Chapter 1
Proteins of Iron Homeostasis

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1 Introduction

Iron is essential for a number of biochemical functions in the body including the transport of oxygen in the blood and energy production in the mitochondria. Therefore, humans require an abundant and regular source of dietary iron to maintain normal health. This is amply demonstrated by data indicating that as many as a third of the world's population suffer from iron deficiency, making it the most common nutrient deficiency disorder. In addition to pathologies associated with iron deficiency, excess iron is highly toxic and can lead to cell and organ damage. Interestingly, while the body levels of other dietary metals can be regulated by excretion in the feces and urine, humans do not possess the capacity to remove excess iron from the body. As a consequence, a number of proteins have evolved which tightly regulate mammalian iron homeostasis. The actions of these proteins control the rate of duodenal iron absorption, its delivery, utilization and storage by metabolically active tissues, and its recycling by reticuloendothelial macrophages. The purpose of this chapter is to introduce the reader to some of the key proteins that maintain iron balance in humans. We have divided these proteins into families based on their function. This includes (1) the proteins that are involved in iron transport across cellular membranes; (2) the reductases and oxidases that facilitate the movement of iron across cell membranes; (3) iron transport in the circulation and its intracellular storage; and (4) the proteins that control iron homeostasis by regulating all of the above processes. Mutations in the genes encoding many of these proteins lead to a wide range of diseases highlighting the varied role that iron plays in human metabolism. Many of these diseases will be discussed in depth in subsequent chapters.

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2 Proteins Mediating Iron Transport Across Cellular Membranes

2.1 Divalent Metal-Ion Transporter 1

The divalent metal transporter, DMT1 – also known as the divalent cation transporter, DCT1 [1], natural resistance–associated macrophage protein, NRAMP2 [2, 3] and solute-linked carrier family 11 (proton-coupled divalent metal ion transporters), member 2 (SLC11A2) – transports ferrous iron across the apical membrane of the intestinal epithelium [1, 4] (Fig. 1.1). In addition to its essential role in dietary non-heme iron absorption, DMT1 is also required for the endosomal release of transferrin-bound iron. The targeted disruption of DMT1 in mice has confirmed its obligate role in both intestinal iron absorption and in the development of erythroid precursors into mature erythrocytes [5]. This function is further underlined by evidence showing that mutations in DMT1 in the *mk*/*mk* mouse [3] and the Belgrade rat [6] and humans [1, 7–10] lead to the development of microcytic anemia.

At least four DMT1 isoforms exist through alternate splicing in exon 16 [11] and the presence of two transcription start sites – in exon 1A and 1B, respectively [12]. Exon 16 splicing gives rise to two variants that differ in their terminal 19–25 amino acids and their 3′ untranslated region (UTR); one variant contains an iron responsive element (IRE) in its 3′ UTR whereas the other lacks this sequence [11]. Isoforms containing a 3′ IRE may be subject to iron-sensitive post-transcriptional regulation via the IRE–IRP system (discussed later in this chapter). All four isoforms can be detected

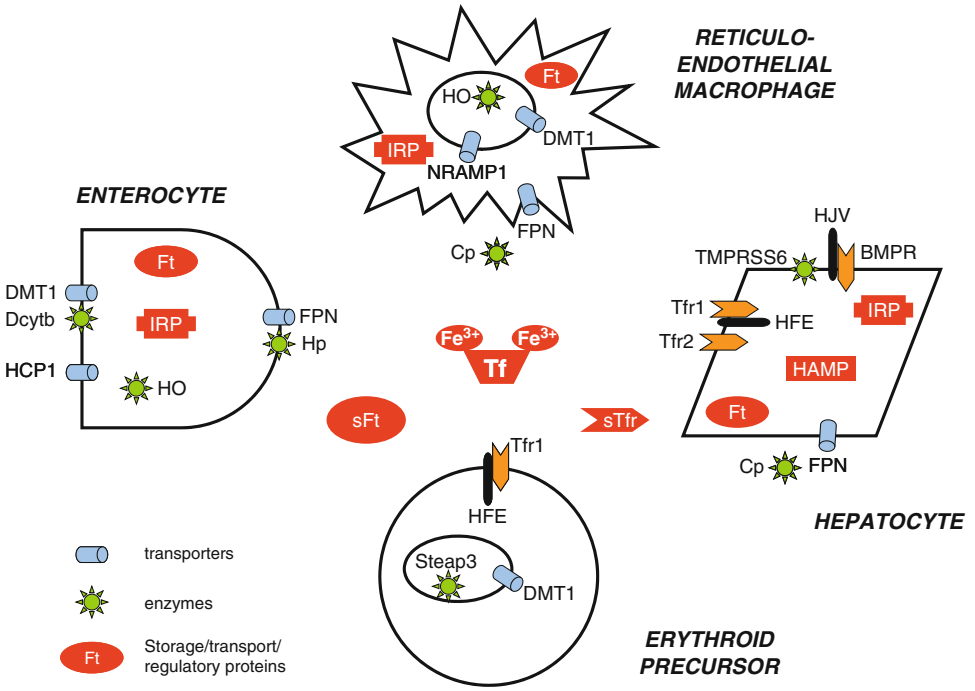

Fig. 1.1 Distribution of iron transport, storage and regulatory proteins in various cell types. *Abbreviations*: *BMPR* bone morphogenetic protein receptor, *Cp* ceruloplasmin, *Dcytb* duodenal cytochrome b, *DMT1* divalent metal transporter 1, *Ft* ferritin, *FPN* ferroportin, *HAMP* hepcidin antimicrobial peptide, *HCP1* heme carrier protein 1, *HFE* hereditary hemochromatosis protein, *HJV* hemojuvelin, *HO* heme oxygenase, *Hp* hephaestin, *IRP* iron regulatory proteins (IRP1/IRP2), *NRAMP1* natural resistance–associated macrophage protein 1, *sFt* serum ferritin, *STEAP3* six transmembrane epithelial antigen of the prostate 3, *sTfr* serum transferrin receptor, *Tf* transferrin, *Tfr1* transferrin receptor 1, *Tfr2* transferrin receptor 2, *TMPRSS6* transmembrane serine protease family member 6 (matriptase 2)

at varying levels in intestinal epithelial cells [12]; and while the exon 1A/IRE-containing variant has been suggested to be the predominant functional isoform with respect to intestinal iron absorption, all four isoforms can transport iron with equal efficiency [13]. Within intracellular endosomes, there appears to be differential localisation of the different DMT1 isoform. The IRE-containing variants are localized to the late endosomes and lysosomes [14–17] whereas non-IRE isoforms are associated with the transferrin receptor-containing early endosomes [15, 17].

