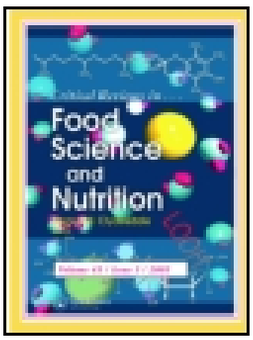

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New perspectives on the regulation of iron absorption via cellular zinc concentrations in humans

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INTRODUCTION

Iron (Fe) and zinc (Zn) are essential trace elements for humans. Together, they play a major role in a wide variety of cellular functions, some of which are proliferation and differentiation, development and maintenance, oxidative metabolism, etc. Systemic Fe and Zn homeostasis is based on tightly coordinated processes and effective communication between the key sites of Fe and Zn uptake, utilization and storage. The enterocytes and the hepatocytes perform the most important roles in homeostasis. Considering the similarities in ionic properties between divalent ferrous Fe and divalent Zn, it is not unexpected that these trace metals influence the transport and absorption of one another across the enterocytes and hepatocytes.

For long, Fe and Zn absorption has been seen as competitive, where Zn had inhibitory effects on Fe absorption and vice versa (Solomons, 1986). However, while high doses of soluble forms of Fe and Zn are capable to produce this negative interaction, the probability of antagonistic interactions appears to be much lower when Zn and Fe intake are closer to 'physiological' concentrations and when they are provided with food (Lonnerdal, 2000; Lopez deRomana et al., 2005; Esamai et al., 2014). The summary review of Fischer-Walker et al. (2005) was one of the first reviews on this topic that provided considerable support for non-competitive absorption between Fe and Zn. Over the years, more and more studies demonstrated evidence suggestive of positive interactions between Fe and Zn in absorption (Smith et al. 1999; Penny et al., 2004; Hininger-Favier et al., 2007; Harvey et al., 2007; Chang et al., 2010). Additionally, a number of data sets have clearly showed a positive correlation between anemia and signs of the risk of Zn

deficiency in adult males, children, and pregnant women (Ece et al., 1997; Ma et al., 2004; Gebremedhin et al., 2014). Zn is shown to be a strong predictor of hemoglobin concentrations (Smith et al., 1999; Gibson et al., 2008; Shamim et al., 2013). Moreover, Fe supplementation, by itself, was not always effective in treatment of anemia (Palupi et al., 1997; Beaton and McCabe 1999; Allen et al., 2000; Neto et al., 2013). Positive effect of provision of Zn in addition to Fe has been proven by Kolsteren et al., 1999; Alarcon et al., 2004; Nishiyama et al., 1996; Nishiyama, 1999 and many others (Shoham and Youdim 2002; Ramakrishnan et al. 2004; Hossain et al., 2011; Kaluza et al., 2014).

Although, there is more and more evidence accumulating showing the positive link between Fe and Zn and a strong positive influence of Zn on Fe absorption and Fe status, there is still a lack of information on the precise mechanisms of Zn involvement in the Fe absorption processes. Therefore, this paper presents a comprehensive overview on the structure and roles of major Fe and Zn transport proteins aimed at clarifying Fe and Zn interactions at these sites, and providing a model of potential mechanism of Zn impact on Fe absorption processes both at the local and systemic level.

1. IRON AND ZINC TRANSPORTERS IN THE HUMAN BODY

1.1. Iron transporters in human enterocytes and hepatocytes

Iron is essential for every living organism. It is a key component of oxygen-carrying proteins, has a pivotal role in cellular metabolism and is essential to cell growth and differentiation.

Intestinal Fe absorption involves a series of proteins including duodenal cytochrome b reductase (Dcytb), divalent metal transporter (DMT1), hephaestin (Heph), and ferroportin 1 (FPN1) (Figure 2).

1.1.1. Divalent Metal Transporter 1 (DMT1) – iron import protein

DMT1 (Divalent Metal Transporter 1), also known as Nramp2 (natural resistance-associated macrophage protein) or Dct1 (divalent cation transporter), is a proton-coupled metal-ion transporter that is commonly expressed in enterocytes. DMT1 is a 12-transmembrane domain protein that transports several divalent metals but operates mostly as a co-transporter of protons and ferrous Fe in the proximal duodenum.

DMT1 is encoded by the mammalian *Solute carrier family 11, member 2* gene (SLC11A2) which gives rise to 4 variant messenger RNA (mRNA) transcripts that differ in their tissue distribution and regulation 1A/+IRE, 1A/-IRE, 2/+IRE and 2/-IRE (Figure 1) (Mackenzie et al., 2007). All four isoforms of human DMT1 function as metal-ion transporters of equivalent efficiency (Garrick et al., 2006). They all transport Fe^{2+} at the same turnover rate and exhibit no differences in their functional properties, permeate ions or rate limiting steps. Similarly, N and C-terminal sequence variations among the DMT1 isoforms do not alter DMT1 functional properties (Mackenzie et al., 2007). Therefore, all of the hDMT1 isoforms are equally efficient in terms of the rate at which they transport iron. It is interesting that although Zn^{2+} is a ligand for both the 1A and 1B isoforms of hDMT1, it is only poorly transported, if at all, relative to Fe^{2+} forms (Mackenzie et al., 2007). DMT1 forms the most stable substance binding with Fe2+. The

historic belief that Fe and Zn compete for absorption at the DMT1 has been proven false. A large number of studies over the years confirmed that Zn2+ is not transported by DMT1 (Table 1). More importantly, Zn has been reported to be a chief element in the regulation of DMT1 gene expression (Table 1).

One explanation for the way Zn affects DMT1 expression is that the promoter region of DMT1contains several metal response elements suggesting that Zn exposure can positively regulate DMT1 mRNA level via metal transcription factor-1 activation (Sacher, 2001; de Benoist et al., 2007) or perhaps through other Zn-dependent transcription factors, such as peroxisome proliferator activated receptor-g, nuclear factor-kB, or activator protein-1 (Meerarani et al., 2003).

1.1.2. Ferroportin (FPN1) – iron export protein

Ferroportin (FPN1), also known as solute carrier family 40 member 1 (SLC40A1) or iron-regulated transporter (IREG1) is a multipass transmembrane protein composed of 571 amino acid residue found on the basolateral membrane of enterocytes (Pietrangelo, 2004). It is present in various forms from 9 -12 trans-membrane domains (Ward and Kaplan, 2009), with the N-terminus present inside the cells (Figure 1).

It is the only known mammalian Fe export protein that is responsible for the movement of Fe from the enterocytes into the circulation. Deletion of FPN1 in intestinal cells in mice results in a near complete block of intestinal Fe absorption and a consequent accumulation of Fe in intestinal enterocytes (Donovan et al., 2005). FPN1 transports Fe in the ferrous form, while plasma

transferrin (Tf) only binds Fe in the ferric form, so ferroxidases (hephaestin) are needed to oxidize ferrous Fe to ferric Fe for transport by FPN1. Without the activity of a hephaestin, FPN1 is internalized and degraded (Fuqua et al., 2012).

The regulation of FPN1 expression is still incompletely understood, but is believed to be mainly controlled at the post-translational level, by hepcidin-mediated internalization and degradation. Following hepcidin binding to FPN1, FPN1 is internalized, phosphorylated and subsequently degraded by lysosomes (McKie et al., 2000; Nemeth et al., 2006; Knutson et al., 2009; Chiabrando et al., 2013). When hepcidin concentration is low, the rate of FPN1 synthesis is greater than its degradation, resulting in higher amounts of FPN1 at the cell surface, thus increasing Fe export. In contrast, high hepcidin levels lead to FPN1 degradation and consequently to Fe retention (Chiabrando et al., 2013).

However, FPN1 can also be regulated by other mechanisms (Zhang et al., 2009), via translational regulation by the iron-regulatory element/iron-regulatory protein (IRE/IRP) system (McKie et al., 2008; Abboud and Haile, 2000; Lymboussaki et al., 2003) and transcriptional regulation in the duodenal mucosa and macrophages (McKie et al., 2000; Knutson et al., 2003).

Importantly, over the years, it has been shown that FPN1 levels are increased when cells are exposed to Zn (Yamaji et al., 2001; Yeh et al., 2004; Kelleher and Lonnerdal, 2006; Iyengar et al., 2009; Aydemir et al., 2009; Iyengar et al., 2012). Zn induces FPN1 transcription through the action of Metal Transcription Factor1 (MTF1). In 2010, Troadec and colleagues showed that Zn leads to MTF1 binding to the FPN1 promoter, while Fe does not. Recently, hypoxia inducible factor HIF2α has been shown to regulate FPN1 expression in intestinal cells (Taylor et al., 2011).

HIF 2α was responsible for adaptive increase of intestinal FPN1 during Fe deficiency in mice (Taylor et al., 2011).

1.1.3. Dyodenal cytochrome b (Dcytb) and Hephaestin (Hp)

Duodenal cytochrome b (Dcytb) is a plasma membrane protein localized predominantly on the duodenal brush-border membrane. It has ferric reductase activity and plays a physiological role in dietary Fe absorption. Dcytb is a member of the cytochrome b561 family, made of 286 amino acids with six trans-membrane domains. Dcytb mRNA and protein are believed to be rapidly induced in response to Fe deficiency and hypoxia, indicating a key role of this molecule in Fe metabolism (McKei, 2008).

