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Review

Iron Balancing Mechanism: Iron Regulatory Element (IRE)-Messenger RNA Metal Sensing

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Abstract

Iron deficiency and overload are both major public health problem worldwide. About 30% of the world population is suffered from iron related disorder. Iron plays a central role in the metabolism of all living organisms and is toxic in excess. Iron is used to catalyse key reactions in DNA synthesis, respiration and metabolism. Free iron accumulated in a protein, ferritin. Iron mis-regulation can result in many diseases such as Anaemia, Thalassemia, Cancer, Diabetes and neurodegenerative disorders Alzheimer's, Parkinson's, Stroke and Cardiovascular. Therefore maintaining the proper amount of iron is a challenge for everyone to avoid the adverse consequences of iron excess or deficiency. Understanding the cellular iron homeostasis is critical for identifying iron related diseases and advancing the treatment of iron disorder. Maintenance of cellular iron homeostasis is accomplished by the coordinated regulation of iron uptake, storage and export by iron regulatory proteins (IRPs). IRPs are key controllers of iron metabolism and post-transcriptionally regulate expression of the major iron homeostasis genes. IRPs bind to iron responsive elements (IREs)-mRNA encoding proteins involved in iron uptake, sequestration and export. Each IRE-mRNA consists of about 30-nucleotide RNA helix. Iron destabilized IRP/IRE-RNA complexes, however, increase the protein biosynthesis. Significant advances have been made in understanding of the cellular and molecular mechanism of iron regulation. This review will provide an overview of the mechanism of iron regulation, iron absorption, transport and storage, and effect of metal ions in the IRE-mRNA regulation. Molecular understanding of these processes and key regulatory molecules involved in maintaining homeostasis will provide novel insights into understanding human disorder associated with iron deficiency and overload.

Keywords: Iron Responsive Elements; Iron Regulatory Protein; Ferritin; Iron Regulation

Introduction

Iron deficiency and overload affects billions of people throughout the world. Iron is an essential element for most life forms, but because it can be toxic at elevated cellular levels, elaborate regulatory systems have evolved to maintain it at sufficient yet safe concentrations. Coordinated regulation of the IRE-RNA/IRP network enables cells to respond to the multiple signals of iron availability and demand in a balanced manner. High level of IRP and iron found in the cells of the patients of Parkinson's disease, Alzheimer's diseases, Diabetes and Cancer. IRPs plays an important regulatory role in the gene regulation of the iron balancing. The reason for accumulation of high level of iron and IRP in neurodegenerative diseases remains unclear. There is some debate about the molecular ratio of the initiations factors involvement in the iron regulation. We have identified that highly organized mRNA and eIF4F complex in translation initiation plays a crucial role for protein biosynthesis [1].

Iron responsive elements (IREs)-mRNA are cis-acting hairpin structures that bind iron regulatory proteins (IRP1, IRP2) [2-4]. An IRE-RNA is about 30 nucleotide structure formed by two RNA helices that are separated by a bulged cytosine residue, and by a six nucleotide loop of the sequence 5'-CAGUGN-3' (N is usually a pyrimidine). The loop and the bulged nucleotide sequence are highly conserved (Figure 1) [5-7]. The two IRPs, which are highly conserved themselves, bind IRE-mRNA structures that appeared at various times during evolution [8] and have been extensively characterized [9-12]. When IRP1 binds the ferritin-IRE-RNA conformational changes occur, based on solution NMR of the free RNA and the crystal structure of the ferritin IRE-RNA/protein complex [13,14]. The tri-loop bases A15 and G16 and helix bulge base C8, present in all IRE-RNAs, are flipped out and a large surface of the RNA remains exposed in the RNA protein complex (Figure 2). The IRE-RNA hairpin in ferritin IRE-RNA contains the conserved terminal loop and the mid-helix bulge C, as well as a ferritin IRE-RNA-specific bulge U that is associated with tighter IRP binding and larger iron responses in vitro and *in vivo* [8,15]. In the IRE-RNA terminal loop, the base pair between conserved C and G bases create an AGUtriloop [16]. The three main protein/RNA contact sites in IRE-RNA complexed to IRP1 are in the terminal

