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Metabolismus železa v nádorových buňkách
Iron metabolism in cancer cells

Bakalářská práce

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V ..... dne ..... podpis .....

Abstrakt:

Rakovina je jednou z nejčastějších příčin úmrtí na světě. Vědecký výzkum se tedy mimo jiné zaměřil na metabolismus železa a možné využití cílů souvisejících s metabolismem železa jako prostředku k zastavení nekontrolovaného množení a šíření těchto rakovinných buněk. Tato práce by měla sloužit jako stručná literární rešerše zabývající se metabolickými procesy železa od jeho absorpce z výživy a recyklace odumřelých buněk po jeho využití v proteinech obsahujících hem či železo-sirná centra nebo jeho uskladnění v podobě ferritinu. Zároveň pak podtrhává odlišnosti v těchto reakcích, které vykazují právě rakovinné buňky, a stručně se zmiňuje o poruchách metabolismu ústících ve vyčerpání železa v těle či naopak přetížení organismu tímto esenciálním prvkem. Také se snaží naznačit možné odchylky, které mohou nebo nemohou být využity pro léčbu a v neposlední řadě zmiňuje vyhlídky tohoto výzkumu a budoucí práci, která může přinést nový přístup a metody v tomto oboru.

Klíčová slova: železo, rakovina, metabolismus železa, hepcidin

Abstract:

Cancer is one of the major causes of death in the present world. As the research of this disease has progressed, the attention of some scientists has been focused on a metabolism of iron and how it can be used to fight these rapidly proliferating invasive cells and stop their spreading. This work should serve as a brief review of iron metabolic processes from the iron absorption from dietary resources and recycled cell iron, to its usage in heme- or Fe/S clusters-proteins and storage in a form of ferritin, while highlighting the points that differ in cancer cells. It also gives a modest overview on the regulatory pathways of iron uptake and use, and mentions iron metabolism disorders such as iron-depletion and overload. Simultaneously it is denoting possible differences that could be targeted in tumor treatment, and, at least but not last, the perspectives and future work that could bring a new methods and approaches to this matter.

Keywords: iron metabolism, iron, cancer, hepcidin

Contents

List of abbreviations

β2m β2macroglobulin

ABCB6 ATP-binding cassete B6 transporter

AD Altzheimer's disease

ALA 5-aminolevulinic acid

ALAD ALA dehydratase

ALAS ALA synthase

Atm1 ABC transporter B family member 23

ATP adenosine triphosphate

BFR bacterioferritin

BMP bone morphogenetic protein

CD163 scavenger receptor cysteine-rich type 1 protein M130

CD91 alpha2-macroglobulin endocytic surface receptor

cdk cyclin-dependent kinase

CIA cytosolic iron-sulfur protein assembly

CoASH coenzyme A

Dcytb duodenal cytochrome b

DFO desferrioxamine

DMT1 divalent metal transporter 1

DNA deoxyribonucleic acid

dNTP deoxynucleotide triphosphate

Dp44mT di-2-pyridylketone 4,4-di-methyl-3-thiosemicarbazone

Dps DNA-binding protein from starved cells

E2F1 transcription factor E2F1

eALAS erythroid ALAS

ERFE erythroferrone

FBXL15 F-box/LRR-repeat protein 15

FC ferrochelatase

GADD45α Growth arrest and DNA damage-inducible protein GADD45 alpha

GDF15 Growth/differentiation factor 15

GPI glycophosphatidylinositol

Grx5p Monothiol glutaredoxin-S14

GSH glutathione

GTP guanosin triphosphate

HAMP hepcidin

HCP1 heme carrier protein 1

HEPH hephaestin

HFE hereditary hemochromatosis protein

HH hereditary hemochromatosis

HHE 4-hydroxy-2-hexanal

HIF hypoxia inducible factor

HJV hemojuvelin

HNE 4-hydroxylnonenal

HO heme oxygenase

Hpx hemopexin

IDA iron-deficiency anemia

IL interleukin

IRE iron responsible element

IRP iron regulatory protein

IscS cysteine desulfurase IscS

IscU iron-sulfur cluster assembly enzyme IscU

Isu1p/Isu2p iron-sulfur cluster assembly protein

Jac1p J-type co-chaperone Jac1

JAK1/2 tyrosine-protein kinase JAK

LDL low density lipoprotein

LIP labile iron pool

mRNA messenger RNA

NADH nicotinamide adenin dinucleotide

Nfs1p cysteine desulfurase

NTBI not-transferrin-bound iron

p53R2 R2 subunit of p53

PBG porphobilinogen

PCBP poly C-binding protein

PD Parkinson's disease

PHD prolyl-hydroxylase

PIH pyridoxal isonicotinoyl hydrazone

pRb retinoblastoma-associated protein

RNA ribonucleic acid

ROS reactive oxygen species

RR ribonucleotide reductase

sHJV soluble hemojuvelin

SMAD mothers against decapentaplegic homologue

Ssq1p iron sulfur cluster assembly protein

STAT signal transducer and activator of transcription

Tf transferrin

TfR transferrin receptor

TMPRSS6 matriptase 2

TWSG1 twisted gastrulation protein

UTR untranslated region

UVA ultraviolet light A

Yah1p ferredoxin

1. Introduction

Iron, beeing an essential element for almost all life on Earth, has a wide use in intracellular structures.

Its metabolism influences a lot of cellular processes, including Krebs cycle, hemoglobin synthesis, DNA replication and repair, cellular respiration etc. Any disruptiom may therefore lead to a cell malfunction and potentially also to organ failure and even death.

This fact lead to an idea to exploit it in fighting cancer, one of the most frequent cause of death causes in the world.

By studying the differences between normal and tumor cell metabolism of iron, numbers of scientists have suggested new anti-cancer approaches and treatments that might represent a future of tumor therapy.

This thesis should outline the basic context of iron metabolism from the gastrointestinal uptake through the intrabody transport, to intracellular usage, traficking and export.

It also denotes the most common deffects, affecting millions of people, of iron homeostasis regulation, orchestrated by hormone hepcidin and many other regulatory proteins controlling its expression.

The abnormalities of cancer cells are discussed and eplained in this thesis, pointing out the potential problems and future work and research.

2. Iron metabolism

Mammalian iron absorption concentrates in the proximal small intestine - the duodenum and proximal jejunum, where the surface is covered by specialized polarized cells, enterocytes, with a brush-border apical side and flat basolateral side (figure 2.1). The apical side forms microvilli, increasing the active surface and therefore boosting the nutriens uptake. The whole tissue is organized in the same manner - the cells make a bigger form of microvilli, the villi. This arrangement intesifies the effectivity of digestive processes and the absorption of nutrients.

The cells in intestinal tract are arranged in a single layer, where the one side is in contact with nourishment coctail in the intestine, while the other with the bloodstream.

When a cell is formed in the Lieberkühn crypt, it is not ready yet to absorb and transport the iron to the plasma, it can only devour a small amount for its own purposes (as a construction unit). After its maturation, when the cell is pushed from the crypt to the top of the villus by newly formed cells, it serves as a sieve and transports the iron through its cytoplasm to the circulation. When it completes its life cycle, it is peeled off the villus top and it leaves the organism with the rest of food residue and the iron it contents is lost.

Interestingly, the human body is essentialy unable to excrete iron. The only iron loss (which is about 14 μg/kg per day in man) is due to the exfoliation of gastrointestinal epithelia or skin cells, or in temporary cases like pregnancy, lactation and menstruation.

Iron can be absorbed in a form of heme iron or inorganic iron. The latter is poorly accepted by intestinal cells and needs an organic enhancers to improve the uptake, like ascorbate and citrate, which solubilize the iron in duodenum (Crichton, 2016). But normal western diet is full of inhibitors of inorganic iron absorption, like phytates in cereals and nuts (Hurrell and Egli, 2010) or polyphenols and tannins in vegetables, fruits, coffee, wine and tea (as cited in Abbaspour et al., 2014).

On the other hand, heme iron in hemoglobin and myoglobin from meat, fish and poultry diet is well absorbed and is only modestly influenced by iron status whereas the non-heme absorption is highly regulated by iron store levels (Crichton, 2016).

The molecular mechanism of iron absorption from dietary sources can be divided into three individual steps:

1. uptake
2. transport
3. release

The iron uptake at the apical pole of brush-border cell uses two separate pathways. One for heme iron and the second one for non-heme iron. The first one is not well explored but there are some assumptions that it could be a receptor-mediated pathway where a low affinity heme transporter HCP1 (heme carrier protein 1) might play a role (Shayeghi et al., 2005).

The second one - non-heme iron uptake is mediated by DMT1 (divalent metal trans-

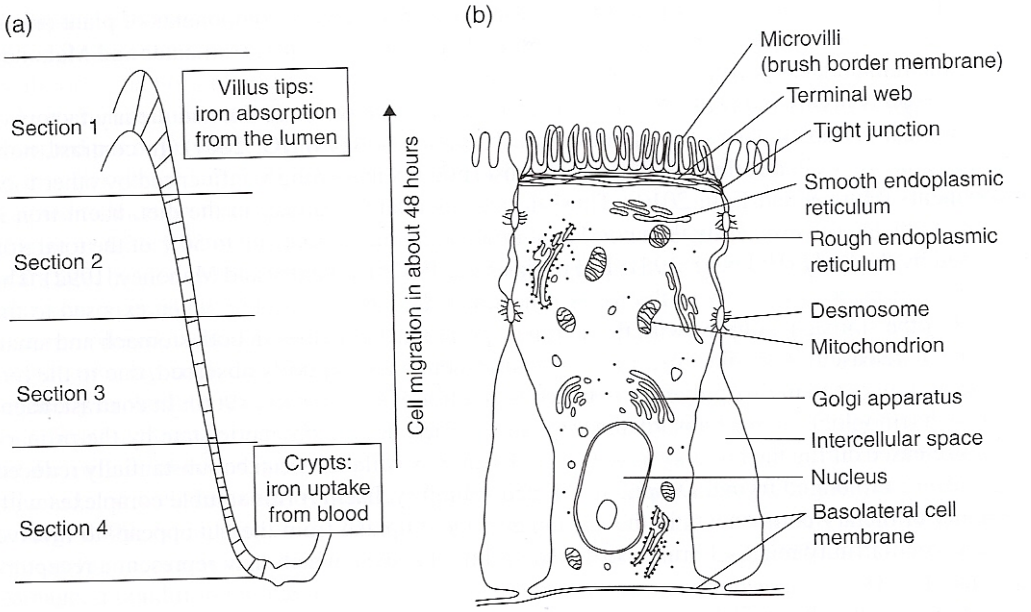


Figure 2.1: (a) Lieberkühn crypt - stem cells in the bottom of the crypt, sensing the iron blood levels, are relatively rapidly proliferating and while they are differentiaing, they are pushed up to the villus top, where they can absorp dietary iron from the intestinal lumen; (b) Enterocyte (adapted from Crichton, 2016))

porter) and Dcytb (duodenal cytochrome b). It is necessary for the transport that Fe^{3+} must be reducted by Dcytb to Fe^{2+}. Dcytb is a brush-border ferroreductase, similar to six transmembrane helices cytochromes b561 (Crichton, 2016), which use an ascorbate from duodenum as an elektron donor. It seems to play an important role in the regulation of absorption because its expression is increased and decreased depending on plasma iron levels.

