Biochimica et Biophysica Acta 1823 (2012) 1459–1467

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Review

Cellular and mitochondrial iron homeostasis in vertebrates ☆

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ARTICLE INFO

Article history:
Received 17 November 2011
Received in revised form 7 January 2012
Accepted 7 January 2012
Available online 18 January 2012

Keywords:
Iron
Transferrin
Non-transferrin bound iron
Mitochondrion
Mitoferrin

ABSTRACT

Iron plays an essential role in cellular metabolism and biological processes. However, due to its intrinsic redox activity, free iron is a potentially toxic molecule in cellular biochemistry. Thus, organisms have developed sophisticated ways to import, sequester, and utilize iron. The transferrin cycle is a well-studied iron uptake pathway that is important for most vertebrate cells. Circulating iron can also be imported into cells by mechanisms that are independent of transferrin. Once imported into erythroid cells, iron is predominantly consumed by the mitochondria for the biosynthesis of heme and iron sulfur clusters. This review focuses on canonical transferrin-mediated and the newly discovered, non-transferrin mediated iron uptake pathways, as well as, mitochondrial iron homeostasis in higher eukaryotes. This article is part of a Special Issue entitled: Cell Biology of Metals.

1. Introduction

Iron is a cofactor that plays critical roles in numerous biological processes including oxygen transport, electron transport, gas sensing and DNA synthesis. Despite its essential role for all eukaryotes, free iron is highly toxic due to its intrinsic ability to generate free radicals through Fenton reactions. Thus, organisms have developed sophisticated pathways to import, chaperone, sequester, and export this metal ion. Disruption of iron homeostasis leads to either iron deficiency or iron overload and contributes to such medical problems as anemia, hemochromatosis, and neurodegenerative disorders [1,2]. Research over the past decade has led to a detailed description of many molecules involved in the transport of iron. In this review, we will focus on both canonical and noncanonical iron uptake pathways as well as mitochondrial iron homeostasis in vertebrates. For iron metabolism in yeast and plants, we refer readers to reviews by Drs. Caroline Philpott and Mary Lou Guerinot in this issue, respectively.

Vertebrates have high requirements for iron, the majority of which is used by red blood cells for hemoglobin production. In humans, although all tissues have a requirement for iron, 65% to 75% of the total body iron is present as heme iron in erythrocytes [3]. Under normal condition, the body iron is derived exclusively from dietary iron through intestinal absorption. It has been proposed that dietary iron is reduced by the duodenal ferric reductases, such as Dcytb [4]; however, the deficiency of Dcytb in engineered knockout mice had no impact on body iron stores, even in the setting of iron deficiency, suggesting that other mechanisms must be available for the reduction of dietary iron [5]. Iron in the intestinal lumen is transported into the enterocytes by the divalent metal transporter DMT1 [6,7]. A fraction of iron is sequestered in the iron storage protein ferritin, while the rest is translocated into the bloodstream by the iron exporter ferroportin1 [8–10]. Ferrous iron in the circulation is oxidized by ceruloplasmin or hephaestin followed by association with the high-affinity iron binding protein transferrin (Tf).

2. Iron uptake through transferrin cycle

Iron uptake through transferrin–transferrin receptor (Tf–TfR) system is essential for maintaining the body iron homeostasis (Fig. 1; see also reviews [11–13]). Besides intestinal absorption, iron recycled from senescent red blood cells contributes significantly to the Tf iron pool. Normally, almost all serum iron is bound to Tf. Iron-loaded Tf (diferric Tf) binds to the Tf receptor (TfR) on cell surface, and the complexes are endocytosed through clathrin-dependent pathway. When pH decreases during the endosome maturation, the ferric iron dissociates from the Tf and the rest of the complex returns to plasma membrane. Once exposed to the neutral pH at cell surface, apotransferrin and TfR dissociates and are ready for another round of iron uptake. Mammals have two TfRs. TfR1 is ubiquitously expressed in almost all types of cells, and TfR2 is predominantly expressed in hepatocytes [14,15]. In addition to its function as a receptor for Tf, TfR2 also senses

☆ This article is part of a Special Issue entitled: Cell Biology of Metals.

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doi:10.1016/j.bbamcr.2012.01.003

and regulates the organismal iron status through modulating the expression of the iron-regulatory hormone hepcidin [16,17].

One of the key factors that control efficient iron uptake is the varying binding affinities of Tf to iron and to TfR at different pH. In the extracellular medium, which has a pH of ~7.4, apotransferrin has very high affinity for ferric iron [18,19]. The diferric Tf binds TfR1 with ~10 and ~100 fold higher affinity than that of monoferric Tf and apotransferrin, respectively [20]. In the acidic environment of endosomes, iron dissociates from Tf due to reduced binding affinity [21,22]. The resultant apotransferrin has significantly higher affinity to TfR1 at the acidified endosomal pH [23,24]. This increased interaction facilitates the recycling of apotransferrin back to the circulation.

This Tf cycle involves membrane trafficking along specific cellular compartments including plasma membrane, early endosomes, and recycling endosomes. It is likely that each step requires specific sorting machinery to ensure the efficiency of cycling [25]. For example, Sec15L1, a protein identified from a hemoglobin deficit (*hbd*) mouse, regulates TfR1 recycling in red blood cells [26,27]. Sec15L1 localizes to the recycling endosomes and interacts with Rab11 [28]. Defective exocytosis, but not endocytosis, of Tf was observed in the *hbd* mouse [29]. The heme synthesis defect can be rescued by supplying the *hbd* reticulocytes with Fe complexed with salicylaldehyde isonicotinoyl hydrazone (SIH), a membrane-permeable iron chelate that can be taken up by cells through TfR-independent pathway [30].

