

## Review Article

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# Iron transport & homeostasis mechanisms: Their role in health & disease

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**Iron is an essential trace metal required by all living organisms and is toxic in excess. Nature has evolved a delicately balanced network to monitor iron entry, transport it to sites of need, and serve as a unique storage and recycling system, in the absence of an excretory system, to remove excess iron. Due to the unique nature of iron metabolism, iron homeostasis is achieved by integrated specialized mechanisms that operate at the cellular and organism level. The use of positional cloning approaches by multiple researchers has led to the identification and characterization of various proteins and peptides that play a critical role in iron metabolism. These efforts have led to elucidation of the molecular mechanisms involved in the uptake of iron by the enterocytes, transportation across the membrane to circulation, and delivery to diverse tissues for use and storage and sensor system to co-ordinate and achieve homeostasis. Molecular understanding of these processes and the key regulatory molecules involved in maintaining homeostasis will provide novel insights into understanding human disorders associated with either iron deficiency or overload.**

**Key words** Anaemia - haemochromatosis - hepcidin - homeostasis - iron - trace metal

### Introduction

As early as 1842, von Leibig grasped the significance of chemical functions in life processes and proposed the theory that human nourishment consists of three classes of food: carbonaceous, nitrogenous, and mineral salts, essential for the construction of bones and teeth<sup>1</sup>. The analysis of elemental composition of living organisms of diverse species indicated the majority presence of metallic elements. Most of the metals are barely detectable at trace levels, while a few

are found in significant amounts. The metallic composition of organisms is fairly similar across the species, not in quantity, but in the proportion of various metals each species or tissue may contain. The so called macro elements calcium, sodium, potassium, and magnesium are present in large quantities, and the trace metals (also known as micronutrients) presenting a wide range of distribution among tissues and species. There are currently eight trace metals (iron, zinc, copper, manganese, selenium, cobalt, molybdenum, and chromium) that are nutritionally required for normal

human health and each of these metals contributes to <0.01 per cent to the total body weight<sup>2</sup>.

Metal ions are vital to life and participate in numerous metabolic processes in every living cell with considerable specificity and selectivity as components of enzymes and other molecular complexes. The living cell has developed an elegant and highly regulated system to utilize these metals based on their redox-active state to protect the cellular and organelle systems. The non-redox ions such as Ca and Zn participate in enzyme complexes involved in DNA metabolism; mRNA transcription prevents any redox-metal ion associated radical reactions that can cause damage to nucleic acids. Other redox-active ions such as Fe, Cu, Co, and to some extent Mn are utilized in enzyme complexes that participate in redox reactions and the conversion of active oxygen containing components<sup>3</sup>. The living cell has developed homeostatic mechanisms to maintain balance for competition for metal ions among numerous proteins, and to prevent accumulation to abnormal concentrations that could cause damage to the very proteins that compete for these metal ions. These trace metals have to be compartmentalized, and maintained at a fixed level to avoid any toxic effects. A delicately balanced homeostasis of metals is achieved by co-ordinated interaction among highly evolved and regulated uptake, storage, and secretion processes. Shortage or excess of metal ion availability due to nutritional imbalance or presence of toxic metals can tilt this balance with deleterious effects including the very survival of the cell. Nature has positioned in place homeostatic mechanisms at multiple levels starting from the regulation of uptake into the body to the cellular level, from the transport and maintenance of physiologically relevant concentrations at the cellular level that regulate the operation of molecular switches at the transcription level, translation and stability of RNA for metal ion regulatory proteins<sup>4-6</sup>. Numerous human disorders of altered metal homeostasis have been identified. For example, excessive uptake of iron has been implicated in the pathologies of hereditary haemochromatosis, and Parkinson's disease due to the influence of environmental factors or life style<sup>7</sup>.

Membrane proteins that serve as gatekeepers for cells and organelles controlling uptake and efflux of varied substances from nutrients to metal ions and drugs are generally referred to as transporters. The transporters can be passive transporters or facilitated transporters that allow passage of solutes (*e.g.*, glucose, aminoacids, urea) across membrane down their electrochemical

gradient or active transporters that create an ion/solute gradient across membrane and utilize diverse energy-coupling systems to transport against the gradient. Ion pumps are active transporters, while ion channels are passive transporters. These proteins appear to be conserved across the species and diversification might have occurred more than 600 million years ago before the initial separation of vertebrates from invertebrates<sup>8</sup>. The availability of genome sequence information for numerous model organisms and widespread use of expression cloning in the recent years had led to molecular identification of transporters across the species. These efforts led to identification of candidate genes for 47 solute carrier (SLC) protein families<sup>9</sup>. The SLC proteins relevant to metal transport are presented in Table I. It is becoming increasingly evident that natural resistance-associated macrophage protein (NARMP) is highly conserved (from bacteria to man) and plays a major role in the transport and homeostasis of metals<sup>3</sup>. Similarly a number of metal-specific storage proteins, other than transferrin or metallothionin have been identified and characterized. A comprehensive discussion on metal transport and homeostasis mechanisms and their role in health and disease is beyond the scope of this review article. To have a focused discussion on this important aspect of metal biology, we will restrict our discussion on one of the essential metals, *viz.*, iron (Fe).