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loop A15, terminal loop G16 and helix bulge C8 [16]. Large RNA surface remains exposed, inviting RNA interactions with other molecules and ions even while complexed to IRP1. Probing IRE-mRNA with metalloncleases such as Cu1+ -1,10–phenathroline or Mg ion [17,18] to locate metal sensitive sites, combined with the X-ray crystal structure of the IRE-RNA/IRP1 complex shows that some metal sites are located on exposed surfaces that are available for additional RNA interactions even in the IRP1 complex [15]. Metal binding to selective sites on IRE-RNA by small, shapely molecules such as Cu1+- phenathroline and or Ru2+-tris (bipyridyl) has been known for some time [18]. In the presence of dioxygen and the RNA-bound complexes, reactive intermediates form, which cleave the folded RNA at the binding site of the metal-complex; sequencing of the cleaved RNA identifies the RNA binding sites. Such studies showed specific binding of the metallo-complexes at sites near helix distortions or in the terminal loop of the IRE-RNA structure [18,19]. The possibility of iron binding to IRE-RNA in ferritin mRNA is suggested by the results of IRE-RNA cleavage with iron and hydrogen peroxide. The unusual pattern of hypersensitive cleavage sites was initially explained as solvent access prevented by RNA folding [20], a supposition that was not supported by NMR structures obtained later [21]. Alternatively, the hypersensitive sites could indicate iron/RNA binding sites. Some of the IRE RNA-NMR metal sites observed by metallonuclease probing are at bases that are on the exposed RNA surfaces in the IRE-RNA conformation observed in the IRP/IRE-RNA crystal structure and thus could be accessible to metal ions while the IRE-RNA is bound to IRP. Recent studied with 2AP substituted IRE-RNA showed that iron altered the conformation of both the terminal IRE loop: A15 was less stacked, and the bulge C8 was more ordered. A15 and C8 are part of the two IRE-RNA/IRP1 contact sites and have multiple contacts [16]. Recent studies showed [22] that metal ions directly weaken IRE-RNA/IRP1 stability. Metal ions directly modulate the function of many RNA classes, e.g., tRNA [23], rRNA [24], ribozymes [25,26], riboswitches in bacterial mRNAs, where metals contribute both to RNA function and to metal sensing [27,28], and possibly hammerhead, mammalian mRNAs [29]. Ferritin IRE-RNA binds metal ions with a 1:2 stoichiometry [16]; Magnesium ion also binds to many rRNAs, tRNAs, riboswitches and ribozymes. In addition, the IRE-RNA also binds shape specific metal complexes [1,10] phenanthrolene and Ru(tpybpy) and other small molecules at even more specific sites [19].

Iron responsive element (IRE) mRNA and iron regulatory proteins (IRPs) are key players in the control of iron metabolism in animal cells. Iron increases rates of ferritin protein synthesis in animals by facilitating mRNA binding; metabolic iron, i.e., "free" iron in cells, is considered to be ferrous [30]. IRP proteins bind with different affinities to IRE-RNAs family [12,22], creating a hierarchal set of mRNA responses to iron *in vivo*. Deletion of the 30 nucleotide IRE-RNA not only removes IRP regulation, but also decreases the rate of IRP-independent protein synthesis [31]. A number of current models of IRE-RNA/IRP regulation feature iron-dependent degradation/modification of the IRP proteins as the main control point [11]. Regulation by metabolic iron binding to IRE-mRNA, in order to increase activator protein (eIF4F)-RNA interactions and decrease inhibitor protein (IRP)-RNA interactions, is a novel genetic regulatory mechanism in eukaryotes. The IRE-mRNA riboregulator illustrates the potential of specific metal interactions with folded, noncoding mRNA structures for gene regulation. The selective role of iron in dissociating IRE-RNA from the protein synthesis inhibitor, IRP1, identified the chemical nature of the biological iron signal [22], and complemented regulatory models of IRP protein degradation by iron sensitive E3 ligase [32,13] or IRP inhibition of IRE-RNA binding by insertion of a [4Fe-4S] cluster at the RNA binding site. Insertion of the [4Fe-4S] cluster converts IRP1 to c-aconitase [9,33]. How the IRE-RNA enhances protein synthesis rates in the absence of IRP [29] has remained obscure.

