionophore effect was independent of DMT1[46]. Despite these discrepancies regarding the mechanism of action, these findings suggest that nifedipine could possibly be beneficial in iron overload cardiomyopathy.

Alternative iron uptake systems

Some specific cell types have additional means to take up iron, usually involving receptor-mediated endocytosis of other forms of protein-bound iron. For example, survival of kidney cells in culture has been shown to be regulated by lipocalin 2-dependent endocytosis of an iron-laden siderophore via the SLC22A17 lipocalin receptor [47]. In addition, some cells can uptake serum ferritin through the Scara5 (scavenger receptor class A, member 5) and TIM-2 (T

cell immunoglobulin and mucin domain containing 2) ferritin receptors [48, 49]. Another source of iron for some specialized cells is represented by heme. For example, SLC48A1 has been identified as a heme import molecule [50]. Macrophages can uptake heme indirectly, through phagocytosis of senescent and dying red blood cells (RBC). Finally, in plasma, hemoglobin and free heme arising from intravascular hemolysis are cleared by specific scavenger systems: hemoglobin forms a complex with haptoglobin that is delivered to macrophages via CD163-mediated endocytosis [51, 52], while free heme binds to hemopexin and the complex is endocytosed via the CD91 receptor present on the surface of macrophages, hepatocytes, and other cell types [53].

2.1.2 Cellular iron storage

Once uptaken into the cell, iron that is not immediately utilized or exported is stored within ferritin, the major iron-storage protein in the body. Ferritin is a conserved protein that assembles into a large shell-like structure. Its structure possesses a cavity that provides space to accommodate up to 4500 iron atoms**.** Thus, ferritin can sequester excess intracellular iron in a redox inactive form to achieve the purpose of storage and detoxification. Ferritin is composed of a combination of 24 subunits of heavy (FtH) and light (FtL) chains with the expression ratio of FtH/FtL chains varies depending on the tissue. For example, FtH is highly expressed in the heart, while FtL expression is increased in the liver. The FtH/FtL ratio also changes in response to inflammation and infection. Functionally, FtH has a potent ferroxidase activity that catalyzes the oxidation of ferrous iron, essential for iron internalization and packing into the mineral core. In turn, the FtL subunit is involved in iron nucleation and protein stability. Iron stored in ferritin is thought to be bioavailable and is mobilized for cellular utilization mainly during lysosomal and proteasomal turnover [54]. Serum ferritin is mostly composed of the FtL isoform and as such, contains negligible iron levels but is useful as a marker for body iron storage levels if no inflammatory or infectious conditions are present

[55]. In addition to this major form of ferritin, mitochondria also contain a nuclear-encoded H-type ferritin homopolymer, designated mitochondrial ferritin (FtMt) [56]. FtMt is mostly detected in mitochondrial-rich tissues, including heart, pancreas and kidney. Under normal conditions, FtMt is not involved in mitochondrial iron utilization. However, the expression of FtMt in patients suffering from sideroblastic anaemia rises significantly in iron-loaded ring erythroblasts (sideroblasts), indicating that FtMt may play an important role in the detoxification of iron within this organelle [57].

2.1.3 Iron usage in the mitochondria

Mitochondria perform an important role in the control of cellular iron metabolism. They constitute the major subcellular site of iron utilization, as the sole site for heme biosynthesis[58], as well as the major site for Fe–S cluster protein assembly [59]. The exact mechanism(s) by which mitochondria acquire iron is not fully understood. The inner mitochondrial membrane contains iron transporters that traffic iron from the cytosol into the mitochondrial matrix. Mitoferrin has been recently identified as a mitochondrial iron importer important for iron uptake in both erythroid and non-erythroid cells [60, 61], as exemplified by the fact that mutating mouse mitoferrin leads to impaired heme synthesis due to defective mitochondrial iron uptake [60]. In addition to mitoferrin, an endogenous mammalian siderophore, namely 2,5-dihydroxybenzoic acid (2,5-DHBA), has been proposed to be involved in mitochondrial iron uptake. In this case, iron is imported into the mitochondria in the form of an iron-siderophore complex [62]. Several studies suggest that in erythroid cells, iron-loaded endosomes directly deliver iron to mitochondria through a transient and rapid contact, in a process that has been termed “kiss-and-run” [63].

Once iron enters the mitochondria, it can be used for heme biosynthesis, Fe–S cluster protein assembly or it is stored in mitochondrial ferritin. For heme synthesis, iron is exported toward

the cytosol and further inserted into hemoproteins, such as hemoglobin or in cytochromes. In response to iron deficiency, mammalian cells enhance mitochondrial iron uptake to maintain vital mitochondrial functions that are Fe-dependent [64], and suppress multiple Fe-dependent pathways, such as Fe-S cluster scaffold proteins, Nfu1 and Isa1, to limit the utilization of cellular iron [65]. A matrix protein called frataxin [66] has been identified as a possible regulator of mitochondrial iron export [67] and storage [68]. In humans, inappropriate expression of frataxin due an intronic GAA triplet repeat expansion causes the neurodegenerative disorder Friedreich ataxia [69], an autosomal recessive disease in which accumulation of iron within the mitochondria has been reported both in humans [70] and animal models [71, 72].

2.1.4 Cellular iron export

Ferroportin (FPN, also known as Ireg1 and MTP1) is the only known mammalian iron exporter identified so far [73-75]. While FPN is ubiquitously expressed, higher levels are found in cells that are crucial for cellular iron homeostasis, such as duodenal enterocytes, macrophages, hepatocytes and, interestingly, also in cells of the central nervous system. Intestinal enterocytes are responsible for iron transfer into the body, while macrophages are the major sites for iron recycling. FPN-mediated iron export depends on the activity of the copper-dependent ferroxidase hephaestin in enterocytes, while all other cells require ceruloplasmin, another copper-dependent ferroxidase present in the plasma, to successfully export iron. Cherukuri et al proposed ceruloplasmin could share responsibility with hephaestin for iron absorption under stress in the intestine [76]. These ferrioxidases convert Fe^{2+} to Fe^{3+}, the iron form that can then bind plasma transferrin. Mice with hephaestin deficiency develop anemia due to defective iron-export from enterocytes into the body to complete the process of intestinal iron absorption [77]. In humans, mutations in the ceruloplasmin gene result in iron accumulation in macrophages, hepatocytes, and cells of the central nervous system, causing

iron-restricted anemia and neurodegeneration, as seen in aceruloplasminemia, an autosomal recessive disorder [78, 79].

FPN-mediated iron-export is highly regulated and this regulation is essential for iron acquisition, utilization and storage. At the transcriptional level, FPN mRNA levels are augmented in response to increased erythropoiesis [80], iron, heme and other transition metals [81-84], and conversely, mRNA levels are inhibited during inflammation [85]. At the translational level, expression of FPN is controlled by the iron regulatory proteins (IRPs) 1 and 2. FPN can additionally be regulated at the post-translational level by a small antimicrobial peptide called hepcidin [86]. Last but not least, in addition to FPN-mediated iron export, cells may also export iron in the form of heme and ferritin, as previously mentioned.

2.1.5 Regulation of cellular iron homeostasis

To achieve cellular iron homeostasis, iron uptake, storage, utilization, and export must be coordinately regulated. The post-transcriptional regulation of these processes by IRPs through their interaction with iron-responsive elements (IREs), the so-called IRE/IRP system, is very well characterized. IRPs belong to the iron-sulfur cluster (ISC) isomerase family and include two IRP homologue proteins, IRP1 and IRP2. IRPs bind to evolutionarily-conserved *cis*-regulatory hairpin structures called IREs, which are located at the 5′ or 3′ mRNA untranslated regions (UTRs) [52]. Cellular iron levels regulate the interaction between IREs and IRPs through different mechanisms. More specifically, IRP1 is regulated in a reversible manner through Fe/S assembly/disassembly, while IRP2 is regulated in an irreversible manner, at the level of protein stability. In the presence of high intracellular iron levels, IRP1 binds to a [4Fe-4S] cluster, namely cytoplasmic aconitase, leaving the IREs free. When cellular iron levels are low, IRP1 binds to IREs [87]. The conversion between holo-IRP1 and apo-IRP1 mainly depends on mitochondrial iron availability and Fe/S cluster production. Although IRP2 does not contain a Fe/S cluster, several signals including activity of a

2-oxoglutarate-dependent oxygenase(s) may be involved in IRP2 degradation in response to iron[88]. Non-iron signals are also involved in IRE/IRP binding regulation. For example, nitric oxide (NO) favors apo-IRP1 formation and inhibits IRP2 degradation while hypoxia conditions induce holo-IRP1 formation and stabilize IRP2.

How intracellular iron regulates iron uptake and storage via the IRE/IRP system is best exemplified by the regulation of TfR1 and ferritin [52]. For TfR1, IREs are located at the 3′ UTR mRNAs, while ferritin IRE is located at the 5′ UTR of the mRNAs encoding FtH and FtL chains. In iron-deficient cells, IRE/IRP interaction with the 3′ UTR stabilizes TfR1 mRNA [89]. However, 5′ UTR binding of IRPs to their IREs inhibits the early steps of the translation process [90]. As a result, TfR1 expression levels are increased and *de novo* ferritin synthesis is suppressed, leading to increased cellular iron uptake via TfR1 and decreased iron storage to counteract iron deficiency. In iron-abundant cells, the binding of IREs to IRPs is inhibited, resulting in TfR1 mRNA degradation and ferritin mRNA translation. This will limit iron uptake via TfR1 preventing iron excess while augmenting ferritin synthesis to safely store iron within the cell.

Besides their presence in TfR1 and ferritin, IREs have been identified also at the 5′ UTR of mRNAs encoding ALAS2 (iron utilization), an enzyme that catalyzes the first step of heme synthesis in the mitochondria of erythroblasts. Both IRPs when bound to ALAS2 IRE can inhibit ALAS2 mRNA translation [91]. IREs are also present at the 5′ UTR of mRNAs encoding ferroportin (export) [73], mitochondrial aconitase (a citrate cycle enzyme) [92] and hypoxia-inducible factor- (HIF-) 2α [93], and at the 3′ UTR of mRNAs encoding DMT1 (uptake) [94]. In contrast to TfR1, the mRNAs encoding TfR2 and mitochondrial ferritin do not possess IREs, and as such are not regulated through the IRE/IRP system.

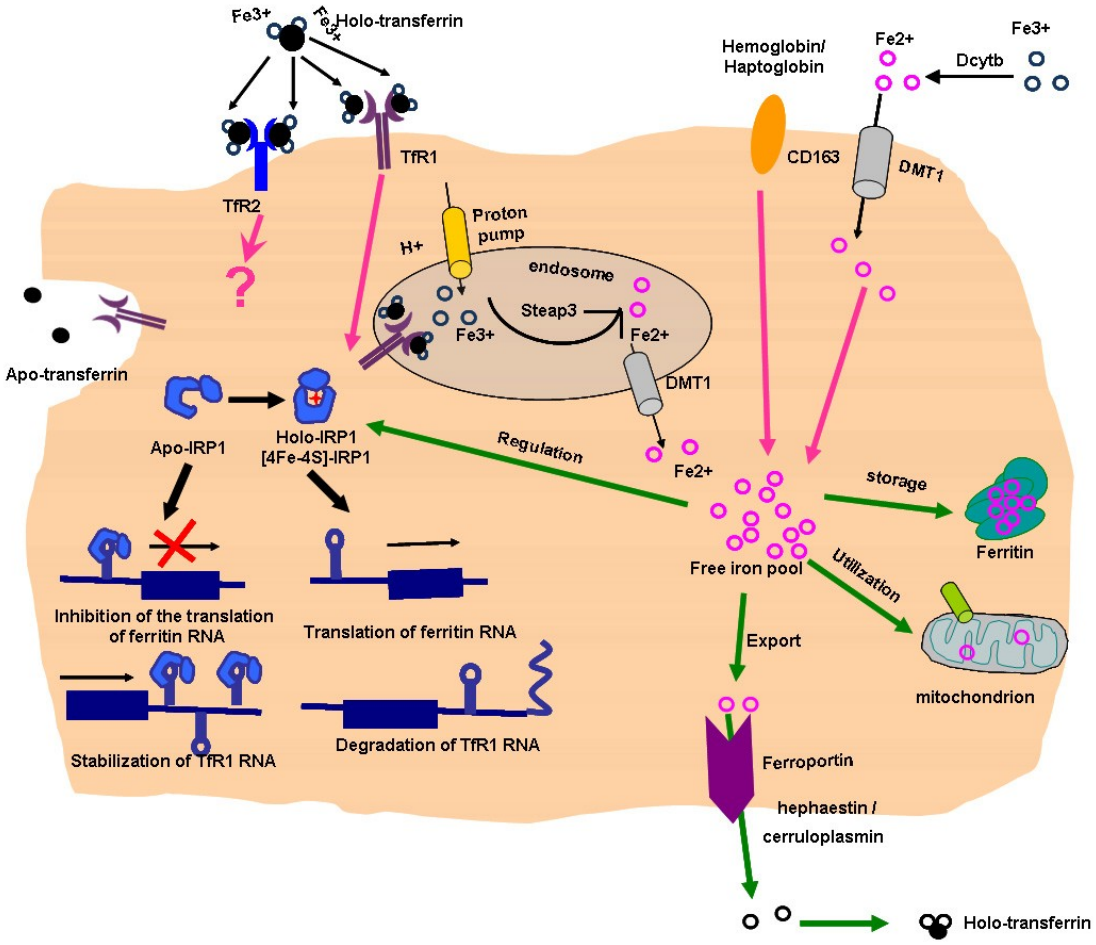

Figure 3 Depicition of iron import (top) and export (bottom) pathways in a generic mammalian cell.

Iron uptake, storage and export mechanisms and some regulatory elements are shown. TfR1 - transferrin receptor-1; TfR2 - transferrin receptor-2; DMT1 – divalent metal transporter 1; Dcytb - duodenal cytochrome b-like ferrireductase; CD163 - hemoglobin scavenger receptor; IRP-1 - iron regulatory protein-1. Steap-3 - six-transmembrane epithelial antigen of prostate 3. Modified from [95].

2.2 Systemic iron homeostasis

Because mammals have no regulated mechanism for the elimination of excess iron, the control of iron balance requires a fine adjustment in iron absorption by intestinal enterocytes and the recycling of iron by macrophages. Regulatory effectors that modulate intestinal iron absorption probably also regulate the release of iron from tissue macrophages and hepatocytes.

The levels of intestinal iron absorption are regulated by both systemic and local enterocyte iron status. Changes in enterocyte iron concentrations serve as a fine-tuning mechanism of intestinal iron absorption through the "mucosal block" that occurs several hours after consumption of a large dose of iron [96]. The decrease in intestinal iron absorption occurring following an oral dose of iron is associated with increased enterocyte iron levels, as assessed by IRP activity and ferritin immunoblotting. Reduced iron absorption is also accompanied by a rapid decrease in expression of the mRNAs encoding the brush border iron transport molecules Dcytb and DMT1 containing the IRE splice variant[97]. Thus, in the brush border, but not in the basolateral border, iron transport components are regulated locally by enterocyte iron levels, which support the hypothesis that systemic stimuli exert their primary effect on basolateral transport molecules.

More recently, Bruno Galy et al[98] found that IRPs inhibit the expression of both FPN and DMT1 in adult mice. However, intestine-specific IRP-deficient mice fail to absorb adequate amounts of iron in spite of the high expression levels of the iron transporters DMT1 and FPN. IRP ablation also results in iron retention in enterocytes. For over a half century, people have believed enterocytic ferritin can limit dietary iron intake [99, 100]. Thus, the mucosal block in IRP-deficient mice likely results from iron withholding by the large excess of mucosal ferritin. Bruno Galy et al further showed ferritin actually only traps a minor fraction of the iron transiting through enterocytes. Their results suggest a dual function of IRPs in the regulation

of iron absorption: IRPs limit apical and basolateral iron transport and also ensure sufficient passage of iron through the enterocyte by counteracting the diversion of the metal into ferritin.

Besides the IRP/IRE interaction, some iron sensing proteins are additionally involved in the regulation of iron absorption. HIF2α is shown to be involved in directly regulating the transcription of the gene encoding DMT1, the major intestinal iron transporter [101] and FPN, the only known iron exporter [98, 102]. Intestinal specific deletion of HIF2α results in the decrease of hepatic and serum iron levels [101]. HIF2α levels are dependent on prolyl hydroxylase domain (PHD) enzymes that require ferrous iron for their enzymatic activity [103]. Therefore, PHD enzymes serve as iron sensing proteins that regulate iron absorption.

Intestinal iron absorption is modulated by 4 major regulators: body iron stores, erythropoiesis, hypoxia and inflammation [104]. The regulation of these factors results in the variation of hepcidin levels, a small antimicrobial peptide secreted by hepatocytes [105, 106] which functions as an inhibitor of iron absorption [107]. The discovery of hepcidin and its central role in iron metabolism have clarified the pathological mechanisms of most common iron disorders. Mice deficient in hepcidin develop iron overload [108], while mice overexpressing it present profound iron deficiency [109]. A change in hepcidin levels subsequently targets the expression of the iron-exporter FPN [107] in duodenal absorptive cells and macrophages, and consequently the amount of iron entering the body and being recycled by macrophages.

3. Hepcidin

The discovery of the iron regulatory hormone hepcidin has provided a consistent model of iron homeostasis that allows for a better understanding of the importance of sensors at the cellular level for iron needs, as well as of the pathophysiology of hereditary iron metabolism disorders such as hemochromatosis and anemia of inflammation (or anemia of chronic disorders) [110].

3.1 The discovery of hepcidin

Hepcidin, also named LEAP-1, was initially identified as an antimicrobial peptide in human urine and serum by two independent groups [105, 111]. The link between hepcidin and iron metabolism was first discovered by Sophie Vaulont's group [108]. The group determined that USF2 (upstream stimulatory factor 2) knockout mice unexpectedly experienced severe iron overload with iron deposition in the liver and pancreas. The iron dysregulation observed in USF knockout mice was found to be related to the accidental knockout of a neighbor gene, identified as hepcidin, in the construct used for generating the USF2 knockout mice [108]. Further evidence of hepcidin involvement in iron metabolism was then demonstrated with the generation of transgenic mice overexpressing hepcidin under the control of a liver-specific promoter, leading to the development of severe iron deficiency anemia and premature death of transgenic mice [109].

Further studies showed that the hepcidin gene is evolutionarily conserved across vertebrate species from fish to humans [112]. The human genome contains only one copy of the hepcidin gene [112], whereas there are duplicate copies in the mouse (Hepc1 and Hepc2) genome [106,

108] and two or more copies in certain fishes [113, 114]. In mice, both Hepc1 and Hepc2 are homologous to human hepcidin, but only hepc1 plays a role in iron metabolism [115]. Hepc2 is not an inactive pseudogene since it is expressed in high levels and subjected to certain regulation. It shares some structural similarities with fish hepcidin-like peptides, which suggests Hepc2 serves a similar function as these fish hepcidin-like peptides, that is, antimicrobial activity [115].

The human hepcidin gene (*HAMP*) maps to chromosome 19q13.1. It comprises two introns and three exons encoding a precursor protein of 84 aa, called preprohepcidin. Preprohepcidin undergoes enzymatic cleavage during its export from the cytoplasm resulting in a 64 aa prohepcidin that is released into the endoplasmic reticulum [116]. Next, a 39 aa pro-region peptide is post-translationally removed by a furin-like proprotein convertase which results in the production of mature bioactive hepcidin-25 (25-aa form), identified both in blood and urine [105, 111]. In urine, two N-terminally truncated isoforms of 22-aa and 20-aa have also been detected. These two isoforms do not seem to have any identified iron-regulatory activity, suggesting that they are degradation products of the hepcidin-25 form [105].

Further studies using nuclear magnetic resonance spectroscopy (NMR) showed that hepcidin-25 it's a simple hairpin-shaped, cysteine-rich peptide with distorted β-sheets linked by four disulfide bridges. Three disulfide bridges stabilize the two anti-parallel strands while the fourth bridge is located in the vicinity of the hairpin loop which is more stressed and could have greater chemical reactivity [117] (**Figure 4**). Structure function analysis indicated that the iron regulatory activity requires the full-length peptide (hepcidin-25). The 20-aa, N-terminally truncated hepcidin has no iron-regulating bioactivity, indicating that the five N-terminal amino acids are crucial for this activity [118, 119].

Hepcidin structure closely resembles the cysteine-rich antimicrobial peptide defensins. It has a positively charged hydrophilic residue located on the concave side, and a hydrophobic residue distributed on the convex side, a typical structure of antimicrobial peptides known to disrupt bacterial membranes [120]. Several studies have shown that hepcidin indeed possesses antimicrobial properties [105, 116, 121, 122]. However, very high levels of hepcidin are required in order to achieve antimicrobial activity, about 100-fold higher than necessary for its iron regulatory activity[105].

Several studies suggest that the hepcidin gene is expressed in multiple tissues. The highest expression is predominantly in the liver of mammals and most fishes [105, 111-113, 123-126], and weaker expression can also be detected in the heart, lungs, spinal cord, stomach, intestine, pancreas, adipocytes, skeletal muscle, and testis [127-133]. Myeloid cells, including monocytes, macrophages and neutrophils, also express hepcidin [134-138]. Hepcidin expression is regulated differently in macrophages and in hepatocytes. Direct iron-loading does not induce hepcidin production in mouse macrophages [134], much as BMP6 treatment fails to, but LPS and IL-6 stimulation leads to hepcidin induction in these cells [139]. In addition, hepcidin can also be induced in macrophages by gram-positive and gram-negative bacteria wall constituents, through toll-like receptor (TLR) 2 and TLR4, which favors iron retention in macrophages and promotes host defense [135, 140]. Moreover, TLR-mediated hepcidin induction requires signaling through the MyD88-dependent pathway [140].

