

## A PRECIOUS METAL: IRON, AN ESSENTIAL NUTRIENT FOR ALL CELLS

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**ABSTRACT:** *Iron is an important cofactor required for a number of essential cell functions and hence is a vital nutrient. However, iron can also be dangerous as a catalyst of free radical reactions. Accordingly, intracellular iron homeostasis and body iron balance are tightly regulated. In this review, we presented an overview of the remarkable new insights that over the last years have been gained into the multifaceted and sophisticated molecular mechanisms controlling iron acquisition, storage and release. We also reviewed the data about nutrition-related abnormalities of iron metabolism, such as iron overload and deficiency. Finally, we discussed how pathogenic microorganisms and host cells compete for iron, a battle whose outcome has a relevant role in infectious disease*

**KEY WORDS:** Anemia, Hemochromatosis, Infection, Iron, Overload

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### INTRODUCTION

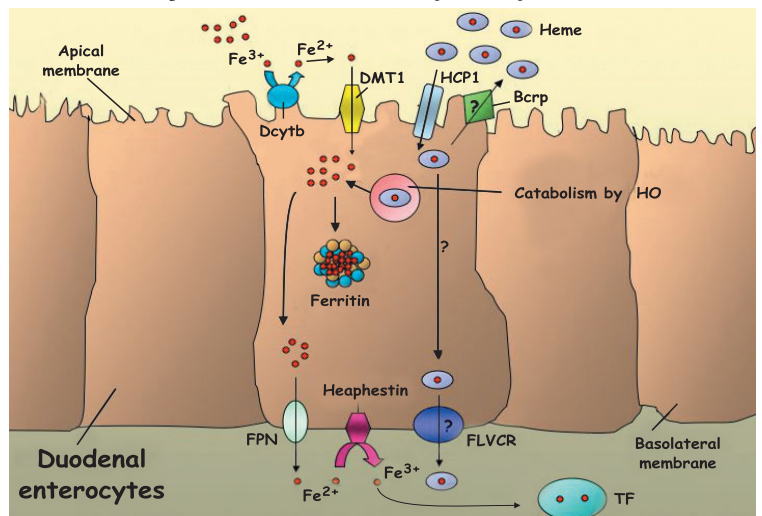
Iron is an essential cofactor for a wide variety of important cellular processes, such as oxygen transport, respiration, the tricarboxylic acid cycle, lipid metabolism, gene regulation and DNA synthesis. In order to execute such multiple tasks iron is incorporated in the heme group of hemoglobin, myoglobin and cytochromes, or is associated to nonheme moieties (e.g., in ribonucleotide reductase) or Fe-S motifs (functionally versatile prosthetic groups associated with proteins that sustain fundamental life processes). Therefore, iron can be considered an essential nutrient for virtually all organisms. However, acquiring iron from the environment is both difficult and energy consuming; thus, only the required amount should be taken up. Moreover, iron is toxic at high concentrations; hence, the organisms have developed a tight regulatory control of iron homeostasis (Hentze et al., 2004). From a nutritional viewpoint, both iron scarcity and excess have dramatic consequences and great epidemiological significance. Indeed, iron deficiency, which affects two billion people, is the primary nutritional disorder in the world, whereas hereditary hemochromatosis, which causes excess iron accumulation, is one of the most common genetic disorders.

Progress has been made in understanding iron metabolism: the past few years have seen an expansion in the number of proteins directly or indirectly involved in iron homeostasis (see Table 1), but much remains to be known about its regulation. In this review, after an overview of the multifaceted and sophisticated molecular mechanisms controlling iron acquisition, storage and release, we will focus on some aspects of iron metabolism more nutrition-related, such as iron overload and deficiency. Finally, we will also consider recent evidence revealing new aspects of the competition between pathogenic microorganisms and host cells for iron acquisition, a fact with relevant effects in various pathologic conditions.

Iron metabolism at the systemic level consists of three main processes, which reflect similar steps at the cellular level: uptake, storage and release. For both the single cell and the whole organism, the pathways of iron entry and deposition are understood reasonably well, whereas the mechanisms and the regulation of iron efflux are relatively less characterized and only in recent years new genes involved in iron release have been identified.

### Figure 1. Intestinal iron absorption

Iron absorption in the duodenal mucosa of the gut requires the transport across both the apical and basolateral membranes of duodenal enterocytes. Dietary nonheme iron present in the duodenal lumen is reduced by the Dcytb ferrireductase and thus made available for DMT1 that transports Fe (II) across the apical brush-border membrane. Heme iron absorption at the apical side is mediated by HCP1. The portion of iron not retained in the cell inside the iron storage protein ferritin is then transferred to the bloodstream. Basolateral release of nonheme iron (also derived from heme catabolized by heme oxygenase, HO) is mediated by ferroportin (FPN), which transports the metal across the membrane, in conjunction with Heaphestin which re-oxidizes iron, a necessary step for binding to the plasma carrier protein transferrin (TF). It is also possible that a portion of heme be released intact through the action of either of two heme exporters (Bcrp and FLVCR).