Iron Absorption and Transport

Iron, an essential nutrient for human beings, plays an important role in biochemical activities. Iron deficiency is the most prevalent nutrition disorder in the world [34,35]. However, iron overload can damage body tissue [36] and related to increasing risk of Alzheimers, Parkinsons, Diabetes and Cancer. Because several of the regulators of intestinal iron absorption have also been localized to the central nervous system. Ferroportin has also was isolated in the central nervous system where it is expressed in many cell types like neurons, astrocytes, oligodendrocytes, and the cells that comprise the blood-brain barrier such as choroids plexus and central nervous system blood vessels [14]. Iron enters our body through the diet. Iron in food is found in heme and nonheme forms. Heme iron is derived predominantly from meat products and nonheme (ferric forms) iron from both meat and plant sources [37]. Since there is no regulated excretion of iron through the kidneys or liver, iron balance is primarily controlled at the level of intestinal absorption. Iron absorption takes place in the proximal portion of the duodenum, where polarized cells are arranged in the fingerlike villi that protrude into intestinal lumen to maximize absorptive surface area [38]. Each of the absorptive cell or enterocyte has a microvilli brush border at the luminal surface [39]. For adults, male body iron is about 3.8 gram and female body iron is about 2.3 grams [40]. In order to replenish iron loss from sweat, urine, sloughed intestinal mucosal cells, and other blood loss, person need to absorb 1-2 mg of iron daily from food [41]. Humans absorb iron through enterocyte in the small intestines by the divalent metal transport 1 (DMT1). Only the divalent iron forms, ferrous are suitable for adsorption. However, 90% of dietary iron is in the ferric form and the other 10% is in the heme form [42]. In the intestinal lumen, ferric form is reduced to ferrous by an enzyme ferric reductase a duodenal cytochrome B [43,44]. DMT1 transports the divalent ionic iron across the enterocyte apical membrane.The heme iron is transported to the enterocyte by unidentified heme transporters. In the enterocyte, transported heme is metabolized into ferrous (Figure 3). After iron is absorbed into the enterocyte, internalized ferrous iron remains accessible in

a labile iron pool (LIP) by binding to unknown molecules, is stored in the iron storage protein ferritin or exits the enterocyte. Ferrous iron is exported across the basolateral membrane into the plasma by ferroportin. Ferroportin iron export is coupled with oxidizing ferrous iron back into ferric iron by the transmembrane copper-dependent ferroxidase, hephaestin. The reoxidized ferric iron is circulated in the plasma by binding with transferrin (Tf) [45], and the resulting diferric transferrin complex circulates through the plasma to deliver iron to tissues.Transferrin not only maintains iron in a soluble form but also as the main iron vehicle delivers iron to different cells. Circulating diferric-Tf pool serves as the major source of iron. This diferric-Tf binds to transferrin receptor 1 (TfR1) on the plasma membrane, and the resulting diferric-TfR1 complex internalizes into the endosome. Release of ferric iron from diferric-Tf facilitated by the conformational changes in Tf and TfR1 by the ATPase mediated acidification. Ferric iron is reduced to ferrous iron by the endosomal ferrireductase. Ferrous iron is then transported by DMT1 into the cytosol. The apo-Tf-TfR1 complex is unstable because apo-Tf has a very low affinity for TfR1. After release of iron, both Tf and TfR1 recycle back to the cell surface. Divalent metal transporter 1 can also directly transport non-transferrin-bound iron into the cells, in conditions of hemochromatosis and haemolytic anaemia while serum iron levels exceed the binding affinity to Tf [46]. In humans, plasma transferrin iron saturation indicates iron levels: transferrin iron saturation of around 30% shows normal iron level: transferrin iron saturation below 16% shows iron deficiency and transferrin iron saturation above 45% shows iron overload. If plasma transferrin iron saturation goes over 60%, the accumulated free non-protein binding iron can damage organs [47]. Transferrin bound iron enters the target cells by transferrin receptor 1 (TfR1) mediated endocytosis. The TfR1 has approximately 500-fold higher affinity for iron loaded transferrin (holo-Tf) than for iron released transferrin (apo-Tf) [45]. The TfR1 and Tf affinity changes help apo-Tf dissociation with TfR1 and enable recapture of ferric iron. In the target cell, released ferric iron is reduced to ferrous by metallo-reductase [48]. Ferrous substrate transport through ferritin protein cage where internal iron-oxy minerals form [49].