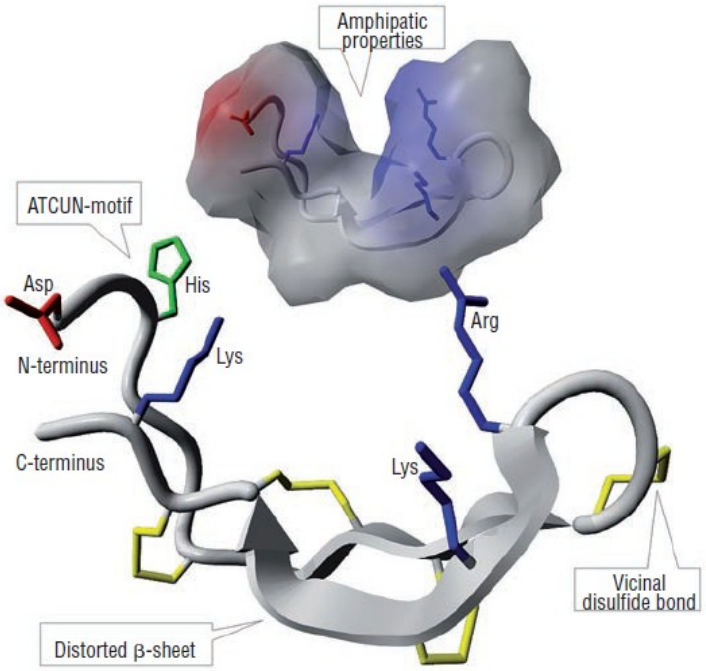

Figure 4 Hepcidin-25 structure

Front: overview of the structure of hepcidin-25. Distorted β-sheets are shown as grey arrows, and the peptide backbone is colored gray. The disulfide bonds are colored yellow, highlighting the position of an unusual vicinal bond between adjacent cysteines at the hairpin turn. Positive residues of arginine (Arg) and lysine (Lys) are pictured in blue, the negative residue of aspartic acid (Asp) in red, and the histidine-containing Cu^{2+}- Ni^{2+} (ATCUN)-binding motif in the N-terminal region is colored green. *Background*: hepcidin-25 molecule displayed with solvent-accessible surface that illustrates the amphipathic structure of the molecule. The molecule is colored gray, except for the side-chains of positive (blue) and negative (red) residues. Figure obtained from [141] (permission obtained from Journal Haematologica).

3.2 Regulation of hepcidin synthesis

Considering the close relationship between iron metabolism and hepcidin, the same factors that influence dietary iron absorption, namely iron stores, erythropoietic activity, hemoglobin, oxygen content and inflammation, are also capable of regulating the expression of hepcidin in hepatocytes. Recent advances in understanding how hepcidin is regulated at the molecular level have expanded our knowledge about the pathways regulating iron metabolism.

3.2.1 Iron-sensing pathway of hepcidin regulation

The regulation of hepcidin by body iron levels acts through a negative feedback mechanism. In both mice and men, hepcidin expression is induced by iron loading [106, 142] leading to inhibition of intestinal iron absorption and iron release from macrophages. Conversely, hepcidin expression is suppressed in iron deficiency [143], promoting intestinal iron absorption and iron release from macrophages into the circulation. Hepcidin is mainly expressed in the liver, and it is therefore most likely that iron-sensing takes place in this organ. However, direct iron treatment of both primary hepatocytes and hepatocytes cell lines leads to reduced hepcidin expression rather than activation [142], indicating that iron-sensing by hepatocytes *in vivo* involves the collaborative interaction between cell types, such as Kupffer cells. Studies in patients with an iron-overload disease, hereditary hemochromatosis (HH) and juvenile hemochromatosis (JH), revealed several proteins that are involved in hepcidin regulation by iron. HH and JH are a group of genetic disorders caused by mutations in the HFE, TfR2, hepcidin, and hemojuvelin (HJV) genes [144-149]. Despite iron accumulation in several organs in patients with HH and JH, hepcidin expression is inappropriately defective, clearly indicating that all molecules involved (HFE, TfR2, hepcidin and HJV) are crucial for hepcidin activation by iron.

3.2.2 Inflammatory pathway of hepcidin regulation

Hepcidin synthesis is also significantly increased during inflammation due to infection, autoimmune disease, and cancer. Excess hepcidin production in these settings inhibits dietary iron absorption and sequesters iron inside macrophages, resulting in low levels of circulating iron in the plasma, or hypoferremia. The finding that hepcidin-deficient mice do not develop hypoferremia upon inflammatory stimuli further demonstrates that hepcidin is a crucial player in the hypoferremic response accompanying inflammation [150]. Hypoferremia is often understood as a host defense response aimed at limiting iron availability to microorganisms. However, sustained hypoferremia over significant time may lead to the development of the anemia of chronic disease, or functional anemia [151].

Hepcidin induction by inflammatory stimuli is predominantly mediated by interleukin-6 (IL-6) [150, 152]. IL-6 administration in mice and humans rapidly (within hours) induces hepcidin excretion, and is concomitantly accompanied by a significant decrease in serum iron and transferrin saturation [150]. How does hypoferremia develop so rapidly? The plasma transferrin compartment contains approximately 2–4 mg of iron, which accounts for 1% of total body iron content. This transit compartment flows about 20 mg of iron each day, thus, the entire content of iron in plasma turns over every 3–4 hours[9]. Only 1 to 2 mg of iron are obtained from intestinal absorption, and most iron is recycled from senescent erythrocytes. If hepcidin could completely block iron recycling, in one hour plasma iron would drop by at least 25%, consistent with the degree of hypoferremia observed in humans infused with IL-6 [150]. *In vitro* studies further demonstrate that primary hepatocytes treated with IL-6 are also capable of increasing hepcidin synthesis, an induction that can be efficiently blocked by pre-treatment with anti-IL-6 antibodies [150].

IL-6 has been believed to be the only cytokine responsible for hepcidin stimulation in human

hepatocytes [152] and Hep3B cells [150]. However, later studies showed that IL-6-deficient mice still respond to LPS and produce hepcidin [153]. Therefore, other cytokines must also contribute to the regulation of hepcidin in hepatocytes. In murine hepatocytes, IL-1α and IL-1β are able to stimulate hepcidin transcription, while IFN-β shows an inhibitory effect [154]. Similarly, in other cell types, hepcidin is also regulated by cytokines. In human monocytes, IL-6 and IFN-α are capable of inducing hepcidin, while TNF-α can inhibit hepcidin induction by IL-6 [155]. A recent study found that hepcidin expression is induced by both IFN-γ and IL-6 in human airway epithelial cell lines [156]. The study also demonstrates that hepcidin induction by IFN-γ is independent of IL-6 and that it signals via the JAK/STAT1 cascade to activate hepcidin.

3.2.3 Regulation of hepcidin in response to erythropoietic demand

Erythropoiesis is the process by which RBCs are produced, by consuming most of the iron absorbed from the diet or recycled from hemoglobin by macrophages. Increased erythropoietic activity suppresses hepcidin expression promoting dietary iron absorption and mobilizing iron release from storage sites, resulting in more iron being available for erythropoiesis. Both hypoxia and anemia stimulate erythropoiesis through the production of erythropoietin (EPO). Accordingly, EPO administration in humans and mice suppresses hepcidin expression [157, 158]. Recent studies have shed some light on whether EPO acts directly or indirectly to suppress hepcidin expression. In mice that received full-body irradiation to block erythropoiesis, EPO-mediated suppression of hepcidin is prevented [159]. Similarly, when mice that were rendered anemic by bleeding or chemical hemolysis are pretreated with erythropoiesis inhibitors, such as carboplatin or doxorubicin, EPO-mediated hepcidin suppression is also abolished [159, 160]. These studies indicate that EPO acts indirectly, possibly through soluble mediators, to mediate hepcidin suppression and it requires erythropoiesis activity. More recent studies suggest that growth differentiation factor 15

(GDF15), and twisted gastrulation protein (TWSG1) both of which are released by erythroid precursors during accelerated erythropoiesis, are the most likely candidates mediating hepcidin suppression in response to erythropoietic demand [161, 162]. GDF15, a member of the transforming growth factor-β superfamily, was found at high levels in the serum of patients suffering from β-thalassemia and congenital dyserythropoietic anemia (CDA) [161, 163, 164]. Furthermore, human hepatocytes treated with serum from β-thalassemia patients showed a decreased hepcidin expression, which could be reversed by depletion of GDF15 [161]. Thus, GDF15 acts as an erythroid factor by down-regulating hepcidin expression in these patients. However, immunoprecipitation of GDF15 in the sera of β-thalassemia patients did not completely abolish hepcidin suppression in exposed hepatocytes, suggesting that GDF15 is likely not the sole mediator of hepcidin suppression. In fact, further studies identified yet another mediator, TWSG1, found to be elevated in a murine model of β-thalassemia major [162]. TWSG1 is a BMP-binding protein that can suppress hepcidin expression in human hepatocytes through a BMP-dependent mechanism. Further studies showed that this regulation involved the inhibition of BMP-dependent activation of the SMAD-mediated signal transduction pathway [162].

Both *in vivo* and *in vitro* studies have additionally showed that hepcidin expression is also suppressed by hypoxia [143, 158]. Hypoxia *per se* induces EPO production in the kidney and liver, and as such would contribute to increase erythropoiesis and consequently suppress hepcidin production. Hypoxia-inducible transcription factors (HIFs) are the central mediators of hypoxia-induced erythropoiesis. HIFs consist of an O2 and iron-sensitive α subunit (HIF-1α and HIF-2α,) and a constitutively expressed β subunit, HIF-β. Under normoxic conditions, HIF is subjected to degradation by the von-Hippel–Lindau (VHL) ubiquitin ligase, a tumor suppressor protein via an iron-dependent mechanism[165]. Under hypoxic conditions, HIFs are stabilized and function as transcriptional factors to adapt to oxygen change [166]. Hepatic deletion of HIF1α in mice leads to increased hepcidin expression even when mice are rendered

iron-deficient through an iron-depleted diet. Furthermore, mice with liver-specific deletion the VHL gene express extremely low levels of hepcidin [167]. Thus, the VHL/HIF axis is essential for oxygen sensing and hepcidin regulation. The study of Mastrogiannaki M et al. indicated that hepcidin suppression by hepatic HIF-2 required enhanced erythropoietic activity [168]. Similarly, Liu Q et al found that *Hamp1* suppression requires Epo-induced erythropoiesis [169]. The human hepcidin promoter contains several consensus-binding sites for HIF suggesting that hypoxia may also directly regulate hepcidin via HIF at the transcriptional level [167]. In addition, ROS are also involved in the hypoxic regulation of hepcidin. Last but not least, HepG2 cells incubated in hypoxic conditions markedly increased ROS levels and decreased hepcidin expression, and anti-oxidant treatments can reverse this inhibition. Further studies examined the role played by several transcriptional factors and found that C/EBPα and STAT3 dissociate from hepcidin under hypoxic conditions, an effect that also can be reversed by anti-oxidant treatment. Thus, ROS may suppress hepcidin expression via preventing C/EBPα and STAT3 binding to the hepcidin promoter during hypoxia [170] .

3.3 The hepcidin/ferroportin axis

Substantial progress has been made recently in elucidating the mechanism of action of hepcidin. Hepcidin acts by inhibiting the expression of the FPN protein both in enterocytes and macrophages [171, 172].

The concentration of FPN on the cell surface is controlled by both the rate of FPN synthesis and degradation among other factors. *In vitro* studies demonstrated that hepcidin mediates FPN internalization and degradation [86]. Hepcidin-mediated FPN internalization requires the activity of two of FPN lysines that may be targets of ubiquitination [173]. Internalized FPN is subjected to mono-ubiquitination and is targeted for degradation [86, 172]. The hepcidin-FPN

interaction forms an iron homeostasis loop: iron induces hepcidin production, which then regulates FPN concentration at the cell surface. When iron levels are high, hepcidin is produced and targets FPN for degradation on the basolateral membrane of absorptive enterocytes, thereby blocking the transfer of iron from enterocytes into the plasma. Within two days, short-lived absorptive enterocytes are shed into the intestinal lumen and the iron sequestered in these cells is consequently removed from the body [174]. Similarly, in macrophages, hepcidin-mediated FPN degradation results in iron sequestration within these cells.

Besides post-translational regulation, FPN expression is additionally regulated at the transcriptional level by iron and heme [75, 134, 175-177]. Iron, and some other transition metals including zinc, copper, manganese, cobalt, and cadmium, can directly induce FPN transcription via unknown mechanism(s) [178, 179]. It has been observed that animal products such as meat, fish and poultry enhance non-heme iron absorption in humans. A proposed mechanism involves the induction of FPN expression by heme at the transcriptional level via the transcription factor Bach1 [81]. Finally, and as already mentioned, FPN levels are also regulated post-transcriptionally via the IRE/IRP system [180, 181].

3.4 Molecular signaling pathways regulating hepcidin expression

Intensive research during the past 10 years has been conducted to identify the signaling pathways involved in hepcidin transcriptional regulation. At the present, two major signaling pathways have been identified: the BMP/SMAD iron-sensing pathway and the IL-6/STAT3 inflammatory pathway, depicted in Figure 7.

3.4.1 BMP/SMAD4 signaling pathway

BMPs are a group of secreted molecules that belong to the transforming growth factor-β

superfamily. The SMAD proteins are homologs of both the drosophila protein "mothers against decapentaplegic" (MAD) and the Caenorhabditis elegans protein SMA, with its name deriving from a combination of the two. I*n vivo* and *in vitro* studies demonstrated that several BMPs, such as BMP2, 4, 6, and 9 can activate *HAMP* transcription [182, 183]. Further studies in liver-specific *Smad4*-knockout mice revealed that lack of *Smad4* in hepatocytes results in a 100-fold lower hepcidin transcriptional expression than wild-type mice accompanied by massive hepatic iron overload [184]. Furthermore, knockdown of *Smad4* in mouse primary hepatocytes also ablated the hepcidin induction by BMP4 [184]. These results establish that the BMP/SMAD4 pathway is important for hepcidin regulation through the iron-sensing pathway. The BMP/SMAD4 iron-sensing pathway can be described as follows: iron induces BMP6 expression, which then binds to cell-surface BMP receptor (BMPR) complexes that include type I and II (BMPRI and BMPRII) serine/threonine kinase receptors. Upon ligand binding, BMPRII phosphorylates BMPRI, leading to further phosphorylation of SMAD1/5/8 in the cytosol. Phosphorylated SMDS1/5/8 then form a complex with the common mediator SMAD4, translocate into the nucleus and bind to BMP-responsive elements (BMPRE) located in the hepcidin promoter and activate hepcidin transcription.

Several elements participating in the iron-sensing pathway mediated by SMAD4 signaling have been identified, including BMP6, HJV, TfR2, HFE and TMPRSS6.

BMP6*:* BMP6 has emerged to be the essential molecule sensing iron for the BMP/SMAD4 pathway. *In vitro* studies showed that BMP2, 4, 6, and 9 can all induce hepcidin production [183, 185], however only BMP6 transcription is actually regulated by dietary iron *in vivo* [186]. Accordingly, mice with targeted disruption of BMP6 have reduced phosphorylated Smad1/5/8 levels, develop massive iron overload and show undetectable levels of hepcidin mRNA in the liver [187, 188]. Finally, administration of exogenous BMP6 directly into mice has been shown to induce hepcidin [187]. Collectively, these studies demonstrate that BMP6 is essential for hepcidin induction and is a key player in iron sensing.

HJV: HJV is a GPI-linked cell-surface protein also known as repulsive guidance molecule C (RGMC). HJV interacts with BMP6, BMP4, and BMP2 and functions as a BMP co-receptor [189]. Thus, HJV regulates hepcidin expression by enhancing BMP/SMAD4 signaling [190]. Mutations in the HJV gene cause juvenile hemochromatosis (JH) in humans and mice, characterized by severe iron overload in the liver, pancreas and heart [149, 191]. There are two forms of HJV with opposing effects: a membrane-bound form (m-HJV) that can induce hepcidin expression and a soluble form (s-HJV) that suppresses hepcidin expression. s-HJV is the product of cleavage at the C-terminus of m-HJV by a proprotein convertase, furin [192]. Neogenin, a receptor membrane protein, is then required for the release of the s-HJV [193]. Neogenin binds to HJV and this interaction is crucial for BMP-dependent signaling of hepcidin expression [194]. In addition, s-HJV can also bind to BMPs, thereby preventing the interaction between m-HJV and BMPs [190] and functioning as a competitive antagonist of m-HJV. Importantly, s-HJV is increased during iron deficiency and hypoxia, possibly due to furin activation in these conditions [195]. Conversely, increased iron concentrations reduced s-HJV expression in cells overexpressing HJV[195].

TfR2: TfR2 is a homolog of TfR1 mainly expressed in hepatocytes [33]. Similarly to TfR1, TfR2 also binds to iron-loaded transferrin, but with much weaker affinity [33, 34]. Homozygous mutations in the TfR2 gene cause type 3 HH in humans. Accordingly, mouse models with mutated or knockdown of TfR2 present a phenotype similar to human HH, associated with reduced hepcidin expression [146, 196]. Unlike TfR1, TfR2 transcription is up regulated by increased serum iron possibly due to the stabilization of the TfR2 protein upon binding to diferric transferrin [197]. TfR2-mediated hepcidin regulation occurs through its interaction with HFE [198].

HFE: Mutations in the HFE gene, encoding a β2-microglobulin-dependent MHC class I-like molecule, are associated with type I HH [199, 200]. HFE competes with transferrin for

binding to TfR1 and consequently inhibits cellular iron uptake [201, 202]. In addition, HFE is also able to bind to TfR2. The presence of iron-loaded transferrin displaces HFE from TFR1, and allows HFE to rather bind to TfR2 and form the HFE/TfR2 complex. The HFE/TfR2 complex subsequently induces SMAD1/5/8 phosphorylation expression via the Erk (extracellular signal-regulated kinase) activation pathway, and hence induces hepcidin expression [203]. Thus, it has been proposed that BMP6, BMPR, HFE, TfR2 and HJV form a super complex at the hepatocyte surface that senses body iron levels [204] and activates hepcidin expression through a SMAD4-dependent pathway.

TMPRSS6*:* TMPRSS6 (transmembrane protease, serine 6**),** also called matriptase-2, is a transmembrane serine protease mainly expressed in the liver [205] that is required for sensing iron deficiency. Once activated during iron deficiency, it cleaves m-HJV into small fragments [206], resulting in the suppression of BMP signaling and hence hepcidin expression. Alternatively, TMPRSS6 is involved in an yet to be identified transmembrane signaling pathway activated by iron deficiency, which would then bind to inhibitory elements located in the *HAMP* promoter region [207]. The mechanisms through which TMPRSS6 senses iron deficiency are not fully elucidated, however mutations in this gene lead to iron-refractory iron deficiency anemia (IRIDA) in humans and mice [207-209].

3.4.2 IL-6/STAT3 signaling pathway

As mentioned previously, IL-6 is a crucial cytokine that induces hepcidin during inflammation. Further studies have demonstrated that this induction is mediated by STAT3 [210-212]. One study showed that IL-6 directly induces hepcidin and activates STAT3 in mouse hepatocytes, as assessed by measuring phospho-STAT3 expression. Furthermore, in mice with disrupted gp130, the signal transducing unit in the IL-6/IL-6 receptor (gp80), both hepcidin induction and STAT3 phosphorylation are blunted. This blunted response suggests that STAT3 is the

key transcription factor responsible for IL-6-mediated activation of hepcidin gene expression in the liver [212]. Additional studies identified a STAT3 binding motif located at position –64/–72 on the hepcidin promoter, which is required for IL-6-mediated hepcidin activation and even for basal hepcidin mRNA expression [210, 211]. These studies identified STAT3 as necessary and sufficient for IL-6 responsiveness of the hepcidin promoter. So far, the IL-6/STAT3 signaling pathway has been described as follows: upon stimulation with inflammatory molecules such as LPS [150] and other TLR ligands [213], IL-6 is induced in macrophages. IL-6 then binds to its membrane-bound receptor (gp80) on hepatocytes and interacts with gp130. Subsequently, the formation of gp130-containing complexes activates Janus kinases (JAKs), which in turn, phosphorylate tyrosine residues in the cytoplasmic domain of gp130. These phosphotyrosines serve as docking sites for STAT3. Recruited STAT3 then becomes phosphorylated on a single tyrosine residue (Y705) and phosphorylated STAT3 forms a dimer that translocates into the nucleus, binds to the STAT-responsive element on the hepcidin promoter and activates hepcidin transcription [210-212].

3.4.3 Crosstalk between signaling pathways

The BMP/SMAD and IL-6/STAT3 signaling pathways are synergistic through a mechanism(s) that remains to be elucidated. Several studies suggest that there is a crosstalk between both pathways. For example, *in vitro* studies using Hep3B cells showed that dorsomorphin, a BMP receptor inhibitor, could also inhibit hepcidin induction by IL-6 [214]. Even more suggestive of such a crosstalk, is the finding that the abolishment of the BMP pathway, as seen in liver-specific $Smad4^{-/-}$ mice, leads to blunted responses to IL-6-mediated hepcidin transcription [184].