Hephaestin (Hp) is a transmembrane bound ceruloplasmin homologue that functions as ferroxidase (McKie et al., 2000). Hp ferroxidase activity is necessary for effective release of Fe following transport through the basolateral membrane by the Fe transporter FPN1.

There are findings which demonstrate that the duodenal ferroreductase Dcytb and ferroxidase Hp mRNA expression are not significantly altered by variations in Fe homeostasis and are not affected by Fe status (Sturt et al., 2003; Frazer et al., 2003; Zoller et al.2003; Gunshin et al., 2005).

Similarly, it has been demonstrated that Dcytb is most highly up-regulated by hypoxia in the duodenum, through the activity of HIF-2 (Mastrogiannaki et al., 2009; Shah et al., 2009). It is still not completely clarified whether or not duodenal ferric reductase and oxidase mRNA expression have a pivotal role to play in Fe metabolism (Gleeson et al., 2005; Dada et al., 2008).

In addition, it has been demonstrated that hephaestin protein stability is sensitively regulated through polyubiquitination and proteosomal degradation, a process that is prompted by Zn exposure (Wu et al., 2003; Nittis and Gitlin, 2004). Increased hephaestin levels are demonstrated in Zn supplemented pups after weaning (Kelleher and Lonnerdal, 2006). Although, there is still no conclusive evidence about the effect of Zn on Dcytb or hephaestin activity some initial findings show that Zn may have a role in determining stability and function of these proteins.

1.2. Zinc transporters in human enterocytes and hepatocytes

Zinc (Zn) is an important trace element for many biological functions. It is present in over 300 enzymes as a catalytic metal and in at least 3000 proteins as a structural metal (King et al., 2006). Zn is also an important regulator of enzyme activity; acting as an activator or inhibitor ion. These pleiotropic actions require very tight regulation. Zn homeostasis in humans is achieved through a balance between intestinal absorption and excretion involving adaptive mechanisms programmed by levels of dietary Zn (King et al., 2010; Ou et al., 2013).

There are 24 Zn transporters in various parts of the human body that handle uptake, efflux and intracellular trafficking (Thambiayya, 2012). Half of these proteins are expressed in the enterocytes or enterocyte-like cell lines (Cousins, 2010).

There are two families of Zn transporters: ZnT and Zip members. The ZnT family of transporters is responsible for decreasing intracellular Zn levels by transporting Zn from the cytoplasm to the lumen of organelles or to the extracellular space (Lichten and Cousins, 2009). The ZIP family is responsible for increasing intracellular Zn levels by either transporting the metal from the

extracellular space or the organellar lumen into the cytoplasm (Mocchegiani, 2012). Intestinal concentrations of Zn are regulated by the activity of Zn transporters (mainly Zip4 Zip5, ZnT1 and ZnT5) and Zn binding proteins such as metallothioneins (MTs) (Figure 2) (Mocchegiani, 2012). The expression and function of these proteins act in response to a variety of physiological stimuli and/or dietary conditions, while others appear to be constantly expressed (Aydemir et al., 2012).

The function of most of the Zn transporters is primarily transcription regulated by the metal responsive element binding transcription factor 1 (MTF1) (Lichten and Cousins 2009; Iyengar et al., 2012).

1.2.1. Zip 4 - The most dominant zinc import protein in human enterocytes

The Zip 4 (SLC39A4) protein is the main ZIP family transporter responsible for the uptake of dietary Zn into intestinal enterocytes. It is localized at the apical plasma membrane of enterocytes. This transporter has eight trans-membrane (TM) domains; most loops between TM domains are very short; a longer loop region is frequently found between TM domains three and four (Figure 1). This longer region often contains a histidine-rich domain with the sequence (HX) n where n ranges from 3 to 5.

The function of this domain is not clear (Eide, 2004). Transmembrane domains four and five are particularly amphipathic and contain conserved histidine residues frequently with adjacent polar or charged amino acids. Given their sequence conservation and amphipathic nature, TM four and

five are predicted to line a cavity in the transporter through which the substrate passes and as such these regions are essential for function (Eide, 2004).

Zip4 localization and expression are thought to be the primary regulators of intestinal Zn absorption in humans (Jou et al., 2010). This transporter is the only Zn transporter that adequately compensates for the Zn deficiency in acrodermatitis enteropathica that result from hereditary defects in Zip4 (Illing et al., 2012). Zip4 is expressed in the stomach, small intestine, colon and most likely in the cecum (Liuzzi et al., 2004; Dufner-Beattie et al., 2004; Cousins, 2010).

Expression of Zip4 appears to be regulated by both transcriptional and post-transcriptional mechanisms in response to Zn availability (Table 2). The abundance of Zip4 mRNA, cellular localization and turnover of this protein are regulated by Zn availability in the intestine. The transcription factor Kr uppel-like factor 4 (KLF4) is an important component of the mechanism responsible for the transcriptional up-regulation of Zip4 (Cousins et al., 2000; Cousins, 2010). Important to note is that transcription factors are also regulating the expression of proteins involved in Fe homeostasis (Shah et al., 2009).

1.2.2. ZnT1 – The main Zn export protein

ZnT1 (SLC30A1) is the first Zn transporter discovered. It is the main transporter controlling cellular Zn efflux. The transporter is most highly expressed in tissues involved in Zn acquisition, recycling, or transfer, such as the small intestine (Liuzzi et al., 2003).

ZnT1 is increasingly abundant along basolateral membranes of enterocytes where it participates in Zn transfer into the circulation (McMahon and Cousins, 1998). ZnT1 expression can be influenced differentially by the dietary Zn supply (Table 2). In addition, it was also shown that ZnT1 regulation is under the control of the transcription factor MTF-1, which is Zn-responsive (Lichten and Cousins, 2009; Langmade et al., 2010).

1.2.3. Zip 14 – the main Zn import protein in human hepatocytes

Zip 14 (SLC39A14) is a part of LZT subfamily members, distinguished from other ZIP transporters by their consensus sequence HEXPHEXGD in TMD V (Liuzzi et al., 2006). Amino acid sequence analysis of human Zip14 revealed a slightly altered motif, EEXPHEXGD in TMD V. Multiple studies and tissue array data show that Zip 14 is mainly expressed in the liver, though also found in the pancreas and heart (Girijashanker et al., 2008).

In addition to Zn, the Zip 14 was identified as a protein capable of transporting Fe (Luizzi et al., 2006; Pinilla-Tenas et al., 2011). DMT1 transports Fe optimally at low pH 5.2-5.5 (Mackenzie and Takanaga, 2007) while Zip14 exhibits maximal Fe transport at pH 7.5 (Pinilla-Tenas et al., 2011), making it suitable for Fe uptake from the plasma, such as from non-transferrin-bound Fe (NTBI) during Fe overload. In addition, it has been demonstrated that Zip14 is found at the apical and basolateral membrane of enterocytes (Liuzzi et al., 2006; Cousins, 2010).

1.2.3.1. The important role of Zip14 in inflammation

Inflammation is initiated by pro-inflammatory cytokines that have powerful effects on nutrient metabolism and function. During the acute phase response the liver prioritizes nutrient flows toward production of acute phase proteins (Gifford et al., 2012). Trace elements are among those nutrients that exhibit atypical metabolic profiles during inflammation and infectious episodes (King and Cousins, 2006).

Using a global screening approach for the ZnT and Zip transporter genes it was shown that Zip14 was the gene most profoundly up-regulated by these pro-inflammatory conditions (Aydemir et al., 2012). Northern analysis showed that Zip14 up-regulation was specific for the liver (Liuzzi et al., 2005). Abundant Zip14 mRNA was also found in duodenum and jejunum (Liuzzi et al.,2006).

Induction of Zip14 leads to enhanced expression of Zip14 in the hepatic plasma membrane and is associated with increased Zn transport into hepatocytes via interleukin 6 (IL-6) and other mediators (Figure 3.) Under these conditions, serum Zn levels drop and liver levels rise in a likely effort to withhold this essential metal nutrient from the invading pathogen (Eide, 2006). This suggests that induction of Zip14 by IL-6 is responsible for the serum hypozincemia associated with infection. The binding sites of metallothioneins (MT), particularly those that comprise the N-terminal (β-cluster) metal- binding domain of the protein (Zangger et al., 2001) are the ultimate recipients of Zn ions lost from the plasma pool during stress and inflammation. During infections, multiple cytokines may contribute to hepcidin regulation (Liuzzi et al., 2005). The inflammation produces increases in IL-6 signal, which transduction requires STAT3 that is activated by Jak-kinase 1 (JAK) to up-regulate Type II acute-phase genes (Liuzzi et al., 2005). In addition, hepcidin is upregulated by lipopolysaccharide (LPS) (Lee and Beutler, 2009). LPS

stimulated cytokines such as tumor necrosis factor (TNFα), interleukins IL-1α, as well as IL-1β, which, sequentially, stimulate IL-6 production (Weston et al., 2009).

