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A General Map of Iron Metabolism and Tissue-specific Subnetworks

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Abstract

Iron is required for survival of mammalian cells. Recently, understanding of iron metabolism and trafficking has increased dramatically, revealing a complex, interacting network largely unknown just a few years ago. This provides an excellent model for systems biology development and analysis. The first step in such an analysis is the construction of a structural network of iron metabolism, which we present here. This network was created using CellDesigner version 3.5.2 and includes reactions occurring in mammalian cells of numerous tissue types. The iron metabolic network contains 151 chemical species and 107 reactions and transport steps. Starting from this general model, we construct iron networks for specific tissues and cells that are fundamental to maintaining body iron homeostasis. We include subnetworks for cells of the intestine and liver, tissues important in iron uptake and storage, respectively; as well as the reticulocyte and macrophage, key cells in iron utilization and recycling. The addition of kinetic information to our structural network will permit the simulation of iron metabolism in different tissues as well as in health and disease.

Keywords

iron; liver; macrophage; reactive oxygen species; red blood cells

Introduction

Understanding of iron metabolism and trafficking in eucaryotes has increased exponentially in recent years. New genes important in cellular and systemic iron homeostasis have been identified, and their roles have been intensively investigated (see1–3, for review). These studies have unveiled a complex, interacting network largely unknown just a few years ago. As such, the cellular iron network provides an excellent model for systems biology development and analysis. The first step in such an analysis is the construction of a network model, which we present here.

Iron metabolism is highly regulated at both the organismal and cellular levels. Iron is an essential element, required for the function of many enzymes and transport proteins. In these proteins, iron functions as a requisite cofactor, frequently in the form of iron-sulfur clusters or heme. Well-known proteins that require iron for their function include hemoglobin and

myoglobin, which function in oxygen transport; cytochromes, necessary for both respiration and detoxification; aconitase and other proteins required for oxidative metabolism; ribonucleotide reductase, which catalyzes the rate-limiting step in DNA synthesis, and many others.

Iron is also a redox active element with substantial potential toxicity. It can facilitate the formation of oxygen free radicals through the Fenton reaction, in which reduced iron (FeII) reacts with hydrogen peroxide to produce the hydroxyl radical, a highly reactive radical capable of damaging DNA, lipids and proteins. Iron is also extremely insoluble (10^{-18} M) in aqueous solution4. Thus the proper distribution and sequestering of iron is a challenge at both the cellular and organismic level.

The importance of appropriate iron homeostasis becomes clear when conditions of iron imbalance are considered. For example, hemochromatosis is a disease of iron overload characterized by excess uptake of dietary iron5. In patients with hemochromatosis, iron can accumulate not only in the liver, its normal storage site, but in the pancreas and heart, where it can lead to diabetes or heart disease. More prevalent than these conditions of iron overload are conditions of iron insufficiency, which can lead to iron-deficiency anemia. Indeed, according to the World Health Organization, iron deficiency is the most common and widespread nutritional disorder in the world: 2 billion people – over 30% of the world's population – are anemic, many due to iron deficiency6.

At the level of the whole organism, iron homeostasis is regulated at sites of absorption, utilization (and re-utilization) and storage. There is no excretory pathway for iron, although small amounts are lost through the skin, intestinal mucosa, and menstruation in women. Major sites of iron regulation are the duodenum, where dietary iron is absorbed; the bone marrow, where red blood cells are produced; the liver, where excess iron is stored; and the spleen and other cells of the reticuloendothelial system (RES), where red blood cells are catabolized and iron is extracted for reuse. Cell types that exert these functions are the enterocyte in the duodenum; reticulocyte in the bone marrow; and macrophages in the spleen, liver and RES.

At the cellular and molecular level, the regulation of iron is achieved through the concerted action of iron importers, iron storage proteins, and iron efflux pumps. Collectively, these act to deliver iron to its sites of utilization within proteins, while minimizing the ability of iron to participate in oxygen free radical-generating reactions. Within the cell, iron is also shuttled among different compartments. For example, following its uptake through transporters located on the plasma membrane, iron is found in early/recycling/late endosomes. It is subsequently delivered to mitochondria for heme biosynthesis and formation of iron-sulfur clusters. Iron storage proteins, which minimize the formation of ROS (reactive oxygen species), as well as iron regulatory proteins, which provide an important feedback mechanism when iron is low, are located in the cytoplasm. Iron-containing proteins are also present in the lysosome, where they are degraded; the iron from these proteins is then recycled back into the cytoplasm. In our models, we have illustrated these major cellular compartments of iron, as well as iron localized to the extracellular space. This extracellular compartment represents iron delivered through the blood as a complex with the transport protein transferrin, as well as extracellular iron present as other species. To simplify drawing the diagram, we do not include the nucleus and instead depict transcription in the cytoplasm, but the reader should bear in mind that this is of course implicit.

The complexities of iron regulatory pathways suggest that mathematical approaches might be useful in understanding the interactions among iron-dependent species, identifying key regulatory nodes, simulating their response to various stimuli, and understanding how these

differ in various cell types. As a first step towards that goal, we present a structural model of iron metabolism in a mammalian cell using CellDesigner v.3.5.2.

CellDesigner7 is a diagram editor for creating biochemical networks and has been used to construct maps of other important pathways8,9. CellDesigner is not merely a pictorial tool, but a platform for the creation of biochemical network models in the SBML language. The SBML files created by CellDesigner can be used to generate kinetic simulations of the network using a system of ordinary differential equations or through stochastic simulation algorithms with software packages such as COPASI10,11 and others (see 12 for an example of CellDesigner network construction followed by kinetic simulation through the SBML platform).

Results

General network

Using CellDesigner, we first created a general iron network (Fig. 1). In this network we do not depict the reactions that occur in a particular cell type, but rather a culmination of the reactions occurring in cells of numerous tissue types as described in the published literature. Sometimes, multiple studies report conflicting results, and often the precise reaction details are not yet understood. For those cases, we have made a judgment call for the purpose of our network and indicate the controversy in the detailed Description of Reactions, below.

Our general network of iron metabolism contains 151 chemical species and 107 reactions and transport steps. When a chemical species is present in more than one compartment, multiple individual species are present in the map (thus they actually represent “pools”). There are 57 proteins, 7 mRNAs, and 7 genes represented explicitly. Our main interest lies in the protein level, and hence we only represent the corresponding genes or mRNAs if there is an iron-related modification of transcription or translation (*i.e.* all other transcription/translation events are left implicit). Additionally, we have 46 complexes which include protein-protein and protein-iron complexes. The set of reactive oxygen species (ROS) is also represented by the symbol for complexes but in this case any ROS is meant (*i.e.* they are not in complexes in the cell). 10 iron ions exist as well as 22 metabolites (“simple molecules” in the notation of CellDesigner), such as the intermediates in porphyrin formation. Degradation occurs in two compartments, which accounts for 2 species.

Tissue- specific networks

We then focused more closely to create subnetworks specific to cells important in iron metabolism: the hepatocyte, reticulocyte, enterocyte, and macrophage. We first assessed the expression of each of the species depicted in the General Network (Fig. 1) in these specific cell types (Table 1). We included those genes and proteins for which there was evidence of expression in relevant mammalian tissues, although in some cases only cell line data was available. The general criteria for the entry of each mRNA or protein in the tissue-specific network are documented in Table 1; specific judgment calls related to individual species are discussed in detail below. Tissue-specific networks created in this way were the hepatocyte (Fig. 2), reticulocyte (Fig. 3), enterocyte (Fig. 4) and macrophage (Fig. 5).

Description of reactions in the general network

Reactions included in the general network are those involved in iron/heme import (e.g. TfR1, DMT1, lactoferrin receptor, 24p3R, HCP1); iron/heme export (ferroportin, FLVCR, ABCG2); and iron storage (ferritin). Synthetic reactions involving iron, including those involved in synthesis of iron sulfur clusters (ISU) and heme (ALAS, ferrochelatase), are described under the heading of mitochondria, although some of the reactions in these pathways occur in the

cytoplasm. Feedback regulatory reactions that operate at the level of the cell (IRPs) or whole organism (hepcidin) are also included.