Afterwards, iron is transported across the membrane via DMT1 (Mackenzie and Garrick, 2005). It uses a slightly acidic microenvironment around enterocytic cells for H^+ electrochemical gradient to drive the Fe^{2+} transport. This protein is present ubiquitously among organisms and is able to transport also other divalent metal ions like Mn^{2+} or Cd^{2+} but it does not transport Ca^{2+} and Mg^{2+} (Mackenzie et al., 2006). This fact is really important because the high concentration of these ions in duodenum would interfere with Fe^{2+} transport and no ferrous iron would be able to get in (Shawki and Mackenzie, 2010). The molecule of DMT1 is considered a main regulatory point of intestinal iron absorption due to the presence of iron regulatory elements (IREs) on its mRNA.

When the iron molecule enters the cell, it goes right to the labile iron pool in the cytosol. The closer destiny of this atom is not known but it is transported across the cytoplasm to the basolateral side of the mucosal cell. If the iron enters ferritin cycle, it is either used for the cell purposes or it remains in the ferritin form and it is discarded eventually after the cell exfoliation.

On the basolateral enterocyte membrane, iron ions are excreted by ferroportin (FPN1; see Cellular uptake and export) and oxidized to Fe^{3+} by multicopper ferroxidase, similar to ceruloplasmin (Cp) (Syed et al., 2002), hephaestin (HEPH) inserted by GPI (glycosylphosphatidylinositol) anchor into the membrane.

2.1 Cellular uptake and export

The iron uptake of a cell is maintained mainly by receptor-mediated endocytosis (figure 2.4) in which the transferrin and transferrin receptor play the major role. However, there are alternative ways of iron transportation such as transport of heme, ferritin and NTBI (non-transferrin-bound iron), mediated by potential transporter ZIP14 (Liuzzi et al., 2006). The latter still remains to be clarified but heme translocation has been studied and the studies showed that when hemoglobin is released into plasma, it dissociates in $\alpha\beta$dimers. Due to its oxidative properties, this heterodimers must be instantly captured by haptoglobin (an acute-phase protein) (Shim et al., 1965) or hemopexin (Hpx). Hemoglobin-haptoglobin complexes are rapidly bound to CD163 scavenger receptors of macrophages (Kristiansen et al., 2001) and degraded but hemopexin acts as an extracellular heme transporter across the tissues. Complex like this is then internalized via CD91 receptors and receptor-mediated endocytosis. Heme is used in the cell whereas the Hpx is recycled and returned back to the plasma.

Ferritin is discussed below.

2.1.1 Transferrin

Transferrin (Tf), also referred to as serotransferrin, is a monomeric glycoprotein essential for non-heme iron trafficking in all multicellular animals. The family of transferrins constitutes of a large number of proteins, nevertheless, not all of them have the same function in organism. Some, now called transferrin homologues, have evolved through the time and participate in different kinds of reactions such as immune response (Lambert, 2012). One of this molecules is lactoferrin. It can be found in numerous body liquids (saliva, milk, tears etc.), secreted by mucosal epithelia and polymorphonuclear granulocytes (Ward et al., 2003), and prevents the proliferation of invading microorganisms (as cited in Crichton, 2016). Although this protein is able to bind iron, it does not play a major role in iron homeostasis (Ward et al., 2003) due to its inability to bind to transferrin receptors (as cited in Adlerova et al., 2008).

Human serum apotransferrin, the major non-heme transporter, has around 80 kDA and 680 amino acid residues. It is formed by two homologous globular lobes (C and N), connected through a short loop with one iron-binding site at each lobe (Crichton, 2016). Both lobes consist of two domains (1 and 2) which together form the iron-binding site. It can acquire two conformational states - open (without bound iron) and close (with two ferric iron ions) (Baker and Baker, 2012). Interestingly, the iron atoms cannot be bound without a synergistic anions - in this case a carbonate anion (figure 2.1). This important molecule provides stability and rigidity to the complex by preventing binding of water in the coordinate sphere and therefore avoiding hydrolysis (Surgenor et al., 1949) and brings two oxygen ligands which are used by metal ion. It also helps neutralize the positive charge of a binding site (Crichton, 2016). It was shown that in absence of carbonate, other anions like small carboxylic acids can replace it (Schlabach and Bates, 1975).

Iron is not the only metal ion that can be bound to transferrin. Mn^{3+}, Ti^{4+}, Cr^{3+}, Ru^{3+} and Bi^{3+} can also function as a ligand and be transported via transferrin and its receptor (TfR) (Crichton, 2016).

There is no other way how can transferrin-bound iron enter the cell than via transferrin receptors (figure 2.3 (a)) which are therefore found ubiquitously across organisms.

The TfR molecule is present in two forms - TfR1 and TfR2. The first one is the most

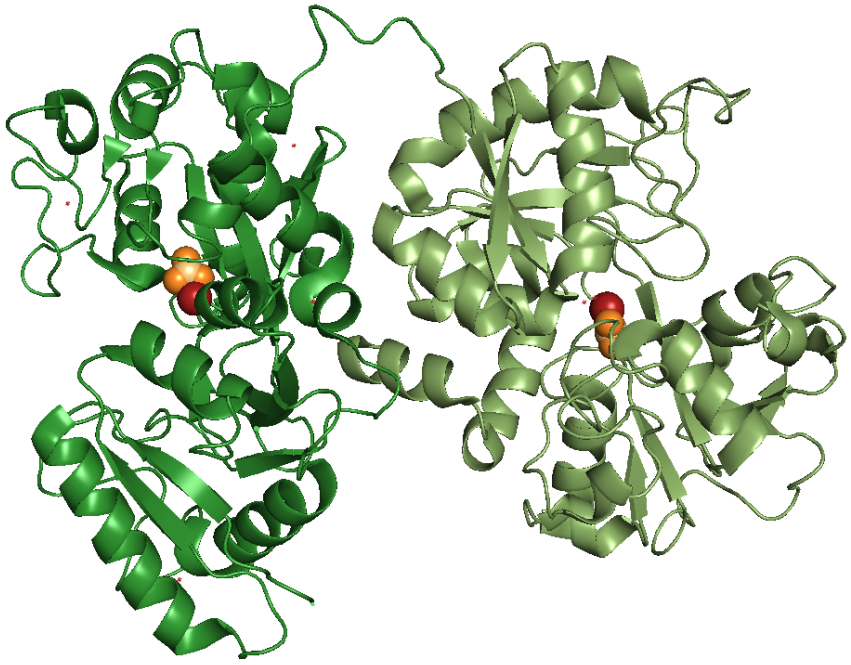


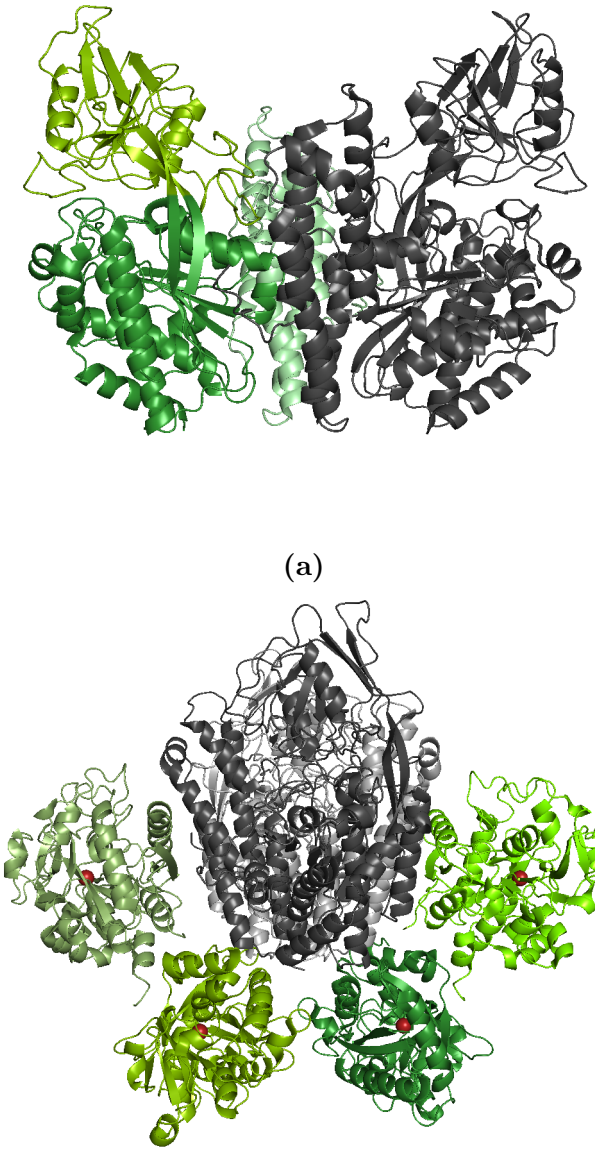
Figure 2.2: Human serum holotransferrin - two subunits in different shades of green, two Fe atoms are shown in red, two CO_3^{2-} anions in orange (source - PDBsum, PDB code: 3qyt

common type, abundant in all iron-requiring cells and expressed in almost every human cell type. TfR1 mRNA contains IREs binding IRPs (iron regulatory proteins), which stabilize the transcript and therefore increase the expression when intracellular iron pool is low.

TfR is a homodimeric membrane-bound protein, where each monomer is made of three domains - protease-like, apical and helical. One homodimer can bind two molecules of Fe_2-Tf, one at each monomer (figure 2.2). This reaction is species-specific which means that for example porcine transferrin-bound iron cannot interact with human transferrin receptor (Kawabata et al., 2004).

The latter of homologues mentioned above, TfR2, has much lower affinity for Fe_2-Tf (Kawabata et al., 2000) which suggest that it is not able to compensate for TfR1 in a case of *TFRC* gene knockout (Fleming et al., 2000). Importantly, this homologue does not contain IREs and cannot be regulated by IRP proteins (Kawabata et al., 2000).

When the Fe_2-Tf-TfR complex (figure 2.3 (b)) is formed, TfR stabilizes the open apo-Tf conformation through the long-range conformational change, to accelerate the iron release within the cell (Giannetti et al., 2005). This complex formation does not require energy, nevertheless, the contact between Fe_2-Tf and TfR initiates clathrin proteins assembly to make a clathrin coated pit (figure 2.4) which encage the transferrin receptor complex with polyhedral framework. This phase of receptor-mediated endocytosis is ended by a cut between membrane and vesicle, processed by small GTP-binding proteins dynamins. The clathrin coat is removed after internalization and the vesicles continue through cytoplasm with smooth surface so they can fuse with membrane of early endo-



(a)

(b)

Figure 2.3: (a) homodimer of transferrin receptor, one subunit in grey, second one in shades of green according to individual domains (apical in yellow-green, helical in light green, protease-like in grass-green) (source - PDBsum, PDB code: 1cx8); (b) Fe_2-Tf-TfR complex, TfR subunits in shades of grey, Tfs in shades of green, Fe atoms in red (source - PDBsum, PDB code: 1suv)

somes(Crichton, 2016).

