Biochimica et Biophysica Acta 1820 (2012) 188–202

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Review

Regulation of iron transport and the role of transferrin ☆,☆☆

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ARTICLE INFO

Article history:
Received 18 September 2011
Received in revised form 27 October 2011
Accepted 30 October 2011
Available online 4 November 2011

Keywords:
Transferrin
Hepcidin
Iron metabolism

ABSTRACT

Background: Iron is utilized by several proteins as cofactor for major biological processes. However, iron may also harm cells by catalyzing the generation of free radicals and promoting oxidative stress. Acquisition, transport, utilization and storage of iron are tightly controlled to meet physiological needs and prevent excessive accumulation of the metal within cells. Plasma transferrin has been known for years as a central player in iron metabolism, assigned to circulate iron in a soluble, non-toxic form and deliver it to the erythron and other tissues. Recent data uncovered an additional role of transferrin as an upstream regulator of hepcidin, a liver-derived peptide hormone that controls systemic iron traffic.
Scope of review: Here, we review basic features of iron metabolism, highlighting the function of transferrin in iron transport and cellular iron uptake. We further discuss the role of hepcidin as an orchestrator of systemic iron homeostasis, and the mechanisms underlying hepcidin regulation in response to various physiological cues. Emphasis is given on the role of transferrin on iron-dependent hepcidin regulation.
Major conclusions: Transferrin exerts a crucial function in the maintenance of systemic iron homeostasis as component of a plasma iron sensing system that modulates hepcidin expression.
General significance: Proper expression of transferrin and hepcidin are essential for health, and disruption of their regulatory circuits is associated with iron-related disorders. This article is part of a Special Issue entitled Transferrins: Molecular mechanisms of iron transport and disorders.

1. Introduction

The unique ability of iron to serve both as an electron donor and acceptor renders this metal irreplaceable for various physiological and metabolic pathways. Vital biochemical activities, including oxygen transport, energy production and cellular proliferation depend on iron-containing cofactors, such as heme, or iron sulfur clusters (ISC). However, although beneficial at normal levels, excess iron may become toxic due to its ability to catalyze the generation of free radicals and damage cellular macromolecules [1]. Mammals have evolved complex homeostatic circuits and specialized molecules to ensure safe and balanced iron acquisition, transfer and storage. Proteins involved in iron transport and in regulation of iron metabolism are summarized in Tables 1 and 2, respectively.

Plasma transferrin (Tf) is a powerful chelator, capable of binding iron tightly but reversibly [2,3]. A molecule of Tf can bind two atoms of ferric iron (Fe^{3+}) with high affinity ($K_d = 10^{-23}$ M) [2], which is higher in the extracellular pH of 7.4 and decreases in the acidified endosomes, allowing the dissociation of Fe^{+3}. Tf belongs to a family of homologous iron-binding glycoproteins that encompasses lactoferrin (found both intracellular and in secretions, including milk), melanotrasferrin (present on melanoma cells) and ovotransferrin (present in egg white) [4]. They are all monomeric proteins of 76–81 kDa and consist of two structurally similar lobes (termed N- and C-lobes), each containing a single iron-binding site.

Iron chelation by transferrin serves three main purposes: i) it maintains Fe^{3+} in a soluble form under physiologic conditions, ii) it facilitates regulated iron transport and cellular uptake, and iii) it maintains Fe^{3+} in a redox-inert state, preventing the generation of toxic free radicals. Tf has an indirect defensive role against systemic infections by depriving the potential pathogens of extracellular iron, which is essential for their growth [5]. Moreover, diferric holo-Tf exerts a key regulatory function in the expression of hepcidin (encoded by the *HAMP* gene), a small hepatic peptide hormone that controls intestinal iron absorption and hepatic and macrophage iron release [6–8]. This review focuses on the functions of Tf and the

☆ This article is part of a Special Issue entitled Transferrins: Molecular mechanisms of iron transport and disorders.
☆☆ This work was supported by a grant from the Canadian Institutes for Health Research (CIHR; MOP-86514).
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0304-4165/$ – see front matter © 2011 Published by Elsevier B.V.
doi:10.1016/j.bbagen.2011.10.013

Table 1
Proteins involved in iron metabolism.

Protein	Abbreviation	Function in iron metabolism	Disruption phenotype
Iron acquisition			
Transferrin	Tf	Plasma iron transfer	Iron deficiency anemia with tissue iron overload
Transferrin receptor 1	TfR1	Internalization of holo-Tf	Embryonic lethality in homozygotes. Iron deficiency anemia in heterozygotes
Six transmembrane epithelial antigen of the prostate 3	STEAP3	Ferric reductase in the endosomes of erythroid cells	Iron deficiency anemia
Divalent metal transporter 1	DMT 1	Epithelial/endosomal transport of ferrous iron	Iron deficiency anemia
Duodenal cytochrome b	Dcytb	Apical membrane ferric reductase of enterocytes	Little or no impact on body iron stores
Heme oxygenase 1	HO-1	Degradation of hemoglobin for Fe^{2+} release	Anemia with low serum iron and hepatic and renal iron overload
Cubilin	CUBN	Reabsorption of Tf from the glomerular filtrate	No significant impact with regards to Tf reabsorption
Haptoglobin	HP	Hemoglobin binding and endocytosis via CD163	Increased intestinal iron absorption with splenic and renal iron overload
Hemopexin	HPX	Heme binding and endocytosis via CD91	Regional CNS iron overload
HRG-1 protein	HRG-1	Heme transport	
Zrt-Irt-like protein 14	Zip14	NTBI uptake by hepatocytes	
L-type voltage-depended calcium channels	LVDCC	NTBI uptake by cardiomyocytes	
Lipocalin 2	Lcn 2	Kidney iron uptake	No apparent defects on iron metabolism
Ferritin	Ft	Cellular iron storage and uptake	Embryonic lethality (H-ferritin)
T-cell immunoglobulin domain and mucin-domain	TIM-2	Endocytosis of ferritin	
Scavenger receptor class A, member 5	Scara5	Endocytosis of ferritin	
Intracellular iron transport			
Mitoferrin1/2	MFRN1/2	Import of ferrous iron to mitochondria	Embryonic lethality with profound anemia
Iron efflux			
Ferroportin	FPN1	Export of ferrous iron	Embryonic lethality in homozygotes. Iron overload with low Tf saturation in heterozygotes (HH type 4)
Hephaestin	HEPH	Membrane-bound ferroxidase	Severe hypochromic anemia & mucosal iron retention
Ceruloplasmin	CP	Plasma ferroxidase	Mild anemia with hepatic and regional CNS iron overload
Feline leukemia virus, subgroup C, receptor	FLVCR	Heme export	Macrocytic anemia with erythropoietic disorders

regulation of Tf expression in the context of cellular and systemic iron homeostasis.

2. Tissue distribution and intestinal iron absorption

2.1. Tissue and plasma iron pools

The adult human body contains approximately 3–5 g of iron (about 55 mg and 44 mg per kilogram of body weight for males and females respectively), with more than two thirds (>2 g) incorporated in the hemoglobin of developing erythroid precursors and mature red blood cells [9–11]. Most of the remaining body iron is found in a transit pool in reticuloendothelial macrophages (~600 mg) or stored in hepatocytes (~1000 mg) within ferritin, an iron storage protein. A smaller fraction is present in muscles within myoglobin (~300 mg), while only a minuscule amount (~8 mg) is constituent of other cellular iron-containing proteins and enzymes.

Iron bound to plasma Tf corresponds to less than 0.1% of total body iron (~3 mg), but represents in kinetic terms the most active pool (Fig. 1). More than 2 million new erythrocytes are produced every second by the bone marrow, requiring a daily supply of at least 20–30 mg of iron. To meet bone marrow needs, plasma Tf turns over >10 times a day. It has been calculated that an iron atom entering the plasma Tf pool will remain for only 90 min in systemic circulation before being taken up by the bone marrow [12]. Of the approximately 30 mg of Tf-bound iron circulating every day, more than 80% is delivered to bone marrow erythroblasts [13]. Since the amount of dietary absorbed iron is limited to 1–2 mg daily, the basic source of plasma iron is the reticuloendothelial macrophage system. Senescent erythrocytes are phagocytized by macrophages in the spleen, liver and bone marrow. Macrophages degrade hemoglobin and catabolize heme in a reaction catalyzed by *heme oxygenases* (HO-1 or HO-2) that liberates inorganic ferrous iron (Fe^{2+}) and generates CO and biliverdin [14]. Macrophages export Fe^{2+} through the transmembrane transporter ferroportin (FPN1), in a process coupled by re-oxidation of Fe^{2+} to Fe^{3+} by ceruloplasmin and followed by loading of Fe^{3+} to Tf (Fig. 2) [11]. Recycled iron can then be stored in ferritin or released back to plasma Tf at a rate that correlates with the iron needs for erythropoiesis [13]. Finally, approximately 5 mg of the daily plasma iron turnover is allocated to non-erythroid cells such as hepatocytes, macrophages *etc.*

