Multiple regulatory mechanisms act in concert to control ferroportin expression and heme iron recycling by macrophages

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The daily production of new erythrocytes by the bone marrow is a process which makes big demands on body iron stores and the erythroid activity of the bone marrow regulates the control of iron homeostasis. Tissue macrophages, especially in the spleen and liver, play a major role in controlling the bioavailability of iron for erythropoiesis. These cells phagocytose senescent red blood cells (RBCs) and recycle heme iron back to the plasma thereby providing the 20-25mg iron required for the daily production of new erythrocytes.[1] Following binding of a senescent red blood cell to the macrophage membrane, the formation of a phagocytic vesicle leads to the internalization of the red blood cell which is subsequently degraded by the proteolytic machinery of the phagolysosome. The heme molecule reaches the cytosol where it can act as a sensor molecule regulating the transcription of several genes, or be degraded by heme oxygenase 1 (HO-1) to release CO, biliverdin and iron in its ferrous Fe(II) form. Iron will subsequently be stored into the ferritin molecules for further use or exported back to the plasma by ferroportin (FPN), the sole iron exporter in mammals. FPN is highly expressed at the plasma membrane of macrophages but also in duodenal enterocytes where it contributes to intestinal iron absorption, and in several other cell types such as hepatocytes and tubular kidney cells, albeit at a much lower level. The presence of FPN in erythroid cells has also been reported, although its function has still not been clarified.[2]

The amount of iron that is actually recycled by macrophages is very much dependant on the amount of functional FPN at the plasma membrane. Recent evidence shows that this results from a complex interplay between several transcriptional and posttranscriptional regulatory mechanisms.

FPN structure and function

The topology of FPN is not yet fully elucidated but it is considered to be a twelve transmembrane domain protein with both the N and the C terminus in an intracellular position.[3,4] For many years, whether FPN functions as a monomeric[4-6] or as an oligomeric form[7] has remained controversial and conclusions have not yet been reached. Several biochemical studies performed on transfected cells expressing tag-FPN molecules or on recombinant FPN in solution have yielded conflicting results. Evidence based on type 4 hemochromatosis, an autosomal dominant iron overload disorder due to *FPN* mutations, suggest that FPN behaves as a dimer, the mutated FPN exerting a dominant negative effect on the FPN molecule synthesized by the normal allele, thereby impairing iron transport.[8] It is thought that haploinsufficiency alone is not sufficient to impair iron recycling by macrophages and induce tissue iron overload. This issue is still a matter of debate.

The metal transport activity of FPN has not been studied in detail but all the evidence points to a ferrous Fe(II) transport activity, although FPN can also transport other divalent metals but with a lower affinity. The export of ferrous iron is concomitant with the oxidation to the ferric Fe(III) state by proteins of the multi-copper oxidase family. Ceruloplasmin synthesized by hepatocytes and secreted in the plasma, also present as a GPI-anchored form in macrophages, or hephestin in duodenal enterocytes catalyze the oxidation of ferrous to ferric iron prior to its binding by serum transferrin (Figure 1). When the oxidase is deficient, as in aceruloplasminemia, iron is retained in the macrophages, probably because the pro-oxidant ferrous iron remains bound to FPN and induces its degradation.[9]

Regulation of FPN expression

Transcriptional regulation by heme

Little has been known up to now about the transcriptional regulation of *FPN* gene expression. A previous study carried out on primary cultures of bone marrow derived macrophages had already proposed that erythrophagocytosis induces FPN, HO-1 and ferritin mRNA expression by a heme-mediated pathway followed by stimulation of FPN and ferritin mRNA translation by an iron-dependant mechanism.[10] In this issue of the journal, Marro *et al.* add new insights into the mechanism by which heme activates the transcription of the *FPN* gene.[11]

Heme is a multifunction molecule, serving as the prosthetic group of various types of proteins, such as hemoglobin, myoglobin and cytochromes but also acting as a sensor molecule regulating the expression of several genes.[12] For instance, in erythroid cells, heme deficiency activates the heme-regulated inhibitor (HRI), a general repressor of mRNA translation, thereby limiting the production of globin chains in conditions of insufficient heme supply. In hepatocytes, accumulation of intracellular uncommitted heme will repress the mictochondrial transport of delta amino-levulinic acid synthase 1, the first and rate-limiting enzyme of the heme biosynthetic pathway. Heme is also a potent transcriptional activator, stimulating the transcription of several genes such as *HO-1*, FTL and FTH genes. Under heme-rich condition, heme binds to cysteine-proline motifs of the transcriptional repressor Bach1, inhibiting its DNA-binding activity. Bach1 is a basic leucine zipper transcriptional repressor which forms heterodimers with the small Maf proteins such as MafK and binds to Maf Recognition Elements (MARE) present in upstream regulatory regions of several target genes.