In the intestine, DMT1 expression is increased when body iron levels are depleted [1, 18, 19]. This may reflect the presence of the IRE in the 3′ UTR [1] which can bind cytosolic IRP [20]. In addition to these longer term changes in expression, there is good evidence that DMT1 levels respond more rapidly to changes in dietary composition. The so-called "mucosal block" hypothesis was formulated more than 50 years ago following studies which demonstrated that a large oral dose of iron could reduce subsequent iron absorption ([21, 22]; reviewed in [23]). It was argued that due to the short time interval between doses, the initial dose must have acted directly on the mature enterocytes rather than the crypt cells. More recent studies with rodents given an iron bolus [24–26] and in cell culture models [16, 27] suggest that the primary mechanism for mucosal block arises from redistribution of DMT1 between the plasma membrane and intracellular compartments. Our evidence suggests that DMT1 is trafficked from the apical membrane of intestinal epithelial cells to late endosomes/lysosomes [16]. Such a mechanism may be important physiologically for optimizing iron absorption from a meal so that it matches the body's metabolic requirements.

Rapid transcriptional control of iron transport genes may also be important in regulating intestinal iron homeostasis. In this context, two recent studies have identified a role for hypoxia-inducible factors – in particular HIF2α – as important local regulators that respond to decreased intracellular iron levels and low oxygen tension by up-regulating the expression of DMT1 and the ferric reductase Dcytb [28, 29].

2.2 *Heme Transporters*

In addition to non-heme iron, the iron contained within heme also makes an important contribution to dietary iron absorption. Heme is absorbed intact [30] and the iron is liberated intracellularly under the action of heme oxygenase [31]. While there is a great deal of information regarding the mechanisms involved in non-heme iron absorption, much less is known about potential heme transport mechanisms. A number of candidate heme transport proteins have been identified in the intestinal epithelium including the Breast Cancer Resistance Protein, ABCG2 [32], the feline leukemia virus C receptor protein, FLVCR [33] and the heme carrier protein, HCP1 [34]. Of these candidate transporters, only HCP1 acts as a heme importer (Fig. 1.1), while both ABCG2 and FLVCR mediate heme efflux. The high duodenal expression of HCP1 supports a potential role in heme absorption from the diet; however, this has been complicated by recent evidence suggesting that HCP1 may function primarily as a proton-coupled folate transporter [35]. In an interesting recent development, the heme-regulated gene (HRG) family of proteins has been identified in *C. elegans* [36]. These proteins play an essential role in heme transport and homeostasis in nematodes and their orthologues are also expressed in vertebrates, including humans, suggesting that HRG proteins may also play an important role in heme biology in mammals.

2.3 *Ferroportin*

Iron efflux from all tissues is mediated by ferroportin [37] – also known as Iron Regulated Transporter 1 (IREG1) [38], Metal Transporter Protein 1 (MTP1) [39] and solute-linked carrier family 40 (iron-regulated transporter) member 1 (SLC40A1) (Fig. 1.1). To date, ferroportin is the only identified

iron efflux protein and is particularly important for iron release into the circulation from absorptive enterocytes, iron recycling macrophages, hepatocytes and the placental syncytiotrophoblast. Not surprisingly therefore, global deletion of ferroportin in mice is embryonically lethal [37]. Furthermore, mice with intestine-specific ferroportin deletions (achieved by expression of an inducible intestine-restricted villin-cre-ferroportin transgene) develop iron deficiency highlighting the essential role of ferroportin in dietary iron assimilation [40].

Studies with *Xenopus laevis* oocytes overexpressing ferroportin indicate that this protein mediates the unidirectional efflux of ferrous iron [37, 38]. However, in order for exported iron to be loaded onto transferrin for transport in the circulation, it must be oxidized to its ferric form. Therefore, ferroportin forms only half of the iron efflux pathway, working in concert with the ferroxidases hephaestin and ceruloplasmin (discussed later). Ferroportin is primarily localized to the plasma membrane of cells; in polarized epithelia such as the duodenum and placenta, it is expressed at the basolateral membrane of cells [37, 38] which is consistent with its function as an iron export protein. Interestingly, there have been reports of ferroportin protein expression on the apical membrane of enterocytes [41], but this remains controversial. In iron-recycling macrophages, ferroportin appears to reside in intracellular vesicles but following iron loading [42] or erythrophagocytosis [43, 44], ferroportin is rapidly translocated to the plasma membrane. A recent study has suggested that the trafficking of ferroportin in macrophages may be under the control of Mon1A which plays a fundamental role in the macrophages secretory apparatus [45].

The regulation of ferroportin expression is complex. Ferroportin mRNA contains a single IRE in the 5′ UTR [38, 39] which is predicted to raise protein expression under high iron conditions. In agreement with this hypothesis, iron loading increases ferroportin expression in the liver [39], lung [46] and macrophage [44]. However, the response to changes in iron status is tissue specific. In the duodenum, ferroportin is elevated by iron deficiency but not by iron loading [38]. Recently, a second ferroportin transcript (termed FPN1B) has been identified which lacks the IRE and is not repressed in iron-deficient conditions [47]. While the FPN1A and FPN1B transcripts give rise to identical protein products, their tissue distribution is different; FPN1B is more highly expressed in the duodenum and in erythroid precursors. The identification of the FPN1B transcript provides an explanation of the lack of IRE/IRP-dependent regulation of duodenal iron absorption in the face of systemic iron deficiency.

