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Ferroportin-mediated iron transport: expression and regulation

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Abstract

The distinguishing feature between iron homeostasis in single versus multicellular organisms is the need for multicellular organisms to transfer iron from sites of absorption to sites of utilization and storage. Ferroportin is the only known iron exporter and ferroportin plays an essential role in the export of iron from cells to blood. Ferroportin can be regulated at many different levels including transcriptionally, post-transcriptionally, through mRNA stability and post-translationally, through protein turnover. Additionally, ferroportin may be regulated in both cell-dependent and cell-autonomous fashions. Regulation of ferroportin is critical for iron homeostasis as alterations in ferroportin may result in either iron deficiency or iron overload.

Keywords

ceruloplasmin; ferroportin; hepcidin; homeostasis; internalization; iron

1.0 Introduction

Iron is essential and while one of the most abundant of the transition metals, it is generally biologically unavailable. Organisms must go to great efforts, in terms of energy expenditure and expression of specific proteins to generate iron in a bioavailable form. Iron is used for a variety of oxidation/reduction reactions and when complexed with protoporphyrins forms heme, the major oxygen-binding molecule. Iron can be found bound to proteins as iron, oxo-diiron (Fe-O-Fe), oxo-iron-zinc (Fe-O-Zn), iron-sulfur clusters (Fe-S) and heme. The ability of iron to participate in oxidation/reduction reactions is the feature that makes iron so useful in enzymatic reactions. That same feature of facile electron transfer makes iron potentially dangerous, as iron can donate electrons to O_2 and H_2O_2 generating superoxide anion and the hydroxyl radical (for review see [1]. These molecules can oxidize proteins, lipids and nucleic acids rendering them defective. Iron in high concentrations can replace other transition metals in their binding to proteins resulting in defective protein function and inactive enzymatic activity. This is most notably seen when iron replaces manganese as the metal cofactor for superoxide dismutase 2 [2]. Thus, while iron is essential, its concentration in biological fluids must be tightly controlled.

In eukaryotes, iron acquisition can be regulated, but once acquired there is no regulated mechanism of iron loss. In contrast, excess copper can be exported through bile [3]. The rate of iron loss is essentially invariable and is due to epithelial cell loss, menstruation and child-bearing. In the absence of an export mechanism, malregulation of iron import can lead to

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iron overload, tissue damage and organ dysfunction. While the penetrance of genetic iron overload disorders is subject to debate [4, 5], there is no question that secondary iron overload or transfusion-related iron overload carries a significant burden of morbidity and mortality.

There is no regulated mechanism of iron loss from an organism, however, multicellular organisms face the issue of transferring iron from one tissue to another [6]. This requires the active export of iron from cells to biological fluids, most notably plasma. Four tissues are critically important for cellular iron homeostasis. The placenta and intestine are responsible for net iron transfer to the organism while macrophages and parenchymal tissue like hepatocytes, represent sites of iron recycling. The majority of iron in vertebrates is found as heme in circulating erythrocytes. Erythrocytes have a defined life span and in humans the average erythrocyte lives for 120 days. Aged or damaged erythrocytes are cleared from the circulation through phagocytosis by macrophages. Once ingested by macrophages, erythrocytes are degraded in lysosomes, heme is released, iron extracted from heme by the ER-localized enzyme heme oxygenase and the released iron can be stored or exported. Most of the iron that enters plasma daily comes from macrophage-recycled iron. Intestinal iron uptake, under normal conditions, accounts for only a fraction of total plasma iron. Ferroportin (Fpn) emerges as a critical transporter in terms of iron acquisition and transfer of iron between cell types, as it is the only known transporter that exports elemental iron from cells. Fpn is essential to distribute iron between tissues and for iron absorption into the organism.

2.0 Function of Fpn in mammalian health

To date, Fpn is the only known mammalian iron exporter and is essential for transport of iron from one cell type to another. Three different groups reported the identification of Fpn nearly simultaneously [7–9]. One of the groups that discovered Fpn did so through analysis of a zebrafish mutant [9]. The mutant resulted from a large-scale ENU mutagenesis screen and showed a severe defect in hemoglobinization in early zebrafish embryos. The mutant gene was shown to be FPN containing a premature stop codon. The Fpn protein was not expressed in erythrocytes but rather in the yolk syncytial layer, suggesting that the defective protein prevented the movement of iron from iron stores to the circulation. Studies with older mutant embryos showed increased iron content in intestinal cells and macrophages, suggesting a defect in iron transport [10]. These results were confirmed in mice with a targeted deletion in the *Fpn1* gene [11]. These findings show that Fpn is essential for early embryonic development. The loss of Fpn in the extraembryonic visceral endoderm resulted in embryonic death. In contrast, expression of Fpn hypomorphs, notably $Fpn^{ffe/-}$, where *ffe* is the name of a mouse with a missense mutation of Fpn (H32R), resulted in severe neural tube defects [12]. The severity of the defect was a function of the expression of Fpn and could be phenocopied by iron limitation using iron chelators [13]. These results underscore the importance of Fpn and iron in vertebrate development.