#### *Iron (Fe)*

Iron (Fe) is a micronutrient and its daily intake in milligram amounts is adequate for normal health. Despite such a low dietary requirement, iron deficiency due to malnutrition is a global health problem. An average adult human body has a typical iron content of approximately 4g and about 50 per cent of which is in haemoglobin, about 25 per cent is stored in liver and rest constitute myoglobin and numerous other iron containing proteins. Mammals obtain iron exclusively from their diet, whereas haem iron, primarily from animal sources in the form of haemoglobin or myoglobin is absorbed efficiently, absorption of inorganic non-haem iron present in wide variety of diets is very inefficient. Humans ingest approximately 12-18 mg/day of dietary iron, of which only 1-2 mg is absorbed<sup>10</sup>. Systemic iron status in the organism is maintained by regulation of iron absorption and storage, but there is no known regulated mechanism for iron excretion from the body. Over the past decade, the sequencing of numerous genomes including human has facilitated cross species comparative genome analysis,

**Table I.** Proteins involved in the transport of metal ions

Gene family	Protein name	Prominent substrate (ions)	Transport type	Distribution	Link to disease
SLC11 (2**)	NRAMP1	Mn, Fe & other divalent metal ions	C/H+	Macrophages neutrophils	Polymorphisms linked susceptibility to bacterial infections and autoimmune diseases
	DMT1	Fe, Cd, Co, Cu, Mn	C/H+	Widespread (intestine, erythroid cells, kidney, lung, brain, testis, thymus)	Hereditary haemochromatosis
SLC30 (11)	ZNT1	Zinc	Unknown	Widespread	Embryonic lethal
	ZNT3	Zinc	Unknown	Glutamatergic neurons	Seizures, Alzheimer's
	ZNT5	Zinc	Unknown	Secretory glands	Bone abnormalities, heart failure
	ZNT8	Zinc	Unknown	Brain, liver	
SLC31 (2)	HCTr1	Copper	Energy independent/potassium dependent	ubiquitous	Unknown
SLC39 (14)	hZIP1	Zinc	Unknown	Widespread (plasma membrane and intracellular vesicles)	Unknown
	hZIP2	Zinc	Unknown	Prostate, uterus, cervical epithelium	Unknown
	hZIP4	Zinc	Unknown	Small intestine, stomach, colon, kidney	Acrodermatitis enteropathica
	KE4	Manganese	Unknown	Widespread, endoplasmic reticulum	Unknown
	BIGM103	Zinc	Unknown	Widespread	Unknown

Source: Ref 9, 79-82. \*\* No. of genes within the family.

C, co-transporter; DMT1, divalent metal-ion transporter-1; NRAMP1, natural resistance-associated macrophage protein-1; SLC, Solute Carriers; ZIP, zinc-iron permease family of proteins; ZNT, zinc transporter proteins

and positional cloning of many of these genes led to the identification of novel molecular players involved in iron uptake, transport, regulation and understanding of signaling pathways involved in iron homeostasis mechanisms, and their role in disease conditions.

#### *Intestinal absorption and transport of iron*

Dietary iron is predominantly absorbed in the proximal small intestine, near the gastro-duodenal junction. Three pathways are proposed to mediate the absorption process. The inorganic Fe is not efficiently absorbed, but the molecular pathways involved in the absorption of this form of Fe are extensively characterized<sup>11</sup>. Each transmembrane transport step is mediated by specific set of transport proteins and accessory enzymes that change the oxidation state of iron to facilitate the transport process. Although, the most efficient means of Fe uptake is from haem, the mechanisms of iron uptake from this source have not been elucidated<sup>12</sup>. Another less understood Fe uptake mechanism is mucin-integrin mobilferrin pathway<sup>13</sup>.

The non-haem iron or elemental iron in the diet is solubilized in the acidic environment of the gastric and duodenal lumen. The Fe (III) is converted to Fe (II) by duodenal cytochrome b (DCYTB), the first identified intestinal ferrireductase present on the apical surface of the enterocytes. The observation of increased expression of DCYTB in iron deficiency indicated its importance in apical iron uptake<sup>14</sup>. Fe(II) is transported across the cellular membrane by a 12-transmembrane-segment protein, divalent metal transporter 1 (DMT1), also known as SLC11A2, NRAMP2 and DCT1 (Table I). DMT1 also transports other divalent metals including zinc, manganese, cobalt, copper, cadmium, nickel and lead by a proton-coupled mechanism. DMT1, the only known Fe transporter in the intestine, which is highly conserved across the species, is also expressed in the endosomes of all cells. The significant role of DMT1 in intestinal absorption is evident from studies in microcytin anaemic mice and Belgrade rats<sup>15</sup>. A spontaneous mutation (G185R) found in both strains caused significant defects in intestinal iron absorption

and assimilation of iron by erythroid precursor cells<sup>15</sup>. Also, the targeted mutation of murine DMT1 gene (Slc11a2<sup>-/-</sup> mice) further confirmed its role in intestinal iron absorption. But these studies also suggested that DMT1 is not essential for placental transfer of iron or acquisition of iron by other tissues<sup>16</sup>. Mutations in DMT1 observed in humans with congenital anaemia did not result in interference with intestinal iron absorption. This effect may be compensated through efficient absorption of haem-iron, but has been associated with hepatic iron overload.