Ferric iron released from the Tf–TfR complex is reduced within endosomes. The six-transmembrane epithelial antigen of the prostate member 3 (Steap3) and other Steap proteins may function as endosomal ferrireductases in erythroid and non-erythroid cells, respectively [31,32].

Once reduced, the iron will be transported across the endosomal membrane. It is well known that the endosomal-localized DMT1 is responsible for mobilizing iron out of endosomes [7,33]. It was recently shown that the transient receptor potential mucolipin 1 (TRPML1) may function as another iron release channel. TRPML1 predominantly localizes to late endosomes and lysosomes (LELs) [34,35]. Mutations of TRPML1 are associated with human mucolipidosis type IV disease — a neurodegenerative lysosomal storage disorder. Studies using patch-clamp recordings showed that addition of Fe^{2+} induced large inwardly rectifying currents on the membranes of TRPML1-positive LELs, indicative of transport [36]. Skin fibroblasts from homozygous $TRPML^{-/-}$ patients displayed significantly lower Fe^{2+} in the cytoplasm, whereas their lysosomes retained higher Fe^{2+} levels than the $TRPML1^{+/+}$ or $TRPML1^{+/-}$ cells [36]. In addition, transport assays using TRPML1(V432P), a mutant form that localizes to both LELs and plasma membrane, indicate that TRPML1 can also transport other divalent metals including Mn^{2+} and Zn^{2+}, but not Fe^{3+}. These results suggest that TRPML1 may function as an iron transporter in endolysosomal systems in parallel to DMT1. A third protein that may be implicated in endosomal iron release is the zinc transporter ZRT/IRT-like protein 14 (Zip14). Zip14 localizes to plasma membrane as well as Tf-positive endosomes in HepG2 cells [37]. Overexpression of *Zip14* in HEK293T cells increased the iron uptake delivered by Tf, whereas its knockdown in HepG2 cells reduced the Tf iron uptake [37]. However, as discussed later in this review, other studies have shown that Zip14 mainly localizes to plasma membrane. Thus, it's more likely that Zip14 plays an important role in Tf-independent iron uptake rather than endosomal iron translocation.

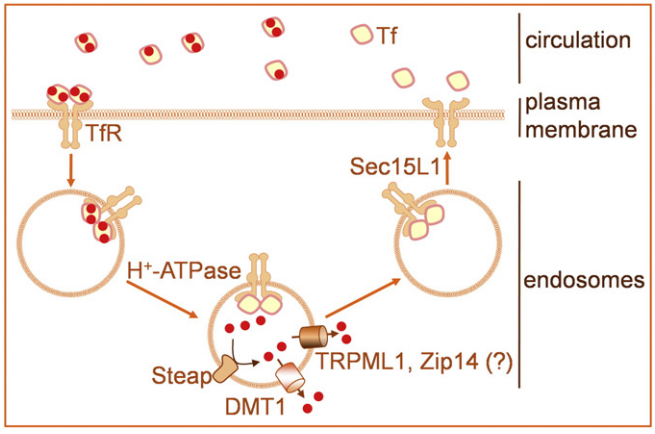

Fig. 1. Iron uptake through transferrin cycle. Diferric Tf binds TfR on the plasma membrane. The Tf–TfR complex is internalized through clathrin-dependent endocytosis. Acidification of endosomes by proton-ATPase lowers the affinity of Tf to ferric iron, which is subsequently released into the lumen. The released ferric iron is reduced by Steaps and exported out of endosomes by divalent metal transporter DMT1. The transient receptor potential mucolipin TRPML1 and the zinc transporter ZRT/IRT-like protein Zip14 may also be involved in endosomal iron release. Within acidic environment, apotransferrin remains bound to TfR. The complex is recycled to the cell surface, where Tf dissociates from TfR. The trafficking molecule Sec15L1 is required for the efficient recycling of Tf–TfR. Details of the basic trafficking machinery such as clathrin, adaptors, and Rab proteins are not shown in the figure.

The mechanism immediately following the endosomal iron release is not well understood. Within the cytoplasm, a proportion of iron is incorporated into the cytoplasmic iron-containing proteins and extramitochondrial iron–sulfur (Fe–S) clusters [38–40]. Excess amount of iron is stored in ferritin. Cytosolic ferritin is composed of 24 H- and L-subunits, and stores up to 4500 iron atoms. A cytosolic iron chaperone poly (rC) binding protein 1 (PCBP1) and its paralog PCBP2 may mediate the iron delivery to cytoplasmic iron-containing proteins. PCBP1 binds ferrous iron with a stoichiometric ratio of one PCBP1 to three iron atoms [41]. It also interacts with cytoplasmic iron-containing proteins including ferritin, iron-dependent prolyl hydroxylases PHDs, and the asparaginyl hydroxylase FIH1 [41,42]. Knockdown of PCBP1 or 2 specifically impairs iron incorporation into these metalloproteins, suggesting that PCBP1 and 2 may serve as iron chaperones responsible for delivering iron to the cytoplasmic proteins [41,42]. It remains to be investigated whether PCBP1 and 2 acquire iron directly from endosomal iron transporters such as DMT1.