All ligands that are mentioned to be taken into the cell are sorted to undergo a specific processes. "First sorting stop" is at the cell surface. A decision is made whether the molecules are directed into the dynamic or the static early endosome. The latter are present in greater numbers and act more steady, whilst the dynamic endosomes mature quickly into late endosomes and are predominantly intended for ligands destined for degradation such as LDL and its receptor.

Nevertheless, TfR complexes enter both types of endosomes indiscriminately (Lakadamyali et al., 2006). The pH in early endosomes is maintained by ATP-dependent

proton pumps and held at about 5.5 - 6.0. This slightly acidic pH force Tf to release iron atoms. As was said earlier, the Fe_2-Tf-TfR complex is more likely to release iron because of the conformational changes within this molecule after the receptor binding than the diferric transferrin alone (Bali and Aisen, 1991). After its release, the ferric iron is reduced by ferrireductase (Ohgami et al., 2005) and transported into cytosol via DMT1 (divalent metal transporter 1) (Gunshin et al., 1997).

Unlike most other ligands, Tf-TfR linkage is not broken under acidic endosomal pH (Dautry-Varsat et al., 1983) and the apo-complex is in the vesicle taken back to the plasma membrane, where the two membranes fuse. The environment here has pH about 7.4 which disconnects the two molecules and Tf is free to undergo another cycle (Leverence et al., 2010).

While the recycled ligands are being deprived of its cargo and returned to the plasma membrane, the molecules destined for degradation remain in the early endosome which becomes late endosome, and are destroyed later.

This whole cycle can run one direction only.

2.1.2 Ferroportin

Iron export from cells is not completely understood and so far the only exporter identified is ferroportin. It is a product of gene *SLC40A1* and is expressed mostly in four most important tissues regarding to iron homeostasis - intestine, placenta, hepatocytes and macrophages.

Its structure has not been determined yet but a model of 12 transmembrane domains has been suggested (Liu et al., 2005). Its mRNA contain IREs and the expression is therefore responsive to iron and also to inflammatory stimuli (Crichton, 2016).

The mechanism of iron export itself remains elusive, nontheless it has been shown that it interacts with hepcidin (as cited in Ganz and Nemeth, 2006), the most important iron homeostasis regulator.

Other important proteins involved in cellular iron export are ceruloplasmin and hephaestin. These multicopper oxidases are required for the release of iron from hepatocytes and macrophages. They mediate the release of Fe^{2+} from cells and indirectly make sure it is further incorporated in the Fe^{3+} form. This system serves as a protection against harmful radicals due to its high affinity of Cp to O_2 and prevent the spontaneous oxidation.

2.2 Utilization

The destiny of iron atoms differ from cell to cell. Basicaly, it can be used to function in active sites of proteins (heme proteins, Fe/S clusters proteins, non-heme proteins or non-iron-sulfur proteins), it can be stored in a form of ferritin and released when it is needed, or it can stay in the cytosol, not bound to anything, and form a potentially risky labile iron pool (LIP).

2.2.1 Ferritin

About 25 % of body iron is stored, mostly in ferritin that exists in every mammalian cell. This protein seems to be highly conserved: it has been found in all organisms except for yeast and some archaebacteria (Crichton, 2016).

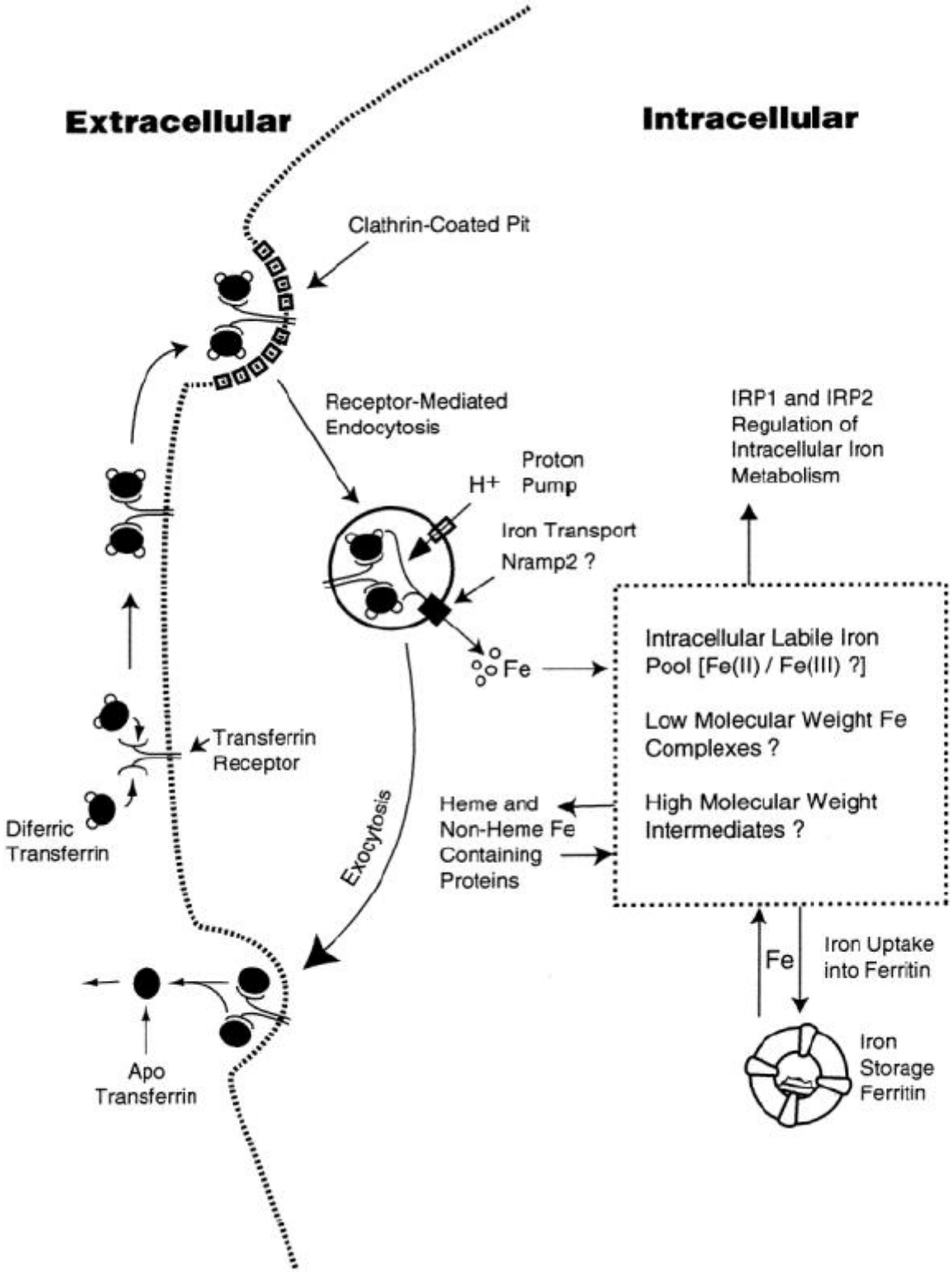


Figure 2.4: Internalization of $2\,Fe_2$-Tf-TfR complex via receptor-mediated endocytosis, using clathrin coated vesicles, release of iron in the early endosome and recyclation of Tf-TfR complex back to the membrane (adapted from Ponka, 1999)

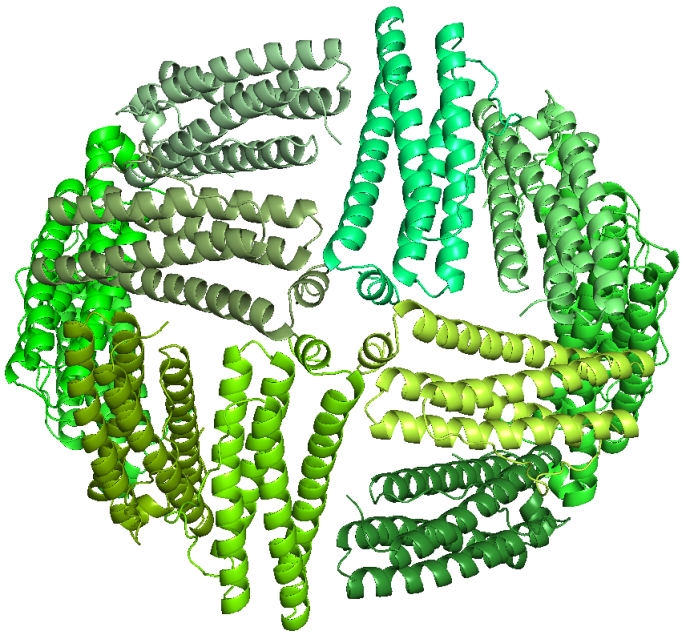


Figure 2.5: Bacterioferritin from *E. coli* (source - PDBsum, PDB code: 1bcf)

It is an oligomeric hollow protein shell with relative molecular weight about 500 kDa, having an outside diameter cca 12-13 nm and inside diameter cca 7-8 nm. One ferritin molecule can store up to 4500 Fe(III) atoms (Ford et al., 1984). It can be found mainly in cytoplasm but low amounts are present in blood plasma too (as cited in Harrison and Arosio, 1996).

Each apoferritin (protein without the iron atoms) consists of 24 structurally equivalent subunits (Harrison and Arosio, 1996). These subunits are of two types - type L (as light, typical for organs storing iron like liver and spleen) and type H (as heavy, typical for heart and brain). Therefore, ferritins can differ in the subunit composition but no H-chain homopolymers were isolated although L-chain homoproteins have been found in the human serum ferritin (Arosio et al., 1977). The subunits consist of 1 short helix, long extended loop and bundle of 4 long helixes (as cited in Harrison and Arosio, 1996).

We can distinct between three types of ferritins - (1) 24-meric ferritins, (2) heme-containing 24-meric bacterioferritins (BFRs) of procaryotes (figure 2.5) and (3) 12-meric DNA-binding Dps proteins (Crichton, 2016).

The mechanism of heme binding to the protein is not described in detail but it binds at the inter-subunit site near the cavity surface. It seems to facilitate the reductive iron release from ferritin (Harrison and Arosio, 1996).

The protein cavity is about 80 Å in diameter (Harrison and Arosio, 1996) and the composition is quite variable, notably due to different content of inorganic phosphate (Treffry et al., 1987). Due to a large numbers of intra- and inter-subunit salt bridges and hydrogen bonds, the apoferritin molecule is highly resistant to heat (Harrison and Arosio, 1996).

The mechanism of iron loading is not yet elucidated in details yet, however, there were two promising models introduced. In both cases, Fe(II) is oxidized by ferroxidase activity of ferritin (it has been shown that all ferritins sequenced contain conserved residues with

this type of activity - when the iron is supplied as a Fe(II) salt it is oxidized (Macara et al., 1972) using dioxygen. In the next step, ferric iron undergoes hydrolysis to form ferrihydrate ($(Fe^{3+})_2O_3 \cdot 0.5\,H_2O$).

The first, crystal growth model, presents that Fe(II) is bound and oxidized at catalytic centers on the protein, then the Fe(III) clusters are formed in the cavity of the protein forming stable nucleus of the crystalline mineral. This nuclei then provides another binding site (growing surface) onto which Fe(II) can be deposited and oxidized (Macara et al., 1972).