Hypoferremia is also associated with inflammation and infection. Mechanisms that result in reduced serum Fe in response to both acute and chronic stimuli focus on the regulatory peptide hepcidin. Hepcidin controls Fe levels via regulation of the FPN1 through a mechanism that regulates degradation of the transporter. However, there may well be an additional mechanism responsible for low Fe levels. A decreased concentration of Zn in enterocytes caused by reduced expression of Zip4 and consequentially low expression of DMT1 and FPN1 transporters could also contribute to reduced plasma Fe concentrations (see discussion below).

1.2.4. Zip8 (SLC39A8)

ZIP8 was first identified in 2002 in a screen of monocyte cDNAs induced by infection and inflammation (Begum et al., 2002). This protein is mainly expressed in the lung, kidney, testis, liver, brain and small intestine (Lichten and Cousins, 2009).

Among the 14 mammalian ZIP family members, ZIP8 is most closely related to ZIP14 (Jenkitkasemwong et al., 2012). Mouse ZIP14 and ZIP8 are similar in length (489 *versus* 462 amino acids), ~50% of their amino acids are identical, and they each contain a long extracellular N-terminal region with multiple potential glycosylation sites. Notably, Zip14 and ZIP8 are 90% identical (Wang et al., 2012).

Wang et al. in 2012 demonstrated that Zip8 can transport Fe at physiologic pH. Zip8 is expressed at the cell surface, and mediates the uptake of Fe from ferric citrate, the predominant form of non-transferrin bound iron (NTBI) in the plasma of individuals with Fe overload.

The data from the H4IIE hepatoma cells are showing that Fe loading increases plasma membrane levels of Zip8 which suggests that similarly to Zip14, Zip8 can also be recruited to the cell surface to enhance the uptake of NTBI. The up-regulation of the Zn/Fe importer Zip8 in response to Fe loading may partially account for hepatic Zn accumulation that occurs during Fe overload (Wang et al., 2012). Notably, the Fe transport activity of Zip8 decreases with decreasing pH. At pH 6.5, the pH by which~50% of the Fe dissociates from transferrin-transferrin receptor complex in endosomes Zip 8 shows the highest transport activity, while at pH 5.5, Zip8 shows no Fe transport activity.

1.2.4.1. The key role of Zip 8 in Fe transport across the placenta

It is still unknown how Fe is transported across the placenta to the developing fetus (McArdle et al. 2011). What is known is that DMT1 and Zip14 are not required for materno-fetal Fe transfer because DMT1 and Zip14 knock-out mice are born alive with adequate amounts of Fe (Gunshin et al., 2005). These observations leave ZIP8 as the only remaining known Fe transport protein that could function in the placenta.

The uptake of NTBI is increased during the Fe loading of hepatocytes (Scheiber-Mojdehkar et al., 2003, Richardson et al.,1999). The up-regulation of cell-surface ZIP8 by Fe loading suggests that ZIP8 may contribute to NTBI uptake (Wang et al., 2012).

Recently, it was shown that ZIP8 hypomorphic mice (which carries a neomycin resistance gene in intron 3 of Zip8) exhibit severe anemia and embryos and neonates and do not survive more than 48 h after birth (Galvez-Peralta et al., 2012).

The anemia appears to result from Fe deficiency as indicated by low serum Fe, total Fe binding capacity, and low hepatic Fe. The Fe-deficient phenotype of the hypomorphic mice strongly suggests that ZIP8 plays a direct physiologic role in Fe metabolism.

What is interesting is that Zip8 mRNA levels do not change according to Fe status in rat liver; and are not affected by Fe loading, whereas ZIP8 protein levels increase, which suggest a post transcriptional regulation of this transporter (Nam and Knudson, 2012).

1.2.5. Fe and Zn transporters - cellular location and tissue-specific expression

Iron and zinc transporters are expressed all over the intestinal tract. DMT1 and FPN1 transporters are most highly expressed in the proximal duodenum, but they are also found in the distal parts and colon, although with a much lower expression rates (Table 2). Zn transporters are produced in the epithelium lining of the entire gastrointestinal tract, so it is likely that some Zn absorption occurs in the entire intestinal tract (Cousin, 2010). Zip 14 is mainly expressed in the liver but it is also found in the duodenum and jejunum (Litchten and Cousins, 2009). Similarly,

DMT1 and FPN1 are expressed in the liver cells. As previously discussed, besides being regulated by other mechanisms (hepcidin, hypoxia, inflammation, hormones) major Fe and Zn import and export transporters are all responsive to cellular Zn concentrations.

1.2.6. The role of pancreas in Zn homeostasis

The pancreas is well-known as a site of high Zn turnover, with pancreatic acinar cells having much higher Zn turnover rates than islet tissue (Langmade, 2000). Pancreatic release of Zn by acinar cells is through the secretory process at the apical membrane and involves transporters ZnT2 and ZnT1, respectively (Cousins, 2000). Under normal dietary conditions, 1–2 mg/d Zn enters the digestive tract via zymogen granules secreted from pancreatic acinar cells (Hambidge, 2010). These zymogen granules contain enzymes necessary for digestion and their activity is Zn dependent (Vehik & Dabelea, 2011). Enterocytes and acinar cells constitutively express Zip5 at the basolateral membrane, which serve as a monitor of Zn status and play a role in Zn import into the cell.

Dietary Zn restriction significantly decreased the Zn concentration over 50% in both pancreatic cell cytoplasm and in zymogen granules and was correlated with decreased expression of ZnT1 and ZnT2 (Liang et al., 2010). These two transporters function closely in acinar cell Zn secretion and produce an important component of the entero-pancreatic Zn circulation.

Up-regulation of Zip4 in enterocytes and concurrent down-regulation of ZnT1 and ZnT2 in pancreatic acinar cells when dietary Zn intake is low are the key factors that may balance the

intestinal intake with endogenous loss via pancreatic secretions (Liuzzi et al., 2004).

The pancreas has the potential to act as a key component of Zn homeostasis in humans, which may also mean that the pancreatic Zn is also a major determinant of Fe absorption, however this requires further investigation, as the molecular mechanisms, pathways, and the transporters for pancreatic Zn secretion and their role in Fe absorption are still not entirely clear.

2. THE MECHANISMS OF LOCAL AND SYSTEMIC REGULATION OF IRON ABSORPTION UNDER PHYSIOLOGICAL AND PATHOLOGICAL CONDITIONS. THE IMPERATIVE ROLE OF ZINC IN THESE PROCESSES

2.1. The effect of iron/zinc depletion/supplementation on the expression of iron transporters and their storage proteins

The most recently published study that looked at Fe and Zn interaction at the transporter level during Fe and/or Zn depletion/ supplementation conditions was completed by Iyengar et al. in 2012. The authors used Caco-2 cells, an established model of absorptive enterocytes, to investigate Fe/Zn interaction; they reported the following:

Apical DMT1 expression increased upon Zn supplementation while Fe supplementation did not have any significant effect on the localization of DMT1. Depletion of Fe and Zn leads to movement of DMT1 away from the apical membrane and accumulation towards the center of the cell. Expression of FPN1 increased upon supplementation with Fe or Zn and upon depletion of Zn. These findings suggest that Zn supplemented cells not only take up more Fe but also transfer the excess Fe out of the cell. Total cellular IRP1 protein expression did not change upon supplementation or depletion of Fe and/or Zn (Iyengar et al., 2012). The RNA bound form decreased upon Fe supplementation while notably increased upon Zn supplementation.

An interesting finding from this study is that Zn supplementation increased both the RNA bound form of IRP1 and IRP2 expression, resulting in increased DMT1, which suggests that the observed changes in DMT1 expression upon Zn supplementation may be under the control of the IRP/IRE system (Iyengar et al., 2012).

Over the years, the evidence for increased expression of DMT1 during Zn supplementation was also provided by Yamaji et al. (2001), Leong et al. (2003), Kelleher and Lonnerdal (2006) and many others (see Table 1).

Comparable results were reported for the activity of basolateral transporter, the expression of FPN1 increased in Zn treated cells (Yamaji et al., 2001; Yeh et al., 2004; Kelleher and Lonnerdal,2006; Iyengar et al., 2009; Aydemir et al., 2009).

However, inconsistencies exist regarding the mechanism that controls the activity of Fe transporters in the presence of Zn. Iyengar et al. (2012), show some evidence for the involvement of the IRE/IRP system in the control of DMT1. Others believe that the effect is post translational and that the IRE/IRP system is unlikely to play a role in the regulation (Sharp et al., 2002; Frazer

et al., 2003). Transcriptional regulation of both Fe transporters has also been demonstrated (Yamaji et al., 2001, Zoller et al., 2001; McKie et al., 2000; Knutson et al., 2003). It is tempting to postulate that the activity of various mechanisms involved in the regulation of Fe absorption is coordinated by a common component. We believe that cellular Zn concentrations, in addition to intracellular Fe levels, may be the key element that controls and modulate the function of the entire Fe absorption machinery (see later sections).