Deletion of *Fpn1 in utero* resulted in viable mice, however, the mice showed stunted growth and anemia shortly after birth. These mice accumulated iron in enterocytes, macrophages, and hepatocytes [11]. The data are consistent with an essential role for Fpn in cellular iron export, as the lack of Fpn resulted in iron accumulation in iron-exporting tissues. Elemental iron, iron not bound to protein, is taken up as Fe^{2+} by the H^+/M^{2+} (divalent transition metals) symporter DMT1 present on the luminal surface of the enterocyte (for review see [14]). DMT1 is not specific for iron and will transport other transition metals. The importance of DMT1 in iron acquisition is shown by the severe defect in intestinal iron absorption shown by mice with mutations in DMT1 [15]. Once iron is taken up by DMT1 it is found in the cytosol of enterocytes. To enter the blood and have access to the rest of the

tissues of the body, iron needs to cross the basolateral membrane. Transport across the basolateral membrane is mediated by Fpn. The enterocyte has a short life span and iron accumulated within the enterocyte will be lost to the body when the cells are sloughed off. Thus, iron acquisition is dependent on transfer of iron across both the apical and basal surfaces. As will be described below, Fpn-mediated iron transport is highly regulated and this regulation provides the coordination between iron acquisition, utilization and storage.

Mice with a targeted deletion of *Fpn1* in macrophages show a relatively mild anemia [16]. Most of the iron entering blood comes from macrophages recycling iron from senescent red blood cells, so it was unexpected that the anemia was mild. The *Fpn*$^{ffe/}$ mouse also shows a mild anemia [12]. One explanation is that macrophages can export heme via the Feline Leukemia Virus C Receptor (FLVCR) transporter [17], which might compensate for the loss of Fpn.

The importance of Fpn has been underscored by studies of humans with iron overload diseases and studies of genetic or acquired iron-related anemias. In both instances there are alterations in the level of the peptide hormone hepcidin. Studies in mice, discussed below, first showed that hepcidin is a negative regulator of iron acquisition and acts by altering Fpn levels. Most forms of human hereditary hemochromatosis result from a deficiency in hepcidin expression and are inherited as recessive disorders (for review see [18]). Deficiencies in hepcidin result in increased iron acquisition. In contrast, increased expression of hepcidin results in iron-limited anemias. In 1999 Pietrangelo and colleagues identified patients with iron overload disease but the patients had no defects in the HFE gene, which is responsible for most instances of hepcidin-deficiency related iron overload disease [19]. They demonstrated that this iron overload disorder was dominantly inherited in contrast to disorders due to hepcidin deficiency. They reported that many patients with this disorder showed a unique phenotype of primarily Kupffer cell iron loading and relatively low to normal transferrin saturation (referred to as “classic” Fpn disease). Other patients showed a phenotype indistinguishable from classic hereditary hemochromatosis, which results from mutations in genes that lead to hepcidin deficiency. It was later shown that both phenotypes resulted from mutations in Fpn [20]. Mechanisms that explain both the inheritance pattern and phenotypes are described below.

3.0 Structure of ferroportin

Ferroportin has 9–12 transmembrane domains (multitopic). The topology of Fpn has not been defined with precision and the number of transmembrane domains is still undefined. A model proposed by Liu et al [21] suggesting 12 transmembrane domains has been most often used as a working model for Fpn structure [22]. Studies indicate that the amino terminus of Fpn is cytosolic [21–24]. The location of the carboxyl-terminal, however, is unclear. Studies using epitope-tagged proteins have suggested that the carboxyl-terminal is cytosolic [21, 25] but other studies, including a recent study using monoclonal antibodies, have suggested that the carboxyl terminal is extracellular [26]. These authors suggested that the presence of the epitope might affect the location of the carboxyl terminal. While this is possible, epitope-tagged Fpn is fully functional with respect to transport activity and regulation.

A second controversial issue regarding Fpn structure is whether Fpn is a monomer or dimer. Many reports using similar approaches including chromatography, cross-linking and physical techniques such as birefringence, have led to contrasting results, with some studies showing Fpn as a monomer [20–22] and others showing Fpn as a dimer [23, 25, 27]. Support, for the dimer model comes from genetic data. Human diseases due to mutations in Fpn show dominant inheritance. There are a number of possible mechanisms that explain the

dominant inheritance. The two most probable possibilities are haploid insufficiency, in which the required level of a gene product necessitates production from both parental alleles. In the absence of one allele, the level of gene product is not sufficient for function. A second possible explanation is that Fpn is a multimer. A mutant Fpn can participate in multimer formation and affect the activity of the complex, thus becoming dominant negative. In this model, a cell carrying a mutant allele would have three different Fpn multimers, a wild type homodimer, a mutant homodimer and a wild type-mutant heterodimer. Only the wild type homodimer would have activity, giving the cell 25% of normal activity. There is a critical distinction between the two models: a null allele or missense mutation would lead to haploid insufficiency but would not affect wild type Fpn activity versus a multimer acting in a dominant negative mechanism. Analyses of Fpn mutations in human patients have shown that all mutations identified to date are missense mutations, no nonsense mutations have been reported. Further, a targeted deletion of *Fpn1* in mice, which is embryonic lethal when homozygous, shows no phenotype when heterozygous [11]. Young $Fpn^{null/+}$ heterozygous mice were indistinguishable from controls in all measurements. At age 6 months, $Fpn^{null/+}$ mice were not anemic, although reticulocytes and mature erythrocytes had decreased cellular hemoglobin and decreased cell volume indicative of iron-restricted erythropoiesis [11].