The alternative model (less likely to be true) is that all Fe(II) enters the ferritin molecule and is catalytically oxidized before reaching the cavity (as cited in Harrison and Arosio, 1996).

The fact, that many more Fe(II) binding sites are present in ferritin than in apoferritin (Watt et al., 1985), talks for the first hypothesis, since in the second one, we cannot expect any increase in binding sites after iron acquisition.

An interesting fact is, that in absence of ferritin, α- or γ-iron(III) oxide-hydroxide (FeOOH) is produced in otherwise identical conditions (Harrison et al., 1967).

2.2.2 Heme metabolism

The molecule of heme consists of protoporphyrin IX and Fe2+ ion. This structure functions as a prosthetic group of dozens of hemoproteins, and takes a part in a significant number of reactions, such as oxygen binding (hemoglobin, myoglobin), oxygen metabolism (oxidases, peroxidases, catalysis) or electron transfer (cytochromes) etc. (Ponka, 1999).

Another important function is also the negative feedback regulation of its own synthesis (Burnham and Lascelles, 1963) and other important proteins (globin, transferrin receptor, cytochromes etc.) (Ponka, 1999). Most of the heme synthesizing pathway is located in mitochondrial matrix in almost every type of cell, however, the most rapid biosynthesis occurs in immature erythroid cells and hepatocytes (Ponka, 1999).

Vast majority of heme in hepatocytes is used for hemoprotein synthesis (80 %), the rest is converted into bile pigments such as biliverdin, bilirubin and phycobilin (Grandchamp et al., 1981).

The first step of heme biogenesis (figure 2.6) that takes place in mitochondria is a condensation of succinyl-CoA and glycine to form a 5-aminolevulinic acid (ALA) and carbon dioxide and CoASH (Ponka, 1999). This rate-limiting reaction (Carvalho et al., 1997) is catalyzed by 5-aminolevulinic acid synthase (ALAS) attached to mitochondrial cristae with disulfide bonding (Whiting and Elliott, 1972). There are two different isoforms of the enzyme - ALAS1, ubiquitously expressed in all cells and ALAS2 (eALAS), specialized for erythroid cells (Ponka, 1999).

For further processing, ALA must be transported to cytosol. There is not much known about this transport but there is some indirect evidence that it is mediated by ABCB6 transporter in mitochondrial membrane (Krishnamurthy et al., 2006).

In cytosol the ALA dehydratase (ALAD) forms monopyrrole porphobilinogen (PBG) from two molecules of ALA and four molecules of PGB are in two reactions converted into tetrapyrrole uroporphyrinogen III which is decarboxylated to coproporhyrinogen III. In this step, the molecule is transported back to the mitochondrion and in 2 other reactions it is converted into protoporphyrin IX. This is the final precursor for heme molecule. In the last part, ferrochelatase (FC) inserts Fe2+ ion into the protoporphyrin IX and forms the heme (Ponka, 1999).

Regulation of this process is maintained mainly by regulating the ALAS1 and ALAS2 activity. ALAS1, the "housekeeping" ALA synthase, is highly likely supressed on transcriptional level by heme which decreases the mRNA's half-life and inhibits the translocation of ALAS1 precursor protein to mitochondria (Tyrrell and Marks, 1972). In erythroid cells, ALAS2 transcription is induced by erythroid-specific transcription factors. The mRNA contains IRE sites at the 5' untranslated region and is therefore regulated by the IRE/IRP system. This isoform associates with succinyl-CoA synthase which functions as an anchor for ALAS2, and simultaneously provides succinyl-CoA, therefore facilitating the reaction. This isoform is not inhibited by heme, nevertheless, heme does block the iron uptake from transferrin.

The product of ALAS - 5-aminolevulinic acid seems to be one of important ROS (reactive oxygen species) generating molecules. Its intracellular oxidation creates higly reactive oxygen radicals. There is a possibility that increased levels of ALA might correlate with IRP1 activation which further regulates ALAS2. The heme biosynthetic pathway is considered to be the major consumer of iron in the cell and when the intracellular iron levels are low, the rate of ALA also decreases (Carvalho et al., 1997).

Almost every eukaryotic cell (with some exceptions) is able to degrade heme and the only way, cells proceed this reaction, is by heme oxygenase (HO). However, this enzyme is mostly active n the spleen (where the senescent erythrocytes are sequestreted and destroyed) and liver (where the blood is purified). It catalyzes a cleavage of heme at the α-methene bridge, forming a linear tetrapyrrole (biliverdin, a bile pigment), ferrous iron and carbon monoxide (Tenhunen et al., 1969). Biliverdin is then converted to bilirubin by the cytosolic enzyme biliverdin reductase. The heme degradative activity was found to be very useful in stress situations like UVA (ultraviolet A light) or an influence of oxidizing agents like hydrogen peroxide. The heme destruction is a part of an additional pathway of protecting the cells by increasing an antioxidant defense - removing a prooxidant heme and forming antioxidant pigments (Keyse and Tyrrell, 1989).

CO, the other product of HO reaction, in low amounts serves as a signal molecule and neurotransmitter. In high concentrations, on the other hand, inhibits heme protein activity.

The iron atom released from heme molecule represent a threat to the cell in the form of ROS generated in Fenton reaction with iron. This can lead to deleterious cellular consequences (Crichton, 2016), however, cells have mechanisms how to avoid this scenario, for example by oxidizing ferrous iron to ferric and incorporating it into ferritin.

Hemeoxygenase has two isoforms (HO-1, HO-2), encoded by the *HMOX1* and *HMOX2* genes, which differ not only in molecular composition and structure (Maines et al., 1986) but also in rate of expression: HO-1 is expressed ubiquitously in mammalian tissues and functions as a response to oxidative stress while HO-2 is expressed constitutionaly and is not inducible (Gozzelino et al., 2010).

2.2.3 Fe-S clusters synthesis

The Fe-S clusters are synthesized (figure 2.7) de novo in mitochondria and this process seems to be highly conserved in evolution (Loiseau et al., 2003). Enzymes with iron-sulfur clusters are fundamental in a wide range of cellular processes such as amino acid synthesis (isopropylmalate isomerase), vitamin biosynthesis (biotin and lipoate synthase), DNA replication (primase), Krebs cycle (aconitase) and cellular respiration (electron transport chain complexes) (Sheftel and Lill, 2009).

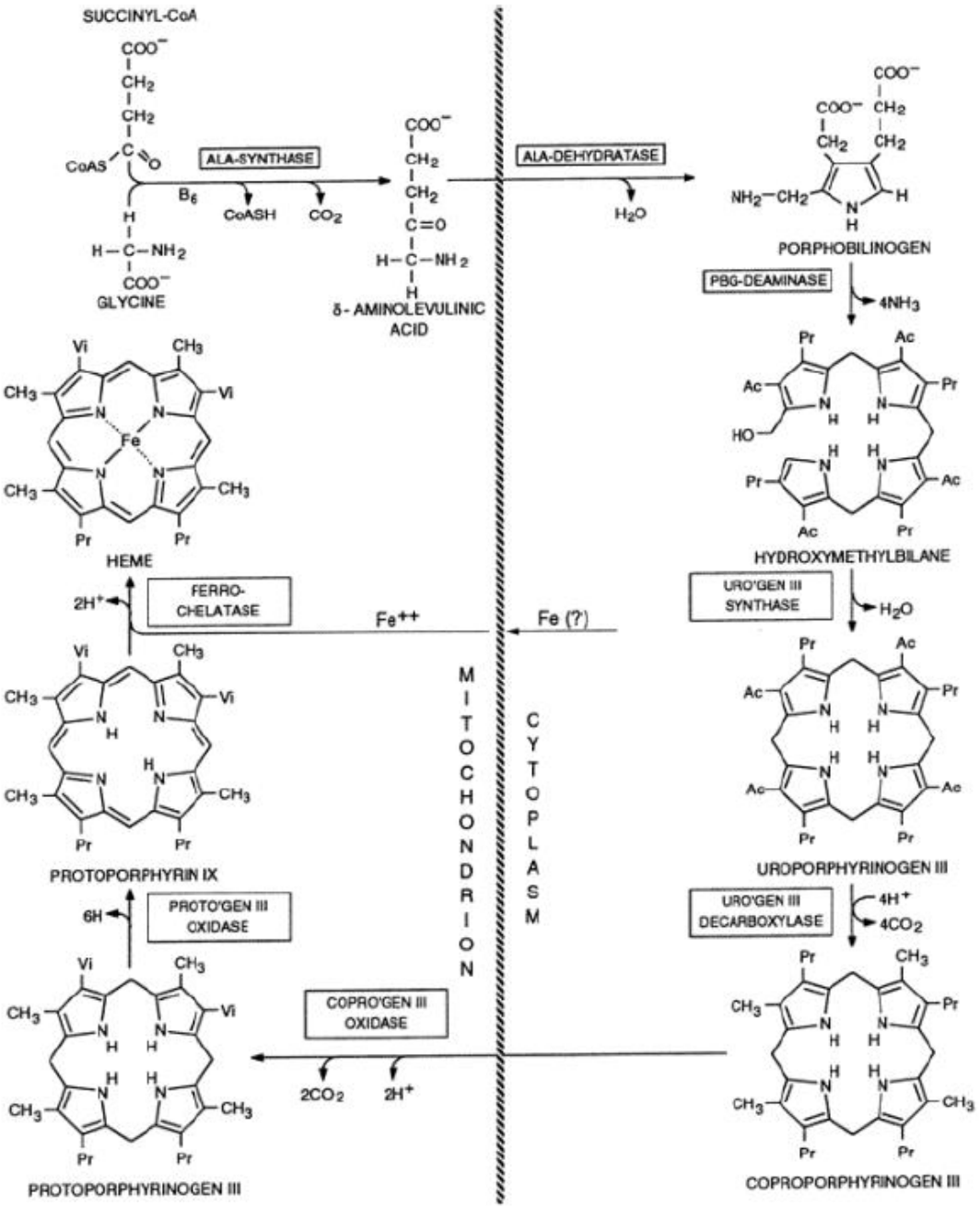


Figure 2.6: The pathway of heme biosynthesis ((adapted from Ponka, 1999))

The environment of mitochondria is highly reductive hence the ferrous iron is not oxidized after entering the matrix and can be further utilized for Fe-S cluster assembly. For its stabile binding to IscU protein, the presence of sulfur is required, so it is released from L-cysteine (L-alanine is formed simultaneously) by IscS protein - the cysteine desulfurase. The released sulfur is in sulfan state and has to be reduced to sulfide. This reaction has been studied in yeast on its homologuous proteins Isu1p/Isu2p (homologues with IscU) and Nfs1p (homologue with IscS) (Mühlenhoff et al., 2003). In bacteria, the IscS desulfurase is bound to IscU, forming (in vitro) weakly bound heterotetrameric protein and providing the sulfur for the assembly (Agar et al., 2000b). These two proteins form a one-electron chain, using NADH as a reduction equivalent, which reduces the sulfane sulfur to sulfide that is further used by Isu1p. This protein serves as a scaffold for the iron-sulfur cluster assembly (Mühlenhoff et al., 2003). The bacterial homologue IscU forms homodimer which is able to handle two $[2\,\mathrm{Fe}{-}2\,\mathrm{S}]^{2+}$ clusters at once (Agar et al., 2000a).