2.2. The effect of iron/zinc depletion/supplementation on the expression of zinc transporters

Zinc absorption is influenced by dietary Zn intake, not Zn status (King, 2010, Ou et al., 2013). Expression of Zip4 appears to be regulated by both transcriptional and post-transcriptional mechanisms in response to Zn availability (Kim et al., 2004; Weaver, 2007; Andrews, 2008). In addition, Iyengar et al. (2012) reported that Zip14 transcript level decreased with Zn supplementation, with no change in Zip1 transcript levels. Supplementation with Fe and Zn significantly increased ZnT1 expression; the increase with Zn supplementation was higher when compared with Fe supplementation. No significant changes in ZnT1 localization (cell height at maximum amplitude) were observed except for diffuse punctuate fluorescence upon Zn supplementation. Similarly, Zn supplementation resulted in a marked increase in discrete punctate ZnT4 fluorescence (Iyengar et al., 2012).

2.3. The transporter models of iron and Zn uptake

Diverse models have been proposed for Fe and Zn absorption. The uptake of Fe is dependent on a two component model while a three compartment model is suggested for Zn uptake (Iyengar et al., 2009). There are two transporters capable of transporting Fe, one of which is the well-known DMT1 transporter.

Based on the Ki (the inhibition constant) value, which indicates high affinity for Zn, the second Fe transporter is a Zn influx transporter (most likely it is Zip14 that is able to transport NTBI along with Zn) (Iyengar et al., 2009). In the presence of concomitant Zn, the second Fe transporter is less capable to transport Fe, thus halving Fe uptake. Upon Zn pre-treatment the second transporter becomes non-functional. The Zn absorption model contains three compartments. Component I of this model represents paracellular uptake that is diffusion limited, component 2 characterizes Zn influx (most likely Zip4 transporter) and component 3 is an efflux transporter (probably ZnT1).

The gastrointestinal tract is the major site for regulation of Zn homeostasis. Unlike Fe, Zn absorption is influenced by dietary Zn intake, not Zn status (King et al., 2006). Fractional Zn absorption is inversely related to dietary Zn intake, whereas intestinal absorptive capacity is high at low levels of Zn intake.

During periods of higher levels of Zn intake, there is a decrease in absorptive efficiency but an increase in the total amount of Zn absorbed. At intakes below 9 mg/day, Zn absorption occurs primarily by a saturable process involving ZIP4, ZnT1, and other transporters. There is no evidence that past Zn intakes, or status, influence Zn absorption. Instead, current Zn intake is the chief determinant of Zn absorption (King et al., 2010). However, an upper limit exists where increasing Zn intake >20mg in solution does not result in greater Zn absorption (Jou et al., 2010).

The Fe absorption is also saturable with a limited number of carriers mediating uptake; it is twofold greater at pH 5.5 than at pH 7.4 (Bannon et al., 2003).

2.4. The mechanism of local regulation of intestinal iron absorption

Besides systemic regulation (via hepcidin) Fe absorption is also regulated at the local level, in intestinal enterocytes. The Fe responsive element (IRE)/iron regulatory protein (IRP) system is the major player in this regulation, which affects the post-transcriptional regulation of proteins involved in Fe metabolism (Wang and Pantopoulos, 2011).

The mRNAs encoding TFR1 and two isoforms of DMT1 have IREs in their 3'UTRs which are believed to be stabilized under Fe deficient conditions when an IRP (IRP1 or IRP2) binds to them (Zhang et al., 2005). This stabilization increases the half-life of the mRNA and consequently increases the amount of protein translated (Figure 4).

Ferritin and one isoform of FPN1 have IREs in their 5' UTR. In this location, stabilization of the stem loop by IRP binding blocks translation (Figure 4).

The major form of FPN1 in intestinal cells, however, lacks the 5'IRE and thus FPN1 protein levels can be maintained even if Fe levels in the cell are low (Zhang et al., 2009). Interestingly, the FPN1 transcript IRE that is present in the 5′UTR when activated via IRP binding should lead to decreased FPN1 expression. However, an increase is noted, implying that FPN1 may not be under the control of the IRP/IRE system (Zhang et al., 2005), or that some other factor is also controlling the expression of the FPN1 transporter. Similarly, two of four isoforms of DMT1 and Dcytb mRNA has no IRE, and therefore their regulation has also never been adequately

explained by the IRE/IRP system. Clearly, local regulation of Fe absorption does not rely solely on intestinal Fe concentrations, but an additional mechanism is involved.

A molecular basis by which elements other than Fe can selectively modulate Fe metabolism in cells and tissues in the presence or absence of changes in Fe availability is based on the regulation of IRP function through changes in their phosphorylation state (Shreedhar et al., 2005). IRPs bind to Fe responsive elements in the untranslated regions of mRNA and regulate translation of key proteins involved in Fe metabolism, ferritin, and transferrin receptor. The formation or loss of the Fe-S cluster is a means by which changes in cellular Fe status could be transmitted into alterations in RNA binding capacity of IRP1 (Eisenstein and Blemings, 1998). An enzyme, aconitase has a central role in determination of the labile Fe pool and overall regulation of the intracellular Fe homeostasis. In order for the Fe-S cluster of IRP to serve as a biosensor, its assembly and/or disassembly needs to be adequately regulated.

However, the Fe-S clusters do not disassemble spontaneously. In other words, Fe deficiency per se is not sufficient to promote loss of the cluster (Eisentein and Blemings, 1998; Brown et al., 1998). Consequently, we postulate that cellular Zn concentration, in addition to Fe, can affect the stability of the Fe-S cluster and determine if the cluster is going to be removed or not. Further support to this idea comes from the studies that demonstrated an inhibiting effect of Zn on aconitase, showing low aconitase activity during Zn supplementation periods (Shreedhar and Nair, 2004, Shreedhar et al., 2005; Iyengar et al., 2012).

Similarly, there is some evidence that Zn can replace/alter 4Fe-4S clusters in proteins; Zn has an intrinsic preference for the fourth site over Fe (Butt et al., 1991). In addition, newly identified

IRP mRNAs fall into the category of "metal ion binding" proteins that are shown to interact selectively and non-covalently with Zn ions (Sanchez et al., 2011).

Recently, Iyengar et al. (2012) showed that Zn supplementation increased the RNA-bound forms of both IRP1 and IRP2 expression, resulting in increased DMT1 activity (Iyengar et al., 2012). Similarly, the expression of FPN1 was also increased upon supplementation with Zn (Yamaji et al., 2001; Yeh et al., 2004; Kelleher and Lonnerdal, 2006; Iyengar et al., 2012). Furthermore, it appears that Zn is able to block the Fe storage capacity of ferritin (Harrison, 1996; Niereder, 1990, Iyengar et al.,2012).

Besides, it has been shown that the induction of the DMT1 IRE isoform by Fe depletion was completely dependent on HIF 2, suggesting molecular crosstalk between HIF 2 and IRP signaling, where IRP1 and/or IRP2 are critical for basal gene expression of DMT1 IRE isoform (Shah et al., 2009) and HIF 2 is required for transcriptional response (see figure 5 for an association between Zn and HIF 2). These results noticeably raise the probability that fine-tuned coordination of Fe and Zn metabolism may also take place via the IRE/IRP system, but this requires further investigation.

When low concentrations of Zn are present in the cells, Zn is not able to bind to ferritin and does not block its storage capacity, which in turn, contributes to increased accumulation of Fe in the enterocytes. This implies that the availability of Zn in the cells plays a crucial role in Fe absorption and transfer to circulation. Although, the Fe may be supplied in sufficient amounts it cannot be absorbed (Zn deficiency leads to DMT1 delocalization) and transported out of the enterocyte (as ferritin storage capacity increases) without an adequate supply of Zn.

However, the increased expression of FPN1 and ZnT1 has also been noted during Zn depletion (Iyengar et al., 2012). Briefly, this initial increase in FPN1 and ZnT1 expression in response to Zn deficiency, in our opinion, exists in order to improve the transfer of Fe and Zn to systemic circulation, which helps in minimizing the difference between Fe and Zn supply and demand, and alleviates negative consequences of prolonged Fe and Zn depletion. Similarly, Zn plays a protective role against Fe induced oxidative damage, so decreased concentration of Zn in the cells indicate that the levels of free Fe in the cells should be reduced in order to avoid oxidant induced damage, which additionally explains the increased storage capacity of ferritin, as well as increased FPN1 expression, during the periods of cellular Zn depletion.

2.5. The regulation of iron absorption during pathological conditions (Inflammation, Hypoxia, IRIDA and iron overload conditions)

2.5.1. Hypoxia

Fe is essential for supplying cells and tissues with oxygen. Hypoxia (oxygen deprivation) creates a strong stimulus to Fe absorption, regulating the function of Fe transporters by hypoxia inducible factors (HIF 1 and HIF 2). HIFs are a part of a transcription factor complex that binds to promoters containing HIF responsive elements (HREs) and induces transcription. Fe metabolism genes that contain HREs include Dcytb, DMT1, and FPN1. HIF 2 levels, and thus the transcription of target genes, rise when oxygen levels decrease.

