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Ana Rita Ladeira Courelas da Silva, MSc

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Citrate Defines a Regulatory Link Between Energy Metabolism and the Liver Hormone Hepcidin

Referees: Dr. Alexander Aulehla

Prof. Dr. Michael Boutros

Ao meu Pai e à minha mãe, que sempre me deram força para todos os desafios ao longo da minha vida.

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Summary

Iron plays a critical role as an oxygen carrier in hemoglobin as well as a constituent of iron-sulfur clusters. Increasing evidence suggests that mechanisms maintaining iron homeostasis cross-talk to intermediary metabolism. The liver hormone hepcidin is the key regulator of systemic iron metabolism. Hepcidin transcriptional control is linked to the nutrient-sensing mTOR pathway, proliferative signals, gluconeogenic responses during starvation and hormones that modulate energy metabolism. The aim of my PhD project was to investigate links between hepcidin regulation and cellular metabolism. My work describes for the first time that citrate metabolism controls hepcidin expression.

A previously reported genome-wide RNAi screen generated a first comprehensive atlas of hepatocytic hepcidin regulators. To study metabolic signals that control hepcidin expression, I selected putative hepcidin regulators from the screening data, noting that several are specifically involved in citrate metabolism. I show that the siRNA-mediated knockdown of the enzymes that catalyze citrate-consuming reactions ACO1 (cytosolic aconitase), ACO2 (mitochondrial aconitase) and ACLY (ATP citrate lyase), as well as the inhibition of their enzymatic activities by small molecules, increases hepcidin mRNA expression in primary murine hepatocytes (PMH). Treatment of PMH with citrate furthermore results in the increase of citrate in cells and increased hepcidin expression. I further demonstrate that citrate acts intracellularly to regulate hepcidin via the BMP/SMAD signaling pathway. Injection of citrate into the tail vein of mice supports my findings that citrate increase in the liver augments hepcidin expression as well as the activity of BMP/SMAD signaling also *in vivo*.

All together, my results uncover the cross-talk between iron homeostasis and intermediary metabolism, and may help to explain why iron levels are altered in several metabolic disorders.

Zussamenfassung

Eisen ist ein essentieller Bestandteil von Enzymen, die am Zellstoffwechsel beteiligt sind. Beispiele hierfür sind die Cytochrom-c-Oxidase, die Fettsäure-Desaturase, die Lipoxygenase sowie der Komplex I der Atmungskette. Der systemische Eisenstoffwechsel wird maßgeblich durch das Leberhormon Hepcidin reguliert. Es gibt Hinweise, dass der Mechanismus, der die Eisenhomöostase aufrechterhält, funktionell mit dem intermediären Stoffwechsel verknüpft ist. Die Hepcidin Transkription wird reguliert durch den mTOR Signalweg, Proliferationssignale, die Reaktion der Glykolyse auf Nährstoffmangel sowie durch Hormone, die den Energiestoffwechsel modulieren. Das Ziel meiner Doktorarbeit war es, eine funktionelle Verknüpfungen zwischen der Hepcidinregulation und dem Zellstoffwechsel zu untersuchen. Meine Ergebnisse zeigen, dass Zitrat die Expression von Hepcidin kontrolliert.

Ein genomweiter RNAi Screen, welcher einen ersten umfassenden Atlas hepatozytärer Regulatoren des Hepcidins generierte, stellte eine wichtige Grundlage für meine Doktorarbeit dar. Um metabolische Signale zu identifizieren, die die Expression von Hepcidin kontrollieren, fokussierte ich meine experimentelle Arbeit auf Hepcidinregulatoren, die speziell in den Zitratzyklus involviert sind. Ich konnte zeigen, dass sowohl der siRNA vermittelte ‚knockdown' der Enzyme ACO1 (zytosolische Aconitase), ACO2 (mitochondriale Aconitase) and ACLY (ATP-Citrat-Lyase), welche Reaktionen des Zitratzyklus katalysieren, als auch die Hemmung ihrer enzymatischen Aktivität, die Expression von Hepcidin mRNA in primären, murinen Hepatozyten (PMH) steigern. Des Weiteren führt eine Behandlung der PMHs mit Zitrat zu einer Akkumulation von Zitrat in den Zellen und einer gesteigerten Expression von Hepcidin. Weiterhin zeigen meine Arbeiten, dass Zitrat intrazellulär die Regulation von Hepcidin mittels des BMP/SMAD Signalweges bewirkt. Die Injektion von Zitrat in die Kaudalvene von Mäusen unterstützt meine zellulären Befunde insoweit, als dass dieses sich in der Leber anreichert, die Aktivität des BMP/SMAD Signalweges in vivo erhöht sowie die Expression von Hepcidin steigert.

Zusammenfassend zeigen meine Ergebnisse ein Zusammenspiel der Eisenhomöostase und des intermediären Stoffwechsels, welches dazu beitragen kann, dass der Eisenspiegel bei häufigen Stoffwechselerkrankungen verändert ist.

Abbreviations

ACD	Anemia of chronic diseases
ACLY	ATP citrate lyase
Acetyl-coA	Acetyl-coenzyme A
ACO1	Cytosolic aconitase
ACO2	Mitochondrial aconitase
Act-R	Activin receptor
ATP	Adenosine triphosphate
BMP	Bone morphogenic protein
BMPR	BMP receptor
BMPRE	BMP responsive element
CREBH	Cyclic AMP response element binding protein H
CS	Citrate synthase
ChIP	Chromatin immunoprecipitation
Dcytb	Duodenal cytochrome B
DMT1	Divalent metal transporter 1
DNA	Deoxyribonucleic acid
dNTPs	deoxy-ribonucleotides triphosphate
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetra-acetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EPO	Erythropoietin
ER	Endoplasmic reticulum
ERFE	Erythroferrone
ETC	Electron transport chain
$FADH_2$	Reduced flavin adenine dinucleotide
FCS	Fetal calf serum
Fe^{2+}	Ferric iron
Fe^{3+}	Ferrous iron
Fe-S	Iron-Sulfur
Ferritin L	Light chain of ferritin
FPN	Ferroportin
F1,6BPase	Fructose 1,6-bisphosphatase
F1,6P	Fructose 1,6-bisphosphate
g	Grams
GDF15	Growth differentiation factor 15
GLUT4	Glucose transporter type 4
HATs	Histone acetyltransferases
h	Hour
Hb	Hemoglobin
Hamp1/Hepc	Hepcidin
HCl	Hydrochloric acid
HGF	Hepatocyte growth factor
HH	Hereditary hemochromatosis
HJV	Hemojuvelin
HO1	Hemoxygenase I
HuH7	Hepato-cellular carcinoma cell line

HXK2	Hexokinase-2
LiCl	Lithium chloride
IL6	Interleukine 6
ISCU	Iron-sulfur cluster assembly enzyme
IN	Input
IRE	Iron-responsive element
IRIDA	Iron-refractory iron deficiency anemia
IRP	Iron regulatory protein
IV	Intravenous
JAK	Janus kinase
LD	Lethal dose
LIP	Labile iron pool
MCV	Mean corpuscular volume
Mfrn	Mitoferrin
M	Molar
min	minutes
mg	Milligrams
ml	Milliliters
μl	Microlitters
mRNA	Messenger RNA
mm	Milimeters
mM	Milimolar
μM	Micromolar
μm	Micrometer
MPK	Mitogen-activated protein kinase
MPV	Mean platelet volume
mTOR	Mammalian target of rapamycin
MTT	Methyl-thiazolyl-tetrazolium
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NADH	Reduced nicotinamide adenine dinucleotide
nM	Nanomolar
nm	Nanometer
NPCs	Non-parenchymal cells
NTBI	Non-transferrin bound iron
PBS	Phosphate buffered saline
PDGF-BB	Platelet-derived growth factor-BB
PDH	Pyruvate dehydrogenase
PFK1	Phosphofructokinase-1
PMH	Primary murine hepatocytes
PK	Pyruvate kinase
PKD	Polycystic kidney disease
PRPN	Prion-protein ferrireductase
p-SMAD	Phosphorylated SMAD
Ras-RAF	Rapidly accelerated fibrosarcoma
qPCR	Quantitative real-time PCR
RBC	Red blood cell
RDW	Red blood cell distribution
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SDH	Succinate dehydrogenase
SDS	Sodium dodecyl sulfate
siRNA	Small interfering-RNA

SMAD	Sma and mother against decapentaplegic
p-SMAD	Phosphorylated SMAD
STAT	Signal transducer and activator of transcription
Tf	Transferrin
TfR	Transferrin receptor
TGF-β	Transforming growth factor-b
Tris	Tris(hydroxymethyl)aminomethane
TMPRSS6	Matriptase-2
TWSG1	Twisted gastrulation BMP signaling modulator
UTR	Untranslated regions
WBC	White blood cell
WT	Wild type
ZIP14	ZRT/IRT-like protein 14
°C	Degree Celsius

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Introduction

1 Introduction

1.1 Iron Metabolism

1.1.1 The Yin and Yang of Iron

Iron is one of the most abundant elements in the Earth's crust and essential for almost all forms of life. Its ability to shift between different oxidation states (-2 to +6) makes iron a highly versatile metal, suitable for numerous biochemical reactions (reviewed in [1]). As a constituent of heme, iron is necessary for oxygen transport and storage (in hemoglobin (Hb) and myoglobin respectively), and required for the electron transport chain (cytochrome P450) and energy production (cytochrome C and cytochrome C oxidase). In non-heme iron-containing proteins, iron is essential for oxygen sensing (hypoxia-inducible factor prolyl-hydroxylases), DNA synthesis (ribonucleotide reductase), protein metabolism (amino-acid oxidases), as well as for fatty acid biosynthesis (fatty acid desaturases) and metabolism of eicosanoids (lipoxygenases). In the mitochondrial complex I, iron-sulfur (Fe-S) clusters are crucial for energy production (reviewed [2]). The fluctuation between oxidation states not only allows iron to participate in vital reactions, but it also potentiates the generation of free radicals from superoxide and hydrogen peroxide (Fenton's reaction). These reactive oxygen species induce protein, lipid and DNA damage [3]. Since iron is both essential and toxic to cells, organisms have to efficiently regulate iron in order to meet the metabolic demand whilst preventing iron excess.

1.1.2 Iron Distribution and Utilization

The body of a healthy human adult contains an average of 3-5 g of iron, distributed among different cell types. In the blood, 2.5 g of iron are present in erythrocytes (in hemoglobin) and 2-4 mg circulate bound to the high affinity iron plasma protein transferrin (Tf). Macrophages recycle 20 mg of iron daily from

senescent or damaged erythrocytes, which is later used for a new process of erythropoiesis. In cells, 5 mg of iron are utilized as a cofactor for iron-containing proteins. The human body looses 1-2 mg of iron a day due to bleeding, sweating, epithelial cell desquamation and urinary excretion, however, there is no controlled mechanism for iron elimination. These iron losses are then compensated by dietary iron absorption - which is a highly regulated process (reviewed in [4, 5]).

Duodenal enterocytes are responsible for taking up dietary iron. The inorganic iron (mostly ferric iron (Fe^{3+})) is reduced to ferrous iron (Fe^{2+}) by the ferrireductase duodenal cytochrome B (Dcytb) [6] at the apical membrane side. The iron is then imported to the cytoplasm by the divalent metal transporter 1 (DMT1) [7]. The organic iron in the form of heme is absorbed by the enterocytes [8, 9] and then released from heme by the action of hemoxygenase I (HO1) [10]. The mechanism of heme absorption by the enterocytes is not yet completely understood. In the cytoplasm, the iron that is not used immediately for the cellular metabolism (labile iron pool or LIP) is delivered by the chaperone poly (rC)-binding protein 1 [11] to ferritin for storage. Ferritin is a hollow protein composed of several light and heavy chains which can store a high amount of iron. The heavy chains of ferritin are important for oxidation of ferrous iron into ferric iron for storage, whereas the light chains support the formation of the core of the Fe^{3+} complexes inside ferritin (reviewed in [12]). To supply iron when needed, ferritin undergoes lysosomal degradation resulting in iron release [13]. The free iron is then exported into the blood by ferroportin (FPN), the only-known iron exporter [14], which is located at the basolateral membrane of enterocytes [15]. The iron export from enterocytes to the blood stream by ferroportin and subsequent loading onto transferrin (Tf) is dependent upon the oxidation of Fe^{2+} to Fe^{3+} by the ferroxidase hephaestin [16] (Figure 1.1). Iron bound to transferrin can be carried to all tissues to be used or stored.

The uptake of iron bound to transferrin is mediated by the transferrin receptor 1 (TfR1) [17]. When transferrin binds to TfR1, the complex undergoes clathrin-mediated endocytosis and the acidification of the vesicle allows iron to be released from transferrin. Iron is then reduced to Fe^{2+} by the ferrireductase STEAP3 [18] and exported out of the endosome by DMT1 [19], while transferrin and TfR1 are recycled

to the plasma membrane for a new round of iron transport and uptake [20] (Figure 1.1).

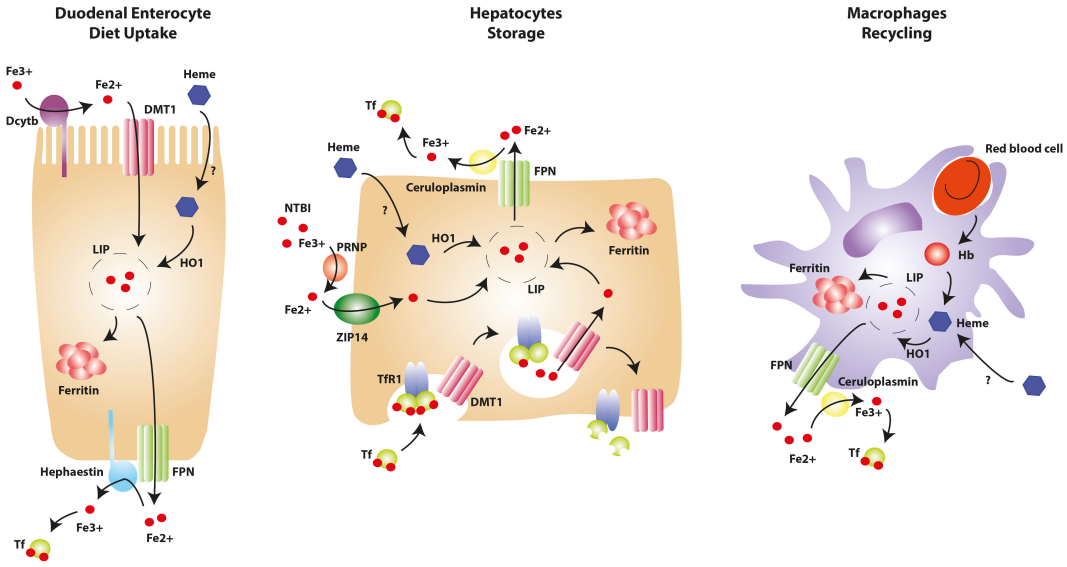

Figure 1.1: Cell types involved in iron uptake, storage and recycling.
Dietary uptake: Duodenal enterocytes are responsible for taking up dietary organic and inorganic iron. Inorganic iron (ferric iron, Fe^{3+}) is reduced to Fe^{2+} (ferrous iron) by duodenal cytochrome b (Dcytb) at the apical membrane of enterocytes and transported into the cell by the divalent metal transporter 1 (DMT1). The iron that is not used for metabolic processes is stored in ferritin until it is required, whereupon it is exported into the blood via FPN. Hephaestin oxidizes Fe^{2+} into Fe^{3+} allowing iron to be loaded into transferrin (Tf) where it can be delivered to cells. Transferrin delivers iron to sites where it is needed or stored. It is not known how heme iron is absorbed by enterocytes. By the action of homoxygenase 1 (HO1), iron is released from heme and contributes to the labile-iron pool (LIP).
Iron storage: Hepatocytes are able to store a large amount of iron. Iron is taken up via the TfR1. The iron bound Tf binds to the TfR1 and this complex undergoes endocytosis. The iron is released from transferrin and exported from the endosome to the cytoplasm via DMT1. In the cytoplasm, iron can be either utilized, stored in ferritin or exported to the blood via FPN. Ceruloplasmin oxidizes Fe^{2+} to Fe^{3+} in order for iron to be loaded into transferrin. The ZRT/IRT-like protein 14 (ZIP14) and its partner ferrireductase prion-protein (PRNP) mediate the uptake of NTBI in hepatocytes under iron-overload conditions.
Iron recycling: Macrophages are responsible for recycling iron. They digest aged red blood cells and release hemoglobin (Hb), which in turn releases heme. HO1 removes iron from heme, which can then be stored in ferritin or exported to the blood to support erythropoiesis via FPN. Ceruloplasmin oxidizes Fe^{2+} to Fe^{3+} in order for iron to be loaded into transferrin. Adapted from Darshan *et al*, 2010.

Most iron inside the cell is used for heme and Fe-S cluster biogenesis in the mitochondria (reviewed in [21, 22]). Iron is imported into the mitochondria via the inner membrane protein mitoferrin (Mfrn) 1 [23] or 2 [24], in erythroid and non-erythroid cells respectively. The mitochondrial inner membrane ATP-binding cassette transporter ABCB10 enhances the stability of Mfrn1 and, as a consequence, the iron flux to the mitochondria increases [25]. The synthesis of heme and Fe-S clusters are coordinately regulated by iron availability (reviewed in [26]). The ABC7 mitochondrial transporter is implicated in the export of Fe-S clusters from the mitochondria to the cytoplasm [27]. Studies on the human frataxin homologue in yeast show that it is required for mitochondrial iron export [28] and storage [29]. A mechanism for heme export from the mitochondria is not yet known.

Consistent with their role in transporting oxygen in the body, red blood cells need a large amount of iron. To cope with the high demand of iron for erythropoiesis and knowing that only a small percentage of iron is taken up daily from the diet, macrophages have an imperative role in recycling iron from aging erythrocytes (reviewed in [30]). Macrophages engulf and lyse the senescent erythrocytes so that heme iron is released [31]. The compartment where iron is released from heme is still not completely determined. Two hypotheses have been suggested where either a) in the phagolysosome HO1 releases iron from heme which is then exported to the cytosol both by DMT1 and Nramp1 (similar to DMT1 but specifically expressed in macrophages) [32], or b) that heme catabolism may occur in the cytoplasm by the action of HO1 with heme being exported from the phagolysosome via the heme transporter HRG1 [33]. Despite different possible routes for heme catabolism, iron is either stored in ferritin or exported by FPN to support erythropoiesis. Ceruloplasmin oxidizes Fe^{2+} to Fe^{3+} for loading onto transferrin (Figure 1.1).