## IRON ACQUISITION

### Systemic

The duodenal mucosa is the site of dietary iron absorption. Iron is a large, charged ion that cannot freely cross lipid-rich cellular membranes. In the past decade the major proteins involved in transport of non-heme iron across both the apical and basolateral membranes of duodenal epithelial cells have been identified and characterized (Figure 1). Divalent metal transporter1 (DMT1), which sits on the surface of the intestine that comes in contact with food, is responsible for almost all dietary nonheme iron uptake (reviewed by Mackenzie and Garrick, 2005). The process can be summarized as follows: on the luminal side of the duodenum the ferric reductase Dcytb reduces dietary Fe(III), which has not been previously reduced by reductants like ascorbate, to ferrous iron that can then be internalized in mucosal cells by means of DMT1. The role of this protein is underlined by the demonstration that mice lacking DMT1 in all tissues survive for a few days after birth but have severe iron-deficiency anemia (Fleming et al., 1997). Selective DMT1 inactivation in individual mouse tissues showed that, while DMT1 is needed for iron uptake in the intestine, it also plays an important role in bringing iron into red blood cell precursors for hemoglobin biosynthesis (Gunshin et al., 2005). In contrast, DMT1 is not necessary for iron transfer across the placenta or for iron uptake by macrophages or liver cells. Another transporter, ferroportin (also designated IREG1/MTP1) (see below), exports across the basolateral membrane into the bloodstream the iron not stored in or utilized by the absorptive epithelial cells. In this process, ferroportin is assisted by hephaestin, an oxidase highly homologous to ceruloplasmin, which oxidizes iron back to the ferric form that is bound and carried by serum transferrin (see Wessling-Resnick, 2006 for a recent review). In addition, recent findings indicated that the role of ceruloplasmin, which is required for iron release from tissue stores, may also play a critical function in basolateral iron transport under stress (Cherukuri et al., 2005). Only recently the identification and cloning of the gene coding for the transporter of heme iron has filled a gap in our understanding of iron metabolism (Shayeghi et al., 2005). This finding has a great importance for nutrition, as heme iron, albeit constituting only a minor part of total dietary iron, is absorbed very efficiently and hence is the major source of dietary iron for mammals (Lynch et al., 1989). This heme carrier protein (HCP1) transports heme iron across the apical membrane of duodenal epithelial cells; the metal is then possibly released through the action of heme oxygenase (HO) and made available for transport to the bloodstream, probably by ferroportin. Alternatively, all or part of the heme may remain intact and be exported into the serum by the recently discovered heme exporter FLVCR (Quigley et al., 2004). Another transporter (Bcrp), which specifically binds heme (Krishnamurthy et al., 2004) and is located in the apical plasma membrane (van Herwaarden and Schinkel, 2006), might extrude heme into the gut lumen, thus reducing net intestinal heme iron uptake. In spite of the fact that RNA subtraction techniques to isolate genes differentially expressed in normal and hypotransferrinemic-anemic mice were used to clone HCP1, regulated trafficking of the HCP1 protein between the apical and internal membrane compartments seems the major molecular mechanism that the body uses to control heme iron uptake

(Shayeghi et al., 2005). This result suggests that the control of the subcellular locations of the components of the iron traffic network may be an additional mechanism involved in the maintenance of iron homeostasis.

Physiological regulators of intestinal absorption are the body iron stores, the rate of erythropoiesis and hypoxia (Finch, 1994). Total body iron content is inversely related to duodenal iron absorption, whereas increased red blood cells production and hypoxic conditions enhance iron uptake. Accordingly, proteins involved in iron transport across mucosal cells have iron responsive elements (IRE) (see below) in the untranslated regions of their mRNAs (e.g. DMT1, ferroportin) or hypoxia inducible factor (HIF1) binding sites in their promoter regions (e.g. Bcrp). These molecular features may suggest the existence of an intestine-centered sensing of body iron content and consequent regulation of absorption, but the emerging role of hepcidin as a central mediator of iron absorption (see below) has challenged this view, indicating instead a key role for systemic signaling.

### Cellular

Under normal circumstances nearly all the iron in the blood plasma and interstitial fluid is transported by transferrin and therefore, at the cellular level, internalization of iron-laden transferrin by the transferrin receptor (TfR1) is the major route of iron acquisition. The “transferrin cycle” has been the subject of extensive biochemical investigations and localization studies at the light and electron microscope, so that it now constitutes a model for studies of ligand-independent receptor trafficking (see Aisen, 2004 for a recent review). Uptake of transferrin-bound iron involves internalization of diferric transferrin within an endocytic vesicle by receptor-mediated endocytosis and the release of iron at the slightly acidic pH of the endosome. Iron is then transferred to the cytosol by means of DMT1 while the complex between apotransferrin and TfR1 is returned back to the cell surface where transferrin dissociates from TfR1. A homologous receptor (TfR2), which is predominantly expressed in the liver and not regulated by iron, has been cloned (Kawabata et al., 1999). Although its affinity for transferrin is about 25-fold lower than that of TfR1, TfR2 can mediate cellular iron uptake in transfected cells, but cannot replace the function of TfR1 because deletion of TfR1 in mice is lethal (Levy et al., 1999). Overall, although the physiological function of TfR2 in iron homeostasis is still obscure, it but must be relevant because mutations in this gene are associated with a rare form of hemochromatosis (see below) and targeted mutagenesis produces hemochromatosis in mice (Fleming et al., 2002).

A possible alternative way for the cell to acquire large amounts of iron is through internalization of ferritin, the major iron storage protein (see below). The recent identification of TIM-2, a member of a family of receptors of evident importance in immunity, as a receptor for H ferritin opens new avenues to *investigate the role of this source of iron* (Chen et al., 2005).

Uptake of iron bound to low molecular weight ligands (defined as non-transferrin-bound, NTBI) can lead to iron loading of hepatocytes in severe iron overload states such as hemochromatosis or transfusional siderosis when the binding capacity of transferrin is fully saturated.

## IRON STORAGE

### Systemic

Approximately 20% of the 3-4 grams of iron in the human body correspond to stored iron, which is mainly deposited in parenchymal liver cells and reticuloendothelial macrophages. The latter, which are predominantly located in the spleen, liver and bone marrow, remove senescent or damaged erythrocytes and thus acquire mainly heme iron, although they also express TfR1. In addition to iron bound to transferrin (in physiological conditions) and NTBI (in pathological conditions of iron overload), hepatocytes take up heme iron bound to haptoglobin (Kristiansen et al., 2001) or hemopexin (Hvidberg et al., 2005).

