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Study of the role of the adaptor protein MyD88 in the iron-sensing pathway and of the effect of curcumin in the development of anemia in a DSS-induced colitis mouse model

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RÉSUMÉ

Étude du rôle de la protéine adaptatrice MyD88 dans la voie de détection du fer et de l'effet de la curcumine dans le développement de l'anémie dans un modèle murin de colite induite par le DSS

L'hepcidine est l'hormone peptidique essentielle pour la régulation systémique de l'homéostasie du fer, en déclenchant la dégradation de la ferroportine et en évitant le développement d'une surcharge ou d'une carence en fer. L'expression de l'ARNm de l'hepcidine HAMP est contrôlée par deux voies principales, la voix de signalisation inflammatoire et la voie de détection en fer, BMP/SMAD4.

Il a été rapporté que MyD88, la protéine adaptatrice impliquée dans l'activation des cytokines via la stimulation des récepteurs toll-like (TLRs), contribue à l'expression de l'hepcidine dans la voie inflammatoire. Ainsi, comme les deux voies de signalisation révèlent un chevauchement de l'induction de l'hepcidine, nous avons étudié le rôle potentiel de MyD88 dans la détection du fer en analysant le métabolisme du fer chez des souris MyD88 déficients (*MyD88*$^{-/-}$). Nous avons analysé le mécanisme d'action potentiel de MyD88 dans la voie de signalisation BMP/SMAD4 pour l'induction de l'hepcidine. Nous avons étudié si l'inhibition de MyD88 causée par la curcumine, rapportée dans différentes études, pourrait être liée au mécanisme de déséquilibre du fer éventuellement déclenché par les propriétés de chélation du fer de la curcumine. De plus, nous avons évalué les effets de l 'équilibre systémique du fer et de l'expression de l 'hepcidine, dans un modèle de souris colite à base de sodium dextran sulfate (DSS) avec un régime alimentaire suffisant en fer complété de curcumine.

Nous avons constaté que les souris *MyD88*$^{-/-}$ ont une concentration élevée de fer dans le foie en raison de leur incapacité à contrôler les niveaux d'hepcidine, et ont une faible concentration de la protéine Smad4. De plus, nous avons démontré que MyD88 interagit avec SMAD4 et induit l'expression de HAMP. De plus, nous avons rapporté que l'inhibiteur de SMAD (SMAD6) induit également la dégradation protéomique de MyD88, en agissant comme un mécanisme de rétroaction négative pour limiter l'induction de l'hepcidine. Nous

avons montré que la mutation de MyD88^{L265P}, fréquemment rencontrée dans les lymphomes, augmente l'expression de l'hepcidine et l'accumulation de fer dans les lymphocytes B. Nous avons ainsi souligné un nouveau rôle pour MyD88 dans la voie de signalisation SMAD et la régulation de l'homéostasie du fer en dehors du contexte de la signalisation TLRs. Enfin, nous avons trouvé que les souris nourries avec de la curcumine avant administration de DSS, ont une colite aggravée et une anémie légère, suite à une déplétion en fer sur les tissus de la rate et du foie et diminution de l'expression de l'hepcidine.

En conclusion, notre étude révèle une nouvelle compréhension de la régulation des voies de détection du fer et une meilleure compréhension et gestion des potentielles cibles thérapeutiques comme la curcumine dans les maladies chroniques telles que les maladies inflammatoires chroniques de l'intestin.

Mots clés: MyD88, SMAD4, hepcidine, la voie de détection en fer, curcumine

ABSTRACT

Hepcidin is the peptide hormone essential for the regulation of systemic iron homeostasis. Hepcidin binds and triggers the degradation of ferroportin, the only known iron protein exporter, avoiding, in this case, the development of iron overload or iron deficiency. Hepcidin mRNA expression is controlled by two major pathways, the inflammatory and iron sensing signaling pathways.

It has been reported that MyD88, the adaptor protein involved in the activation of cytokines via toll-like receptor stimuli, contributes to hepcidin expression in the inflammatory pathway. Thus, since both pathways, the iron sensing and the inflammatory, reveal overlap in hepcidin induction, we investigated the potential role of MyD88 in iron sensing by analyzing iron metabolism in MyD88-deficient mice (*MyD88*$^{-/-}$). We also analyzed the potential mechanism of action of MyD88 in the BMP/SMAD4 signaling pathway for hepcidin induction. Furthermore, we investigated the modulation of MyD88 protein expression in chronic inflammation settings associated with systemic iron imbalance. We evaluated the effect of the anti-inflammatory and MyD88 modulator agent curcumin, the active ingredient of the rhizome Curcuma longa that also has iron chelating properties in a murine model of inflammatory bowel disease (IBD). We analyzed the effects of systemic iron balance and hepcidin expression in a dextran sulfate sodium (DSS)-defined colitis mouse model fed on an iron-sufficient diet supplemented with curcumin.

We found that *MyD88*$^{-/-}$ mice have a high concentration of iron in the liver due to their inability to control hepcidin levels, and they revealed low Smad4 protein. Furthermore, we showed that MyD88 interacts with SMAD4 and induces hepcidin mRNA expression. Moreover, we reported that BMP6 stimulation, which increases SMADs inhibitor SMAD6 expression, also induces MyD88 proteasomal degradation as a negative feedback mechanism to limit hepcidin induction. We showed that the MyD88 gain-of-function L265P mutation, frequently encountered in B-cell lymphomas, enhances hepcidin expression and iron accumulation in B cells. In this way, we highlighted a new role for MyD88 in the SMAD signaling pathway and iron homeostasis regulation outside the

context of toll-like receptor signaling. Finally, we found that mice fed with curcumin prior to DSS administration developed an aggravated colitis and mild anemia following iron depletion in the spleen and liver tissues and the decrease of hepcidin expression.

In conclusion, these studies provide new insights into iron-sensing pathway regulation and a better understanding and management of the potential therapeutic target of curcumin in chronic disease such as inflammatory bowel disease.

Keywords: MyD88, SMAD4, hepcidin, iron-sensing pathway, curcumin

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List of acronyms and abbreviations:

ABCB	ATP-binding cassette, subfamily B
ACD	Anemia of chronic disease
ACO1	Aconitase 1
ACO2	Aconitase 2 (Mitochondrial aconitase)
AD	Adrenaline
ALAS2	δ-aminolevulinic acid synthase 2
Alk	Activin-like
ARNT	Aryl hydrocarbon receptor nuclear trans-locator
β2m	β_2- microglobulin
BMP	Bone morphogenetic protein
CAT	Catalase
CBCL	Cutaneous diffuse large B cell lymphoma
CD	Cluster differentiation
C/EBPα	CCAAT-enhancer binding protein
CHO	Chinese hamster ovary
CLL	Chronic Lymphocytic Leukemia
CO	Carbon monoxide
CRP	C-reactive protein
CTRP15	C1q/TNF-related protein family member 15
DAMP	Damage-associated molecular pattern
DcytB	Duodenal Cytochrome B
DD	Death domain
2,5-DHBA	2,5-dihydroxybenzoic Acid
DFP	Deferiprone
DFO	Deferoxamine
DFX	Deferasirox

DPC4	Deleted in pancreatic cancer-4
DMT1	Divalent metal transporter 1
DNA	Deoxyribonucleic acid
DSS	Dextran sulfate sodium
EPO	Erythropoietin
ERFE	Erythroferrone
EXOC6	Exocytosis protein 6
FBXL5	F-box and leucine-rich repeat protein 5
Fe	Iron
Fe/S	Iron-sulfur
FtH	Ferritin heavy chains
FtL	Ferritin light chains
FtMt	Mitochondrial ferritin
FLCVR	Feline leukaemic virus receptor
FPN-1	Ferroportin-1
GDF15	Growth differentiation factor 15
GLRX5	Glutathione redoxine 5
GPx	Glutathione peroxidase
GSH	Glutathione
HAMP	Hepcidin antimicrobial peptide
Hb	Hemoglobin
HCP1	Heme carrier protein 1
HFE	Hemochromatosis gene
HLA-H	Human leukocyte antigen- H
HGF	Hepatocyte growth factor
HH	Hereditary hemochromatosis
HRE	Hypoxia responsive-element
HIF-2α	Hypoxia-inducible factor-2α

HIV	Human immunodeficiency virus
HFN4α	Hepatocytes nuclear factor 4-α
HJV	Hemojuvelin
HO-1	Hemoxigenase 1
HO•	Hydroxil radical
H_2O_2	Hydrogen peroxide
Hx	Hemopexin
IBD	Inflammatory bowel disease
ID	Intermediate domain
IL	Interleukin
IRI	Ischemia-reperfusion injury
IRAK	Interleukin- (IL-) 1 receptor-associated kinase
IRE	Iron responsive element
IREB2	Iron responsive element binding protein 2
IREG1	Encoded iron-regulated 1
IDA	Iron deficiency anemia
IRIDA	Iron-refractory iron deficiency anemia
IRP	Iron regulatory protein
ISC	Iron Sulfur Cluster
Jak2	Janus kinase 2
JNK	Jun N-terminal kinase
KO	Knockout
LEAP1	Liver-expressed antimicrobial peptide
LIP	Labile iron pool
LO•	Alkoxy radical
LOO•	Peroxy radical
LPL	Lymphoplasmacytic lymphoma
LPS	Lipopolysaccharide

LRP	Low density lipoprotein
MAD	Malonyldialdehyde
MAPK	Mitogen-activated protein kinase
Mb	Myoglobin
MCH	Mean corpuscular hemoglobin
MCV	Mean corpuscular volume
MD-2	Myeloid differentiation factor 2
Mfrn1	Mitoferrin 1
MHC-1	Major histocompatibility complex class-1
MTP1	Metal transporter protein 1
MyD88	Myeloid differential factor 88
NADH	Nicotinamide adenine nucleotide
NASH	Nonalcoholic steatohepatitis
NE	Norepinephrine
NF-κB	Nuclear factor- κB
NIF	Nitrogen fixation
NTBI	Non-transferrin bound iron
NRAMP1	Natural resistance-associated macrophages protein 1
NRAMP2	Natural resistance-associated macrophages protein 2
O_2^-	Superoxide anion
PAMP	Pathogen-associated molecular pattern
PCFT	Proton-couple folate transporter
PCNSL	Primary central nervous system lymphoma
PDG	Platelet-derived growth factor
PHD	Prolyl-hydroxylases
RAF	Rapidly accelerated fibrosarcoma
RBC	Red blood cell
RNA	Ribonucleic acid

ROOH	Hydroxyl peroxide
ROS	Reactive oxygen species
SCD	Sickle-cell disease
SCF	SKP1-CUL1-F-box
SDH	Succinate dehydrogenase
SOD	Superoxide dismutase
SLC	Solute carrier
SMAD	Small mother against decapentaplegic
STEAP3	Six-transmembrane epithelial antigen of prostate 3
Tf	Transferrin
TfR/TFRC	Transferrin receptor
TIR	Toll-interleukin 1 receptor
TIRAP	Toll-interleukin 1 receptor domain containing adaptor protein
TLR	Toll-like receptor
TMPRSS6	Transmembrane protease serine 6
TNF	Tumor necrosis factor
TRAF	Tumour necrosis factor (TNF) receptor-associated factor
TAK	TRAF-activated kinase
TRAM	TRIF-related adaptor molecule
TRIF	TIRAP inducing IFN-β
TWSG1	Twisted gastrulation BMP signaling modulator 1
USF2	Upstream stimulatory factor 2
V-ATPase	Vacuolar-type H^+-ATPase
vHL	von Hippel-Lindau
WM	Waldenström macroglobulinemia
ZIP	Zinc transporter protein or
ZRT/ IRT	Zinc-regulated transporter/Iron regulated transporter

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CHAPTER 1

1. INTRODUCTION

1. Iron as an essential element in living organisms

Iron is a fundamental trace element in living organisms [1-3]. This transition metal is the second most abundant metal element in the world after aluminum and the fourth most abundant element in the earth's crust behind oxygen, silicon and aluminum [4]. Moreover, the earth's crust is composed of approximately 5% iron**.**

Iron exists fundamentally mainly in two different states, the reduced ferrous iron II (Fe^{2+}) and the oxidized ferric iron III (Fe^{3+}). Ferrous iron is more soluble in water than ferric iron at physiological pH, 10^{-1} M and 10^{-18} M respectively [2, 5]. However, despite its geological abundance, iron is difficult to uptake by the organisms since in contact with oxygen it forms oxides, which are highly insoluble, hence limiting its capture in biologically useful forms [6].

The importance of iron as a chemical element can be explained by its requirement in different protein structures and its usage as a basis for multiple metabolic processes that are indispensable for life such as oxygen and electron transport, cellular respiration, deoxyribonucleic acid (DNA), and heme synthesis [7-10]. However, since iron has the property to take and give electrons in different metabolic processes [11, 12], it can also lead to the creation of free radicals (reactive oxygen species [ROS]) and oxidative stress. In turn, ROS can damage the DNA and cause lipid peroxidation and oxidative stress, and cell death [13-16]. This means that iron deregulation may play an important role in the development and progression of several diseases [10, 17]. Iron needs therefore to be tightly regulated both at the cellular and systemic levels [3, 10, 18].

To conclude, it is fundamental to better understand the mechanisms by which iron is regulated and identify iron-binding proteins and key regulatory molecules that coordinate iron homeostasis, in order to find multiple therapeutic opportunities to prevent iron overload or deficiency.

1.1. Iron-containing proteins

Iron-binding proteins are highly conserved between species and are classified fundamentally into three distinct classes: hemoproteins, iron-sulfur proteins, and iron-binding proteins.

1.1.1. Heme proteins

Nearly half of all the proteins in nature are metalloproteins [19, 20] and in this group heme proteins have an essential role by accomplishing primordial and different functions that are fundamental for life of aerobic organisms [21, 22]. Heme proteins are expressed ubiquitously, and containing iron in the reduced ferrous form Fe^{2+} in the center of a highly hydrophobic, planar, protoporphyrin IX ring, a tetrapyrrole core at the center of which is encased an iron atom [23]. The prosthetic group is called hemin when the iron atom is present in the oxidized ferric form Fe^{3+}. The formation of protoporphyrin IX consists of a series of conserved enzymatic reactions, with the last step culminating in the enzymatic insertion of iron into the protoporphyrin IX ring [24, 25].

Heme proteins are able to perform a large range of biological functions from electron transfer (cytochrome c [cyt c] and cytochrome b5 [cyt b5]) [26]**,** oxygen binding and transport (myoglobin [Mb] and hemoglobin [Hb]) [27, 28]**,** catalysis (cytochrome P450 [CYP]) [29, 30], and signaling (e.g., carbon monoxide CO sensor heme protein, known as CooA) [31]. Hemoglobin, present in circulating erythrocytes, represents roughly two-thirds of the total body iron [19, 20].

1.1.2. Iron-sulfur proteins

Iron-sulfur proteins (Fe/S), are characterized by iron bound *via* sulfur-containing ligands (clusters), more precisely, iron with variable states of oxidation linked to sulfide to form commonly [2Fe-2S], [4Fe-4S] and [3Fe-4S] clusters [32-34]. Iron-sulfur clusters (ISC) contain different kinds of proteins, such as ferredoxins, frataxin, nicotinamide adenine dinucleotide (NADH) dehydrogenase, hydrogenases, coenzyme Q, cytochrome c reductase, succinate, and nitrogenase**.** These proteins are essential cofactors involved in a variety of biological processes such as enzymatic reactions, electron transport,

photosynthesis, nitrogen fixation [35], DNA replication and repair, gene expression regulation, and antiviral response [36-39]. In eukaryotes, Fe/S proteins are present in various cellular compartments (mitochondrion, endoplasmic reticulum, cytosol, and the nucleus) [40-42].

1.1.3. Iron-binding proteins (Non-heme/Non-ISC)

Iron-binding proteins, also known as non-heme/non-iron sulfur clusters (ISCs), are proteins in which iron is directly associated with proteins. Iron-binding proteins are important for various cellular activities such as DNA synthesis, cell proliferation and differentiation (ribonucleotide reductase) gene regulation, drug metabolism, and steroid synthesis [43]. Other proteins, of this category, such as ferritin and lactoferrin play a fundamental role on iron transportation and storage [43-45].

1.2. Iron toxicity

Iron homeostasis is tightly regulated to orchestrate the distribution of iron in the body but also to prevent the possibility of free iron becoming cytotoxic. During biological redox reactions, free iron has the potential to become cytotoxic when electron exchanges with oxygen are unrestricted and catalyze the production of ROS via Fenton-Haber Weiss reactions (see Table 1) [11, 15, 46-48]. ROS are responsible for macromolecular damage and play an important role in the progression of various diseases' pathophysiology (diabetes, atherosclerosis, inflammation, ischemia-reperfusion injury [IRI], and neurologic disorders such as Parkinson's or Alzheimer's disease) [16, 49-51]. ROS creation also occurs during cellular response to xenobiotics, cytokines and bacterial invasion.

1.2.1. ROS (Reactive Oxygen Species)

ROS are radical and non-radical highly reactive oxygen species such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO•) formed by partial reduction of oxygen during the oxidative mitochondrial process and other redox reactions in the cells. An excess of ROS, compared to the cellular capability to swing with an antioxidant response, leads to oxidative stress.

In normal cell conditions, superoxide anion (O_2^-) is detoxified by superoxide dismutase SOD [52] to form hydrogen peroxide (H_2O_2) that is further reduce to oxygen and water by the enzymatic reactions catalyzed by catalase CAT and glutathione peroxidase GPx [53-55]. However, during oxidative stress, superoxide anion (O_2^-) in the presence of divalent ferrous Fe^{2+} forms hydrogen peroxide (H_2O_2), more reactive and hydroxyl radicals (HO•), the most reactive and deleterious ROS, and causes carbohydrate, protein, and nucleic acid damage. Iron can also generate lipid radicals through the Fenton reaction (as alkoxy LO• or peroxy LOO• radicals), which are responsible for deleterious damage to lipid membranes. Moreover, it has been shown that the reaction of hydroxyl radicals with the 8-hydroxyguanine nucleic acid (8-OHG) base is strongly correlated with teratogenicity and carcinogenicity due to oxidative stress [56]. Another potent ROS that shows reactivity similar to hydroxyl radicals is the lipid hydroxyl peroxide (ROOH).

To counteract ROS activity, mammalian cells have developed a system of defense composed of potent antioxidants such as SODs, catalase, GPxs, and glutathione reductase, together with a number of low molecular-weight antioxidants such as ascorbate, α-tocopherol and glutathione [11, 57].

Paradoxically, under select conditions, anti-oxidant agents as ascorbate and glutathione, that are able to maintain body redox balance, can for ferrous reductions in the same manner as superoxides (Reaction 4 in Table 1) and in such cases contribute to oxidative stress [58-62]. In fact, it has been demonstrated that, especially at high concentration, ferric iron Fe^{3+} reduced to Fe^{2+} by ascorbate or glutathione, can participate to Haber-Weiss reaction leading to the generation of hydroxyl radicals [60-62].

Reaction 1:	$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO\bullet + HO^-$
Reaction 2:	$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^-$
Reaction 3:	$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$
Reaction 4:	$Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2$
Reaction 5:	$O_2^- + H_2O_2 \rightarrow O_2 + HO\bullet + HO^-$

Table 1: Fenton-Haber Weiss reactions, adapted from [63]. Hydroxyl radical formation from hydrogen peroxide (Reaction 1); Generation of superoxide anion (O_2^-) from ferrous Fe^{2+} reduction with dioxygen (Reaction 2); Formation of hydrogen peroxide (H_2O_2) from superoxide anion dismutase, either enzymatically by SODs or non-enzymatically (Reaction 3); Superoxide anions can reduce ferric Fe^{3+} to form dioxygen and regenerate ferrous Fe^{2+} (Reaction 4); The Fenton-Haber Weiss reaction is the sum of Reaction 1 and Reaction 4 (Reaction 5).

1.2.2. Heme toxicity

Aside from heme's physiological function, it can be highly cytotoxic with its potent ability to oxidize lipids and proteins and to damage DNA when it is released from hemoproteins [64, 65]. Intracellular levels of labile heme, the free pool of heme in transit between proteins, must be tightly regulated since it is able to generate ROS [16, 66].

Heme toxicity is closely related to hemolytic diseases such as β-thalassemia, sickle-cell disease (SCD), and malaria, in which there is a high number of hemoproteins out of their physiological environments [67].

2. Iron homeostasis

Iron homeostasis is tightly regulated due to the lack of a regulated iron excretory pathway and the pro-oxidant activity of iron that promotes ROS activities and leads to cell death and tissue damage [68, 69]. Animals, plants, fungi, and bacteria have developed specific mechanisms for iron regulation and have acquired efficient mechanisms to maintain adequate levels of iron through the actions and interconnections of different proteins at the cellular and systemic levels [18, 70]. In iron homeostasis, these are responsible for acquisition, distribution and storage of iron in a variety of organs, tissues and intracellular organelles.

2.1. Iron distribution in the body

The human body contains approximately 3–5 g of iron, corresponding to 55 mg/kg for males and 44 mg/kg for females [10, 71, 72]. Iron in the body is mainly present as heme in hemoglobin (2 g), representing approximately 70% of iron in the body, and myoglobin (300 mg), representing approximately 2.5% [73, 74]. Bone marrow and macrophages in the spleen contain a transient fraction of iron (~ 600 mg), while excess iron is stored in the liver hepatocytes within ferritin, the iron storage protein (~1000 mg) [74]. Iron is released from the body mostly by mucosal sloughing in the gut, and to a lesser extent through skin cell desquamation, blood loss, menstruation, and urinary excretion (1–2 mg/day) [73, 75, 76].

Circulating transferrin (Tf) proteins, part of the most dynamic iron pool in the body, are responsible for delivering iron to erythroblasts and most tissues; they carry ~3 mg of iron. Bloodstream iron is predominantly furnished from reticuloendothelial macrophages that recycle iron via phagocytosis of senescent erythrocytes [73, 74]. Moreover, a small amount of iron (~1–2 mg/day) is obtained by absorption from heme or non-heme in the diet, mediated by intestinal enterocytes in the lumen [73, 77] (Figure 1).

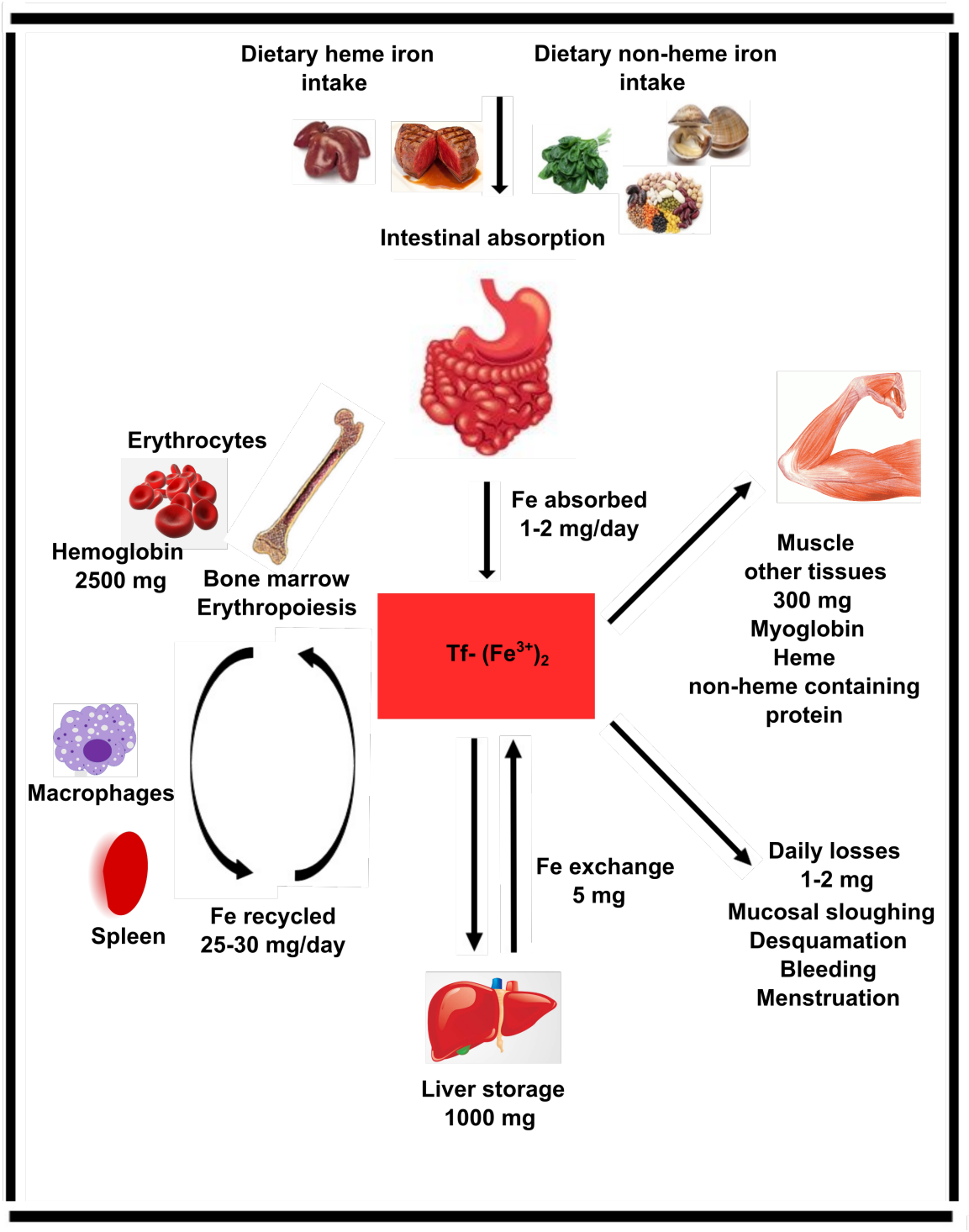

Figure 1: Body iron distribution. Schematic depiction of the physiological distribution of iron in the body, including dietary iron absorption, iron recycling, and losses.

2.2. Iron absorption

Iron absorption is the most critical checkpoint for iron distribution, regulation and maintenance, providing the right amount of iron in the body at all times. Iron absorption is regulated almost exclusively at the intestinal level [78, 79]. A human adult can absorb around 1–2 mg of iron per day to compensate for non-specific losses such as cell desquamation, menstrual bleeding or other blood loss [73-76]. Iron absorption occurs in the duodenum or upper jejunum and varies depending on iron demand by the body, increasing during iron deficiency and decreasing during iron overload [77, 80, 81]. Moreover, mucosal degradation of the duodenum due to various pathological conditions (celiac disease, tropical sprue, Crohn's disease, adenomatous polyposis, cancer, and ulcers of the duodenum) leads to decreased iron absorption.

2.2.1. Non-heme absorption

The mechanism for non-heme absorption is well investigated [78, 81]. Iron absorption begins in the acidic region of the duodenum, where the insoluble aggregates are degraded by proteolytic digestion. Non-heme dietary iron, mainly found in cereals and vegetables [82], is absorbed in the apical region of the duodenal enterocytes [83] via divalent metal transporter (DMT1, also known as solute carrier family 11 member 2 (SLC11A2) or natural resistance-associated macrophages 2 (NRAMP2) [84]. This fundamental protein contains 12 transmembrane domains with both terminals in the cytoplasm [85, 86]. DMT1 has also been localized in hepatocytes and erythrocytes, where it is implicated in Tf recycling in endosomes [87, 88]. Besides iron, DMT1 does not seem to be implicated in the uptake of other metals.

Prior to iron uptake via DMT1, ferric iron (Fe^{3+}) is reduced to ferrous iron (Fe^{2+}) by the iron-regulated ferrireductase DcytB (duodenal cytochrome B, also known as cytochrome B reductase 1 [CYBRD1] or cytochrome B_{561} member 2 [CYB561A2]) [89]. DcytB mediates extracellular ferric iron reduction by using intracellular ascorbate as an electron donor [90, 91]. It has been reported that DcytB may be the only iron ferrireductase present at the brush border of the enterocytes given that DcytB knockout (KO) mice (*DcytB*$^{-/-}$)

showed abnormal iron absorption [92]. However, different studies have proposed that the reduction of non-heme iron in the extracellular environment also implicates non-enzymatic ferrireduction driven by reductant agents [93] such as ascorbate [94-96], superoxide [97, 98] or possibly amino acids such as cysteine [79]. Since a deficiency in ascorbate synthesis in mammals leads to a dysregulation of iron absorption [99-101], ascorbate is likely an important component of cellular iron absorption [102].

Moreover, other transmembrane proteins are involved in iron uptake such as the zinc transporter protein 14 (ZIP14), also known as solute carrier family 39 member 14 (SLC39A1), and zinc-regulated transporters/iron-regulated transporters such as ZRT/IRT-like protein [103, 104].

In addition to intestinal duodenal absorption, it has been shown that iron uptake can also occur via the vesicular pathway of transcytosis on the duodenal brush border membrane [105-107].

In the enterocytes, iron is either stored in the cytosolic protein ferritin [9] or released to the circulating plasma by the iron protein exporter ferroportin (FPN-1), also known as iron-regulated 1 (IREG1), metal transporter protein 1 (MTP1), and solute carrier family 4 member 1 (SLC40A1) through the basolateral membrane enterocytes [108-111].

In the bloodstream, iron circulates in a non-reactive form bound to Tf, an 80 kDa liver-derived glycoprotein [63, 112]. Prior to loading to Tf, ferrous iron oxidizes to the ferric form (Fe^{2+} to Fe^{3+}) through the ferroxidase activity of hephaestin, a membrane-bound multi-copper oxidase homologous to ceruloplasmin [113, 114]. It has been shown that hephaestin-deficient mice display an iron-deficient anemia phenotype with iron retention on the mucosal enterocytes [114, 115]. As Hephaestin, ceruloplasmin, is responsible for oxidation of ferrous iron to ferric iron Fe^{3+} [116]. Ceruloplasmin-deficient mice ($CP^{-/-}$) and humans accumulate iron in hepatocytes and macrophages, while aceruloplasminemia, an autosomal recessive disorder, causes anemia, diabetes, a late-onset disorder of the basal

ganglia, and retinal degeneration [117]. Non-transferrin bound iron (NTBI) can also be taken up by cells especially in iron overload when Tf saturation is more than 45% [118].

2.2.2. Heme absorption

Heme iron is absorbed independently of non-heme iron; however, the molecular mechanisms of its absorption remain uncertain [7, 119]. Heme-iron derived from animal food sources such as meat or seafood is more easily and better absorbed than non-heme iron and contributes to the greater part of total iron derived from the diet [120].

One of the proteins proposed as a potential heme transporter on the duodenum is the proton-couple folate transporter/heme carrier protein 1 (PCFT/HCP1, also known as SLC46A1), responsible for carrying iron-containing porphyrin heme on the lumen of the duodenum enterocytes [111, 112, 121, 122]. As DMT1, PCFT is expressed in the apical region of epithelial cells. However, it has been shown to have a higher affinity to folate [122-124]. It has been suggested, also, that PCFT/HCP1 is negatively regulated by iron, since it is highly expressed during iron deficiency and located in intracellular endosomes in iron-replete cells [122].

Heme iron export from the basolateral portion of the enterocytes is mediated by FPN-1. In addition, it has been shown that cells can release iron in the heme form. Feline leukaemic virus receptors (FLCVR) and ATP-binding cassette ABC transporter (ABCG2), in hematopoietic cells and erythroid cells, are capable of releasing iron in heme form, particularly under stress (hypoxic conditions) to prevent heme toxicity [125, 126].
Moreover, uptake of heme in the cells is not confined to the enterocytes, since multiple studies have reported that other cell types (hepatocytes and hematopoietic cells) can uptake iron through energy or passive dependent mechanisms [119, 127-129].

Following heme endocytosis in enterocytes, through passive pinocytosis or active receptor mediation [130], ferrous iron is released intracellularly by protoporphyrin ring degradation mediated by hemoxygenase1 (HO-1) [131, 132]. The molecular mechanisms for the

intracellular traffic of heme from heme receptors to heme degradation sites are not yet clearly understood [7, 119, 132].

Furthermore, in the context of hemolysis, heme forms a complex with an acute-phase 63 KDa glycoprotein called hemopexin (Hx), the major heme-binding protein of serum, mainly expressed in the liver [133-135]. The complex heme/Hx is then recognized by hemopexin receptors on the cell surface, supposedly the low-density lipoprotein receptor LRP/CD91 [136] or potentially toll-like receptors 2/4 in macrophages [137]. Heme is then rapidly degraded via HO-1 or HO-2 within endocytic vesicles, while Hx is recycled [138-140].

Consequently, the cellular labile iron pool (LIP), a group of chelatable and redox-active iron, can participate in regulatory processes or be stored in ferritin [141].

2.3. Iron recycling

Macrophages are responsible for recycling iron, since less than 10% of iron is absorbed from the diet, while erythropoiesis, a highly iron-dependent process, requires about 25 mg of iron daily for hemoglobin synthesis in bone marrow [73, 74].

The splenic macrophages of reticuloendothelial cells (known as the mononuclear phagocyte system) engulf aged or damaged erythrocytes by phagocytosis, while HO-1 catabolizes heme iron to release ferrous iron Fe^{2+} [142-144]. DMT1 and its homologous NRAMP1, expressed in the phagolysosomal membrane, are responsible for ferrous iron export from phagocytic vesicles [145, 146].

Ferroportin 1 (FPN-1) is responsible for the release of ferrous iron from macrophages for erythropoiesis [147].

2.3.1. Iron utilization: Erythropoiesis

Erythropoiesis is a dynamic process that involves multiple factors such as cytokines, nutrient availability and the cellular environment of erythroid progenitors for red blood cell

(RBC) production [73, 148]. Erythrocytes are responsible for the acquisition and manipulation of large amounts of iron; they require 2,500 mg of iron daily, while about 25 mg of iron is recycled every day [74].

Erythrocytes acquire iron mainly from Tf receptor 1 (TfR1) but also from erythroblastic islands (ferritin released from macrophages). Erythroblastic islands contribute less than TfR1 to iron uptake, as shown by reports that TfR1 deficiency in mice and humans causes severe iron deficiency anemia [149].
It is fundamental for the body to maintain a normal level of circulating erythrocytes, avoiding pathological conditions such as anemia and the consequent negative impact of hypoxic stress. On the other hand, high levels of erythrocytes circulating in the bloodstream (polycythemia or erythrocytosis) can lead to hyperviscosity and potentially lethal thrombotic events [150].

One of the most critical factors involved in RBC production is the hypoxia-inducible factor 2α (HIF-2α). HIF2α belongs to the HIF-α family, a group of heteromeric transcription factors, the expression of which is regulated by oxygen and iron [151, 152]. The level of HIF-2α increases erythropoietin (EPO) production in the kidney but also liver and brain and regulates other genes required in iron absorption. As a result of this activity, erythropoiesis is enhanced [153-158]. The increase in erythropoiesis activity leads to hepcidin suppression and iron acquisition from reticuloendothelial macrophages after degradation of senescent erythrocytes. In mice, hepcidin suppression, which is essential to maintain normal iron absorption and efficiency in erythropoiesis, is mediated by erythroferrone (ERFE) [159]. HIF-2α upregulates DMT1 [160] and DctyB expression on the apical surface of enterocytes to increase dietary iron absorption from the lumen of enterocytes [80]. Intestinal-specific *Hif2α*$^{-/-}$ mice have decreased DMT1 and FPN-1 expression and fail to promote iron absorption [155, 160]. In humans, it has been found that the HIF-2α gain-of-function mutations that cause polycythemia [161] and its dysregulation are associated with cancer [162-164], demonstrating the essential role of this protein in erythropoiesis.

2.3.1.1. Mitochondrial iron uptake

Mitochondrial iron uptake is critical for iron-sulfur cluster (ISC) biogenesis and heme synthesis [42, 165]. Iron is transported in the inner mitochondrial membrane by the membrane protein mitoferrin 1 (Mfrn1/ SLC25A37, solute carrier family 25, member 37) but also by direct inter organellar transfer, the so-called "kiss-and-run mechanism," by bypassing the cytosol [166, 167]. The ABCB10 (ATP-binding cassette, subfamily B, member 10) protein, highly expressed in erythroid precursors, stabilizes Mfrn1 in the process [105]. This stabilization promotes an efficient heme synthesis via ferrochelatase activity, the enzyme responsible for iron insertion in protoporphyrin IX to form heme [168]. Moreover, it has also been proposed that mitochondrial iron uptake can involve other proteins such as 2,5-dihydroxybenzoic acid (2,5-DHBA), an endogenous mammalian siderophore that imports iron to the mitochondria in an iron siderophore-like complex [169].

Iron bioavailability coordinates the synthesis of the heme precursor protoporphyrin IX via the post-transcriptional regulation of δ-aminolevulinic acid synthase 2 (ALAS2) [170], the erythroid-specific first enzyme of protoporphyrin IX synthesis, through the iron-responsive element/iron-regulatory protein (IRE/IRP) system. The ALAS2 mutation causes sideroblastic anemia, whereas porphyrias (a group of disorders characterized by hereditary abnormalities of porphyrin) result in the haploinsufficiency of other enzymes in the pathway due to the accumulation of toxic heme precursors [171].

The molecular mechanism of heme secretion from mitochondria remains unclear.

Abnormal iron deposition in the mitochondria due to excessive iron not being used in heme or Fe/S cluster biogenesis provokes an imbalance of iron import and export in the cells. In such cases, erythroblasts with perinuclear iron accumulations appear as "ringed" sideroblasts, also known as mitochondrial ferritin (FtMt) [172]. Ringed sideroblasts are characteristic of various disorders such as X-linked sideroblastic anemia caused by ALAS2 deficiency [173] or the mitochondrial transporter of the ALA substrate glycine SLC25A38 (solute carrier family 25, member 38), an autosomal recessive deficiency [174-176].

Anemia and ringed sideroblasts also occur in deficiencies of Fe/S cluster biogenesis, which could be due to an impairment of a protein essential for mitochondrial iron uptake, GLRX5 (glutaredoxin 5) [177] or the ATP-binding cassette protein ABCB7 [174, 178].

In normal conditions, erythroblasts store iron in ferritin or export iron via FPN-1 [3, 73]. Moreover, erythroblasts are capable of exporting heme (when globin synthesis is limited) *via* the feline leukaemic virus receptor (FLVCR), a multi-transmembrane protein receptor of a virus that causes severe aplastic anemia in cats [179]. FLVCR has the function of protecting erythroblasts from heme toxicity, above all during early stages of differentiation. FLCVR deficiency in mice causes severe hyperchromic, macrocytic anemia, reticulocytopenia, and a block in erythroid maturation at the proerythroblast stage, thus FLCRV expression is essential in mice [179]. FLVCR and ABCG2, essential to preventing protoporphyrin accumulation in erythroblasts, are also expressed in other cells such as enterocytes, where it has been speculated that they work as apical heme exporters to prevent toxicity [179, 180].

2.4. Iron uptake

In the bloodstream, Tf is normally abundant and unsaturated, binding iron absorbed by the diet, released from reticuloendothelial macrophages, or from other body stores. Tf is therefore the crucial glycoprotein for the safe distribution of iron in the cells and the avoidance of ROS formation. Tf binds a maximum of two ferric iron Fe^{3+} forming a mono- or diferric-Tf, known as holo-transferrin [112]. Under normal physiological conditions, Tf saturation in the serum is about 20–30% and the affinity of Tf for Fe^{3+} is high and ph-dependent, with maximum binding at pH 7.4 [181-183].

Iron uptake then occurs through clathrin-dependent endocytosis to cell surfaces *via* TfR1. The Tf-Fe_2/TfR1 complex undergoes structural changes due to proton influx that acidifies endosomes (pH 5.5), promoting iron release [9, 184]. Reduced by the iron ferrireductase STEAP3 (six-transmembrane epithelial antigen of prostate 3) from ferric iron to ferrous

iron, Fe^{2+} is then transported to the cells by DMT1, while Tf is released as apo-transferrin (iron-free) and then recycled for further use [185, 186].

Transferrin receptor 2 (TfR2), which has a lower affinity for iron than TfR1, is expressed in enterocytes, erythrocytes and duodenal crypt cells. TfR can also mediate cellular iron uptake. It has been speculated that TfR2 acts as a systemic sensor more than as an iron uptake protein since, when Tf reaches peak capacity, a pool of circulating NTBI is formed [118, 187].

2.4.1. TfR1-mediated iron uptake

As mentioned above, TfR1 (also known as cluster differentiation, CD71) is a 95 kDa glycoprotein that plays an essential role in iron homeostasis; acting as a cellular iron "gate," it controls iron entry into the cells [188]. Characterized by a homodimeric type I transmembrane protein linked by disulfide-bonds, it efficiently binds two holo-Tf molecules (Tf-Fe_2) [189, 190]. The determination of the crystal structures' form by the Tf-Fe_2/TfR1 complex shows an overlap with the hemochromatosis protein (HFE) that acts as the cofactor of this interaction. TfR1 forms a homodimer binding Tf-Fe_2 at each of its subunits [191-193].

It has been proposed that, in hepatocytes, TfR1 limits the iron sensing pathway through HFE sequestration, debilitating TfR2-HFE interactions. TfR2-HFE interaction has not been completely demonstrated [73].

The affinity of TfR1 for Tf is determined by the iron status of Tf, which is referred as holo-Tf when Tf is iron saturated, and as apo-Tf when not bond to iron. On the cell surface, the affinity of TfR1 with Tf-Fe_2 is about 10 to 30 times higher than that of TfR2 [194]. Moreover, TfR1 has a 20-fold higher affinity for holo- compared to apo-Tf at pH 7.4 [195]. Internalization of the TfR1 and Tf complex is an energy-dependent process of clathrin-mediated endocytosis [195, 196]. Consequently, endosome acidification (pH 5.3–5.6 [197]) due to the activity of an ATP-dependent proton pump of vacuolar-type H^+-ATPase

(V-ATPase) triggers conformational changes in both Tf and its receptor that promote the release of ferric iron from Tf [198-200]. Vesicles with the apoTf/TfR1 complex are recycled back to the plasma membrane, while the trafficking of the complex to the cell surface is dependent on the EXOC6 member of the exocytosis protein [201]. Increased pH allows apo-Tf/TfR1 (iron-free Tf) separation and TfR1 is now available for a new cycle [184, 202]. DMT1 mediates the transport of ferrous form Fe^{2+}, reduced from ferric form Fe^{3+} by the metallo-reductase STEAP3 to the cytosol or, as Lane *et al.* suggested, the members of cytochrome b_{561} family [90, 185, 186, 203]. Recently, it has been suggested that ZIP8 and ZIP14, capable of mediating NTBI uptake at the plasma membrane, are also implicated in iron exit from endosomes [103, 204, 205]. Moreover, it has been reported that TfR1 can bind and internalize ferritin [187].

TfR1 is ubiquitously expressed except in mature erythrocytes and plausibly microglia, astrocytes and oligodendrocytes [206, 207]. Thus, TfR1 inactivation leads to tissue damage and dysfunction in many organs. The importance of TfR1 is underlined by the fact that *Tfr1*$^{-/-}$ mice die with severe anemia and neurologic abnormalities, and Tfr1 haploinsufficiency causes microcytic anemia [208]. Recently, it has been reported that missense mutation in the transferrin receptor gene (TFRC), encoding TfR1, decreases T and B lymphocyte proliferation and causes combined immunodeficiency [209]. Inactivation caused by genetic manipulation of TfR1 expression in cardiomyocytes causes severe cardiomyopathy leading to death in the second week of life [210]. Additionally, in skeletal muscle, TfR1 deficiency leads to a dysfunction of systemic metabolism and disorder in skeletal muscle metabolism [52]. It has been shown, in a variety of neurodegenerative diseases, that the expression of TfR1 is decreased [211] and, in dopaminergic neurons, loss of TfR1 leads to brain injury [212]. All these conditions indicate the fundamental role of TfR1 to accomplishing the functions of hematopoietic but also non-hematopoietic cells rich in mitochondrial protein with sulfur-cluster structures. Interestingly, a loss of TfR1 in the intestinal epithelium showed that TfR1 is required for the homeostatic maintenance of intestinal epithelium [213], which may be related to the potential role of TfR1 as a mitochondrial regulator [214].

A TfR1-independent pathway has also been identified, where holo-Tf uptake occurs via endocytosis or pinocytosis on the hepatocytes cell membrane, with the consequent release of iron in the cytosol [215].

2.4.2. Transferrin receptor 2 (TfR2)

Like TfR1, TfR2 has been well documented [216]. Similarly to TfR1, TfR2 is a transmembrane protein used for iron uptake; however, they have significant differences and bind Tf through different mechanisms [217].

The TfR2 gene is transcript in two main isoforms TfR2-α (full length) and TfR2-β (short form without a transmembrane domain) and probably released as a soluble receptor in the plasma [216]. TfR2-α has been shown to be implicated in hepcidin regulation, whereas TfR2-β has been reported to control spleen iron efflux via FPN-1 regulation [218]. Embryonic lethality of *TfR1*$^{-/-}$ mice reveals that TfR2 cannot rescue mice from mortality [208]. This may be related to the low affinity of TfR2 to form a complex with Tf-Fe_2 compared to TfR1 [194]. Importantly, in contrast to TfR1, TfR2 is not upregulated or downregulated in iron deficiency and iron overload, respectively [219]. Since TfR2 mutation leads to Type III hereditary hemochromatosis, a hereditary iron overload disorder [220], it has been suggested that TfR2 function is related to iron sensing and maintenance of iron homeostasis rather than to iron transport [221].

As in hepatocytes, TfR2 is highly expressed in erythroblasts, and various studies suggest that TfR2 may be involved in adapting red blood cell production to iron levels in the body [222]. It has been shown that in liver hepatocytes, TfR2 expression is mediated by hepatocytes nuclear factor 4-α (HFN4α) [223], a member of the nuclear receptor superfamily that activates numerous genes involved in liver-specific functions [224]. However, other transcription factors, such as GATA-1, the erythroid-specific transcription factor essential for erythrocytic differentiation in early-stage differentiation [225], and CCAAT-enhancer binding protein (C/EBPα), highly expressed in the liver [226], have been reported to activate TfR2 transcription by binding to consensus sequences in the proximal and distal promoter regions [227].