The lack of an obvious phenotype for $Fpn^{null/+}$ mice could be discounted as mice being different from humans, however, a missense mutation in mice *flatiron* (Fpn H32R) showed the phenotype of “classic” ferroportin disease and the expected dominant genetic transmission [12]. The mutant protein, when expressed in mammalian cells or when analyzed in cultured *flatiron* mouse macrophages, showed impaired iron transport activity. These results provided support for the model that Fpn is a dimer. Further support for a dominant negative dimer model came from studies in zebrafish. Expression of wild type human or mouse Fpn cDNA in zebrafish had no discernible phenotype [28]. In contrast, expression of missense mutations that result in defective Fpn activity led to anemia due to iron insufficiency. As zebrafish express endogenous Fpn, the observation that mutant Fpn can affect the function of wild type endogenous Fpn provides strong support for the dominant negative model of genetic transmission and Fpn multimerization.

Mutations that result in defective Fpn transport activity will lead to the phenotype of “classic” Fpn disease, which is macrophage iron loading and normal to low transferrin saturation. What is unclear is the exact mechanism by which a specific Fpn mutation will lead to decreased iron transport. Expression of mutant Fpn in some cultured cell types results in defects in Fpn trafficking where the mutant protein never reaches the cell surface [21, 23]. In other cell types, the mutant Fpn was shown to accumulate at the cell surface but had defective transport activity [22, 24]. The exact reasons for the differences in observed behavior of Fpn mutants has yet to be resolved.

4.0 Metal transport activity of Fpn

The role of Fpn in iron transport has been readily shown by expressing Fpn in a variety of different cell types, including cultured mammalian cells, *Xenopus* oocytes and zebrafish embryos. Fpn-mediated iron transport activity, in mammalian cells, has been assessed by measuring the levels of the iron storage protein ferritin. Cells incubated with iron show increased cytosolic iron and concomitantly increased levels of ferritin [8, 29]. When iron is removed, cellular ferritin is high and expression of Fpn, either endogenously or from transfected plasmids, results in iron export and decreased ferritin, which can be assayed by ELISA or Western blot. Fpn activity can also be assayed by measurement of the egress of radioactive iron from cells [30, 31]. All of these measurements support the genetic data, that Fpn is an iron transporter and may well be the only vertebrate cellular iron exporter.

Little is known about the mechanism of Fpn-mediated iron transport. It is inferred that the substrate for Fpn is Fe(II), based on the observation that iron transport requires an extracellular ferroxidase activity. This ferroxidase is provided by the multicopper oxidases ceruloplasmin (Cp) and/or hephaestin. The role of Cp in facilitating entry of iron into blood was reported in the mid 1800's in which copper salts were shown to be efficacious in treating anemia in young women (see [32] for an engaging history of the relationship between copper and iron). Starting in the mid 1950's, studies by Cartwright [33] and later Frieden and co-workers [34, 35] proved that copper-deficient animals were also iron-deficient and that the copper-containing protein Cp was required to release iron from macrophages and hepatocytes. Vulpe et al identified a Cp homologue hephaestin as the gene defective in sex-linked anemia in mice [36]. Hephaestin is a membrane bound multicopper oxidase, which is highly expressed in the intestine. In the absence of hephaestin, mice are anemic due to defective iron export from the intestine.

Hephaestin, Cp, zyloklopen (a newly identified mammalian multicopper oxidase [37]) and the orthologous multicopper oxidase Fet3 in fungi and algae have a similar mode of action. These molecules contain four to six atoms of copper which store electrons as Fe(II) is oxidized to Fe(III). The stoichiometry of the reaction is

$$4\mathrm{Fe}^{2+}+4H^{+}+O_2 \rightarrow 4\mathrm{Fe}^{3+}+2H_2O$$

The multicopper oxidases oxidize one iron atom at a time storing the extracted electron. When the fourth atom is oxidized the stored electrons reduce molecular oxygen in one concerted reaction. The oxidation of ferrous to ferric iron can occur non-enzymatically. There are, however, two reasons that the multicopper oxidases are important for iron transport. First, the reaction carried out by the multicopper oxidases does not generate oxygen radicals in contrast to spontaneous oxidation of iron. Second, at low oxygen tension spontaneous oxidation of iron is slow and multicopper oxidases, due to their high affinity for oxygen, dramatically increase the rate of oxidation [38]. The requirement for a ferroxidase has been shown genetically and in a number of systems by demonstrating a requirement for a multicopper oxidase in Fpn-mediated iron export. In several cell types including macrophages [39] and neural derived cells [40], the lack of a multicopper oxidase impairs iron transport. Some studies have reported a physical interaction between Fpn and Cp [40] or hephaestin [26]. The fact that a soluble form of the yeast Fet3 can restore iron egress in mice with a targeted gene deletion in Cp suggests that if there is an interaction between Fpn and a multicopper oxidase, it need not be stable [41].

