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The roles of iron in health and disease

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Abstract

Iron is vital for almost all living organisms by participating in a wide variety of metabolic processes, including oxygen transport, DNA synthesis, and electron transport. However, iron concentrations in body tissues must be tightly regulated because excessive iron leads to tissue damage, as a result of formation of free radicals. Disorders of iron metabolism are among the most common diseases of humans and encompass a broad spectrum of diseases with diverse clinical manifestations, ranging from anemia to iron overload and, possibly, to neurodegenerative diseases. The molecular understanding of iron regulation in the body is critical in identifying the underlying causes for each disease and in providing proper diagnosis and treatments. Recent advances in genetics, molecular biology and biochemistry of iron metabolism have assisted in elucidating the molecular mechanisms of iron homeostasis. The coordinate control of iron uptake and storage is tightly regulated by the feedback system of iron responsive element-containing gene products and iron regulatory proteins that modulate the expression levels of the genes involved in iron metabolism. Recent identification and characterization of the hemochromatosis protein HFE, the iron importer Nramp2, the iron exporter ferroportin1, and the second transferrin-binding and -transport protein transferrin receptor 2, have demonstrated their important roles in maintaining body's iron homeostasis. Functional studies of these gene products have expanded our knowledge at the molecular level about the pathways of iron metabolism and have provided valuable insight into the defects of iron metabolism disorders. In addition, a variety of animal models have implemented the identification of many genetic defects that lead to abnormal iron homeostasis and have provided crucial clinical information about the pathophysiology of iron disorders. In this review, we discuss the latest progress in studies of iron metabolism and our current understanding of the molecular mechanisms of iron absorption, transport, utilization, and storage. Finally, we will discuss the clinical presentations of iron metabolism disorders, including secondary iron disorders that are either associated with or the result of abnormal iron accumulation. © 2001 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Iron represents approximately 35 and 45 mg/kg of body weight in adult women and men, respectively (Andrews, 1999a,b; Bothwell et al., 1995). The majority of total body iron, about 60–70%, is present in hemoglobin in circulating erythrocytes. Another 10% of essential body iron is present in the forms of myoglobins, cytochromes, and iron-containing enzymes, amounting to no more than 4–8 mg of iron. In healthy individual, the remaining 20–30% of surplus iron is stored as ferritins and hemosiderins in hepatocytes and reticuloendothelial macrophages (Conrad et al., 1999).

Even though the amount of iron bound to transferrin is less than 1% (approximately 4 mg) of the total body iron store, it is the most significant iron pool and has the highest turnover. The turnover of transferrin-bound iron is about 25 mg/day. Of this transferrin-bound iron, 80% is transported to the bone marrow for hemoglobin synthesis in developing erythroid cells (Conrad et al., 1999). From these sites, reticulocytes are released into the circulation and within a day, they develop into mature erythrocytes that circulate in the blood for about 120 days. Since erythrocytes have a high daily requirement of about 20 mg of iron, the majority of iron for the synthesis of hemoglobin comes from the destruction of red blood cells and from recycled iron in plasma. Reticuloendothelial macrophages ingest senescent red blood cells and release heme molecules from hemoglobins into circulation. Iron targeted to mitochondria is incorporated into protoporphyrin IX by ferrochelatase to form heme molecules, thus completing an important cycle of heme biosynthesis (Bottomley et al., 1995; May et al., 1995). Hence, iron plays a crucial role in oxygen transport.

Being one of the most abundant metals in the human body, iron plays important roles in cellular processes such as the synthesis of DNA, RNA, and proteins; electron transport; cellular respiration; cell proliferation and differentiation; and regulation of gene expression (Andrews et al., 1999; Boldt, 1999; Conrad et al., 1999; Gerlach et al., 1994; Wessling-Resnick, 1999). Iron metabolism takes place in specialized tissues: testes, brain, intestines, and placenta, and skeletal muscle. High levels of iron is found in the liver, brain, red blood cells, and macrophages (Andrews et al., 1999; Yehuda and Youdim, 1989; Youdim, 1988). Importantly, formation of myelin and development of neuronal dendritic tree (Ben-Shachar et al., 1991; Youdim et al., 1990, 1991) require the participation of iron. Therefore, iron homeostasis is critical for normal brain function, especially in learning and memory (Gerlach et al., 1994; Youdim et al., 1989, 1990).

By supporting transcription of certain genes, iron affects cell cycling and differentiation (Boldt, 1999). Transcription of three mammalian genes, *protein kinase C- β* , type 5 isoenzyme of *acid phosphatase* or tartrate-resistant *acid phosphatase*, and *p21*, which is a cyclin-dependent kinase inhibitor, are dependent on iron (Boldt, 1999). The protein kinase C- β , a member of the protein kinase C family of intracellular signaling pathways, is required for cell cycle progression and differentiation (Macfarlane and Manzel, 1994; Tonetti et al., 1994). Iron is required for the expression of protein kinase C- β in many different types of cells, including hematopoietic and human leukemic cells (Alcantara et al., 1991). The type 5 isoenzyme of acid phos-

phatase is an iron-containing molecule that is expressed mainly in cells of monocytes and macrophages (Grimes et al., 1993; Lord et al., 1990). Although the function of type 5 isoenzyme of acid phosphatase is not defined, it might be encoded by the *uteroferrin* gene, which codes for a putative iron-transport molecule in the placenta (Ling and Roberts, 1993a; Ling and Roberts, 1993b). Sequence analysis reveals that an iron-responsive transcription activation site and heme responsive elements are present in the promoter region of the type 5 isoenzyme of *acid phosphatase* gene (Reddy et al., 1996, 1998). It is therefore possible that iron and heme impose opposite effects on the expression of this gene (Boldt, 1999; Reddy et al., 1996, 1998). p21 is a member of the cyclin-dependent kinase inhibitor family of cell cycle regulatory proteins (Harper et al., 1993). Induction of p21 results in the inhibition of cyclin-dependent kinase functions that are needed for controlling cell cycling, hence causing cell cycle arrest at G1 phase (Harper et al., 1993). Indeed, cellular differentiation of monocytes and macrophages requires G1 phase cell cycle arrest, which is mediated by transcriptional induction of p21 and protein kinase C- β (Boldt, 1999). Under iron-deficient conditions, p21 mRNA in monocytes and macrophages is not induced. Cells are not blocked at the G1 phase (Boldt, 1999), but rather arrested at the S phase of the cell cycle. As a result, monocytes and macrophages undergo extensive apoptosis (Boldt, 1999). These findings suggest that iron is required for cellular differentiation, a process that involves the induction of p21 and protein kinase C- β (Boldt, 1999).

Iron is also the key component of many cellular enzymes, such as oxidases, catalases, peroxidases, cytochromes, ribonucleotide reductases, aconitases, and nitric oxide synthases (Boldt, 1999; Conrad et al., 1999; Ponka, 1999). These enzymes are not only critical in many basic cellular processes, i.e., DNA and RNA synthesis, electron transport, and cell proliferation, but they also contribute to the development of a wide number of diseases, ranging from iron disorders, cancers, neurodegenerative diseases, and aging. Interestingly, in cells loaded with high levels of iron, mRNA and protein levels of apolipoprotein B100 are decreased by 50%, whereas mRNA levels of semaphorin cd100 and aldose reductase are increased (Barisani et al., 2000). These studies demonstrate that iron has a significant impact on many key cellular processes; however, the molecular mechanisms of iron's effect on these cellular processes remain to be determined.

Most importantly, iron plays a crucial role in maintaining cellular iron homeostasis by regulating gene expression at the posttranscriptional level (Haile, 1999). In mammalian cells, synthesis of many of the key molecules involved in iron metabolism is tightly controlled by intracellular iron levels. Iron binds directly to iron regulatory proteins and interferes with binding of iron regulatory proteins to iron responsive elements, which in turn, determines the expression levels of iron responsive element-containing genes. Molecular mechanisms of how iron interacts with iron regulatory proteins and how iron regulatory proteins affect the expression of iron responsive element-containing genes are discussed in Section 2. At least five genes with major functions in iron metabolism contain iron responsive elements that are located in the untranslated regions of their mRNAs (Table 1). Expression of these genes is regulated by the levels of iron in cells (Haile, 1999; Kuhn, 1996). As a

Table 1
Iron responsive element-containing genes in humans^a

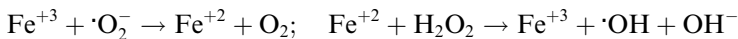
Gene ^b	IRE location	Function
Ferritin light chain	5'	Iron store
Ferritin heavy chain	5'	Iron store
Ferroportin1	5'	Iron exporter
TfR1	3'	Transferrin-binding and -transport molecule
Nramp2	3'	Iron importer
5'-aminolevulinate synthase	5'	Erythroid-specific key enzyme in heme synthesis
Aconitase	5'	Enzyme in citric acid cycle
K–Cl co-transporter	3'	Erythroid-specific K–Cl co-transporter
Hexokinase III	5'	Iron transduction signalling pathway?
Aminopeptidase A	3'	Degradation of iron-related proteins?
Uracil DNA Glycosylase	5'	DNA synthesis
MRP	5'	Multiple drug transporter
Acid phosphatase	5'	Iron transduction signalling pathway?

^a Abbreviations used are: IRE – iron responsive element; MRP – multi-drug resistance-associated protein; Nramp2 – natural resistance-associated macrophage protein 2; TfR1 – transferrin receptor 1.

^b Iron responsive element-containing genes with known or potential functions in iron metabolism are listed. By bioinformatics searches using several public and private sequence databases, at least ~70 novel genes in human genome has been identified to contain at least one iron responsive element at their 5' or 3' untranslated regions.

result of changes in the expression levels of these genes, the uptake, transport, storage, and utilization of intracellular iron are altered accordingly.

The biological importance of iron is in its chemistry. Existing in both ferrous (Fe^{+2}) and ferric (Fe^{+3}) oxidation state, iron is capable of accepting and donating electrons. Iron participates in the oxidation–reduction reaction known as the Fenton reaction (Wessling-Resnick, 1999):



These redox actions are essential for the biochemical functions of iron that participate in many diverse cellular metabolic processes. Iron levels must be well maintained within cells, because excess iron is highly toxic. The deleterious effect of iron is related to its ability to generate reactive oxygen species via the Fenton reaction (McCord, 1998). Highly reactive oxygen species, such as hydroxyl (OH^-) and superoxide radicals ($\cdot\text{O}_2^-$), are highly toxic, owing to their ability to react rapidly with high affinity with almost every molecule found in living cells (McCord, 1998). The net effects are DNA damage; impaired synthesis of proteins, membrane lipids, and carbohydrates; induction of proteases; and altered cell proliferation (Halliwell, 1992; Halliwell and Gutteridge, 1992; McCord, 1998; Schaich, 1992; Smith et al., 1992). In addition, free iron can react directly with unsaturated fatty acids and induce lipid hydroperoxides to form alkoxyl and/or peroxy radicals and, in turn, severely impair cellular integrity and cause cell death (Gerlach et al., 1994; McCord, 1998). This destructive potential of iron has led to the suggestion that iron might play a role in the multi-step processes of carcinogenesis; pathogenesis of atherosclerosis; or neurodegenerative disorders, such as Parkinson's or Alzheimer's diseases (Connor et al.,

1992; Dexter et al., 1991; Jenner, 1991; Sofic et al., 1988; Youdim, 1988). To minimize these potential toxic effects, highly sophisticated mechanisms and specialized molecules for the acquisition, transport, and storage of iron in soluble, non-toxic forms have evolved to meet cellular iron requirements and to control the body's iron homeostasis.

Physiological iron loss from the body includes excretion of iron in bile, urine iron, and the daily loss of cells from the skin and gut, which represents about 1 mg/day in the adult human (Andrews, 1999a,b; Andrews et al., 1999). Because of menses and childbirth, women usually lose additional iron from hemoglobins that contain high concentrations of iron. In healthy adults, iron losses due to excretion are balanced by absorption of sufficient dietary iron, from 1 to 2 mg daily, to maintain a relatively constant amount of body iron throughout life. Though iron excretion is important in maintaining iron balance, the absorptive process in the proximal small intestine plays a more active role in regulating iron homeostasis.

2. Regulation of iron uptake and storage

Since iron is required for a number of diverse cellular functions, a constant balance between iron uptake, transport, storage, and utilization is required to maintain iron homeostasis. Due to the fact that excessive iron can lead to the formation of reactive radicals and cause cell damage, iron imbalance can contribute to the development of numerous iron disorders, neurodegenerative diseases, and possibly some cancers (Smith et al., 1992; Sussman, 1992). In mammalian cells, the expression of many key molecules that participate in iron metabolism is regulated by the levels of intracellular iron via a feedback regulatory mechanism that entails specific mRNA-protein interactions in the cytoplasm (Haile, 1999; Kuhn, 1996). These interactions feature iron regulatory proteins 1 and 2, whose expression and function are modulated by intracellular iron. In iron deficiency conditions, iron regulatory proteins bind mRNA that contains stem-loop structures, termed iron responsive elements, with high affinities and specificities. By increasing mRNA stability or inhibiting translation of targeted iron responsive element-containing genes, iron regulatory proteins control expression of target genes post-transcriptionally. This iron-mediated feedback regulatory mechanism maintains an appropriate level of intracellular iron that is critical for numerous cellular processes.

2.1. Iron-mediated feedback regulation

The stem-loop structure of iron responsive elements consists of a terminal hexanucleotide loop region and a base-paired stem structure that are interrupted by a conserved unpaired cytosine nucleotide (Fig. 1) (Haile, 1999; Mikulits et al., 1999). Mutations, deletions, or insertions of nucleotides in the hexanucleotide loop as well as in the position of the bulged cytosine nucleotide significantly alter the binding affinity of iron regulatory proteins to iron responsive elements (Theil et al., 1994, 1999). Thus, both sequences and structures of iron responsive elements dictate the

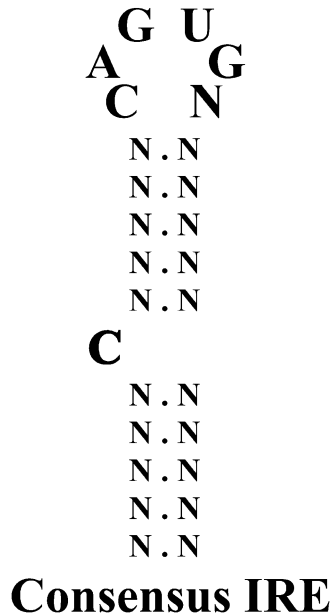


Fig. 1. Sequence and structure of a consensus iron responsive element. The stem loop structure of iron responsive element (IRE) is consisted of a terminal hexa-nucleotide loop region and a base-paired stem structure that is interrupted by a conserved un-paired cytosine nucleotide. Iron regulatory proteins bind iron responsive elements with varied affinities and selectivities, depending on the specific sequence of the iron responsive element.

binding affinity of iron regulatory proteins to iron responsive elements (Kim et al., 1996; Rouault et al., 1990; Samaniego et al., 1994).

Iron responsive elements are present in the 5' untranslated region of many genes, including mammalian iron store proteins, *ferritin* heavy and light chains, iron exporter *ferroportin1*, erythroid-specific *5-aminolaevulinate synthase* (Cox et al., 1991; Harigae et al., 1999), mitochondrial *aconitase* (Gray and Hentze, 1994; Kim et al., 1996), and *Drosophila melanogaster succinate dehydrogenase* subunit β (Kohler et al., 1995). On the other hand, *transferrin receptor 1* and *Nramp2* mRNAs (Theil, 1998) have iron responsive elements in their 3' untranslated regions (Table 1). Depending on the locations of iron responsive elements present in the mRNA, iron responsive elements act as repressors or enhancers of translation (Ponka et al., 1998; Theil, 1994). Binding of iron regulatory proteins to iron responsive elements either sterically prevents the recruitment of 43S translation preinitiation complexes, thereby repressing initiation of protein synthesis, or increases mRNA stability, thereby increasing protein synthesis (Fig. 2) (Gray and Hentze, 1994; Meleforts and Hentze, 1993; Muckenthaler et al., 1998a). For example, under conditions of iron deficiency, binding of iron regulatory proteins to iron responsive elements in the 5' untranslated region of *ferritin* mRNA blocks translation of ferritin (Rouault et al., 1990). Concurrently, binding of iron regulatory proteins to iron responsive elements in the 3'

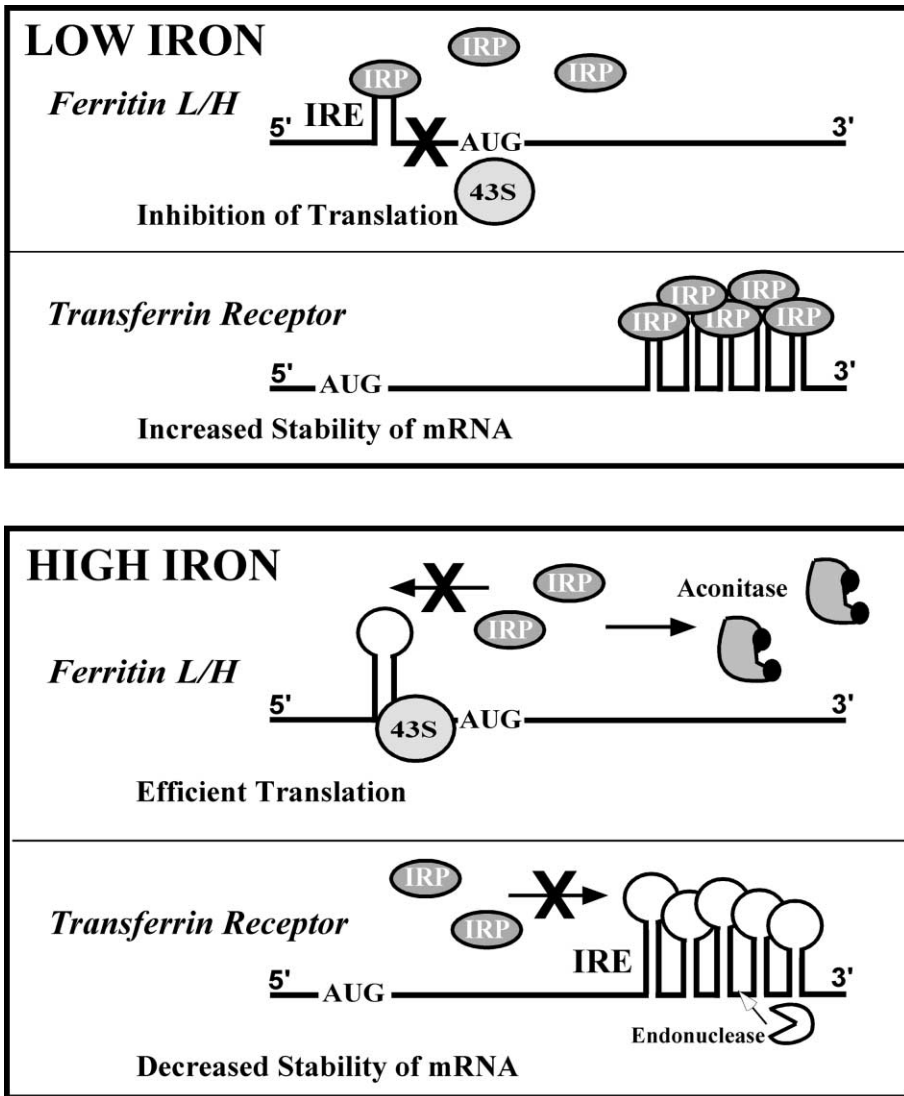


Fig. 2. Post-transcriptional regulation of iron responsive element-containing gene expression. Intracellular iron concentration determines binding of iron regulatory proteins (IRP) to iron responsive elements (IRE). Binding affinity of iron regulatory proteins to iron responsive elements inversely correlates with intracellular iron concentration. For example, a single iron responsive element is located in the 5' untranslated region of *ferritin* mRNA, whereas five iron responsive elements are present in the 3' untranslated region of *transferrin receptor 1* mRNA. Under conditions of iron deficiency, iron regulatory proteins bind to the iron responsive element in the 5' untranslated region of *ferritin* mRNA. This binding sterically prevents the recruitment of 43S translation pre-initiation complex and arrests translation of ferritin. On the other hand, binding of iron regulatory proteins to the iron responsive elements in the 3' untranslated region of transferrin receptor 1 mRNA increases mRNA stability and synthesis of transferrin receptor 1. In contrast, under high concentration of intracellular iron, iron regulatory proteins possess aconitase activity but lack RNA-binding activity. In the absence of binding of iron regulatory proteins to iron responsive elements, *ferritin* mRNA is translated efficiently, while *transferrin receptor 1* mRNA is rapidly degraded, probably by iron responsive element-specific endonucleases.

untranslated region of *transferrin receptor 1* mRNA results in increased stability of *transferrin receptor 1* mRNA and, thereby, increased expression of transferrin receptor 1 at the cell surface (Casey et al., 1989; Mullner and Kuhn, 1988). On the other hand, a high concentration of intracellular iron inhibits binding of iron regulatory proteins to iron responsive elements, resulting in efficient translation of ferritin and a decrease in the stability of *transferrin receptor 1* mRNA (Muckenthaler et al., 1998a,b). Hence, the binding of iron regulatory proteins to iron responsive elements has opposite effects on the expression of ferritin and transferrin receptor 1. Conceivably, expression of *Nramp2*, *ferroportin1*, and other iron responsive element-containing genes are regulated in a similar manner. The tight regulation of this process is necessary to maintain a balance between iron uptake, transport, storage, and utilization in response to cellular metabolic needs.

2.2. *The iron responsive element-binding proteins: iron regulatory proteins*

Iron regulatory proteins 1 and 2 bind directly to iron responsive element-containing RNA (Rouault et al., 1990). Iron regulatory protein 1 is present in all tissues with high expression levels in liver, kidneys, and intestine (Guo et al., 1994; Henderson et al., 1993). Sequence analysis of iron regulatory protein 1 reveals that it is identical to cytoplasmic aconitases and has a high degree of homology to other mitochondrial aconitases (Hentze, 1994; Kaptain et al., 1991; Rouault et al., 1991). Aconitase is an enzyme that functions in the citric acid cycle and catalyzes the conversion of citrate to isocitrate. Purified iron regulatory protein 1 has aconitase activity with a specific activity similar to that of mitochondrial aconitase (Haile, 1999). Under conditions of normal iron supplies, iron regulatory protein 1 contains an iron-sulfur cluster bound to three cysteine residues (Emery-Goodman et al., 1993; Kaptain et al., 1991). This form of iron regulatory protein 1 possesses aconitase activity but lacks RNA-binding activity. Under conditions of iron deficiency, iron regulatory protein 1 cannot mature to function as the holoprotein of aconitase and instead an apoprotein that lacks an iron-sulfur cluster, accumulates in the cell (Basilion et al., 1994; Emery-Goodman et al., 1993). This apoprotein form of iron regulatory protein 1 does not have aconitase activity but is capable of binding iron responsive elements with high affinity (Haile et al., 1992; Rouault et al., 1992). Therefore, the iron responsive element-binding activity and the aconitase activity of iron regulatory protein 1 are reciprocally regulated, depending on the presence or absence of the iron-sulfur cluster.

A second iron responsive element-binding protein, termed iron regulatory protein 2, has greater than 50% amino acid homology with iron regulatory protein 1 and binds to iron responsive elements with similar affinity (Guo et al., 1994; Henderson et al., 1993). Iron regulatory protein 2 is expressed in all tissues but in most cells has less abundant expression than iron regulatory protein 1 (Guo et al., 1994; Henderson et al., 1993). In vivo, under conditions of iron deficiency, the iron responsive element-binding activity of iron regulatory protein 2 is increased in a manner similar to iron regulatory protein 1. Whereas, binding of iron regulatory protein 2 to iron responsive elements is decreased when intracellular iron is in excess (Guo et al., 1994;

Samaniego et al., 1994). In vitro, iron regulatory protein 2 is able to inhibit ferritin translation (Pantopoulos et al., 1995). In general, binding of iron regulatory protein 2 to iron responsive elements in response to changes in cellular iron levels is similar to the characteristics of iron regulatory protein 1.