A. Iron/heme importers

1. TfR1: Most iron circulates in the blood bound to transferrin, a protein with two binding sites for ferric iron13,14. In healthy adults, transferrin is approximately 30% saturated with iron15, while increased transferrin saturation levels (>45%) are associated with the iron overload disorder hereditary hemochromatosis16. The binding and release of iron to transferrin is pH dependant13,17,18, which allows transferrin to bring iron into cells through a receptor-mediated process. Transferrin receptor 1 (TfR1) is a ubiquitous protein expressed on the cell surface19,20. TfR1 is a homodimer that binds holo-transferrin in a 2:2 stoichiometry21, and this complex is internalized into an early endosome. As the endosome matures, a proton pump facilitates a drop in acidity. In the acidic environment, transferrin undergoes a conformational change22 and TfR1 facilitates the release of iron from transferrin18,23. The endosome recycles TfR1 back to the cell surface, and transferrin re-enters the blood. The kinetics of TfR1 receptor-mediated endocytosis of iron bound to transferrin have been the subject of multiple studies17,22,24–26.

Holo-transferrin competes with another protein, HFE, for the binding of transferrin receptor. The gene associated with HFE is mutated in type 1 hemochromatosis27, and its protein is expressed in a wide variety of tissue types28. At this point, we understand that HFE can bind with TfR1 to lower its affinity for holo-transferrin21,29–32. The stoichiometry for the HFE/TfR1 complex can be 2:2 or 1:221, and a complex with holo-transferrin, HFE, and TfR1 might exist30, although this is not shown in our network. Some authors suggest that the HFE/TfR1 complex enters the endosome29,33 although this is not fully established, and another main study suggesting this was retracted34. Our network does not show HFR/TfR1 inside the endosome, and in general HFE's role in iron metabolism has not been settled upon35.

2. Divalent metal transporter 1: Once free in the endosome, ferric iron is reduced by STEAP3 36 and is exported into the cytoplasm by DMT1. Divalent metal transporter 1 (DMT1, also called Nramp2, SLC11A2, and DCT1) is a ubiquitous protein which plays an important role in the transport of iron across membranes37,38. DMT1 is highly expressed in the duodenum37,39 where it transports ferrous iron into the cell40,41 assisted by the ferrireductase duodenal cytochrome B (Dcytb)42–45. Dcytb reduces dietary ferric iron to ferrous iron. In many types of cells, DMT1 is located both on the plasma membrane and in the endosome 40. In both locations, DMT1 transports ferrous iron into the labile iron pool46.

3. Lactoferrin Receptor: Lactoferrin is a member of the transferrin family that was originally found in human milk47 and other epithelial secretions. Lactoferrin is also secreted by neutrophils48. Because it tightly sequesters iron, lactoferrin is suggested to have many biological roles including defense against microbial infection and inflammation49. Unlike transferrin, lactoferrin does not lose its iron in mild acidity but instead requires pH<3.5 for iron release47. Lactoferrin also has 2.5–3 times higher affinity for ferric iron than does transferrin50. This fits with the function of lactoferrin being to hold on to iron and transferrin's function being iron transport. The structure and kinetics of binding and release of ferric iron to lactoferrin have been the focus of many studies50–53. While neither TfR1 nor TfR2 bind to lactoferrin54, a lactoferrin receptor is present on the plasma membrane of intestinal cells55 and many other tissue types56,57. Once lactoferrin and ferric iron are complexed with lactoferrin receptor, the complex is not affected by the acidic pH of the early and recycling endosome environment. In our network, we show iron being released from lactoferrin only in the lysosome, an iron-rich environment that degrades many iron-containing proteins and whose pH is significantly lower than the early/recycling endosome58.

4. 24p3R: Neutrophil gelatinase-associated lipocalin (NGAL, also called lipocalin 2) was first found in human neutrophils59 and is secreted by multiple types of cells during inflammation60,61. NGAL is a member of the lipocalin family and binds siderophores, which are high affinity iron chelators62,63. NGAL serves two functions in the delivery of iron to/from a cell, and both are mediated by a cell surface receptor 24p3R (also called SLC22A17). 24p3R derives its name from the mouse homologue of NGAL, which is called 24p364. First, the holo-NGAL complex consisting of ferric iron, siderophore, and NGAL can bind to 24p3R and enter receptor-mediated endocytosis. Iron does not begin to be released from the NGAL—24p3R complex until the pH decreases to 5, and this occurs slowly until the pH falls lower than 565. In this acidified compartment, ferric iron is reduced to ferrous iron, which does not bind to the acidified siderophore. Hence, ferrous iron dissociates from the NGAL—24p3R complex. This iron then enters the labile iron pool66. Like lactoferrin, holo-NGAL is targeted to the late endosome rather than the early/recycling endosome for transferrin65. Second, 24p3R can uptake apo-NGAL. In this case, apo-NGAL picks up a siderophore and then complexes with ferric iron from within the cell. This iron is exported out of the cell and 24p3R is recycled to the plasma membrane64. It is possible that 24p3R also recycles after mediating the uptake of holo-NGAL65, but this is yet to be shown. Megalin (also called gp330) can facilitate the uptake of NGAL67 and its function is understood to be the same as 24p3R.

5. Heme carrier protein 1: Following the discovery of the iron transporter DMT1 in the duodenum, many researchers speculated that a heme transporter must exist in the duodenum68,69. Heme carrier protein 1 (HCP1, also called SCL46A1, hPCFT) was identified as a heme importer on the apical membrane of the duodenum70. Later, researchers found that HCP1 transports folate with greater affinity and suggest that folate transport could be this protein's primary function71–73. Since its discovery, HCP1 has been found in many other human organs including brain, breast, kidney, prostate, and liver74,75. We show HCP1 transporting heme as it may play a role in iron homeostasis for many tissue types. Once heme is imported into a cell, heme oxygenase 176–79 and heme oxygenase 280 break down heme and the iron can enter the labile iron pool. While our network only shows heme and oxygen as reactants with ferrous iron as a product, biliverdin is also produced. In a second reaction, biliverdin is converted to bilirubin, a powerful antioxidant. We have chosen to omit both biliverdin and bilirubin as our main interest lies in the metabolism of iron.

6. TfR2: Holo-transferrin can also bind to transferrin receptor 2 (TfR2)81,82, a homologue of TfR1 that has only recently come into the picture82. The role of TfR2 is not fully determined, but we do know that TfR2 is essential for iron homeostasis: a defective TfR2 gene leads to a non-HFE form of hemochromatosis83–87. Expression of TfR2 is largely restricted to the liver82,85,88. Like TfR1, TfR2 does mediate the endocytosis of holo-transferrin89,90, but there are known differences between the two receptors. TfR2 has an affinity for holo-transferrin that is 25-fold lower than of TfR1 and does not bind to HFE81. It has been suggested that TfR2 acts primarily as an 'iron sensor' rather than an iron importer: its protein expression is increased by holo-transferrin both *in vitro*16,86,91,92 and *in vivo*91. The exact mechanism of this regulation is not known, but it is likely to be post-transcriptional92. A recent study suggests that holo-transferrin might facilitate the recycling of TfR289.

B. Iron/heme exporters

1. Ferroportin: Ferroportin (also called MTP1, IREG1, SLC40A1) is a mammalian iron exporter located on the plasma membrane and is expressed in a wide variety of human tissue types93–97. Once translated, intracellular ferroportin is transported to the plasma membrane by mon1a98 where it can function as an iron exporter. Ferroportin exports ferrous iron that is then oxidized to ferric iron by a ferroxidase. This oxidation is catalyzed by either ceruloplasmin,

a circulating protein produced by the liver99–102, or hephaestin40,44,103–105 in the duodenum.