To date it has been shown that all known Fe(II) transporters can also transport other transition metals. Expression of Fpn can protect cells from zinc [42] or manganese [43] toxicity and does so by lowering the concentration of those metals in cells. As described below, transcription of *Fpn1* can be induced by zinc and other metals providing support for a physiological role for Fpn-mediated transport of other transition metals.

Fpn can be found in eukaryotes other than vertebrates. Morrissey et al identified FPN in *Arabidopsis* based on positional cloning of a mutant *Arabidopsis* gene that showed increased cobalt accumulation in shoots [44]. The mutation was identified as a premature stop codon in a putative FPN. Further analysis showed that *Arabidopsis* has two FPN loci that encode proteins with different tissue and subcellular locations and function. FPN1 is expressed in the plasma membrane of the stele, which is the vascular system of plants. FPN2 is expressed on the vacuolar membrane of the root and its levels change in response to iron deficiency. Studies in mutant and transgenic plants, as well as expression of *Arabidopsis* FPN1 in yeast,

showed that the two plant FPNs can differ in metal transport activity [44]. FPN2 was shown to transport cobalt whereas FPN1 showed a preference for iron.

The nematode *Caenorhabditis elegans* has three Fpn genes. Fpn1.1 expressed in mammalian cells was capable of transporting iron and zinc [45]. The substrate preferences of the other two Fpn are unknown. There are three striking features about Fpn in non-vertebrate species. First, vertebrates have only one Fpn gene; non-vertebrates may express multiple genes. Second, non-vertebrate Fpn may be localized on subcellular membranes, whereas, vertebrate Fpn appears to function at the plasma membrane. Finally, invertebrates do not appear to express hepcidin, the major peptide hormonal regulator of Fpn levels. These findings suggest that during evolution Fpn might have had selective pressure resulting in a more restricted subcellular distribution and metal selectivity.

5.0 Transcriptional regulation of Fpn expression

Ferroportin transcription has been shown to be regulated by iron deficiency hypoxia, transition metals, heme and inflammatory cytokines. The extensive regulation underscores the important role of Fpn in iron homeostasis.

5.1 Regulation of Fpn in response to hypoxia

One of the major physiological cues for increased iron absorption is hypoxia/anemia. Increased erythropoiesis leads to increased iron absorption and elevated levels of Fpn mRNA. McKie et al's discovery of Fpn was based on increased expression of Fpn mRNA in the duodenum of the hypotransferrinemic mouse [4]. This mouse is severely anemic and shows increased hypoxic response. Hypoxia leads to wide changes in transcription including genes involved in iron metabolism. The basis for the hypoxic response is the stabilization of members of the HIF family of transcription factors (for review see [46]). Under normal conditions Hif1α and Hif2α are cytosolic proteins that are rapidly degraded by the proteasome. The signal for degradation is the hydroxylation of Hif proline residues. Proline hydroxylation is mediated in an iron, oxoglutarate and oxygen-dependent reaction by prolyl hydroxylases. In the absence of these reagents, Hif1α or Hif2α are not hydroxylated and not degraded but accumulate in the nucleus where they partner with aryl hydrocarbon receptor nuclear translocator (Arnt) (also referred to as Hif1β) to form an active transcription factor. Deletion of *Arnt* was shown to decrease iron-mediated induction of DMT1 and Fpn in the intestine [47]. Furthermore, an intestine-specific deletion of Hif2α reduced transcription of Dmt1 and Fpn1 while deletion of Hif1a did not [48]. The Fpn promoter contains HIF-Responsive Elements (HRE) and an *Fpn* reporter construct responded to low oxygen [49]. Mutation of the HREs prevented that response. Finally, Chromatin immunoprecipitation (CHiP) studies performed with mouse duodenum showed that Hif2α could bind to the Fpn promoter region. These results show that Hif2α is a direct activator of Fpn transcription.

The importance of transcriptional regulation of *Fpn1* in intestinal iron homeostasis was shown using mice with a targeted deletion in *Hamp*, the gene that encodes hepcidin [50]. In the absence of hepcidin there is increased expression of intestinal iron transporters, DMT1 and Fpn, as well as systemic iron overload. An intestinal specific deletion of *Hif2α* in $Hamp^{-/-}$ mice resulted in decreased levels of *DMT1* and *Fpn1* mRNA, showing the importance of transcriptional regulation of *Fpn1*[51]. It was noted that deletion of *Hif2α* reduced transcription of *Fpn1* in mice placed on a low iron diet for a short period of time (two weeks). In contrast, there was increased *Fpn1* transcription in *Hif2α*-deleted mice chronically maintained on a low iron diet, indicating the importance of other transcription factors in regulating *Fpn1*.