However, the mechanisms responsible for the changes in iron responsive element-binding activity between iron regulatory protein 1 and iron regulatory protein 2 are distinct. The main difference is that iron regulatory protein 2 is degraded rapidly in high iron conditions (Guo et al., 1995; Pantopoulos et al., 1995). Though the mechanism for the degradation of iron regulatory protein 2 is unclear, a unique region of 70 amino acid residues in iron regulatory protein 2 is responsible for its degradation in response to iron (DeRusso et al., 1995). Degradation of iron regulatory protein 2 is inhibited by specific proteasome inhibitors, indicating that ubiquitination and degradation by the proteasome (Yang et al., 1992) are involved in this regulation (DeRusso et al., 1995; Guo et al., 1995). Although iron regulatory proteins 1 and 2 bind the typical iron responsive elements with similar affinities, their binding specificities to distinct sequences of iron responsive elements differ significantly (Guo et al., 1994; Henderson et al., 1993). For example, unlike iron regulatory protein 1, iron regulatory protein 2 is able to bind the iron responsive elements in transferrin receptor 1 during rat liver regeneration (Cairo and Pietrangelo, 1994). In addition, iron regulatory protein 2 is missing some of the amino acid residues that are critical for the aconitase activity of iron regulatory protein 1. As a result, iron regulatory protein 2 lacks the aconitase activity found in iron regulatory protein 1 (Phillips et al., 1996; Samaniego et al., 1994). Even though iron regulatory protein 2 contains a number of conserved cysteine residues that are required for the formation of the iron–sulfur cluster, its RNA binding activity has not been reported to be regulated by iron–sulfur clusters (Guo et al., 1994). Together with the fact that responses of the iron regulatory proteins to reactive oxygen species are different for each, these differences between iron regulatory proteins 1 and 2 suggest that in response to cellular iron status, they perform distinct functions, probably by acting on different target genes. However, iron regulatory protein 1-deficient mice show no abnormalities in iron metabolism (Rouault and Klausner, 1997). It seems likely that iron regulatory protein 2 is able to compensate for a deficiency in iron regulatory protein 1 function.

2.3. Regulation of iron regulatory proteins by oxidative stress

A distinct role in modulating the function of iron regulatory proteins by iron-independent pathways has been reported for reactive oxygen intermediates, such as nitric oxide and hydrogen peroxide (Domachowske, 1997; Drapier et al., 1993). Induction of nitric oxide synthesis by interferon or bacterial endotoxin in macrophages results in an increase in the binding of iron regulatory protein 1 to iron responsive elements and a functional loss of cytoplasmic aconitase activity (Henry et al., 1993; Lancaster and Hibbs, 1990). In addition, induction of nitric oxide by the gene coding for the inducible form of nitric oxide synthase or the treatment of cells with nitric oxide-releasing compounds results in activating the binding of iron

responsive elements by iron regulatory proteins 1 and 2 (Drapier et al., 1993; Pantopoulos and Hentze, 1995b). As a result of activation of iron regulatory protein 1 binding by nitric oxide, expression of ferritin or transferrin receptor 1 is reduced or increased, respectively, leading to increased intracellular iron availability (Pantopoulos and Hentze, 1995a; Phillips et al., 1996). Thus, nitric oxide seems to regulate the binding of iron regulatory protein 1 by disassembling iron–sulfur clusters or by acting as a cytoplasmic iron chelator that results in a loss of aconitase activity (Drapier et al., 1993; Gardner et al., 1995).

Hydrogen peroxide also activates iron regulatory protein 1 and induces its binding to iron responsive elements, thereby resulting in a decrease or an increase in the expression levels of ferritin or transferrin receptor 1, respectively (Martins et al., 1995; Pantopoulos and Hentze, 1995b). Activation of iron regulatory protein 1 by hydrogen peroxide is more rapid than activation by nitric oxide and can be blocked by okadaic acid, a type I/IIa protein phosphatase inhibitor, suggesting an involvement of protein phosphatase (Pantopoulos and Hentze, 1995b). Treatment of cells with phorbol-12-myristate-13-acetate results in phosphorylation and binding of iron regulatory protein 1 to iron responsive elements. Because iron regulatory protein 1 is a substrate for phosphorylation at serine residues, phosphorylation might influence its RNA binding activity (Eisenstein and Blemings, 1998). These findings suggest that specific kinase and/or phosphatase signal transduction pathways might be involved in the iron regulatory protein-mediated iron homeostasis (Pantopoulos and Hentze, 1995a). In contrast to nitric oxide, hydrogen peroxide has no effect on the activation of iron regulatory protein 2 (Pantopoulos and Hentze, 1995b). The molecular mechanisms of how these reactive oxygen intermediates affect the binding activity of iron regulatory proteins to iron responsive elements and the functional link between iron metabolism and reactive oxygen intermediates are still not entirely understood.

2.4. *The iron store proteins: ferritin heavy and light chains*

In most cells, the source of stored intracellular iron is ferritin, an important detoxification machinery that prevents free iron from forming reactive oxygen species. Ferritin exists in two subunits, heavy and light chains that form a protein shell that can store up to 4500 molecules of iron (Theil, 1998). Synthesis of ferritin is induced when iron is available, whereas in conditions of iron deficiency, ferritin synthesis is repressed (Gdaniec et al., 1998; Ke et al., 1998; Zahringer et al., 1976). Sequence analysis reveals that the 5' untranslated regions of the heavy and light chains of ferritin mRNAs are necessary and sufficient to permit this iron-mediated regulation (Aziz and Munro, 1987). The regulatory RNA sequences that determine the fate of the mRNAs for the heavy and light chains of ferritin are iron responsive elements (Casey et al., 1988). In addition, a cis-acting element, termed the acute box, is present in the 5' untranslated region downstream of the iron responsive elements of the heavy and light chains of *ferritin* mRNAs. The acute box regulates ferritin synthesis independently of iron regulatory protein-mediated pathways (Rogers et al., 1994). A trans-acting RNA binding protein in hepatic cells is known to bind to the

acute box (Thomson et al., 1999). Mutations of this acute box appear to alter binding affinity of the RNA binding protein to the acute box and interferes with formation of the preinitiation complex, hence reduces the level of ferritin synthesis (Rouault et al., 1987). Therefore, regulation of ferritin synthesis is likely to involve at least two cis-acting mRNA elements and two sets of RNA binding proteins that act together or independently in response to different stimuli. Interestingly, translation of ferritin in hepatic and endothelial cells is also mediated by interleukin 1 (Rogers et al., 1994), which is the major inflammatory cytokine that lowers levels of blood serum iron by increasing ferritin synthesis (Gordeuk et al., 1992a,b). A clinical association between serum iron levels, serum ferritin levels, intracellular ferritin, and cytokine-induced inflammation has been established (Rogers et al., 1994). Elevated serum ferritin levels commonly serve as a diagnostic indicator for detecting inflammation and other diseases (Thomson et al., 1999).

2.5. Iron regulatory proteins and neurological diseases

Iron plays an important role in the biosynthesis of neurotransmitters and myelin in the brain (Beard et al., 1993a,b; Hill et al., 1985). Increased iron levels have been reported in many common neurological diseases, including Alzheimer's disease and Parkinson's disease (Beard et al., 1993a,b; Dexter et al., 1991; Riederer et al., 1989). In patients with Alzheimer's disease, iron accumulates in certain regions of the brain, such as the cerebral cortex and hippocampus, without a concomitant increase in ferritin (Beard et al., 1993a,b; Riederer et al., 1988). Interactions between iron regulatory proteins and iron responsive elements differ significantly in the brains between Alzheimer's disease patients and normal controls (Pinero et al., 2000a,b). In the patients, the complexes of iron responsive elements and iron regulatory proteins are more stable than complexes in the normal brain (Pinero et al., 2000a,b). As a consequence of this altered stability, cellular iron uptake might be excessively increased without increasing ferritin synthesis in the brain of Alzheimer's patients (Pinero et al., 2000a,b). Interestingly, expression patterns of iron regulatory protein 2 is strikingly different between Alzheimer's disease-afflicted brains and normal brains (Bouton et al., 1998). Iron regulatory protein 2 is mainly expressed in intraneuronal lesions, neurofibrillary tangles, and senile plaques, consistent with the histopathological changes that occur in Alzheimer's disease patients. It is conceivable that iron regulatory proteins play a pathophysiological role in the development of a number of neurological diseases, including Alzheimer's disease.

2.6. Iron responsive element-associated diseases

Patients with the hereditary hyperferritinemia–cataract syndrome, an autosomal dominant disorder, suffer from early onset bilateral cataracts and eventually become visually impaired. In these patients, a single point mutation in the light chain of the *ferritin* gene on chromosome 19q13.1 has been identified and characterized (Beaumont et al., 1995). Because this single nucleotide A to G change takes place in the highly conserved hexa-nucleotide loop region of the iron responsive element in the

ferritin light chain, the affinity of iron regulatory proteins for this mutant iron responsive element is dramatically reduced. This reduced affinity results in overproduction of the ferritin light chain under conditions of iron deficiency (Beaumont et al., 1995; Bonneau et al., 1995). High levels of serum ferritin are evident in patients with hereditary hyperferritinemia-cataract syndrome, however, they have normal serum iron levels and transferrin saturation (Dandekar and Hentze, 1995). Other mutations in the iron responsive elements of the ferritin light chain also affect binding of iron regulatory proteins to a certain extent, leading to variable degrees of hyperferritinemia, irrespective of cellular iron status (Cazzola et al., 1997; Mumford et al., 1998).

3. Dietary iron absorption

The intestine is the major site of iron regulation in controlling the uptake of dietary iron across the brush border and the release of absorbed iron across the basolateral membrane to the circulation (Fig. 3). In the intestinal lumen, iron exists in the forms of ferrous and ferric iron salts. Because ferric iron becomes insoluble at pH values above 3, ferric irons must be reduced or chelated by amino acids or sugars to be efficiently absorbed (Conrad et al., 1999). Most ferrous iron remains soluble even at pH 7 (Conrad et al., 1999); hence, absorption of ferrous iron salts is more efficient than absorption of ferric iron salts (Conrad et al., 1966). However, most dietary inorganic iron is in the form of ferric iron (Conrad et al., 1999). Reduction of ferric irons becomes necessary for efficient dietary iron absorption and is mediated by a mucosal ferrireductase that is present in the intestines (Ekmekcioglu et al., 1996; Riedel et al., 1995). Inhibition of ferrireductase activity in intestinal cells reduces iron absorption (Han et al., 1995; Nunez et al., 1994), demonstrating the importance of ferric iron reduction in dietary iron import. Alternatively, uptake of ferric irons might be mediated by the para-ferritin pathway, though less efficiently.

3.1. Non-heme iron uptake

Nutritional absorption of both heme and non-heme iron occurs predominantly in the proximal small intestine, specifically in the crypt cells of the duodenum and jejunum (Conrad et al., 1987; Wood and Han, 1998). Enterocytes located on the intestinal villus are highly specialized, polarized absorptive cells that control the passage of dietary iron in the lumen of the gut and the transfer of iron into the body's circulation (Wood and Han, 1998). To enter the body's circulation, dietary iron must cross three cellular barriers: iron absorption across the apical membrane, intracellular iron translocation across the cytosol, and iron export across the basolateral membrane and into the circulation. Unlike other nucleated cells in the body, the luminal surface of absorptive enterocytes contain no transferrin receptors (Pietrangolo et al., 1992). Thus, iron must enter these cells via a mechanism that is different from the classical transferrin–transferrin receptor pathway. Absorption of iron across the apical membrane of the enterocytes is mediated by a divalent cation transporter, termed Nramp2 (or DCT1 or DMT1) (Fig. 3) (Fleming et al., 1997;

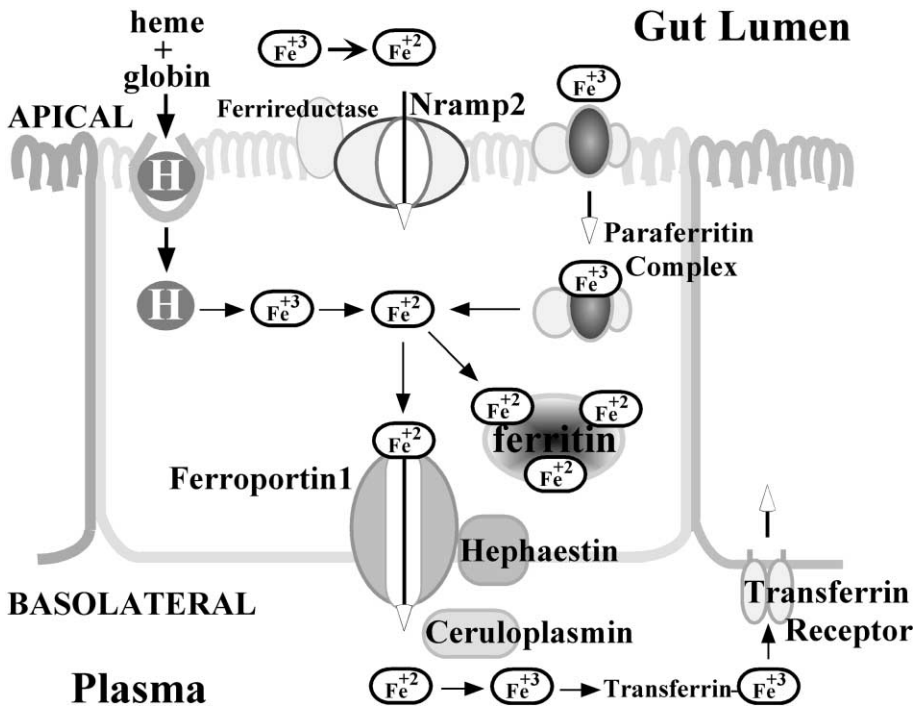


Fig. 3. Pathways of heme and non-heme iron uptake and transport in the intestine. Most dietary iron in the ferric state is reduced to ferrous iron (Fe^{+2}) or solubilized by mucin, ascorbate, or other reducing agents. Ferric iron (Fe^{+3}) in the gut lumen is reduced by ferrireductase or in the cytoplasm, by flavin mono-oxygenase. Majority of ferrous iron is transported into the cell by an apical cell surface iron transporter, Nrap2. Ferric and ferrous iron can also enter absorptive enterocytes via a pathway mediated by paraferitin complex, consisting of β integrin, mobilferrin, and flavin mono-oxygenase. Heme is processed in the gut lumen and enters enterocytes as an intact metalloporphyrin (H). Mechanism of heme iron transport is not well defined, probably mediated by an endocytic pathway. Once inside the cytoplasm, heme is degraded by heme oxygenase to release inorganic iron. Intracellular iron is either stored as ferritin or transported across the basolateral membrane by ferroportin1 into plasma. Hephaestin appears to facilitate iron export in concert with ferroportin1. Ferroportin1 might also load iron onto transferrin with assistance from a soluble plasma ferroxidase, ceruloplasmin.

Gunshin et al., 1997). The name Nrap is abbreviated from the phrase “natural resistance-associated macrophage protein”, because Nrap2 is highly homologous to Nrap1, a molecule that plays an important role in host defense against pathogen infection (Gruenheid et al., 1995; Vidal et al., 1993). Nrap2 is most likely to be responsible for iron transport from the duodenum lumen into the cytoplasm of epithelial cells.

3.2. The iron importer: Nrap2

The *Nrap2* gene in humans encompasses more than 36 Kb (Lee et al., 1998) and encodes at least two different spliced forms of mRNA (Fleming et al., 1998; Lee et al.,

1998). The 3' untranslated region in the *Nramp2* isoform I contains an iron responsive element, which is similar to the iron responsive elements present in the 3' untranslated region of the mRNA of *transferrin receptor 1* (Fleming et al., 1998; Tandy et al., 2000). In contrast, the *Nramp2* isoform II lacks the iron responsive element. Consequently, expression of *Nramp2* isoform I is up-regulated in iron-deficient animals and human intestinal cells, whereas expression of *Nramp2* isoform II is not (Fleet, 1998; Fleming et al., 1999). The presence of a functional iron responsive element in the *Nramp2* isoform I suggests that its expression, unlike the expression of isoform II, is controlled post-transcriptionally by intracellular iron concentration.

Nramp2 is expressed in many different tissues, with high expression levels at the duodenum brush border, which is consistent with its role in intestinal iron absorption (Cannon-Hergaux et al., 1999; Gruenheid et al., 1995; Gunshin et al., 1997). At subcellular levels, *Nramp2* is located on the plasma membrane as well as on subcellular vesicular compartments characterized as late endosomes or lysosomes (Gruenheid et al., 1999; Su et al., 1998; Tabuchi et al., 2000). The *Nramp2* protein is predicted to consist of 12 transmembrane domains with potential glycosylation sites between transmembrane domains 7 and 8 (Andrews, 1999a,b). Functional studies demonstrate that *Nramp2* acts as a proton-coupled divalent cation transporter (Gunshin et al., 1997). *Nramp2* is capable of transporting not only ferrous iron, but also a broad range of divalent cations: Zn^{+2} , Mn^{+2} , Co^{+2} , Cd^{+2} , Cu^{+2} , Ni^{+2} , and Pb^{+2} (Gunshin et al., 1997). In addition, *Nramp2* function is pH dependent, optimal at low pH (pH < 6) (Gunshin et al., 1997). Microcytic anemic *mk/mk* mice and Belgrade (*blb*) rats are defective in intestinal iron absorption and hemoglobin production, resulting from a missense mutation (G185R) in transmembrane domain 4 of *Nramp2* that interferes with *Nramp2* function (Fleming et al., 1997, 1998; Gunshin et al., 1997). In addition, Belgrade (*blb*) rats have a defect in iron transport within endocytic vesicles in reticulocytes (Bowen and Morgan, 1987; Edwards et al., 1986). Because *Nramp2* is subcellularly colocalized with transferrin, a role for *Nramp2* in transporting transferrin-bound iron across the membrane of endosomes into the cytoplasm is suggested (Gruenheid et al., 1999; Su et al., 1998).

3.3. Heme iron uptake

Hemoglobin iron from food is absorbed more efficiently than inorganic iron (Majuri and Grasbeck, 1987; Parmley et al., 1981a,b). The absorption of iron from myoglobin and hemoglobin differs from that of inorganic iron (Conrad et al., 1999). Initially, hemoglobin iron is enzymatically digested in the intestinal lumen, and the heme molecule enters the absorptive cell as an intact metalloporphyrin (Majuri and Grasbeck, 1987; Parmley et al., 1981a,b). It has been suggested that heme molecule enters the cell via a heme receptor-mediated internalization process (Mills and Payne, 1995). Within the enterocyte, heme is degraded by heme oxygenase and releases inorganic iron, which is either stored as ferritin or transported across the basolateral membrane to enter the body's circulation (Fig. 3). As the enterocyte completes its life cycle, iron that remains in the form of ferritin will be sloughed with the senescent cells and will leave the body through the gastrointestinal tract. Since

humans have a limited means of eliminating iron, this process represents an important mechanism of iron loss.

3.4. *Paraferritin-mediated iron uptake*

Although ferrous iron is more efficiently transported by Nramp2, ferrous and ferric iron can also enter enterocytes via different pathways (Conrad et al., 1999; Umbreit et al., 1998). A 520-kDa membrane complex called paraferritin, which contains β -integrin, mobilferrin (a calreticulin homologue), and flavin mono-oxygenase, participates in the mucin-mediated iron uptake in the gut lumen (Fig. 3) (Umbreit et al., 1998). Factors bound to mobilferrin interact with integrins at the surface of erythroleukemia cells (Conrad et al., 1994a,b). Treatment of erythroleukemia cells with an anti- β_2 -integrin monoclonal antibody blocks 90% of ferric citrate uptake but has little effect on the uptake of ferrous iron (Conrad et al., 1999). Thus, ferric iron is seemingly absorbed via the paraferritin-mediated pathway. Although the exact mechanism is still undefined, it is postulated that ferric iron is solubilized by mucin in the gut lumen, transferred to the mobilferrin- and β -integrin-containing paraferritin complexes, and then internalized (Conrad et al., 1999). Following internalization, flavin mono-oxygenase becomes associated with the complexes, and ferric iron is reduced to ferrous iron in concert with the activity of NADPH. Interestingly, the β -integrin- and mobilferrin-containing paraferritin complex also interacts with β_2 microglobulin. Mobilferrin and β_2 microglobulin have been demonstrated to play critical roles in the development of iron overload in hemochromatosis animals (Rothenberg and Volland, 1996; Sadasivan et al., 1996).

3.5. *The iron exporter: ferroportin1*

By positional gene cloning, a novel iron transporter gene named *ferroportin1*, which is responsible for hypochromic anemia in zebrafish (Donovan et al., 2000), has recently been identified. Sequence analysis of both mouse and human *ferroportin1* reveals the presence of a stem-loop structure, typical of iron responsive elements, in the 5' untranslated region of *ferroportin1* (Donovan et al., 2000; McKie et al., 2000). Binding of this iron responsive element to iron regulatory proteins 1 and 2 has been demonstrated (McKie et al., 2000), indicating that expression of ferroportin1 is regulated by intracellular iron levels. The human ferroportin1 has a predicted open reading frame of 562 amino acids (Donovan et al., 2000). Functional studies of ferroportin1 demonstrate that ferroportin1 mediates iron efflux across membranes by a mechanism that requires an auxiliary ferroxidase activity (Donovan et al., 2000; McKie et al., 2000). Ferroportin1 is expressed highly in the placenta, liver, spleen, macrophages, and kidneys (Donovan et al., 2000). Subcellularly, ferroportin1 is located on the basolateral membrane of duodenal enterocytes (McKie et al., 2000), strongly suggesting that ferroportin1 is likely to function as an iron exporter in the enterocytes (Fig. 3). Because ferroportin1 is located on the basal surface of placental syncytiotrophoblasts, a role for ferroportin1 in iron transport into the embryonic circulation is also suggested (Donovan et al., 2000).

Ferroportin1 is thought to function in concert with the membrane-resident ferroxidase hephaestin and serum ceruloplasmin (McKie et al., 2000). Hephaestin is highly similar to ceruloplasmin, which is a multi-copper oxidase with ferroxidase activity that is required for the release of iron into blood and the binding to transferrin (Harris et al., 1998). Like ceruloplasmin, hephaestin is not a transporter, but it facilitates the transport of iron from enterocyte into the body's circulation (Harris et al., 1998; McKie et al., 2000). In mice with sex-linked anemia, hephaestin is defective (Vulpe et al., 1999). The sex-linked anemic mice display normal dietary iron absorption into the enterocytes but suffer from a defect in transport of iron from duodenum to the blood (Vulpe et al., 1999). As a consequence of the defective hephaestin, iron export from enterocytes to the circulation is severely impaired, resulting in microcytic anemia in animals. The mechanisms by which ferroportin1 mediates the passage of iron across the basolateral membrane and by which it interacts with other factors, such as hephaestin and ceruloplasmin, remain to be defined.

3.6. Regulation of dietary iron absorption

Iron absorption by enterocytes in the lining of the gastroduodenal junction is regulated in several ways. First, it can be modulated by the amount of iron recently consumed in the diet, a mechanism referred to as the “dietary regulator” (Andrews, 1999a,b). For several days, after the consumption of dietary iron, absorptive enterocytes are resistant to acquiring additional iron. This phenomenon is called “mucosal block” (Andrews, 1999a,b). This blocking action probably results from the accumulation of intracellular iron that has met the iron requirements of the body. A second regulatory mechanism senses the body-stored iron levels rather than the status of dietary iron. This mechanism is termed the “stores regulator” (Finch, 1994). The stores regulator can influence the amount of iron uptake by about two to three factors in iron-deficient conditions (Finch, 1994). It is likely that dietary iron absorption is indirectly influenced by the saturation of plasma transferrin with iron. However, the exact molecular details of the activity of the stores regulator are presently not known. The third regulatory mechanism, known as the “erythropoietic regulator”, has a greater capacity to increase iron absorption than the stores regulator. The erythropoietic regulator does not respond to the cellular iron levels at all (Finch, 1994), but rather modulates iron absorption in response to the requirements for erythropoiesis. It is possible that the erythropoietic regulator requires a soluble signal that is carried by plasma from the bone marrow to the intestine. Further studies are needed to expand our understanding of the molecular mechanisms of intestinal iron absorption.

4. Transferrin receptor-mediated iron uptake

Within the body, between sites of absorption, storage, and utilization, iron is transported in the plasma by a plasma protein, called transferrin, which has high

affinity for ferric iron. The majority of non-intestinal cells acquire iron from transferrin. Cellular iron uptake from transferrin first involves the binding of transferrin to transferrin receptors, which are the key surface receptors that mediate transferrin-bound iron uptake. Although transferrin receptors do not interact directly with iron, they control iron uptake and storage by most cells in the organism. There are at least two types of transferrin receptors, each has its own distinct cell- and tissue-specific expression pattern. Transferrin receptor 1 is a cell membrane glycoprotein that is expressed in all cells, except for mature erythrocytes. Transferrin receptor 2, a homologue of transferrin receptor 1, is specifically expressed in the liver, particularly in the hepatocytes. Following binding, complexes of transferrin receptor–transferrin–iron are internalized via the classical endocytic pathway, and iron is released from transferrin within the acidic endosomal compartments. Iron then passes through the endosomal membrane and enters the intracellular labile pool. Intracellular iron can then be utilized for the synthesis of heme- and non-heme-containing proteins or stored within ferritin, the iron store protein. The transferrin receptor-bound transferrin is recycled back to the cell surface for reuse, completing a cycle of highly specific and efficient cellular iron uptake.

4.1. The iron-binding and -transport protein: transferrin

Transferrin is one of the major serum proteins in eukaryotes and plays a critical role in binding and transporting iron (Hoefkens et al., 1996), hence reducing the toxic side effects of iron. Transferrin is a single polypeptide chain of 80-kDa glycoprotein, consisting of two globular domains. Each domain contains a high-affinity binding site for one iron molecule (Yang et al., 1984). Affinity of transferrin for iron is pH dependent, in which iron is released from transferrin as the pH is lowered below pH 6.5. In addition to iron, transferrin might be involved in the transport of a number of metals, such as aluminum, manganese (Davidsson et al., 1989a,b), copper, and cadmium (Moos et al., 2000). However, iron has the highest affinity to transferrin and will displace other metals.

