CHAPTER EIGHT

Hepcidin and its multiple partners: Complex regulation of iron metabolism in health and disease

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Abstract

The peptide hormone hepcidin is central to the regulation of iron metabolism, influencing the movement of iron into the circulation and determining total body iron stores. Its effect on a cellular level involves binding ferroportin, the main iron export protein, preventing iron egress and leading to iron sequestration within ferroportin-expressing cells. Hepcidin expression is enhanced by iron loading and inflammation and suppressed by erythropoietic stimulation. Aberrantly increased hepcidin leads to systemic iron deficiency and/or iron restricted erythropoiesis as occurs in anemia of chronic inflammation. Furthermore, insufficiently elevated hepcidin occurs in multiple diseases associated with iron overload such as hereditary hemochromatosis and iron loading

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anemias. Abnormal iron metabolism as a consequence of hepcidin dysregulation is an underlying factor resulting in pathophysiology of multiple diseases and several agents aimed at manipulating this pathway have been designed, with some already in clinical trials. In this chapter, we assess the complex regulation of hepcidin, delineate the many binding partners involved in its regulation, and present an update on the development of hepcidin agonists and antagonists in various clinical scenarios.

1. Introduction

Iron is an essential element for almost all living organisms, from unicellular organisms to mammals. It forms the core of molecules such as hemoglobin, myoglobin, cytochromes and nitric oxide synthase as well as in multiple enzymes required ubiquitously for cellular ATP generation. Two to three million red blood cells (RBCs) are produced every second and require 30–40 mg of iron delivered to the bone marrow to make 30 pg of hemoglobin per cell, a total of 6 g of hemoglobin daily. Thus, of the total 3–4 g of iron in the human body, 2–2.5 g are present within hemoglobin, 0.5–1 g within macrophages and hepatocytes, and 0.5 g total between myoglobin, ferritin, and iron-containing enzymes in other cells. Only 2–4 mg of iron is typically found in the circulation bound to transferrin, the main iron transport protein. Because the majority of iron is found in the hemoglobin compartment, erythropoiesis dominates systemic regulation of iron metabolism and the two are inextricably intertwined, leading to decreased hemoglobin synthesis and anemia when iron is in short supply. Such hypoferremia, when severe, can also compromise function in non-erythroid lineage cells, causing epithelial changes in the nails, tongue, and esophagus; lead to cognitive deficits and diminished muscle function; and impair immune responses (Pasricha, Tye-Din, Muckenthaler, & Swinkels, 2021). In contrast, elevated plasma iron concentration can exceed the transferrin iron binding capacity and result in the formation of non-transferrin bound iron (NTBI) which is taken up by parenchymal cells, over time leading to iron deposition and organ damage (Pantopoulos, 2018). As a consequence, iron availability must be tightly regulated to prevent shortfalls and iron deficiency, resulting in anemia, as well as iron excess, resulting in the generation of free iron and consequent reactive oxygen species (ROS), causing tissue injury and organ failure.

As systemic iron loss is not physiologically regulated, the stable concentration of circulating iron is maintained by baseline dietary absorption, storage, and recycling of iron. Most of the iron found in circulation is recycled from erythrophagocytosis of RBCs within macrophages in the spleen and

liver. Recycled iron within macrophages can be exported to carrier proteins in the circulation. Transferrin-bound iron in circulation leads to transferrin saturation of iron-binding sites, calculated as a ratio of serum iron to total transferrin iron-binding capacity, approximately 20–45% under normal conditions. Iron trafficking is a dynamic process, using transferrin to transport iron between sites of absorption, recycling, and storage and to sites of iron utilization. Transferrin-iron is taken up by binding transferrin receptor 1 (TFR1) which is ubiquitously expressed on all cells. The highest concentration of TFR1 is found on erythroid precursors as a consequence of the highest iron requirements necessary for hemoglobin production in these cells.

Pathological blood loss and some forms of hemolysis are the main mechanisms of iron loss. As a consequence, phlebotomy is used as a currently standard therapeutic approach for non-anemic diseases, both diseases of iron overload as well as some erythrocytotic conditions. In response to our enhanced understanding of the regulation of iron metabolism, additional strategies are being evaluated to expand therapeutic options for iron-loaded anemias as well as primary iron overload and other disorders of erythropoiesis, as a useful alternative to the easy and efficacious but relatively ancient therapeutic phlebotomy approach. Because iron absorption is greatly increased in response to phlebotomy, a more mechanistic approach would circumvent the unintended physiological compensatory responses and enable more targeted effects. To elucidate physiology and pathophysiology of iron metabolism by exploring the relationships between hepcidin and its partners, this chapter aims to (1) describe our current understanding of iron metabolism and multiple partners involved in its regulation, (2) delineate the mechanistic relationship between iron metabolism and erythropoiesis, (3) elucidate abnormalities in regulation of iron metabolism in various diseases, and (4) discuss potential therapeutic modalities in development.

2. Regulation of iron metabolism

2.1 Systemic iron metabolism regulation

Complex living organisms have developed sophisticated mechanisms to obtain, distribute, and sequester iron. The peptide hormone hepcidin, secreted primarily by hepatocytes, is the principal regulator of iron homeostasis (Ganz, 2005; Krause, Neitz, Magert, et al., 2000; Park, Valore, Waring, & Ganz, 2001), including its regulation of dietary iron absorption, iron recycling by macrophages, and the release of iron from hepatic stores (Fig. 1A). Hepcidin is a negative regulator of iron flows. Thus, high hepcidin

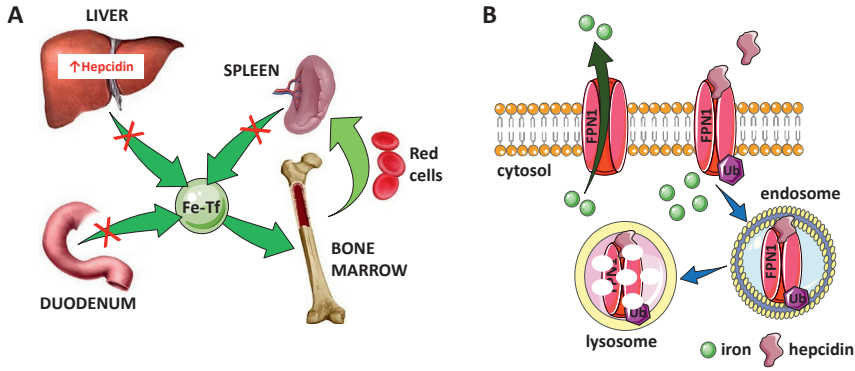

Fig. 1 Hepcidin is central to the regulation of iron metabolism. (A) Systemically, hepcidin is a negative regulator of iron flows such that increased hepcidin synthesis (which mainly occurs in the liver) leads to hypoferremia by decreasing iron absorption in the duodenum, iron recycling from splenic macrophages, and iron release from hepatocyte stores. (B) The mechanism of action of hepcidin involves binding to and occluding ferroportin, induction of ferroportin ubiquitination, followed by endocytosis and lysosomal degradation of the ferroportin:hepcidin complex. Fe-Tf = transferrin-bound iron; FPN1 = ferroportin1; Ub = ubiquitination.

concentration typically results in blockade of iron absorption and sequestration of iron in hepatocytes and macrophages. Hepcidin down-regulates iron release into plasma by binding to and functionally down-regulating ferroportin 1, the sole exporter of intracellular iron (Donovan, Lima, Pinkus, et al., 2005; Nemeth, Tuttle, Powelson, et al., 2004) (Fig. 1B). Ferroportin has been conserved during evolution and is found in microbes, invertebrates, plants and animals (Taniguchi et al., 2015). In humans, ferroportin is found in all cells involved in iron transport, including duodenal enterocytes, macrophages, and hepatocytes. In addition, erythroblasts in the bone marrow also express ferroportin (Zhang, Ghosh, Ollivierre, Li, & Rouault, 2018), an important and interesting finding that, while it is incompletely understood, presumptively enables excess iron release from cells to avoid iron-mediated toxicity and/or enable sharing of a limiting resource for hemoglobin synthesis.