Iron Storage Protein

Once iron enters the cell, the excess iron that is not needed for immediate use is stored in the ferritin, an iron storage protein (Figure 4). Ferritin is a large protein composed of 24 structurally similar light and heavy subunits, where approximately 4500 iron atoms can accommodated as a macroinorganic iron complex. Ferritin is a 12 nm diameter spherical protein with an 8 nm diameter hollow centre [50]. Iron atoms can diffuse into and out of ferritin through eight channels located at the axis of the ferritin (Figure 4) [50]. Main function of the ferritin is to detoxify and store cellular iron by coupling iron and oxygen to form a stable but biologically available ferric oxide mineral at nonreactive sites inside the ferritin cavity. The protein cage prevents other biological molecules in the cytosol from reacting with iron minerals [51,52]. From microorganism to human, ferritin is the protein which to stores cellular iron in a water soluble, non-toxic way [53]. Ferritin subunits are classified into two subunit types, the H and L subunits. Both types of subunits have similar 5 α helices structure but different amino acids sequence [53]. The H subunit has ferroxidase activity, oxidizing ferrous iron into ferric iron. The L subunit lacks oxidizing activity but it provides the ligand for ferric binding and initiates iron nucleation [54]. In mammals, ferritin can be assembled in different proportions of H and L subunits which are tissue specific. High proportion of H subunit ferritin is found in heart, kidney and bone marrow and high proportion of L subunits ferritin is found in liver and spleen [47]. Besides iron utilization and storage, 3-5% of the total cellular iron forms an accessible and transient iron ion pool, which is named as liable iron pool (LIP) [55]. The LIP consists of both ferrous and ferric ion associated with lower molecular weight chelators, such as citrate, phosphate and other organic ions [56]. The cytosolic LIP homeostasis is directly related to the ferritin expression. Ferritin has a dual role in LIP homeostasis. As the main iron storage protein, ferritin binds to iron to keep iron in a soluble state and away from harmful redox reaction. Ferritin can also release iron into LIP to stabilize the cellular iron level under low iron conditions [57].

Structure of Iron Responsive Element (IRE) RNA

Iron responsive elements (IREs) RNA are a conserved 30 nucleotide structure formed by two RNA helices that are separated by a bulged cytosine residue and a six nucleotide loop of the sequence 5'CAGUGN3' (usually N is a pyrimidine). The name IRE (iron responsive element) was developed based on effects of increasing iron in animals and most models depended on increased IRP degradation [9,33]. Reporters usually used to monitor iron effects were entry of IRE-RNA to polysomes and protein accumulation and more recently changes in RNA mobility with cell extracts. Models that developed reflected iron induced protein degradation of both IRP repressors and Fe-S cluster insertion to IRP1.