Transferrin is synthesized primarily in the liver (Morgan, 1983) but significant amounts are also produced in the brain, testis, lactating mammary gland, and in some fetal tissues during development (Dickson et al., 1985; Takeda et al., 1998). Transferrin exists as a mixture of iron-free (apotransferrin), one iron (monoferric transferrin), and two irons (diferric transferrin) forms of the molecule. The relative abundance of each form depends on the concentration of iron and transferrin present in blood plasma. Under normal conditions, most of the iron molecules in blood plasma are bound to transferrin, and iron–transferrin complexes enter cells via a transferrin receptor-mediated endocytic pathway. The primary function of transferrin is to accept iron from plasma and to transport iron to various cells and tissues.

4.2. The transferrin-binding and -transport protein: transferrin receptor 1

Transferrin receptor 1 is a transmembrane homo-dimer that consists of two identical subunits. With a molecular weight of approximately 90 kDa, each mono-

mer is joined by two disulfide bonds at cysteines residue 89 and 98 (Jing and Trowbridge, 1987) and consists of three domains: a 61-residue amino-terminal domain, a 28-residue transmembrane region that helps to anchor the receptor into the membrane, and a large extracellular carboxyl-terminus of 671 amino acid residues (Fig. 4) (McClelland et al., 1984; Schneider et al., 1984). As a type II membrane protein, the carboxyl-terminal ectodomain of the transferrin receptor 1 is critical for transferrin binding. Indeed, replacement of the carboxyl-terminal, 192 amino acid

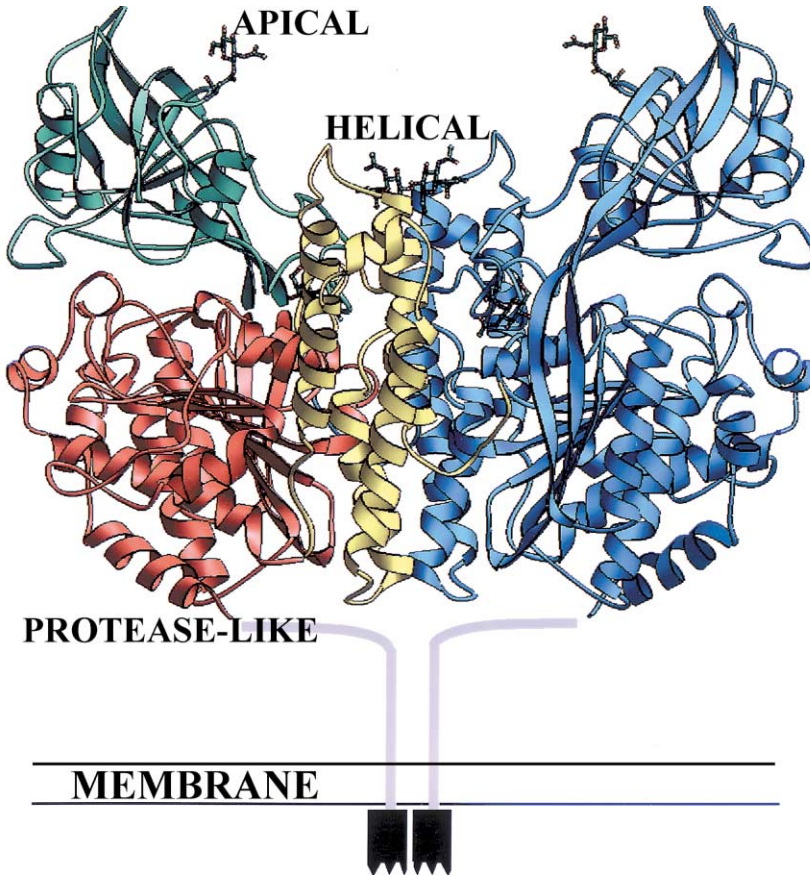


Fig. 4. Crystal structure of the transferrin receptor 1. A ribbon diagram of the dimeric ectodomains of the human transferrin receptor 1 reveals a three-domain subunit. The transferrin receptor 1 monomer contains three distinct domains, organized in a butterfly-like shape. The first, protease-like domain in red is closely related to carboxy- or amino-peptidases. The second, apical domain in green resembles a β sandwich in which the two sheets are splayed apart, with a helix running along the open edge, and is related to the domain 4 of aconitase. One principal function of the third, helical domain in yellow appears to be transferrin receptor dimerization. The monomer on the left is colored according to domain, and the other is blue. The cytoplasmic domain is black, and the stalk is purple. The stalk region is connected to the putative membrane-spanning helices (kindly provided by Dr. Stephen C. Harrison of Harvard Medical School, Massachusetts, USA).

residues of the human transferrin receptor 1 with the corresponding region of the chicken transferrin receptor, dramatically reduces or completely abolishes its binding affinity for transferrin (Buehgger et al., 1996). Because each ectodomain contains a binding site for the transferrin molecule, a homodimer of transferrin receptor 1 can bind up to two molecules of transferrin simultaneously.

Transferrin receptor 1 is synthesized in the endoplasmic reticulum and undergoes a number of post-translational modifications. The extracellular domain contains three N-linked glycosylation sites and one O-linked glycosylation site (Omary and Trowbridge, 1981). The N-linked glycosylation sites of transferrin receptor 1 are vital for its correct folding; mutations at these glycosylation sites impair transferrin-binding activity. Elimination of the O-linked glycosylation at threonine 104 enhances the cleavage of transferrin receptor 1 and promotes the release of its ectodomain (Rutledge and Enns, 1996; Williams and Enns, 1991). The transmembrane segment of transferrin receptor 1, which consists of 18 hydrophobic amino acids, is also subjected to post-translational modifications. The hydrophobic transmembrane segment is covalently bound to fatty acids and undergoes acylation with palmitate, which presumably helps to anchor the receptor to the plasma membrane (Omary and Trowbridge, 1981; Schneider et al., 1984).

The cytoplasmic portion of transferrin receptor 1 is required for the clustering of the receptor into the coated pits of plasma membrane and, subsequently, for endocytosis (Iacopetta et al., 1988; Rothenberger et al., 1987). Interestingly, the rate of endocytosis of the human transferrin receptor 1 is much higher than that of the hamster transferrin receptor (Alvarez et al., 1990). This difference is due to a single amino acid variation in the primary sequence of the cytoplasmic portions of the human and hamster transferrin receptor 1 (Alvarez et al., 1990). A tyrosine residue at position 20 is found in the human transferrin receptor 1, whereas a cysteine residue is in the hamster transferrin receptor. Replacement of the tyrosine with the cysteine residue results in a dramatic decrease in the rates of endocytosis and appears to account for the different rates of endocytosis between the human and hamster transferrin receptors 1 (Alvarez et al., 1990). Further functional characterization demonstrates that a conserved internalization signal (YTRF) within the 61 amino acid residues of the cytoplasmic portion of transferrin receptor 1 is critical for high-efficient endocytosis of the receptor (Collawn et al., 1990, 1993). Phosphorylation and dephosphorylation had been proposed as potential signals for internalization of transferrin receptor 1 (Salter-Cid et al., 2000a). Indeed, transferrin receptor 1 undergoes phosphorylation at the serine 24 residue (Kuhn, 1996; Schneider et al., 1982). Replacement of the serine 24 residue by an alanine in a mutant transferrin receptor 1 seemed not to affect receptor distribution (Zerial et al., 1987), nor was the internalization rate of the mutant transferrin receptor 1 significantly altered (Rothenberger et al., 1987). These results seemed to suggest that phosphorylation at Serine 24 residue did not affect the recycling kinetics of transferrin receptor 1. However, in cells treated with protein phosphatase inhibitors, the uptake of transferrin is inhibited by more than 85% (Beauchamp and Woodman, 1994). Protein phosphatase inhibitors (Beauchamp and Woodman, 1994) also affect recycling of transferrin to the cell surface from intracellular compartments. Because transferrin

receptor 1 contains several potential phosphorylation sites in its cytoplasmic portion in addition to the serine 24 residue, phosphorylation at other sites on transferrin receptor 1 might interfere with the function of transferrin receptor 1 (Beauchamp and Woodman, 1994). Indeed, our recent study demonstrates that phosphorylation of transferrin receptor 1 appears to interfere with the functions of transferrin receptor 1 in iron–transferrin binding, transferrin-receptor internalization, and probably, transferrin recycling (Salter-Cid et al., 2000a). Therefore, the functional significance of the phosphorylation of transferrin receptor 1 requires further investigation.

Crystallographic studies of human transferrin receptor 1 reveal that it is a tightly associated homo-dimer (Fig. 4). Each transferrin receptor 1 monomer consists of three distinct globular domains, organized in a butterfly-like shape (Lawrence et al., 1999). These domains, identified as the protease-like, apical, and helical domains, form a lateral cleft, which is most likely to be in contact with the docked transferrin molecule (Lawrence et al., 1999). The overall shape of the homo-dimer suggests that transferrin could bind to either side with no contact between the two transferrin molecules. The ectodomain of transferrin receptor 1 is separated from the membrane by a stalk, which presumably includes residues involved in disulfide bond formation and O-linked glycosylation (Fuchs et al., 1998). Amino acid sequences of all three of these globular ectodomains show significant similarity (approximately 28%) to the sequence of membrane glutamate carboxypeptidase II, which hydrolyzes N-acetyl-L-aspartyl-L-glutamate, the most prevalent mammalian neuropeptide (Lawrence et al., 1999). Because membrane glutamate carboxypeptidases are similar to aminopeptidases, transferrin receptor 1 might have evolved from a peptidase that is related to membrane glutamate carboxypeptidases (Bzdega et al., 1997). However, as revealed by its crystal structure, transferrin receptor 1 lacks peptidase activity, showing that the catalytic site is covered by the apical domain (Bzdega et al., 1997; Lawrence et al., 1999).

4.3. Expression of transferrin receptor 1

With exceptions of mature erythrocytes and other terminally differentiated cells, transferrin receptor 1 is expressed in all cells but differs in levels of expression (Davies et al., 1981; Enns et al., 1982). Transferrin receptor 1 is expressed on rapidly dividing cells, with 10,000 to 100,000 molecules per cell commonly found on tumor cells or cell lines in culture (Inoue et al., 1993). In contrast, in non-proliferating cells, expression of transferrin receptor 1 is low or frequently undetectable. A possible link between the high expression levels of transferrin receptors and cell proliferation is the enzyme, ribonucleotide reductase. By producing the four deoxyribonucleotides from the corresponding ribonucleotides (Jordan and Reichard, 1998), ribonucleotide reductase is the rate-limiting factor in DNA synthesis. Nevertheless, studies have not yet shown that this enzyme is the major factor in consuming high levels of intracellular iron in rapidly dividing cells.

A basal level of transferrin receptor 1 is expressed in epithelial cells of various organs, including tongue, esophagus, cervix, kidneys, stomach, endocrine pancreas,

hepatocytes, testes, pituitary gland, and breast (Gatter et al., 1983). Cells and tissues that express the highest levels of transferrin receptor 1 are immature erythrocytes, placental tissue, the liver, and rapidly dividing cells (Ponka, 1999). Erythroid cells require high amounts of iron for hemoglobin synthesis and for cellular division via a transferrin receptor-dependent pathway. Similarly, placental syncytiotrophoblasts require large amounts of iron for transporting to the developing fetus. Within the brain, transferrin receptor 1 is expressed in capillary endothelial cells (Kalaria et al., 1992), choroid plexus epithelial cells (Giometto et al., 1990), and neurons (Broadwell et al., 1996; Moos and Morgan, 2000). Expression levels of transferrin receptor 1 on brain cells change with the stages of development and iron status (Moos et al., 2000). For example, in capillary endothelial cells, the numbers of transferrin receptor 1 are greatest at the time of the most rapid brain growth, during replication of endothelial cells, as well as under conditions of iron deficiency (Taylor et al., 1991; Taylor and Morgan, 1991). Iron transport into the brain is regulated primarily by the expression of transferrin receptor 1 at the blood-brain and the blood-cerebral spinal fluid barriers (Moos, 1996). The transport of iron across these barriers is most likely the result of receptor-mediated endocytosis of transferrin-bound iron by capillary endothelial cells and choroid plexus epithelial cells (Moos et al., 2000). The function of transferrin receptor 1 is thought to reflect the need for iron in neurons with a high respiratory rate that coupled to the oxidative respiratory chain in the mitochondria (Morris et al., 1995; Morris and Edwardson, 1994).

Human transferrin receptor 1 is encoded by a single gene that extends over an entire 32-Kb region on chromosome 3 (Enns et al., 1982). Interestingly, chromosome 3 also encodes genes responsible for synthesis of plasma transferrin and a cell surface-resident transferrin-like molecule, paratransferrin (Brown et al., 1982). The *transferrin receptor 1* gene gives rise to a major 5-Kb mRNA species, containing an unusually large 3' untranslated region (Casey et al., 1989; Schneider et al., 1984). Nucleotide sequence analysis of the *transferrin receptor 1* gene reveals a moderate degree of similarity with the sequence of prostate-specific membrane antigen, a transmembrane glycoprotein that is expressed by normal and neoplastic prostate cells (Evans and Kemp, 1997; Israeli et al., 1993). Nonetheless, the biological significance between these homologies remains to be investigated.

Sequence analysis of the transferrin receptor 1 promoter reveals that a region of about 100 bp upstream from the transcriptional start site is required for basal and activated transcription in proliferating, non-erythroid cells (Miskimins et al., 1986). A number of regulatory elements have been identified, including the AP1/CRE-like (cyclic AMP-responsive element) element, SP1/GC-rich sequences, and the recently identified hypoxia-response element (Lok and Ponka, 1999; Tacchini et al., 1999). Mutational analysis demonstrates that the AP1-like sequence (GTGACGCA, -73 to -86) is critical for the promoter activity of transferrin receptor 1. The AP1/CREB-like factors and the Ku autoantigen, a DNA-binding protein that binds non-specifically to DNA ends, bind to the AP1 binding site of transferrin receptor 1 (Roberts and Griswold, 1990; Roberts et al., 1989). However, the functional roles of these factors in regulating expression of transferrin receptor 1 have not been demonstrated.

Expression of transferrin receptor 1 in non-erythroid cells is regulated at the post-transcriptional level by interactions of iron regulatory proteins and iron responsive elements in the 3' untranslated region of transferrin receptor 1 mRNA. Transferrin receptor 1 mRNA has an usually large 3' untranslated region of about 2500 nucleotides (Klausner et al., 1993; Theil et al., 1994), which contains five stem-loop structures of the iron responsive elements that are essential for iron-regulated mRNA degradation. Iron modulates the stability of mRNA by binding directly to iron regulatory proteins, which recognize and bind to iron responsive elements. Under low intracellular iron conditions, each of these iron responsive elements can be bound by one cytoplasmic iron regulatory protein, resulting in the stabilization of transferrin receptor 1 mRNA (Fig. 2) (Hentze and Kuhn, 1996; Klausner et al., 1993). Binding of iron regulatory proteins presumably blocks an endonucleolytic cleavage site found within the vicinity of the iron responsive elements from being recognized by an unidentified endonuclease (Kuhn, 1996; Thomson et al., 1999). As a result of stabilizing transferrin receptor 1 mRNAs, synthesis and cell surface expression of transferrin receptor 1 molecules are increased. The net effect is a higher level of iron uptake into the cell. On the other hand, when intracellular iron levels are high, iron regulatory proteins are unable to bind to iron responsive elements (Thomson et al., 1999). In the absence of binding, the endonuclease site is exposed and recognized by endonuclease, resulting in a decrease in the half-life of transferrin receptor 1 mRNAs (Hentze, 1996; Thomson et al., 1999). A decrease in transferrin receptors at the cell surface leads to a reduction in cellular iron uptake. It can be concluded that a critical function of iron is its role in regulating the expression of the transferrin receptor and ferritin at the post-transcriptional level (Ponka et al., 1998; Testa et al., 1993). The feedback mechanism of regulation of ferritin biosynthesis by iron regulatory proteins is opposite to the regulation of transferrin receptor 1 biosynthesis. Hence, the tight regulation of this process is essential in maintaining the balance between the levels of intracellular iron needed by the cells and in preventing the formation of deleterious radicals due to excess iron. The interactions between iron, iron regulatory proteins, and iron responsive elements reveal a fascinating system for controlling iron uptake, storage, and utilization in maintaining iron homeostasis in mammalian cells.

In contrast to non-erythroid cells, intracellular iron levels do not have a major effect on the expression of *transferrin receptor 1* mRNA in erythroid cells. Rather, transferrin receptor 1 expression is up-regulated at the transcriptional level during erythroid differentiation, and the iron responsive element and iron regulatory protein feedback mechanism is not involved (Chan et al., 1994). Two specific motifs, the Ets-binding site and CRE-like motifs, are critical for the transcriptional activation of transferrin receptor 1 expression in non-proliferating, hemoglobin-producing murine erythroleukemia cells (Lok and Ponka, 2000). Clearly, regulation of the gene expression of transferrin receptor 1 differs significantly between erythroid and non-erythroid cells.

Serum or mitogenic stimulants also up- or down-regulate expression of transferrin receptor 1 transcriptionally (Hirsch et al., 1996; Miskimins et al., 1997). Whereas expression of *transferrin receptor 1* mRNA is transcriptionally up-regulated during T

and B lymphocyte activation (Seiser et al., 1993) and during erythroid differentiation (Busfield et al., 1997; Chan et al., 1994), its expression is down-regulated during terminal differentiation of myeloid and lymphoid leukemic cell lines (Alcantara et al., 1989). The mitogenic activation of the *transferrin receptor 1* gene is linked to the activity of a phosphatidylinositol 3-kinase, a tyrosine kinase that is activated by the stimulation of growth factors in quiescent cells and is important for the induction of DNA synthesis (Miskimins et al., 1997). It can be concluded that expression of the *transferrin receptor 1* gene is regulated at both transcriptional and posttranscriptional levels.

4.4. Function of *transferrin receptor 1*

The well-defined function of transferrin receptor 1 is to mediate cellular uptake of iron from plasma transferrin. The current model of iron uptake from transferrin via receptor-mediated endocytosis in mammalian cells is shown in Fig. 5. The first step involves the binding of transferrin to the transferrin receptor 1 on the cell surface by a physical interaction that does not require temperature or energy (Ciechanover et al., 1983; Ponka et al., 1998). The iron status of transferrin affects the affinity of transferrin for its receptor: diferric transferrin has the highest affinity, followed by monoferric transferrin, and apotransferrin has the lowest affinity (Young et al., 1984). The transferrin receptor 1 have high affinity for diferric transferrin with an estimated dissociation constant of 2–7 nM (Feder et al., 1998; Ponka et al., 1998). Since the concentration of plasma diferric transferrin is about 5 μ M under physiological conditions, most surface transferrin receptors become saturated with transferrin (Feder et al., 1998; Ponka et al., 1998). Each transferrin receptor can bind to two molecules of transferrin, and thus, the homo-dimeric transferrin receptor can mediate a maximum uptake of four atoms of iron at a time.

The transferrin receptor–transferrin-iron complexes interact with adaptor proteins within the clathrin-coated pit, and are then internalized by the cells via a receptor-mediated endocytic pathway (Odorizzi and Trowbridge, 1997). The tyrosine internalization motifs located on the cytoplasmic portions of transferrin receptors are required to mediate high-affinity binding to the adaptor protein complexes on the plasma membrane (Richardson and Ponka, 1997). This process is temperature and energy dependent (Ponka, 1999). Once within the endosome, a yet unidentified ATPase proton pump mediates acidification of the endosome and results in the release of iron from transferrin. The iron-free transferrins remain attached to the transferrin receptors and return to the cell surface, where the apotransferrin is released from the cells. The binding between transferrin and the transferrin receptor is pH dependent, which is critical to both membrane uptake and the release of transferrin. Dissociation of apotransferrin from its receptor takes place at neutral pH at the cell surface, making both the ligand and receptor available for further rounds of iron absorption. After iron is released from transferrin, iron passes through the endosomal membrane via the iron transporter, Nramp2, also known as the divalent cation transporter DCT1 (Fleming et al., 1998), into the cytoplasm. Iron that enters the cell can be utilized in the synthesis of heme or incorporated in iron-

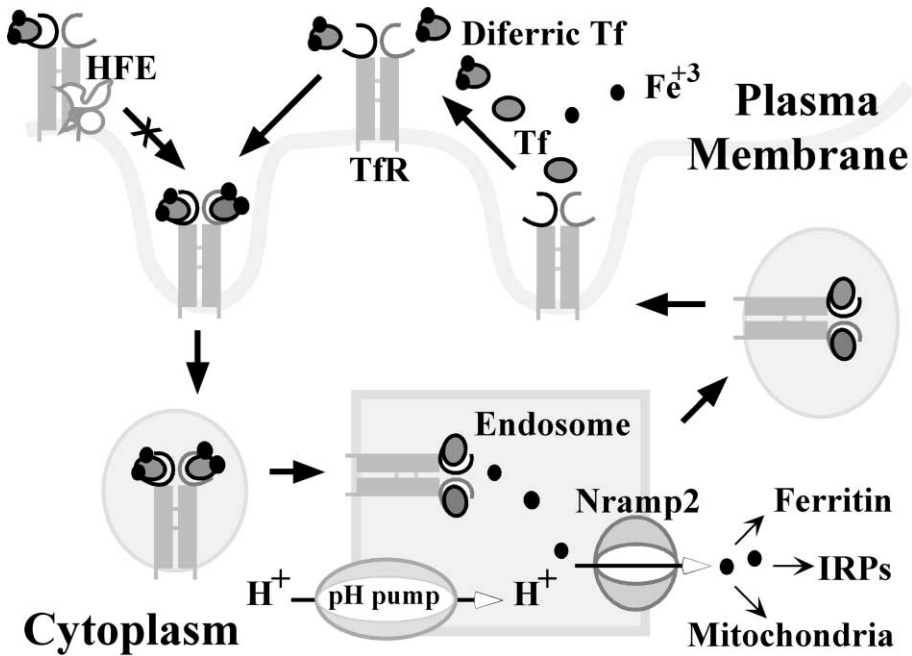


Fig. 5. Cycle of transferrin receptor- and transferrin-mediated cellular iron uptake. In plasma, iron is sequestered by transferrin (Tf), which can bind up to two molecules of iron (Fe^{+3}). In most cells, diferric transferrin binds to transferrin receptor (TfR) on the cell surface. The complex, consisting of iron, transferrins, and transferrin receptors, is internalized via a classic receptor-mediated endocytic pathway. By binding to transferrin receptor, HFE, the hemochromatosis protein, appears to inhibit transferrin receptor endocytosis. A proton pump decreases the pH within the endosomes, resulting in the release of iron from transferrin. Iron is then transported across the endosome membrane into the cytoplasm by the iron transporter, Nramp2. Apo-transferrin and transferrin receptor complexes are recycled to the cell surface for another cycle of iron uptake. In erythroid cells, intracellular iron is transported into the mitochondria and incorporated into heme. In non-erythroid cells, iron is either stored as ferritin or targeted to iron containing molecules, such as iron regulatory proteins (IRPs).

containing molecules. Intracellular iron can also be stored in the ferritin complexes or can modulate the activity of iron regulatory proteins (Fig. 5).

Since the identification of the gene for transferrin receptor 1, much has been learned about its functions. Yet, its functional significance in the biological process was not demonstrated until the recent studies of transgenic mice that lack one or both functional copies of the *transferrin receptor 1* gene (Levy et al., 2000). Mice heterozygous for the disrupted *transferrin receptor 1* gene show abnormalities in erythropoiesis and iron homeostasis. They develop microcytic, hypochromic erythrocytes, a typical symptom of iron deficiency (Levy et al., 2000). The *transferrin receptor 1*-deficient mice with homozygous disruption of the *transferrin receptor 1* gene die in utero. As expected, these mice have defective erythropoiesis and neurological development (Levy et al., 1999). These findings clearly demonstrate a significant role of transferrin receptor 1 in regulating iron homeostasis. The

neurological defect observed in *transferrin receptor 1*-deficient mice raises questions as to whether the neurological defect is caused by the lack of iron or by the lack of transferrin receptors that have some additional, yet unidentified, functions that are not related to iron. In this regard, transferrin has been shown to be required for the normal completion of neurulation in the mouse embryo (Copp et al., 1992).

4.5. *Second transferrin-binding and -transport protein: transferrin receptor 2*

The homologue to transferrin receptor 1, termed transferrin receptor 2, has recently been identified. The *transferrin receptor 2* gene is located on chromosome 7q22 (Kawabata et al., 1999) and gives rise, presumably by alternative splicing, to two transcripts of approximately 2.9 and 2.5 Kb in length (Kawabata et al., 1999). Amino acid sequence analysis reveals that like transferrin receptor 1, transferrin receptor 2 is a type II transmembrane glycoprotein that shares 66% similarity in its extracellular domain with transferrin receptor 1 (Kawabata et al., 1999). Although the cytoplasmic portion is highly divergent from transferrin receptor 1, transferrin receptor 2 also contains an internalization motif, YQRV, which is similar to the YTRF motif in transferrin receptor 1. Sequence analysis of the transferrin receptor 2 coding and non-coding region reveals that transferrin receptor 2 does not possess iron responsive elements (Kawabata et al., 1999). It is most likely that expression of transferrin receptor 2 is not regulated by the iron regulatory protein-mediated feedback regulatory mechanism in response to cellular iron status.