### Cellular

Inside the cell, iron is deposited into ferritin, an evolutionarily conserved, ubiquitous protein destined to store iron. Mammalian ferritin is a heteropolymer composed of 24 subunits of two types (H and L), which assemble to make a hollow spherical shell in which iron accumulates in a readily available but non-toxic form (Harrison and Arosio, 1996). H and L chains assemble in a tissue-specific way to generate a family of isoferritins with different metabolism and functions. L subunit-rich isoferritins predominate in iron storing tissues, whereas H subunit-rich ferritins are preferentially found in cells which rapidly take up and release iron. The recently discovered human mitochondrial ferritin (MtF), unlike other mammalian ferritins, is a homopolymer of 24 subunits having a high degree of sequence homology with human H-chain ferritin (Levi et al., 2001). The expression of MtF, which is structurally and functionally similar to the cytosolic ferritins, seems restricted to the testis and few other tissues, and is abundant in the erythroblasts of patients with sideroblastic anemia, where it may protect the mitochondria from the damage caused by iron overload (Cazzola et al., 2003).

Changes in iron availability regulate ferritin gene expression at multiple levels. Although transcriptional induction has been documented, (Cairo et al., 1985; White and Munro, 1988), the major control mechanism consists of iron regulatory proteins (IRPs)-mediated repression of ferritin translation under conditions of iron scarcity and, conversely, active ferritin synthesis when iron is abundant and IRPs are inactive (see below). This mechanism allows the cell to finely tune ferritin content with iron availability (Cairo and Pietrangelo, 2000; Hentze et al., 2004). Since cellular environmental conditions may require an adjustment in iron homeostasis without a change in the exogenous levels of the metal, ferritin responds to regulatory influences in addition to iron (Torti and Torti, 2002). In particular, in consideration of the influence of intracellular iron levels on the susceptibility to oxidative stress, an emerging role of ferritin as antioxidant protein is increasingly recognized. In fact, the cell keeps reactive oxygen species (ROS) levels under control also by means of the modulation of iron availability, because the appropriate sequestration of iron may allow the physiologic roles of the relatively safe  $O_2^-$  and  $H_2O_2$  to take place without the production of highly reactive  $\cdot OH$ . In the case of oxidative stress, increased ferritin protein levels are associated not only with IRP2 downmodulation (Cairo et al., 2002a) but also with higher transcription of the H and L genes (Cairo et al., 1995; Tsuji et al., 2000; Hintze and Theil, 2005), which is

apparently related to the presence of antioxidant response elements in the promoter (Wasserman and Fahl, 1997; Tsuji et al., 2000; Hintze and Theil 2005).

Notably, the capacity of ferritin to store iron, in particular that of the H subunit, has been shown to play a central role in promoting cell survival. The antioxidant activity of NF- $\kappa$ B, which is required to antagonize programmed cell death induced by the proinflammatory cytokine tumor necrosis factor alpha, has been shown to depend upon upregulation of H ferritin (Pham et al., 2004). Cell growth-related genes such as the oncogene c-myc (Wu et al., 1999) or the oncosuppressor VHL (Alberghini et al., 2005) also regulate ferritin expression at both the transcriptional and translational level.

## IRON RELEASE

### Systemic

Under physiological conditions, organismal iron balance is essentially determined by duodenal absorption because the body does not have a regulated pathway of iron excretion. Because of this latter feature, genetic abnormalities of iron uptake lead to iron overload disease. The intestine is the main route of iron loss, which results from bleeding, biliary excretion and epithelial exfoliation.

### Cellular

Contrary to what happens at body level, cellular iron excretion is tightly regulated and many cells have the capacity to export and donate iron. Ferroportin has been shown to be important both for moving iron out of intestinal lining cells (see above), hepatocytes and macrophages into the bloodstream. The macrophages play a critical role in body iron distribution because they recover used iron from old red blood cells and return it to the circulation for binding to transferrin. The oxidase activity of ceruloplasmin is necessary to oxidize iron to Fe(III), which is the form bound by transferrin. Mice that lack the ferroportin protein in all tissues die during embryonal life, apparently because the embryo could not obtain iron from the mother, while animals in which ferroportin expression was inactivated in all cells of developing embryos but preserved in extraembryonic tissues survived through birth but developed severe iron deficiency and anemia in the first days of life (Donovan et al., 2005). To clarify where ferroportin function is important, Andrews and colleagues developed mice that lacked ferroportin expression in selected tissues and confirmed that ferroportin is the major (or only) molecule involved in exporting iron from intestinal absorptive cells and from macrophages (Donovan et al., 2005). It also contributes to iron export from liver cells that harbour a substantial amount of the body's iron stores. Ferroportin is subjected to transcriptional and posttranscriptional regulation by a number of factors such as iron content, hypoxia, inflammation (reviewed in Pietrangelo, 2004a) and is also regulated at the posttranslational level by hepcidin (see below).

## IRON HOMEOSTASIS

### Systemic

Over the last years, hepcidin, a peptide produced and secreted by hepatocytes, turned out to be the long sought-after central

regulator of iron homeostasis. Hepcidin exerts its function by controlling the presence of ferroportin on the plasma membrane of cells important in iron metabolism. Hepcidin binding induces ferroportin internalization and degradation (Nemeth et al., 2004b; De Domenico et al., 2005), thus inhibiting iron release from enterocytes, macrophages, hepatocytes and placental cells and resulting in cellular iron retention. Inadequate hepcidin expression relative to body iron stores is at the basis of the excessive duodenal iron absorption that characterizes most genetic iron overload disorders (see below). The molecular pathway leading to hepcidin regulation in response to other stimuli such as inflammatory cytokines has been clarified (see below), but, in spite of several studies (Nemeth et al., 2005; Montosi et al., 2005; Lou et al., 2005), the details of the stimulatory effect of iron on hepcidin synthesis *in vivo* are still obscure. However, recent work has shown that hepcidin is under the transcriptional control of the TGF- $\beta$ /SMAD4 pathway and that SMAD4-mediated hepcidin expression in turn controls the levels of proteins involved in duodenal iron uptake such as Dcytb, DMT1 and ferroportin (Wang et al., 2005). The regulation and molecular control of systemic iron homeostasis, including the role of HFE, the gene whose mutation leads to the disruption of iron homeostasis in the majority of hemochromatosis patients, will be further discussed in the section dedicated to hemochromatosis (see below).