Interestingly, it has been reported that in chinese hamster ovary (CHO) cells stably transfected with TfR2 (CHO-TRVb), the overexpression of TfR2 increases iron uptake by receptor-mediated endocytosis of Tf and NTBI [228], highlighting the dual role of TfR2 as an iron sensor and, in certain circumstances, iron uptake protein.

2.4.3. Non-transferrin-bound iron (NTBI)

As explained above, under physiological conditions, all iron in the plasma is bound to Tf in a mono (apo-) or diferric (holo-) form. However, when there is an excess of iron in the bloodstream, a pool of non-transferrin-bound iron is formed [118, 229, 230]. NTBI has been primarily related to iron disorders, such as hereditary hemochromatosis (HH), hypotransferrinemia and hemolytic anemia as in
β-thalassemia, in which iron exceeds Tf-binding capacity and results in saturation [118, 231-233]. However, this pool of NTBI, known as the low molecular-mass (low-M_r) pool of iron bound to small organic chelators, is also present in healthy individuals (< 1 μM). NTBI increases during severe iron overload (10-20 μM) [231].

It has been implied that NTBI is complexed in the plasma by organic chelators, mostly citrates [234, 235] but also organophosphates as ATP [231]. NTBI uptake can occur in a wide range of cells - mainly hepatocytes [118, 236] - but also erythroid cells [237], intestinal epithelial-like cells [238], certain types of brain cells [96, 239], skin fibroblasts [240], cancer cells (HeLa and K562) [94, 241, 242], monocytic cells [95], cardiomyocytes, and pancreatic cells [118]. Several studies suggest that trans plasma membrane electron molecules such as ferricyanide reductase or duodenal ferrireductase Dcytb are responsible for iron reduction from ferric to ferrous form before uptake in the cells [243, 244]. NTBI could be reduced by an ascorbate-stimulated reductase plasma membrane before DMT1 ferrous iron transportation in the cytosol [90, 94, 96]. Another study implicates TfR2 in NTBI cellular uptake, at least during TfR2 overexpression [228]. It has also been hypothesized that NTBI ferric iron could be internalized by endocytosis, reduced to ferrous iron by a reductase in the lumen, and stored in the cytosol [96].

2.4.4. Hemochromatosis protein (HFE)

HFE protein, also known as human leukocyte antigen HLA-H, is a major histocompatibility complex I (MHC-I)-like protein involved in intracellular iron uptake and expressed in hepatocytes, macrophages and intestinal crypt cells [245-247]. Like other (MHC-I) molecules, HFE associates with β_2-microglobulin (β2m) for its appropriate expression at the cell surface [248]. It has been shown that *Hfe*$^{-/-}$ and *β2m*$^{-/-}$ mice develop liver iron overload due to hyperabsorption of iron in the duodenum, the same phenotype of patients with hereditary hemochromatosis [248, 249].

HFE competes with TfR1 for Tf interaction, limiting Tf-Fe_2/TfR1 affinity and negatively regulating iron uptake [250]. HFE binds TfR1 with less affinity, implying a different mode of interaction. This divergence on the stoichiometry has been reported via crystallographic structure characterization: TfR:HFE (2:1) and TfR:Tf (2:2) [251]. Moreover, it has been proposed that, in the case of holo-Tf saturation, HFE displaces from HFE-TfR1, causing the initiation of hepcidin expression [252].

Finally, interaction of HFE with TfR2 through overexpression of the protein in hepatoma cell lines has also been reported [221, 253]. However, conversely to *in vitro* studies, other studies have suggested that HFE and TfR2 need not interact to regulate hepcidin synthesis [73, 254-256].

2.5. Iron storage

Ferritin is the major iron storage protein, its function consisting of the sequestration of iron in a nontoxic form to minimize the oxidation-like activity of iron to form ROS. It is located intracellularly and in circulating plasma. Ferritin is composed of 24 subunits of heavy chains (FtH) that act as a potent ferroxidase to catalyze ferrous iron Fe^{2+} oxidation [257] and light chains (FtL), which are important for iron nucleation and stability [209]. The ratio of the aforementioned is tissue-specific, and the subunits can be modified by several diseases and stimuli such as inflammation and infection [258]. Ferritin can contain up to

4,500 iron ions in an inactive form (ferrihydrite mineral) with phosphate and oxo hydroxide anions.

Ferritin expression is increased in iron-replete cells by transcriptional and post-transcriptional controls that increase cellular ferritin in response to environmental cues and high iron levels [259]. Additionally, a small amount of ferritin is released into the plasma. Plasma ferritin is rich in L-subunit and glycosylated, it is used as an indicator of iron stores helpful in diagnosing iron overload [260, 261]. The role of plasma ferritin remains unclear, and it has been speculated that it may act on angiogenesis regulation [262]. While high levels of plasma ferritin (>400 ng/mL) may indicate iron overload, however it can also be found in severe non-alcoholic steatohepatitis (NASH), inflammation, cancer, and liver disease (hematophagocytosis, hepatitis).

Fferritin-like molecules are also present in the mitochondria (FtMt), mostly in mitochondrial-rich tissues [172]. FtMt is encoded by intron-less gene present in mitochondrial DNA, resulting in 22 kDa ferritin-like protein aim to sequester potentially deleterious free iron using ferroxidase activity [263]. FtMt is present in high energy and oxygen requirements tissues such as brain, heart, skeletal muscles and less expressed in iron storage tissues (liver and spleen. FtMt levels increase in patients with sideroblastic anemia and in several iron deficient phenotype [264].

2.6. Iron export

FPN-1 (also known as Ireg1 or MTP1) is the only iron exporter protein identified thus far in mammals [108, 265]. FPN-1 facilitates dietary iron export from the basolateral membrane of duodenal enterocytes into the systemic circulation, the recycling of iron from senescent erythrocytes into the blood stream, and the release of iron storage in ferritin from hepatocytes. FPN-1 acts in tandem with the copper-dependent ferroxidase hephaestin in enterocytes and ceruloplasmin in other cells that are capable of converting Fe^{2+} to Fe^{3+} to allow iron to be taken up by Tf in the plasma [113, 114].

FPN-1 expression is regulated at the posttranslational level by hepcidin, a small peptide hormone secreted mainly by the liver in response to different stimuli (iron overload, inflammation, hypoxia, and erythropoiesis) [17]. Hepcidin binds to FPN-1 and induces its internalization and degradation via a ubiquitination process, provoking the blocking of iron efflux from cells into the blood stream [266-268].

FPN-1 expresses two different transcripts, FPN-1A and FPN-1B. Both transcripts encode ferroportin 1 with the same open reading frame but are expressed differently in the body and have an identical physiological role. The FPN-1B transcript, expressed in enterocytes and erythroid precursor cells, has a promoter located upstream of the FPN-1A transcript (FPN-1 transcript that contains the IRE) [269, 270]. In turn, the ubiquitously expressed FNP-1A contains an IRE, hence can be repressed by IRE/IRP machinery. In erythroblasts, the FPN-1 promoter is regulated through putative GATA and EKLF (members of the KLF family) [271] binding sites, and its expression is impaired during erythroblast differentiation [270].

2.7. Regulation of cellular iron homeostasis

Cellular iron homeostasis is achieved through a balancing of the IRE/IRP post-transcriptional expression [3, 18, 272]. The complex form of IRE/IRP responds to cellular iron levels and coordinates iron protein expression by blocking ribosome-binding proteins or avoiding protein nuclease degradation [273].

More precisely, iron regulatory proteins 1 and 2 (IRP1 and IRP2 also known as aconitase1, ACO1 or iron-responsive element binding protein 2, IREB2, respectively) are RNA-binding proteins that directly interact with iron regulatory elements (IRE) sequences present in mRNAs [274]. IRE is conserved stem loop structures, or *cis*-regulatory hairpin, in the 5′ or 3′ untranslated regions (UTRs) of target mRNAs.

IRE/IRP1-2 interactions regulate mRNAs that encode iron protein acquisition (TFR1, DMT1), storage (ferritin L or H chain, H [FtH] and ferritin L [FtL]), utilization (erythroid

5-aminolevulinic acid synthase, mitochondrial aconitase [ACO2], Drosophila succinate dehydrogenase [SDH], hypoxia-inducible factor 2α [HIF2α/EPAS1]), and export FPN-1 [3, 18, 275-277]. IRPs may influence the amount of iron released from the body by the modulation of the quantity of iron retained in sloughing mucosal cells. Additional mRNAs regulated by IRP1-2 have been identified, but their functions remain unclear [278-280]. Interestingly, it has been demonstrated that deficiency of IRP2 but not IRP1 leads to dysregulated iron metabolism, highlighting potential functional differences between them [281-283].

2.7.1. Iron-responsive element (IRE) structure

IREs are composed of a canonical sequence of an unpaired cytosine separated from a CAGUGN loop (N = U, C or A) by a 5 base pair upper stem-loop plus a lower stem-loop of variable length [18]. Interestingly, it has been shown that DMT1 and HIF2α mRNA have a noncanonical additional bulk on the 3′ strand of the upper stem-loop. The high specificity of IRP1/IRE binding activity is determined by two separated sites, each binding multiple RNA proteins at the IRE terminal loop or C bulge [284]. Moreover, multiple regions of the IRE stem, not predicted to contact IRP1, help establish binding affinity, contributing to the effectiveness of the response between IRE-controlled mRNAs and IRP regulation [285]. Multiple studies have suggested that the regulatory activity of IRP may be involved in another biological context beyond the current list of IRE-containing mRNAs, implicating different RNA binders [286, 287].

2.7.2. IRE/IRP function

In iron-replete conditions, IRP2 (and apo-IRP1) form a complex with the hemerythrin-like FBXL5 (F-box and leucine-rich repeat protein 5) adaptor protein that recruits a SCF (SKP1-CUL1-F-box) E3 ligase complex, leading to IRP proteasome degradation by a ubiquitination process [288, 289]. Additional molecular regulations control IRP1 binding to IREs in iron-replete cells. IRP1 (but not IRP2) interacts with the 4Fe-4S cluster that induces IRE binding [18, 284]. Additionally, 4Fe-4S IRP1 aconitase function in the cytosol, making the protein bifunctional. In cells lacking iron, IRP1 changes its conformation, losing, in this case, the Fe/S cluster and aconitase activity.

The molecular mechanisms of this iron-regulated Fe/S cluster assembly/disassembly are not yet defined [290]. The ratio between the different conformations of IRP1 depends mainly on mitochondrial iron availability and Fe/S cluster synthesis, while cytosolic iron sensing involves primarily IRP2. Complete loss of IRP1 and IRP2 in mice causes embryonic lethality [291, 292]. Conversely, mice lacking one of the proteins are still viable and fertile. IRP2 deficiency causes mild microcytic anemia and dysregulation in body iron distribution [281, 282]. Cytosolic aconitase activity is not primordial for cells, since it has been reported that *Irp1*$^{-/-}$ mice are asymptomatic and, in hypoxia, IRP1 is inactivated due to hypoxic signals [283, 293], whereas IRP2 is stabilized as a result of the oxygen requirement for iron-mediated FBXL5 degradation [3, 283, 293].

HIF-2α mRNA is repressed by IRP1 in hypoxic stress [294], highlighting the role of IRP1-HIF-2α in the regulation of erythrocytes production as well as tissue responses to iron deficiency and hypoxia. Furthermore, in cultured cells, new mechanisms of HIF regulation have been reported, including programmed changes in their synthesis [162, 277, 295, 296].

FPN-1 is also regulated by the IRP/IRE post-transcriptional mechanism [110, 111, 297]. In iron deficient cells the FPN-1 5′ untranslated region (UTR) IRE is bound by IRPs repressing FPN-1 translation and consequently reducing FPN1 protein expression, allowing cells to retain intracellular iron. In contrast, it has been shown that, in the duodenum of mice maintained on an iron-deficient diet, FPN-1 expression is upregulated, since enterocytes need to provide more iron for systemic demands in this condition [110, 111]. Thus, in enterocytes, the FPN-1 transcript (FPN-1B) lack IRE in its 5′ UTR [298], underlying FPN-1 upregulation in iron-deficient conditions [270]. Nevertheless, this isoform is susceptible to hepcidin degradation and thus can be regulated by systemic iron availability.

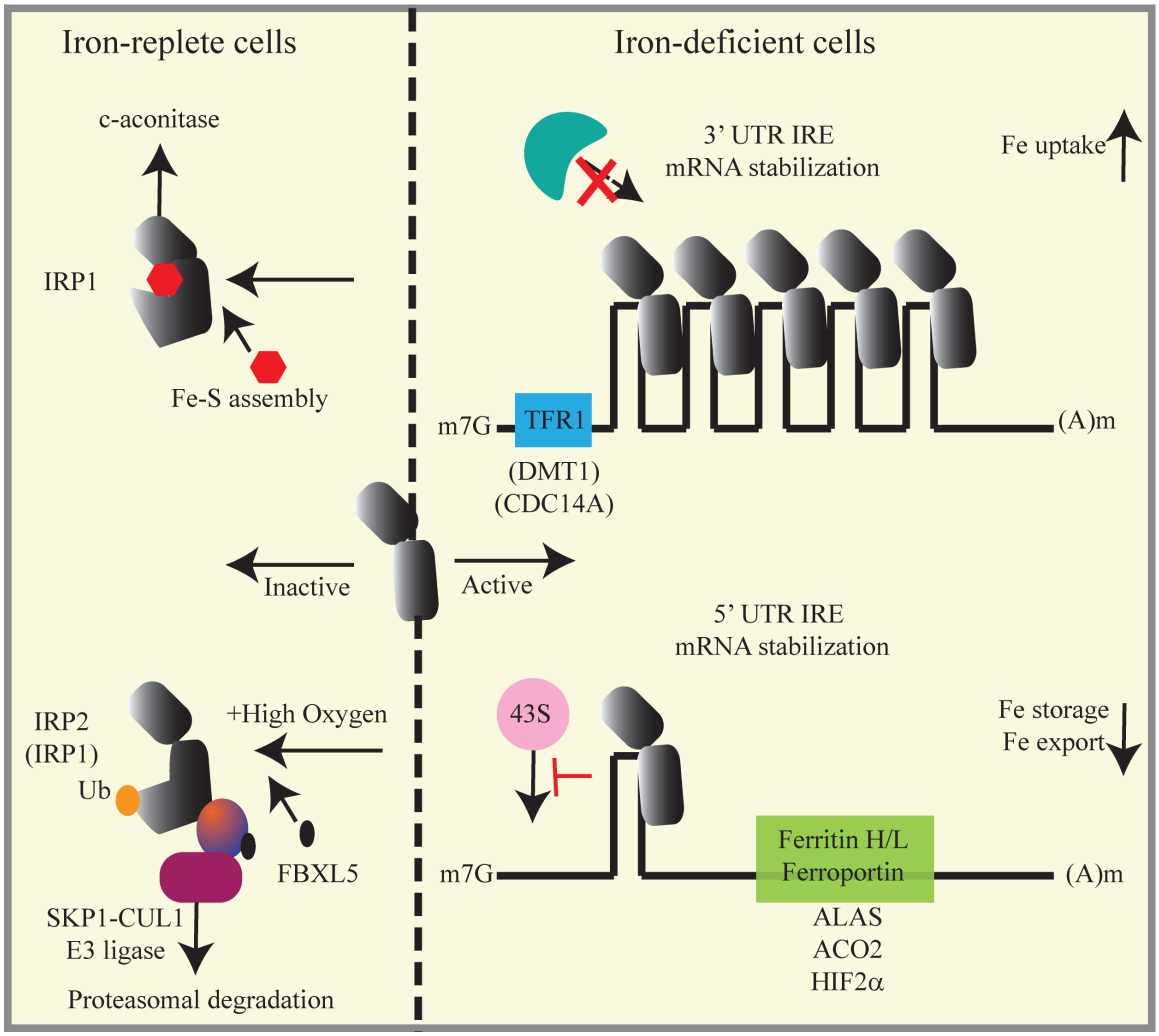

Figure 2: Regulation of cellular iron metabolism (figure adapted from [3]). Schematic depiction of IRP1/2 regulation in iron-replete and iron-deficient cells. Permission obtained from Cell journal.

2.8. Crosstalk between cellular and systemic iron homeostasis

To maintain control of iron metabolism, cellular IRE/IRP and systemic iron homeostasis (hepcidin/FPN-1) are tightly coordinated [3, 272]. FPN-1, HIF2α and DMT1 expression defines the level of interconnection between cellular and systemic iron homeostasis.

FPN-1, essential for iron efflux, exerts control at both levels, while at the systemic level iron status stimulates hepcidin expression to manage FPN-1 expression via post-

translational changes, whereas intracellular iron concentration regulates changes through the 5′ IRE of the FPN-1 mRNA.
IRP1/2 are both important for iron export, since it has been reported that duodenal double KO mice *Irp1Irp2*$^{-/-}$ were impaired in their ability to downregulate FPN-1 expression, leading to cellular iron deficiency [292]. On the other hand, IRP target HIF2α mRNA expression [277], which regulates DMT1 expression in the apical surface of duodenal enterocytes [155].

Finally, hepcidin, HIF2α, TfRs and IRP activity are also associated to ensure adequate systemic iron homeostasis. In fact, it has been reported that, in response to hypoxia or iron deficiency, hepcidin transcription is possibly controlled by HIF2α.

To conclude, TfR1 expression is promoted by elevated IRP activity and, consequently, IRP may control the equilibrium of the amount of plasma iron "sensing" TfR1 and the iron "sensor" TfR2, essential for hepcidin production, indirectly affecting hepcidin expression in hepatocytes.

3. Systemic iron homeostasis

Systemic iron homeostasis is regulated by three cell types that control iron efflux in the plasma (duodenal enterocytes, reticuloendothelial macrophages and liver hepatocytes) [10, 299], while hepcidin, also known as liver-expressed antimicrobial peptide 1 (LEAP-1), acts as the orchestrator of the process [300, 301]. Thus, hepcidin is considered as the key regulator of systemic iron homeostasis in mammals [70, 266, 299, 302, 303].

Hepcidin levels are similar in men and postmenopausal women and lower in girls and young women, with high correlation with ferritin and serum levels [304, 305]. Moreover, during pregnancy, hepcidin levels are low to ensure iron bioavailability to mothers and fetus [306].
Systemic iron homeostasis is regulated at the transcriptional (hepcidin expression) and posttranslational levels (FPN-1 degradation mediated by hepcidin) [266-268]. However, other protein activities, such as diferric Tf (Tf-Fe_2) saturation, contribute as determinant

indicators of systemic iron homeostasis. Diferric Tf determines the amount of intestinal iron absorption, iron recycled from senescent red blood cells due to reticuloendothelial macrophages and iron used for erythropoiesis [272]. In addition, diferric Tf affects hepcidin mRNA expression [307]. Thus, both iron-bound Tf and hepcidin are important regulators of systemic iron homeostasis [308].

3.1. Hepcidin: the systemic iron regulatory hormone

Hepcidin mRNA expression (HAMP), regulated by different stimuli such as systemic iron levels and inflammatory stimuli, controls FPN-mediated blood iron efflux by modulating its expression on iron-recycling macrophages and enterocytes that absorb dietary iron from the intestinal lumen (Figure 3).

Hepcidin is a defensin-like peptide hormone with strong links to innate immunity. Three laboratories simultaneously identified the antimicrobial peptide activity of hepcidin against fungi and bacteria 17 years ago [299-301, 309].
The implication of hepcidin in iron metabolism was then determined by the discovery of severe iron overload in the liver and pancreas in upstream stimulatory factor 2 (USF2) knockout mice, in which the hepcidin gene was abrogated [310]. Furthermore, the development of severe iron deficiency anemia and premature death in transgenic mice overexpressing hepcidin under the control of a liver-specific promoter has been reported [311].

Hepcidin is mainly synthesized in the liver, secreted from hepatocytes, circulates in plasma bound to α2-macroglobulin or not specifically to albumin [312], and is excreted in urine [300, 301, 309]. The interaction between α2-macroglobulin and hepcidin appears to decrease hepcidin urinary excretion [313]. Besides hepatocytes, other tissues have been reported to express hepcidin at much lower levels, such as the heart, lungs, stomach, intestine, pancreas, adipocytes, skeletal muscle, testis, and myeloid cells, including monocytes, macrophages, neutrophils, lymphocytes [299, 314-319].
Hepcidin clearance occurs via the kidney or by co-degradation with FPN-1. triggering internalization and ubiquitination that leads to the lysosomal degradation of the protein iron exporter [266].

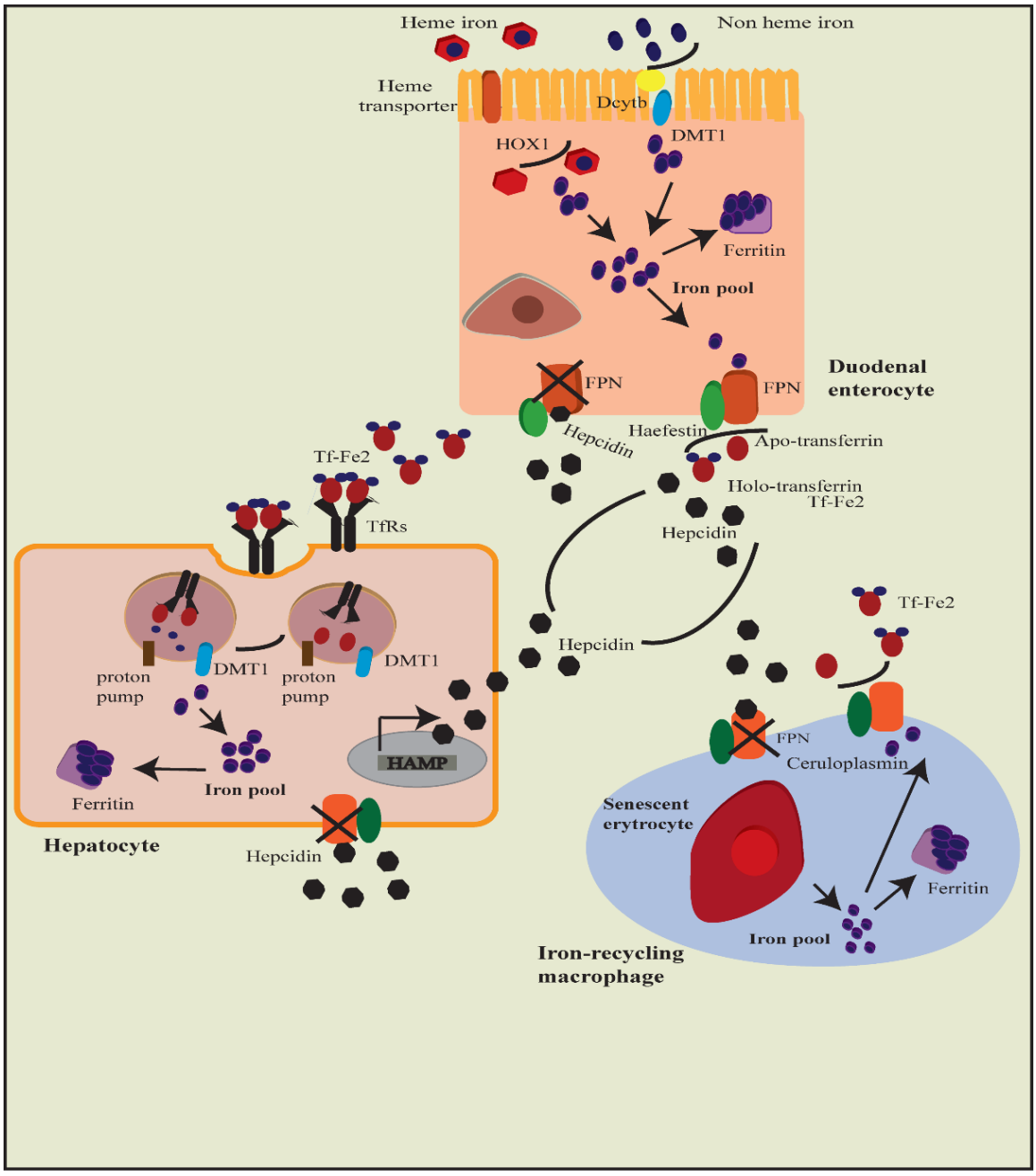

Figure 3: Hepcidin-mediated regulation of systemic iron homeostasis. Hepcidin mRNA (*HAMP*) expression in hepatocytes is regulated by systemic iron levels and inflammatory stimuli. Hepcidin controls FPN-mediated blood iron efflux by modulating its expression on iron-recycling macrophages and enterocytes that absorb dietary iron from the intestinal lumen.

3.2. Hepcidin structure

Human *HAMP* gene, positioned on chromosome 19q13.1, is composed of two introns and three exons encoding prepropeptide, also called preprohepcidin. Preprohepcidin comprises 84 amino acids with an N-terminal endoplasmic reticulum targeting signal sequence (25 amino acids) and proregion (pro) with a consensus furin cleavage site (39 amino acids), immediately followed by the C-terminal 25 amino acid, the bioactive form of the hormone [320, 321]. Undergoing intracellular and plasmatic post-translational modification mediated by furin convertase, preprohepcidin is converted to prohepcidin (60 amino acids) and then the active form of hepcidin (25 amino acids) [322, 323]. The consensus site of furin in prohepcidin is conserved in mammals and fish, highlighting its crucial role in hepcidin processing [321, 324]. It has been indicated that $\alpha 1$ – antitrypsin may regulate preprohepcidin and prohepcidin posttranslational modifications to prevent an excess of hepcidin maturation from furin cleavage [325]. The hepcidin human bioactive form release in the plasma is composed of 25 amino acids composed of eight cysteines with distorted β-sheets and four disulfide bonds [326]. Three disulfide bonds are capable of stabilizing the anti-parallel strands while the fourth disulfide bridge, located near the hairpin loop, has been reported to be more reactive [324, 327] (Figure 4).

The urinary secreted form of hepcidin is composed of two N-terminally truncated isoforms of 22 and 20 amino acids. It has been suggested that these isoforms are direct degradation products of the hepcidin-25 form since they lack iron-regulatory activity [301].

Conversely to the human genome that has only one gene-encoded hepcidin [321, 324], mice have two genes of encoded hepcidin, HAMP1, analogous to humans, and HAMP2 [311]. Via genetic modifications overexpressing hepcidin-1, hepcidin-2 or both in mice hepatocytes, it has been shown that overexpression of hepcidin-1 leads to severe iron-deficiency anemia with consequent death of mice shortly after birth, indicating an inhibition in placental iron transport from the mother to the affected fetus. In contrast, hepcidin-2 transgenic mice show no abnormalities in iron absorption, suggesting a non-essential role in iron regulation [311, 328]. Finally, mosaic mice survivors remained severely iron-deficient after birth, indicating impaired duodenal iron absorption [311].

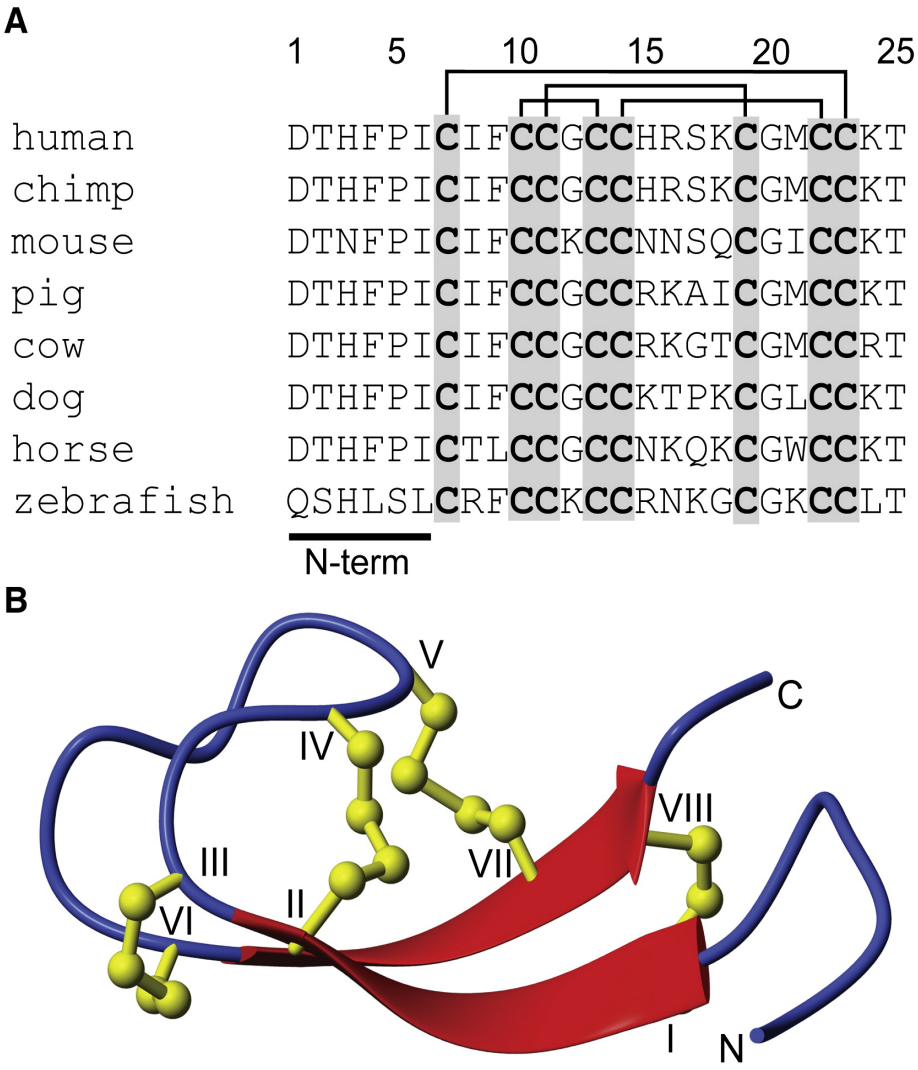

Figure 4: Sequences and tridimensional structure of hepcidin adapted from [324] (A) Hepcidin sequence alignment from different species highlights the high sequence conservation and N-terminal region essential for biological activity. Hepcidin is composed of eight conserved cysteines that form four disulfide bonds, indicated by the lines above the sequence list. (B) The three-dimensional illustration of hepcidin structure showing the β sheet structure (broad arrows) and the four disulfide bonds (ball and sticks). Permission obtained from the Chemical Biology (Chem Bio) journal.

3.3. Hepcidin regulation

Hepcidin acts as an acute-phase protein that regulates systemic iron levels following enterocyte iron absorption or macrophage iron recycling [266, 329]. Hepcidin expression increases following iron absorption in response to high circulation and tissue levels iron or due to systemic inflammation and infection [17, 70]. On the other hand, hepcidin is inhibited by iron deficiency, erythropoiesis and hypoxia [3, 330, 331]. Hepcidin is strictly correlated with serum ferritin levels and its interconnection with bloodstream iron has been fully observed in several studies, during which oral iron administration increased hepcidin within one day [329, 332-334].

Hemojuvelin (HJV) has a critical effect on hepcidin expression and its mutation leads to quasi suppression of hepcidin expression causing severe HH [335]. HJV, mostly expressed in the liver, is a glycophosphatidylinositol-linked protein, homologous to repulsive guidance molecules, that has been shown to act as a bone morphogenetic protein (BMP) coreceptor [335]. HJV is associated with the transmembrane protease serine 6 (TMPRSS6), also known as matriptase 2, that causes HJV release by furin-mediated cleavage when both proteins physically interact on the cell surface [336]. Moreover, Silvestri et al. also reported that furin mRNA expression is regulated by iron and hypoxia [337]. Soluble HJV (sHJV) antagonizes BMP-dependent hepcidin activation. Importantly, HJV cleavage by other proteases does not seem to be redundant with that of TMPRSS6 since a lack of TMPRSS6 activity causes iron deficiency in humans and mice [336].

Neogenin, also plays a role in hepcidin regulation by stabilizing HJV to enhance BMP signaling and HAMP expression. *Neogenin*$^{-/-}$ mice exhibit hepatic iron overload, low hepcidin levels, and reduced BMP signaling [338].

Transcriptional regulation of hepcidin is associated with a multitude of molecules, including HFE, TfR2, HJV, BMP6, and TMPRSS6, and their mutations lead to hepcidin dysregulation. In fact, mutations of HFE, TfR2 and HJV lead to hepcidin deficiency and different forms of hereditary hemochromatosis (HH). Animal studies further demonstrate the importance of these molecules in iron metabolism. For example, double knockout mice

HfeTfr2$^{-/-}$ and *Hfeβ2m*$^{-/-}$ had a more severe phenotype than single knockout mice [339, 340]. HFE, TfR2 and HJV form a multiprotein complex at the surface of hepatocytes. Mutations in TMPRSS6 decrease in iron-deficient mice and are associated with iron-refractory iron deficiency anemia (IRIDA). Double knockout mice *Tmprss6Hjv*$^{-/-}$ cause iron overload, suggesting that TMPRSS6 acts upstream of HJV [336, 341]. Bone morphogenetic protein *Bmp6*$^{-/-}$ [342-344] and liver-specific small mother against decapentaplegic *Smad4*$^{-/-}$ mice also developed severe iron overload and low HAMP mRNA expression [345]. BMPs are members of the TGF-β family and well known for playing a crucial role in iron metabolism [346]. Finally, the importance of hepcidin and FPN-1 regulation is highlighted by the fact that they are involved in various pathogenesis of diseases such as hereditary hemochromatosis (HH) [328, 331, 347], anemia of chronic disease [348] and β-thalassemia [349] (See Table 2).

3.3.1. Iron sensing pathway: BMP/SMAD4 signaling pathway

The signaling pathway BMP/SMAD4 is the main iron-dependent modulatory pathway [346]. It initiates through the BMPs, and BMP6 acts as a central regulator of hepcidin expression in the liver *in vivo* [342, 343]. How iron stimulates BMPs' expression is not yet well understood. Other BMPs, such as BMP9 and BMP4 are capable of activating hepcidin, and recently it has been shown that BMP2 activates the pathway independently of BMP6 [350-352] . BMPs bind to their receptors at the hepatocyte cell surface, which is a complex of type I (activin-like, Alk2 and Alk3) and type II (ACTRIIA serine-threonine kinase receptors also called BMPRI and II [353] and coreceptors (HJV) [335, 354]. The complex BMP/HJV induces the phosphorylation of SMAD1/5/8 also known as regulatory SMADs (R-SMAD) in the cytoplasm, which forms a complex with SMAD4 [345]. This complex translocates into the nucleus to modulate transcription of target genes, including HAMP. Responsive elements at the proximal and distal position of the HAMP promoter, BMP-RE1 at position -84/-79 and BMP-RE2 at -2,255/-2,250 are essential for transcription of the peptide [341, 355].

The administration of bone morphogenetic proteins, BMP2 and BMP6 in mice induces hepcidin mRNA and decreases serum iron levels, while BMP antagonists (such as

dorsomorphins) inhibit hepcidin mRNA expression and increase serum iron levels [356]. As mentioned before, these proteins are responsible for SMAD1/5/8 phosphorylation, the so-called R-SMADs, that interact with SMAD4 to form a complex that translocates into the nucleus to induce hepcidin expression. R-SMAD phosphorylation is attenuated in mice lacking HFE, suggesting that HJV and HFE act together to activate hepcidin transcription via the BMP-SMAD pathway [357, 358]. *Tfr2*$^{-/-}$ mice have also been reported to have reduced pSMAD1/5/8 levels in the liver, suggesting a modulation of TfR2 to regulate hepcidin via BMP6 upregulation to activate the BMP–SMAD pathway [359, 360].

Finally, the inhibitory SMAD7 and SMAD6 are responsible for hepcidin transcriptional suppression [361, 362].

3.3.2. Inflammatory pathway: IL-6/STAT3 signaling pathway

Inflammatory cytokines such as interleukin IL-6, IL-1α and tumor necrosis TNF-α are important inducers of hepcidin expression [329, 332]. The pathway is triggered during infections and inflammation. For example, lipopolysaccharide (LPS) and the irritant turpentine oil stimulate inflammatory cytokine-enhanced hepcidin expression [329, 331, 332, 334]. High hepcidin expression during infection is a defense by limiting iron availability, since most micro-organisms need iron to proliferate. Moreover, it has also been suggested that increased hepcidin expression and iron deprivation may represent defense mechanisms against excessive cell proliferation and cancer, as iron is fundamental for cell proliferation.

In this pathway, inflammatory cytokines such as IL-6 promote the activation of the Janus kinase (JAK)/signal transducer and activator of the transcription (STAT3) signaling pathway [363, 364]. The pathway initiates by the binding of IL-6 to glycoprotein 130 kDa (gp-130) receptors' complex followed by STAT3 phosphorylation mediated by JAK1/2 and translocation to the nucleus to activate the hepcidin promoter via STAT-binding motif at the proximal region of the HAMP promoter [364, 365]. Interestingly, another member of the TGF-β family, Activin B, has been shown to activate HAMP expression during

inflammation via the activation of the BMP signaling pathway *in vitro* [366, 367], highlighting the interaction between the pathways.

Finally, endoplasmic reticulum stress (ER) also increases hepcidin expression by regulating the transcription factor cyclic AMP response element-binding protein H (CREBH) [368] or by the stress-inducible transcription factors CHOP and CAAT enhancer-binding protein α (C/EBPα) [369].

3.3.2.1. Crosstalk between signaling pathways

As mentioned previously, interaction/crosstalk between iron and the inflammatory pathway to enhance *HAMP* expression has been reported by several studies [370].
For example, hepcidin transcription upon IL-6 stimuli is abrogated in liver-specific *Smad4*$^{-/-}$ mice [345, 355], whereas LPS counteracts diminished hepcidin expression in response to iron deficiency, suggesting that the two signals are integrated at the hepcidin promoter and that inflammatory and iron stores regulators operate independently rather than following a strict hierarchy [371]. Mutations in BMP-RE1 impair inflammatory pathway induction through IL-6 activation, suggesting a dual control by BMP-RE1 that is iron sensing but also inflammatory [372]. Furthermore, *Alk3*$^{-/-}$ mice with type I BMP receptors were impaired to enhance HAMP expression after inflammatory stimuli by IL-6 [373].

3.3.3. Hepcidin regulation by erythropoiesis

As mentioned above, erythropoiesis requires considerable amounts of iron; therefore, hepcidin inhibition by erythropoietic signals is critical for the physiological condition.
However, the molecular mechanism and transcriptional factor responsible for such activities are still poorly understood.

Erythroid factors such as growth differentiation factor 15 (GDF15) [374], twisted gastrulation BMP signaling modulator 1 (TWSG1) [375] and erythroferrone ERFE [159] seem to play a critical role in hepcidin regulation by erythropoiesis [376, 377]. GDF15 is

detected at high concentrations in the serum of patients with impaired erythropoiesis such as β-thalassemia [374]. Moreover, GDF15 can suppress hepcidin transcription in cell models, but the underlying molecular mechanism has not yet been characterized. And low doses of GDF15 fail to suppress hepcidin in cellular models and are supposedly invaluable in patients with sickle-cell anemia, myelodysplastic syndrome and ACD. TWSG1 produced during early erythroblast maturation and its expression is increased in thalassemic mouse models. In cellular models, the BMP-binding protein TWSG1 inhibits BMP-dependent activation of Smad-mediated signal transduction that leads to hepcidin expression [375]. Hepcidin suppression in response to phlebotomy or hemolysis depends on intact erythropoietic activity in mouse models, since irradiation and cytotoxic inhibition of erythropoiesis prevent hepcidin suppression in these settings [378]. Finally, the erythroferrone ERFE, also known as myonectin or C1q/TNF-related protein family member 15 (CTRP15) has been suggested to play a role in hepcidin suppression by erythropoiesis following EPO stimulation in certain conditions as β-thalassemia but not in others such as IRIDA [159].

3.3.4. Hepcidin regulation by hypoxia

Hepcidin expression is regulated also by hypoxia, a pathological condition resulting from a failure of oxygenation at the tissue level [331]. In this disorder, gene expression involved in erythropoiesis and iron metabolism (such as *Tf* and *TfRs*) but also angiogenesis are upregulated [379]. The liver-specific hypoxia-inducible factor (HIF)-1 plays a critical role in the pathway by directly binding to the hypoxia response elements (HREs) of target genes [379]. HIF-1 is a heterodimer form of two subunit HIF-1αs, the oxygen-responsive component, and a constitutively expressed HIF-1β subunit, also known as the aryl hydrocarbon receptor nuclear translocator (ARNT) [379].

At normal oxygenation levels, iron-dependent prolyl-hydroxylases (PHDs) hydroxylate HIF-1α on two of the prolines, inducing the recruitment of the von Hippel-Lindau (vHL) tumor-suppressor protein that triggers HIF degradation through the ubiquitin-proteasome pathway. In hypoxic conditions, prolyl-hydroxylase activity is inhibited with the

consequent accumulation of HIF-1α and its translocation to the nucleus and interaction with ARNT/HIF-1β, allowing the transcription of HIF-dependent genes [379].

HIF1 and HIF2 stabilization decreases hepcidin expression in hepatoma cells, and it has been shown that chemical HIF stabilizers suppress hepcidin mRNA expression in hepatoma cells. It has also been suggested that HIF degradation can act as hepatic iron sensors [380]. In mouse models of hypoxia and in mice treated with phenylhydrazine (PHZ) to induce erythrocyte lysis, both conditions that induce erythropoietin (EPO) synthesis and stimulates erythropoiesis, hepcidin levels are reduced and can override signals that activate hepcidin expression, such as LPS treatment [371, 378, 381]. A low dosage of injected EPO in human volunteers decreases urinary excretion of hepcidin [382]. Since this effect can be prevented by erythropoiesis inhibitors, it has been suggested that EPO indirectly suppresses hepcidin expression through stimulation of erythropoiesis [378]. A recent study observed an increase of platelet-derived growth factor (PDGF)-BB in response to hypoxic conditions with consequent decrease of hepcidin levels, suggesting that PDGF-BB could be the hypoxic mediator of HAMP expression [383]. The authors found that, in response to hypoxic conditions in mice, levels of C/EBPα, CREB and CREB-H are decreased and liver HAMP expression is not downregulated in CREB-H knockout mice injected with PDGF-BB. These results indicate that CREB-H is required for PDGF-BB-to mediate hepcidin suppression [383].

3.3.5. Other hepcidin regulatory mediators

Hormones such as estrogen, testosterone and cortisol have been shown to negatively regulate hepcidin expression [298, 384, 385]. Growth factors such as hepatocyte growth factor (HGF) and epidermal growth factor downregulate HAMP expression by limiting the amount of pSMAD1/5/8 translocating to the nucleus [386]. Moreover, iron metabolism plays a key role in certain diseases, and recently it has been reported that during the progression of hypertension, adrenaline (AD) and norepinephrine (NE) act on hepatic hepcidin regulation via the STAT3 pathway [387].

Finally, *in vitro* experiments identified another signaling pathway of rapidly accelerated fibrosarcoma (Ras–RAF), mitogen-activated protein kinase (MAPK) and mammalian target of rapamycin (mTOR) as HAMP regulators [388].

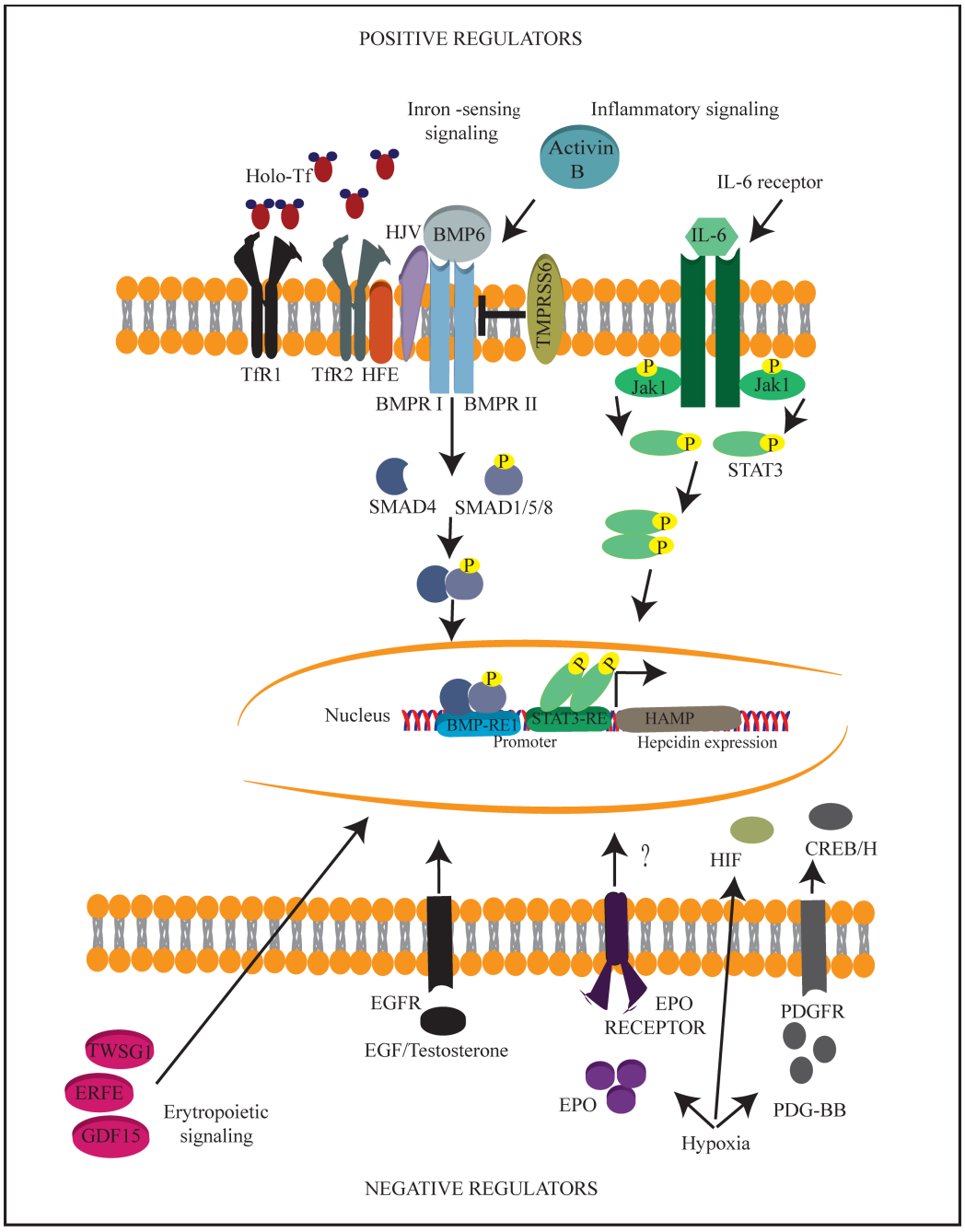

Figure 5: Hepcidin mRNA (HAMP) positive and negative regulators. Figure adapted from [389]. HAMP positive signaling pathway comprises the inflammatory, mainly induced by IL-6 and the iron sensing, commonly triggered by BMP6, which includes the iron sensor complex (HFE which is regulated by transferrin-bound iron Holo-Tf, TfR2 and HJV). Negative regulators comprise erythropoietic signaling, several hormones and hypoxic regulators. Permission obtained from the Bioscience Reports journal.