2. FLVCR and ABCG2: While ferroportin is the only known iron exporter, we currently know of two heme exporters located on the cell surface: feline leukemia virus C receptor (FLVCR) and ATP binding cassette protein G2 (ABCG2, also BCRP)69. FLVCR was first cloned as a receptor for feline leukemia virus subgroup C106,107 and was later shown to export heme from the cell108. High levels of both the mRNA and protein for FLVCR are present in small intestine, liver, and red blood cells, and FLVCR is also expressed in many other tissue types108. A recent study showed FLVCR is necessary for iron homeostasis and exports heme *in vivo*109. ABCG2 was cloned from MCF-7 cells as a multi-drug resistance transporter110. Like other ABC transporters, ABCG2 can transport substrates against a concentration gradient111. ABCG2 interacts with both heme and porphyrins and prevents heme/porphyrin build up within the cell111,112. The expression of ABCG2 is highest in the placenta with high expression levels also in the liver and small intestine110,113. ABCG2 is expressed at different levels in other tissue types but is not a ubiquitous protein113. While the story is not completely understood for either FLVCR or ABCG2, our network presents both proteins acting as heme exporters.

C. Storage of iron

Ferritin: Excess free ferrous iron can be toxic to the cell. In the presence of hydrogen peroxide, ferrous iron can be oxidized to ferric iron via the Fenton reaction which forms dangerous hydroxyl radicals114,115. The ferritin protein can mineralize and store excess ferrous iron to prevent hydroxyl radical production. Each molecule of ferritin can hold 4500 iron atoms inside a chamber with a diameter of 8nm116. Ferritin is comprised of 24 subunits of two distinct types: H117,118 and L119,120; the number of ferritin H versus L subunits depends on tissue-type121. Ferritin H and L are the products of distinct genes. The subunits have different functions in the storage of iron: H-chain ferritin has ferroxidase activity while L-chain does not122. Many studies have explored the kinetics and molecular mechanisms of the storage of iron in ferritin116,123–128. Recently, a research group found that poly (rC)-binding protein 1 (PCBP1) might deliver cystolic iron to ferritin129; however, since this work was performed primarily in yeast, we do not depict PCBP1 in our map. Iron is stored as mineral core (FeOOH) in ferritin by at least three reactions, as listed below128.

$$2Fe^{2+}+O_2+4H_2O \rightarrow 2FeOOH+H_2O_2+4H^+ \quad \text{A}$$

$$4Fe^{2+}+O_2+6H_2O \rightarrow 4FeOOH+8H^+ \quad \text{B}$$

$$2Fe^{2+}+H_2O_2+2H_2O \rightarrow 2FeOOH+4H^+ \quad \text{C}$$

In reaction A, ferrous iron is oxidized at the ferroxidase site of H-chain ferritin. This reaction occurs almost exclusively when iron levels in ferritin are low and continues at lower levels throughout the iron loading process126,128. Reaction C governs iron loading in the middle stages of iron loading while reaction B dominates when iron in ferritin is high128. Since the kinetics of reaction A changes from low (< 48Fe/Protein) to intermediate/high (>48 Fe/Protein) iron in ferritin126 and reaction B is the sum of reactions A and C, we represent two distinct species of the ferritin-iron complex in our network: Low iron ferritin (<48 Fe/Protein) and

High iron ferritin (>48 Fe/Protein) and depict reactions A and C. Once iron is stored in ferritin, it is tightly sequestered, and some studies suggest that ferritin must be degraded in the lysosome for iron to be released130,131. Another group of researchers propose that iron chelators and reducers can reach the iron mineral core through the eight pores in the ferritin protein132–134. The specific details of iron moving out of the pores are not understood, but this is thought to occur in iron deficiency and hemoglobin production133. Additionally, ferritin pores are mostly closed both *in vivo* and *in vitro*134. Because we lack definitive information regarding the ferritin pores, we exclude this mechanism of releasing iron from ferritin in our map.

D. Mitochondria and iron—Mitochondria are essential in cellular iron homeostasis as most metabolic processes involving iron depend on the mitochondrion. Ferrous iron enters a mitochondrion from the labile iron pool. Mitoferrin (also called SLC25a37) was recently proposed to be an iron importer located on the membrane of the mitochondrion135.

1. Mitochondrial ferritin: Like cystolic ferritin, mitochondrial ferritin stores iron as mineral core to control the amount of free ferrous iron present. While mitochondrial ferritin is also comprised of 24 subunits, this protein is a homopolymer whose subunits behave somewhat like ferritin H subunits125,136,137. Both mitochondrial ferritin and H-ferritin contain a ferroxidase center that oxidizes ferrous iron in the process of mineral core formation136–138. In mitochondrial ferritin, 12 of the 24 ferroxidase sites are inoperative125 and mitochondrial ferritin has a slower rate of mineral core formation125,137. Moreover, the switch in kinetics occurs at 24 Fe/Protein rather than 48 Fe/Protein125. Thus, in our network “Low Iron Mtf” is <24Fe/Protein and “High Iron Mtf” is >24Fe/Protein. Reaction C listed for cystolic ferritin does occur for mitochondrial ferritin in high iron loading, but the overall mineral core formation is governed by two reactions instead of three. The stoichiometry is slightly different for the reaction corresponding to H-ferrtin’s reaction A, and there is no reaction consuming hydrogen peroxide as in reaction B125.

2. Frataxin, ferrochelatase, ISU: Frataxin was discovered through its connection with the neurodegenerative disease Fredreich ataxia139,140. Researchers are not in full agreement on the precise cellular functions of frataxin in mammalian cells, and many studies focus on the yeast homolog Yfh1p. We do understand that frataxin is a mitochondrial protein that facilitates the formation of iron-sulfur clusters and heme140–147. Specifically, frataxin binds ferrous iron and delivers it to ISU142,143,146 for the formation of iron-sulfur clusters. The iron-sulfur clusters are then either used in mitochondrial iron-sulfur cluster proteins or exported to the cytoplasm for use throughout the cell. The ABC transporter ABCB7, a functional orthologue of yeast Atm1p148–150, is thought to facilitate this transport148,151. While some studies suggest that iron-sulfur cluster formation can also occur in the cytoplasm152, other authors stress the importance of the mitochondrion in iron-sulfur cluster formation151. Moreover, as far as frataxin is concerned, the mature protein is located solely in mitochondria143,153, and a recent study showed that deletion of frataxin in mice affected three extra-mitochondrial iron-sulfur proteins143. We have elected to show iron-sulfur formation occurring only in the mitochondrion, and our network represents a simplified picture of the complex iron-sulfur biogenesis process151.

3. Heme synthesis: Frataxin also appears to deliver iron to ferrochelatase for the production of heme141,147. Ferrochelatase catalyzes the final step in heme production154–157, which involves the insertion of iron into protoporphyrn IX (PPIX). We have included all of the steps of heme biosynthesis in our network. Before iron is added, a series of reactions occurs, beginning and ending in the mitochondrion and with middle steps occurring in the cytoplasm. The heme biosynthesis pathway has been well understood for some time now and can be found in textbooks158, pathway databases159,160, and other sources161. One detail worth

mentioning is the transport of coproporphyrinogen III (COPRO III) from the cytoplasm into the mitochondrion, which is catalyzed by the transporter ABCB6162. After the final step in heme production, heme is exported out of the mitochondrion into the cytoplasm for use in heme proteins. The protein responsible for this transport has not been determined although speculations exist163. Once in the cytoplasm, heme can also be degraded by heme oxygenase 1 and 2, as previously discussed.