In sharp contrast to the ubiquitous expression pattern of transferrin receptor 1, transferrin receptor 2 is predominantly expressed in the liver, where expression of transferrin receptor 1 is relatively low. Transferrin receptor 2 is expressed at high levels in the hematopoietic cell line, K562, which can be induced to synthesize hemoglobin, and in HepG2 cells, a hepatoblastoma cell line (Kawabata et al., 1999). Upon transfection of the *transferrin receptor 2* gene in Chinese hamster ovary cells, which lack transferrin receptor 1, transferrin binding and iron uptake are increased, suggesting that transferrin receptor 2 is able to bind transferrin and plays a role in cellular iron uptake (Kawabata et al., 1999). Like transferrin receptor 1, binding of transferrin receptor 2 to transferrin is also pH dependent. The binding of apo-transferrin to transferrin receptors 1 and 2 only takes place at acidic pH (Kawabata et al., 1999). Expression levels of both transferrin receptors 1 and 2 also correlate with the stages of cell cycle, which is related to the requirement for iron during DNA synthesis (Kawabata et al., 2000). Nevertheless, transferrin receptor 2 differs from transferrin receptor 1 in its binding properties with transferrin and regulation of expression. Holotransferrin has lower affinity for transferrin receptor 2 than for transferrin receptor 1 (Kawabata et al., 2000; West and Bjorkman, 2000). Expression of transferrin receptor 2 is not regulated by cellular iron status. In cells treated with ferric nitrate (iron loading) or desferrioxamine (an iron chelator), expression of transferrin receptor 1 mRNA is increased in the presence of desferrioxamine and decreased with ferric nitrate (Kawabata et al., 2000). In contrast, neither ferric nitrate nor desferrioxamine has any effect on expression of transferrin receptor 2

mRNA. This difference seems to be due to the fact that transferrin receptor 2 mRNA lacks iron responsive elements (Kawabata et al., 2000).

Transferrin receptors 1 and 2 are likely to not only be regulated through distinct pathways but also to mediate iron uptake and storage by a different, yet undefined, mechanism. Transferrin receptor 1 seems to play a general role in cellular iron uptake. On the other hand, transferrin receptor 2 appears to play a specific role in iron uptake and storage in the liver, due to its high expression in hepatocytes. It has been reported that in vitro, soluble, recombinant protein of transferrin receptor 2 fails to interact with soluble, recombinant HFE protein, the hemochromatosis protein (Kawabata et al., 2000). However, in vivo, transferrin receptor 2 is able to associate with HFE in a manner similar to that of transferrin receptor 1 (Y. Yang, unpublished results). In addition, transferrin receptor 2 is capable of forming a heterodimer with transferrin receptor 1 (Y. Yang, unpublished results). These results indicate a complex iron–transferrin function that is mediated by transferrin receptors 1 and 2. Further studies are required to distinguish the respective functional roles of transferrin receptors 1 and 2 in iron uptake and iron stores in tissues, such as the liver and intestines.

In mice, the transferrin receptor 2 is abundantly expressed in the liver (Fleming et al., 2000). Like human transferrin receptor 2, expression of murine transferrin receptor 2 is not regulated by the status of iron, which is in agreement with the finding that iron responsive elements are absent in murine *transferrin receptor 2* mRNA (Fleming et al., 2000). In both normal and hepatic iron-overload mice, levels of mRNA for murine transferrin receptor 2 are higher than levels of mRNA for murine transferrin receptor 1 (Fleming et al., 2000). Consequently, transferrin receptor 2 might contribute to iron overloading by mediating the uptake of transferrin-bound iron in hepatocytes, despite the high levels of iron that would have already down-regulated the expression of transferrin receptor 1 (Fleming et al., 2000). The observation that *transferrin receptor 1*-deficient mice led to embryonic lethal demonstrates that transferrin receptor 1 is not redundant and that the presence of transferrin receptor 2 can not compensate for the functional loss of transferrin receptor 1 (Levy et al., 1999).

4.6. *Transferrin receptors in disease development*

Transferrin exerts a proliferative effect on cells by supplying iron for key synthesis processes required for cell growth, hence, it plays an essential role in stimulating the growth of lung-metastasizing tumor cells, prostatic carcinomas, and a number of human carcinoma cell lines (Cavanaugh and Nicolson, 1990; Inoue et al., 1993). As expected, in many cell types, expression of transferrin receptors is frequently associated with cellular proliferative activity (Inoue et al., 1993; van Muijen et al., 1990). Quiescent lymphocytes have a low dependence on iron and low numbers of transferrin receptor molecules, but when stimulated to proliferate, the number of transferrin receptor molecules increases markedly (Seiser et al., 1993; Testa et al., 1991). Consistent with this observation is the finding that anti-transferrin receptor 1 anti-

bodies are able to block the activation of lymphocytes and suppress the proliferation of tumor cells (Kemp et al., 1987; Taetle et al., 1983). Expression of the transferrin receptor 1 and the proliferative response to transferrin correlate well with the metastatic capability of breast cancer cells (Cavanaugh et al., 1999). Indeed, expression of human transferrin receptor 1 in a poorly metastatic rat mammary adenocarcinoma cell line, which expresses low endogenous levels of rat transferrin receptor 1, results in a significant proliferative response. In contrast, the control cells show no proliferative activity (Cavanaugh et al., 1999). Moreover, the transferrin receptor-transfected cell line forms larger lesions and lung metastases *in vivo* than the control cells (Cavanaugh et al., 1999). Thus, expression of transferrin receptor 1 in tumor cells appears to affect their growth response to transferrin *in vitro* and their ability to grow at a secondary site *in vivo*. It is noted that on the membranes of the human malignant melanoma cell line, SK-MEL-28, high concentrations of the transferrin homologue, p97, or melanotransferrin, and transferrin receptor 1 are expressed (Hedley et al., 1985; Seligman et al., 1979). The *melanotransferrin* gene is located on chromosome 3, the same chromosome as the *transferrin* and *transferrin receptor* genes. The melanotransferrin protein has approximately 38% sequence homology with human serum transferrin (Richardson and Baker, 1990); nevertheless, its functional significance in iron transport in melanoma cells remains to be demonstrated (Richardson and Baker, 1990).

The biological significance of transferrin receptor 2 is evident in humans with a homozygous non-sense mutation in the *transferrin receptor 2* gene. These patients develop a non-*Hfe*-linked form of hemochromatosis (Camaschella et al., 2000a,b). Functional studies of transferrin receptor 2 from *transferrin receptor 2*-deficient mice and compound mutant mice will shed light on the precise biological role of transferrin receptor 2 in maintaining iron homeostasis and will potentially identify additional factors that regulate the expression and function of transferrin receptor 2.

5. The hemochromatosis protein HFE in iron metabolism

Hereditary hemochromatosis is an inherited disorder that results from an accumulation of excess iron in many organs, which is manifested by liver cirrhosis, cardiomyopathy, diabetes mellitus, hypogonadism, arthritis, skin pigmentation, and if left untreated, death (Andrews and Levy, 1998; Bacon and Schilsky, 1999). The gene responsible for hereditary hemochromatosis is closely linked to the locus for the human leukocyte antigens and has been identified as a major histocompatibility complex-encoded class I-like HFE (Feder et al., 1996; Salter-Cid et al., 2000a). This discovery allows geneticists to track the genetic trait within families with hereditary hemochromatosis. Many genetic studies have subsequently characterized hereditary hemochromatosis as a common autosomal recessive disorder that is found mainly in people of Northern European descent (Feder et al., 1996; Merryweather-Clarke et al., 1997). An estimated 10% of the population is heterozygous for the gene, with 1 in 400 having the condition (Bradley et al., 1996; Edwards et al., 1988; Phatak et al., 1998).

Hereditary hemochromatosis is more prevalent than other inherited diseases, such as cystic fibrosis, sickle cell anemia, phenylketonuria, and Tay–Sachs disease (Fodinger and Sunder-Plassmann, 1999; Olynyk et al., 1999). Individuals who are homozygous for hereditary hemochromatosis absorb three to four times (3–4 mg) more iron per day than normal individuals (Fodinger and Sunder-Plassmann, 1999). Because the human body has very limited means of excreting iron, increased absorption leads to the buildup of iron stores in various organs, predominantly in the liver, pancreas, pituitary, synovium, and heart and others (Crawford et al., 1998; Powell et al., 1998). Although manifestations of the disease occur in later years of life, an excess accumulation of free iron can contribute to intracellular redox reactions, generating toxic reactive oxygen species that can cause damage or death to cells and organs.

5.1. The hemochromatosis gene: *Hfe*

The first clue that *Hfe* is involved in iron metabolism came from studies using $\beta 2$ microglobulin-deficient mice, which develop iron-overload syndromes with characteristics similar to those seen in humans with hereditary hemochromatosis (De Sousa et al., 1994; Rothenberg and Volland, 1996). The fact that $\beta 2$ microglobulin is physically associated with major histocompatibility complex-encoded class I molecules (Bjorkman and Parham, 1990; Lawlor et al., 1990; Rammensee et al., 1993; Salter-Cid et al., 2000a) indicates a functional role for $\beta 2$ microglobulin and/or for a $\beta 2$ microglobulin-associated molecule in iron homeostasis and the pathogenesis of the disease. Indeed, positional gene cloning demonstrates a linkage between the gene responsible for hereditary hemochromatosis to the ‘HLA-A3’ locus on chromosome 6q21.3 (Simon et al., 1976; Simon and Brissot, 1988). In 1996, a strong correlation between the mutation of a novel gene encoding a major histocompatibility complex class I-like molecule (Salter-Cid et al., 2000a) and the occurrence of hereditary hemochromatosis was reported (Feder et al., 1996). The identified gene was initially termed HLA-H and since renamed *Hfe*. More than 80% of the hereditary hemochromatosis patients that were examined contained mutations in *Hfe* (Beutler, 1997; Feder et al., 1996; Jazwinska et al., 1996; Jouanolle et al., 1996). Particularly, genetic analyses shows that most patients are homozygous, with a missense mutation at nucleotide position 845 by a G-to-A transition, resulting in a cysteine-to-tyrosine substitution at residue 282 (C282Y) (Feder et al., 1996, 1997). A second missense mutation in *Hfe* is in the coding sequence of the gene, with a C-to-G transition at nucleotide 187, changing a histidine at position 63 to aspartic acid (H63D) (Fig. 6) (Feder et al., 1996, 1997). Patients with hereditary hemochromatosis who are heterozygous for C282Y are frequently found to be heterozygous for H63D (Feder et al., 1996, 1997). Although the H63D mutation is less prevalent, its presence in individuals heterozygous for C282Y is strongly associated with the development of hereditary hemochromatosis (Beutler and Gelbart, 1997). In addition to the C282Y or H63D mutations, three other mutations (S65C), (I105T), and (G93R) have been detected in *Hfe* (Barton et al., 1999). These mutations in *Hfe* are also associated with

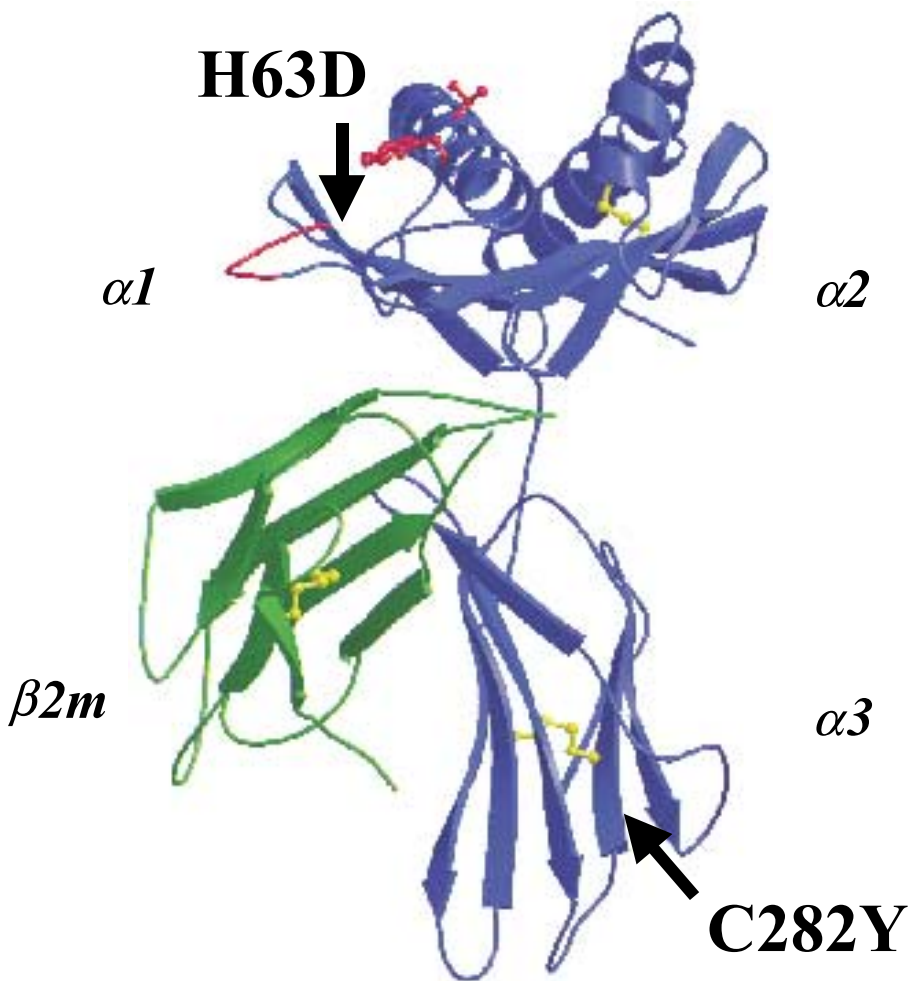


Fig. 6. Crystal structure of the hemochromatosis protein HFE. A ribbon diagram of the HFE structure. HFE functions as a heterodimer of a heavy chain and a non-covalently-bound light chain, $\beta 2$ microglobulin ($\beta 2m$). The heavy chain consists of three extracellular domains $\alpha 1$, $\alpha 2$, and $\alpha 3$, a transmembrane region, and a cytoplasmic tail. The locations of the C282Y and H63D mutations, commonly found in the *Hfe* gene of hereditary hemochromatosis patients, are indicated (kindly provided by Dr. P.J. Bjorkman of California Institute of Technology, California, USA).

the development of iron overload, especially in individuals who are heterozygous for C282Y or H63D (Barton et al., 1999).

Direct evidence that the *Hfe* gene is involved in regulating iron metabolism came from studies of the defective *Hfe* gene in mice (Zhou et al., 1998). *Hfe*-deficient mice exhibit high levels of saturated serum transferrin and excessive iron accumulation in the liver (Zhou et al., 1998). Similar to human hereditary hemochromatosis, high iron levels are also found in the hepatocytes of *Hfe*-deficient mice (Cox, 1996). The

biochemical abnormalities and histopathology of *Hfe*-deficient mice are almost identical to those observed in human hereditary hemochromatosis. Thus, loss of *Hfe* function results in the development of hereditary hemochromatosis. These findings strongly suggest that HFE is a hemochromatosis molecule that is responsible for regulating iron homeostasis.

5.2. The hemochromatosis protein: HFE

The *Hfe* gene encodes a 343-amino-acid protein and belongs to the non-classical major histocompatibility complex class I family (Fourie and Yang, 1998; Salter-Cid et al., 2000a; Yang et al., 1996). The heavy chain of major histocompatibility complex class I molecules consists of three extracellular domains ($\alpha 1$, $\alpha 2$, and $\alpha 3$), a transmembrane region, and a short cytoplasmic tail (Bjorkman and Parham, 1990; Fourie and Yang, 1998). Major histocompatibility complex class I molecules are known to bind short antigenic peptides and function in antigen presentation to T cells (Fourie and Yang, 1998; Garcia et al., 1999). Like most major histocompatibility complex class I molecules, HFE is a heterodimer, consisting of a heavy chain encoded by the *Hfe* gene and a non-covalently bound light chain encoded by the $\beta 2$ *microglobulin* gene (Fig. 6) (Bjorkman and Parham, 1990; Lawlor et al., 1990; Rammensee et al., 1993; Salter-Cid et al., 2000a). Crystallographic studies have shown that the HFE molecule resembles major histocompatibility complex class I molecules (Feder et al., 1997; Fourie and Yang, 1998; Lebron et al., 1998). However, the structure of HFE does not possess a functional peptide-binding groove (Feder et al., 1997; Lebron et al., 1998) because the position of the HFE extracellular domain forms a narrow groove that prevents peptide binding (Feder et al., 1996; Lebron et al., 1998). Therefore, HFE is unlikely to play a role in antigenic peptide presentation to T cells.

One of the most conserved features of major histocompatibility complex class I molecules is the four cysteine residues in the $\alpha 2$ and $\alpha 3$ domains that are required for the development of intra-molecular disulfide bonds (Bjorkman and Parham, 1990; Fourie and Yang, 1998). Mutation at amino acid 282, in the $\alpha 3$ domain, with the substitution of a tyrosine at the conserved cysteine, disrupts the non-covalent interaction with $\beta 2$ microglobulin (Bjorkman and Parham, 1990; Feder et al., 1996). Consequently, the C282Y mutant HFE is not properly processed and is retained in the endoplasmic reticulum and Golgi compartments (Feder et al., 1997; Waheed et al., 1997). The C282Y mutant HFE is also degraded more rapidly than wild-type HFE (Waheed et al., 1997); mutation at this residue seems to disrupt the integrity of the structure of HFE and abolish cell-surface expression of the HFE molecules (Feder et al., 1997; Waheed et al., 1997). These results are consistent with the study of the $\beta 2$ *microglobulin*-deficient mice, in which the absence of $\beta 2$ microglobulin alters the expression and function of HFE, and mice develop iron overload. The H63D mutation in HFE shows no detectable changes in the interaction between $\beta 2$ microglobulin and HFE heavy chains and/or in intracellular processing. The pathophysiological roles of the H63D, S65C, I105T, and G93R mutations in iron metabolism remains to be determined (Feder et al., 1996, 1997).

5.3. Regulation of HFE

Hfe is widely expressed in all tissues but at a low level, with no expression detected in the brain. The highest expression of *Hfe* is found in the liver and small intestine (Feder et al., 1996), indicating a potential role for HFE in intestinal iron absorption and transport as well as in iron storage in the liver. At the cellular level, HFE is expressed at the cell surface of epithelial cells throughout the gastrointestinal tract, but such expression is varied among cell types (Parkkila et al., 1997a,b). HFE is highly expressed in crypt cells, suggesting that HFE may respond to the iron status of the body and regulate according to the amount of dietary iron absorbed. At the subcellular level, HFE is localized to the basolateral surface of the epithelial cells in the stomach and colon. However, in crypt cells of the small intestine, expression of HFE is intracellular and perinuclear (Parkkila et al., 1997a; Waheed et al., 1999), indicating that HFE might be in compartments destined for degradation or for recycling of transferrin–transferrin receptor complexes. HFE is also expressed on the apical plasma membrane of the syncytiotrophoblasts, suggesting another role of HFE in regulating iron transport from maternal blood to the fetus (Parkkila et al., 1997a). Interestingly, induction of HFE expression in HeLa cells results in activating iron regulatory proteins (Riedel et al., 1999). Increased activities of iron regulatory proteins are accompanied by decreased ferritin biosynthesis and up-regulation of the expression of transferrin receptor 1 (Corsi et al., 1999; Riedel et al., 1999). Expression of HFE also has a reciprocal effect on expression of Nramp2, another key regulator of intracellular iron homeostasis. Thus, HFE expression can up- and down-regulate expression of iron responsive element-containing molecules, and, in turn, affect iron metabolism.

5.4. Physical association of HFE and transferrin receptor 1

The molecular mechanism of the role of HFE in iron metabolism was addressed when HFE was found to physically associate with transferrin receptor 1 (Feder et al., 1998; Parkkila et al., 1997a; Salter-Cid et al., 1999, 2000a). Physical association between HFE and transferrin receptor 1 is demonstrated in cultured human embryonic kidney 293 cells and HeLa cells as well as in syncytiotrophoblasts (Parkkila et al., 1997a; Salter-Cid et al., 1999, 2000a). HFE also binds to transferrin receptor 1 in duodenal crypt enterocytes (Bacon et al., 1999; Parkkila et al., 1997a; Waheed et al., 1999), further supporting a role for HFE in regulating the absorption of dietary iron in the intestines. Upon forming an association complex in the endoplasmic reticulum, HFE co-trafficks with transferrin receptor 1 through the Golgi reticulum network to reach the cell surface, where they remain firmly associated (Gross et al., 1998; Roy et al., 1999; Salter-Cid et al., 1999, 2000a). In vitro, HFE and transferrin receptor 1 can associate in different ratios; At a ratio of 2:1, a transferrin receptor 1 homodimer with one HFE molecule (Lebron et al., 1998); at a 2:2 stoichiometry, one HFE molecule contacts each polypeptide chain of the transferrin receptor 1 homodimer on the same membrane (Bennett et al., 2000). In vivo, all three molecules, diferric iron–transferrin, transferrin receptor 1, and HFE can co-immunoprecipitate

in a 1:2:1 ratio, indicating that all three molecules can interact simultaneously to form a ternary complex consisting of one iron–transferrin, one transferrin receptor 1 homodimer, and one HFE heterodimer (Gross et al., 1998; Lebron et al., 1998; Lebron and Bjorkman, 1999; Salter-Cid et al., 2000a). Turnover of non-transferrin receptor 1-bound HFE is more rapid than turnover of transferrin receptor 1-bound HFE (Gross et al., 1998). Therefore, binding of HFE to transferrin receptor 1 appears to be required for the intracellular transport, stabilization, and surface expression of HFE (Gross et al., 1998; Salter-Cid et al., 1999, 2000a). While wild-type HFE molecules firmly associate with transferrin receptor 1, the C282Y mutant HFE failed to associate with transferrin receptor 1 (Feder et al., 1998). The fact that a mutation in HFE abolishes its interaction with binding partners suggests that patients with HFE mutations might have pathological iron regulation, possibly via an altered transferrin receptor 1-dependent iron metabolic pathway. In most cells, the major route for iron uptake from plasma to cells is by the transferrin receptor 1-mediated internalization in the form of diferric iron–transferrin. A physical interaction between HFE and transferrin receptor 1 provides an important functional link in studying how HFE influences the function of transferrin receptor 1 and regulates transferrin receptor 1-dependent iron uptake (Salter-Cid et al., 2000a,b). The interaction between HFE and transferrin receptor 1 appears to be pH dependent (Feder et al., 1997; Lebron et al., 1998). At pH 7.5, HFE binds transferrin receptor 1 with high affinity, whereas very weak binding or no binding at all is observed at pH 6.0 (Feder et al., 1997; Lebron et al., 1998). The results that neutral pH at the cell surface favors the association of HFE and transferrin receptor 1, and that HFE is most likely not associated with transferrin receptor 1 in acidic endosomal compartments, strongly suggest that the primary action site for HFE is on the plasma membrane, where HFE affects transferrin receptor 1 functions.

Crystallographic studies of transferrin, HFE, and transferrin receptor 1 complexes also reveal that HFE does not interact with transferrin and that HFE binding to transferrin receptor 1 is independent of transferrin binding (Bennett et al., 2000; Feder et al., 1998). Most importantly, upon HFE binding, conformational changes of transferrin receptor 1 are induced (Salter-Cid et al., 2000a), as revealed by the crystal structural study of transferrin receptor 1 and HFE complexes (Fig. 7) (Bennett et al., 2000). By inducing conformational changes of transferrin receptor 1, binding of iron–transferrin and HFE to transferrin receptor 1 homo-dimer possibly alters the symmetric structure of transferrin receptor 1 and creates only a single HFE-binding site on transferrin receptor 1.