4. Disorders of iron metabolism

Dysregulation between iron uptake and storage leads to several common nutrition-related pathologies that debilitate quality of life, representing a global health problem [390, 391]. Disorders of iron metabolism, inherited genetically or acquired, are among the most common diseases of humans worldwide with diverse clinical manifestations from anemia (iron deficiency) to hereditary hemochromatosis (iron overload) [8, 392]. Moreover, iron metabolism disorders can exacerbate multiple other diseases or conditions such as ischemia-reperfusion injury [393, 394], neurological disorders [395-398], muscle diseases such as Duchenne's muscular dystrophy, and stroke damage**,** in which iron excess leads to tissue toxicity [16, 399]. Excess of iron combined with UV radiation can also be toxic for the skin due to the formation of free radical-mediated injury on human skin [400]. However, iron deficiency that leads most predominantly to anemia can also play an aggravating role on muscle, epithelia and nervous system disorders.

4.1. Iron overload

Iron overload disorders are most common in industrialized countries, where red meat consumption and iron fortification of food are often abused [391]. The etiology of iron overload tissues dysfunction can be attributed, at least partially, to the production of ROS and decreased antioxidant storage [16, 399]. Iron overload may aggravate liver diseases (cirrhosis, hepatic fibrosis, hepatocellular cancer), and can also affect the development of other diseases such as diabetes mellitus, arthritis, cardiomyopathy, hypogonadism, and hypothyroidism. Several diseases associated with iron overload such as hereditary hemochromatosis (HH), β-thalassemia and SCD are treated with phlebotomy and iron chelators [401].

4.1.1. Iron overload (Hemochromatosis)

The term hereditary hemochromatosis (HH) is used to designate several diseases characterized by systemic iron overload of genetic origin [233, 402, 403]. Most forms of

HH (types 1, 2, 3 and 4B hemochromatosis) are related to hepcidin dysregulation. The most frequent form of HH is type 1, a form that is associated with HFE deficiency [249]. The excess of iron accumulation in the liver, heart and pancreas provokes tissues damage from oxidative stress [402, 404], leading to the development of different malignancies such as cirrhosis or other liver diseases, hepatocellular carcinoma, diabetes mellitus, arthropathy, hypogonadism, and cardiomyopathy [403, 405]. HH type 1 is most commonly genetically transmitted in Northern Europe and males are affected more [121, 406].

HH disorders are classified in four classes and six molecular subtypes caused by mutations in six genes [407]: type 1 - HFE [249], type 2A - hemojuvelin (HJV) [408], type 2B - HAMP [347, 406], type 3 - TfR2 [220], type 4A - FPN-1, and type 4B – ceruloplasmin (CP) [409, 410]. Types 2A and 2B are also known as juvenile hemochromatosis (JH), and type 4B as hereditary aceruloplasminemia.
HFE type 1 results from HFE missense mutations on chromosome 6, mostly a conversion of cysteine to tyrosine at amino acid 282 (C282Y), due to a 845G-->A change [248, 249]. As mentioned before, HFE, needs to bind to β_2-microglobulin (β2m) for its proper folding. The C282Y mutation impedes the formation of the β_2-microglobulin/HFE complex, leading to inefficient trafficking and expression of HFE at the cell surface. Other HFE missense mutations such as HFE_{H63D} (histidine to aspartic acid mutation at position 63) that do not affect β2m-HFE binding and HFE_{S65C} (serine to cysteine mutation at position 65) are rare. These mutations abrogate the interaction between TfR and HFE [245, 411, 412]. Patients with HH due to HJV gene mutations on chromosome 1 or rare HAMP gene mutation on chromosome 19 are phenotypically similar [407]. HJV patients develop, at an early age, irreversible hypogonadism, refractory heart failure and even death [413]. Autosomal recessive inheritance due to mutations in the TfR2 gene on chromosome 3 presents a less severe phenotype [220, 412]. Finally, autosomal dominant inheritance with heterozygous mutations in the FPN-1 gene (N144, Y64N and C326) on chromosome 2 causes a hemochromatosis-like disease with high plasma iron and liver iron overload [406, 407, 414]. The FPN-1 mutations cause a hepcidin failure to bind FPN-1 or impairment in internalization and degradation of FPN-1 following hepcidin association [414], resulting in enhanced cellular iron export.

4.2. Iron deficiency

Iron deficiency is the most common iron disorder with a high incidence in infants, preschoolers, women of childbearing age, and pregnant women in both developed and developing countries that affects more than two billion people worldwide [415, 416]. Iron deficiency may contribute to cognitive developmental retardation in children, since it affects mitochondrial biogenesis, promoting poor physical performance and unfavorable pregnancy outcomes [417]. The causes of iron-deficiency in developing countries compared to developed countries are different. In the former, iron-deficiency and iron deficiency anemia result from a deficiency in dietary uptake and loss of blood due to bacterial and parasitic colonization, while in the latter, the most common causes are eating habits (vegetarian diet or no intake of red meat) or pathological conditions (chronic blood loss) [418]. To conclude, iron deficiency is based on iron homeostasis tightly regulated by hepcidin; inappropriately low hepcidin levels therefore cause iron overload, while high hepcidin expression lowers plasma iron levels (due to diminished iron release by macrophages and lower iron absorption) and causes anemia of chronic diseases (ACD) and genetic iron-refractory iron deficiency anemia (IRIDA).

4.2.1. Iron deficiency anemia

Iron-deficiency anemia (IDA) is a microcytic hypochromic form of anemia. It refers to the reduction of iron stores that precedes overt iron-deficiency anemia, a more severe condition in which low iron is associated with anemia, i.e. reduction of hemoglobin (Hb) levels (generally Hb <12 g/dL for women and Hb <13 g/dL for men). The hallmarks of iron-deficiency anemia are the suppression of hepcidin expression and the lack of normal oxygenation in the tissue (hypoxia) consequent to microcytic anemia. Serum ferritin and transferrin saturation are the key tests in IDA [419], and IDA is considered severe when transferrin saturation is about 16% and serum ferritin is about < 12 ng/ mL. Other values as the mean corpuscular volume MCV, the average size of the RBC, and the mean corpuscular hemoglobin MCH, the average mass of hemoglobin per RBCs, are decreased in IDA (<80 fL and <27 pg/cell respectively) [419].

In certain cases, iron deficiency may be compensated through iron administration to saturate Tf and stimulate NTBI uptake. Furthermore, DMT1 expression in the gut is increased to facilitate intestinal iron uptake through HIF2α activation.

The degree of iron repletion in the body is determined by the rapidity of activation of these molecules to counteract the condition. However, in certain cases of chronic blood loss, such as in IBD [420], a drastic reduction in iron absorption must be monitored closely.

4.2.1.1. Anemia in Inflammatory bowel disease

Inflammatory bowel disease (IBD) are a group of chronic gastrointestinal illnesses, such as ulcerative colitis and Crohn's disease. The symptoms of IBD included diarrhea, weight loss, ulceration, perforation and complete obstruction of the gastrointestinal tract.

In IBD patients iron deficiency is the most common systemic complication and it is associated with high mortality and clinical complication of the disease [420-422]. Anemia in IBD is caused by rectal bleeding, and it can be characterized by functional IDA, related to low iron absorption, or absolute IDA due to insufficient iron intake from the diet and blood loss from ulcerated intestinal mucosa [420].

While iron deficiency is the most common cause of anemia in IBD patients, anemia of chronic disease micronutrients deficiency (vitamin B_{12} and folic acid), pro-inflammatory cytokines effects, hemolysis, drug therapies, and myelosuppression, have also been identified as the origin of the condition in other patients [420]. Beside IDA and micronutrients, another potential cause of anemia in IBD is anemia of chronic disease (ACD), characterized by the upregulation of inflammatory cytokines that will lead to the increase of hepcidin expression followed by restricted iron availability for erythropoiesis [423]. Determination of the ferritin index, hepcidin and inflammatory markers levels has been taken as a tool to distinguish between iron deficiency anemia, anemia due to chronic disease, or mixed anemia in IBD patients [424, 425].

4.2.2. Anemia of chronic disease

Anemia of chronic disease (ACD), also called anemia of inflammation, is a normocytic normochromic anemia caused by high level of hepcidin and functional iron deficiency due to chronic inflammation [348, 423, 426, 427].

ACD is a multifactorial, functional anemia characteristic of chronic inflammation, chronic immune infection and malignancy.

ACD occurs as a host defense reaction against infection. It is characterized by a normal or high level of ferritin and low body iron host defenses during which inflammatory cytokines (IFN-γ, IFN-α, TNF-α and IL-1) are capable of shortening the erythrocytes' lifespans by activating macrophages, prioritizing leukocyte production in the marrow [348, 428]. Cytokines also mediate the down-regulation of erythroid progenitor EPO-receptor expression and contribute to inhibiting erythroid proliferation by suppressing the growth and differentiation of the erythroid progenitor and iron uptake [423, 429, 430].

As in IDA, in ACD transferrin saturation decreased, in contrary serum ferritin increased, indicating macrophages iron sequestration [419]. Finally, measurement of inflammatory markers as C-reactive protein CRP are important tools used to differentiate ACD to IDA [424].

4.2.3. Other inborn errors in iron metabolism

As previously described, multiple proteins are involved in iron homeostasis; consequently, mutations of these proteins result in defective iron homeostasis (Table 2).

The divalent protein-coupled metal ion transporter 1 (DMT1) mutation in animal models (mice and rats) and humans causes severe microcytic, hypochromic anemia [431]. In mice, the mutation leads to a defect in intestinal iron uptake, acquisition and utilization for RBC precursors and other tissues. It is most generally characterized by G185R in the transmembrane domain 4 of the mouse protein [87, 432]. In humans, the mutation leads to a decrease in erythroid iron utilization but also an increase in liver iron storage with high

levels of transferrin saturation and serum ferritin [433]. Moreover, only three human patients have been reported with DMT1 mutations, namely E399D, G1285C and R416C; in these patients, anemia was present from birth with variable severity [434].

β-thalassemia is one of the major and rare inherited recessive disorders of hemoglobin synthesis resulting in transfusion-dependent anemia. β-thalassemic patients are characterized by decreased RBC production due to a defect in hemoglobin β-chain synthesis [349].

IRIDA is an autosomal recessive disorder cause by the mutation of the protease TMPRSS6, which by HJV cleavage inhibits the signaling pathway that activates hepcidin. Loss of function of TMPRSS6 leads to a constitutively high hepcidin expression with a consequent block of iron efflux from intestinal enterocytes to the plasma [435, 436].

Disorder	Gene mutated	Hepcidin level
Hypotransferrin	Tf	Low levels of hepcidin
Hereditary Hemochromatosis	TfR2	Low levels of hepcidin
	HFE	Low or inappropriately normal despite iron load
	HJV	
	Hepcidin	Hepcidin expression abrogated
	Ferroportin	
Iron-refractory iron deficiency anemia (IRIDA)	TMPRSS6	High or normal, despite iron deficiency
β-Thalassemia	β-globin	Low, unless transfused
Anemia of chronic disease (ACD)		High or inappropriately normal

Table 2: Frequent human and mouse disorders that cause hepcidin dysregulation.

5. Iron chelators

Iron chelators are a group of varied compounds that have the ability to bind iron very tightly in a nontoxic form and consequently modulate the excretion of excess iron in the body via urine and stool [437, 438]. Iron chelators, either synthetic or natural, are used to decrease iron toxicity and as treatment of iron overload disorders, especially in patients that receive multiple blood transfusions. In these patients, iron chelators are usually administrated after 10–12 transfusions or when ferritin levels rise above 1,000 ng/ml [439, 440]. Moreover, iron chelators have also been reported to have anti-inflammatory, anti-cancer and cardio- and neuroprotective properties and are used to counteract the deleterious effects of oxidative stress [437, 441, 442]. As a result, they have been used in recent years for the treatment of a number of clinical conditions such as β-thalassaemia but also cancer, anthracycline-mediated cardiotoxicity, malaria, HIV, and severe neurodegenerative diseases such as Friedreich's ataxia [437-439, 442]. The most used iron chelators available for clinical therapy are deferoxamine [138], deferiprone (DFP) and deferasirox (DFX) [443, 444]. DFO is effective to treat secondary iron overload in β-thalassemia; however, it can procure several side effects such as pain, erythema, swelling, induration, burning, pruritus, and rash [443-446]. A combination of iron chelators (DFP with DFO) has been shown to improve their pharmacological properties as iron sequestering agents, which cannot be achieved through either drug alone without increasing their toxicity [443, 445, 446]. It has been shown that iron chelators, especially DFO, are able to detoxify NTBI by chelation and hemoproteins by the reduction of ferryl states and ROS, which are responsible for macromolecular damage and progression in oxidative stress-induced diseases [447, 448]. Chelators such as DFO [449] and the flavonoid quercetin [450] are capable of inhibiting HIF-1α hydroxilation through the removal of iron form PHD, which increases the levels of HIF-1α [442, 444].

Iron chelators can be classified using different criteria, including their origin (natural versus synthetic chelators), their interactions with solvents such as water (hydrophilic versus hydrophobic chelators) and their stoichiometric interaction (bidentate versus hexadentate). All these parameters are very important for the impact of the chelators' activity as therapeutic agents [442, 444]. While the chemical structure of iron chelators differs, they

all contain iron bound to oxygen, nitrogen or sulfur atoms as donor atoms [441]. The donor atoms can bind either Fe^{2+} (ferrous) or Fe^{3+} (ferric) iron with high affinity.

5.1.1. Siderophores

Siderophores are natural, small iron chelator molecules secreted by some bacteria for iron uptake and storage [451-453]. Iron is an essential micronutrient for almost all microorganisms and its acquisition from the host environment is fundamental for the growth of commensal and pathogenic bacteria [453]. A similar strategy of iron acquisition from the colonized environment is also used by other living organisms such as fungi [451]. In nature, more than 260 microbial siderophores (such as enterobactin or enterochelin) have been identified and studied [453-456]. Their chemical structures comprise typically metal-binding motifs - catecholates, hydroxamates and α-hydroxycarboxylic acids - allowing them to bind iron with high affinity.

To limit iron availability for the growth of microorganisms, the immune systems in mammals have evolved a line of defense using the secretion of various proteins that act as iron-sequesting molecules, such as lipocalin 2, also known as siderocalin and neutrophil gelatinase-associated lipocalin (NGAL) that binds ferric enterobactin [457, 458]. Among many functions, lipocalin 2, secreted by different cell types in response to inflammation or infection, can sequester *Escherichia coli (E. coli)* catecholate-type and *Mycobacterium tuberculosis* (*M. tuberculosis*) salicylate-derived siderophores [457, 458]. Another important and potent iron chelator secreted by neutrophil granules is the transferrin family glycoprotein with a high affinity for the ferric iron, lactoferrin. It is induced by neutrophil granules at the sites of infection and inflammation [45].

5.1.2. Natural phytochemical chelators

Various phytochemicals and herbal remedies have been reported for decades to possess anti-oxidants, due to their iron-chelating properties [437, 459, 460]. In fact, compounds such as proanthocyanins, epicatechins, flavonoids, and anthocyanin contain iron-binding

motifs that confer on them the ability to sequester iron acting as iron chelators [461, 462]. Multiple clinical trials have been conducted to highlight the importance of healthy foods as good antioxidants [463] while herbal remedies such as curcumin from the rhizome *Curcuma Longa* [464, 465], kolaviron from *Garcinia kola* African seeds [466], baicalein from the Chinese herb *Scutellaria baicalensis* [467], and many others have also been reported to have iron-chelating properties [437].

5.1.2.1. Curcumin

The yellow spice curcumin (diferuloylmethane) is the active ingredient in the rhizome turmeric *Curcuma Longa* (from the *Zingiberaceae* family) [468]. Curcumin is a hydrophobic polyphenolic compound bis α,ß unsaturated ß-ketone known as diferuloylmethane or 1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione. Its molecular formula is $C_{21}H_{20}O_{6,}$ and it consists of a linear diarylheptanoid with 2-oxy-substituted aryl moieties connected through a 7-carbon chain [468, 469]. Curcumin has been reported as a free radical scavenger and hydrogen donor and has been studied for decades for its multiple biological properties, including anti-inflammatory, anti-oxidant [470, 471], anti-tumor [469], anti-septic, and anti-thrombosis properties. Revealingly, curcumin has been used for centuries as a herbal remedy [472].

As an anti-cancer agent, curcumin has been shown to inhibit cancer development and progression [469], in which its iron-chelating properties seem to play a role [464]. Curcumin is considered to be an anti-cancer natural extract since it has chemotherapeutic activity and chemo preventive effects in animal models of induced cancer [473-475]. Numerous studies reported several beneficial effects of curcumin on cancer cell development such as in breast, prostate, pancreatic, lung, uterine, ovarian, kidney, renal, brain, non-Hodgkin lymphoma, and leukemia cancers [469, 475-477]. Moreover, curcumin has shown anti-proliferative effects, acting as an inhibitor of NF-κB and STAT3 related pathways (including fas, p53, VEGF, Bcl-2, and MMP-9) in multiple myeloma [474-476]. It has also been reported that curcumin can decrease apoptosis and angiogenesis [478].

As an anti-inflammatory agent, curcumin decreases various pro-inflammatory cytokines such as NF-κB, decreasing the release of interleukins, TNF, inducible nitric oxide synthase, hypoxia-inducible factor-1, and nuclear factor erythroid-2-related factor 2 (Nrf2) [474, 476].

As an anti-oxidant, it activates antioxidant systems such as erythroid 2-related factor-2 and members of the vitagene family (heat shock protein 70) (HO-1) and thioredoxin [12]. Moreover, different studies have demonstrated that curcumin inhibits toll-like receptor 4 (TLR4) and its adaptor protein MyD88 (TLR4/MyD88 pathway), an important activator of NF-κB transcription [479].

Curcumin and turmeric derivatives are considered to be safe by the U.S. Food and Drug Administration (FDA) and the Food and Agriculture Organization (FAO) / World Health Organization (WHO). Curcumin is a hydrophobic polyphenol that can be rapidly degraded and presents a reduce bioavailability [480]. However, since it is nontoxic with relatively few side effects, multiple studies have considered high dosages of curcumin (six g/day orally for four to seven weeks) to be safe and beneficial [472, 481-483]. Because of all of the above, curcumin is considered to be a good candidate as a therapeutic agent. Currently, curcumin is in use in a variety of clinical trials for different diseases including IBD, pancreatitis and neurodegenerative disorders such as Alzheimer's or Parkinson's diseases [474, 484, 485], and may represent an option in the management of free-radical-related disorders [486].

5.1.2.2. Curcumin as an iron chelator

In vitro and *in vivo* chelating activity of curcumin has been reported in several studies [464, 465, 487, 488].

In iron overload conditions, it has been shown that curcumin decreases liver and spleen iron content and lipid peroxidation markers such as malonyldialdehyde (MDA) and nitric oxide (NO) in rats [489]. Consistent with these reports, curcumin reduced NTBI in a mouse model of β-thalassemia [490]. Furthermore, it has been demonstrated that curcumin administration in rats restored decreased anti-oxidant activities in hepatic and splenic tissues [489]. Jiao et al. reported for the first time the chelating property of curcumin in mice fed with the compound for 12 weeks (2% curcumin supplementation corresponding to 7–8 g/day in the human diet). Under these conditions, curcumin dramatically affected iron homeostasis by decreasing ferritin storage, increasing TfR1 and activating iron-regulatory protein IRPs, indicators of iron-depleted conditions [465]. A study further demonstrated the ability of curcumin to modulate iron homeostasis in mice fed with a normal iron diet and 2% curcumin over six months. In this study, dietary curcumin was associated with a decline in erythroid parameters, serum iron and Tf saturation, and the appearance of hypochromic red blood cells [464]. Moreover, under these conditions, curcumin downregulated HAMP mRNA expression [464]. This study demonstrated for the first time that curcumin may impair iron status particularly in mice with low iron diet (5 mg iron/kg diet) and slightly less in mice fed with sufficient iron diet (12 to 50 mg iron/kg diet), leading to iron-deficiency. Moreover, Chin et al. also demonstrated that long-term dietary curcumin (12 months) at a low concentration (0.2% of dietary curcumin) may affect iron homeostasis leading to mild anemia [488]. These studies open up the controversy of using curcumin as a therapeutic agent in chronic diseases in which iron levels are critically low without further monitoring of iron status [491].

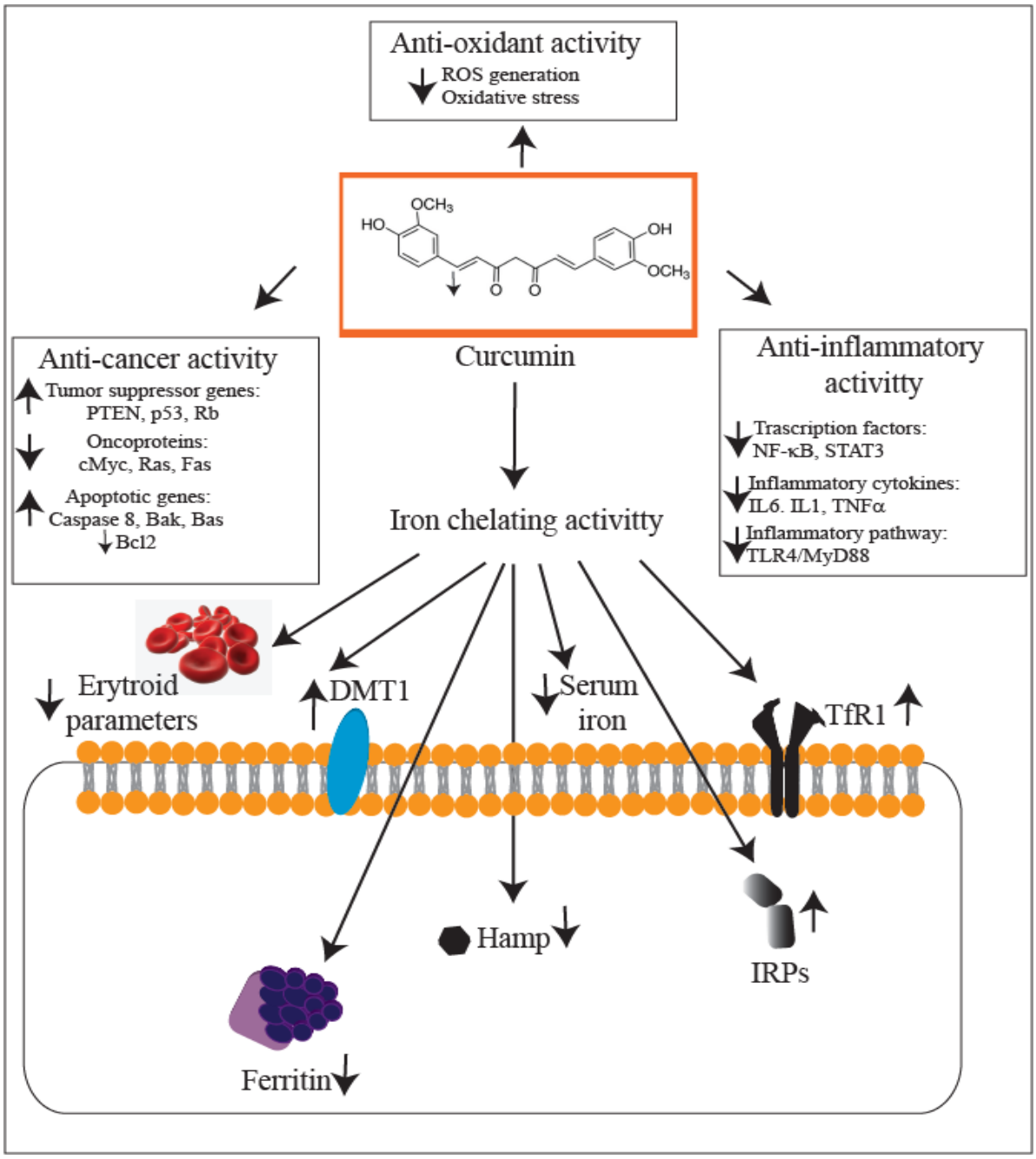

Figure 6: Schematic representation of multifactorial curcumin properties as an anti-cancer, anti-inflammatory and iron chelator agent. Curcumin downregulates transcriptional factors and inflammatory cytokines and inhibits the TLR4/MyD88 pathway. Curcumin is also involved in the regulation of tumor suppressors, oncoproteins and apoptotic genes. As an iron chelator, curcumin represses oxidative stress.

6. Interaction between iron metabolism and innate immunity

The interaction between iron metabolism and innate immunity, the first defense of the host against microorganisms, can be detailed using different examples.

As previously mentioned, iron is first and foremost a critical micronutrient for almost all microbial growth and proliferation. Consequently, iron homeostasis plays an important role in the host's defense mechanism to fight pathogens. In this context, innate immunity uses several proteins that are important for iron sequestration during infection such as lipocalin 2 or lactoferrin [45, 457, 458, 492]. Both proteins are considered to be critical components of innate defense, since both show the ability to inhibit bacterial growth [492].

Inflammatory cytokines have been shown to influence iron homeostasis, promote iron sequestration within macrophages and contribute to the development of functional anemia [493]. Accordingly, hepcidin regulation via the inflammatory pathway is mediated by multiple inflammatory cytokines, such as IL-6 and TNF-α [423].

Finally, the toll-like receptor / myeloid differentiation factor 88 (TLRs/MyD88) pathway that plays a critical role in innate immunity has been associated with iron metabolism in response to microbial infections via hepcidin regulation [494-496].

In this section, the TLR and MyD88 roles, functions and implication in iron metabolism are presented.

6.1. Toll-like receptors

Toll-like receptors (TLRs) are a group of pattern recognition receptors (PRRs) that have the ability to recognize molecular patterns present in microorganisms called pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) [497-499]. Since their activation depends on the recognition of pathogen-associated ligands such as glycoproteins, LPS, flagellin, and viral double-strand or single-

strand RNAs or DNAs, TLRs are consider the keystones of innate and adaptive immunity [495].

The evolutionarily-conserved TLRs are type I transmembrane proteins composed of an extracellular domain (leucin-rich repeats ectodomain known as LRR) and a cytoplasmic domain homologous to IL-1R [495, 496]. A total of 13 members of mammalian TLRs has been identified, 10 in humans (TLR1 toTLR10) and 12 in mice (TLR1 to TLR9, TLR11 to TLR13), depending on the type of pathogen (bacterial, fungal, parasite or viral) to which they are capable of detecting and responding [495, 496] . In humans, TLRs 1, 2, 5, and 6 are localized on the cell surface, and they recognize lipo-, glycol-, and acyl-peptides present on the surfaces of many Gram-positive and Gram-negative bacteria and mycobacteria [495]. TLR4 identifies bacterial lipopolysaccharide (LPS), the main components of the cell wall of Gram-negative bacteria, through its coreceptor, the monomeric recombinant myeloid differentiation factor MD-2, essential for the surface expression of TLR4 [495, 500]. TLR4 also recognizes endogenous ligands such as heat-shock proteins, extracellular matrix components including fibronectin, hyaluronic acid, and heparin sulphate in response to tissue injury, also known as DAMPs [495, 500, 501]. In contrast, TLRs 3, 7, 8, and 9, also known as the nucleic acid-sensing subfamily, are localized on the endosomal membrane where they single- or double-stranded nuclear materials (ssRNAs, dsRNAs and dsDNAs) in response to viral insults [495, 499]. Following molecular recognition and binding of pathogen ligands, TLRs dimerize and initiate downstream intracellular signaling cascades through TLR adaptor molecules that lead to the production of inflammatory cytokines such as nuclear factor B (NF-κB) or interferon β (IFN-β). Four TLR adaptor molecules have been identified, the myeloid differentiation response protein 88 (MyD88), the toll-interleukin 1 receptor domain containing adaptor protein (TIRAP also known as MAL), the TIRAP inducing IFN-β (TRIF), and the TRIF-related adaptor molecule (TRAM). TLR signaling pathways are classified into either MyD88 dependent or MyD88 independent pathway [495].

6.2. Protein adaptor MyD88

The myeloid differentiation factor 88 (MyD88) is the universal adaptor protein used by all TLRs (except TLR3) and the IL-1Rs family (IL-1R, IL-18R, and IL-33R), and it is essential for the induction of inflammatory cytokines; it is therefore critical to the host immune response against pathogens [495, 499, 502, 503]. Human MyD88 is well characterized; it maps to chromosome 3p22-p21.3 with five exons. Full-length MyD88 encodes 296 amino acids forming a 33 kDa protein [504, 505], whereas the murine homologue MyD88 maps to chromosome 9q119 and contains five exons encoding 296 amino acids forming a 33 kDa protein. MyD88 is composed of two principal conserved protein domains, a C-terminal toll-interleukin 1 receptor TIR domain [506] and an N-terminal death domain (DD), as well as a shorter linker of the intermediate domain (ID) localized between them [505, 507]. The MyD88 TIR domain is responsible for TLR upstream signal propagation through a TIR-TIR interaction [508]. It has been found that the structural conformation called BB loop in the MyD88 TIR domain is crucial for its homodimerization [494]. The DD domain is important for MyD88 oligomerization [509, 510] and interaction with the serine–threonine kinase, the IL1-receptor associated kinases (IRAKs), which also involve the ID domain [511].

6.2.1. MyD88 dependent TLR signaling

All TLRs, except TLR3, use the MyD88 dependent pathway. Upon TLR stimuli, MyD88 is activated via the TIR-TIR interaction [508, 512], then MyD88 oligomerization activates interleukin- (IL-) 1 receptor-associated kinase 4 (IRAK4) through their DD domains [500, 509, 510, 512]. IRAK1 and IRAK2 are then activated and phosphorylated by IRAK4 to form the Myddosome (also known as MyD88 signaling complex) [510, 513]. This structure interacts with the E3 ubiquitin ligase tumour necrosis factor (TNF) receptor-associated factor 6 (TRAF6), *via* the TRAF binding domain [510, 514]. The ubiquitin activity of TRAF6 leads to TRAF-activated kinase 1 (TAK1) activation [515]. TRAF6 interacts with TAK1 and IRAK1/2 to form a complex that will lead to the phosphorylation and activation of the IκB kinase (IKK complex) with the consequent activation of the NF-κB pathway. MyD88 downstream pathway will also leads to the activation of the mitogen-activated

protein kinase (MAPK) pathway and the interferon regulatory factors (IRFs) [514, 516]. MAPK activates c-Jun N-terminal kinase (JNK) to stimulate the Jun family of transcription factors activity [517]. The NF-κB factor depends on the state of activation of the cells; in resting cells, NF-κB is sequestered in an inactive form in the cytoplasm by the IκB protein [495, 500], while during NF-κB activation, IκB is degraded by proteasomal activity IκB kinase β (IKKβ), activated by TAK1 and followed by the release of NF-κB [500].

MyD88-indipendent pathway, also called TRIF-dependent pathway, is originated from TLR3 but also from TLR4 for type I interferon activation [500]. TLR3 uses exclusively TRIF adaptor protein. When recruited to the TLR4-TRAM-TRIF complex by TRIF, TRAF3 is polyubiquitinated, that will lead to the activation of TRAF family member-associated NF-*κ*B activator- (TANK-) binding kinase- (TBK-) 1 and IKKε. The signal will lead to interferon regulator factor 3 (IRF3) activation and IFN-β production [500, 514].
The TLR signaling cascades are summarized in Figure 7.

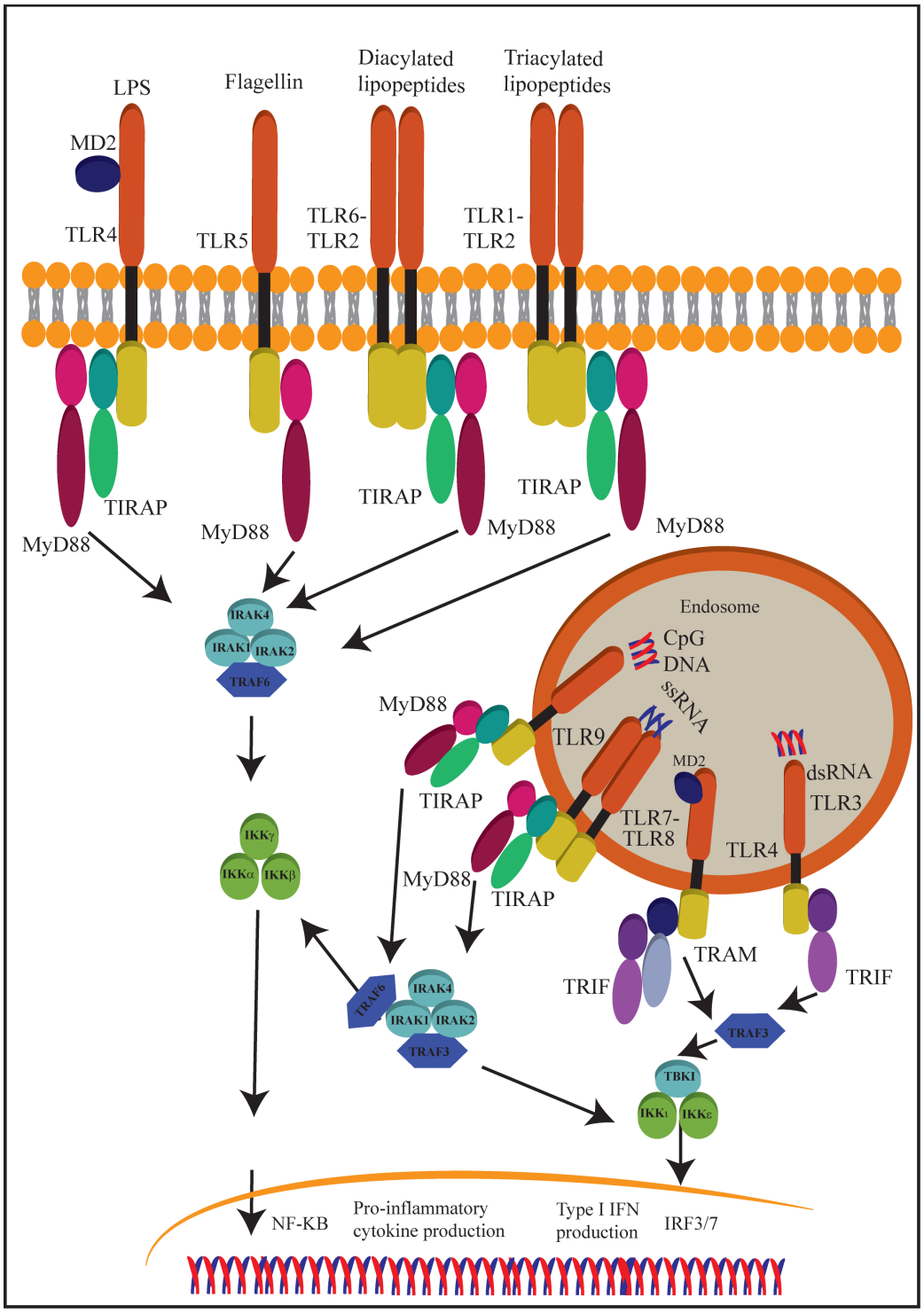

Figure 7: Toll-like receptors and downstream signal transduction of MyD88 dependent and independent pathways, adapted from [518]. Permission obtained from Frontiers in Immunology (Front Immunol) journal

6.2.2. Role of MyD88 in inflammation

As previously mentioned, MyD88 is critical for a variety of biologically important signal transduction pathways in innate immunity, since it is an essential protein for the induction of NF-κB and MAPK signaling pathways upon TLR or IL1-18 stimuli [497]. Consequently, MyD88 signaling is important in inflammatory regulation during pathogenic infection and cancer progression [518]. Hence, MyD88 deficiency and mutations are associated to recurrent life-threatening bacterial infections and susceptibility to a broad range of pathogens and immunodeficiency. Some of these conditions are lethal for MyD88-deficient mice (*MyD88*$^{-/-}$) and can cause life-threating organ dysfunction in humans [518, 519].

MyD88$^{-/-}$ mice are susceptible to a wide range of pathogens, including: 27 bacteria, 8 viruses, 7 protozoa, and 4 fungi [519]. Moreover, it has been shown that *MyD88*$^{-/-}$ mice are unable to adequately induce acute phase proteins and cytokine production in response to inflammatory stimuli. *MyD88*$^{-/-}$ mice also have defects in T-cell proliferation, with abrogation in the increase of INF-γ production and natural killer cell activation in response to IL-18 and IL-1 stimuli [520]. On the other hand, MyD88 ablation has been reported to be beneficial for various infection-related disorders such as the severe periodontitis causes by the Gram-negative bacteria *Aggregatibacter actinomycetemcomitans* [521].

In humans, autosomal recessive MyD88 deficiency has been associated with primary immunodeficiency, which confers a predisposition to severe bacterial infections such as meningitis, sepsis, arthritis, osteomyelitis, and abscesses [522]. These occurrences of pyogenic bacterial infections (mostly caused by *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*) are due to an impaired TLR canonical pathway with a lack of IL-6 production by leukocytes and cluster differentiation 62 ligand (CD62L), important components of granulocyte response [522].

The contribution of the TLR/MyD88 pathway in iron metabolism has been emphasized in several studies. First, it has been demonstrated that hepcidin induction upon LPS stimulation in macrophages and hepatic cells is specifically dependent on TLR4 *via* the

MyD88 dependent pathway [523-525]. In fact, it has been shown that hepcidin expression, in *MyD88*$^{-/-}$ and *Tlr4*$^{-/-}$ mice, is completely abrogated upon LPS stimulation macrophages. Recently, it has been reported that hepcidin expression is also impaired in primary hepatocytes from *Tlr4*$^{-/-}$ mice in response to LPS [523-525]. In this context, Layoun *et al.* demonstrated the essential role of MyD88 for endotoxin-induced acute hypoferremic response *in vivo* [524]. Xiong *et al.* recently demonstrated that hepcidin expression is upregulated *via* the TLR4/MyD88 pathway in an intracerebral hemorrhage (ICH) mouse model [526].

Finally, also TLR2 ligands, the synthetic tryacilated and dyacilated lipopeptides Pam3CSK4 and FSL1, have been shown to induce hepcidin mRNA *Hamp* expression in murine peritoneal macrophages. More precisely, Layoun *et al.* demonstrated that Hamp expression was increased upon TLR2 ligands stimulation and was impaired in *tlr2*$^{-/-}$ mice. They highlighted the role of MyD88 pathway for hepcidin induction by TLR2 since Hamp expression was completely abrogated in *MyD88*$^{-/-}$ mice upon TLR2 ligands stimulation [523].

6.2.3. MyD88 mutation: MyD88^{L265P}

Besides inflammation, MyD88 is also associated with hematological malignancies when mutated [518]. The most frequent mutation found in the MyD88 gene is a somatic mutation, consisting in genetic alteration acquired by a cell that can be passed to the progeny of the mutated cell in the course of cell division, (T-C) at position 38182641 in chromosome 3 at 3p22.2 locus. This single point mutation leads to an amino-acid substitution from leucine to proline in position 265 (MyD88^{L265P}) positioned on the hydrophobic core of the MyD88 TIR domain [527-529]. In B cell tumors, this mutation leads to the permanent activation of MyD88 downstream signaling pathways in the absence of cognate ligands, promoting the assembly and functional activity of IRAK1 and IRAK4 protein complexes, followed by the elevation of NF-κB signaling, JAK kinase activation and production of IL-6, IL-10 and IFN-β, which results in increased malignant cellular proliferation and survival [527, 530, 531].

MyD88^{L265P} is observed in a variety of B cell tumors, with the most striking incidences in lymphoplasmacytic lymphoma and Waldenström macroglobulinemia (WM), a lymphoproliferative disorder characterized by lymphoplasmacytic bone marrow associated with IgM paraprotein, in which it was detected in more than 90% of patients in different studies [518, 530, 532]. It has also been detected in 60% of patients with Immunoglobulin (Ig) M monoclonal gammopathy of undetermined significance (also known as IgM MGUS) [532, 533]. These data were confirmed by whole genome sequencing and Sanger sequencing [423, 534-536]. Moreover, using an RNA interference screen followed by high-throughput re-sequencing and comparative genomic hybridization, Ngo et al. discovered that MyD88^{L265P} was present in 39% of patients with activated B-cell (ABC) of diffuse large B-cell lymphoma (DLBCL) tumor biopsies and rare or absent on Burkitt's lymphoma and in other DCBL [527]. Furthermore, more recently, it has been shown that MyD88 L265P is also present in 2–10% of patients with chronic lymphocytic leukemia (CLL), 69% of patients with cutaneous diffuse large B cell lymphoma (CBCL) and 38% of patients with primary central nervous system lymphoma (PCNSL) [537-539].

In peripheral blood B cells from patients with WM, hepcidin was produced at levels higher than in healthy patients [315]. While hepcidin has been identified as an unequivocal contributor to anemia in WM, its production by B cells and monocytes is about 4 to 20 times lower than hepatocytes [316]. In monocytes [314] and lymphocytes [540], hepcidin produced by these cells may act as an autocrine regulator of iron accumulation.

CHAPTER 2

2. THESIS RATIONALE AND STUDIES OBJECTIVES

Hepcidin, an antimicrobial peptide belonging to the defensin family, is the major regulator of systemic iron homoeostasis, is regulated through the inflammatory STAT3/IL-6 and iron-sensing BMP/SMAD4 pathways [70, 299, 331, 389]. Our studies concentrate on the iron-sensing pathway, which is triggered mainly by the bone-morphogenetic 6 (BMP6) and 2 (BMP2) [342, 343, 350, 351]. Upon BMPs stimulation, phosphorylated SMAD1/5/8 in the cytoplasm forms a complex with SMAD4 [345]. This complex translocates into the nucleus to modulate hepcidin (HAMP) transcription [372] and activates other genes, including the inhibitory SMADs (SMAD6-7) [362].

Chapter 3: MyD88 adaptor protein is required for appropriate hepcidin induction in response to dietary iron overload in mice

The immune system is directly related to iron metabolism. In fact, cytokines influence iron homeostasis and deregulation of cytokines production is closely linked to iron-related disorders [541-543]. In innate immunity, an important role is played by the Toll-like receptors (TLRs), which recognize molecular patterns present in micro-organisms. MyD88 is the universal adaptor protein that is used by almost all TLRs and is essential for the induction of inflammatory cytokines [518, 544]. Previous studies have shown that during inflammation hepcidin can be regulated through Toll-like receptors (TLRs) *via* the MyD88-dependent signaling pathway (TLR4/MyD88) in macrophages and hepatocytes [523-525]. Interestingly, in our laboratory it has been observed that MyD88 deficient (*MyD88*$^{-/-}$) mice were unable to sustain hypoferremia in response to lipopolysaccharide (LPS) induction, due to defective hepcidin expression [524].

Interestingly, several studies highlighted a crosstalk between inflammatory and iron-sensing pathways involved in systemic iron metabolism [355, 370]. In fact, multiple *in vitro and in vivo* studies demonstrated that the inhibition of molecular components of iron-sensing pathway impaired hepcidin induction by inflammatory stimuli and specific pro-

inflammatory cytokines upregulate hepcidin expression through SMAD signaling pathways [345, 356, 366, 367, 545].

Thus, since the molecular components of the inflammatory IL-6/STAT3 signaling and the iron-sensing BMP/SMAD pathways overlap for hepcidin induction, we hypothesized that MyD88 could also be involved in the iron-sensing pathway, playing a potential role in hepcidin induction.

The objective of this study was to evaluate the potential role of MyD88 in iron-sensing pathway, by characterizing the iron metabolism phenotype of MyD88-deficient (*MyD88* $^{-/-}$) mice fed with an iron supplemented diet.

Chapter 4: MyD88 regulates the expression of SMAD4 and the iron regulatory hormone hepcidin in hepatoma cells

In our previous study, we demonstrated for the first time that MyD88 plays a role in the iron-sensing pathway. In fact, we reported that *MyD88*$^{-/-}$ mice were unable to appropriately regulate hepatic hepcidin levels in response to dietary iron overload.

Since this phenotype was associated to significantly reduced Smad4 protein levels in *MyD88*$^{-/-}$ compared to wild type mice [546], we hypothesized that MyD88 could interact with SMAD4 expression to regulate hepcidin expression.

Moreover, previous studies reported that SMAD6, involved in inhibiting SMAD inhibitory pathway and suppressor of Hamp expression in primary hepatocytes [362, 547, 548], acts as a MyD88 inhibitor by recruiting SMURF1/2, which are E3-ubiquitin ligases and degrade MyD88 *via* ubiquitination process when macrophages are stimulated with TGF-β1 [549]. We hypothesized that members of the TGF−β superfamily, such as BMP6 and Activin-B, could be implicated in the negative feedback loop of hepcidin expression through MyD88 degradation.

Finally, anemia is a common problem in patients with lymphoma, and in lymphoplasmacytic cells hepcidin is overexpressed and is associated with anemia [315, 534]. The mutated MyD88 gene MyD88^{L265P} is a highly characterized marker of human

lymphoma [518, 530, 536]. Gain-of-function mutation in MyD88^{L265P} promotes cell survival through nuclear factor κB (NF-κB) activation in the above cell line [527, 530, 531]. We hypothesized that the gain of function mutation in MyD88^{L265P} gene is responsible for the augmentation of hepcidin and in turn the underlying cause of the manifestation of anemia.