Hepatocytes are known for storing a large amount of iron and providing it when needed [34]. Apart from TfR1, hepatocytes also express TfR2 (homologue to TfR1). In contrast to TfR1, TfR2 does not substantially contribute to the iron-uptake and contributes more to regulating iron homeostasis [35], an aspect that will be covered in detail in the Section 1.1.4. When the saturation of transferrin exceeds 60%, its capacity to bind to iron is reduced and leads to increased “free” iron, or non-

transferrin bound iron (NTBI) (reviewed in [22]). The ZRT/IRT-like protein 14 (ZIP14) is increased upon increased levels of iron and it is described to mediate the uptake of NTBI in the liver thus contributing to the high amount of iron taken up by this organ [36, 37]. The prion-protein (PRNP) is a partner of ZIP14 and reduces NTBI to Fe^{2+} by its ferrireductase activity thus mediating its transport to hepatocytes [38] (Figure 1.1).

1.1.3 Cellular Iron Homeostasis

Organisms require iron for their vital processes, however an excess of iron is also toxic at the cellular level and therefore cells must regulate iron uptake and release. Cellular iron homeostasis is achieved by the iron-responsive element/iron regulatory protein (IRE/IRP) network (Figure 1.2). This system controls mRNA stability or translation of genes important for the uptake, storage and export of iron (iron-related genes) in a coordinated fashion (reviewed in [22]). IREs are characterized by hairpin structures present at untranslated regions (UTR) of mRNA from iron-related genes. Two cytosolic IRPs have been identified to bind to IREs. IRP1 is a bifunctional protein, it can either function as an iron regulatory protein or acquire an Fe-S cluster and function as an cytosolic aconitase, an enzyme involved in citrate metabolism [39]. IRP2 is a homologue to IRP1 but lacks the aconitase activity [40].

When cells are iron-depleted, the IRPs serve as RNA-binding proteins and bind to IREs at the 5'UTR of the heavy (H) and light (L) chain of ferritin [41] and FPN [15] mRNAs, thus inhibiting their translation [42]. As a consequence, iron is not stored in ferritin and remains inside the cell in order to be utilized. In the same conditions, IRPs bind to the 3'UTR of TfR1 and DMT1 mRNAs protecting them from degradation ([43, 44] and reviewed in [45]). The stabilization of the TfR1 and DMT1 mRNAs leads to the increase in both systemic and dietary iron uptake. Conversely, in iron-rich cells the activity of the IRPs is inhibited. IRP1 acquires an Fe-S cluster and dissociates from target mRNAs [46, 47], whereas IRP2 is degraded by the proteasome [48]. The inactivation or degradation of the IRPs allows the mRNA

of TfR1 and DMT1 to be degraded, decreasing iron uptake, and ferritin and FPN to be translated, stimulating iron storage and export from the cell.

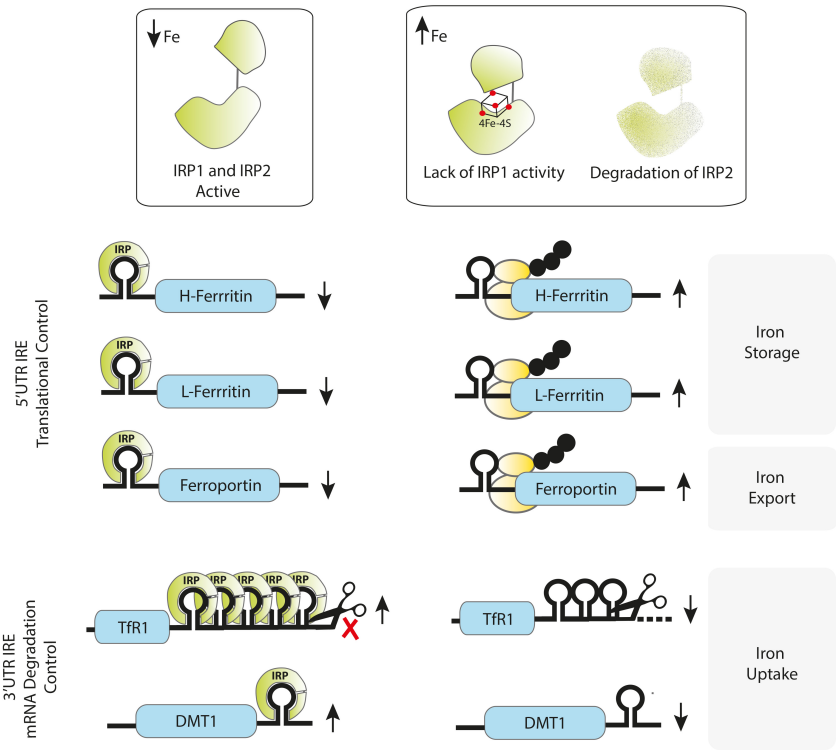

Figure 1.2: Cellular iron regulation.
In iron-deficient cells, the binding of IRPs to the 5'UTR of the mRNAs for ferritin L and H-chain and FPN prevents their translation thus preventing iron from exiting the cell and potentiating the iron release from the stores. IRPs stabilize the mRNA of TfR1 and DMT1, which leads to an increase in iron uptake. **In iron-rich cells**, IRP1 acquires an Fe-S cluster and loses its ability to bind to IREs whereas IRP2 is degraded in the proteasome. This inactivation of the IRPs leads to the increase in FPN and ferritin L and H-chain mRNA, thus stimulating iron export and sequestration of iron in ferritin. mRNA of TfR1 and DMT1 are degraded shutting down iron uptake from the blood and diet respectively. Adapted from Hentze *et al*, 2004.

1.1.4 Systemic Iron Homeostasis

It is of great importance that dietary iron uptake from duodenal enterocytes, storage and release of iron by hepatocytes, and the recycling of iron from aged

erythrocytes by macrophages are regulated. Systemic iron homeostasis is achieved by the production of a hepatic hormone which controls iron fluxes - the peptide hormone hepcidin (mouse - *Hamp1*, human - *Hepc*).

Discovery and function of hepcidin

Hepcidin was first discovered after being isolated from human urine and blood samples as a defensin-like peptide with antimicrobial activity against several pathogens [49, 50]. Hepcidin was found to be synthetized mostly by the liver as an 84 amino acid long pre-pro-peptide. However, only the post-translationally processed 20 and 25 amino acid forms were prevalent in the urine [50]. Although the first entry of hepcidin in UniProt (Universal Protein Resource) dates December 1998 (http://www.uniprot.org/uniprot/P81172), it was only in 2001 when it was suggested that hepcidin could have a role in iron homeostasis. Early work describes that the mRNA of hepcidin was increased in the liver of mice fed with a high-iron diet or iron-dextran injected mice [51]. It was also shown that disruption of the hepcidin gene leads to an iron-overload phenotype in mice [52]. Work throughout the years has elucidated in more detail the role of hepcidin in systemic iron control.

Hepcidin is produced by hepatocytes in response to increased iron levels. The convertase furin cleaves the immature 84 amino acid protein into an active 25 amino acid form before it is secreted by hepatocytes [53]. In the blood, hepcidin associates with α-2-macroglobulin, which promotes its efficient targeting to cells [54]. It acts by binding to an extracellular loop of FPN expressed in hepatocytes, macrophages and enterocytes [55]. The interaction between hepcidin and ferroportin triggers the ubiquitination of ferroportin. This post-translational modification directs FPN for internalization by endocytosis and its degradation in the lysosome [55, 56]. Thus, the hepcidin-ferroportin axis is crucial for systemic iron homeostatic control. High systemic iron levels induce hepcidin expression, which in turn decreases iron export from cells by degrading ferroportin. In iron deficiency, hepcidin expression is reduced so that iron can be released from cells via FPN (Figure 1.3).

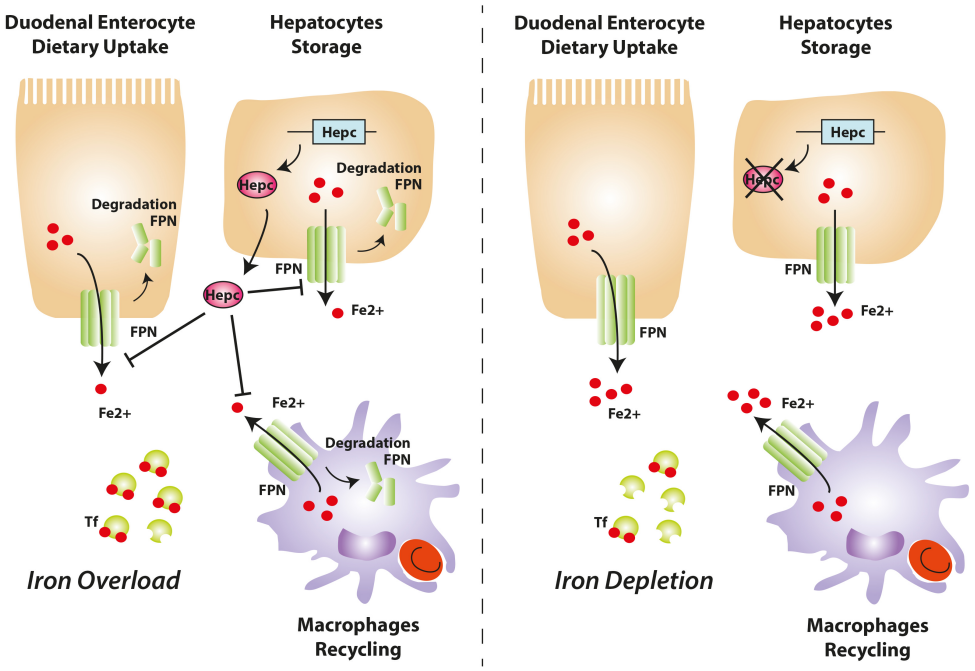

Figure 1.3: Systemic iron regulation.
Hepatocytes activate the expression of hepcidin in response to high iron levels. Hepcidin is secreted to the serum where it binds to the ferroportin present at the membrane of hepatocytes, duodenal enterocytes and macrophages. The binding of hepcidin to ferroportin triggers its degradation leading to the reduction of iron export. Conversely, hepcidin expression is reduced under iron depletion so that ferroportin remains stable at the cell membrane and iron is exported to the serum.

Regulation of Hepcidin

Hepcidin is the master regulator of systemic iron homeostasis, and therefore hepcidin must be tightly controlled. To date, the only known mechanism for hepcidin control is the regulation of its mRNA expression. The hepcidin promoter responds to a variety of different signals. Three major stimuli for the hepcidin regulation are the iron status, inflammation and erythropoietic signals, although increasing evidence has shown that the hepcidin transcriptional regulation extends beyond these three core regulatory processes (Figure 1.4).

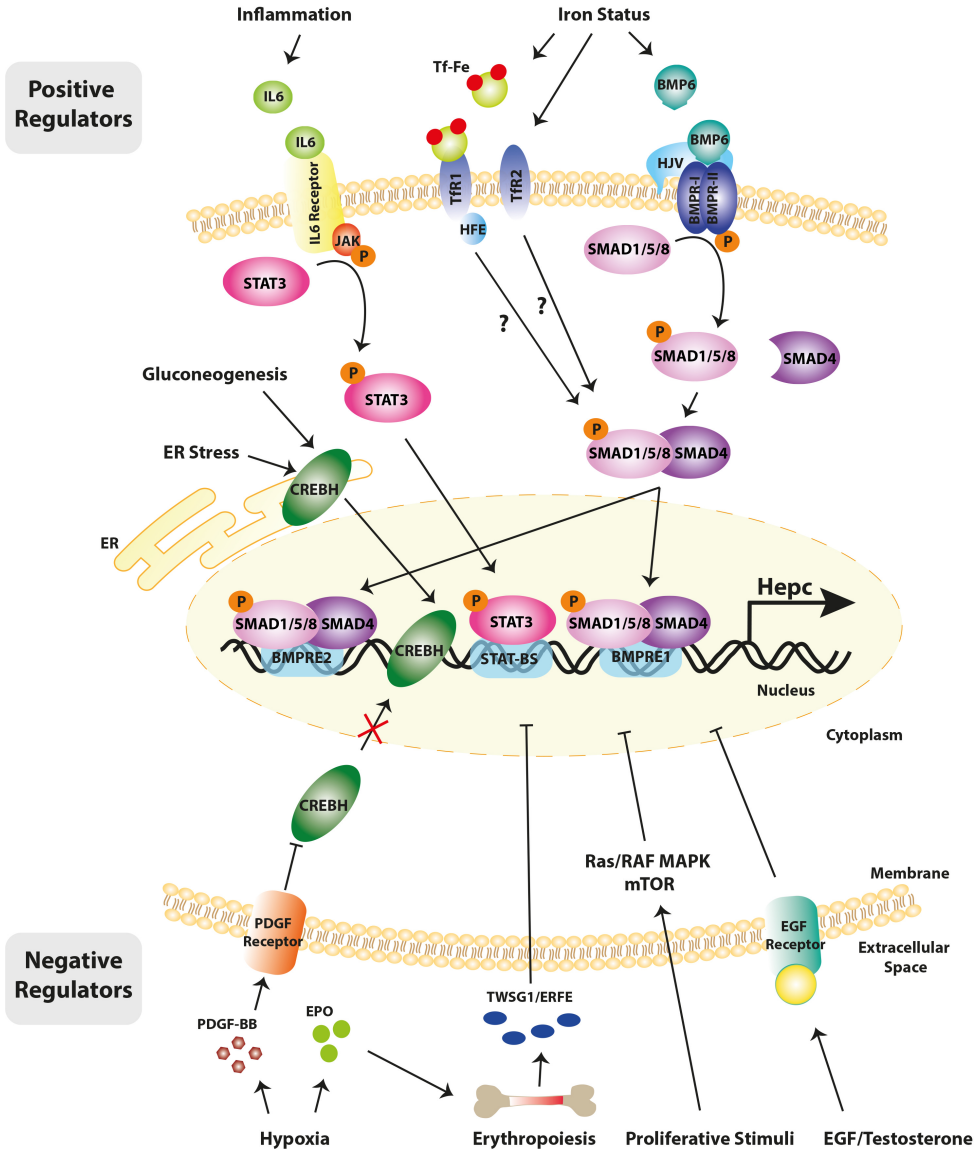

Figure 1.4: Regulation of hepcidin transcription.
In **high iron conditions**, BMP6 binds to BMPR-I and BMPR-II triggering the phosphorylation of the transcription factors SMAD1/5/8. p-SMAD1/5/8 associates with the co-activator SMAD4 and is translocated into the nucleus. This complex binds to the BMPREs present at the promoter of hepcidin, activating its transcription. The complex of TfR1, TfR2 and HFE sense iron levels and cross-talk to the BMP pathway to up-regulate hepcidin transcription. **In inflammatory conditions**, IL6 binds to its receptor triggering the phosphorylation of the STAT3 transcription factor. p-STAT3 is transported to the nucleus and binds to the STAT-BS present at the promoter of hepcidin. The binding of STAT3 to the STAT-BS induces hepcidin transcription. Both **gluconeogenesis and ER stress** induce hepcidin expression by activating the CREBH transcription factor. EPO stimulates erythropoiesis **during hypoxia**. In these conditions, hepcidin suppressors such as TWSG1 and ERFE are produced and act on the liver to negatively regulate hepcidin transcription. In addition, PDGF-BB binds to the PDGF receptor, preventing CREBH from activating hepcidin. The growth factor EGF, the hormone testosterone and proliferative stimuli due to growth and nutrient signals, suppress hepcidin expression. Adapted from Rishi *et al*, 2015.

Hepcidin regulation by the iron status

The signaling pathway which responds to iron levels and is involved in modulating hepcidin expression in the liver is the bone morphogenic protein (BMP)-sma and mother against decapentaplegic (SMAD) pathway. BMPs are secreted cytokines which belong to the transforming growth factor-β (TGF-β) family and are involved in regulating many basic cellular processes (reviewed in [57]). In response to increasing systemic iron levels, non-parenchymal cells (NPCs) of the liver produce and secrete BMP6 [58], the predominant BMP involved in hepcidin regulation [59]. The mechanism by which NPCs sense iron and activate BMP6 transcription is yet to be elucidated. Although hepatocytes also have detectable levels of *Bmp6* mRNA and therefore could control hepcidin expression in a paracrine manner, it is believed that the BMP6 produced by the NPC is the major contributor to the BMP6-induced hepcidin expression in hepatocytes [58]. Secreted BMP6 binds to the BMP receptors at the cell membrane of hepatocytes. Functional BMP receptors are composed of two type I and two type II subunits. The relevant receptors for iron homeostasis are the activin receptor-like kinase 2 (ALK2) and BMP receptor 1a (BMPR-1A/ALK3) (type I receptors), and the BMPR-II and activin type II receptor (ActR-II) [60-62]. The binding of BMP6 to these type I receptors triggers the activation of a serine-threonine kinase located in the type II receptors, which in turn recruits and phosphorylates the SMAD1, SMAD5 and SMAD8 transcription factors (SMAD1/5/8) [57, 63]. Phosphorylated SMAD1/5/8 (p-SMAD1/5/8) then associates with the co-activator SMAD4. This complex is translocated into the nucleus where it binds to the promoter of hepcidin thus activating its transcription. The expression of SMAD7 is induced by BMP6 and it acts as a suppressor in a negative feedback loop for the suppression of hepcidin transcription [64]. There are two conserved BMP responsive elements (BMPRE) in the human hepcidin promoter. These BMPREs are located at different positions, with the BMPRE1 being proximal (-84/-79) and the BMPRE2 being distal (-2255/-2250) (Figure 1.4). Mutations in these regions strongly reduce hepcidin expression, showing that these motifs are crucial for the regulation of hepcidin [65]. In the mouse promoter, one BMPRE has been identified at the position -200/-140 [66].

Other molecules interact with the BMP/SMAD pathway and contribute to the iron-induced hepcidin regulation. The membrane protein hemojuvelin (HJV) is a BMP co-receptor necessary for the stimulation of hepcidin transcription [67]. The matriptase-2 (TMPRSS6) exerts an inhibitory effect on the BMP pathway by cleaving the HJV into a soluble form [68]. The cleaved HJV then competes with BMP6 for the binding to its receptor resulting in a reduction of the p-SMAD1/5/8 and hepcidin expression.