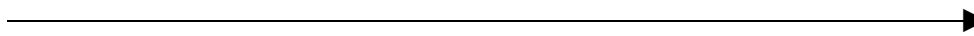
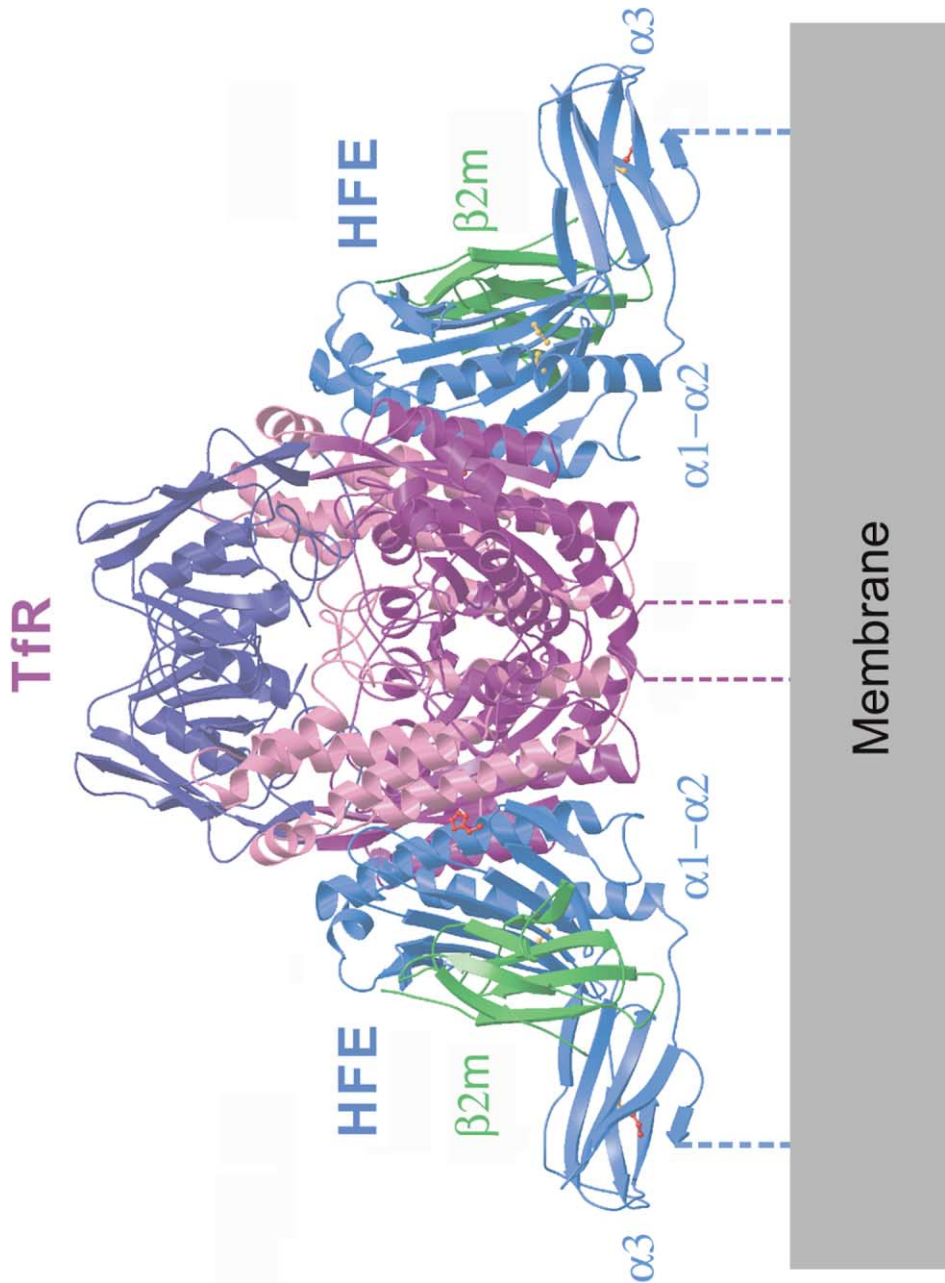


Fig. 7. Crystal structure of the HFE-transferrin receptor 1 complex. A ribbon diagram of a HFE-transferrin receptor 1 complex between the ectodomains of human HFE and transferrin receptor 1 shows two HFE molecules which grasp each side of a transferrin receptor 1 homo-dimer on the same membrane. The complex has two-fold symmetry with a 2:2 stoichiometry, revealing one HFE molecule contacts each polypeptide chain of the transferrin receptor 1 homo-dimer. Conformational changes in transferrin receptor 1 induced by HFE binding appear to influence transferrin receptor 1 function in binding transferrin and in inhibiting transferrin receptor internalization, thus altering cellular iron homeostasis. The membrane bilayer is represented by a gray box on the bottom (kindly provided by Dr. P.J. Bjorkman of California Institute of Technology, California, USA).



5.5. Consequence of the HFE-transferrin receptor 1 interaction

Both iron-bound transferrin and HFE can bind to transferrin receptor 1 simultaneously to form a ternary complex, indicating that HFE and iron–transferrin complexes are not competing for the same site on transferrin receptor 1 (Feder et al., 1998; Gross et al., 1998; Lebron et al., 1998, 1999). Interaction of HFE with transferrin receptor 1 appears to decrease the binding affinity of transferrin receptor 1 to transferrin by 5- to 10-fold, whereas the mutant C282Y HFE fails to exert such an effect (Feder et al., 1998; Gross et al., 1998). The dissociation constant of transferrin receptor 1 to iron-bound transferrin is approximately 5 nM in the absence of HFE and 12–75 nM in the presence of HFE (Feder et al., 1998). However, these results are not consistent with the fact that under physiological conditions, the concentration of transferrin in plasma is in the micromolar range, approximately 5 μ M (Salter-Cid et al., 1999). Consequently, the binding of diferric transferrin to its receptor is saturated even in the presence of HFE. Together with the fact that transferrin receptor 1-bound transferrin complexes undergo rapid recycling (Salter-Cid et al., 1999), decreasing transferrin receptor 1 affinity to transferrin by HFE is not an efficient means for inhibiting iron uptake and is not a principal effect imposed by HFE on transferrin receptor 1.

Overexpression of HFE has been found to alter the rate of transferrin recycling from the endosome to the cell surface in hepatoma cells. It is not clear whether this action is direct or indirect, because HFE dissociates from the receptor at acidic pH (Lebron et al., 1998). If these results are confirmed, HFE might function to block release of iron from the transferrin–transferrin receptor 1 complex within the endosomal compartments. On the other hand, it has been reported that HFE binding does not alter transferrin receptor 1 endocytosis or recycling (Roy et al., 1999). However, detailed analyses of rate constants of internalization and externalization of transferrin receptor 1-bound transferrin demonstrates that HFE expression impairs transferrin receptor 1-transferrin internalization but has no effect on recycling rates (Salter-Cid et al., 1999, 2000a). The relative amount of transferrin that remains on the surface HFE-expressing cells is higher than those that do not express HFE, and the uptake of iron-bound transferrin is slower in cells expressing HFE (Salter-Cid et al., 2000a). In HFE-expressing cells, cell surfaced-expressed transferrin receptor 1 is increased by 40%, due to the accumulation of non-functional transferrin receptor 1 by HFE (Salter-Cid et al., 1999). As a consequence, the uptake of transferrin-bound iron by cells and the accumulation of intracellular iron are reduced in the presence of HFE.

Transferrin receptor 1 possesses an internalization domain at the cytoplasmic N-terminus, which contains a conserved tyrosine-based internalization signal (YTRF) (Collawn et al., 1990, 1993) and several conserved serine residues. Phosphorylation of transferrin receptor 1 can take place at serine residues (Castagnola et al., 1987; Davis et al., 1986; Johnstone et al., 1984a,b; May and Cuatrecasas, 1985). In cells expressing HFE, the intensity of phosphorylated serine on transferrin receptor 1 is at least two times higher than those without HFE expression (Salter-Cid et al., 2000a). The rate of intracellular uptake of transferrin is reduced when cells are treated with

phosphatase inhibitors and/or when HFE expression is induced (Salter-Cid et al., 2000a). Thus, HFE-induced phosphorylation of transferrin receptor 1 appears to inhibit endocytosis of transferrin receptor 1. Consequently, non-functional transferrin receptor 1 accumulates at the cell surface.

Unlike many other diseases, hereditary hemochromatosis is a treatable, chronic iron-disorder disease. If detected early with accurate diagnosis, individuals with this potentially fatal disorder can experience a normal life expectancy. Therefore, identifying the *Hfe* gene is important for implementing a molecular diagnostic screening for individuals who have the disease and for predicting family members who are likely to be at risk for developing iron overload. In addition, analysis of the function of HFE might provide some insights into the processes controlling the regulation of transferrin receptor-dependent iron metabolism. The observations that HFE is expressed in the crypt enterocytes of the duodenum and is physically associated with transferrin receptor 1 suggest that the HFE protein might participate in regulating intestinal iron absorption (Waheed et al., 1999). In hereditary hemochromatosis patients, increased intestinal iron absorption results in the massive overload in other tissues, suggesting that interactions between HFE and transferrin receptor 1 must regulate iron homeostasis at the level of dietary iron uptake by the enterocytes. Mutations in *Hfe* disrupt this function, impair iron uptake by the crypt cells, and lead to iron accumulation; however, the mechanisms of HFE interaction and its effect on the function of transferrin receptor 1 is still not defined and requires further investigation.

It is puzzling that enterocytes of hereditary hemochromatosis patients are iron deficient. Yet, the loss of HFE function in cells promotes iron uptake via the transferrin–transferrin receptor 1 pathway. How the opposite effect might be elicited in intestinal cells to regulate dietary iron absorption remains to be investigated. Nevertheless, much has been learned about different factors that are involved in regulating iron metabolism since the identification of the *Hfe* gene 4 years ago. Many questions are still unanswered: Is HFE an iron “sensor?” How do crypt cells, which uptake the transferrin-bound iron from plasma, communicate and regulate villus cells to absorb dietary iron in response to the body’s iron status? Are there additional molecules involved in iron homeostasis? One can imagine that the near-completed effort of sequencing the human genome will aid in finding additional novel factors involved in iron homeostasis and that many of these questions will be answered in the near future.

6. Additional factors involved in iron metabolism

The balance required to maintain appropriate levels of iron in cells and tissues has led to the evolution of multiple mechanisms to precisely regulate iron uptake from transferrin and low-molecular-weight iron chelates. Beyond current knowledge as described in this review, the complexity of the iron metabolic pathways is significantly greater than is presently appreciated. For example, iron metabolism has been functionally linked to copper metabolism and possibly to the metabolism of other

metals. Several molecules, such as ceruloplasmin and the stimulator of iron transport protein, appear to be indirectly involved in copper and iron metabolic pathways, but the molecular mechanism for this type of involvement remains to be elucidated.

6.1. *The multi-copper oxidase: ceruloplasmin*

Ceruloplasmin is a multi-copper oxidase that contains more than 95% of the copper found in plasma. Although each molecule can bind up to six atoms of copper, ceruloplasmin does not play a role in copper transport (Gitlin, 1998). Rather, ceruloplasmin catalyzes the conversion of ferrous to ferric iron and assists the incorporation of ferric iron onto transferrin. A role for ceruloplasmin in mammalian iron metabolism is suggested by its potent ferroxidase activity in catalyzing the conversion of ferrous iron to ferric iron. Knowledge of this activity has been gained through the identification of yeast copper oxidases homologous to ceruloplasmin that facilitate high affinity iron uptake and studies of aceruloplasminemic animals who have extensive iron deposits in multiple tissues (Mukhopadhyay and Fox, 1998). In human adults, the primary site of ceruloplasmin synthesis is the liver, but it is also synthesized by cells of monocytic origin. Under iron deficiency conditions in human cells, ceruloplasmin mRNA expression and protein synthesis increase significantly. The increase in ceruloplasmin mRNA is due to an increased rate of transcription. Interestingly, in monocytic cells, interferon induces the synthesis of ceruloplasmin mRNA and protein. Induced synthesis of ceruloplasmin is terminated by a mechanism involving transcript-specific translational repression. The inhibitory factor bound to the 3' untranslated region of ceruloplasmin mRNA is required for complex formation as well as for silencing translation. This translational silencing of ceruloplasmin transcripts following cytokine activation provides another example of the interplay between iron metabolism and immune function.

In addition to the ferroxidase activity, ceruloplasmin is also an acute-phase protein with amine oxidase, and pro- and antioxidant activities. Functional studies have shown that the principal function of ceruloplasmin is its serum ferroxidase activity (Mukhopadhyay et al., 1998). In individuals afflicted with hereditary ceruloplasmin deficiency, high levels of iron accumulate in most tissues, reflecting the requirement of ceruloplasmin for the mobilization of iron from tissues and the incorporation of iron into ferric transferrin (Gitlin, 1998). Thus, ceruloplasmin is involved in iron metabolism, although its exact role in the cellular iron process is unclear. Using anti-transferrin receptor 1 antibodies that block the binding of transferrin to its receptor and inhibit transferrin-mediated iron uptake in intact ceruloplasmin-expressing cells, studies have suggested that ceruloplasmin stimulates iron uptake by a transferrin-independent pathway (Gitlin, 1998). However, the discovery of transferrin receptor 2 and, especially, the discovery of cells used in experiments express high levels of transferrin receptor 2 cast serious doubt on this conclusion. Transcriptional induction of ceruloplasmin or of regulated transporters is possible, because actinomycin D and cycloheximide block the induction of ceruloplasmin-stimulated iron uptake. Ceruloplasmin-stimulated iron uptake is completely blocked by unlabeled ferric iron and by other trivalent cations, including

Al^{3+} , Ga^{3+} , and Cr^{3+} , but is not blocked by divalent cations. These results indicate that ceruloplasmin might utilize a trivalent cation-specific transporter.

6.2. *The hypoxia responsive element-binding protein: hypoxia-inducible factor-1*

Plasma ceruloplasmin increases markedly in several conditions of anemia, for example, iron deficiency, hemorrhage, renal failure, sickle cell disease, pregnancy, and inflammation. However, little is known about the cellular and molecular mechanisms that are involved in ceruloplasmin increases. Sequence analysis has revealed that the 5' flanking region of the *ceruloplasmin* gene contains three pairs of consensus hypoxia responsive elements. Deletion and mutation analysis shows that a single hypoxia responsive element is necessary and sufficient for activating the *ceruloplasmin* gene. The hypoxia-inducible factor-1 α and β subunits are able to bind the hypoxia responsive elements in the ceruloplasmin promoter in vitro. Because iron deficiency and hypoxia do not activate expression of the *ceruloplasmin* gene in cells that are deficient in hypoxia-inducible factor-1 α and because iron deficiency increases plasma ceruloplasmin in vivo, hypoxia-inducible factor-1 plays an important role in regulating ceruloplasmin expression and function in iron metabolism.

Interestingly, sequence analysis of the promoter region of transferrin receptor 1 has also identified a functional hypoxia response element that contains a binding site for hypoxia-inducible factor-1. A motif with multipartite organization that is similar to the hypoxia response element of a number of hypoxia-inducible genes, such as erythropoietin, is present within a 100-base pair sequence upstream of the transcriptional start site of the transferrin receptor 1 gene. Exposure of cells to hypoxia results in a two- to three-fold increase in the expression of transferrin receptor 1 mRNA. Mutations in this motif attenuate the hypoxic response by 80%. Transient co-expression of the hypoxia-inducible factor-1 α and β enhances the activity of the wild-type transferrin receptor 1 promoter, but promoters with a mutated hypoxia response element yield no such response. Because hypoxia-inducible factor-1 is stimulated and bound to the hypoxia response element of the transferrin receptor 1 upon hypoxic challenge, the gene that encodes the transferrin receptor 1 is also a target for hypoxia-inducible factor-1.

6.3. *The Friedreich's ataxia protein: frataxin*

Frataxin is a small, nuclear-encoded mitochondrial protein that appears to be involved in mitochondrial iron homeostasis (Gibson et al., 1996; Koutnikova et al., 1997). Frataxin deficiency is the result of hyper-expansion of a polymorphic GAA trinucleotide repeat, which severely inhibits transcription and results in decreased levels of mature frataxin mRNA (Campuzano et al., 1996; Durr et al., 1996). As a consequence of frataxin deficiency, patients develop Friedreich's ataxia, which is characterized by a progressive neurodegenerative disorder and cardiomyopathy that leads to premature death (Askwith and Kaplan, 1998; Machkhas et al., 1998). Recent studies in cultured cells from patients with Friedreich's ataxia have shown that frataxin deficiency results in accumulation of mitochondrial iron with increased

oxidant stress and respiratory insufficiency, which are ultimately responsible for the neuronal and cardiac cell damage of the disorder.

6.4. The transferrin-like iron-binding protein: melanotransferrin

Melanotransferrin is a membrane-bound protein that shows high homology to transferrin (Richardson and Baker, 1994) and is capable of binding iron. Melanotransferrin mRNA is widely expressed in many tissues and is expressed at highest levels in the salivary gland (Richardson, 2000). It is also expressed at high levels in malignant melanoma cells. Levels of melanotransferrin mRNA do not change in the presence or absence of an iron chelator or iron source. Thus, unlike transferrin receptor 1 and transferrin, expression of melanotransferrin mRNA is not regulated according to cellular iron levels (Richardson, 2000). Functional studies have shown that melanotransferrin transports iron from iron-citrate complexes but not from iron–transferrin complexes (Richardson, 2000). Because depletion of melanotransferrin from HeLa cell membranes has little effect on iron uptake from iron-citrate complexes (Kriegerbeckova and Kovar, 2000), melanotransferrin appears to play a minor role in iron uptake. In contrast to transferrin and transferrin receptors, melanotransferrin expression is not altered in the liver of iron-overload disordered animals (Sciot et al., 1989). In addition, the distribution of melanotransferrin mRNA differs from the distribution of transferrin or transferrin receptor 1 mRNAs (Richardson, 2000). Additionally, melanotransferrin is reported to be expressed in amyloid plaques in brains of patients with Alzheimer's disease (Kennard et al., 1996; Rothenberger et al., 1996). Defining the role of melanotransferrin in the human brain will require additional studies. Although melanotransferrin does not play a major role in iron uptake, it might have roles in iron metabolism that are yet to be defined.

6.5. The stimulator of iron transport protein

Stimulator of iron transport is a transmembrane protein that enhances uptake of both transferrin-bound and non-transferrin-bound iron in cultured cells (Gutierrez et al., 1997). Stimulator of iron transport requires copper for its full activity (Yu et al., 1998). The nature of stimulator of iron transport-mediated uptake is different from the iron uptake mechanisms associated with Nramp2 activity. Stimulator of iron transport stimulates both ferric and ferrous iron at neutral pH, whereas Nramp2 prefers ferrous iron at low pH (<6) (Gunshin et al., 1997). Expression of stimulator of iron transport seems to be inversely regulated by cellular iron levels. At low cellular iron levels, stimulator of iron transport is highly expressed (Yu et al., 1998); in the liver of patients with hemochromatosis, this protein is up-regulated (Yu et al., 1998). Though stimulator of iron transport is most likely an integral component of the cellular iron-transport pathway, its exact role in iron metabolism and its function in relation to HFE, transferrin receptors, Nramp2, ferroportin1, and other iron-regulated proteins remain unclear.

6.6. The Menkes' and Wilson's disease proteins: ATPase 7A and 7B

Copper, an essential transition metal that permits the facile transfer of electrons in a series of critical biochemical pathways, is a heavy metal ion required for the activity of a variety of enzymes in the body. In excess, copper is a very toxic ion; therefore, efficient regulation of its metabolism is required. Copper-mediated human diseases include the genetic disorders of X-linked Menkes' syndrome and autosomal recessive Wilson's disease. Both Menkes' and Wilson's causative genes encode homologous cation copper-transporting P-type ATPase proteins. The Menkes' protein ATPase 7A is expressed in most tissues, except liver. In contrast, the Wilson's protein ATPase 7B is abundantly expressed in liver tissues. Intracellular localization of those proteins was investigated. Both ATPase 7A and 7B are localized in the trans-Golgi network and post-Golgi vesicular compartment in cells. This intracellular localization is altered by the copper content and, possibly, the iron content in the cell. ATPase 7A is an inner mitochondrial protein involved in energy-dependent transport of a wide variety of substrates across cell membranes. ATPase 7A is highly homologous with the gene in *Saccharomyces cerevisiae* that is thought to be involved in heme transport (Shimada et al., 1998). Mutations in the *ATPase 7* gene are functionally linked to the development of an inherited form of X-linked sideroblastic anemia with ataxia (Shani et al., 1997). Patients with this disease show an accumulation of mitochondrial iron, which seems to be responsible for the cellular damage that causes impaired neuronal and erythroid functions in this iron disorder (Allikmets et al., 1999; Shimada et al., 1998). Definitive functions of ATPase 7A and 7B are yet to be determined. ATPase 7A and 7B are hypothesized to be involved in cellular copper transport and play an indirect role in iron metabolism.

Despite striking differences in the clinical presentation between iron- and copper-mediated diseases, in the iron and copper metabolic pathways, participation of molecules, such as ceruloplasmin and stimulator of iron transport, indicates a functional link between iron and copper metabolism. Elucidation of the basic defect in copper-disordered diseases has also provided a valuable heuristic paradigm for understanding the mechanisms of cellular iron homeostasis. Like iron, copper participates in the pathogenesis of neuronal injury in Alzheimer's disease and the prion-mediated encephalopathies. Further elucidation of the mechanisms of iron and copper trafficking and their metabolism within the nervous system will be of direct relevance to our understanding of the pathophysiology and treatment of neurodegenerative diseases.

The use of differential gene display and DNA microarray technologies to evaluate the effects of iron deficiency and overload on gene expression has uncovered a number of genes whose expression is affected by cellular iron levels (Y. Yang, unpublished results) (Barisani et al., 2000). These genes, not previously known to be involved in iron metabolism, might play significant roles in this process. In addition, by employing a computer algorithm for detecting the presence of the consensus iron responsive element in the human genome with the use of the near completed human genome sequence database and other public and private sequence databases, we have also uncovered at least 70 novel genes that contain iron responsive elements

(Y. Yang, unpublished results). Expression of these iron responsive element-containing genes is thought to be modulated by the feedback regulatory mechanism of iron regulatory proteins in response to the body's iron status; however, the direct or indirect involvement of these molecules in iron metabolism is presently unknown. Interestingly, several novel iron transporter genes have been identified in human genome by extensive bioinformatics searches. (P.T. Lieu, Y. Yang, unpublished results). It remains to be seen whether these gene products are actively expressed, whether they express in a tissue- and cell-type-specific fashion, and whether they participate in iron metabolism. The applications of bioinformatics, DNA microarray, and proteomics technologies to study deficiency and overload conditions of iron and copper will ultimately reveal the functional roles these molecules play in iron metabolic pathways.

7. Iron metabolism disorders in animals

Despite the clinical prevalence of iron metabolism disorders in humans, the mechanisms by which dietary iron is absorbed into the body and by which cellular iron homeostasis is controlled are still poorly understood. Important insights into the molecular modulators of iron absorption, transport, and storage are being provided by genetic mapping to identify gene mutations in animals with phenotypic abnormalities in iron metabolism and by altering genes involved in the iron metabolic pathways of animals. Thus, iron metabolism-disordered animals are ideal models for investigating these poorly understood, yet clinically important, iron metabolic processes. Here, a list of animals with abnormalities in iron metabolism is shown in Table 2, and the characteristics in the phenotypic changes of the respective animals are discussed.

7.1. *Transferrin receptor 1-deficient mice*

Circulating plasma iron is bound to transferrin, which solubilizes ferric iron and attenuates its reactivity. Diferric transferrin interacts with cell-surface transferrin receptors to undergo receptor-mediated endocytosis into specialized endosomes. Endosomal acidification leads to iron release, and iron is transported out of the endosome by the iron transporter Nramp2. Transferrin and transferrin receptors then return to the cell surface for reuse, completing a highly efficient cycle (Fig. 5) (Ponka and Lok, 1999). The transferrin cycle seems to be the general mechanism for cellular iron uptake. Disruption of the *transferrin receptor 1* gene in mice eliminates the transferrin cycle, but leaves other transferrin functions intact (Levy et al., 1999). In addition, mice lacking the *transferrin receptor 1* gene have defects in both erythropoiesis and neurological development, thus displaying a more severe phenotype than the hypotransferrinemic $\text{Trf}^{\text{hpx/hpx}}$ mice (Trenor et al., 2000). However, haploinsufficiency for transferrin receptor 1 in mice results in impaired erythroid development and abnormal iron homeostasis. Thus, the transferrin cycle is necessary for the development of erythrocytes and the nervous system.

Table 2
Phenotypic changes of iron metabolism-disordered animals^a

Name	Defect	Symptom
<i>Trf</i> ^{hpx/hpx}	Transferrin	hypotransferrinemia iron overload in all non-hematopoietic tissues
<i>Hfe</i> -/-	<i>HFE</i>	hemochromatosis
<i>TfR1</i> -/-	TfR1	impaired erythropoiesis and neurologic development abnormal iron homeostasis
<i>mk/mk</i>	Nramp2	microcytic anemia, defects in intestinal iron transport erythroid iron utilization
Belgrade (<i>b/b</i>) rat	p45 NF-E2	decreased globin production and iron deficiency
<i>sla/sla</i>	Nramp2	hypochromic, microcytic anemia
<i>Ceruloplasmin</i> -/-	hephaestin	hypochromic, microcytic anemia
β -thalassemia	ceruloplasmin	aceruloplasminemia; abnormal iron compartmentation
α -thalassemia	$\beta 1/\beta 2$ globin	iron overload; homozygous mice die perinatally
$\beta 2$ m -/-	α -hemoglobin	iron overload due to iron absorption defects
<i>TCR</i> δ -/-	$\beta 2$ m	iron overload
<i>RAG1</i> -/-	TCR δ	iron accumulation in the liver
	RAG1	no mature lymphocytes; iron accumulation in the liver

^a Abbreviations used are: $\beta 2$ m – $\beta 2$ microglobulin; mk – microcytic anemia; Nramp – natural resistance-associated macrophage protein; RAG1 – recombination-activating gene 1; sla – sex-linked anemia; TCR – T-cell receptor; TfR1 – transferrin receptor 1; Trf – transferrin.