The objective of this study was to analyze the potential mechanisms of action of MyD88 in the SMAD/BMP signaling pathway for hepcidin regulation and the potential implication of MyD88 mutation on the manifestation of anemia in hematological malignancies.

Chapter 5: Curcumin induces mild anemia in a DSS-induced colitis mouse model maintained on an iron-sufficient diet

Previous studies demonstrated the modulation of MyD88 and hepcidin (Hamp) expression by curcumin, a phytochemical compound, important for the variety of beneficial biological properties [479]. Specifically, the anti-inflammatory properties of curcumin have been shown to downregulate the TLR4/MyD88 pathway with consequent decrease in NF-κB expression [479, 550]. In addition, curcumin has been shown to have iron chelating properties, and this has been suggested as an explanation for the decrease of Hamp expression in mice fed with iron sufficient or iron deficient diet (5-12 and 50 mg iron diets) supplemented with 2% or less concentration of curcumin [464, 488].

Given the iron-chelating properties of curcumin, we questioned whether curcumin may induce or exacerbate anemia in chronic inflammatory diseases, such as colitis, in which body iron stores may be compromised due to intestinal bleeding and hepcidin induction though the inflammatory pathway.

The objective of this study was to investigate the effects of curcumin on systemic iron balance using a dextran sodium sulfate (DSS) induced colitis mouse model induced in mice fed with an iron sufficient diet with/without supplemented curcumin.

CHAPTER 3

MyD88 adaptor protein is required for appropriate hepcidin induction in response to dietary iron overload in mice

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Running title: MyD88 involvement in iron sensing pathway

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Abstract

Iron homeostasis is tightly regulated to provide virtually all cells in the body, particularly red blood cells, with this essential element while defending against its toxicity. The peptide hormone hepcidin is central to the control of the amount of iron absorbed from the diet and iron recycling from macrophages. Previously, we have shown that hepcidin induction in macrophages following Toll-like receptor (TLR) stimulation depends on the presence of myeloid differentiation primary response gene 88 (MyD88). In this study, we analyzed the regulation of iron metabolism in *MyD88*$^{-/-}$ mice to further investigate MyD88 involvement in iron sensing and hepcidin induction. We show that mice lacking MyD88 accumulate significantly more iron in their livers than wild-type counterparts in response to dietary iron loading as they are unable to appropriately control hepcidin levels. The defect was associated with inappropriately low levels of Smad4 protein and Smad1/5/8 phosphorylation in liver samples found in the *MyD88*$^{-/-}$ mice compared to wild-type mice. In conclusion, our results reveal a previously unknown link between MyD88 and iron homeostasis, and provide new insights into the regulation of hepcidin through the iron-sensing pathway.

Key words: iron, hepcidin, MyD88, Smad4, hemochromatosis, Bmp6, Bmp2

Introduction

Iron homeostasis in mammals is tightly regulated to meet body requirements while preventing iron toxicity, as iron can participate in the generation of harmful free radicals (Hentze et al., 2004;Sheftel et al., 2011). Most of this regulation takes place at the level of intestinal iron absorption, which is negatively controlled by levels of the peptide hormone hepcidin, encoded by the *HAMP* gene (Ganz and Nemeth, 2012). Hepcidin also plays a major role in regulating iron release from macrophages. These cells are responsible for iron recycling, with high hepcidin levels ultimately leading to iron accumulation in macrophages (Ganz and Nemeth, 2012). Erythropoietic activity, hypoxia, host defense and multiple signals reflecting systemic iron stores and circulating levels converge to regulate hepcidin production, mostly in the liver, and affect body iron homeostasis (Huang et al., 2009).

Two major pathways that contribute to hepcidin regulation are the inflammatory pathway mediated through IL-6/STAT3 signaling (Nemeth et al., 2004;Verga Falzacappa et al., 2007), and the iron-sensing pathway, which is mediated through BMP/SMAD signaling (Rishi et al., 2015). BMP/SMAD signaling is modulated in response to body iron status: increased dietary iron levels stimulate the production of bone morphogenetic protein 6 (BMP6) (Kautz et al., 2008;Meynard et al., 2009;Corradini et al., 2011), which then binds to heteromeric complexes containing type II and type I BMP receptors (BMPRI/II) (Parrow and Fleming, 2014). The binding of BMP6 to its receptors results in the recruitment and subsequent phosphorylation of SMADs 1, 5, and 8 (SMAD1/5/8). In turn, SMAD1/5/8 binds to SMAD4 to form a transcriptional complex that translocates to the nucleus and binds to specific DNA elements in the hepcidin promoter (Casanovas et al., 2009). While BMP6 is a key regulator of hepcidin expression and systemic iron homeostasis (Andriopoulos et al., 2009;Meynard et al., 2009), more recent work identified BMP2 as another BMP ligand indispensable for iron homeostasis *in vivo* that is nonredundant with BMP6 (Canali et al., 2017;Koch et al., 2017).

Hepcidin regulation through IL-6/STAT3 signaling occurs during inflammation or infection, when the host's innate immune response activates a sequence of events that limits iron availability by sequestering iron and down-regulating intestinal iron absorption (Weiss, 2009). Production of cytokines, particularly IL-6, leads to STAT3 phosphorylation, translocation into the nucleus, and binding to STAT3-recognizing DNA elements located in the hepcidin promoter (Verga Falzacappa et al., 2007).

Previously, we have shown that hepcidin induction in macrophages following stimulation of *Toll-like receptors* (*TLRs*) depends on the presence of myeloid differentiation primary response gene 88 (MyD88) (Layoun and Santos, 2012). In addition to macrophages, TLRs

and MyD88 are also expressed in hepatocytes (Liu et al., 2002) and it has been demonstrated that LPS stimulation induces hepcidin expression in hepatocytes via a MyD88-dependent signaling pathway (Lee et al., 2017).

Almost all TLRs use MyD88 as a universal adapter protein to activate the transcription factor NF-κB and cytokine production through the common MyD88-dependent signaling pathway. TRIF is another adapter protein used by TLR3 and TLR4 to activate NF-κB through the TRIF-dependent signaling pathway (Yamamoto et al., 2003). Both MyD88 and Trif-deficient mice have impaired production of inflammatory cytokines (Takeuchi and Akira, 2010). In previous work, we showed that MyD88-deficient mice are unable to sustain an acute hypoferremic response triggered by lipopolysaccharide (LPS), a TLR4 ligand (Layoun et al., 2012). The contribution of TLRs/MyD88 signaling for hepcidin expression through the inflammatory pathway has been further demonstrated using a variety of cellular and animal models (Wang et al., 2009;Xiong et al., 2016;Lee et al., 2017).

Since both the iron-sensing pathway and inflammatory pathways reveal overlap in hepcidin induction when converging at SMAD1/5/8 phosphorylation and SMAD4 binding, we investigated the potential role of MyD88 in iron sensing by analyzing iron metabolism in MyD88-deficient mice (*MyD88*$^{-/-}$).

Materials and Methods

Animals

This study was carried out in accordance with Canadian Council on Animal Care guidelines. The protocol was approved by the institutional Animal Care Committee of the CRCHUM.

C57BL/6 (B6) wild-type (Wt) and Trif-deficient mice (C57BL/6JTicam1^{Lps2}/J or Trif$^{LPS2/LPS2}$, B6 background), were purchased from Jackson Laboratories (Bar Harbor, ME). *MyD88*$^{-/-}$ mice in the B6 genetic background were kindly provided by Dr. Shizuo Akira (Research Institute for Microbial Diseases, Osaka University, and Japan Science and Technology Agency, Tokyo, Japan) and were maintained as described previously (Adachi et al., 1998). Mice were maintained under standard 12:12 h light/dark conditions at the Centre de recherche du CHUM (CRCHUM). The animals used in the experiments were female and were permanently housed under specific pathogen-free conditions.

Animal treatments

Mice were fed a commercial diet containing approximately 200 mg of iron per kg (Teklad Global 18% protein rodent diet; Harlan Teklad, Madison, WI). Dietary iron overload was produced by feeding 8-week-old mice the same commercial diet supplemented with 25 g carbonyl iron per kg (2.5% wt/wt carbonyl iron; Sigma-Aldrich, St. Louis, MO, USA) for two weeks (Jiang et al., 2008). All animals were 10-weeks old at the end of the experiments.

Hematologic 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 was assessed by colorimetric assay with the Kodak Ektachem DT60 system (Johnson & Johnson, Ortho Clinical Diagnostics, Mississauga, ON).

Measurement of tissue iron concentration

Spleen, and liver iron concentrations (LIC) were assessed by acid digestion of tissue samples, followed by iron quantification with atomic absorption spectroscopy (Miranda et al., 2004).

Serum ferritin assay

Ferritin was measured in serum with an enzyme-linked immunosorbent assay (ELISA) kit as per manufacturer's instructions (mouse Ferritin ELISA kit, Kamiya Biomedical, Seattle).

Histology
Liver tissue sections were stained with Perls' Prussian blue for ferric iron detection (iron stain kit; Sigma Immunochemicals).

Quantitative RT-PCR
Total RNA was isolated with Trizol reagent (Invitrogen, Burlington, ON), and reverse transcription was performed with the Omniscript RT kit (QIAGEN, Mississauga, ON). mRNA expression levels were measured by real-time PCR in a Rotor Gene 3000 Real Time DNA Detection System (Montreal Biotech, Kirkland, QC) with QuantiTect SYBRGreen I PCR kits (QIAGEN, Mississauga, ON) as described (Makui et al., 2005). The primers used in this study are presented in Table 1. Expression levels were normalized to the housekeeping gene β-actin (*Actb*).

SDS–PAGE 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). Total cell lysates and, when indicated, nuclear protein extracts were separated by 10% SDS–PAGE and blotted onto nitrocellulose membranes (Bio-Rad Laboratories, Mississauga, Ontario). The membranes were immunoblotted with the following antibodies: ferritin (FTH1) (1:1000) (Alpha Diagnostic International, San Antonio, TX), phospho-Smad5 (1:1000) (Abcam, Cambridge, MA), Smad1 (1:1000) (Cell Signaling, Danvers, MA), Smad4 (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-Stat3 (1:1000) (Cell Signaling, Danvers, MA), Stat3 (1:1000) (Cell Signaling, Danvers, MA), HDAC1 (1:1000) (Santa Cruz, Biotechnology, Santa Cruz, CA), β-Tubulin (1:1000) (Cell Signaling, Danvers, MA) and β-actin (1:10000) (Abcam, Cambridge, MA). Anti–rabbit IgG (1:5000) (Cell Signaling, Danvers, MA) or anti–mouse IgG (1:5000) (GE Healthcare, Amersham Biosciences, Baie d'Urfe, QC, Canada) was used as secondary antibody. Antigen-antibody complexes were visualized with the ECL Western Blotting Detection Reagent (GE Healthcare).

Statistical analysis
All statistics were calculated with Prism software (GraphPad, San Diego, CA), with a pre-specified significant *P* value of 0.05. Multiple comparisons were evaluated statistically by one-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparison test.

Results

Increased liver iron stores in *MyD88*$^{-/-}$ mice

We have previously reported that mice deficient in MyD88, unlike Trif-deficient mice, are unable to maintain LPS-induced, acute hypoferremic response as they fail to divert iron from the circulation into the spleen (Layoun et al., 2012). To further investigate the role of MyD88 in iron metabolism, we first examined MyD88-deficient mice and assessed iron levels in the liver, the major iron storage organ in the body. Compared to Wt and Trif-deficient mice that were used as controls, *MyD88*$^{-/-}$ animals presented consistently higher iron concentrations in the liver (**Figure 1A**). In addition, *MyD88*$^{-/-}$ mice had approximately twice the level of serum ferritin (**Figure 1B**) and higher levels of hepatic H-ferritin protein (**Figure 1C**) than the levels observed in Wt mice, further confirming that MyD88 deficiency results in elevated liver iron storage. The increased iron concentration in *MyD88*$^{-/-}$ livers was not due to altered erythropoiesis or circulating iron since the erythroid parameters and serum iron levels in *MyD88*$^{-/-}$mice were similar to those in Wt and Trif-deficient mice (**Figures 1D and 1E**).

MyD88$^{-/-}$ mice exhibit inability to appropriately regulate hepcidin in response to dietary iron loading

Next, we investigated whether MyD88 deficiency might impact the regulation of hepcidin with consequent storage of excess iron in the liver. Mice were challenged with an iron-enriched diet for two weeks to mimic chronic iron overload. This treatment resulted in a 5.6-fold increase of iron accumulation in the livers of *MyD88*$^{-/-}$ mice compared to the 3.5-fold increase observed in Wt mice (**Figure 2A**), and was further confirmed by Perls' staining for ferric iron in liver samples (**Figure 2B**). In contrast, iron levels in the spleen of iron-loaded *MyD88*$^{-/-}$ mice were similar to Wt mice (**Figure 2C**), indicating an inability to proportionately accumulate iron in this organ. These results suggest that *MyD88*$^{-/-}$ mice may have a defect in adjusting the levels of the iron regulatory hormone hepcidin (Ganz and Nemeth, 2012); therefore, we next assessed mRNA expression levels of liver hepcidin. Despite showing increased hepcidin mRNA expression upon chronic dietary iron-loading, *MyD88*$^{-/-}$ mice have comparatively lower levels of hepcidin than iron-loaded Wt mice (**Figures 2D-E**).

Altogether, our data suggest that mice lacking functional MyD88 adaptor molecules present insufficient hepcidin induction in response to dietary iron loading.

Defective signaling through the BMP/SMAD pathway in *MyD88*$^{-/-}$ mice

In vivo, iron loading induces the expression of *Bmp6* mRNA in the liver (Kautz et al., 2008), which is believed to be an initial step in the activation of the BMP/SMAD1/5/8 signaling cascade for hepcidin induction in response to iron overloading (Meynard et al., 2009). Thus, we next sought to understand whether BMP6 activation was compromised in *MyD88*$^{-/-}$ mice. Liver *Bmp6* mRNA expression was induced by iron in both Wt and *MyD88*$^{-/-}$ mice, and was even higher in iron-loaded *MyD88*$^{-/-}$ mice, demonstrating that the absence of MyD88 did not affect the induction of *Bmp6* (**Figure 3A**). Since BMP2 has also been

shown to be necessary for iron homeostasis *in vivo* (Canali et al., 2017;Koch et al., 2017), we also measured *Bmp2* mRNA expression in the liver. We found that *Bmp2* mRNA levels were induced by dietary iron to similar levels in both Wt and *MyD88*$^{-/-}$ mice (**Supplementary Figure 1**). However, induction of BMP ligands was not accompanied by the expected rise in hepcidin. Consequently, the ratio between *Hamp* mRNA and *Bmp6* mRNA was persistently lower in *MyD88*$^{-/-}$ mice compared to Wt mice (**Figure 3B**), and indicated a defect in the downstream signaling transmission initiated by Bmp6. Measurements of the mRNA levels of *Atoh8* and *Id1*, two genes shown to be induced by iron through the BMP6/SMAD4 pathway (Kautz et al., 2008), further reinforced the idea that the main defect in *MyD88*$^{-/-}$ mice resides in the BMP6/SMAD4 down-stream signaling pathway of targeted genes. In fact, the mRNA levels of both genes were significantly lower in *MyD88*$^{-/-}$ mice compared to wild-type mice in response to dietary iron loading (**Figures 3C-D**).

BMP6 signals through the phosphorylation of SMAD proteins 1, 5, and 8 (Kersten et al., 2005), which translocate into the nucleus where they regulate the transcription of specific target genes, including hepcidin (Pantopoulos et al., 2012). Hence, we next analyzed phosphorylation levels of Smad5 in liver nuclear extracts from dietary iron-loaded *MyD88*$^{-/-}$ and Wt mice. Dietary iron loading induced phosphorylation of liver Smad5 proteins in Wt mice, as expected (Besson-Fournier et al., 2017;Canali et al., 2017). In contrast, despite higher hepatic iron levels found in *MyD88*$^{-/-}$ mice, no significant differences regarding Smad5 phosphorylation were detected in this mouse strain when comparing the standard diet with the carbonyl iron supplemented diet (**Figures 4A-B**).

For the activation signal to be successfully transmitted, phosphorylated SMAD1/5/8 form heteromeric complexes with the common mediator SMAD4 before translocation into the nucleus. Thus, we next investigated whether the lack of MyD88 affected the amount of Smad4. As shown in **Figures 5A-B**, we found that Smad4 protein levels in nuclear extracts were significantly lower in *MyD88*$^{-/-}$ mice compared to Wt mice, both in mice fed the standard diet and the carbonyl iron supplemented diet (**Supplementary Figure 2**).

To exclude the influence of MyD88 mediated inflammatory response on hepcidin expression we measured Stat3 phosphorylation levels in the liver. As shown in **Figure 6A-B**, no significant differences were found regarding Stat3 phosporylation levels between mouse strains (Wt *vs. MyD88*$^{-/-}$ mice) or treatments (standard diet *vs.* carbonyl iron supplemented diet).

Discussion

Previous studies have shown that MyD88 is required for a sustained LPS-induced hypoferremic response (Layoun et al., 2012). To further study the role of MyD88 in iron sensing *in vivo*, we analyzed iron metabolism in MyD88-deficient mice. Unlike Trif-deficient mice, *MyD88*$^{-/-}$ mice kept on a standard diet exhibited increased iron levels in the liver compared to Wt mice. Accordingly, *MyD88*$^{-/-}$ mice also showed higher serum and liver ferritin levels (Lee and Means, 1995). Ferritin can be abnormally elevated in a wide range of disease states including malignancy, diabetes, infection, inflammation, and chronic iron-overload syndromes (Acton et al., 2006; Jacobs et al., 2009; Lee and Means, 1995; Torti and Torti, 1994; Turnbull et al., 1997). Since *MyD88*$^{-/-}$ mice present higher liver iron stores, the increased ferritin levels in the serum most likely reflected excess iron accumulation in the liver.

High levels of iron and ferritin reflecting increased iron stores are encountered in the liver and serum of a number of disorders that are collectively called iron-loading anemias. These include thalassemia syndromes, congenital dyserythropoietic anemias, and sideroblastic anemias, all of which have the presence of robust but inefficient erythropoiesis (Nemeth and Ganz, 2006). To rule out the possibility that *MyD88*$^{-/-}$ iron burden was due to augmented erythropoiesis activity (Papanikolaou and Pantopoulos, 2017) we measured several erythroid parameters and found no differences between *MyD88*$^{-/-}$, Wt and Trif-deficient mice, hence ruling out a possible defect in erythropoiesis activity in *MyD88*$^{-/-}$ mice.

Increased body iron stores are also found in the different forms of hereditary hemochromatosis (HH) (Pietrangelo, 2006). All these hereditary disorders have a common feature of inappropriately low hepcidin levels with consequential excessive iron accumulation in the liver with relative sparing of the spleen. These features are reproduced in other mouse models with hepcidin deficiency, such as *Hfe*$^{-/-}$ and *Hjv*$^{-/-}$ mice (Makui et al., 2005;Kent et al., 2015). Similarly, *MyD88*$^{-/-}$ mice challenged with dietary iron were unable to appropriately control hepcidin levels, resulting in significantly higher liver iron accumulation when compared to Wt mice. These results suggest a defect in *MyD88*$^{-/-}$ mice in the iron-sensing pathway leading to insufficient hepcidin induction. However, unlike other mouse models of iron overload, iron accumulation in the liver occurs in the absence of increased serum iron levels. A possible explanation for this difference could be enhanced ability to transport iron into hepatocytes involving heightened expression and/or activity of iron uptake molecules.

In vivo, hepcidin expression correlates with *Bmp6* gene expression in mice fed an iron-enriched or iron-deficient diet (Andriopoulos et al., 2009;Ryan et al., 2010). Accordingly,

Bmp6-deficient mice present insufficient hepcidin levels and consequent massive iron overload (Meynard et al., 2009). Besides Bmp6, there is increasing evidence that Bmp2 also plays an important role in iron homeostasis, since mice models lacking expression of Bmp2 in liver endothelial cells develop an iron loading phenotype similar to *Bmp6*$^{-/-}$ mice (Canali et al., 2017;Koch et al., 2017). In *MyD88*$^{-/-}$ mice, we show that the appropriate Bmp6 and Bmp2 induction in response to iron loading is disassociated from hepcidin production. The defect was associated with the levels of Smad4 protein found in nuclear extracts from liver samples, which were diminished by about half in the *MyD88*$^{-/-}$ mice compared to Wt mice. This finding is in line with previously reported data that described Smad4 deficiency in mice causing iron overload due to insufficient hepcidin activation (Wang et al., 2005). In addition, our results indicate that the levels of Smad5 phosphorylation were too low in *MyD88*$^{-/-}$ mice when taking into account iron and Bmp6 levels in the liver (Kautz et al., 2008), suggesting a potential defect in Smad1/5/8 phosphorylation.

A connection between the SMAD and the MyD88 pathway has been reported that concerns the role of SMAD6 as a negative regulator of the transforming growth factor β (TGF-β) family signaling pathway, which also involves BMP (Goto et al., 2007;Lee et al., 2011). The study by Lee et al. showed SMAD6 physically interacts with MyD88. Potentially, MyD88 interactions with SMAD4 and SMAD6 may be relevant for the regulation of hepcidin, given the reported role of SMAD6 in hepcidin suppression (Lee et al., 2017).

More recently, interaction between the TLR4/MyD88 and BMP2-induced BMP1SMAD signaling has been demonstrated in osteoblasts, where the two pathways were found to play conflicting roles in the regulation of BMP-2-induced osteoblast differentiation (Huang et al., 2013). In their study, Huang et al. showed that LPS-mediated inflammatory environment inhibits BMP-2-induced osteogenic differentiation through the crosstalk between TLR4/MyD88/NF-κB and BMP/SMAD signaling, which contrasts with our present findings. However, cross-talk between the inflammatory and SMAD pathways could be cell-context specific, since receptor expression seems to modulate the specificity of TGF-β signaling pathways (Murakami et al., 2009). In fact, stimulation of SMAD1/5/8 signaling and hepcidin induction during inflammation by other TGF-β superfamily members, such as activin B (Besson-Fournier et al., 2012), has been shown to be unique in liver cells (Canali et al., 2016). The exact nature of the cross-talk between MyD88 and SMAD pathways remains to be defined, and further studies are needed to understand whether MyD88 may, for example, stabilize SMAD4 and/or have a role in the translocation of the SMAD4/phosphorylated SMAD1/5/8 complexes into the nucleus.

In summary, we report here for the first time that MyD88 is critical for appropriate nuclear Smad4 protein expression, Smad1/5/8 phosphorylation, and for the induction of hepcidin

upon challenge with dietary iron. Together, our data identify a new role for MyD88 as a potential key molecule in the BMP signaling pathway mediated by SMAD proteins.

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Author contributions

MSM, AL, GF and AC contributed to the investigation, validation, methodology, and formal analysis. MSM, AL and MMS additionally contributed to the conceptualization and writing of the original draft of the manuscript. MMS additionally contributed to the visualization, supervision, and funding acquisition of the study.

Disclosure of conflicts of interest

All authors declare no competing financial interests.

Tables

Table 1. Primers used for qPCR analysis of mRNA levels

Gene		Sequence
Actb	Forward	TGTTACCAACTGGGACGACA
	Reverse	GGTGTTGAAGGTCTCAAA
Hamp	Forward	CCTATCTCCATCAACAGATG
	Reverse	AACAGATACCACACTGGGAA
Atoh8	Forward	CACCATCAGCGCAGCCTTC
	Reverse	AATCCAGCAGGTCAGCAAAG
Id1	Forward	ACCCTGAACGGCGAGATCA
	Reverse	TCGTCGGCTGGAACACATG
Bmp6	Forward	GACAAGGAGTTCTCCCCACA
	Reverse	CCAGCCAACCTTCTTCTGAG
Smad4	Forward	CCCACTGAAGGACATTCGAT
	Reverse	GCCCTGAAGCTATCTGCAAC
Bmp2	Forward	GCCTGCACCCTGTTCTCTGA
	Reverse	ATGTTCAAACACATATCCCTGGAA

Figure Legends

Figure 1. Increased liver iron stores with normal erythropoietic parameters in MyD88-deficient mice. Wild-type (Wt), MyD88-deficient ($MyD88^{-/-}$) and Trif-deficient ($Trif^{Lps2/Lps2}$) mice were fed a standard diet. (A) Liver iron concentration measured by atomic absorption spectroscopy. (B) Serum ferritin levels. (C) Representative western blot showing H-ferritin levels in the liver of Wt and *MyD88*$^{-/-}$ mice. The blot was stripped and reprobed with an antibody to β-actin as loading control. (D) Erythroid parameters: red blood cells (RBC), hemoglobin (Hb), hematocrit (HCT) and mean corpuscular volume (MCV). Results are presented as mean ± SEM. (E) Serum iron.

Results are presented as combined data from two independent experiments performed with $n \geq 5$ mice per group. Statistical analysis was performed with one-way ANOVA. n.s. = not significant compared to Wt mice.

Figure 2. Inability to appropriately upregulate hepcidin in response to iron challenge in MyD88-deficient mice. Wild-type (Wt) and MyD88-deficient ($MyD88^{-/-}$) mice were fed a standard diet (SD) or carbonyl iron supplemented diet (CI) for two weeks. (A) Liver iron concentration measured by atomic absorption spectroscopy. (B) Ferric iron staining in liver sections. *Left panels*: Basal iron levels in the liver of mice fed SD, detected by DAB-enhanced Perl's staining (Smith et al., 1997) (brown) with hematoxylin (blue) and eosin (Pantopoulos et al.) counterstaining. *Right panels:* Iron levels in the liver of mice fed CI, detected by Perls' Prussian blue staining. Scale bar = 50μm. (C) Spleen iron concentration. (D) Liver hepcidin mRNA levels. (E) Ratio hepcidin / liver iron concentration (LIC).

Results are representative of a minimum of three independent experiments using n = 4-6 mice per group in each experiment. Statistical analysis was performed with one-way ANOVA. $*P < 0.01$, $**P < 0.001$, and $***P < 0.0001$, compared with mice fed SD; n.s. = not significant.

Figure 3. Defective signaling though the BMP6/SMAD pathway in response to dietary iron-loading in *MyD88*$^{-/-}$ mice. Wt and $MyD88^{-/-}$ mice were fed a standard diet (SD) or carbonyl iron supplemented diet (CI) for two weeks. (A) *Bmp6* mRNA levels in the liver. (B) Hepcidin/Bmp6 mRNA ratio. (C) *Atoh8* mRNA levels in the liver. (D) *Id1* mRNA levels in the liver. The results are representative of a minimum of three independent experiments using n = 4-6 mice per group in each experiment. Statistical analysis was performed with one-way ANOVA: $*P < 0.01$, $***P < 0.0001$ and n.s. = not significant compared with mice fed SD.

Figure 4. Lack of Smad5 phosphorylation in response to dietary iron-loading in *MyD88*$^{-/-}$ mice. Liver nuclear extracts from mice fed standard diet (SD) and carbonyl iron supplemented diet (CI) were analyzed by western blots. (A) One representative blot is presented that was probed with antibodies against phosphorylated Smad5 (pSmad5) and total Smad1. (B) Graphic depicting densitometric quantification of western blots from three independent experiments. Statistical analysis was performed with one-way ANOVA: n.s. = not significant compared with Wt mice fed the same diet.

Figure 5. Defective signaling in response to dietary iron-loading in *MyD88*$^{-/-}$ mice involves diminished Smad4 protein in the liver. Liver nuclear extracts from mice fed standard diet (SD) and carbonyl iron supplemented diet (CI) were analyzed by western blots. (A) One representative blot probed with an antibody against Smad4. Blots were stripped and reprobed with an antibody to HDAC1 (nuclear marker) and to β-tubulin (cytoplasmic marker). Total = liver total extract; Cyt = liver cytoplasmic extract. (B) Graphic depicting densitometric quantification of western blots from three independent experiments. Statistical analysis was performed with one-way ANOVA: n.s. = not significant compared with mice fed SD.

Figure 6. Stat3 phosphorylation in response to dietary iron-loading remains unchanged in both Wt and in *MyD88*$^{-/-}$ mice. Liver nuclear extracts from mice fed standard diet (SD) and carbonyl iron supplemented diet (CI) were analyzed by western blots. (A) One representative blot probed with antibodies against phosphorylated Stat3 (pStat3) and total Stat3 (Stat3). (B) Graphic depicting densitometric quantification of western blots from three independent experiments. Statistical analysis was performed with one-way ANOVA: n.s. = not significant compared with mice fed SD.

Supplementary Figure 1. Liver *Bmp2* mRNA levels. Wt and MyD88$^{-/-}$ mice were fed a standard diet (SD) or carbonyl iron supplemented diet (CI) for two weeks. *Bmp2* mRNA levels in the liver. The results are representative of three independent experiments using *n* = 4-8 mice per group in each experiment. Statistical analysis was performed with one-way ANOVA: n.s. = not significant compared with Wt mice fed the same diet.

Supplementary Figure 2. Liver Smad4 protein levels. Liver nuclear extracts from mice fed (A) standard diet – SD; and (B) carbonyl iron supplemented diet - CI were analyzed by western blots. One representative blot is shown probed with an antibody against Smad4. Blots were stripped and reprobed with an antibody to β-actin.

Figures

Figure 1.

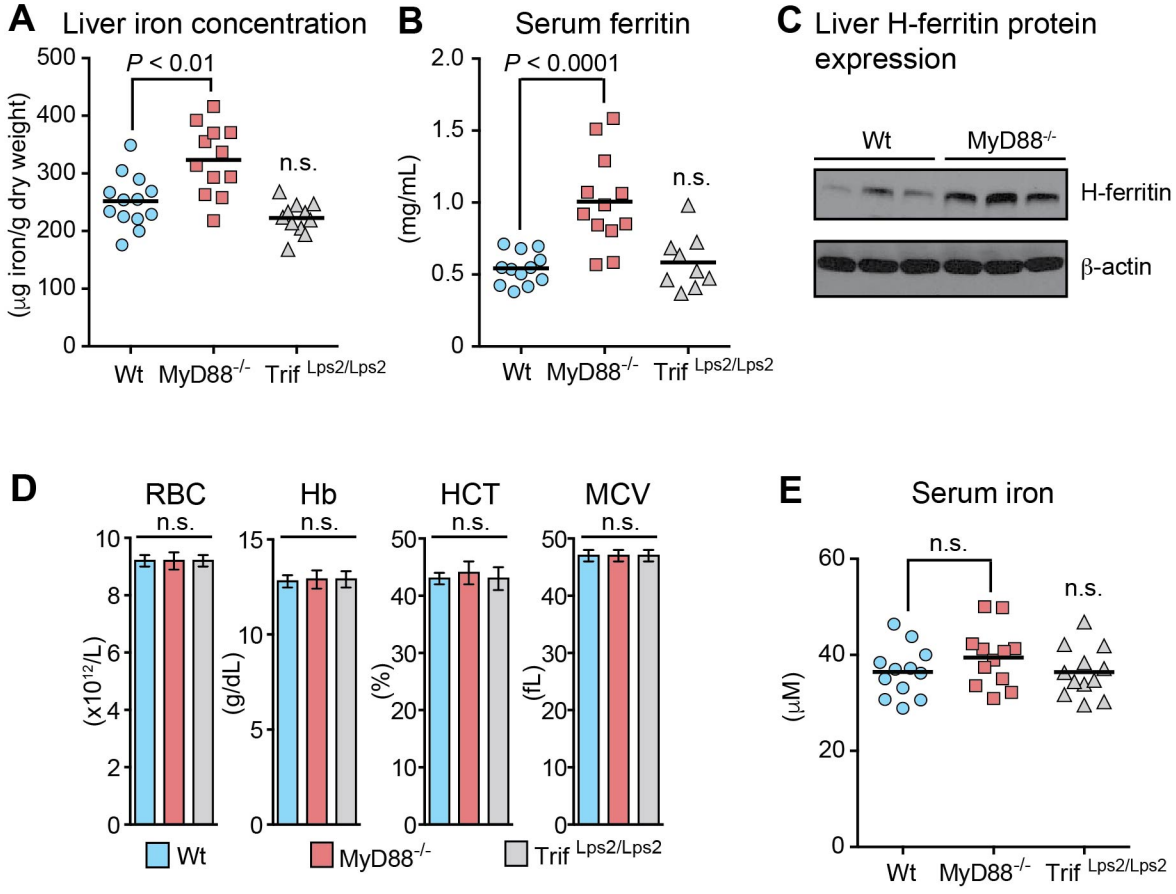


Figure 2.

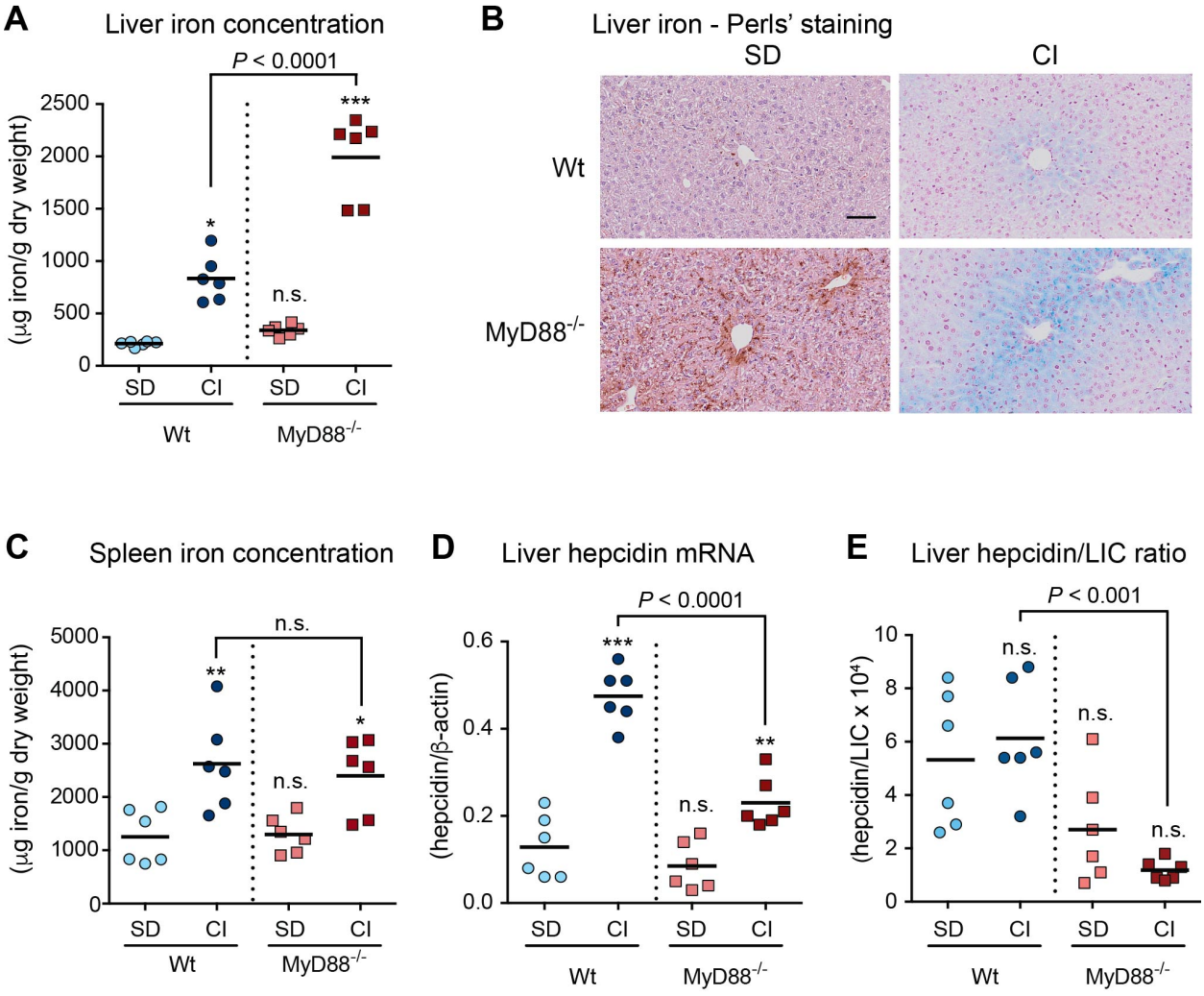

A
Liver iron concentration
P < 0.0001
(μg iron/g dry weight)
2500
2000
1500
1000
500
0
***
*
n.s.
SD
CI
SD
CI
Wt
MyD88-/-
B
Liver iron - Perls' staining
SD
CI
Wt
MyD88-/-
C
Spleen iron concentration
(μg iron/g dry weight)
5000
4000
3000
2000
1000
0
n.s.
**
n.s.
*
SD
CI
SD
CI
Wt
MyD88-/-
D
Liver hepcidin mRNA
P < 0.0001
(hepcidin/β-actin)
0.6
0.4
0.2
0.0
***
n.s.
**
SD
CI
SD
CI
Wt
MyD88-/-
E
Liver hepcidin/LIC ratio
P < 0.001
(hepcidin/LIC x 10⁴)
10
8
6
4
2
0
n.s.
n.s.
n.s.
SD
CI
SD
CI
Wt
MyD88-/-

Figure 3.

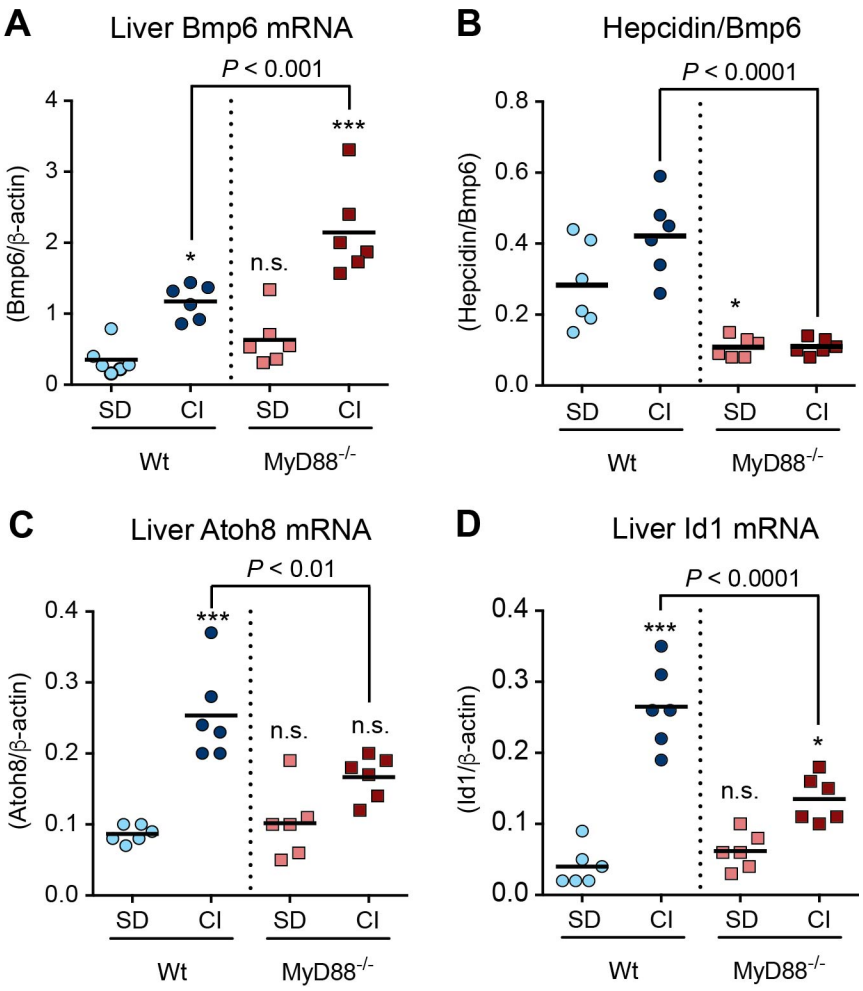

A
Liver Bmp6 mRNA
P < 0.001
(Bmp6/β-actin)
4
3
2
1
0
*
n.s.
***
SD
CI
SD
CI
Wt
MyD88-/-
B
Hepcidin/Bmp6
P < 0.0001
(Hepcidin/Bmp6)
0.8
0.6
0.4
0.2
0.0
*
SD
CI
SD
CI
Wt
MyD88-/-
C
Liver Atoh8 mRNA
P < 0.01
(Atoh8/β-actin)
0.4
0.3
0.2
0.1
0.0
***
n.s.
n.s.
SD
CI
SD
CI
Wt
MyD88-/-
D
Liver Id1 mRNA
P < 0.0001
(Id1/β-actin)
0.4
0.3
0.2
0.1
0.0
***
n.s.
*
SD
CI
SD
CI
Wt
MyD88-/-

Figure 4.

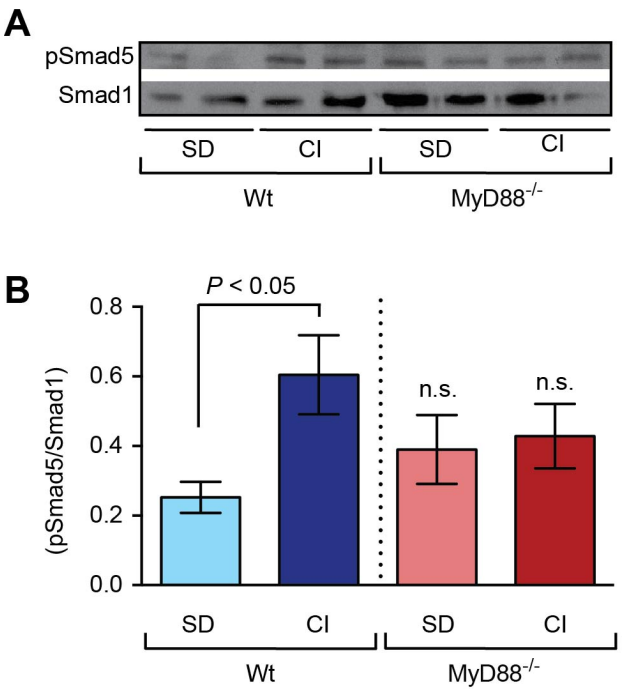

A
pSmad5
Smad1
SD
CI
SD
CI
Wt
MyD88-/-
B
P < 0.05
n.s.
n.s.
(pSmad5/Smad1)
0.8
0.6
0.4
0.2
0.0
SD
CI
SD
CI
Wt
MyD88-/-

Figure 5.

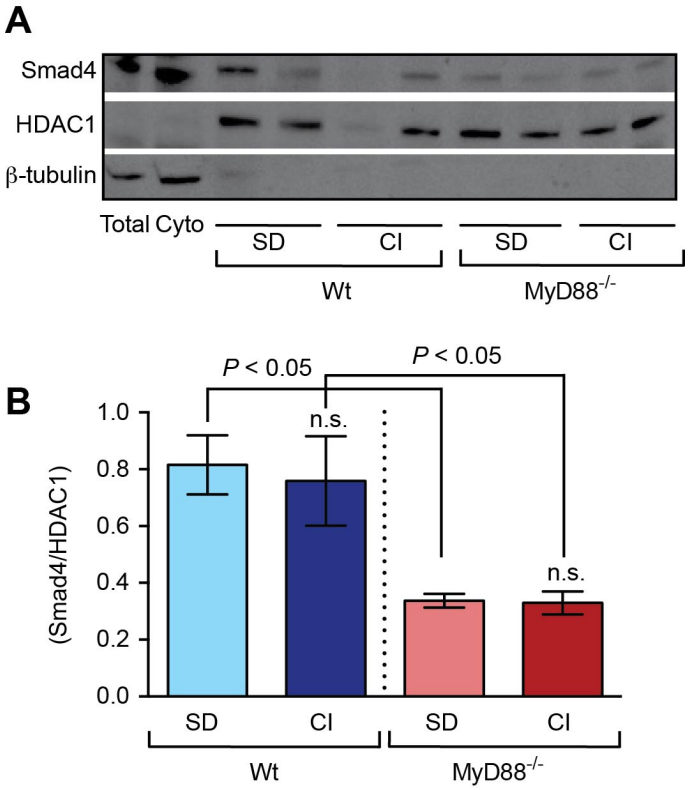
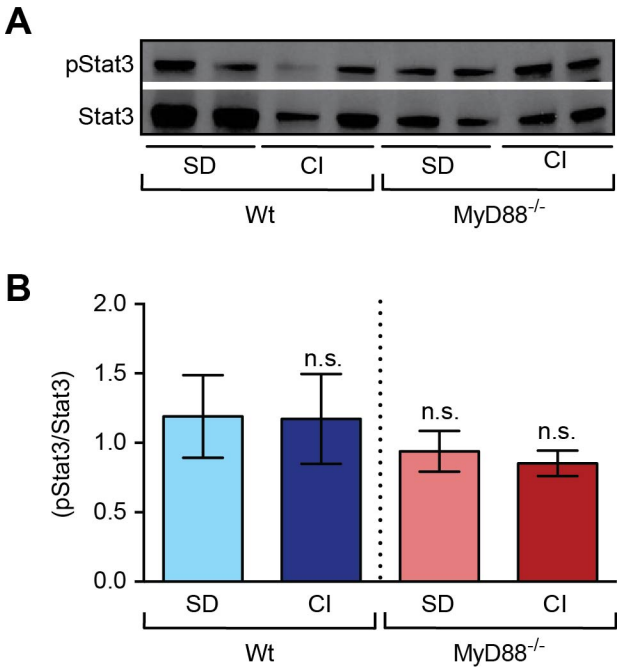
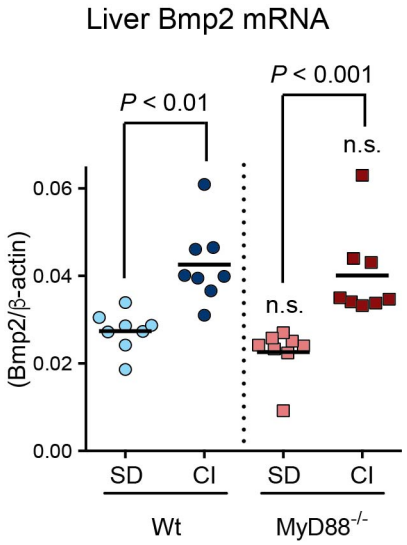

A
Smad4
HDAC1
β-tubulin
Total Cyto
SD
CI
SD
CI
Wt
MyD88-/-
B
P < 0.05
P < 0.05
n.s.
n.s.
(Smad4/HDAC1)
1.0
0.8
0.6
0.4
0.2
0.0
SD
CI
SD
CI
Wt
MyD88-/-

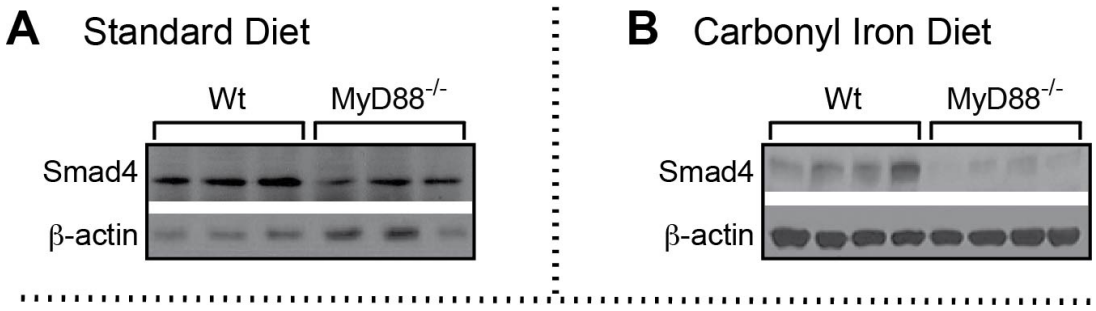
Figure 6.