The communication between iron levels and the expression of the hepcidin gene is not only accomplished by means of BMP6 stimulation. TfR1, TfR2 and the membrane protein HFE in hepatocytes sense circulating iron levels through iron bound-transferrin. Upon binding of iron-loaded transferrin, HFE is displaced from TfR1. The dissociation of HFE from TfR1 not only increases the affinity of this receptor to transferrin but also triggers hepcidin expression [69, 70]. It has been suggested that HFE, TfR2 and HJV form a complex, which could be important for the regulation of hepcidin [71, 72]. Contrary to this, it was suggested that these proteins do not need to interact to modulate hepcidin [73, 74]. Therefore, further investigation must be carried out in order to clarify the involvement of this complex in regulating hepcidin levels. Despite the unclear role of HFE/TfR2/HJV in the control of hepcidin as a complex, it is well documented that HFE stabilizes BMPR1A leading to the up-regulation of hepcidin transcription [75]. Furthermore, *Hfe*- and *Tfr2*-null mice present diminished p-SMAD1/5/8 reflecting the importance of these proteins for the regulation of hepcidin via the BMP/SMAD signaling pathway [76] (Figure 1.4).

Hepcidin regulation by inflammatory stimuli

Pathogen growth depends upon iron availability. Therefore, upon inflammation, the transcription of hepcidin is up-regulated in order to limit the iron level in the blood [51]. In response to inflammatory stimuli, the circulating cytokine interleukin 6 (IL6) increases hepcidin levels via the janus kinase (JAK)-signal transducer and activator of transcription 3 (STAT3) signaling pathway [77, 78]. The binding of IL6 to its receptor activates the JAK kinase resulting in the

phosphorylation of the STAT3 transcription factor (reviewed in [79]). The phosphorylated form of STAT3 is then transported to the nucleus where it binds to the promoter of hepcidin at a region known as the STAT-binding site (position -72/-64 in the human promoter) [80] (Figure 1.4).

Studies have uncovered an intertwined network involving the BMP/SMAD and the JAK/STAT pathways. Hepatocytes lacking *Smad4* in the liver [81] or mice administered with a BMPR1A inhibitor [82] do not efficiently up-regulate hepcidin when challenged with IL6. In addition, mice treated with lipopolysaccharide (which triggers inflammation) show increased p-SMAD1/5/8 compared to control mice and it is believed that this response involves the binding of the cytokine activin B to BMP receptors [83].

Hepcidin regulation by erythropoietic signals

Iron is an element present in hemoglobin, which is essential for red blood cells to carry out their function as oxygen transporting cells. Thus, increased erythropoiesis requires a supply of iron. For this reason, hepcidin has to be regulated according to the erythropoietic demand. This process involves communication between different organs in a coordinated manner. Erythropoietin (EPO) is a cytokine synthesized by the kidneys in hypoxic conditions, which controls erythropoiesis in the spleen and bone-marrow (reviewed in [84]). As a result, a stimulation of the recently identified secretory protein erythroferrone (ERFE) occurs, which in turn acts directly on the liver to suppress hepcidin [85] (Figure 1.4). The suppression of hepcidin leads to an increase in iron availability in the serum, which is used for erythropoiesis. Little is known about the mechanism by which erythroferrone suppresses the transcription of hepcidin. So far, one initial study shows that the suppression of hepcidin does not involve the BMP/SMAD pathway [85] whereas more recent work suggests that the hepcidin inhibitor TMPRSS6 is necessary to attenuate the activity of the BMP/SMAD pathway in response to ERFE [86].

Apart from ERFE, two other molecules are described to play a role in hepdicin down-regulation upon high erythropoietic demand. Erythroblasts, precursors of red blood cells, produce both the twisted gastrulation BMP signaling modulator 1 (TWSG1) and the growth differentiation factor 15 (GDF15). While it is known that TWSG1 suppresses the expression of hepcidin by reducing the activity of the BMP/SMAD pathway in hepatic cells [87], whether GDF15 has a role in suppressing hepcidin expression is controversial [88, 89] (Figure 1.4).

In addition to EPO stimulation, low oxygen levels induce the expression of the platelet-derived growth factor (PDGF)-BB. The PDGF-BB factor has been described as a repressor of hepcidin under hypoxic conditions. The PDGF-BB-mediated suppression of hepcidin is achieved by the binding of PDGF-BB to PDGF receptors, which inactivate the CREBH transcription factor [90] (Figure 1.4).

Other regulators of hepcidin

In addition to the three major stimuli that regulate hepcidin (iron status, inflammation and erythropoietic signals), hepcidin modulation has been linked to sex hormones, proliferation and growth factors as well as to metabolism. Testosterone is reported to repress hepcidin by acting on the epidermal growth factor receptor (EGFR) of hepatocytes [91] (Figure 1.4). Contradicting work has shown hepcidin being both up- or down-regulated in response to increased estrogen levels [92, 93]. Several proliferative stimuli have been reported to suppress hepcidin expression. It has been demonstrated that growth factors involved in tissue formation such as the hepatocyte growth factor (HGF) and epidermal growth factor (EGF) suppress hepcidin mRNA (Figure 1.4), possibly by blocking the translocation of p-SMAD1/5/8 to the nucleus in hepatocytes [94]. Consistently, it has been shown that growth signals involving the rapidly accelerated fibrosarcoma (Ras-RAF) mitogen-activated protein kinase (MAPK) signaling pathway, target of HGF and EGF, repress hepcidin transcription [95] (Figure 1.4). Proliferative signals involving the nutrient-dependent mammalian target of rapamycin (mTOR) signaling pathway have also been linked to the repression of hepcidin [95] (Figure 1.4). Furthermore, gluconeogenic signals

increase hepcidin levels through the activation and cooperative binding of CREBH and the peroxisome proliferator and activated receptor gamma coactivator 1-α (PGC-1α) to the promoter of hepcidin [96] (Figure 1.4).

1.1.5 Iron-Related Diseases

Perturbation of systemic iron homeostasis is associated with disease. The common cause for most of the iron-related disorders is the dysregulation of the hepcidin-ferroportin axis. Iron-related diseases are divided into an iron-overload or iron-deficiency disorders, depending on whether iron is in excess or depleted in the body.

Iron-overload disorders

Accumulation of iron in the body leads to hereditary hemochromatosis (HH), which is characterized by high oxidative stress and organ failure. HH is a genetically heterogenous autosomal recessive disorder, divided into four types according to its severity (reviewed in [97, 98]) (Table 1.1). The HH type I is typically a relatively mild and late onset form and it is the most frequent type of HH, more than 80% of all HH cases. It is characterized by mutations in the *Hfe* gene [99] leading to an inability of HFE to be expressed on the cell surface [100]. It has been described that the mutated HFE protein fails to prevent ALK3 receptor's ubiquitination and proteasomal degradation [75]. Therefore, it may be the cause of inappropriately low hepcidin levels in patients with HH type I [101, 102]. HH type II is a more severe type of HH. Patients present first symptoms of iron overload during childhood (juvenile HH). HH type II is characterized by mutations either in the *Hjv* gene (type II-a) [103] or in the hepcidin gene (type II-b) [104]. Both cases of HH type II are associated with extremely low hepcidin levels. The HH type III is a rare form of HH. This type of HH is similar to HH type I in terms of disease progression however the mutation is

present in the *Tfr2* gene [105]. Similarly to other types of HH, a mutation in this gene impairs the capacity of liver cells to sense iron levels and results in low hepcidin expression. The genetics of the HH type IV differs from other types of HH. The HH type IV is autosomal dominant. Contrasting to other types of HH, which affect the expression of hepcidin, HH type IV is associated with mutations in the ferroportin gene [14]. Two different types of mutations have been identified, which lead to different phenotypes. One type results in a gain of function of ferroportin causing resistance to hepcidin [106]. As a consequence, iron is continuously exported from the iron-releasing cells hence generating iron overload in the serum [107]. The second type of mutation results in the loss of the ferroportin function [108]. The lack of a functional ferroportin leads to iron retention and accumulation in ferroportin-expressing cells but decreased iron in the serum.

Patients with HH can be treated by regular phlebotomy (blood withdrawal) in order to remove the iron-rich red blood cells. In the ferroportin disease, iron chelation can be an alternative treatment (reviewed in [97]).

Table 1.1: Hereditary Hemochromatosis types.

Hereditary Hemochromatosis	Mutated Gene	Pattern of Inheritance	Age of Symptoms Onset
Type I	*Hfe*	Autosomal recessive	30-40 years-old
Type II-a	*Hjv*	Autosomal recessive	10-20 years-old
Type II-b	*Hepc*	Autosomal recessive	10-20 years-old
Type III	*Tfr2*	Autosomal recessive	30-40 years-old
Type IV	*Fpn*	Autosomal dominant	30-40 years-old

Iron-deficiency disorders

Iron-deficiency can be caused by continuous blood loss or inadequate dietary iron absorption (reviewed in [109]). Genetically, low serum iron can be due to mutations in the *Tmprss6* gene. These mutations cause inappropriately high levels of hepcidin, preventing iron from being absorbed from the diet or released from stores. This phenotype leads to the iron-refractory iron deficiency anemia (IRIDA) [110].

Iron-deficiency can also occur as a consequence of persistent intestinal bleeding. Furthermore, disorders such as chronic inflammation or chronic liver and kidney diseases highly increase hepcidin expression, leading to a particular iron-deficiency type designated anemia of inflammation (AI) or anemia chronic diseases (ACD) [111]. There is no specific treatment for IRIDA or AI/ACD. Erythropoietic stimulators and supplementation of iron intravenously in patients are usually not effective. Thus, the control of hepcidin expression by pharmacological drugs holds a more promising avenue. Among these hepcidin antagonists are inhibitors targeting the BMP/SMAD and JAK/STAT pathways. Injections with soluble HJV have shown to efficiently down-regulate hepcidin levels. Although the preclinical results are encouraging, all these options are still being tested only in animal models. More advanced studies involve the production of humanized anti-hepcidin antibodies or antibodies against the hepcidin binding site on ferroportin and are currently under phase I of clinical trials (reviewed in [112]).

1.2 Cellular Metabolism

1.2.1 Overview of Metabolism

Metabolism is a life-sustaining process divided into two phases: i) catabolism is the energy (adenosine triphosphate (ATP))-producing process in which carbohydrates, fats and proteins are degraded to precursor molecules, and ii) anabolism is an ATP-consuming process in which precursor molecules are used to build up complex macromolecules. In order for cells to achieve a metabolic balance, they have to coordinate energy production and expenditure. These processes can be regulated in various ways. Enzymatic activities can be modulated by the availability of their substrates, by allosteric binding of small molecules that signal the metabolic state of the cell (such as ATP, citrate), or by the coordination of the metabolic activity between different tissues, which involve the production of growth factors and hormones (reviewed in [113]).

The citric acid cycle (also called the tricarboxylic acid (TCA) cycle or Krebs' cycle) occurs in the mitochondria and is central to energy metabolism. It accounts for the degradation of carbohydrates, fats and proteins to generate ATP while it also functions as a biosynthetic pathway to produce glucose, fatty acids and non-essential amino acids.

1.2.2 Citrate Metabolism

Citrate (citric acid) is an intermediate of the citric acid cycle that is generated as a result of the reaction of acetyl-coA with oxaloacetate, which is catalyzed by the citrate synthase (CS) enzyme in the mitochondria. The oxidation of citrate within the citric acid cycle accounts for the major source of cellular ATP production. The generation of citrate stimulates the flow of the cycle, which leads to the generation of reduced nicotinamide adenine dinucleotide (NADH), reduced flavin adenine dinucleotide ($FADH_2$) and succinate. These molecules are crucial for ATP production by the electron transport chain (ETC) and oxidative phosphorylation. Hence, the production of citrate correlates with the production of ATP, which means that high levels of citrate indicate that ATP levels are elevated (reviewed in [114, 115]). Under this condition, citrate adjusts the activity of the citric acid cycle enzymes, as well as the breakdown of glucose (glycolysis), to meet the cellular need for ATP (Figure 1.5).

High citrate levels slow-down the citric acid cycle by directly repressing the activity of the pyruvate dehydrogenase (the enzyme that generates acetyl-coA from pyruvate) (PDH) [116], and both the citric acid cycle enzymes citrate synthase (rate-limiting) [117] and succinate dehydrogenase (SDH) [118]. As a consequence of its accumulation in the mitochondria, citrate can also be transported to the cytoplasm. There, it regulates important key enzymes for glycolysis, gluconeogenesis and fatty acid synthesis. Citrate allosterically inhibits the activity of phosphofructokinase-1 (PFK1), the rate-limiting enzyme in glycolysis, which converts fructose 6-phosphate into fructose 1,6-bisphosphate (F1,6P) (reviewed in [119]). F1,6P is an activator of the pyruvate kinase (PK), which generates pyruvate. Therefore, by decreasing the levels of F1,6P, citrate indirectly reduces the activity of PK (reviewed in [120]). The

inhibition of PFK1 and PK activities leads to the reduction in pyruvate and acetyl-coA levels, resulting in reduced production of ATP. Apart from regulating glycolysis, citrate is also able to regulate the gluconeogenic process by inducing the activity of the fructose 1,6-biphosphatase (F1,6BPase). Therefore, citrate has a dual role in regulating glucose metabolism in the cytoplasm. It represses glycolysis and energy production whilst it can stimulate gluconeogenesis (reviewed in [119]).

High levels of citrate in the cytoplasm also stimulate fatty acid biosynthesis. Citrate is metabolized to oxaloacetate and acetyl-coA by the ATP citrate lyase (ACLY) enzyme. While oxaloacetate is converted to pyruvate and enters a new round of oxidation in the citric acid cycle, the acetyl-coA serves as a precursor for fatty acid synthesis (reviewed in [114]).

Another layer of complexity for citrate-dependent regulatory mechanisms emerges from the ability of citrate to regulate transcription. The regulation of gene expression is achieved by histone modifications, such as acetylation. Histone deacetylases and acetyltransferases target specific regions in the genome exposing or constraining promoters to the binding of transcription factors (reviewed in [121]). The activity of histone acetyltransferases (HATs) is limited by acetyl-coA levels. ACLY is found in the nucleus where it converts citrate to acetyl-coA, serving as a substrate for acetylation reactions. Among genes regulated by ACLY activity are genes encoding glycolytic enzymes, such as glucose transporter type 4 (GLUT4), hexokinase-2 (HXK2), PFK1 and lactate dehydrogenase A (LDHA) ([122]). Hence, citrate is postulated to control glycolysis directly by modulating the enzymatic activity of some of the enzymes involved in this pathway as well as by controlling their gene expression.

Other enzymes apart from citrate synthase and ACLY are involved in citrate metabolism. The mitochondrial aconitase (ACO2) acts in the citric acid cycle and converts citrate into iso-citrate in the mitochondria whereas the cytosolic aconitase (ACO1) is the functional homologue of ACO2 in the cytoplasm (reviewed in [123]). ACO1 can also act as an iron-binding protein depending upon the iron availability in the cell (previously described in Section 1.1.3). Citrate metabolism is summarized in Figure 1.5.

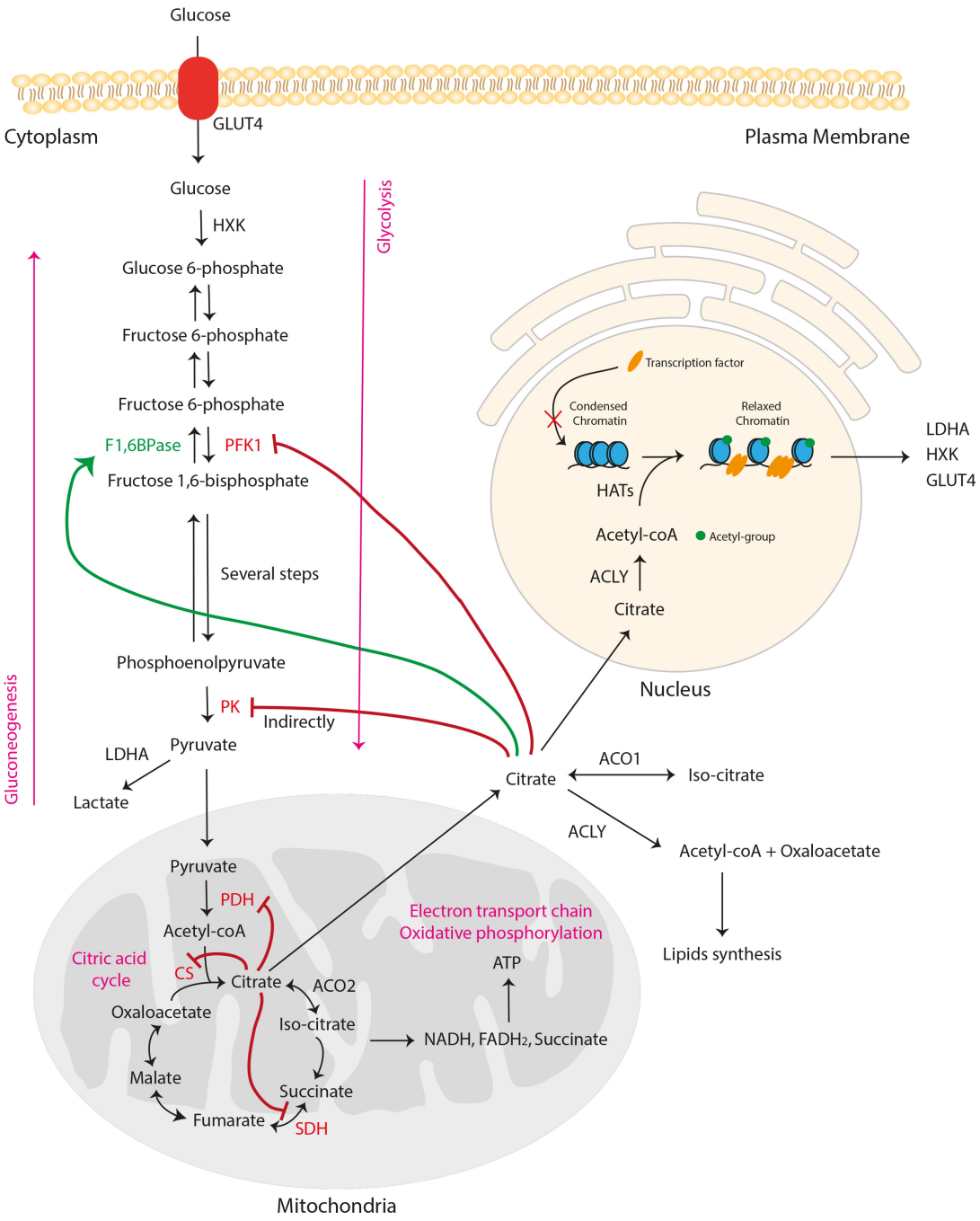

Figure 1.5: Citrate metabolism.
Glycolyis is stimulated in response to abundant glucose. As a consequence, pyruvate is synthesized in the cytoplasm and transported to the mitochondria where it is converted to acetyl-coA, which enters the citric acid cycle. Citrate is an intermediate synthesized in the citric acid cycle as a result of the condensation of oxaloacetate with acetyl-coA catalyzed by the CS enzyme. The enzyme ACO2 is the second enzyme of the citric acid cycle and converts citrate into iso-citrate. High flux through the citric acid cycle leads to the generation of ATP. Therefore, citrate and ATP levels correlate. When the cellular demand for ATP is fulfilled, citrate accumulates in the mitochondria and represses the enzymatic activity of PDH, CS and SDH thus reducing the flow of the cycle and ATP production. At the same time, citrate is transported to the cytosol where it inhibits the activity of the glycolytic enzymes PFK1 and

PK. This repression results in the attenuation of glycolysis and the supply of acetyl-coA to the citric acid cycle. Citrate can stimulate F1,6BPase and thus promotes gluconeogenesis. Citrate can be metabolized to iso-citrate by ACO1 or to acetyl-coA and oxaloacetate by ACLY in the cytoplasm. The acetyl-coA formed in the cytosol promotes lipogenesis. Citrate and the ACLY enzyme can also be found in the nucleus where they promote acetylation of histones and activate the transcription of genes involved in glycolysis, such as HXK, GLUT4 and LDHA. Adapted from Icard *et al,* 2012.