Using a murine macrophage cell line, Marro *et al.* show that heme, in the form of hemin or as hemoglobin, activates *FPN* transcription in an iron-independent manner, similarly to what is observed with the *HO-1* gene.[11] This activation relies

on the presence of a functional MARE element located 7 kb upstream of the *FPN* gene transcription start site. Knockdown of Bach1 by siRNA strongly increased both *HO-1* and *FPN* gene expression whereas its overexpression decreased both mRNAs. When Bach1 is replaced by the transcriptional activator Nrf2, which also dimerizes with a small Maf protein, on the MARE element, transcriptional activation of the target genes follows (Figure 1). A similar mechanism has been previously described for ferritin genes[13] and it is likely that these coordinated regulations aim at protecting the cells from heme-mediated toxicity and at allowing rapid recycling of heme iron. Posttranscriptional regulations will also further increase this cellular response.

Posttranscriptional regulations by iron

Changes in intracellular iron content are also detected by Iron Regulatory Proteins called IRP1 and IRP2, which act as iron sensors and regulate target mRNAs by binding to Iron Responsive Elements (IREs) present in the non-coding regions of these mRNAs.[14] Both ferritin H and L chain and FPN mRNAs contain an IRE in their 5' non-coding region and their synthesis is repressed in conditions of low iron content. Increased availability of intracellular iron will induce conformational changes in the IRPs that will loose their IRE binding affinity and favor recruitment of ferritin or FPN mRNAs in the translational machinery.[10,11] This mechanism will stimulate both ferritin and FPN synthesis following heme degradation by HO-1 and iron will be either sequestered in the ferritin molecule or exported to the plasma by the newly synthesized FPN (Figure 1). It is not yet clear as to what is controlling the distribution of iron between the storage compartment and the "export" compartment. It is possible to speculate that the relative affinity for iron of these two iron-accepting proteins (ferritin and FPN) is different and/or that their respective level of expression favors one pathway or the other. Furthermore, it has been proposed that the net amount of FPN present at the cell membrane, which is tuned to the erythroid need of the bone marrow by systemic signaling, facilitates iron mobilization from the ferritin-associated iron stores.[15]

Finally, the importance of the IRP-mediated control of FPN mRNA translation has been challenged recently by the observation that the selective inactivation of IRP2 in mouse macrophages had no consequences on FPN expression nor on macrophage iron content and that iron management by macrophages was more dependant on systemic signaling.[16]

Membrane trafficking

FPN is mostly found at the plasma membrane of macrophages or duodenal enterocytes but it is also present in intracellular vesicles when over-expressed by transfection or following stimulation of its synthesis by iron. The mechanisms controlling its membrane targeting or its sorting to baso-lateral membrane in epithelial cells have not been studied in detail.

One protein has been reported to control the amount of FPN targeted to the plasma membrane. By quantitative trait locus analysis (QTL), Mon1a, a cargo protein implicated in vesicular trafficking, was identified as a gene modifier of iron macrophages through its ability to control membrane targeting of FPN in iron-recycling macrophages. A gain-of-function mutation in Mona1a in C57BL/6 strain of mice was associated with more FPN at the cell surface and reduced macrophage iron stores as compared to other strains of mice.[17]