3. Mitochondrial iron homeostasis

The mitochondrion serves as the center for cellular iron homeostasis since iron is primarily consumed by this organelle for the synthesis of heme and Fe–S clusters [43,44] (Fig. 2). It is likely that a PCBP1-like cytosolic chaperone may exist to facilitate the transfer of endosomal iron, exported by DMT1, to mitochondria. Alternatively, the iron-loaded endosomes may directly interact with mitochondria for targeted iron delivery. This transient “kiss-and-run” mechanism has been observed in developing erythroid cells, which have high iron demand for mitochondrial heme synthesis [45,46]. Although the molecular basis is still lacking, transient interaction of two organelles may bypass the cytoplasm and ensure sufficient iron supply for the heme synthesis. Intracellular ferritin may provide another source of iron for mitochondria [47] (Fig. 2). Under iron-limiting conditions, ferritin complexes are degraded and ferritin iron is recycled. The degradation of cytosolic ferritin takes place in proteasomes or lysosomes, depending on the type of iron chelation [48–50]. The mechanisms responsible for the transfer of ferritin iron from these compartments to mitochondria are unclear.

By working on the anemic zebrafish mutant *frascati*, Shaw et al. discovered the mitochondrial iron importer mitoferrin 1 (Mfrn1; Slc25a37) and its paralog mitoferrin 2 (Mfrn2; Slc25a28) [51]. These two transporters, located primarily on the mitochondrial inner membrane, play essential roles in supplying iron for the biosynthesis of heme and Fe–S clusters in the mitochondria. Knockout of *Mfrn1* in mouse early erythroid cells resulted in defects in both mitochondrial iron import and heme synthesis [51,52]. In addition to vertebrate Mfrn, its homologs in yeast and plants also play conserved role in transporting iron into the mitochondria. Deletion of the yeast

ortholog, MRS3–MRS4, reduced mitochondrial iron import as well as the synthesis of mitochondrial Fe–S clusters and heme [53–55]. Suppression of the Mfrn1 ortholog in rice, mitochondrial iron transporter (MIT), led to a reduction in mitochondrial iron accumulation and Fe–S cluster biogenesis [56].

The expression of *Mfrn*1 is regulated at both transcriptional and posttranslational levels. The erythroid transcription factor GATA1 can bind two *cis*-regulatory elements upstream of mouse *Mfrn1* and mediates its erythroid-specific expression [57]. During erythroid differentiation, Mfrn1 protein is also stabilized by interacting with another mitochondrial protein, the ATP binding cassette protein Abcb10 [58]. Additionally, the final enzyme in heme synthesis pathway, ferrochelatase, forms an oligomeric complex with Mfrn1 and Abcb10 to enhance the coordinated iron utilization by heme synthesis [59].

In contrast to the strong expression of *Mfrn1* in hematopoietic tissues, *Mfrn2* is ubiquitously expressed in all tissues at low levels, suggesting that it may serve as the mitochondrial iron importer in non-erythroid cells [51]. In non-erythroid cells, these two *Mfrns* are functionally interchangeable [60]. However, *Mfrn2* does not rescue the heme defect in *Mfrn1*-deficient erythroid cells or *frascati* fish mutant [51,60]. This is because that Abcb10 selectively interacts with and stabilizes Mfrn1, but not Mfrn2, in erythroid mitochondria [58].

Iron within the mitochondria is either utilized for the biosynthesis of heme and Fe–S clusters, or stored in mitochondrial ferritin (MtF). MtF was identified as a Ferritin H subunit-like protein expressed in testes and erythroid cells and localized to the mitochondrial matrix [61]. It has both ferroxidase and iron binding activities [61,62]. Overexpression of MtF caused iron redistribution from cytosol to mitochondria [62,63]. Within mitochondria, MtF sequesters iron and limits the iron consumption by heme and Fe–S cluster synthesis [63]. Thus, abnormal expression of MtF in erythroid cells associates with the X-linked sideroblastic anemia [64]. Most of these observations were made in overexpression systems. Further studies are required to reveal the precise role of MtF in iron homeostasis.

Although non-erythroid cells need heme for incorporation into a wide variety of hemoproteins such as cytochromes, erythroid tissues have extremely high demand for heme synthesis, and thus mitochondrial iron supply. Heme biosynthesis is a highly conserved pathway which involves eight enzymatic reactions [44]. It is tightly controlled by many factors such as iron availability and oxygen levels.