Theoretically, under constant rates of Tf production and catabolism, the saturation of Tf with iron is influenced by: i) the amount of iron absorbed from the diet, ii) the amount of iron recycled and released by reticuloendothelial macrophages, and iii) the amount of iron utilized by the bone marrow and other tissues (Fig. 1). Tf saturation is an indicator of body iron stores, but also reflects the balance between reticuloendothelial iron release and bone marrow uptake. Under normal conditions, approximately 30% of the Tf iron-binding sites are saturated. The relatively low Tf saturation in conjunction with its high affinity for iron, allow Tf to efficiently buffer alterations of plasma iron levels and capture unshielded iron, minimizing the risk of toxicity. In humans, values of Tf saturation <15% indicate iron deficiency, whereas >45% are consistent with iron overload [15]. In disorders of severe iron overload (hemochromatosis), Tf saturation exceeds 60%. Under these conditions, the levels of redox-active non-transferrin bound iron (NTBI) increase dramatically (up to 10–15 μM or higher), and excess iron accumulates in tissue parenchymal cells and leads to organ damage [16,17]. Although the exact chemical nature of NTBI remains elusive, it is thought to circulate in the plasma in a form that is loosely bound to albumin or small organic acids, such as citrate [18,19]. The liver is the most prominent site of NTBI deposition, although considerable amounts of NTBI accumulate in further tissues, such as heart and pancreas.

Table 2
Proteins involved in regulation of iron metabolism.

Protein	Abbreviation	Iron regulatory function	Disruption phenotype
Systemic iron metabolism			
Hepcidin	HEPC	Inhibition of ferroportin-mediated iron efflux	Early onset severe iron overload with suppressed hepcidin expression (HH type 2A)
Bone morphogenetic protein 6	BMP 6	Regulation of hepcidin expression in response to hepatic iron	Iron overload with decreased levels of hepcidin
Hemojuvelin	Hjv	Enhancement of BMP signaling to hepcidin	Early onset severe iron overload with suppressed hepcidin expression (HH type 2A)
Transferrin	Tf	Plasma iron transfer	Iron deficiency anemia with tissue iron overload
High Fe (hemochromatosis) protein	HFE	Regulation of hepcidin expression by plasma iron	Iron overload with decreased levels of hepcidin (HH type 1)
Transferrin receptor 2	TfR2	Regulation of hepcidin expression by plasma iron	Iron overload with decreased levels of hepcidin (HH type 3)
SMAD4	SMAD4	Signaling to hepcidin	Decreased hepcidin levels and iron overload
Matriptase-2	TMPRSS6	Proteolytic cleavage of Hjv	Macrocytic anemia with low iron stores and increased hepcidin
Neogenin	NEO	Regulation of Hjv	Hepatic iron overload with decreased hepcidin levels and BMP-signaling
Cellular iron metabolism			
Iron regulatory protein 1	IRP1	Post-transcriptional regulation of IRE-containing mRNAs	No apparent impact on iron metabolism
Iron regulatory protein 2	IRP2	Post-transcriptional regulation of IRE-containing mRNAs	Microcytic anemia with increased iron loading of the liver and the duodenum
F-box and leucine-rich repeat protein 5	FBXL5	Iron-dependent ubiquitination and degradation of IRP2	Embryonic lethality. Liver-specific knock-out mice develop iron overload and die with liver-failure when fed an iron-rich diet

2.2. Iron absorption

Dietary iron absorption occurs in the duodenum and the upper portions of the jejunum. Absorbed iron normally equals to obligate iron losses, estimated to approximately 1–2 mg/day. Iron is lost through sweating, epidermal sloughing, urinary and intestinal epithelial exfoliation or bleeding. Since humans have no physiologic pathway for the excretion of redundant iron, maintenance of iron homeostasis is dependent on the regulation of intestinal iron absorption [20,21].

In order to reach systemic circulation, dietary iron has to cross the apical and basolateral membranes of absorptive enterocytes. Gastric acidity promotes the chelation of iron to soluble compounds, such as amines, amino acids and sugars, and is critical for iron bioavailability since prolonged achlorhydria leads to iron deficiency [22].

Heme iron is more bioavailable than inorganic iron and is absorbed by a distinct, yet not well-defined mechanism, which most likely involves a specialized heme carrier. A recently proposed candidate (*heme carrier protein 1*; HCP1) [23] was found to be predominantly a folate transporter and humans harboring mutations in HCP1 suffer from megaloblastic anemia associated with folate deficiency [23,24]. Heme may be translocated across the apical membrane of enterocytes either via direct transport or by receptor-mediated endocytosis [25,26]. Subsequently heme is thought to undergo degradation inside enterocytes by heme oxygenases to release Fe^{2+}, which follows the fate of absorbed inorganic iron (see below). Heme may also be exported as an intact molecule across the basolateral membrane to plasma via the putative heme exporter FLVCR (*feline leukemia virus, subgroup C, receptor*), and then scavenged by circulating hemopexin (Hpx) [27,28].

Inorganic (non-heme) iron, which roughly accounts for 90% of dietary iron, predominates within the intestinal lumen in the insoluble oxidized Fe^{3+} form (Fig. 2). To cross the apical membrane, nutritional Fe^{3+} undergoes reduction by brush border ferrireductases, of which *duodenal cytochrome b* (Dcytb) [29] is a candidate, and gets subsequently transported as Fe^{2+} by the proton-coupled *divalent metal transporter 1* (DMT1) [30–32]. The latter is a member of the solute carrier (SLC) family of membrane transport proteins and is also known as SLC11A2. Experiments in mice showed that DMT1 is indispensable for dietary iron absorption [33]. On the other hand, the disruption of Dcytb did not significantly affect body iron stores [34], suggesting the existence of alternative mechanisms for the reduction of iron in the intestinal lumen.

The next step in the process involves the intracellular traffic of newly acquired Fe^{2+}. Although several models have been proposed, some of them involving chaperons and transcytosis or vesicular transfer of iron, the exact mechanisms are still poorly understood [35]. In the basolateral membrane, cytosolic iron is exported to the circulation by ferroportin (FPN1), which also belongs to the SLC transporter family (SLC40A1) [36–38]. The export of iron and its subsequent loading to apo-Tf requires the re-oxidation of Fe^{2+} to Fe^{3+}, catalyzed by the membrane-bound ferroxidase hephaestin (Fig. 2). The severe hypochromic anemia, associated with mucosal iron retention that characterizes hephaestin mutant (sla) mice [39], illustrates the importance of this ferroportin-associated oxidase for the efflux of iron across the basolateral membrane of enterocytes. Studies on phlebotomized ceruloplasmin null mice have shown that under stress conditions that may exceed the oxidative capacity of hephaestin, the re-oxidation of Fe^{2+} to Fe^{3+} upon its release from intestinal enterocytes is accomplished by its plasma homologue ceruloplasmin [40]. It has been proposed that circulating gastrins, peptide hormones that stimulate the secretion of gastric acid, may act as transient Fe^{3+} chaperones that facilitate the loading of apo-Tf with iron [41].

3. Cellular iron uptake

3.1. Transferrin-mediated mechanisms

Erythroid progenitor cells and other rapidly dividing cell populations acquire their metabolic iron from plasma Tf by receptor-mediated endocytosis, following interaction of iron-loaded Tf with the cell surface *transferrin receptor 1* (TfR1). This transmembrane glycoprotein forms a disulfide-bonded homodimer, which can bind one Tf molecule at each of its subunits [42,43]. Interestingly the iron status of Tf impinges on its affinity for TfR1; thus, diferric Tf binds with 30- and 500-fold higher affinity to TfR1, as compared to monoferric and apo-Tf, respectively [44]. TfR1 is expressed in many cell types. Cells with increased needs for iron such as developing erythroid and intestinal epithelial cells, placental syncytiotrophoblasts and neoplastic cells are among those demonstrating the highest TfR1 levels [13,43].