Increased expression of HIF 2 in the intestine in genetically modified mice leads to increased DMT1 and Dcytb expression and increased Fe absorption, whereas intestinal knockout of HIF 2 leads to low levels of DMT1, Dcytb and FPN1 and systemic Fe deficiency despite low levels of hepcidin (Mastrogiannaki et al., 2009). Dcytb and DMT1 are the genes most highly up-regulated by hypoxia in duodenum and both transporters are regulated by HIF 2 (Shah et al., 2009).

HIFs (hypoxia-inducible factors) have been shown basically to control the cellular response to hypoxia. Recently, it was shown that NF-κB (nuclear factor κB) is a modulator of HIF 2 expression in the presence of normal oxygen pressure (Haase et al., 2010, Willam, 2014). NF-κB indirectly controls HIF 2α through its control of HIF 1β (van Uden et al., 2011).

A number of studies over the years confirmed that Zn plays an important role in the activation of NF-kappaB (Prasad et al., 2001; Bao et al., 2007). Under low Zn conditions, NF-kappaB nuclear binding activity is lower (Oteiza et al., 2001; Ho and Ames, 2002; Butcher et al., 2004; Liu et al., 2009). The initial reduction in NF-kappaB binding most likely reflects an early response to Zn deficiency-induced oxidative stress (Oteiza, 2001).

At times of prolonged Zn deprivation, oxidative stress (ROS-reactive oxygen species) continues to activate NF-kappaB which increases the production of growth factors, antiapoptotic molecules and inflammatory cytokines. In these situations, the provision of adequate concentrations of Zn to the cells is crucial, as Zn has been shown to decrease ROS by several mechanisms. One mechanism by which Zn reduces inflammatory cytokine production involves the Zn-induced up-regulation of a Zn-finger protein, A20, which inhibits NF-kB activation via TRAF pathway (Prasad, 2009). Thus, Zn functions as an antioxidant and as an important anti-inflammatory agent.

2.5.2. IRIDA

Iron refractory iron deficiency anemia (IRIDA) is an inherited Fe deficiency disorder manifested by a profound anemia that is unable to respond effectively to oral Fe therapy. The gene mutated in most such cases is transmembrane protease serine 6 (Tmprss6), a hepatocyte plasma membrane protease that degrades HJV and effectively acts to repress hepcidin production. When Tmprss6 is mutated, hepcidin levels are relatively high and body Fe intake declines.

2.5.3. Iron overload conditions

Iron overload conditions (e.g. thalassemia, hereditary hemochromatosis) are characterized by increased Fe absorption despite the presence of adequate or increased body Fe stores. They are mainly caused by mutations in the genes encoding, HFE, TFR2, JH, and they share the common feature of reduced or absent hepcidin expression (Lagnel et al., 2011).

Under normal circumstances and during Fe deficiency, the liver acquires Fe via receptor-mediated endocytosis of transferrin, the circulating Fe transport protein (Morgan et al., 1986). During conditions of Fe overload, the Fe-carrying capacity of transferrin is exceeded, giving rise to non-transferrin bound iron (NTBI). As previously discussed, Zip14 mediates the uptake of NTBI into hepatocytes (Liuzzi et al., 2006).

The role of Zip14 in HFE-mediated Fe overload was examined in HepG2 cells (Gao et al., 2008). The expression of HFE in HepG2 cells resulted in a lower abundance of Zip14, possibly by a

post-transcriptional mechanism. Interestingly, Fe uptake was unaffected by HFE expression after Zip14 knockdown, implying that HFE has a direct effect on Zip14-mediated Fe transport.

2.6. The mechanism of systemic iron regulation: the important role of hepcidin (major iron regulatory protein in hepatocytes)

Hepcidin is a small peptide, composed of 25 amino acids, produced mainly by hepatocytes in the liver. It is a major regulator of Fe homeostasis that acts at two sites, tissue macrophages and the small intestine. While there is general agreement on the essential role of hepcidin in Fe absorption, there is still no conclusive evidence about the exact site of hepcidin action in enterocytes. Hepcidin has been shown to induce a significant reduction in intestinal Fe transport and DMT1 protein levels, but no change in FPN1 levels (Frazer et al., 2003; Mena et al., 2008, Yamaji et al., 2004).

On the other hand, there are studies that demonstrated a link between decreased hepcidin and elevated intestinal FPN1 expression (Leong and Lonnerdal, 2004; Frazer, 2003; Muckenthaler et al., 2003).

Looking at the structural properties of this molecule, the first five N-terminal amino acids are essential for regulation of Fe metabolism (Rivera, 2005; Ganz and Nemeth, 2006). The N-terminal region of hepcidin is essential for binding to FPN1 because sequential truncation of the N-terminal residues results in a progressive loss in activity of the peptide. However, the N-terminal region alone is not sufficient to induce FPN1 internalization (Nemeth et al., 2006). In

addition, it has been shown that Cys326 of FPN1 is required for the interaction between hepcidin and FPN1 (Liu et al., 2005; Fernandes et al., 2009).

The regulation of hepcidin production is complex. Animal models and human diseases that lead to inappropriate Fe levels have helped in the clarification of hepcidin regulatory mechanisms, although some of these mechanisms are still incompletely understood (Fuqua et al., 2012). It is now clear that there are several pathways involved in the regulation of this hormone, including inflammation, infection, erythropoietic demand, hypoxia and body Fe status (Zhang, 2009). Hepcidin synthesis by the liver is increased by inflammation and Fe stores and is decreased by hypoxia and anemia.

Among the multiple mechanisms that regulate hepcidin expression the BMP6/SMAD pathway takes the central role (Silvestri et al., 2008; Fuqua et al., 2012; Ganz, 2011). BMP6 is a transforming growth factor beta family cytokine produced and secreted by hepatocytes in proportion to their Fe load.

The BMP6 receptor complex includes a co-receptor called hemojuvelin (HJV) that is critical for this pathway to function (Zhang and Enns, 2009; Fuqua et al., 2012). Individuals with a congenital defect preventing HJV synthesis have very low hepcidin levels and develop the severe Fe loading disease juvenile hemochromatosis. HJV is normally found bound to the plasma membrane of cells, but a soluble form can be produced by the action of the protease furin (Ganz, 2011). Soluble HJV can compete with membrane-bound HJV for BMP binding and thus is able to ameliorate BMP/SMAD signaling (Zhang and Enns, 2009).

Tmprss6, a cell surface serine proteases that degrades HJV and is able to reduce BMP signaling (Silvestri et al., 2008). Tmprss6 is an essential component of a pathway that detects Fe

deficiency and blocks HAMP transcription, permitting enhanced dietary Fe absorption. Tmprss6 is a Zn dependent endopeptidase. Therefore, inadequate Zn levels lead to improper function of Tmprss6 (Ramsay et al., 2009; Knutson, 2009). In this situation, Tmprss6 cannot bind HJV which accordingly increases hepcidin production and lowers the absorption of Fe (Figure 6).

2.6.1. The role of intracellular zinc concentrations on hepcidin expression and regulation of intestinal FPN1 transporter

The inhibitory role of hepcidin is believed to be initiated by a direct binding of this peptide to FPN1 transporters (Nemeth et al., 2006; Fernandes et al., 2009). However, there is more and more evidence showing the absence of the hepcidin effect on FPN1 in the intestine (Zhang et al., 2009; Abboud and Haile, 2000, Taylor et al., 2011).

The explanation could be the presence of a second FPN1 isoform that is unable to interact with hepcidin. A new FPN1B transcript lacking the IRE sequence has been described recently in enterocytes (Zhang et al., 2009); however it is still unknown how the protein expression of this isoform is controlled.

The FPN1B form represents 25% of total FPN1 mRNA in duodenum (Zhang and Enns, 2009). The translation of FPN1B is insensitive to Fe but the protein can export Fe under conditions of Fe deficiency (Zhang et al., 2009). Similarly, *in vitro* studies using either hepatoma cell lines or primary hepatocytes have failed to demonstrate increased synthesis of hepcidin in response to Fe loading (Nemeth et al., 2006; Fuqua et al., 2012). Moreover, analysis of hepcidin mRNA and protein levels in hepatoma cells suggests that its expression is regulated by divalent metal ions,

with Zn inducing maximal effects on hepcidin levels (Balesaria et al., 2010). MTF 1, a divalent metal ion sensitive transcription factor, regulates hepcidin transcription by binding to its cognate response elements within the hepcidin promoter. Out of four elements which activity was tested (Fe, Zn, Co, Cu) only Zn was shown to induce metal sensitivity fully (Balesaria et al., 2010). In addition, hepcidin belongs to the family of metallothioneins (MTs), proteins regulated by intracellular Zn ion levels through MTF1/MREs interactions (Westin, 1988). MTs are cysteine-rich, metal-binding proteins involved in Zn homeostasis and are also involved in protection against oxidative stress. MTF1 has been shown as important in the Zn-mediated induction of FPN1 mRNA (Figure 1). Troadec et al. in 2010 demostrated that MTF1 binds to the FPN1 promoter in the presence of Zn and initiates its transcription. It has also been shown that although some other metals (cadmium, cobalt) can induce metallothionein expression by MTF1, only Zn can activate the DNA binding activity of this transcription factor by its reversible interactions with specific Zn fingers (Troadec et al., 2010).