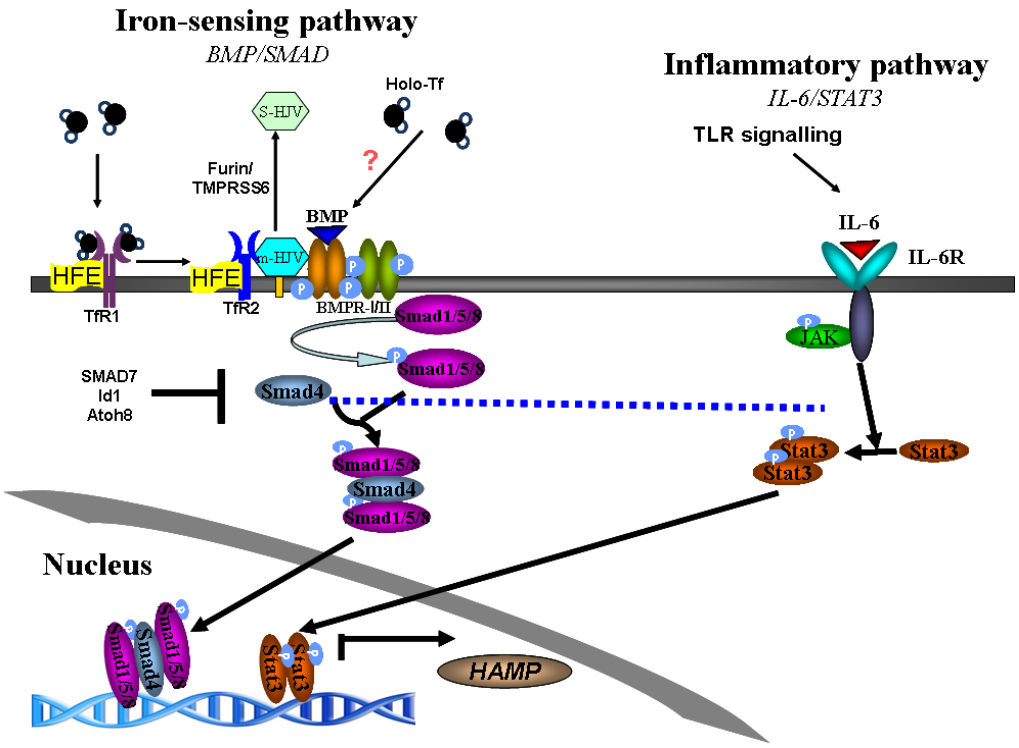

Figure 5 Regulatory signaling pathways in hepcidin expression

In the iron-sensing BMP/SMAD pathway, the binding of BMPs to the BMP receptor induces receptor-regulated Smads (Smad1/5/8). Following phosphorylation, Smad1/5/8 heterodimerize with Smad4 and co-migrate to the nucleus where it binds to the BMP-response elements (BMP-RE) in the hepcidin promoter. Smad1/5/8 can be inhibited by inhibitory Smads (Smad6 and Smad7), Id1 and Atoh8. The iron sensor complex includes HFE, TfR2 and HJV. HFE is regulated by transferrin-bound iron (Holo-Tf). Holo-Tf regulates BMP signaling through unknown mechanisms. There are two forms of HJV: membrane-bound and soluble (m-HJV and s-HJV). s-HJV is obtained following furin cleavage or proteolytic cleavage by the membrane-bound TMPRSS6.

4. Interactions between immune system and iron metabolism

Defense against microbes is mediated by the early reactions of innate immunity and the later responses of adaptive immunity. Innate immunity, also known as non-specific immunity, is the first line of defense, which protects the host from infection by other organisms in a non-specific and generic manner. Adaptive immunity, also known as acquired immunity, is composed of antibody responses and cell-mediated responses, which eliminate or prevent pathogenic growth. It has long been observed that in response to various inflammatory stimuli, iron metabolism changes dramatically [215, 216]. Since iron is an essential nutrient for nearly all infectious microorganisms as well as for their hosts [217], iron metabolism is targeted as a host defense mechanism to prevent the growth of microbial pathogens. Iron metabolism changes have been implicated in both innate immunity and adaptive immunity. Once the host encounters a pathogen, iron is rapidly sequestered within macrophages and bound to specialized extracellular proteins via different mechanisms. This sequestration in turn restricts iron availability to certain pathogens at the systemic and cellular levels and thus contributes to host defense by delaying pathogen growth [217]. In the next section, several examples of proteins involved in iron metabolism having a role in innate immunity are presented.

Lactoferrin

Lactoferrin is a transferrin family glycoprotein with a high affinity for ferric iron. Synthesized by epithelial cells, lactoferrin is found at high levels in mucosal secretions, including tears, saliva, bile, nasal secretions and breast milk. It is also present in neutrophil granules and is strongly induced at the sites of infection and inflammation [218, 219].

Lactoferrin functions as a potent iron-chelator having the ability to retain iron over a wide range of pH. Unlike transferrin, iron bound to lactoferrin cannot be released at the acidic pH

[220] prevalent in infected or hypoxic areas. In addition, lactoferrin exhibits a direct effect on pathogens, and is thus considered a critical component of innate defense. In fact, both *in vivo* and *in vitro* studies show that exogenous lactoferrin effectively inhibits bacterial growth [217]. The bacteriostatic effect of lactoferrin is due to its ability to sequester iron and as such, deprive pathogens of iron absolutely necessary for their growth. Furthermore, lactoferrin has been shown to induce the production of proinflammatory cytokines *per se*, and to promote phagocytosis and cell adherence [221, 222]. Despite the strong correlation between exogenous lactoferrin and inhibition of bacterial growth, lactoferrin knockout mice do not exhibit major abnormalities in the immune system, and do not display enhanced susceptibility to pathogens such as *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis* when compared to wild-type animals [217, 223]. These studies suggest that the bacteriostatic effect of lactoferrin may be redundant, at least in these mouse models.

Nramp1

Nramp1 (Natural resistance-associated macrophage protein-1), is a divalent metal-proton co-transporter located on the membrane of macrophage and neutrophil phagosomes [224]. Nramp1 expression is transcriptionally induced by interferon-γ (IFN-γ) and lipopolysaccharide (LPS) via a mechanism involving interferon regulatory factor 8 (IRF-8) [225]. Nramp1 acts by pumping Fe^{2+}, Co^{2+} and Mn^{2+} cations out of the phagosomal compartment into the cytosol, resulting in the depletion of these nutrients, which could otherwise be exploited by intracelluar pathogens. Macrophages represent a choice site for the establishment of intracellular phagosomal pathogens such as *Salmonella, Mycobacteria, Leishmania* and *Chlamydia.* Therefore, an alteration in intra-macrophage iron content and distribution by Nramp1 results in the inhibition of the growth of pathogens that live within the phagosome [224, 226]. Furthermore, Nramp1-mediated changes in iron distribution may suppress the expression of the inhibitory cytokine IL-10 and induce iNOS production by macrophages, thereby restricting

microbial growth [227-229]. These mechanisms suggest that functional Nramp1 is required to maintain the resistance to intracellular phagosomal pathogen infection.

Lipocalin 2

Lipocalin 2 has several other names such as siderocalin, neutrophil gelatinase associated lipocalin (NGAL) or, in mice, 24p3. Structural studies indicated that lipocalin 2 can bind to catecholate-type and salicylate-derived siderophores, such as enterobactin secreted by *E. coli* and mycobactin secreted by *M. tuberculosis*, respectively [230, 231]. Siderophores are small, high affinity iron chelators secreted by microbes to acquire iron from their environment. Siderophores can cross the outer membrane of the cell through receptor reorganization [232, 233]. Lipocalin represents a host defense strategy to prevent siderophore-mediated iron utilization by microbes. Lipocalin 2 is secreted by neutrophils, epithelial cells, macrophages and other cell types in response to inflammation or infection [228, 234, 235]. It acts as an acute-phase protein and sequesters iron to subvert pathogen iron acquisition by certain types of siderophores. Lipocalin 2 binds iron as ferric siderophore complexes to limit the growth and virulence of microbial pathogens. *In vitro* studies have shown that culture media containing recombinant lipocalin 2 could inhibit the growth of both *E. coli* and *M. tuberculosis* [231, 234, 236] . Furthermore, lipocalin 2 knockout mice also display increased tissue bacteria numbers and mortality in response to *E. coli* and *M. tuberculosis* infections [234, 237]. Lipocalin 2 exerts selective bacteriostatic pressure on pathogenic bacteria, since several pathogenic bacteria have developed lipocalin 2 resistance mechanisms to maintain a parasitic relationship with the host. These mechanisms include producing multiple and/or modified siderophores to escape the inhibitory activity of lipocalin 2 [230, 238, 239]. Lipocalin 2 can also bind to endogenous ligands including urinary catechol and its relative 2,5-dihydroxybenzoic acid, which indicates lipocalin 2 may sequester host systemic free iron within these complexes to limit its availability to pathogens [62, 240]. Beside its role in innate immunity, lipocalin 2 has

been shown to also be involved in a range of other biological processes, such as neoplasia, kidney development and repair, implantation and involution of the uterus and mammary gland, possibly through iron-delivery to certain types of cells[241-245]. However, studies in lipocalin 2-deficient mice showed no obvious defects in these biological processes, indicating that lipocalin 2 function in these biological processes may be redundant [234].

Compared to the fast response of innate immunity, it takes more time (days to weeks) to develop adaptive immunity. Adaptive immunity involves the proliferation and differentiation of antigen-specific B and T lymphocytes that are required for the antibody- and cell-mediated responses, respectively. The implication of iron in adaptive immunity is mainly due to its proliferative effect on immune cells and its interference with cell-mediated immune effector pathways and cytokine activities. The development of lymphocytes depends on their ability to obtain iron via TfR1, also known to immunologists as the differentiation marker CD71. Studies indicate that iron deficiency may alter the cytokine expression profile of activated lymphocytes, resulting in higher expression of IFN-γ and lower expression of IL-4 [246]. An interesting observation that remains to be fully understood is that the number of circulating lymphocytes, especially CD8+ T cells, is decreased in patients with *HFE*-associated HH [247, 248]. Finally, Hfe-deficiency in mice has been associated with altered cytokine production [249, 250].

5. Disorders of iron metabolism

Since iron is bioactive and excess free iron is toxic in the body, conditions resulting in excessive iron accumulation and overload in body tissues may cause serious damage. Iron overload may result from inherited (primary) and acquired (secondary) causes. Inherited iron overload is caused by genetic disorders of iron metabolism that lead to excessive absorption of dietary iron and/or impaired iron transport within the body, such as HH [251]. Acquired iron overload is often related to the conditions that bypass normal iron absorption and metabolism pathways, such as repeated blood transfusions or high doses of supplemental iron, and may be aggravated by increased dietary iron absorption in response to inefficient erythropoiesis, as seen in β-thalassemia major[252, 253] . A list of causes of iron overload disorders is presented in Table 1.

Table 1 Causes of iron overload disorders

Category	*Condition*
Primary iron overload	Hereditary disorders of iron metabolism • Hemochromatosis • Hypotransferrinemia/atransferrinemia • Aceruloplasminemia
Secondary iron overload	Prolonged ingestion of iron-containing supplements Excess parenteral iron supplements Chronic transfusion therapy • β-thalassemia major • sickle cell anemia • myelodysplastic syndromes • Diamond-Blackfan anemia • marrow replacement • maintenance chemotherapy • myeloablative hematopoietic stem cell transplantation • hemolytic anemias • RBC membrane defects • RBC enzyme abnormalities • hemoglobin molecule defects

Adapted from [254]

5.1 Hereditary hemochromatosis

The most common form of inherited iron overload is HH [251, 255]. HH is an autosomal recessive disorder characterized by excessive intestinal absorption of dietary iron leading to progressive and pathological increases in total body iron stores. Excess iron accumulates in parenchymal cells of vital organs, primarily in the liver, leading to organ damage. In the early stage of the disease, the signs and symptoms of iron overload are nonspecific. They may include a wide range of common symptoms such as chronic fatigue, joint pain, impotence, osteoporosis, non-specific abdominal problems, and cardiac problems [256, 257]. In the advanced stages of the disease, symptoms are more typical, and may include diabetes, bronzing of the skin, hepatomegaly, and arthropathy, especially of the second and third meta-carpophalangeal joints.

Classical HH is caused by mutations in the *HFE* gene [258]. Almost 10% of the Caucasian population carries the most prevalent C282Y *HFE* mutation, however disease penetrance is much lower: 2 to 38% among men and 1 to 10% among women [259]. The C282Y mutation in the *HFE* gene appears to have originated in central Europe [260]. Migration of Europeans to USA, Canada, South Africa, and Australia accounts for the high prevalence of HH in these countries [261]. The heterozygosity of the H63D mutation combined with heterozygosity of the C282Y mutation in the *HFE* gene may also cause iron overload [257]. Unlike the low disease penetrance of *HFE* gene mutation, loss of functions of *HJV*, hepcidin, *TfR2*, and *FPN* result in hemochromatosis. These forms of hemochromatosis are defined by numbers (type 2, type 3 and type 4). Type 2 HH, also called juvenile hemochromatosis (JH), results from mutations in genes encoding *HJV* or hepcidin (in rare cases), and is characterized by the early onset of clinical complications [149, 262]. Type 3 HH is caused by *TFR2* mutations and shows a more severe form of HH with an earlier presentation [196, 263]. Combined mutations of *HFE* and *TFR2* also present JH phenotypes [264]. Type 4 HH, also called ‘ferroportin disease’,

results from mutations of the ferroportin gene [265, 266]. It has distinct genetic, biochemical, clinical and histological features from hemochromatosis [267].

In mice, deletion of the iron hormone hepcidin and any of genes that regulate its biology, including *Hjv*[145, 148], *Bmp6* [187, 188], *Hfe* [144], *TfR2*[146] , *Smad4*[184] , *neogenin* [268], *C/EBP alpha* [269], and *Fpn* all lead to reduced hepcidin synthesis (except *Fpn*) and systemic iron overload that resembles human hemochromatosis.

The most common treatment of HH is phlebotomy. Iron removal by regular phlebotomies can reverse fibrosis in the liver and cardiomyopathy, as well as improve symptoms such as fatigue and skin pigmentation [256, 270, 271]. The major side effects of phlebotomy treatments are problems with venous access and the time consuming nature of the treatment [272]. An alternative therapy of phlebotomy is therapeutic erythrocytapheresis, which is the removal of erythrocytes only instead of whole blood removal. Compared to phlebotomy, it reduces the bleeding volume, increases iron removal per session and reduces side effects [273]. Iron chelation is another routine therapy. However, it is more expensive and has more severe adverse side effects. It is mainly used when phlebotomies cannot be performed, either because venous access cannot be obtained, and in patients with heart failure or iron-loading anemia. Alcohol consumption should be limited since alcohol has toxic effects on the liver and may further suppress hepcidin expression [270, 274].

5.2 β-thalassemia

β-thalassemia syndromes are a group of hereditary blood disorders characterized by defects in hemoglobin β-chain synthesis, resulting in reduced hemoglobin in RBC, decreased RBC production and anemia [275]. β-thalassemia is one of the most common autosomal recessive

disorders in humans; it affects multiple organs and is associated with considerable morbidity and mortality. About 1.5% of the world's population may be carriers of β-thalassemia and about 60 000 infants are born every year with the severe form of the disease [276]. High prevalence is present in populations in the Mediterranean area, the Middle East, the Indian subcontinent, the Far East and in tropical Africa. Intensive epidemiological and case-controlled studies strongly suggest that the high gene frequency of β-thalassemia in these regions is most likely related to the selective pressure from *Plasmodium falciparum* malaria, since β-thalassemia carriers have a survival advantage against the invasion of *Plasmodium falciparum* [277]. Because of population migration, β-thalassemia is also present in populations in Northern Europe, North and South America, the Caribbean, and Australia.

In adult humans, about 97% of hemoglobins contain 2α- and 2β-globin chains (α2, β2), combined with the heme moiety, which has the ability to bind oxygen [278]. At each stage of development, the production of α- and β- globins is balanced. In β-thalassemia, mutations in the β-globin gene abolish or reduce the synthesis of β-globin chains, which result in imbalanced production of α- and β- globins. Relative excess α-globin chains accumulate in erythroid precursors, resulting in their premature death and in ineffective erythropoiesis [279]. At the molecular level, β-thalassemia-related mutations are heterogeneous. So far, more than 200 different mutations have been identified in the disease. The majority of mutations are point mutations or small insertions or deletions of a few bases [280, 281]. Rarely, β-thalassemia can result from gross gene deletion involving part of or the whole β-globin gene. Point mutations influence β-globin expression at three different levels: gene transcription, mRNA processing and mRNA translation.

The severity of β-thalassemia depends partly on the extent of the reduction of β-chain output. Clinical expression can thus be severe, mild or silent. Nevertheless, individuals with identical mutations in β-globin genes may present very different phenotypes, ranging from mild to

severe. The clinical phenotype of β-thalassemia includes thalassemia major, thalassemia intermedia, thalassemia minor, dominant β-thalassemia and β-thalassemia associated with other hemoglobin anomalies [282]. Thalassemia major develops in homozygotes or compound heterozygous mutation carriers.

Anemia in patients with β-thalassemia requires periodic blood transfusions, leading to excessive iron accumulation in the body (secondary hemochromatosis). Therefore, monitoring iron status is crucial in thalassemia patients, since it helps evaluate the clinical relevance of iron overload, the need for treatment, and the timing and monitoring of chelation therapy. Iron-chelators bind to excess iron in the body, which allows iron exit through urine and stool. In general, after 10-12 transfusions, or when ferritin levels rise above 1000 ng/ml, patients begin iron chelation therapy [283]. The first drug available for chelation therapy was *deferoxamine* (DFO). The most common side effects of DFO are local reactions at the site of infusion, such as pain, swelling, induration, erythema, burning, pruritus, wheals and rash. However, treatment with DFO significantly reduces the morbidity and mortality of β-thalassemia. Two other chelators *deferiprone* (DFP) and *deferasirox* (DFX) are also available for clinical use. DFP is a bidentated oral chelator. DFP combined with DFO could achieve levels of iron excretion that cannot be achieved by either drug alone without increasing toxicity [284, 285]. The side effects include neutropenia, agranulocytosis, arthropathy, and gastrointestinal symptoms which demand close monitoring [282].

Bone marrow transplantation (BMT) from an HLA-identical sibling is the definitive cure currently available for patients with β-thalassemia. The outcome of BMT is related to pretransplantation clinical conditions, specifically the presence of hepatomegaly, extent of liver fibrosis, history of regular chelation and hence the magnitude of iron accumulation. A lower survival rate is correlated with the presence of these risk factors. About 5–8% of patients develop chronic graft-versus-host disease (GVHD) with variable severity. The major

issue of allogenic BMT is the lack of an HLA-identical sibling donor for the majority of affected patients. Cord blood transplantation from a related donor offers a good alternative and is associated with a low risk of GVHD.

5.3 Anemia of chronic diseases

The anemia of chronic disease (ACD), also termed anemia of inflammation, is the most prevalent anemia in hospitalized patients worldwide and the second most prevalent worldwide anemia after iron deficiency [286]. ACD often develops in the setting of chronic disease, infections or malignancy. The hallmark of ACD is that body iron stores can be normal or even high, but the iron available for erythropoiesis is very much restricted [286-288]. Anemia develops due to the impaired mobilization of iron from diet and stores rather than inadequate iron intake. ACD is classically mild-to-moderate anemia (Hgb 70–120 g/L) and the symptoms may include: weakness and fatigue, headaches, paleness and shortness of breath. The development of anemia is associated with detrimental effects especially in relation to cardiac function, quality of life, growth and mental development [289]. Strong evidence has suggested that ACD has a negative impact on the quality of the life and that ACD can independently worsen morbidity and mortality of many disorders [290, 291]. However, hypoferremia and iron restriction in the circulation may also have some potentially positive effects, especially for patients with cancer and infectious diseases [292]. Three major pathophysiological mechanisms, all related to immune system activation, have been identified so far as underlying ACD.

One of the mechanisms involves decreased erythrocyte lifespan. Early studies suggest that transfused ACD erythrocytes have a normal lifespan in normal recipients but transfused normal erythrocytes have a decreased lifespan in ACD recipients [293]. The decreased lifespan is related to the activation of macrophages, which prematurely removes aging

erythrocytes from the blood stream. In addition, the increased production of cytokines, such as IFN-γ, IFN-α, TNF-α and IL-1, can inhibit the proliferation and differentiation of erythroid progenitor cells [294, 295]. Several other factors, such as the cytokine-mediated down-regulation of EPO-receptor expression on progenitor cells and direct toxic effects of cytokine-inducible radicals, also contributes to the inhibition of erythroid progenitor cell proliferation [296]. Finally, acute-phase proteins, such as α1-antitrypsin and α2-macroglobulin, also can play a role by suppressing the growth and differentiation of erythroid progenitor cells via inhibiting iron uptake [297, 298].

A second mechanism is linked to suboptimal EPO activity resulting from diminished EPO production and blunted response to EPO. Often, EPO levels in ACD patients are well below the expected range based on the degree of anemia [288, 299]. Inflammatory cytokines such as IL-1 and TNF-α have been shown to inhibit EPO gene expression *in vitro* [300], mainly through increased binding activity of inflammatory cytokines to the transcription factor GATA and resulting inhibition of EPO gene promoter activity [301]. In addition, IL-1 and TNF-α also induce toxic radicals that damage EPO-producing cells [300].

The third and most important mechanism involves iron-restricted erythropoiesis. Under inflammatory conditions, pro-inflammatory cytokines, such as TNF-α can increase iron levels in monocytes/macrophages through transcriptional induction of ferritin expression [302, 303]. The mechanism of increased iron accumulation by RES is additionally related to the stimulation of erythrophagocytosis by macrophages. Studies have shown that TNF-α leads to shortening of erythrocyte half-life and faster clearance of these cells via erythrophagocytosis [304]. Another cytokine, IFN-γ, also affects iron storage in monocytes/macrophages as it modulates ferritin transcription and translation via nitric oxide (NO) formation. NO induction by IFN-γ depends on the iron status of cells, resulting in either stabilization or degradation of ferritin via IRE and IRP2 activity modulation [305-310]. Cytokines also can directly

down-regulate ferritin transcription, leading to blockage of iron export from macrophages. Last but not least, hepcidin released by monocytes/macrophages during inflammation targets ferroportin in an autocrine fashion, further promoting iron retention in these cells [138, 311, 312]. Summarizing, cytokines promote iron accumulation and retention within macrophages via stimulation of various iron acquisition pathways and hepcidin induction. While iron retention in macrophages results in limited iron availability to pathogens, thus functioning as a defense mechanism of the immune system against invading pathogens, this innate immune response also results in limited iron availability to the fundamental process of erythropoiesis, and in the development of ACD.