### Cellular

The fine adjustment of intracellular iron levels is mainly obtained by means of divergent but coordinate regulation of the iron storage protein ferritin and the iron uptake protein TfR1. Although transcriptional regulation of the ferritin H and L subunits (see Cairo and Pietrangelo, 2000 for review) and of the TfR1 gene (Bianchi et al., 1999; Lok and Ponka, 1999) in response to iron has been described, these two proteins are mainly controlled at post-transcriptional level through the IRE/IRP system.

IRPs (IRP1 and IRP2), cytoplasmic proteins belonging to the aconitase superfamily, regulate iron metabolism by binding with high affinity and specificity to conserved IRE in the untranslated regions of ferritin and TfR1 mRNAs (Hentze and Kuhn, 1996; Hanson and Leibold, 1999; Cairo and Pietrangelo, 2000). Under conditions of iron deficiency, IRPs actively bind to IRE and stabilize the mRNA for TfR1 while also decreasing translation of mRNA for ferritin, eventually increasing iron uptake and availability within the cell. Conversely, high iron levels decrease IRE-binding activity, leading to efficient translation of ferritin mRNA and decreased stability of TfR1 mRNA, ultimately enhancing iron sequestration over uptake. IRP1 is the cytoplasmic counterpart of mitochondrial aconitase, the enzyme which converts citrate to isocitrate through a *cis*-aconitate intermediate in the tricarboxylic acid cycle by virtue of a catalytic [4Fe-4S] cluster (Beinert and Kennedy, 1993). In iron-replete cells the cluster is assembled and IRP1 displays aconitase activity; in iron depleted cells the cluster is lacking and IRP1 functions as an RNA-binding protein. This reversible switch between a cluster-containing holoprotein and a cluster-deficient apoprotein therefore allows aconitase/IRP1 to constantly sense iron levels and to adapt them to cell requirements. IRP2 is highly homologous to IRP1 but lacks aconitase activity, probably because of its inability to assemble a [4Fe-4S] cluster; the protein

accumulates in iron deficient cells and is rapidly targeted for proteasomal degradation in iron replete cells (Iwai et al., 1998). IRP2 binds consensus IRE sequences with affinity and specificity similar to that of IRP1 (Hentze and Kuhn 1996; Henderson et al., 1996 and references therein), but it has been shown to recognize an exclusive IRE subset (Henderson, 1996). Furthermore, growing evidence suggests that IRP2 may play a specific role independent of aconitase/IRP-1. In addition to a specific modulation in response to stimuli and agents other than iron, such as hypoxia (Hanson et al., 2003; Meyron-Holtz et al., 2004b), oxidative stress characterized by enhanced production of ROS (Cairo et al., 1995; Minotti et al., 2001; Corna et al., 2004) or reactive nitrogen intermediates (RNI) (Cairo et al. 2002a; Cairo et al., 2002b), IRP2 has a characteristic pattern of tissue expression (Hentze and Kuhn, 1996; Henderson et al., 1996), is more sensitive than IRP1 to variations of iron in the diet (Chen et al., 1997) and is preferentially expressed in most cell lines (Recalcati et al., 1999). Moreover, when abundantly (Recalcati et al., 1998) or uniquely (Schalinske et al., 1997) expressed, IRP2 can act as the major or only modulator of intracellular iron metabolism.

Gene targeting experiments have provided additional information about the respective role of the two IRPs. Early embryonic lethality in mice doubly deficient for both IRPs (Smith et al., 2004) shows that the IRP/IRE regulatory system is essential. However, mice lacking either IRP1 or IRP2 are viable. *Irp2*<sup>-/-</sup> mice display mild microcytosis and compromised hematopoiesis with abnormal body iron distribution (Galy et al., 2004; Galy et al., 2005a; Galy et al., 2005b; Cooperman et al., 2005) and neurodegeneration (LaVaute et al., 2001), whereas mice lacking IRP1 present with no detectable phenotypic abnormality (Meyron-Holtz 2004a). Therefore, IRP2 seems to dominate the regulation of iron homeostasis in animal models. Extensive investigation of the comparative expression of the two IRPs in human tissues and blood cells suggested that IRP2 is the key regulator of intracellular iron homeostasis also in humans (Recalcati et al., 2006). The above indications of a minor role of IRP1 have been challenged by recent results obtained in anemic zebrafish showing an important role for IRP1 in the control of the IRE-containing mRNA for erythroid aminolevulinic synthase (eALAs) and hence for heme synthesis (Wingert et al., 2005).

It should be remembered in fact, that, in addition to modulating ferritin and TfR1 levels, IRP can regulate mRNAs for other enzymes closely related to iron utilization (eALAs), availability (mitochondrial aconitase), uptake (DMT1), and release (ferroportin). Hence, the influence of IRPs on the iron status of the cell extends over a number of regulatory pathways (Cairo and Pietrangelo, 2000; Hentze et al., 2004).

New insights into cellular iron metabolism have been provided by the recognition that certain diseases are associated with mitochondrial iron overload and by the discovery of mitochondrial ferritin (MtFt) (see above) and a number of mitochondrial iron transporters (see Table 1). An increasing body of data has recently pointed at the role of the mitochondrion as a crossroad of iron traffic (reviewed in Napier et al., 2005; Chitambar, 2005; Rouault and Tong, 2005). In fact, an iron transit through the mitochondrion is required for biosynthesis of both heme and Fe-S clusters. Recently, an important study provided evidence that

these two biosynthetic pathways are connected. In fact, in anemic mutant zebrafish, deficiency of the mitochondrial glutaredoxin 5 enzyme impaired assembly of Fe-S clusters, thus leading to IRP1 activation and consequent block of heme biosynthesis catalyzed by ALAS (Wingert et al., 2005).