1.3 Iron and Metabolism

Increasing evidence suggests that the mechanisms involved in controlling iron homeostasis may influence metabolic processes. A single IRE sequence was identified in the 3'UTR of the glycolate oxidase mRNA (involved in ROS detoxification) by analysis of total poly(A)+ mRNA precipitated bound to recombinant IRP1 [124]. By computational analysis of the consensus sequence of IREs, IREs were identified in the 5'UTR of mRNA coding for enzymes such as δ-aminolevulinate synthase 2 (heme biosynthesis) [125], mitochondrial aconitase (citric acid cycle) and succinate dehydrogenase (citric acid cycle) [126]. Furthermore, cancer cells treated with iron show increased activity of citric acid cycle enzymes such as mitochondrial aconitase, citrate synthase and iso-citrate and succinate dehydrogenases compared to untreated cells [127]. Conversely, cells treated with desferrioxamine (an iron chelator) show reduced enzymatic activities of the same enzymes. Therefore iron availability has an impact on the citric acid cycle activity, which in turn impacts on energy production [127]. Consistent with this, others have shown that rats fed with an iron deficient diet show reduced activities of the cytochrome c, cytochrome c oxidase I and succinase dehydrogenase enzymes (crucial for the electron transport chain - energy production) in skeletal muscle cells [128].

In turn, several studies have shown that metabolic changes are able to regulate iron homeostasis. Epinephrine and norepinephrine are hormones that increase the rate of energy metabolism. Treatment of hepatocytes, skeletal muscle cells and mice with epinephrine/norepinephrine stimulates the binding of IRP1 to IRE sequences located in TfR1 and ferritin mRNAs. This leads to the stabilization of the TfR1 transcript, and

increased transferrin bound iron uptake. In addition, ferritin translation is inhibited, which causes reduced iron storage. The resulting increase in iron availability supports the activity of iron-dependent proteins, which are involved in the citric acid cycle and electron transport chain [129].

Iron metabolism and hepcidin transcriptional control have been linked to the nutrient-sensing mTOR pathway. The mTOR pathway regulates crucial metabolic processes such as cell size, proliferation, survival and lipid synthesis (reviewed in [130]). A siRNA screen of the human signaling proteome in HeLa cells identified members of the mTOR signaling pathway which control transferrin uptake. siRNA-mediated knockdown of tuberous sclerosis complex 2 and phosphatase and tensin homolog (known mTOR suppressors) enhances transferrin uptake whereas knockdown of pyruvate dehydrogenase kinase 1, RAC-alpha serine/threonine-protein kinase and mTOR (known activators) suppresses transferrin uptake [131]. Furthermore, it has been described that incubation of mouse preadipocytes and lung fibroblasts with rapamycin (inhibitor of the mTOR signaling pathway) induces the phosphorylation of the iron-sulfur cluster assembly enzyme (ISCU). The stabilization of the ISCU protein results in an increase in its abundance in the mitochondria thus potentiating the assembly of Fe-S clusters [132]. Moreover, siRNA-mediated knockdown of activators of the mTOR signaling pathway increase hepcidin gene expression. Consistently, treatment of primary hepatocytes and hepatocytic cell lines with rapamycin lead to the activation of hepcidin transcription [95]. Work in mice has shown that mice in starvation, a condition in which gluconeogenesis is stimulated due to the lack of glucose intake, show increased levels of hepcidin compared to control mice [96], suggesting that gluconeogenic stimuli regulate hepcidin expression.

Iron perturbations have also been associated with metabolism-related diseases. Hepcidin mRNA levels are increased in patients with obesity [133]. Patients with metabolic syndrome show increased levels of hepcidin in the blood [134]. In turn, patients with diabetes mellitus show altered hepcidin levels, which can be either increased or decreased, depending upon the inflammatory status of the patient ([135-137].

1.4 Aim of the Study

Links between hepcidin regulation and alterations in metabolism have been identified. However, the underlying mechanisms are poorly understood. In my thesis project I set out to characterize links between cellular metabolism and hepcidin expression.

I took advantage of data obtained from a genome-wide small interfering-RNA (siRNA) screen previously performed in our laboratory [95], which aimed to identify candidates for transcriptional regulators of hepcidin. This screen was performed by co-transfecting the hepato-cellular carcinoma cell line HuH7 with a reporter plasmid, containing the luciferase gene under control of the human full-length hepcidin promoter, and siRNAs targeting the near complete set of human protein-coding genes. Data obtained from this screen (about 1500 activators and 500 repressors) were analyzed by the DAVID tool to identify genes involved in metabolic reactions [95].

My specific aims were to:

I. Select putative hepcidin regulators identified in the RNAi screen, which are involved in metabolism.

II. Validate the putative regulators of hepcidin in primary mouse hepatocytes.

III. Perturb metabolic pathways associated with the validated effectors and investigate their role in hepcidin expression.

IV. Validate the data in mice *in vivo* and assess the physiological consequences of the manipulation of these effectors for hepcidin regulation.

Chapter 2
Experimental Procedures

2 Experimental Procedures

2.1 Frequently used reagents, chemicals and materials

Glycine, Tris-(hydroxymethyl)-methylamine (Tris), sodium chloride (NaCl), methanol, ethanol, isopropanol and sodium dodecyl sulphate (SDS), deoxyribonucleotides triphosphate (dNTPs), chloride acid (HCl), sodium bicarbonate ($NaHCO_3$) and Tween® were bought from Carl Roth GMBH. Citric acid (suitable for cell culture), fumaric acid (suitable for cell culture), succinic acid (suitable for cell culture), α-ketoglutaric acid disodium salt monohydrate, potassium citramalate monohydrate, Dulbecco's phosphate buffered saline (PBS), trypan-blue, dexamethasone, insulin from bovine pancreas, ethylenediaminetetraacetic acid (EDTA), NP40, glycerol, β-merceptoethanol, bromophenol blue, Triton X-100, Empigen BB, lithium chloride (LiCl) and sodium deoxycholate were obtained from Sigma-Aldrich. Opti-MEM®, fetal bovine serum (FBS), sodium pyruvate, liver perfusion medium and liver digest medium were obtained from Gibco. Williams' medium E and penicillin/streptomycin were purchased from Biochrom. Percoll and nitrocellulose membranes were obtained from GE Healthcare. Random primers, salmon sperm DNA and GlutaMAX were purchased from Invitrogen. The compound BMS-30314 was obtained from Tocris. Lipofectamine RNAiMAX Transfection Reagent and RevertAid H Minus Reverse Transcriptase were purchased from Thermo Scientific. Protein A/G magnetic beads were obtained from Pierce. Tungsten carbide beads (3 mm) were purchased from Qiagen. Cell culture multi-well collagen I-coated plates and dishes were obtained from (Corning). Tubes and falcons were obtained from Sarstedt or Greiner Bio-One. TRIzol®Reagent was bought from Ambion. SYBR-Green was obtained from Applied Biosystems.

2.2 Buffers and Solutions

Running buffer for SDS-PAGE:
25 mM Tris
192 mM Glycine
0.1% SDS

Transfer buffer for Western blot:
25 mM Tris
192 mM Glycine
10% Methanol

RIPA buffer for protein extraction:
10 mM Tris-HCl pH 8.0
150 mM NaCl
1 mM EDTA
1% NP-40
0,1% SDS

Laemli Buffer for protein loading (4x):
250 mM Tris-HCl pH 6.8
8% SDS
40% Glycerol
10% β-Mercaptoethanol
0.06% Bromophenol blue

TBS-Tween 0.1% for washing of protein membranes:
100 mM Tris-HCl pH 7.6
150 mM NaCl
0.5% TWEEN®20

RC buffer:
250 mM Saccharose
50 mM KCl
5 mM MgCl2
20 mM Tris HCl pH 7.4

2.3 Oligonucleotides, small-interfering RNAs and antibodies used in this study

All antibodies, oligonucleotides and small-interfering RNAs (siRNAs) used in this study are listed below. All primers were purchased from Sigma-Aldrich.

Table 2.1: List of oligonucleotides used in this study.

Mouse target gene	Forward primer 5'- 3'	Reverse primer 5'- 3'
Acly	aagaaggaggggaagctgat	tcgcatgtctgggttgttta
Aco1	gtcgccaccattctttgaa	ggcatccactatggacttgg
Aco2	gttgggggtgagaaagacct	gaagcccacaccatacttgg
Smad1	cccctaccactataagcgagtg	aaggctgtgctgaggattgt
Smad4	acaccaacaagtaacgatgcc	gcaaaggtttcactttcccca
Bmpr1a	tggctgtctgtatagttgctatg	tgcttgagatactcttacaataa
Hjv	ccaacgctaccaccatcc	cccccattgatagaacccatct
Hfe	caccgtctgtgccatcttctt	acatagccacccatggttcct
Bmp4	tgagtacccggagcgtcc	ctccagatgttcttcgtgatgg
Bmp6	atggcaggactggatcattgc	ccatcacagtagttggcagcg
Bmp7	cgagaccttccagatcacagt	cagcaagaagaggtccgact
Bmp9	ggaagctgtgggtagatgacc	caagtcggtggggatgat
Tgfb1	tggagcaacatgtggaactc	cagcagccggttaccaag
Id1	accctgaacggcgagatca	tcgtcggctggaacacatg
Nact	tcacaaaagcccaagttcaa	ggggtagaacggagttttcc
Nadc3	tggatctccttcctctatggtg	ctgcgtcagctcgtatctttg
Hamp1	ataccaatgcagaagagaagg	aacagataccacactgggaa
Rpl19	aggcatatgggcatagggaagag	ttgaccttcaggtacaggctgtg
*Hamp1*_BMPRE	gagccacagtgtgacatcac	gtctaggagccagtcccagt

Table 2.2: List of antibodies used in this study.

Target protein	Dilution	Provider
Vinculin	1:250	Sigma-Aldrich
SMAD1	1:1000	Cell signaling
p-SMAD1/5/8	1:1000	Cell signaling

Table 2.3: List of siRNAs used in this study.

Mouse siRNA name	Gene symbol/siRNA ID	Provider
FlexiTube siRNA Mm_Smad1_1	*Smad1*/SI00177072	Qiagen
AllStars Negative Control siRNA	NA/1027280	Qiagen
Silencer®Select Negative Control #2 siRNA	NA/4390846	Ambion
Silencer®Select Pre-designed and validated siRNA Bmp4	*Bmp4*/s63022	Ambion
Silencer®Select Pre-designed and validated siRNA Smad4	*Smad4*/s201661	Ambion
Silencer®Select Pre-designed and validated siRNA Bmpr1a	*Bmpr1a*/s63041	Ambion
Silencer®Select Pre-designed and validated siRNA Aco1	*Aco1*/s61844	Ambion
Silencer®Select Pre-designed and validated siRNA Aco1	*Aco1*/s61842	Ambion
Silencer®Select Pre-designed and validated siRNA Hfe2	*Hjv*/s87981	Ambion
Silencer®Select Pre-designed and validated siRNA Hfe	*Hfe*/s67488	Ambion
Silencer®Select Pre-designed and validated siRNA Aco2	*Aco2*/s61847	Ambion
Silencer®Select Pre-designed and validated siRNA Aco2	*Aco2*/s61845	Ambion
Silencer®Select Pre-designed and validated siRNA Acly	*Acly*/s98225	Ambion
Silencer®Select Pre-designed and validated siRNA Acly	*Acly*/s98226	Ambion
Silencer®Select Pre-designed and validated siRNA Slc13a5	*Nact*/s108630	Ambion
Silencer®Select Pre-designed and validated siRNA Slc13a3	*Nadc3*/s100155	Ambion

2.4 Mice

Mice used for the isolation of primary hepatocytes were wild type C57BL6/N, male and aged between eight- and thirteen-weeks old. Mice used for *in vivo* studies were male C57BL6/J WT aged eight weeks old. C57BL6/N mice were housed in the University of Heidelberg animal facility under a constant light-dark cycle and maintained on a standard mouse diet with 200 mg of iron/kg of food (D.Rod18.A07,

LASvendi) under *ad libitum* access to food and water. The isolation of hepatocytes from mice was approved by the University of Heidelberg. C57BL6/J mice were housed in the EMBL animal facility. C57BL6/J were housed under a constant light-dark cycle and maintained on a standard mouse diet with 200 mg of iron/kg of food (Teklad 2018S, Harlan Laboratories) under *ad libitum* access to food and water. *In vivo* experiments were approved by EMBL and conducted according to the guidelines of the EMBL institutional Animal Care and Use Committee.

2.5 Methods

2.5.1 Isolation of primary murine hepatocytes

Hepatocytes were isolated as previously described in literature [138]. Mice were euthanized and the full liver was collected. Liver lobes were cut transversely to expose the portal vein and placed on top of a warm surface. Liver cells were isolated by perfusion of all individual lobes with 100 ml of liver perfusion medium followed by 100 ml of liver digest medium at 37°C by using a 24G cannula connected to a peristaltic pump with a speed of 8 ml/minute. Cells were then filtered with a 100 μm strainer and purified using a 1.06 g/ml diluted in PBS. Cell viability was accessed by trypan-blue exclusion and cells were seeded in William's E medium containing 10% of heat-inactivated FBS, 1% penicillin/streptomycin, 1% GlutaMAX, 0.1 nM dexamethasone and 10 mg/ml insulin into collagen I-coated plates. After 5 hours, the medium was replaced by William's E medium with a different supplement according to the experiment (see sections below). Experiments were either performed immediately or 16 hours after medium change. Hepatocytes were maintained at 37°C with 5% of carbon dioxide (CO_2).

2.5.2 RNAi experiments

Five hours after seeding, hepatocytes (250 000 cells in 12-well plate) were washed 3 times with PBS and then William's medium E with 10% of heat-inactivated FBS and 1% GlutaMAX was added to cells. Transfections of siRNAs were performed by adding 12 pmol of commercially available siRNAs (Table 2.3) and 2 ml of Lipofectamine RNAiMAX Reagent into cells according to manufacturer's recommendation. The medium was replaced after 24 hours of transfection by William's E medium containing 10% of heat-inactivated FBS, 1% penicillin/streptomycin and 1% GlutaMAX and cells were collected 72 hours after transfection for total RNA extraction. When combined with citrate treatment, the medium was changed to William's E medium with 1% penicillin/streptomycin and 1% GlutaMAX (without FBS) 38 hours after transfection. Cells were incubated with this medium for 1 hour and then 5 mM of a citrate solution or water was added for 9 hours. Hepatocytes were collected then for total RNA extraction.

2.5.3 Treatment of hepatocytes with small molecules

Five hours after seeding, hepatocytes were washed 3 times with PBS and William's medium E with 10% of heat-inactivated FBS, 1% penicillin/streptomycin and 1% GlutaMAX was added to cells. Treatments were conducted after one hour period of starvation from FBS in Williams' medium E with 1% penicillin/streptomycin and 1% GlutaMAX (approximately 16 hours after cell washing and medium replacement).

Potassium citramalate monohydrate (or water) and BMS-30314 (or DMSO) were added to the culture medium at a final concentration of 3 mM and 50 μM respectively. Hepatocytes were collected 3 hours after treatment with citramalate and 90 min after treatment with BMS-30314 for total RNA extraction (250 000 cells in 12-well plate) or measurements of enzymatic activities (750 000 cells in 6-well plate).

Water or citric acid (citrate) were added to the cell culture medium at a final concentration of 1 mM and 3 mM. Hepatocytes were collected 3 hours, 6 hours and 9 hours after treatment for total RNA extraction (250 000 cells in 12-well plate). Hepatocytes were also treated for 6 hours with 1 mM and 3 mM of citrate or water and collected for citrate measurements (750 000 cells in 6-well plate).

Fumaric acid, succinic acid, α-ketoglutaric acid disodium salt dihydrate, oxaloacetic acid and malic acid disodium salt were added to the cell culture medium at a final concentration of 1 mM or 3 mM. Hepatocytes were collected 3 hours, 6 hours and 9 hours after treatment for total RNA extraction (250 000 cells in 12-well plate).

2.5.4 Measurement of ACLY enzymatic activity

The enzymatic activity of ACLY was measured via the malate dehydrogenase-coupled method in protein extracts as described before [139, 140]. Samples were homogenized in RC buffer and the supernatant was collected. Triton-X was added to a final concentration of 0.01% and the supernatant was incubated for 20 min on ice. 200 µl of reaction mix (1 mM ADP, 10 mM potassium phosphate, 1 mM oxaloacetate, 0.25 mM acetyl-coA, 100 mM 5,5'-dithio-bis(2-nitrobenzoic acid) was added to 20 µl of the cell lysate and the absorbance was measured at 412 nm on kinetic mode (SpectraMax Plus 384, VWR). ACLY activity was calculated using the speed of reaction automatically measured by the spectrophotometer and normalized with regards to the total protein concentration in the cell lysate.

2.5.5 Measurement of ACO1 and ACO2 enzymatic activities

The activity of cytosolic and mitochondrial aconitase was performed by using a colorimetric aconitase assay kit (Sigma-Aldrich) according to manufacture's instructions. Hepatocytes were homogenized in ice-cold assay buffer and centrifuged

to remove insoluble material. Half of the supernatant was directly used for the cytosolic aconitase assay whereas the remaining sample was re-centrifuged. The resulting pellet was dissolved in assay buffer, sonicated and used for the mitochondrial aconitase assay. All samples were activated by adding the aconitase activation solution and loaded into a 96-well plate. The reaction mix was added to every sample and standard and the absorbance was measured at 450 nm against blank in a spectrophotometer (SpectraMax-M2e, VWR). Calculation of the enzymatic activities based on the absorbance was made as indicated by the manufacturer. The activity of ACLY detected in each sample was normalized against the protein content. Protein concentration was determined directly from the cell extracts using the DC Protein assay from Bio-Rad according to manufacturer's instructions.