The severity of anemia is related to the underlying disease process. Thus, initial treatment should be concentrated on the causative disease. This is often sufficient if the symptom is mild or asymptomatic. For patients with severe anemia or with diseases which can't be reversed, such as chronic kidney disease or patients with cancer being treated by chemotherapy, alternative strategies, such as transfusion, erythropoiesis-stimulating agents (ESAs), iron supplementation and blocking the inflammatory responses, are often necessary.

6. Rational and objectives of study

The first objective of this thesis is linked to the observation that, in HH, excessive non-transferrin-bound iron (NTBI) is cleared from the circulation and deposited into hepatocytes. At the molecular level, iron-transporter molecules that may be responsible for iron uptake into hepatocytes in these conditions remain elusive. Lcn2, an acute phase protein [234, 243, 313, 314], is known to be involved in iron trafficking via siderophores [62, 240]. Once Lcn2 is bound to iron-laden siderophores, it serves as an iron donor; conversely, iron-free Lcn2-siderophores function as iron chelators [315, 316]. Thus, Lcn2 has emerged as a candidate iron-transporter, which may be responsible for NTBI accumulation in the liver of HH patients. Hence, the first objective of this study is to evaluate the importance of Lcn2 in the pathogenesis of hepatic iron loading in Hfe knockout mice. To this end, HfeLcn2 double-knockout mice will be generated and phenotyped in regards to iron metabolism (**Chapter 2**).

As mentioned before, hepcidin expression is induced by iron-loading and inflammation while, conversely, being inhibited by anemia and hypoxia. Under certain pathologic situations, hepcidin is regulated by more than one regulator that modulates hepcidin expression in opposing directions. For example, in ACD, hepcidin is co-regulated by anemia (inhibitory effect) and inflammation (stimulatory effect). In iron-loading anemias, disorders characterized by increased inefficient erythropoiesis, such as β-thalassemia, hepcidin is regulated by both anemia (inhibitory effect) and iron-loading (stimulatory effect). Despite intensive research into hepcidin regulation over the last decade, hepcidin regulation by opposing stimuli remains poorly understood. The second objective of my thesis is therefore to investigate how different factors influence hepcidin expression *in vivo*. To this end, hepcidin regulation in the presence of opposing stimuli as well as the contributions of mediators and downstream signaling pathways of hepcidin expression will be examined *in vivo* in mouse models (**Chapter 3**).

Chapter 2 Is the iron-donor lipocalin 2 implicated in the pathophysiology of hereditary hemochromatosis?

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Keywords: NGAl, 24p3, Lcn2, Hfe, hereditary hemochromatosis

List of Abbreviations

DMT1 – divalent metal ion transporter 1; HH – hereditary hemochromatosis; Lcn2 – Lipocalin 2; LVDCCs – L-type voltage-dependent Ca2+ channels; NGAL – neutrophil gelatinase-associated lipocalin;. NTBI-non-transferrin-bound iron

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Abstract

Under normal conditions, iron is taken up by the cells through the transferrin-mediated pathway. However, in hereditary hemochromatosis, a common iron-overloading disorder associated with mutations in the *HFE* gene, iron in plasma exceeds transferrin-binding capacity, and non-transferrin-bound iron appears in the circulation of patients with iron overload. This non-transferrin-bound iron can be taken up by hepatocytes through a transferrin-independent pathway. Lipocalin 2 (Lcn2), a secreted protein of the lipocalin family, has emerged as the mediator of an alternative, transferrin-independent pathway for cellular iron delivery. To evaluate the importance of Lcn2 in the pathogenesis of hepatic iron loading in *Hfe* knockout mice, we generated *HfeLcn2* double-deficient mice. Our studies revealed that deletion of Lcn2 in *Hfe*-knockout mice does not influence hepatic iron accumulation in $Hfe^{-/-}$ mice, or their response to iron loading, as the phenotype of $HfeLcn2^{-/-}$ mice remained indistinguishable from that of $Hfe^{-/-}$ mice. We conclude that Lcn2 is not essential for iron delivery to hepatocytes in hemochromatosis.

Introduction

Under normal conditions, the major pathway for cellular iron uptake is through internalization of the complex of iron-bound transferrin and the transferrin receptor. Thereafter, iron is released from transferrin as the result of acidic pH in endosomes and is transported to the cytosol by divalent metal ion transporter 1 (DMT1; also known as SLC11A2)[30, 317]. However, there is convincing evidence that, in situations of disrupted iron homeostasis, iron can also be delivered to cells by alternative, transferrin-independent mechanisms. For example, mice and humans lacking transferrin, while anemic, develop iron overload in the liver, implicating that iron is being delivered to hepatocytes by transferrin-independent pathway(s) [318, 319]. Likewise, in HFE-related hereditary hemochromatosis (HH), a common disorder of iron homeostasis characterized by excessive iron deposition principally in the liver [320, 321], iron overload exceeds the iron-binding capacity of serum transferrin [38, 106] , and this non-transferrin-bound iron (NTBI) is cleared from the circulation and deposited into hepatocytes [322]. This alternate iron uptake pathway is further brought into evidence by studies in *Dmt1*$^{-/-}$ knockout mice [323]. In fact, despite the presence of a disrupted transferrin cycle, *Dmt1*$^{-/-}$ mice have abnormally high iron liver stores. Moreover, iron-loading with iron dextran leads to significant accumulation of iron in *Dmt1*$^{-/-}$ hepatocytes, further indicating that the liver has alternate mechanisms than the transferrin cycle for iron uptake [323]. The identification of the components of NTBI however, remains elusive.

Lipocalin 2 (Lcn2), also called neutrophil gelatinase-associated lipocalin (NGAL) or 24p3 [324], has been proposed to be a mediator of the transferrin-independent iron delivery pathway [325] after it was found that Lcn2 can bind bacterial ferric siderophores [326], and that Lcn2-siderophore-iron complexes can transport iron into cells during kidney development [327].

Lcn2 has been found to exert a broad range of biological activities, which seems to depend on whether Lcn2 is bound to iron-laden siderophores or not, implicating iron-loaded Lcn2-siderophore as an iron donor and, conversely, iron-free Lcn2-siderophore as an iron chelator [328, 329]. As such, iron-loaded Lcn2-siderophore (Holo-Lcn2) has been shown to be required for mesenchymal-epithelial transition of embryonic kidney [327] and oncogene Ras-transformed epithelial cells [330], as well as for kidney protection from renal failure [331, 332], and delivery of ferric ion to mouse spermatozoa [333]. In contrast, Lcn2–siderophore complex without iron [334] and Lcn2 (Apo-Lcn2) [241] chelates iron from cells and, through iron deprivation, can induce apoptosis of pro-B cells, [241] and inhibit bacterial growth [326, 335, 336] and erythropoiesis [337, 338]. Additional evidence that Lcn2 may assist cellular iron trafficking is provided by the demonstration that iron delivered through Lcn2 has been shown to regulate iron-sensitive genes [334] and the identification of one of its receptors as being megalin [339]. Megalin is also known to bind another iron-binding protein, namely lactoferrin [340, 341]. Thus, Lcn2 emerged as a possible candidate involved in NTBI uptake under iron-overload conditions and, as such, possibly implicated in the pathophysiology of HH [325, 342].

In this report we investigated whether Lcn2, as a component of an alternative iron delivery system, may contribute to the pathophysiology of HH. For this purpose, we generated and then characterized iron metabolism in *HfeLcn2*-double mutant mice.

Materials and Methods

Animals. All procedures were performed in accordance with Canadian Council on Animal Care guidelines and approved by the institutional Animal Care Committee. Control, wild-type mice were C57BL/6 female mice purchased from Charles River Laboratories, Inc. (Wilmington, MA). *Hfe*$^{-/-}$ mice were kindly provided by Dr. Nancy C. Andrews, Howard Hughes Medical Institute and Harvard Medical School, Children's Hospital, Boston, MA, USA [343], in the 129/SvEvTac background, and were backcrossed onto the C57BL/6 (B6) background for 10 generations [344]. *Lcn2*$^{-/-}$ mice generated in the C57BL/6 (B6) background have been described previously [335]. Compound mutants, *HfeLcn2*$^{-/-}$, were obtained by interbreeding *Hfe*$^{-/-}$ and *Lcn2*$^{-/-}$ mice and genotyped by PCR (supplementary Figure 1A and B). The following primers were used: Hfe - 5'-AGTTGGGAGTGGTGTCCGA-3'; 5'-TGGCTACAGTGTGAGAGGC-3' and 5' CTAGCTTCGGCCGTGACG-3'; Lcn2 - 5'-CCTCAAGGACGACAACATCA-3'; 5'-ACCCATTCAGTTGTCAATGC-3'; 5'-TTGGGTGGAGAGGCTATTC-3' and 5'-AGGTGAGATGACAGGAGATC-3'. All mice used in the experiments were genotyped by PCR assay performed on DNA prepared from mouse tails. Absence of Lcn2 expression in *Lcn2*$^{-/-}$ and *HfeLcn2*$^{-/-}$ mice was confirmed by western blotting, using a monoclonal anti-mouse Lipocalin-2/NGAL antibody (R&D Systems Inc. Minneapolis, MN) (supplementary Figure 1C).

Diets. Mice were given a commercial diet (Harlan Teklad, Madison, WI), or, when indicated, an iron-supplemented diet containing 2.5% (w/w) carbonyl iron (Sigma Immunochemicals, St. Louis, MO*).*

Measurement of serum iron, transferrin saturation, and tissue iron concentration. Serum iron, total iron-binding capacity and transferrin saturation were assessed by colorimetric assay

[345]. Iron levels in livers, spleens, heart and kidneys were measured by acid digestion of tissue samples followed by iron quantification by atomic absorption spectroscopy [345].

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Total RNA was isolated with Trizol reagent (Invitrogen, Burlington, ON, Canada), and RT was performed with the Thermoscript RT-PCR system (Invitrogen). Hepcidin 1 (*Hamp 1*) and *β-actin* mRNA levels were measured by real-time PCR in a Rotor Gene 3000^{TM} Real Time DNA Detection System (Montreal Biotech Inc., Kirkland, QC, Canada) with the QuantiTect SYBRGreen I PCR kit (Qiagen, Mississauga, ON, Canada), as described [345], and expression levels of hepcidin were normalized to the housekeeping gene *β-actin*. The primers employed were: *β-actin* – 5'-TGTTACCAACTGGGACGACA-3' and 5'-GGTGTTGAAGGTCTCAAA-3'; *Hamp 1* – 5'AGAGCTGCAGCCTTTGCAC3' and 5'GAAGATGCAGATGGGGAAGT3'.

Statistical analysis. All statistics were calculated using SigmaStat® 3.1 (Systat Software, Richmond, CA). All values in the figures are expressed as means ± SD. Multiple comparisons were evaluated statistically by 1-way analysis of variance (ANOVA), followed by the Bonferroni multiple comparison test.

Results

Hfe$^{-/-}$ mice develop iron overload characterized by high circulating iron levels and deposition of excess iron in the liver, but with resistance to iron loading in the spleen due to deficient iron storage in macrophages [343]. To determine whether Lcn2 participates in iron delivery to the liver in Hfe-deficient mice, we interbred *Hfe*$^{-/-}$ and *Lcn2*$^{-/-}$ mice. Circulating iron levels, assessed by measuring serum iron and transferrin saturation, as well as iron deposition in the liver and spleen were analyzed at 10 and 20 weeks of age (Figure 1). Iron parameters in Lcn2-single knockout mice were similar to those in wild-type mice (B6), while *Hfe*$^{-/-}$ and *HfeLcn2*-double mutants had higher amounts of circulating and liver iron than B6 mice (~1.5-2-fold higher; $P < 0.05$; Figure 1A-1C). In contrast, spleen iron content, while slightly increased with age, was lower in 10-week-old *Hfe*$^{-/-}$ and *HfeLcn2*$^{-/-}$ mice compared to B6 (31% lower; $P < 0.05$) or *Lcn2*$^{-/-}$ mice (Figure 1D). No significant differences were found in heart iron (Figure 1E), while iron levels in kidneys were slightly increased in both, *Hfe*$^{-/-}$ and *HfeLcn2*$^{-/-}$ mice, compared to B6 mice (Figure 1F). Thus, we found no significant differences between *Hfe*$^{-/-}$ and *HfeLcn2*$^{-/-}$ mice regarding iron parameters. These results indicate that iron accumulation in the liver of *Hfe*$^{-/-}$ mice did not improve in the absence of Lcn2, suggesting that Lcn2-mediated cellular iron delivery is not essential in iron uptake by the liver in *Hfe*$^{-/-}$ mice.

To further investigate the responses to dietary iron loading in compound mutants, we challenged the mice with a 2.5% wt/wt carbonyl iron-supplemented diet for 2 weeks (Figure 2). B6 and *Lcn2*$^{-/-}$ mice fed the iron-enriched diet showed significant increments of serum iron, transferrin saturation (>50% increase; $P < 0.01$), hepatic iron content (>4-fold rise; $P < 0.001$) and spleen iron content (>2.5-fold elevation; $P < 0.001$) compared to mice on the control diet (Figure 2A-2C). While *Hfe*$^{-/-}$ and *HfeLcn2*$^{-/-}$ double knockout mice also manifested heightened liver iron content over the already-elevated levels observed on the control diet in response to

dietary iron loading (>2-fold rise; $P < 0.001$), the increase in iron loading of the spleen was considerably more modest than what was seen in B6 ($P < 0.01$) or *Lcn2*$^{-/-}$ mice on the iron-supplemented diet (Figure 2D).

Because augmented iron absorption in *Hfe*$^{-/-}$ mice has been related to inappropriate expression levels of hepcidin [144], the principal regulator of systemic iron homeostasis, we also measured hepatic hepcidin 1 mRNA. As illustrated in Figure 2E, we found lower amounts of hepcidin in both *Hfe*$^{-/-}$ and *HfeLcn2*-double mutants than in B6 and *Lcn2*$^{-/-}$ mice on the control diet (~30% decrease). Hepcidin levels rose about 2.5-3.5-fold in response to iron loading in all mouse strains ($P < 0.01$), but remained significantly lower in *Hfe*$^{-/-}$ and *HfeLcn2*$^{--/-}$ mice compared to B6 ($P < 0.05$) or *Lcn2*$^{-/-}$ mice. Since the response to iron-loading was indistinguishable between *Hfe*$^{-/-}$ and *HfeLcn2*$^{--/-}$ mice, these results further confirm that Lcn2 is not essential for iron delivery to the liver and the regulation of hepcidin in HH.

Discussion

While under normal conditions hepatocytes acquire iron mostly through the transferrin-receptor pathway, the existence of uptake of NTBI in HH is now well established. Several candidates have been proposed, including Lcn2, but clear demonstration of its participation and importance in HH has not been yet provided.

In this report, we set out to determine whether Lcn2 represents a physiologically-relevant mechanism of iron uptake by the liver in HH, an iron overloading disease. For this purpose, we generated and characterized iron metabolism in *HfeLcn2*-double knockout mice. The $Hfe^{-/-}$ mice have increased plasma NTBI levels and, importantly, hepatocytes from $Hfe^{-/-}$ mice have been shown to uptake significantly more NTBI than control mice [38]. We found that basal iron status and iron metabolism changes induced by iron diet supplementation were indistinguishable between *Hfe*-single knockout and *HfeLcn2*-double knockout mice. Our results indicate that Lcn2 is dispensable for NTBI uptake by hepatocytes in HH. However, they do not exclude that the Lcn2-mediated iron delivery pathway may be involved in other pathologies.

The identification of the components of non-transferrin bound iron delivery pathways remains important for the understanding of the patophysiology of HH and other iron overload diseases such as hypotransferrinemia and thalassemia, because in these situations plasma iron exceeds the binding capacity of transferrin and uptake of non-transferrin bound iron significantly contributes to iron accumulation in the liver, pancreas and heart [346].

Non-transferrin-bound iron uptake mechanism have been described in a variety of cell lines [347, 348], including hepatocytes [349]. In hepatocytes, this system requires the reduction of Fe^{3+} to Fe^{2+} [349]. Iron salts, such as iron ascorbate, citrate, or nitrilotriacetate

have been suggested as candidates for low-molecular-weight non-transferrin iron carriers, but the transporter(s) of non–transferrin-bound iron remains to be identified. Beside Lcn2, which the present study suggests it is redundant, potential candidates include L-type voltage-dependent Ca2+ channels (LVDCCs) [350] and Zip14 [351] .

LVDCCs have been identified as key transporters of iron into cardiomyocytes and neuronal cells under iron-overload conditions [350, 352]. Further support for a role for cardiac LVDCCs in myocardial non-transferrin bound iron uptake under conditions of iron overload comes from the demonstration that LVDCCs blockers are protective and able to attenuate myocardial iron accumulation in iron-overloaded mice [353].

Zip14 is a zinc transporter and member of the SLC39A metal ion transporter family [354], and is highly expressed in hepatocytes. Recent studies have shown that mouse Zip14 transports both iron and zinc in cultured hepatocytes [351].

In conclusion, the work presented here shows that Lcn2 is dispensable for iron delivery to hepatocytes in the context of *Hfe*-deficiency. Further studies will be necessary to establish whether other candidates, namely LVDCCs and Zip14, are involved in the uptake of non-transferrin-bound iron by hepatocytes under iron-overload conditions such as HH.

Acknowledgments

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Authorship

Contribution: H.H. designed and performed experiments, analyzed data and contributed to the writing of the manuscript. S.A. provided $Lcn2^{-/-}$ mice; and M.M.S. designed the experiments, analyzed data, and wrote the manuscript.

Figures and Legends

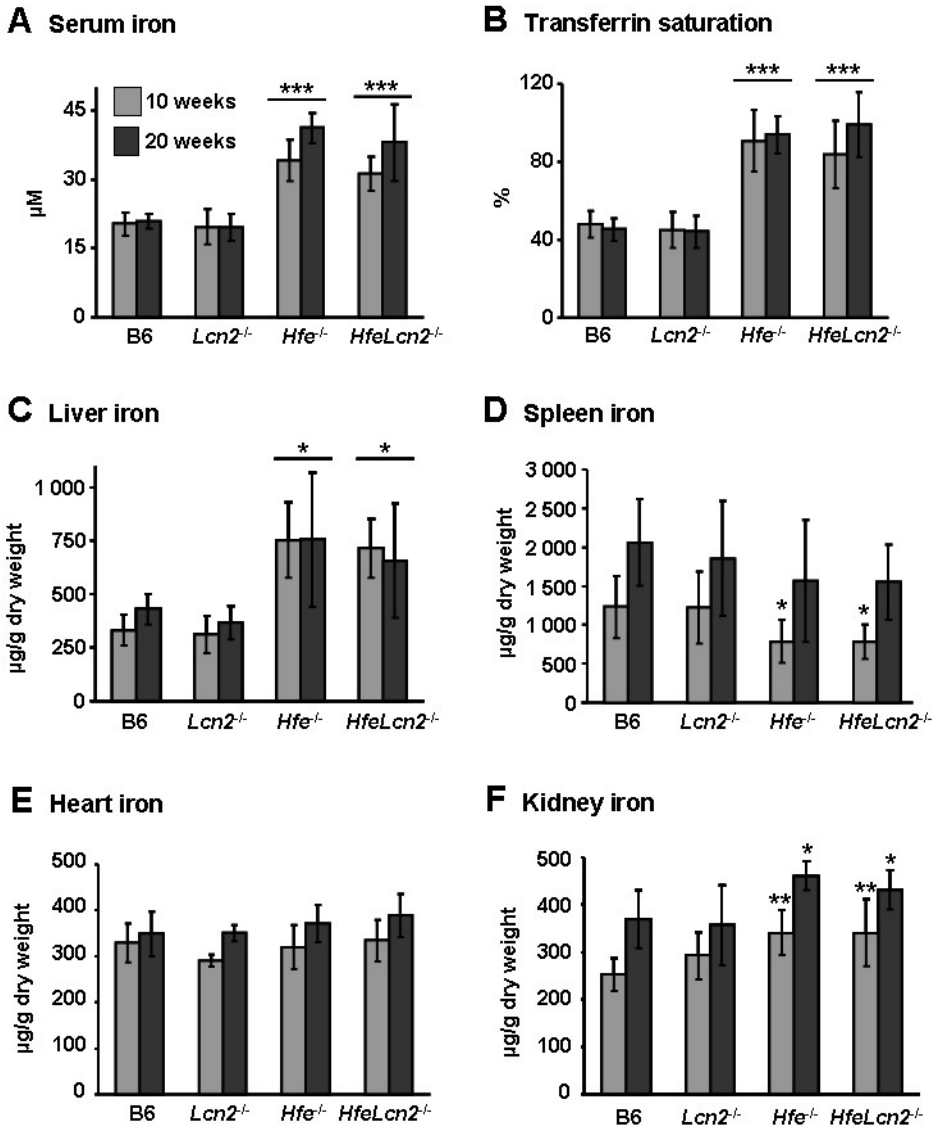


Figure 1. Iron parameters in 10 week-old and 20 week-old wild-type (B6) and mutant mice. Serum iron (**A**), transferrin saturation (**B**), iron concentration in the liver (**C**), spleen (**D**), heart (**E**) and kidney (**F**). The results are presented as means ± SD from *n*=6-8 mice per group. (*, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$ and mutant mice *versus* B6 same age).