The events that follow the binding of holo-Tf to TfR1 are schematically illustrated in Fig. 3. The holo-Tf/TfR1 complex undergoes endocytosis via clathrin-coated pits. Acidification of the endosome by a proton pump ATPase to pH 5.5 triggers a conformational change in Tf resulting in the release of Fe^{3+} [45]. This is facilitated by the fact

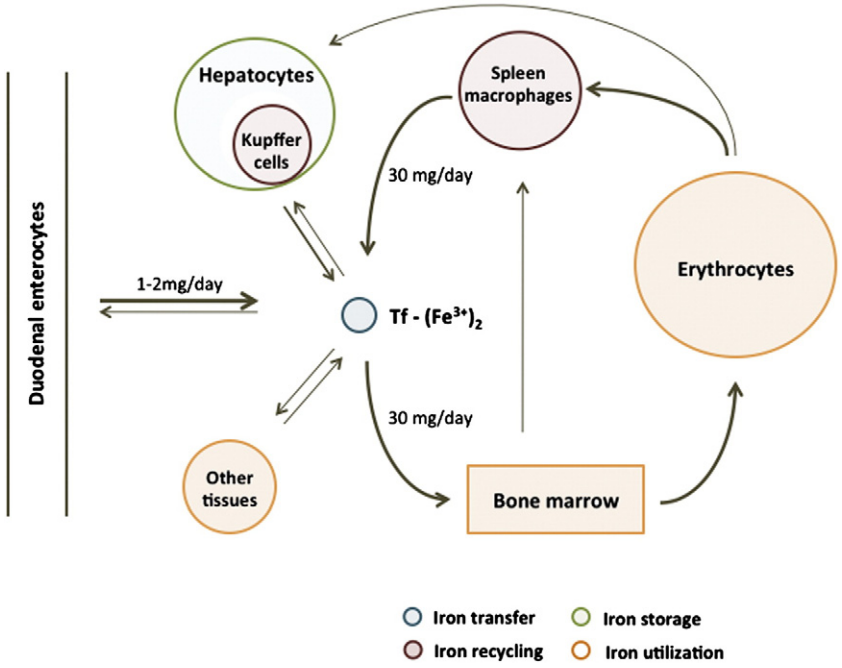

Fig. 1. Distribution and traffic of iron in the body. Most of the body iron is utilized in hemoglobin of developing erythroid precursors and mature red blood cells. Reticuloendothelial macrophages recycle iron from ingested senescent erythrocytes and release it to plasma Tf for delivery to the bone marrow and other tissues. Likewise, dietary iron that is absorbed by duodenal enterocytes also reaches plasma Tf, which circulates 30 mg of iron per day. Excess of body iron is stored in liver hepatocytes.

that Tf remains bound to TfR1, since a mildly acidic pH alone would not have been enough to remove iron efficiently from both Tf binding sites [46]. Liberated Fe^{3+} needs to be reduced before transportation, via DMT1 [47], across the endosomal membrane to the cytosol. The ferric reductase STEAP3 (*six-transmembrane epithelial antigen of the prostate 3*) has been shown to perform this function in immature erythroid cells [48]. Finally, the apo-Tf/TfR1 complex returns to the cell membrane, through a process involving the trafficking protein Sec15l1 [49], where apo-Tf is recycled back to the bloodstream, available to recapture iron. The half-life of Tf in humans is around 8 days, while iron delivery via the Tf/TfR1 cycle can be completed in approximately 5–20 min, depending on the cell type [50]. In other words, each Tf molecule may accomplish hundreds of cycles of iron binding and delivery to cells during its life span.

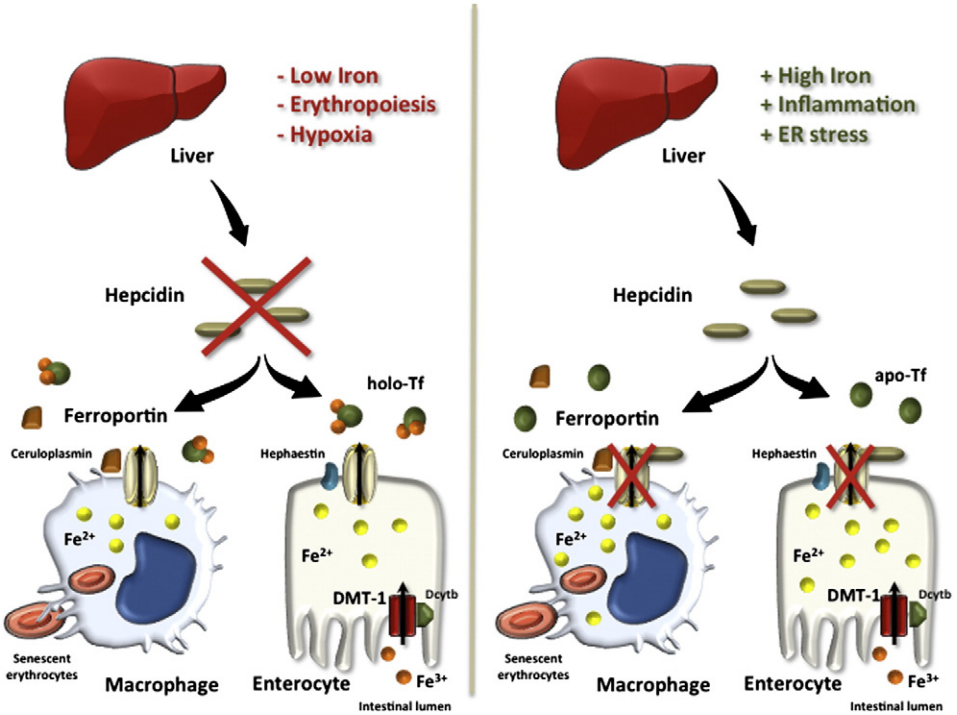

Fig. 2. Regulation of systemic iron traffic by hepcidin. Duodenal enterocytes assimilate dietary iron from the intestinal lumen via DMT1, following reduction of Fe^{3+} to Fe^{2+} by Dcytb. Reticuloendothelial macrophages recycle iron from phagocytized senescent red blood cells. Both cell types release Fe^{2+} to plasma via ferroportin, which is then re-oxidized to Fe^{3+} by hephaestin or ceruloplasmin, and captured by circulating apo-Tf. Liver-derived hepcidin inhibits iron efflux from these cells by binding to ferroportin, which promotes its internalization and lysosomal degradation. The expression of hepcidin is induced by iron, inflammatory signals or ER stress, and suppressed by iron deficiency, erythropoietic signals or hypoxia.

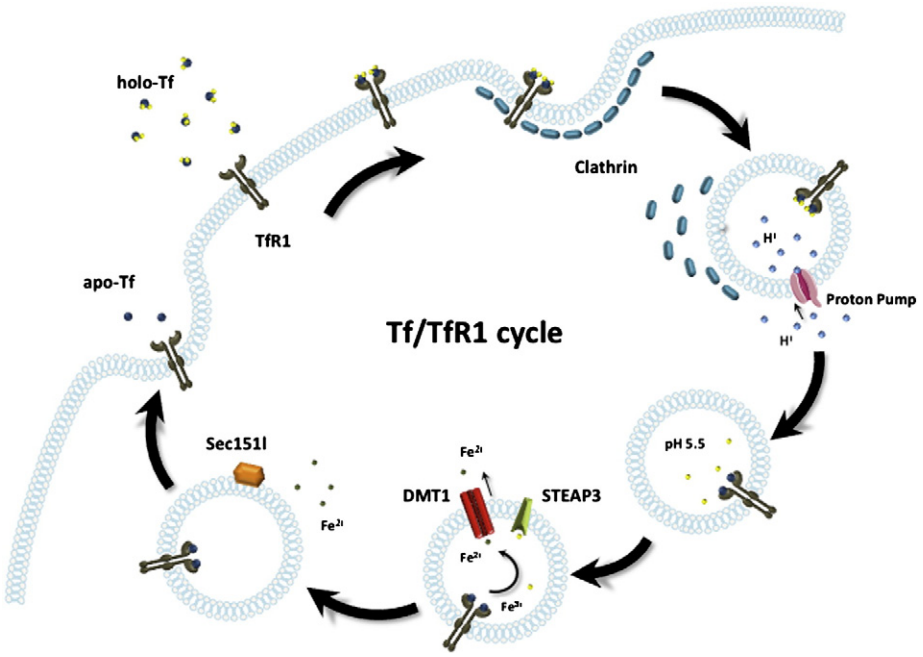

Fig. 3. Cellular uptake of Tf-bound plasma iron. Circulating diferric Tf binds to TfR1 and the Tf/TfR1 complex undergoes endocytosis via clathrin-coated pits. A drop of pH to 5.5, mediated by a proton pump, triggers the release of Fe^{3+} from Tf. Following reduction by STEAP3, Fe^{2+} gets transported across the endosomal membrane to the cytosol via DMT1. Apo-Tf is recycled back to the cell surface and released from TfR1 to plasma for another iron uptake cycle.

Although this high-affinity pathway accounts for most of the uptake of Tf-bound iron in cells, alternative low-affinity mechanisms have also been described [51]. Studies in Huh7 hepatoma cells provided evidence for internalization of Tf-bound iron in a low-affinity and TfR1-independent manner [52]. The contribution of such mechanisms to cellular iron acquisition depends on the concentration of Tf and the cell type. At low Tf concentrations (<0.3 μmol/L) TfR1 mediated uptake predominates, while at higher Tf concentrations low-affinity mechanisms take over [53]. It should be noted that the physiological concentration of diferric holo-Tf in human plasma is ~5 μmol/L, representing ~10% of total circulating Tf [13]. This indicates that TfR1-independent pathways may dominate the uptake of holo-Tf in many cells. Experiments showed that hepatocytes and intestinal crypt cells acquire Tf-bound iron by utilizing both high- and low-affinity systems, whereas holo-Tf enters immature erythroid cells exclusively via the TfR1 route [53,54].