FPN1 is the main hepcidin receptor (Nemeth et al., 2004; Donovan et al., 2005; Ganz, 2011). Acute hepcidin exposure has little effect on FPN1 while chronically high levels of hepcidin lead to loss of intestinal FPN1 (Muckenthaler et al., 2003; Folgueras et al., 2008; Chung et al., 2009). The alterations in Fe transport activity in enterocytes seems to result from the changes in DMT1 protein levels (Frazer et al., 2003; Mena et al., 2008, Yamaji et al., 2004; Lagnel et al., 2011). How hepcidin affects DMT1 levels is not clear, but it is believed that the process is guided by ubiquitin-dependent proteasome degradation of DMT1 (Lagnel et al., 2011). The inconsistent evidence about the exact site of hepcidin action in the intestine could be explained by the fact that changes at the apical side of the enterocyte most likely contribute to the changes at the

basolateral side and vice versa; and possibly indicate the presence of an additional mechanism controlling the entire process. The presented findings demonstrate that cellular Zn concentrations modulate the expression and function of the transporters.

2.7. The mechanism of iron absorption under physiologically favorable conditions (adequate supply of both minerals Zn and Fe, without inflammation or infection); the significance of suitable cellular zinc concentrations.

Zinc adequacy: Zip4 and ZnT1 respond positively to adequate dietary Zn intake and transport Zn inside the enterocytes (Zip4) and outside of it, to the plasma (ZnT1) (Figure 7). The satisfactory levels of Zn in the cells, in addition to Fe, stimulate the expression of both DMT1 and FPN1 transporters. Consequently, there is a transfer of Fe across the enterocytes (Fe is not stored as ferritin), Fe goes out to the plasma. Due to the presence of adequate levels of Zn in the hepatocytes, Tmprss6 is functioning properly, hepcidin production is decreased (HJV, BMP pathway) and FPN1 and DMT1 expression increases, so the cells are able to absorb Fe further. In order for the cells to respond appropriately to Fe deficiency the presence of adequate intracellular concentrations of Zn is vital.

Zn deficiency: Dietary Zn intake is the main determinant of Zn absorption (Figure 7). During the states of prolonged marginal intake of Zn, homeostatic adjustments are not sufficient to replace Zn losses and a negative Zn balance occurs. Even if Fe is supplied in sufficient amounts it cannot be absorbed without an adequate supply of Zn. Low intracellular Zn concentrations cause DMT1

delocalization, reduce FPN1 expression and there is no transport of Fe into the enterocytes. Additionally, in an effort to adjust to low dietary Zn intake, the system will start to withdraw Zn from the tissues (plasma, pancreas and liver are among the most affected organs). Low concentrations of Zn in the liver will affect the activity of Tmprss6 (HJV will not be degraded) which will cause an increased hepcidin synthesis and consequently, through limited expression and activity of DMT1 and FPN1 transporters lead to reduced Fe uptake.

3. CONCLUDING REMARKS

In recent years, substantial progress in the understanding of Fe and Zn absorption and interaction processes in humans has been made. The mode of Fe absorption is complex and it is challenging to distinguish between the different players involved in the process. There are still missing pieces of information to fully explain certain inconsistencies.

This paper describes the main Fe and Zn transporters found in major sites of Fe and Zn uptake and storage, the enterocytes and the hepatocytes. It also explains Fe and Zn interactions at the transporter level. Finally, this review, for the first time, proposes the entire mechanism of Zn involvement in the Fe absorption processes (both at the local and systemic level) and further clarifies some of the discrepancies related to Fe and Zn absorption and interactions.

DMT1 is not the site of negative interactions between Fe and Zn. Zip14 is a second Fe transporter that can transport both NTBI Fe and Zn. Cellular Zn dictates the course of events that determine the expression of proteins involved in cellular Fe metabolism. Zn controls the expression of both DMT1 and FPN1, as well as the expression of Zip4, Zip14 and ZnT1 transporters. Anemia develops due to impaired mobilization of Fe from diet and stores rather

than inadequate dietary intake, which demonstrates the crucial role of Zn on Fe transporters. In addition, the major protein of systemic Fe regulation, hepcidin, is coordinated by intracellular Zn ion levels through MTF1/MREs interactions. Tmprss6, an essential component of a pathway that detects Fe deficiency, is a Zn dependent endopeptidase.

In conclusion, this review clarifies the role of cellular Zn status in determination of Fe/Zn interactions and illuminates mechanisms by which Zn could possibly affect the control of Fe absorption.

The challenge is certainly to further utilize this new information, to provide a more comprehensive understanding of the link between Zn and Fe absorption pathways using both *in vitro* and *in vivo* models. Finally, research into the mechanism by which Zn status influences Fe absorption in humans is, and will remain, an important and interesting area of investigation for many years to come.

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Table 1. A summary of studies (published between 2000 and 2015) showing that Zn is not transported via DMT1 (Divalent Metal Transporter 1) and that intracellular Zn concentrations regulate the expression of DMT1 transporter.

Authors	Material used	Findings
Bishop et al. (2010)	Astrocytes	Zn(2+) is not transported through DMT1
Deng et al. (2009)	Epithelial cells	Iron and zinc can enhance the uptake of each other
Espinoza et al. (2012)	Caco-2 cells	Zinc is not transported by DMT1
Garrick et al. (2006)	HEK 293 cells	DMT 1 has very low activity for Zn (almost no activity)
Iyengar et al. (2009)	Caco-2 cells	Zinc does not inhibit transport of iron, and is not transported via DMT1
Iyengar et al. (2012)	Caco-2 cells	DMT1 is not a site of iron and zinc interaction, DMT1 expression increases with zinc supplementation
Kordas and Stoltzfus (2004)	Neural tissue	DMT1 is not a site of Zn absorption
Leong et al. (2003)	Rat pups	DMT 1 changes in accordance to intestinal Zn concentrations
Lopez de Romana et al. (2003)	Caco-2 cells	Zn is not absorbed via DMT1
Mackenzie et al. (2007)	Xenopus laevis	DMT 1 does not mediate zinc transport
Olivares et al. (2012)	A review of studies	No negative effect of zinc on iron absorption
Rashed Abd (2011)	Caco-2 cells	Zinc significantly increases DMT1 expression
Sacher et al. (2001)	Caco 2 cells	Km and Vmax of iron and zinc uptake are very different, no competition for a single transporter
Sacher et al. (2004)	Xenopus laevis	DMT1 does not transport Zn
Tallkvist et al. (2000)	Caco 2 cells	DMT 1 is not regulated by intestinal iron concentrations
Tandy et al. (2000)	Caco-2 cells	Zinc does not inhibit transport of iron and is not transported by DMT1

Wang et al. (2005)	Epithelial cells	DMT1 mRNA increases with zinc exposure
Yamaji et al. (2001)	Caco-2 cells	Zinc increases DMT1 expression and Fe2+ uptake

Table 2. Fe and Zn transporters: cellular location, tissue-specific expression and the mechanism of regulation. Data modified from: Litchten and Cousins, 2009; Wang and Zou, 2010; Myers et al., 2012

Transporter name	Cellular location	Tissue expression	The mechanism of regulation	Zn dependent regulation	Expression along intestinal axis
DMT1	Apical membranes	Small intestine, kidney, liver	Cellular Zn, Hepcidin, Hypoxia, Inflammation	Increased expression and activity by Zn treatment	Expressed all over the intestinal tract; and liver, most strongly in the proximal duodenum
FPN1	Plasma membrane	Ubiquitously expressed	Hepcidin, Zn, Fe, Hypoxia, Inflammation	Increased expression and activity by Zn treatment	Expressed all over the intestinal tract and liver most strongly in the proximal intestine, lower expression in distal parts and colon
Zip4	Apical membranes	Small intestine, stomach, colon, kidney, brain	Dietary Zn, Transcriptional and post-transcriptional regulation	Up-regulated by Zn deficiency	Abundantly expressed throughout the small intestine
Zip14	Plasma	Ubiquitously	IL-6 and IL-1,	-	Expressed in small

	membranes	expressed, liver cells, heart, kidney	nitric oxide		intestine (found in duodenum and jejunum)
ZnT1	Basolateral membranes	Ubiquitously expressed, small intestine	Dietary Zn supply	Down-regulated by Zn deficiency	Most abundant in the proximal small intestine, cecum
ZnT2	Vesicles, secretory granules	Small intestine, liver, pancreatic acinar cells, kidney	Dietary Zn and hormones	Down-regulated by Zn deficiency	Expressed in small intestine

Figure 1.