#### *Iron export to plasma*

Identification and characterization of numerous animal models carrying spontaneous mutations exhibiting iron deficiency or overload phenotype have greatly contributed to understanding the role of diverse proteins involved in the export and transport of iron. A detailed analysis of these studies, and the biochemical and functional characterization of these proteins will be beyond the scope of this review, but a glimpse of these proteins is provided in Table II. Once internalized, iron can be stored in the cytosolic iron-storage molecule ferritin or exported into plasma by basolateral iron exporter protein. Three independent groups simultaneously identified basolateral iron transporter proteins known as iron-regulated protein 1 (IREG1), ferroprotein 1 or MTP1<sup>17-19</sup>. In addition, another protein hephaestin is required for iron export at the basolateral membrane. Hephaestin is closely related to serum multi-copper oxidase, ceruloplasmin and acts as a ferroxidase<sup>20</sup>. The current working hypothesis is that the diffusion of Fe(II) across the basolateral membrane is facilitated by iron regulated protein 1 (IREG1)/ferroprotein1 (FPN)/MTP1 and hephaestin, a membrane-bound protein that promotes oxidation of Fe(II) to Fe(III) prior to its release from transporter molecule. The ferric iron then binds to apotransferrin (the iron-free form of transferrin) in the plasma to form Fe(III)-transferrin (TF) complex, which is the major type of iron present in blood. The TF is an 80 kDa glycoprotein with homologous N- and C-terminal iron binding domains, is synthesized in liver, retina, testis and brain. The 3-5 µg/ml of iron present in serum is predominantly bound to TF. The very small amount of iron bound to albumin or other small molecular weight ligands such as citrate constitutes the so called non transferrin bound iron. Plasma TF plays two important roles in iron physiology: (i) facilitating the transport of iron to cells that express TF receptors, and (ii) binding iron to limit the ability of iron to generate toxic radicals

thereby protecting the organ systems from the toxic effects of Fe. There is a clear species-specific difference in the iron saturation of TF, as high as 80 per cent in mice and 30 per cent in humans. The saturation of TF also varies due to diurnal cycle and local circumstances such as high saturation of TF in the blood in portal circulation and low in the blood leaving bone marrow<sup>21,22</sup>.

#### *Delivery of iron to tissues and intracellular transport*

The cellular uptake, storage and export of iron depend on functional demands of different cell types. In the majority of eukaryotic cells, iron uptake occurs primarily by TF-Transferrin receptor (TFR)-mediated endocytic pathway. The molecular events associated with the TF-bound iron and release of apotransferrin to the plasma for its reutilization or cycling is very well established. There are two TF receptors, namely TFR-1 and TFR-2. The TFR-1, with high affinity binding to iron-bound TF, is the most predominant receptor involved in iron uptake in the majority of cells, while TFR-2 is expressed primarily in liver and binds TF-Fe(III) complex at a much low affinity. Embryonic lethality observed in TFR-1 knockout mice further reinforces the important role of TFR-1 in cellular iron uptake<sup>23</sup>.

Once Fe(III)-TF binds to its receptor at the cell surface, the TF-TFR-1 complex is internalized in clathrin-coated pits that form endocytic vesicles. Inside the cells, the internalized complex in the endosome is acidified by a vacuolar H<sup>+</sup>-ATPase (V-ATPase) that lowers the luminal pH to about 5.5. This acidification process induces conformational changes in TF-TFR-1 complex with consequent release of iron<sup>24</sup>. Positional cloning studies in the *nm1054* mutant mouse model, which exhibits impaired haemoglobin synthesis due to decreased iron uptake, identified a six-transmembrane epithelial antigen of the prostate-3 (STEAP3) as the endosomal ferrireductase responsible for reduction in endosomal ferric iron to ferrous iron<sup>25</sup>. Mutations in STEAP3 have been implicated in microcytic anaemia due to a defect in the delivery of iron in reticulocytes<sup>26</sup>. However, STEAP3 is not required for efficient iron uptake in other cell types, suggesting the existence of several other ferrireductases remain to be identified<sup>13</sup>. The endosomal DMT1 transports the ferrous iron to the cytosol. This leads to the next important step of iron transport, *i.e.* TF recycling, to carry fresh cargo of iron for transport. At acidic pH apotransferrin remains bound to TFR1, and the complex is recycled to the cell surface. At the more neutral pH of the plasma, apotransferrin dissociates from TFR1 and is ready to