2.5.6 MTT assay

Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described in literature but with some modifications [141]. 5 mg/ml of MTT solution was added to the culture and cells were incubated for 2 hours and 30 min at 37°C with 5% CO_2. 10% SDS was added to the culture medium and cells were incubated for 16 hours at 37°C. The absorbance was measured at 570 nm in a spectrophotometer (SpectraMax-M2e, VWR) and the background absorbance at 690 nm was subtracted.

2.5.7 Citrate measurements

Citrate measurements were performed by using a colorimetric citrate assay kit from Sigma-Aldrich according to manufacture's instructions. Hepatocytes (750 000 cells) or tissues (about 20 mg) were homogenized in citrate assay buffer and centrifuged to isolate insoluble material. The supernatant and standards were added to the reaction mix and the absorbance was measured at 570 nm against blank in a

spectrophotometer (SpectraMax-M2e, VWR). Calculation of the citrate level based on the absorbance was made as indicated by the manufacturer. The citrate level in the cell extracts was normalized to the protein content. Protein concentration was determined directly from the cell extracts using the DC Protein assay from Bio-Rad according to manufacturer's instructions. The citrate level in extracts from liver was normalized to the tissue weight.

2.5.8 RNA extraction, RT-PCR and quantitative real-time PCR

Total RNA was isolated from cells using the RNeasy Plus Mini kit (Qiagen) according to manufacturer's instructions. Total RNA (500 ng) was reverse-transcribed in a 25 ml reaction mixture using RevertAid H Minus Reverse Transcriptase and random primers. Total RNA was isolated from tissues by using TRIzol®Reagent according to manufacture's instructions. Approximately 20 mg of tissue was lysed in TRIzol and homogenized with 3 mm tungsten carbide beads in a tissue lyser (Qiagen). Total RNA was then purified by phenol-chloroform and washed with 70% ethanol. RNA (1 mg) was reverse-transcribed in a 25 ml reaction mixture using RevertAid H Minus Reverse Transcriptase and random primers. SYBR-green quantitative real-time PCR (qPCR) was performed using the ABI StepONE Plus real-time PCR system with the primers listed in Table 2.1. Relative mRNA expression was normalized to *Rpl19* (ribosomal protein L19) mRNA. Results were calculated by using the ΔΔCt method.

2.5.9 Protein extraction and Western blot analysis

Proteins were extracted by lysing about 20 mg of tissue with RIPA buffer with 1x protease inhibitor cocktail (Roche) and phosphatase inhibitors (Roche). After 30 min, extracts were centrifuged to remove insoluble material and the supernatant was used to measure protein content. Protein concentration was determined by using the

DC Protein assay from Bio-Rad according to manufacturer's instructions. Cell protein extracts (80 mg) or tissue protein extract (50 mg) were then used for Western blot analysis. Extracts were mixed with 1x Laemmli buffer and boiled at 95 °C for 10 min. Proteins were separated by size in a 10% SDS-PAGE and transferred to a nitrocellulose membrane by a wet system. Membranes were blocked with 5% milk powder in TBS-0.1% tween for 1 hour at room temperature. Primary antibodies are listed in Table 2.2 and were incubated overnight at 4°C except for the vinculin antibody, which was incubated for 1 hour at room temperature. Membranes were washed with TBS-0.1% tween and incubated with anti-IgG of the respective source of production for 1 hour in 5% milk in TBS-0.1% tween (listed in Table 2.3). After washing, proteins were detected by using ECL-plus reagent and visualized with a Fusion FX system (Peqlab). Proteins were normalized to vinculin. Protein quantification was performed by using the ImageJ software available online (https://imagej.nih.gov/ij/).

2.5.10 Tail vein injections

Mice were administered 10.5 mg of citrate/kg of body weight or vehicle (PBS) via the tail vein. The citrate concentration of the citrate solution used for the injections was 2 ng of citrate/ml of PBS pH 7.0 and the volume administered in the mouse was an average of 130 µl. Mice were fasting for 4 hours before citrate or PBS administration and euthanized with isoflurane 2 hours after injections. The blood was removed by cardiac puncture and the liver was collected for downstream applications.

2.5.11 Measurement of blood parameters

The blood was collected from PBS and citrate injected mice by cardiac puncture and added to tubes with heparin to prevent coagulation. All hematological parameters were determined by introducing each blood sample to the ScilVet ABC

blood analyzer machine. After measurement of hematological parameters, the blood was centrifuged 10 min at 2000xg to isolate plasma. The plasma was then collected to measure hepcidin levels.

2.5.12 Measurement of hepcidin in the plasma

Hepcidin levels in the plasma were assessed by using the Hepcidin-Murine Compete ELISA (Intrinsic Lifesciences) according to manufacturer's instructions. After measuring the absorbance of the samples and standard, the levels of hepcidin were calculated based on the equation of a 4PL plot (curve expert program), as recommended, and represented as ng/ml.

2.5.13 Statistical Analysis

Experiments with primary murine hepatocytes represent a mean of at least 3 independent experiments. *In vivo* experiments are represented by mean of 8 mice per group in a total of two independent experiments. All graphs are represented as relative expression or fold-change plus standard error of the mean (SEM). A two-tailed Student's t-test was used for estimation of statistical significance except for when more than one variable was being taken into consideration. In this case, a two-way ANOVA was used to estimate statistical significance. Data with a P value less than 0.05 was considered significant ($*P<0.05$,$**P<0.001$,$***P<0.0001$).

Chapter 3
Results

3 Results

3.1 Citrate metabolism cross-talks to the regulation of hepcidin mRNA levels

Genome-wide RNAi screens are a powerful technique to unveil new effectors involved in particular processes. The unbiased siRNA screen performed in HuH7 cells in our laboratory identified about two thousand genes, which potentially affect hepcidin expression [95]. By applying DAVID analysis, the candidate hepcidin regulators were classified according to various annotation terms, including gene ontology and biochemical and signaling pathways. The DAVID analysis enabled the identification of metabolic processes contained within the putative regulators of hepcidin. These functional categories varied from broad to more specific and are represented in Table 3.1.

The "citrate metabolic process" category was selected for this study because i) citrate is central to energy metabolism, and ii) citrate is the substrate of the iron-regulatory protein 1, involved in the regulation of cellular iron metabolism. This category was contained within the putative hepcidin activators and included three genes: ACO1 (cytosolic aconitase), ACO2 (mitochondrial aconitase) and ACLY (ATP citrate lyase). ACO1 and ACO2 metabolize citrate into iso-citrate in the cytosol and mitochondria, respectively, whereas ACLY produces oxaloacetate and acetyl-coA from citrate in the cytosol (reviewed in [114, 123]). I next set out to investigate whether perturbation of citrate metabolism influences hepcidin transcriptional control.

The screening data in HuH7 cells showed a validation rate of 50% [95]. I further validated the initial findings in HuH7 cells by using primary murine hepatocytes (PMH), which are a more physiological system compared to the cancer cell line HuH7 cell.

Table 3.1: List of enriched metabolic groups obtained from DAVID analysis of the RNAi screening data. Obtained from Mleczko-Sanecka *et al*, 2014.

Category - hepcidin activators	**No. of genes**	***P* value**
Macromolecule metabolic process	462	$1.5x10^{-2}$
Citrate metabolic process	3	$1.2x10^{-2}$
Protein metabolic process	237	$2.2x10^{-2}$
Porphyrin metabolic process	3	$4.8x10^{-2}$
Proton-transporting ATPase activity	5	$8.8x10^{-2}$
Cellular lipid metabolic process	21	$1.6x10^{-2}$
Lysophosphatase activity	4	$5.5x10^{-2}$
Fatty acid binding	7	$6.0x10^{-2}$
Category - hepcidin suppressors	**No. of genes**	***P* value**
Cellular lipid metabolic process	21	$1.6x10^{-2}$
Regulation of macromolecules metabolic process	25	$4.4x10^{-2}$
Fatty acid metabolism	4	$7.6x10^{-2}$
Carbohydrate metabolic process	18	$9.3x10^{-2}$
Vitamin A metabolic process	3	$9.4x10^{-2}$
Oxidoreductase activity, acting on NADH, NADPH, quinone or similar as acceptor	5	$2.4x10^{-2}$
Regulation of catalytic activity	27	$6.9x10^{-2}$
Regulation of cell biosynthetic process	21	$3.6x10^{-2}$
Isoprenoid metabolic process	4	$7.7x10^{-2}$
NAD or NADH binding	4	$8.9x10^{-2}$
Acylglycerol metabolic process	4	$9.9x10^{-2}$

To validate ACO1, ACO2 and ACLY as regulators of hepcidin, I first performed siRNA-mediated knockdown in PMH. As positive controls, components of the BMP/SMAD signaling pathway were subjected to RNAi. BMP receptor (BMPR1A) is an activator of hepcidin expression whereas SMAD7 is a negative control of the pathway [59, 62, 64]. Thus, hepatocytes from wild-type (WT) mice were transfected with a single scrambled siRNA, one single siRNA targeting *Bmpr1a* and *Smad7* (positive controls) or two independent siRNAs each for *Acly*, *Aco2* and *Aco1*. The scrambled siRNA was designed to present limited sequence similarity to known genes of the mouse and will be further referred to as unrelated control siRNA. The knockdown efficiency of each gene and endogenous hepcidin mRNA expression were determined by qPCR analysis. As depicted in Figure 3.1 (panel A), the knockdown of *Bmpr1a* (hepcidin activator) reduced hepcidin mRNA levels whilst

knockdown of *Smad7* (hepcidin repressor) resulted in an up-regulation of hepcidin mRNA expression. This result shows that the pathway that regulates hepcidin transcription in the liver is well preserved in primary hepatocytes. Knockdown of *Acly*, *Aco2* and *Aco1* significantly diminished *Acly*, *Aco2* and *Aco1* mRNA expression (to <25% of the control), which correlated with a significant increase in hepcidin mRNA expression (mean of 3-, 1.8- and 2-fold in *Acly*, *Aco1*, and *Aco2* knockdown, respectively) (Figure 3.1, panel B). In the case of the *Aco2* knockdown, only one siRNA was successful to alter hepcidin mRNA levels. Together, these results indicate that ACLY, ACO1 and ACO2 may be regulators of hepcidin expression in PMH. As shown in Table 3.1, *Aco1*, *Aco2* and *Acly* are contained within the group of putative hepcidin activators in HuH7 cells. Therefore, it was expected that the knockdown of these genes would lead to the down-regulation of hepcidin mRNA expression. However, my data show that knockdown of *Aco1*, *Aco2* and *Acly* up-regulates hepcidin in PMH. This discrepancy may be explained by the fact that primary cells show different physiology, which may have an impact on hepcidin regulation.

One disadvantage of using RNAi experiments is the possible occurrence of off-target effects. To consolidate my findings, I treated PMH with inhibitors of the enzymatic activities of ACLY, ACO1 and ACO2. The fetal bovine serum contains BMPs and other factors such as interleukins, which are known to strongly up-regulate hepcidin transcription (see Section 1.1.4). To avoid confounding effects of BMPs and interleukins on hepcidin mRNA expression, all subsequent experiments were performed without addition of serum to the cell culture medium.

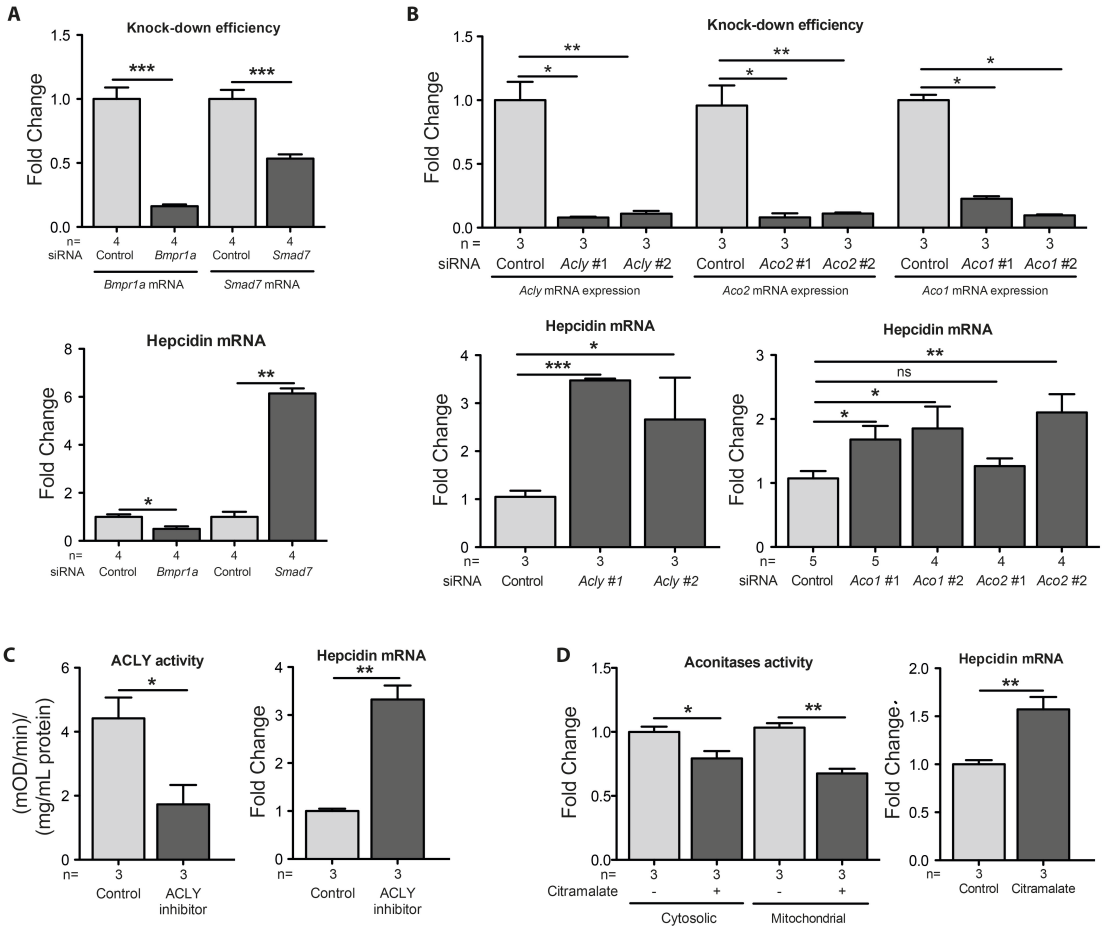

Figure 3.1: Inhibition of the activity of enzymes involved in citrate metabolism alters hepcidin mRNA levels in primary murine hepatocytes.

A) Hepatocytes were transfected with an unrelated control siRNA or siRNAs for *Bmpr1a* or *Smad7* for 72 hours; top graph: mRNA analysis of *Bmpr1a* and *Smad7*; bottom graph: mRNA analysis of hepcidin; **B)** PMH were transfected with an unrelated siRNA or 2 independent siRNAs (#1 and #2) for *Acly*, *Aco1* or *Aco2* for 72 hours; top graph: mRNA analysis of *Acly*, *Aco1* and *Aco2*; bottom left and right graphs: mRNA analysis of hepcidin. **C)** PMH were starved from serum for one hour and treated for 90 min with 50 μM of the ACLY inhibitor (BMS-30314); left graph: measurement of the activity of ACLY; right graph: analysis of the hepcidin mRNA level. **D)** Hepatocytes were starved from serum for one hour and treated for 3 hours with 3 mM of citramalate (inhibitor of the activity of aconitases); left graph: measurement of the activities of the cytosolic and mitochondrial aconitase; right graph: hepcidin mRNA levels analysis; *Rpl19* was used as reference gene. Data are represented as fold-change (+SEM) compared to the control except for the left graph in panel C, which is represented as optical density/minute normalized against the protein content (+SEM). Significant changes are marked with *(P<0.05), **(P<0.001) and ***(P<0.0001). ns - not significant.

The commercially available compound BMS-30314 has been described as an inhibitor of the ATP citrate lyase activity [142] *in vitro* and *in vivo*. Consistently, treatment of hepatocytes with this inhibitor resulted in a 50% decrease of ACLY activity (Figure 3.1, panel C, left graph). Concomitantly, the hepcidin mRNA level was elevated by 3.4-fold (Figure 3.1, panel C, right graph). The measurement of the ACLY activity was done in collaboration with Dr. Sven W. Sauer (University of Heidelberg). Citramalate has been suggested to be an inhibitor of the activity of mitochondrial aconitase in plants [143]. Curiously, PMH treated with citramalate showed reduced activity of both cytosolic and mitochondrial aconitase, 35% and 40% of inhibition respectively (Figure 3.1, panel D, left graph). In this condition, hepcidin mRNA expression was significantly up-regulated (Figure 3.1, panel D, right graph). These results support the data from the RNAi experiments in PMH, which identified ACLY, ACO1 and ACO2 as suppressors of hepcidin mRNA expression.

All together, these results show that citrate metabolism cross-talks to hepcidin expression as both suppression of the mRNA levels and inhibition of the enzymatic activity of ACLY, ACO1 and ACO2 leads to the increase in hepcidin mRNA levels.

3.2 Increased intracellular citrate levels regulate hepcidin mRNA expression

Cytosolic aconitase (ACO1), mitochondrial aconitase (ACO2) and ATP citrate lyase (ACLY) are enzymes that catalyze citrate-consuming reactions. ACO1 and ACO2 convert citrate into iso-citrate in the cytosol and mitochondria respectively, and ACLY metabolizes citrate into acetyl-coA and oxaloacetate in the cytosol (reviewed in [114, 122]). I have shown that inhibition of cytosolic and mitochondrial aconitases as well as of the ATP citrate lyase results in the up-regulation of hepcidin mRNA expression. If these three enzymes function by consuming citrate and converting it into other intermediates, it is tempting to hypothesize that the inhibition of their activity would lead to the accumulation of citrate in the cell. In fact, it has been described that the siRNA-mediated knockdown of *Acly* in cells increases

intracellular citrate levels [144]. It has also been shown that prostate cells show high citrate levels due to limiting activity of mitochondrial aconitase ([145] and reviewed in [146]). In addition, treatment of plants with citramalate (mitochondrial aconitase inhibitor) results in increased citrate levels in fruits [143]. Furthermore, isolated cells from citrus fruit calli grown in iron-free soil show elevated citrate levels compared to cells grown in iron-rich soil [147]. This is most likely due to reduced aconitase activity of IRP1 upon iron-deficiency [147]. Therefore, I tested whether increasing citrate levels in cells by supplementing citrate in the medium would up-regulate hepcidin mRNA expression in PMH.