Functional IRE RNAs have been identified in the 5' untranslated regions (UTR) of ferritin H and L-chain, erythroid aminolevulinate synthase (eALAS), mitochondrial aconitase and succinate dehydrogenase (SDH) mRNAs, as well as the 3' UTR of transferring receptor (TfR) mRNA [58]. The conserved structure of IRE RNA family are a double stranded RNA helix of 9/10 base pairs with a terminal hexaloop and an unpaired C8 residue in the helix creating a 5 base pair upper stem and a variable length of lower stem. The terminal loop is spanned by a G-C base pair which effectively creates a triloop of AGU that is exposed to solvent. In the terminal loop, C14 and G18 is base paired and create a pseudotriloop of AGU [16,17,59]. G16 and U17 are disordered. Base pairing and sequence around the unpaired C8 residue varies between different IRE RNAs, separating IRE RNAs into two groups, one with an internal loop constructed from the unpaired C8, and other with an isolated unpaired C, an unpaired base at position 6, 5' UTR to the unpaired C, and an additional base pair between them. Helix distortion around the conserved C8 residue plays an important role in selective repressor binding [17]. Five base pairs separate

the conserved apical loop from a bulge, which is a conserved C residue (Figure 1). Each IRE RNA have three specific structural components that are contribute to the signal responses observed *in vivo* [60]; helix bulge/loop; flanking structure and helix base pairs that modulate and shared features of the short RNA helix and terminal loop structure. Difference among the helix sequence, flanking structure and helix bulge/loop are associated with variation in IRP binding *in vitro*, and changes in the protein synthesis *in vivo* [60]. The helix distortion, which surrounds the conserved C residue, influences the binding of IRP2 much more than IRP1. In a large group of IRE RNAs, mitochondrial aconitase, erythroid aminolevulinate synthase, ferroportin, and DMT1, and in isolated transferring receptor 1 IREs, the unpaired C is a bulge in the helix that makes a relatively weak IRP2 recognition site. When the unpaired C is separated from an unpaired U by a G-C pair, as in ferritin IREs or in a loop induced by an AU rich and linked to four other IRE structures by sequences with conserved motifs, IRP2 binding is enhanced [61]. Interactions of IRE-RNA/IRP are more stable than typical enzyme-substrate complexes. Sequence comparisons of different animal IRE-RNAs and footprinting reveal that the IRP binding site is 30 oligonucleotides long. The importance of helix structure around the unpaired C8 is illustrated by the effect of deleting a single unpaired U6, characteristic of ferritin IRE RNA, which decreases the stability of the IRP1 and IRP2 complexes significantly, with the consequence of decreasing translational repression [17,62]. IRE RNA flanking sequences serve an important role in ferritin mRNA. The length of the base paired flanking region differs among different ferritin IRE RNAs. Binding of ferritin IRE RNA with IRP increases the helicity of the flanking regions, showing the conformational change between IRE RNA and the flanking region helix [20]. Changes occurred on IRE RNA structure after binding with IRP. The residues A15 and G16 of terminal loop were extruded from the IRE RNA molecule and extended into a large protein cavity, with U17 completing the helix stack (Figure 2). However, A15 was stacked over the C13-G18 base pair, and G16 with U17 were disordered in solution. Second RNA binding site was unpaired C8 in the helix, which was disordered in free RNA in solution [16,63]. RNA was extended completely away from the stem and inserted into a protein pocket, however unpaired U6, which was stacked in an RNA cavity of the free RNA, was flipped out toward the protein surface in the IRP1 complex (Figure 2). The correct sequence and structure of IRE RNA stem-loop is important in human genetic disorder such as hereditary hyperferritinemia-cataract syndrome, which arises from point mutations or deletions within the IRE or flanking regions in the ferritin 5'UTR. Mutations clustered in the terminal loop and within the helix loop of ferritin IRE RNA affect IRP binding.