Hepcidin:ferroportin binding leads to both occlusion of the ferroportin channel (Aschemeyer et al., 2018) as well as induction of a conformational change, leading to ferroportin ubiquitination, endocytosis of the complex (Qiao et al., 2012), and its ultimate lysosomal degradation (Nemeth et al., 2004) (Fig. 1B). From a systemic perspective, this block in cellular iron efflux leads to hypoferremia in circulation as iron uptake continues, predominantly for hemoglobin synthesis in bone marrow erythroblasts, leading to its

consumption in the circulation if it is not replaced by iron efflux from enterocytes, macrophages, and hepatocytes. Taken together, maintaining a stable supply of iron in the circulation is dependent on hepcidin-mediated post-translational regulation of ferroportin. In addition, ferroportin expression by macrophages is also transcriptionally regulated by heme (Marro et al., 2010) and under translational regulation (i.e., iron response elements on messenger RNA bound to iron response proteins) by iron (Zhang, Hughes, Ollivierre-Wilson, Ghosh, & Rouault, 2009) independent of hepcidin regulation.

Ferroportin expression on the basolateral side of duodenal enterocytes, on reticular endothelial cells (i.e., splenic macrophages and Kupffer cells in the liver), and on hepatocytes enables hepcidin regulation of iron absorption, recycling, and storage, respectively. Because hepcidin is a negative regulator of iron metabolism, decreased hepcidin concentration results in recovery from iron deficiency and insufficiently increased hepcidin leads to iron overload. Both scenarios are a consequence of increased iron absorption and increased release of iron from intracellular compartments in hepatocytes and macrophages. In some pathological conditions (see below), insufficiently increased hepcidin results in the release of iron into the circulation to such a degree that it overwhelms transferrin's iron binding capacity and results in the generation of non-transferrin bound iron (NTBI) (Esposito et al., 2003). NTBI, in particular its redox active form, labile plasma iron (LPI), is the cause of clinically significant iron overload (Cabantchik, Breuer, Zanninelli, & Cianciulli, 2005). NTBI/LPI is unavailable for erythropoiesis, is taken up by non-hematopoietic cells in a dysregulated manner, causes parenchymal iron deposition (Jenkitkasemwong et al., 2015), and can result in free radical damage leading to the morbidity and mortality of iron overload diseases. Because of the limited expression of ferroportin in most cells, dysregulated iron deposition in such cells is not physiologically reversible and requires therapeutic intervention (e.g., iron chelation therapy). A more complete handling of this point is discussed below.

2.2 Cellular regulation of iron metabolism

Iron is required for homeostatic function in all cells. Iron is essential for the production of heme and iron-sulfur clusters, which are components of proteins/enzymes involved in respiration, nucleic acid replication and repair, metabolic reactions and host defense. Specifically, iron is involved in enzymatic reactions as part of the electron transport chain and the tricarboxylic

acid cycle, as well as in reactions catalyzed by microsomal cytochromes involved in detoxification of drugs and other foreign substances. The majority of iron functions as an oxygen carrier in the heme groups of hemoglobin and myoglobin molecules. In large quantities and when unbound, iron can be highly toxic to cells. As a consequence, cellular iron trafficking is highly coordinated to enable its safe utilization and avoid potential toxicity. Here, we focus on several specific cell types that play a central role in iron metabolism.

(1) *Duodenal enterocytes*: The primary site of dietary iron absorption involves enterocytes within the villous tips of the duodenum. These polarized cells have an apical side, in contact with the gut lumen and dietary contents, and a basolateral side, in contact with blood in circulation. In these enterocytes, non-heme iron is imported from the lumen by the apical divalent metal transporter 1 (DMT1) (Fleming et al., 1997; Gunshin et al., 1997). DMT1 is a metal transporter that takes up iron after duodenal cytochrome B reductase (DCYTB) (McKie et al., 2001) (and possibly other reductases) reduces iron from ferric (Fe^{3+}) to ferrous (Fe^{2+}) states on the luminal side of the enterocyte (Fig. 2A). Iron that is not used for cellular homeostasis is either stored within ferritin or exported by FPN1 on the basolateral surface to be loaded onto transferrin in the circulation. As transferrin-bound iron is obligate Fe^{3+}, intracellular Fe^{2+} must first be oxidized by the basolateral ferroxidase hephaestin, a transmembrane-bound ceruloplasmin homolog, to enable its export (Vulpe et al., 1999). Finally, iron that remains within the enterocyte at the end of its life-cycle—iron that is not exported at the enterocyte's basolateral surface into the circulation (i.e., absorbed iron)—is lost during mucosal shedding. The concentration of hepcidin in circulation regulates iron absorption at the basolateral surface such that low hepcidin levels enable increased iron absorption to enable recovery from iron deficiency (see below) while high hepcidin levels prevent additional iron absorption (Fig. 1A and B).

(2) *Reticuloendothelial macrophages*: At the end of their 120-day life cycle, RBCs undergo erythrophagocytosis by macrophages in the spleen and liver and the iron is recovered. In addition, hemolysis of RBCs leads to the release of hemoglobin into the circulation, where it is bound by haptoglobin, and circulating heme:hemopexin complexes, taken up by macrophages via CD163 and CD91, respectively. Macrophages are equipped with mechanisms to recover iron from heme through heme oxygenase 1 (HMOX1) (Fig. 2B). Depending on systemic requirements, a fraction of the recovered iron is stored

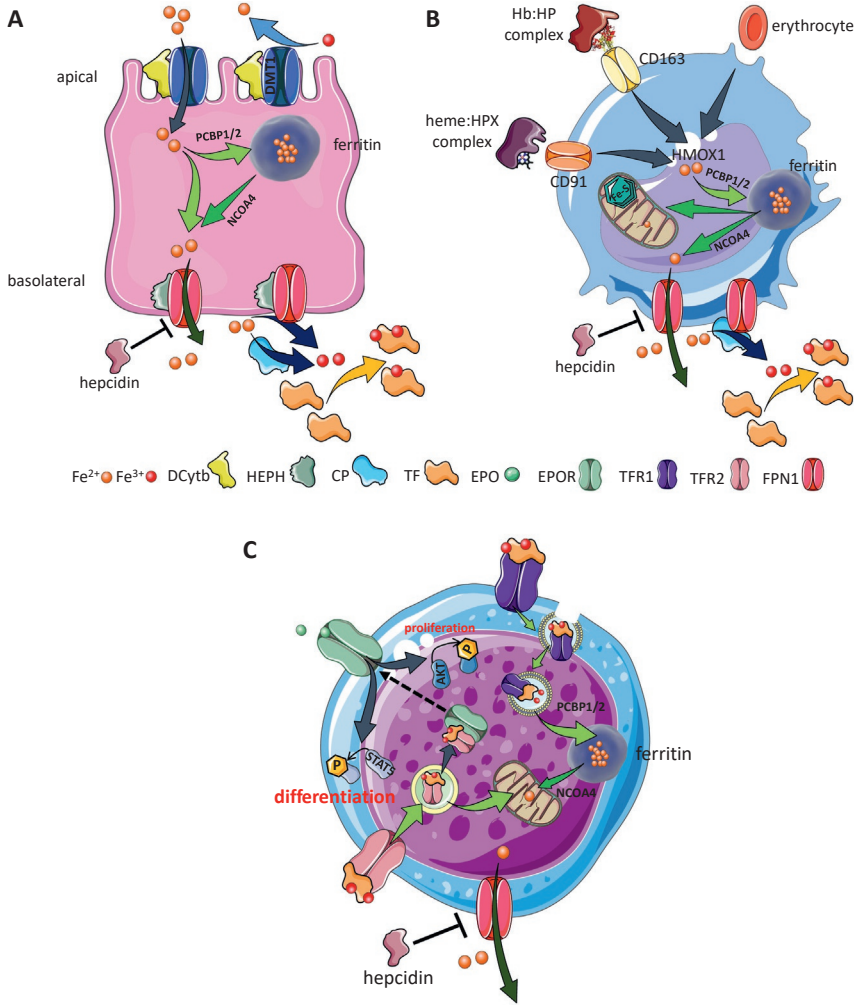

Fig. 2 Cellular iron metabolism. Intracellular iron homeostasis is balanced by coordinated iron uptake, utilization, storage and export. The three main cells of interest include duodenal enterocytes (involved in systemic iron absorption), reticuloendothelial macrophages (involved in systemic iron recycling), and erythroblasts (main location of systemic iron utilization for hemoglobin synthesis during erythropoiesis). (A) *Duodenal enterocyte*: Absorbed inorganic ferric iron (Fe^{3+}) must be first converted to ferrous iron (Fe^{2+}) via ferrireductase Dcytb and subsequently taken up by iron importer DMT1. Once inside the cell, iron is shuttled to ferritin via iron chaperones PCBP1/2 and stored there or shuttled out of ferritin by NCOA4 for export via FPN1. During iron export, Fe^{2+} must be oxidized to Fe^{3+} by HEPH or CP and loaded onto TF for transport in the circulation. Hepcidin prevents iron export at the basolateral cell membrane and results in ferritin iron accumulation within the enterocyte. (B) *Macrophage*: Splenic and liver macrophages

(Continued)

in macrophages, bound for intracellular storage in cytosolic ferritin. Iron is exported via FPN1 back into the circulation to bind transferrin when needed to maintain equilibrium or when erythropoiesis is increased (Fig. 2B).