**Table II.** Salient feature of critical proteins (well characterized) involved in iron metabolism

Protein	Characteristics	Protein	Characteristics
DCYTB	Duodenal cytochrome-b; a potential ferrireductase enzyme present on the apical surface of enterocytes, reduces ferric iron to ferrous iron for absorption.	Hepcidin/ HAMP	A 25-amino acid peptide, also known as hepcidin antimicrobial peptide. Primarily produced in liver, a circulating peptide hormone playing a key role in iron homeostasis mechanisms.
DMT-1	Also known as DCT1, Nramp2, identified as homologue of Nramp1, a protein that confers resistance to intracellular pathogens. A glycosylated protein of ~90kDa, is ferrous iron transporter that absorbs dietary iron at the apical surface of the enterocytes, also facilitates release of iron from endosomal vesicles.	BMP	Bone morphogenetic protein
FPN	Ferroportin, an iron exporter protein, also known as IREG-1 (iron-regulated transporter-1); or metal transport protein 1 (MTP1). This protein is responsible for iron export from the basolateral membrane of enterocytes and release of iron from hepatocytes and macrophages. FPN is ~62kDa protein containing 9-10 transmembrane domains with N-terminal protein on the intracellular side of the membrane; contains a functional IRE at its 5'UTR.	HFE	It is an atypical major histocompatibility complex protein ubiquitously expressed at relatively low levels in tissues. It complexes with TFR1 and plays a role in iron transport and homeostasis.
TF	Transferrin, a primary iron transport protein in the serum that reversibly binds iron with high affinity. An 80kDa glycoprotein synthesized mainly in liver, and has a bilobal structure. TF can also bind Mn, Co, Cu, Cd with low affinity.	HJV	Haemojuvelin, is part of repulsive guidance molecule and its precise function is unknown. The multiple protein motifs on HJV suggest that it could function as membrane bound receptor and found to coprecipitate with BMP2. It is expressed in liver, heart and skeletal muscle and expression is linked to hepcidin. Mutations in this gene are linked to pathogenesis similar to hepcidin.
TFR1	Transferrin receptor 1 is required for cellular up take of TF-bound iron. A homodimeric glycoprotein of ~90kDa is expressed in most cells except mature erythrocytes. TFR1-TF interaction is reversible and is dependent on pH and iron content of TF.	IRE	Iron regulatory elements are RNA stem-loop structures found in non-coding sequence.
TFR2	Transferrin receptor 2 has 66 per cent sequence similarity with TFR1. It is predominantly expressed in liver and localized on the basolateral membrane domain of hepatocytes. TFR2 cannot compensate TFR1 for iron transfer.	IRP	Iron regulatory binding proteins (IRP-1, IRP-2) bind to IRE sequence and control gene expression.
STEAP3	Six transmembrane epithelial antigen of the prostate-3, is an endosomal ferrireductase responsible for the uptake of TF-bound iron in erythroid cells.	Hephaestin	Hephaestin was identified as protein responsible for sex-linked anaemia (sla) in mice. It is a multicopper oxidase homologous to ceruloplasmin and involved in the basolateral membrane of enterocytes.
Ferritin	Ferritin is a ubiquitous and highly conserved major iron binding protein. A 24-subunit polymer composed of varying ratios of polypeptide chains: L-ferritin and H-ferritin	Cerulo- plasmin	A serum ferroxidase contains 95 per cent of copper present in plasma. A 132 kDa glycoprotein synthesized predominantly in hepatocytes, undergoes N-linked glycosylation before release into serum. Acts in conjunction with FPN to mediate iron release and also plays a role in iron homeostasis.

take next shipment of iron. It should also be recognized that there exist certain TF-independent iron transporting systems facilitating transport of iron in other tissues<sup>27</sup>.

#### *Iron storage and recycling*

The cellular uptake, storage and export of iron are dictated by distinct physiological and metabolic needs of different cell types. Not all iron is utilized in metabolic processes and some is stored as reserve for use when iron levels are low and to prevent toxic effects of free iron in the cell. Hepatocytes serve as major depots for iron storage, although TF cycle may be involved in iron acquisition, but non transferrin bound iron uptake pathways appear to play an important role when serum iron levels exceed TF binding capacity. The stored iron accounts for 20-30 per cent of body iron and majority of it is bound to ubiquitous and highly conserved iron binding protein, ferritin. Ferritin is a multimer of 24 subunits comprised of two subtypes H (heavy, 21kDa) or L (light, 19kDa) that constitute the central core that contains up to 4,500 atoms of iron<sup>28</sup>. An important feature of H subunit is its ferroxidase activity that facilitates the oxidation of Fe<sup>2+</sup> (predominant form in the cytosol) to Fe<sup>3+</sup> to be bound to ferritin. The two ferritin subunits are highly conserved and show different combination of the two subunits depending on tissue. For example, liver and spleen are rich in L subunits, whereas heart is rich in H subunits. Iron is also stored in an insoluble form in a poorly defined storage protein, haemosiderin, presumed to be derived from the lysosomal degradation of ferritin. Under iron overload conditions the content of this protein was found to increase dramatically, particularly in liver, pancreas and heart<sup>29</sup>.