Binding of Iron Responsive Element (IRE)-RNA to IRP

Binding of Iron regulatory protein with target mRNAs regulates key aspects of cellular iron metabolism. IRP1 is a unique protein. IRP1 Not only binds to IRE RNA, but also binds to [4Fe-4S] cluster confers cytoplasmic aconitase activity. IRP protein that selectively recognize IRE-mRNA structures are aconitase homologues. The

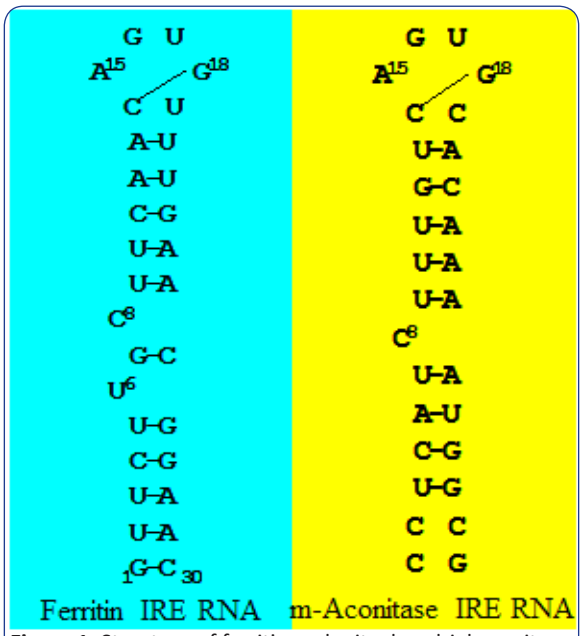

Figure 1: Structure of ferritin and mitochondrial aconitase IRE-RNA

IRP effect is generally related to the position of the IRE in the mRNA, with IREs near the 5' cap repressing ribosome binding, and IREs after the mRNA coding region and stop codon repressing mRNA degradation. All members of the aconitase family have a four domain organization. The first three domains of typical mitochondrial aconitase are in tight association to nestle the Fe-S cluster, while the fourth is bound through a long amino acids (~60) linker. Binding of IRE RNA creates conformational changes on IRP1 (Figure 4). IRP1 protein molecule adopts an L-shaped conformation after binding to IRE RNA, and RNA is bent. IRP1/IRE RNA complex is achieved with two widely separated contact sites [16].

In the complex, the RNA is inserted between protein domains 1–2 and 4 [16], using the numbering of aconitases. Aconitases are globular structures with close interactions among all four domains. IRE RNA stem loop is tethered across the concave opening of IRP1, with direct contacts from the IRE RNA terminal loop and the lower stem. Exposed terminal loop residues A15 and G16 extend into an IRP protein cavity at the interface between domains 2 and 3. A15, G16, and U17 bonds provide the stability of the IRE RNA/IRP complex. IRP tethering to the stem of the IRE RNA is centred on the C8 oligonucleotide, which inserts into a pocket on the inner face of domain 4. Bending of 31° in the IRE RNA redirects the path of the lower stem along the same face of domain 4, which binds the side of the IRE RNA lower stem farthest from the terminal loop (Figure 5) [7,16]. The terminal loop and C8 of the IRE RNA are separated by 30 A°, held in their orientations by the 5 base pair helix of the upper stem. IRE RNA and IRP protein held together by twenty-two bonds, 10 contacts are formed between A15 and G16 in the pseudotriloop at the IRE RNA terminus and amino acids, such as residues Ser371 and Lys379 in a pocket generated