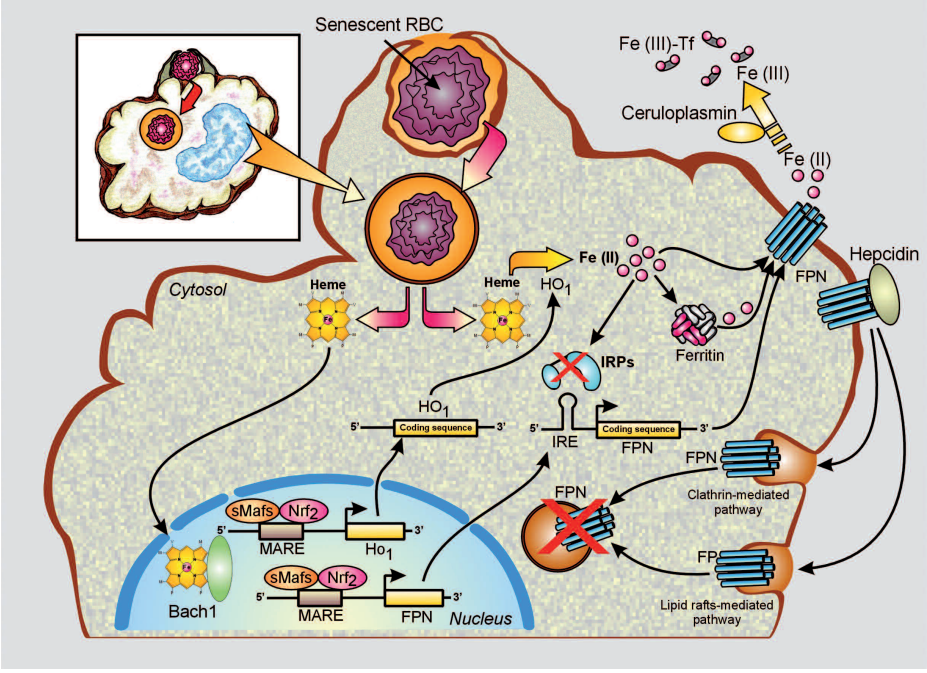

Figure 1. Schematic representation of the different regulatory steps that control the amount of cell surface FPN expression in tissue macrophages. Following phagocytosis of a senescent red blood cell (RBC), heme is released into the cytosol. It can then enter the nucleus to inactivate Bach1 and relieve the transcriptional repression of HO-1 and *FPN* gene expression, allowing the recruitment of the transcriptional activator Nrf2 complexed to small Maf proteins. The HO-1 protein will subsequently degrade heme and release iron that will in turn inactive the Iron Regulatory Proteins (IRPs) and allow the translation of the Iron Responsive Element (IRE)-containing mRNAs (FPN and ferritin chains). Iron is then stored into ferritin or exported back to the plasma by FPN. Finally, serum hepcidin can bind cell surface FPN and induce its internalization and degradation by two different mechanisms. Illustration by Jean-Pierre Laigneau, INSERM.

Systemic regulation by hepcidin

Besides these intrinsic regulations, the amount of FPN at the plasma membrane is tightly controlled by systemic regulations relying on FPN-hepcidin interactions. Hepcidin is a 25 amino-acid peptide synthesized primarily by hepatocytes, secreted into the plasma and filtered into urines. Hepcidin is a negative regulator of intestinal iron absorption and recycling of heme iron by macrophages. Hepcidin was discovered at the turn of the century and aroused a wave of enthusiasm among the scientific community, rapidly leading to the evidence that this molecule plays the role of the two long sought after signaling molecules: the so-called "iron store regulator" and the "erythroid regulator".[18] Hepcidin is able to play this dual role through exquisitely complicated regulatory pathways.[19]

Several studies have shown that hepcidin binds to FPN and induces its internalization and subsequent degradation, thereby limiting iron efflux from the target cells.[20,21] The hepcidin binding site is highly dependant on the cystein in position 326 in the extra cellular loop present between TM 7 and 8 of the FPN molecule.[22] Hepcidin binding induces the binding and activation of protein kinase Jak2 that in turn phosphorylates either of two adjacent tyrosines, Y302 and Y303, present in a large intracellular loop of FPN while it is resting at the cell surface.[23] FPN is subsequently internalized in a clathrin-coated pit, dephosphorylated and ubiquitinated. Trafficking through the multivesicular body will finally send FPN for degradation into lysosomes.

A challenging mechanism of FPN internalization is reported in the paper by Auriac *et al.* published in this issue of the journal.[24] Using primary cultures of bone marrow derived macrophages or a macrophage cell line, the authors propose that FPN is associated with microdomain-like structures in the macrophage membrane consisting in lipid rafts. These membrane regions are short-range ordered structures enriched in cholesterol, sphingolipids and a large variety of membrane proteins, including GPI-anchored proteins or conventional transmembrane proteins. This association of FPN with lipid rafts seems necessary for hepcidin-mediated internalization of FPN since the sequestration of cholesterol by filipin, a drug inhibiting lipid raft-dependant endocytosis, significantly reduced the proportion of FPN that was internalized following hepcidin addition to the culture medium. Interestingly, inhibition of the clathrin-mediated endocytic pathway by chlorpromazine did not prevent hepcidin-mediated internalization, at odds with the previous studies showing clathrin-mediated internalization of FPN following hepcidin addition.[25] Cell specificities of this hepcidin-mediated internalization of FPN could account for these apparent discrepancies since most studies on the phosphorylation and clathrin-mediated internalization of FPN were performed in transfected HEK293 cells, an immortalized epithelial cell line, whereas the study presented here showing the association of FPN with lipid rafts was performed in both primary and immortalized macrophage cultures. Interestingly some recent reports have suggested that hepcidin action on duodenal enterocytes, another type of polarized epithelial cells, could involve a different intracellular signaling pathway.[26,27] Clearly, more studies are required to confirm the respective role of the Jak2-mediated or lipid raft-mediated pathways in FPN internalization and degradation.