Intestinal absorption accounts for only a fraction of TF-bound iron in the circulation. Recovery of iron from senescent erythrocytes also plays an important role in iron maintenance. At the end of 120-day lifespan, human erythrocytes undergo surface alterations that mark them to be phagocytosed and digested by macrophages in the spleen and liver. In the macrophages iron is predominantly recovered from haem by the action of induced form of haem oxygenase<sup>30</sup>. Metal transporters, NRMP-2 and DMT-1 transport the recovered iron through the phagosomal membrane to the cytoplasm to be stored in the macrophage bound to ferritin. This stored iron will be eventually transported to TF facilitated by the participation of ferroportin and ceruloplasmin proteins<sup>31,32</sup>. The observation of severe anaemia and rapid accumulation of iron in FPN-

deficient mice suggested that FPN is essential for iron recycling<sup>33</sup>. Although this recycling is fundamental in the overall biology of iron, this is the least understood process of iron metabolism.

#### *Iron homeostasis mechanisms and regulation*

Balancing the iron levels in the body has to be meticulously achieved to provide iron as needed and the amount required to avoid toxicity associated excessive accumulation of iron. This is a co-ordinated act regulated by signals from cells and tissues in response to various physiological inputs. In the absence of an established mechanism for excretion, iron absorption and storage, homeostasis mechanisms operate to balance iron both at whole organism, or systemic at the cellular level that is mediated by transcriptional and post-transcriptional mechanisms.

#### *Systemic iron homeostasis*

The systemic iron homeostasis is achieved by regulating iron absorption and storage and recycling mechanisms. Intestinal iron absorption is regulated in response to iron need and availability. The studies by Hahn *et al*<sup>33</sup> and Stewart *et al*<sup>34</sup> four decades ago indicated that a large oral dose of iron (mucosal block) reduced the absorption of smaller dose of iron administered several hours later. This is found to be mediated by downregulation of DMT1 transport protein in the brush border<sup>35</sup> without altering basolateral transport of iron in the intestine. These observations suggest that enterocytes may receive signals from other tissues or cells that are involved in either consumption (erythroid precursors) or storage (hepatocytes, duodenal enterocytes, macrophages) in maintaining homeostasis. Signals that originate from storage sites to balance intestinal absorption are termed *storage regulators*, while *erythroid regulators* signal when the consumption demand for iron, in bone marrow, erythroid precursors and circulating erythrocytes, exceeds the amount present in stores. On the other hand, *inflammatory regulators* communicate signals in response to infection or inflammation, resulting in accumulation of iron in macrophages. Iron homeostasis is also found to be altered due to *hypoxia regulatory signals*. Adding to the complexity, these diverse regulatory signals may not be completely independent of each other and elicit quantitative differences in response through a common molecule(s). The regulators that modulate intestinal absorption may also regulate release of iron from hepatocytes or macrophages, by humoral or plasma proteins that might act at multiple sites.

The observation of iron-overload in the upstream stimulatory factor 2 (USF-2) knockout mice, led to the serendipitous discovery of hepcidin (HAMP) gene which codes for a antimicrobial peptide and mediator of innate immunity<sup>36</sup>. Hepcidin is a 25- amino acid circulating peptide hormone, a member of a family of defensins primarily secreted by liver and is conserved across the species<sup>37</sup>. There are two hepcidin genes in mice, but only one gene, hepcidin-1, appears to have biological activity<sup>38</sup>. Targeted deletion of hepcidin gene in mice or mutations in human gene result in elevated body iron stores, presumably due to hyperabsorption associated with decreased iron in tissue macrophages<sup>39</sup>. Iron deficiency observed in a transgenic mouse strain that constitutively expresses hepcidin suggests that hepcidin mediating stores regulator function (attenuating both intestinal iron absorption and macrophage iron release). On the other hand, very little expression of hepcidin in iron-loading, observed in TF gene mutated (Trf<sup>hpx</sup>) mice suggests that hepcidin is also a factor in erythroid regulator signaling<sup>40</sup>. The decreased expression of hepcidin in response to non anaemic hypoxia and its increased expression in mice and humans with inflammation suggest that hepcidin may also participate in the mediation of hypoxia and inflammatory regulatory cascades<sup>41</sup>. Hepcidin is also found to control iron levels by directly interacting with FPN1, leading to internalization and degradation of FPN1 when iron levels are high, consequently blocking release of iron from storage sites, hepatocytes, enterocytes and macrophages<sup>42</sup>. Although no direct interaction between hepcidin and duodenal transport proteins, DMT1 and DCYTB is established, some studies indicate that these proteins are also negatively regulated by hepcidin<sup>43</sup>.

Under basal conditions, hepcidin gene expression is mediated through bone morphogenetic protein (BMP) and SMAD signaling pathway<sup>43</sup>. Hepcidin gene expression is in part regulated by four genes, *viz.*, hemojuvelin (HJV), TFR2 and HFE, an atypical major histocompatibility complex proteins, and the molecular mechanisms of these interactions are discussed in detail later. HJV is a member of membrane-bound BMP co-receptor and binds to BMP ligands (BMP1 or BMP2). The complex of HJV-BMP2 induces intracellular BMP signaling cascade mediated by SMADs and activates expression of hepcidin. The liver-specific knockout of *smad4* abrogates hepcidin gene transcription resulting in iron overloading<sup>44</sup>. Inflammatory mediators such as IL-6 and other cytokines induce transcription of

hepcidin in hepatocytes through activation and binding of STAT-3 to hepcidin gene promoter. STAT-3 activation requires SMAD4 and deletion of SMAD4 blocks STAT-3 mediated expression of hepcidin<sup>45</sup>. The promoter region of HAMP in mice and humans has hypoxia-inducible factor (HIF) family of transcription factor binding sites suggesting the negative regulation of hepcidin expression in hypoxic conditions may be mediated by HIF family members<sup>46</sup>.