(3) *Erythroblasts*: As much as 90% of transferrin-bound iron in the circulation is targeted to RBCs in the bone marrow, and ~90% of this is eventually incorporated into heme within developing erythroblasts (Finch et al., 1970). Iron uptake for erythropoiesis is the result of transferrin:transferrin receptor 1 (TFR1) binding (Fig. 2C). Transferrin protein, with two iron molecules (also known as holo-transferrin or diferric transferrin), binds with high affinity to TFR1 at physiologic pH7.4; apo-transferrin (lacking iron) does not. Furthermore, monoferric transferrins, which bind TFR1 with affinity intermediate between holo- and apo-transferrin, are the most abundant transferrin moieties in the circulation (Dautry-Varsat, Ciechanover, & Lodish, 1983; Iacopetta, Morgan, & Yeoh, 1983; Klausner, Ashwell, van Renswoude, Harford, & Bridges, 1983; Klausner, Van Renswoude, et al., 1983; Luck & Mason, 2012; Parrow et al., 2019; van Renswoude, Bridges, Harford, & Klausner, 1982); recent evidence demonstrates that the regulatory consequences

Fig. 2—Cont'd are specifically equipped with mechanisms to enable direct erythrophagocytosis, uptake of Hb:HP complexes via CD163, and heme:HPX complexes via CD91. The heme extracted from these pathways is processed by HMOX1 to liberate iron that is then either incorporated into ferritin or exported from the cell via FPN1 and loaded onto TF for delivery to iron-requiring cells. (C) *Erythroblast*: Iron loaded TF binds to TFR1 on the surface of cells with erythroblasts expressing the highest concentration of TFR1 relative to other cells in light of their high iron requirements. These complexes localize to clathrin-coated pits that invaginate to form specialized endosomes where proton pumps decrease the pH and transported Fe^{3+} is reduced by STEAP3 for export from the endosome via DMT1. Erythroblasts shuttle much of their iron to the mitochondria by an incompletely understood mechanism where it is incorporated into protoporphyrin. FPN1 is also expressed on erythroblasts but purpose of iron export in erythroblasts is incompletely understood. Finally, iron loaded TF also binds TFR2 which is thought to function as an iron sensor to coordinate iron supply with erythropoietic output by modulating EPOR localization and consequently EPO responsiveness; a detailed mechanistic understanding of TFR2's role in erythropoiesis (DMT1 = divalent metal transporter 1; Dcytb = duodenal cytochrome B reductase; FPN = ferroportin 1; HEPH = hephaestin; CP = ceruloplasmin; TF = transferrin; Fe^{3+} = ferric iron; Fe^{2+} = ferrous iron; Hb = hemoglobin; HP = haptoglobin; HPX = hemopexin; CD91 and 169 = cluster of differentiation 91 and 163; HMOX1 = heme oxygenase 1; TFR1 and 2 = transferrin receptor 1 and 2; EPO = erythropoietin; EPOR = EPO receptor; PCBP1 = poly(rC)-binding protein 1; NCOA4 = nuclear receptor coactivator 4; pSTAT5 = phosphorylated signal transducer and activator of transcription 5; pat = phosphorylated protein kinase B).

of monoferric transferrin influence EPO responsiveness in a manner independent of its iron delivery (Parrow et al., 2019). The transferrin: TFR1 complexes are internalized by receptor-mediated endocytosis (Iacopetta et al., 1983; Klausner, Ashwell, et al., 1983; Klausner, Van Renswoude, et al., 1983). Acidification of the endosome results in the release of iron from transferrin (Dautry-Varsat et al., 1983; Klausner, Ashwell, et al., 1983; Klausner, Van Renswoude, et al., 1983; van Renswoude et al., 1982). Several hypotheses have been tested to ascertain how iron it transported within cells, the most compelling of which involves the cytosolic chaperone Poly(rC)-binding protein 1 (PCBP1) which delivers iron to ferritin (Leidgens, Bullough, Shi, et al., 2013; Ryu, Zhang, Protchenko, Shakoury-Elizeh, & Philpott, 2017) (Fig. 2C) with evidence from *Pcbp1* knockout mice, with microcytosis and anemia, that iron delivery to ferritin is required for normal erythropoiesis (Ryu et al., 2017). In addition, PCBP2 is also required for ferritin complex formation (Leidgens et al., 2013). While the mechanistic understanding of iron chaperones following endocytosis remains controversial, there is more consensus that an autophagic process to extract iron from the ferritin core is mediated by nuclear receptor coactivator 4 (NCOA4), a selective cargo receptor for autophagic ferritin turn-over, critical for regulation of intracellular iron availability (Dowdle, Nyfeler, Nagel, et al., 2014; Mancias, Wang, Gygi, Harper, & Kimmelman, 2014) (Fig. 2C). In iron replete states, PCBP1 and PCBP2 expression is enhanced while NCOA4 is targeted to the proteasome for degradation (Mancias, Pontano Vaites, Nissim, et al., 2015). This process, termed ferritinophagy, is believed to provide iron to the mitochondria, the main organelle involved in heme and hemoglobin synthesis during erythropoiesis.

A second transferrin receptor, TFR2, has been shown to mediate signaling events unrelated to meeting cellular iron needs. Specifically, recent studies reveal a novel iron-sensing function of TFR2 in erythropoiesis, via its interaction with EPOR (Fig. 2C) (Forejtnikova et al., 2010; Fouquet et al., 2021; Lee, Hsu, Welser-Alves, & Peng, 2012; Nai et al., 2015; Rishi, Secondes, Wallace, & Subramaniam, 2016). However, the effect of TFR2 on EPO sensitivity remains incompletely understood. While studies in cell culture systems suggest that TFR2 increases EPO sensitivity by enhancing transferrin-mediated increase in cell surface EPOR and downstream signaling (Forejtnikova et al., 2010; Fouquet et al., 2021), mice with TFR2 knockout in the bone marrow demonstrate an increase, rather than the

predicted decrease, in EPO sensitivity—but only during iron deficiency (Rishi et al., 2016). Likewise, iron deficient mouse chimeras with *Tfr2*-deficient hematopoietic cells, demonstrate increased EPO sensitivity, including erythrocytosis and activation of the JAK2-STAT5 and AKT pathways (Nai et al., 2015). We anticipate that the effect of TFR2 on EPO sensitivity depends upon the relative transferrin forms available for binding in the circulation and therefore reflect systemic iron status on erythropoiesis.

3. Physiological regulation of hepcidin expression

Hepcidin expression is predominantly regulated by iron in a feedback loop, a process that is further modulated by inflammation and erythropoiesis (Fig. 3A). Hepcidin is produced mainly by hepatocytes, where *HAMP* transcription is upregulated by iron loading, and suppressed by iron deficiency as well as expanded or ineffective erythropoiesis. The regulation of hepcidin by iron is incompletely understood, but murine models suggest hepatocytes

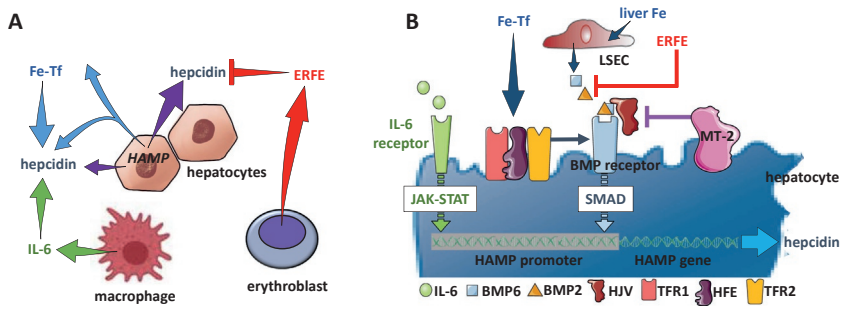

Fig. 3 Hepcidin regulation. (A) Systemically, hepcidin is stimulated by transferrin-bound iron in the circulation and liver iron stores as well as by systemic inflammation and suppressed by enhanced erythroid activity. (B) Regulation of hepcidin expression in the hepatocyte involves JAK-STAT signaling as a consequence of IL-6 receptor stimulation and SMAD signaling as a consequence of a BMP receptor complex stimulation. IL-6 and BMP2/6 binding their receptors, respectively, leads to stimulation of hepcidin expression. Stimulation of erythropoiesis leads to the expression and secretion of erythroferrone which sequesters BMP2/6 to suppress SMAD signaling, decreasing hepcidin. Additional coregulation via matriptase-2 and hemojuvelin as well as systemic iron sensing byTFR1, HFE, and TFR2 enhance BMP receptor stimulation and increase hepcidin expression. Fe-Tf = transferrin-bound iron; IL-6 = interleukin 6; *HAMP* = gene name for hepcidin; ERFE = erythroferrone; LSEC = liver sinusoidal endothelial cell; TFR1/2 = transferrin receptor 1 and 2; HFE = homeostatic iron regulator; HJV = hemojuvelin; MT-2 = matriptase 2; JAK-STAT = janus kinase and signal transducer and activator of transcription; SMAD = small mothers against decapentaplegic.

sense local and systemic iron status by binding bone morphogenetic proteins (BMPs), primarily BMP6, BMP2 and/or their heterodimers via BMP receptors, and transferrin-bound iron via TFR1 and TFR2, leading to signaling to induce hepcidin expression (Fig. 3B) (Camaschella, Nai, & Silvestri, 2020).