Intracellular heme provides important regulation of its own production and degradation. Heme's regulation of its production occurs via delta aminolevulinate synthase (ALAS), which catalyzes the first step in the formation of the porphyrin ring164. ALAS comes from two different genes: ALAS2 in erythroid and ALAS1 in non-erythroid cells165. We only depict ALAS1 in the general epithelial network. In mammals, heme inhibits ALAS1 translationally166 as well as at the protein level. The regulation at the protein level involves an inhibition of the transport of ALAS1 protein from the cytoplasm into the mitochondrion167–170. Heme also regulates its own degradation by inducing heme oxygenase 171. Heme oxygenase 1 is also induced by reactive oxygen species78,172,173 and by decreased levels of heme oxygenase 2174.

E. Feedback regulation

1. Iron regulatory proteins: In mammalian cells, a fundamental mechanism for controlling iron metabolism occurs post-transcriptionally through iron regulatory proteins (IRPs) and iron responsive elements (IREs)175. The IRE is a nucleotide hairpin located in the untranslated region (UTR) of mRNAs encoding certain proteins of iron metabolism176–179. Two different iron regulatory proteins, IRP1 and IRP2, bind to the IRE with high affinity when intracellular iron levels are low180. The IRE can be located in the 5′ UTR in which case the binding of IRPs blocks translation175,181. When the IRE is located in the 3′ UTR, the binding of IRPs to IRE stabilizes the mRNA and hence more protein is translated175. IRP1 and IRP2 have distinct IRE binding sites182,183 and are regulated by iron levels differently184. IRP1 is active when iron levels are low and functions as cystolic aconitase when iron levels are high185–189. The cystolic aconitase form of IRP1 contains a 4Fe-4S iron-sulfur cluster, and one iron molecule detaches from this cluster to activate IRP1185,190. This activation occurs in response to low intracellular iron levels and oxidative stress187,190,191. IRP1 can be phosphorylated by protein kinase C, which increases its affinity for IRE binding192–194. IRP2, which shares 57% homology to IRP1195, also binds to IRE elements. Increased levels of iron cause the ubiquitination and degradation of IRP2196–199. Two studies found that IRP2 can be phosphorylated193,200, although they give conflicting statements as to the effect of phosphorylation on IRE binding. Due to the lack of agreed upon evidence, we do not depict the phosphorylation of IRP2.

For the genes in our network, the mRNA for ferritin, ferroportin, and HIF2-alpha contain IREs in the 5′ UTR while TfR1 and DMT1 mRNA contain IREs in the 3′ UTR. It is well-accepted that the IREs in ferritin mRNA and TfR1 mRNA are functional201–203. Despite their similarity to ferritin and TfR1, the mRNA of mitochondrial ferritin and TfR2 lack IREs138. At the transcriptional level, ferritin is induced by reactive oxygen species204 and inflammatory mediators205,206. The IRE in the mRNA for HIF2-alpha has only recently been discovered207. The ability of iron regulatory proteins to affect translation of DMT1 and ferroportin is disputed. Iron regulatory proteins (IRPs) can bind to the IRE is ferroportin mRNA96, and ferroportin's IRE is known to be functional in certain tissue-types208,209. Moreover IRP activity is necessary to obtain the required ferroportin levels in the duodenum210, and ferroportin expression is increased in an iron-rich environment211. We do depict IRPs modifying ferroportin translation in our iron metabolic network.

The human DMT1 gene includes alternative 3′ exons: one contains an IRE in the 3′ UTR and one does not212. Alternative 5′ exons were also discovered213 yielding four different isoforms of DMT1214. The isoform expressed varies depending on tissue-type and subcellular location213–216, however all four isoforms operate as iron transporters with the same efficiency214. Thus, in our model we only distinguish between +IRE and −IRE forms of DMT1. The +IRE isoform of DMT1 is typically located on the plasma membrane of epithelial cells while the −IRE isoform is found on the membrane of the endosome215. The functionality of the IRE in the +IRE DMT1 mRNA could depend on tissue-type210,217. In the duodenum, DMT1 is greatly increased in iron-starved animals37,39,218, and intracellular iron levels could affect the transcription of DMT1219, although we do not depict this in our network.

2. Hepcidin: Hepcidin is an antimicrobial peptide that is produced and secreted by the liver in response to systemic iron levels220,221. Overexpression of hepcidin leads to low plasma iron levels and anemia while underexpression of hepcidin leads to high plasma iron levels and hemochromatosis222. Hepcidin circulating in the blood can regulate plasma iron levels in two ways. The first is through its interaction with ferroportin. Hepcidin binds to ferroportin and causes its internalization and degradation222–225. When ferroportin levels are reduced, less iron is exported into the blood. Until very recently, this was the only known regulatory mechanism of hepcidin. However, two studies suggest that in the duodenum, hepcidin does not affect ferroportin but rather inhibits DMT1 production transcriptionally223,226. In cells expressing GPI-linked ceruloplasmin, this protein prevents internalization and degradation of ferroportin in a hepcidin-independent pathway227.

The production of hepcidin is regulated at the level of transcription and involves an intricate BMP signaling cascade222,228–231. Normally, hemojuvelin is GPI-linked and this form activates this BMP signaling cascade228,229,231,232. In abnormal states, soluble hemojuvelin inhibits the BMP signal229,233,234. In our network, we omit the BMP signaling cascade and represent both forms of hemojuvelin. Hemojuvelin and its gene HFE2 are fundamental to iron metabolism since defects in HFE2 cause juvenile hemochromatosis235–237. HFE and TfR2 are suspected of participating in the regulation of hepcidin, possibly through a signaling process, but we have not found direct evidence of this85,222,238,239.

3. HIF Pathway: HIF (hypoxia inducible factor) is a transcription factor that plays a central role in the cellular adaptation to low oxygen levels (see240 for review). Consequences of HIF activity are profound: HIF is a transcription factor, and its stabilization induces the transcriptional activation of over 100 genes. Key targets of HIF include EPO (erythropoietin), which controls red blood cell production, VEGF (vascular endothelial growth factor), which regulates angiogenesis, and many others. HIF activity is regulated by iron, and moreover HIF activity affects levels of iron.

First, iron controls the stability of HIF. HIF is a heterodimer composed of an unstable alpha subunit (e.g. HIF1alpha, HIF2alpha) and a stable beta subunit (e.g. HIF1beta). Under normoxic conditions, the alpha subunit of HIF is degraded. Degradation occurs due to the action of members of the prolyl hydroxylase domain (PHD) family. These proteins hydroxylate HIF at conserved proline residues, creating a recognition site for ubiquitin ligases that target HIF for degradation. The PHD proteins are Fe(II) and 2-oxoglutarate-dependent dioxygenases whose activity is dependent on oxygen as well as iron. Thus, under conditions of low oxygen or low iron, prolyl hydroxylase activity is decreased, and HIF becomes stabilized.

Iron also controls the activity of HIF. In addition to prolyl hydroxylation, HIF1alpha activity is regulated by asparaginyl hydroxylation, a modification that decreases the activity of HIF by reducing its affinity for coactivators. Asparaginyl hydroxylation is also catalyzed by a PHD family member termed FIH1241 (also called HIF1AN). HIF2alpha (also called EPAS1) is more

resistant to this mechanism of inactivation242. As previously mentioned, the mRNA for HIF2alpha contains an IRE, and thus iron controls the translation of HIF. This mechanism has been postulated to act as a feedback loop to restrict HIF2alpha expression when iron is scarce.

HIF, in turn, affects the transcription of genes in the iron metabolic network. There is evidence for HIF1alpha modifying transcription for genes heme oxygenase 1243, hepcidin244, ceruloplasmin245, and transferrin receptor246. Moreover, HIF2alpha induces the frataxin gene247.

Description of reactions in specialized cells

Most of the body's iron is found in hemoglobin, where it carries out oxygen transport. Two types of specialized cells play a key role in this fundamental aspect of body iron metabolism: the reticulocyte and the macrophage. Many of the species and reactions from our general mammalian cell model are present in these cells, as shown in Table 1. However, the reticulocyte and macrophage also express additional genes that are involved in reactions that do not occur in other mammalian cells.