#### *Cellular iron homeostasis*

As discussed above, the numerous proteins involved in iron uptake, export, storage and recycling have to be regulated in a co-ordinated fashion. This is achieved by intricately co-ordinated regulatory mechanisms that orchestrate their expression, stability, translation and post-translational modifications. The intracellular iron homeostasis is achieved by co-ordinated regulation of iron storage protein, ferritin and iron uptake protein, transferrin receptor 1 (TFR1). The cloning of H and L subunits of ferritin led to the identification of iron-responsive elements (IREs) in the untranslated regions (UTRs)<sup>12</sup>. The IREs are stem shaped structures located in the 5' and 3' UTRs of mRNA that display remarkable structural similarity. Canonical IREs have a six nucleotide apical loop with a consensus sequence of 5'-CAGUGN-3'<sup>47</sup>. The cytosolic proteins that specifically recognize and bind IREs, called iron regulatory proteins (IRP-1 and IRP-2), control expression of genes containing IREs. The IRP1 was soon recognized as counterpart of mitochondrial aconitase due to its ability to assemble a [Fe-4S] cluster<sup>48</sup>. It also exists as apo-IRE1. The switch between the two forms is mainly regulated by the labile iron pool and participation of different factors that mediate transcriptional regulation. Depending on the position of IREs, IRP-IRE binding exerts diverse regulatory responses (Table III). For example, the binding of IRP to IREs found in the 5' UTR of mRNAs encoding ferritin, ferroportin, and the haem biosynthetic enzyme, aminolevulinic synthase interferes with the initiation of translation. On the other hand, if the IREs are in the 3' UTR of mRNA, as in the case of TFR-1, the binding of IRPs to multiple IREs in the 3' UTR of TFR1 leads to stabilization of TFR-1 mRNA by inhibiting its degradation by nuclease digestion<sup>49</sup>.

As discussed above, under iron deficiency conditions, IRPs actively bind IREs and stabilize TFR1 mRNA and simultaneously decrease translation of ferritin mRNA, consequently increasing the uptake and

**Table III.** Genes regulated by iron responsive element (IRE)\*.

Gene	IRE location	Function
L-Ferritin	5'	Iron storage
H-Ferritin	5'	Iron storage
Erythroid 5-ALA synthase	5'	Haem synthesis
Nramp2	3'	Iron importer
FPN (IREG1)	5'	Iron exporter
DMT1	3'	Iron transporter
TFR1**	3'	TF binding and intracellular transport

\*Adopted from Sargent *et al*<sup>83</sup>; \*\*Contains multiple iron responsive elements (IREs).

availability of iron in the cell. Conversely, when the iron levels are high, decreased IRE binding facilitates efficient translation of ferritin mRNA and decreases the stability of TFR1 mRNA, leading to iron sequestration over uptake. The majority of these regulatory mechanisms were observed from *in vitro* studies and their role *in vivo* remain uncertain. Studies with IRP-1 or IRP-2 knockout mice are mixed and inconclusive in establishing their role<sup>49</sup>. Inflammatory mediators such as TNF- $\alpha$ , IL-6, IFN- $\gamma$  also have been found to regulate the expression of proteins involved in cellular iron homeostasis (ferritin, TFR-1), including DMT-1 through IRP1/IREs mediated mechanisms<sup>50,51</sup>.

Transgenic knockout mouse models of IRP1, IRP2 indicate that the double knockout is embryonically lethal<sup>52</sup>. Observation of no overt phenotype for IRP1/- knockout mouse is rather surprising and suggests that IRP2 can compensate for the loss of IRP1<sup>49</sup>. Mouse models of IRP2 knockout exhibited increased iron content and expression of DMT1, ferritin and ferroportin, increased serum ferritin and liver iron content<sup>53</sup>. These observations suggest that other unidentified factors may participate along with IRPs in cellular iron homeostasis.

#### *Iron homeostasis in disease conditions*

The role and participation of various protein and peptide molecules in maintaining the delicate balance of iron homeostasis suggests that disruption or overexpression of any of these entities may lead to significant health consequences. Studies from various spontaneous or transgenic animal models in zebrafish, mice, and rat and certain human genetic disease conditions (Table IV) implicate mutations in HFE, TFR2, hepcidin, HJV, IREG1, TF, ceruloplasmin and ferritin to some form of iron overloading pathology.