First, hepatocytes sense iron indirectly in response to iron-induced bone morphogenic protein (BMP) production by liver sinusoidal endothelial cells (Enns et al., 2013). The BMP pathway is critical for the regulation of hepcidin expression by iron (Babitt et al., 2007; Truksa, Peng, Lee, & Beutler, 2006). BMP6 and BMP2 stimulate hepcidin expression by binding hepatocellular BMP receptor complexes (BMPRc) which include BMPR2 and either of two BMPR1 components: ALK3 or ALK4. BMP:BMPR binding triggers phosphorylation and signaling via SMAD1/5/8 which, coupled with SMAD4, translocate to the nucleus to induce *HAMP* expression (Fig. 3B). Second, several hepatocellular surface molecules modulate *HAMP* activation in response to iron status, enabling hepatocytes to directly sense iron via expression of TFR1, TFR2, and HFE. To briefly delineate, HFE associates with TFR1 under low iron conditions is displaced when TFR1 binds transferrin (Bennett, Lebrón, & Bjorkman, 2000; Feder et al., 1998; Giannetti & Björkman, 2004; Lebrón et al., 1998). Although a mechanistic understanding of how TFR2 contributes to hepcidin regulation remains unclear, we surmise that as serum iron concentration increases, TFR2 expression exceeds that of TFR1 and transferrin binding increases TFR2 stability (Johnson & Enns, 2004; Robb & Wessling-Resnick, 2004) on the membrane and possibly induces HFE binding to TFR2. This HFE:TFR2 complex interacts with hemojuvelin (HJV), the iron-specific BMP co-receptor, and potentiates the BMP signaling pathway and hepcidin transcription (Fig. 3B). Finally, the pathway is negatively regulated by the transmembrane serine protease matriptase 2 (i.e., TMPRSS6) which suppresses hepcidin by cleaving HJV and possibly other components of the signaling pathway to hepcidin expression (Wahedi et al., 2017). Thus, both TFR2 and HFE:TFR1 complex function as the main iron sensors (Robb & Wessling-Resnick, 2004; Schmidt, Toran, Giannetti, Bjorkman, & Andrews, 2008) and communicate systemic iron status to modify hepatocyte hepcidin production and secretion with multiple co-factors modulating this signal.

Hepatocytes may also increase *HAMP* expression in response to stored intracellular iron. Evidence to support this premise comes from the positive correlation between BMP6 expression and iron loading *in vivo* (Kautz et al., 2008). However, the mechanisms of this regulation of BMP6 by intracellular iron stores is not known.

4. Hepcidin regulation by inflammation

Hepcidin expression is increased during infections and systemic inflammatory diseases, reflecting the role of hepcidin in innate immunity and the context for its initial identification as a central mediator of systemic iron metabolism. Enhanced hepcidin production during inflammation is typically mediated by IL-6 (but also IL-1β and IL-22; Lee et al., 2006), signaling via JAK1/2:STAT3 pathway to induce *HAMP* expression in hepatocytes (Fig. 3A), and results in iron sequestration within iron-recycling macrophages and hypoferremia early in infection and inflammation (Smith et al., 2013; Wrighting & Andrews, 2006) (Fig. 1A). Iron sequestration results in decreased iron availability for erythropoiesis, decreasing both hemoglobin synthesis and EPO sensitivity to cause anemia (Bullock et al., 2010; Khalil et al., 2018) (see below). More recent data suggests that IL-6 may have a secondary suppressive effect on erythroid precursors (McCranor, Kim, Cruz, et al., 2014). This process of iron sequestration during infection may have evolved to prevent the generation of NTBI that may occur when suppression of erythropoietic output concurrently with increased RBC destruction leads to systemic iron in excess of erythropoietic demand, thereby increasing iron availability for pathogens (Kim et al., 2014). Thus, hepcidin in host defense prevents the rapid growth of NTBI-dependent extracellular infectious agents.

Some crosstalk between IL-6 induced STAT3 signaling and BMP/SMAD signaling to hepcidin has been documented. For example, the SMAD binding site on the hepcidin promoter remains essential for IL-6 mediated hepcidin expression (Fleming, 2007; Huang, Constante, Layoun, & Santos, 2009; Verga Falzacappa, Casanovas, Hentze, & Muckenthaler, 2008), and liver-specific Smad4 knockout mice demonstrate diminished hepcidin responsiveness to IL-6 (Wang, Li, Xu, et al., 2005). *In vitro* experiments reveal that methods blocking BMP receptor signaling inhibit IL-6-mediated hepcidin expression (Babitt, Huang, Wrighting, et al., 2006; Babitt et al., 2007), providing a rationale for modulating the BMP pathway to alter hepcidin expression in the context of inflammation and its associated anemia.

5. Hepcidin regulation by erythropoiesis

Because hemoglobin synthesis in erythroblasts requires large amounts of iron, erythropoiesis dominates regulation of iron metabolism requiring significant crosstalk. For instance, iron absorption increases, often

dramatically, during stress erythropoiesis to accommodate increased iron demand. Recent data provides mechanistic evidence of what is termed the iron restriction response, demonstrating regulation of erythroid precursor proliferation and differentiation during iron restriction (Bullock et al., 2010; Khalil et al., 2018), in addition to an expected decrease in per cell and total hemoglobin synthesis. Conversely, disease states of excess iron are often associated with expanded RBC size and higher cellular hemoglobin concentrations as a functional utilization of iron within a non-toxic compartment (McLaren et al., 2007). Furthermore, iron loading anemias exhibit complicated regulation schema that remain incompletely understood. Such diseases of concurrent iron overload and expanded or ineffective erythropoiesis (e.g., β-thalassemia, myelodysplastic syndromes, and dyserythropoietic anemias) exhibit lower than expected hepcidin expression, despite increased iron stores. In fact, insufficiently elevated hepcidin expression is implicated in iron overload in these diseases and predicted the existence of an "erythroid factor" regulating iron metabolism (Gardenghi et al., 2007; Ginzburg et al., 2009).

To explore how erythropoiesis regulates hepcidin and thus iron metabolism required separating whether EPO, hypoxia, anemia, reticulocytosis, or erythropoiesis itself are involved. Prior experiments demonstrate that phlebotomy, EPO administration, and hemolysis all resulted in decreased hepcidin expression (Nicolas, Chauvet, et al., 2002; Nicolas, Viatte, et al., 2002; Vokurka, Krijt, Sulc, & Necas, 2006). To separate the effect of erythropoiesis from that of anemia and iron stores, chemotherapeutic agents, radiation, and EPO-blocking antibodies were used to ablate erythropoiesis comparing the effect of hemolysis, bleeding, or EPO injection (without ablation of erythropoiesis) on hepcidin expression in vivo. Results revealed that bone marrow ablation prevents hepcidin suppression in response to hemolysis, bleeding, or EPO injection (Pak, Lopez, Gabayan, Ganz, & Rivera, 2006; Vokurka et al., 2006), strongly supporting the hypothesis that erythroid regulation of hepcidin is a consequence of expanded number of erythroid precursors during stress or ineffective erythropoiesis.