7.2. Hypotransferrinemic *Trf*^{hpx/hpx} mice

The hypotransferrinemic *Trf*^{hpx/hpx} mouse is a mutant strain exhibiting transferrin deficiency, marked anemia, hyper-absorption of iron, and elevated hepatic iron stores (Bernstein, 1987). Hypotransferrinemic *Trf*^{hpx/hpx} mice have a severe deficiency in serum transferrin as the result of a spontaneous mutation linked to the murine transferrin locus. The gene defective in *Trf*^{hpx/hpx} mice was mapped to the *hpx* locus on chromosome 9. Further studies show that the *transferrin* gene in the *hpx* allele of *Trf*^{hpx/hpx} mice contains a single point mutation, altering an invariable nucleotide in the splice donor site after exon 16 (Trenor et al., 2000). As a consequence, normal *transferrin* mRNA is not made from the *hpx* allele of *Trf*^{hpx/hpx} mice. Mice with hypotransferrinemia have little or no plasma transferrin. *Trf*^{hpx/hpx} mice have massive tissue iron overload in all non-hematopoietic tissues, while they continue to have severe iron-deficiency anemia, indicating that the transferrin cycle is essential for iron uptake by erythroid cells. Other tissues, however, are generally normal, and there is a paradoxical increase in intestinal iron absorption and iron storage.

Despite their severe transferrin deficiency, *Trf*^{hpx/hpx} mice that are initially treated with exogenous transferrin injections or red blood cell transfusions can survive after weaning without any further treatment. Transfusion of erythrocytes obtained from littermate controls is able to increase hemoglobin levels and reduce reticulocyte counts in recipient mice (Trenor et al., 2000). Iron absorption in homozygotes, in contrast to littermate controls, is not reduced by hyperoxia. Transferrin injections, in the short term, increase delivery of iron to the marrow and raise hemoglobin levels. While mucosal iron transfer and total iron uptake are reduced at the higher

transferrin doses, total uptake is still higher than in controls. Daily injections of transferrin for 3 weeks from weaning normalize hemoglobin values and markedly reduce liver iron and intestinal iron absorption values in $\text{Trf}^{\text{hpx/hpx}}$ mice. When daily-injected mice are untreated for a week to allow transferrin clearance, iron absorption values are significantly enhanced; in contrast, hemoglobin or hepatic iron levels are not significantly altered. Thus, hyper-absorption of iron in $\text{Trf}^{\text{hpx/hpx}}$ mice is not solely because of the anemia. Transferrin levels per se do affect iron absorption, possibly via a direct effect on the intestinal mucosa.

The liver iron burden in $\text{Trf}^{\text{hpx/hpx}}$ mice is 100-fold greater than that of normal wild-type mice and 15- to 20-fold greater than the liver iron burden of mice lacking the hemochromatosis gene, *Hfe* (Trenor et al., 2000). Because the amount of tissue iron in $\text{Trf}^{\text{hpx/hpx}}$ mice greatly exceeds that found in mice defective in the *Hfe* gene, it is clear that in the absence of HFE, some regulation of intestinal iron absorption must exist. Interestingly, unlike human iron-overload disorders, the tissues of $\text{Trf}^{\text{hpx/hpx}}$ mice do not develop cirrhosis and pancreatic fibrosis. One explanation for the lack of tissue damage is that such changes might require long-standing iron deposition. Therefore, $\text{Trf}^{\text{hpx/hpx}}$ mice provide an animal model with a defined molecular defect for studying genetic disorders of iron metabolism.

7.3. Microcytic anemic *mk/mk* mice

Microcytic anemic *mk/mk* mice, displaying a phenotypic abnormality in iron metabolism, have inherited defects in intestinal iron transport and erythroid iron utilization that result in severe iron-deficiency anemia (Fleming et al., 1997; Su et al., 1998; Wood and Han, 1998). These *mk/mk* mice and mice with hypoplastic anemias (*W/W^v*, *Sll/Sld*, *an/an*) have mild-to-moderate increases in the expression levels of 5'-aminolevulinate dehydratase, uroporphyrinogen-I synthase, and protoporphyrin IX in erythrocytes. Elevated enzyme activities and protoporphyrin IX correlate well with the number of reticulocytes and probably reflect increased frequency of young red cells that are still active in heme biosynthesis. Because all mice with anemias possess elevated levels of 5'-aminolevulinate dehydratase, uroporphyrinogen-I synthase, and protoporphyrin IX, independent of differences in their genotypes, the increase in these parameters is not likely to be the result of a specific gene defect.

Positional gene cloning in *mk/mk* mice identifies that hypochromic, microcytic anemia is a consequence of a missense (G185R) mutation in the *Nramp2* gene (Su et al., 1998). The G185R mutation causes almost total loss of *Nramp2* function that cannot be fully explained by a decreased amount of protein, indicating that G185R disrupts iron transport by altering *Nramp2* function. Functional studies characterize *Nramp2* as an iron-regulated, proton-coupled divalent cation transporter. *Nramp2* is likely to be the iron transporter that controls iron absorption across the apical membrane of intestinal epithelial cells and the iron transporter (Fig. 3) that functions to transport the endosomal, free ferrous iron into the cytoplasm in the transferrin cycle (Fig. 5).

In addition, *mk/mk* mice also carry a missense mutation that causes substitution of valine to alanine at amino acid 173 of the p45 NF-E2 molecule (Peters et al.,

1993). NF-E2, a nuclear DNA-binding protein, functions as an erythroid-specific transcription factor and is thought to mediate the erythroid enhancer activity of the α - and β -globin locus control regions and participates in controlling the activities of genes that encode two enzymes of heme biosynthesis (porphobilinogen deaminase and ferrochelatase). P45 NF-E2, a major component of NF-E2, is a 45-kDa protein that is specifically expressed in hematopoietic progenitor cells and differentiated cells of the erythroid, megakaryocyte, and mast cell lineages (Andrews et al., 1994). Interestingly, expression of p45 NF-E2 is detected in the duodenum of normal and severely anemic β -thalassemic (*Hbbd-th3/Hbbd-th3*) mice. The gene encoding p45 NF-E2 has been mapped to mouse chromosome 15. Mutations in p45 NF-E2 result in an impaired form of NF-E2 that seems to fail to regulate both globin production and iron metabolism, resulting in the iron deficiency and anemia of mk/mk mice.

7.4. Belgrade (*blb*) anemic rat

The Belgrade (*blb*) rat has an autosomal recessively inherited, microcytic, hypochromic anemia that is associated with abnormal reticulocyte iron uptake and gastrointestinal iron absorption (Fleming et al., 1998). The *blb* reticulocyte defect appears to be failure of iron transport out of endosomes within the transferrin cycle. The phenotypic characteristics indicate that the gene defective in the *blb* rat is essential both for normal intestinal iron absorption and for transport of iron out of the transferrin cycle endosome. Aspects of this phenotype are similar to those reported for the microcytic anemia mutation in the mk/mk mouse. Genetic mapping of the phenotype of the *blb* rat shows a linkage to the centromeric portion of rat chromosome 7, which exhibits synteny to the chromosomal location of *Nramp2* in the mouse (Fleming et al., 1998). Indeed, a glycine-to-arginine missense mutation (G185R) is found in the *blb Nramp2* gene, but not in the normal allele (Su et al., 1998). This amino acid alteration is the same as the alteration seen in the mk/mk mouse. Functional studies of the protein that is encoded by the *b* allele of rat *Nramp2* demonstrate that the mutation disrupts iron transport. Thus, *Nramp2* is the gene defective in the *blb* rat. Expression of HFE and *Nramp2* are reciprocally regulated by cellular iron status in intestinal cells, and these molecules might work in concert to regulate intestinal iron absorption.

7.5. Sex-linked anemic (*sl/sla*) mice

The sex-linked anemic (*sl/sla*) mouse carries an anemia that results from an inherited defect of intestinal iron absorption (Anderson et al., 1998; Vulpe et al., 1999). Although these mice take up iron from the intestinal lumen into mature epithelial cells normally, the subsequent exit of iron into the circulation is diminished. As a result, iron accumulates in enterocytes and is lost during turnover of the intestinal epithelium. Because *sl/sla* mice have a block in intestinal iron transport, they develop moderate-to-severe microcytic hypochromic anemia. The

sla locus is mapped to the central region of the X chromosome. Within this *sla* locus, a gene named *hephaestin*, which encodes a transmembrane-bound ceruloplasmin homologue, is mutated in the *sla/sla* mice (Vulpe et al., 1999). Hephaestin is highly expressed in intestines and a loss of hephaestin function results in the *sla* phenotype. The hephaestin protein appears to function as a multicopper ferroxidase that is necessary for iron egress from intestinal enterocytes into the circulation. The identification of mutant *hephaestin* as the gene responsible for the sex-linked anemia in *sla/sla* mice provides an important link between copper and iron metabolisms in mammals.

7.6. β -thalassemic *Hbbd(th3)/Hbbd(th3)* mice

Maintenance of iron homeostasis must balance the demand for iron due to heme synthesis, which is driven by hematopoiesis and the restricted intestinal uptake of iron that otherwise limit absorption of this toxic element. The consequences of perturbed iron homeostasis are witnessed in inherited forms of β -thalassemia in which erythroid hyperplasia results in enhanced intestinal iron absorption despite tissue iron overload (Van Wyck et al., 1987). A mouse model of β -thalassemia has both the $\beta 1$ and $\beta 2$ *globin* genes deleted (Yang et al., 1995). Mice homozygous for this *Hbbd(th3)/Hbbd(th3)* deletion die perinatally, similar to the most severe form of Cooley's anemia in humans. Homozygous β -thalassemic mice show many of the features seen in human β -thalassemia, such as decreased hemoglobin and hematocrit values, decreased red blood cell count, as well as increased reticulocyte count (Garrick et al., 1989; Yang et al., 1995). They also exhibit splenomegaly and decreased osmotic fragility of red cells. Accumulation of iron occurs in the spleen, liver, and kidneys but not in the heart. An increase in tissue iron occurs primarily in the spleen, even before weaning, despite the low iron content of milk. Iron accumulation in the absence of blood transfusion is of interest because iron overload is the major cause of death in human β -thalassemia. Mice heterozygous for the deletion appear normal, but their hematological indexes show characteristics typical of severe thalassemia, including dramatically decreased hematocrit, hemoglobin, and red blood cell counts; decreased mean corpuscular volume and mean corpuscular hemoglobin concentration; and dramatically increased reticulocyte counts, serum bilirubin concentration, and red cell distribution widths. Tissue and organ damage typical of β -thalassemia, such as bone deformities and splenic enlargement due to increased hematopoiesis, are also seen in the heterozygous animals, as is spontaneous iron overload in the spleen, liver, and kidneys. Several intestinal factors are induced when iron homeostasis is disrupted in β -thalassemic *Hbbd(th3)/Hbbd(th3)* mice. One induced factor is sucrose isomaltase. Expression of this intestinal hydrolase is increased in response to iron overload. This β -thalassemia mouse model should be of great value in developing therapies for the treatment of thalassemias in utero. The heterozygous mice will be useful for studying the pathophysiology of thalassemias and have the potential of generating a model of sickle cell anemia when mated with appropriate transgenic animals.

7.7. α -thalassemic mice

Mice with hereditary α -thalassemia are also a good model for studying clinical disorders of spontaneous iron overload (Van Wyck et al., 1984). As in the human condition, mice heterozygous for a radiation-induced α -hemoglobin gene deletion exhibit a mild hemolytic anemia, with microcytosis, reticulocytosis, splenomegaly, and chemical evidence of defective α -hemoglobin synthesis. Total iron content in the spleen, liver, and kidneys, but not in the heart or lungs, of adult α -thalassemic mice is greater than that in unaffected littermates. Iron concentration is also increased in the liver, spleen, kidneys, and heart; in general, the greater the iron concentration in the liver, the greater the iron concentration in the spleen, kidneys, and heart. In mice examined 8 months postoperatively, splenectomy, as compared to a sham operation, significantly raises iron content in extrasplenic tissues, but does not affect total body iron. At 10–11 weeks of age α -thalassemic mice show higher rates of iron absorption than age-matched controls; this effect is no longer present at 12–14 weeks. Thus, α -thalassemic mice display an early occurring iron-absorption defect, leading to a modest, sustained, non-progressive iron overload, and thereby represent a valuable model for exploring disorders of iron homeostasis.

7.8. *Aceruloplasminemic ceruloplasmin-deficient mice*

Aceruloplasminemia is an autosomal recessive disorder of iron metabolism. Affected individuals evidence iron accumulation in tissue parenchyma in association with absent serum ceruloplasmin (Harris et al., 1998, 1999). Genetic studies of such patients reveal inherited mutations in the *ceruloplasmin* gene (Vulpe et al., 1999). In an animal model of aceruloplasminemia, *ceruloplasmin*-deficient mice are normal at birth, but accumulate iron progressively such that by one year of age, all *ceruloplasmin*-deficient mice have a prominent elevation in serum ferritin and a three- to six-fold increase in the iron content of the liver and spleen. Iron stores within reticuloendothelial cells and hepatocytes are also evident. *Ceruloplasmin*-deficient mice show no abnormalities in cellular iron uptake but a striking impairment in the movement of iron out of reticuloendothelial cells and hepatocytes. Compared to normal wild-type mice, *ceruloplasmin*-deficient mice have equivalent rates of iron absorption and plasma iron turnover, suggesting that iron accumulation is caused by an altered compartmentalization within the iron cycle. Thus, ceruloplasmin plays an essential physiological role in controlling the rate of iron efflux from cells with mobilizable iron stores.

7.9. $\beta 2$ microglobulin-deficient mice

Hereditary hemochromatosis is an inherited disorder of iron absorption, mapping to the non-classical class I *Hfe* gene within the human *major histocompatibility complex* region (Fourie and Yang, 1998; Salter-Cid et al., 2000a; Yang et al., 1996). Because $\beta 2$ microglobulin is an essential component of a functional HFE molecule (Salter-Cid et al., 1999), it is not surprising to learn that in mice, mutations in the $\beta 2$

microglobulin gene causes hepatic iron overload that recapitulates hereditary hemochromatosis in man. Due to increased iron absorption, the $\beta 2$ *microglobulin*-deficient mouse, a first mouse model of hereditary hemochromatosis, has high levels of circulating iron and abnormally high transferrin saturation (Rothenberg and Voland, 1996; Santos et al., 1996). Pathological iron depositions occur predominantly in liver parenchymal cells. Reconstitution with normal hematopoietic cells redistributes the iron from parenchymal to Kupffer cells, but does not correct the mucosal defect. Mucosal uptake of ferric iron, but not of ferrous iron, by $\beta 2$ *microglobulin*-deficient mice is significantly higher when compared with control wild-type mice. Mucosal transfer of iron into the plasma in $\beta 2$ *microglobulin*-deficient mice is higher, independent of the iron form tested. No significant differences are found in iron absorption between control and $\beta 2$ *microglobulin*-deficient mice when anemia is induced either by repetitive bleeding or by hemolysis through phenylhydrazine treatment. However, iron absorption in mice made anemic by dietary deprivation of iron is significantly higher in $\beta 2$ *microglobulin*-deficient mice. The $\beta 2$ *microglobulin*-deficient mice manifest an impaired capacity to down-modulate iron absorption when dietary or parenterally iron-loaded. The higher iron absorption capacity in $\beta 2$ *microglobulin*-deficient mice might involve the initial step of mucosal uptake of ferric iron and the subsequent step of mucosal transfer of iron to the plasma. The $\beta 2$ *microglobulin*-deficient mice fail to limit the transfer of iron from mucosal cells into the plasma. Thus, iron metabolism is defective in the gut mucosa as well as the liver of $\beta 2$ *microglobulin*-deficient mice, providing functional support for a causative role of *Hfe* mutations in hereditary hemochromatosis.

7.10. Hemochromatosis *Hfe*-deficient mice

Hereditary hemochromatosis is a prevalent human disease caused by a defective *Hfe*, which encodes a non-classical major histocompatibility complex class I molecule involved in the regulation of intestinal iron absorption (Salter-Cid et al., 2000a). The *Hfe*-deficient mice with a disrupted *Hfe* gene show profound differences in parameters of iron homeostasis (Bahram et al., 1999; Zhou et al., 1998). By 10 weeks of age, the fasting transferrin saturation of *Hfe*-deficient mice is significantly elevated compared with normal wild-type littermates, and hepatic iron concentration is eight-fold higher than that of normal wild-type littermates. Hepatic iron in the *Hfe*-deficient mice is predominantly in hepatocytes in a periportal distribution. In the spleen, heart, and kidneys, iron concentration is not significantly different. Erythroid parameters are normal, indicating that the anemia does not contribute to the increased iron storage. Positional gene cloning in humans with hereditary hemochromatosis has identified several mutations in the *Hfe* gene. Mice carrying mutations in the murine *hemochromatosis* gene *Hfe* locus are also made available. The first mutation deletes a large portion of the coding sequence, generating a null allele. The second mutation introduces a missense mutation (C282Y) into the *Hfe* locus, but otherwise leaves the gene intact. This C282Y mutation is identical to the disease-causing mutation in humans with hereditary hemochromatosis. Homozygosity for either mutation results in postnatal iron loading. The effects of the null mutation are more

severe than the effects of the C282Y mutation. Mice heterozygous for either mutation accumulate more iron than normal wild-type controls. Interestingly, although liver iron stores are greatly increased, splenic iron is decreased. Thus, *Hfe*-deficient mice lacking *Hfe* expression faithfully mimic human hemochromatosis.

Hereditary hemochromatosis in humans is characterized by excess absorption of dietary iron and progressive iron deposition in liver and several tissues. Liver disease resulting from iron toxicity is the major cause of death in patients with hereditary hemochromatosis. Hepatic iron loading in hereditary hemochromatosis is progressive, despite down-regulation of the classical transferrin receptor 1. In normal mice and murine models of dietary iron overload, dietary iron deficiency, and hereditary hemochromatosis (*Hfe*-deficient), studies on the expression of transferrin receptor 1 and of transferrin receptor 2, which is a recently identified transferrin receptor 1 homologue that binds holotransferrin and mediates cellular uptake of transferrin-bound iron, have provided some clues on the potential role for transferrin receptor 2 in iron uptake by liver tissues. Transferrin receptor 2 is expressed highly in the liver where transferrin receptor 1 expression is low. In particular, expression of transferrin receptor 2 is abundant in hepatocytes. In contrast to transferrin receptor 1, transferrin receptor 2 expression in the liver is not increased in iron deficiency. Hepatic expression of transferrin receptor 2 is not down-regulated with dietary iron loading or in the *Hfe*-deficient mice (Fleming et al., 2000). Transferrin receptor 2 appears to allow continued uptake of transferrin-bound iron by hepatocytes even after transferrin receptor 1 has been down-regulated by iron overload. This transferrin receptor 2-mediated iron uptake might contribute to the susceptibility of liver to iron loading in hereditary hemochromatosis.

In duodenal crypt cells, because HFE physically interacts with transferrin receptor 1 but interacts weakly with transferrin receptor 2, mutations in *Hfe* might attenuate the uptake of transferrin-bound iron from plasma by duodenal crypt cells, which would lead to up-regulation of iron transporters for dietary iron. The iron importer *Nramp2* expresses as two classes of transcripts in the duodenum: one containing an iron responsive element, called *Nramp2* (IRE) and one containing no iron responsive elements, called *Nramp2* (non-IRE). Expression of duodenal *Nramp2* (IRE) is up-regulated under conditions of dietary iron deficiency. In *Hfe*-deficient mice, duodenal *Nramp2* (IRE) mRNA is increased approximately 7.7-fold, despite their elevated transferrin saturation and hepatic iron content. Duodenal expression of *Nramp2* (non-IRE) is not increased, nor is hepatic expression of *Nramp2* increased (Fleming et al., 2000). Thus, *Hfe* mutations appear to lead to inappropriately low crypt cell iron, with resultant stabilization of *Nramp2* (IRE) mRNA, up-regulation of *Nramp2*, and increased absorption of dietary iron. Thus, the primary defect in *Hfe*-deficient mice could, indeed, be traced to an augmented duodenal iron absorption.

In parallel, measurement of the gut mucosal iron content as well as iron regulatory proteins allows a more informed evaluation of various hypotheses regarding the precise role of HFE in iron homeostasis. Future studies on the expression of the iron exporter ferroportin1 in this mutant mouse model of hereditary hemochromatosis will facilitate investigation into the pathogenesis of increased iron accumulation in

hereditary hemochromatosis and provide opportunities to evaluate therapeutic strategies for prevention or correction of iron overload.

7.11. *T-cell receptor δ -deficient mice*

The functional linkage between genetic hemochromatosis and immune function became evident when the *major histocompatibility complex*-encoded, hemochromatosis protein, HFE, was identified (Salter-Cid et al., 2000a). HFE performs an unusual, yet essential, function in iron metabolism; however, our understanding of HFE function in the immune system remains elusive (Salter-Cid et al., 2000a). A recent study on the immune function in *Hfe*-deficient mice had suggested that there was no evidence for a function of HFE in immune responses (Bahram et al., 1999). Yet, some of the data presented seem to support the notion that, at least at the level of the intestine, HFE plays a role in influencing the T cell repertoire (Bahram et al., 1999).

As mutations in the *Hfe* gene per se are insufficient to explain the physiopathology of hereditary hemochromatosis, other genetic and environmental factors must play a role in modulating iron metabolism. The first clue that iron metabolism plays a role in influencing the immune function came from the observation that lymphocyte populations in the lymphocytic compartments of patients with hereditary hemochromatosis are abnormal (Elshof et al., 1999; Salter-Cid et al., 2000a). It has been known that growth of intestinal epithelial cells is dependent on the differentiation of intraepithelial lymphocytes expressing the γ/δ T-cell receptor. Liver iron loading correlates inversely with the ability of the mice to generate a tumor necrosis factor α response by intraepithelial lymphocytes. In *T-cell receptor δ -deficient mice*, the level of liver iron is high, compared to *T-cell receptor α -deficient mice*, whose iron levels do not differ from the levels of normal wild-type control mice (Elshof et al., 1999). These findings suggest a potential role for γ/δ + intraepithelial lymphocytes in the regulation of iron absorption. Iron loading in intestinal epithelial cells is possibly communicated to intraepithelial lymphocytes via the HFE molecule, resulting in a release of tumor necrosis factor α by γ/δ + intraepithelial lymphocytes. Tumor necrosis factor α in turn up-regulates ferritin expression in intestinal epithelial cells.

7.12. *Recombination-activating gene 1-deficient mice*

Under conditions of high dietary iron levels, *recombination-activating gene 1-deficient mice* that lack mature lymphocytes have been reported to accumulate iron in their liver in a pattern similar to that seen in *$\beta 2$ microglobulin-deficient mice* (De Sousa et al., 1994). Moreover, in comparison to *$\beta 2$ microglobulin-deficient mice*, *$\beta 2$ microglobulin and recombination-activating gene 1 double-deficient mice* develop a more severe phenotype and display increased iron absorption and accumulation in the liver, heart, and pancreas (Salter-Cid et al., 2000a). These findings suggest that lymphocytes might influence cellular iron storage and iron-mediated cellular damage.

7.13. Compound mutant mice

Crossbred *Hfe*-deficient mice carrying other mutations that impair normal iron metabolism have also provided insights into the pathogenesis of iron metabolism disorders (Levy et al., 2000). Compound mutant mice that lack both *Hfe* and $\beta 2$ microglobulin have more iron deposited in tissues than mice lacking *Hfe* only, strongly suggesting that another $\beta 2$ microglobulin-interacting molecule(s) might be involved in iron regulation. It is possible that major histocompatibility complex-encoded class I molecules might have a role in iron metabolism (Salter-Cid et al., 2000a). Consistent with the findings from *T-cell receptor* δ -deficient mice, a recent review discussing the interplay between the immune function and iron metabolism hypothesizes that cytokines from T cells play a functional role in regulating expression of *Hfe* and *transferrin* and in modulating iron status through intestinal iron absorption and transport. Because *Hfe*-deficient mice that carry mutations in the iron transporter *Nramp2* fail to load iron, hemochromatosis appears to involve *Nramp2*-mediated iron flux. Similarly, compound mutants deficient in both *Hfe* and *hephaestin* show less iron loading than do *Hfe*-deficient mice, indicating that iron absorption in hemochromatosis involves the function of hephaestin as well. Mutant mice lacking both *Hfe* and the transferrin receptor 1 accumulate more tissue iron than do mice lacking *Hfe* alone, consistent with the idea that interaction between these two molecules contributes to the control of normal iron absorption. Accordingly, each of these genes is a candidate modifier of the hereditary hemochromatosis phenotype.