A. Reticulocyte—The reticulocyte is an immature erythroid cell that still has mitochondria and ribosomes. Once mature, a circulating erythroid cell transports both oxygen and carbon dioxide and contains mostly hemoglobin. In our network, we show the synthesis of hemoglobin A (HbA), the type primarily synthesized in adults. HbA is composed of α and β peptide globin chains and is regulated by heme-regulated eIF2α kinase (HRI) and α hemoglobin-stabilizing protein (AHSP). Activated HRI inhibits globin synthesis through phosphorylation of eIF2α, and HRI is regulated by heme248. When intracellular heme levels are high, heme binds to and deactivates HRI. In heme deficiency, heme detaches from HRI which activates the protein248,249. Thus, HRI ensures that the amount of globin produced matches the amount of heme present. The mechanism by which AHSP regulates hemoglobin production is different. AHSP binds to α hemoglobin (αHb), stabilizing αHb for hemoglobin synthesis250. In certain conditions, for instance in β thalassemia, excess αHb accumulates in the reticulocyte and AHSP can bind to this αHb to protect the cell250.

The main way that reticulocytes obtain iron for hemoglobin production is through the Tf-TfR pathway251–253. A cell-surface receptor for ferritin, however, also exists in this cell type254. FtR likely mediates endocytosis of soluble ferritin255. Once internalized, the soluble ferritin is degraded by the lysosome256 and the iron enters the labile iron pool, affecting IRP activity255,257. There are some major differences in the regulation of proteins involved in iron metabolism of the reticulocyte. First, ALAS2, the erythroid-specific form of ALAS, contains an IRE in the 5′ UTR258,259 and neither its synthesis nor its activity is inhibited by heme260. In reticulocytes, heme inhibits the internalization of transferrin by TfR1261,262. The non-IRE form of DMT1 dominates in reticulocytes and is found on the membranes of endosomes containing TfR1263. DMT1 is not thought to import iron directly into the reticulocyte, but evidence of the +IRE isoform does exist at the mRNA level264. We show DMT1+IRE on the plasma membrane just like in the general model. However, we do not show it transporting iron, as we have no evidence of this. DMT1+IRE could possibly be on the membrane of the endosome as well.

B. Macrophage—In terms of iron homeostasis, the main function of the macrophage is to recycle iron from hemoglobin back into ferrous iron for circulation and use throughout the body. Macrophages are present in most tissues throughout the body, and we do not distinguish between different types of macrophages in our model. The two ways that the macrophage ingests hemoglobin are CD163-mediated endocytosis of haptoglobin:hemoglobin (Hp-Hb) complexes265,266 and phagocytosis of senescent erythroid cells267. CD163 is a member of

the scavenger receptor cystein-rich (SRCR) family of receptors expressed in macrophages. CD163 is a cell-surface receptor that mediates the endocytosis of Hb-Hp complexes265,268. The Hb-Hp complex enters the lysosome where heme is released and subsequently degraded by heme oxygenase 1 and 2269. CD163 can detach from the plasma membrane of macrophages to contribute to the soluble CD163 (sCD163) in the plasma, but the function of sCD163 is not known266,270.

An important function of the macrophage in iron metabolism is phagocytosis of senescent erythroid cells. This process begins when cell-surface receptor binds to the senescent red blood cell267, which activates the receptor. The receptor signals the macrophage to initiate phagocytosis, and the red blood cell is absorbed into a vesicle called a phagosome. This phagosome fuses with a lysosome, forming a phagolysosome267. In our network, we depict the phagosome and phagolysosome as a single compartment. At this point, hydrolytic enzymes degrade the red blood cell and then hemoglobin is degraded to release heme271. We show heme being exported into the cytoplasm for degradation by the heme oxygenase system. Additionally, heme could be degraded in the phagolysosome. In macrophages, Nramp1 is expressed on the membrane of the phagosome and, like DMT1 (Nramp2), transports ferrous iron. Nramp1 transports recycled iron out of the phagosome272. It is possible that Nramp1 transports iron bidirectionally and moreover affects other proteins involved in iron metabolism273, but this is still under debate and is not depicted in our network. HRI is present in macrophages at a much lower level than in erythroid cells and this protein is functional133. The role of HRI in the iron metabolism of macrophages is to stimulate phagocytosis133. Macrophages recycle iron primarily through ferroportin, but some studies suggest that macrophages secrete ferritin255. As previously discussed, this soluble ferritin may deliver iron to reticulocytes for hemoglobin production.

Discussion

We believe we are the first to use CellDesigner to map one metabolic network in multiple types of cells. In the construction of the tissue/cell specific networks, we see both redundancy and uniqueness. The redundancies are essential elements in the metabolism of iron for different types of cells. The unique species and reactions give a cell the ability to perform its special functions. A number of insights can be gained from inspection of these networks.

In graph theoretic terms, we may use the degree (number of edges) of a node as a measure of a species' significance in the overall network274,275. In all networks, the nodes for LIP, cystolic heme, and cystolic ROS have the highest degrees (19, 8, and 11, respectively, for the general network). Interestingly, the ratio of reactions involving LIP to those involving heme shifts according to cell type: the LIP is involved in over 2.3 times as many reactions as cystolic heme in epithelial cell types, while involved in only 1.6 and 1.7 times as many reactions in the macrophage and reticulocyte, respectively (the ratio of reactions involving LIP to those involving heme is 19/8 for epithelial cells, 20/12 for macrophages, and 17/10 for reticulocytes). This may illustrate the fact that the macrophage and reticulocyte have a greater need for heme, as compared to the epithelial cell.

A main observation gathered from the iron metabolic network is the large number of feedback loops involving the labile iron pool (LIP) and intracellular species (see Fig. 6). IRP activity participates in multiple negative feedback loops beginning and ending at the LIP, whereas HO1 participates in positive feedback loops for the LIP. Perhaps surprisingly, given the extensive differences between hepatocytes, enterocytes, and macrophages, the feedback loops in all these cell types are virtually identical (Fig. 6A). However, the feedback loops for the erythroid cell are different (Fig. 6B).

The four cell types exhibit a different distribution of species in their compartments. For instance, the macrophage has the smallest percentage of species in the mitochondrion and the largest percentage of species in the lysosome, as compared to the other cell types. The macrophage also has the largest number of reactions (112 in total) of any cell type and the highest percentage of transport reactions involving iron (20.5%). The additional transport is due, in part, to the sixth compartment—the phagosome/phagolysosome—that exists only in the macrophage. The reticulocyte has significantly fewer membrane-bound proteins—both in number (8) and in percentage of proteins in network (19%)—participating in iron metabolism, but this could be due to the lack of expression data for certain genes/proteins in the reticulocyte. The general network has an equal number of transcriptional and translational modifications. However, for any given tissue type, there are more translational modifications in the iron metabolic network. For the general network as well as all four tissue types, there are more modifers of transcription than modifiers of translation. This is due in part to the fact that most translational modification coming from IRE/IRP system. The reaction with the greatest number of modifiers is the transcription of the HO1 gene, which is affected by five different species in all tissue types. In the liver and general network, transcription of the HAMP gene also has five modifiers.

However, a limitation of this interpretation is that the number of times a reaction or species is studied in a given cell type may influence its apparent importance in that cell's network. A natural next step in this modeling activity will therefore be to include kinetic information about each of the reactions in the iron metabolism map. In an advanced stage, this will then lead to different models of iron metabolism specific for the different cell types. These models would allow us to quantitatively determine the importance of the various species and reactions for the different cell types. Perhaps this analysis would support our frequency observations above, but surely one would learn new information regarding the metabolism of iron. A perceived problem with this path is the lack of kinetic data for many of the reactions depicted in these maps, even though for many reactions such information is available, as indicated in the text above. An alternative to using detailed enzyme kinetics data is to use a generic rate law274 to substitute kinetics that are not known in detail, as has been used previously in metabolic models275. Once kinetic information has been added to the model, it can then be simulated using a software package like COPASI11. The modeling effort is facilitated by the fact that CellDesigner stores the model using the Systems Biology Markup Language (SBML276), which is also understood by simulation software like COPASI. We hope to further understand, through modeling efforts, how the common and distinguishing mechanisms interlock with one another to produce cells that are functionally very different in terms of iron metabolism. Additionally, one could use a modeling framework to shed light on how iron is metabolized differently in various states (e.g. health versus inflammation or malignancy).