Furthermore, an erythroid factor secreted by erythroid precursors, functioning as a hormone to suppress hepcidin expression in the liver, was predicted several decades prior to its discovery. Although several candidates have been proposed, recent data confirmed that multiple factors may correlate in pathological conditions of expanded or ineffective erythropoiesis but not with physiological regulation of iron by erythropoiesis. Circulating growth differentiation factor 15 (GDF15) for example is increased in patients

with several congenital and acquired anemias and correlates with concurrent low hepcidin (Tanno et al., 2007). However, studies in phlebotomized mice (Casanovas et al., 2013) and in MDS patients (Santini et al., 2011) have shown poor correlation between GDF15 and hepcidin levels, suggesting that mechanisms of hepcidin suppression may be disease specific. Recently, a potential physiologic erythroid regulator of hepcidin, erythroferrone (ERFE), has been identified (Kautz et al., 2014). However, ERFE appears to play a minimal role in baseline erythropoiesis and iron regulation with ERFE knockout mice exhibiting only mild anemia during the postnatal period (Kautz et al., 2014); its main function is likely to facilitate iron mobilization during recovery from transient anemia. A mechanistic understanding of ERFE's regulation of hepcidin has also recently been elucidated with evidence that ERFE is able to sequester BMPs, especially BMP2 and BMP6 which are central to hepcidin regulation, resulting in decreased BMP:BMPR binding, diminished BMP:SMAD signaling, and suppressed hepcidin expression (Fig. 3B) (Arezes et al., 2018; Wang et al., 2020). As a consequence, elevated ERFE enables the increased iron absorption and release from intracellular iron stores needed to meet the iron requirements of the temporary expansion of erythropoiesis during recovery from transient anemia.

6. Hepcidin regulation and hepcidin-independent regulation of iron absorption by hypoxia

Hypoxia regulates hepcidin both via EPO-dependent and EPO-independent mechanisms. Transcription factors known as hypoxia-inducible factors (HIFs) enable physiological responses to hypoxia. HIFs bind hypoxia-response elements to regulate a broad range of genes including those involved in modulating erythropoiesis (e.g., EPO), angiogenesis, and metabolism. HIF hydroxylation by propyl hydroxylases in normoxic conditions results in degradation of HIFs in the proteasome. Because propyl hydroxylase function requires iron and propyl hydroxylase inactivating mutations result in increased HIF concentration and induction of erythrocytosis, this pathway overrides inflammation induced upregulation of hepcidin (Peyssonnaux et al., 2007), resulting in hepcidin suppression (Piperno et al., 2011). Although several *in vitro* studies suggest direct hepcidin regulation by HIFs (Braliou et al., 2008; Peyssonnaux et al., 2007), subsequent *in vivo* studies identified EPO-dependent pathways (e.g., via ERFE) as critical for

HIF-induced hepcidin suppression (Liu, Davidoff, Niss, & Haase, 2012; Mastrogiannaki et al., 2012). In addition, hypoxia induces iron absorption directly, in part independently of regulation by hepcidin and ferroportin. The typically hypoxic environment and increased HIF2α in the small intestine induce upregulation of intestinal mRNAs encoding factors important for iron uptake at the apical side of enterocytes and iron transport at the basolateral side to enhance iron absorption independently of modulating hepcidin (Shah, Matsubara, Ito, Yim, & Gonzalez, 2009) (Fig. 2A). Lastly, recent *in vivo* data indicates that by regulating HIF2α, iron restriction enhances hypoxia responsiveness of iron absorption (Frise et al., 2016).

7. Hepcidin-ferroportin axis regulates iron flows

Iron absorption is predominantly regulated at the basolateral surface of the duodenal enterocyte by control of iron export via ferroportin into plasma in a hepcidin-dependent manner. To initiate iron absorption, iron is taken up into the duodenal enterocyte on the apical membrane via DMT1 and is stored or exported during the few day enterocyte life span (Fig. 2A). If iron retained within the enterocyte is not exported, it will be lost during normal duodenal enterocyte shedding in the gastrointestinal tract. In addition to enterocytes, ferroportin is also expressed on macrophages, physiologically necessary in light of their involvement in erythrophagocytosis of senescent RBCs, break down of hemoglobin, and consequent recycle iron. The major populations of macrophages responsible for steady-state erythrophagocytosis include Kupffer cells, residing in the liver sinusoids, and red pulp macrophages in the spleen. As in duodenal enterocytes, iron liberated from hemoglobin within macrophages is either exported via ferroportin or sequestered within cytosolic ferritin. As ferroportin mediates all cellular iron export, hepcidin binding regulates both the acquisition of dietary iron and the release of iron from macrophages (Fig. 2B). In conditions associated with high hepcidin, iron absorption and recycling are decreased, resulting in a decrease in circulating serum iron concentration and transferrin saturation and an increase in serum ferritin concentration (Fig. 4). Because serum ferritin concentration is proportional to the increased intracellular ferritin, classically driven by increased intracellular iron through the IRE:IRP post-transcriptional regulation, elevated serum ferritin reflects increased iron sequestration in high hepcidin conditions.

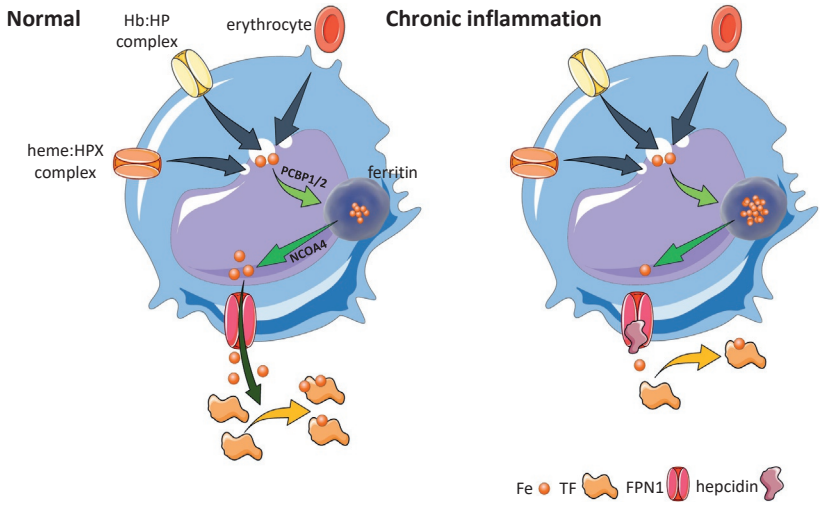

Fig. 4 Effects of inflammation of iron recycling. Under normal conditions, iron recycling from multiple sources within macrophages leads to export of iron via ferroportin back into the circulation where it is loaded onto transferrin and delivered to cells with iron requirements (e.g., for hemoglobin synthesis in erythroblasts during erythropoiesis in the bone marrow). Increased hepcidin in states associated with chronic inflammation lead to binding to and occlusion of the ferroportin channel, preventing iron egress from cells involved in iron recycling (e.g., splenic red pulp macrophages), leading to the accumulation of iron within cellular ferritin core, and causing decreased iron-bound transferrin (low transferrin saturation). This decreased availability of iron for erythropoiesis results in anemia of chronic inflammation. Fe = iron; FPN = ferroportin 1; TF = transferrin; Hb = hemoglobin; HP = haptoglobin; HPX = hemopexin; PCBP1 = poly(rC)-binding protein 1; NCOA4 = nuclear receptor coactivator 4.

8. Hepcidin-ferroportin axis in disease

The discovery of hepcidin as a central regulator of iron metabolism and erythroid regulation of hepcidin by ERFE have enabled a more comprehensive exploration of aberrant iron metabolism in many diseases.

8.1 Hereditary hemochromatosis

Hereditary hemochromatosis (HH) is a common genetically inherited disorder of iron metabolism. Although gene frequency is as high as 5–7%, low penetrance results in only 1:300 to 1:400 affected individuals. All of four types of HH causing disorders result in increased intestinal iron absorption as a consequence of inadequate hepcidin, or hepcidin insensitivity, relative to the degree of systemic iron (Bridle et al., 2003; Muckenthaler et al., 2003;

Nicolas et al., 2003). The overall heterogeneous severity of HH is dependent on the degree of hepcidin suppression (Katsarou & Pantopoulos, 2018). The most common type of HH, Type I HH, results from C282Y or H63D mutation in the HFE gene. HFE is an important membrane protein involved in communicating systemic iron status to the hepatocyte and affects HJV/BMP signaling pathway to positively influence hepcidin production. Both C282Y and H63D mutations lead to reduced cell surface HFE on hepatocytes, preventing appropriate iron sensing and result in a dampened hepcidin response to iron load, resulting in increased iron absorption and transferrin saturation. As a consequence, homozygous C282Y mutation accounts for more than 80% HH patients. Clinically relevant disease is occasionally present as a compound heterozygote mutation with a second synergistic H63D mutation. Overall, HFE-related HH leads to a relatively mild disease phenotype with symptoms associated with systemic iron depositions/overload typically presenting after the fourth decade of life.