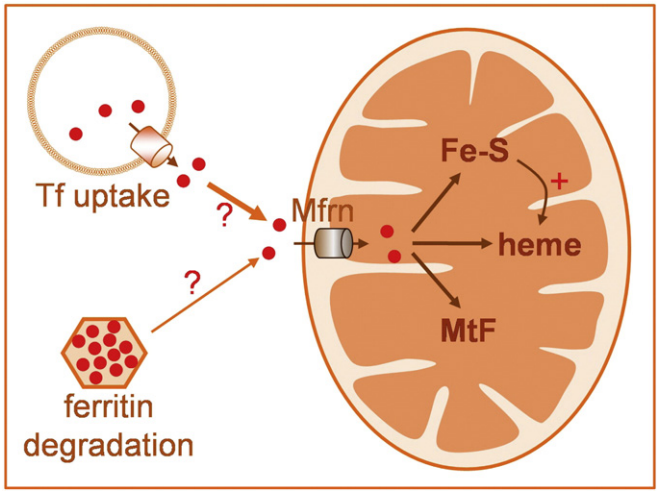

Fig. 2. Mitochondrial iron import. The iron imported through Tf–TfR cycle can be readily utilized by mitochondria. Cytosolic ferritin may also provide iron for mitochondria. Tf iron is released into endosomes, whereas ferritin is degraded in either proteasomes or lysosomes. Iron is delivered to mitochondrial outer membrane through unknown mechanisms. Mitoferrin (Mfrn) imports iron into the mitochondria, where iron is either utilized for the synthesis of heme and Fe–S clusters, or stored in mitochondrial ferritin (MtF). Fe–S clusters may regulate erythroid heme synthesis through modulating the activity of iron regulatory protein 1 and the expression of δ-aminolevulinate synthase 2.

Mutations in heme synthesis genes result in human hematological disorders characterized by defective cellular iron homeostasis [65]. Defects in genes relating to heme biosynthesis, such as ferrochelatase [66] and C-terminal deletion of δ-aminolevulinate synthase 2 (ALAS2) [67] are associated with porphyrias. In addition, proteins that impact on the phenotypic expression of porphyrias include heme-regulated eIF2-kinase [68], iron regulatory protein 2 (IRP2) [69], and abnormal expression of Mfrn1 [52,70]. Previous human studies have identified *ALAS2*, *FXN*, *ABCB7*, and *SLC25A38* as the genetic causes of congenital sideroblastic anemias [71,72].

Heme synthesis is also regulated by the availability of Fe–S clusters (Fig. 2). Fe–S clusters are prosthetic groups that play essential roles in basic cellular metabolism such as mitochondrial electron transport and catalysis. Defects in the assembly of mitochondrial Fe–S clusters are associated with mitochondrial iron overload and blood disorders [43,73]. For example, patients with Friedreich ataxia exhibit mitochondrial iron overload due to genetic defects in the mitochondrial Fe–S cluster iron chaperone Frataxin [74]. Likewise, mutations in *glutaredoxin-5*, a gene required for Fe–S cluster synthesis [75], resulted in mitochondrial iron accumulation and microcytic anemia [76,77]. The resultant mitochondrial iron accumulation may lead to abnormal heme synthesis. In addition, Fe–S clusters may directly control heme synthesis through regulating the activity of iron regulatory protein 1 (IRP1) and the expression of *ALAS2*, the rate limiting enzyme in erythroid heme synthesis [78]. When the level of Fe–S clusters is low, IRP1 associates with the iron regulatory element (IRE) in the 5′ untranslated region (UTR) of *ALAS2* mRNA, blocking its translation as well as heme synthesis. More recently, a systems biology study found that two genes known to be involved in Fe–S cluster biosynthesis, *Isca1* and *Iba57*, are essential for erythroid heme biosynthesis [79]. Morpholino knockdown of either gene in zebrafish resulted in profound anemia, possibly due to defects in heme synthesis.

In addition to the functional requirement of heme and Fe–S clusters within mitochondria, a large number of hemoproteins and Fe–S containing proteins are present in extramitochondrial compartments [80,81]. Thus, these two iron-containing cofactors must be able to exit mitochondria. These export mechanisms may be indispensable for maintaining mitochondrial iron homeostasis. The ATP binding cassette transporter ABCB7 is functionally required for the export of mitochondrial Fe–S clusters. Mutation of ABCB7 caused X-linked sideroblastic anemia in humans [82,83]. Knockdown of ABCB7 resulted in mitochondrial iron overload accompanied with cellular iron deficiency [84]. The pathway responsible for mitochondrial heme export remains elusive. A potential porphyrin transporter ABCB6 might be involved [85]. ABCB6 is an outer mitochondrial membrane protein initially identified as a mammalian ortholog of the yeast mitochondrial iron transporter ATM1 [86]. Its expression strongly correlates with that of heme synthesis genes [79]. ABCB6 interacts with both heme and porphyrin compounds [85]. Overexpression of ABCB6 in human erythroleukemia (K562) cells significantly increased the population of protoporphyrin IX-positive cells and the cytosolic heme levels [85,87]. Further investigation is required to dissect the biological role of ABCB6 in heme homeostasis and its subcellular localization [88].

4. Trafficking of non-transferrin bound iron

Although Tf cycle is indispensable for hematopoiesis, most other cells may take up iron independent of this cycle. In hypotransferrinemic mice, only hematopoietic cells are deficient of iron, while other cells even exhibited iron accumulation [89]. Similarly, mice lacking TfR1 displayed severe anemia and embryonic lethality due to iron deficiency in erythroid tissues, but the development of other organs

appeared to be normal [90,91]. Studies with chimera mouse embryos showed that TfR1$^{-/-}$ stem cells can populate many organs including bladder, gonad, liver, lung, kidney, and intestine [91], indicating that these cells are able to utilize iron through mechanisms that are distinct from Tf–TfR pathway. Non-transferrin bound iron (NTBI) refers to the circulating pool of iron that is not bound to Tf. A number of molecules, such as citrate, acetate, and albumin, have been proposed to bind NTBI. It was recently found that mammals secrete siderophore-like compounds that can bind iron in the circulation [92,93]. The cellular uptake of siderophore iron, heme, and ferritin may represent main iron uptake pathways that are independent of Tf cycle (Table 1). Heme trafficking is not covered in this review since it is discussed by Drs. Iqbal Hamza and Harry Dailey in this issue.