Materials and Methods

Construction of the general iron network

The general iron network was created in CellDesigner, a diagram editor for drawing biological networks7. CellDesigner involves Kitano's graphical notation276 and stores files in Systems Biology Markup Language (SBML277). Networks created with CellDesigner can be exported as a picture file for network visualization, and the models can also be simulated or analyzed by software packages like Copasi11 and others. CellDesigner features a graphical, point-and-click user interface with different symbols for the various types of molecular species and reactions. The specific symbols we used in the iron network are listed in the legend of Fig. 1. A complete list of symbols used in CellDesigner is available on the CellDesigner website (http://www.celldesigner.org). For clarity, we note that the transport arrow is used to signify

movement of a species from one compartment to another, or movement between the interior and transmembrane locations of a single compartment.

The reactions occurring in the network are based on the published literature. A PubMed search was performed using broad search terms such as iron metabolism, heme production, iron transport, Fenton reaction, iron and gene expression, and iron regulation. In addition, PubMed searches were performed for each specific species identified in Fig. 1. Searches were also conducted for gene expression in specific cell types (macrophages, hepatocytes, enterocytes, reticulocytes). Reference lists from recent articles were used as a secondary method to assure the completeness of the literature reviewed. Due to space limitations, not all key references used in the construction of this network are cited in the text.

Construction of Table 1 and tissue-specific networks

To create tissue-specific networks, we considered each species in the general network, and evaluated evidence for its expression in specific tissues. Our first choice for data in determining positive or negative gene expression was data from tissues including human, mouse, and rat. When this was unavailable, we looked for expression in cell lines. For the liver and intestine, we also relied upon NCBI's database Unigene278 when no other data was available. Unigene is based on expressed sequence tags (ESTs) and reports the number of transcripts per million for many tissue types including the liver and intestine. For the macrophage, we included evidence from all types of macrophages, for instance Kupffer cells, histiocytes, or cultured macrophage cell lines. For the reticulocyte, we included evidence from erythroid precursors, erythroids, reticulocyte lysates, erythroid colonies (CFU_E), and erythroblasts. We did not, however, use expression data from cultured erythroleukemia cell lines, like K562 or Friend cells, because these are disease states and may not represent true gene expression in healthy reticulocytes.

Note that "--" in Table 1 does not necessarily indicate absent expression. Some studies report quantitative measurements of RNA for both the liver and intestine 36,220,279, and moreover Unigene also gives a quantitative report for the liver and intestine. Since very low levels of gene expression often give rise to proteins that are not functionally present, we report "--" if the level of one tissue type is less than 8% of the level measured in the other. The 8% cut off is somewhat arbitrary, although it was chosen so that, according to Unigene, ferrochelatase would be present in both the liver and intestine, and hephaestin would not be present in the liver (matching the tissue data 105). Sometimes we have no evidence (positive or negative) of a gene's expression in a certain tissue/cell type. This is shown in the table when all the entries are blank for a given gene and tissue/cell type. In our tissue-specific networks, we exclude the species for which we have no evidence of expression. The exception to this rule involves species in the heme biosythesis pathway, since it is believed that it occurs in almost all types of mammalian cells. For the macrophage, we found evidence for presence of ferrochelatase, frataxin, and ISU, three mitochondrial proteins involved in heme production. We thus infer the presence of the other proteins in the heme biosythesis pathway. Similarly, we did not find any published evidence for each protein involved in heme biosynthesis in the reticulocyte, but these are included in the reticulocyte network. Another exception involves HIF in reticulocytes. We have postive evidence of PHD and FIH1 in reticulocytes, and we infer the presence of HIF1alpha and HIF2alpha. Currently, little is known about gene expression in the reticulocyte, and perhaps in the future more of the species will prove to be present in this cell type.

In depicting the tissue-specific networks, we used a lighter color to shade the species for which we found no positive evidence of expression. In most cases, we also lightened all subsequent reactions involving said species as we assume those reactions do not occur. Sometimes, however, we kept the reactions in our network, for instance ABCB6 was not detected in the intestine. While the ABCB6 protein is lightened, we still show the transport of

coproporphyrinogen III (COPRO III) from the cytoplasm into the mitochondrion during heme biosynthesis as we assume another protein must catalyze this reaction in the intestine. As mentioned in the discussion, hepcidin has two possible interactions: the internalization and degradation of ferroportin and the inhibition of DMT1 translation. The first is the standard, accepted interaction which does not occur in the intestine and the second has been found only in the intestine. We therefore lightened the pathway depicting hepcidin's internalization and degradation of ferroportin in the intestinal cell network, and lightened the inhibition of DMT1 in all other tissue-specific networks.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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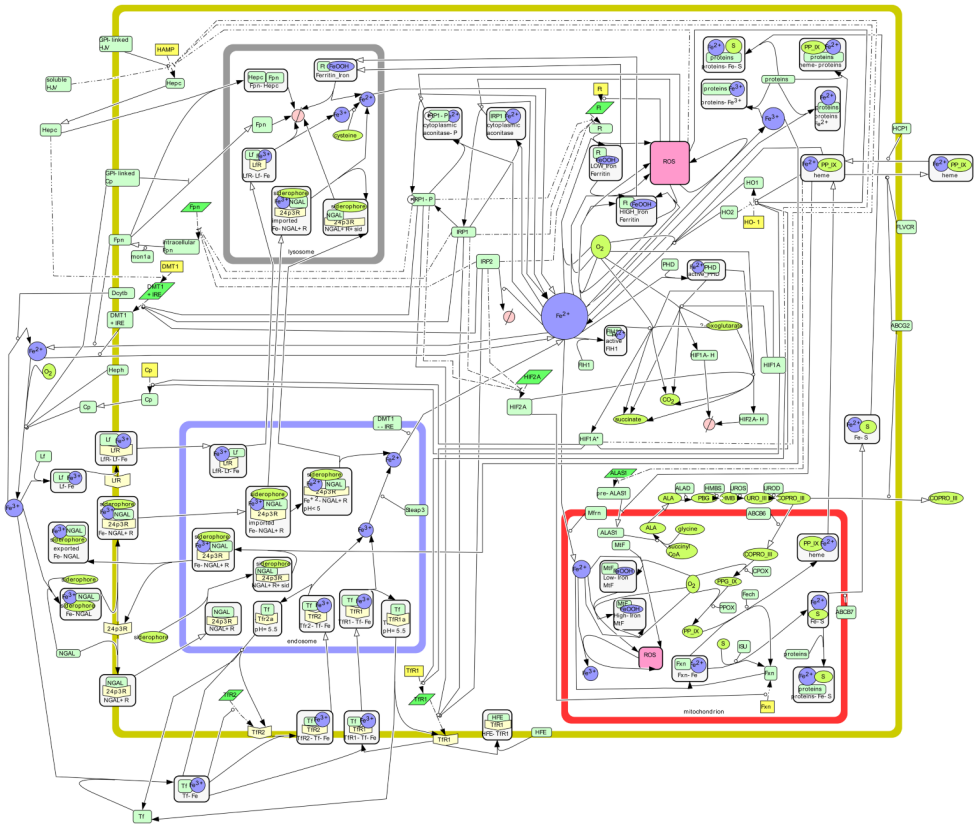

Figure 1. The Iron Metabolic Network

This structural network contains a culmination of reactions occurring in epithelial cells of numerous tissue types. Symbols used in the network include protein, receptor, gene, RNA, simple molecule, ion, complex, stste transition, transcription, translation, transport, catalysis, inhibition, transcriptional inhibition, translational inhibition, and degradation. A complete list of the symbols used in CellDesigner can be found on the CellDesigner website (http://www.celldesigner.org).