The exact mechanisms for TfR1-independent uptake of Tf-bound iron have not been well characterized. One of them could potentially involve *transferrin receptor 2* (TfR2) that is mainly expressed in hepatocytes, and binds holo-Tf with a 25-fold lower affinity compared to its homolog TfR1 [55,56]. However, the limited tissue distribution of TfR2 does not support a broad role of this receptor in low-affinity iron uptake. Along these lines, there are cell types demonstrating considerable TfR1-independent uptake of Tf-bound iron, such as the intestinal crypt cells, which do not express TfR2 [53]. Moreover, mutations of TfR2 in both humans and mice have not been associated with defects in iron delivery to tissues [57,58], whereas ablation of the TfR1 gene impairs erythropoiesis and neurologic development in mice and leads to embryonic lethality [59]. TfR2 mutations cause hereditary hemochromatosis [57,58], indicating a critical role of this protein in the regulation of systemic iron homeostasis rather than cellular iron uptake, which will be discussed later in this review.

In the kidney, renal proximal tubules acquire Tf-bound iron via the membrane receptor cubilin, which operates conjugated to its co-receptor megalin [60]. Cubilin is expressed on the apical (urine facing) membrane of the proximal tubule cells and mediates the re-absorption of filtered Tf from the glomerular filtrate [61]. Other potential candidates as receptors for low-affinity uptake of Tf-bound iron are glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and proteoglycans, which have been reported to mediate endocytosis of holo-Tf in macrophages and hepatocytes, respectively [51,62]. However, the overall contribution of these molecules to cellular iron-uptake *in vivo* is currently unclear and awaits validation.

TfR1-mediated endocytosis is the only route for iron delivery to erythroid precursors. Its importance is highlighted by the embryonic lethality of mice with targeted disruption of TfR1, which is associated with hematopoietic and neuronal defects [59]. Moreover, heterozygous *Tfr1*$^{+/-}$ mice manifest severe microcytic hypochromic anemia [59]. Likewise, hypotransferrinemic mice, which express pathologically low levels of Tf (<1% compared to healthy animals) due to a splicing defect, develop severe iron deficiency anemia, accompanied by massive iron overload in all non-hematopoietic tissues [63]. Thus, regardless of the existence of multiple pathways of Tf-bound iron uptake, the Tf/TfR1 cycle is indispensable for the delivery of Tf-bound iron to hemoglobin-synthesizing developing erythroid cells.

The Tf/TfR1 route is also essential for iron transport to the central nervous system (CNS), which is separated from the circulation by the blood brain barrier. This consists of capillary endothelial cells, a basement membrane and endfeet of astrocytes that form tight junctions and do not allow the free passage of nutrients and proteins, including Tf [64]. It appears that brain capillary endothelial cells acquire iron from plasma Tf via TfR1 and release it to the brain interstitium, possibly within vesicles [65]. Astrocytes play a crucial role in providing iron to neurons [66] and are capable of exporting the metal via ferroportin, with the aid of a membrane-bound ceruloplasmin isoform [67]. Exported iron can bind to brain Tf, which is locally produced by the choroid plexus. Brain Tf delivers iron to TfR1-expressing cells, such as developing oligodendrocytes [68] and neurons [69]. However, because the concentration of Tf in the brain is considerably lower compared to that in plasma and, moreover, brain Tf is fully saturated with iron [70], it is very likely that the delivery of iron to CNS cells also involves transferrin-independent mechanisms.

The transport of iron to the developing fetus during pregnancy is mediated by the binding of maternal circulating Tf to TfR1 on the

placental microvillar membrane [71], which is followed by efflux of iron via ferroportin [72], for binding to fetal Tf.

3.2. Transferrin-independent mechanisms

Tf is the principal source of iron for tissues. However, as discussed above, specific cell types may assimilate iron via alternative Tf-independent routes. For example, macrophages acquire high amounts of iron through phagocytosis of senescent erythrocytes. Following intravascular hemolysis, hemoglobin or free heme are released and circulate in plasma. Haptoglobin (Hp), a liver-derived plasma protein binds to free hemoglobin and promotes its endocytosis in macrophages, upon recognition by the CD163 receptor [73,74]. Likewise, free heme is scavenged by hemopexin and the resulting complex is endocytosed via the CD91 receptor, present on the surface of macrophages, hepatocytes and other cell types [28,75]. Specialized cells may possess the capacity to acquire heme iron via direct transport of heme across their plasma membrane [15,25,27]. Members of the conserved family of HRG-1 (SLC48A1) proteins, which were shown to mediate heme transport in *Caenorhabditis elegans* and to be essential for erythropoiesis in zebrafish [76], are good candidates for this activity. Nevertheless, further studies are required to establish the functional significance of HRG-1 proteins in mammalian heme homeostasis. The putative heme chaperone HRG-3 was recently reported to supply maternal heme to developing *C. elegans* embryos [77], but it is currently unclear whether mammalian HRG-3 homologs exist, which could potentially be involved in intercellular pathways for heme delivery.

In iron overload states (hereditary hemochromatosis, hypotransferrinemia/atransferrinemia, transfusional siderosis *etc.*), NTBI that accumulates in the circulation is transported to tissue parenchymal cells by Tf-independent mechanisms [19]. DMT1 was initially proposed to operate as a major NTBI transporter in the liver, which is the principal site of NTBI clearance. However, liver-specific *Dmt1*$^{-/-}$ mice are not protected against hepatic iron accumulation [33]. This is consistent with the lack of significant iron transport activity by proton-dependent DMT1 at neutral pH, where NTBI uptake occurs. Moreover, it implies the existence of other pathways. The zinc transporter Zip14 (Zrt-Irt-like protein 14 — also known as SLC39A14) exhibits a capacity for NTBI uptake in cultured cells [78], but the role of this protein in liver iron loading remains to be established. Interestingly, apart from its NTBI transporting activity, Zip14 appears to also facilitate cellular assimilation of Tf-bound iron in cells [79].

Cardiomyocytes may take up considerable amounts of NTBI through L-type voltage-depended calcium channels (LVDCC) [80]. Indeed, treatment of iron overloaded mice with calcium channel blockers such as amlodipine or verapamil led to attenuation of myocardial iron accumulation and oxidative stress, and improved the cardiac function [80]. These results not only validated the involvement of LVDCC in myocardial iron uptake but also uncovered a new potential target for the treatment of heart disease, which develops secondary to iron overload disorders. A similar role of voltage-gated calcium channels has been proposed in the context of iron delivery to neuronal cells [81].

In the kidney, iron-loaded lipocalin 2 (Lcn2) can be endocytosed by the receptors 24p3R (also known as SLC22A17) [82] and megalin [83]. These define further Tf-independent iron uptake mechanisms, which operate during organ development [84], injury [85] or inflammation [82]. *Lcn2*$^{-/-}$ mice develop normally and do not exhibit any iron metabolism defects [86], excluding a major non-redundant function of the Lcn2 pathways in iron uptake. Finally, serum ferritin has been implicated in iron uptake, especially in pathological states where tissue damage results in release of iron-rich intracellular ferritin into plasma. Its endocytosis is mediated by the TIM-2 (T-cell immunoglobulin-domain and mucin-domain 2) [87] and the Scara5 (scavenger receptor class A, member 5) [88] receptors, while H-ferritin can also be internalized upon specific binding to TfR1 [89]. Whether other ferritin uptake pathways exist, remains to be determined.

4. Cellular iron metabolism

4.1. Iron trafficking, utilization and storage

The trafficking of iron inside the cells is perplexing and despite significant recent advances several aspects remain poorly understood. It is believed that following its DMT1-mediated transport across the endosomal membrane to the cytosol, newly acquired iron from the Tf/TfR1 cycle enters the *labile iron pool* (LIP) [90]. This is a transient pool of redox-active iron, presumably associated with several low molecular-weight chelates, such as citrate, ATP, AMP, pyrophosphate or various peptides. Although it represents only a minor fraction of total cellular iron (estimated to be approximately 3–5%), the cytosolic LIP reflects the cellular iron status [91]. Thus, fluctuations of LIP levels are monitored by intracellular sensors, which trigger homeostatic adaptive responses.