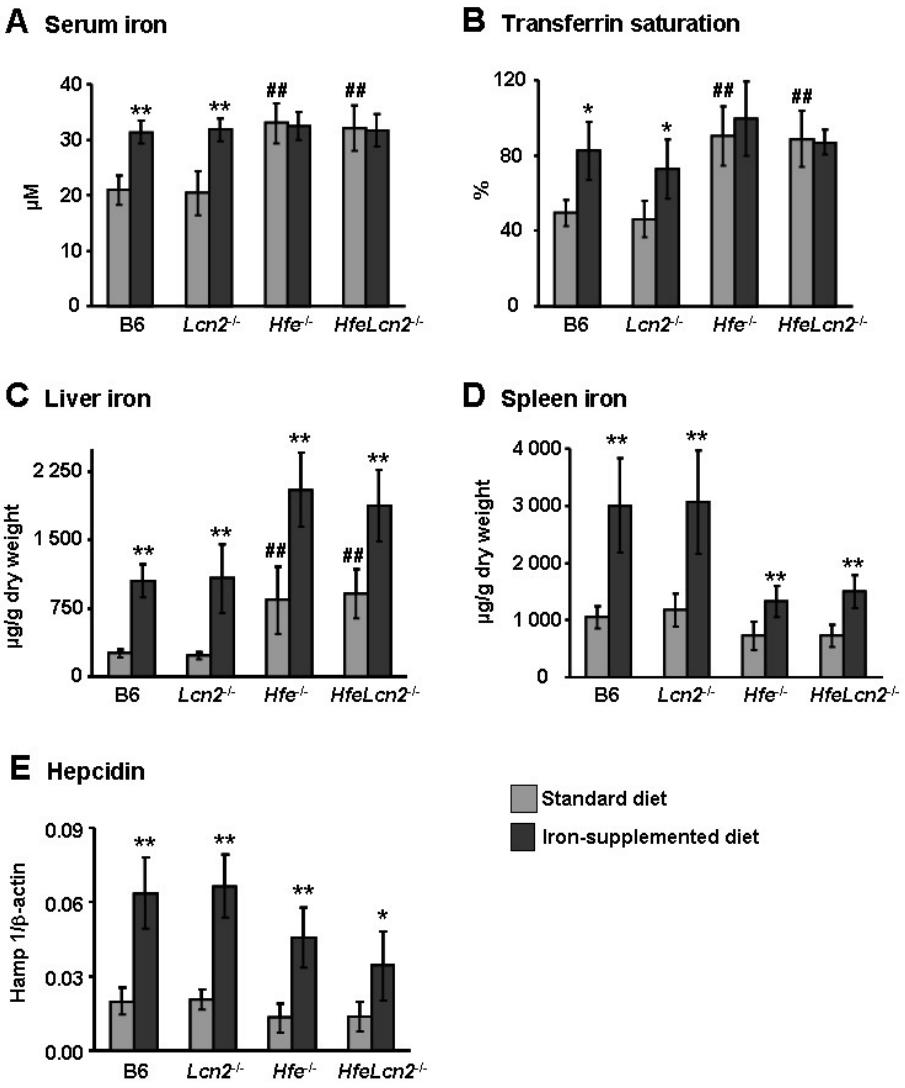
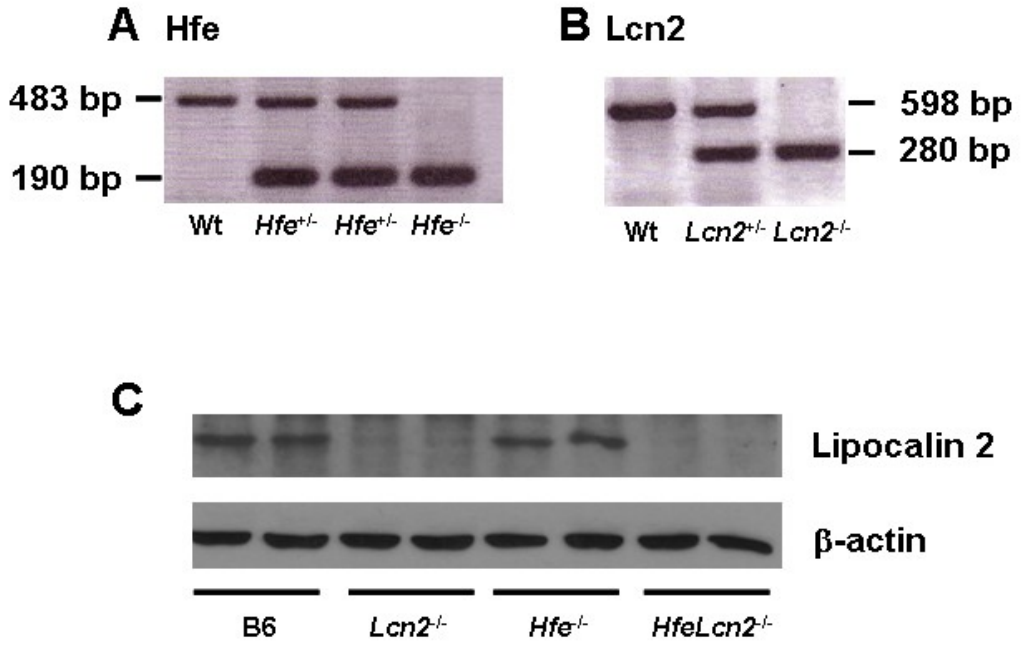


Figure 2. Response to iron-loading in wild-type (B6) and mutant mice. Serum iron (**A**), transferrin saturation (**B**), iron concentration in the liver (**C**) and spleen (**D**) and hepatic hepcidin 1 mRNA expression *(*Hamp 1) (**E**) in mice on a standard diet (light-shaded bars) and mice on a diet supplemented with 2.5% carbonyl iron for 2 weeks (dark-shaded bars). The results are presented as means ± SD from *n*=6-8 mice per group. (##, $P < 0.001$ mutant mice *versus* B6; *, $P < 0.01$ and **, $P < 0.001$ iron-supplemented *versus* standard diet).



Supplementary Figure 1 (**A and B**) Mouse genotyping using genomic DNA extracted from mouse tails and the primers described in materials and methods. (**C**) Immunoblot analysis of kidney protein extracts using a monoclonal anti-lipocalin 2 antibody.

Chapter 3 Contribution of STAT3 and SMAD4

pathways to the regulation of hepcidin by opposing stimuli

Short title: Hepcidin regulation by opposing stimuli

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Preface

In chapter 2 we evaluated the importance of Lcn2 in the pathogenesis of hepatic iron loading in *Hfe* knockout mice. We conclude that Lcn2 is dispensable for iron delivery to hepatocytes in the context of *Hfe*-deficiency. As mentioned before, at the site of inflammation/infection, Lcn2 is released by neutrophils to sequester bacterial ferric siderophores. Lcn2 comprises a critical component of innate immunity to exogenous bacterial infections. Hepcidin, stimulated by inflammation, by inducing sequestration of iron in macrophages, and limiting iron uptake from gut, robs bacteria of this element. Therefore, both Lcn2 and hepcidin, sequester iron, but via different mechanisms, as part of host defense mechanisms. The regulation of hepcidin has been studied extensively. In the next chapter, we will further investigate hepcidin regulation by opposing stimuli.

Abstract

Hepcidin, a key regulator of iron metabolism, is a small antimicrobial peptide produced by the liver that regulates intestinal iron absorption and iron recycling by macrophages. Hepcidin is stimulated when iron stores increase and during inflammation, and, conversely, is inhibited by hypoxia and augmented erythropoiesis. In many pathological situations, such as in the anemia of chronic disease (ACD) and iron-loading anemias, several of these factors may be present concomitantly and may generate opposing signaling to regulate hepcidin expression. Here, we addressed the question of dominance among the regulators of hepcidin expression. We show that erythropoiesis drive, stimulated by erythropoietin but not hypoxia, down-regulates hepcidin in a dose-dependent manner, even in the presence of lipopolysaccharide (LPS) or dietary iron-loading, which may act additively. These effects are mediated through down-regulation of phosporylation of Stat3 triggered by LPS and of Smad1/5/8 induced by iron.

In conclusion, hepcidin expression levels in the presence of opposing signaling are determined by the strength of the individual stimuli rather than by an absolute hierarchy among signaling pathways. Our findings also suggest that erythropoietic drive can inhibit both inflammatory and iron-sensing pathways, at least in part, via the suppression of STAT3 and SMAD4 signaling *in vivo*.

Introduction

Body iron stores are kept in balance by controlling the level of absorption from the diet, because in mammals iron is not efficiently excreted [355]. Thus, considerable work in the past was directed at identifying factors that influence intestinal iron absorption. Those studies established that both the size of iron stores and the rate of erythropoiesis influence the amount of iron being absorbed in the intestine [356]. Information about the amount of iron in the body is transmitted to the intestine where iron absorption takes place such that, if the body is iron-depleted, the amount of daily iron absorption is augmented, whereas if there is an excess of iron, absorption is diminished to prevent iron-overloading (referred to as the *store regulator*). Iron absorption is also independently regulated by erythropoiesis: it increases when erythropoietic activity rises (*erythroid regulator*) [356]. Additional systemic stimuli that also affect intestinal iron absorption include tissue oxygenation (*hypoxia regulator*) [357], and inflammatory cytokines (*inflammatory regulator*)[358]. During hypoxia, iron absorption increases [359], while, conversely, it may decline during inflammation [358].

More recent work identified hepcidin, a small antimicrobial peptide [360, 361], as an effector molecule common to all these four regulators of iron absorption [362, 363]. Hepcidin expression is stimulated when iron stores increase [362] as well as during inflammation [363]. Conversely, it is inhibited by anemia/hypoxia and heightened erythropoiesis drive [363, 364].

In many pathological situations however, antagonistic signaling for hepcidin regulation can co-occur. For example, anemia may co-occur with inflammation, as seen in the anemia of chronic disease (ACD) [365] arising in chronic inflammatory conditions such as autoimmune diseases, chronic infectious diseases and cancer. ACD is associated with excessive production of cytokines produced by macrophages and T-lymphocytes which ultimately leads to iron

withdrawal from circulation and iron sequestration in reticuloendothelial macrophages and thus, to impaired iron mobilization from stores [365]. This may explain why patients with ACD may become iron-deficient when inhibition of intestinal iron absorption by high hepcidin levels is sustained for a long period. When treating the underlying disease is not feasible, erythropoietin (EPO) agents may help stimulate erythropoiesis and improve hemoglobin levels in patients with ACD [365]. However, whether the therapeutic effect of EPO also involves suppression of hepcidin expression in inflammatory conditions has not yet been demonstrated. Previously, we showed that hepcidin expression is induced in iron-deficient mice after lipopolysaccharide (LPS) challenge even in iron-depleted mice [366]. Similarly, in humans treated with endotoxin, serum hepcidin levels also increased despite the presence of slightly decreased serum iron levels [367].

Anemia may also co-occur with iron overload as observed in disorders characterized by robust but inefficient erythropoiesis [368]. These disorders, collectively called iron-loading anemias, include among others: thalassemia syndromes, congenital dyserythropoietic anemias and sideroblastic anemias that have ineffective erythropoiesis in common, leading to the development of anemia and consequent hypoxia [369]. The second common characteristic of these disorders is the development of iron-overload, which can be severe as a consequence of inappropriately-increased intestinal iron absorption [356]. Indirect evidence suggests that serum factors in β-thalassemia patients might override the potential effect of iron-overload on hepcidin expression and thus explain the inappropriate intestinal iron absorption levels. In fact, serum from β-thalassemia individuals has been reported to inhibit hepcidin mRNA expression in hepatoma cells [370].

Because of the central role of hepcidin in iron metabolism and its direct contribution to the pathogenesis of several common disorders, it is important to understand how different factors influence hepcidin expression *in vivo* and lead to its misregulation when present concomitantly.

In this study we examined how hepcidin is regulated in the presence of opposing stimuli *in vivo* in mice as summarized in Figure 1, and further investigated the contribution of mediators and downstream signaling pathways of hepcidin expression.

Materials and Methods

Animals

All procedures were performed in accordance with Canadian Council on Animal Care guidelines after approval by the institutional Animal Care Committee of the CRCHUM. C57BL/6 female mice aged 6 weeks were purchased from Charles River Laboratories, Inc. (Wilmington, MA).

Animal treatments

Erythroid regulator-Erythropoiesis was induced by treating mice with 50 U of human biosynthetic erythropoietin-α (EPO) (epoetin alfa, Ortho Biotech, Bridgewater, NJ) dissolved in phosphate-buffered saline (PBS). Mice were injected i.p. daily for four days and were sacrificed on day 5. Control mice were similarly injected with an equivalent volume of PBS. *Immune regulator*- Acute inflammation was produced by a single dose of lipopolysaccharide (LPS, *Escherichia coli* serotype 055:B5 – 1 mg/kg i.p., Sigma-Aldrich, St. Louis, MO). In other experiments, mice were injected with recombinant mouse IL-6 (1 µg i.p., Cederlane Laboratories Ltd., Hornby, Ontario, Canada). Control mice were similarly injected with an equivalent volume of sterile saline solution (0.09% NaCl). The animals were sacrificed six hours after the LPS and three hours after the IL-6 injection. *Stores regulator*- Control mice were given a commercial diet containing approximately 226 mg of iron per kg (Teklad Global 18% protein rodent diet, Harlan Teklad, Madison, WI). Dietary iron overload was produced by giving 8-week-old mice the same commercial diet supplemented with 25 g carbonyl iron (2.5% w/w carbonyl iron from Sigma-Aldrich) for 2 weeks. *Hypoxia.* Normobaric hypoxia was established by diluting ambient air with nitrogen in a special ventilated chamber in which N_2-enriched air supply was controlled with an O_2 sensor-driven inlet valve. Mice were maintained in the chamber and exposed to normobaric hypoxia (10% O_2) for three days. An

oxygen analyzer was used to monitor the oxygen concentration in the hypoxic chamber. Age-matched control mice were kept under normoxia (room air) in the same room in which the hypoxic chamber was placed.

Hematological measurements and transferrin saturation

Red blood cell (RBC) count, hemoglobin (Hb), hematocrit (HCT) and mean corpuscular volume (MCV) were measured with an automated cell counter calibrated for murine samples (ABC vet counter, ABX hématologie, Montpellier, France). Serum iron, total iron-binding capacity and transferrin saturation were assessed by colorimetric assay with the Kodak Ektachem DT60 system (Johnson & Johnson, Ortho Clinical Diagnostics, Mississauga, ON, Canada).

Measurement of tissue iron concentration

Liver iron concentrations were assessed by acid digestion of tissue samples, followed by iron quantification with atomic absorption spectroscopy [371].

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

Total RNA was isolated with Trizol reagent (Invitrogen, Burlington, ON, Canada), and RT was performed with the Thermoscript RT-PCR system (Invitrogen). *Hepcidin* and *β-actin* mRNA levels were measured by real-time PCR in a Rotor Gene 3000^{TM} Real Time DNA Detection System (Montreal Biotech Inc., Kirkland, QC, Canada) with QuantiTect SYBRGreen I PCR kits (Qiagen, Mississauga, ON, Canada) as described [345]. The primers employed were: *β-actin* – 5'-TGTTACCAACTGGGACGACA-3' and 5'-GGTGTTGAAGGTCTCAAA-3'; *hepcidin* – 5'AGAGCTGCAGCCTTTGCAC3' and 5'GAAGATGCAGATGGGGAAGT3'; *IL-6* – 5'TGTGCAATGGCAATTCTGAT3' and 5'CCAGAGGAAATTTTCAATAGGC3'. Expression levels were normalized to the housekeeping gene *β-actin*.

IL-6 assay

IL-6 was measured in serum and in supernatants of liver homogenates with an ELISA kit as per manufacturer's instructions (mouse IL-6 ELISA kit, catalog # M6000B, R & D Systems, Minneapolis, MN). Snap-frozen liver samples were thawed, weighed, and homogenized in solutions containing 1 ml of protease inhibitor cocktail (Complete; Boehringer Mannheim, Indianapolis, IN). The resulting supernatants were analyzed and standardized to the weight of the liver sample.

SDS-polyacrylamide-gel electrophoresis and Western blot analysis

Livers were removed, rinsed in ice-cold PBS and used to prepare liver nuclear extracts with Nuclear Extract Kits (Active Motif, Carlsbad, CA). Nuclear protein extracts were separated on 10% SDS-polyacrylamide gel and blotted onto nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK). The membranes were immunoblotted with the following antibodies: phospho-Stat3, Stat3, phospho-Smad1/5/8 (Cell Signaling, Danvers, MA), Smad1/5/8 (Santa Cruz Biotechnology, Santa Cruz, CA) and β-actin (Abcam, Inc., Cambridge, MA). As a secondary antibody, anti-rabbit IgG (Cell Signaling) or anti-mouse IgG (GE Healthcare) were used. Antigen-antibody complexes were visualized with the ECL^{TM} Western Blotting Detection Reagent (GE Healthcare).

Statistical analysis

All statistics were calculated with SigmaStat® 3.1 (Systat Software, Richmond, CA). Multiple comparisons were evaluated statistically by 1-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparison test. When data failed the equal variance test, the Kruskal-Wallis 1-way ANOVA on ranks was used followed by Student-Newman-Keuls or Dunn's post hoc test. Correlation coefficients were determined by Spearman's correlation coefficients.

Results

Erythropoietin but not hypoxia blocks hepcidin induction by LPS

EPO: Previous studies have demonstrated that when erythropoiesis is stimulated by erythropoietin (EPO), hepcidin mRNA expression is inhibited [364, 372], and, conversely, LPS induces it [362]. To assess whether increased erythropoiesis could affect the induction of hepcidin by LPS, mice were treated for four consecutive days with EPO to stimulate erythropoiesis, followed by a single injection of LPS on day 5 and were examined after 6 h. EPO treatment stimulated erythropoiesis, as revealed by the elevation of hematological indices (RBCs – 12.4%; HGB – 11.5% and HCT – 19.9% increase). As shown in Figure 2A, EPO alone led to a significant inhibition of mRNA hepcidin expression (92% reduction). When compared to LPS-treated controls, EPO pre-treatment considerably reduced the ability of LPS to evoke hepcidin mRNA expression (LPS *vs.* EPO+LPS; $P<0.001$). However, the observed reduction was not absolute, because hepcidin levels remained higher in mice with the combined EPO and LPS treatments than in mice treated with EPO alone (EPO *vs.* EPO+LPS; $P<0.0001$).

Hypoxia: Hypoxia has been identified as an independent regulator of iron absorption [373] and, consistently, exposure to hypoxia has been shown to suppress hepcidin mRNA both in mice *in vivo* and in isolated hepatocytes *in vitro* [363], suggesting that hypoxia may directly modulate hepcidin expression. To test whether hypoxia could, similarly to EPO, block LPS induction of hepcidin expression, mice were exposed to normobaric hypoxia (10% O_2) for three days and received a single LPS injection 6 h before being analyzed. Hypoxia stimulated erythropoiesis, as judged by the elevation of hematological indices when compared to control, normoxic mice (RBCs – 23%; Hb – 21.8% and HCT – 23% increase). As expected [363], hypoxia alone led to suppression of hepcidin mRNA levels in the liver (~72% inhibition) but unlike EPO, was insufficient to block induction by LPS (~112% induction compared to control, normoxic mice,

Figure 2B).

Erythropoietin inhibits LPS-mediated hepcidin induction independently of IL-6 production

LPS injection leads to the production of inflammatory cytokines, including IL-6 [374], which has been identified as a major hepcidin inducer [375]. Because EPO has been shown to inhibit LPS-induced secretion of IL-6 in cell lines [376], we next set out to determine whether the increase in IL-6 that occurs *in vivo* after LPS injection would be blocked in animals previously treated with EPO. We found that IL-6 mRNA as well as serum and intra-hepatic IL-6 levels were similar in mice treated with LPS alone and mice treated with both EPO and LPS (Figure 3A-C). Furthermore, EPO treatment was able to completely inhibit IL-6-induced hepcidin expression (Figure 3D). These data reveal that *in vivo*, an IL-6-deficit is unlikely to explain EPO inhibition of hepcidin expression induced by LPS.

Erythropoietin but not hypoxia partially inhibits phosphorylation of Stat3 mediated by LPS

IL-6 production causes the activation of signal transducer and activator of transcription 3 (STAT3) in hepatocytes which in turn induces hepcidin [377, 378]. To analyze whether EPO would inhibit Stat3 activation by LPS in mice, we undertook phosphoimmunoblotting of Stat3 in liver nuclear extracts (Figure 4A) followed by chemiluminescence quantification and normalization to β-actin as loading control (Figure 4B). We found that EPO treatment by itself reduced basal phosphorylation levels of Stat3 in the liver ($P<0.01$), whereas LPS induced both total Stat3 and its phosphorylation in the liver. Importantly, the phosphorylated Stat3/β-actin ratio was 54% lower in mice pretreated with EPO before LPS injection compared to animals treated with LPS alone.

We similarly examined activation levels of Stat3 in hypoxic mice treated with LPS. As shown

in Figure 4C and 4D, hypoxia alone led to a reduction of basal Stat3 phosphorylation levels in the liver ($P<0.01$). However, unlike EPO, hypoxia was insufficient in influencing LPS-induced Stat3 activation. Taken together, these data suggest that mechanistically, EPO pretreatment may result in deficits in LPS-induced Stat3-signaling pathways without affecting IL-6 production.

Erythropoietin but not hypoxia blocks hepcidin induction by dietary iron supplementation

EPO: Next, we tested whether EPO treatment could similarly block the induction of hepcidin by iron, another well-established hepcidin inducer [362]. Mice were placed on a carbonyl-iron (CI)-supplemented diet, and treated with EPO for five days before being analyzed, as described in Materials and Methods. As expected, iron-loaded mice presented a 5.2-fold increase in liver iron concentrations compared to mice kept on the standard diet, reflecting a significant rise in iron stores (from 233±8 in control mice to 1,207±303 µg iron/g dry weight in iron-loaded mice; $P<0.0001$). Hepcidin elevation evoked by iron-loading was substantially blocked by EPO treatment (~86% inhibition, Figure 5A, CI *vs.* EPO+CI; $P<0.001$). However, as similarly observed with LPS, the reduction was not complete since hepcidin levels remained higher in mice with the combined EPO and CI treatments than in mice treated with EPO alone (EPO *vs.* EPO+CI; $P<0.001$).