Interestingly, iron regulatory protein 1-deficient mice display no symptoms and abnormalities of iron metabolism disorders. Iron homeostasis is such an essential physiological process that is delicately and tightly regulated. To minimize any potential risk of abnormal alterations of iron metabolism, redundant pathways that are independent of iron regulatory protein 1 must have evolved. Mice with iron metabolism disorders provide unique animal models to study both iron deficiency and iron overload. Information from these mutant mice is complementary to those from molecular biology, biochemistry, structural biology, molecular medicine, and clinical study of humans with iron metabolism disorders. In future studies, the use of mice that are defective in iron exporter *ferroportin1* or *transferrin receptor 2* or that are cross-bred shall shed light on how iron homeostasis is regulated. These studies will lead to therapeutic strategies for use of these genes as targets to manipulate, with methods directed toward specific cells and tissues, the processes of iron absorption, transport, and storage.

8. Diagnosis of human iron disorders

Iron is essential for a variety of biologic functions, including oxygen transport, mitochondrial electron transfer, DNA synthesis, di-oxygen processing, and enzymatic reactions that require cofactors with redox properties. Reduced iron is also a key participant in oxygen-mediated toxicity involving O_2 -derived free radicals.

Consequently, disturbances of iron homeostasis, leading to either iron deficiency or iron overload, can have significant clinical consequences. To minimize iron-mediated harmful effects and to reduce cost of treatment, early diagnosis of these conditions in patients is a must. Here, diagnostic parameters routinely used to assess and evaluate the status of iron metabolism disorders in humans are described and discussed.

8.1. Parameters for diagnosing iron deficiency

Globally, iron deficiency is the most common cause of anemia. Typically, in iron deficiency anemias, all three red cell indices, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration, are low. These low values are due to defective hemoglobin synthesis. Iron deficiency, although the most common, is not the sole cause of defective hemoglobin synthesis. Hemoglobinopathies, such as thalassemias, and probably certain forms of congenital dyserythropoietic anemias, induce similar conditions. Thus, the diagnosis of these conditions is based on the findings from hemoglobin electrophoresis and cytologic observations.

The hallmarks of iron-deficient erythropoiesis are an increase in free erythrocyte protoporphyrin and microcytosis, which are defined by mean corpuscular volume less than 83 femtoliters per cell. Two major categories of anemia present with these findings: iron deficiency anemia and the anemia of chronic disease. In both, serum iron concentration is low (less than 40 $\mu\text{g}/\text{dl}$) and transferrin saturation is below 15–20% (Cook, 1999). In iron deficiency anemia, serum ferritin values are below 20 $\mu\text{g}/\text{l}$, whereas a serum ferritin above 100 $\mu\text{g}/\text{l}$ is indicative of anemia of chronic disease. High serum ferritin values are encountered also when excess ferritin is released of damaged tissues, as occurs with acute hepatitis. Free erythrocyte protoporphyrin increases in iron deficiency, but increased levels are also seen in lead poisoning, sideroblastic anemia, and erythropoietic porphyria, and tests for these values are not commonly used for differential diagnosis of anemias (Savage et al., 2000; Tancabelic et al., 1999). Serum concentration of the transferrin receptor 1 tends to be within a normal range (5–6 mg/l) in anemia of chronic disease (Flowers et al., 1989), whereas in iron deficiency anemia, this concentration is elevated three-fold (Cook, 1999). However, since the serum concentration of the transferrin receptor 1 reflects the turnover of transferrin receptors in the erythrocyte progenitors in marrow and indirectly measures erythropoietic activities, elevated values are typical of hemolytic anemias and myelodysplasia, as well. Therefore, the transferrin receptor 1–ferritin index, calculated as a ratio of transferrin receptor/log ferritin, is used to evaluate patients with high serum transferrin receptor levels. The normal range of transferrin receptor–ferritin index is 0.076 (± 0.062), whereas in iron deficiency, the range is 3.739 (± 3.413) (Punnonen et al., 1997).

8.2. Parameters for diagnosing iron overload

When iron overload is suspected, serum iron concentration, transferrin saturation, and serum ferritin should be measured. Elevated serum iron level (greater

than 20 μM), transferrin saturation (greater than 50–60%), and serum ferritin (greater than 400 $\mu\text{g/l}$ in males and greater than 300 $\mu\text{g/l}$ in females) are typical of hereditary hemochromatosis patients (Mura et al., 2000). In 98% of hemochromatosis patients, the transferrin saturation is higher than 45% (McLaren et al., 1988); in fact, elevated serum transferrin saturation is an early symptom in hemochromatosis. Serum ferritin concentration reflects more accurately the accumulation of iron. However, serum ferritin values might also be high in conditions not related to increased body iron. For instance, in Gaucher's disease, which is caused by an inherited defect in the β -glucocerebrosidase enzyme, serum ferritin concentration is greater than 1000 $\mu\text{g/l}$ (Beutler, 1997; Punnonen et al., 1997). Moreover, the haptoglobin phenotype affects serum markers of iron status in males; the haptoglobin 2-2-phenotype is associated with elevated serum ferritin (Langlois et al., 2000). The desferrioxamine iron excretion test can be used to evaluate the amount of chelatable iron.

The accumulation of iron in the liver can be estimated by using dual-energy computed tomography and magnetic resonance imaging (Chapman et al., 1980; Jensen et al., 1994); liver biopsy can also be used. These measures provide information regarding not only the iron content of the liver, but also the distribution of iron in the tissues, as well as the presence of putative damage, such as cirrhosis. The hepatic iron content is proportional to the age of the individual, which is why the hepatic iron index is used to assess the iron load of the liver. This value is calculated as micromoles of iron per dry weight gram of liver divided by the patient's age. In hereditary hemochromatosis, the iron in the liver is characteristically in the parenchyma, whereas in other forms of iron storage diseases, it concentrates in the macrophages and Kupffer cells. When the parameters for iron status indicate iron overload, genetic studies might be undertaken to confirm the diagnosis. On the other hand, the absence of mutations in the known hemochromatosis-associated genes, *Hfe*, and *transferrin receptor 2*, does not rule out the hemochromatosis diagnosis (Beutler, 1998). Clinical manifestations of the less precisely characterized forms of hemochromatosis are identical and require the same type of treatment.

9. Iron metabolism disorders in humans

Iron metabolism-related diseases in humans fall into three major categories: (I) diseases associated with defective regulation of iron absorption, (II) diseases caused by erroneous tissue and/or subcellular compartmentalization of iron, and (III) secondary disorders induced by altered iron content in cells and/or tissues (Table 3). Discovering new genetic defects leading to disturbances in iron metabolism and elucidating the molecular basis of these disturbances has produced unexpected insights into the pathophysiology of iron metabolism-related diseases in humans.

Table 3
 Characteristics of iron metabolism disorders in humans

Gene ^a	Chromosome	Defect ^b	Symptom
<i>Ferritin L</i>	19q13	M in IRE ^a	Hypertransferrinemia with cataract
<i>Transferrin</i>	3q21	Null	Congenital atransferrinemia
<i>TfR2</i>	7q22	M	HFE 3 type hemochromatosis
<i>Hfe</i>	6p21	M	Hereditary hemochromatosis
<i>URO-D</i>	1p34	M, I, D	Porphyria cutanea tarda
<i>Ferrochelatase</i>	18q21.3	M	Erythropoietic protoporphyria
<i>Ceruloplasmin</i>	3q21–q24	M, I, D	Aceruloplasminemia
<i>ALAS2</i>	Xp11.21	M	Sideroblastic anemia
<i>ABC7</i>	Xq13	M	Sideroblastic anemia and ataxia
<i>ATPase 7A</i>	Xq13–q21.1	M	Menkes disease; occipital horn syndrome
<i>ATPase 7B</i>	13q14.3	M	Wilson's disease
<i>FRDA</i>	9q13	GAA, M	Friedreich's ataxia
Unknown	1q	Unknown	Juvenile hemochromatosis
Unknown	15q5.1–15.3	Unknown	Congenital dyserythropoietic anemia type I
Unknown	20q11.2	Unknown	Congenital dyserythropoietic anemia type II
Unknown	15q21–25	Unknown	Congenital dyserythropoietic anemia type III
Unknown	Non-MHC ^a	Unknown	African dietary iron overload
Unknown	Unknown	Unknown	Atransferrinemia
Unknown	Unknown	Unknown	Familial hypoferremic microcytic anemia

^a Abbreviations used are: ABC – ATP-binding cassette transporter; ALAS – erythroid-specific 5'-aminolaevulinate synthase; ferritin L – ferritin light chain; FRDA – frataxin; IRE – iron responsive element; MHC – major histocompatibility complex; TfR – transferrin receptor; URO-D – uroporphyrinogen decarboxylase.

^b M – point mutation; I – insertion; D – deletion; GAA – expansion of GAA trinucleotide repeats.

9.1. Defects in iron absorption

9.1.1. Hereditary hemochromatosis

Hereditary hemochromatosis is an autosomal recessive disorder with a high prevalence in Caucasians (0.05–1.20%) (Bell et al., 1997; Cogswell et al., 1998). The condition is characterized by the absorption of iron in excess. The body iron stores accumulate slowly, and the majority of patients present with clinical symptoms at the age of 40–60 years. The classic tetrad, skin pigmentation, diabetes, liver disease, and gonadal failure can be seen in homozygotes diagnosed at older age, whereas the most common complaints are more non-specific, such as weakness, lethargy, and joint and abdominal pain (Crawford et al., 1998; Dooley et al., 1997). A malfunction in the maximal urinary concentration of the kidney is also associated with hereditary hemochromatosis and other iron-overload conditions (Zhou et al., 1996). The iron overload is parenchymal, first affecting hepatocytes but later building up in the pancreas. By the time of diagnosis, the body iron has increased 15- to 20-fold from the normal amount, which is approximately 1 g. Preceding the increase in the body

iron stores, plasma iron concentration and transferrin saturation rise. When the iron load in the liver increases, plasma ferritin concentration also begins to elevate. The excessive iron load induces serious morbidity and mortality and can easily be treated by phlebotomy. In *Hfe* heterozygotes, hereditary hemochromatosis is incompletely expressed: the transferrin saturation is only moderately increased, and the alteration in the body iron stores is minimal (Crawford et al., 1998). Because hereditary hemochromatosis is relatively common in Caucasians of European ancestry, with at least 0.5% of the population estimated to be homozygous and 10% heterozygous for the HFE C282 mutation (Cogswell et al., 1998), screening for early diagnosis might prove cost-effective.

Hereditary hemochromatosis is known as a disease entity long before the causative gene *Hfe* was identified. The facts that patients with inherited hemochromatosis frequently have the HLA-A3 allele (Simon et al., 1987) and that β_2 microglobulin-deficient mice develop an iron-overload syndrome similar to human hereditary hemochromatosis (De Sousa et al., 1994) led to the assumption that the hereditary hemochromatosis gene is linked to the *HLA* gene locus at chromosome 6p21. In 1996, a gene, first named HLA-H but later renamed *Hfe* with similarity to classical HLA class I genes was shown to be mutated in 87% of patients with hereditary hemochromatosis (Feder et al., 1996). The predominant mutation is the substitution of cysteine at amino acid residue 282 to tyrosine (C282Y). The second mutation, histidine to aspartic acid at residue 63 (H63D), is enriched in patients who are concomitantly heterozygous for the C282Y mutation. Mutations at S65C, I105T, and G93R in *Hfe* have been detected recently. In addition, some allelic variations at the intron areas of the *Hfe* gene have been found; these might have relevance for the *Hfe* gene function (Barton et al., 1999). Interestingly, in the United States, there remains a subpopulation of clinically indistinguishable hereditary hemochromatosis patients who do not have any of these *Hfe* mutations, which suggests that other, probably HLA-linked, genes control iron absorption (Barton et al., 1997).

Individuals homozygous to the *Hfe* gene mutations have significantly higher levels of blood lead than wild-type *Hfe* carriers. In heterozygotes, the blood lead is moderately elevated. In individuals with hereditary hemochromatosis, cobalt is also absorbed in excess (Valberg et al., 1969). Evidently, iron-deficient individuals, hereditary hemochromatosis homozygotes, and heterozygotes absorb increased amounts of lead and/or other metal ions (Barton et al., 1994). Animals defective in *Hfe* only develop hereditary hemochromatosis if the iron transporter *Nramp2* and the *hephaestin* gene products are intact, supporting the view that the pathogenesis of hereditary hemochromatosis involves excessive iron absorption from the gut (Levy et al., 2000). Consistent with this notion, in hereditary hemochromatosis patients, a sustained iron regulatory protein 1 activity is reported to enhance the expression of the transferrin receptor 1 and to hinder the expression of ferritin (Cairo et al., 1997). *Nramp2* is also up-regulated in patients with hereditary hemochromatosis (Zoller et al., 1999). Recently, wild-type *Hfe* has been demonstrated to bind to the transferrin receptor 1 and to co-traffick with the transferrin receptor 1 to the cell surface. This physical interaction not only lowers transferrin receptor affinity to bind iron–

transferrin (Feder et al., 1998), but also inhibits transferrin receptor internalization (Salter-Cid et al., 1999, 2000a). Possibly, hereditary hemochromatosis patients lack this down-regulatory mechanism, thus altering iron absorption activity.

9.1.2. HFE3-type hemochromatosis

A second type of hereditary hemochromatosis, mapping to the HFE3 locus on 7q22, has been recently described. Patients with the HFE3-type hereditary hemochromatosis have a homozygous nonsense mutation in the gene that encodes for the transferrin receptor 2. The role of transferrin receptor 2 in iron metabolism is poorly understood (Camaschella et al., 2000a,b). Two different *transferrin receptor 2* transcripts have been described, the α -transcript translates to a transmembrane protein and the β -transcript, which is a result of alternative splicing, that translates to an intracellular protein (Kawabata et al., 1999). It seems possible that transferrin receptor 2 function is associated with iron regulation rather than directly involved in iron uptake. Seemingly, functional transferrin receptor 2 is not necessary for iron export, since the excess iron in patients with HFE3-type hemochromatosis can be mobilized from the liver by phlebotomias (Camaschella et al., 2000a,b).

9.1.3. Juvenile hemochromatosis

Juvenile hemochromatosis is an autosomal-recessive disorder that is characterized by an early onset of severe iron overload (Camaschella, 1998). The clinical manifestations, hypogonadotropic hypogonadism, abdominal pain, and cardiac dysfunction, typically appear before the age of 30 years. The spectrum of atypical hemochromatosis includes two distinct familial forms: juvenile hemochromatosis and a novel form of familial iron overload, with apparently autosomal-dominant inheritance, predominant Kupffer cell siderosis, and possible minimal dyserythropoiesis on bone marrow exam. Forms of atypical hemochromatosis appear unrelated to either *Hfe* or β_2 *microglobulin*. The locus for juvenile hemochromatosis has been identified on the long arm of chromosome 1, but the gene that is affected is still unknown (Roetto et al., 1999). Detailed linkage studies are required to identify the involved genes, which might encode novel molecules crucial to regulating iron metabolism.

9.1.4. African dietary iron overload

The dietary iron overload among Africans is a distinct disease entity. Recent evidence indicates that it is caused by an interaction of increased dietary iron and a genetic defect that is not associated with the HLA locus (Gordeouk et al., 1992; Kasvosve et al., 2000). The increase in iron load can be comparable with that in hereditary hemochromatosis, with similar clinical manifestations. In this disease, macrophages accumulate significant amounts of iron, which has a special consequence in the regions of Africa where tuberculosis is endemic: The iron-laden macrophages are less cytotoxic to microbes, making iron-overloaded tuberculosis patients extremely vulnerable (Moyo et al., 1997).

9.1.5. Iron deficiency-induced anemia

Iron deficiency-induced anemia is the most common iron metabolism-related disease globally. Iron deficiency can be attributed to either inadequate dietary iron absorption, which includes low dietary availability, abnormally high gastric pH, and loss or dysfunction of absorbing enterocytes, or to increased iron losses that are contributed by blood losses associated with malignant and inflammatory diseases, hormonal disorders or trauma. As a first step, iron deficiency leads to the depletion of iron stores, as reflected by low serum ferritin levels. Thereafter, iron-deficient erythropoiesis gives rise to increased levels of protoporphyrin and zinc protoporphyrin in erythrocytes and elevates serum transferrin receptor 1 concentration (Andrews and Fleming, 1999). Because of high demand and frequent heme-iron losses, young children and premenopausal women, respectively, are at the highest risk to develop the condition. Differentiating iron deficiency anemia from other types of anemias is usually uncomplicated. Elevated serum transferrin receptor level is a sensitive indicator of iron depletion. However, erythroid marrow activity is also reflected in serum transferrin receptor 1 concentration, elevated levels being associated with hyperplastic anemia or myelodysplasia. The ratio of transferrin receptor 1/log ferritin has recently been shown to be a more specific discriminator between iron deficiency anemia and chronic disorders (Punnonen et al., 1997).

9.2. Defects in iron transport

9.2.1. *Porphyria cutanea tarda*

Porphyria cutanea tarda is an example of hepatic-type porphyrias. There is a sporadic and a familial form, the latter presenting as an autosomal-dominant trait. Uroporphyrinogen decarboxylase deficiency is responsible for the genetic cutaneous porphyria, which is characterized by photosensitive cutaneous lesions and hepatic dysfunction and is precipitated by various exogenic factors. In hepatoerythropoietic porphyria, the same enzyme has a defect, but this disorder is transmitted as a recessive trait and is more severe than porphyria cutanea tarda. At least 39 mutations in the *uroporphyrinogen decarboxylase* gene are known (Mendez et al., 2000). As a result of decreased or lacking uroporphyrinogen decarboxylase activity, porphyrins are increased in the liver, plasma, urine, and stool. Mild-to-moderate iron overload is found in most patients with porphyria cutanea tarda. Iron overload seems to be a cofactor for the clinical manifestations of porphyria cutanea tarda. Although no known hemochromatosis-inducing genetic defects are detected in all porphyria cutanea tarda patients, an underlying alteration in iron metabolism is possibly necessary for the uroporphyrinogen decarboxylase deficiency to become symptomatic (Bulaj et al., 2000).

9.2.2. *Erythropoietic protoporphyria*

Ferrochelatase catalyzes the terminal step of the heme biosynthetic pathway by incorporating a ferrous ion into protoporphyrin. The expression of mammalian ferrochelatase is regulated by intracellular iron levels (Taketani et al., 2000). Defects in the human *ferrochelatase* gene seem to lead to erythropoietic protoporphyria, a

rare autosomal-dominant inherited disorder characterized by skin photosensitivity and by overproduction and accumulation of protoporphyrin IX in erythrocytes, plasma, and liver (Schneider-Yin et al., 2000). Therapeutic inhibition of the enzymatic activity of ferrochelatase has been proposed to be beneficial in cancer treatment. The resulting accumulation of protoporphyrin IX would make cancer cells photosensitive, providing a new approach in eliminating malignant cells (Bhasin et al., 1999).

9.2.3. *Familial hypoferremic microcytic anemia syndrome*

Microcytic anemia with iron malabsorption is an inherited disorder of iron metabolism. In the absence of any gastrointestinal disorder or blood loss, patients present with severe hypoproliferative microcytic anemia and iron malabsorption. There is severe microcytosis (mean corpuscular volume 48 fl, hemoglobin 7.5 g/100 ml) with decreased serum iron, elevated serum total iron binding capacity, and decreased serum ferritin, even despite of prolonged treatment with oral iron. Despite lifelong severe hypoferremia, the growth, development, and intellectual performance of these patients are normal (Andrews, 1999a,b; Pearson and Lukens, 1999). Treatment with intravenous iron dextran leads only to a partial correction of the hemoglobin, hematocrit, and microcytosis.

The features of the disorder resemble those found in the microcytic anemia mk/mk mouse, which also has severe microcytic anemia and iron malabsorption that only partially responds to parenteral iron. The ferrokinetics, however, differ from those of the mk/mk mouse, which has a missense mutation in *Nramp2*. In familial hypoferremic microcytic anemia syndrome, once iron enters the plasma, its subsequent metabolism, transfer into erythroid bone marrow cells, and ensuing incorporation into erythrocyte hemoglobin are all normal. The underlying defect seems to be a yet undefined abnormality in mobilization of iron into the plasma from both the intestinal mucosal and reticuloendothelial cells.

9.2.4. *Congenital dyserythropoietic anemias*

Congenital dyserythropoietic anemias are a heterogenous group of rare iron disorders characterized by ineffective erythropoiesis and dysplastic changes in erythroblasts (Andrews and Fleming, 1999; Rothenberg et al., 2000). Type I congenital dyserythropoietic anemia is inherited as an autosomal recessive trait. The causative gene is mapped on chromosome 15q15.1–15.3. Neonatal manifestations are anemia, hepatosplenomegaly, transient cardiac abnormalities, and in some cases dysmorphic features. Most adults have iron overload, which increases progressively by the age. Type II congenital dyserythropoietic anemia, which is the most common of this group of disorders, is also autosomally recessively inherited. The affected gene is located on chromosome 20q11.2. The degree of anemia varies. Plasma bilirubin is usually high, due to ineffective erythropoiesis and peripheral hemolysis. Iron overload is occasionally severe. Type III congenital dyserythropoietic anemia is the least common of the three classic types of the disorder. There are two forms of type III congenital dyserythropoietic anemia, one is inherited as autosomal dominant trait, whereas the other is sporadic. The disease gene is located on chromosome 15q21–25.

The phenotypic features of the inherited form include fatigue, episodes of abdominal pain and jaundice, increased prevalence of monoclonal gammopathy. Iron overload does not develop probably because of iron loss from hemosiderinuria.

9.2.5. *Aceruloplasminemia*

Aceruloplasminemia is an autosomal-recessive disorder resulting from deficient ceruloplasmin ferroxidase activity, due to mutations in the *ceruloplasmin* gene (Harris et al., 1998). The lack of ferroxidase results in defective oxidation of ferrous iron to ferric iron. This in turn reduces iron binding to transferrin, impairing the transport of iron from intracellular stores to plasma. Similar to hereditary hemochromatosis patients, affected individuals present with parenchymal iron accumulation. The manifestations of this disorder also include decreased serum iron and microcytic anemia. The condition leads to diabetes, retinal degeneration, and neurologic symptoms. Unlike other iron-overload syndromes, aceruloplasminemia is dominated by neurologic manifestations, with patients eventually dying from the effects of iron accumulation in the basal ganglia. The underlying disorder results from an imbalance in the compartmentalization of iron rather than from an increase in the absolute amount of body iron. Ceruloplasmin, through its plasma ferroxidase activity, appears to provide a gradient for the cellular efflux of ferrous iron. A similar ferroxidase activity has been found to be associated with the *FET3* gene product in yeast and with the *hephaestin* gene product in murine (Vulpe et al., 1999).

Consistent with the above findings, ceruloplasmin has been reported to play an essential role in the movement of stored iron from reticuloendothelial cells and hepatocytes (Harris et al., 1999). Because the predominant symptoms in aceruloplasminemic patients come from the basal ganglia, ceruloplasmin seems to play a role in controlling the iron efflux from the storage sites in the central nervous system. The increased oxidative stress associated with aceruloplasminemia is due to the excessive iron accumulation and subsequent increased amount of oxygen-free radicals (Yoshida et al., 2000). There is evidence that aceruloplasminemia leads to increased lipid peroxidation in the brain. Aceruloplasminemia has also been reported to be associated with Parkinson's disease (Kohno et al., 2000). The presence of neurological symptoms in patients with aceruloplasminemia is in concordance with the recent findings indicating that astrocyte specific *ceruloplasmin* gene expression is important for neuronal survival in the retina and basal ganglia (Gitlin, 1998).

9.2.6. *Wilson's disease*

Wilson's disease is an autosomal-recessive genetic disorder, resulting from an inability to transfer copper into the secretory pathway to form holoceruloplasmin. The gene defective in Wilson's disease, *ATPase 7B*, encodes for a copper-transporting P-type adenosine triphosphatase (Petrukhin et al., 1994). *ATPase 7B* is abundantly expressed in the liver. More than 100 different mutations of the Wilson's disease gene have been reported so far, and the affected individuals are either homozygous or compound heterozygous. Mutations in this gene lead, not only to excessive accumulation of hepatic copper because of impaired excretion of biliary copper, but also to defective ceruloplasmin biosynthesis. Ceruloplasmin is an α_2 -

serum glycoprotein, each molecule binding up to six atoms of copper. In the absence of copper, a non-stable apoprotein is secreted and rapidly degraded, resulting in low serum ceruloplasmin and a defect in iron metabolism. Excretion of copper by the trans-Golgi network-resident ATPase 7B protein appears to be achieved by ligand-induced apical sorting (Roelofsen et al., 2000) and/or by excretion via lysosomes (Harada et al., 2000). Overexpression of *ATPase 7B* is known to be associated with cisplatin resistance in certain tumors (Komatsu et al., 2000).