Cytosolic iron can enter the mitochondria via the SLC transporter mitoferrin (Mfrn or SLC25A37), localized to the inner mitochondrial membrane [92]. This is expressed in two isoforms, the erythroid-specific mitoferrin-1 [93] and the ubiquitous mitoferrin-2 [94]. Recent studies suggested an involvement of 2,5-dihydroxybenzoic acid (2,5-DHBA), a ligand of Lcn2, in mitochondrial iron transport [95]. Cells unable to synthesize this mammalian siderophore accumulate excessive quantities of cytoplasmic iron and exhibit mitochondrial iron deficiency, suggesting a requirement of 2,5-DHBA for iron entry to mitochondria. Other experiments in hemoglobin-synthesizing erythroid cells have provided evidence that iron can be transported directly from the endosomes to the mitochondria via a “kiss-and-run” mechanism involving a direct contact between the two organelles [96,97]. Once inside the mitochondria, iron is utilized for the biosynthesis of heme or iron–sulfur clusters, which are pathways of paramount importance for cellular metabolism [98–100].

Cellular iron that is not required immediately for metabolic purposes can be sequestered in the cytosol within ferritin, for storage and detoxification. Each molecule of ferritin consists of 24 subunits of H- (heavy) and L- (light) chains, which are encoded by distinct genes and assemble into a shell-like structure [101]. A distinct ferritin isoform is expressed in mitochondria of specific cells [102]. The function of mitochondrial ferritin (FtMt) is to detoxify excessive iron that accumulates in these organelles under pathological conditions, such as in sideroblastic anemia. Iron stored within ferritin is redox-inert and can be mobilized following ferritin degradation by lysosomes or the proteasome [103,104]. The essential role of ferritin in cellular iron metabolism is highlighted by the early embryonic lethality of H-ferritin null mice [105].

Excess of intracellular iron can also be exported via ferroportin [106]. In addition, heme iron may be released from cells expressing FLVCR [107].

4.2. Coordinate regulation of cellular iron homeostasis

Cellular iron uptake, utilization, storage and transport are coordinately controlled by post-transcriptional mechanisms involving the *iron responsive element* (IRE)/*iron regulatory protein* (IRP) system. Several proteins of iron metabolism are encoded by mRNAs containing one or more IREs in their untranslated regions (UTRs). These phylogenetically conserved hairpin structures constitute binding sites for two homologous iron regulatory proteins, IRP1 and IRP2, which are activated for IRE-binding in iron-starved cells [108–112].

IRE/IRP interactions inhibit translation of the mRNAs encoding H- and L-ferritin, ALAS2 (erythroid-specific *5-aminolevulenic acid synthetase 2*; a key enzyme for heme biosynthesis), ferroportin,

HIF-2α (*hypoxia inducible factor 2α*) and others, which contain a single IRE in their 5′ UTR. In addition, IRE/IRP interactions stabilize TfR1 and DMT1 mRNAs, containing IREs in their 3′ UTR. Under conditions of iron deficiency, these homeostatic adaptations allow increased iron uptake and transport via DMT1 and prevent storage of scarce iron into ferritin and efflux via ferroportin. Moreover, under iron-limiting conditions, the IRP-mediated translational suppression of ALAS2 [113] and HIF-2α [114] is thought to block erythroid heme biosynthesis and expression of HIF-2α transcriptional target genes of iron metabolism and erythropoiesis, respectively. In specialized cell types, alternatively spliced non-IRE-containing isoforms of ferroportin [115] and DMT1 [116] mRNAs bypass post-transcriptional regulation by IRPs.

An increase in iron supply prevents IRPs from binding to IREs and allows opposite homeostatic responses. In IRP1, this involves the reversible insertion of a cubane 4Fe-4S cluster that converts the protein into a cytosolic aconitase [108–110,112]. By contrast, IRP2 undergoes iron- and oxygen-dependent degradation following ubiquitination by FBXL5 (*F-box and leucine-rich repeat protein 5*), an E3 ubiquitin ligase that senses iron levels via an Fe–O–Fe center within its hemerythrin domain [117–119]. Iron deficiency promotes the removal of IRP1's iron–sulfur cluster [120] and the stabilization of IRP2 [121], resulting in increased IRE-binding activity.

IRPs also respond to iron-independent signals. Thus, both IRP1 and IRP2 can be induced upon exposure of cells to H_2O_2 [122,123] or NO [124,125], stimulating TfR1 expression and iron uptake [126,127]. Sustained H_2O_2 may also activate TfR1 expression and iron uptake in an IRP1-independent manner [128]. Hypoxia results in IRP2 stabilization [129], while it decreases the IRE-binding activity of IRP1 and modulates cellular iron homeostasis [130]. Taken together, these data provide links between iron metabolism, inflammation and hypoxic responses.

The IRE/IRP system is essential for life [131]. Targeted disruption of both IRPs causes embryonic lethality [132,133], highlighting the importance of these proteins in early development. The viability of single *Irp1*$^{-/-}$ or *Irp2*$^{-/-}$ animals indicates a high degree of functional redundancy between IRP1 and IRP2. *Irp1*$^{-/-}$ mice are asymptomatic under standard laboratory conditions [134], whereas *Irp2*$^{-/-}$ mice develop hypochromic microcytic anemia and exhibit abnormal body iron distribution (iron overloaded duodenum and liver, iron deficient spleen) [135,136]. Experiments with tissue specific disruption of both IRP1 and IRP2 revealed an important role of IRPs in regulation of intestinal iron transport [133] and iron sufficiency in hepatocellular mitochondria [137].

5. Regulation of Tf expression

Tf is expressed predominantly in the fetal and adult liver, but lower amounts can be synthesized in other tissues such as the brain and the testis [138]. The expression of the Tf gene is controlled by transcriptional mechanisms. The levels of Tf mRNA increase steadily in liver during fetal development, reaching a plateau shortly after birth, and remain high in adult life [13]. By contrast, Tf expression declines rapidly after birth in other tissues such as the kidney, the spleen, the lung, the heart and the muscles [13].

Tissue specificity of Tf expression is accomplished by the recruitment of different combinations of transcription factors in specific subsets of cells [138]. Two binding sites, PRI and PRII (proximal regions I and II respectively), localized adjacent to the −125/+1 promoter region, exhibit a key regulatory role in hepatic Tf transcription [139,140]. PRI interacts with HNF-4 (*hepatocyte nuclear factor 4*) [141], while PRII contains a CCAAT sequence that binds to C/EBP (*CCAAT enhancer-binding protein*) [142]. Both these transcription factors positively regulate Tf expression in the liver. By contrast, Tf transcription in Sertoli cells and in the brain is regulated by different factors [140]. An enhancer region 3.6 kb upstream from the Tf mRNA transcriptional start site that interacts with HNF-3α and other ubiquitous factors, was found to be active in hepatocytes but not in Sertoli cells, explaining the 10-fold lower amount of Tf mRNA found in the latter [140,141]. Studies in hepatoma cells revealed that the −1000/−819 upstream region is able to repress the Tf promoter [141]. In neuronal cells and oligodendrocytes the expression of Tf is regulated by a different combination of transcriptional factors. These include C/EBP, CRI-BP (*central region I binding protein*) and COUP-TF (*chicken ovalbumin upstream promoter transcription factor*), with the two first having a positive regulator function and COUP-TF acting as a repressor [13].

In iron deficiency, the rate of Tf synthesis in the liver increases significantly (2- to 4-fold) but the underlying mechanism is unknown [143]. In contrast, Tf gene expression in the kidney, testis or brain is not affected by iron [143]. Furthermore, inflammatory or immunologic stimuli may decrease the levels of circulating Tf, even though interleukin-6 (IL-6) and other inflammatory cytokines were shown to upregulate the expression of Tf gene in hepatoma cells [144]. Finally, *in vitro* studies identified two adjacent *hypoxia response elements* (HREs) within the Tf gene enhancer, which are binding sites of HIF-1 (*hypoxia-inducible factor-1*) [145]. This is consistent with the known increase in levels of circulating Tf under hypoxia [15–17], a response that may facilitate iron supply for erythropoiesis.

6. Regulation of systemic iron homeostasis

6.1. Hepcidin, the iron-regulatory peptide hormone

Tight and accurate regulation of iron absorption in humans is critical to prevent systemic excess or deficiency. This complex task is accomplished by hepcidin, a liver-derived peptide hormone that responds to multiple regulatory cues, including, iron availability, erythropoietic activity, anemia, inflammatory signals and hypoxia (Fig. 2) [15,146].

Hepcidin exerts its biological action by binding to ferroportin and promoting its phosphorylation, internalization and lysosomal degradation [147]. Since ferroportin is the unique cellular iron exporter in mammals [72], its expression in enterocytes and macrophages determines the degree of intestinal iron absorption and reticuloendothelial iron release. Therefore hepcidin's physiological role is to act as a negative regulator of iron absorption and release.