Iron overload is a condition in which inborn errors of iron metabolism (e.g. hemochromatosis) or other factors (secondary or acquired iron overload) lead to progressive body iron overload and multi-organ failure.

## IRON OVERLOAD

**TABLE 1. Proteins of iron metabolism**

Protein	Major biochemical activity	Role	References
<b>Iron storage</b>			
Ferritin H chain	Fe (II) oxidation	Cytosolic storage-antioxidant	Reviewed in Harrison and Arosio, 1996
Ferritin L chain	Fe mineralization	Cytosolic storage	Reviewed in Harrison and Arosio, 1996
Mitochondrial ferritin	Fe (II) oxidation	Mitochondrial storage-Antioxidant (?)	Levi et al., 2001
<b>Iron transport</b>			
Transferrin	Fe(III) binding	Transport in plasma	Reviewed in Aisen, 2004
Lactoferrin	Fe(III) binding	Antimicrobial	Reviewed in Brock, 2002
Ferroportin	Membrane transporter	Cellular export	Reviewed in Pietrangelo, 2004a
DMT1	Membrane transporter	Cellular import	Reviewed in Andrews, 2000
Nramp1	Membrane transporter	Export from phagosomes	Reviewed in Fortier et al., 2005
HCP1	Membrane transporter	Intestinal heme import	Shayeghi et al., 2005
Bcp	Membrane transporter	Intestinal heme export	Reviewed in van Herwaarden and Schinkel, 2006
FLVCR	Membrane transporter	Intestinal heme export	Quigley et al., 2004
ABC7	Membrane transporter	Mitochondrial export	Reviewed in Lill and Kispal, 2001
Sideroflexin	Membrane transporter	Mitochondrial traffic	Fleming et al., 2001
Mitoferrin	Membrane transporter	Mitochondrial traffic	Shaw et al., 2006
Hemopexin	Heme binding	Plasma heme transport	Reviewed in Muller Eberhard, 1988
Haptoglobin	Hemoglobin binding	Hemoglobin transport	Reviewed in McCormick and Atassi, 1990
Lipocalin2	Siderophore iron binding	Traffic of siderophore-bound iron	Reviewed in Richardson, 2005
<b>Receptors</b>			
Transferrin receptor 1	Transferrin binding	Transferrin iron uptake	Reviewed in Aisen, 2004 and Hentze et al., 2004
Transferrin receptor 2	Transferrin binding	Transferrin iron uptake	Kawabata et al., 1999
TIM-2	H ferritin receptor	H ferritin uptake	Chen et al., 2005
LRP/CD91	Hemopexin receptor	Hemopexin uptake	Hvidberg et al., 2005
CD163	Haptoglobin receptor	Haptoglobin uptake	Kristiansen et al. 2001
24p3R	Lipocalin2 receptor	Lipocalin uptake	Devireddy et al. 2005
<b>Regulators</b>			
IRP1	RNA binding	Control of cellular iron	Reviewed in Hentze and Kuhn, 1996
IRP2	RNA binding	Control of cellular iron	Reviewed in Cairo and Pietrangelo, 2000
Hepcidin	Ferroportin binding	Control of systemic iron	Reviewed in Ganz and Nemeth, 2006
HFE	TfR1 binding	Control of hepcidin expression (?)	Feder et al., 1996
HJV	Neogenin binding	Control of hepcidin expression	Reviewed in Camaschella, 2005
Frataxin	Iron binding	Chaperon for Fe-S synthesis	Reviewed in Pandolfo, 2002
SMAD4	Transcription factor	Control of hepcidin expression	Wang et al., 2005
<b>Oxireductases</b>			
Ceruloplasmin	Fe(II) oxidation	Facilitates transferrin iron loading in serum	Reviewed in Hellman and Gitlin, 2002
Hephaestin	Fe(II) oxidation	Facilitates transferrin iron loading in duodenum	Vulpe et al., 1999
Dcytb	Fe(III) reduction	Facilitates duodenal transport by DMT1	McKie et al., 2001

### Genetic

Hemochromatosis, which is the most common inherited autosomal recessive disorder in the white population, affecting about 1 in 200 of northern European ancestry, is attributable to inappropriately increased iron absorption (see Pietrangelo, 2004b for a recent review). In hemochromatosis, iron loading of parenchymal cells in the liver, pancreas, heart, and other organs impairs the function and damages the structure of these organs leading to cirrhosis, liver cancer, heart disease, and diabetes. Hemochromatosis is caused by mutations in genes encoding proteins that regulate or carry out intestinal absorption of dietary iron. At least four genes may now be associated with a disease phenotype fulfilling this definition. The most common form of hemochromatosis results from a mutation in HFE (Feder et al., 1996), a protein that somehow helps to control the efficiency of intestinal iron absorption (reviewed in Pietrangelo, 2002). HFE usually forms a protein-protein complex with the Tfr1, which picks up circulating transferrin containing iron and brings it inside the cell. The mutation most commonly found in the hemochromatosis patients does not allow the interaction between HFE and  $\beta$ 2-microglobulin, thus preventing the transport of HFE to the cell membrane and its interaction with Tfr1 in endocytic vesicles. It is not clear how the HFE-Tfr1 complex controls the activity of Tfr1. Mice that produce no functional HFE protein and mice that produce a mutant HFE protein analogous to that seen in most human patients with HFE hemochromatosis have been shown to be reasonably faithful models of the human disease (Zhou et al., 1998; Levy et al., 1999). Mice lacking normal HFE have a defect in the production of hepcidin (Muckenthaler et al., 2003), which in turn controls how cells release iron, by binding to the ferroportin and causing it to be destroyed. Inadequate hepcidin production could explain why patients with hemochromatosis develop iron overload (Bridle et al. 2003; Gehrke et al., 2003).