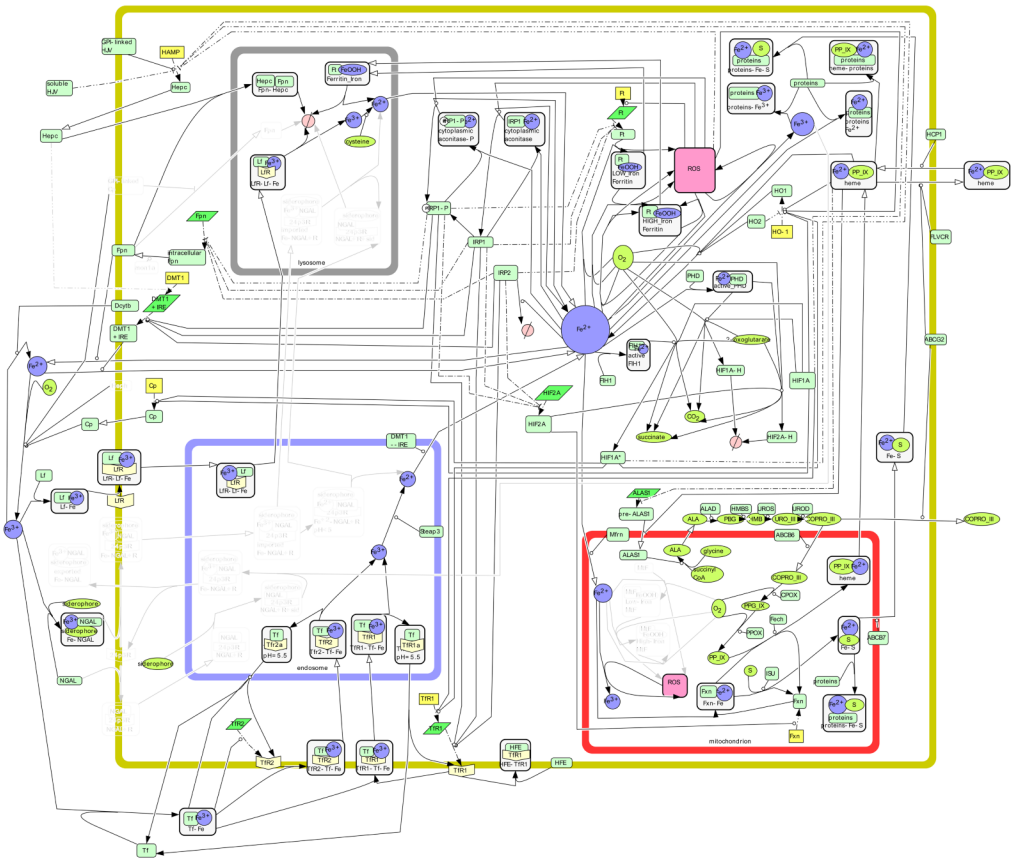

Figure 2. The Liver Cell

Using gene/protein expression information listed in Table 1, iron metabolism in the liver epithelial cell is illustrated. Species are lightened when there is no published positive evidence of said species in the liver cell, as described in Materials and Methods.

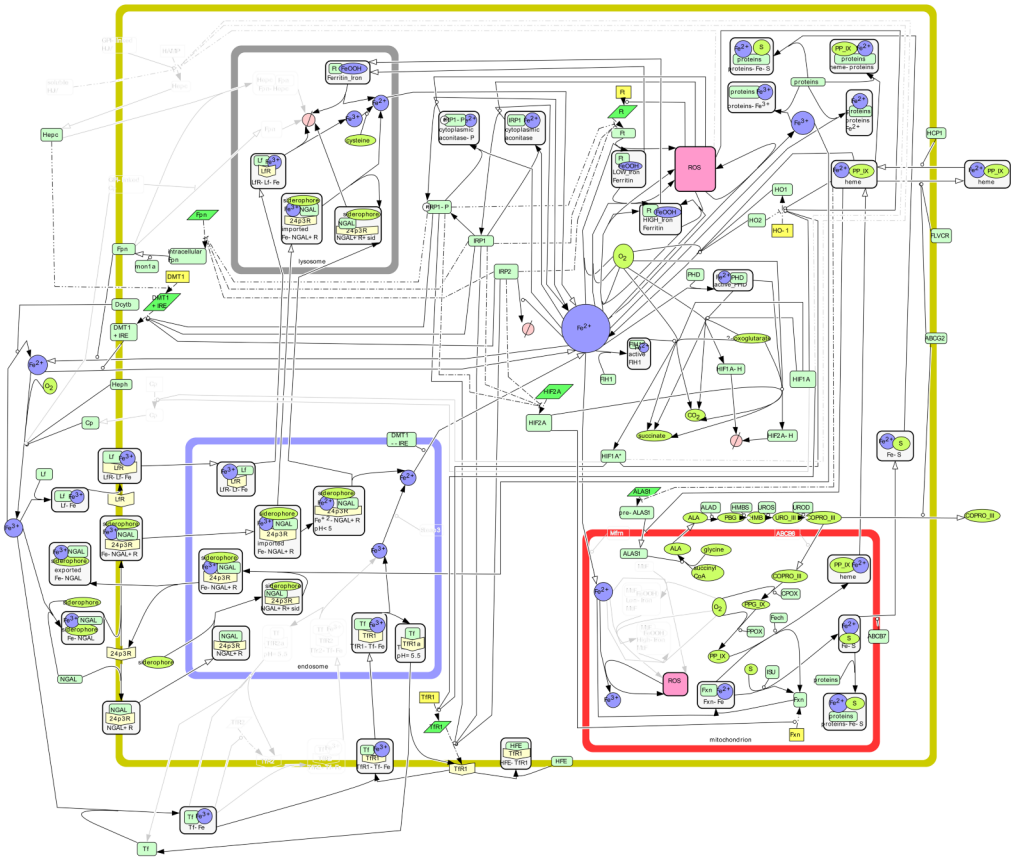

Figure 3. The Intestinal Cell

Using gene/protein expression information listed in Table 1, iron metabolism in the intestinal epithelial cell is illustrated. Species are lightened when there is no published positive evidence of said species in the intestinal cell.