A
pStat3
Stat3
SD
CI
SD
CI
Wt
MyD88-/-
B
2.0
1.5
1.0
0.5
0.0
(pStat3/Stat3)
n.s.
n.s.
n.s.
SD
CI
SD
CI
Wt
MyD88-/-

Supplementary Figure 1



Supplementary Figure 2



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CHAPTER 4

MyD88 regulates the expression of SMAD4 and the iron regulatory hormone hepcidin in hepatoma cells

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Running title: MyD88 involvement in iron sensing pathway

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Abstract

The myeloid differentiation primary response gene 88 (MyD88) is an adaptor protein that is essential for the induction of inflammatory cytokines through almost all the Toll-like receptors (TLRs). TLRs recognize molecular patterns present in microorganisms called pathogen-associated molecular patterns. Therefore, MyD88 plays an important role in innate immunity since its activation triggers the first line of defense against microorganisms. Herein, we describe the first reported role of MyD88 in an interconnection between innate immunity and the iron-sensing pathway (BMP/SMAD4). We found that direct interaction of MyD88 with SMAD4 protein activated hepcidin expression. The iron regulatory hormone hepcidin is indispensable for the intestinal regulation of iron absorption and iron recycling by macrophages. We show that MyD88 induces hepcidin expression in a manner dependent on the proximal BMP responsive element on the hepcidin gene (*HAMP*) promoter. We identified the Toll/interleukin-1 receptor (TIR) domain of MyD88 as the domain of interaction with SMAD4. Furthermore, we show that BMP6 stimulation, which activates SMAD6 expression, also induces MyD88 proteosomal degradation as a negative feedback mechanism to limit hepcidin induction. Finally, we report that the MyD88 gain-of-function L265P mutation, frequently encountered in B-cell lymphomas such as Waldenström's macroglobulinemia, enhances hepcidin expression and iron accumulation in B cell lines. Our results reveal a new potential role for MyD88 in the SMAD signaling pathway and iron homeostasis regulation.

Keywords

MyD88, BMP6, SMAD4, iron, hepcidin, L265P mutation, SMAD6, anemia, Waldenström's macroglobulinemia

Introduction

The myeloid differentiation primary response gene 88 (MyD88) plays an important role in the mammalian host defense (Deguine and Barton, 2014). This universal adaptor protein is part of the family of signal transduction molecules required for the production of pro- or anti-inflammatory cytokines (Akira, 2003;Deguine and Barton, 2014) in response to IL-1R1 (Muzio et al., 1997;Burns et al., 1998) and Toll-like receptor (TLR) signaling (Medzhitov et al., 1998). TLR3 is the exception as it exclusively uses the adaptor molecule Toll/interleukin-1 receptor (TIR)-domain-containing adapter-inducing interferon-β (TRIF) (Yamamoto et al., 2003).

TLRs/MyD88 signaling has been shown to also contribute to the regulation of hepcidin, a molecule involved in cellular and systemic iron metabolism (Wang et al., 2009;Xiong et al., 2016;Lee et al., 2017). Hepcidin, encoded by the *HAMP* gene, is the major regulator of intestinal iron absorption and iron recycling from macrophages (Hentze et al., 2010). Hepcidin modulates cellular iron export by binding to Ferroportin 1, the only known cellular iron exporter in vertebrates, and inducing its degradation (Donovan et al., 2000;McKie et al., 2000). Since Ferroportin 1 is expressed on duodenal enterocytes absorbing dietary iron and on macrophages in liver and spleen, high hepcidin levels leads to the suppression of intestinal iron absorption and the accumulation of iron in macrophages (Rishi et al., 2015). Prolonged activation of hepcidin with consequent iron sequestration manifested by hypoferremia, may lead to the development of anemia of chronic diseases (ACD) or anemia of inflammation (Ganz and Nemeth, 2009). ACD is characterized by the presence of adequate iron stores, as defined by conventional criteria, but with insufficient iron mobilization from stores to appropriately support erythropoiesis. ACD is prevalent in patients suffering from infections, malignancies and auto-immune disorders, and it is linked with immune activation, exemplifying the interplay between iron metabolism and immune function (Weiss, 2009).

Besides being regulated by inflammatory stimuli though IL-6/STAT3 signaling (Wrighting and Andrews, 2006;Verga Falzacappa et al., 2007), hepcidin expression is also modulated though an iron-sensing pathway involving bone morphogenetic proteins (BMP), such as BMP6, and SMAD4 signaling (Ganz and Nemeth, 2012;Sheftel et al., 2012). BMPs are part of the transforming growth factor-β (TGF-β) superfamily of proteins, which includes TGF-βs and activins, among others. The iron signaling-pathway can initiate with BMP6, which is activated by increased iron stores (Kautz L et al., 2011). BMP6 binds to the heteromeric complexes containing type I and type II BMP receptors (BMPRI/II) (Parrow and Fleming, 2014), resulting in the recruitment and subsequent phosphorylation of SMADs 1, 5, and 8 (SMAD1/5/8) in the cytoplasm (Kautz et al., 2008). In turn, phosphorylated SMAD1/5/8 proteins (pSMAD1/5/8) form heteromeric complexes with SMAD4 that translocate into the nucleus to modulate the transcription of target genes, including *HAMP* (Casanovas et al., 2009). In addition to BMP6, other molecules such as BMP2 (Canali et al., 2017;Koch et al., 2017) and the peptide hormone Activin B have

also been shown to induce hepcidin expression through SMAD1/5/8 signaling (Besson-Fournier et al., 2012;Canali et al., 2016).

As with other genes regulated through the TGF-β/BMP/SMAD signaling pathway, hepcidin is also regulated through a negative feedback loop by inhibitory SMADs, SMAD6 and SMAD7 (Mleczko-Sanecka et al., 2010;Vujic Spasic et al., 2013), which antagonize the activation of receptor-regulated SMADs. Inhibitory SMADs associate with activated TGF-β superfamily type I receptors, thereby preventing phosphorylation of receptor-regulated SMADs (Itoh and ten Dijke, 2007). SMAD7 inhibits both TGF-β/activin and BMP signaling, while SMAD6 efficiently inhibits BMP signaling but only weakly inhibits TGF-β/activin signaling (Hata et al., 1998;Ishisaki et al., 1999;Hanyu et al., 2001). The expression of inhibitory SMADs 6 and 7 is induced by activin/TGF-β and BMP signaling, thus creating a negative regulatory feedback loop (Imamura et al., 1997) (Nakao et al., 1997).

Previously, we have shown that MyD88 plays an important role in the development of endotoxin-induced hypoferremia in mice (Layoun et al., 2012). More recently, we reported that *MyD88*$^{-/-}$ mice are unable to appropriately regulate hepatic hepcidin levels in response to dietary iron overload (Layoun, Samba-Mondonga et al., 2018). This was associated with significantly reduced Smad4 protein levels in the livers of *MyD88*$^{-/-}$ mice compared to wild-type mice. In the present study, we further investigated the link between MyD88, the SMAD/BMP signaling pathway, and hepcidin regulation.

Materials and Methods

Cell culture and treatments

MyD88 knockout (KO) human embryonic kidney 293 cells (HEK293-I3A) were a kind gift from G. Stark (Department of Molecular Genetics, Lerner Research Institute, Cleveland, OH, USA) (Li et al., 1999). HEK293-I3A cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Wisent Inc., Montreal, QC, Canada). Huh7 human hepatoma cells (ATCC) were maintained in DMEM. HepG2 human hepatoma cells (ATCC) were maintained in Eagle's minimal essential medium (EMEM; Wisent Inc.). Namalwa cells and Raji B cells were a kind gift from R. Bertrand and W. Mourad, respectively (Centre de Recherche du Centre Hospitalier de l'Université de Montréal, CRCHUM, Montréal, QC, Canada), and were cultured in Roswell Park Memorial Institute medium 1640 (RPMI1640; Wisent Inc.) Cell lines were supplemented with 10% fetal bovine serum (FBS; Wisent Inc.) and penicillin/streptomycin (Wisent Inc.), and were incubated at 37^{0}C with 5% CO_2. Cells were treated, where indicated, with 12.5, 25 and 50 ng/ml of either Activin B (R&D systems, MN, USA) or BMP6 (R&D systems), or with 15 ng/ml of TGF-β (R&D systems) for 24 hr. Huh7 cells were treated with 10 μM of the proteasome inhibitor MG132 (Sigma-Aldrich, St. Louis, MO, USA).

Plasmids

Plasmid pCMV-HA-MyD88, also referred as pMyD88, contained full length MyD88 with hemagglutinin (HA) tag and was a gift from B. Beutler (Addgene plasmid #12287); pRK-DPC4-Flag contained SMAD4 with a Flag tag and was a gift from R. Derynck (Addgene plasmid #12627); pCI-His-hUbi contained ubiquitin with a histidine tag and was a gift from A. Winoto (Addgene plasmid #31815); pCS2-HA-SMAD6 contained SMAD6 with a HA tag (pSMAD6) and was a gift of J. Massague (Addgene plasmid #14962); and pRK-Myc-SMURF1 contained SMURF1 with a Myc tag (pSMURF1) and was a gift from Y. Zhang (Addgene plasmid #13676). All were purchased through Material Transfer Agreements (MTAs) with Addgene (Cambridge, MA). Plasmid pCMV was created by removing MyD88 from pCMV-HA-MyD88 by digestion with restriction enzyme. The wild-type (-1234/+73) *HAMP* promoter reporter construct HAMP-Luc (*Firefly* luciferase) and *Renilla* luciferase reporter phRL-TK plasmid were used in the present study as previously described (Bagu and Santos, 2011). The QuickChange II site-directed mutagenesis kit (Agilent Technologies, ON, Canada) was used to generate the HAMP-LucΔBMP-RE1 construct, in which the BMP-RE1 site was mutated from GGCGCC to AGAACC (Verga Falzacappa et al., 2008). The site-directed mutagenesis kit was also used to create the pCMV-HA-MyD88 deletion mutants (ΔTIR, ΔDD and ΔID). The glutathione S-transferase (GST)-SMAD4 fusion protein was produced by cloning SMAD4 from pRK-DPC4-Flag into pGEX-2TK (kind gift from I. Royal, CRCHUM). All constructs were confirmed by direct sequencing. Ready-made psiRNA Kit (ksirna42-hmyd88) containing psi-RNA-hMyD88 (psiRNA42-hMyd88-LucGl3), and the control plasmid psi-RNA-LucGL3 (psiRNA42-LucGl3) was purchased from InvivoGen (San Diego, CA, USA).

psiRNA42-hMyd88LucGl3 sequences (F): AACUGGAACAGACAAACUAUCUCAA, (R):UUGACCUUGUCUGUUUGAUAGGAG. psiRNA42-LucGl3 sequences (F): GACUUACGCUGAGUACUUCGAUCAA, (R): uuCUGAAUGCGACUCAUGAAGCUGAG. The mutated form of the pCMV-HA-MyD88 plasmid, pCMV-HA-MyD88L265P, which results from a T to C mutation at position 794, was obtained using the QuickChange II site-directed mutagenesis kit (Agilent Technologies). The plasmid HAMP-MetLuc2 encoding a sequence-optimized, secreted luciferase was obtained by cloning the *HAMP* promoter reporter construct from HAMP-Luc into pMetLuc2-Reporter Vector (Clontech Laboratories, Mountain View, CA, USA). All plasmids were verified by digestion with restriction enzymes and sequencing (McGill University and Génome Québec Innovation Centre).

Transfection and co-immunoprecipitation assays

MyD88-deficient HEK293-I3A and Huh7 cells were transiently transfected using Lipofectamine™ 2000 (Invitrogen, Burlington, ON, Canada) as recommended by the manufacturer with indicated plasmids. The total amount of DNA was kept constant. For assessment of SMAD4 and MyD88 interactions, MyD88 KO HEK293-I3A cells were used. Twenty-four hours after transfection, cells were lysed in 1 mL RIPA buffer containing 50 mM Tris (pH 8), 150 mM NaCl, 1% NP40, 0.5% deoxycholic acid, 0.1 % SDS, and protease inhibitor cocktail (Complete Mini, Roche, Mannheim, Germany). Cell lysates were then incubated with indicated antibodies (anti-Flag, anti-HA, or anti-MyD88 antibody) for 3 hr at 4°C, after which EZview Red Protein A Affinity Gel (Sigma-Aldrich) was added for another 2 hr. For control reactions, mouse IgG1 (Santa Cruz) was used. The immune complexes were precipitated and washed thoroughly with RIPA buffer. Immunoprecipitated proteins were then eluted by adding sample buffer and were subsequently fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) and visualized by immunoblotting with anti-Flag and anti-HA antibodies. Lysates were also immunoblotted for expression of transfected SMAD4-Flag (pRK-DPC4-Flag) and HA-MyD88 (pCMV-HA-MyD88).

si-RNA transfection

Huh7 cells were transiently transfected with control psiRNA-LucGL3 (si-Ctrl) or psiRNA-hMyD88 (si-MyD88). Twenty-four hours after transfection, the cells were incubated with Zeocin at 400 µg/ml and BMP6 at 25 ng/ml for 24 hr. Cell lysates were used for western blot analysis to verify the efficacy of protein knockdown by siRNA.

Luciferase reporter assay

Huh7 cells were seeded at 1.1 x 10^5 cell/ml onto 24-well plates. Cells were transiently cotransfected by lipofection using Lipofectamine™ 2000 (Invitrogen). Lipofection included *Renilla* luciferase (phRL-TK) as the control reporter and the *Firefly* luciferase under the control of the *HAMP* promoter (HAMP-Luc) (Bagu and Santos, 2011;Bagu et al., 2013) in combination with pCMV-HA-MyD88 (pMyD88) or empty vector pCMV-HA

(also referred as pCMV). The total amount of DNA was kept constant. After 24 hr, cells were harvested and luciferase activity was measured by the Dual-Luciferase reporter assay system (Promega, Mississauga, ON, Canada). In all cases, the data were normalized for transfection efficiency by dividing *Firefly* luciferase activity by *Renilla* luciferase activity. Namalwa and Raji B cells were seeded at $1x10^6$ cell/ml and were transfected by electroporation with pCMV-HA, pCMV-HA-MyD88 or pCMV-HA-MyD88L265P along with HAMP-MetLuc2 using the Gene Pulser (Bio-Rad Laboratories, Mississauga, ON, Canada). After 24 hr, cells were harvested and luciferase activity was measured by using the Ready-To-Glow Secreted Luciferase Reporter Assay (Clontech Laboratories) with a Victor3 1420 Multilabel Counter (Perkin Elmer life and Analytical Sciences, Turku, Finland). For long-term experiments, Huh7 cells were stably co-transfected with HAM-MetLuc2 along with pCMV or pMyD88 and were selected using G418 antibiotic (Invitrogen; 200 mg/ml), which was added in the culture medium 24 hr post-transfection. Luciferase activity was measured at 24 hr, 48 hr, 2 weeks, 3 weeks, and 4 weeks after transfection.

GST pull-down assays

The GST-SMAD4 fusion protein was induced with 0.1 mM IPTG in BL21 (C2530H) *Escherichia coli* competent cells (New England BioLabs, NEB, MA, USA)) transformed with pGEX-2TK-SMAD4 and after 4 hr, the bacteria were lysed in 2% sarkosyl-STE buffer (10 mM Tris pH 8, 150 mM NaCl, 1 mM EDTA) and sonicated. HEK293-I3A cells were transfected with pCMV-HA-MyD88 or one of the three pCMV-HA-MyD88 deletion constructs. Total cellular lysate was extracted with RIPA buffer. Ten micrograms of GST-SMAD4 fusion protein or GST (as control) were incubated with Glutathione Sepharose 4B (GE Healtcare) for 1 hr at 4ºC. Beads were washed three times in TIF buffer (150 mM NaCl, 20 mM Tris pH 8, 1 mM MgCl2, 0.1% NP-40, and 10% glycerol), incubated with 50 µg of total cellular lysate for 1 hr at 4ºC, and washed again three times in TIF buffer. Pulled-down proteins were eluted by adding sample buffer, then fractionated by SDS-PAGE and visualized by immunoblotting with anti-HA and anti-GST antibodies. Lysates were also immunoblotted for expression of transfected HA-MyD88.

SDS–PAGE and western blot analysis

Cells were lysed in RIPA lysis buffer. Nuclear extracts were prepared with Nuclear Extract Kits (Active Motif, Carlsbad, CA, USA). Total cell lysates or nuclear and cytosol protein extracts were separated by 10% SDS–PAGE and blotted onto nitrocellulose membranes (Bio-Rad Laboratories). The membranes were immunoblotted with antibodies against the following: SMAD4 (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), MyD88 (1:1000) (Cell Signaling, Danvers, MA, USA), phosphorylated SMAD5 (1:1000) (Abcam, Cambridge, MA, USA), SMAD1 (1:1000) (Cell Signaling), His (1:1000) (Genscript, Piscataway, NJ, USA), Flag (1:5000) (Genscript), HA (1:5000) (Genscript), Ubiquitin FK2 (1:100), Ferroportin 1 (1:1000) (Novus Biologicals, Littleton, CO, UCA), GST (1:1000) (Genscript), and β-actin (1:10000) (Abcam, Cambridge, MA, USA). For secondary antibodies, anti–rabbit IgG (1:5000) or anti–mouse IgG (1:5000) were used. Antigen-

antibody complexes were visualized with the ECL Western Blotting Detection Reagent (Invitrogen).

Ubiquitination assays

Huh7 cells were co-transfected with His-Ubi (pCI-His-hUbi) and HA-MyD88 or the empty vector pCMV-HA. After 24 hr, cells were treated with Activin B and BMP6 at 25 ng/ml for 6 hr. Immunoprecipitation was performed using anti-His antibody (Genscript). For endogenous ubiquitin assay, Huh7 and HepG2 cells were transfected with HA-tagged MyD88 plasmid alone. After 24 hr, cells were treated with Activin B and BMP6 at 25 ng/ml for 6 hr. Immunoprecipitation was performed using anti- ubiquitin (FK2) antibody (EMD Millipore).

Intracellular iron concentration

Namalwa and Raji B cells were lysed in 1 mL RIPA buffer, and iron concentration in lysates was determined using the QuantiChromTM Iron Assay Kit (BioAssays Systems, Hayward, CA, USA). The number of live/dead cells was determined by flow cytometry using propidium iodide dye (Invitrogen).

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

Total RNA was isolated with Trizol reagent (Invitrogen), and reverse transcription was performed with the Omniscript RT kit (QIAGEN, Mississauga, ON, Canada). mRNA expression levels were measured by real-time PCR in a Rotor Gene 3000 Real Time DNA Detection System (Montreal Biotech, Kirkland, QC, Canada) with QuantiTect SYBRGreen I PCR kits (QIAGEN) as described (Makui et al., 2005). The following primers were used: hepcidin - (F) CTCTGCAAGTTGTCCCGTCT and (R) ACCAGAGCAAGCTCAAGACC; β-Actin - (F) AGAAAATCTGGCACCACACC and

(R) AGAGGCGTACAGGGATAGCA.

Statistical analysis

All statistics were calculated with Prism software (GraphPad, San Diego, CA, USA), with a pre-specified significant *P*-value of 0.05. Student's *t*-test was performed or multiple comparisons were evaluated statistically by one-way analysis of variance (ANOVA), followed by the Bonferroni multiple comparison test.

Results

MyD88 expression influences SMAD4 levels in hepatoma cells

We previously found that Smad4 protein levels were diminished in the livers of dietary iron-loaded *MyD88*$^{-/-}$ mice (Layoun, Samba-Mondonga et al., 2018). Therefore, we tested whether an *in vitro* system of hepatoma cells could demonstrate a similar association between MyD88 levels and SMAD4/hepcidin expression. We transfected Huh7 and HepG2 cells with HA-tagged MyD88 (pMyD88) or with empty plasmid (pCMV) and assessed the levels of endogenous SMAD4 protein. As shown in **Figure 1A-B** and **Supplementary figure 1 A-B and 2 A-B,** SMAD4 levels increased when MyD88 was overexpressed. The SMAD4 pathway modulating hepcidin expression involves the phosphorylation of SMAD proteins 1, 5, and 8 (Kersten et al., 2005), which translocate into the nucleus where they regulate the transcription of hepcidin (Pantopoulos et al., 2012). Hence, we next analyzed Smad5 phosphorylation levels in pMyD88 transfected Huh7 cells. As shown in **Supplementary figure 3,** MyD88 overexpression did not influence SMAD5 phosphorylation.

Next, we investigated whether the repression of MyD88 expression in Huh7 cells would result in a reduction of endogenous SMAD4. We generated a knockdown of endogenous MyD88 in the Huh7 cell line using the si-RNA MyD88 plasmid (si-MyD88) and compared with the control, scrambled si-RNA (si-Ctrl) (**Figure 1C-D**). Consistently, MyD88 knockdown resulted in a reduction of endogenous SMAD4 protein expression (**Figure 1C-E**).

MyD88 expression levels modulate hepcidin expression in hepatoma cells

To further understand the link between MyD88 expression levels and hepcidin regulation, we measured endogenous levels of hepcidin in cells overexpressing MyD88. As shown in **Figure 2A** and **Supplementary figure 1C,** MyD88 overexpression resulted in an increase of endogenous hepcidin mRNA levels, and this increase was further enhanced with the addition of BMP6, a signaling molecule activated by dietary iron-loading *in vivo* (Andriopoulos et al., 2009). Conversely, repression of MyD88 resulted in the abolishment of endogenous hepcidin mRNA induction by BMP6 (**Figure 2B**).

Next, we examined the effects of MyD88 on the SMAD4-dependent activation of hepcidin with a luciferase reporter-gene controlled by the human *HAMP* promoter (HAMP-Luc) (Bagu and Santos, 2011). In line with previous studies (Bagu and Santos, 2011;Besson-Fournier et al., 2012), both BMP6 and Activin B, two distinct BMP-signaling activators relevant to hepcidin induction, were able to activate the HAMP-Luc reporter gene (**Figure**

2C and Supplementary figure 1D). In turn, MyD88 transfection also led to HAMP-Luc activation (**Figure 2C and Supplementary figure 2 C-D**). To further establish a link between MyD88 and the BMP-signaling pathway for hepcidin induction, we mutated the BMP-responsive element (BMP-RE1) located at position -84/-79 of the *HAMP* promoter (HAMP-LucΔBMP-RE1), which is essential for BMP6-mediated hepcidin activation. The mutation of this vital responsive element abolished the activation of *HAMP* promoter by MyD88 (**Figure 2C and Supplementary figure 1D**) and, as expected by BMP6 and Activin B (Verga Falzacappa et al., 2008;Besson-Fournier et al., 2012).

Taken together, data in Figures 1 and 2 show that MyD88 expression levels affect SMAD4 protein levels in Huh7 hepatoma cells, as well as hepcidin activation induced by BMP6 and Activin B through BMP-RE elements located on the *HAMP* promoter.

MyD88 directly interacts with SMAD4 through the Toll/Interleukin-1 receptor (TIR) domain of MyD88 affecting hepcidin expression

Next, we hypothesized that MyD88 may influence SMAD4 levels by physically interacting with SMAD4. We transiently transfected Flag-tagged SMAD4 and HA-tagged MyD88 (HA-MyD88) expression vectors in the MyD88 KO cell line HEK293-I3A (Li et al., 1999), and performed immunoprecipitations. As shown in **Figure 3A**, HA-MyD88 co-immunoprecipitated with Flag-tagged SMAD4, and reciprocally, Flag-tagged SMAD4 co-immunoprecipitated with HA-MyD88, showing that SMAD4 can associate with MyD88. The SMAD4-MyD88 interaction was further confirmed by co-immunoprecipitating endogenous MyD88 with Flag-tagged SMAD4 and, reciprocally, endogenous SMAD4 with HA-tagged MyD88 in Huh7 cells (**Figure 3B**). Co-immunoprecipitations also detected interactions between endogenous SMAD4 and endogenous MyD88 in both Huh7 and HepG2 cells **(Figure 3C and Supplementary figure 1H).**

To identify the regions of MyD88 that bind to SMAD4, the MyD88 protein was divided into three regions (**Figure 3D and E**) that have been previously described: the death domain (DD) at its N terminus; a C-terminal TIR domain required for TLRs and IL-1 receptor interaction; and a short connecting linker or intermediate domain (ID) (Bonnert et al., 1997;Beutler, 2009). We generated HA-tagged MyD88 deletion mutants specifically lacking one of these domains and then tested the ability of the MyD88 deletion mutants to directly interact with SMAD4 using GST pull-down assays. For this, we constructed a GST-fusion protein for SMAD4 (GST-SMAD4). As shown in **Figure 3D**, the overexpressed HA-MyD88 lacking the TIR domain (HA-MyD88ΔTIR) was the only deletion mutant that could not be pulled-down with GST-SMAD4, thus demonstrating that MyD88-SMAD4 interaction was mediated by the TIR domain of the MyD88 protein.

Since MyD88-SMAD4 interaction was mediated by the TIR domain of the MyD88 protein, we further analyzed the functional role of the MyD88 TIR domain in the BMP/SMAD4 pathway in hepatocytes. We transfected Huh7 cells with pMyD88 or the HA-tagged MyD88 deletion mutant lacking ΔTIR domain (pMyD88ΔTIR) and analyzed the expression of endogenous SMAD4 protein. Compared to wild-type MyD88, the MyD88ΔTIR mutant failed to upregulate SMAD4 protein levels (**Figure 3F-G**). In addition, we found that the defective ΔTIR domain MyD88 mutant also failed to activate the *HAMP* promoter when compared to wild-type MyD88, as assessed by luciferase assay (**Figure 3H**). Similar results using the MyD88ΔTIR mutant were obtained in HepG2 cells (**Supplementary figure 1 E-G**).

These results show that the TIR domain of the MyD88 adapter protein is essential for its role in modulating SMAD4 and hepcidin expression levels.

MyD88 regulation through BMP6 and Activin B-induced degradation

The BMP/SMAD signaling pathway is regulated by a negative feedback loop involving the inhibitory SMADs, particularly SMAD6 (Goto et al., 2007), which is upregulated in response to dietary iron-loading (Kautz L et al., 2011). Previous studies have shown that in macrophages, SMAD6 negatively regulates MyD88 through degradation driven by the SMAD6-SMURFs (SMAD ubiquitin regulator factor proteins) pathway (Lee et al., 2011). We hypothesized that MyD88 levels may also be regulated by a negative feedback loop in hepatocytes involving BMP6 and Activin B signaling. Therefore, we assessed the ability of BMP6 and Activin B to trigger MyD88 degradation in Huh7 cells. First, we examined the ubiquitination and degradation of HA-MyD88 in cells that were co-transfected with HA-MyD88 and histidine-tagged ubiquitin (His-Ubi). Both BMP6 and Activin B treatments induced MyD88 ubiquitination and degradation (**Figure 4A and Supplementary figures 4 and 5**). Accordingly, endogenous MyD88 levels in Huh7 cells were reduced by BMP6 and Activin B treatments (**Figure 4B**), and MyD88 degradation was inhibited by the proteasomal inhibitor MG132 (**Figure 4C**). We also show that overexpression of SMAD6 and SMURF1 in hepatoma cells inhibited HAMP-Luc activity (**Figure 4D and Supplementary figure 1I**), while endogenous MyD88 protein expression diminished (**Figure 4E and Supplementary figure 1J**). Furthermore, HAMP-Luc activity that was inhibited by SMAD6 and SMURF1 overexpression could be rescued by treatment with MG132 **(Supplementary figure 6**).

These results indicate that signaling initiated by BMP6 and Activin B regulates MyD88 through a negative feedback loop relevant to the modulation of hepcidin expression in hepatocytes.

The L265P mutation of MyD88 results in enhanced hepcidin production

In humans, genomic MyD88 mutations are extremely rare (Picard et al., 2011). In contrast, significantly higher rates of somatic MyD88 mutations have been identified in a variety of mature B cell tumors, with the most prevalent mutation being the Leu265Pro (L265P) missense substitution (Rossi, 2014). Most MyD88 mutations, including the L265P mutation in B cell tumors, cluster in the TIR domain, thus coinciding with the domain that we have identified as essential for interaction with SMAD4. Hence, we questioned whether the L265P mutation could affect hepcidin production in B cell lines. We generated an HA-MyD88 construct carrying the L265P mutation and used immunoprecipitation assays to show that the MyD88 L265P mutant could still interact and bind to SMAD4, similar to wild-type MyD88 (**Figure 5A**). We then co-transfected the wild-type MyD88 or MyD88 L265P mutant with HAMP-MetLuc2 in two B cell lines (Namalwa and Raji) and found that *HAMP*-driven luciferase activity was significantly higher in cells expressing the MyD88 L265P mutant compared to the wild-type (**Figure 5B-C**), indicating a gain-of-function of the L265P mutation in regards to hepcidin activation. When these experiments were repeated in Huh7 cells and the MyD88 KO cell line HEK293-I3A, we observed a similar enhanced induction of hepcidin by the MyD88 L265P mutant (**Supplementary figure 7**).

In macrophages, hepcidin has been shown to regulate intracellular iron levels in an autocrine manner (Theurl et al., 2008). We reasoned that hepcidin produced in B cells may similarly regulate intracellular iron levels. Therefore, we measured the iron levels in the Namalwa and Raji B cell lines transfected with wild-type MyD88 or the MyD88 L265P mutant. As shown in **Figure 5D-E**, intracellular iron levels were significantly higher in B cell lines transfected with the MyD88 L265P mutant compared to wild-type MyD88 or the control pCMV vector, suggesting that this mutation of MyD88 results in enhanced accumulation of iron in B cells. In addition, we found that Ferroportin1 protein expression was significantly lower in Namalwa B cells transfected with MyD88 L265P mutant **(Figure 5F-G**), which is consistent with hepcidin modulating Ferroportin 1 expression through its internalization and degradation (Nemeth et al., 2004) resulting in enhanced intracellular iron accumulation.

Taken together these results indicate that the MyD88 L265P mutation leads to higher hepcidin expression and enhanced iron accumulation in B cells.

Discussion

In previous studies, we found that *MyD88*$^{-/-}$ mice cannot appropriately regulate hepcidin activation in response to iron-loading and that Smad4 expression is decreased in the nuclear extracts of these mice (Layoun, Samba-Mondonga et al., 2018). Here, we further investigate the involvement of MyD88 in the regulation of SMAD4 and hepcidin expression.

We show that MyD88 overexpression in Huh7 hepatoma cells increases endogenous SMAD4 protein levels, while conversely, suppression of MyD88 results in lowering SMAD4 levels. SMAD4 is pivotal to hepcidin regulation *in vivo*, as evidenced by mice with liver-specific Smad4 disruption that express very low levels of hepcidin in the liver, causing severe iron overload in several organs (Wang et al., 2005). Furthermore, transcriptional activation of hepcidin in response to known strong hepcidin stimulators such as iron, BMP and IL-6 in SMAD4-deficient hepatocytes from these mice is completely abrogated (Wang et al., 2005). Here, we show that MyD88-modulated SMAD4 expression in Huh7 also affects hepcidin expression: MyD88 overexpression induced hepcidin while repression of MyD88 inhibited hepcidin mRNA expression. Furthermore, MyD88 overexpression upregulated hepcidin promoter activity and, importantly, this was abolished in the absence of a functional BMP-responsive site on the HAMP promoter. These results show that, in our *in vitro* system, MyD88 modulates SMAD4 and consequent hepcidin expression through the BMP/SMAD4 signaling pathway.

We hypothesized that MyD88 could regulate SMAD4 levels through direct binding to SMAD4. Using co-immunoprecipitation assays, we show that, indeed, SMAD4 physically interacts with MyD88 protein. The physical interaction between SMAD4 and MyD88 protein could potentially be essential for translocation to the nucleus to prevent SMAD4 degradation or may facilitate SMAD4 interaction with the hepcidin promoter. We further identified the TIR domain of the MyD88 protein as the region involved in this interaction. Accordingly, the deletion of the TIR domain not only abolished the MyD88-SMAD4 interaction, but also MyD88-mediated stimulation of SMAD4 expression and hepcidin promoter activity. TIR domains of adapter proteins are known to assemble signaling components to trigger activation of transcription factors such as NF-κB and AP-1, as well as the overexpression of genes involved in the immune response (Bonnert et al., 1997;Ohnishi et al., 2009). Ultimately, interactions between adaptor proteins containing TIR domains activate transcription factors that regulate the expression of various proinflammatory cytokines (IL-1, IL-6, IL-8 and TNF-α) and chemokines (Narayanan and Park, 2015).

In addition to its role in innate immunity, the TIR domain of MyD88 directly interacts with SMAD6 (Lee et al., 2011) to negatively regulate the transforming growth factor β (TGF-β) family signaling pathway, particularly BMP signaling (Goto et al., 2007). Protein degradation by the ubiquitin-proteasome pathway plays a vital role in monitoring the abundance of many regulatory proteins. SMAD6 inhibits BMP signaling through reduced phosphorylation of SMAD2 and SMAD5, via competition with SMAD4, and through downregulation of SMAD4 with SMURF1 (Imamura et al., 1997;Hata et al., 1998;Moren et al., 2005). In addition to its conventional role as a negative regulator of TGF-β/BMP signaling, SMAD6 negatively regulates TLR-4 signaling through several mechanisms (Choi et al., 2006). In macrophages, SMAD6 induces MyD88 degradation by mediating the recruitment of SMURF proteins, which have E3-ubiquitin ligase activity (Lee et al., 2011). These previous studies led us to investigate if similar SMAD6-dependent negative feedback loop mechanisms would be applicable for the regulation of hepcidin, particularly mechanisms involving proteolytic-dependent degradation induced by BMP6 and Activin B, which are specifically relevant to the BMP/SMAD pathway (Andriopoulos et al., 2009;Besson-Fournier et al., 2012). Indeed, we show that both BMP6 and Activin B induced the ubiquitination and degradation of MyD88 and that this negative feedback loop is relevant to the modulation of hepcidin production in hepatocytes. Our results reveal for the first time, a potential mechanism of hepcidin negative feedback loop that involves ubiquitin-proteolytic degradation of modulatory proteins.

Our identification of the TIR domain of MyD88 as essential for the MyD88-SMAD4 interaction led to the question of the possible relevance of MyD88 mutations in the regulation of hepcidin expression and its consequent impact on cellular iron metabolism. However, genomic MyD88 mutations in humans are extremely rare, with approximately two dozen patients from six different countries identified thus far (Picard et al., 2011). In contrast, somatic MyD88 mutations, particularly the L265P missense substitution (Rossi, 2014), are frequent in a variety of mature B cell tumors. The most striking incidences are seen in Waldenström macroglobulinemia (WM) (Treon et al., 2012) where the mutation is detectable in more than 90% of patients (Gertz, 2015). There is increasing evidence that overactivation of TIR domain-mediated signaling is involved in inflammatory diseases and cancer growth (Ota et al., 2012). In B cell tumors, mutant MyD88 consistently results in a gain-of-function, leading to the activation of TLR downstream signaling pathways in the absence of cognate ligands and, ultimately, the elevation of NF-κB activity. In turn, this results in increased proliferation and survival of tumor cells (Ngo et al., 2011;Treon et al., 2012;Ansell et al., 2014). We therefore tested whether this mutation would affect hepcidin activation since the L265P mutation is located in the same TIR domain that is essential for the MyD88-SMAD4 interaction. We show that, in B cell lines, the L265P mutation of MyD88 can still bind to SMAD4 and results in enhanced SMAD4 expression to similar levels as seen with Wt MyD88. Furthermore, the gain-of-function of the L265P MyD88 is manifested by enhanced hepcidin expression and consequent iron accumulation in B cells. As a direct consequence of hepcidin activation, the expression of the iron

exporter Ferroportin 1 (Nemeth et al., 2004) was significantly reduced in B cells that overexpressed the MyD88 L265P mutant, resulting in iron accumulation in these cells.

Importantly, previous studies report that hepcidin is produced by peripheral blood B cells from patients with WM and further show that hepcidin expression levels in B cells are also higher in WM patients compared to healthy donors (Ciccarelli et al., 2015). While hepcidin has been identified as an unequivocal contributor to anemia in WM, its production by B cells and monocytes is significantly lower than hepatocytes (data not shown and (Zhang and Rovin, 2010)). However, as shown for monocytes (Theurl et al., 2008) and lymphocytes in general (Pinto et al., 2010), hepcidin produced by these cells may act as an autocrine regulator of iron accumulation. Accordingly, we show that B cell lines transfected with the MyD88 L265P mutant accumulated more iron when compared to wild-type MyD88. Our results therefore suggest a novel mechanism by which gain-of-function somatic MyD88 mutations may interfere with iron re-utilization by sequestrating iron and hence, contribute to anemia in WM (Weiss and Goodnough, 2005;Treon et al., 2013). This may help explain why, in many WM patients, anemia is of a severity out of proportion to bone marrow disease involvement (Treon, 2015). In addition, since iron is essential for cell survival, particularly for highly active cells such as tumor cells, increased hepcidin expression leading to higher cellular iron availability may further fuel tumor growth. In fact, there is increasing evidence that tumor cells manipulate hepcidin expression and regulation to meet their metabolic needs (Vela and Vela-Gaxha, 2018).

In summary, we report a new interaction between MyD88 and SMAD4 proteins that affects hepcidin induction though the BMP6/SMAD4 signaling pathway. Together, our data identify MyD88 as a potential player molecule in the BMP signaling pathway mediated by SMAD proteins. These findings may contribute to the identification of pathways and interacting elements that will provide further insight into the cross-regulation between iron metabolism and the immune system (Reuben et al., 2017).

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Author contributions

MSM and AC contributed to the investigation, validation, methodology, and formal analysis. FAM provided technical input regarding ubiquitination experiments and data analysis. MSM and MMS additionally contributed to the conceptualization and writing of the original draft of the manuscript. MMS additionally contributed to the visualization, supervision, and funding acquisition of the study.

Disclosure of conflicts of interest

All authors declare no competing financial interests.

Figure legends

Figure 1. MyD88 expression levels regulate endogenous SMAD4 expression in Huh7 hepatoma cells

(**A-B**) MyD88 overexpression enhances endogenous SMAD4 expression. (**A**) Huh7 cells transiently transfected with an empty vector (pCMV) or HA-tagged MyD88 plasmid (pMyD88). Total cell lysates were analyzed by western blotting for endogenous SMAD4 expression. Expression of the β-actin protein was used as a loading control. (**B**) Densitometric quantification of SMAD4 levels in western blots from three independent experiments.

(**C-E**) MyD88 repression lowers endogenous SMAD4 expression. (**C**) Representative western blot of MyD88 and SMAD4 expression in Huh7 cells transiently transfected with control psiRNA-LucGL3 (si-Ctrl) or psiRNA-hMyD88 (si-MyD88). Expression of the β-actin protein was used as a loading control. (**D-E**) Densitometric quantification of (**D**) MyD88 and (**E**) SMAD4 protein levels from three independent experiments.

Results are presented as mean ± SEM. Statistical analyses were performed with Student's *t*-test.

Figure 2. MyD88 expression levels regulate endogenous hepcidin expression in Huh7 cells

(**A**) MyD88 overexpression enhances endogenous hepcidin expression. Huh7 cells were transiently transfected with an empty vector (pCMV) or HA-tagged MyD88 plasmid (pMyD88) and treated with (+) or without (-) BMP6. Hepcidin (*HAMP)* mRNA levels were assessed by RT-PCR. Results are presented as mean ± SEM. $*P < 0.01$ compared with vector (pCMV) transfected cells and $\&P < 0.01$ compared to cells without BMP6 treatment.

(**B**) MyD88 knockdown lowers endogenous hepcidin expression in Huh7 cells**.** Hepcidin mRNA levels in Huh7 cells were transfected with si-Ctrl or si-MyD88 and treated with BMP6 (+). Results are presented as mean ± SEM. $*P < 0.05$ compared with si-Ctrl transfected cells and $\&P < 0.05$ compared to cells transfected with si-MyD88 and treated with BMP6; n.s. = not significant compared to untreated si-Ctrl transfected cells.

(**C**) Mutation of the BMP-RE1 in the HAMP promoter abolishes HAMP-Luc induction by MyD88. Huh7 cells were transiently cotransfected with HAMP-Luc or mutated HAMP-LucΔBMP-RE1 along with phRL-TK (*Renilla* Luciferase) as an internal control, and MyD88 plasmids (pCMV or lMyD88). BMP6 and Activin B treatments were used as controls. Luciferase activity was assessed 24 hr after transfection. Results are presented as mean ± SEM of the relative activity (*Firefly*/*Renilla* ratio). $**P < 0.001$, $***P < 0.0001$, and n.s.= not significant compared with empty plasmid (pCMV).

The results are representative of at least three independent experiments. Statistical analysis was performed with one-way ANOVA.

Figure 3. MyD88 directly interacts with SMAD4 through the Toll/Interleukin-1 receptor (TIR) domain of MyD88.

(**A-C**) Co-immunoprecipitation of MyD88 with SMAD4. (**A**) MyD88 KO HEK293 cells (HEK293-I3A) were transfected transiently with HA-tagged MyD88 together with Flag-tagged SMAD4. Cell lysates were subjected to immunoprecipitation (IP) with the anti-Flag, anti-HA or normal IgG antibody (as a control) and analyzed by immunobloting (IB) with an anti-HA antibody to detect MyD88 and an anti-Flag antibody to detect SMAD4. (**B**) Co-immunoprecipitation of endogenous MyD88 with Flag-tagged SMAD4 and, reciprocally, of endogenous SMAD4 with HA-tagged MyD88. (**C**) Co-immunoprecipitation of endogenous MyD88 with endogenous SMAD4 in Huh7 cells treated with BMP6 (+BMP6) for 24 hr.

(**D-E**) The TIR domain of MyD88 is necessary for its interaction with SMAD4. HEK293-I3A cells were transiently transfected with pCMV-HA-MyD88 or one of the three MyD88-deletion plasmids as shown in (E). **(D)** Total cellular lysates (TCL) were extracted, incubated with GST-SMAD4, and analyzed by western blot with the indicated antibodies. (**E**) Schematic representation of plasmids encoding different truncated forms of MyD88. FL - full length; DD - death domain; TIR - Toll/Interleukin-1 receptor domain; ID - intermediate domain.

(**F-H**) Defective MyD88 mutant (ΔTIR domain) abolishes the induction of endogenous SMAD4 and HAMP promoter activation by MyD88 overexpression. Huh7 cells were transfected with HAMP-Luc and pCMV or HA-tagged MyD88 vector (pMyD88) or the MyD88 vector lacking the TIR domain (pMyD88ΔTIR). (**F**) Expression of endogenous SMAD4 and transfected HA-tagged MyD88 was analyzed by western blotting. β-actin protein was used as a loading control. (**G**) Densitometric quantification of SMAD4 levels in western blots from three independent experiments. (**H**) Luciferase activity assessed 24 hr after transfection. Results are presented as mean ± SEM of the relative activity (*Firefly*/*Renilla* ratio). Data are representative of a minimum of three experiments. Statistical analysis was performed with one-way ANOVA; n.s.: not significant compared to pCMV.

Figure 4. MyD88 is regulated through BMP6- and Activin B-induced degradation.

(**A**) BMP6 and Activin B induce MyD88 ubiquitination. Huh7 cells were co-transfected with HA-tagged MyD88 and His-tagged Ubiquitin (His-Ubi), and treated with BMP6 or Activin B. HA-MyD88 ubiquitination was examined by immunoprecipitation (IP) using

anti-His antibody, followed by immunoblotting (IB) with anti-HA antibody. Total cell lysates (TCL) before IP were immunoblotted with anti-HA and anti-β-actin antibodies. (**B**) BMP6 and Activin B reduce endogenous MyD88 protein levels. Huh7 cells were left untreated (-) or were treated (+) with BMP6 or Activin B at the specified doses (12.5, 25, and 50 ng/ml). TGF-β was used as a control (15 ng/ml). TCL were analyzed by immunoblotting using an anti-MyD88 antibody. Expression of the β-actin protein was used as a loading control. (**C**) The proteasome inhibitor MG132 prevents the degradation of MyD88 induced by BMP6 and Activin B. Huh7 cells were treated with BMP6 or Activin B (25 ng/ml) in the presence (+) or absence (-) of the proteasome inhibitor MG132 (10 μM) for 4 hr. (**D-E**) Overexpression of SMAD6 and SMURF1 in Huh7 cells inhibits HAMP-Luc activity and correlates with changes in endogenous MyD88 expression. (**D**) Huh7 cells were transiently cotransfected with HAMP-Luc in combination with empty plasmid (pCMV), pSMAD6, or pSMURF1. Luciferase activity was assessed 24 hr after transfection. Results are presented as mean ± SEM of the relative activity (*Firefly*/*Renilla* ratio). Statistical analysis was performed with one-way ANOVA. ***$P < 0.0001$. (**E**) TCL were analyzed by immunoblotting using an anti-MyD88 antibody. Expression of the β-actin protein was used as a loading control. All data are representative of at least three independent experiments.