The mechanism of conversion of two $[2\,\mathrm{Fe}{-}2\,\mathrm{S}]^{2+}$ clusters into one $[4\,\mathrm{Fe}{-}4\,\mathrm{S}]^{2+}$ cluster is proven to be a condensation of those two $[2\,\mathrm{Fe}{-}2\,\mathrm{S}]^{2+}$ clusters present at one homodimer simultaneously. It has not been clarified if the $[4\,\mathrm{Fe}{-}4\,\mathrm{S}]^{2+}$ cluster functions as a bridge between the two monomers or if it is bound to only one of them. In either way, the cluster stays bound to the homodimer which suggests that some dislocating mechanism is needed to transfer the cluster from Isu1p to apoprotein (Mühlenhoff et al., 2003).

Nonetheless, some Fe-S clusters that functions in nucleic and cytosolic Fe-S proteins are also assembled in the cytosol via an additional pathway. This pathway, termed CIA (cytosolic iron-sulfur protein assembly) machinery and aquires sulfur from sulfur-containing compounds that are transported by Atm1 ABC transporter from the mitochondria and whose identity has not been incovered yet (as cited in Stehling et al., 2014). Both ISC and CIA pathways thus cooperate in order to provide functional Fe-S clusters for numerous iron-containing proteins.

The dislocation of the assemled Fe-S clusters to the tarer apoprotein seems to be catalyzed by a chaperone system consisting of ATP-dependent chaperone Ssq1p, co-chaperone Jac1p and monothiol glutaredoxin Grx5p. There are few hypothesis about the function of these three proteins but the one most likely to be true is that they play an important role in the dislocation and/or transfer of the cluster onto apoprotein.

Rather striking finding shows that Yah1p, an essential protein in the early iron-sulfur cluster biogenesis, contain the Fe-S cluster itself. Although this might suggest some separate pathway in Fe-S cluster biosynthesis, none has been found yet. (Mühlenhoff et al., 2003).

2.2.4 Labile iron pool

The vast majority of iron in a cell, is in protein-bound form. The rest is denoted as a labile iron pool (LIP), which is only vaguely defined as an chelatable pool of iron inside the cell.

This iron "compartement" is made predominantely of Fe^{2+}, but also of Fe^{3+} and different kinds of low-molecular-weight LIP ligands (the major cytosolic ligand for Fe^{2+} has been proposed to be in vitro reduced glutathion (GSH) Hider and Kong (2013). It is considered as an interchange station for further iron movement inside the cell, because almost every iron ion that enters the cell, in other than transferrin form, intially goes to the LIP (Shvartsman and Cabantchik, 2012) from where it is transfered to either mitochondria or other organells, to be used as a cofactor in non-heme iron enzymes, stored in a ferritin

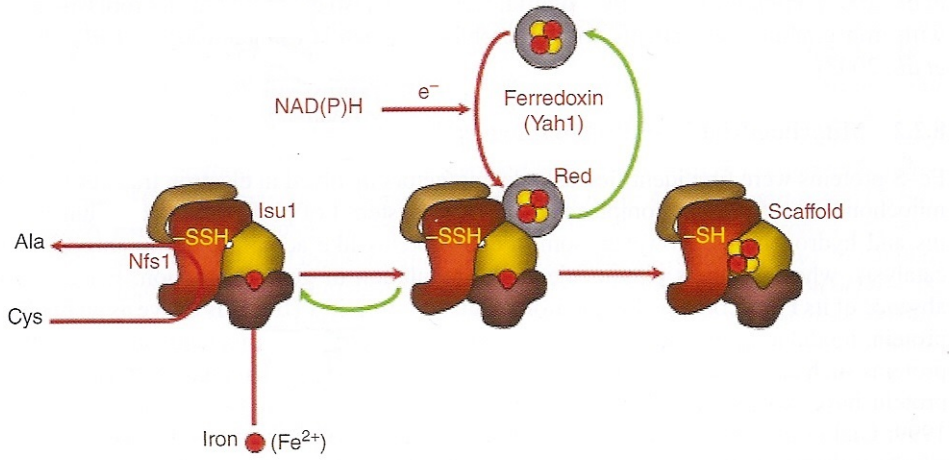


Figure 2.7: The Nfs1 cystein desulfurase releases S from L-cysteine forming L-alanine. Yah1 is reduced using NAD(P)H as a reduction equivalent and it subsequently reduces sulfane sulfur to sulfide. Fe^{2+} is take up from the mitochondrial matrix and through the stage of two $2\,[Fe{-}2\,S]^{2+}$ clusters is one $[4\,Fe{-}4\,S]^{2+}$ cluster formed ((adapted from Crichton, 2016)

form or serve in a prosthetic group in hemoproteins or iron-sulfur-clusters proteins.

The translocation of cytosolic LIP iron is managed by PCBPs (poly C-binding proteins) (Philpott and Ryu, 2015) that possess chaperone activity for ferritin (Shi et al., 2008). As it has been shown lately, PCBP2 physically interacts with DMT1 and FPN1 transporters (Yanatori et al., 2014) that suggests its major role in intracellular iron trafficking.

The exception to this rule could be the transferrin-bound iron as an alternative hypothesis called "kiss and run". After the receptor-mediated endocytosis, iron is released from transferrin inside the early endosome compartement. To prevent iron from encountering the oxygenous environment of cytosol, early endosomes perform this "kiss-and-run" mechanism of a content exchange and for a moment, mitochondrial and endosome's membranes fuse to translocate free iron to the mitochondrial matrix (Sheftel et al., 2007).

Nonetheless, the rest of labile free iron in the cytoplasm represents a potential risk of forming reactive oxygen species. The interaction with highly oxygenous environment of cytosol sometimes leads to a Fenton reaction as discussed in the section 3.1.1.

2.3 Regulation

The regulation of iron homeostasis is maintained on two levels, to control iron uptake, utilization and export from a single cell, and to provide a sufficient amount of iron to all tissues by supervising the duodenal iron absorption on the level of a single cell and also on the systemic level where erythroid needs have to be met and iron uptake in the duodenum and iron storage in spleen and liver have to be properly balanced.

2.3.1 Cellular level

The majority of regulatory processes are based on the IRE/IRP system (figure 2.8) which controls the homeostasis on protein level. This system is considered the general

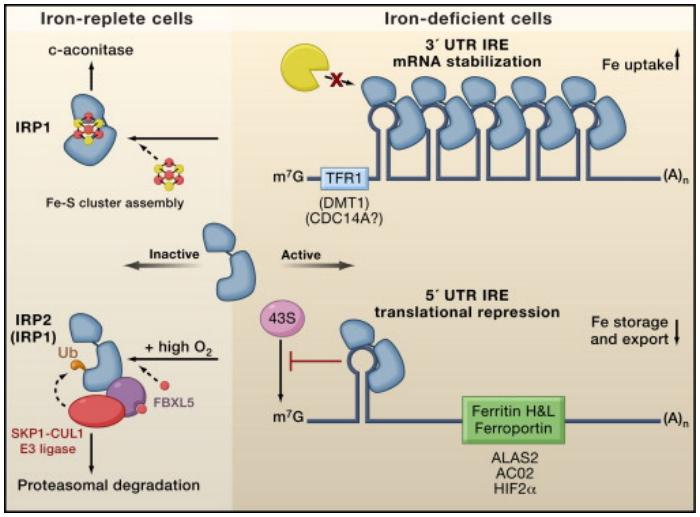


Figure 2.8: (left) Iron-replete cells - IRP1 acquire Fe-S cluster, looses its mRNA affinity and promotes its aconitase activity. IRP2, on the other hand, is ubiquitinilated by FBXL15 complex and subsequently destroyed; (right) Iron-depleted cells - IRPs bind to mRNA's 3'UTR and 5'UTR, where, in the first case, stabilize the transcript and thus prevent degradation, while in the second case, IRPs prevent the ribosome association and therefore the mRNA transcription (adapted from Hentze et al., 2010)

post-transcriptional regulatory mechanism (Casey et al., 1988).

IRE is an iron regulatory element, present in several copies, in a great number of mRNA of proteins involved in iron regulation like ferritin, transferrin receptor, eALAS (Cox et al., 1991), ferroportin(Abboud and Haile, 2000), hypoxia inducible factor 2α HIF2α (Sanchez et al., 2007), one of the splice variants of divalent metal transporter (DMT1) (Kühn, 2015) to name a few. This IRE sites, located either on the 5'-untranslated region (5'-UTR) of mRNA or 3'-UTR, are recognized by iron regulatory proteins (IRPs) that bind to these sequences under low iron conditions, and either prevents the mRNA translation or the mRNA degradation (depending on the position of the UTR).

IRE forms a conserved hairpin structure with upper and lower paired stem of nucleotides, conserved loop CAGUG and at least one conserved unpaired C. By turning outwards, the unpaired bases provide a specific contact sites for IRP1 which covers the IRE hairpin using two sites and about dozen interactions (Crichton, 2016).

The 5'-UTR IREs exist within 40 or less nucleotides from the 5'm^{7}G cap and when the IRPs are bound to them, it prevents the small ribosomal unit from association with the mRNA, hence preventing mRNA from translation (Gray and Hentze, 1994).

The 3'-UTR IREs are present mostly in several copies (5 copies in case of TfR). When the iron concentrations are high, the cleavage, mediated by RNase, occurs between them and the mRNA is degraded.

In the cytosol we can find two types of IRP proteins which are IRP1 and IRP2. These two iron regulatory proteins have 56% sequence identity and are both ubiquitously expressed (nontheless, IRP is predominantly expressed in kidneys, liver and brown fat whilst IRP2 dominates in central nervous system). It has been shown that regarding iron homeostasis, they are able to compensate for each other in a case when the first or the second is dysfunctional (Meyron-Holtz et al., 2004).

Although they have both 4 globular domains, their structure shows diversity and subsequently they differ also in function.

When the iron level is high, IRPs loose their affinity to IREs, but they are not both completely inactivated. IRP1 is able to acquire a [4Fe-4S] cluster together with an cy-

tosolic aconitase activity - with its Fe/S centre, attached to 3 cysteine residues, it binds the solvent and substrate with its remaining free iron. IRP2, on the other side, does not possess the enzymatic activity and when it is not needed to maintain the iron homeostasis, it is ubiquitinated and degraded by proteasome (Guo et al., 1994).

The mechanism of IRE/IRP system is quite simple - when the iron concentration decreases, IRPs bind to the IREs importantly of ferritin and transferrin receptors. While in the first case it reduces iron storage, in the second case, it stabilizes the mRNA and accelerate the TfR synthesis and increase the iron uptake. After the cellular iron homeostasis is restored, the Fe-S clusters are assembled again and seized by IRP1, inactivating its IRE binding activity. Meanwhile, from the same reason, di-iron centers are inserted into FBXL15 protein which is therefore able to combine with another subunits and form an E3 ubiquitin ligase complex inducing IRP2 degradation (Salahudeen et al., 2009).