Humans with nonsense mutations in the hepcidin gene (*HAMP*), suffer from *juvenile hemochromatosis* (JH), a disease characterized by early onset of severe iron overload [148]. JH is a subtype of hereditary hemochromatosis (HH), a genetically heterogeneous disorder where hepcidin deficiency or ferroportin resistance to hepcidin leads to increased iron absorption and progressive systemic iron accumulation [149,150]. Clinical complications of iron overload include fibrosis and cirrhosis of the liver, hypogonadism, arthritis, cardiomyopathy, diabetes and skin hyperpigmentation. The majority of adult cases are attributed to mutations in HFE or, more rarely, to mutations in TfR2 and ferroportin (SLC40A1). On the opposite side in the spectrum, patients with mutations in matriptase-2 (encoded by the *TMPRSS6* gene), a transmembrane serine protease, manifest pathologically increased hepcidin levels and develop a form of iron deficiency anemia that is refractory to oral iron administration (iron refractory iron deficiency anemia - IRIDA) [151]. Similarly, *Tmprss6*$^{-/-}$ and *mask* mice exhibit growth retardation, alopecia and severe anemia, as a result of hepcidin upregulation [152]. Under chronic inflammatory conditions, increased hepcidin expression contributes to the anemia of chronic disease [153].

Biologically active hepcidin is a peptide of 25 amino acids [154,155]. It is generated primarily in hepatocytes, and at much lower levels in other cell types, upon cleavage of a larger 84 amino acid pro-peptide by the pro-hormone convertase furin [156]. Mature hepcidin is secreted to plasma and circulates bound to α2-macroglobulin [157]. Hepcidin

levels increase in response to iron [158] or inflammation [159], the latter considered as a mechanism of innate immunity to deprive iron from rapidly growing invading pathogens. Conversely, hepcidin levels drop with iron deficiency, hypoxia and increased erythropoietic demand for iron [160]. Hepatocytes integrate diverse signals deriving from these stimuli, which result in transcriptional activation of hepcidin by as yet partially characterized mechanisms. Basal hepcidin transcription requires C/EBPα (*CCAAT enhancer-binding protein α*), which binds to a CCAAT promoter element [161]. Regulation of hepcidin expression in response to different stimuli is discussed below.

6.2. Iron-dependent regulation of hepcidin expression

Clinical studies in humans and experiments in mice have provided evidence that hepcidin expression can be modulated by both hepatic iron stores and circulating iron. Patients with HFE- or TfR2-related hemochromatosis show blunted hepcidin responses after oral iron challenge, as compared to healthy controls [162,163]. Moreover, their hepcidin levels are inappropriately low relative to iron load, as expressed by the hepcidin/ferritin ratio [164–167]. Similar findings have been reported in mouse models of hemochromatosis [58,165,168,169].

6.2.1. Regulation by iron stores

Hepatic iron is thought to induce hepcidin expression via *bone morphogenetic protein* (BMP) signaling (Fig. 4) [170,171]. Activation of the BMP receptor upon BMP binding [172] eventually leads to phosphorylation of the intracellular SMAD1, SMAD5 and SMAD8 proteins (*homologs of both the drosophila protein mothers against decapentaplegic and the C. elegans protein SMA*). Subsequently, activated p-SMAD1/5/8 interacts with SMAD4 and the complex translocates

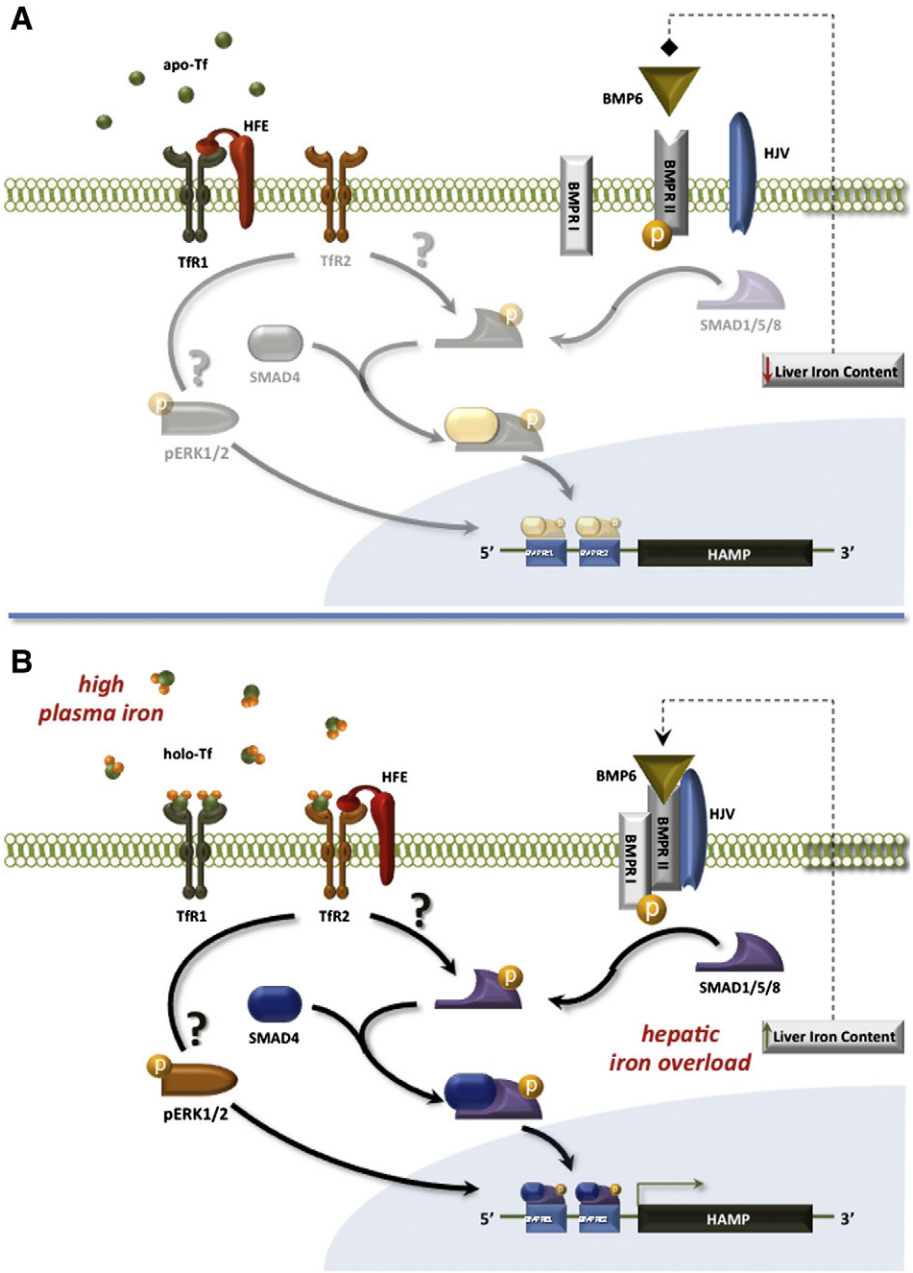

Fig. 4. Models for iron-dependent regulation of hepcidin. (A) When the levels of serum iron are low, TfR1 sequesters HFE, preventing its interaction with TfR2, which is necessary for signaling to hepcidin. Likewise, low hepatic iron content precludes expression and release of BMP6 for SMAD signaling. (B) When plasma iron levels increase, holo-Tf displaces HFE from TfR1, allowing its interaction with TfR2. The resulting, HFE/TfR2 complex activates the SMAD signaling cascade and/or the MAPK pathway, which activates hepcidin transcription. Hepatic iron overload triggers the expression and release of BMP6, which activates the SMAD signaling cascade upon binding to BMP type I and II receptors and Hjv.

to the nucleus and binds to two *BMP responsive elements* (BMP-RE1 and BMP-RE2) at proximal and distal sites of the hepcidin promoter, thereby activating its transcription [173,174]. In agreement with this model, liver-specific *Smad4*$^{-/-}$ mice misregulate hepcidin expression and develop iron overload [175]. Biochemical experiments showed that inhibitory SMAD7 blocks the interaction of p-SMAD1/5/8 with SMAD4 and attenuates hepcidin expression [176]. Among the various BMPs, which induce hepcidin *in vitro* (BMP2, 5, 6, 7 and 9) [170,175,177,178], BMP6 is the most physiologically relevant. Mice with targeted disruption of BMP6 in the liver exhibit iron overload and express nearly undetectable hepcidin levels, excluding a possible redundant compensatory role of other BMPs [179,180]. Injection of recombinant BMP6 into mice increased hepcidin expression and reduced serum iron levels [179], whereas administration of a BMP6 neutralizing antibody [179] or the BMP6 antagonist dorsomorphin triggered opposite effects [181]. The expression of BMP6 mRNA is induced by iron via a yet unknown mechanism, and BMP6 mRNA levels correlate with hepatic iron content, suggesting that BMP6 senses alterations in hepatocellular iron [7,171].