Figure 5. MyD88 L265P mutation enhances hepcidin expression and intracellular iron accumulation in B cell lines. (**A**) MyD88 L265P mutant interacts with SMAD4. MyD88 KO HEK293T cells (HEK293-I3A) were transfected transiently with either pCMV (control) or HA-tagged MyD88 (as Wt) or HA-MyD88L265P (as L265P), together with Flag-tagged SMAD4. Cell lysates were subjected to immunoprecipitation (IP) with the anti-Flag antibody and analyzed by immunobloting (IB) with an anti-HA antibody to detect MyD88 and an anti-Flag antibody to detect SMAD4. Total cell lysates (Input) before IP were immunoblotted with anti-HA, anti-Flag, and anti-β-actin antibodies.

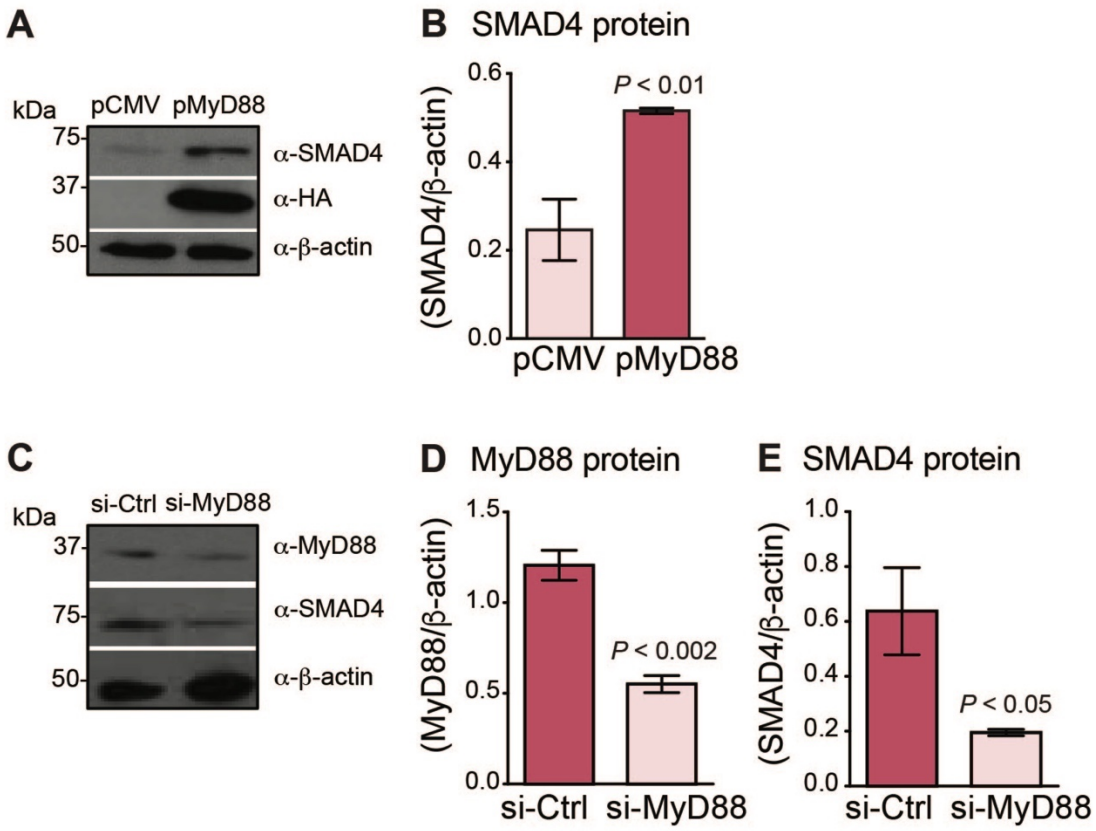
(**B-C**) MyD88 L265P mutant upregulates hepcidin expression in B cell lines. (**B**) Namalwa and (**C**) Raji B cell lines were co-transfected with HAMP-MetLuc2 and control (pCMV) or MyD88 plasmids (pMyD88): wild-type (Wt) or mutated MyD88 (L265P). Luciferase activity was assessed 24 hr after transfection. Data are from a minimum of three independent experiments.

(**D-E**) MyD88 L265P mutant increases intracellular iron content in B cell lines. (**D**) Namalwa and (**E**) Raji B cell lines were co-transfected with HAMP-MetLuc2 and control (pCMV) or MyD88 plasmids (pMyD88): wild-type (Wt) or mutated MyD88 (L265P). Intracellular iron concentrations were determined using the QuantiChrom Iron Assay Kit. Concentrations of iron are given in μg iron per 10^6 live cells and data are from a minimum of three independent experiments.

(**F-G**) MyD88 L265P mutant decreases Ferroportin 1 expression. (**F**) Namalwa cells were co-transfected with HAMP-MetLuc2 and control (pCMV) or MyD88 plasmids (pMyD88): wild-type (Wt) or mutated MyD88 (L265P). Total cell lysates were analyzed by western blotting for ferroportin 1 and β-actin as loading control. (**G**) Densitometric quantification of ferroportin 1 levels in western blots from three independent experiments.

Statistical analysis was performed with one-way ANOVA. Results are presented as mean ± SEM. **P* < 0.05, ***P* < 0.001, ****P* < 0.0001, and n.s. = not significant compared with empty plasmid (pCMV).

Figure 1.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kDa
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pMyD88
75
37
50
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α-HA
α-β-actin
B
SMAD4 protein
(SMAD4/β-actin)
0.6
0.4
0.2
0.0
P < 0.01
pCMV
pMyD88
C
kDa
si-Ctrl
si-MyD88
37
75
50
α-MyD88
α-SMAD4
α-β-actin
D
MyD88 protein
(MyD88/β-actin)
1.5
1.0
0.5
0.0
P < 0.002
si-Ctrl
si-MyD88
E
SMAD4 protein
(SMAD4/β-actin)
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0.8
0.6
0.4
0.2
0.0
P < 0.05
si-Ctrl
si-MyD88

Figure 2.

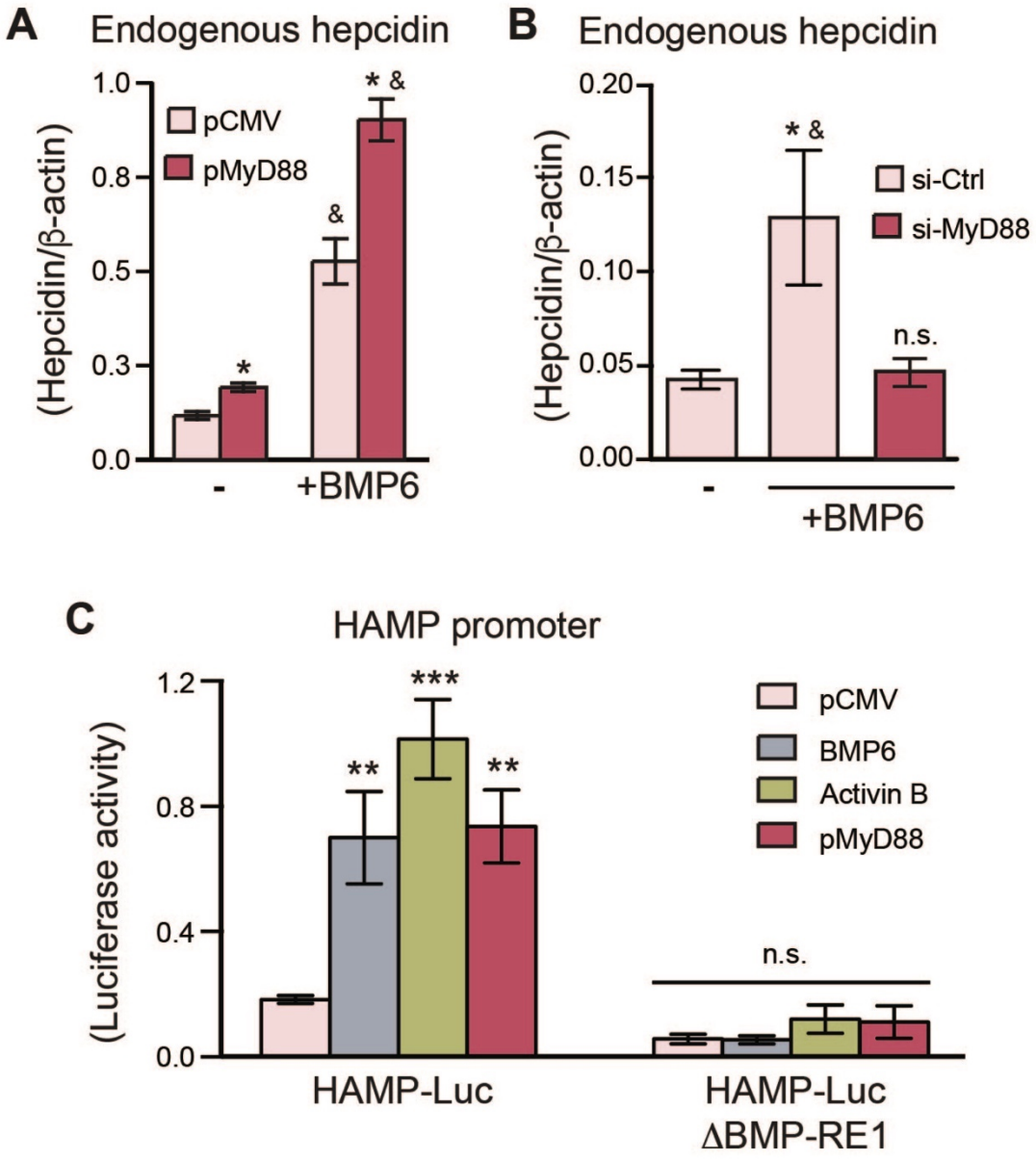
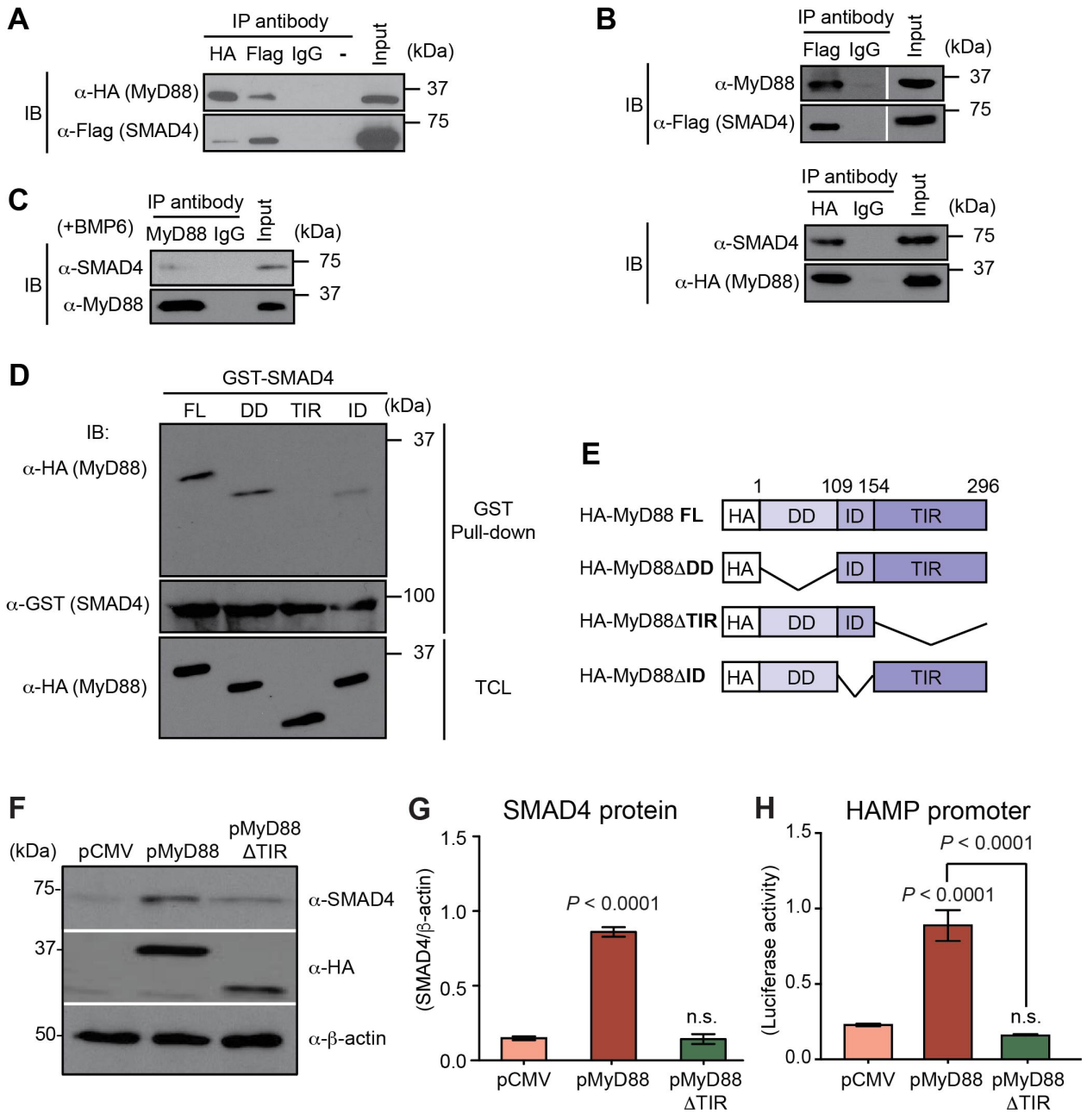

A
Endogenous hepcidin
(Hepcidin/β-actin)
1.0
0.8
0.5
0.3
0.0
pCMV
pMyD88
*
&
* &
-
+BMP6
B
Endogenous hepcidin
(Hepcidin/β-actin)
0.20
0.15
0.10
0.05
0.00
si-Ctrl
si-MyD88
* &
n.s.
-
+BMP6
C
HAMP promoter
(Luciferase activity)
1.2
0.8
0.4
0.0
**
***
**
pCMV
BMP6
Activin B
pMyD88
n.s.
HAMP-Luc
HAMP-Luc
ΔBMP-RE1

Figure 3.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IP antibody
HA Flag IgG -
Input
(kDa)
IB
α-HA (MyD88)
α-Flag (SMAD4)
37
75
B
IP antibody
Flag IgG
Input
(kDa)
IB
α-MyD88
α-Flag (SMAD4)
37
75
IP antibody
HA IgG
Input
(kDa)
IB
α-SMAD4
α-HA (MyD88)
75
37
C
(+BMP6)
IP antibody
MyD88 IgG
Input
(kDa)
IB
α-SMAD4
α-MyD88
75
37
D
GST-SMAD4
FL DD TIR ID
(kDa)
IB:
α-HA (MyD88)
α-GST (SMAD4)
α-HA (MyD88)
37
100
37
GST Pull-down
TCL
E
1 109 154 296
HA-MyD88 FL
HA-MyD88ΔDD
HA-MyD88ΔTIR
HA-MyD88ΔID
HA DD ID TIR
F
(kDa)
pCMV
pMyD88
pMyD88 ΔTIR
75-
37-
50-
α-SMAD4
α-HA
α-β-actin
G
SMAD4 protein
(SMAD4/β-actin)
1.5
1.0
0.5
0.0
P < 0.0001
n.s.
pCMV
pMyD88
pMyD88 ΔTIR
H
HAMP promoter
(Luciferase activity)
1.5
1.0
0.5
0.0
P < 0.0001
P < 0.0001
n.s.
pCMV
pMyD88
pMyD88 ΔTIR

Figure 4.

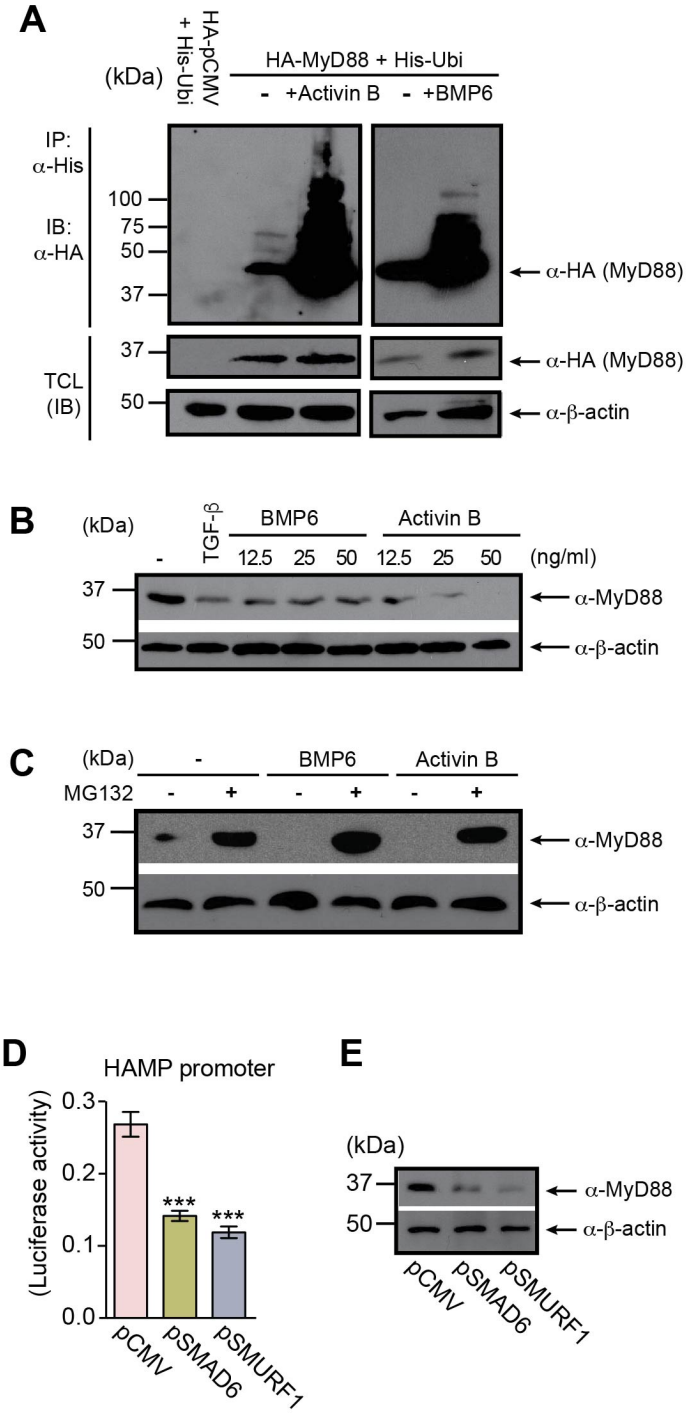
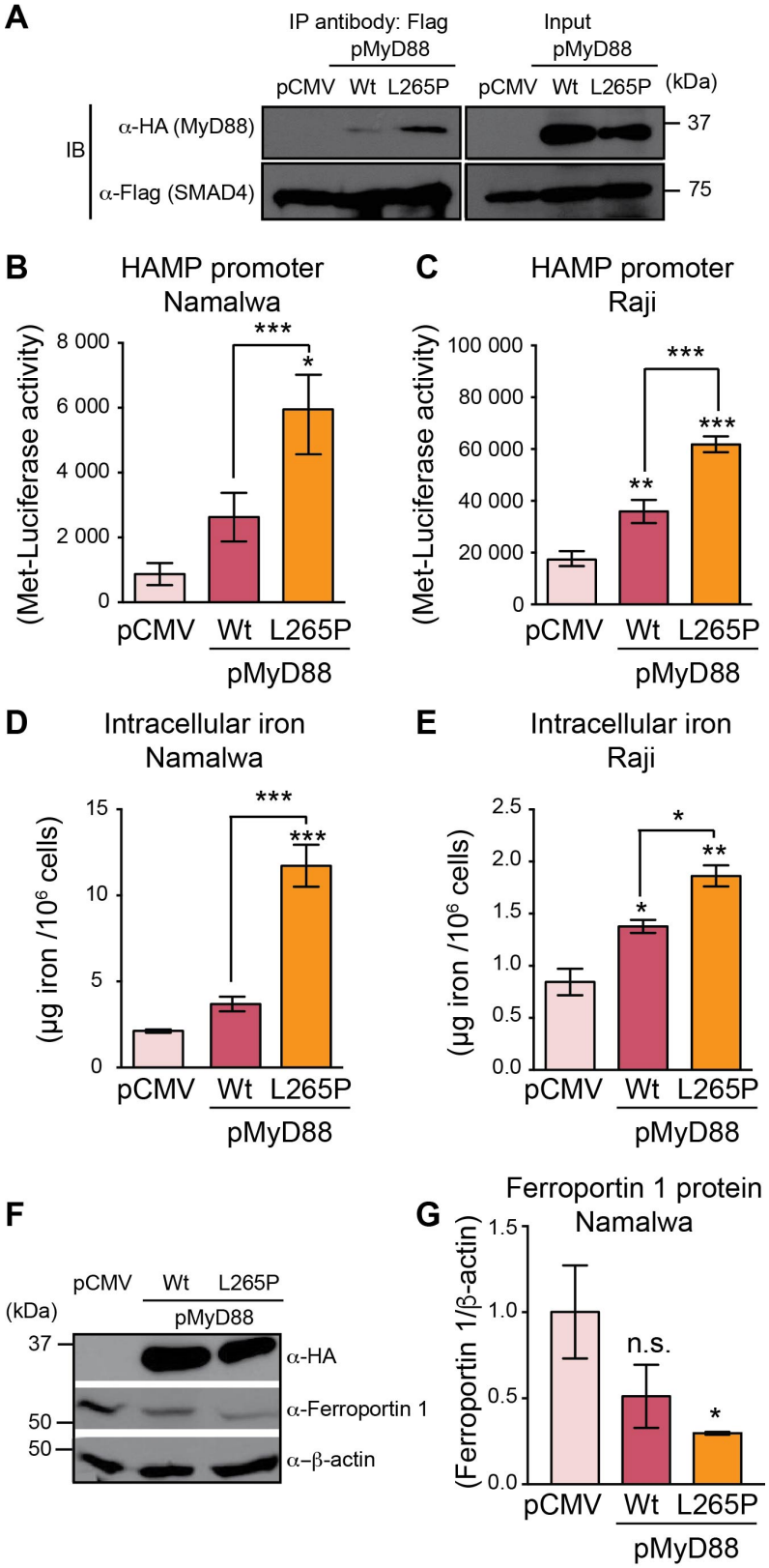
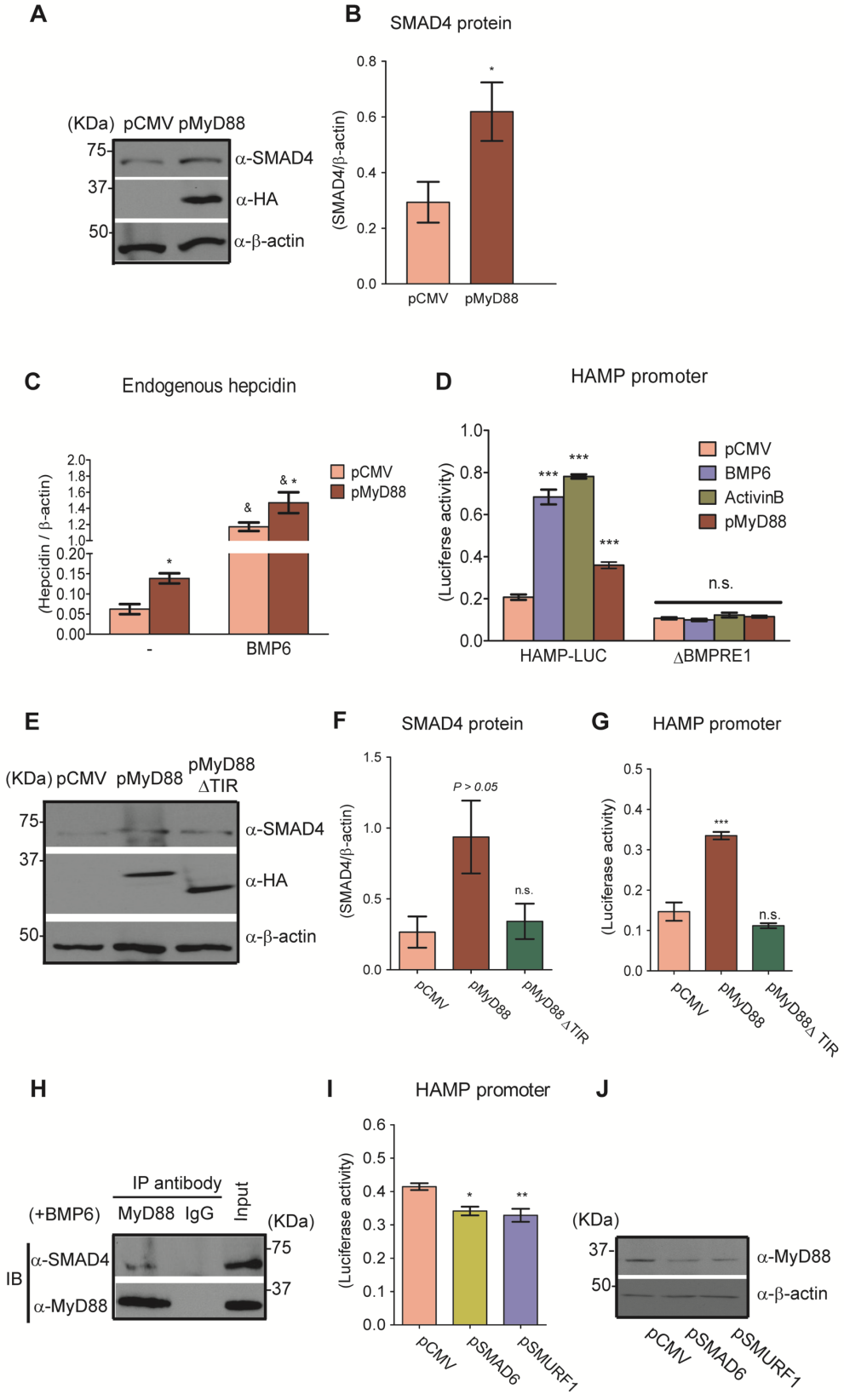

A
(kDa)
HA-pCMV + His-Ubi
HA-MyD88 + His-Ubi
- +Activin B - +BMP6
IP: α-His
IB: α-HA
100
75
50
37
α-HA (MyD88)
TCL (IB)
37
50
α-HA (MyD88)
α-β-actin
B
(kDa)
TGF-β
BMP6
Activin B
- 12.5 25 50 12.5 25 50 (ng/ml)
37
50
α-MyD88
α-β-actin
C
(kDa)
MG132
- + - + - +
37
50
α-MyD88
α-β-actin
D
HAMP promoter
(Luciferase activity)
0.3
0.2
0.1
0.0
***
***
pCMV
pSMAD6
pSMURF1
E
(kDa)
37
50
α-MyD88
α-β-actin
pCMV
pSMAD6
pSMURF1

Figure 5

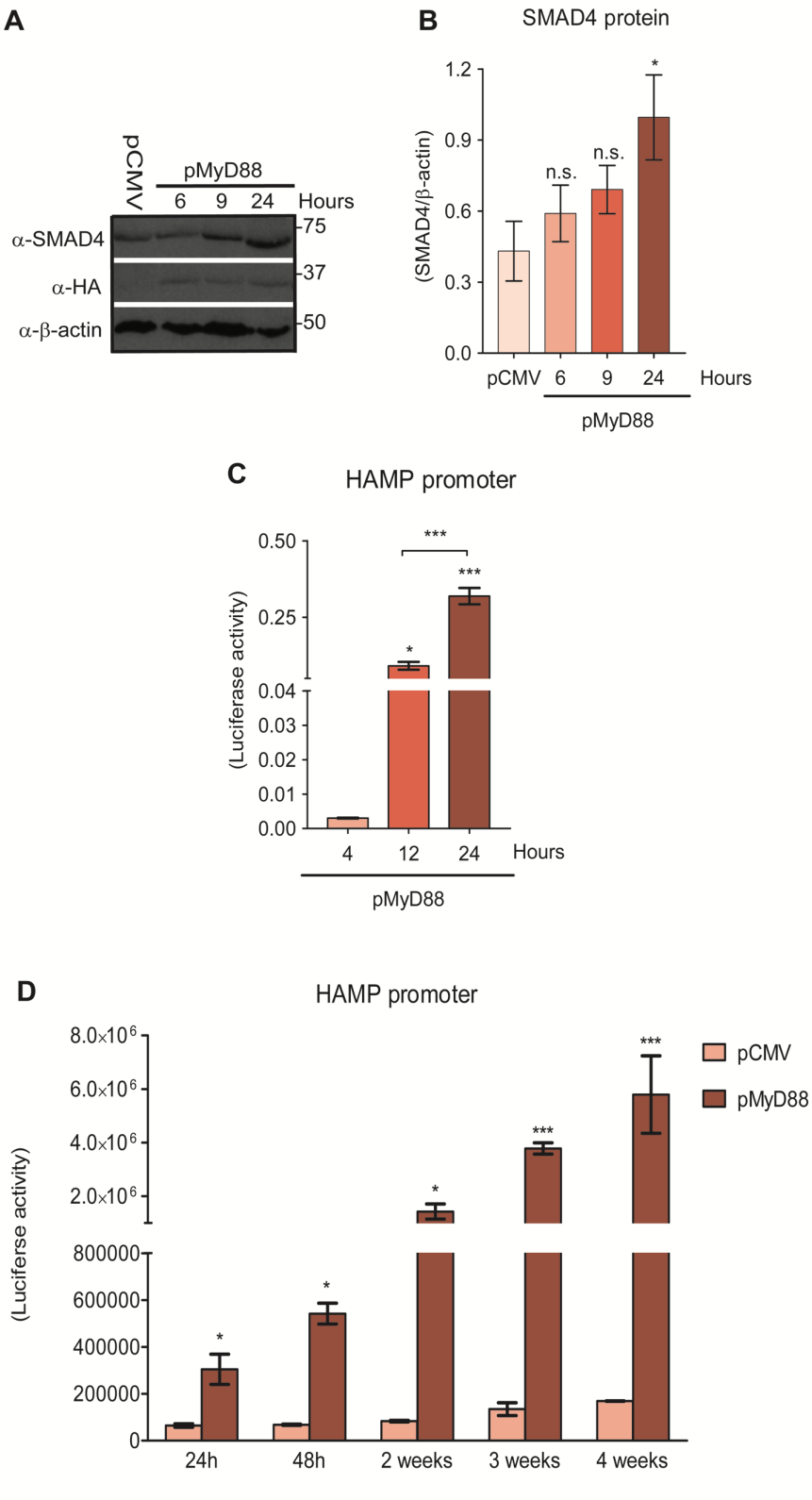

A
IP antibody: Flag
pMyD88
Input
pMyD88
pCMV Wt L265P
pCMV Wt L265P
(kDa)
IB
α-HA (MyD88)
α-Flag (SMAD4)
37
75
B
HAMP promoter
Namalwa
(Met-Luciferase activity)
8 000
6 000
4 000
2 000
0
***
*
pCMV Wt L265P
pMyD88
C
HAMP promoter
Raji
(Met-Luciferase activity)
100 000
80 000
60 000
40 000
20 000
0
***
**
***
pCMV Wt L265P
pMyD88
D
Intracellular iron
Namalwa
(µg iron /10⁶ cells)
15
10
5
0
***
***
pCMV Wt L265P
pMyD88
E
Intracellular iron
Raji
(µg iron /10⁶ cells)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
**
pCMV Wt L265P
pMyD88
F
pCMV Wt L265P
(kDa)
pMyD88
37
50
50
α-HA
α-Ferroportin 1
α–β-actin
G
Ferroportin 1 protein
Namalwa
(Ferroportin 1/β-actin)
1.5
1.0
0.5
0.0
n.s.
*
pCMV Wt L265P
pMyD88

Supplementary figure 1



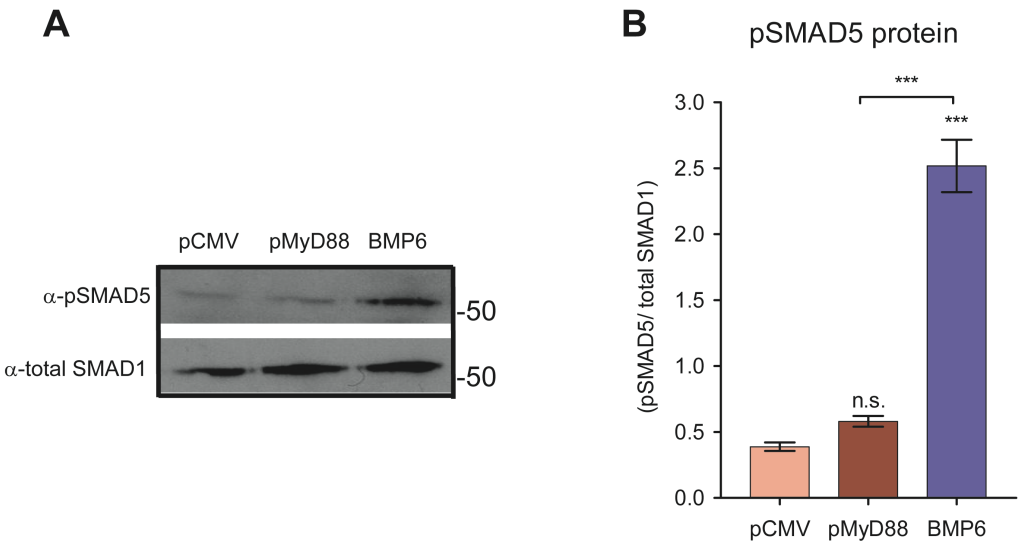
Supplementary figure 1. (A-D) MyD88 overexpression enhances SMAD4 and hepcidin expression. HepG2 cells were transiently transfected with an empty vector (pCMV) or HA-tagged MyD88 plasmid (pMyD88). (**A**) Total cell lysates were analyzed by western blotting for endogenous SMAD4 expression. Expression of the β-actin protein was used as a loading control. (**B**) Densitometric quantification of SMAD4 levels in western blots from three independent experiments. Results are presented as mean ± SEM. Statistical analyses were performed with Student's *t*-test. **P < 0.01* compared with empty vector (pCMV) (**C**) HepG2 cells were transiently transfected with an empty vector (pCMV) or HA-tagged MyD88 plasmid (pMyD88) and treated without (-) or with BMP6. Hepcidin (*HAMP)* mRNA levels were assessed by RT-PCR. Results are presented as mean ± SEM. **P < 0.01* compared with empty vector (pCMV) transfected cells. (**D**) **Mutation of the BMP-RE1 in the HAMP promoter abolishes HAMP-Luc induction by MyD88**. HepG2 cells were transiently co-transfected with HAMP-Luc or mutated HAMPLucΔBMP-RE1 along with phRL-TK (*Renilla* Luciferase) as an internal control, and MyD88 plasmids (pCMV or MyD88). BMP6 and Activin B treatments were used as controls. Luciferase activity was assessed 24 hr after transfection. Results are presented as mean ± SEM of the relative activity (*Firefly*/*Renilla* ratio). ****P* < 0.0001, and n.s. = not significant compared with empty plasmid (pCMV). **(E-G) Defective MyD88 mutant (ΔTIR domain) abolishes the induction of endogenous SMAD4 and HAMP promoter activation by MyD88 overexpression.** HepG2 cells were transfected with HAMP-Luc and pCMV or HA-tagged MyD88 vector (pMyD88) or the MyD88 vector lacking the TIR domain (pMyD88ΔTIR). **(E)** Expression of endogenous SMAD4 and transfected HAtagged MyD88 was analyzed by western blotting. β-actin protein was used as a loading control. **(F)** Densitometric quantification of SMAD4 levels in western blots from three independent experiments. **(G)** Luciferase activity assessed 24 hr after transfection. Data are representative of a minimum of three experiments. Results are presented as mean ±SEM of the relative activity (*Firefly*/*Renilla* ratio). Statistical analysis was performed with one-way ANOVA for (**F**) and (**G**). ****P < 0.0001* and n.s.= not significant compared to pCMV. (**H**) Co-immunoprecipitation of endogenous MyD88 with endogenous SMAD4. HepG2 cells were treated with (+BMP6) for 24 hr. Cell lysates were subjected to immunoprecipitation (IP) with the anti-MyD88 or normal IgG antibody (as a control) and analyzed by immunoblotting (IB) with SMAD4 and MyD88 antibodies. (**I**) HepG2 cells were transiently co-transfected with HAMP-Luc in combination with empty plasmid (pCMV), pSMAD6, or pSMURF1. Luciferase activity was assessed 24 hr after transfection. Results are presented as mean ± SEM of the relative activity (*Firefly*/*Renilla* ratio). Statistical analysis was performed with one-way ANOVA. **P < 0.05*, ***P < 0.001*. (**J**) Total cell lysates were analyzed for endogenous MyD88 by immunoblotting using an anti-MyD88 antibody. Expression of the β-actin protein was used as a loading control. All data are representative of at least three independent experiments.

Supplementary figure 2



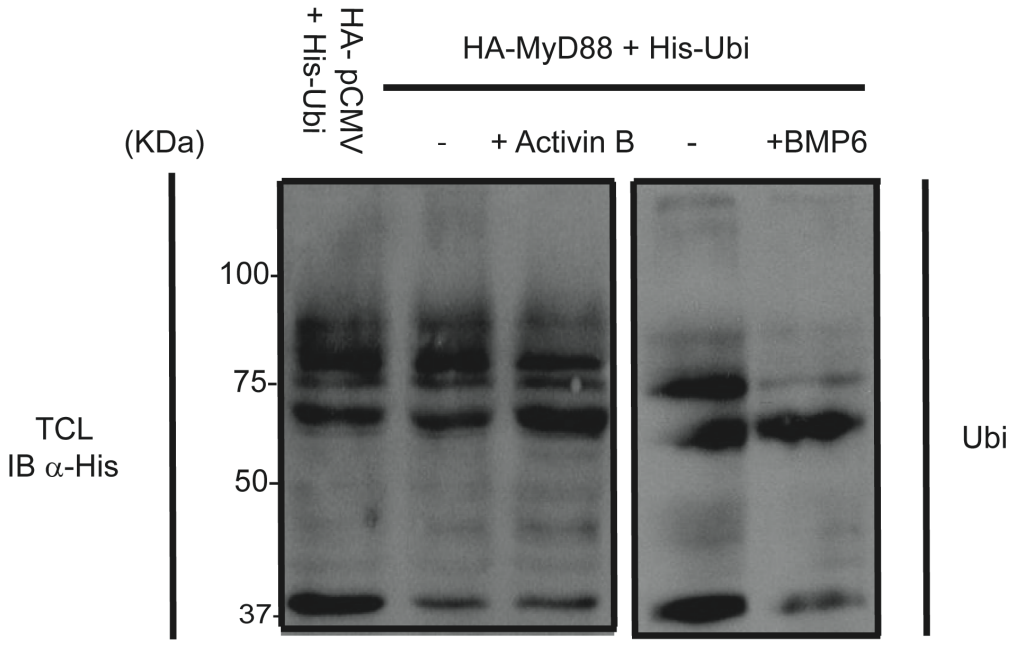
Supplementary figure 2. **MyD88 expression influences SMAD4 levels in Huh7 hepatoma cells.** (**A**) Huh7 cells were transiently transfected with HA-tagged MyD88 plasmid (pMyD88) for 6, 9 and 24 hr or were transfected with the empty plasmid pCMV as negative control. Total cell lysates were analyzed by western blotting for endogenous SMAD4 expression. Expression of the β-actin protein was used as a loading control. (**B**) Densitometric quantification of SMAD4 levels in western blots from three independent experiments. Results are presented as mean ± SEM. *$P < 0.05$, and n.s. = not significant compared with empty plasmid (pCMV). (**C**) Huh7 cells were transiently co-transfected with HAMP-Luc along with phRL-TK (*Renilla* Luciferase) as an internal control, and pMyD88 plasmids. Luciferase activity was assessed after 4, 12 and 24 hr after transfection. Results are presented as mean ± SEM of the relative activity (*Firefly*/*Renilla* ratio). *$P < 0.05$ and ***$P < 0.0001$ compared with empty plasmid (pCMV). The results are representative of at least three independent experiments. Statistical analysis was performed with one-way ANOVA. (**D**) Huh7 cell lines were stably co-transfected with HAMPMetluc2 along with pCMV or pMyD88. Cells were grown under selection using G418 (200 μ g/ml), which was added in the culture medium 24 hours post-transfection. Luciferase activity was measured at 24 hr, 48 hr, 2 weeks, 3 weeks and 4 weeks after transfection.
Results are presented as mean ± SEM of the relative activity (*Firefly*/*Renilla* ratio). *$P < 0.05$ and ***$P < 0.0001$ compared with empty plasmid (pCMV). Statistical analysis was performed with two-way ANOVA.

Supplementary figure 3



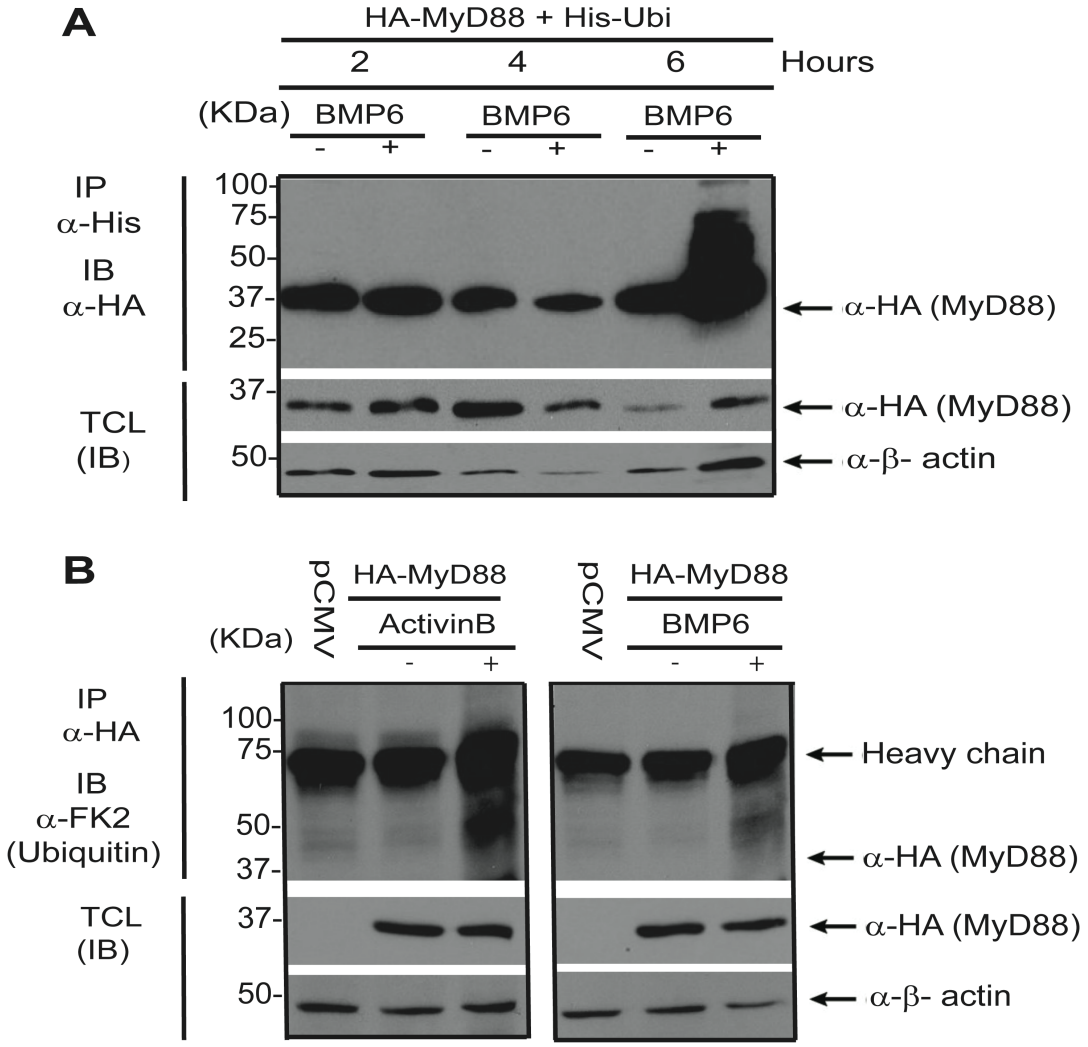
Supplementary figure 3. (**A**) Huh7 cells were transiently transfected with an empty vector (pCMV) or HA-tagged MyD88 plasmid (pMyD88) or with pMyD88 or treated with BMP6 (BMP6) as a positive control. Total cell lysates were analyzed by western blotting for endogenous phosphorylated SMAD5 (pSMAD5) and total SMAD1. (**B**) Densitometric quantification of pSMAD5 levels in western blots from three independent experiments. Statistical analysis was performed with one-way ANOVA. Results are presented as mean ± SEM. ****P < 0.0001* and n.s. = not significant compared with empty plasmid (pCMV).