In addition to regulation by iron, it is now know that ferroportin is the cellular target for the regulatory actions of hepcidin (discussed in detail in subsequent chapters). However, recent work suggests that there may also be subtle tissue-specific differences in ferroportin–hepcidin interactions in the duodenum and macrophage [48–50].

The original identification of ferroportin arose from a study of mutations associated with impaired iron metabolism in zebrafish (*Danio rerio*). One of these fish with impaired hematopoiesis, named *weissherbst*, resulted from mutations in the zebrafish homologue of mammalian ferroportin [37]. Subsequently, a number of mutations have been identified in human ferroportin that give rise to an iron loading syndrome that has been referred to as either type IV hemochromatosis or ferroportin disease (see Chap. 20).

2.4 Transferrin Receptors

Transferrin receptors are the main route for iron entry into most cells. To date, two distinct transferrin receptors have been identified, TfR1 (the subject of many reviews) which is expressed on all proliferating cells to permit iron acquisition for the cell cycle and TfR2 [51] which is expressed mainly on hepatocytes [52] (Fig. 1.1).

TfR1 exists on the surface of cells as a disulfide-bonded homodimer of 760-residue subunits (reviewed in [53]). At physiological pH (7.4), TfR1 binds circulating transferrin (either mono-ferric or di-ferric) at the cell surface and the receptor-transferrin complex is endocytosed via a clathrin-mediated mechanism (reviewed in [54]). The transferrin-containing endosome is acidified by recruitment of a V-type proton ATPase permitting the release of iron from transferrin (apotransferrin remains bound to its receptor at the acid pH of the endosome). In erythroid precursors, the iron is reduced by STEAP3 ([55], discussed below) and exits the endosome through DMT1 [6]. The apotransferrin/TfR1 complex is recycled back to the cell surface where at pH 7.4 it dissociates from its receptor and re-enters the circulation. Iron uptake via this pathway may be regulated by the HFE protein, which competes with transferrin for a common binding site on TfR1 [56, 57].

Studies with Tfr1 knockout mice and zebrafish expressing the *chianti* phenotype highlight the essential role of Tfr1, particularly in development and functioning of erythroid tissue. Murine Tfr1 deletion is embryonically lethal and is characterized by severely disrupted erythropoiesis and neurological development [58]. Furthermore, heterozygous mice carrying only one copy of the Tfr1 allele exhibit impaired erythroid development and abnormal iron homeostasis [58]. Zebrafish, unlike humans and mice, contain two Tfr1-like genes. A mutation in Tfr1a, which is highly expressed in erythroid tissue, gives rise to the *chianti* phenotype characterized by microcytic hypochromic anemia [59]. Interestingly, over-expression of mouse Tfr1, mouse Tfr2 and zebrafish Tfr1b partly rescued the *chianti* phenotype, suggesting that they could permit transferrin-bound iron uptake by erythroid precursors for hemoglobin synthesis [59].

Tfr1 mRNA expression is highly regulated by iron status and this is mediated by the presence of five IRE motifs in the 3′ UTR of the mRNA sequence [60]. Under iron-deficient conditions, the IRE sequences are bound by iron regulatory proteins, protecting the mRNA from endonucleolytic degradation and thus increasing mRNA half-life. As a consequence, Tfr1 protein expression is up-regulated in iron deficiency. In contrast, IRE are unbound under iron replete conditions, leading to rapid degradation of Tfr1 mRNA and a reduction in cellular protein expression [60–62].

Tfr2 has significant sequence homology to Tfr1 and can also bind and transport transferrin-bound iron, albeit with a much lower affinity than Tfr1 [51, 63]. Unlike Tfr1 which is ubiquitously expressed, the expression of Tfr2 is restricted to the liver, and normal and neoplastic hematopoietic cells [51, 64]. Interestingly, mutations in Tfr2 lead to severe hepatic iron overload, termed type III hemochromatosis [65], highlighting its importance in iron homeostasis. These findings have been further confirmed by disruption of the Tfr2 gene in mice [66–68], which also leads to hepatic iron loading.

Tfr2 mRNA expression does not appear to be regulated in response to changes in iron status and its transcript does not contain the IRE motifs that are characteristic of Tfr1 mRNA [51]. Studies using cell culture models [63, 64] as well as in vivo studies in mice [69] report no changes in Tfr2 mRNA levels in response to altered iron status. However, Tfr2 protein expression is up-regulated in response to elevated levels of di-ferric transferrin [70, 71], which binds to Tfr2and increases its membrane stability [70, 72]. Binding of di-ferric transferrin also alters the cellular fate of Tfr2; increasing the levels associated with recycling endosomes and decreasing the fraction of Tfr2 targeted to the lysosomes for degradation [73]. Because of this tight regulation by di-ferric transferrin, Tfr2 has been proposed as a sensor of iron status that monitors changes in circulating transferrin saturation. Intriguingly, two recent studies suggest that the HFE protein interacts with Tfr2 and may therefore be an important component of this iron-sensing pathway [74, 75].

In addition to membrane-associated cellular Tfr1, a soluble form (sTfr) exists in human serum. During the maturation process, erythroid precursors shed transferrin receptors from their cell surface into the circulation [76, 77]. The shedding process is primarily mediated by an integral membrane protease belonging to the disintegrin and metalloprotease (ADAM) family [78] and results in the proteolytic cleavage of Tfr1 at Arg-100 within the transmembrane stalk [79, 80]. Serum transferrin receptor concentrations are increased in patients with elevated levels of immature erythroid cells and in individuals with iron deficiency, but are not altered (or are lower than normal) in patients with the

anemia of chronic disease [81, 82]. As such, sTfr has emerged as a powerful biomarker to distinguish between these two forms of anemia (especially when used in conjunction with measurement of serum ferritin). Interestingly, recent data have shown that increasing transferrin saturation decreases the release of sTfr and this effect is mediated by a direct molecular interaction between transferrin and its receptor, indicating that sTfr does not only reflect the iron demand of the cells but also the iron availability in the bloodstream [83].