5.2 Regulation of Fpn transcription by heme and metals

Fpn transcription was shown to be induced by iron, heme and other transition metals. Studies have shown that erythrophagocytosis results in *Fpn1* transcription in macrophages [52, 53]. Subsequent work has shown that both heme and iron can induce *Fpn1* transcription. Different results, however, were obtained using different cell types. In some instances heme and iron were shown to act independently on *Fpn1* transcription. For example, Marro et al showed that in RAW264.7 mouse macrophages, *Fpn1* transcription was induced by heme or by iron-free protoporphyrin IX [53]. Addition of iron salts had no effect on *Fpn1* transcription. They further showed that the heme induction of *Fpn1* transcription was due to the release of the heme-sensitive transcriptional inhibitor Bach1 and the accumulation of the transcription factor Nuclear Factor Erythroid 2 (Nrf2). The authors demonstrated the presence of binding sites for these transcription factors in the promoter region of *Fpn1*. Mutation of those elements in reporter constructs abrogated transcription. In contrast, Knutson et al showed that in the mouse macrophage-like cell line J774 heme-induced Fpn transcription was due to the release of heme iron [31, 52, 54]. For example, *Fpn1* transcription could be reduced by the iron-chelator salicylaldehyde isonicotinoyl hydrazone (SIH) and increased in response to iron salts, suggesting that iron was the critical factor in regulating transcription. Delaby et al, investigating heme-dependent transcription of *Fpn1* in cultured bone marrow macrophages, concluded that heme-induced *Fpn1* transcription required the release of iron from heme [55]. Inhibition of iron release from heme prevented *Fpn1* transcription and incubation of cells with protoporphyrin was unable to induce *Fpn1* transcription. *Fpn1* mRNA and *Fpn1* reporter constructs, expressed in cultured cells or bone marrow macrophages, responded to iron salts suggesting transcriptional activation rather than mRNA stabilization [42]. It is clear that different results are found in different cell types. In the case of iron-induction of *Fpn1* transcription, the relevant transcription factor has not been identified. One possible explanation that might reconcile these results is that Nrf2, which belongs to the basic leucine zipper (b-Zip) transcription factor family, has a central role in protecting cells against oxidative stress. Electrophilic agents can induce Nrf2-dependent *Fpn1* transcription in an iron-independent manner [56]. It might be possible that iron salts can also induce Nrf2-dependent transcription through iron's effect on oxidant stress.

Fpn can also be induced by activation of the transcription factor MTF-1 [42]. MTF-1 targets genes relevant to heavy metal loading such as metallothionein-1 (MT-1) or the zinc efflux transporter Znt1. MTF-1 can also mediate the induction of MT genes in response to stress situations such as oxidative stress and hypoxia. Troadec et al determined that MTF-1 is important in the zinc-mediated induction of *Fpn1* mRNA [42]. MTF-1 binds to the *Fpn1* promoter in the presence of zinc and mutagenesis of the two Metal Responsive Elements (MREs) in the *Fpn1* promoter abolishes the MTF-1 zinc responsiveness. They further demonstrated that cadmium induced *Fpn1* transcription in an MRE-dependent manner. Cadmium can directly displace zinc from metallothionein or damage metallothionein by generating oxidative radicals that would act on metallothionein and release zinc. The released zinc can then bind to MTF-1 and induce transcription.

5.3 Inhibition of Fpn transcription by inflammation

Hepcidin expression is induced during inflammation, commonly by bacterial products acting through Toll-like receptors. Inflammation can also affect transcription of Fpn. Injection of bacterial-produced lipopolysaccharide (LPS) into mice or rats resulted in decreased *Fpn1* transcription in spleen and intestine [50]. The effect on *Fpn1* transcription was independent of specific cytokines, as mice with gene deletions in *IL-6*, *Tnf-α* and *IL-1* all respond to LPS with hypoferremia and reduced levels of *Fpn1* mRNA [57]. Interestingly, the effect of LPS attenuation on intestinal *Fpn1* mRNA levels was decreased by the addition of an iron

chelator [58]. Most recently, Harada et al showed that activators of the redox-sensitive transcription factor Nrf2 reduced the LPS-induced suppression of *Fpn1* mRNA in human and mouse macrophages [56]. It is tempting to speculate that iron chelators might function as Nrf2 activators, which would unify these findings.