Supplementary figure 4



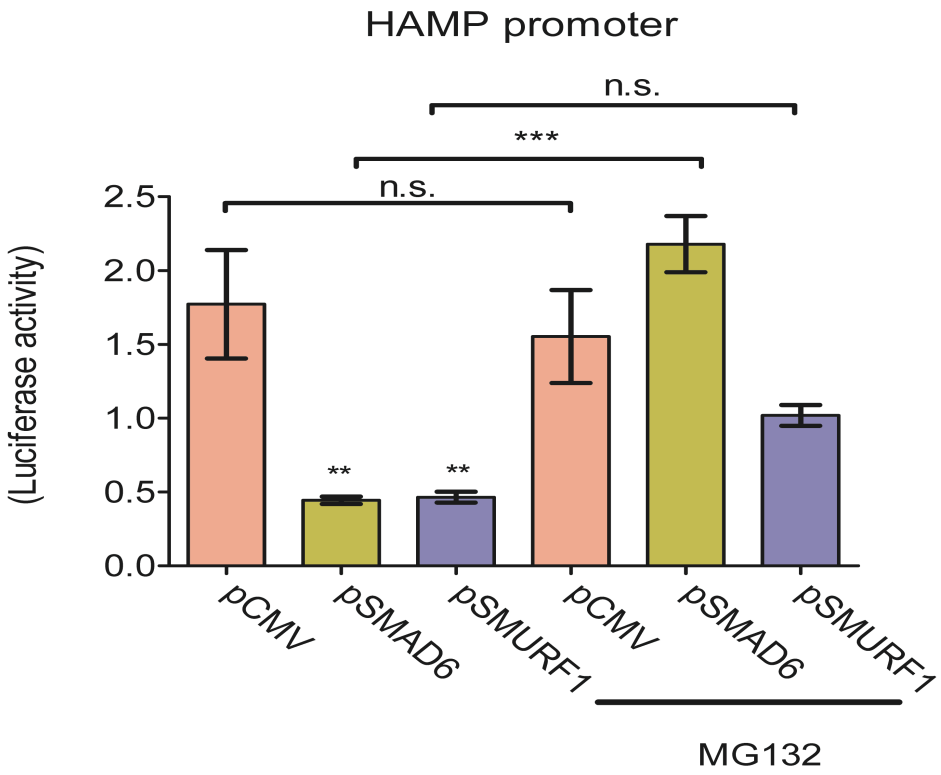
Supplementary figure 4: Huh7 cells were co-transfected with HA-tagged MyD88 and His-tagged Ubiquitin (His-Ubi), and treated with BMP6 or Activin B. Total cell lysates (TCL) were immunoblotted with anti-His (Ubi) antibody as a control for Figure 4A.

Supplementary figure 5



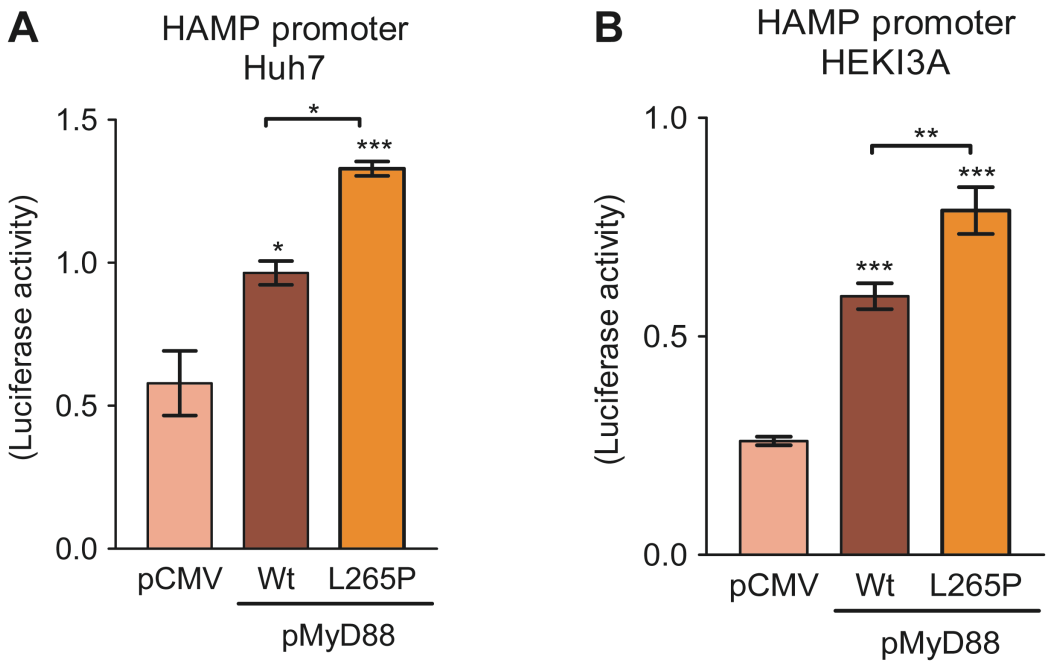
Supplementary figure 5. (A) Huh7 cells were co-transfected with HA-tagged MyD88 and His-tagged Ubiquitin (His-Ubi) for 24 hr prior to BMP6 treatment (25 ng/ml) for 2, 4 and 6 hours. HA-MyD88 ubiquitination was examined by immunoprecipitation (IP) using anti-His antibody, followed by immunoblotting (IB) with anti-HA antibody. Total cell lysates (TCL) before IP were immunoblotted with anti-HA and anti-β-actin antibodies. **(B)** Huh 7 cells were transfected with the empty plasmid pCMV or HA-tagged MyD88 for 24 hr and treated with Activin B or BMP6 for 6 hr. Endogenous ubiquitination was examined by immunoprecipitation (IP) using anti-HA followed by IB with anti-FK2 (ubiquitin).

Supplementary figure 6.



Supplementary figure 6. Huh7 cells were co-transfected with HAMP-luc and phRL-TK (Renilla Luciferase) along with pCMV, pSMAD6 or pSMURF1. After 24 hr, cells were treated with MG132 (10 mM) for 4 hours. Luciferase activity was assessed and results are presented as mean ± SEM of the relative activity (*Firefly*/*Renilla* ratio). Statistical analysis was performed with one-way ANOVA. Results are presented as mean ± SEM. ***P* < *0.001*, ****P* < *0.0001* and n.s. = not significant compared with empty plasmid (pCMV).

Supplementary figure 7



Supplementary figure 7. (**A**) Huh7 and (**B**) HEKI3A cells were co-transfected with HAMP-Luc and control (pCMV) or MyD88 plasmids (pMyD88): wild-type (Wt) or mutated MyD88 (L265P). Luciferase activity was assessed 24 hr after transfection. Results are presented as mean ± SEM. *$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$.

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V. CHAPTER 5

Curcumin induces mild anemia in a DSS-induced colitis mouse model maintained on an iron-sufficient diet

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Running Title: Effects of curcumin in DSS-induced colitis mouse model (Submitted)

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Abstract

Anemia is frequently encountered in patients with inflammatory bowel disease (IBD), decreasing the quality of life and significantly worsening the prognosis of the disease. The pathogenesis of anemia in IBD is multifactorial and results mainly from intestinal blood loss in inflamed mucosa and impaired dietary iron absorption. Multiple studies have proposed the use of the polyphenolic compound curcumin to counteract IBD pathogenesis since it has significant preventive and therapeutic properties as an anti-inflammatory agent and very low toxicity, even at high dosages. However, curcumin has been shown to possess properties consistent with those of an iron-chelator, such as the ability to modulate proteins of iron metabolism and decrease spleen and liver iron content. Thus, this property may further contribute to the development and severity of anemia of inflammation and iron deficiency in IBD. Herein, we evaluate the effects of curcumin on systemic iron balance in the dextran sodium sulfate (DSS) model of colitis in C57Bl/6 and BALB/c mouse strains that were fed an iron-sufficient diet. In these conditions, curcumin supplementation caused mild anemia, lowered iron stores, worsened colitis and significantly decreased overall survival, independent of the mouse strain. These findings suggest that curcumin usage as an anti-inflammatory supplement should be accompanied by monitoring of erythroid parameters to avoid exacerbation of iron deficiency anemia in IBD.

Keywords: Curcumin, iron, colitis, inflammatory bowel disease (IBD), MyD88

Core tip

Our study shows that curcumin supplementation of an iron-sufficient diet causes mild anemia, depletes iron stores, worsens colitis and decreases survival, independent of the mouse strain. These findings demonstrate that curcumin may have a potential adverse activity in chronic inflammatory diseases, such as IBD, since the iron chelating properties of curcumin may play a role in iron deficiency and the severity of colitis.

Introduction

For patients with inflammatory bowel disease (IBD), anemia is one of the major causes of hospitalization (27, 32) and has a debilitating effect on the quality of life (QoL) (47, 54), increasing disease morbidity and tightly associating the disease with mortality (12). IBD is an inflammatory disease consisting of a group of gastrointestinal tract disorders, namely ulcerative colitis and Crohn's disease, which are characterized by blood loss from the intestinal mucosa and reduced iron absorption. Up to two-thirds of patients with IBD develop anemia, with the most common types being iron-deficiency anemia and anemia of chronic disease, which often overlap (27, 40, 46).

IBD pathophysiology includes the activation of inflammatory cytokines such as TNF-α (34, 42). Therefore, many studies on IBD therapies have focused on anti-inflammatory treatments or natural compounds such as curcumin that have anti-inflammatory properties to mitigate the disease (5, 13).

Curcumin, the yellow pigment obtained from the rhizome of *Curcuma longa* (turmeric), is commonly used as a spice and food-coloring agent (14). Curcumin features complex and multifactorial mechanisms of action that have demonstrated a variety of therapeutic properties, including those described as anti-oxidant, anti-infection, anti-tumor, and anti-inflammatory (49). Moreover, curcumin use has no major side effects and has low toxicity at high dosages (8-12 g/day) (8). Anti-cancer activities of curcumin are mediated by a variety of biological pathways in mutagenesis, oncogene expression, cell cycle regulation, apoptosis, tumorigenesis, and metastasis. Additionally, the effects of curcumin as an anti-

inflammatory agent have been previously associated with the regulation of different inflammatory cytokines (extracellular matrix metalloproteinase inducer, EMMPRIN; matrix metalloproteinase-9, MMP-9; IL-1β; and mitogen-activated protein kinase, MAPK) (6, 39, 51). Recently, Kong *et al.* demonstrated curcumin's mechanism of action in macrophages during the inflammasome response (26). Furthermore, different studies have highlighted curcumin's properties to inhibit the toll-like receptor 4/myeloid differential factor 88 (TLR4/MyD88) pathway via the repression of TLR4 homodimerization and the subsequent decrease of MyD88 expression (58, 59). TLR4 has a critical role in the inflammatory response inducing nuclear factor-kappa B (NF-κB) expression via protein adaptor MyD88 stimulation.

Curcumin has also been shown to have anticoagulant and antiplatelet activities (24), which may sustain or prolong active bleeding (25) and has been proposed to have the properties of an iron chelator (4, 22, 23). Consistent with its iron chelating properties, curcumin has been reported to reduce spleen and liver iron stores in mice (9, 23). Moreover, curcumin has been also shown to affect hepcidin expression (23), the main regulator of iron homeostasis (18). Hepcidin, encoded by the *HAMP* gene, controls the levels of intestinal iron absorption and plays a major role in regulating iron release from macrophages. These cells are responsible for iron recycling and, in inflammatory settings, will accumulate iron at high hepcidin levels (53).

The potentially detrimental effects of curcumin on iron homeostasis in the inflammatory context could exacerbate anemia and iron deficiency; however, this aspect has been often ignored in studies of gastrointestinal disorders and IBD mouse models, which exhibit marginal or depleted iron stores. Herein, we investigated the effects of curcumin on the dextran sodium sulfate (DSS)-induced colitis mouse model in the context of an iron-sufficient diet.

Materials and Methods

Animals

All procedures were performed in accordance with the Canadian Council of Animal Care guidelines after approval by the Institutional Animal Care Committee of the Centre de recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM). Female C57Bl/6 and BALB/c mice aged 8 weeks old were purchased from Charles River Laboratories (Wilmington, CA, USA). Animals were maintained under standard 12:12 light/dark conditions at the CRCHUM.

Animal treatments, diet, and induction of colitis

Two weeks before DSS treatment, 8 weeks old female C57BL/6 or BALB/c mice received *ad libitum* a control diet containing 50 mg/kg of iron in the form of iron sulfate (Teklad TD.120515; Envigo, IN, United States) with or without 2% (wt/wt) curcumin supplementation (Teklad TD.140182). Colitis was induced by the administration of DSS (molecular weight 40000; TdB Consultancy, Uppsala, Sweden) at 1% w/v for C57BL/6 and 2% w/v for BALB/c, in drinking water for 5 days, followed by 7 days of rest for 3-4 cycles of DSS (10).

Erythroid parameters and serum iron

Red blood cell, hemoglobin, hematocrit, and mean corpuscular volume levels were measured with an automated cell counter calibrated for murine samples (ABC vet counter; ABX Hématologie, Montpellier, France). Serum iron was measured by a colorimetric assay

with the Kodak Ektachem DT60 system (Johnson & Johnson, Ortho-Clinical Diagnostics, Mississauga, ON, Canada).

Histology

Histological scoring was assessed on colon samples from each mouse. The samples were fixed in 10% neutral buffered formalin (Chapter Chemicals, Montreal, QC, Canada), cut, fixed and stained with hematoxylin and eosin. All histological evaluations were assessed in a blinded fashion. Histological scoring was calculated as follows: presence of occasional inflammatory cells in the lamina propria (assigned a value of 0); increased numbers of inflammatory cells in the lamina propria (value of 1); confluence of inflammatory cells, extending into the submucosa (value of 2); and transmural extension of the infiltrate (value of 3). For *tissue damage score:* no mucosal damage (value of 0); lymphoepithelial lesions (value of 1); surface mucosal erosion or focal ulceration (value of 2); and extensive mucosal damage and extension into a deeper structure (value of 3) (21).

Disease activity index

The disease activity index was scored accordingly to previous studies (11, 15). The index consists of the sum of all scores attributed to weight loss (0, none; 1, 1%–5%; 2, 5%–10%; 3, 10%–20%; 4, >20%), stool consistency (0, normal; 2, soft; 4, diarrhea) and fecal blood (0, negative; 2, blood in the stool; 4, gross bleeding), divided by 3.

SDS-PAGE and western blot analysis

Protein concentrations were measured using a Pierce BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Same concentrations of protein from colon or liver extracts were loaded to perform electrophoresis. More precisely, equivalent amounts of proteins were boiled in loading buffer containing 4% SDS, 20% glycerol, and bromophenol blue for 5 minutes. Proteins were resolved on 10% SDS-PAGE gels and then were transferred onto nitrocellulose membranes (GE Healthcare; Amersham Biosciences, Baie d'Urfé, QC, Canada). The membranes were blocked with 5% non-fat dry milk solution and incubated with antibodies against lipocalin 2 (R&D Systems, Minneapolis, MN, USA), MyD88 (Cell Signaling, Danvers, MA, USA) and β-actin (Abcam, Cambridge, MA, USA). To detect the formation of immunocomplexes, peroxidase-conjugated anti-goat IgG (Santa Cruz, Dallas, TX, USA) and anti-mouse IgG (R&D Systems, Minneapolis, MN, USA) were used as secondary antibodies. Staining intensity was developed with an Amersham enhanced chemiluminescence system (GE Healthcare, Amersham Biosciences, Baie d'Urfé, QC, Canada).

Quantitative RT-PCR

Total RNA from tissue samples was isolated by phenol chloroform using the TRIzol reagent (Invitrogen, Burlington, ON, Canada) as recommended by the manufacturer, and reverse transcription was performed with an Omniscript RT-PCR system (Qiagen, Mississauga, ON, Canada). The mRNA levels of selected genes were measured by real-time PCR with a Rotor-Gene 3000 real-time DNA detection system (Montreal Biotech,

Kirkland, QC, Canada) and QuantiTect SYBR Green I PCR kits (Qiagen, Mississauga, ON, Canada) as previously described (35). Expression levels were normalized to the housekeeping gene β-actin. The following primers were used: *Hamp* (F) CCTATCTCCATCAACAGATG; *Hamp* (R) AACAGATACCACACTGGGAA; β-Actin (F) TGTTACCAACTGGGACGACA; β-Actin (R) GGTGTTGAAGGTCTCAAA.

Measurement of iron in the liver and the spleen

Non-heme iron concentrations were assessed by acid digestion of liver and spleen tissue samples (56), followed by measurement by colorimetry using the ferrozin reagent and measuring absorption at 560 nm (17).

Statistical analysis

All statistics were calculated using Prism GraphPad (GraphPad, San Diego, CA) with a pre-specified significant *P*-value of 0.05. Student's *t*-test (two-tailed) was used for comparisons between two groups, and multiple comparisons were evaluated by one-way analysis of variance (ANOVA), followed by the Bonferroni multiple comparison test.

Results

Curcumin supplementation of an iron-sufficient diet causes mild anemia in a DSS-induced colitis mouse model

We investigated the potential contribution of curcumin in the systemic iron balance in an IBD setting using the DSS-induced colitis mouse model. To evaluate such effects, colitis was induced by administration of DSS in C57Bl/6 mice with or without 2% curcumin supplementation of an iron-sufficient diet (50 mg/kg chow). Repeated cycles of DSS administration in the drinking water of mice were intercalated with resting periods of 7 days with untreated water to model the chronic pattern of IBD (45, 55).

We found that erythroid parameters including red blood cells, hemoglobin, mean corpuscular volume and hematocrit values were significantly lower in DSS-treated mice on the curcumin-supplemented diet (Curcumin-DSS), compared with mice treated with DSS without curcumin supplementation (Ctrl-DSS) **(figure 1A-D)**. In control conditions with non-colitic mice (Ctrl and Curcumin, without DSS), only the mean corpuscular volume was lowered by curcumin supplementation, with all other erythroid parameters remaining similar in both groups. The lowest values for serum iron levels were found in the Curcumin-DSS group, with levels significantly lower compared to non-colitic Ctrl mice **(figure 1E)**.

Next, we measured liver hepcidin mRNA expression *(Hamp*), the major regulator of iron homeostasis (18). As shown in **figure 1F,** Curcumin-DSS mice had the lowest hepcidin

expression of all groups, and the values were also significantly lower when compared to Ctrl-DSS mice that did not received curcumin supplementation.

We then analyzed the iron concentration in the spleen and liver and found that splenic iron concentrations in mice fed with 2% curcumin with or without DSS treatment were significantly lower compared with their respective controls (Ctrl and Ctrl-DSS; **figure 1G**). In regards to liver concentration, both groups treated with DSS had lower liver iron content compared to non-DSS mice, indicating that DSS treatment lowers liver iron concentrations **(figure 1H)**. The lowest liver iron content was found in the Curcumin-DSS group when compared to all other treatment groups.

Taken together, these findings indicate that mice fed an iron-sufficient diet supplemented with curcumin develop mild anemia accompanied by marked lower iron levels in the spleen and liver in the DSS-mouse model. In contrast, mice treated with DSS alone do not develop anemia despite a modest lowering of serum and liver iron concentrations.

Curcumin supplementation aggravates colitis in the DSS-mouse model

We evaluated the effect of curcumin supplementation on colitis severity. Curcumin supplementation in DSS-treated mice contributed to a greater weight loss compared to Ctrl-DSS mice **(figure 2A)**. This result was in line with survival **(figure 2B)**, where both Ctrl-DSS and Curcumin-DSS groups demonstrated mortality. However, only Curcumin-DSS

mice showed a steady decrease in survival reaching 60% compared to 90% in the Ctrl-DSS group at the end of the experiment.

The disease activity index, consisting of the sum of the scores attributed to body weight loss, stool consistency and observance of blood in feces divided by three, was measured according to previous studies (11, 15). We found that Curcumin-DSS mice had an increased disease activity index score compared to Ctrl-DSS mice **(figure 2C)** after 30 days of treatment. The stool consistency score, highest for diarrhea, reflected the same trend and showed a significant increase in Curcumin-DSS mice compared to Ctrl-DSS **(figure 2D)**.

Spleen weight was significantly higher in both groups of mice treated with DSS (Ctrl-DSS and Curcumin-DSS) than non-colitic mice **(figure 2E)**, with no significant differences found between Ctrl-DSS and Curcumin-DSS. We additionally analyzed the colon length as a marker of disease severity and found that it was significantly shorter in DSS-treated mice (Ctrl-DSS and Curcumin-DSS) than in non-colitic mice, but curcumin treatment did not did not appear to enhance the shortening of the colon **(figure 2F)**.

Overall, these results show that, in our experimental setting, curcumin supplementation decreases survival and is associated with higher scores of disease activity in DSS-induced colitis.

Curcumin supplementation in DSS-treated mice enhances inflammation in the colon

We assessed protein levels of lipocalin 2 in the colon as it is highly expressed in response to injury and inflammation and is used as a biomarker of intestinal inflammation (7). Our results showed that curcumin supplementation of DSS-treated mice was associated with enhanced lipocalin 2 expression **(figure 3A-B)** compared to Ctrl-DSS mice, indicating heightened inflammation.

We then assessed the severity of DSS-induced colitis using histology scoring (see materials and methods). We found that cellular infiltration and tissue damage followed by epithelial destruction were more severe in mice supplemented with curcumin (Curcumin-DSS) compared to mice treated with DSS without curcumin supplementation (Ctrl-DSS; **figure 3C-D)**.

We also analyzed curcumin modulation of MyD88 protein expression. MyD88 is the major adaptor protein essential for the inflammatory cytokine activation upon stimulation of almost all the TLRs except for TLR3 (28). As shown in **figure 4A-B**, DSS treatment resulted in MyD88 activation (Ctrl vs. Ctrl-DSS groups). Curcumin supplementation significantly decreased MyD88 expression in colonic tissues in both Curcumin and Curcumin-DSS groups compared to Ctrl and Ctrl-DSS mice. In contrast to the colon, no significant modulation of MyD88 was observed in the liver between the four mouse groups **(figure 4C-D)**.

These findings show that when mice are fed an iron-sufficient diet, curcumin enhances inflammation and aggravates colitis induced by DSS. In addition, curcumin treatment suppresses MyD88 protein expression in the colon but not in the liver and is independent of DSS induction of colitis.

Curcumin supplementation of an iron-sufficient diet induces mild anemia independent of mouse strain

To investigate whether the aggravation of colitis in mice by curcumin is dependent on the mouse strain, we assessed the DSS-induced colitis mouse model on BALB/c mice using the same iron-sufficient diets with or without curcumin supplementation at 2% (w/w).

We found that BALB/c mice showed, as previously reported (37), higher resistance to the DSS treatment, recovering more readily from the successive DSS-treatment cycles. However, around day 35, body weight loss and survival decreased in Curcumin-DSS mice compared to Ctrl-DSS mice **(figure 5A-B)**. Disease activity index was higher in Curcumin-DSS mice compared to Ctrl-DSS **(figure 5C)**, whereas no significant differences were found in the colon length and spleen weight **(figure 5D-E)**. Lipocalin 2 expression and histology scoring in the distal colon were higher in mice supplemented with curcumin (Curcumin-DSS) than in Ctrl-DSS mice **(figure 5F-I)**, indicating more severe inflammatory cell infiltration and tissue damage in Curcumin-DSS mice.

Regarding erythroid parameters, we found that Curcumin-DSS mice had significantly lower red blood cells numbers as well as lower hemoglobin and hematocrit values, while mean corpuscular volume remained unaffected when compared to Ctrl-DSS mice **(figure 6A-D)**. Significant differences were also found in serum iron levels and liver hepcidin expression, with lower values found in Curcumin-DSS than in Ctrl-DSS mice **(figure 6E-F)**. Finally, spleen and liver iron concentrations were significantly lower in Curcumin-DSS mice compared to Ctrl-DSS mice **(figure 6G-H)**.

These results show that curcumin in an iron-sufficient diet causes mild anemia in a mouse model of IBD, reduces iron stores in the spleen, worsens colitis and decreases survival even in a DSS-resistant mouse strain.

Discussion

In this study, we aimed to investigate the potential of curcumin's chelating activity to affect body iron stores and anemia development in a murine model of IBD. We found that curcumin supplementation in DSS-treated mice led to a decrease of several erythroid parameters, including the number of red blood cells, hemoglobin, mean corpuscular volume, and hematocrit, indicating the development of mild anemia. We also showed that Curcumin-DSS mice developed splenomegaly, which is indicative of extramedullary erythropoiesis in mice responding to iron-deficiency anemia (3). These changes were accompanied by a reduction in liver hepcidin mRNA levels, further indicating that mice become anemic since hepcidin levels are inhibited by anemia (44). Hepcidin levels decreased despite the presence of inflammation, which has the opposite effect of anemia and activates hepcidin expression, confirming previous studies showing that erythropoietic drive can inhibit hepcidin activation through the inflammatory pathway (19). Our results are in agreement with previous work reporting that curcumin supplementation decreases hepcidin levels both in mice (9) and humans (29). This regulation has been associated with the inhibition of phosphorylation of the signal transducer and activator of transcription 3 (STAT3) (16, 29) and TNFα activation (48). MyD88 has also been implicated as an important factor for hepcidin regulation as MyD88-deficient mice are unable to appropriately upregulate hepcidin expression when iron-challenged and develop iron-loading in the liver (31). However, we show that curcumin does not affect MyD88 levels in the liver; hence, MyD88 would not be expected to further interfere with hepatic hepcidin expression in our experimental settings. The fact that MyD88 expression in the liver was not affected and contrasted with its downregulation in colonic tissue, indicates that

curcumin regulation of MyD88 expression could be more relevant locally, within the intestine.

Our finding that curcumin modulates iron status is in line with its iron chelating properties that have been demonstrated both *in vitro* (4) and *in vivo* (22, 23). In long-term experiments in mice, curcumin supplementation has been shown to significantly lower liver and spleen iron levels (9). Others have reported that the usage of a high dosage of curcumin in a low iron diet modulates erythroid and iron parameters, exacerbating iron deficiency symptoms (23). Our present work strengthens and adds to these previous studies by demonstrating that the effect of curcumin in iron metabolism is of importance in the context of chronic intestinal inflammation.

Under our experimental conditions, Curcumin-DSS mice exhibited more severe symptoms of intestinal inflammation. This contradicts previous reports on curcumin's anti-inflammatory effects in colitis, which had been linked to the attenuation of the TLR4/MyD88/NF-κB inflammatory pathway by inhibiting TLR4 homodimerization (58) and decreasing MyD88 expression (33). Although the reasons for these differences are not clear at present, it is worth mentioning that MyD88 deficiency in mice seems to increase susceptibility to DSS-induced colitis. Araki *et al.* reported that MyD88 is crucial in intestinal homeostasis by playing a protective role against the development of colitis since DSS induced a more severe colitis in *MyD88*$^{-/-}$ mice (1). In their experiments, they observed that mice lacking MyD88 in the colon had a subsequent higher intestinal

permeability, causing more severe infiltration of bacterial products from the lumen (1). Similarly, our data show that MyD88 downregulation by curcumin in DSS-treated mice resulted in an aggravation of the inflammatory responses in our experimental conditions. In fact, DSS-curcumin mice showed more severe colonic tissue damage compared to DSS-treated mice that were not supplemented with curcumin. While the precise mechanism of action remains to be investigated, the susceptibility of MyD88-deficient mice to colitis has been linked to gut microbiota composition, which is altered in IBD (36). Furthermore, *MyD88*$^{-/-}$ mice, in contrast with wild-type mice, are unable to respond to treatment with probiotic bacteria in the context of DSS-induced colitis, further highlighting the link between MyD88 and the gut microbiota (50). Overall, the implications of these studies are that bacterial components may play both detrimental (36) and protective roles, at least partially, in a MyD88-dependent manner (1, 50).

Regarding colitis severity, our study is in contrast with previous reports revealing a protective effect of curcumin in DSS-induced acute (13, 57) and chronic (20) colitis in mice. Such differences may be due to the amount of iron found in standard rodent chow, which tends to have excess iron ranging from 350 mg/kg up to 900 mg iron/kg diet in some related studies (20). This strikingly contrasts with our iron-sufficient diet that contained 50 mg/kg, which is more in accordance to mouse iron requirements (41). Excess dietary iron may compensate for the iron chelating effect of curcumin (2) and presumably avoids the development of iron deficiency and anemia in this model. In addition to iron, other components of the rodent diet, such as fermentable fibers, may have a role in altering the effects of curcumin in DSS-induced colitis. Indeed, recent studies have shown that the

presence of fermentable fibers in the diet can ameliorate low-grade inflammation while exacerbating disease severity in response to acute colitis (38).

Previous studies have reported that the efficacy of dietary curcumin in trinitrobenzene sulfonic acid (TNBS)-induced colitis, another rodent model for IBD, may vary depending on the mouse strain (5). Furthermore, mouse strain has also been shown to influence the severity of DSS-induced colitis (37). We tested BALB/c mice since they are known to be substantially more resistant to DSS acute colitis in comparison to C57BL/6 mice (37). We found that curcumin aggravated colitis and induced an iron-deficiency anemia phenotype in BALB/c mice as well, indicating that the detrimental effect of curcumin in the context of an iron-sufficient diet is independent of mouse strain.

In conclusion, we found that long-term curcumin administration in mice has potentially adverse effects in a DSS-induced model of ulcerative colitis, lowering iron stores and leading to the development of anemia. While beneficial effects of curcumin as an anti-inflammatory agent have been documented in animal models as well as in patients with mild to moderate ulcerative colitis (30, 52), the iron chelating properties of curcumin should be taken into account. This is particularly pertinent in situations of iron-deficiency, a condition that is found in up to 78% of Crohn's disease patients with active inflammation (43). Our study highlights the potential risks of curcumin, which is commonly taken as an over-the-counter supplement without monitoring erythroid and iron status parameters.

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Author roles

MSM and MC designed and performed experiments, analyzed and interpreted data, and contributed to the writing of the manuscript. GF performed experiments and analyzed data. AC provided technical support in some experiments. MMS designed experiments, interpreted data and contributed to the writing of the manuscript. All authors read and approved the final manuscript.

Figure Legends

Figure 1. Curcumin supplementation of an iron-sufficient diet causes mild anemia in a DSS-mouse model. C57BL/6 mice were fed an iron-sufficient diet (50 mg/kg chow; Ctrl) or an iron-sufficient diet supplemented with 2% curcumin (Curcumin). For dextran sodium sulfate (DSS) treatment, mice were fed an iron-sufficient diet with or without curcumin, starting 2 weeks before administration of 1% w/v DSS in drinking water. (A-D) Erythroid parameters: red blood cells, hemoglobin, mean corpuscular volume, and hematocrit. (E) Serum iron levels. (F) Liver hepcidin (*Hamp)* mRNA expression against housekeeping β-actin mRNA. (G-H) Iron content in spleen (G) and liver (H).

Results are representative of a minimum of three independent experiments; n = 9-10 mice per group. Statistical analysis was performed with one-way ANOVA. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and ns = not significant between Curcumin to Ctrl groups and when indicated between non-DSS and DSS groups.

Figure 2. **Curcumin supplementation of an iron-sufficient diet aggravates colitis in a DSS-mouse model**. C57BL/6 mice were fed an iron-sufficient diet (Ctrl) or iron-sufficient diet supplemented with curcumin (Curcumin). For dextran sodium sulfate (DSS) treatment, mice were fed an iron-sufficient diet with or without curcumin, starting 2 weeks before administration of of 1% w/v DSS in drinking water. (A) Body weight. (B) Survival. (C) Disease activity index (DAI). (D) Stool consistency. (E) Spleen weight. (F) Colon length.

Results are representative of a minimum of three independent experiments; n = 9-10 mice per group. Statistical analysis was performed with one-way ANOVA. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and ns = not significant between Curcumin to Ctrl groups and when indicated between non-DSS and DSS groups.

Figure 3. Curcumin supplementation in DSS-treated mice enhances inflammation in the colon. (A) Representative western blot of colon protein extracts probed with antibodies against lipocalin 2 and β-actin. (B) Graphic depicting densitometric quantification of western blots from three independent experiments. Data are presented as mean ± SEM. (C) Representative hematoxylin and eosin staining of mouse colon histological sections. (D) Graphic depicting quantification of colonic histology scores. Statistical analysis was performed by two-tailed Student's *t*-test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; ns = not significant; n = 8 mice per group.

Figure 4. Curcumin decreases MyD88 protein expression in the colon but not in the liver. (A-B) Colonic MyD88 protein expression in C57BL/6 mice treated with or without dextran sodium sulfate (DSS) and fed an iron-sufficient diet (Ctrl) or iron-sufficient diet supplemented with curcumin (Curcumin). (A) Representative western blot of colon protein extracts probed with antibodies against MyD88 and β-actin. (B) Graphic depicting densitometric quantification of western blots from three independent experiments. (C-D) Hepatic MyD88 protein expression. (C) Representative western blot of liver protein extracts probed with antibodies against MyD88 and β-actin. (D) Graphic depicting densitometric quantification of western blots from three independent experiments.

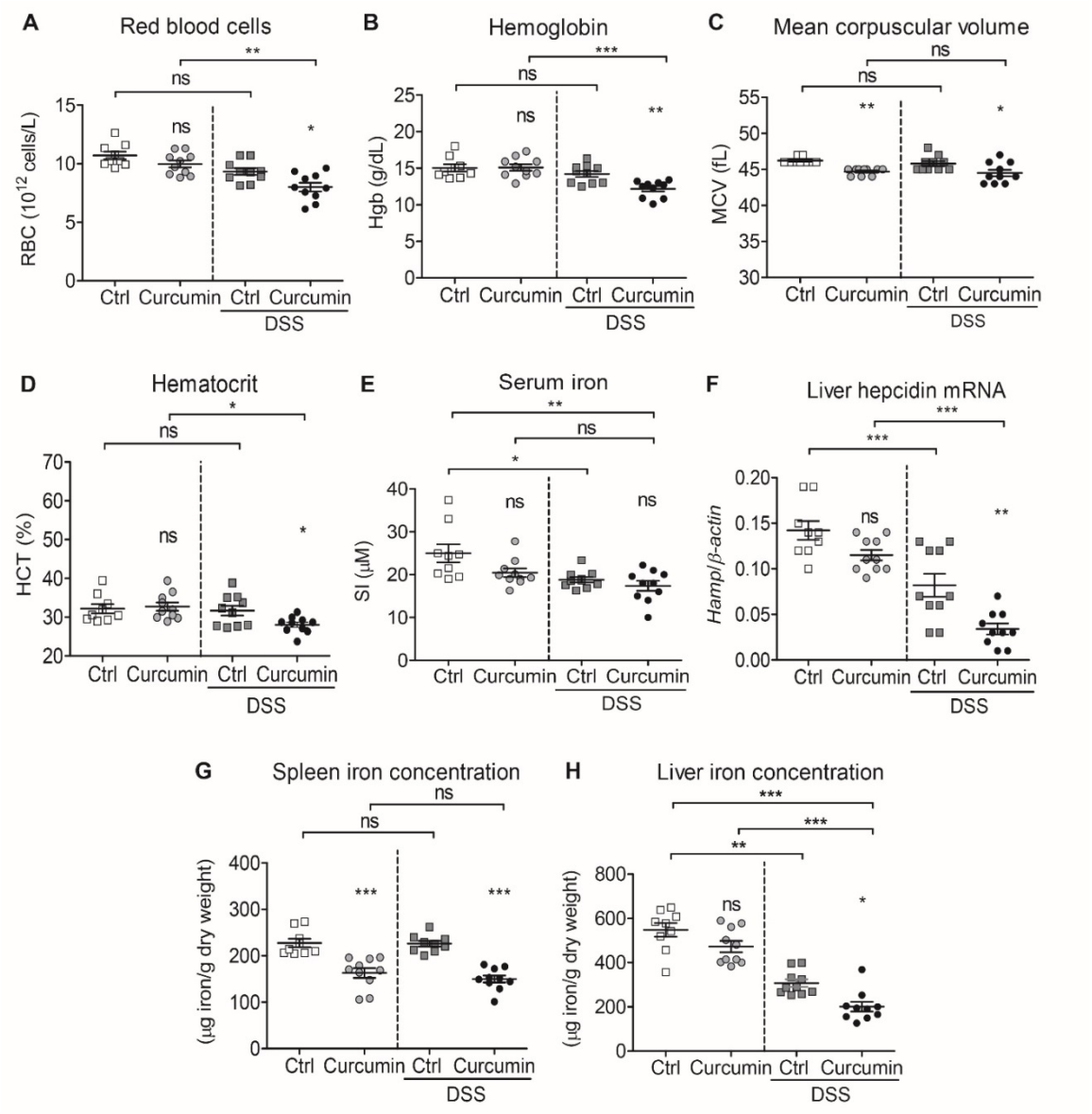
Statistical analysis was performed with one-way ANOVA: data in B and C are presented as mean ± SEM. Statistical analysis was performed by two-tailed Student's *t*-test. $*P < 0.05$, $**P < 0.01$; ns = not significant; n = 8 mice per group.

Figure 5. Curcumin supplementation of an iron-sufficient diet in BALB/C mice exacerbates DSS-induced colitis. BALB/c mice were fed an iron-sufficient diet (50 mg/kg

chow; Ctrl) or iron-sufficient diet supplemented with 2% curcumin (Curcumin) starting at 2 weeks before administration of 2% w/v DSS in drinking water. (A) Body weight. (B) Survival. (C) Disease activity index (DAI). (D) Colon length. (E) Spleen weight. (F-G) Colonic lipocalin 2 expression. (F) Representative western blot of colonic lipocalin 2 and β-actin expression. (G) Graphic depicting densitometric quantification of western blots from three independent experiments. Data are presented as mean ± SEM. (H) Representative hematoxylin and eosin staining of mouse colon histological section. (I) Graphic depicting quantification of colonic histology scores. Results are representative of a minimum of three independent experiments; n = 8 mice per group. Statistical analysis was performed by two-tailed Student's *t*-test. $*P < 0.05$; ns = not significant.

Figure 6. Curcumin supplementation of an iron-sufficient diet induces mild anemia independent of mouse strain. BALB/c mice were fed an iron-sufficient diet (50 mg/kg chow; Ctrl) or iron-sufficient diet supplemented with 2% curcumin (Curcumin) starting at 2 weeks before administration of 2% w/v DSS in drinking water. (A-D) Erythroid parameters: red blood cells, hemoglobin, mean corpuscular volume and hematocrit. (E) Serum iron levels. (F) Liver hepcidin (*Hamp)* mRNA expression against housekeeping β-actin mRNA. (G-H) Iron content of spleen or liver. Statistical analysis was performed by two-tailed Student's *t*-test. $*P < 0.05$, $**P < 0.01$; ns = not significant; n = 8 mice per group.

Figure 1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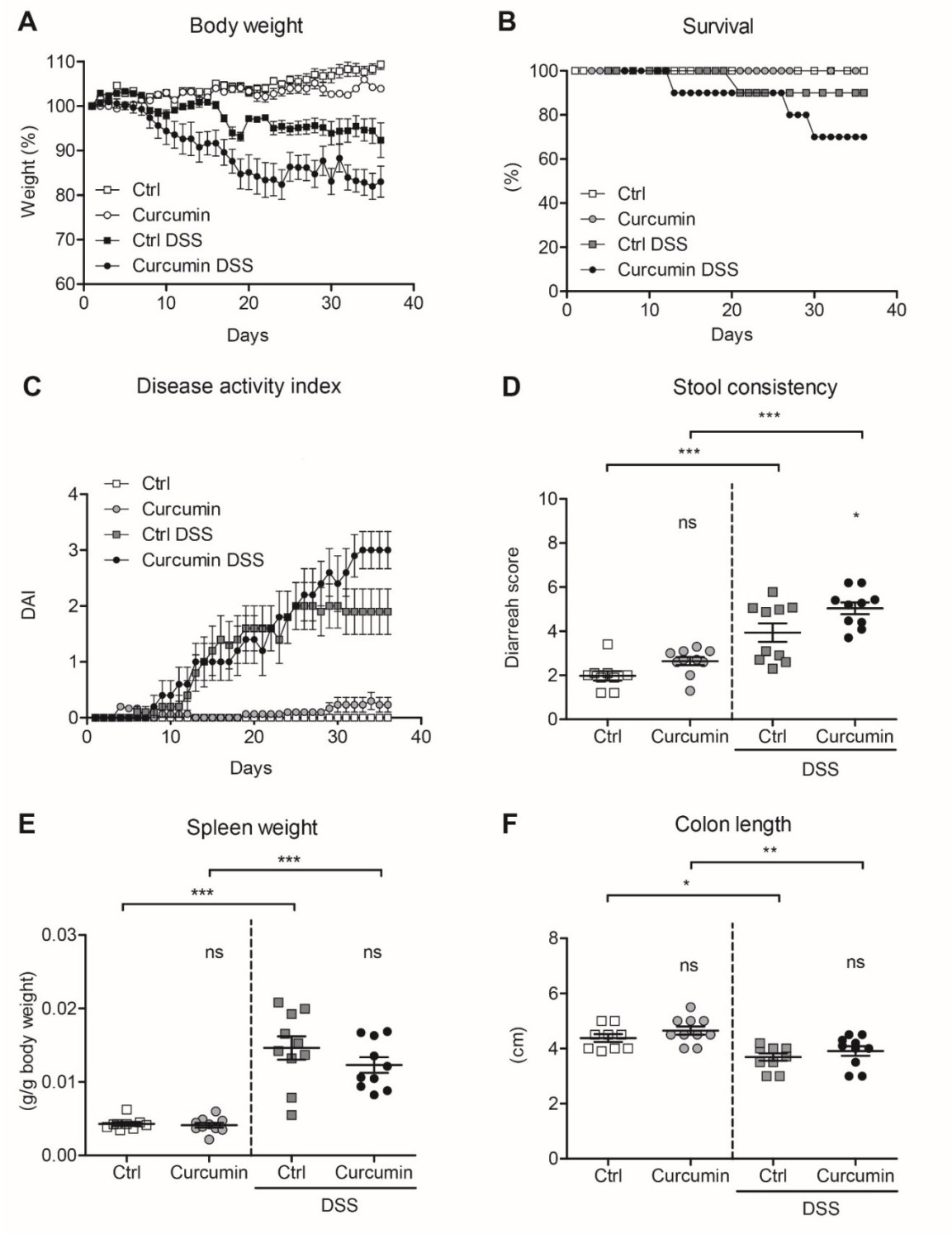
Red blood cells
RBC (10^12 cells/L)
B
Hemoglobin
Hgb (g/dL)
C
Mean corpuscular volume
MCV (fL)
D
Hematocrit
HCT (%)
E
Serum iron
SI (μM)
F
Liver hepcidin mRNA
Hamp/β-actin
G
Spleen iron concentration
(μg iron/g dry weight)
H
Liver iron concentration
(μg iron/g dry weight)
Ctrl
Curcumin
Ctrl
Curcumin
DSS
ns

Figure 2


A
Body weight
Weight (%)
Days
Ctrl
Curcumin
Ctrl DSS
Curcumin DSS
B
Survival
(%)
Days
C
Disease activity index
DAI
Days
D
Stool consistency
Diarreah score
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*
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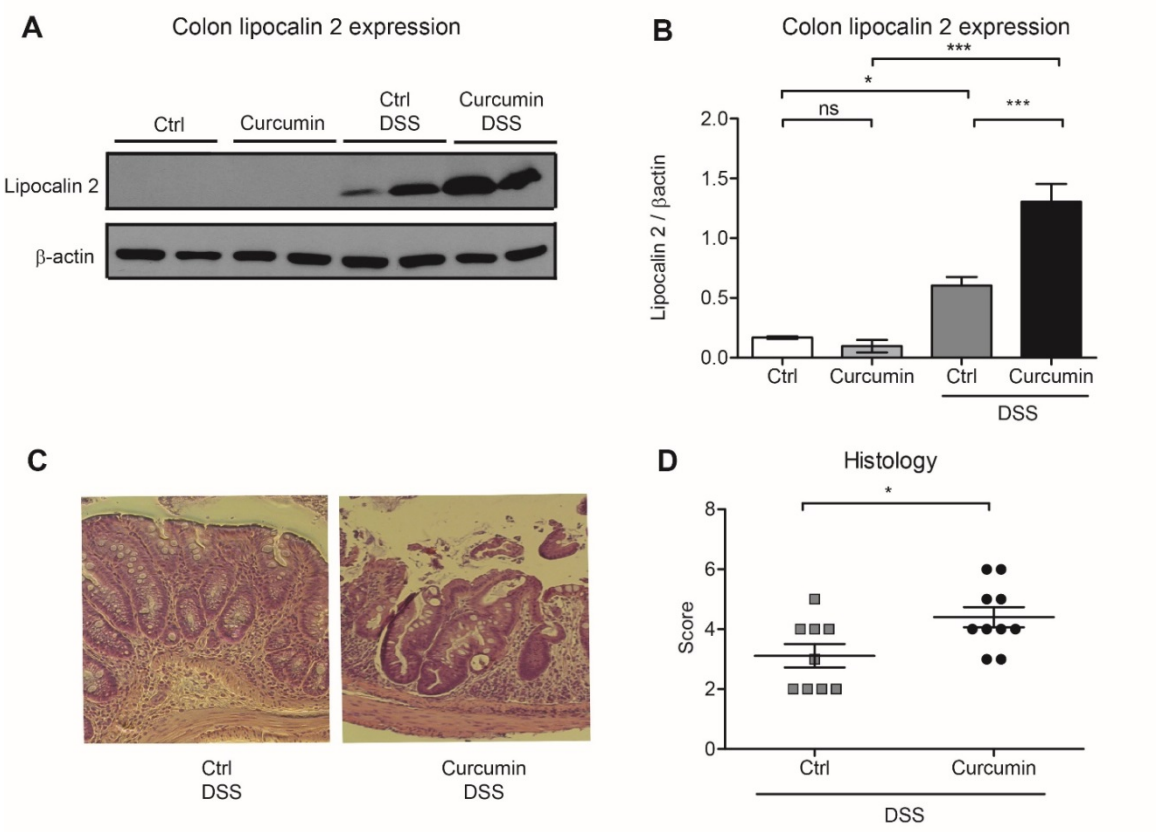
E
Spleen weight
(g/g body weight)
F
Colon length
(cm)
**
DSS