Hepatocytes from WT mice either remained untreated or were treated with 1 mM and 3 mM of citrate. 3, 6 and 9 hours after treatment PMH were collected for further analyses. As depicted in Figure 3.2 (panel A, left graph), hepatocytes supplemented with citrate in the cell culture medium significantly up-regulated the mRNA expression of hepcidin up to 3.8-fold in a time- and dose-dependent fashion.

To investigate whether the hepcidin-response to citrate requires citrate uptake, I measured citrate levels in PMH untreated or treated with 1 mM or 3 mM of citrate for 6 hours. At this time-point hepcidin mRNA levels start to be strongly and significantly increased. As shown in Figure 3.2 (panel A, middle graph), intracellular citrate levels were significantly elevated in a dose-dependent manner compared to the control. The intracellular citrate concentration varies from 100-400 μM (up to 4x more) in mammalian cells [145]. The fact that citrate levels only increased by less than 1.5-fold after 6 hours of citrate supplementation compared to control, shows that citrate in PMH increased within the physiological range.

Citrate can reduce cell viability in high doses. For instance, cancer cells treated with citrate concentrations of 10-20 mM show a dramatic reduction of cell viability 24 hours after treatment [148, 149]. To analyze whether citrate supplementation reduces cell viability in PMH, I determined the cell viability by the methyl-thiazolyl-tetrazolium bromide (MTT) assay. The MTT assay is based on the ability of living and metabolically active cells to cleave the MTT into formazan, thus, the cell viability and degree of activation is proportional to the production of formazan. Due to its colorimetric property, formazan can be quantified by a

spectrophotometer [150]. As shown in Figure 3.2 (panel A, right graph), treatment of hepatocytes with 1 mM or 3 mM of citrate for 3, 6 or 9 hours does not result in a significant decrease in cell viability.

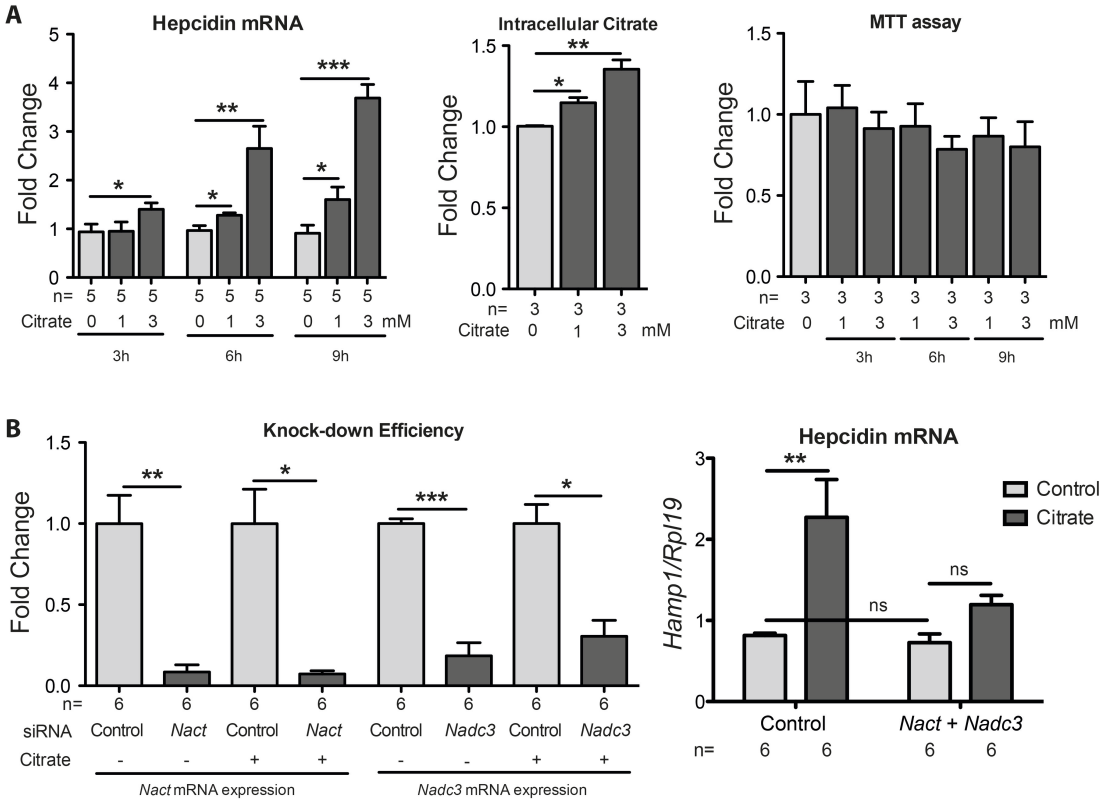

Figure 3.2: Increased intracellular citrate levels activate hepcidin expression.
A) left graph: hepcidin mRNA analysis of PMH treated with 1 mM or 3 mM of citrate for 3, 6 and 9 hours; middle graph: measurement of intracellular citrate levels of hepatocytes treated with 3 mM of citrate for 6 hours; right graph: analysis of cell viability by the MTT assay after treatment with 1 mM or 3 mM of citrate for 3, 6, and 9 hours**; B)** Hepatocytes were transfected with an unrelated control siRNA or siRNAs for *Nact* and *Nadc3* for 39 hours followed by 5 mM citrate treatment for further 9 hours. left graph: mRNA analysis of *Nact* and *Nadc3*; right graph: mRNA analysis of hepcidin. *Rpl19* was used as reference gene. Data are represented as fold-change (+SEM) compared to the control or as relative expression (*Hamp1*/*Rpl19*) (+SEM). Significant changes are marked with *(P<0.05), **(P<0.001) and ***(P<0.0001). ns - not significant.

The results presented above (Figure 3.2, panel A) show that hepatocytes externally treated with citrate up-regulate hepcidin mRNA expression likely due to

increased intracellular citrate concentration. If this is the case, inhibition of citrate uptake should prevent the citrate-induced hepcidin mRNA increase. Citrate is taken up in different cell types via the sodium-coupled dicarboxylate and citrate transporters, members of the SLC13 family. These plasma membrane transporters import citrate as well as other metabolites generated in the citric acid cycle with different affinities (reviewed in [151]). Particularly in the liver, the NaCT transporter (SLC13A5) is expressed in the sinusoidal membrane of hepatocytes [152] and shows high affinity for citrate whilst it transports succinate and malate with lower affinity [152, 153]. The NaDC3 (SLC13A3) transporter is also expressed in hepatocytes [154], however it has equal affinity for a wider range of citric acid cycle components. It transports citrate, succinate, fumarate, malate, oxaloacetate and α-ketoglutarate [155].

The citrate transporters NaCT and NaDC3 are expressed in hepatocytes [151, 152]. I next transfected PMH with either an unrelated control siRNA or siRNAs targeting the *Nact* and *Nadc3* mRNAs. A concentration of 3 mM citrate was applied in the time course supplementation experiment shown in Figure 3.2 (panel A). Because 3 mM citrate treatment in combination with siRNA-mediated transfections does not significantly alter the hepcidin mRNA expression (data not shown), a higher concentration of citrate was used. 39 hours after RNAi transfection, hepatocytes were treated with 5 mM citrate for a further 9 hours. The knockdown of *Nact* and *Nadc3* significantly reduced the expression of *Nact* and *Nadc3* (to 10% and 25% of the control respectively) (Figure 3.2, panel B, left graph). As represented in Figure 3.2 (panel B, right graph), hepatocytes transfected with a control siRNA responded to citrate by significantly up-regulating hepcidin mRNA expression (from a relative expression of 0.8 to 2.2). However, the knockdown of *Nact+Nadc3* attenuated the increase of hepcidin mRNA levels from 0.7 to 1.2 in response to citrate. The knockdown of *Nact+Nadc3* in absence of citrate treatment does not have any impact on hepcidin mRNA levels. These results show that the citrate transporters are necessary for the response of hepcidin to citrate.

The citrate transporters NaCT and NaDC3 transport other intermediaries of the citric acid cycle [151, 152]. To test whether succinate, malate, fumarate, α-

ketoglutarate and oxaloacetate have a role in regulating hepcidin expression, I treated PMH with 1 mM or 3 mM of these compounds for 3, 6 and 9 hours. As shown in Figure 3.3, hepcidin mRNA expression did not change with these treatments except for malate in one particular concentration and time-point (3 mM, 6 hours). Therefore, I conclude that the response of hepcidin to the supplementation of intermediaries of the citric acid is specific for citrate.

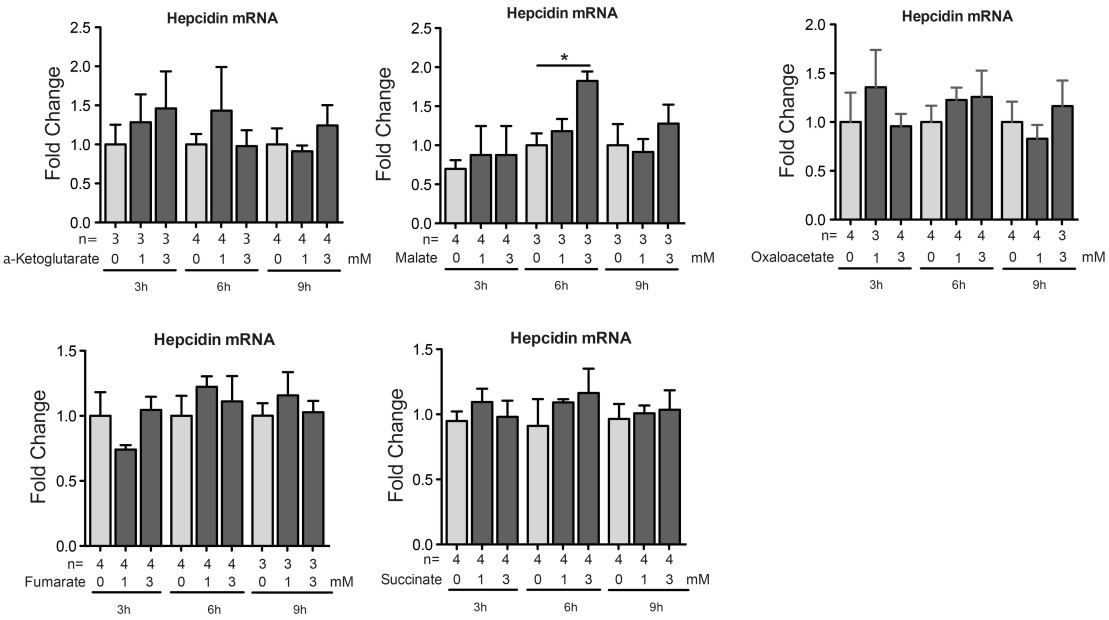

Figure 3.3: Citrate specifically regulates hepcidin mRNA levels.
mRNA analysis of PMH treated with 1 mM or 3 mM of α-ketoglutarate, malate, oxaloacetate, fumarate or succinate for 3, 6 and 9 hours. *Rpl19* was used as reference gene. The data is represented as fold-change (+SEM) compared to the control. Significant changes are marked with *(P<0.05).

3.3 The hepcidin response to citrate involves the BMP/SMAD signaling pathway

The BMP/SMAD signaling pathway controls hepcidin activation in response to iron levels and inflammatory stimuli [59, 83]. Furthermore, suppression of hepcidin mRNA levels by the nutrient-sensing mTOR signaling pathway requires functional

BMPREs, suggesting the involvement of the BMP/SMAD pathway in this response [95]. When this pathway is activated, BMPR1A induces the phosphorylation of the SMAD1/5/8 proteins, which then associate with the co-activator SMAD4, and bind to the promoter of hepcidin [57, 63].

Since the BMP/SMAD signaling pathway regulates hepcidin expression upon a variety of stimuli, I investigated whether the BMP/SMAD signaling pathway mediates the response of hepcidin to citrate. To test this hypothesis I first analyzed the mRNA expression of *Id1* in RNA samples from PMH treated with citrate for 3, 6 and 9 hours (the same samples used to generate the graph in Figure 3.2, panel A). *Id1* is a target gene of BMP/SMAD signaling and it is co-regulated with hepcidin following pathway stimulation [63]. Although the gene response pattern of *Id1* differed from the one of hepcidin (Figure 3.2, panel A, left graph), the mRNA of *Id1* was significantly up-regulated upon citrate treatment (Figure 3.4, panel A). The data suggest that the BMP/SMAD signaling pathway plays a role in regulating hepcidin upon citrate supplementation.

To understand the involvement of the BMP/SMAD signaling pathway in the citrate-mediated hepcidin response, I transfected PMH either with an unrelated control siRNA or with siRNAs targeting *Bmpr1a*, *Smad4* and *Smad1* mRNAs, and either treated cells or not with citrate for 9 hours. The knockdown of the *Bmpr1a*, *Smad4* and *Smad1* mRNAs resulted in the reduced mRNA expression of the *Bmpr1a*, *Smad4* or *Smad1* genes (to 10%, 5% and 15% of the control, respectively) (Figure 3.4, panel B) and in decreased levels of hepcidin mRNA (Figure 3.4, panel C). This result shows that the hepatocytes correctly responded to stimuli that regulate hepcidin expression. Consistent with previous results (Figure 3.2), PMH transfected with control siRNA and treated with citrate responded by significantly increasing hepcidin mRNA levels (Figure 3.4, panel C). However, knockdown of *Bmpr1a*, *Smad4* and *Smad1* resulted in a complete inability of hepcidin to respond to citrate (Figure 3.4, panel C). These results show that the BMP pathway either is mediating the response of hepcidin or must be functional for hepcidin to be regulated by citrate.

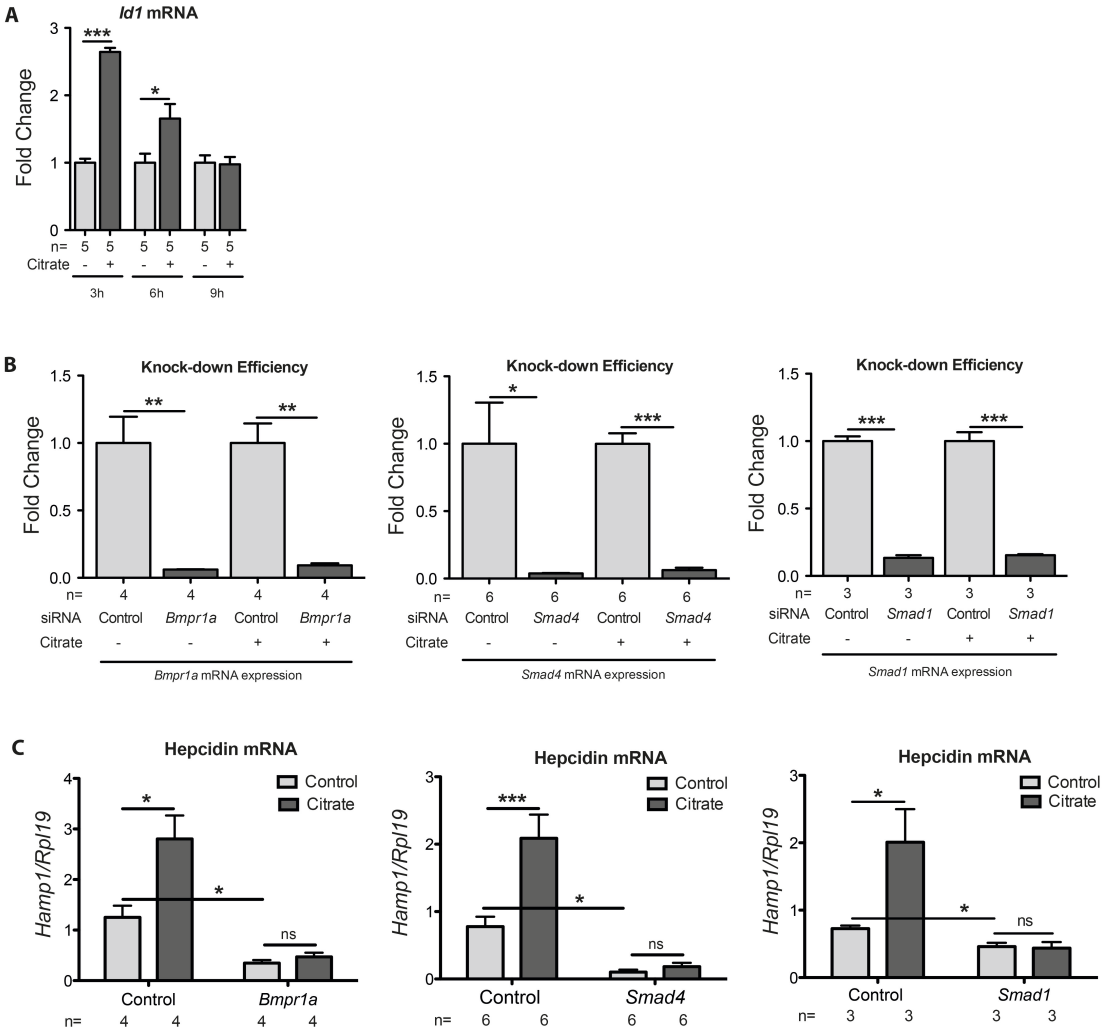

Figure 3.4: BMP/SMAD signaling and the hepcidin response to citrate.
A) *Id1* mRNA analysis of PMH treated with 3 mM of citrate for 3, 6 and 9 hours. Hepatocytes were transfected with an unrelated control siRNA or siRNAs for *Bmpr1a*, *Smad4* and *Smad1* for 39 hours following 9 hours of treatment with 5 mM of citrate. **B)** Cells were analyzed for the mRNA expression of *Bmpr1a* (left graph), *Smad4* (middle graph*)*, *Smad1* (right graph). **C**) Cells were analyzed for the mRNA expression of hepcidin. *Rpl19* was used as reference gene. Data are represented as fold-change (+SEM) compared to the control or in relative expression (*Hamp1*/*Rpl19*) (+SEM). Significant changes are marked with *(*P*<0.05), **(*P*<0.001) and ***(*P*<0.0001). ns - not significant.

To analyze whether the BMP/SMAD signaling pathway has an active role in regulating hepcidin upon citrate treatment, I treated PMH with citrate for 90 min, 3 hours and 6 hours, and performed Western blot analysis to detect the phosphorylation level of the SMAD1/5/8 transcription factors. The phosphorylation status of these proteins indicates the degree of activation of the BMP/SMAD pathway.

Unfortunately, phosphorylated SMAD proteins in extracts from untreated or citrate-treated cells were not detectable (data not shown). This may be explained by the fact that the citrate supplementation experiments were performed under serum-free conditions, which lack basal stimulators of this pathway that would usually be present in the serum. Without these stimulators, most likely the phosphorylation level of the SMAD proteins was below the limit of detection.