Perspectives

Taken together, these transcriptional and posttranscriptional regulations of FPN expression play a major role in controlling iron delivery for erythropoiesis and a defect in any of these regulatory steps can theoretically contribute to iron-related disorders. Indeed, increased iron efflux from macrophages due to abnormally low hepcidin levels contribute to increased transferrin saturation and tissue iron loading in genetic hemochromatosis. Ferroportin mutations altering either the hepcidin binding site or the iron transport activity are responsible for an iron overload disease.[28] Finally, increased erythrophagocytosis by activated macrophages combined with the presence of elevated serum hepcidin levels contribute to macrophage iron retention and iron restricted erythropoiesis in chronic inflammatory disorders.[29]

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Inherited bone marrow failure syndromes

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The inherited bone marrow failure syndromes are a heterogeneous group of disorders characterized by bone marrow failure usually in association with one or more somatic abnormality. The bone marrow failure (which can involve all or a single cell lineage) often presents in childhood but may not do so until adulthood in some cases. Furthermore, some patients initially labeled as having "idiopathic aplastic anemia" actually have cryptic presentations of these genetic syndromes. Over the last two decades there have been considerable advances in the genetics of these syndromes with 33 genes having been identified to date. These advances have provided a better understanding of normal hematopoiesis and how this is disrupted in patients with bone marrow failure. They have also provided important insights into fundamental biological pathways: DNA repair-FA/BRCA pathway; telomere maintenance- dyskeratosis congenita-related genes; ribosome biogenesis-Shwachman Diamond syndrome (SDS) and Diamond-Blackfan anemia (DBA) genes. Additionally, as these disorders are usually associated with developmental abnormalities and an increased risk of cancer they are providing insights into human development and the genesis of cancer.

The features of some of the classical inherited bone marrow syndromes are summarized in Tables 1 and 2. A brief outline of each syndrome is given below.

Table 1. Characteristics of the inherited bone marrow failure syndromes.

	FA	DC	SDS	DBA	CAMT	SCN
Inheritance pattern	AR, XLR AD	XLR, AR	AR	AD AR	AR	AD
Somatic abnormalities	Yes	Yes	Yes	Yes	Rare	Rare
Bone marrow failure	AA (>90%)	AA (~80%)	AA (~20%)	RCA[a]	Meg[b]	Neut[c]
Short telomeres	Yes	Yes	Yes	No	?	?
Cancer	Yes	Yes	Yes	Yes	Yes	Yes
Chromosome instability	Yes	Yes	Yes	?	?	?
Genes identified	13	6	1	9	1	3

FA: Fanconi anemia; DC: dyskeratosis congenita; SDS: Shwachman-Diamond syndrome; DBA: Diamond-Blackfan anemia; CAMT: congenital amegakaryocytic thrombocytopenia; SCN: severe congenital neutropenia; AD: autosomal dominant; AR: autosomal recessive; XLR: X-linked recessive; RCA[a]: Red cell aplasia although some patients can develop global bone marrow failure. Meg[b]: low megakaryocyte count which can progress to global bone marrow failure. Neut[c]:usually low neutrophils count.

Fanconi anemia

Fanconi anemia is usually inherited as an autosomal recessive trait but in a small subset of patients it can be an X-linked recessive disorder. The condition is clinically heterogeneous, but characteristic features include the progressive development of bone marrow failure and an increased predisposition to malignancy.[1] Affected individuals may also have one or more developmental abnormality including skin, skeletal, genitourinary, gastrointestinal and neurological anomalies. Approximately 30% of patients with Fanconi anemia have no overt somatic abnormalities. The majority of patients present towards the end of the first decade of life. However, increasingly some patients are being diagnosed in adulthood and many