Furthermore, iron overload diseases are also associated with mutation in genes coding for other proteins involved in iron sensing and hepcidin regulation. Type II HH results from HJV or hepcidin mutations, and patients with mutated HJV/HAMP exhibit enhanced iron absorption and rapid iron accumulation leading to clinically relevant disease at a young age. *HJV* mutations, like mutations in *hepcidin* itself, result in nearly absent hepcidin expression and result in the most severe clinically form of HH termed Juvenile Hemochromatosis. Type III HH results from a mutation in the gene encoding TFR2 with intermediate clinical phenotype compared to HFE-related HH and Juvenile Hemochromatosis forms. Lastly, type IV HH is an autosomal dominant mutation in the ferroportin gene. Because mutations in the ferroportin gene may affect its membrane concentration as well as hepcidin binding potential, there are two clinical features of this genetic disorder. Patients with mutation leading to reduced ferroportin membrane expression (*loss-of-function* mutation) develop low transferrin saturation and Kupffer cell iron loading due to limited iron export; these patients may become anemic when treated with phlebotomy since they have a diminished capacity to mobilize their iron stores. Although ferroportin on duodenal enterocytes is likely also affected, the transport of 2–4 mg of iron during iron absorption may be easier to accomplish by compensatory mechanisms than the 20 mg of iron recycled daily by macrophages. The other type of ferroportin mutation is characterized by hepcidin insensitivity (*gain-of-function* mutation) and is associated with high transferrin saturation and hepatocyte iron loading. Supporting evidence from a ferroportin mutated cell line demonstrates normal iron efflux activity but no respond to hepcidin. Thus, "hepcidin-resistant" HH is phenotypically similar to hepcidin deficiency in other types of HH.

Taken together, because HH mutations are associated with hepcidin suppression or hepcidin insensitivity, hepcidin-mimetic agents may be applicable as novel therapies in HH patients, either in conjunction or in place of therapeutic phlebotomy. Supporting evidence for this approach comes from pre-clinical studies, using hepcidin injections in $Hfe^{-/-}$ mice and demonstrate reversal of the increased iron absorption and forced expression of hepcidin corrects the hemochromatosis phenotype. These findings implicate insufficient hepcidin stimulation in HFE mutant hepatocytes leading to increased iron absorption and iron overload in HH patients.

8.2 Iron-loading anemias

Hereditary or acquired anemias, such as β-thalassemias, congenital dyserythropoietic anemias, sideroblastic anemias, and myelodysplastic syndrome, are all associated with bone marrow erythroid hyperplasia, ineffective erythropoiesis, extramedullary erythropoiesis and splenomegaly. In severe cases, patients are often treated with chronic RBC transfusions to ameliorate anemia and suppress extramedullary erythropoiesis but result in secondary iron overload. In many cases of inherited iron-loading anemias, withholding RBC transfusion results in expanded erythropoiesis with progressive hepatosplenomegaly and bone deformities, due to expansion of extramedullary and intraosseous erythropoiesis, respectively. In milder cases (e.g., patients with β-thalassemia intermedia), only intermittent RBC transfusions are required but iron overload occurs due to increased intestinal iron absorption. If left untreated, iron overload results in progressive iron deposition, leading to multiple organ dysfunction and accounts for the majority of deaths in this disease.

Ineffective erythropoiesis in iron-loading anemias results in increased expression of ERFE and possibly other pathological regulators of iron metabolism, dampening hepcidin responsiveness to iron. Because increased hepcidin levels are expected in diseases of iron overload to prevent continued iron absorption and exacerbation of iron loading, insufficient hepcidin expression, relative to the degree of iron overload, is implicated as the cause of iron overload observed in iron-loading anemias. Thus suppressed hepcidin results in further increased iron absorption, ultimately exceeding transferrin iron-carrying capacity and leading to the formation of NTBI which is not available for erythropoiesis and deposits in the parenchyma of non-hematopoietic tissue.

The largest body of data on the crosstalk between erythropoiesis and iron metabolism comes from work done in β-thalassemia. Serum hepcidin concentration in β-thalassemic patients is increased in correlation with

hemoglobin and increases in response to RBC transfusion, suggesting that enhanced erythropoietic activity induces hepcidin suppression. Furthermore, the exposure of HepG2 cells to sera from patients with β-thalassemia major after RBC transfusion resulted in higher hepcidin levels relative to the cells exposed to sera of the same patients prior to the next RBC transfusion (Kemna et al., 2008; Weizer-Stern et al., 2006). These findings suggest that hepcidin suppression in β-thalassemic patients results from the secretion of a soluble factor the concentration of which is proportional to the degree of erythroid activity. Recent evidence supports that ERFE is this erythroid regulator of hepcidin which in β-thalassemia is stronger than the regulation of hepcidin by iron and leads to the exacerbation of iron overload. Supporting preclinical evidence reveals increased ERFE in mouse models of β-thalassemia and relatively increased hepcidin expression and decreased iron overload in β-thalassemic/ERFE knockout relative to β-thalassemic mice (Kautz et al., 2014).

Manipulating the ERFE:hepcidin:ferroportin axis to increase hepcidin or block ERFE or ferroportin could help limit intestinal iron absorption and sequester iron in macrophages and hepatocytes to reverse or prevent iron overload. Furthermore, multiple lines of evidence support the beneficial effects of iron deficiency on ineffective erythropoiesis which could reverse splenomegaly and decrease RBC transfusion requirements by reducing RBC destruction in the spleen or improve erythroid differentiation and enucleation in β-thalassemia (Gardenghi et al., 2010; Gelderman et al., 2015; Li et al., 2017, 2010). Thus, in addition to preventing or reversing iron overload, modulating the ERFE:hepcidin:ferroportin axis may provide significant iron restriction within the erythroid compartment and enable more effective erythropoiesis in iron loading anemias.

8.3 Anemia of chronic inflammation

Anemia of chronic inflammation (ACI) is the result of a heterogeneous group of chronic inflammatory disorders, including infections, inflammatory diseases, chronic renal insufficiency, obesity, and/or cancer. In addition, anemia in the elderly is often grouped in this category likely as a consequence of our currently insufficient understanding of its underlying pathophysiology. ACI is characterized as a mild, normocytic normochromic anemia with hemoglobin between 8 and 10 g/dL, elevated inflammatory markers (e.g., CRP and ESR), and decreased serum iron concentration and transferrin saturation despite ample iron stores (e.g., serum ferritin >100 ng/mL). The pathophysiology of ACI is multifactorial, involving

inflammatory cytokines which induce erythrophagocytosis by splenic macrophages, suppress erythroid precursor differentiation and EPO responsiveness (Grigorakaki, Morceau, Chateauvieux, Dicato, & Diederich, 2011; Means Jr., Dessypris, & Krantz, 1992; Wang, Udupa, & Lipschitz, 1995), and decrease EPO production and renal excretion of hepcidin (Jelkmann, 1998; Krajewski, Batmunkh, Jelkmann, & Hellwig-Burgel, 2007; Vannucchi et al., 1994). Recent consensus supports altered iron metabolism at the center of ACI pathophysiology.

Within hours of infection or inflammatory stimuli, plasma iron concentration decreases in humans, other mammals, and lower vertebrates. As a consequence of immune mediators, such as IL-6 and possibly other cytokines involved in host defense, induced as part of the underlying disease, hepcidin-induced hypoferremia results in iron sequestration within the reticuloendothelial system, decreasing iron availability for erythropoiesis and causing anemia (Fig. 4). In preclinical studies, the loss of IL-6 or hepcidin in mice results in absence or attenuation of hypoferremia, a milder anemia, and more rapid recovery of hemoglobin in a well-established mouse model of ACI (Gardenghi, Renaud, Meloni, et al., 2014). Furthermore, IL-6 knockout mice exhibited faster bone marrow recovery relative to hepcidin knockout mice. Taken together, although treatment of the underlying disease remains at the center of therapy for ACI, recent advances in molecular understanding of the hepcidin-ferroportin axis are stimulating the development of hepcidin antagonists that would be potentially beneficial in select ACI cases.