2.5 Mitoferrin

Once taken up by cells in its various forms, iron is utilized for a number of metabolic functions including the synthesis of heme, the production of hemoglobin and the assembly of iron-sulfur clusters. Many of these processes take place in the mitochondrion but the mechanisms by which mitochondria accumulate and store iron has only recently been discovered. Mitoferrin, a member of the vertebrate mitochondrial solute carrier family (SLC25A37) was identified by positional cloning, and is highly expressed in hematopoietic tissue [84]. A mutation in the mitoferrin gene in zebrafish is responsible for the *frascati* phenotype that shows profound hypochromic anemia and the arrest of erythroid maturation owing to defects in mitochondrial iron uptake. Mitoferrin orthologues (MRS3 and MRS4) also exist in yeast and disruption of these genes causes defects in hemoprotein production and the mitochondrial synthesis of iron-sulfur clusters [85–88]. Interestingly, work by Shaw et al. [84] indicates that expression of murine mitoferrin can rescue the defects in iron metabolism exhibited in the *frascati* zebrafish, and furthermore that introduction of zebrafish mitoferrin can complement the yeast MRS3/4 mutant, indicating that the function of the gene may be highly conserved.

2.6 Natural Resistance–Associated Macrophage Protein 1

The NRAMP family of proteins has two mammalian members; NRAMP1 (SLC11A1) which confers resistance to infection by mycobacteria [89]; and NRAMP2 (DMT1 or SLC11A2) [2] which transports iron across the apical membrane of duodenal enterocytes and the membrane of transferrin-containing endosomes [1, 3, 6] (Fig. 1.1). NRAMP orthologues exist in yeast (SMF1 and SMF2) [90] and in *Drosophila melanogaster* (malvolio) [91] and are all thought to act as metal ion transporters [92].

NRAMP1 is almost exclusively expressed in macrophages and neutrophils where upon activation it is recruited to phagosomal membranes [93]. In inbred mouse models, increased susceptibility to infections by intracellular pathogens is associated with a single amino acid substitution (glycine to aspartic acid) at position 169, which lies in the predicted fourth transmembrane domain of the protein [94]. Like other members of the NRAMP family, NRAMP1 is a metal ion transport protein but its mode of action remains unclear. Gros and colleagues have proposed that NRAMP1 acts as a membrane efflux pump in phagosomes, thereby restricting the availability of essential metals such as Mn^{2+} and Fe^{2+} to the pathogen [95–97]. In contrast, there is evidence from other groups that NRAMP1 acts as a metal influx pump to increase the production of oxygen radicals through Fenton-type chemistry [98, 99]. Furthermore, studies in Xenopus oocytes have suggested that NRAMP1 could act as proton-coupled antiporter [100], unlike its family member DMT1 which acts as a symporter [1, 4]. Interestingly, recent evidence suggests that both NRAMP1 and DMT1 are required for efficient macrophage iron recycling following erythrophagocytosis [101, 102].

NRAMP1 polymorphisms are distributed along the entire NRAMP1 genomic sequence and a complex linkage disequilibrium pattern exists within and around the NRAMP1 locus [103, 104].

It is becoming increasingly apparent (>100 papers) that NRAMP1 polymorphisms may predispose individuals to a number of human infections (some of the evidence is reviewed in [105, 106]). We have recently shown that hypoxia inducible factor (HIF-1) regulates allelic variation in SLC11A1 expression by directly binding to microsatellite (GT/AC)n dinucleotides during macrophage activation by infection. Therefore it is assumed that HIF-1 influences heritable variation in SLC11A1-dependent innate resistance to infection and inflammation within and between populations [107]. Determining the rationale for these associations remains a major challenge for the future.

3 Iron Reductases and Oxidases That Facilitate the Movement of Iron Across Membranes

3.1 Duodenal Cytochromes b (Dcytb)

Non-heme iron is present in the diet mainly as ferric salts and oxides. However, these compounds are not bioavailable and iron must be reduced to the ferrous form prior to absorption by duodenal enterocytes. A number of dietary factors contribute to the conversion of Fe(III) to Fe(II), notably ascorbic acid [108] and a number of meat digestion products [109–112]. In addition, several studies have demonstrated that the brush-border surface of duodenal enterocytes and cultured intestinal cells possess ferric reductase enzymic activity [113–115]. The enzyme responsible for this process, named Dcytb (duodenal cytochrome b) (Fig. 1.1), a homologue of cytochrome b_{561}, was cloned from mouse duodenal mRNA using a subtractive hybridisation strategy [116]. Dcytb is expressed at the apical membrane of duodenal enterocytes, the major site for the absorption of dietary iron and like other members of the cytochrome b_{561} family is a heme-containing, ascorbate requiring protein [116, 117].

The mRNA expression of Dcytb is highly regulated by dietary iron status, hypoxia and in hemochromatosis [116, 118], suggesting that it plays an important role in the maintenance of body iron homeostasis. In vitro studies show a dramatic increase in iron uptake in cultured cell lines overexpressing Dcytb [119, 120]. In contrast, the targeted disruption of the *Cybrd1* gene (which encodes Dcytb) in mice does not lead to an iron-deficient phenotype [121], casting doubt on the absolute requirement of Dcytb for intestinal iron absorption. An important caveat to these studies is that humans rely totally on the diet to provide vitamin C, whereas mice can synthesize abundant quantities of vitamin C *de novo* from glucose, and as such may have less need for a duodenal surface ferric reductase. However, it is interesting to note that, unlike DMT1 and ferroportin where a number of disease-causing mutations have been identified, only one recent report has linked a single nucleotide polymorphism in Dcytb to impaired iron metabolism [122].