6.0 Post-transcriptional regulation of Fpn

Abboud and Haile discovered Fpn through the identification of an mRNA that had an iron-responsive element (IRE) in the 5′-region of the mRNA [8]. The FPN mRNA was identified by its ability to bind to Iron Regulatory Protein 1 (IRP1). (For review see [59–61]). Subsequent studies showed that translation of *Fpn1* or Fpn reporter constructs containing the IRE was inhibited by low iron and increased by high iron. The importance of the 5′-IRE was underscored by the discovery of a mouse that had a mutation that deleted the IRE. A radiation-induced mutation in mice was identified that resulted in a 58 bp microdeletion in the *Fpn1* promoter region [62]. This deletion altered the transcription start site and eliminated the 5′-IRE, resulting in increased duodenal and hepatic Fpn1 protein levels during early postnatal development. Mice carrying this mutation show a complex phenotype including polycythemia (increased red blood cell production) at birth followed by iron overload as adults and anemia as the mice age [62–64]. Increased red cell expansion (polycythemia) was related to defective maternal-to-fetal iron transport. Fetal iron deficiency was attributed to decreased placental Fpn protein and mRNA. It is unclear why *Fpn1* mRNA levels were decreased. Iron overload in adult mice was attributed to increased Fpn protein expression due to the absence of the 5′-IRE. Age-related anemia was ascribed to a loss of splenic stromal cells. The reason for this loss was not determined.

Analysis of mRNA transcripts in mouse duodenum and erythroblasts [55] and human erythroblasts [56] identified *Fpn1* transcripts which lacked the 5′-IRE, termed *Fpn1B*. Translation of *Fpn1B* is insensitive to iron. The reading frame of the protein, however, is identical to that of the IRE-containing transcript and the protein can transport iron and respond to hepcidin. *Fpn1B* accounted for 25% of total *Fpn1* mRNA in duodenum but constituted a much higher percent of *Fpn1* transcripts in erythroblasts [65]. It was suggested that the Fpn1B in the intestine might still export iron even under conditions of iron deficiency. In the developing erythroblast, iron-insensitive translation of *Fpn1* might make erythropoiesis sensitive to hepcidin levels and thus sensitive to systemic iron deficiency [66].

7.0 Post-translational regulation of Fpn

The expression of Fpn can also be regulated post-translationally. Once Fpn is expressed, it is targeted to the cell surface. The concentration of cell surface Fpn determines the amount of iron exported. The level of cell surface Fpn is highly regulated by the rate of synthesis, the rate of internalization and the rate of degradation. Below we describe how the hormone hepcidin regulates Fpn levels and how Fpn levels can also be regulated independent of hepcidin.

7.1 Hepcidin-mediated Fpn internalization

The roles of hepcidin and Fpn were cemented by the striking observation that mice with a targeted deletion in *Hamp* were massively iron-loaded [67]. In contrast, overexpression of hepcidin in mice led to severe iron deficiency [68]. A study in humans showed that overexpression of hepcidin, as seen in a severe example of the Anemia of Chronic Inflammation, resulted in hypoferremia [69]. In contrast, the recessive iron overload diseases were due to decreased levels of hepcidin [70]. These studies showed that hepcidin affected entry of iron into blood. How this occurred was elucidated by Nemeth et al, who

demonstrated that hepcidin bound to and induced the internalization of Fpn expressed in cultured cells [29]. The observation that Fpn levels were affected by hepcidin explained both recessive iron overload disorders resulting from decreased hepcidin and iron deficiency disorders resulting from increased levels of hepcidin. Hepcidin levels are increased by inflammation and increased iron stores and are decreased by hypoxia and by increased erythropoiesis (for review on regulation of hepcidin transcription see [18, 71].

The finding that hepcidin resulted in a loss of Fpn was confirmed in vivo by examining Fpn levels in mouse tissues. Increased levels of Fpn were seen in intestine, spleen and liver in *Hamp*$^{-/-}$ mice compared to control mice. Hepcidin also induces the loss of Fpn in cultured hepatocytes [72] and macrophages [12]. The relationship between hepcidin and intestinal Fpn, however, remains cloudy. Chronically high levels of hepcidin lead to loss of intestinal Fpn, but studies have shown that acute changes in hepcidin have a modest effect on intestinal Fpn levels. At the same time, however, acute changes in hepcidin have a significant effect on Fpn levels in splenic macrophages [73, 74]. Analogous results were reported in intestine-like cell lines; acute hepcidin exposure had little effect on Fpn levels but did affect iron transport activity [75]. The alteration in iron transport activity was suggested to result from changes in DMT1 levels. How hepcidin affects DMT1 levels is unclear. To date the only known hepcidin receptor is Fpn. There are data that show that hepcidin binding to Fpn has effects beyond inducing the internalization of Fpn, including marked transcriptional changes [76]. Such transcriptional effects may extend to DMT1. One approach to test this possibility is to measure the effect of hepcidin on DMT1 levels in mice with a targeted deletion of Fpn in intestine.