Lastly, evidence of susceptibility to demise with siderophilic bacteria (e.g., *Vibrio vulnificus* and *Yersinia enterocolitica* but also the significantly more common *Klebsiella pneumonia* and *Escherichia coli*) as a consequence of circulating NTBI in diseases of iron overload highlights the potential clinical application of hepcidin agonists as protection from such susceptibility (Ganz, 2018; Stefanova et al., 2018). However, because some intracellular pathogens (e.g., Salmonella) target macrophages, it would be reasonable to consider possible increase in susceptibility to injection in response to hepcidin agonists. *In vivo* and *in vitro* studies have not yet provided definitive resolution of this question (Chlosta et al., 2006; Stefanova et al., 2017; Willemetz et al., 2017) but it remains clear that hepcidin agonists may play a significant clinical role in preventing demise from some severe acute systemic infections.

8.4 Polycythemia vera

Polycythemia vera (PV) is one of the classical BCR-ABL negative myeloproliferative neoplasms (MPNs), a heterogeneous group of clonal hematopoietic

stem cell disorders with constitutively activated physiologic signal-transduction pathways (Spivak, 2017). PV is characterized by erythrocytosis, bone marrow hyperplasia, and a variety of both characteristic and non-specific symptoms. Complications of PV include a significantly increased risk of thrombosis and the potential for evolution to myelofibrosis and MPN-blast phase, significantly reducing survival (Gruppo Italiano Studio Policitemia, 1995; Passamonti et al., 2004; Stein et al., 2015). The most common JAK2 driver mutation is *JAK2 V617F* which results in EPO-hypersensitive JAK-STAT signaling and upregulation of genes downstream of the JAK-STAT pathway (Akada et al., 2010; Baxter et al., 2005; James et al., 2005; Kralovics et al., 2005; Lu, Huang, & Lodish, 2008; Rampal et al., 2014).

Most patients with PV present with iron deficiency at diagnosis (Gianelli et al., 2008; Thiele et al., 2001), prior to the onset of treatment with therapeutic phlebotomy, and iron deficiency is often exacerbated by repeated phlebotomies. Several studies demonstrate that cytoreductive therapy may be associated with correction of iron deficiency in PV patients, either by decreasing phlebotomy requirement or by directly influencing regulators of iron metabolism. For example, a recent phase III clinical trial in PV patients demonstrated significant reductions in HCT levels, splenomegaly, and PV-related symptoms in the ruxolitinib-treated group relative to those receiving best available therapy (Verstovsek et al., 2017). Notably, ruxolitinib treatment resulted in normalization of standard iron-related parameters in PV patients with baseline iron deficiency and hepcidin increased to a greater extent in ruxolitinib-treated PV patients relative to those treated with best available therapy. However, analysis of HepG2 cells treated with ruxolitinib *in vitro* led to decreased hepcidin expression at high concentration mainly by decreasing signaling via JAK1/2:STAT3 (Asshoff et al., 2017), suggesting that *enhanced* hepcidin expression in ruxolitinib-treated PV patients is due to suppression of erythropoiesis and thus a presumed reduction in ERFE levels. It is conceivable that reversal of PV-related symptoms in ruxolitinib-treated patients is a consequence solely of suppressing erythropoiesis. However, it is also possible that, because iron deficiency- and PV-related symptoms often overlap, ruxolitinib-treated patients improve symptomatically due to the consequent decreasing in phlebotomy requirements, reversing iron deficiency in general, or specifically increasing hepcidin levels, resulting in iron sequestration without exacerbating systemic iron deficiency (Fig. 4). A more recent clinical trial using Ropeginterferon α2b (Ropeg-IFN) in PV patients demonstrates a small decrease in phlebotomy requirement relative to control and a trend toward increased serum ferritin after 12 months on trial (Barbui et al., 2021).

Previous reports suggest that hepcidin expression is higher than expected in PV patients (Tarkun et al., 2013), possibly due to the known inflammation associated with MPNs, thus preventing recovery from iron deficiency. We previously demonstrate that despite increased ERFE and relative suppression of hepcidin, JAK2 mutated patients with erythrocytosis exhibit relatively more iron restricted erythropoiesis (Ginzburg et al., 2018). This is surprising because hepcidin suppression is expected to result in iron influx into the circulation to enable recovery from iron deficiency. If iron restriction normally serves as a brake on erythropoiesis when iron availability is limited, erythropoiesis in PV may hijack iron for hemoglobin synthesis at the expense of iron requirements for all other cell functions, depleting iron stores. Thus, hepcidin suppression without recovery from iron deficiency suggests that erythropoiesis in PV may be *hypersensitive* to iron in a similar manner to the well-known EPO hypersensitive erythropoiesis in this disease (Fig. 5). Finally, a currently on-going phase II trial using a hepcidin-mimetic agent in PV patients is demonstrating phlebotomy independent hematocrit control, reversal of systemic iron deficiency, and improvement in PV-associated symptoms.

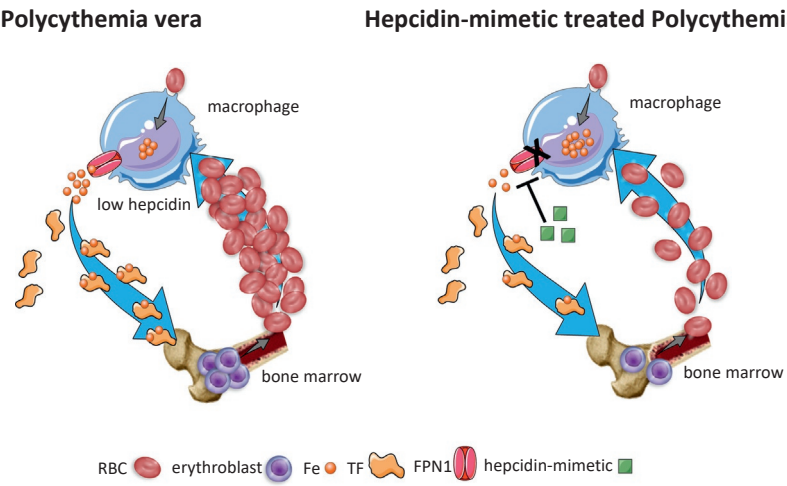

Fig. 5 Effects of hepcidin-mimetic on erythropoiesis in polycythemia vera. In polycythemia vera, where hepcidin levels are low, iron recycling within macrophages leads to export of iron via ferroportin back into the circulation where it is loaded onto transferrin and delivered to the bone marrow for hemoglobin synthesis in erythroblasts during erythropoiesis. Exogenous hepcidin-mimetic agents or inducers of endogenous hepcidin lead to binding to and occlusion of the ferroportin channel, preventing iron egress from macrophages involved in iron recycling, leading to the accumulation of iron within cellular ferritin core, and causing decreased iron availability for erythropoiesis, suppressing RBC production in the bone marrow. RBC = red blood cell; Fe = iron; TF = transferrin; FPN = ferroportin 1.

9. Targeting the hepcidin:ferroportin axis for therapeutic purposes

Novel therapeutic approaches are needed for patients with β-thalassemia for whom state-of-the-art therapy includes chronic iron chelation therapy and splenectomy in addition to lifelong RBC transfusion. Iron chelation therapy has been shown to reduce mortality from secondary iron overload due to recurrent RBC transfusion. Although RBC transfusions suppress ineffective erythropoiesis, thus decreasing ERFE expression and increasing serum hepcidin concentration relative to pre-transfusion levels, the effects are transient (Pasricha, Frazer, Bowden, & Anderson, 2013). Thus, hepcidin suppression, even in transfusion-dependent β-thalassemia major patients, may in part contribute to iron overload, both by enabling increased iron absorption between RBC transfusions as well as preventing sequestration of iron within macrophages and hepatocytes, resulting in increased circulating iron, generation of NTBI, and parenchymal iron overload. Furthermore, multiple lines of investigation suggest that increasing hepcidin and the consequent reduction of iron availability for erythropoiesis improves ineffective erythropoiesis, reversing splenomegaly and increasing hemoglobin, in mouse models of β-thalassemia intermedia (Casu et al., 2016; Guo et al., 2013; Li et al., 2010), in addition to ameliorating iron overload. Several hepcidin-mimetic agents are currently in clinical trials to treat both transfusion-independent β-thalassemia intermedia and in some cases also transfusion-dependent β-thalassemia major (Longo, Piolatto, Ferrero, & Piga, 2021).