4.1. Lipocalin–siderophore system

Bacteria, fungi, and graminaceous plants are able to obtain iron by secreting a group of small, high-affinity iron binding molecules called siderophores [94,95]. Hydroxamic acids and catechols are common functional moieties found in siderophores. Once loaded with Fe^{3+}, these siderophores can be taken up by cells through cell surface receptors.

Vertebrate animals also produce siderophores. A recent chemical screen on protein-free urine filtrates identified a group of active catechol-like compounds with high affinity for Fe^{3+} [92]. Devireddy et al. independently discovered 2,5-dihydroxybenzoic acid (DHBA) as another iron-binding compound that is excreted by murine pro-B lymphocytic FL5.12 cells [93]. 2,5-DHBA is analogous to 2,3-DHBA, the functional core in bacterial siderophore enterobactin [95]. Importantly, EntA, the enzyme responsible for the synthesis of bacterial 2,3-DHBA, is conserved in vertebrates as a β-hydroxybutyrate dehydrogenase (BDH2) [93]. Silencing of BDH2 in mouse erythroleukemia (MEL) cells impaired the production of heme and hemoglobin [93]. BDH2 knockdown with morpholinos in zebrafish resulted in hypochromic anemia [93]. Taken together, these results suggest that vertebrate siderophores may contribute to iron homeostasis in erythroid tissues.

Vertebrate siderophores bind lipocalin 2, which is also known as α2-microglobulin-related protein, neutrophil gelatinase-associated lipocalin, 24p3, and siderocalin. Lipocalin 2 belongs to lipocalin family — a group of small, secreted proteins that bind small hydrophobic compounds [96,97]. Human lipocalin 2 is highly expressed in bone marrow and trachea [98]. The expression and secretion of lipocalin 2 is also induced under pathological conditions such as renal injury [99,100]. Lipocalin 2 was shown to bind catechol-type siderophores, and the binding affinity is affected by its iron status [92,93]. For example, the presence of Fe^{3+} allosterically increased the catechol–lipocalin 2 interaction by ~100 fold [92]. When introduced separately into mouse serum, lipocalin 2 and catechol-Fe^{3+} quickly associate [92].

Lipocalin 2 is also able to bind siderophores secreted by bacteria or mycobacteria with high affinity. When expressed in *Escherichia coli*, lipocalin 2 was copurified with bacterial siderophore, ferric enterobactin [101]. Additionally, lipocalin 2 can bind soluble siderophores from *Mycobacterium tuberculosis*, carboxymycobactins [102]. Subsequent studies showed that it plays an important role in antibacterial innate immune response by sequestering iron. Its expression was significantly induced by lipopolysaccharide and infection [103,104]. Presence of lipocalin 2 specifically inhibits bacteria growth in an enterobactin-dependent manner, whereas lipocalin 2 deficient mice had significantly increased bacteria burden and lethality upon infection with pathogenic *E. coli* [103,105]. Interestingly, these lipocalin 2 knockout mice appear to have normal organ development [103], suggesting that the contribution of siderophore iron to organogenesis may be dispensable during the development, or other pathways may exist to mediate the uptake of siderophore iron. Another lipocalin, lipocalin Q83, originally identified in quail fibroblasts, was also shown to bind siderophore enterobactin with affinity comparable to that of lipocalin 2 [106]. Lipocalin Q83 has 87% and 23% sequence identities to chicken homolog Ch21 and human lipocalin 2, respectively [106]. However, this lipocalin also binds unsaturated fatty acids, suggesting that it may play a dual role in the metabolism of iron and fatty acid [107].

Table 1
Candidate molecules involved in the uptake of non-transferrin bound iron.

Name of protein or molecule	Description and other names	Function or putative function	Reference
Catechol		Mammalian siderophore	[92]
2,5-DHBA	2,5-dihydroxybenzoic acid	Mammalian siderophore	[93]
Lipocalin 2	α2-microglobulin-related protein, neutrophil gelatinase-associated lipocalin, 24p3, siderocalin	Mammalian siderophore-iron binding protein	[92,93,101, 102,108]
BOCT	Brain type organic cation transporter	Lipocalin 2 receptor	[110]
Megalin	Low-density lipoprotein receptor 2	Lipocalin 2 receptor	[109]
Scara5	Scavenger receptor class A member 5	L-ferritin receptor	[91]
Tim-2	T cell immunoglobulin and mucin domain-containing protein-2	H-ferritin receptor in mouse	[119,120]
TfR1	Transferrin receptor 1	H-ferritin receptor in human	[123]
Zip14	ZRT/IRT-like protein 14	Fe^{2+} transporter	[126,127]
DMT1	Divalent metal ion transporter 1	Fe^{2+} transporter	[129–131]
TRPC6	Transient receptor potential protein canonical 6	Regulator of NTBI uptake	[135]
LVDCC	L-type voltage-dependent calcium channel	Regulator of NTBI uptake	[136]