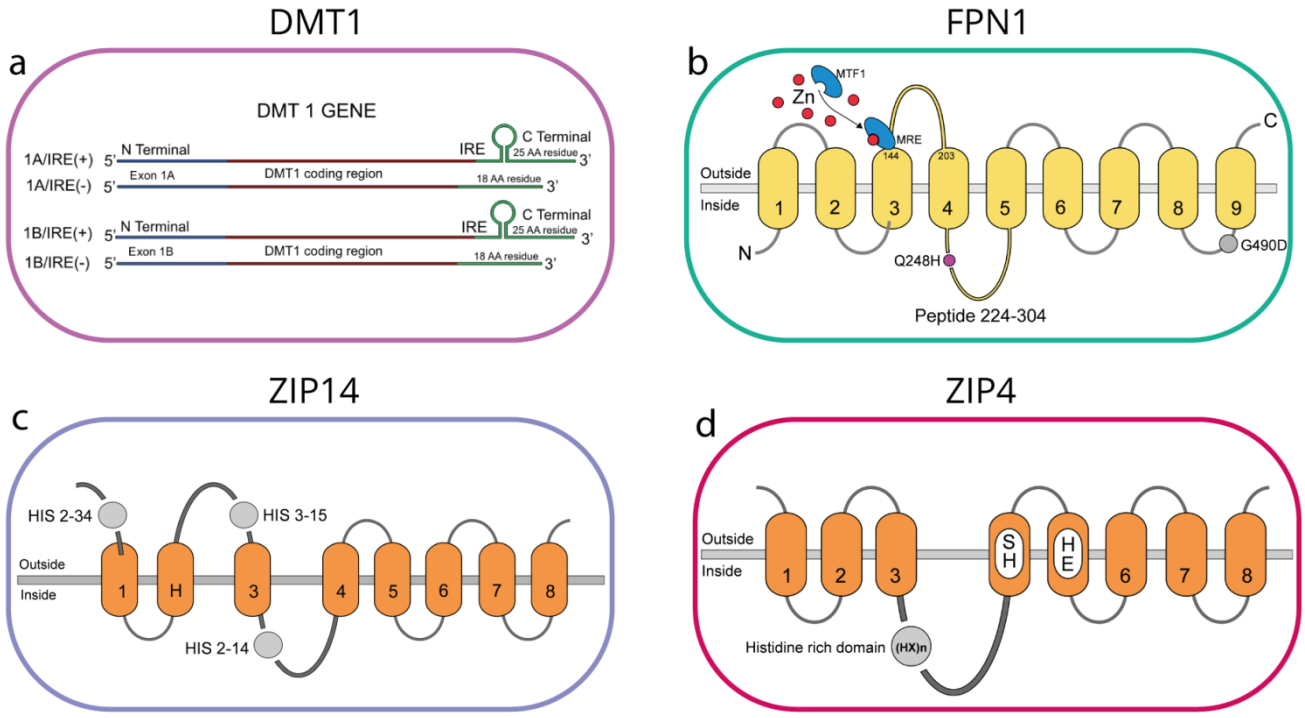

Figure 1. DMT1, FPN1, Zip 4 and Zip 14 gene. **a**. Four DMT1 isoforms that differ in their N- and C-termini arise from mRNA transcripts that vary both at their 5'-ends (exon 1A or 1B) and at their 3'- ends giving rise to mRNAs containing (+) or lacking (−) the 3'- IRE (iron-responsive element). The exon 1A/IRE (+) isoform is the predominant form in duodenal enterocytes. **b**. C terminal oriented towards extracellular medium. The extracellular loop 144-203 is a possible hepcidin bind The Zip4 gene has eight trans-membrane domains. **c**. Zip 14 contains three histidine-rich repeats and six to eight trans-membrane domains. **d**. A histidine rich region within

the large intracellular loop between putative transmembrane domains 3 and 4 plays a role in the response of Zip4 to zinc by regulating endocytosis and ubiquitination. Zinc induces FPN1 transcription through the action of MTF1.

Figure 2.

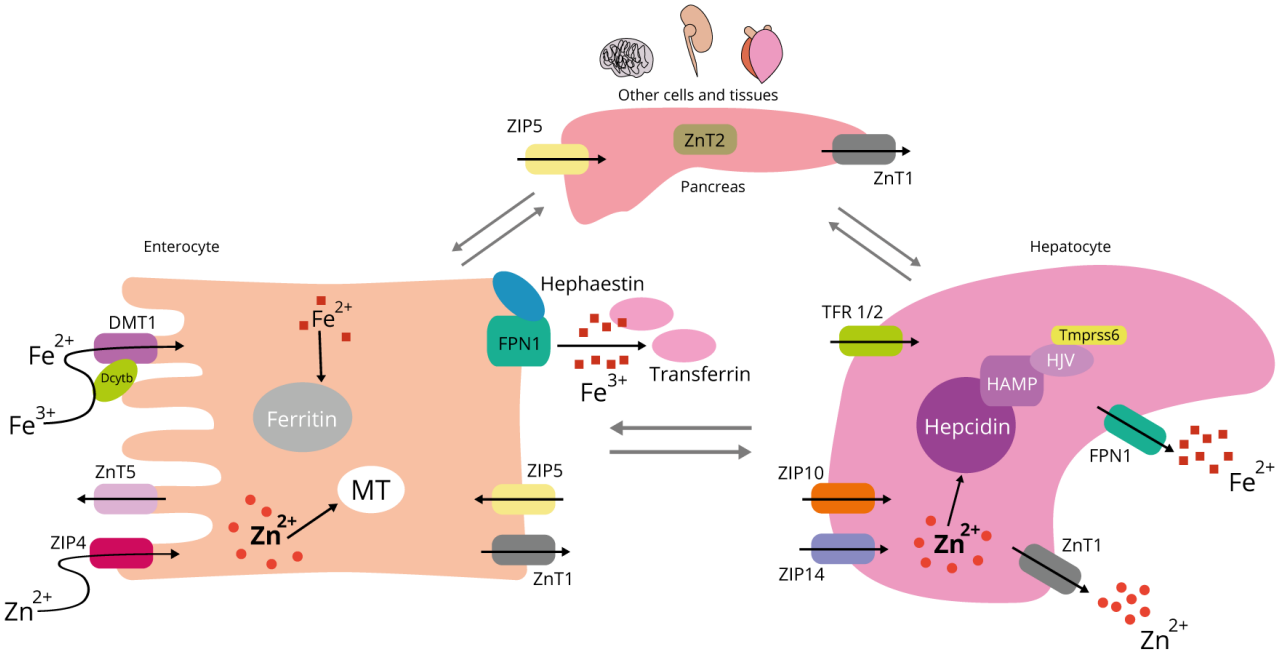

Figure 2. The mechanism of Fe and Zn absorption in human enterocytes and hepatocytes

Most Fe is moved across the enterocyte brush border membrane by the divalent metal-ion transporter 1 (DMT1), a process enhanced by the prior reduction of the Fe (ferric reductase activity) by duodenal cytochrome B (DcytB). Enterocyte Fe is exported to the blood via ferroportin 1 (FPN1) on the basolateral membrane. This transporter acts in partnership with the ferroxidase hephaestin that oxidizes exported ferrous Fe to facilitate its binding to plasma transferrin. Diferric transferrin binds to its specific receptor (TFR) and is endocytosed. Ferritin binds to its specific receptor and is endocytosed. At the high level of iron in plasma, NTBI is reduced to Fe2+ by ferric reductase and is rapidly transported into the hepatocytes via Zip14

transporter. Zip4 is the most important transporter in the enterocytes responsible for the uptake of Zn into the cells, while ZnT1 is the main Zn export transporter. The activity of Zip4, Zip10, ZnT1 is regulated via dietary Zn intake.

Figure 3.

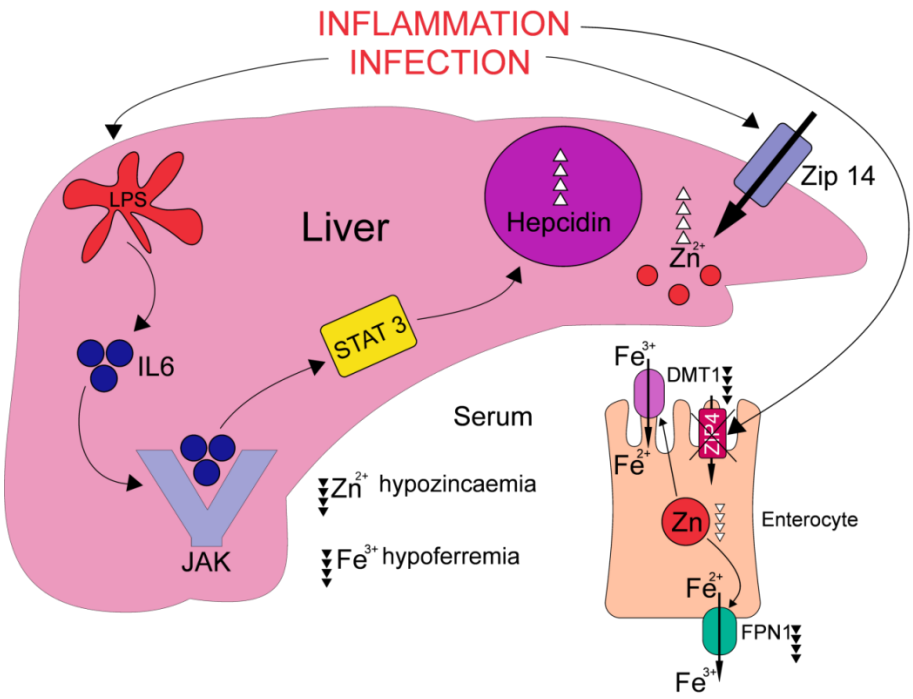

Figure 3. The effect of inflammation on iron and zinc absorption in the intestine and liver

Inflammation → increased Zip14 expression → increased levels of Zn in the liver and decreased in the serum (hypozincaemia) and hypoferrimia. Zip 4 reduced to nearly undetectable levels so there is no Zn uptake in the enterocytes. Infection →interleukin (IL1b)→IL6→ JAK/ STAT3→ increased hepcidin. Hepcidin can also be upregulated by LPS. Low concentrations of Zn in enterocytes + high hepcidin→ low expression of FPN1 and DMT1→ reduced absorption of iron→ anemia of inflammation. During the inflammation pH in the duodenum increases which decreases Fe solubility.