9.2.7. *Menkes disease*

Menkes disease is an X-linked genetic disorder, where the cation copper-transporting P-type ATPase gene, *ATPase 7A*, is mutated. *ATPase 7A* is expressed in a variety of tissues, except liver. The protein is localized in the trans-Golgi network and post-Golgi vesicular compartment in the cell (Suzuki and Gitlin, 1999). Defects in *ATPase 7A* prevent copper efflux into the secretory pathway, disturbing stable ceruloplasmin synthesis and deranging iron compartmentalization. The primary metabolic defect causes accumulation of dietary copper in the intestine, whereas the blood, liver, and brain are in a state of copper deficiency (Kodama et al., 1999). Clinically, Menkes disease presents as an early onset neurodegenerative disorder. The characteristic features, including the neurological disturbances, arterial degeneration, and hair abnormalities, can be attributed to the decrease in cuproenzyme activities. More than 150 point mutations have now been identified in the *ATPase 7A* gene. Most of these mutations lead to the classic form of Menkes disease. The milder form of *ATPase 7A*-mediated disease, the occipital horn syndrome, seems to result from mutations located in the splice-donor site of intron 6, occasionally allowing the transcription of minimal amounts of normally spliced *ATPase 7A* (Moller et al., 2000). Interestingly, expression of *ATPase 7A* is suppressed in colon cancer (Cao et al., 1997).

9.2.8. *Sideroblastic anemias*

X-linked sideroblastic anemia is caused by mutations in the erythroid-specific *5'-aminolevulinate synthase* gene (Cox et al., 1991; Raskind et al., 1991). The encoded protein is a key enzyme in heme synthesis. Mutations in erythroid-specific *5'-aminolevulinate synthase 2* lead to ineffective erythropoiesis, an increased number of erythroblasts, and erroneously increased iron absorption. Eventually, the gene defects cause the unused iron to accumulate in the liver, heart, pancreas, and brain, leading to typical clinical manifestations. At least 15 different point mutations have been found in the erythroid-specific *5'-aminolevulinate synthase 2* gene. Pyridoxine, which is metabolized to pyridoxal 5' phosphate, a cofactor of the erythroid-specific *5'-aminolevulinate synthase*, usually ameliorates the condition. This response suggests that the defective erythroid-specific *5'-aminolevulinate synthase 2* gene products still have some enzyme activity, which can be boosted by an overdose of the cofactor. Reversal of the iron overload by phlebotomy results in higher hemoglobin concentrations during pyridoxine supplementation in certain probands.

9.2.9. Sideroblastic anemia and ataxia

X-linked sideroblastic anemia and ataxia is a distinct type of sideroblastic anemia. This recessive disorder is characterized by an early onset of non-progressive cerebellar ataxia and mild anemia with hypochromia and microcytosis. *ATP-binding cassette transporter 7*, the gene responsible for the disease, has been mapped to Xq13 (Allikmets et al., 1999; Shimada et al., 1998). The *ATP-binding cassette transporter 7* gene is an ortholog of the yeast *ATMI* gene, whose product localizes to the mitochondrial inner membrane and is involved in heme transport from the mitochondria. In the absence of the wild-type *ATP-binding cassette transporter 7* gene product, mitochondria accumulate iron, with resulting injury especially to neuronal and erythroid cells.

9.2.10. Friedreich's ataxia

Friedreich's ataxia is a progressive and degenerative autosomal-recessive disease, which is caused by mutations in the *FRDA* gene that encodes frataxin, a nuclear-encoded mitochondrial protein (Campuzano et al., 1996; Durr et al., 1996). The gene has been mapped on chromosome 9q13–21 (Bidichandani et al., 1998; Cossee et al., 1997). Frataxin is required for normal iron efflux from mitochondria and in the absence of the functional wild-type protein, mitochondria accumulate iron, which leads to increased generation of free-radicals and cellular damage (Radisky et al., 1999). Affected individuals are usually homozygous for the expansion of a GAA-triplet repeat within the *FRDA* gene, but compound heterozygosity for a point mutation and GAA-repeat expansion are also possible (De Castro et al., 2000). The importance of the iron load for the etiopathogenesis of Friedreich's ataxia is underlined by the finding that the iron chelator, deferoxamine, is effective both in decreasing brain iron stores and in inhibiting the progression of neurological symptoms (Miyajima et al., 1997).

9.2.11. Hyperferritinemia with autosomal-dominant congenital cataract

Hyperferritinemia with autosomal-dominant congenital cataract is a disorder where the regulatory role of iron in the translational control of the iron storage protein, ferritin, is disrupted by multiple point mutations in the iron responsive element of the *ferritin* light chain mRNA (Beaumont et al., 1995). Consequently, the ferritin light chain is translated in excess, and the serum ferritin levels are moderately elevated. The only clinical manifestation seems to be bilateral cataract. Interestingly, high levels of *ferritin* light chain mRNA are found in the human lens, whereas the amount of ferritin light chain protein is negligible (Cheng et al., 2000). In individuals carrying mutations in the iron responsive elements of *ferritin* light chain mRNA, the levels of mRNA and protein of *ferritin* light chain appear to be changed accordingly. As a consequence of increased ferritin deposit, cataract is induced.

9.2.12. Atransferrinemia

Atransferrinemia is a rare autosomal-recessive condition, where plasma transferrin is either very low or absent. Consequently, iron enters the portal system as

non-transferrin bound iron, and accumulates in the liver. Because of the defective transport mechanism the physiologic need of iron for erythropoiesis is not met. Clinical manifestations include severe microcytic anemia and iron deposits in the parenchymal organs (Hamill et al., 1991).

9.3. Secondary iron disorders

9.3.1. Chronic inflammation-associated anemia

Chronic inflammation is associated with anemia, which has features in common with iron-deficiency anemia. Typical findings are low serum iron levels, low iron-binding capacity, increased serum ferritin, and normocytic, or slightly microcytic, anemia (Andrews, 1999a,b). In chronic inflammation-associated anemia, while there is an increased tendency toward ferritin conversion to haemosiderin, which is less available for iron efflux than ferritin, the labile or chelatable iron pool is diminished (Hoy and Jacobs, 1981). Under the conditions of altered iron recycling, the erythropoiesis is iron-deficient. The decreased release of iron from macrophages to plasma, reduced red cell life span, and inadequate erythropoietin response to anemia together participate in the pathogenesis of the conditions. The mechanism of the altered iron metabolism in chronic inflammation and infection is not fully clarified, but the importance of cytokines and nitric oxide as mediators of the process has been recognized.

Inflammatory cytokines, such as interleukin 1, interleukin 6, tumor necrosis factor α , and interferon γ , seem to regulate the binding of iron–transferrin to cells (Fahmy and Young, 1993). Tumor necrosis factor α , possibly via activation of interleukin 6 and interferon γ , induce a primary stimulation of ferritin synthesis, resulting in a decrease in the release of tissue iron (Feelders et al., 1998). An intracellular, chelatable pool of iron regulates the transcription of interleukin 1 β (O'Brien-Ladner et al., 2000), which in turn enhances the synthesis of both ferritin heavy and light chains (Hirayama et al., 1993). Lipopolysaccharide and interferon γ increase the binding activity of iron regulatory protein 1, whereas RNA binding of iron regulatory protein 2 is decreased. The decrease of iron regulatory protein 2 binding and/or protein levels is associated with an inducible nitric oxide synthase-dependent decrease in transferrin receptor 1 mRNA levels. Interferon γ also enhances the synthesis of ferritin (Kim and Ponka, 2000). Moreover, polymorphonuclear cells secrete apolactoferrin at the site of inflammation, locally changing the availability of iron. This protein binds iron in an acidic milieu, restricting its availability to microbes, inhibiting hydroxyl radical formation, but at the same time leading to hypoferrinemia on the host side. Lactoferrin seems to be anti-inflammatory as well, probably because of its inhibitory effect on local production of tumor necrosis factor α and on migration of epidermal Langerhans' cells (Cumberbatch et al., 2000). Conceivably, the changes that the inflammatory process induces on iron metabolism are part of the host defence against invading pathogens and tumor cells. The effects of cytokines on the expression of ferritin and iron regulatory proteins are another examples of how immune function is intimately associated with iron metabolism functionally, and vice versa.

9.3.2. Alcohol-induced iron abnormalities

Alcohol abuse is associated with excessive iron accumulation, which is due to unregulated, increased iron absorption via the non-carrier-mediated paracellular route (Duane et al., 1992). Further, iron accumulation is probably due to pancreatic insufficiency, which leads to failure to neutralize the gastric effluent and as a consequence, increases the availability of the soluble form of ferrous iron (Sherwood et al., 1998). The metabolism of ethanol has been proposed to form free radicals, suggesting that the toxicity of ethanol might be potentiated by iron overload, and vice versa. Concordantly, it has been reported that hepatic iron enhances ethanol-induced liver lesions, but not those caused by an inflammatory process. In addition to the increased iron load, the clinical expression of acute hepatic porphyrias can also be triggered directly by alcohol, because of induction of δ -aminolevulinic acid synthase in the liver. In chronic hepatic porphyrias, alcohol potentiates the disturbance of the decarboxylation of uro- and hepta-carboxyporphyrinogen, which leads to their accumulation (Doss et al., 2000). Interestingly, serum ferritin levels are increased in men using large quantities of alcohol. This can be used as a marker of significant alcohol intake, but the mechanism and biologic relevance of the finding remain to be determined.

9.3.3. Chronic hepatitis C

Chronic hepatitis C is often associated with excessive iron accumulation in the liver (Di Bisceglia et al., 1992; Snover, 2000). Serum and liver markers of iron overload are elevated more often in patients with chronic hepatitis C than in patients with other chronic liver diseases. The iron deposits are predominantly found in hepatocytes in patients with chronic hepatitis C. The mechanism of the chronic hepatitis C-associated iron accumulation is unclear, although increased intestinal iron absorption (Snover, 2000) and ineffective erythropoiesis have been proposed. The increased iron load in the hepatocytes of chronic hepatitis C patients is mediated by cytokines. In these patients, lipid peroxidation in hepatocytes is elevated. Iron accumulation and lipid peroxidation might contribute to the pathogenesis of chronic hepatitis C. Supporting this hypothesis, iron load and lipid peroxidation simultaneously decrease in patients who respond to interferon γ treatment (Kageyama et al., 2000).

9.3.4. Tumorigenesis

Anemia is a frequent symptom in malignant disorders, but the underlying pathogenetic mechanism is not clear. It might be related directly to the malignant process, but often it seems to be caused by multiple mechanisms that associate with the growth of cancer, chronic infection, and/or inflammation. One of the causes of cancer-related anemia is shortened red blood cell survival, which is not being compensated by erythropoiesis. In addition, the levels of erythropoietin in the serum of cancer patients are low in relation to the degree of anemia. Even though erythropoietin treatment ameliorates the anemia, the response of bone marrow of cancer patients is weaker than the response of healthy individuals (Zucker et al., 1974). The erythropoiesis might be inhibited by cytokines, such as interleukin 1, interleukin 6,

tumor necrosis factor α , and interferons. Patients with malignancies have characteristic alterations in iron metabolism. Serum ferritin levels are elevated, and serum iron concentrations are decreased, suggesting a shift of iron toward storage sites, as in chronic inflammation (Dorner et al., 1983; Koller et al., 1979). Elevated levels of serum ferritin correlate with tumor progression in head and neck carcinomas and can be used as a follow-up marker (Rosati et al., 2000). In renal cell carcinoma patients, the serum ferritin concentration of the renal vein is correlated with tumor size and is proposed as a prognostic marker (Kirkali et al., 1999). The diagnostic value of bronchoalveolar lavage fluid ferritin as a lung tumor marker has been confirmed as well (Fracchia et al., 1999). Given the fact that ferritin in cerebrospinal fluid is produced by glioblastoma cells, it is evident that ectopic expression of ferritin is relatively common in transformed cells.

Despite the altered iron compartmentalization and/or accompanying microcytic anemia encountered in cancer patients, elevated iron levels are considered a risk factor for malignancies. Excessive iron load in the liver, pancreas, skin, and connective tissue has been linked to an increased risk for carcinomas and sarcomas, respectively (Weinberg, 1999), probably because of increased oxidative stress. Iron has also been reported to be a risk factor for colon cancer. Individuals with serum ferritin levels higher than 70 $\mu\text{g/l}$ have an increased risk of recurrence of colon adenoma as compared to those with lower values (Tseng et al., 2000). It is apparent that, rather than the total body iron load, the luminal exposure to excessive iron increases the risk of colon cancer in combination with a high-fat diet.

Iron is necessary for proliferating cells and recently is of interest in therapeutic iron deprivation as a component of cancer therapy. Gallium nitrate and the iron chelator desferrioxamine have been tested in the clinical setting, whereas anti-transferrin receptor 1 antibodies are in the early phase of clinical trials. Gallium is taken into cells via transferrin binding (Dobson et al., 1998), but significant transferrin-independent cellular uptake also takes place (Luttropp et al., 1998). Gallium might interfere with cellular iron metabolism, probably disturbing endosomal acidification, which is necessary for the release of ferric iron from transferrin (Chitambar and Seligman, 1986). Alternatively, gallium might limit the availability of iron to the ribonuclease reductase M2 subunit, thus decreasing intracellular deoxyribonucleotide pools (Chitambar et al., 1988). Desferrioxamine enters cells passively and binds the labile pool of iron. The principal effect of desferoxamine treatment appears to be inhibiting ribonucleotide reductase activity (Lederman et al., 1984), thus preventing DNA synthesis during tumorigenesis.

9.3.5. Neurodegenerative diseases

9.3.5.1. Parkinson's disease. Irreversible and regionally specific neurodegeneration and the presence of Lewy bodies are the essential pathological hallmarks of idiopathic Parkinson's disease. In Parkinson's disease patients, the iron content in brain tissue is increased. In particular, the ferric iron is accumulated in brains (Sofic et al., 1988). The transferrin/iron ratio is decreased in the globus pallidus and caudate of Parkinson's disease patients, suggestive of a defect in iron mobilization (Loeffler

et al., 1995). The number of ferrotransferrin binding sites is increased in patients with Parkinson's disease, probably due to increased iron uptake capacity of dopaminergic nerves or of brain microvessels. The ultimate cause of neuronal death in patients with Parkinson's disease is not clear, but a growing body of evidence indicates that increased oxidative stress is one of the main culprits. However, whether oxidative stress is a primary or secondary event is unclear. By its enhancing effect on the formation of free radicals and on the generation of oxidative stress in cells, iron might to play a central role in the development of Parkinson's disease and other neurodegenerative diseases.

The selective damage to melanin-containing substantia nigra neurons in Parkinson's disease is possibly initiated by the interaction between iron and neuromelanin, resulting in the accumulation of ferric iron. Substantia nigra neurons might be at a special risk for damage by free radicals because of their dopamine metabolism. Dopamine auto-oxidation leads to the formation of hydrogen peroxide as a by-product and, if not effectively detoxified by glutathione, hydrogen peroxide might potentially induce the generation of highly reactive hydroxyl radicals in the presence of excess iron. The amount of neuromelanin, formed by polymerized oxidized dopamine, seems to direct the vulnerability of neurons. To investigate the process of neurodegeneration, oxidative stress induced by the neurotoxins 6-hydroxydopamine and N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine has been used in animal models. Radical scavengers and iron chelators are found to be neuroprotective. Because the pathogenesis of Parkinson's disease involves several cellular processes and metabolic pathways, including dopamine metabolism, nitric oxide synthesis, activation of calcium channels, and inflammatory processes with activated $\text{NF}\kappa\beta$ and cytotoxic cytokines, success in treating Parkinson's disease with agents that prevent iron accumulation has not been achieved desirably (Grunblatt et al., 2000).

9.3.5.2. Hallervorden–Spatz syndrome. Hallervorden–Spatz syndrome, a neurodegeneration with an accumulation of brain iron, is a rare disorder characterized clinically by Parkinsonism, cognitive impairment, pseudobulbar features, as well as cerebellar ataxia. Neuropathological findings include neuronal loss, gliosis, and iron deposition in the globus pallidus, red nucleus, and substantia nigra, with axonal spheroids as hallmark lesions (Galvin et al., 2000). The relationship between iron and the pathogenesis of Hallervorden–Spatz syndrome remains to be investigated.

9.3.5.3. Alzheimer's disease. β -amyloid deposits, the hallmarks of Alzheimer's disease, contain advanced glycosylation end products and copper and iron ions. The formation of covalently cross-linked, high-molecular-weight, β -amyloid peptide oligomers is accelerated by the presence of micromolar amounts of copper and iron ions (Loske et al., 2000). The amount of ferritin iron in basal ganglia is increased in Alzheimer's disease at its onset, suggesting that the accumulation of iron might have a role in the pathogenesis of the disease (Bartzokis and Tishler, 2000). Iron has been shown to accumulate in Alzheimer's disease without a concomitant increase in ferritin. The lack of increase in the amount of translated ferritin might be due to the fact that iron regulating protein 1 seems to form more stable complexes with the iron

responsive elements of *ferritin* and *transferrin receptor 1* mRNA in the brain of Alzheimer's disease patients than of healthy individuals, resulting in an inhibition of ferritin translation (Pinero et al., 2000a,b). The pool of free iron might thus be increased, leading to enhanced vulnerability to oxidative damage. Indeed, Alzheimer's disease is characterized by signs of major oxidative stress in the neocortex, with simultaneous deposition of β -amyloid proteins. This metalloprotein converts molecular oxygen into hydrogen peroxide by reducing copper or iron, potentially leading to the generation of free radicals through the Fenton reaction (Lynch et al., 2000). The connection between iron metabolism and Alzheimer's disease is further supported by the fact that the *C2 transferrin* allele is more frequently associated with patients with Alzheimer's disease than with controls. Because the presence of the *C2 transferrin* allele in patients with Alzheimer's disease seemingly shifts the onset of the disease to an earlier age, transferrin might be involved in the pathogenesis of Alzheimer's disease (van Rensburg et al., 2000).

9.3.5.4. Huntington's disease. Huntington's disease is a progressive and fatal neurological disorder caused by the expansion of a CAG trinucleotide repeat in exon 1 of the gene coding for huntingtin, the Huntington's disease protein. The function of the *huntingtin* gene product is unknown, as is the exact cause of neuronal death in Huntington's disease. Toxicity and apoptosis induced by oxidative stress are hypothesized to be involved (Gutekunst et al., 2000). Because basal ganglia iron levels are elevated early in the disease, the accumulation of iron might play a causative role in the pathogenesis of the disease (Bartzokis et al., 1999).

10. Conclusions and future perspectives

“Biology of iron overload and new approaches to therapy” has been identified as one of the priority areas in the national “Healthy People 2000” initiative by the Public Health Service of the United States of America. The identification of *Hfe* as a hereditary *hemochromatosis* gene and *ferroportin1* as an iron exporter represents a major breakthrough and creates a strong impetus for rapid advances in the molecular understanding of the pathophysiology of hereditary hemochromatosis, the mechanisms of iron absorption and transport, and the cellular regulation of iron metabolism.

Iron is transported through the plasma by transferrin, an iron carrier molecule, which binds to transferrin receptors at the cell surface. Transferrin receptors provide the main route for the entry of iron into the cells via receptor-mediated endocytosis. Recent identification of liver-specific transferrin receptor 2 suggests that transferrin receptor 2 might mediate iron uptake through a distinct, yet unidentified, pathway. The hemochromatosis protein, HFE, binds to transferrin receptors and negatively modulates the receptor's activity. Once the complex, consisting of iron, transferrins, and transferrin receptors, is internalized within endosomal compartments, iron is released from transferrin as the endosomal compartment becomes acidified. Iron is

then transported across the endosomal membrane into the cytoplasm by the divalent cation transporter, Nramp2. Nramp2 is also responsible for iron uptake at the brush border in the intestinal duodenum. The iron exporter, ferroportin1, resides at the basolateral surface of duodenal enterocytes and is responsible for exporting iron into plasma. In addition, ceruloplasmin and hephaestin are essential for mobilizing iron from tissues into systemic circulation. Once inside the cell, iron can be utilized for the synthesis of heme and incorporated into enzymes or sequestered within ferritin, the iron storage protein.

The balance between iron uptake and storage is tightly controlled by the feedback regulatory mechanism of the iron responsive elements and iron regulatory proteins 1 and 2. By binding to iron responsive element-containing mRNAs, such as ferroportin1, transferrin receptor 1, and ferritin, iron regulatory proteins either prevent the translation or degradation of mRNAs with which they associate, resulting in up- or down-regulating expression of iron responsive element-containing genes in response to the body's iron status. This feedback mechanism of iron responsive elements and iron regulatory proteins is, so far, the only known regulatory function in sensing iron status in cells, tissues, and body. It is interesting to note that almost all molecules that play major roles in iron metabolism express both forms of iron responsive element-containing and non-containing mRNAs, probably in a cell-type and/or tissue-specific fashion. The identification of many novel iron responsive element-containing genes, achieved by searching consensus motifs for iron responsive elements, reminds us that our accomplishments in characterizing the regulatory mechanisms of iron homeostasis are only rudimentary.

Though excessive iron in specific cells and tissues promotes the development of infections, neoplasia, cardiomyopathy, and, possibly, various neurodegenerative disorders, iron is also a nutrient for invading microbial and neoplastic cells. Hosts have evolved an iron-withholding defense system to suppress microbial growth, but the system can be compromised by a number of factors. Thus, routine screening for iron loading in populations that are exposed to certain diseases could provide valuable information in epidemiological, diagnostic, prophylactic, and therapeutic studies of emerging infectious diseases. A variety of genetic, medicinal, immunological, and behavioral methods are being developed to prevent abnormal iron loading and to reduce its subsequent, detrimental effects. With expression profiles that are closely correlated to the characteristics of the transferrin cycle, the iron status of the body, and the rates of erythropoiesis and cell proliferation, transferrin receptors have invaluable diagnostic and therapeutic potentials. Plasma levels of transferrin receptors increase with elevated tissue iron deficiency or with elevated erythropoiesis. In uncovering iron deficiency that is associated with anemia of chronic diseases, measuring levels of plasma transferrin receptors has advantages over measuring plasma ferritin, which is generally used to gauge body iron stores. For example, in the case of rheumatoid arthritis, the plasma ferritin level is often increased as a result of inflammation. Consequently, in patients with anemia of chronic diseases, a measurement of plasma transferrin receptors will have diagnostic value in evaluating erythropoietic activity and body iron stores. In activated lym-

phocytes, an elevated level of transferrin receptors is commonly used as a reference for monitoring lymphoproliferative abnormalities. In detecting malignancies, plasma levels of transferrin receptors can be used as a diagnostic tool, as transferrin receptors are a marker protein for cell proliferation.

Because levels of surface transferrin receptors are generally greater in malignant cells than in normal cells, antibodies that specifically recognize and block transferrin receptors from binding to transferrin have been used to selectively deliver cytotoxic or static agents to the malignant cells of patients in several clinical settings. In tumor therapy, delivering chemotherapeutic drugs conjugated to transferrin or antibodies against the transferrin receptors have been used to target and kill actively proliferating cells with satisfactory results. Moreover, antibodies against transferrin receptors are able to cross the blood–brain barrier and are deposited in the brain parenchyma. As the delivery of drugs to the brain is inefficient by the tightly apposed capillary endothelial cells, these anti-transferrin receptor–drug conjugates could deliver drugs across the blood–brain barrier. In gene therapy, exogenous DNA that has been coupled to transferrin can be targeted to proliferating and hemopoietic cells and internalized via endocytic pathways for efficient delivery and expression of foreign genes in the desired cell nucleus.

The imminent completion of the Human Genome Project's sequencing efforts will certainly identify more candidate disease genes that contribute to the disorders of iron metabolism. Confirming the involvement of these genes in iron metabolism will require research-based genetic association studies to link suspected genes to diseases. In addition, by using functional genomic tools, such as bioinformatics, DNA microarrays, and proteomic platforms, studies on these candidate genes will also generate invaluable information that will unequivocally confirm an association between specific genetic alterations and susceptibility to iron disorders. Moreover, the translation of these disease-genotype associations to rapid, sensitive, specific, inexpensive routine diagnostic clinical practice will have dramatic applications. In the molecular medicine era, these new genotyping methods will be used for diagnosing symptomatic iron-disordered patients, but more importantly, for identifying pre-symptomatically individuals who are at risk for iron metabolism disorders and for whom effective preventative interventions are available.

The recent advances in iron disorders demonstrate a complex, yet intimate, relationship between iron and copper metabolism and the central nervous system and immune system. Current progress is a reminder of the tasks that await us in identifying and characterizing the structural and functional relationships of these gene products in iron metabolism. Many important questions remain unanswered: are there other iron homeostasis molecules, such as iron transporters and their regulators, yet to be discovered? Which molecule functions as an iron sensor? What are the functions of these novel iron responsive element-containing genes? Even though our basic knowledge of the genetics and biochemistry of iron metabolism disorders has been growing exponentially, these questions indicate our naïve status in treating iron metabolism disorders. However, we are confident that advances in the molecular and biochemical pathways of iron metabolism and in our understanding of the fundamental mechanisms of iron sensor activities will lead to therapeutic

strategies to manipulate iron absorption, transport, and storage. These forms of interventions will have tremendous benefits for patients with iron metabolism disorders.

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