3.2 The Six Transmembrane Epithelial Antigen of the Prostate (STEAP) Family

A second family of reductase proteins – the STEAP family – has recently been identified and, with the exception of STEAP1, these proteins act as iron reductases in vitro [55, 123]. One of these proteins, STEAP 3, acts as the endosomal ferric reductase in erythroid precursors, converting iron liberated from transferrin from ferric to ferrous so that it can exit endosomes via DMT1 [55] (Fig. 1.1). A mutation in STEAP 3 in *nm1054* mice leads to hypochromic, microcytic anemia due to the inability of erythroid precursors to utilize transferrin-bound iron. This essential role of STEAP 3 was confirmed following the generation of STEAP 3 knockout mice, which like their spontaneous mutant (*nm1054*) counterparts also exhibited anemia.

3.3 Ceruloplasmin

The essential role played by copper in the regulation of iron metabolism has been recognized for many years (reviewed extensively by [124, 125]). However, it is only relatively recently that we have begun to understand the molecular basis for the biological interactions between these two metals. In experimental animals, it is possible to generate anemia by both copper deficiency and iron deficiency, which display remarkably similar hematological features [126, 127]. The common factor in the etiology of both of these diseases was identified as ceruloplasmin, a multicopper binding protein with serum oxidase activity [128]. Subsequent studies revealed that ceruloplasmin acted as a ferroxidase converting Fe^{2+} to Fe^{3+} [129] and increased the rate of loading of iron onto transferrin [130] (Fig. 1.1). Further studies with perfused liver preparations showed that ceruloplasmin markedly stimulated iron efflux from the liver suggesting that it is a crucial factor for the mobilization of iron from the body stores for metabolic utilization [131]. More recently, the key role of ceruloplasmin in iron metabolism has been confirmed in studies on human patients and mice displaying disrupted ceruloplasmin production. In patients with aceruloplasminemia, and in ceruloplasmin-null mice, there is accumulation of iron in a number of organs, including the liver and various regions of the brain [132], as well as hypoferremia and impaired erythropoiesis.

While ceruloplasmin is often thought of as a plasma protein, synthesized and secreted by the liver, a second form of the enzyme, which is bound to the cell membrane by a glycosylphosphatidylinositol (GPI)-anchor (GPI-ceruloplasmin), is localized to the surface of astrocytes in the central nervous system (CNS) [133]. GPI-ceruloplasmin is produced by alternative splicing of the ceruloplasmin gene [134, 135] and is essential for iron efflux from cells in the CNS [136]. Mechanistically, GPI-ceruloplasmin and soluble ceruloplasmin may be important for the stabilization of the ferroportin efflux transporter at the plasma membrane. Recent studies have shown that the loss of ceruloplasmin activity prevents ferroportin-mediated iron export in cultured cells [137].

The essential role of copper in the regulation of iron metabolism is also evident in lower eukaryotic species. Genetic studies of iron metabolism in the yeast *Saccharomyces cerevisiae* have shown that a copper-binding protein Fet3, which has sequence homology to ceruloplasmin, is required for high-affinity iron uptake [138, 139]. Like ceruloplasmin, Fet3 has ferroxidase activity suggesting that oxidation and reduction of iron are crucial to its movement across biological membranes in yeast as well as in mammals.

3.4 Hephaestin

The link between copper and iron metabolism has been further enhanced by studies carried out in *sla* (sex-linked anemia) mice. The *sla* phenotype is characterized by normal iron absorption from the diet but defective transfer of iron into the plasma. The *sla* locus is present on the X-chromosome; this region has subsequently been mapped [140] and the candidate gene mutated in the *sla* mice identified. The gene encodes the protein hephaestin [141], a copper-containing protein with homology to ceruloplasmin (Fig. 1.1). Hephaestin expression is not limited to the duodenum, and is widely expressed along the length of the gastrointestinal tract, suggesting that it may have other physiological functions in addition to the regulation of iron absorption [141–143]. Recent work has confirmed that hephaestin, like ceruloplasmin, exhibits significant ferroxidase activity [144]. Further modeling of the protein predicts that the mutation present in the *sla* mice would lead to protein misfolding and reduced ferroxidase activity [144, 145]. The cellular localisation of the hephaestin protein is intriguing. Based on the actions of ceruloplasmin, one would predict that hephaestin would localize to the basolateral membrane of duodenal enterocytes where it could interact with ferroportin to oxidise

iron leaving the enterocytes so that it could be loaded onto transferrin. However, in normal enterocytes, in addition to some staining on the basolateral membrane [146], there is abundant expression of hephaestin protein within intracellular structures [142]. Taken together, these studies suggest that in normal enterocytes hephaestin may traffic between intracellular organelles and the cell surface. In contrast, in *sla* mice, hephaestin is localized exclusively to the supranuclear compartment of enterocytes [146] suggesting that the mutation leads to mislocalisation of hephaestin protein and results in the functional deficiency in iron efflux from the *sla* intestine.

4 Iron Transport and Storage Proteins

4.1 Transferrin

Approximately 3–4 mg of iron circulates in the plasma bound to a specific binding protein, transferrin (Tf) (Fig. 1.1). Serum Tf is a member of the Tf superfamily of proteins that includes lactoferrin (found in milk and other secretory fluids), ovotransferrin (found in avian egg white) and melanotransferrin. Whereas the primary function of serum transferrin – the transport of iron to sites of utilization and storage within the body – is clearly defined, the functions of the other major members of Tf superfamily are less clear [147]. There are approximately 19 Tf variants, however only Tf C can be found in the majority of humans [148]. The Tfs are synthesized primarily in the liver and are formed of single polypeptide chains of approximately 80 kDa, which can bind two atoms of ferric iron [149]. The binding of iron to transferrin is reversible and pH-dependent, with complete association above pH 7 but increasing dissociation at acid pH (below pH 6.5). The equilibrium constant for iron–transferrin binding is 10^{26}–10^{30} [150].