Mammalian cells are capable of utilizing extracellular lipocalin 2-siderophore-Fe^{3+} complex for iron homeostasis. Bao et al. showed that the complex formed in mouse serum could be captured by kidney, lung, and liver [92]. Incubation of ^{59}Fe-labeled lipocalin 2 with Madin Darby Canine Kidney (MDCK) and other kidney-derived cell lines resulted in a time-dependent accumulation of cellular ^{59}Fe [108]. This iron source can contribute to cellular iron metabolism since iron-loaded lipocalin 2 induced the ferritin expression and repressed TfR1 expression [108]. Similar to Tf–TfR system, lipocalin 2–catechol–Fe^{3+} complexes are stable at neutral pH, and dissociate upon acidification of the environment [92]. After iron is released within acidic endosomes, lipocalin 2 is not degraded, suggesting that it may be recycled [108]. While the mechanisms of iron release and protein recycle are likely to be similar to Tf cycle, the internalization step is different. Upon entering the cells, the majority of lipocalin 2 is localized to the compartments that are distinct from Tf [108]. Furthermore, the uptake of lipocalin 2 and Tf does not compete with each other [108], suggesting that other receptors exist for the internalization of lipocalin 2–catechol–Fe^{3+} complexes.

Two cell surface receptors may be implicated in the trafficking of lipocalin 2. Megalin, also known as low-density lipoprotein receptor 2, is an endocytic molecule that binds both apo- and hololipocalin 2 with high affinity [109]. A rat yolk sac cell line expressing megalin specifically takes up lipocalin-2 — an activity that is blocked by anti-megalin antibody [109]. By screening the cDNA library of murine FL5.12 cells for lipocalin 2 binding activity, Devireddy et al. discovered brain type organic cation transporter (BOCT) as another lipocalin 2 receptor [110]. BOCT is widely expressed in mouse tissues such as kidney, placenta, and heart. HeLa cells stably expressing BOCT exhibited specific internalization of ^{32}P-labeled or fluorophore-labeled lipocalin 2. Incubation of BOCT-expressing HeLa cells with iron-

loaded lipocalin 2 significantly increased the cellular iron uptake, as indicated by changes in ferritin expression and intracellular iron levels.

Interestingly, when BOCT-expressing cells were exposed to apolipocalin 2, the intracellular iron levels were reduced [110]. This result is consistent with a study by Li et al. which show that iron-loaded lipocalin 2 donates iron whereas apolipocalin 2 chelates iron when incubating with HEK293 cells [111]. Iron chelation of apolipocalin 2 within the cells is dependent on the presence of siderophores. When the siderophore synthesis gene BDH2 was knocked-down in murine FL5.12 cells, apolipocalin 2 had dramatically reduced activity to bind or chelate intracellular iron [93]. These results suggest that mammalian siderophores could also associate with iron in the cytoplasm. Thus, lipocalin 2 may play a dual role in regulating cellular iron homeostasis.

4.2. Secreted form of ferritin and associated receptors

The iron-storage protein ferritin may also be involved in cellular iron import. Ferritin is present in the cytoplasm, nucleus and mitochondrion within the cell. It is also found in the circulation, and the levels of serum ferritin are widely used to monitor the body iron status in humans. Cohen et al. showed that mouse serum ferritin forms a complex of 24 subunits, which are composed of mostly L-ferritin with detectable levels of H-ferritin [112]. The majority of serum ferritin comes from secretion by macrophages [112,113], the cells primarily responsible for iron recycling from senescent red blood cells. The ferritin chains are secreted through classic secretory pathway and this may be mediated by the ER-targeting sequence contained at the amino terminus [114,115]. The secretion is inducible under conditions when ferritin proteins are produced but the cytoplasmic iron is limiting [114].

A number of studies have shown that cells can utilize extracellular ferritin as an iron source. Cationic ferritin, a derivative of horse spleen ferritin, can be endocytosed to lysosomes for degradation in human fibroblast [116]. The macrophage-derived ferritin iron can be utilized by hepatocytes and erythroid precursor cells for the production of heme and hemoglobin [113,117]. Supplementation of H-ferritin can stimulate the growth and differentiation of oligodendrocyte progenitor cells that were deprived of Tf [118].

Li et al. found that mesenchymal tissues, capsule and stroma of the kidney are able to take up iron independent of TfR1, and further identified the scavenger receptor class A member 5 (Scara5) as a receptor for L-ferritin in the kidney [91]. Transient expression of Scara5 in Trvb cells, a cell line that lacks Scara5 and TfR1, resulted in the internalization of L-ferritin. Knockdown of Scara5 in mouse Sertoli cell line (MSC-1) cells by shRNA inhibited the ferritin endocytosis. The Scara5-expressing cells can also internalize haptoglobin–hemoglobin complex, but not H-ferritin or Tf. Uptake studies with ^{59}Fe-Ferritin confirmed that the imported ferritin iron stimulates the growth and proliferation of Trvb and capsular cells.

Two molecules were suggested to function as receptors for H-ferritin. Mouse T cell immunoglobulin and mucin domain-containing protein-2 (Tim-2) may serve as a receptor for H-ferritin in splenic T cells and oligodendrocytes [119,120]. TIM-2 localizes to plasma membrane and endosomes [119,121]. It specifically interacts with and internalizes H-ferritin, but not L-ferritin [119,120]. TCMK-1, a mouse kidney epithelial cell line, transfected with Tim-2 had significantly increased uptake of ^{55}Fe-loaded H-ferritin [121]. The uptake was confirmed in a mouse lymphocyte cell line (A20). Receptor-mediated endocytosis is responsible for the internalization of H-ferritin [121]. Since serum ferritin is present as 24-subunit nanocages [112], they need to be disassembled before receptor binding and internalization take place. It is unclear how ferritin disassembly is achieved extracellularly. Upon reaching lysosomes, H-ferritin is degraded and the released iron enters the cytoplasmic iron pool [121].