Studies in mouse models of HH also demonstrate that hepcidin overexpression prevents iron overload (Gardenghi et al., 2010; Nicolas et al., 2003). Although phlebotomy therapy in HH is relatively easy, it is not free. The cost can be circumvented by enabling these patients to donate blood at donor centers but may be limited at some donor centers only to patients who would otherwise be eligible to donate blood (i.e., meeting all blood donor criteria for sexual preference, vital signs, concurrent illnesses (e.g., HCV), travel history, and medications). In addition, a small fraction of patients are intolerant to phlebotomy. Lastly, there is clear evidence that relative hepcidin deficiency leading to iron overload increases susceptibility of HH patients to siderophilic bacteria such as *V. vulnificus* and *Y. enterocolitica* (Khan, Fisher, & Khakoo, 2007; Vadillo, Corbella, Pac, Fernandez-Viladrich, & Pujol, 1994). Mouse models of HH and iron loading in wild type mice revealed increased susceptibility to these infections as well as

Klebsiella pneumoniae (Arezes et al., 2015; Horseman & Surani, 2011; Michels et al., 2017; Stefanova et al., 2017). We and others anticipate that increasing hepcidin to sequester iron within macrophages would decrease pathogen access to circulating iron and increase survival in infected iron overloaded patients (Brissot, Ropert, Le Lan, & Loréal, 2012). It is yet unclear how targeting the hepcidin:ferroportin axis would affect pathogenesis of intracellular organisms during infection. Several industry partners are currently exploring whether hepcidin-mimetics would provide a useful alternative therapeutic strategy for HH patients.

In addition, iron sequestration as a consequence of increased hepcidin may be clinically beneficial for PV patients with high phlebotomy requirements. Although the pathophysiological cause for iron deficiency in newly diagnosed PV remains to be clarified, increasing hepcidin would be expected to induce a kind of "chemical phlebotomy" by preventing iron accessibility for erythropoiesis. In fact, administration of exogenous hepcidin (Casu et al., 2016), hepcidin-inducing agents (Casu et al., 2021), and ferroportin inhibitor (Kubovcakova et al., 2018) have been shown to reverse erythrocytosis and splenomegaly in *JAK2 V617F* mice. The mechanism of action appears to be iron sequestration in splenic macrophages, inducing iron restricted erythropoiesis (Fig. 5).

To support the potential clinical utility of increasing hepcidin in PV, we look to another disease of systemic iron deficiency caused by excess hepcidin, iron-refractory iron deficiency anemia (IRIDA) (Heeney & Finberg, 2014). IRIDA presents in childhood as an extremely microcytic anemia with very low serum ferritin and transferrin saturation. Despite this, IRIDA patients have significantly fewer signs and symptoms relative to true iron deficiency, and long-term follow-up reveals normal growth and cognitive development (unlike the concerns in systemic iron deficiency, leading to universal infant screening programs) (Baker & Greer, 2010; Melis et al., 2008; Pearson & Lukens, 1999). These differences suggest that iron sequestered in macrophages is available for cellular function and homeostasis, even during periods of rapid growth, but the quantities of available iron are insufficient to enable robust erythropoiesis. We hypothesize that redistribution of iron that is expected to result from increased hepcidin in PV would be expected to sequester iron within macrophages to halt constitutive and aberrant erythropoiesis, decrease phlebotomy requirements, and together reduce systemic symptoms of iron deficiency.

As a consequence of systemic iron restriction, the use of hepcidin mimetics may appear counter intuitive. However, because PV is associated

with increased red cell mass and a larger than typical proportion of body iron is contained within hemoglobin, using hepcidin mimetics would enable a redistribution of iron by sequestering it within hepatocytes and macrophages, preventing depletion of iron stores, restricting iron availability for erythropoiesis, and reversing symptoms of systemic iron deficiency. Studies in *JAK2 V617F* mice demonstrate the potential of exogenous hepcidin to reverse erythrocytosis, decrease splenomegaly, and sequester iron in splenic macrophages (Casu et al., 2016) and suggest that the use of such "hepcidin mimetic agents" may be beneficial in low risk PV patients.

In addition to hepcidin-mimetics, several companies are taking the approach of endogenous hepcidin inducing agents. For example, silencing *Tmprss6*, important for physiological hepcidin suppression, enhances hepcidin expression, preventing iron overload and improving erythropoiesis in β-thalassemic mice (Finberg, Whittlesey, & Andrews, 2011; Nai et al., 2012). Both antisense oligonucleotides as well as siRNAs against *Tmprss6* have been successfully developed, enabling liver targeting of these compounds to enable robust suppression of *Tmprss6* and significant multi-fold induction of hepcidin production. Efficacious preclinical experiments with such agents for multiple indications have stimulated the pursuit of targeting the hepcidin:ferroportin axis for translational purposes. Taken together, several strategies are being used to exogenously delivery hepcidin mimetics, induce endogenous hepcidin production, and ferroportin inhibitors in clinical trials (Casu, Nemeth, & Rivella, 2018) (Table 1).

Hepcidin antagonists have been postulated as potentially effective novel therapy for ACI and anemia of cancer, diseases in which ESAs and IV iron are either scarcely effective or contraindicated. This category of potential therapeutics includes hepcidin sequestering antibodies, anticalins, and aptamers, BMP/SMAD and IL6/STAT3 inhibitors, hepcidin mRNA degrading and protein sequestering agents, or ferroportin stabilizers (Fung & Nemeth, 2013; Leung, Luan, Manetta, Tang, & Witcher, 2012). Specifically, first-in-human phase 1 study of fully humanized neutralizing anti-hepcidin monoclonal antibody, LY2787106, in anemia of cancer was recently published (Vadhan-Raj et al., 2017). Results demonstrate that LY2787106 is well tolerated and leads to increased serum iron and transferrin saturation within 24 h, returning to baseline after 8 days. In addition, pre-clinical studies in cynomolgus monkeys using the small protein-based anticalin PRS-080 demonstrate high affinity and specificity for hepcidin, 43 h half-life, and dose-dependent hepcidin suppression and iron mobilization (Hohlbaum et al., 2018). Furthermore, the anti-hepcidin Spiegelmer,

Table 1 Hepcidin mimetics in development for PV patients.

Name	Industry sponsor	Type of agent	Route of administration	Stage of development
Rusfertide	Protagonist Therapeutics	Synthetic hepcidin mimetic	Subcutaneous	*Phase II* (REVIVE: NCT04057040; PACIFIC: NCT04767802) *Phase III* (VERIFY: NCT05210790)
Sapablursen	Ionis Pharmaceuticals	Liver-targeted ASO against TMPRSS6	Subcutaneous	*Phase II* NCT05143957
SLN124	Silence Therapeutics	Liver-targeted siRNA against TMPRSS6	Subcutaneous	*Phase II* for patients with β-thalassemia and MDS (GEMINI II; NCT04718844); *Phase II* in PV in planning stage
VIT-2763 (Vamifeport)	Vifor (International), Inc	Ferroportin inhibitor	Oral	*Phase II* for patients with β-thalassemia (NCT04938635) and SCD (NCT04817670)

lexaptepid, a synthetic compound designed to inhibit hepcidin without nuclease and immune system clearance susceptibility, resulted in a dose-dependent increase in serum iron and transferrin saturation and was well tolerated in healthy subjects (Boyce et al., 2016). This agent is being developed for ACI.

Other approaches, such as anti-BMP6 antibody, was ineffective as a consequence presumably of compensation by other BMPs (Corradini et al., 2010) while inhibitors of the HJV blocked SMAD phosphorylation and decreased hepcidin expression in normal rodents (Andriopoulos et al., 2009; Babitt et al., 2007) as well as an ACD rat model, inducing recovery from anemia (Theurl et al., 2011). Anti-IL6 (siltuximab) and anti-IL6 receptor (tocilizumab) antibodies demonstrate decreased hepcidin in patients with

Castleman's disease, improving anemia (Song et al., 2010; van Rhee et al., 2010). In addition, several agents that prevent STAT3 phosphorylation decreased IL6-mediated hepcidin expression in mice (Fatih et al., 2010; Zhang, Wang, Wang, & Liu, 2011). Several excellent reviews were recently published on this topic (Katsarou & Pantopoulos, 2018; Poli, Asperti, Ruzzenenti, Regoni, & Arosio, 2014). In addition, silencing of HJV and TFR2 by siRNA as well as induction of purported erythroid regulators of hepcidin (e.g., ERFE) may be additional future targets to explore.

10. Conclusion

The discovery of hepcidin as a central regulator of iron metabolism and erythroid regulation of hepcidin by ERFE enabled a mechanistic exploration of aberrant iron metabolism in many hematopoietic and non-hematopoietic diseases. This enhanced understanding has within a relatively short timeframe lead to the development of novel compounds manipulating this pathway to support both exogenous agonist and antagonist function with multiple agents already undergoing clinical trials for several indications.

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