**Table IV.** Selected genes that exhibit inherited defects in iron metabolism.

Gene	Phenotype	Species
Ceruloplasmin	Plasma hypoferraemia with tissue iron loading	H, M1
DMT1	Iron deficiency	H, M2, R, Z
H-Ferritin	Iron loading	H
L-Ferritin	Bilateral cataracts	H
HO-1	Plasma hypoferraemia with tissue iron overloading	M1
HJV	Iron loading	H
HAMP	Iron loading	H, M2
Hephaestin	Iron deficiency anaemia	M2
HFE	Iron loading	H
IREG-1	Plasma hypoferraemia with tissue iron overloading	H, Z
TF	Plasma hypoferraemia with tissue iron overloading	H, M1, M3
TFR1	Severe anaemia	M2
TFR2	Iron loading	H, M1

H, human and all mutations are spontaneous; M1, mouse mutations are engineered; M2, induced mutations in mouse; M3, spontaneous mutations in mouse; R, induced mutations in rat; Z, induced mutations in zebrafish

These new observations have aided in gaining better understanding of molecular mechanisms involved in inherited iron overload diseases<sup>45</sup>.

### **Hereditary iron-overload disorders**

#### *Hereditary haemochromatosis*

Haemochromatosis was first coined by Recklinghausen in 1889 for the clinical conditions of 'bronze diabetes with cirrhosis' (reported by Trousseau in 1865)<sup>54</sup>, due to its association with deposition of iron containing pigment in liver. The cloning of the HFE gene in 1996<sup>55</sup> and understanding of its interaction with other proteins involved in iron metabolism have helped in the molecular understanding of the disease processes. The primary iron-overload disease or hereditary haemochromatosis (HH) appears to be mediated by mutational events in four genes leading to perturbations in iron acquisition. Depending on the mutation in the gene(s) involved in iron homeostasis and the clinical presentation of HC symptoms, HH can be classified in to four phenotypes. The type 1 HH is the most common iron-overload disorder that occurs due to mutations in HFE gene. The major histocompatibility protein transcribed by this gene forms a complex with  $\beta$ 2-microglobulin, a component of MHC. Similarly this protein



is also known to complex with TFR1<sup>55</sup>. Two predominant mutations in this gene<sup>56</sup> have been found to interfere with its ability to complex with  $\beta$ 2-microglobulin and also presumably alter complexation with TFR1. The role and involvement of hepcidin in the regulation of this complexation is not understood well. In this type, increased iron absorption and deposition occurs in liver, heart, pancreas and skin, eventually leading to liver cirrhosis, fibrosis and diabetes.

The type 2 HH is characterized with severe cardiac and endocrine dysfunction, due to increased iron overload leading to mortality before the age of 30, and is also known as juvenile haemochromatosis. Mutations in hepcidin (HAMP) and or haemojuvelin (HJV, also known as HFE2) genes are implicated in the disease process. As discussed above, HJV acts as a co-receptor for BMP, and through this regulatory mechanism increases hepcidin synthesis leading to iron overloading. The type 3 HH is caused by mutations in TFR2, a homologue of TFR1, with limited tissue distribution. The iron overload in this disease is slow and not fatal. The studies on TRF2 targeted gene deletions in mice was found to be associated with decreased hepcidin production and subsequent iron overload<sup>45</sup>. The fourth type of HH occurs due to mutations in ferroportin gene. This disease exhibits two types of phenotypes: accumulation of iron in macrophages and low TF saturation, iron-limited erythropoiesis, or increased hepatic iron accumulation and high saturation of TF. Targeted gene deletion studies in mice and zebrafish indicated that mutant ferroportin proteins are not trafficked to cell surface or become transport incompetent causing iron overloading in macrophage<sup>57,58</sup>. Mutant ferroportin proteins also become resistant to hepcidin-induced internalization and lead to increased accumulation of iron in hepatocytes<sup>59</sup>.

#### *Iron-overload neuronal disorders*

The loss of function mutations in the ceruloplasmin gene lead to autosomal recessive disorder of iron homeostasis, aceruloplasminaemia<sup>60</sup>. The unique features of this disease are characterized by iron overload in brain tissue with excess iron deposition in glia and neurons, a mechanism similar to iron overload due to absence of ceruloplasmin<sup>61-63</sup>. The patients with this disease present progressive dementia, dysarthria and dystonia, secondary to glial iron accumulation<sup>64</sup>. The observations of synthesis of ceruloplasmin in astrocytes<sup>65</sup> suggest that this membrane-bound

ferroxidase may facilitate release of iron from storage sites within the brain and play a critical role in iron homeostasis of the central nervous system. Studies from mouse models suggest that the neuronal loss observed in this disease may be due to iron deficiency, secondary to impaired iron movement from astrocytes<sup>66</sup>.