However, iron level is not the only variable important for IRE/IRP system, oxygen pressure also affects the IRPs activity. When the oxygen levels are high in the iron-depleted cells, IRP1 is more active while IRP2 is degraded (the FBXL15 is highly unstable protein dependent on oxygen concentrations). On the contrary, when the oxygen levels are low in iron-depleted cells, IRP1 activity is more easily inactivated while IRP2 remains intact.

2.3.2 Systemic level

It is necessary to ensure availability of iron in different places in the body to either store it or use it.

Most circulating iron can be found in hemoglobin in erythrocytes (cca 30 mg $\mathrm{Fe} \cdot \mathrm{kg}^{-1}$) and vast majority of this iron is recycled in the spleen by splenic macrophages.

The major organs of iron consumption are liver, spleen, bone marrow and muscles, where it is either used as hemoproteins, iron-sulfur proteins, non-heme proteins and non iron-sulfur proteins (cca 2 mg $\mathrm{Fe} \cdot \mathrm{kg}^{-1}$) or stored in a form of ferritin, hemosiderin and myoglobin (cca 14-16 mg $\mathrm{Fe} \cdot \mathrm{kg}^{-1}$) in men and about 9 mg $\mathrm{Fe} \cdot \mathrm{kg}^{-1}$ in women).

Almost every pathway, leading to changes of systemic iron availability, passes through hepcidin (figure 2.9) - an antimicrobial peptide hormone produced predominantly by liver hepatocytes and secreted to the circulation (Krause et al., 2000); nontheless, monocytes (Theurl et al., 2008), macrophages (Nguyen et al., 2006), kidney (Kulaksiz et al., 2005), brain (Wang et al., 2008), adipose tissue (Berk et al., 1969) and heart (Merle et al., 2007) are also able to produce small amounts.

This iron-controlling hormone has a distorted antiparallel β-sheet structure with disulfide bonds between cysteine residues (Crichton, 2016). This form of the terminal protein is a 25-residue hepcidin which underwent two cleavages from 84-residue prepropeptide and 60-residue propeptide (Krause et al., 2000); however, there are two other hepcidin forms - the 20- and 22-residue hepcidins with no iron regulatory functions but more effective antimicrobial activity. Both of them are found in urine (Nemeth et al., 2006).

As it circulates freely in the plasma, it binds to its receptor - ferroportin (the only known iron exporter) and iniciates its degradation (figure 2.10), controlling the iron excretion from cells to the bloodstream (Nemeth et al., 2004b). When it is no longer needed, it is cleared from the plasma by kidneys (Park et al., 2001). As a very important molecule, hepcidin is very tightly regulated by numerous processes.

The most important is the systemic iron availability, which gives a negative feedback, using the bone morphogenetic protein (BMP) signaling pathway (figure 2.7). BMP lig-

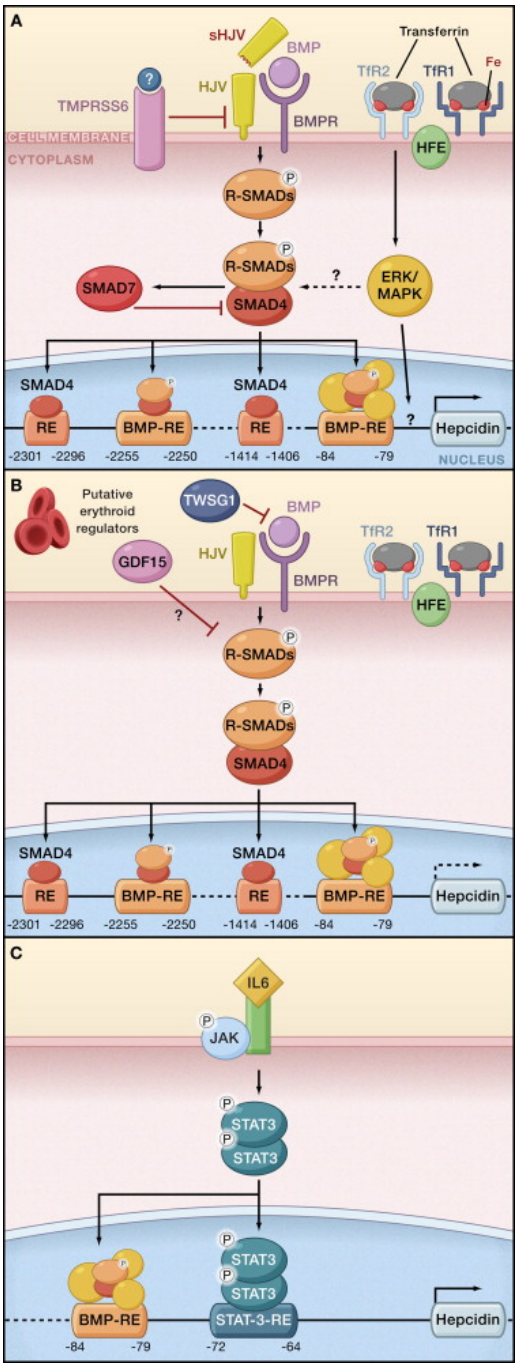


Figure 2.9: (A) Regulation by systemic iron availability - HFE is displaced by high iron concentrations from TfR1 and interacts with TfR2 that further activates expression of hepcidin via intermediate activator. Simultaneously, BMP ligand binds to BMP receptor associated with HJV its kinase activity and therefore phosphorylating R-SMADs that translocate into the nucleus to induce hepcidin expression. This process may be inhibited by sHJV that can bind to BMP ligands, or by TMPRSS6 that cleaves the necessary cofactor HJV or in the cytoplasm by SMAD7; (B) Regulation by erythropoietic signals - GDF15 and TWSG1 from erythroid precursors inhibit the BMP/SMAD signalling pathway; (C) Regulation by inflammatory stimuli - IL6 activates the JAK kinase that phosphorylates STAT3 protein and thus induce hepcidin expression (adapted from Hentze et al., 2010)

ands (such as BMP6) bind to the BMP type I and type II receptors - the serine-threonin kinases. After such activation, BMP type I receptor phosphorylates the BMP type II receptor which further phosphorylates receptor-associated SMAD (mothers against decapentaplegic homologue) proteins 1, 5 and 8 present in the cytoplasm. These three molecules form a complex with SMAD4, which is then capable of translocation into the nucleus, where it regulates the *HAMP* (hepcidin encoding gene) expression (Crichton, 2016).

In this process, the HJV (hemojuvelin) protein functions as a coreceptor for BMP receptors to modulate it for iron regulatory purposes (Babitt et al., 2006). This molecule seems to play a central role in iron homeostasis, since experiments with knockout mice, where HJV was the only protein, apart from mutations of the hepcidin itself, that when mutated, caused a profound decrease of hepcidin expression (Hentze et al., 2010). It interacts with inhibitory serin protease matriptase-2 (TMPRSS6) that cleaves HJV on the plasma membrane, thus inactivating it. Similar reaction is performed by furin that also cleaves HJV but on a different place. It is suggested, that it forms a soluble form of HJV (sHJV) that acts as a hepcidin expression inhibitor, probably by binding to BMP ligands before they can reach BMP receptors (Andriopoulos Jr et al., 2009).

This pathway is strongly regulated by an "iron-sensing complex" (Crichton, 2016), consisting of hemojuvelin (HJV), hereditary hemochromatosis protein (HFE) and both types of TfR (TfR1 and TfR2). The proposed mechanism of iron sensing is based on the location of active sites for HFE binding on TfR molecules. HFE is constantly bound to TfR1. However, the binding sites for HFE and Fe_2-Tf are overlapping and when the Fe_2-Tf ligand comes, it pushes the HFE away from TfR1 and it associates with TfR2. This receptor is able to bind both ligands at the same time and additionaly, the bond with HFE increases its Fe_2-Tf affinity. This HFE-transferrin receptor 2 complex then induces the hepcidin transcription (Hentze et al., 2010).

Hepcidin is also regulated by inflammatory stimuli. In order to sequester the iron from pathogenes, it is withdrawn from the circulation by increased hepcidin concentration.

When a pathogene invades the organism, inflammatory cytokines, IL-6 (Nemeth et al., 2004a) and IL-12 (Armitage et al., 2011) are released. IL-6 binds to the gp130-protein-receptor complex (membrane glycoprotein 130) and activates tyrosine-protein kinases JAK1/2 which phosphorylates STAT3 (signal transducer and activator of transcription 3). This transription factor translocates to the nucleus where associates with hepcidin promoter and induces hepcidin expression and upregulation (Pietrangelo et al., 2007).

Iron, being an essential element for erythropoiesis, must be accessible for erythroid synthesis. Therefore, the availability is regulated by the erythropoietic demands, using probably GDF15 (growth and differentiation factor 15) and TWSG1 (twisted gastrulation protein 1) as a downregulators of hepcidin concentrations, secreted by erythroid precursor cells (Pak et al., 2006). Lately, a new protein involved in this processes was discovered - erythroferron (ERFE, FAM132B). It seems to mediate hepcidin supression during stress erythropoiesis, being produced by erythroblasts in response to erythropoietin (Kautz et al., 2014).

Another important regulator of hepcidin is hypoxia. The low pressure of oxygen stimulates erythropoiesis which, as has been said above, decreases the hepcidin levels. Simultaneously, it causes an expression of hypoxia inducible factors (HIF-1, HIF2), which are heterodimeric transcription factors controlling the gene expression of Tf, TfR, Cp, DMT1 and other proteins.

Under normall conditions, α-subunit's prolines of the HIF are hydroxylated by prolyl-

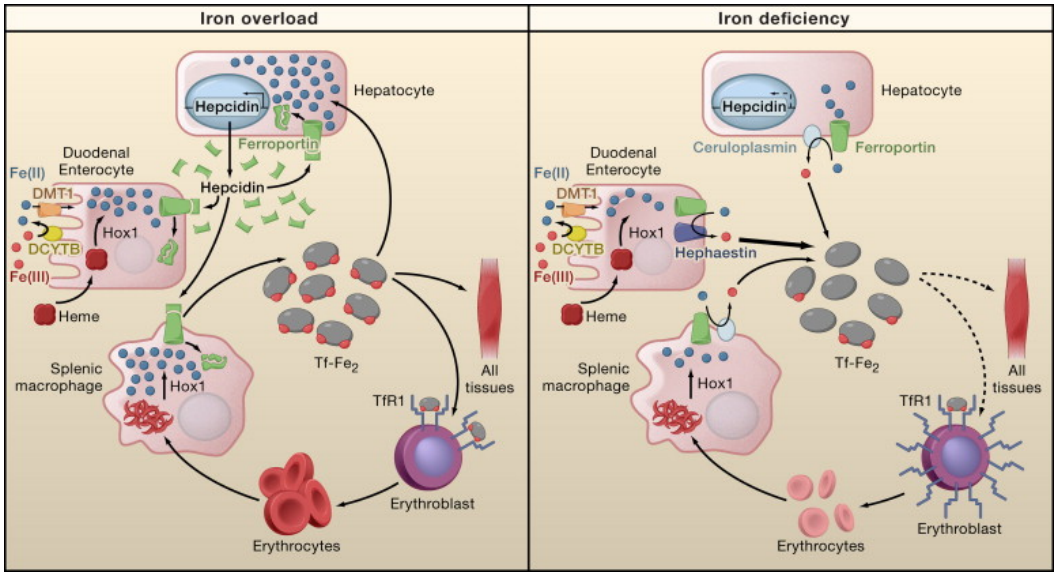


Figure 2.10: Incoming Fe(III) is reduced by Dcytb on the brush-border side of enterocyte.It is subsequently taken up by DMT1 and transported across the membrane. Heme is transported into the cellular lumen and metabolised by heme oxygenase 1 (Hox1). Fe is afterward able to be excreted by ferroportin that is regulated by hepcidin. Hepcidin is secreted by hepatocytes to the bloodstream. Iron is provided in a form of transferrin and internalized via transferrin receptors. (left) Iron overload - in normal cells, hepcidin levels are increasing, causing inhibition and subsequent internalization of ferroportins; (right) Iron deficiency - hepcidin expression is supressed, ferroportin is expressed on the cell surface and Fe is released into the circulation (adapted from Hentze et al., 2010)

hydroxylase (PHD). These hydroxyprolines are eventually recognised by the von Hippel-Lindau E3 ubiquitin ligase, ubiquitinylated and degraded in proteasome (Maxwell et al., 1999). But in hypoxia state, PHDs are inhibited by low oxygen pressure and HIFs cannot be marked to be destroyed and thus it is stabilized.