The mechanism of hepcidin-mediated Fpn internalization in macrophages and many other cultured cell types has been described. The binding site for hepcidin has been identified as an extracellular loop in Fpn in which the residue Cysteine 326 is absolutely required for hepcidin binding [77, 78]. Mutation of C326 leads to hepcidin-resistant hemochromatosis in patients. As described above, evidence suggests that Fpn is a dimer and that each monomer of Fpn must bind hepcidin for hepcidin-mediated internalization. Hepcidin binding to Fpn was shown to lead to the binding of the cytosolic Janus kinase (Jak2), a non-receptor tyrosine kinase and member of the JAK-STAT signaling family [79]. Bound Jak2 is autophosphorylated and then phosphorylates Fpn on either of two adjacent tyrosines [80]. Mutation of these two tyrosines to phenylalanines resulted in a loss of hepcidin-mediated Fpn internalization [80, 81].

Phosphorylated Fpn is internalized by clathrin-coated pits. Phosphorylation is a transient event and once internalized, the phosphate(s) on Fpn is removed by an unidentified phosphatase. Hepcidin-resistant Fpn mutants that are capable of binding hepcidin show defects in Jak2-mediated Fpn phosphorylation. Jak2 can bind to the Fpn mutants but it is not activated and does not phosphorylate Fpn [79]. Cooperativity between the two Fpn monomers is required for Jak2 binding and phosphorylation. A dimer composed of a wild type Fpn monomer and a hepcidin–resistant monomer will bind Jak2 but will not be phosphorylated or internalized. A different example of cooperativity is shown by the human Fpn mutant D157G; this mutation results in hepcidin-independent activation of Jak2 and constitutive internalization of Fpn [82].

One of the consequences of Jak2 activation is a transcriptional response resulting from Jak2 phosphorylation.[83]. In isolated macrophages as well as mice, hepcidin addition and binding to Fpn leads to a transcriptional response that results in the suppression of inflammation. This suppression is mediated in part through induction of Suppressor of cytokine 3 (Socs3) [76]. These studies are supported by work in mice with a targeted deletion in macrophage Fpn. The hepcidin-Fpn1 macrophage signaling is abrogated, which

mimics the low hepcidin situation and leads to enhanced TLR4 signaling and an increased proinflammatory response [74]. A particularly elegant study showed that mice with mutations in TransMembrane PRoteaSe Serine 6 (TMPRSS6) show a blunted response to an inflammatory stimulus [84]. Mutations in TMPRSS6 result in high levels of hepcidin and severe iron-limited anemia. This result distinguishes between the effects of iron and hepcidin on transcription and shows that high levels of hepcidin can modulate inflammation independent of iron.

Hepcidin-mediated internalized Fpn is degraded in the lysosome, but to gain entry to the lysosome Fpn must enter the lumen of multivesicular bodies (MVB). The MVB then fuses with lysosomes and Fpn is then exposed to lysosomal hydrolases. Entry of membrane proteins into the MVB requires their ubiquitination and recognition of protein bound ubiquitin by a set of cytosolic proteins termed ESCRT proteins. Reductions in many of the ESCRT proteins by RNAi leads to decreased degradation of Fpn and the accumulation of Fpn in intracellular vesicles [80, 85]. Tsg101 (Vps23p) is a component of the ESCRT-I complex, which is required for recognition of ubiquitinated cargos destined for the MVB. Ubiquitination of internalized Fpn is a requirement for entry into the MVB. Prevention of ubiquitination, as occurs in a mutant cell with a temperature-sensitive E1 ligase, leads to hepcidin-mediated accumulation of Fpn in large intracellular vesicles, similar to that seen in Tsg101-silenced cells. The residue that is ubiquitinated on hepcidin-mediated internalized Fpn was identified as lysine 253 [80]. This residue is on the same large intracellular loop that contains the Jak2 phosphorylated tyrosines. The E-3 ubiquitin ligase Nedd4-2 was identified as being responsible for the ubiquitination of hepcidin-internalized Fpn [45].

Kono et al described an interesting interaction between hepcidin and multicopper oxidases [81]. Increased expression of transfected Cp in cultured cells or addition of Cp to medium, antagonized hepcidin-mediated Fpn internalization, as much higher doses of hepcidin were required to internalize Fpn in Cp expressing cells. Expression of mutant Cp, with diminished oxidase activity did not suppress hepcidin-mediated internalization. The mechanism by which Cp influences hepcidin-mediated internalization remains unclear.

7.2 Hepcidin-independent internalization of Fpn

Hepcidin is the sole identified ligand that can induce Fpn internalization. Studies, however, have shown that Fpn internalization can occur in a hepcidin-independent manner. As described above, Cp plays a role in cellular iron egress. Mice with a targeted deletion in *Cp* show brain iron overload similar to that seen in humans with aceruloplasminemia. In the absence of Cp, iron is not exported from cells [40]. Further studies have shown that Fpn is degraded in the absence of Cp. In macrophages and neural cells, the loss of functional Cp, either by RNAi silencing or copper deprivation [33] or expression of a dominant-negative form of Cp [86], resulted in the loss of Fpn on the cell surface. Inhibition of endocytosis revealed that Fpn was being synthesized but was being rapidly internalized and degraded. When endocytosis was inhibited, Fpn accumulated at the cell surface. Fpn internalization, due to the absence of Cp, was independent of hepcidin and Jak2 but was dependent on the ubiquitination of K273 and Nedd4-2 [45].