Hypoxia: To test whether hypoxia could also inhibit the induction of hepcidin expression in dietary iron-loading, mice were placed on an iron-enriched diet (2.5% CI) for two weeks. For combined treatment, the animals were simultaneously exposed to hypoxia for the last three days of the experiment. Again, feeding mice the iron-enriched diet led to a 5.5-fold increase in iron stores. As shown in Figure 5B, suppression of hepcidin mRNA levels by hypoxia was completely inhibited in iron-loaded mice, as hepcidin rose to similar levels as in mice exposed to the iron-enriched diet alone (2.7 and 2.4-fold induction, respectively).

Erythropoietin but not hypoxia inhibits phosphorylation of Smad1/5/8 mediated by iron

Regulation of hepcidin expression by the iron-sensing pathway involves signaling through the bone morphogenetic protein and Sma- and Mad-related protein 4 (BMP/SMAD4) pathway that regulates hepcidin via the Smad1, Smad5 and Smad8 set of Smad proteins [379]. To ascertain the potential role of this signaling pathway in the regulation of hepcidin by antagonistic stimuli, we examined the effects of dietary iron-loading on hepatic Smad1/5/8 activation. As shown in Figure 6, iron-loading led to a 3-fold induction of phosphorylated Smad1/5/8 in the liver compared to basal levels in control mice. Most importantly, EPO treatment of iron-loaded mice completely inhibited this increase in Smad1/5/8 phosphorylation (Figures 6A and 6B). In contrast, activation levels of the SMAD4 pathway in hypoxic mice challenged with iron-loading were similar to those induced by dietary iron-loading alone, indicating that hypoxia is insufficient to block activation of the SMAD4 pathway by iron (Figures 6C and 6D).

Suppression of hepcidin by the erythroid regulator depends on the degree of erythropoiesis activity

While EPO significantly blocked hepcidin induction by LPS and dietary iron, this inhibitory effect was not total, as shown in Figures 2A and 5A. Furthermore, we observed that spleen/body weight ratio, as an indicator of extramedullary erythropoiesis activity, was significantly higher in mice treated with EPO than in hypoxic mice (Figure 7A). Because EPO production is also stimulated during hypoxia [380], we questioned whether dominance of the erythroid over the immune and stores regulator as opposed to hypoxia, depends on the extent of erythropoietic activity. To this end, we treated mice with increasing amounts of EPO for four days, from a total of 25 U (4x6.25 U) to 200 U (4x50 U), alone or in combination with LPS injections or with the CI-supplemented diet. We found a dose-like increase in the spleen/body weight ratio that was indistinguishable between mice treated with EPO alone and

mice treated with EPO in combination with LPS or CI (Figure 7B), indicating that discrete degrees of erythropoietic activity were attained in all treatment groups. Importantly, the capacity of EPO to block hepcidin induction by LPS or CI was strongly dependent on EPO dosage, with lower EPO dosages being significantly less effective in suppressing hepcidin expression compared to higher dosages (Figure 7C). The dose-dependent effect of erythropoietic activity on the inhibition of hepcidin in the presence of opposing stimuli was further supported by the strong inverse correlation of hepcidin mRNA concentration with the spleen/body weight ratio found in each treatment group (Figure 7 D-F; Spearman's R=-0.747 for EPO alone; R=-0.862 for EPO +LPS; and R=-0.815 for EPO + CI; P<0.0001), indicating that dominance of the erythroid regulator over inflammatory and store regulators depends on the degree of erythropoiesis activity. Finally, when both LPS and dietary iron treatments were combined to antagonize EPO-mediated hepcidin suppression, an additive effect of LPS and dietary iron counteracting EPO treatment was observed, with significantly higher hepcidin levels found in mice with the triple treatment, i.e. EPO, LPS and CI, compared to double treatments, i.e. mice treated with EPO + LPS or EPO + CI ($P < 0.05$; Figure 8).

Discussion

Hepcidin has emerged as a central negative regulator of intestinal iron absorption and distribution that can be induced through at least two major pathways, namely, the inflammatory pathway and the iron-sensing pathway [381, 382]. In this study, we investigated its regulation through these two pathways in the presence of antagonistic stimuli.

Inflammatory pathway: In the first set of experiments, we compared the capacity of erythropoietic drive stimulated by EPO and hypoxia to suppress hepcidin induction by inflammation, which was triggered by LPS injections in our model. Of note, LPS appears to be a relatively weak inflammatory stimulus in mice compared with other inflammatory conditions [363, 383], since in our experiments, it induced only a 2-fold increase in hepcidin mRNA levels. Even though our results show that hypoxia alone may be insufficient to inhibit hepcidin induction by LPS. In contrast, EPO led to inhibition of hepcidin induction by LPS, an effect that we found to be dependent on the degree of erythropoietic activity. The induction of hepcidin during inflammatory and infectious states can be mediated, at least in hepatocytes, by cytokines, mainly IL-6 [375], and subsequent STAT3 signaling. This pathway may be initiated by Toll-like receptor signaling leading to the induction of inflammatory cytokines [366],including IL-6, which binds to its membrane-bound receptor (gp80) on hepatocytes and interacts with gp130, resulting in Stat3 activation and binding to a regulatory element in the hepcidin promoter [212, 377, 378]. Recent studies have demonstrated that EPO potentially has anti-inflammatory properties for example, against brain injury [384], myocardial dysfunction induced by ischemia/reperfusion [385] and chronic heart failure [386], and that EPO protection involves the suppression of inflammatory cytokines, including IL-6 [386, 387]. In our models, IL-6 production after LPS injection remained unaffected by EPO pretreatment, indicating that an IL-6 deficit is unlikely to explain EPO suppression of hepcidin in an inflammatory context. However, we found that EPO inhibits downstream signaling of both

basal and LPS-induced levels in the liver by suppressing Stat3 phosphorylation. To our knowledge, this is the first evidence that EPO might modulate hepatic hepcidin expression via suppression of STAT3 signaling in the liver *in vivo*.

Iron-sensing pathway: In the second set of experiments, we evaluated the ability of hypoxia and EPO to block the up-regulation of hepcidin induced by dietary iron-loading, thus counteracting the iron-sensing pathway. We found that hypoxia was insufficient to block hepcidin up-regulation by iron. However, stimulation of erythropoiesis by EPO partially suppressed hepcidin expression in iron-loaded mice, indicating that the iron-sensing pathway can be blocked by erythropoietic drive.

The iron-sensing pathway involves the activation of BMP/SMAD4 signaling, which is initiated after binding of BMP cytokines to BMP receptors, leading to the generation of phosphorylated RSmads, which dimerize with Smad4. The RSmad/Smad4 heterodimers translocate into the nucleus and presumably activate transcription of the hepcidin gene [379]. We found that dietary iron-loading in mice results in activation of the SMAD4 pathway, as judged by the induction of Smad1/5/8 phosphorylation. Similar results have been reported with parenteral iron challenge [214]. Importantly, we show that EPO pretreatment inhibits the activation of this pathway by iron, providing evidence that *in vivo,* EPO effects on hepcidin expression are mediated by the suppression of SMAD4 signaling.

EPO has been shown to be able to modulate hepcidin expression directly in hepatocytes or indirectly through serum mediators. In fact, *in vitro*, EPO may directly suppress hepcidin expression in a dose-dependent manner in the human hepatocyte cell line HepG2 [388]. *In vivo*, however, it has been demonstrated that during erythropoiesis stimulated by EPO, phlebotomy or phenylhydrazine, hepcidin suppression is blocked in mice in which erythropoiesis is simultaneously inhibited, indicating that the regulation of hepcidin by EPO is indirect and that

it requires increased erythropoiesis and possibly the release of some mediator(s) [389, 390]. Alternatively, EPO may interfere with signal transduction at the IL-6/gp80 receptor level and downstream signaling via gp130, which has been shown to be essential for hepcidin activation through STAT3 [212].

Dominance between regulators for hepcidin expression: Recent studies in patients with thalassemia syndromes revealed that, despite elevated iron parameters present in patients with thalassemia intermedia, their urinary hepcidin levels are severely depressed, indicating that the erythropoietic drive may have a dominant effect over the iron signal [391, 392]. However, several confounding factors, including the effect of iron chelation, inflammatory conditions such as hepatitis C, presence or absence of splenectomy and the presence of iron-induced end-organ damage may render the assessment of the influence of opposing stimuli for hepcidin regulation more difficult in these patients [391]. In animal models, assessment of the influence of opposing signals for hepcidin expression has been performed by using only single dosages of the antagonistic stimuli. These experiments found that iron-loaded mice subjected to experimentally induced anemia showed reduced hepcidin expression [363], while iron-deficient mice injected with LPS up-regulated hepcidin expression [366], suggesting dominant effects of the erythroid and inflammatory regulators over iron stores.

Our present results further advance those previous studies by showing that hepcidin expression levels in the presence of opposing signals, namely LPS and dietary iron, depend on the dosage of EPO used. Furthermore, we found that LPS and dietary iron can act additively to induce hepcidin *in vivo*, a finding that is in agreement with recently reported data of a synergistic induction of hepcidin expression by BMPs and IL-6 *in vitro* [393]. Taken together, these data indicate that final hepcidin levels are determined by the individual strength of the regulators rather than by an absolute hierarchy among the pathways. This notion is further supported by the finding that these effects are mediated, at least partially, through inhibition of STAT3 and

BMP/SMAD4 signaling *in vivo*, and thus offering an explanation as to how EPO suppression of hepcidin expression in the presence of antagonistic stimuli is dose-dependent. Dose-dependency also may explain why hypoxia seems to be unable to antagonize LPS or dietary iron, since much lower endogenous EPO levels are elicited by hypoxia compared with the high pharmacologic doses of EPO used in our experiments.

In summary, the present data contribute to the elucidation of dominance among the regulators of hepcidin expression and provide insights into the mechanisms by which erythropoietic drive, stimulated by EPO, affects both inflammatory and iron-sensing pathways via suppression of STAT3 and SMAD4 signaling *in vivo*.

Acknowledgements

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Authors' Contributions.

H.H. designed research, performed research, analyzed data and wrote the paper. M.C. performed research and analyzed data. A.L performed research and analyzed data. M.M.S. designed research, analyzed data and wrote the paper.

Conflicts of Interest: There are no conflicts of interest to declare by any author.

Figures and Legends

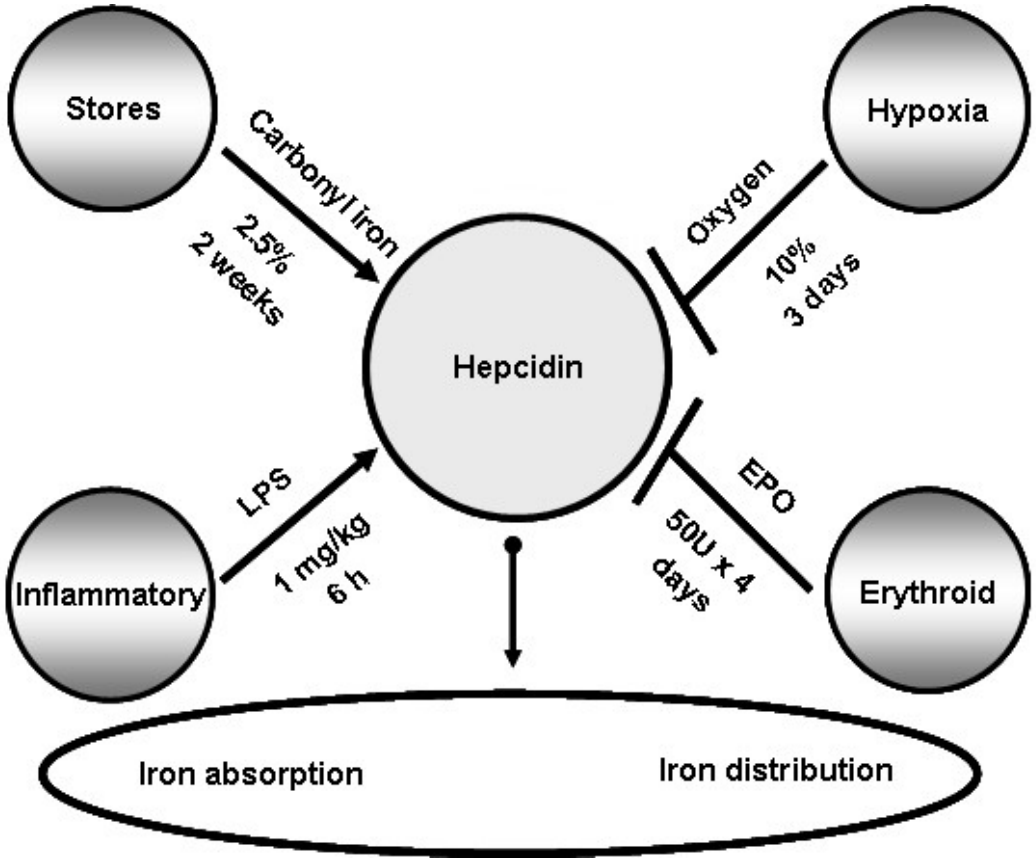

Figure 1 Factors affecting mRNA hepcidin expression in the liver. Hepcidin levels are regulated by iron levels (***store regulator***), immune mediators (***inflammatory regulator***), hypoxia (***hypoxia regulator***) and erythropoietic demand (***erythroid regulator***). Pointed arrows indicate up-regulation of hepcidin and blunt arrows inhibition of its mRNA expression. For each regulator, the treatments used in this study to stimulate or suppress hepcidin expression are shown.

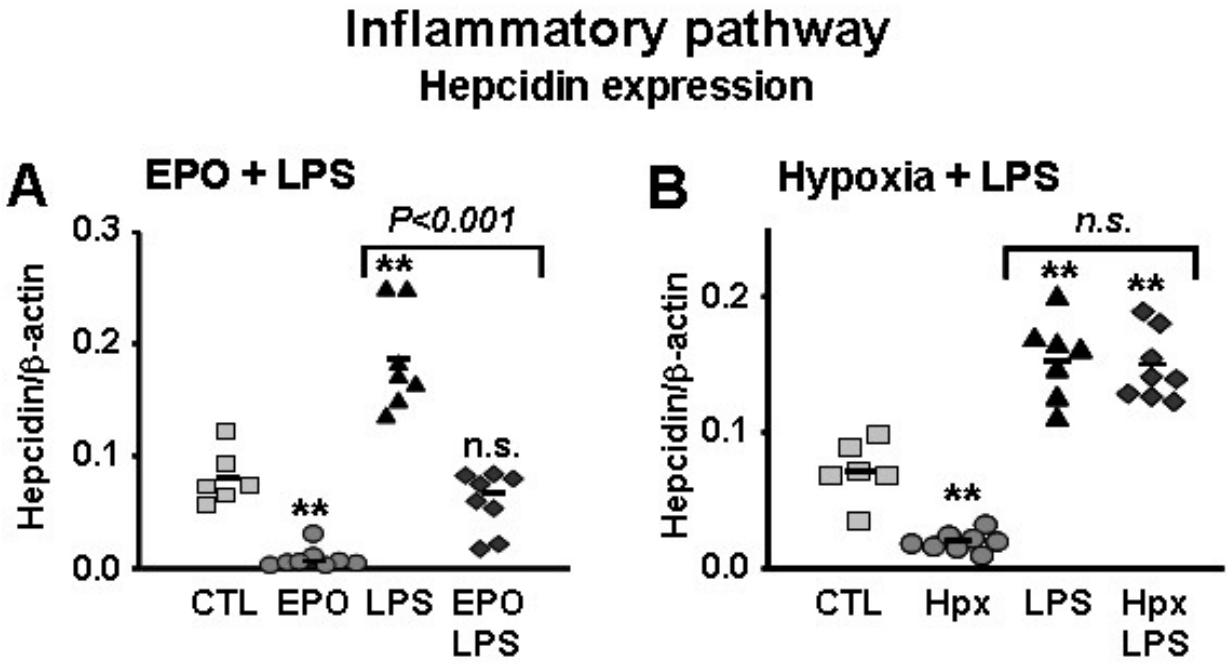

Figure 2 Erythropoietin but not hypoxia inhibits hepcidin induction through the inflammatory pathway. **(A)** Hepcidin mRNA levels in the liver of mice treated with saline (control, CTL), erythropoietin (EPO), lipopolysaccharide (LPS) and mice with combined treatments (EPO+LPS). **(B)** Hepcidin mRNA levels in the liver of mice treated with saline (control, CTL), mice subjected to 10% oxygen (hypoxia, Hpx), LPS and mice with combined treatments (Hpx+LPS). Hepatic hepcidin expression was quantified by real-time RT-PCR and normalized to β-actin. The hepcidin/β-actin ratios are shown, each symbol representing 1 mouse. Statistical analysis was performed by 1-way ANOVA; ***P*<0.001 for comparison with control mice. n.s. indicates not significant.

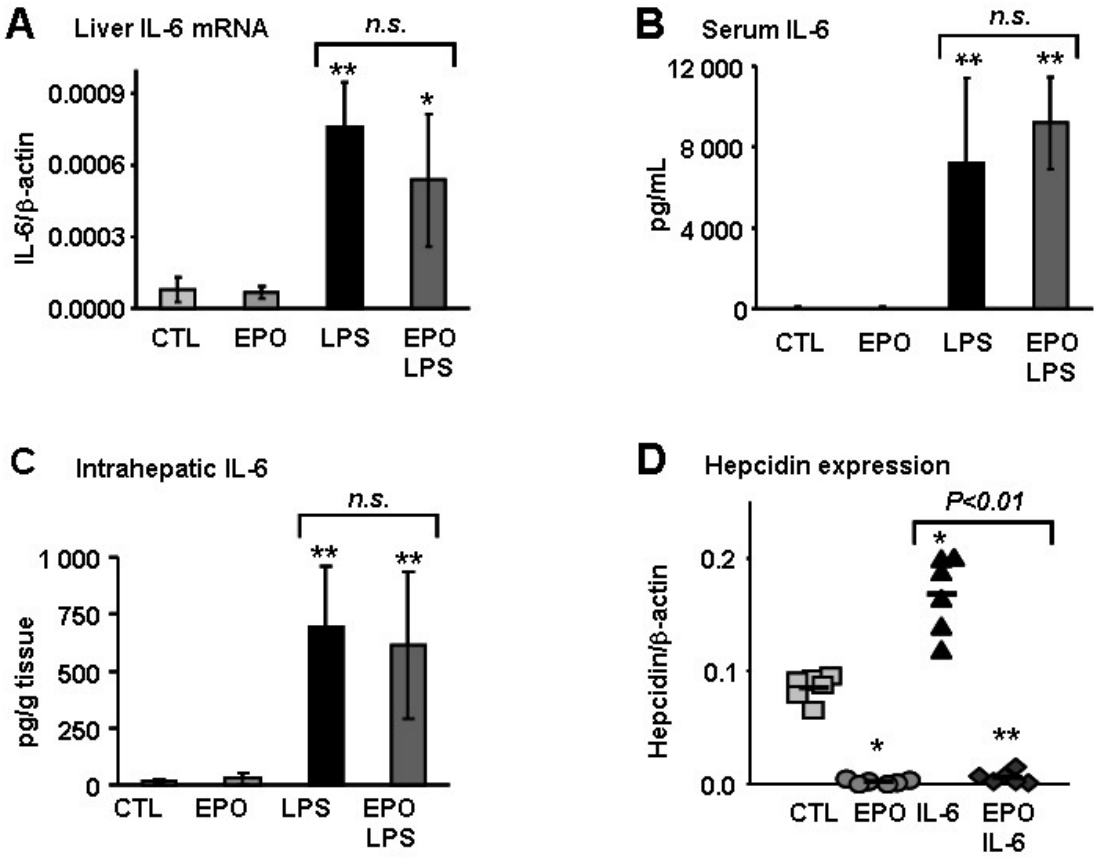

Figure 3 Erythropoietin inhibits LPS-mediated hepcidin induction independently of IL-6 production. (A) IL-6 mRNA levels in liver, **(B)** IL-6 levels in serum, and **(C)** Intra-hepatic IL-6 protein levels in mice treated with saline (control, CTL), erythropoietin (EPO), lipopolysaccharide (LPS), and mice with combined treatments (EPO+LPS). **(D)** Hepcidin mRNA levels in the liver of mice treated with saline (CTL), EPO, mouse recombinant IL-6 (IL-6),, and mice with combined treatments (EPO+IL-6). IL-6 and hepcidin mRNA levels were quantified by real-time RT-PCR and normalized to β-actin. The IL-6/β-actin and hepcidin/β-actin ratios are shown. IL-6 protein levels were measured by ELISA. Statistical analysis was performed by 1-way ANOVA; **P*<0.01; and ***P*<0.001 for comparison with control mice. n.s. indicates not significant. Data are presented as means ± SD with n=6 mice per group in **A**, **B** and **C** and as individual mice in **D**.