Other genes are mutated in some patients with hemochromatosis. Patients with mutations in the gene encoding hepcidin develop very severe, early onset iron overload (juvenile hemochromatosis) (Roetto et al., 2003). Recently, it was reported that mutations in a novel gene, designated hemojuvelin, also cause severe hemochromatosis early in life (Papanikolaou et al., 2004; Lanzara et al., 2004; Lee et al., 2004). Little is known of the function of this protein, which has been found to interact with neogenin, a receptor involved in a variety of cellular signalling processes (Zhang et al., 2005). There is evidence that hemojuvelin may be part of the same regulatory network as HFE and hepcidin. Mice lacking hemojuvelin develop severe hemochromatosis early after birth (Huang et al., 2005; Niederkofler et al., 2005). As they produce almost no hepcidin, high amounts of ferroportin are present in duodenal cells and macrophages. As a result, intestinal iron absorption is greatly increased and very little iron is stored in macrophages, leading to excess iron in the circulation. Mutations in Tfr2 have been found to be associated with rare cases of hemochromatosis (Camaschella et al., 2000). Both patients and Tfr2 mutant mice manifesting a phenotype of hemochromatosis have low levels of hepcidin (Nemeth et al., 2005; Kawabata et al., 2005) but how Tfr2 regulates expression and release of hepcidin is still obscure.

From the findings reported above clearly becomes apparent that a model outlining the mechanisms of iron homeostasis and its alterations in hemochromatosis should have hepcidin as the common pathogenic denominator (Camaschella, 2005; Pietrangelo, 2005). In fact, the different players (i.e. HFE, Tfr2, hemojuvelin) of the systemic iron regulatory loop affected in hemochromatosis are all modulators of hepcidin expression, which in turn controls the rate at which duodenal and reticuloendothelial cells release iron into the circulation. The contribution of the three genes to iron homeostasis may be independent but seems complementary, thus possibly resulting in a spectrum of hemochromatosis phenotypes, as demonstrated by the finding that patients with mutations of both HFE and Tfr2 (usually mutated in "adult" hemochromatosis) present with severe clinical manifestation, typical of juvenile hemochromatosis (Pietrangelo et al., 2005).

In addition to having inadequate hepcidin production as a common pathogenetic cause, all the three forms of hemochromatosis described so far seem to share common histopathological (predominant parenchymal iron overload) and clinical features; they are characterised by tissue iron overload, organ disease and requirement for iron removal therapy. Conversely, a new clinical entity showing peculiar features has been recently characterised where the clinical phenotype may be confused with classic hemochromatosis, but the disorder shows autosomal dominant inheritance and the patients do not have mutations in HFE (Pietrangelo et al., 1999). This form is attributable to mutations of the ferroportin gene (Montosi et al., 2001). Additional distinguishing features are anaemia early in life despite increased serum ferritin concentrations and early iron accumulation in reticuloendothelial cells (Pietrangelo, 2004a).

### Secondary

A distinction should be made between hemochromatosis and siderosis (that is, excess iron accumulation in tissues) a condition present for example in patients affected by refractory anemias, in which the increased intestinal absorption triggered by inefficient erythropoiesis is accompanied by the iron burden that indirectly results from blood transfusions, or which is associated to the long term consumption of traditional beverages containing high amounts of iron in sub-Saharan Africa (see Alla and Bonkovsky, 2005 for a recent review).

### IRON DEFICIENCY

Iron balance is critical for normal red blood cell production and general health; indeed, iron deficiency may result in cessation of hemoglobin synthesis in erythroid cells and in induction of apoptosis in both erythroid and nonerythroid cells (Le and Richardson, 2002). Iron deficiency, which rarely results from inherited defects in iron metabolism (Andrews, 2000), is commonly caused by dietary iron insufficiency (or defective uptake) or ongoing blood loss and typically presents with anemia. Iron deficiency anemia (IDA), which is prevalent throughout the world affecting almost a billion people particularly among infants and young women, is included in those micronutrient deficiencies (e.g. vitamin A, iodine, iron) increasingly recognized as a priority nutritional problem. In particular, iron deficiency is the primary

cause of nutritional anemia and the most common nutritional deficiency in the world. Differential analysis of the intestinal proteome in rats with mild dietary iron-deficiency identified significant changes in the levels of proteins belonging to different functional classes (i.e., glucose and fatty acid metabolism, vitamin transporters, etc.) (Tosco et al., 2005). As the identified proteins do not seem to share any self-evident correlation with the known metabolic fates of iron and appear to be part of a more general metabolic response to the nutritional-induced deficiency of this micronutrient, these results give new insights into a possible more general role of iron deficiency implying unforeseen pathologic consequences.

### Iron deficiency anemia

A seminal publication (DeMaeyer and Adiels-Tegman, 1985) gave consistence to fragmented evidence on IDA prevalence establishing that a large proportion of the world population is anemic and about half had IDA. The study also revealed strong relationships between IDA and age, sex and geographic regions with highest prevalence in childhood, women and developing world.

Anemia per se, the most common manifestation of iron deficiency seems less important than liabilities associated with tissue iron deficiency, which result in abnormalities of psychomotor development and cognitive function in infancy, impaired work capacity in adults (with consequent low productivity and economic loss), and increased rate of premature birth. Blood loss (commonly caused by menstrual blood loss in premenopausal women and occult bleeding from the gastrointestinal tract in men and postmenopausal women) is the most common cause of iron deficiency, but chronic insufficiency of dietary iron or deficient absorption can also lead to negative iron balance.