A distinct H-ferritin receptor may exist in humans since Tim-2 is not present in human genome [122]. In addition to serving as Tf receptor, TfR1 was found to be a primary H-ferritin receptor in reticulocytes, activated lymphocytes, and most other cell lines in humans [123]. It specifically interacts with H-ferritin, but not L-ferritin. The interaction can be partially inhibited by the presence of diferric Tf. The H-ferritin–TfR1 complex is endocytosed through an endocytic pathway that is similar to the Tf cycle. However, within the endosomes, H-ferritin is sorted to lysosomes for degradation whereas Tf returns to the plasma membrane.

4.3. Zip14

Zip14 was first identified as a plasma membrane protein with zinc transport activity [124,125]. Liuzzi et al. found that in addition to zinc, ferrous iron is also transported by Zip14 [126]. Expression of *Zip14* in Sf9 cells significantly increased the uptake of ^{59}Fe, which can be inhibited by the addition of either a cell-impermeant ferrous chelator or zinc. Furthermore, knockdown of *Zip14* in mouse hepatocyte AML12 cells significantly reduced the uptake of iron. Expression of *Zip14* in Xenopus oocytes stimulated the uptake of Fe^{2+}, but not Fe^{3+}, in a pH-dependent and calcium-dependent manner [127]. In addition to Zn^{2+} and Fe^{2+}, Zip14 also transports Cd^{2+} and Mn^{2+} [127]. *Zip14* is regulated by the hereditary hemochromatosis protein HFE post translationally [128]. Studies on both HepG2 and HeLa cells showed that overexpression of HFE reduced the uptake of NTBI through decreasing Zip14 protein level [128].

4.4. Other molecules involved in the uptake of NTBI

The well-studied iron transporter DMT1 mediates the uptake of ferrous iron in the intestine [7]. A number of *in vitro* studies suggest that DMT1 is also involved in the uptake of Fe^{2+} in other cells. For example, Fe^{3+} can be reduced by ascorbate and the Fe^{2+} accumulates in primary rat astrocytes in a DMT1-dependent manner [129]. Overexpression of DMT1 significantly increased the uptake of Fe^{2+} independent of Tf cycle in hepatoma and Chinese hamster ovary cells [130,131]. DMT1 also localizes weakly on the plasma membrane, in addition to the cytoplasmic localization, of hepatoma cells [130].

Calcium channels may also be implicated in the regulation of NTBI transport. Extracellular calcium has been shown to stimulate the uptake of NTBI by HeLa and K562 cells [132]. Transient receptor potential protein canonical 6 (TRPC6), an α1-adrenoceptor-operated calcium channel, plays a predominant role in store-independent Ca^{2+} entry [133,134]. Overexpression of TRPC6 in HEK293 cells decreased the fluorescence of calcein, an indicator that is negatively regulated by cytoplasmic iron levels [135]. Another calcium channel involved in iron homeostasis is L-type voltage-dependent calcium channel (LVDCC). Blocking of LVDCC activity with amlodipine and verapamil in chronic iron-overload mice reduced the myocardial iron levels, while overexpression of α1-subunit of LVDDC increased the iron deposition in cardiomyocytes [136]. Similarly, the use of T-type calcium blocker efonidipine reduced the iron uptake by cultured cardiomyocytes [137]. Although it was proposed that the calcium channels transport a wide range of divalent substrates including Fe^{2+} [138,139], they may function as regulators, rather than transporters, in iron homeostasis. Ludwiczek et al. showed that the dihydropyridine-based LVDCC blocker nifedipine regulated DMT1 activity — a mechanism that was responsible for mobilizing iron out of liver in iron-overload mice [140]. Future investigations using systems other than inhibitors or overexpressed cell lines may help to elucidate the physiological role of calcium channels in iron metabolism.

4.5. Utilization of NTBI by mitochondria

Some forms of NTBI may contribute to mitochondrial iron homeostasis. For example, silencing of vertebrate siderophore synthesis gene BDH2 reduced the mitochondrial iron content as well as heme

synthesis [93], indicating that siderophore iron can be utilized by mitochondria. Similarly, iron delivered through extracellular ferritin may also be consumed by mitochondria for the production of heme and hemoglobin [113,117,141]. Evidence is still lacking regarding the significance of iron uptake through Zip14 and calcium channels on mitochondrial iron metabolism. Furthermore, it is unclear whether siderophore iron, ferritin iron, and Tf-iron traffic to mitochondria through similar molecular pathways.

5. Current approaches in identifying iron trafficking regulators

Studies with model organisms zebrafish, mice, rats, and yeast have greatly improved the understanding of iron metabolism. Many iron trafficking molecules and regulators were discovered through such strategies as forward genetics, reverse genetics, expression cloning, bioinformatics, and proteomics (Table 2).