Tf concentration in the circulation is of the order of 30 μM and it is approximately 30–35% saturated with iron in people with normal iron status. Given that there are two iron binding sites on the protein, Tf can exist as iron-free apo-Tf, in the monoferric form, or as di-ferric- or holo-Tf. At normal circulating levels, the majority of iron-bound transferrin is present as mono-Tf, whereas di-ferric-Tf predominates in iron-loading disorders such as hemochromatosis [151]. Interestingly, the binding of di-ferric-Tf to Tfr1 is at least one order of magnitude greater than for monoferric-Tf [152].

4.2 Ferritin

The ferritin molecule is a hollow protein shell, composed of 24 polypeptide subunits, with an overall molecular weight of approximately 500 kDa that can store up to 4,500 ferric iron atoms (reviewed in [153]). Ferritins are the main iron storage molecules in all mammalian tissues and consist of a mixture of two subunits referred to as L- and H- ferritin (Fig. 1.1). In general, L-rich ferritins are characteristic of organs storing iron for a prolonged period (e.g., liver and spleen) and these ferritins usually have relatively high average iron content (1,500 Fe atoms/molecule or more). H-rich ferritins which are characteristic of heart and brain have relatively low average iron contents (less than 1,000 Fe atoms/molecule). H-ferritin chains are important for Fe(II) oxidation whereas L-chains assist in the formation of the ferritin core. Ferritins are not inert and are constantly turned over [154]. The iron stored in ferritin is available for utilization by other functional proteins and can be mobilized following lysosomal degradation of the ferritin complex [155]. The mechanisms by which iron is donated to ferritin for storage have remained elusive. However, a recent study has identified the

poly(rC)-binding protein 1 (PCBP1) as a key component of this pathway [156]. PCBP1 binds iron, and can also bind directly to ferritin to facilitate iron loading. Furthermore, knocking down PCBP1 with siRNA in human cells decreases ferritin–iron levels, increasing the cytosolic iron pool.

Induction of ferritin synthesis in response to iron administration was first observed by Granick [157] in the gastrointestinal mucosa of guinea pigs after iron feeding. The response is rapid and this may reflect the need to limit the cell's exposure to pro-oxidant free iron. The iron-mediated induction of ferritin expression is largely post-transcriptional and involves IRP binding to a stem-loop IRE present in the 5′ untranslated region of both H- and L-ferritin mRNAs [158–161]. Under iron replete conditions, ferritin mRNA is efficiently translated. However, when cellular iron levels decrease, ferritin protein levels are also lowered. This decrease in ferritin is directly attributable to the position of the IRE in the 5′ UTR. The IRE in both ferritin H and L chain is less than 40 bases from the AUG site and binding of IRP to these IRE sequences prevents the binding of the eukaryotic initiation factor (eIF4F) complex to the 43S ribosomal subunit that is necessary for protein translation [162].

Small amounts of ferritin normally circulate in the serum [163, 164]. In humans, serum ferritin appears to consist largely of a glycosylated form of L-ferritin [165, 166], which has a low iron content [167, 168]. In normal healthy subjects, there is a close correlation between serum ferritin and the body iron stores with 1 μg/L serum ferritin being equivalent to approximately 8–10 mg tissue iron [169, 170]. Serum ferritin levels range from 30 to 300 μg/L in men and 15 to 150 μg/L in women. Serum ferritin derives from tissue ferritin and can be secreted from the liver [171, 172] and from lymphoid cells [173]. Interestingly, it was recently observed that glycosylated L-ferritin, akin to that present in serum, can be actively secreted from human hepatoma cells [174].

In 2001, a novel form of ferritin was identified that localized to the mitochondria [175]. Mitochondrial ferritin is encoded by an intronless gene on chromosome 5q23 [175, 176]; however, unlike cytosolic ferritin, the mitochondrial form lacks a 5′ IRE. Mitochondrial ferritin is 79% identical to cytosolic H-ferritin, but has a long amino acid N-terminal mitochondrial import sequence which is cleaved during processing, and exhibits ferroxidase activity [175]. The expression of mitochondrial ferritin mRNA is highest in metabolically active tissues such as the testis and is noticeably absent from tissues associated explicitly with iron storage such as the liver and spleen [175, 177]. While mitochondrial ferritin does sequester iron, these data suggest that the primary role of the protein might be to protect cells that generate high mitochondrial levels of reactive oxygen species during metabolism from the pro-oxidant effects of iron.

Under conditions of iron excess, some cellular ferritin can be converted into another storage form known as hemosiderin [178], which can be clearly identified in tissues associated with iron storage, including the liver, spleen and bone marrow [178–180]. Both ferritin and hemosiderin are found in lysosomal structures that have been termed siderosomes. Hemosiderin is typically insoluble [181] and is generally considered to be a degradation product of ferritin [178, 179]. Hemosiderin particle sizes are smaller than those of cytosolic ferritin cores [182] and are formed following lysosomal degradation of ferritin. The enzymes causing the cleavage have not been identified.

5 Proteins Involved in the Regulation of Iron Status

5.1 Iron Regulatory Proteins

A number of genes associated with the maintenance of iron homeostasis are tightly regulated in response to the prevailing intracellular iron levels through post-transcriptional mechanisms that involve interactions between cytosolic iron regulatory proteins (IRP) and stem-loop structures known

as iron responsive elements (IRE). These IRE motifs exist in either the 5′ or 3′ untranslated region (UTR) of several target mRNA species. Two cytosolic iron regulatory proteins, IRP-1 and IRP-2, are known to exist in most cells and both of these proteins can bind to IRE structures when cellular iron levels are depressed. However, under iron replete conditions, RNA binding is quickly inactivated by either post-translational modification of IRP-1 or degradation of IRP-2.

The mechanism underlying the iron-dependent inactivation of IRP-1 has been studied extensively. Structurally, IRP-1 is very similar to the mitochondrial aconitase [183] that converts citrate to isocitrate in the tricarboxylic acid cycle. Under conditions of iron deficiency, IRP-1 binds avidly to IRE sequences, but when cells are iron replete, IRP-1 acts as a cytoplasmic aconitase. This dual function is controlled by the presence or absence of an iron-sulfur cluster. When cellular iron is high, a 4Fe-4S cluster is inserted into the IRE-binding pocket of IRP-1 and is held in place by three conserved cysteine residues (these residues are also present in the mitochondrial aconitase). Under these conditions, IRP-1 has a closed conformation and cannot bind IREs. The fourth position iron in the cluster is highly labile and is readily removed when cellular iron levels fall, leading to disassembly of the Fe-S cluster, which permits the apoprotein to bind IRE sequences (reviewed in [184–186]).