2.4 Human diseases linked with iron metabolism defects/disorders

2.4.1 Iron-deficiency anemia (IDA)

Iron-deficiency anemia, affecting prevalently women and young children, is the most common nutritional disorder. Main causes of iron-deficiency are, chiefly in developing countries, infectious diseases (malaria, AIDS, hookworm infestation, schistosomiasis), other infections (tuberculosis), deficiencies of other important nutritients (folate, vitamin B_{12}, vitamin A) or genetically inherited disorders (thalassemia).

The ones that are most at risk are unborn children and their mothers that suffer from such deficiency. This disorder may have a negative effect on mental and physical development of a child such as mental delay (Beard, 2007) (caused by reduced foetal brain maturation (Black et al., 2011) and also on mother's health, causing maternal depression (Black et al., 2011).

The iron requirements are significantly increased during pregnacy, although there are iron savings during the first trimester due to the cessation of menstruation. Nevertheless, a lot of women enter the pregnancy with just a small amount of stored iron or virtually zero iron stores at all. Thus, a treatment in a form of oral supplementation is obvious, but the

opinions on this matter differ greatly among the specialists as iron supplementation seems to increase infectious diseases including malaria, in less developed world (Oppenheimer et al., 1986).

General characteristic of IDA is, that erythropoiesis requires more iron that the macrophage and hepatocyte stores together with dietary uptake can supply. It occurs within three phases:
(i) latent iron deficiency - all iron stores are used up (Verloop, 1970) but the hemoglobin levels are in normal range (Beutler and Waalen, 2006) and all laboratory parameters are also in normal, which makes this disorder "diagnostically silent" and therefore it is practically impossible to indentify (Crichton, 2016).
(ii) iron-deficiency eryhtropoiesis - this state already involves an exhaustion of all iron stores, limiting the rate of hemoglobin synthesis and other Fe-dependent processes. Nonetheless, the hemoglobin levels remain in normal range for the general diagnosis (the changes are too small for detection). On the other hand, other laboratory parameters are detectably increased - serum ferritin and serum iron are decreased whether plasma levels of soluble transferrin receptor and serum transferrin (with decreased transferrin saturation) are increased (as cited in Crichton, 2016).
(iii) iron-deficiency anemia - initial hemoglobin levels remain high but they begin to decrease shortly to very low levels. Pathological morphology of erythroblasts and red blood cells occurs.
(iv) functional iron-deficiency - iron levels are not sufficient for matching the increase demand of the bone marrow.

2.4.2 Hereditary iron overload

The manifestation of iron overload is hereditary hemochromatosis (HH). The term hemochromatosis stands for more than one disease, but all are in some way linked with iron-overload. They are all (except ferroportin disease) autosomal recessive and affect prevalently people of european descent (Crichton, 2016).

The general problem lays in excessive uptake of dietary iron, more than can be used up by erythropoiesis, leading to its accumulation in parenchymal cells of various organs such as heart, liver, skin and gonads. This results in cardiomyopathy, cirrhosis, skin pigmentation, hypogonadism and other serous problems.

The organ damage is an inevitable consequence of rapid or gradual iron influx from duodenal entherocytes to plasma. Eventually, the amount of uptaken iron in cells exceeds the storage capacity of intracellular protein ferritin and the Fe ions remain unbound in the cytoplasm. The presence of free iron in cytosol leads to reactive oxygen species generation, damaging intracellular structures.

Cardiac and endocrine systems are the most easily affected tissues, probably due to its low inner antioxidant concentrations (as cited in Pietrangelo, 2010).

Iron uptake is normally down-regulated by iron homeostasis hormone hepcidin, suggesting that overloading is caused by malfunction of this protein or a defect in its interaction with ferroportin (Sham et al., 2005), the only known iron exporter.

Since the activation of hepcidin expression is regulated by numerous metabolic processes, the disorder can have and actually does have more than one cause. In human, there are mutations in HFE (Feder et al., 1996), HJV (Papanikolaou et al., 2004), TfR2 (as cited in Pietrangelo, 2010) and HAMP (Roetto et al., 2003) itself, and every one of these are linked with one type of hemochromatosis - HFE-related (type 1), juvenile

hemochromatosis (type 2), TfR-related HH (type 3) and ferroportin disease associated with FPN1 malfunction, that does not respond to or does not bind hepcidin.

In HH type 1 or 3, the influx of iron to plasma is rather slow but graduating, because HJV is able to compensate for this loss. Nevertheless, in HH type 2 the influx is rapid and massive (Pietrangelo, 2010).

The most common type, the HFE-related HH, that manifests in early adulthood, is explained predominantely as a C282Y mutation of *HFE* gene located on chromosome 6. It results in replacement of cysteine residue 282 to tyrosine. This substitution disrupts disulfide bond between two cysteine residues, resulting in unability of HFE to bind the light chain of β2m (β2-microglobulin) (Feder et al., 1996) and consecutive lower expression on the cell surface (as cited in Pietrangelo, 2010).

Interestingly, the C282Y homozygosity itself is not enough for full-blown hemochromatosis and represents only a predisposition for the disease. The penetration is often associated with environmental and lifestyle factors such as high alcohol consumption etc. whereas mutations in HJV or HAMP genes cause sever organ damage (Pietrangelo, 2010) even without such environmental factors.

2.5 Neurodegenerative diseases

As it was mentioned before, iron accumulation causes generation of highly reactive oxygen species. These harmful reagents were demonstrated to be present in several neurodegenerative diseases, namely Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease, multiple sclerosis, Friedrich's ataxia and others (as cited in Crichton et al., 2011).

ROS generation near phospholipidic cellular membranes may initiate the peroxidation of polyunsaturated acyl chains of phospholipids or n-6 polyunsaturated fatty acids. This leads to formation of 4-hydroxylnonenal (HNE) and 4-hydroxy-2-hexanal (HHE), where HNE is considered as a "product of phospholipid peroxidation with the greatest toxicological potential" and 4-HNE is believed to be one of the most important molecules in ROS mediated cellular damage (as cited in Crichton et al., 2011).

Results of ROS formation are predominantely protein carbonylation (reactive protein carbonyl derivatives generation), oxidation of aminoacid side chains and DNA lesions leading to mutagenesis and sometimes even to cellular death (as cited in Crichton et al., 2011).

AD, characterized by memory loss and behaviour changes, is accompanied by iron accumulation in brain centers of memory and thought processing - the hippocampus and cerebral cortex. The suggestions are, that increased iron deposition may be responsible for free-radical-related neurotoxicity (as cited in Crichton et al., 2011).

In PD, the iron accumulation is located in substantia nigra and lateral globus pallidus with which the PD's symptoms are associated. Due to abnormally low ferritin levels, harmful free iron is stocking in the cytosolic LIP and damaging the intracellular structures (as cited in Crichton et al., 2011).

Research of a cure exploiating this knowledge is currently in progress, using different types of chelators to remove the access iron from organism (as cited in Crichton et al., 2011).

3. Iron metabolism of a cancer cell

Because of elevated nutritient needs of rapidly proliferating cells, the iron metabolism is slightly different in cancer cells than in normal ones. Iron is an essential element for tumor cell growth (as cited in Richardson et al., 2009) and increase or decrease of its levels have important and rather interesting consequences on the cell life. Regarding these abnormalities in iron metabolism of cancer cells, iron is considered as an anti-tumor target in cancer therapy, using chelators as iron-depleting drugs.

3.1 Transferrin receptors

Due to the importance of iron uptake for tumor cells, the transferrin receptor 1, that mediates the interaction with Fe_2-Tf and influence the amount of received iron, is in these cells upregulated (Larrick and Cresswell, 1979). Nevertheless, it has been found out, that there is also some other way of transferrin-iron gain, probably by non-receptor mediated pinocytosis (Richardson and Baker, 1990), which makes the TfR1 less handy in anti-cancer therapy.

Together with TfR1 expression increase, the expression of the only known iron exporter is decreased (Brookes et al., 2006), suggesting that the proliferating cell is gathering all the iron it can for a further use. However, it is not used for storage purposes - ferritin's heavy chain synthesis is reduced by c-myc protein (as cited in Richardson et al., 2009), a proto-oncogene, activating TfR1 expression (O'Donnell et al., 2006), and regarding some studies, this protein is upregulated in cancer cells (as cited in Richardson et al., 2009) and also regulates the IRP2 expression (as cited in Richardson et al., 2009).

Although the transferrin receptor 1 seems to be a good target for anti-tumor treatment, it is not specific enough. Not only the cancer cells have increased expression of this molecule but also other rapidly proliferating cells of normal organismal system like the erythropoietic progenitors. The therapy targeted on this metabolism deflection would strike some of the normal cells as well. Studies have been carried out on this matter, demonstrating that the combination of anti-transferrin receptor antibodies and chelators, was 73% successful but some side effects of the treatment occured, such as increased risk of bacterial infection and tumors had eventually recurred (as cited in Buss et al., 2003)

The TfR2 receptor might present an alternative source of Fe for tumor cells considering that tumor cells with its expression proliferate faster than those ones with only TfR1 expression (Kawabata et al., 2000).

3.2 Ferritin

As has been mentioned above, Fe is not used to be stored, therefore the iron stores are not increased but there is an upregulation of serum ferritin (as cite in Richardson et al., 2009) - it is secreted by neoplastic cell lines for unknown reason. These cells are able to take up the ferritin by receptor-mediated endocytosis (Bretscher and Thomson, 1983) and utilize such iron as alternative source of iron for sustaining their proliferation.

3.3 Ribonucleotide reductase

Ribonucleotide reductase (RR) is an enzyme mediating de novo synthesis of deoxyribonucleotides. It reduces the ribonucleotides with its Fe cofactor, that is used to form tyrosyl radicals from an interaction with molecular oxygen (Kolberg et al., 2004).

The expression and activity is significantly increased in cancer cells to respond to the cell growth needs (Elford et al., 1970) as it is a rate-limiting step in DNA synthesis. Moreover, it is also responsible for DNA damage repair, when it involves the p53R2 subunit that supplies dNTPs for the repair (as cited in Richardson et al., 2009). This mechanism functions in p53 dependent manner but other studies suggest that it can be triggered on its own (Byun et al., 2002).