An important finding was that a Fpn mutant (N174I) incapable of transporting iron was not internalized in the absence of Cp activity [39]. Further, in the absence of Cp, wild type Fpn immunoprecipitated from ^{59}Fe-labeled cells had ^{59}Fe bound to it. No ^{59}Fe was found on Fpn immunoprecipitated from ^{59}Fe-labeled cells expressing Cp. These findings led to the hypothesis that in the absence of Cp, iron could not exit Fpn and iron bound to Fpn affected Fpn conformation. It was further hypothesized that the altered Fpn conformation was recognized by the E3-ligase resulting in Fpn being marked for internalization and degradation. Support for this hypothesis came from two lines of evidence. First, in the

absence of Cp, addition of other multicopper oxidases could restore cell surface Fpn. Most important, addition of iron chelators to cells silenced for Cp could also restore cell surface Fpn. These data support the view that the loss of cell surface Fpn was due to an inability to "offload" bound iron.

The second line of evidence supporting the view that defective iron transport activity leads to Fpn degradation came from the finding that expressed cell surface Fpn was degraded when cytosolic iron pools were reduced [45]. The initial discovery of this phenomenon came from the finding that there was a time-dependent disappearance of Fpn-GFP in cells induced to express Fpn-GFP. Robust cell surface expression of Fpn was lost in 24–36 hours, although there were still high levels of Fpn mRNA. Addition of iron preserved cell surface Fpn, while addition of permeable iron chelators rapidly decreased cell surface Fpn. Similar results were found using cell lines transfected with Fpn-GFP constructs. Transport-defective Fpn was not affected by iron chelators, and an Fpn mutant lacking K253 was also not affected by the iron chelator. Finally, iron-limited internalization of Fpn was dependent on the E3-ligase Nedd4-2. Further evidence that iron-limited "marking" of Fpn reflected a loss of transport substrate was demonstrated by the fact that high levels of zinc or manganese, substrates for Fpn transport, led to Fpn being retained on the cell surface even in the presence of iron-chelators.

Substrate-mediated degradation of membrane transporters has been eloquently articulated by Pelham and colleagues working in yeast [87]. They suggested that Ub-E3 ligases, such as Rsp5, are able to detect misfolded membrane proteins because polar residues in the lipid bilayer are abnormally exposed. One can imagine that substrate transport requires molecular movement of the transporter. The absence of substrate or the presence of bound substrate might trap the transporter in the extremes of these conformational changes increasing the possibility of degradation.

8.0 Conclusion

The importance of Fpn in iron homeostasis is supported by the fact that it is regulated at many different levels. Many of aspects of that regulation remain to be clarified. The transcriptional regulation of Fpn in response to iron or to inflammation requires identification of the critical transcriptional activators or repressors. What also requires clarity is the reason for the cell type variation in transcriptional regulation of Fpn and in the subcellular location of Fpn. Why do different Fpn mutants show differences in subcellular localization in different cell types? Even wild type Fpn shows cell type specific changes in subcellular location. In some cases, particularly in intestinal cells or macrophages, Fpn can be found in subcellular compartments [27]. Iron or other conditions can induce its translocation to the cell surface. Strains of mice that contain a missense allele of *Mon1a* have lower splenic iron levels than other strains of mice [88]. Macrophages cultured from these mice have higher levels of cell surface Fpn in contrast to macrophages from other strains, which have more Fpn in their Golgi than on the cell surface. The function of Mon1a is unknown nor are the mechanisms that control the movement of Fpn from Golgi to cell surface.

The finding that there are two mechanisms for removal of Fpn from the cell surface leads to the question of how do these mechanisms interact? What are the biochemical requirements that differentiate the two forms of Fpn regulation? Notably, is the hepcidin-independent mechanism of greater importance in tissues not bathed by the systemic circulation? While there is some understanding of Fpn regulation at both transcriptional and post-translational levels, there really is little known about the mechanism of Fpn-mediated metal transport. It has been suggested that multicopper oxidases provide a driving force for iron transport. This

hypothesis needs more rigorous testing. Expression of Fpn can deplete iron from ferritin but the affinity of iron for Fpn is unknown. Mutagenesis studies have suggested residues required for iron transport [22] but beyond that finding there is no structural information on how this multitopic protein transports iron. Finally, both the specific residues in Fpn or the evolutionary pressures that determine metal substrate selectivity in Fpn are unknown. These and other issues will surely be addressed before the next special issue of Cell Biology of Metals.

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Highlights

- Ferroportin is the only known iron exporter.
- Malregulation of ferroportin leads to human disease, iron limitation or iron overload.
- Ferroportin is regulated transcriptionally, post-transcriptionally, and post-translationally.
- Post-translational regulation can occur by hepcidin-dependent and hepcidin-independent mechanisms.