Figure 4.

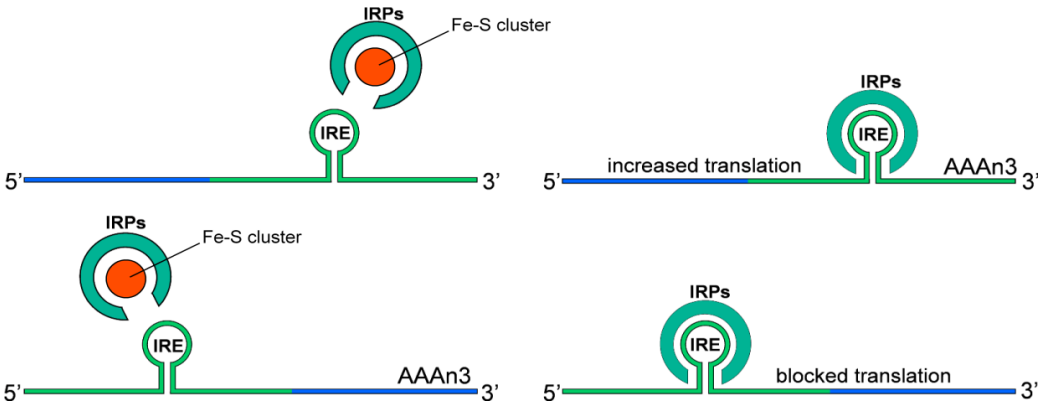

Figure 4. The iron responsive element (IRE)/iron regulatory protein (IRP) system Cytosolic aconitase with a 4Fe-4S-cluster senses the cellular Fe levels and can function as an iron-regulatory protein (IRP). The iron-sulfur cluster cannot assemble under Fe starved conditions, in which case the cytosolic enzyme undergoes a dramatic conformational change; aconitase assumes a 3Fe–4S configuration and loses the enzyme activity. This reorganized form of c-aconitase is called Fe regulatory protein (IRP). IRP bind to IRE in 3'UTRs and so improves stabilization that in turn increases translation of the transporter protein. In contrast, binding of IRPs to IRE in 5'UTRs blocks translation. Fe deficiency per se is not sufficient to promote loss of the cluster. Zn can replace 4Fe-4S clusters in proteins; Zn has an intrinsic preference for the fourth site over Fe.

Figure 5.

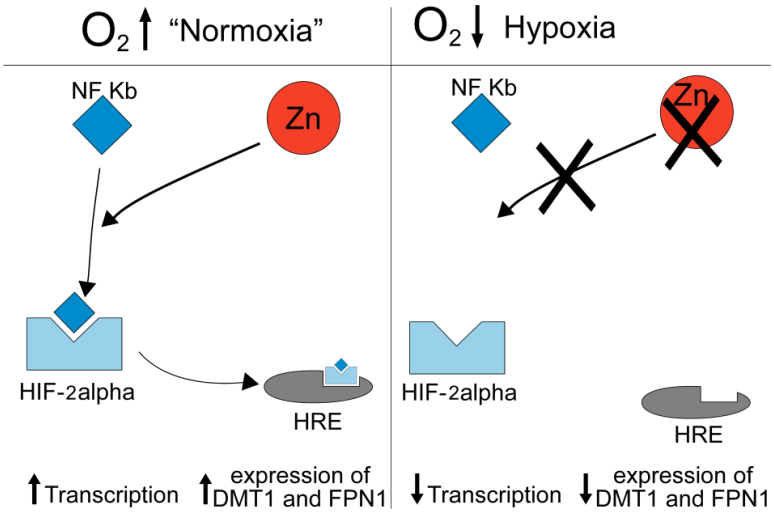

Figure 5. A proposed model for the role of zinc in the cell response to hypoxia Nuclear factor Kb stabilises HIF 1β, HIF 2α binds to HREs and induces transcription, which increases DMT1 expression, Dcytb and FPN1 expression and consequentially increases the levels of Fe and oxygen in the cells. Nuclear factor binding activity declines when Zn concentrations in the cells are decreased.

Figure 6.

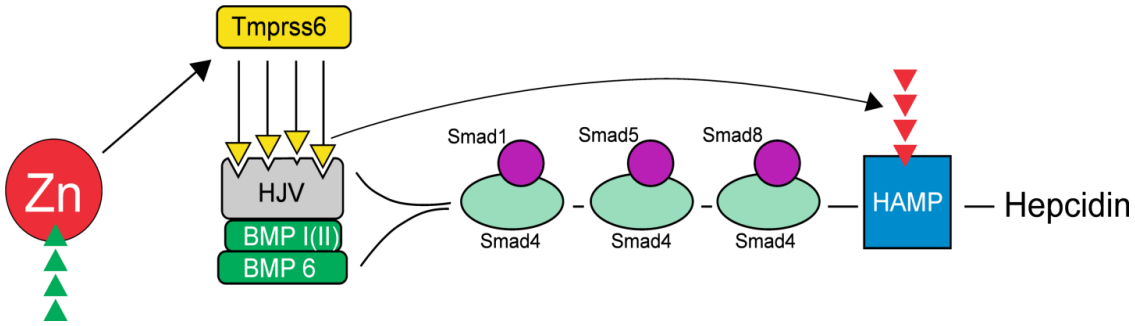

Figure 6. A proposed mechanism for the role of Zn in hepcidin regulation BMP6 binds to a BMP receptor I/II complex on hepatocytes, leading to phosphorylation of receptor-regulated SMADs 1, 5, and 8. Phosphorylation allows these proteins to interact with Smad 4, and the resulting heteromeric complexes stimulate transcription of the gene encoding hepcidin antimicrobial peptide (HAMP). Tmprss6s are Zn dependent endopeptidases, so adequate cellular Zn levels contribute to natural function of Tmprss6 (degradation of HJV) that reduces hepcidin production and consequently contribute to increased Fe absorption.

Figure 7.

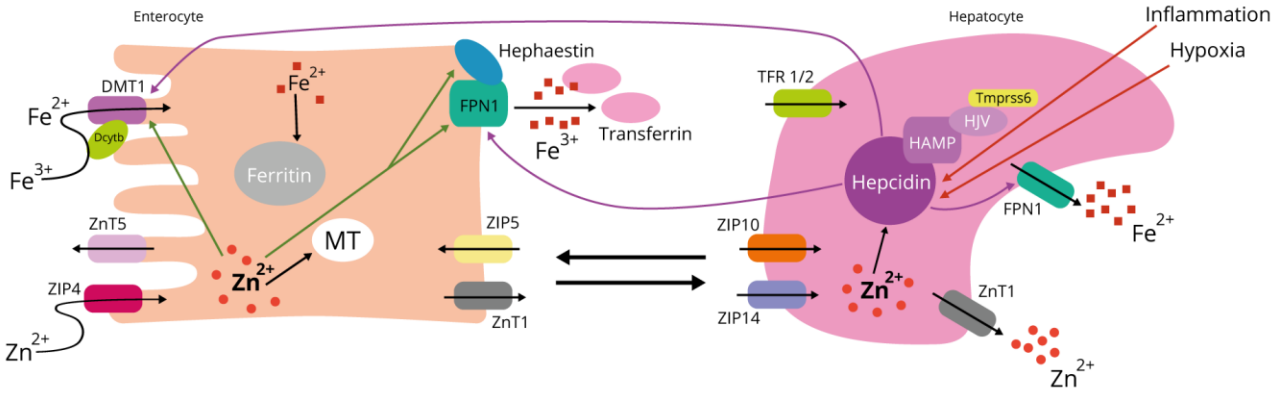

Figure 7. A proposed model for the role of Zn in systemic regulation of Fe absorption In order for the cells to respond appropriately to Fe deficiency the presence of adequate intracellular concentrations of Zn is vital. Cellular Zn concentrations regulate the expression and activity of both intestinal Fe transporters: DMT1 and FPN. In addition, the activity of Zip transporters (Zip4, Znt1, Zip 10) including the Zip 14 transporter is also regulated by Zn. Cellular Zn concentrations are playing a role in hepcidin expression.