IRP-2 is less abundant in cells than IRP-1 and is subject to *de novo* synthesis when cellular iron levels are low but is targeted for proteosomal degradation when iron levels are high [187]. IRP-2 contributes significantly to the total IRP RNA binding activity in several tissues but particularly in the brain [188] and intestine [189]. Both IRP-1 and IRP-2 bind successfully to the consensus IRE sequence; however, evidence suggests that IRP-2 may be able to recognize exclusively a specific subset of IRE sequences [190, 191].

5.2 HFE

Hereditary hemochromatosis is a common inborn error of iron metabolism (approximately 1:200 people mainly of northern European decent are affected) that is characterized by excess iron accumulation and deposition within several tissues, especially the liver. The most common form of hemochromatosis arises from an autosomal recessive mutation that leads to the substitution of tyrosine for cysteine at amino acid 282 (C282Y) of the HFE protein [192]. Other mutations, such as H63D, which is more prevalent than C282Y, and S65C may be associated with mild iron loading. Other rarer mutations include missense mutations in exon 2 of the *HFE* gene (I105T and G93R) and a splice-site mutation (IVS3 + 1G/T) that may contribute to the classical hemochromatosis phenotype (reviewed in [193]).

The HFE protein is a member of the MHC class 1 family of molecules that are involved in antigen presentation to T-cells [192]. Like other class I proteins, HFE contains three extracellular loops (α1, α2, α3) which are essential for its function [194]. The α3 domain is required for HFE to associate with β_2-microglobulin for normal intracellular processing and cell surface expression [195–197]. Recent evidence also suggests that the α3 loop is also the site for HFE/Tfr2 interactions [72]. In addition to its interaction with β_2-microglobulin, HFE also binds to Tfr1, via its α2 loop, regulating the rate at which transferrin-bound iron can enter the cell [195, 198]. Given these interactions, it is not surprising therefore that the HFE protein is highly expressed in a number of tissues that have major roles in body iron metabolism, principally the liver (in Kupffer cells and hepatocytes) [52, 199] (Fig. 1.1) but also the duodenum (where it is found exclusively in the crypts of Lieberkühn) [200], and in tissue macrophages and circulating monocytes [201]. The involvement of HFE in iron metabolism has been further confirmed using Hfe knockout mice, which develop liver iron overload and resemble the human hereditary hemochromatosis phenotype [202].

5.3 Hepcidin

Hepcidin is a major regulator of body iron homeostasis. The HAMP (hepcidin antimicrobial peptide) gene is expressed predominantly in the liver and its mRNA encodes an 84 amino acid pre-pro-peptide which undergoes cellular cleavage [203] to release the active 25 amino acid peptide into the circulation [204, 205]. The mature peptide contains eight cysteine residues that yield four disulphide bonds originally thought to confer a distorted hairpin-like structure [206]. However, more recent analysis has produced an updated structure for hepcidin, comprising a stable β-sheet together with a β-hairpin loop [207]. Hepcidin was first identified as an antimicrobial peptide in human plasma ultrafiltrate and urine [204, 205]. However, it became apparent that hepcidin expression is also associated with the regulation of body iron status in both health and disease. Studies revealed that hepcidin expression is dramatically increased when liver iron is high (following dietary iron loading) [208], and is down-regulated by feeding a low-iron diet [19]. In addition to its modulation by iron, hepcidin expression also responds dramatically to changes in the erythroid requirement for iron. Phlebotomy [209], hemolysis [209, 210] and elevated erythropoietin levels [209], major stimuli for reticulocytosis, all inhibit hepcidin production, and result in increased iron assimilation from the diet.

A role for hepcidin in iron metabolism was first established using knockout mice in which the USF2 transcription factor had been deleted. These animals developed a severe iron overload, strikingly similar to that found in human hemochromatosis and in the *Hfe* $^{-/-}$ mouse [211]. Subsequent examination of the *Usf2* $^{-/-}$ mice revealed that the hepcidin gene had also been disrupted (the two mouse genes are only 1,240 bp apart) [208] and an alternative gene-targeting strategy confirmed that it was the disruption of the hepcidin gene and not USF2 that resulted in the iron overloading phenotype [212]. Recent studies have demonstrated a further link between hepcidin expression and the regulation of human iron metabolism. HAMP gene mutations (93delG and C166T – both homozygous recessive mutations) give rise to a severe iron loading disease that typically affects people in their late teens and early twenties, termed juvenile hemochromatosis (also known as HFE Type 2B) [213]. Further mutations in the hepcidin gene have also been identified which alter the structure and function of the mature peptide (reviewed in [214]).

In addition to null animals, transgenic mice over-expressing hepcidin have also been generated [212]. These animals have severe body iron deficiency and microcytic hypochromic anemia, suggesting a reciprocal relationship between hepcidin expression and iron accumulation. Furthermore, studies in humans have demonstrated that elevated expression of hepcidin is associated with the anemia of chronic disease [205, 209, 215, 216], indicating that pathological changes in hepcidin expression have severe consequences for body iron metabolism.