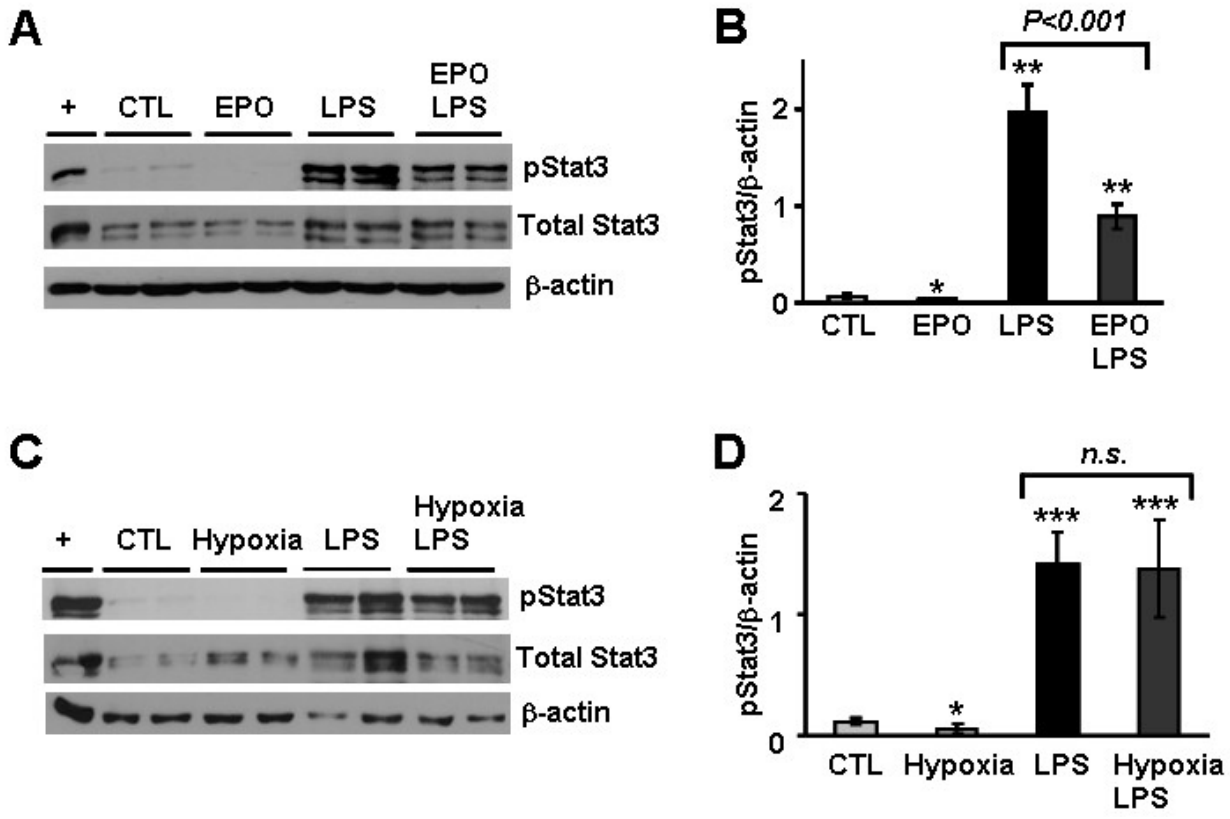

Figure 4 Stat3 phosphorylation induced by LPS is partially inhibited by erythropoietin but not by hypoxia. (A and C) Liver nuclear extracts from mice treated with saline (control, CTL), erythropoietin (EPO), hypoxia, lipopolysaccharide (LPS) and mice with combined treatments (EPO+LPS and Hypoxia+LPS) were analyzed by Western blotting with an antibody to phosphorylated Stat3 and total Stat3. Blots were stripped and reprobed with an antibody to β-actin as loading control. A representative Western blot is shown. Lane "+" is a positive control consisting of total cell extracts from serum-starved HeLa cells prepared with interferon-α treatment. **(B and D)** Quantification of chemiluminescence to calculate the ratio of phosphorylated Stat3 relative to β-actin (pStat3/β-actin). This experiment was repeated twice and the combined results are shown as means ± SD with n=7. Statistical analysis was performed by 1-way ANOVA; *P<0.01; **P<0.001; and ***P < 0.0001 for comparison with control mice. n.s. indicates not significant.

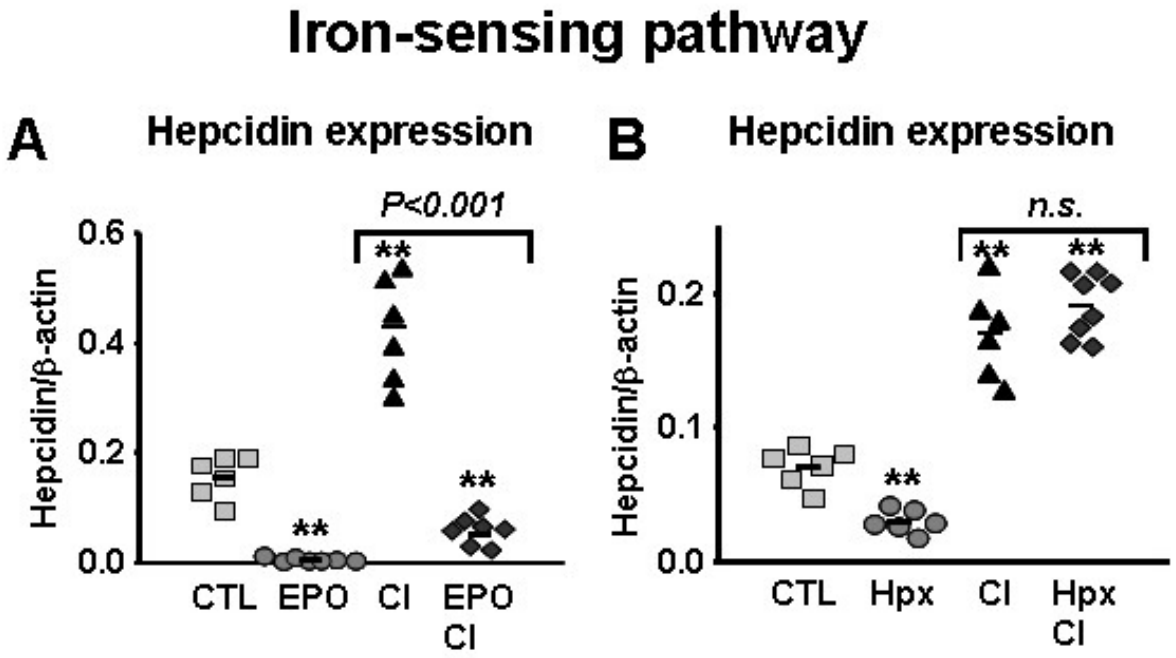

Figure 5 Erythropoietin but not hypoxia inhibits hepcidin induction through the iron-sensing pathway. **(A)** Hepcidin mRNA levels in the liver of mice treated with saline (control, CTL), erythropoietin (EPO), lipopolysaccharide (LPS), carbonyl iron supplemented diet (2.5%, CI) and mice with combined treatments (EPO+CI). **(B)** Hepcidin mRNA levels in the liver of mice treated with saline (control, CTL), mice subjected to 10% oxygen (hypoxia, Hpx), carbonyl iron supplemented diet (2.5%, CI) and mice with combined treatments (Hpx+CI). Hepatic hepcidin expression was quantified by real-time RT-PCR and normalized to β-actin. The hepcidin/β-actin ratios are shown, each symbol representing 1 mouse. Statistical analysis was performed by 1-way ANOVA; ***P*<0.001 for comparison with control mice. n.s. indicates not significant.

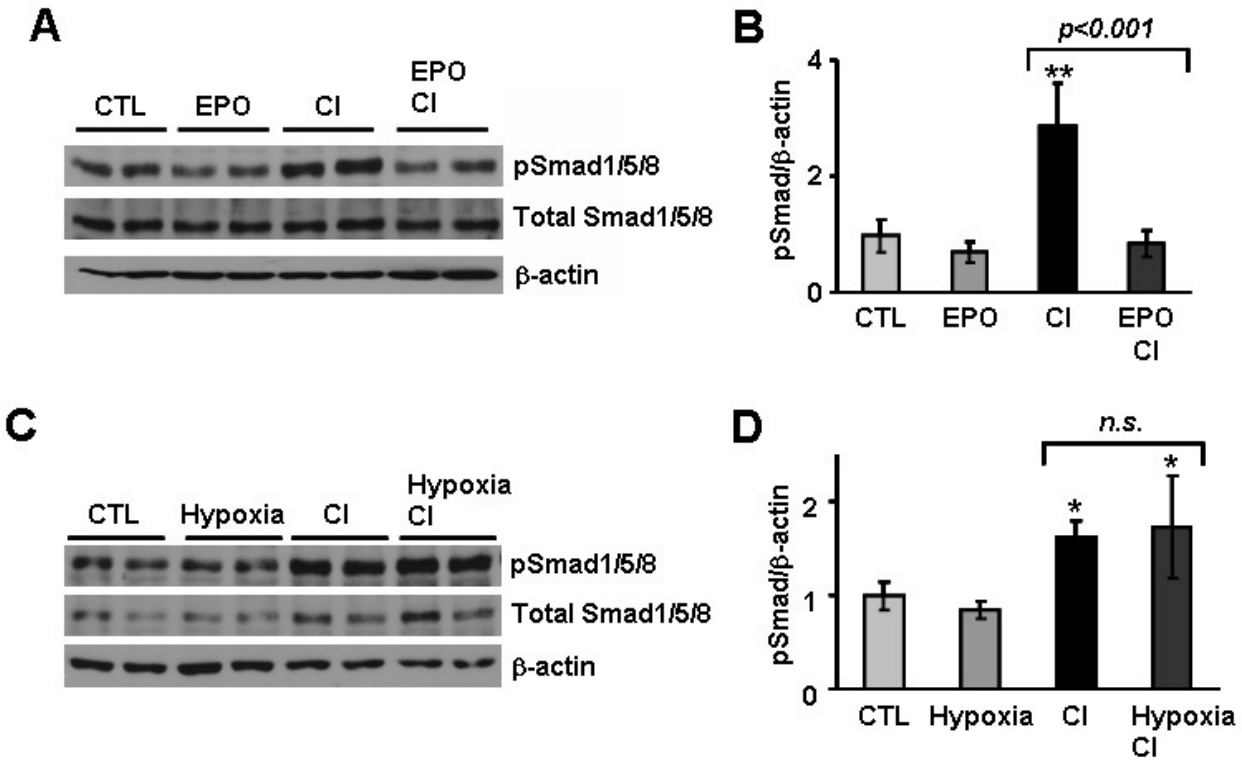

Figure 6 Smad1/5/8 phosphorylation induced by dietary iron-loading is partially inhibited by erythropoietin, but not by hypoxia. (A and C) Liver nuclear extracts from mice treated with saline (control, CTL), erythropoietin (EPO), carbonyl iron supplemented diet (2.5%, CI), hypoxia and mice with combined treatments (EPO+CI and Hypoxia+CI) were analyzed by Western blotting with an antibody to phosphorylated Smad1/5/8 and total Smad1/5/8. Blots were stripped and reprobed with an antibody to β-actin as loading control. A representative Western blot is shown. **(B and D)** Quantification of chemiluminescence to calculate the ratio of phosphorylated Smad1/5/8 relative to β-actin (pSmad/β-actin). This experiment was repeated twice and the combined results are shown as means ± SD with n=7. Statistical analysis was performed by 1-way ANOVA; *P<0.01; and **P<0.001 for comparison with control mice. n.s. indicates not significant.

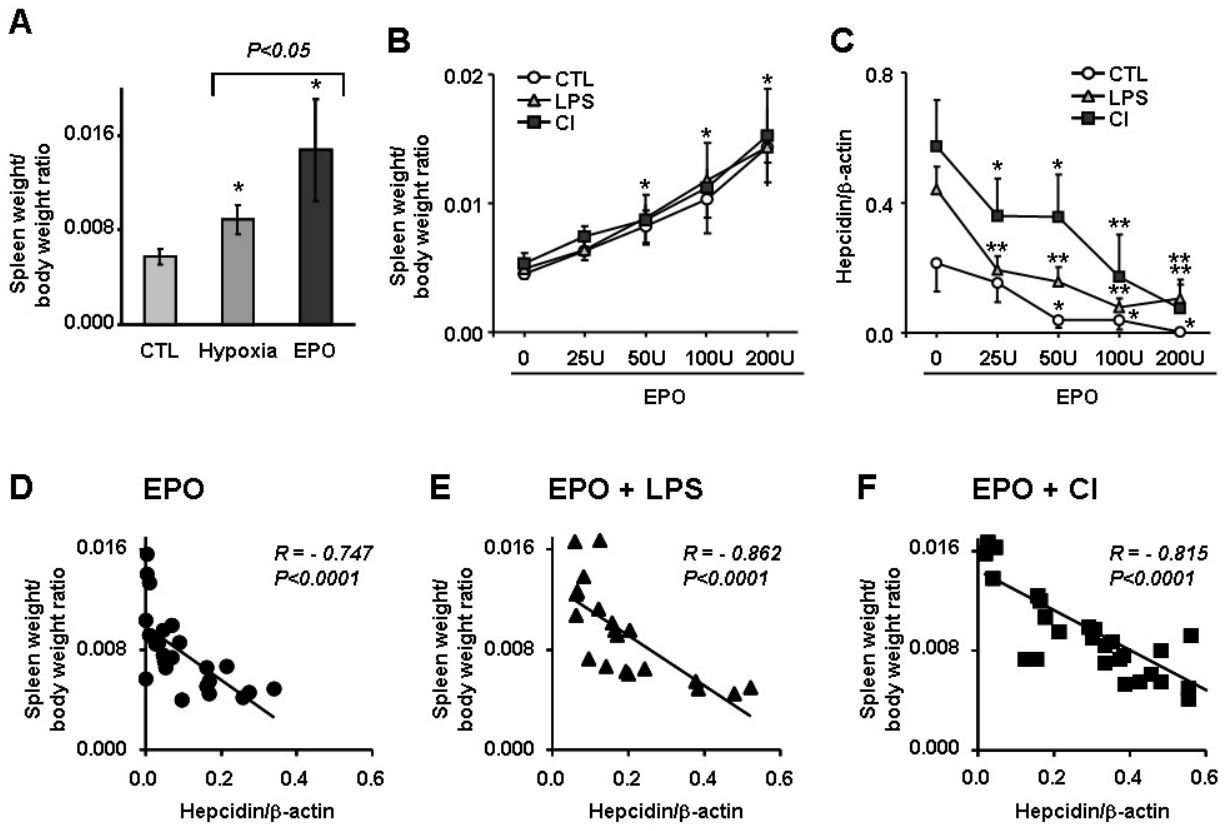

Figure 7 Relationship between erythropoiesis rate and hepatic hepcidin expression. (A) Spleen weight/body weight ratio in control, hypoxic and EPO-treated mice (50U for 4 days). **(B)** Spleen weight/body weight ratio and **(C)** hepatic hepcidin expression in mice treated with increasing amounts of EPO (total dosage over four days is shown) alone (control - CTL) and in combination with LPS (LPS) or CI-supplemented diet (CI). **(A, B and C)** Data are presented as means ± SD with n=5-6 mice per group. Statistical analysis was performed by 1-way ANOVA; **P*<0.01, and ***P*<0.001 for comparison with control mice. **(D, E and F)** Negative correlation between spleen weight/body weight ratio and hepatic hepcidin expression in mice treated with increasing amounts of EPO: **(D)** alone (control - CTL) and in combination with **(E)** LPS (LPS) or **(F)** CI-supplemented diet (CI). Hepatic hepcidin expression was quantified by real-time RT-PCR and normalized to β-actin. The hepcidin/β-actin ratios are shown.

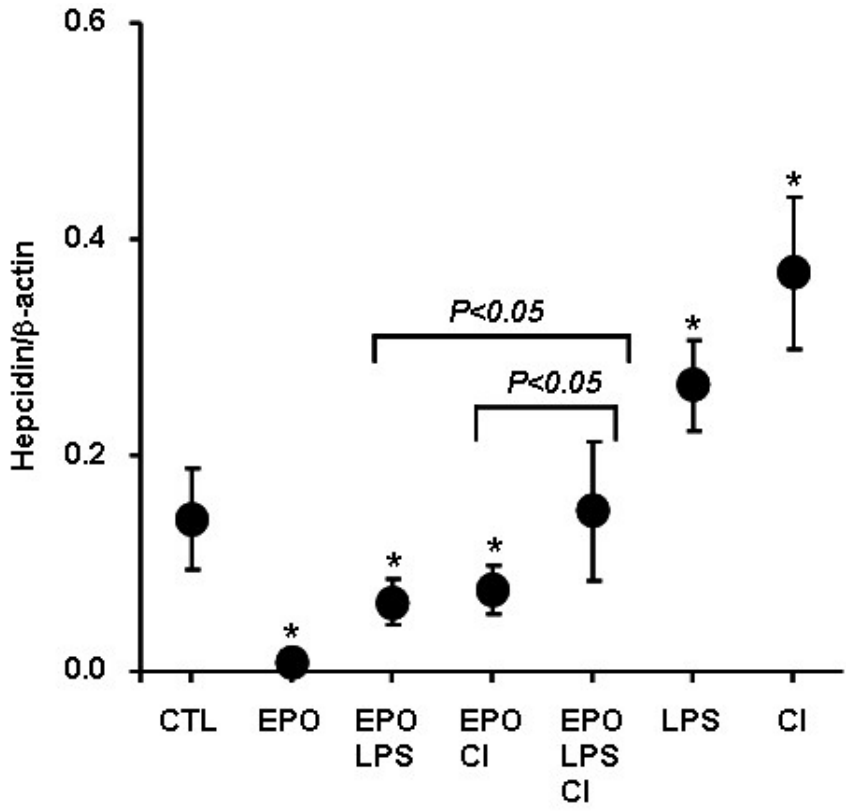

Figure 8 Additive effect of LPS and dietary iron on EPO-mediated hepcidin suppression. Hepatic hepcidin expression in control mice (CTL), mice treated with EPO alone (50U for 4 days; EPO) and in combination with LPS (EPO + LPS), with CI-supplemented diet (EPO + CI), with both LPS and CI (EPO + LPS + CI), with LPS alone (LPS) and CI-supplemented diet alone (CI). Data are presented as means ± SD with n=6 mice per group. Statistical analysis was performed by 1-way ANOVA; **P*<0.05 for comparison with control mice.

Chapter 4 GENERAL DISCUSSION

Iron and immunity are closely linked as many genes and proteins involved in iron metabolism also play a central role in controlling iron flux and preventing bacterial growth by limiting iron availability. Immune cells such as monocytes, macrophages, microglia and lymphocytes, via hepcidin-ferroportin regulation, can be resistant to bacterial invasion by regulating their iron levels and compartmentalization. The crosstalk between iron homeostasis and the immune system has long been studied. However, the molecular basis for their interaction remains poorly understood. Our studies concentrated on two molecules which are involved in both iron homeostasis and the immune response: lipocalin 2 and hepcidin.

Lipocalin 2 and other NTBI transporters

Iron-overload diseases (e.g., thalassemia, HH) are characterized by the appearance in the plasma of NTBI that significantly contributes to iron accumulation in the liver, pancreas, and heart. The identification of the components of NTBI delivery pathway(s) will therefore provide novel targets for therapeutics. Initially, the innate immune molecule Lcn2 was demonstrated to bind bacterial siderophores, and as such, Lcn2 can impair microbial iron acquisition through iron sequestration, hence contributing to limiting bacterial infection. Several studies indicated that Lcn2, once bound to iron-loaded siderophores, might additionally exert an iron trafficking function. Therefore, in our study, we selected Lcn2 as a candidate involved in NTBI uptake in iron overload conditions. More precisely, we investigated whether Lcn2, as a component of an alternative iron delivery system, may contribute to the pathophysiology of HH. For this purpose, we induced and characterized iron overload in *HfeLcn2* double knockout mice. We found that basal iron status and iron metabolism changes induced by dietary iron supplementation were indistinguishable between *Hfe* single knockout and *HfeLcn2* double knockout mice. Our results thus indicate that Lcn2 is dispensable for NTBI uptake by hepatocytes in HH. However, they do not exclude that Lcn2-mediated iron delivery may be involved in other pathologies. For example, it has been reported that enteric infection with *Salmonella* Typhimurium results in luminal accumulation of Lcn2 and that microbial resistance to Lcn2 defines a specific adaptation of *Salmonella* Typhimurium to growth in the inflamed intestine [394, 395]. Nairz, M., et al reported that mice lacking one or both *Hfe* alleles are protected from *Salmonella* Typhimurium septicemia, with prolonged survival and improved control over bacterial replication. Importantly, this resistance was related to increased production of Lcn2 and consequent reduction of iron availability to *Salmonella* within *Hfe*-deficient macrophages [396].

Additional studies on other NTBI candidate transporters in iron overload diseases include Zrt- and Irt-like protein-14 (Zip14) [397], and L-type voltage-gated calcium channels (LVDCCs). Zip14 is abundantly expressed at the major sites of organ damage in iron overload, namely in the liver, heart, and pancreas [398]. Overexpression of Zip14 in embryonic kidney (HEK) 293, SF9, or HeLa cell lines stimulates NTBI uptake [41, 399], while conversely NTBI uptake is inhibited when endogenous Zip14 expression is suppressed in AML12 mouse hepatocytes by small interfering RNA (siRNA) [41]. In a recent study, researchers expressed mouse Zip14 in oocytes and found that the uptake of ^{55}Fe in the presence of L-ascorbate was enhanced, indicating that Zip14 is an iron transporter for ferrous iron (Fe^{2+}) [400]. LVDCCs belong to a group of voltage-gated ion channels found in excitable cells (e.g., muscle, glial cells neurons, etc.) with permeability to Ca^{2+} ions [401]. In iron overload conditions, LVDCCs have been found to function as an iron transporter carrier of NTBI into cardiomyocytes and neuronal cells. Accordingly, LVDCC blockers were shown to be protective and able to attenuate myocardial iron accumulation in iron-overloaded mice. Recent studies also demonstrate a similar protective role of LVDCC blockers in neuronal cells, especially those neuronal cells highly sensitive to iron toxicity [402].

The mechanism(s) of NTBI delivery are not only important for iron overload diseases and as such crucial for the understanding of the pathophysiology of these diseases, but also for iron delivery in the nervous system. The study of Pelizzoni L et al. showed astrocytes have the potential to buffer excess iron, thereby protecting neurons from iron overload [403]. Recent studies found that DMT-1 accounts for at least half of iron accumulation in the brain (mainly in astrocytes) [404]. Thus, DMT-1 mediates ascorbate-dependent ferrous iron uptake by astrocytes with the remaining NTBI uptake occurring via a DMT-1-independent route(s). DMT-1 may also participate in NTBI uptake in Schwann cells, which are responsible for the myelination of the peripheral nervous system (PNS). These findings suggest that iron may play a fundamental role in Schwann cell maturation and, as a consequence, in PNS

myelination [405], further highlighting the importance of identifying and characterizing the molecular players of NTBI cellular uptake systems.