Hemojuvelin (Hjv), encoded by the *HFE2* gene, is a BMP co-receptor and forms complexes with type I and II BMP receptors, which enhance BMP/SMAD signal transduction and augment hepcidin expression [170]. It is expressed in hepatocytes and skeletal muscles and binds to membranes via a C-terminal GPI (glycosylphosphatidylinositol) anchor. Humans with Hjv mutations exhibit profound hepcidin suppression and develop a JH phenotype [166], clinically indistinguishable to that of patients with hepcidin disruption [182]. Mice with complete [183,184] or liver-specific [185,186] ablation of Hjv recapitulate iron overload. Biochemical experiments suggested that membrane-associated hepatocellular Hjv can be inactivated following cleavage by matriptase-2, which thereby attenuates BMP/SMAD signaling and acts as a negative regulator of hepcidin expression [187]. The importance of matriptase-2 is also highlighted by genome wide association studies that show a correlation between matriptase-2 variants, serum iron, transferrin saturation and hemoglobin levels [188–190]. Hypoxia increases matriptase-2 expression, while mice treated with BMP6 or iron were found to up-regulate matriptase-2 mRNA, very likely via induction of Id1 (*inhibitor of DNA binding 1*) [191]. These findings suggest a role for matriptase-2 in fine-tuning hepcidin expression. By acting as a negative feedback regulator, it prevents elevated iron stores from leading to excessive hepcidin accumulation and thereby further deregulating iron homeostasis.

Soluble forms of hemojuvelin (sHjv), lacking the GPI-anchor, have been detected in human [192,193] rat [194] and mouse [195] plasma. One of them, generated by furin-mediated proteolytic cleavage at R332 [196] has been proposed to function as a decoy that inhibits signaling to hepcidin [197]. This concept derives from *in vitro* experiments with a purified recombinant soluble form of Hjv [192] and *in vivo* data with a chimeric sHjv.Fc fusion construct [177]. Other forms of sHjv can be generated upon proteolytic cleavage at R288 by matriptase-2 [187] or lipolytic cleavage of the GPI anchor by phospholipase A [198]. The former does not compete BMP signaling to hepcidin [199]. The origin of sHjv in plasma remains unknown, but given the abundance of Hjv in skeletal muscles [166] and the capacity of differentiating muscle cells to release sHjv [200], it can be hypothesized that circulating sHjv may derive from muscles. Nevertheless, recent results have casted doubt on the physiological relevance of sHjv as a negative regulator of BMP signaling to hepcidin. Thus, mice with conditional disruption of muscular Hjv did not exhibit any obvious alterations in iron metabolism or hepcidin expression [185,186].

6.2.2. Regulation by circulating iron and holotransferrin

Modulation of hepcidin by circulating iron levels is likely mediated by HFE and Tf receptors that sense alterations in plasma Tf saturation (Fig. 4). Injection of holo-Tf into mice caused a pronounced increase in hepcidin mRNA within hours, while control mice, injected with saline or apo-Tf, did not show any analogous effect [7]. Mice with liver-specific disruption of either HFE [201] or TfR2 [169] develop iron overload due to hepcidin suppression, a proof of concept that hepatocellular expression of these proteins is indispensable for proper iron signaling.

Iron-loaded patients with HFE- or TfR2-related hemochromatosis have higher basal levels of hepcidin compared to controls (although greatly suppressed for their iron load), indicating that signaling of iron stores is at least partly preserved [163,202,203]. After oral administration of iron sulfate, normal subjects showed a parallel increase in Tf saturation and hepcidin, while in hemochromatotic patients, the hepcidin response was blunted, despite observed increases in serum iron [163,204]. Similar findings were recorded in mice after acute dietary iron challenge, with *Hfe*$^{-/-}$ mice showing milder impairment than mutant *Tfr2*$^{Y245X/Y245X}$ counterparts [7].

Insights on the interplay between HFE, TfR1, TfR2 and holo-Tf were provided by studies with genetically engineered mice bearing mutations either favoring or preventing HFE/TfR1 interactions. Mice with constitutive HFE/TfR1 interaction, displayed decreased hepcidin mRNA levels and systemic iron overload, similarly to *Hfe*$^{-/-}$ animals [205]. On the other hand, mice bearing a mutation that prevents binding of HFE to TfR1 or mice with enhanced HFE expression developed iron deficiency associated with inappropriately high hepcidin levels [205]. These results imply that TfR1 sequesters HFE and silences its signaling activity, whereas HFE acts to induce hepcidin expression when it is dissociated from TfR1. Interestingly, the binding domains for Tf and HFE overlap in TfR1, suggesting that holo-Tf competes HFE binding, while TfR2 may bind both holo-Tf and HFE simultaneously [206–208]. Finally, TfR2 competes TfR1 for binding of the HFE regardless of the holo-Tf levels [209]. These observations support a model in which TfR1 sequesters HFE and prevents its interaction with TfR2, which is necessary for the induction of hepcidin expression. When the iron saturation of Tf increases, holo-Tf displaces HFE from TfR1, allowing its interaction with TfR2 [205]. According to this view, the HFE/TfR2 complex activates signaling to hepcidin. The stabilization of TfR2 by holo- but not apo-Tf [210,211] provides further support to this model. Nevertheless, *in vivo* experiments with double *Hfe*$^{-/-}$*Tfr2*$^{-/-}$ mice revealed that these animals develop more severe iron overload than single *Hfe*$^{-/-}$ or *Tfr2*$^{-/-}$ counterparts, raising the possibility that HFE and TfR2 exert some independent effects on hepcidin regulation [212].

Several questions arise on the signal transduction pathways that link the TfR2/HFE complex to hepcidin. The treatment of primarily cultured murine hepatocytes with holo-Tf led to ERK/MAP (*extracellular signal regulated kinase/mitogen activated protein*) kinase pathway activation and subsequent treatment with an ERK-specific inhibitor blocked the induction of hepcidin expression by holo-Tf [213]. Consistently, *Hfe*$^{-/-}$, *Tfr2*$^{-/-}$, or double *Hfe*$^{-/-}$*Tfr2*$^{-/-}$ mice exhibited a significant decrease in hepatic phospho-ERK1/2 levels, suggesting that both, HFE and TfR2 signal via the ERK1/2 pathway [212]. Nonetheless, multiple data also support the implication of BMP6/SMAD signaling in HFE- and TfR2-dependent regulation of hepcidin. Hepatocytes treated with holo-Tf demonstrated increased phosphorylation of SMAD1/5/8, which was blocked after ERK inhibition [213], while the levels of SMAD1/5/8 were found significantly reduced in mice lacking HFE [214] or TfR2 [212]. These observations could be explained by the possible existence of a crosstalk between the BMP/SMAD and ERK/MAPK pathways.

Recent studies, investigating the *in vivo* response of hepcidin to isolated increases of either Tf saturation or liver iron content, showed that hepcidin is independently regulated by both stimuli, implying the existence of two distinct regulatory pathways [7,8]. Tf saturation did not have any effect on BMP6 expression, while acute iron administration, which increased Tf saturation without affecting hepatic iron content, resulted to activation of SMAD1/5/8 phosphorylation downstream of BMP6. Interestingly, no involvement of the ERK1/2 pathway was observed in this *in vivo* setting [8]. Taken together, experimental evidence

supports a model in which hepcidin expression is regulated in response to hepatic iron stores via BMP6/SMAD signaling and in response to circulating iron via the HFE/TfR2 complex (Fig. 4). The two pathways appear to crosstalk at the level of SMAD1/5/8, downstream of BMP6.

6.3. Regulation of hepcidin expression by erythropoiesis and the role of transferrin

It is well established that anemia-induced erythropoiesis requires an increase in iron absorption. In his seminal review article on "regulators of iron balance in humans" Clement Finch has proposed the existence of an "erythroid regulator" that stimulates absorption in response to anemia and ineffective erythropoiesis [215]. Unraveling the nature of these signals, which link bone marrow activity and hepcidin expression, is currently a subject of intensive research. The role of erythropoietin (EPO) in hepcidin regulation in anemic states has been addressed by several studies. Injection of EPO in mice [216] as well as treatment of freshly isolated mouse hepatocytes [217] resulted in a dose-dependent decrease of hepcidin levels, while treatment of human volunteers with recombinant EPO reduced considerably urinary hepcidin and increased slightly the expression of GDF15 (*growth differentiation factor 15*) [218]. EPO can directly suppress hepcidin expression by attenuating the binding of C/EBPα to the hepcidin promoter [217]; though the physiological significance of this mechanism *in vivo* remains questionable. Patients with aplastic anemia do not increase iron absorption despite high serum EPO levels [219] and hepcidin's response to exogenous EPO is blocked in mice with prior experimental inhibition of erythropoiesis with carboplatin [220]. In another study, suppression of erythropoiesis with irradiation or post-transfusion polycythemia increased hepcidin mRNA levels, while exogenous post-irradiation EPO administration did not led to hepcidin decrease in mice [221]. These results indicate that hepcidin downregulation is rather triggered by erythropoietic activity and increased iron demand by erythroid precursor cells than directly caused by EPO. It should also be noted that EPO can modulate iron homeostasis by inducing TfR1 expression, cellular iron uptake and subsequently heme biosynthesis in erythroid progenitor cells [222], and by inhibiting pro-inflammatory immune effector pathways known to affect hepcidin and ferritin expression [223].