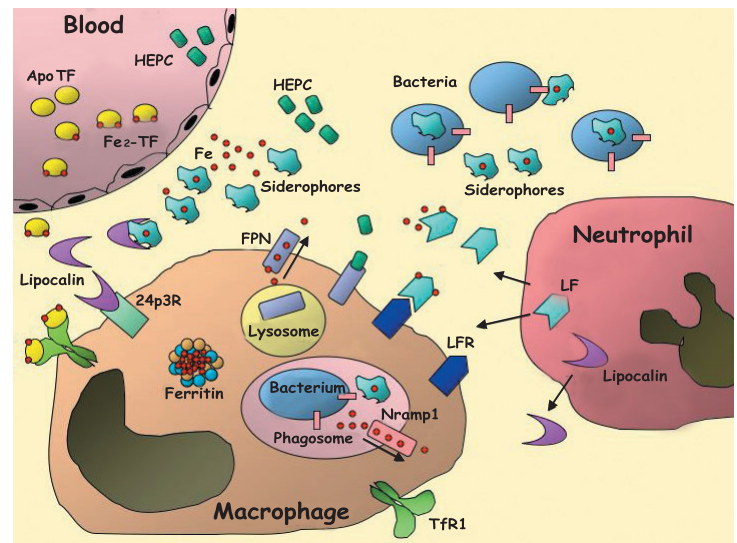
### INFECTION

Another example of the key role of iron as a nutrient in pathological settings is represented by the host-pathogen competition for iron to satisfy their mutual requirement (Barasch and Mori, 2004). Iron is an essential growth factor for most bacteria and parasites but is also required for host metabolism and other important host functions. In particular, iron in macrophages appears to be key for mounting an appropriate response toward pathogens. Inside phagocytic cells the metal is required and used for the respiratory burst with ROS production and iNOS-mediated RNI production; moreover, critical levels of iron are needed for cytokine production and signaling (Weiss, 2005). Therefore, resistance to infection is in part dependent on the successful outcome of a competition for iron between the host and the invading bacteria.

The following examples provide a view of recent advances about the iron-related crosstalk between microorganisms and mammalian host cells. The competition between the host and the invading microorganism (with the double aim of using iron for their purposes and simultaneously to subtract it to the rival) takes place both intracellularly and in the tissue microenvironment.

### Figure 2. Iron metabolism and microbial infection.

A simplified view of the tissural battlefield showing the most important cells (macrophages, neutrophils and bacteria) and molecules involved in the fight for iron. The bacteria synthesize and secrete siderophores (e.g. enterobactin), which, upon iron binding, are subsequently reinternalized by means of siderophore receptors. This is only one of the variety of distinct and specialized iron-acquiring systems utilized by bacteria. The host has a specific mechanism to compete for siderophore-bound iron that is based on Lipocalin2, which is recognized by a specific receptor (24p3R) expressed on macrophages. Additional, less specific, molecules used by the host to subtract iron to bacteria are represented by the avidly iron-binding proteins transferrin (TF) and lactoferrin (LF), both having specific receptors (TfR1 and LFR) on macrophages. The cytokine-mediated increase of hepcidin (HEPC) levels in the circulation, by causing ferroportin (FPN) internalization and degradation, inhibits macrophage iron release and thus decreases iron availability in the microenvironment and TF saturation in the serum. All these mechanisms contribute to reduce the amount of iron available for invading microorganisms and increase iron storage inside reticuloendothelial cells. A similar competition for iron is present inside phagocytic cells, in which internalized bacteria try to acquire iron by means of various strategies, including siderophore-mediated iron-uptake, while the host uses Nrap1 to pump iron out of the phagosome.



### The Host

The host has evolved mechanisms to withhold iron from the extracellular compartment that function at both the systemic and local levels.

#### Extracellular

##### Systemic

The fact that serum transferrin is normally not fully saturated provides a permanent defence, ensuring that circulating free iron is readily bound. Upon microbial invasion, the organism mounts a response to withhold iron into reticuloendothelial cells, hence decreasing serum iron and (indirectly) inhibiting the growth of bacteria. The inflammation-related increase of cytokines, particularly IL-6, triggers the hepatic synthesis and release of hepcidin, which can be considered a classical acute phase protein (Ganz and Nemeth, 2006). Hepcidin binds to ferroportin and induces its internalization and degradation (Nemeth et al., 2004a). The absence of ferroportin, the major (or sole) iron exporter, blocks the release of iron from macrophages, hepatocytes and duodenal cells leading to hypoferremia and low transferrin saturation. In this setting, cytokine-mediated IRP2 degradation allows more efficient ferritin synthesis in macrophages and thus facilitates iron storage (Recalcati et al., 1998).

As a side effect, the consequent restricted availability of transferrin-bound iron for erythropoiesis leads to anemia of chronic disease (ACD) or anemia of inflammation, a particular example of iron-related anemia. ACD is a common disorder that affects

patients with a wide variety of infections and inflammatory conditions, including cancer, and is characterized by inadequate erythrocyte production in the setting of low serum iron, but no body iron deficiency. In fact, ACD is a defect of iron compartmentalization, with the metal mainly sequestered into reticuloendothelial cells (Ganz, 2005).

#### *Local*

In the inflammatory microenvironment, lactoferrin and Lipocalin2 are secreted by neutrophils to sequester iron and interfere with bacterial growth. Lactoferrin, which has been recognized as a secretory product of neutrophils more than 30 years ago, has a well-established antimicrobial role, which is due in part to its ability to chelate iron (Brock, 2002). In body fluids and inflammatory lesions the high capacity of lactoferrin to bind iron (also at slightly acidic pH) makes it an efficient molecule to clear iron from invading microorganisms, and hence lactoferrin represents a first-line innate defence mechanism to prevent infections, including the development of bacterial biofilms. Interestingly, lactoferrin has been shown to counteract the increased susceptibility to tuberculosis caused by iron overload in  $\beta$ -2-microglobulin knock out mice (Schaible et al., 2002). Lipocalin2 (also called siderocalin, 24p3 or NGAL) is an acute phase protein induced in mouse by a sterile inflammatory stimulus like turpentine injection (Liu and Nilsen-Hamilton, 1995). Lipocalin2 specifically binds enterochelin, a bacterial catecholate siderophore (Goetz et al., 2002), i.e. one of the various small iron chelators utilized by bacteria to acquire iron (see below). Upon encountering invading bacteria, the Toll-like receptors on immune cells trigger the expression of Lipocalin2 that then limits bacterial growth by sequestering the iron-laden siderophores (Flo et al., 2004). This event is essential in the innate immune response to bacterial infection as a strikingly higher bacteraemia has been reported in Lipocalin2 knock out mice challenged with *E. coli* (Flo et al., 2004; Berger et al., 2006). Interestingly, in addition to its role as an antibacterial iron chelator, Lipocalin2 has been shown to be involved also in cartilage differentiation (Zerega et al., 2000) and kidney development as an iron donor (Yang et al., 2002), as well as in IL-3 deprivation-dependent apoptosis (Devireddy et al., 2001). These apparently unrelated functions might be explained by the results of a recent study in which the receptor for lipocalin2 (24p3R) has been identified and characterized (Devireddy et al., 2005). In fact, it has been demonstrated that 24p3R can bind and internalize both apo (iron-free) and holo (iron-loaded) Lipocalin2. Upon receptor binding, the internalized holoform releases iron inside the cell, thus possibly regulating iron-dependent developmental processes, whereas the apoform acquires intracellular iron and transfers it out of the cell, thus triggering iron deprivation-dependent apoptosis. These events may also play a role in survival and differentiation of erythroid progenitors and hence in erythropoiesis and anemia (Miharada et al., 2005).