#### *Neuroferritinopathy*

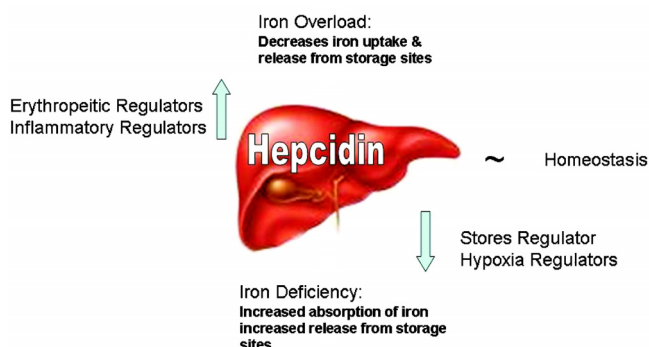
Neuroferritinopathy, an autosomal dominant disease results from mutations in the gene encoding the light chain of ferritin<sup>67</sup>. Presence of ferritin in neuronal cells and their transcriptional regulation by IRP mediated pathways are well known<sup>48</sup>. These mutations appear to impair ferritin assembly leading to loss of iron storage capacity within the neurons and subsequent iron-mediated cell injury<sup>68</sup>. Neuronal loss, degeneration of basal ganglia nuclei, loss of iron and ferritin in both extracellular and cytoplasmic inclusion bodies of microglia were found on the pathological examination of patients. Studies from an IRP deficient murine model suggests that impairment of ferritin transcriptional regulation result in iron accumulation in oligodendrocytes leading to neuronal degeneration and secondary iron deficiency similar to aceruloplasminemia<sup>69</sup>.

#### *Iron-overload anaemia*

The increased intestinal absorption of iron with an ineffective erythropoiesis is implicated in the paradoxical situation of iron-loading anaemia, and this condition gets further aggravated by erythrocyte transfusion. The most common iron-loading anaemias are intermediate and major forms of  $\beta$ -thalassaemia, and rare anaemias include congenital dyserythropoietic anaemia, and X-linked sideroblastic anaemia and anaemias due to mutations in DMT1<sup>70</sup>. The erythropoietic drive, leading to increased absorption of iron, appears to suppress hepcidin synthesis in these conditions as evidenced by low urinary levels of hepcidin in these patients<sup>71</sup>.

#### *Anaemia of chronic disease*

Anaemia of inflammation is a common underlying condition for chronic infections, inflammatory disorders and some cancers. Anaemias resulting from these conditions are characterized by decreased serum iron, decreased iron binding capacity of TF, increased ferritin and increased iron in bone marrow and macrophages due to impaired mobilization of iron from stores suggesting that hypoferraemia and anaemia of inflammation are part of the host response to infections. The common pathway for anaemia of inflammation



**Fig.** Hepcidin is now known to play a critical role in iron homeostasis mechanisms and stands up to the hormonal status conferred on this 25-amino acid peptide. Based on the induced/suppressed expression of hepcidin in liver hepatocytes, and consequently its levels in serum (indicated by the arrow) is increasingly recognized as a key player in mediating information from the diverse signals for iron absorption (efflux from enterocytes/macrophages) and interaction of iron-bound transferrin (TF) with transferrin receptor (TFR) on the cell membrane. Thus either in iron overload (induced or hereditary) or iron deficiency (due to low dietary intake, chronic anaemia due to disease conditions) signals is somehow modulated by hepcidin synthesis in liver and levels in serum.

appears to involve cytokine stimulation of hepcidin synthesis, and iron sequestration resulting from hepcidin-induced loss of ferroportin from macrophages and decreased erythropoiesis, due to low iron limitations in the pathogenesis of anaemia of inflammation<sup>72,73</sup>. It has been recently reported that hepcidin also contributes to anaemia of inflammation by inhibiting proliferation and survival of erythroid progenitor cells<sup>74</sup>.

#### *Influence of lifestyle*

It had been increasingly recognized that certain diseases originating from life-style habits such as alcohol consumption may also perturb iron homeostasis mechanisms. In alcoholic liver diseases such as fatty liver, fibrosis, hepatitis and cirrhosis, increased accumulation of iron was found in hepatocytes and reticuloendothelial (Kupffer) cells<sup>75</sup>. In advanced stage of diseases such as “alcoholic siderosis” iron deposition is dominant in Kupffer cells<sup>76</sup>. In non alcoholic liver disease, which presents similar characteristics described above in the absence of alcohol consumption, an increased association was found for mutations in HFE in certain Australian and North American populations, suggesting the role for iron overload in the aetiology of this disease<sup>77</sup>.

#### *Conclusions*

In the past decade significant advances have been made in our understanding of iron uptake, storage and

homeostasis mechanisms. The efforts to understand the perturbations in iron metabolism in certain inherited disorders have largely contributed to this success. The investigations carried out in numerous animal models that mimic these diseases either due to spontaneous mutations or experimental gene deletion led to identification of a number of key molecules involved in iron metabolism and homeostasis. Identification and characterization of one such molecule, a 25-mer peptide, hepcidin, contributed to the comprehensive understanding of intricate connections among the molecular networks that are involved in maintaining both systemic and cellular homeostasis mechanisms. Our current understanding of the critical role this peptide plays in iron homeostasis is summarized in the Fig. As depicted here, the various hypothetical regulators, storage, erythropoietic, and inflammation/hypoxic regulators, regulate iron homeostasis with hepcidin in the center of this network. Transcriptional regulation of hepcidin appears to be controlled by soluble HJV and this process seems to be under the control of circulating iron<sup>78</sup>. How these signals are sensed and communicated to the regulation of hepcidin synthesis and downstream signaling cascades is still not known.

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