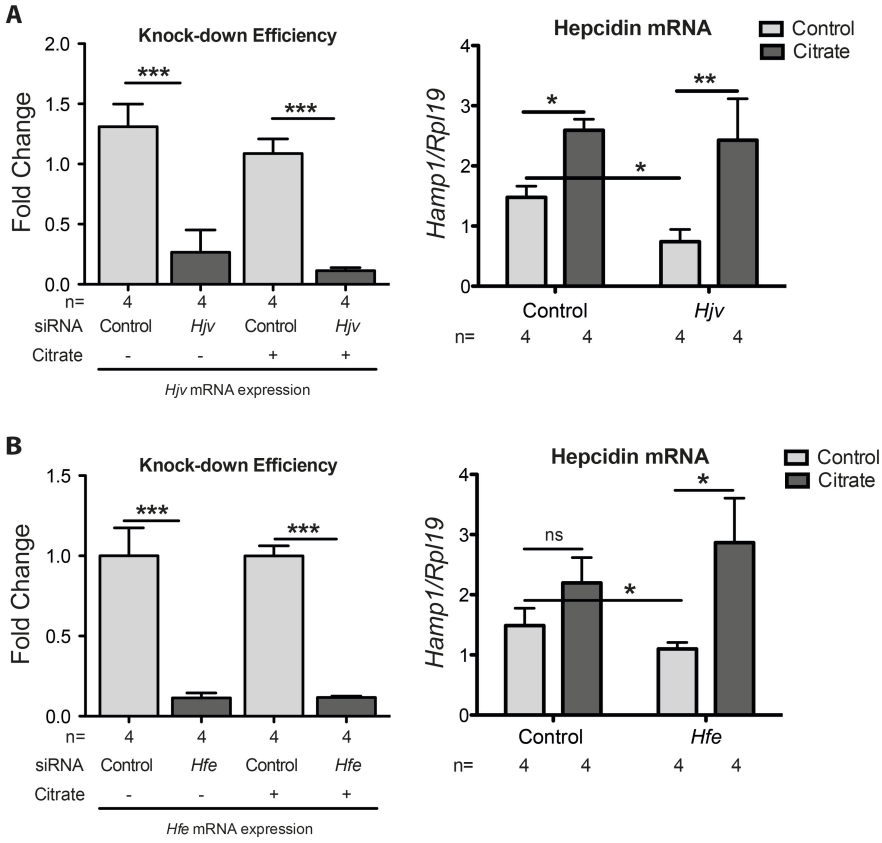

Figure 3.5: The response of hepcidin to citrate is independent of HJV and HFE proteins. Hepatocytes were transfected with an unrelated control siRNA or siRNAs for *Hjv* and *Hfe* for 39 hours following 9 hours of treatment with 5 mM of citrate or water. Cells were analyzed for the mRNA expression of **A)** left graph: *Hjv*, right graph: hepcidin; **B)** left graph: *Hfe*; right graph: hepcidin. The *Rpl19* gene was used a reference. Data are represented as fold-change (+SEM) compared to the control or in relative expression (*Hamp1*/*Rpl19*) (+SEM). Significant changes are marked with *(P<0.05), **(P<0.001) and ***(P<0.0001). ns - not significant.

The hemochromatosis proteins HFE and HJV are important upstream activators of hepcidin by the BMP/SMAD signaling pathway. HJV is a BMP co-receptor [67], which regulates the activity of the pathway [68], and HFE is known to stabilize the BMPR1A [75]. I next investigated whether the citrate response of hepcidin requires HFE and HJV. PMH were transfected with siRNAs targeting *Hjv* and *Hfe*, as well as a control siRNA, and were either treated or not with citrate for 9 hours. The mRNA levels of *Hjv*, *Hfe* and hepcidin were analyzed by qPCR. The mRNA expression of both *Hjv* and *Hfe* genes upon siRNA transfection was significantly reduced to at least 25% of the control, showing that the knockdown of these genes was efficient (Figure 3.5, panel A and B, left graphs). Reflecting the role of HFE and HJV as hepcidin activators, the reduction in *Hjv* and *Hfe* mRNA resulted in decreased hepcidin expression (Figure 3.5, panel A and B, right graphs). Consistent with previous experiments (Figures 3.2 and 3.4), hepatocytes transfected with an unrelated control siRNA and treated with citrate increased hepcidin expression. Interestingly, upon *Hjv* and *Hfe* knockdown, the hepcidin response to citrate was similar to that of control siRNA-transfected cells without citrate treatment (Figure 3.5, panel A and B, right graphs). This result suggests that the regulation of hepcidin expression in response to citrate is independent of the HFE and HJV proteins.

My data show that the BMP/SMAD signaling pathway is involved in regulating the hepcidin response to citrate (Figure 3.4). Several ligands have been identified to bind to BMP receptors and stimulate hepcidin expression. These ligands include BMP6, BMP4, BMP7, BMP9 and TGF-β [59, 156-158]. To investigate whether altered expression levels of BMP ligands play a role in the hepcidin response to citrate, I performed qPCR analysis of *Bmp6*, *Bmp4*, *Bmp9*, *Bmp7* and *Tgfb1* in total RNA extracts from hepatocytes treated with citrate for 3, 6 and 9 hours. *Bmp6* mRNA expression was not detected in control or citrate-treated PMH. As shown in Figure 3.6 (panel A), *Bmp4* was significantly regulated and follows an expression pattern that could be correlated with the pattern of hepcidin expression in the same conditions (Figure 3.2, panel A, left graph). The mRNA levels of *Bmp9*, *Bmp7* and *Tgfb1* did not significantly change when treated with citrate (Figure 3.6, panel A).

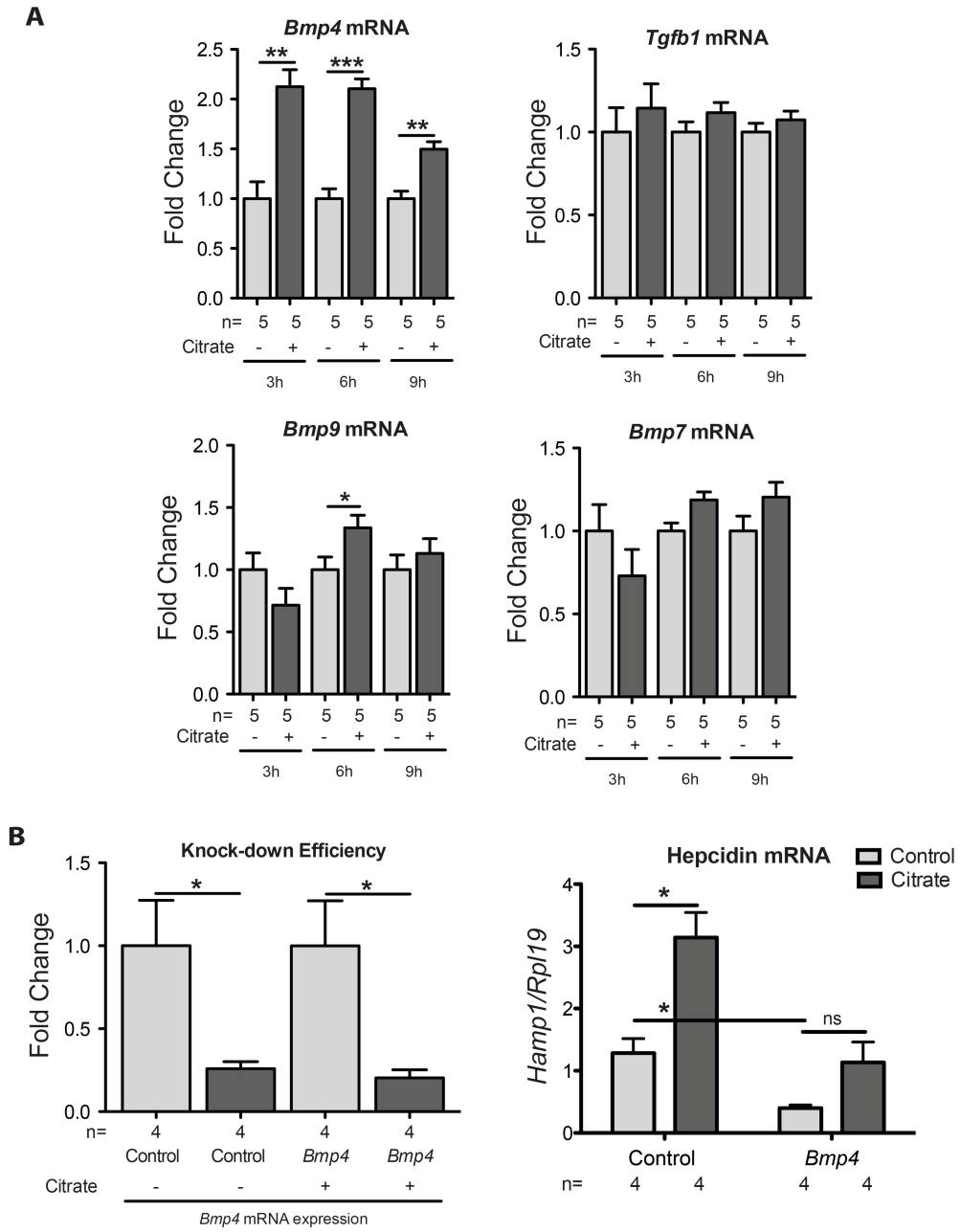

Figure 3.6: The hepcidin response to citrate is independent of BMP6, BMP4, BMP7, BMP9 and TGF-β.
A) RNA extracts from PMH treated with water of citrate for 3, 6 and 9 hours were analyzed for the mRNA expression of *Bmp4*, *Bmp7*, *Bmp9* and *Tgfb1*. **B)** Hepatocytes were transfected with an unrelated control siRNA or siRNA for *Bmp4* for 39 hours following 9 hours of treatment with 5 mM of citrate or water. Cells were analyzed for the mRNA expression of *Bmp4* (left graph) and hepcidin (right graph). The *Rpl19* gene was used as reference. Data are represented as fold-change (+SEM) compared to the control or in relative expression (*Hamp1*/*Rpl19*) (+SEM). Significant changes are marked with *(P<0.05), **(P<0.001) and ***(P<0.0001). ns - not significant.

To test whether BMP4 is involved in the citrate response of hepcidin, I performed siRNA-mediated knockdown of *Bmp4* in PMH under control conditions or upon citrate treatment. The mRNA expression of hepcidin was then analyzed by qPCR (Figure 3.6, panel B).

As depicted in Figure 3.6 (panel B, left graph), the knockdown of the *Bmp4* significantly decreased the mRNA level of *Bmp4*, showing that its knockdown was efficient. Consistent with BMP4 being an activator of hepcidin, the knockdown of *Bmp4* resulted in a decrease in hepcidin expression (Figure 3.6 panel B, right graph). Although the mRNA levels of *Bmp4* were diminished upon knockdown, the hepcidin gene responded similarly to citrate treatment both in control siRNA- or *Bmp4* siRNA-transfected hepatocytes (approximately 3-fold increase) (Figure 3.6, panel B, right graph). This result suggests that the hepcidin response to citrate does not involve BMP4.

3.4 Citrate regulates hepcidin mRNA expression in mice

The cell-based experiments (presented above) show that citrate supplementation increases hepcidin mRNA expression in primary murine hepatocytes and that the hepcidin response to citrate is mediated by the BMP/SMAD signaling pathway. I next investigated whether citrate induces hepcidin expression in the mouse liver and if this response involves BMP/SMAD signaling. Citrate levels in hepatocytes *in vivo* were increased by administering citrate to WT mice. Hepcidin mRNA levels in the liver and plasma, as well as hematological parameters, were assessed.

Tail vein injection was chosen as the route for citrate administration in mice. The LD50 for citric acid has been reported to be 42 mg/kg body weight [159]. To avoid possible hepatic and extra-hepatic toxicity, we intravenously injected 10.5 mg of citric acid/kg body weight. This dose is 4-fold lower than the LD50 and has been

discussed and approved by the EMBL institutional Animal Care and Use Committee. Mice used for the intravenous injection experiments weighed approximately 23 g and were injected with approximately 130 µl of PBS or citrate (below the maximum volume allowed to inject iv in mice). Klaus Schmitt from the EMBL Laboratory for Animal Resources (LAR) performed the tail vein injections. Mice were fasting for 4 hours before injection and were euthanized 2 hours after citrate or PBS injections for blood and organ collection.

To address whether citrate administration leads to changes in the blood chemistry, I measured hematological parameters in citrate- and PBS-injected mice (Table 3.2). As a marker for inflammation, I analyzed the number of white blood cells (WBC), which showed comparable numbers in PBS- and citrate-injected mice. As markers for red blood cell disruption, I analyzed red blood cell number (RBC), mean corpuscular volume (MCV), concentration of hemoglobin, hematocrit and red blood cell distribution (RDW). Similar values for RBC, MCV, RDW revealed that there were no changes in red blood cell numbers upon citrate injection. Together, these results show that citrate did not disrupt blood cells nor induced inflammation at the time point studied. This finding is particularly important because hepcidin is regulated by inflammation and erythropoietic activity (see Section 1.1.4 for more details). Therefore, as RBC and WBC numbers do not change in either PBS- or citrate-injected mice, an erythropoietic and inflammatory stimulus to regulate hepcidin expression upon citrate treatment can be excluded.

To test whether citrate levels increased in the liver following tail vein injections, I performed citrate measurements in the liver from PBS- and citrate-injected mice. I found that citrate levels were significantly increased by 2-fold in the liver (Figure 3.7, panel A). As intracellular citrate concentrations vary from 100-400 mM in mammalian cells [145], the observation that citrate levels increased by 2-fold after injection of citrate compared to control mice show that citrate in the liver increased within the physiological range. Concomitant with the citrate increase in the liver, hepcidin was significantly up-regulated at the mRNA level in the liver (>2-fold) and at the protein level in the plasma of citrate-injected mice (Figure 3.7, panel B). These results confirm my observations in cells (shown in Figure 3.2), in which

supplementation of citrate in the cell culture medium resulted in the accumulation of citrate in primary hepatocytes and in increased hepcidin expression.

Table 3.2: Blood parameters measured in PBS- and citrate-injected mice.
WBC = white blood cells, RBC = red blood cells, MCV = mean corpuscular volume, RDW = red cell distribution, hemoglobin concentration.

Blood Parameter	PBS (n=5)	Citrate (n=6)	*P* value
WBC count (10^9/L)	4.62 ± 1.90	4.67 ± 1.18	0.969
RBC count (10^{12}/L)	10.87 ± 0.64	10.65 ± 0.21	0.781
MCV (fL)	52.40 ± 0.55	53.00 ± 1.10	0.296
Hemoglobin (g/L)	16.58 ± 0.94	16.62 ± 0.26	0.924
Hematocrit (%)	56.16 ± 3.21	56.56 ± 1.39	0.815
Platelet count (10^9/L)	1177.60 ± 473.06	1191.20 ± 223.27	0.414
RDW (%)	13.36 ± 0.21	13.36 ± 0.18	0.984

The data from the cell-based experiments (Figure 3.4) suggests that the hepcidin response to citrate is controlled by the BMP/SMAD signaling pathway. In order to test if the response of hepcidin to citrate in mice also involved the BMP/SMAD signaling pathway, I analyzed the mRNA expression of the *Id1* gene, which is co-expressed with hepcidin and is a target gene of the BMP/SMAD pathway [63]. In agreement with the *in vitro* experiments (Figure 3.4), the mRNA of *ld1* was significantly up-regulated in citrate injected mice (Figure 3.7 panel C), further substantiating the involvement of the BMP/SMAD pathway in the response to citrate.

If the response of hepcidin to citrate in mice involves the BMP/SMAD pathway, then the phosphorylation of the SMAD1/5/8 proteins should be increased upon citrate injection. To test this, I performed Western blot analysis of p-SMAD1/5/8 as well as the total levels of the SMAD1 protein. The analysis of the total levels of SMAD1 was performed to test whether alterations in the protein band of p-SMAD1/5/8 may be due to changes in SMAD1 total levels. As shown in the Figure 3.7 (panel D), the total levels of the SMAD1 protein were unchanged whilst the phosphorylation of p-SMAD1/5/8 increased in citrate-injected mice.

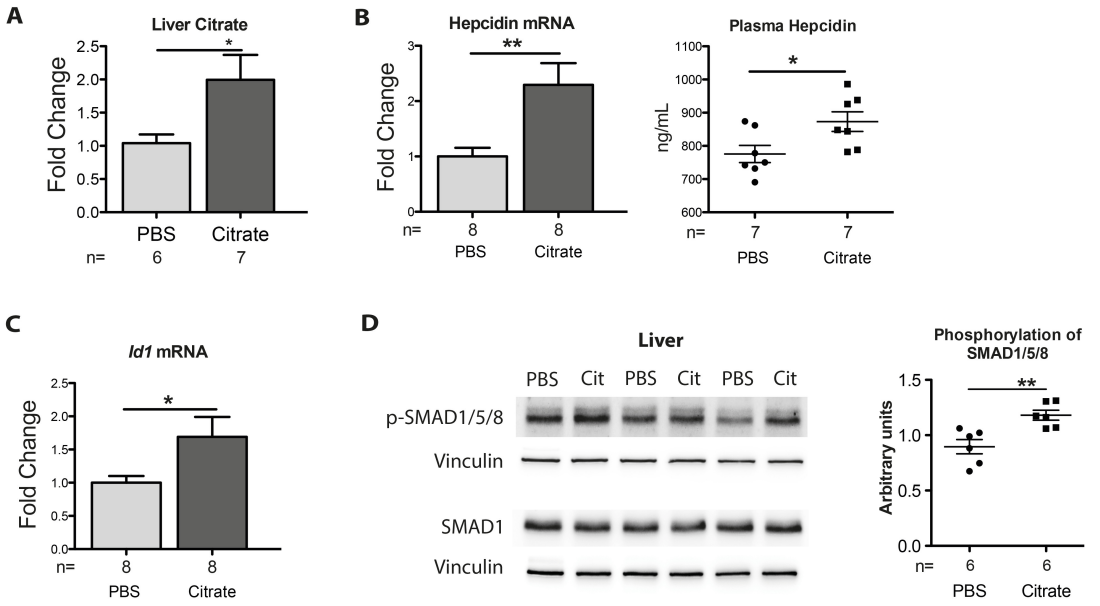

Figure 3.7: Mice injected with citrate via the tail vein present elevated citrate in the liver and induce hepcidin expression via the BMP/SMAD signaling pathway.
Mice were deprived from food for 4 hours, injected with PBS or citrate and blood and liver were collected 2 hours after injections; **A)** Citrate measurements in the liver; **B)** Hepcidin mRNA analysis from total RNA from liver (left graph) and measurements of plasma hepcidin (right graph); **C)** mRNA analysis of *Id1* in total RNA rom liver. The *Rpl19* gene was used as reference. Data are represented as fold-change (+SEM) compared to the control. **D)** Western blot analysis of p-SMAD1/5/8 and SMAD1. Vinculin was used a loading control (left graph), quantification of the Western blot signals for p-SMAD1/5/8)/SMAD1 (right graph). Quantification was performed by using the software ImageJ. Significant changes are marked with *(P<0.05) and **(P<0.001). ns - not significant.