#### *Intracellular*

A protein (Nramp1) which pumps iron across the phagolysosomal membrane is important for the defence response

to pathogens inside the inflammatory cells (reviewed in Fortier et al., 2005). This protein, which was originally discovered as a major regulator of susceptibility to intracellular infections in mice, was also shown to be associated with susceptibility to tuberculosis and leprosy in humans. Nramp1 is uniquely expressed in the membrane of lysosomal vesicles of macrophages and tertiary granules of neutrophils. Phagocytosis triggers the recruitment of this protein to the membrane of phagosomes where it seems to function as a pH-dependent iron transporter to remove the metal from the phagosomal space, although an independent study has obtained evidence that Nramp1 pumps metals into the phagosomal particle to increase ROS production directed against bacteria (Kuhn et al., 2001). The iron export activity may antagonize the capacity of pathogens to express their intracellular survival strategies, such as inhibition of the evolution of phagosomes into bactericidal phagolysosomes. Moreover, Nramp1-mediated depletion of iron would inhibit intracellular bacterial replication by removing a metal essential for microbial metabolic activity and multiplication.

#### ***The microorganism***

On the other hand, bacteria have evolved multiple sophisticated mechanisms for acquiring iron in environments where very little free iron is available. Microbes (e.g. mycobacteria) have evolved several strategies, such as invasion of distinct niches, to have better access to this essential nutrient (reviewed in Schaible and Kaufmann, 2004). These devices include the secretion of iron-binding organic molecules, siderophores, and their reuptake through specific bacterial receptors (the avidly iron-binding drug desferrioxamine is derived from a siderophore). Moreover, it has been recently demonstrated that some pathogenic bacteria may also use heme as a source of iron. After inducing red blood cells lysis, *Staphylococcus aureus* uses a transport system composed of three gene products to take up heme released from hemoglobin and heme oxygenase-like proteins to liberate iron (Skaar et al., 2004). The most highly adapted pathogens even evolved the ability to wrest iron from host iron-binding proteins such as hemoglobin, transferrin, and lactoferrin (reviewed in Andrews et al., 2003). Interestingly, bacterial infection may alter the whole network that maintains iron homeostasis in mammalian cells. In fact, it has been demonstrated that the mRNA expression profile of human epithelial cells infected with *N. meningitidis* resembles that of iron-deficient cells, with the exception of a reduced TfR1 (Bonnah et al., 2004). This may permit the bacteria to better compete with the host for transferrin-bound iron at the cell surface.

Bacteria incur metabolic costs to obtain iron in iron-poor environments, and these may limit growth. Iron deprivation may also inhibit the formation of resistant bacterial biofilms (Schaible and Kaufmann, 2004) and favor the mobile but vulnerable unicellular forms that are better equipped to reach alternative iron sources. Conversely, bacteria grow faster and form biofilms more readily when iron is abundant.

In spite of these efficient uptake mechanisms, microorganisms may undergo iron deficiency. In this case, not only they

transcriptionally activate the so called iron regulon to enhance their capacity of iron uptake (Andrews et al., 2003), but recent work has shown that both bacteria (Massè and Gottesman, 2002; Wilderman et al., 2004) and yeast (Puig et al., 2005), using small RNAs or a protein, respectively, have devised novel gene expression control mechanisms acting at the post-transcriptional level to distribute iron only to essential iron-using proteins (reviewed in Massè and Arguin, 2005). These recent findings advance our understanding of the nutritional needs of the microorganisms (for growth and survival) and the host (for defence) with respect to iron, thus providing guidelines for the development of antimicrobial intervention strategies.

### CONCLUSIONS AND PERSPECTIVES

In summary, from the results discussed in this review regarding the characterization of genes and proteins involved in the maintenance of iron homeostasis three important aspects emerge. First, there is little doubt that iron is an essential nutrient for all types of cells. However, like for many other nutrients, deficiency or excess are dangerous and harmful, as they may result in very common diseases, such as IDA or iron overload, or in altered response to infective agents. Second, in some cases the studies reviewed here have presented novel general mechanisms of transcriptional, posttranscriptional and posttranslational regulation of gene expression that extend the contribution provided by the analysis of iron metabolism to the understanding of the control of gene expression. The previous input is well exemplified by the model of iron-dependent control of intracellular iron homeostasis first advanced by Munro in 1976 (Zahringer et al., 1976) and become one of best-characterized systems of posttranscriptional regulation (Hentze and Kuhn 1996; Cairo and Pietrangelo 2000). Finally, recent findings about the role of iron in pathways such as the activation of the hypoxia inducible factor HIF1, which plays a key role not only in the adaptation to hypoxia (Schofield and Ratcliffe, 2004) but also in tumor development (Semenza, 2003), suggest that this metal will be more and more found to be involved in physiological mechanisms and pathologic settings.

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