Hepcidin signaling pathway

Hepcidin is the master regulatory hormone of systemic iron metabolism. It has emerged as a central negative regulator of intestinal iron absorption and distribution. Two major signaling pathways communicate systemic stimuli to induce hepcidin mRNA expression in hepatocytes, namely, the inflammatory and iron-sensing pathways [148, 406]. In our study, we investigated hepcidin regulation through these 2 pathways in the presence of antagonistic stimuli. The study contributes to the elucidation of the dominance among the regulators of hepcidin expression, and in this regard we show that hepcidin expression levels are determined by the individual strength of opposing stimuli rather than by an absolute hierarchy among signaling pathways, as previously thought. Similar conclusions were later reported by another group in rats [407]. In this study, rats were maintained on a standard or iron-deficient diet and were treated with LPS to induce inflammation, or with phenylhydrazine to stimulate erythropoiesis. The study found that the increase in hepatic hepcidin levels induced by LPS was not affected by phenylhydrazine treatment but was blunted by iron deficiency. In addition, LPS-treated iron-deficient rats also showed lower hepatic inflammatory cytokine production, more specifically, IL-6 and TNF-α production. Hence, these data further support the findings in our study that hepcidin expression levels are determined by the relative strengths of competing stimuli. On the other hand, the generalized effect of iron deficiency in LPS-treated animals regarding cytokine production additionally indicates that adequate iron levels are necessary for a full acute-phase response to take place. Yet another study performed by Catterall, W.A., et al. used rat models of iron deficiency anemia (IDA), anemia of chronic disease (ACD) and ACD with concomitant true IDA to investigate the expression of hepcidin as well as the activation status of upstream signalling pathways [408]. They found that SMAD1/5/8 phosphorylation

and hepcidin expression are activated in ACD, while in ACD/IDA SMAD1/5/8 phosphorylation and hepcidin expression were suppressed. Furthermore, IDA resulted in reduced BMP6 expression and impaired SMAD1/5/8 phosphorylation, presumably effects related to the induction of the inhibitory factor SMAD7 and the reduction of the membrane-bound HJV. Altogether, their studies showed that, depending on the amount of body iron, inflammation-mediated stimulation of hepcidin expression could be blocked, again indicating that it is the strength of the opposing stimuli that determines the levels of hepcidin expression.

A lasting question in our study is how EPO-induced erythropoiesis suppresses both the inflammatory and iron-sensing pathways. Our study showed that the effect of erythropoiesis could be partially blunted by iron overload. In this regard, erythropoietic alterations have been documented in iron overload situations, mostly in HH. These include increases in Hb levels, reticulocyte counts, hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) [409-411] and most likely confirm the close relationship between iron availability for erythropoiesis and erythroid parameters. Interestingly, several binding sites for the erythroid transcription factor GATA-1 are found in the *HFE* promoter, which indicates a possible role for HFE in erythropoiesis [412]. In fact, a recent study investigated the possible involvement of HFE in erythropoiesis using *Hfe*-deficient mice [413]. It was found that *Hfe*-KO mice respond better than wild-type mice to different erythropoietic stress conditions, which could be explained by two distinct, but non-exclusive, mechanisms: 1. Enhanced iron mobilization driven by deficient hepcidin response in Hfe-deficient mice; and 2. Enhanced Tf-bound iron uptake by erythroid cells, since the authors found that HFE inhibits Tf-iron uptake in erythroid cells. Both mechanisms would ultimately contribute to the acceleration of recovery from erythropoietic stress observed in Hfe-deficient mice.

The involvement of HJV - a crucial component of the iron-sensing pathway during erythropoiesis - had also been studied using the Hjv-deficient mouse model. $Hjv^{-/-}$ mice treated with EPO showed a significant decrease in hepcidin expression, similar to that observed in Wt mice, suggesting that HJV is not an essential molecule for EPO-induced hepcidin down-regulation [414]. Another study by the same group revealed that hepatic HJV protein levels are not affected by iron status nor by EPO administration, once again implying that HJV is not an essential component for EPO-mediated hepcidin suppression [415]. Further studies focusing on TMPRSS6, a protease known to cleave HJV [206], also yield similar results. In both Wt, TMPRSS6 deficient and TMPRSS6/HJV deficient mice, hepcidin expression responds to EPO suppression [416]. More recently, the role of different components of the BMP6/SMAD4 signaling pathway have also been examined in the mouse model of chronic stimulated erythropoiesis with secondary iron-loading, using two distinct models: 1. The haemolytic agent phenylhydrazine; and 2. The β-thalassaemia mouse model Hbbth3/+ [417]. Both models rendered similar results, in that SMAD1/5/8 phosphorylation and suppression of hepcidin in the liver are not associated to BMP6 induction. In other words, hepcidin suppression due to erythropoiesis demand is independent of BMP/SMAD4 signaling [415].

The influence of erythropoiesis in iron metabolism is not only limited to the inhibition of hepcidin expression. In monolayers of human intestinal Caco-2 cells, EPO was shown to be able to induce the expression of apical DMT-1 and basolateral FPN1, hence enhancing iron transport across monolayers. Therefore, EPO not only functions as a strong inhibitor of hepcidin expression, but also directly acts on enterocytes to increase iron absorption [418].

Historically, hepcidin inducers have been more rapidly identified, while molecular mechanisms of hepcidin suppression remained to be identified. This state of affairs changed in the last years with the identification of several hepcidin suppressors. For example, two

candidates have been recently identified as being involved in modulating hepcidin suppression in response to increased erythropoietic activity, namely growth differentiation factor-15 (GDF-15) and twisted gastrulation 1 (TWSG1) [161, 162], while SMAD7 has been identified as a general hepcidin suppressor using high throughput siRNA screening [419]. SMAD7 is an inhibitory SMAD protein that blocks TGF-β and BMP signaling through a negative feedback loop. Further studies showed that SMAD7 is co-regulated with hepcidin by BMPs in primary murine hepatocytes and that SMAD7 overexpression completely abolishes hepcidin activation by BMPs and TGF-β. In support of such a role, a distinct SMAD regulatory motif within the hepcidin promoter has also been identified, and this motif was shown to be involved in SMAD7-dependent hepcidin suppression. Heparin, another molecule used at pharmacologic concentrations, was found to strongly inhibit hepcidin expression in hepatic HepG2 cells. This inhibition was associated to sequestration of BMP6 and blocking of SMAD4 signaling [420]. Further evidence that heparin is a hepcidin modulator was provided by the observation that administration of heparin to prevent deep vein thrombosis in 5 patients was accompanied by a strong reduction of serum hepcidin and an increase of serum iron, presumably as a consequence of lower hepcidin levels favoring the release of iron into the circulation [420].

An important aspect of hepcidin regulation that is not yet fully understood is how hypoxia suppresses hepcidin expression. Is it secondary to increased erythropoiesis driven by hypoxia-stimulated EPO or to a more direct effect of hypoxia on hepcidin expression? The answer to this question remains controversial. Several lines of evidence support the possibility that hypoxia induces hepcidin suppression through EPO stimulation, as EPO administration leads to reduced circulating hepcidin levels in humans [157] and to reduced hepcidin gene expression in mice [158, 421]; direct treatment of HepG2 cells with EPO causes a suppression in hepcidin expression [422]. More recent studies revealed that hypoxia-inducible factor-2 (HIF2) is not required for hepcidin suppression under the conditions of an adaptive response to IDA [168]. Liu Q et al group generated a mouse model that dissociates Hif activation from

Epo synthesis. They found that hypoxia/Hif-mediated suppression of the hepcidin gene (*Hamp1*) happens indirectly through stimulation of EPO-induced erythropoiesis [169]. On the other hand, some available evidence supports a direct effect of hypoxia on hepcidin suppression. For example, HIF-recognition elements have been identified in the promoter region of the murine hepcidin gene and binding of HIF-1 was shown to negatively transactivate the hepcidin promoter [167]. Accordingly, hepatic HIF-1 inactivation leads to blunted hepcidin suppression in conditions of iron deficiency [167]. More recently, studies involving von Hippel Lindau (VHL) protein further indicate a direct role for hypoxia in regulating hepcidin. In humans, VHL^{R200W} homozygosity, which is common in Chuvashia, Russia, is characterized by elevated HIF-1α and HIF-2α levels, increased RBC counts, propensity for thrombosis, and early mortality [423]. In these patients, serum hepcidin levels were found to be negatively related to VHL^{R200W} homozygosity but not to serum EPO levels, hemoglobin, or RBC counts. These findings indicate that hypoxic responses leading to decreased expression of hepcidin may be independent of EPO levels and RBC counts [423].

Parallel to these advances regarding the BMP6/SMAD4 signaling pathway for hepcidin expression, some breakthroughs have also been made regarding the inflammatory pathway, initiated by activation of JAK/STAT3 signaling. In fact, another cytokine belonging to the IL-6 family of cytokines, named Oncostatin M (OsM), has been identified as a potent inducer of hepcidin, and this induction is also due to activation of the JAK/STAT signaling pathway [424].

Hepcidin is mainly expressed in liver hepatocytes, and the signaling pathways discussed so far are pertinent to hepcidin production by these cells. However, hepcidin is also expressed by macrophages [134-137]. In these cells, LPS and other bacterial components induce hepcidin through Toll-like receptor- (TLR) signaling [140]. The induction of hepcidin in macrophages results in iron retention in the macrophage by which the host reduces availability of iron from

pathogens and may promote host defense [136, 138]. These studies also indicate that autocrine regulation of iron accumulation in macrophages by hepcidin may affect the levels of proinflammatory cytokine production. More recent studies in macrophage cell lines and primary peritoneal macrophages suggest that BMP4 or BMP6 can only induce hepcidin expression in macrophages if in the presence of LPS [139]. Accordingly, NF-kB inhibitors can abolish BMP/LPS-induction of hepcidin. These results show that in macrophages, unlike hepatocytes, BMPs regulate hepcidin expression in a LPS-NF-kB dependent manner, highlighting the close relationship between the iron-sensing and inflammatory pathways for hepcidin regulation.

Therapeutic applications of hepcidin

Hepcidin plays a central role in iron metabolism, implying that both increased or decreased production of hepcidin potentially can contribute to the development of iron-related diseases. Therefore, targeting hepcidin as a means to appropriately regulating iron metabolism represents an attractive strategy for the treatment of iron-related diseases.

Elevated hepcidin production is one of the pathophysiological mechanisms involved in the development of ACD. Reducing hepcidin production would thus augment iron bioavailability from the diet and from body iron stores to meet erythropoietic demand. Presently, several strategies have been pursued to inhibit hepcidin production, including direct inhibition of hepcidin function, prevention of hepcidin transcription, and enhancing the resistance of ferroportin to hepcidin action.

Direct inhibition of hepcidin function, such as anti-hepcidin antibodies, RNA interference (RNAi) and antisense oligonucleotides against hepcidin, hepcidin-binding proteins and hepcidin spiegelmers, have all been already tested for their ability to inhibit hepcidin function. For example, Ab12B9m, a humanized antihepcidin monoclonal antibody, with proven high affinity to human and monkey hepcidin, has been shown to block hepcidin action on ferroportin[425]. Most recently study with a mouse model of inflammation indicate that the mechanism of action of this antibody is due to an increase in available serum iron leading to enhanced red cell hemoglobinization [426]. The methods of mediating RNAi effect include small interfering RNA (siRNA), short hairpin RNA (shRNA) and bi-functional shRNA. ShRNA directed at hepcidin have also been tested in mouse models to correct ACD [427]. However, RNAi approach is still a long way from achieving similar success in humans, as several obstacles must be bypassed, including effective designing of the RNAi without off-target effects, stability of the RNAi *in vivo*, lack of biocompatibility of the delivery system,

and nonspecific targeted delivery to organs/cells [428]. In summary, antisense oligonucleotides either targeting hepcidin or hepcidin regulators are currently at the discovery stage of development.

Another approach uses knowledge gained about anticalins, which are engineered lipocalins that have high affinity for targeted ligands and block their function [429]. A good example is provided by PRS-080, a recently developed anticalin that specifically targets human hepcidin. PRS-080 has been shown to effectively neutralize the development of short-term hypoferremia induced by injection of synthetic human hepcidin in mice [430]. Further studies are still under investigation.

Last but not least, aptamers have also been investigated for their ability to inhibit hepcidin function. Aptamers are synthetic oligonucleic acid or peptide molecules that bind specific target molecules, and as such can be used to block the function of certain ligands [431]. As an example, NOX-H94 is a newly developed aptamer that binds to human hepcidin. Compared to other hepcidin antagonists, NOX-H94 has shown a high resistance to nuclease activity, good *in vivo* stability, and low immunogenicity [431, 432]. Since NOX-H94 was shown to effectively block hepcidin production in animal models, it has moved onto the clinical stage of testing [433].

Other than directly targeting hepcidin, many research teams have focused on targeting the two major signaling pathways identified for hepcidin regulation, namely the BMP/SMAD4 and IL-6/STAT3 pathways. LDN-193189 is a potent inhibitor of BMP receptor Type I (ALK2 and ALK3) [434] that can inhibit excessive BMP signaling *in vivo* [435]. LDN-193189 can reverse anemia associated with streptococcal peptidoglycan-polysaccharide (PG-APS)-induced chronic arthritis in rats [436] and prevent acute inflammatory anemia induced by turpentine injections in mice [437]. However, LDN-193189 is not a specific inhibitor of the BMP/SMAD

pathway, and it can also block other pathways such as the MAPK/ERK pathway [438].

Soluble HJV is known to negatively regulate the BMP/SMAD pathway. A soluble form of the human HJV protein linked to the constant region of IgG1 (HJV.Fc) has been proved to inhibit BMP-mediated hepcidin expression both *in vitro* and *in vivo [185, 436]*.

Anti-BMP6 monoclonal antibody is another option to inhibit BMP/SMAD pathway. It has been proven that anti-BMP6 antibody could effectively decrease hepcidin expression in both healthy and Hfe transgenic mice [187, 439]. However, the cross-reactivity of anti-BMP6 antibody with other BMPs should be taken into consideration in the development of this therapeutic approach.

The therapeutic strategy of inhibiting the IL-6/STAT3 pathway has mainly focused on blocking the IL-6 ligand/receptor and JAK/STAT3 signaling cascade. The anti-IL-6 receptor antibody (anti-IL-6R) Tocilizumab and anti-IL-6 chimeric monoclonal antibody, Siltuximab could effectively suppress hepcidin production in Multicentric Castleman's Disease, a rare anemia associated with excessive production of IL-6 [440, 441]. The major issue of blockage of IL-6 activity is increasing the risk of infection [442, 443].

The JAK2 inhibitor AG490 inhibits the phosphorylation of STAT3 by JAK2 and synthetic peptide inhibitor of STAT3 (PpYLKTK) disrupts pSTAT3 dimerization. In a mouse hepatic co-culture system, both compounds were able to inhibit STAT3 phosphorylation and thus decrease hepcidin production [444]. So far, these effects have not been tested *in vivo*.

Hepcidin exerts its function through binding to and inducing the degradation of the iron export protein ferroportin. Thus, strategies that stabilize ferroportin on the cell surface are expected to exhibit the same effects as reducing hepcidin production. Cardiac glycosides are molecules

which were found by high-throughput screening approach that have been shown to prevent ferroportin internalization [445]. In addition, an anti-ferroportin mAb has been developed recently, which can block hepcidin-ferroportin interaction while maintaining ferroportin function.

Current therapies for iron overload diseases (HH and β-thalassemia) are largely dependent on phlebotomy and iron chelation. However, both options may have severe side effects and may not be well tolerated by all patients. The loss of hepcidin expression results in or contributes to iron overload in these disorders [446]. Therefore, it is rational to develop hepcidin replacement therapies to prevent and treat iron overload in these diseases. Natural bioactive 25 amino acid long hepcidin is limited by its high cost, short half-life and low absorption rate [447]. Recently, the Ganz's group revealed that a thiol-disulfide interaction between ferroportin C326 and the hepcidin disulfide cage is essential to the stabilization of hepcidin-ferroportin binding [448]. Based on this, they developed several 7–9 N-terminal amino acids forms of hepcidin (minihepcidins) that include a single thiol cysteine, comprising the minimal structure that mimics hepcidin activity in both reporter cells and in mice [448]. They further developed an optimized minihepcidin (PR65) with superior potency, duration of action and reasonable costs. So far, PR65 has been tested in both iron-depleted and iron-loaded hepcidin knockout mice. The results indicate that PR65 could be beneficial for the prevention of iron overload or ameliorate the pre-existing iron overload [449]. The side effect is the dose-dependent iron-restrictive anemia from excess minihepcidin, which should be considered in future therapeutic applications.

Iron metabolism and immune system interaction

A sophisticated network of cells and genes/proteins are involved in both the regulation of iron metabolism and defense against invading pathogens. Changes in iron homoeostasis, either iron deficiency or iron excess, may alter the ability of man to combat pathogen insult. On one hand, these alterations reflect the effects of iron on pathogen growth, and on the other, they may result from iron's influence on the host response to the pathogen.

Macrophages play important roles in both systemic iron homoeostasis and host defense. Iron content inside macrophages affect its ability of combating invading organisms. During the process of phagocytosis of foreign pathogens, NADPH oxidase, a multicomponent enzyme, generates superoxide inside the phagolysosome, and subsequently, produces Fe^{2+}-catalysed hydroxyl radicals, which perform the function of phagocytic microbial killing. Iron levels inside macrophages affect the activity of NADPH oxidase, as iron depletion decreases while iron loading increases its activity [450]. Iron levels inside macrophages also influence the production of inducible nitric oxide synthase (iNOS), which is related to the cytotoxicity of macrophages. Iron loading suppresses while conversely, iron depletion enhances iNOS activity [450]. Microbial survival is related to macrophage iron content [228]. Iron-deficient macrophages, as seen in HH, have a lower susceptibility to bacterial infection. In contrast, iron overloaded macrophages, as seen in β-thalassemia, have higher susceptibility to bacterial infections [451]. The outcome of intracellular pathogens, whether growth or destruction, is dependent on the iron content inside phagosomes. Nramp1 is a phagosomal iron transporter, which transports iron from phagosomes to the cytosol thereby suppressing the growth of intra-phagosomal pathogens. Hence, inactive Nramp1 results in uncontrolled growth of certain pathogens which was related to increased mortality [451]. Several studies have shown, both *in vivo* and *in vitro*, that infected macrophages release more hepcidin. Hepcidin, in this case, may function in an autocrine fashion to modulate local antimicrobial activity and inhibit iron

released from adjacent macrophages [135, 137].

In general, iron deficiency may result in the suppression of immunity and, in fact, iron deficient individuals are more susceptible to infections. This increased susceptibility to infections appears to be related to altered innate immunity, T cell-mediated and adaptive antibody responses, as well as reduced neutrophil function [452]. The outcome of iron deficiency is also affected by variations in baseline iron status, severity of deficiency and other existing nutritional problems [452]. Iron supplementation could correct immune defects in iron deficiency, as it reduces the rate of respiratory infections in infants [452]. However, iron supplementation, particularly in the tropics, can also have deleterious effects on susceptibility to malaria and tuberculosis malaria infection [452, 453].

Iron overload, depending on the pathophysiological mechanism, has a different impact on immunity. In HH, iron is mostly deposited in parenchymal cells. In general, due to the low iron content in macrophages, there is no evident increase in susceptibility to infections. However, HFE-related hemochromatosis may predispose individuals to infection of certain rare pathogens, such as *Yersinia enterocolitica* and *Vibrio vulnificus* [454-456]. On the bright side, it is also associated with an attenuated inflammatory response to *Salmonella* infection [457]. Lowered intracellular iron levels in HFE-related hemochromatosis reduces the biosynthesis of proinflammatory cytokines such as IL-6 and TNF-α which may be related to impaired TLR4 signaling [458]. Mice lacking one or both Hfe alleles are protected from septicemia with *Salmonella Typhimurium*, which is related to enhanced iron-capturing peptide and lipocalin 2 production, thereby reducing iron availability for *Salmonella* growth[396]. Thus, the high prevalence of genetic hemochromatosis may result from evolutionary advantages that enhance host resistance to systemic infections with *Salmonella*. In contrasy, infection is the most prevalent complication in thalassemic patients. In β-thalassemia, iron is mostly deposited in reticuloendothelial cells, thus affecting normal phagocytosis by

macrophages and suppressing killer cell activity, thereby increasing susceptibility to infections [215].

Overall, it is clear now that iron status affects immune system functions. As an essential nutrient to both mammalian cells and pathogens, iron has a direct effect on the growth and virulence of microbial pathogens. Alteration of iron status influences the immune response in multiple ways. The challenge for researchers is to study the impact of iron on lymphocyte biology and adaptive immunity, particularly effector T cell functions. Moreover, newly emerging cytokines and immunomodulators may be potential regulators of iron homeostasis that deserve more attention.

Concluding remarks

Over the last ten years, the understanding of iron metabolism has dramatically increased due to the discovery of hepcidin and many other molecules that are involved in iron metabolism. Our studies contributed to the knowledge of the dominance between regulators of hepcidin expression. We demonstrated that final hepcidin levels are determined by the individual strength of the regulators rather than by an absolute hierarchy among the pathways. We excluded lipocalin 2 as an NTBI transporter in the context of HH. Via different mechanisms, both hepcidin and lipocalin 2 are able to sequester iron and both possess antimicrobial properties. Hepcidin has intrinsic defensin-like antibacterial activity and induces iron sequestration in macrophages while Lcn2 binds siderophores and interrupts enterochelin-specific iron acquisition by bacteria. Future directions inspired by these studies should include the investigation of the mechanisms for hepcidin regulation and the possible impact of other immune cells, such as NKT cells, on iron metabolism. Furthermore, exploring the interaction between the abnormalities of immune functions associated with disorders of iron homeostasis and the actual biochemical reactions affected by changes in cellular iron levels should also yield important information critical for the advancement of the field.

CHAPTER 5 BIBLIOGRAPHY

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