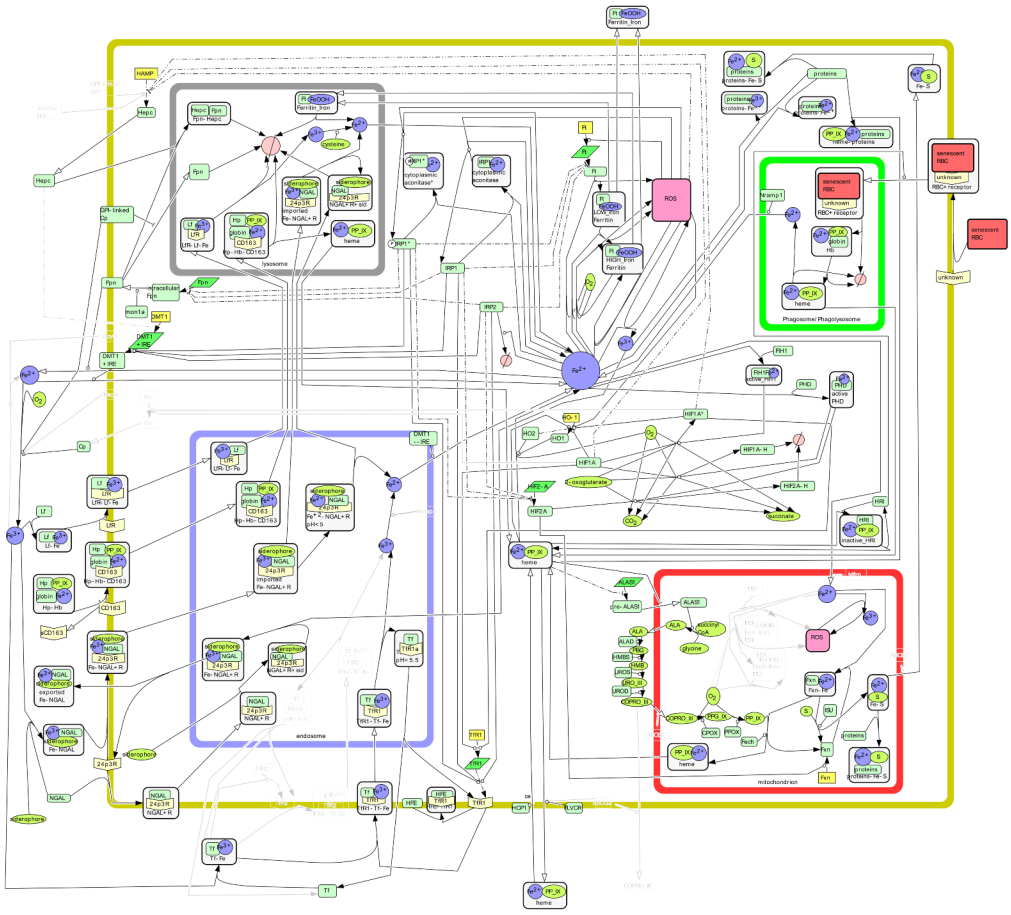

Figure 4. The Macrophage

Using gene/protein expression information listed in Table 1, iron metabolism in the macrophage is illustrated. Species are lightened when there is no published positive evidence of said species in the macrophage. Additional species and reactions are present that do not occur in epithelial cells and are specific to macrophages, as discussed in the text.

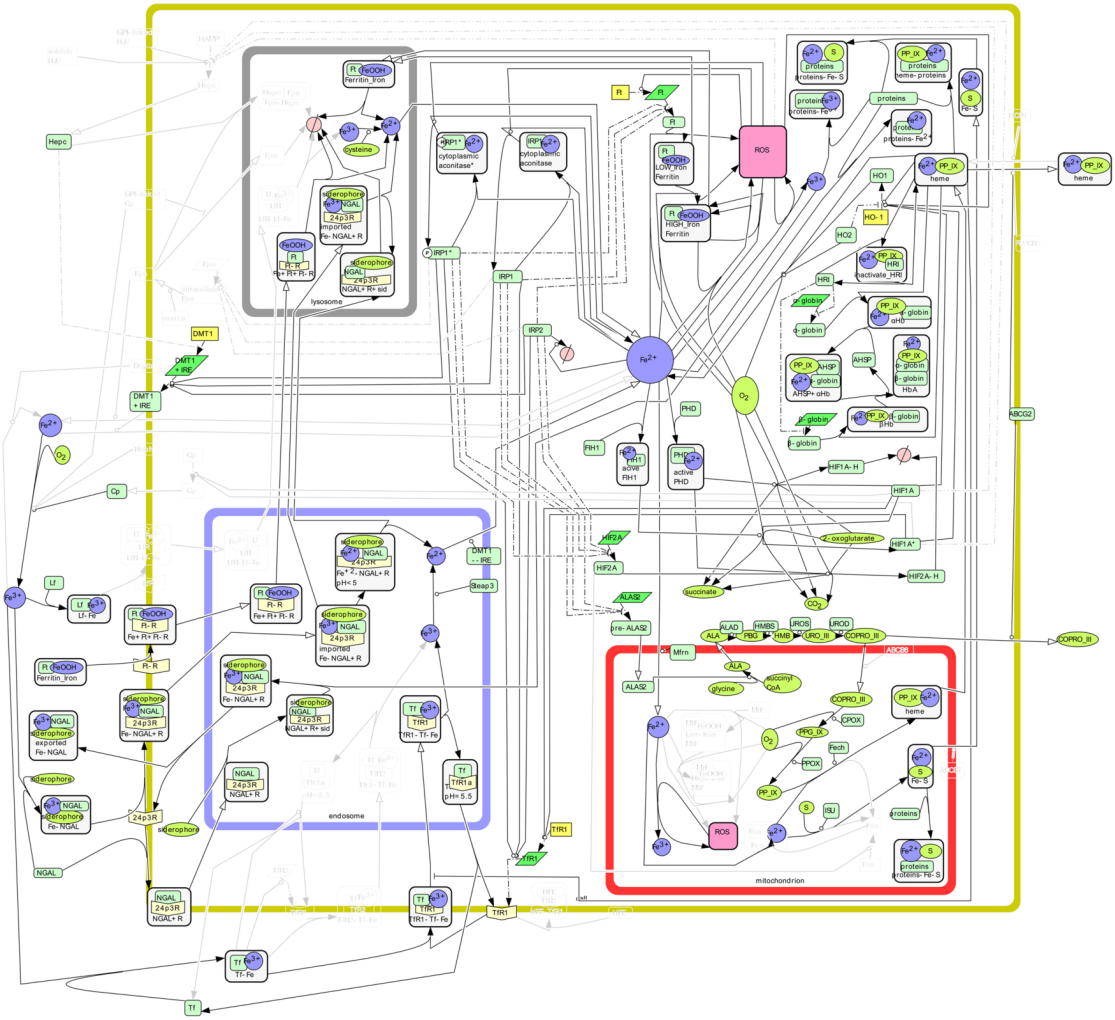

Figure 5. The Reticulocyte

Using gene/protein expression information listed in Table 1, iron metabolism in the reticulocyte is illustrated. Species are lightened when there is no published positive evidence of said species in the reticulocyte. Additional species and reactions are present that do not occur in epithelial cells and are specific to reticulocytes, as discussed in the text.

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Table 1

	Liver			Intestine			Macrophage		Reticulocyte	
	Protein	**RNA**	**Unigene**	**Protein**	**RNA**	**Unigene**	**Protein**	**RNA**	**Protein**	**RNA**
24p3R				--		+		+280		+280
ABCB6		+281,282			--281			--281		
ABCB7	+149	+283			+283					
ABCG2		+279			+279			+284		+285
ALAD			+			+				
ALAS1			+			+				
ALAS2										+285
Cp		+286				--				
Cp (GPI)				+			+227			
Dcytb		+287		+70						
DMT no IRE		+288		+289	+288,289			+290	+263	
DMT+IRE		+288		+289	+288,289			+290		+264
Fech		+291				+	*+292			+285,291
FIH1	+293					+	+293			+285
FLVCR	+109			+109			+109		+108,109	
Fpn	+93	+93		+93	+93		+98			
Ft	+294			+294			+294			+264,285
Fxn		+295		*+296				*+297		
HCP1		+70		+70			+298	+298		
Hepc		+220			--220			+290		
Heph		--105		+289	+105,289					
HFE	+299	+299		+299	+299		+299	+299		
HIF1A, HIF2A		+300			+300		*+301			
HJV		+236			--236					
HMBS			+			+				+285
HO1,2		+302		+302			+302		+303	
IRP1,2	+304			+305	+305		+305	+305		+264
ISU			+			+	*+306	*+306		+285
LfR	+307	+57			+57		+308			

	Liver			Intestine			Macrophage		Reticulocyte	
	Protein	**RNA**	**Unigene**	**Protein**	**RNA**	**Unigene**	**Protein**	**RNA**	**Protein**	**RNA**
Megalin	--309					--		+280		+280
Mfrn		+310			--310				+135	285
Mon1a			--			+	+98			
MtF		--311			--311					--312
PHD	+293			+293			+293		+313	+
PPOX			+			+				+285
Steap3		+36			--36					+36
TfR1	+314			+315			+314		+253	+285
TfR2	+82	+82		--82	--82			*--82		
UROD			+			+				+285
UROS			+			+				