The quantification of the phosphorylation of the p-SMAD1/5/8 proteins was determined by using the ImageJ software. The area of each band of SMAD1 and p-SMAD1/5/8 was determined and divided by the respective area of the loading control (vinculin). Subsequently, the normalized p-SMAD1/5/8 areas were divided by the normalized SMAD1 areas. This normalization allowed for the determination of the proportion of phosphorylated SMAD proteins relative to the levels of total SMAD1 protein. The quantification of the p-SMAD1/5/8 proteins shows that the increase in phosphorylation of these proteins was significantly increased in citrate-injected mice. This result indicates that the BMP/SMAD pathway was more active in mice injected

with citrate and that the response of hepcidin to citrate appears to involve this pathway.

All together, my results show that citrate regulates the mRNA expression of hepcidin via the BMP/SMAD signaling pathway *in vitro* and *in vivo*.

Chapter 4
Discussion and Conclusions

4 Discussion and Conclusions

The regulation of iron homeostasis and cellular metabolism are functionally connected. Iron is a cofactor necessary for the activity of heme- (e.g hemoglobin, cytochromes) and non-heme-iron containing proteins (e.g. ribonucleotide reductase, amino-acid oxidases, lipoxygenases) ([127, 128] and reviewed in [2]). It further regulates the expression of enzymes necessary for metabolic processes (e.g. δ-aminolevulinate synthase 2, mitochondrial aconitase, citrate synthase) [124-126]. Cathecolamines that stimulate energy metabolism also increase intracellular iron levels [129], whilst the mTOR signaling pathway (which responds to nutrient signals) increases iron uptake, reduces the assembly of Fe-S clusters and suppresses hepcidin expression [95, 131, 132]. In addition, proliferative stimuli and gluconeogenesis suppress hepcidin expression [95, 96]. Furthermore, citrate metabolism cross-talks to iron homeostasis due to the function of ACO1/IRP1 as a cytoplasmic aconitase under iron-replete conditions (reviewed in [123]). Specifically, the function of ACO1/IRP1 as an iron-regulatory protein or as an aconitase is determined by the presence or absence of Fe-S clusters that are essential for the catalytic activity of this enzyme in all organisms [160]. While the effect that iron has on the function of ACO1/IRP1 is well understood, little is known about the contribution of citrate, the substrate of this enzyme, to iron homeostasis. In this work I describe for the first time a link between citrate metabolism and the expression of hepcidin, the master regulator of systemic iron homeostasis.

I was able to shown that the siRNA-mediated knockdown of enzymes that catalyze citrate-consuming reactions such as ACO1 (cytosolic aconitase), ACO2 (mitochondrial aconitase) and ACLY (ATP citrate lyase), as well as the inhibition of their enzymatic activities by small molecules, increases hepcidin mRNA expression in primary murine hepatocytes (Figure 3.1). Accordingly, treatment of primary hepatocytes with citrate results in the increase of citrate levels in cells, which correlates with elevated hepcidin expression in a time- and dose-dependent manner (Figure 3.2). I further provide evidence that citrate acts intracellularly to regulate

hepcidin expression (Figure 3.2) and that the hepcidin response to intermediaries of the citric acid cycle is specific for citrate (Figure 3.3). I show that the BMP/SMAD signaling pathway mediates the response of hepcidin to citrate in primary hepatocytes (Figure 3.4), and that HFE and HJV are not required for this response (Figure 3.5). Consistent with the cell-based experiments, citrate injection via the tail vein in mice results in the increase of citrate in the liver as well as the up-regulation of hepcidin expression both at mRNA and protein level (Figure 3.7). Finally, I demonstrate that injection of citrate in mice leads to the increase in SMAD1/5/8 phosphorylation (Figure 3.7), an indicator for activated BMP/SMAD signaling.

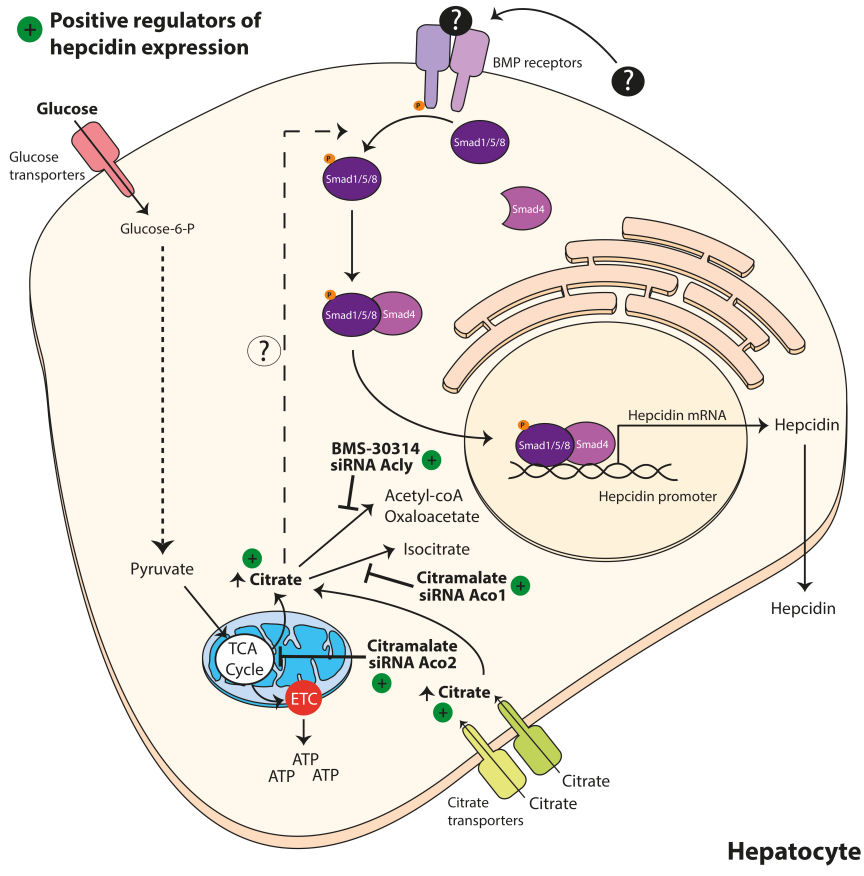

Figure 4.1: Working model.
Citrate levels increase intracellularly by either inhibition of ACO1 (by siRNA/citramalate), ACO2 (by siRNA/citramalate) and ACLY (by siRNA/BMS303-14), or extracellular supplementation. Increased citrate levels lead to the activation of hepcidin expression via the BMP/SMAD signaling pathway. The link between citrate and the BMP/SMAD pathway remains to be elucidated. TCA cycle - citric acid cycle, ETC - electron transport chain.

As summarized in Figure 4.1, my data show that the possible intracellular increase of endogenous citrate levels, either by the mRNA knockdown (siRNA) of ACO1, ACO2 and ACLY or inhibition of their enzymatic activities (citramalate and BMS-30314), as well as by the extracellular supplementation of citrate increases hepcidin expression. The hepcidin response to citrate involves the BMP/SMAD signaling pathway *in vitro* and *in vivo*. However, how intracellular citrate would increase the phosphorylation of the SMAD1/5/8 proteins remains to be elucidated.

Citrate as a regulator of gene expression

The knockdown and the enzymatic inhibition of enzymes that catalyze citrate-consuming reactions (ACO1, ACO2 and ACLY) increase hepcidin mRNA expression in primary murine hepatocytes. It has been described that the inhibition of these enzymes results in an increase of citrate levels in cells [143-147]. Therefore, it is likely that hepcidin expression is regulated due to increased citrate levels. Accordingly, treatment of PMH and mice with citrate results in increased citrate levels in hepatocytes and liver cells, respectively, as well as in increased expression of hepcidin. The ability of citrate to regulate gene expression is an aspect that is well established. Mitochondria-derived citrate can be detected in the nucleus, which serves as a substrate for ACLY to provide acetyl-coA for acetylation reactions. Lysine acetyl-transferases utilize acetyl-coA as a cofactor to donate acetyl groups to lysine residues present in histones (reviewed in [121, 161]). This results in the relaxation of chromatin and in the up-regulation of genes involved in metabolic processes [122] (see Section 1.1.2 for more detail). Interestingly, work published by Miura *et al* [162] and work under revision by Pasricha, S-R *et al* [163] show that hepcidin expression is regulated by histone acetylation. Inhibition of ACLY may result in reduced acetyl-coA levels, while citrate accumulation may provide acetyl-coA for the acetylation of histones. However, both inhibition of ACLY (Figure 3.1) and supplementation with citrate (Figure 3.2) induce hepcidin expression in primary hepatocytes. Therefore, the results presented here do not suggest that hepcidin is regulated by citrate due to

chromatin remodeling. Thus, I hypothesize that a new role of citrate in regulating gene expression independent of histone acetylation may exist.

My data show that citrate acts intracellularly to regulate hepcidin expression (Figure 3.2) and that the hepcidin response to intermediaries of the citric acid cycle (which can be imported by the same plasma transporters) is specific for citrate (Figure 3.3). I speculate that an active role of citrate as an "effector" molecule that integrates energy metabolism and gene expression, a feature that other intermediaries of the citric acid cycle do not share (reviewed in [114, 115, 121]), may contribute to increase hepcidin mRNA expression.

The response of hepcidin to citrate involves the BMP/SMAD signaling pathway and is independent of HFE and HJV

I further explored the signaling pathway that mediates the response of hepcidin to citrate. I have shown that the knockdown of *Bmpr1a*, *Smad4* and *Smad1* leads to the reduced response of hepcidin to citrate compared to the control (Figure 3.4), suggesting the involvement of the BMP/SMAD signaling pathway. Although HFE and HJV are co-activators of the BMP/SMAD signaling pathway, knockdown of *Hfe* and *Hjv* do not affect the hepcidin response to citrate (Figure 3.5). Thus, the BMP/SMAD signaling pathway does not require HFE and HJV for its citrate response. The mRNA expression of hepcidin was significantly reduced upon siRNA-mediated knockdown of *Bmpr1a*, *Smad4*, *Smad1*, *Hfe* and *Hjv*. This result is consistent with the fact that BMPR1A, SMADs and HFE/HJV are strong activators of hepcidin mRNA expression. Importantly, the response of hepcidin to citrate was selectively reduced upon knockdown of *Bmpr1a*, *Smad4* and *Smad1* compared to control siRNA. It is possible that the reduced effect of citrate on hepcidin expression, upon knockdown of *Bmpr1a*, *Smad4* and *Smad1*, could be due to the diminished strength of the BMP/SMAD signaling pathway. However, upon knockdown of *Hfe* and *Hjv*, the response of hepcidin to citrate was similar to the control knockdown. Therefore, the decreased levels of hepcidin upon knockdown of these hepcidin activators do not *per se* determine the strength of the hepcidin response to citrate. I

believe that the weaker response of hepcidin to citrate upon knockdown of *Bmpr1a*, *Smad4* and *Smad1* is due to their specific involvement in mediating this response. In addition, the idea that the BMP/SMAD signaling pathway is involved in the hepcidin response to citrate is supported by the demonstration of increased phosphorylation of SMAD1/5/8 after citrate injection *in vivo*.

To better understand the involvement of the BMP/SMAD signaling pathway in the citrate-induced up-regulation of hepcidin, I am currently optimizing the conditions for chromatin immunoprecipitation (ChIP) analysis of SMAD1. ChIP analysis will enable me to assess whether there is an increase in the SMAD1 transcription factor occupancy on the hepcidin promoter upon citrate treatment.

The fact that citrate involves the BMP/SMAD signaling pathway to regulate the expression of hepcidin raises the question of how citrate stimulates the BMP/SMAD signaling pathway. It is known that citrate can bind allosterically to proteins, such as PFK1 [164, 165] and F1,6BPase [166, 167]. Thus, I postulate that citrate may bind to proteins involved in the BMP/SMAD signaling pathway and potentiate either the interaction of p-SMAD1/5/8 with SMAD4 or mediate the translocation of this protein complex to the nucleus. An alternative hypothesis may be that citrate stimulates the mRNA expression of inducers of the BMP/SMAD signaling pathway in liver cells. Here I demonstrate that the BMP4 ligand of BMP receptors show increased mRNA expression upon citrate treatment (Figure 3.6). However, a functional consequence of increased BMP4 expression in mediating the hepcidin response to citrate could not be demonstrated.

To investigate what is the mechanism by which citrate stimulates the BMP/SMAD pathway it would be interesting to perform protein interactome analysis of the BMPR1A, SMAD4 or SMAD1 proteins in cells untreated and treated with citrate. This experiment would possibly reveal specific partners contributing to the induction of the BMP/SMAD pathway in response to citrate.

Metabolic changes triggered by elevated citrate levels may contribute to increased hepcidin mRNA expression

Citrate is a key metabolite which limits ATP production. When cells show elevated levels of ATP, citrate increases in the mitochondria, where it slows-down ATP production by inhibiting enzymes of the citric acid cycle [116-118] (see Section 1.2.2 for more details). Following citrate increase in the mitochondria, citrate is exported to the cytosol (reviewed in [114, 115]). There, it exerts a negative feedback loop in that it binds to a rate-limiting enzyme for glycolysis (PFK1) ([164] and reviewed in [119]). When citrate is bound to PFK1, the rate of glycolysis is reduced and therefore ATP production is reduced. In the cytosol, citrate also allostericaly binds to the F1,6BPase to stimulate gluconeogenesis ([166, 167] and reviewed in [119]). Based on these findings I speculate that citrate might regulate hepcidin expression as a consequence of altered metabolism. In fact, recent reports show that gluconeogenic signals induce hepcidin expression *in vitro* and *in mice* [96] whilst the nutrient-sensing mTOR signaling pathway suppresses hepcidin expression [95].

To gain a molecular understanding of the functional link between citrate metabolism and hepcidin expression, future experiments will have to investigate the exact role citrate plays within the cell to regulate hepcidin mRNA expression. To address this, a metabolomics analysis of primary hepatocytes following citrate treatment would be crucial to better understand the metabolic changes induced by citrate that may be involved to activate hepcidin mRNA expression. Alternatively, isotope tracing could be performed. In this experimental approach, hepatocytes are treated with an isotope-labeled molecule of citrate (for example - deuterated sodium citrate) for different time-points and collected for mass-spectrometry analysis. The mass-spectrometry analysis would detect the metabolites derived from the isotope labeled citrate. This would allow to identify metabolic pathways the supplemented citrate is involved in.

Such experiments could provide a more in depth understanding as to why patients with metabolic diseases such as diabetes, obesity or metabolic syndrome, often present with perturbations in their body iron status.

Association between the BMP/SMAD signaling pathway and citrate metabolism

To adapt to metabolic changes, cells respond by regulating a variety of signaling pathways. The mTOR pathway responds to the availability of glucose and amino acids, as well as the energy status of the cell, and regulates protein synthesis, autophagy, cell proliferation, mitochondrial function, lipogenesis, ketogenesis and glucose metabolism [168, 169]. The PI3K/AKT pathway regulates cellular oxygen consumption and glucose metabolism [170-172], whereas AMPK signaling senses increased intracellular ADP and promotes catabolic pathways to generate ATP while it inhibits anabolic pathways [173]. This work suggests that the BMP/SMAD signaling pathway, which is predominantly regulated during embryogenesis and in response to iron, could be controlled by alterations in citrate metabolism. This finding opens a new avenue for exploring how metabolism controls the BMP/SMAD signaling pathway or how this pathway can contribute to changes in cellular metabolism in the liver.

Treatment of mice with iso-citrate results in the suppression of hepcidin

It has been reported that multiple-injections of iso-citrate in rats infected with peptidoglycan-polysaccharide polymers of *Streptococcus pyogenes* result in the improvement of anemia and in a suppression of hepcidin expression [174]. However, a similar study has shown that in mice infected with particles of heat-killed *Brucella abortus*, the amelioration of anemia and hepcidin suppression following iso-citrate injections is only transient [175]. Injections of iso-citrate in mice without an inflammatory stimulus were not reported to affect hepcidin mRNA expression [175]. My data show that citrate injections in mice up-regulate hepcidin expression. The differences in the regulation of hepcidin observed in these works could be due to citrate and iso-citrate being structurally different molecules with different fates within the cell.

Citrate as a therapeutic agent

Citrate has emerged as a therapeutic agent for treating polycystic kidney disease (PKD), kidney stones and cancer. Rats and mice with PKD treated with a combination of citric acid and other forms of citrate in drinking water for several months show signs of improved kidney function [176, 177]. In addition, rats with PKD treated with citrate until their death shows that citrate improves the survival of these rats compared to untreated rats with PKD [176]. Dent's disease is characterized by nephrocalcinosis (deposition of calcium salts in the renal parenchyma), nephrolithiasis (presence of stones in the kidneys or lower urinary tract) and progressive renal failure. Mice with Dent's disease fed with high citrate diet show no signs of stones, normal kidney function and no tubular atrophy, compared to mice fed with a normal diet [178]. In the clinical setting, one of the recommended therapies suggested by the American Urological Association to patients with low urinary citrate and/or calcium, uric acid and cystine stones is to intake a solution of potassium citrate for several months (https://www.auanet.org/education/guidelines/management-kidney-stones.cfm). The intake of citrate is expected to replenish citrate levels in case they are low or to reduce the kidney and urinary tract stones by raising the urinary pH to an optimal level.

Due to its suppressive effect on glycolysis and ATP production, citrate has been proposed to be a drug with anti-tumor properties (reviewed in [114]). Citrate has been shown to highly induce apoptosis of human cancer cell lines when continuously administered in excess [149, 179]. Interestingly, few case studies of cancer patients described that the administration of citric acid every day for two weeks to several months improved their clinical features [180-183].

Since the injection of citrate in mice results in up-regulated hepcidin expression, it would be interesting to investigate whether patients undergoing prolonged citrate treatment (either as a therapeutic agent for kidney stones or as an anti-tumor drug) present altered hepcidin levels in the blood. This evidence would be particularly important to determine whether the management of hepcidin levels

should be considered along with the treatment of patients with citrate to prevent alterations of iron levels.

Chapter 5
References

5 References

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