3.4 Metalloproteinases

In metastatic phase of tumor growth, cells secrete enzymes ensuring the movement of the cells through other tissues by degrading extracellular matrix and cell-associated proteins (as cited in Richardson et al., 2009). After a chelation treatment, it was discovered, that the metalloproteinase expression is reduced. Hence, it seems, that chelators could not only inhibit the primary tumor growth (as it is described in greater detail below), but also reduce the metastatic potential of cancer by making it impossible to translocate inside the organism (Richardson et al., 2009).

Additionaly, the metastatic cells have decreased cellular adhesion (Wijnhoven et al., 2000) due to mutations in adhesive proteins such as integrins, cadherins and immunoglobulins. But after a chelation, in some tumor cell lines, the expression of adhesive molecules has decreased. Nevertheless, the mechanism and potential consequences are still unknown and remain to be clarified.

3.5 Cell cycle

In all cells, the cell cycle normally consists of four phases - the G_1-, S- (synthesis), G_2- and M- (mitosis) phase. These involve checkpoints in which the cell must ensure that there are no mistakes in either DNA synthesis or protein expression. These checkpoints are in between the phases and in every one of them the cell decides whether the cycle will continue or the apoptosis pathway will be triggered.

The major cycle regulators are cyclins, cyclin dependent kinases (cdk), p53 and pRb where in every phase, the specialized cyclins and cdks form complexes regulating the cell cycle.

During the S- or M-phase, when a DNA damage occurs, p53 protein activates its downstream target p21 that inactivates cyclin D/cdk4/6 complex or E/cdk2 complex. This subsequently induces GADD45α (growth arrest and DNA-damage-inducible 45α) that stops the cycle.

Other cell cycle regulator is pRb that controls an entry into S-phase. Under normal circumstances, pRb is bound to the E2F1 transription factor. When the cyclin D concentration increases and the cdks4/6 are activated, it is hyper-phosphorylated by this complex and releases the trancription factor. It translocates to the nucleus and induces the S-phase genes expression.

It has been shown that iron-depletion significantly affects the expression of some cyclins (reduced expression of D1, D2 and D3 and increased expression of E (Gao and Richardson, 2001)) and cdks (reduced expression of cdk2 and cdk4 (Kulp et al., 1996)) (Gao and Richardson, 2001). Interestingly, in one case (cyclin D1), although the D1 protein levels were decreased, mRNA levels remained unchanged. This fact suggests that Fe-depletion influences the cyclin concentration on protein level and that it might induce proteasomal degradation (Nurtjahja-Tjendraputra et al., 2007).

Simultaneously, cyclin E levels are elevated after Fe-depletion (Gao and Richardson, 2001). This could suggest that the treatment with chelators does not have to be succesfull, but studies have shown that although the cyclin E is elevated, the cell stops the cell cycle after the depletion.

Additionally, while the Fe chelation downregulates D cyclins expression, the cyclin D/cdk4/6 complex-dependent pRb enzyme is hypo-phosphorylated which does not lead to E2F1 transcription factor release and therefore no S-genes are expressed.

All this(downregulation of cyclin D, hypo-phosphorylation of pRb, inactivation of RR etc.) have consequences in G_1/S and G_2/M arrest (Brodie et al., 1993).

3.6 Angiogenesis

As it has been mentioned before, during hypoxia, HIF-1α and HIF-1β form, in a nucleus, a unified protein, HIF-1. This acts as a transcription factor, associates with HRE (hypoxia response element) that induces the transcription of various genes involved in hypoxia response.

One of the results is an induction of angiogenesis, the formation of new capillaries that supply the cells with nutrients. This process is widely used by rapidly proliferating cells and the hypoxia state can be and is succesfully imitated by Fe decrease, due to the reguirements of prolyl hydroxylase that induce the HIF-1α ubiquitination (Callapina et al., 2005).

3.7 Chelators

Substances, known as chelators, are characterized by their property to bind metal ions. Due to the significant iron metabolism deregulations in cancer cells, chelators, such as DFO (desferrioxamin), deferriprone, thiosemicarbazones and others, have attracted the attention because of their anti-tumor effects (as cited in Merlot et al., 2013). These are attributed to iron-depletion and consequently inhibition of RR, redox activity, apoptosis and cell-cycle modulation (Merlot et al., 2013).

3.7.1 Desferrioxamin

The first chelator used for iron-overload disorder treatment, DFO, is a natural substance produced by bacterium *Streptomyces pilosus* (as cited in Merlot et al., 2013). It binds iron in 1:1 ratio to its oxygen atom. This complex is very stable and prevents the free cytosolic iron from reacting with molecular oxygen or H_2O_2 in Fenton reaction and therefore prevents generation of ROS (Kalinowski and Richardson, 2005).

Although DFO has shown to be efficient in induction of iron-depleted state (it decreases DNA synthesis (Simonart et al., 2002), triggers TfR1 expression and decreases ferritin levels (Brodie et al., 1993), downregulates cyclin D1 (Nurtjahja-Tjendraputra et al., 2007), upregulates p53 and causes cell cycle arrest at $5\,G_0/G_1$ phase (Simonart et al., 2002)), there are some uncomfortable limitations of its use. Due to its hydrophilicity, it is not very membrane permeable and thus the oral administration is not sufficient. It must be used subcutaneously.

Another problem is the molecule's short half-life ($t_{1/2}$ = 5 - 10 min), which leads to low efficacy. For this reason, new iron chelators have been synthesised.

3.7.2 Deferiprone

This syntheticaly made chelator, 1,2-dimethyl-3-hydroxypyrid-4-one, used in treatment of iron-overloade diseases (as cited in Merlot et al., 2013) has a high affinity for iron(III), but alluminum (III) and copper(II) ions act as a competitive inhibitors of iron chelation (as cited in Merlot et al., 2013). Metal is bound to the chelation molecule by carbonyl and hydroxyl oxygen (as cited in Merlot et al., 2013) and in case of micromolar concentrations, it bounds Fe(III) in 3:1 ratio (defferiprone:iron) (Motekaitis and Martell, 1991).

Nevertheless, when the concentration drops, these complexs dissociare, forming 2:1 and 1:1 deferriprone:iron molecules, where iron s not protected from ROS generating reactions and therefore harmfull radicals may be formed (Cragg et al., 1998).

Interestingly, it seems that it is really important whether the complexes are in the former or latter ration because it can change its anti/pro oxidant behaviour (Merlot et al., 2013).

However, this drug was shown to have several side effects (Ceci et al., 2002) - its toxicity is explained by necessary high dosage (otherwise it is rapidly metabolised (as cited in Merlot et al., 2013)) and ROS generation.

3.8 Pyridoxal isonicotinoyl hydrazone (PIH)

This synthetic chelator represents a newer alternative to DFO. It proved to be more efficient, due to the option of oral administration, also more convenient. The difference is in lipophilic nature of PIH that makes it more membrane permeable and therefore orally active (as cited in Merlot et al., 2013).

It has high affinity for ferric iron which it binds, in 2:1 ratio (PIH:iron), to its imine nitrogen, phenoliyc oxygen and carbonyl oxygen (as cited in Merlot et al., 2013). On top of that, it was demonstrated, that PIH has also low affinity for other metal ions such as Mg(II), Zn(II) and Ca(II) (Richardson et al., 1989).

PIH also posses anti-oxidative properties (Brittenham et al., 1994)and protects DNA against oxidative stress (Hermes-Lima et al., 1998) which can be explained as a result

of depletion of reactive iron. Nonetheless, it shows lower anti-proliferative activity than DFO and thus has been found not suitable for cancer therapy (Richardson et al., 1995).

3.8.1 Thiosemicarbazones

This newly synthesised group of chelators was developed in response to the latest anti-tumor research requirements. It posesses a high affinity for several metal ions such as iron(II), copper(II), cobalt(II), galium(III), zinc(II) and manganese(II) (as cited in Merlot et al., 2013) that are subsequently boung trough a sulfur atom, forming complexes in a 2:1 manner (Yu et al., 2009).

Unlike othe anti-cancer chelators, thiosemicarbazones target prevalently ribonucleotide reductase enzyme (Agrawal et al., 1974); however, the exact mechanism remains elusive.

There are few speculations - according to some studies, thiosemicarbazones bind to iron atom that is a part of RR's active R2 subunit and thus inhibiting its activity (Sartorelli et al., 1970). Controversely, other studies are suggesting that the inactivation is caused by iron-deprivation due to chelating effects of thiosemicarbazones (Cooper et al., 1996).

Nevertheless, a complex of this chelator and iron have been found to act as more effective RR inhibitor than chelator alone, denoting alternative explanation of the inhibition mechanism (Shao et al., 2006).

There are some proposals mentioning a possibility that thiosemicarbazone-iron complexes are causing ROS generation that further influence RR's activity or they (ROS) interact with some thiol-related anti-oxidant intermediate that later communicate with RR (Yu et al., 2011).

One of the most effective chelators of this type is Dp44mT (di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone). It was shown to effectively inhibit cellular growth in some cancer cells and simultaneously not to endanger normally developing cells (Merlot et al., 2013). One of the very promising features of this molecule is the fact that it is "pumped" into lysosomes by the ABC transporters and disrupts them. This feature has been experimentally used in the combined treatment where Dmp44T has been delivered together with doxorubicin and such combination proves to be very efficient due to lysosomal destabilization and doxorubicin release. Such compound thus represents a very promising novel anti-cancer agents that are entering clinical trials (Yuan et al., 2004).

4. Conclusion

Cancer is one of the most studied phenomena on the world, resulting in rapid increase of knowledge of this matter.

Although, the research proceeds fast, there is still no satisfactory remedy or a treatment to cure this worldwide occuring disease, causing thousands of deaths a year (American Cancer Society, Inc.).

In this thesis, based on current literature, I have documented that cancer cells show marked difference in their iron metabolism, show enhanced iron uptake and similarly reduced iron efflux. Due to their high iron needs, they are sensitive to iron chelation which results in apoptosis induction. Especially in the cancer of hematopoietic origin several possible anti-tumor treatment targets have been introduced. All of them are focused on iron-depletion, specific for cancer cells, using their increased expression of some iron-related proteins, such as metalloproteinases, ribonucleotide reductase or transferrin receptors. Novel approaches such as synthesis of novel chelators with higher affinity and higher specificity toward cancer cells have been initialized. Also, combining newly developed chelators with other anti-cancer drugs appears to be a viable option that might be utilized in anti-cancer treatment in future.

Furthermore, some studies suggest that gene signature of genes related to iron metabolism could be used for prediction of responders and non-responders in breast cancer which may be of clinical importance as well (Torti and Trusolino, 2011). This is also supported by the data obtained in the laboratory of tumour resistance that suggest that the iron metabolism in cancer stem cells, which are seen as stems of cancer resistance, is markedly altered and warrants further research in this area.

A possible continuation of this work in master's dissertation will be denoted to regulation of human protein matriptase 2 that interacts with HJV molecule and downregulates the hepcidin expression and possibly to the continuation of the research focused on the iron metabolism in cancer stem cells.

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