5.1. Phenotype-driven approaches

A number of novel genes that play fundamental roles in iron metabolism were identified by mutagenesis screens in zebrafish and mouse [142–144]. The only known vertebrate iron exporter, ferroportin1, was initially discovered through characterizing the zebrafish mutant *weissherbst* [9]. This iron exporter was also independently identified using other approaches such as subtractive cloning and RNA pull-down [8,10]. Studies on another zebrafish mutant *frascati* led to the identification of the high-affinity mitochondrial iron importer mitoferrin 1 [51]. Additionally, the iron-related functions of the endosomal ferrireductase Steap3 and the exocyst protein Sec15L1 were uncovered by studying the anemic mutant mice *nm1054* and *hbd*, respectively [26,31]. Current tools such as the next-generation sequencing may be applied to accelerate the identification of genetic lesions in animal mutants or human patients with blood disorders. Classical human genetics recently identified the mitochondrial glycine transporter, SLC25A38, whose defect results in sideroblastic anemia [145].

5.2. Gene-driven approaches

This type of approaches usually starts with a set of interesting genes obtained from DNA sequencing, gene expression studies, or bioinformatics analysis. Genes with similar expression profiles under a certain biological condition are more likely to be involved in the same biological pathway. The phenotypes and functions of these genes can be examined through targeted mutagenesis or gene silencing. To identify new components in mitochondrial iron trafficking, Nilsson et al. screened for the mitochondrial genes that were coordinately coexpressed with the heme synthesis genes from ~35,000 public available cDNA microarrays [79]. Five novel genes (Slc25a39, Slc22a4, Tmem14c, Isca1, and Iba57) were found to be essential for heme synthesis and blood development. Morpholino knockdown of these genes resulted in anemia in zebrafish, and silencing of SLC25A39 in differentiating MEL cells strongly reduced heme synthesis, suggesting that they may be involved in mammalian mitochondrial iron homeostasis.

Table 2
Identification of regulators of iron homeostasis.

	Function	Reference
Phenotype-driven		
DMT1	Divalent iron transporter	[6, 9]
ferroportin1	Vertebrate iron exporter	[9]
mitoferrin 1	Mitochondrial iron importer	[51]
Steap3	Endosomal ferrireductase	[31]
Sec15L1	Trafficking regulator of TfR1	[26]
Gene-driven		
Slc25a39	Regulator of heme synthesis	[79]
Slc22a4	Regulator of heme synthesis	[79]
Tmem14c	Regulator of heme synthesis	[79]
Expression cloning		
DMT1	Divalent iron transporter	[7]
PCBP1	Cytosolic iron chaperone	[41]
BOCT	Lipocalin 2 receptor	[110]
Proteomics		
Abcb10	Regulator of mitoferrin 1	[58]

5.3. Expression cloning

This strategy combines the advantages of both forward and reverse genetics. Usually, after the initial functional screen, the identity of the gene can be quickly uncovered. For example, the screen of a rat duodenal cDNA library in oocytes and a human liver cDNA library in yeast led to the discoveries of the proton-coupled Fe^{2+} transporter DMT1 and the cytosolic iron chaperone PCBP1, respectively [7,41]. DMT1 was also simultaneously identified as an iron transporter by analyzing the microcytic anemic *mk* mice [6]. In addition, by screening a cDNA library prepared from murine FL5.12 cells, Devireddy et al. found that BOCT, the brain type organic cation transporter, acts as a receptor for lipocalin 2 [110].

5.4. Proteomics

Proteomic analysis has been widely used in identifying protein–protein interactions. One successful case of this strategy in iron trafficking is the functional characterization of Abcb10. Abcb10 has long been proposed as a mitochondrial transporter in erythroid tissues [146,147]. The proteomic study by Chen et al. revealed that Abcb10 regulates iron metabolism by stabilizing the mitochondrial iron importer mitoferrin 1 [58]. Furthermore, this study showed that the ferrochelatase interacts with mitoferrin complex in order to enhance the iron utilization by heme synthesis [59].

6. Perspectives

In the near future, we anticipate that additional components will be identified in iron metabolism by using the combination of genetic, genomic, systems biology, and biochemical approaches. Future studies on noncanonical iron uptake pathways will provide important insights in iron homeostasis in nonerythroid tissues. It is an exciting time for current and new investigators to further our understanding of iron homeostasis.The putative porphyrin transporter, ABCB6, previously ascribed to be essential for mitochondrial porphyrin uptake was recently shown to be dispensable for erythropoiesis and specifies the new blood group system Langereis (V. Helias, et al. Nat. Genet. (2012), in press. doi:10.1038/ng.1069). Human patients with somatic mutations in ABCB6 have ocular colobomas (L. Wang, et al. Am. J. Hum. Genet. 90 (2012) 40–48).

Note added in proof

The putative porphyrin transporter, ABCB6, previously ascribed to be essential for mitochondrial porphyrin uptake was recently shown to be dispensable for erythropoiesis and specifies the new blood group system Langereis (V. Helias, et al. Nat. Genet. (2012), in press. doi:10.1038/ng.1069). Human patients with somatic mutations in ABCB6 have ocular colobomas (L. Wang, et al. Am. J. Hum. Genet. 90 (2012) 40–48).

Acknowledgements

This work was supported by grants from the Cooley's Anemia Foundation (C.C.), March of Dimes Foundation (B.H.P.), and the National Institutes of Health Grants R01 DK070838 and P01 HL032262 (B.H.P.).

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