Figure 3


A
Colon lipocalin 2 expression
Ctrl
Curcumin
Ctrl DSS
Curcumin DSS
Lipocalin 2
β-actin
B
Colon lipocalin 2 expression
***
*
ns
***
Lipocalin 2 / βactin
2.0
1.5
1.0
0.5
0.0
Ctrl
Curcumin
Ctrl
Curcumin
DSS
C
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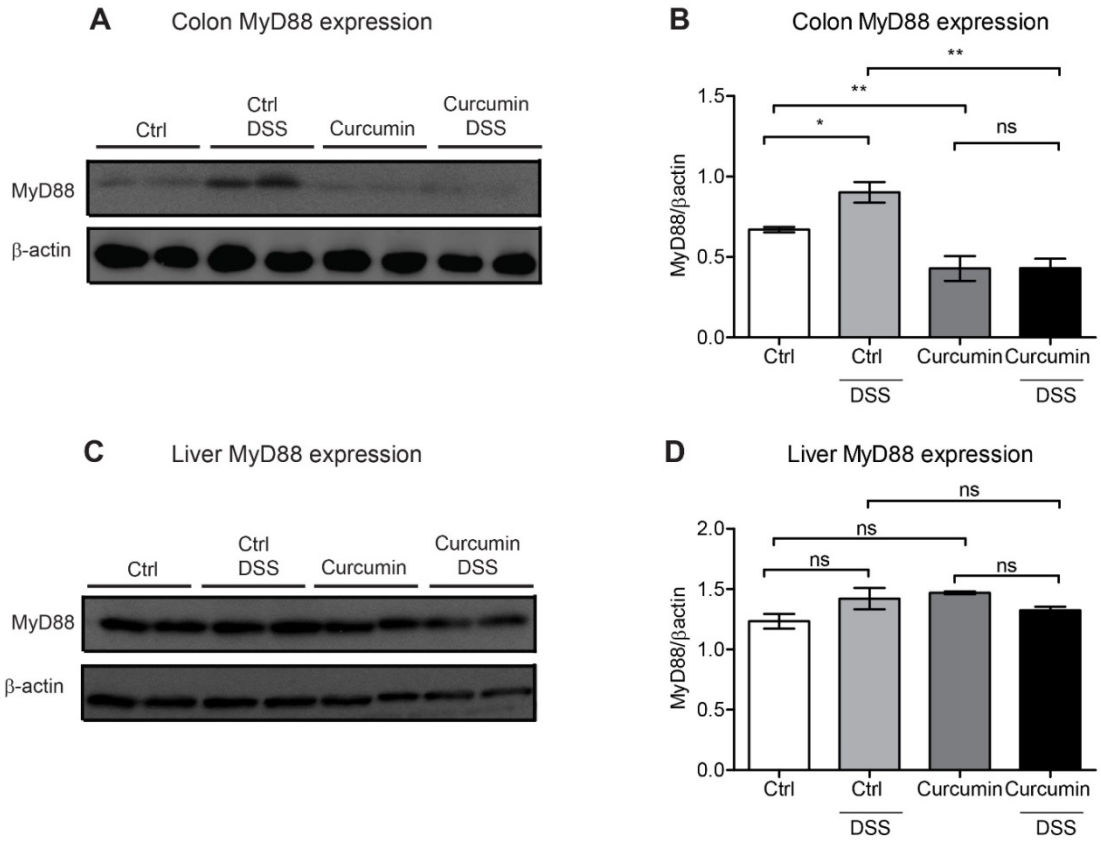
Curcumin DSS
D
Histology
*
Score
8
6
4
2
0
Ctrl
Curcumin
DSS

Figure 4


A
Colon MyD88 expression
Ctrl
Ctrl DSS
Curcumin
Curcumin DSS
MyD88
β-actin
B
Colon MyD88 expression
MyD88/βactin
1.5
1.0
0.5
0.0
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**
*
ns
Ctrl
Ctrl DSS
Curcumin
Curcumin DSS
C
Liver MyD88 expression
Ctrl
Ctrl DSS
Curcumin
Curcumin DSS
MyD88
β-actin
D
Liver MyD88 expression
MyD88/βactin
2.0
1.5
1.0
0.5
0.0
ns
ns
ns
ns
Ctrl
Ctrl DSS
Curcumin
Curcumin DSS

Figure 5

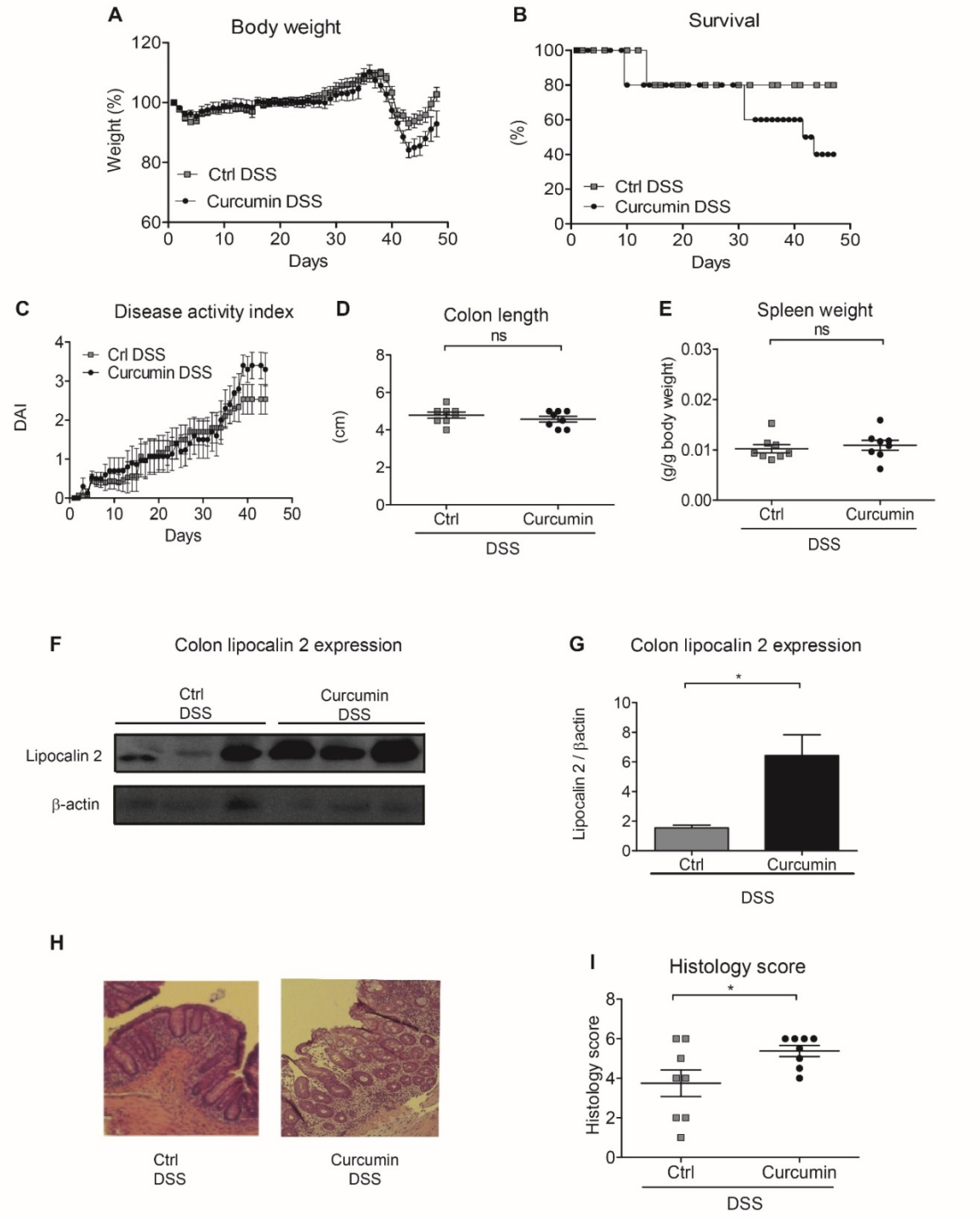
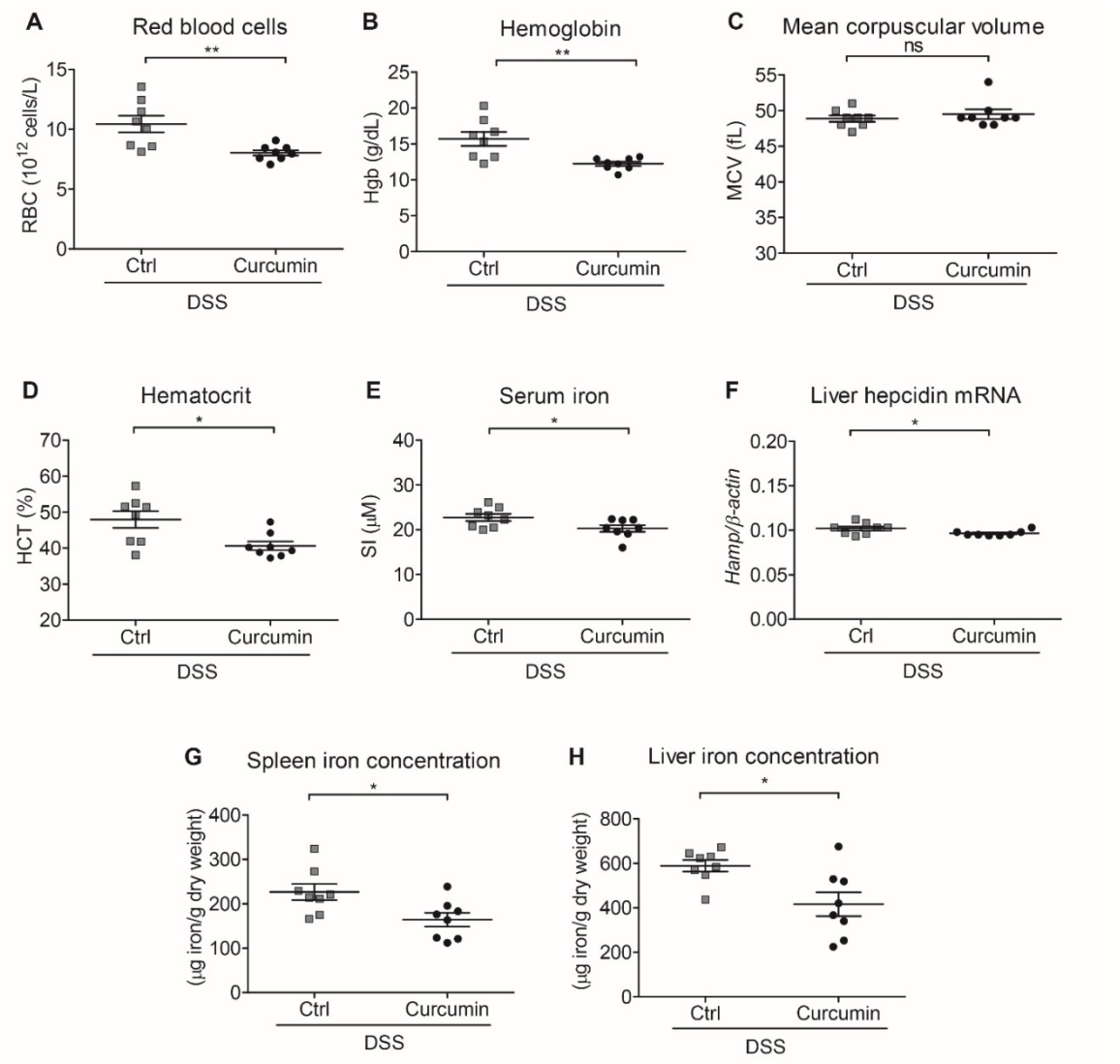

A
Body weight
Weight (%)
Ctrl DSS
Curcumin DSS
Days
B
Survival
(%)
Ctrl DSS
Curcumin DSS
Days
C
Disease activity index
DAI
Crl DSS
Curcumin DSS
Days
D
Colon length
ns
(cm)
Ctrl
Curcumin
DSS
E
Spleen weight
ns
(g/g body weight)
Ctrl
Curcumin
DSS
F
Colon lipocalin 2 expression
Ctrl
DSS
Curcumin
DSS
Lipocalin 2
β-actin
G
Colon lipocalin 2 expression
*
Lipocalin 2 / βactin
Ctrl
Curcumin
DSS
H
Ctrl
DSS
Curcumin
DSS
I
Histology score
*
Histology score
Ctrl
Curcumin
DSS

Figure 6


A
Red blood cells
**
RBC (10^12 cells/L)
15
10
5
0
Ctrl
Curcumin
DSS
B
Hemoglobin
**
Hgb (g/dL)
25
20
15
10
5
0
Ctrl
Curcumin
DSS
C
Mean corpuscular volume
ns
MCV (fL)
55
50
45
40
35
30
Ctrl
Curcumin
DSS
D
Hematocrit
*
HCT (%)
70
60
50
40
30
20
Ctrl
Curcumin
DSS
E
Serum iron
*
SI (μM)
40
30
20
10
0
Ctrl
Curcumin
DSS
F
Liver hepcidin mRNA
*
Hamp/β-actin
0.20
0.15
0.10
0.05
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Crl
Curcumin
DSS
G
Spleen iron concentration
*
(μg iron/g dry weight)
400
300
200
100
0
Ctrl
Curcumin
DSS
H
Liver iron concentration
*
(μg iron/g dry weight)
800
600
400
200
0
Ctrl
Curcumin
DSS

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CHAPTER 6

6. DISCUSSION AND PERSPECTIVES

6.1. General discussion

Since iron is an essential micronutrient present in the organism, iron overload and deficiency disorders are also involved in the incidence of various diseases, acting as the primary cause or secondary manifestation in many diseases. Iron disorders are a wide range group of inherited or acquired diseases all characterized by the deregulation of iron homoeostasis which leads to different phenotypes [7, 203]. Thus, the importance of studying the molecules involved in iron homoeostasis is highlighted by the fact that all these conditions have a large impact on the quality of life, associated with an increased incidence of morbidity of diseases which can have fatal outcomes in patients worldwide [8, 419, 551]

Seventeen years ago, the iron field experienced a major turning point due to the discovery of hepcidin, the key iron regulatory hormone [299-301, 309, 310]. The finding of this peptide unveiled the main mechanisms of systemic iron homoeostasis.

Hepcidin is the negative regulator of iron absorption, also important for iron recycling. Its chronic alterations result in systemic iron metabolism disorders and maldistribution of iron in the body; making it the key factor in the pathogenesis of a wide range of iron disorders (such as hereditary hemochromatosis, anemia of chronic diseases, and β-thalassaemia). It has been shown that dysregulation affecting hepcidin in humans or mice leads to severe iron overload or anemia [299, 303, 389, 551]. In this way, through its fundamental role in iron homoeostasis, hepcidin has been used as a marker associated with multiple iron-related disorders [552, 553].

Nevertheless, while intensive research in the iron field has attempted to explain the regulation of hepcidin, the molecular pathways implicated in the transcription of hepcidin in response to iron status or inflammation are not fully identified.

In this thesis, we focus our research on the iron-sensing pathway responsible for the induction of hepcidin.

6.1.1. Implication of Myeloid differentiation factor 88 (MyD88) in systemic iron homoeostasis

MyD88 is an essential adaptor protein, in innate and adaptive immunity, responsible for the cellular signal transduction pathways upon stimulation of almost all Toll-like receptors (TLRs), and IL1 receptor families [497, 502, 554, 555]. Among the TLRs, TLR4 plays an important role for bacterial host clearance through inflammatory cytokines induction. In previous years, the implication of TLR4/MyD88 pathway in iron homoeostasis has been shown through the regulation of hepcidin upon inflammatory stimuli. In this context, hepcidin expression in macrophages has been shown to be positively regulated by TLR4 but also by TLR2 receptors, *via* the MyD88-dependent signaling pathway, in response to inflammatory stimuli. It has been reported that MyD88 is implicated in the development of endotoxin-induced acute hypoferremic response in mice. Acute hypoferremia is associated with immune system activation and involves a marked reduction in circulating iron levels, coupled with iron sequestration within the macrophages [332, 556]. Macrophages, in iron homoeostasis, play an important role as host immune response factors to counteract bacterial proliferation in the host during infections through hepcidin activation. The TLR4/MyD88 dependent pathway is associated with the modulation of transcription factors, such as NF-κB or AP-1, in response to microbial pathogens or modified endogenous ligands, such as LPS, CD14 and myeloid differentiation factor 2 (MD-2) [497, 554, 555].

Interestingly, it has been also demonstrated that heme activates macrophages to induce the secretion of cytokines such as TNF-α, through the TLR4/MyD88-dependent signaling pathway. This mechanism could be involved in the inflammatory response observed in diseases with increased hemolysis or extensive cell damages, characterized with high amount of free heme [557].

In response to inflammatory stimuli, hepcidin promotes cellular iron sequestration to directly decrease microbial growth by limiting iron availability. Recently, Lee *et al.*

reported that LPS-induced hepcidin expression in hepatocytes is also regulated by TLR4, *via* the MyD88-dependent signaling pathway [525]. Finally, TLR4/MyD88 dependent signaling pathway has been similarly implicated in hepcidin induction in astrocytes of mice model of intracellular hemorrhage (ICH) [526].

HAMP hepcidin gene expression is mainly induced by two different stimuli: dietary iron and inflammation [370]. During inflammation, hepcidin expression is mainly induced by IL-6/STAT3 signaling pathway. However, it has been shown that conditional *Smad4*$^{-/-}$ mice, which develop iron overload in the liver and transcriptional activation of hepcidin expression in response to iron overload, have a strong decrease of IL-6 expression, upon LPS stimulation [345]. Moreover, BMP-RE1 (position -84/-79 bp of HAMP promoter) located immediately adjacent to the STAT3-binding site (position -72/-64 bp of the HAMP promoter), controls BMP/SMAD4-mediated hepcidin promoter activity and response to inflammation [372], since BMP-RE1 mutation leads to a blunted hepcidin expression in response to inflammatory stimuli [373]. BMP-responsive elements regulate hepcidin transcription through two 5'-GGCGCC-3' sequences located at -84/-79 bp and at -2,255/-2,250 position of HAMP promoter.

In the first manuscript, we investigated the possible implication of MyD88 expression in systemic iron homeostasis. By analyzing iron parameters on *MyD88*$^{-/-}$ mice, we found that *MyD88*$^{-/-}$ mice, in basal conditions, have slightly elevated liver iron levels and lower hepcidin induction when kept on a standard diet. Furthermore, we demonstrated that these mice, unlike *Trif* $^{-/-}$ mice, were unable to adequately enhance hepcidin expression after dietary or parenteral iron-loading and consequently accumulate significantly more iron in the liver than wild-type mice.

MyD88$^{-/-}$ mouse model was characterized by a milder version of iron overload compared to other mouse strains such as the *Hfe*$^{-/-}$ and *Hjv*$^{-/-}$ mice that demonstrate increases in both circulating and tissue iron levels. The differences between the phenotypes could be due to the fact that HFE and HJV are direct orchestrators of iron absorption localized on the cell surface of hepatocytes.

Among the most potent inductors of hepcidin through the iron-sensing pathway, there are the bone morphogenetic proteins (BMPs), BMP2, BMP4, BMP5, BMP6, BMP7 and BMP9 [343, 344, 350-352]. Among them, BMP6 acts as a major activator of BMP/SMAD4 signaling pathway, leading to hepcidin gene transcription. In fact, Bmp6 ablation in mice prevents appropriate hepcidin response to iron, resulting in hepcidin deficiency and hemochromatosis [343, 344]. Nevertheless, it has been reported that dietary iron loading in *Bmp6*$^{-/-}$ mice has the residual ability to activate Smad signaling pathway and hepcidin expression, through Bmp2 induction. Moreover, it has been reposted that *Bmp2*$^{-/-}$ display hepcidin deficiency, serum iron overload, and tissue iron loading in liver, pancreas and heart, with reduced spleen iron [350, 351, 558].These data demonstrated that BMP2, produced by liver sinusoidal endothelial cells, can induce hepcidin expression through a distinct pathway independently from BMP6 [351]. Therefore, we evaluated Bmp6 and Bmp2 levels in *MyD88*$^{-/-}$ mice and we found that they were appropriately elevated in response to high iron diet. However, we found that SMAD targets were not appropriately induced, suggesting a defect in downstream SMAD signaling. More precisely, we found that after feeding mice an iron supplemented diet phopho-SMAD1/5/8 levels did not change from baseline in the *MyD88*$^{-/-}$, and nuclear SMAD4 protein levels were reduced compared to wild-type mice.

In the second manuscript we use *in vitro* approaches to describe a direct interaction between MyD88 and SMAD4, *via* the MyD88 TIR domain, influencing SMAD4 expression. MyD88 is composed of three main domains: a death domain (DD) (54 to 109), intermediate domain (INT) (110 to 155), and Toll-interleukin-1 receptor domain (159 to 296). The N-terminal DD is responsible for MyD88 dimerization and interaction with IRAK1and IRAK2. The INT domain is involved in the interaction with IRAK4. Finally, the C-terminal MyD88 TIR domain has been implicated in the interaction with the other TIR adaptors or proteins. In fact, it has been shown that the MyD88 TIR domain can be associated with multiple components in a non-canonical pathway, namely not related to its primary role of cytosolic adaptor protein linked to TLRs stimulation, to in operating different cellular actions. Xie *et al.* demonstrated that due to MyD88 TIR domain conformational changes, MyD88 dissociated with TLR4, upon specific stimuli, to translocate into the nuclei bound to the protein phosphatase 2A Catalytic Subunit α (PP2Ac) to regulate cell survival [559].

Moreover, Lee *et al.* demonstrated that SMAD6 interaction with MyD88, through MyD88 C-terminal region, leads to MyD88 degradation in macrophages [549].

Hepcidin is increased by iron loading providing the homeostatic loop to maintain normal extracellular iron concentrations. Presumably, this loop has to be controlled by inhibitors to maintain adequate iron supply for hemoglobin synthesis and essential metabolic functions. It has been shown that SMADs inhibitors, SMAD6 and SMAD7 are implied in the maintenance of normal extracellular iron concentrations, by interfering with activin signaling pathway and erythroid cell differentiation [560, 561]. Studies by Lee *et al.* in macrophages showed that SMAD6 regulates TGF-β1-induced MyD88 degradation by mediating the recruitment of SMURF1 and SMURF2 and that SMAD6 physically interacts with MyD88 [549]. We therefore investigated the potential role of SMAD6 in targeting MyD88 for proteasomal degradation. We showed that upon BMP6 stimulation, the increased protein level of SMAD6 leads to MyD88 degradation, acting as a negative feedback mechanism to limit hepcidin induction.

Several studies have reported on the ubiquitination and de-ubiquitin-mediated proteasome degradation of the TGF-β superfamily signaling pathway. This has included BMP and Activin B signaling, which are important to functional cellular activity such as heat shock, DNA repair, cell cycle progression, and transcriptional and translational signaling [353, 562-565]. The E3 ubiquitin ligases, SMURF1 and SMURF2, are involved in the mono or poly-ubiquitination and proteasome-mediated degradation of TGF-β, Activin B and BMP signaling components, which include the inhibitory SMAD proteins (SMAD6/7), SMAD4 and regulatory SMADs [566-570]. Furthermore, it has been shown that the inhibitory SMADs, SMAD6 or SMAD7, can act as intermediates for the ubiquitination process by specifically recruiting SMURF1/2 E3 ligases to induce the degradation of TGF-β1 and BMP type 1 receptors [571, 572]. Moreover, the role of BMP and Activin B signaling was highlighted in the Zhou *et al*. study which reported that the ubiquitin-specific protease (USP) 4 de-ubiquitinates the mono-ubiquitinated SMAD4 in a mouse's embryonic stem cells (mESCs), enhancing SMAD4-mediated Activin and BMP pathways [573]. Ubiquitin degradation has also been revealed in the MyD88 pathway where a recent study showed MyD88 poly-ubiquitination *in vivo* and *in vitro* [574].

Thus far, the only identified connection between the SMAD and MyD88 pathways involves the role of SMAD6 as a negative regulator of the TGF-β signaling pathway, which also involves BMP [549, 575]. Hence, we sought to determine whether the proteolytic-dependent degradation induced by TGF-β superfamily members, BMP6 and Activin B, which are also involved in the BMP/SMAD pathway [344, 366], could be involved in hepcidin mRNA downregulation. We showed that both BMP6 and Activin B induced the ubiquitination and degradation of MyD88 and that this negative feedback loop may be relevant to the modulation of hepcidin production in hepatocytes. For the first time, our results revealed a potential hepcidin negative feedback loop mechanism that involved ubiquitin-proteolytic degradation. This negative feedback loop may indirectly regulate the duration of the cellular response to hepcidin expression, thus inhibition mechanism by SMAD6-SMURF1, which may participate for an appropriate hepcidin expression.

Finally, we studied the consequences of a common MyD88 mutation resulting in a gain of function, namely the $MyD88^{L265P}$ mutation. $MyD88^{L265P}$ is the most common and oncogenic representative of MyD88 mutation, highly express in human lymphoma. $MyD88^{L265P}$ is a somatic mutation present on the TIR domain that promotes cell survival through NF-κB activation. Generally, the L265P mutation is associated with extra nodal tumour dissemination and poor clinical outcomes in patients. The somatic gain of function MyD88 mutations were discovered in different B-cell lymphoproliferative disorders such as Waldenström macroglobulinemia (WM), present in almost 100% of WM cases, diffuse large B cell lymphoma (DBCL), and IgM monoclonal gammopathies of uncertain significance (MGUS) [518, 533, 535, 576]. $MyD88^{L265P}$ has also been found in patients with primary central nervous system lymphoma (35%), splenic marginal zone lymphoma (15%), gastric mucosa-associated lymphoid tissue lymphoma (9%), and chronic lymphocytic leukemia (CLL) (3%) [518, 537, 538, 577].

Anemia manifestation in patients with lymphomas is frequent. As an example, in WM anemia (normochromic or normocytic) is considered as one of the clinical features, where it presents at over 80%, resulting in variable phenotypes from severe to clinically

asymptomatic [576]. Anemia in WM was first associated with the reduction of red blood cells (RBC) due to their replacement in the bone marrow (BM) by Waldenström cells. However, since impairment of RBC in the BM occurred only when Waldenström cells production exceeds 40% and patients who are anemic have a very small amount of lymphoma in their bone marrow (15% or less), anemia has been associated with other non-immune-related factors as blood loss. Importantly, it was discovered that in lymphoplasmacytic cells hepcidin is overexpressed and this has been associated with anemia severity. In particular, WM has been associated with hepcidin production and inversely correlated with hemoglobin levels, leading to iron deficiency [315, 534].
Therefore, we investigated whether the $MyD88^{L265P}$ mutation could contribute to the increase in hepcidin. We found that $MyD88^{L265P}$ mutation causes upregulation of HAMP in B cell lines. These findings demonstrated the potential importance of MyD88 in the hepcidin pathway in human lymphomas, providing evidence that MyD88 could play a role in anemia manifestation in human hematological malignancies. The L265P mutation of MyD88 in lymphoplasmacytic lymphoma cells and the upregulation of hepcidin expression leads to cellular iron accumulation. Tumour cells need macro- and micronutrients for their survival and iron is the most essential amongst them. In order to acquire iron, cancer cells change the expression of target protein involved in systemic iron homoeostasis [578].

With the study presented in chapter 3 and 4, we have provided a potential molecular pathway involving the upregulation of hepcidin in lymphomas and we have highlighted the importance of MyD88 in iron homoeostasis.

6.1.2. Ironing out the role of curcumin as iron chelator in colitis

The discovery of MyD88 implication in the iron-sensing pathway for induction of hepcidin led us to investigate its potential role in iron-related disorders associated with the decrease of its expression.

We focused on the biological activity of the pleiotropic molecule curcumin (diferuloyl methane) due to its ability to downregulate MyD88 *in vivo* and its activity as iron chelator. Lubbad *et al.* reported that curcumin downregulates MyD88 protein expression, in a mouse

model with inflammatory bowel disease (IBD) with trinitrobenzene-sulfonic acid (TNBS) [579]. They demonstrated that curcumin's anti-inflammatory activities are related to the inhibition TLR4/Myd88 pathway leading to the repression of NF-κB, whose activation is involved in IBD pathogenesis [579]. For such properties, curcumin has been taken as a promising therapeutic agent for IBD, which includes group of gastrointestinal disease with unknown etiology, such as ulcerative colitis (UC) and Crohn's disease (CD).

Aside from anti-inflammatory properties, curcumin has been also shown to have multiple biological properties including anti-cancer and antioxidant [580] ones. Moreover, due to its low toxicity even at high dosages (8-12 g/day), its marginal side effects and widespread availability, it has been proposed as a promising therapeutic agent, and its usage in a variety of clinical trials for different diseases has grown exponentially in the last decades [483].

Curcumin has also been reported as showing chelating activity for Fe, Cu, Mn and Zn. As an iron chelator, it has been proposed as a potential candidate for iron overload disorders. It has been considered as a potential alternative to iron chelators, commercially available, such as the iron chelators DFO, DFP and DFX, due to their potential adverse effects. These chelators display certain side effects such as systemic allergic reactions following subcutaneous administration of the drugs, sometimes followed by pain and gastrointestinal discomfort in patients, and they are also contraindicated during pregnancy because of teratogenicity [138].

However, several studies demonstrated that a high dosage of curcumin with low iron intake or long-term curcumin administration in a deficient or sufficient iron diet alters iron metabolism, due to its chelating properties [464, 488]. Jiao *et al.* demonstrated that curcumin can substantially affect systemic iron balance *in vitro* and *in vivo*. These studies reported that curcumin lowered iron levels in the spleen and decreased hepcidin expression and IRP activity [464, 465, 488].

Thus, in the third manuscript we aimed to investigate how curcumin affects iron metabolism in IBD settings where the iron content is marginal or altogether absent, due to its decrease associated with chronic blood loss from gastrointestinal lesions. We therefore

analyzed the systemic iron balance to focus on the iron-chelating properties of curcumin. Our mice were fed with an iron sufficient diet. We used the chronic DSS mouse model since it is a well-established model for chemically inducing colitis [581]. Since the molecular weight of DSS can influence the induction and the location of colitis, we chose 40 KDa to induce the most severe diffuse colitis in the middle and distal third of the large bowel [582].

We provided evidence of the modulation of iron homoeostasis by curcumin in mice treated with dextran sulfate sodium (DSS) and fed with an iron sufficient diet. Mice treated with DSS and curcumin developed mild anemia with a depletion of iron stores in the spleen, and a decrease of erythroid parameters and hepcidin levels. Curcumin has also been reported to inhibit the transcription factor STAT3, which plays a crucial role in inflammatory response and in the induction of hepcidin [583]. Laine *et al.* then demonstrated that curcumin decreases serum hepcidin levels in healthy volunteers, which is likely due to STAT3 inhibition [584]. In accordance with these findings, curcumin has been proposed as a potential therapeutic agent against hepcidin overexpression during anemia of inflammation. However, it must be noted that hepcidin levels in IBD patients show conflicting results. Some studies reported that hepcidin levels are upregulated in IBD, probably due to inflammatory cytokines such as IL-6, while others showed a decrease of hepcidin levels which leads to iron deficiency anemia.

We found that the colonic inflammatory responses of curcumin decrease MyD88 protein expression in the colon. Interestingly, and in accordance with our findings, it was shown that *MyD88*$^{-/-}$ mice are more susceptible to developing colitis with DSS due most likely to intestinal microflora immune responses [585]. Intestinal microflora has been shown to play a crucial role in the pathogenesis of IBD [586, 587]. We have excluded the possibility that the aggravation of phenotype in non-colitic mice was due to the intestinal toxicity of curcumin with resultant inhibition of iron absorption, since we did not find differences in the body weight between control group mice fed with/without curcumin and treated with water. More importantly, it was previously reported that curcumin is not toxic to rodents or humans, and has a protective effect on the rodent gastrointestinal tract [469]. It could be interesting to investigate whether curcumin impairs the inhibition of other inflammatory

signals due to the dysregulation of intestinal permeability exacerbating DSS-induced immune response.

Contrary to studies reporting the beneficial effect of curcumin on colitis, our findings showed that curcumin failed to improve the level of colitis in DSS-treated mice fed with curcumin [588]. This could be at least partially explained by major differences between the studies regarding the iron content used in other studies that have a high iron content [589]. High iron content could mask the chelating effects of curcumin. Our study suggests that curcumin's chelating properties may present some limitations on its therapeutic use as an anti-inflammatory or even chemo-preventive adjuvant in diseases which lead to a depletion in iron stores.

Curcumin has been used in multiple clinical trials. However, its implication on iron metabolism has not been taken into consideration. Thus, it may be worthwhile to monitor the iron status of subjects participating in clinical trials in which high dosages of native curcumin are administered.

6.2. Future perspectives

Our results highlight the role of MyD88 in iron metabolism which was not yet explored and could be of important relevance to health. Our findings open new hypotheses and interesting research avenues to explore. In the same way, the relevance of curcumin as iron chelator in chronic iron disorders needs to be further investigated. In this section, possible avenues for addressing our findings, highlighting different questions which would be interesting to analyze through molecular (*in vitro*) or *in vivo* approaches are proposed.

6.2.1. Potential impact of MyD88 interaction with SMAD4

As previously mentioned, we found that MyD88 interacts with SMAD4 in hepatic cell lines. Thus, it could be interesting to investigate the mechanism(s) by which MyD88/SMAD4 interaction affects HAMP regulation: Does MyD88 impact SMAD4 translocation, stabilization and/or prevent SMAD4 degradation or does MyD88 directly influence HAMP transcription by interacting with the BMPRE1 site? In fact, even if the major role of MyD88 is to serve as a protein adaptor of TLRs on the cytoplasm, it has been

also reported to be present in the nuclei of different cells [559, 590]. Thus, the hypothesis that MyD88 could be co-localized in the nuclei with SMAD4 must be considered.
To investigate these points, we can use different approaches. The Electrophoretic mobility shift assay (EMSA) experimental *in vitro* technique can be favourable, and can be a suitable approach for analyzing protein/DNA interactions in crude nuclear or whole cell extract. EMSA exploits the shift or retardation of heavy protein binding-DNA migration, compared to free probe in polyacrylamide or agarose gel electrophoresis. Another interesting approach that can be used is the genome-wide chromatin immunoprecipitation (ChIP) combined with promoter sequence microarray (ChIP-on-chip). It can be an excellent *in vivo* analysis, not simply a method for determining DNA-protein interactions. This experimental technique consists of a probe mixture (immunoprecipitated DNA fragments with a fluorescent dye and genomic DNA labelled with Cy3) applied to a whole genome microarray chip [591]. Using various software, such as CisGenome, the exact location of the interaction [592] can be identified. In conclusion, a reliable tool for detecting protein interaction with DNA is fluorescence resonance energy transfer (FRET) combined with a fluorescence lifetime imaging microscopy (FLIM) [593]. FRET consists of a physical phenomenon in which an exited donor fluorophore transfers the energy, upon interaction, to an acceptor chromophore; subsequently the association with FLIM allows the *in-situ* imaging for protein-DNA interactions in the cell nucleus. Essentially, the technique involves using high affinity nucleic acid fluorophore as an acceptor of donor (protein) energy, all based on a transfer of energy technique. The fluorescence lifetime (the time a molecule spends in its exited state and the return to the ground state) is measured to create the image using the decay rates of the emission.

6.2.2. The relevance of MyD88^{L265P} in hepcidin expression and anemia manifestation

WM and ABC-DLBC are one the most relevant hematological malignancies that display constitutive NF-κB activation due to the MyD88^{L265P} mutation [518, 535, 576]. It is important to investigate the anemia manifestation, which is not well understood at this time, since it impacts the survival rate of the diseases (5-year survival rate of 50% in WM). To investigate the specific role played by MyD88^{L265P} mutation on the manifestation of anemia

in lymphomas, it could be useful to manage the cell model of specific lymphomas or conditional MyD88^{L265P} mouse model already available [518, 535].

Cell models of WM disease such as BCWM.1 and MCWL.1, are available for studies [594]. These cell lines could be useful instruments for evaluating MyD88 induction of hepcidin expression in WM disease, despite their differences. MWCL-1 cells retain similar immunophenotypic and biological properties of the initial WM tumour clone from a 73-year-old WM patient, including the cell surface expression of both B-cell and plasma cell markers. On the other hand, BCWM.1 cell line has an obscure origin. More importantly, both cells have the ability to secrete high levels of IgM protein, one of the main features of WM disease. Suitable mouse models of WM disease are not yet well established. Even if Tompkins *et al.* generated a mouse model that mimics the human WM disease BCL2^{+} IL6^{+} AID^{-} model, the models has some shortcomings to consider, such as low levels of secreted IgM and histopathological changing not visible in WM patients [595]. One possible approach to make will be to silence the mutation on BCL2^{+} IL6^{+} AID^{-} mice model, to investigate hepcidin expression, since analyzing SMAD4 expression in these cells could be informative. It could also be interesting to analyze inhibitory SMAD expressions (especially SMAD6) and their contribution to hepcidin expression when upregulated.

Knittel *et al.* originated the genetically engineered mouse model (GEMM) MyD88^{l252p}, the orthologous mouse allele of human MyD88^{L265P} mutation, in B lymphocytes which develop DLBCL-like neoplasms. They were able to pursue their objective by inducing Cre recombinase activation of the MyD88^{l252p} knock-in gene inserted into the germline Myd88 locus in embryonic stem cells. They expressed MyD88^{L252P} in different B-cell populations by using three different Cre drivers (AID, CD19, and CD22) [577, 596]. This B cell–specific expression of this mutant leads to an LPD and occasional (∼30%) transformation into an aggressive lymphoma that morphologically and immunophenotypically resembles human ABC-DLBCL. It would be interesting to investigate hepcidin level in these cells as well as the relevance of its expression in B-cells for anemia manifestation through engineering conditional knockout mouse models of MyD88^{L265P} in B cells could be useful for validating the expression of this mutation in hepcidin upregulation. More precisely,

hepcidin expression could be measured by RT-PCR or ELISA, and hematological parameters such as hemoglobin and red blood cells could be assessed

6.2.3. MyD88 downregulation by curcumin may influence the intestinal microbiota

In the third manuscript, one factor that has not been yet investigated was the gut microbiota composition. In fact, the gut microbiota has been shown to influence the responsiveness in the DSS-induced colitis mouse model, and more importantly to be crucial in IBD pathogenesis. It has been reported by several studies that IBD patients have a distinct intestinal flora. Moreover, MyD88 signaling has been shown as essential to the protective process in the inflammation. Araki *et al.* demonstrated that *MyD88* $^{-/-}$ develop severe colitis due to the protective role of MyD88 signaling, likely exerting a protective role against the alteration of the mucosa in association with IBD [585]. In our study, we showed that curcumin downregulation of MyD88 protein expression in the colon was correlated with the development of the disease.

It will be interesting to investigate whether the modulation of gut microbiota by curcumin might play a role in the observed severity of colitis. Characterization of the gut bacterial composition by 16S ribosomal RNA (rRNA) gene sequencing may provide additional insight into the group's role in the increased severity of colitis found in curcumin-fed mice.

Finally, it will be interesting to consider other models of IBD, also due to their association with colon cancer development (IL-10 mice and DSS/AOM) to investigate whether, by modulating systemic iron balance, curcumin can exert its role as a chemo-preventive agent. This kind of study could be important for further understanding curcumin chelating versus chemo-preventive activities.

6.3. Concluding remarks

MyD88 is an adaptor protein that plays an important role in innate and acquired immunity, usually leading to pro-inflammatory cytokines. Its implication with iron sensing pathways could potentially lead to promising therapeutic molecular targets to counteract iron disorders linked to hepcidin dysregulation. We reported a new role for MyD88 in the SMAD signaling pathway and iron homoeostasis regulation outside the context of TLR signaling. A better understanding of this pathway is important in order to design and test new approaches for treatment. Furthermore, we also demonstrated that a better understanding of curcumin's chelating properties is essential for its manipulation in chronic diseases related to systemic iron imbalance as IBD.

6.4. Summary of major findings

Our study reported, for the first time, MyD88 implication in iron-sensing pathways. We have shown that MyD88 interacts with SMAD4 on the TIR domain of MyD88 and acts as an enhancer of the expression of HAMP, dependently of the BMP-RE1 hepatocytes. We further demonstrated that BMP6 and Activin-B downregulate the expression of MyD88, and upregulates SMAD6 (inhibitor of SMAD proteins, increasing MyD88 ubiquitination. This might be due to the activation of SMURF1 and SMURF2 *via* SMAD6. The downregulation of MyD88 inhibits the expression of HAMP which shows the presence of a negative feedback loop on MyD88 (Figure 8).

We also found that MyD88 somatic mutation, ($MyD88^{L265P}$) highly present in lymphomas, leads to upregulation of hepcidin with a consequent upregulation of intracellular iron concentration in B cells. Therefore, we concluded that MyD88 is implicated in the positive and negative regulation of the BMP signaling pathway for the regulation of hepcidin.

In the third appended manuscript, we evaluated the effects of curcumin on systemic iron balance in a mouse model with colitis, induced through the administration of dextran sodium sulfate (DSS), to mice fed an iron-sufficient diet. We found that curcumin lowers body iron stores and lowers erythroid parameters. Moreover, curcumin worsens colitis independently from the mouse strain.

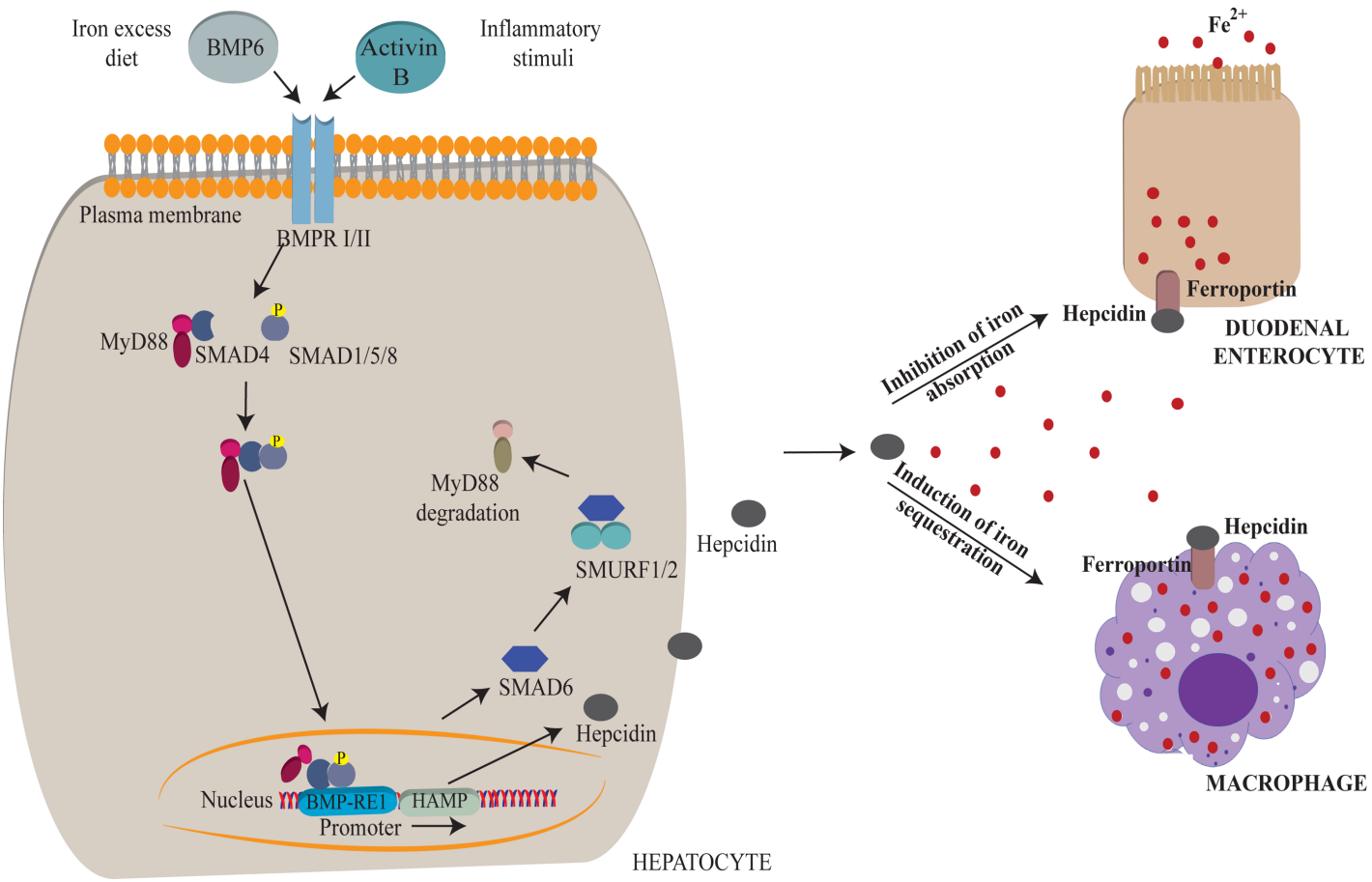

Figure 8: Schematic representation of the proposed role of MyD88 in iron-sensing through the BMP/SMAD signaling pathway. BMP ligands such as BMP6 (induced by dietary iron) and Activin B (induced during inflammation) bind to type-I and/or type-II BMP receptors (BMPI/II). Once BMP ligands are bound to the receptor complex, intracellular signaling proteins SMAD1/5/8 are phosphorylated. The phospho- SMAD1/5/8 interacts with SMAD4, which binds to MyD88. This complex translocates into the nucleus where it regulates expression of target genes such as hepcidin. Hepcidin is secreted by the hepatocytes and regulates iron absorption levels as well as iron retention within macrophages. Acting as a negative feedback regulator, inhibitory SMAD6 recruits SMURF1/2 and facilitates ubiquitination and degradation of MyD88. Finally, hepcidin induces iron absorption and iron sequestration on the basolateral level of duodenal enterocytes and macrophages respectively by interacting with the iron exporter ferroportin and leading to its degradation.

CHAPTER 7

7. BIBLIOGRAPHY

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CHAPTER 8

8. PERMISSIONS

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Hentze, M.W., et al., *Two to tango: regulation of Mammalian iron metabolism.* Cell, 2010. **142**(1): p. 24-38.

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Clark, R.J., et al., *Understanding the structure/activity relationships of the iron regulatory peptide hepcidin.* Chem Biol, 2011. **18**(3): p. 336-43.

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-Figure 5 (p.37) Hepcidin mRNA (HAMP) positive and negative regulators.

Rishi, G., Daniel F. Wallace, and V N. Subramaniam, *Hepcidin: regulation of the master iron regulator.* Bioscience Reports, 2015. **35**(3): p. e00192

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-Figure 7 (p.55) Toll-like receptors and downstream signal transduction of MyD88 dependent and independent pathways.

Wang, J.Q., et al., *Toll-Like Receptors and Cancer: MYD88 Mutation and Inflammation.* Front Immunol, 2014. **5**: p. 367.

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