Hepcidin is thought to exert its effects on iron metabolism by inhibiting iron efflux through the ferroportin transporter. A number of in vitro studies suggest that hepcidin binds directly to ferroportin, rapidly (within 1–4 h) inducing the internalization and degradation of transporter protein [217–220]. This in turn impairs the release of iron from its target cells (namely, the reticuloendothelial macrophages and the duodenal enterocytes) into the circulation. While both in vivo and in vitro studies support this rapid mode of action in macrophages [42, 43, 48, 49], recent data from our laboratory [48, 50] and others [49] suggest that intestinal iron transport is not affected by hepcidin over the same time scale. We have proposed that upon its release into the circulation, hepcidin initially targets iron recycling macrophages resulting in down-regulation of ferroportin protein levels and as a consequence hypoferremia. The inhibitory effects of hepcidin on duodenal iron transport and enterocyte ferroportin levels are only evident following chronic exposure to hepcidin. Since the reticuloendothelial macrophages recycle some 20–25 mg Fe/day from senescent red blood cells, compared with only 1–2 mg Fe/day assimilated from the diet by the duodenal enterocytes, we believe the fact that macrophages respond more acutely to a hepcidin challenge is fully consistent with their paramount importance in maintaining body iron homeostasis.

5.4 TMPRSS6

A number of investigators have identified iron-deficient individuals that do not respond to iron supplementation therapy. Recent studies [221–223] have identified several mutations in the TMPRSS6 gene which gives rise to this phenotype. TMPRSS6 encodes a member of the type II transmembrane serine protease family known as matriptase-2. Its full role in controlling body iron status is unclear; however, two mouse models have recently been used to address this issue – the *mask* mouse (which arose from a chemically induced recessive mutation in *Tmprss6*) [224], and the *Tmprss6* knockout mouse [225]. Both models are characterized by microcytic anemia and a progressive loss of hair from the body but not the face of the mouse. TMPRSS6 appears to be a suppressor of hepcidin expression [224], possibly acting via the cleavage of the regulatory protein hemojuvelin [226]. In *mask* and *Tmprss6−/−* mice [224, 225], or in human subjects with TMPRSS6 mutations [223, 227–229], the lack of functional matriptase 2 results in inappropriately high hepcidin levels. Modulation of hepcidin expression in turn leads to downstream effects on intestinal iron absorption and macrophage iron recycling. Recent genome-wide association studies have also identified TMPRSS6 as a strong candidate gene for determining iron status [230–233].

5.5 Hemojuvelin

Juvenile hemochromatosis can be divided into two distinct subtypes; type 2B is associated with mutations in hepcidin, while type 2A occurs as a consequence of mutations in hemojuvelin (HJV). The HJV protein is attached to the surface of hepatocytes and skeletal and cardiac myocytes through a GPI anchor and shares significant homology to the repulsive guidance molecule family. Interestingly, HJV expression is greatest in skeletal and cardiac muscle, suggesting that these organs also may play a major role in regulating body iron metabolism. While a number of mutations in HJV have been identified (reviewed in [214]), one particular mutation (G320V) is significantly more frequent than others [234–240]. Iron overload associated with HJV mutations is accompanied by greatly diminished hepcidin levels in human patients [236] and in mice [241, 242].

Interestingly, there is evidence that HJV can be shed from the cell surface through the action of a pro-protein convertase [243–245]. Intriguingly soluble HJV (sHJV) when added to hepatoma cells can decrease hepcidin expression, suggesting that sHJV may regulate the effects of the cell-associated HJV protein on hepcidin expression [246]. HJV, like other members of the repulsive guidance molecule family, acts as a co-receptor for bone morphogenetic protein (BMP) receptors [247]. Activation of BMP receptor/HJV with BMP2/4 leads to phosphorylation of SMAD proteins (SMAD 1, 5 and 8 are involved in the HJV pathway) which form heteromeric complexes with SMAD 4 and act as transcription factors. Disruption of this pathway via the liver-specific deletion of Smad 4 in mice recapitulates the juvenile hemochromatosis phenotype [248]. In addition, mutations in HJV impair BMP signaling and down-regulate hepcidin expression in hepatocytes [247]. While several BMPs can interact with HJV to regulate hepcidin expression, BMP6 has emerged as the major regulator of iron homeostasis. BMP6 expression in hepatocytes is regulated by iron status [249]. Furthermore, deletion of the BMP6 gene in mice results in massive iron overload with markedly reduced hepcidin expression [250, 251], suggesting that other endogenous BMPs cannot compensate for the loss of BMP6.

5.6 Frataxin

Friedreich's ataxia is an autosomal recessive neuro- and cardio-degenerative disorder affecting 1 in 40,000 Caucasians. The majority of patients have trinucleotide repeat (GAA) within the first intron of the gene encoding frataxin, which leads to iron accumulation within the mitochondria (reviewed in [252–254]). Frataxin is a 210-amino acid protein located predominantly within the mitochondria – associated with the mitochondrial membrane and present as a free soluble protein [255]. The initial link between frataxin and iron metabolism was established in studies using the yeast frataxin homologue (Yfh1) [256]. Yeast lacking Yfh1p accumulate iron [256] and exhibit decreased ability to synthesize iron-sulfur clusters [257]. Interestingly, frataxin can substitute for Yfh1p in yeast, suggesting that they are functional homologues [256, 258]. Human metabolism is reliant on a number of enzymes containing iron-sulfur clusters and frataxin has been suggested to act as a chaperone for iron in mitochondrial iron-sulfur cluster assembly [259, 260]. One key iron-sulfur protein is ferrochelatase, the enzyme responsible for insertion of iron into protoporphyrin IX to form heme. Recent work has suggested that frataxin may act as a high-affinity binding partner for ferrochelatase [261], indicating that frataxin is central to both iron-sulfur and heme protein synthesis.

It is noteworthy that the identification and characterisation of many of the genes and proteins discussed in this chapter has taken place in the past 10–15 years. The roles of these proteins in maintaining iron homeostasis, and the pathological implications of gene mutations will be discussed in more depth in subsequent chapters. Novel proteins and regulatory pathways continue to be identified, for example growth differentiation factor 15 (GDF15) [262, 263] and twisted gastrulation (TWSG1) [264], both members of the TGF-β superfamily that are proposed to act as negative regulators of hepcidin expression; and neogenin which is a putative regulator of membrane HJV levels [265–267]. It seems likely that the next 15 years will be an equally productive era in unraveling the complex pathways that regulate iron homeostasis.

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