Insights on the link between Tf and erythropoietic control of hepcidin were provided by studies in hypotransferrinemic mice (hpx) and humans suffering from hypotransferrinemia/atransferrinemia, a disorder of severe deficiency in serum Tf. Patients and animals have severe anemia because of impaired iron delivery to erythroid tissue, which is associated with massive parenchymal iron overload, increased iron absorption and suppressed hepcidin expression [224–226]. Administration of Tf to hpx mice corrected anemia, decreased iron overload and restored hepcidin expression. Moreover, transfusion of hpx mice with washed red blood cells (RBCs) was associated with hepcidin induction after restoration of anemia [226]. In a case study of a patient with hypotransferrinemia treated with plasma transfusions, time course measurements showed that Tf administration increased serum iron, hemoglobin and hepcidin [227]. These observations indicate that hepcidin suppression results from iron-restricted erythropoiesis due to Tf deficiency and that exogenous Tf administration restores hepcidin levels by ameliorating bone marrow iron supply. Along the same line, Tf administration to a mouse model of β-thalassemia intermedia normalized labile plasma iron concentrations and increased hepcidin and hemoglobin production, leading to a marked improvement of the thalassemic phenotype [228]. Furthermore, α-globin precipitation was reduced in RBCs of treated mice, contributing to the observed increase in RBC lifespan. The exact mechanism by which Tf improves erythropoiesis remains unknown but it is conceivable that an increase in monoferric Tf, may render iron accessible to more erythroid precursors, albeit at smaller amounts. This is also consistent with the observed further decrease in mean corpuscular hemoglobin (MCH) of RBCs [228].

Thalassemic patients have low hepcidin relatively to their iron load, demonstrating that increased erythropoietic demand for iron, dominates over iron stores in hepcidin regulation [229,230]. Thalassemias and other iron-loading anemias are characterized by ineffective erythropoiesis, where expanded proliferation of erythroid progenitor cells is associated with their limited maturation and premature death. The failure of these cells to maturate and their subsequent death is attributed to several factors, including imbalances of globin synthesis, heme and iron supply [231]. The proposed "erythroid regulator" is thought to comprise one or multiple molecules that signal for hepcidin suppression. GDF15 and TWSG1 (*twisted gastrulation-1*) are good candidates as mediators of bone marrow signaling. The former is a member of the TGF-β (*transforming growth factor β*) superfamily expressed by erythroid precursors. Serum levels of GDF15 are increased in the sera of patients with β-thalassemia, congenital dyserythropoietic anemias and sideroblastic anemia [232–235]. Serum from thalassemic patients or recombinant GDF15 incompletely suppressed hepcidin in human hepatocyte cultures [232]. Similarly, TWSG1 that also derives from erythroid precursors has been shown to increase in thalassemia and to suppress hepcidin expression *in vitro* [236]. Studies in both human cell cultures and thalassemic mouse models demonstrated that TWSG1 acts indirectly by inhibiting the BMP/SMAD signal transduction that leads to hepcidin transcriptional activation [236]. Interestingly, high expression of GDF15 and TWSG1 was also observed in hpx mice that decreased after treatment with Tf [226]. Nonetheless, GDF151 did not correlate with hepcidin levels in a cohort of patients recovering from hematopoietic stem cell transplantation [237], suggesting that GDF15 is unlikely to function as a more general "erythroid regulator" of hepcidin.

6.4. Inflammation and hepcidin expression

Inflammatory cytokines and stress signals modulate hepcidin expression, and this response is associated to the pathogenesis of the anemia of chronic disease. IL-6 promotes the phosphorylation of STAT3 (*signal transducer and activator of transcription 3*), which translocates to the nucleus and activates hepcidin transcription upon binding to a proximal promoter element [238–240]. Likewise, mice injected with lipopolysaccharide (LPS) demonstrate increased hepcidin expression in the liver, attributed to activation of the STAT3 pathway [241]. Interestingly, experiments with mouse and rat models showed that erythropoietic drive [241] or iron deficiency [242] can antagonize hepcidin induction during inflammation, which is indicative of a hierarchy among signals. Myeloid cell types such as macrophages and neutrophils may produce hepcidin in response to LPS or other bacterial pathogens, mediated by TLR4 (*toll-like receptor 4*) signaling [243]. IL-1β is another potent inducer of hepcidin expression by employing both C/EBPα and BMP/SMAD signaling pathways [244]. Hepcidin transcription is also activated by endoplasmic reticulum (ER) stress. This involves the transcription factors CREBH (*cyclic AMP response element-binding protein H*) and/or the stress-inducible CHOP (*C/EBP homologous protein*); the latter operates by modulating the C/EBPα activity [245,246]. Finally, *in vitro* studies have suggested the existence of a putative p53 response element (p53RE) within the hepcidin gene promoter [247]. Thus, during malignancy the activation of the p53 tumor suppressor protein would be expected to result in upregulation of hepcidin. This pathway uncovers an alternative link between malignancy and anemia of chronic disease and is possibly part of a defense mechanism against cancer, through iron deprivation of the malignant cells.

6.5. Hypoxia and hepcidin expression

Hypoxia and oxidative stress are further signals that decrease hepcidin expression. Studies in mice with liver-specific ablation of the expression of VHL (*von Hippel-Lindau*) factor, a tumor suppressor

protein regulating HIFα (hypoxia inducible factor α) subunit levels, proposed a key role for HIF1/HIF2 in the hypoxic pathway of hepcidin expression regulation [248]. However, biochemical data did not support a possible direct transcriptional function of HIFs on the hepcidin promoter, since the deletion of the HIF candidate binding motifs in hepatoma cells did not alter the response of hepcidin to different hypoxic stimuli [249–251]. Thus, the binding of HIFs to the hepcidin promoter remains controversial. Hypoxia is also known to promote EPO synthesis, which consequently decreases the expression of hepcidin, as discussed earlier. Finally, oxidative stress promotes the hypoacetylation of histones and decreases the binding of the two positive regulators C/EBPα and STAT3 to the promoter of hepcidin [249,252].

7. Perspectives

Not too many years ago, the field of iron metabolism was synonymous to the study of Tf and ferritin. The identification of TfR1 and the Tf/TfR1 cycle, and the characterization of the IRE/IRP system in the 80s of the last century, paved the ground for understanding basic mechanisms of cellular iron acquisition and homeostasis. The molecular cloning of iron transporting molecules (DMT1 and ferroportin) and, moreover, the discovery of hepcidin as the principal orchestrator of body iron homeostasis at the dawn of the new millennium, provided a framework for a thorough comprehension of systemic iron traffic and its regulation. It is now well-established that hepcidin expression is controlled by diverse stimuli including iron availability, erythropoietic demand for iron, inflammation and hypoxia. Molecular mechanisms underlying these responses are now at least partially understood. The function of novel players in hepcidin regulation, such as BMPs, Hjv, HFE, TfR2, matriptase-2 and others, is a subject of rigorous investigations. The elucidation of complex hepcidin regulatory pathways is already providing new therapeutic concepts for the treatment of disorders of iron homeostasis, and is expected to lead to the development of new drugs. Recent studies revealed that the plasma iron carrier Tf plays a central role in hepcidin regulation by triggering its transcriptional activation via hemochromatosis-associated proteins (HFE and TfR2) [226]. Studies involving Tf administration to hypotrasferrinemic patients and thalassemic mice, yielded novel and important clues for the treatment of anemias with ineffective erythropoiesis. Exogenous Tf mobilized iron from stores and altered its distribution from tissue parenchymal cells to hemoglobin synthesizing erythroid precursors [228]. An alternative approach, for protection of iron-overloaded tissues from iron's toxicity emerged, based on redistribution of the metal to utilization sites, rather than its simple chelation and excretion. The observed induction of hepcidin following Tf administration to thalassemic mice would favor this, by antagonizing typical uncontrolled iron egress from reticuloendothelial macrophages and intestinal enterocytes in these animals due to low hepcidin levels. Considering that hepcidin suppression contributes to systemic iron overload in iron-loading anemias, the development of Tf-based pharmacological strategies, possibly in combination with classical iron chelation regimens, may offer therapeutic benefits to patients and find future applications in ordinary clinical practice.

Acknowledgements

This work was supported by a grant from the Canadian Institutes for Health Research (MOP-86514). KG is supported by doctoral awards from the J. Latsis and A. Onassis Public Benefit Foundations. KP holds a *Chercheur National* career award from the *Fonds de la Recherche en Santé du Quebéc* (FRSQ).

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