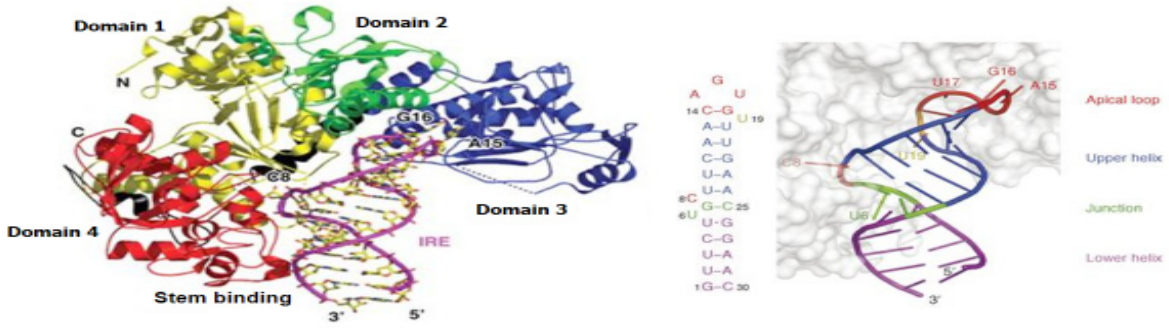

Figure 2: Structure of ferritin IRE-RNA complex with IRP1 (Modified from Ref 14, 63)

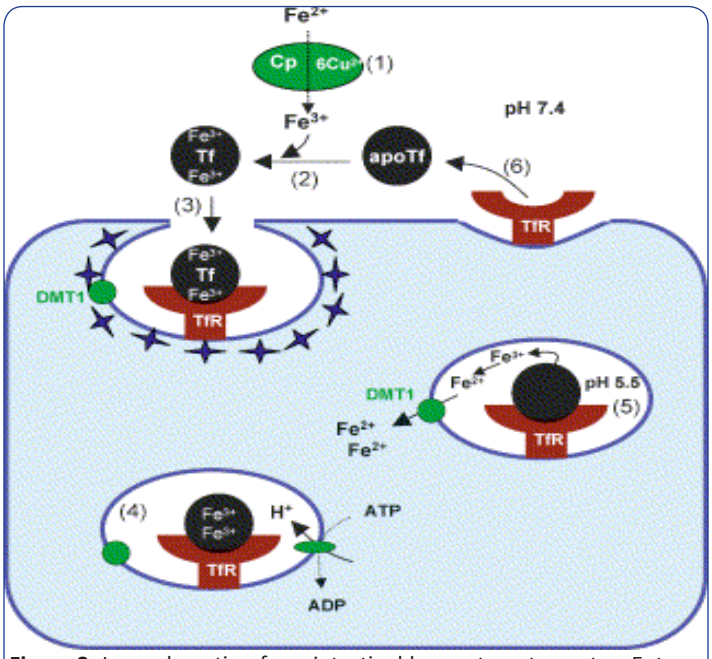

Figure 3: Iron adsorption from intestinal lumen to enterocytes. Enterocytes absorb inorganic or heme iron from the diet by DMT1 and excrete iron through ferroportin protein. Iron is reoxidized into ferric form in the body plasma and binds to Tf(Ref 45).

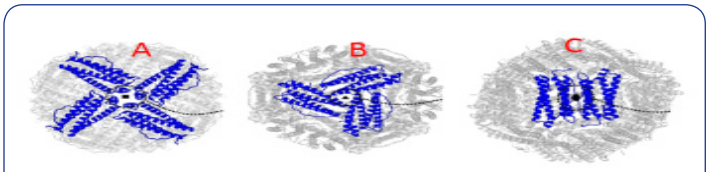

Figure 4: Ferritin molecular symmetry. The whole ferritin shell is represented with the subunits participating in the formation of the (A) four, (B) three and (C) twofold pores (Ref 50).

in domain 3 at a site that is blocked by domains 1 and 2 in the globular form [16]. Eight bonds are formed between IRE-RNA C8 and amino acid residues Arg713 and Arg780 in Domain 4 of IRP1; four additional bonds occur between amino acids Ser681, Pro682, Asp781, and Trp782 in domain 4 of IRP1 and the IRE stem below C8. In addition to flipping out of terminal loop bases A15 and G16 and helix bulge C8, [63] in the IRP/IRE-RNA complex, the IRE-

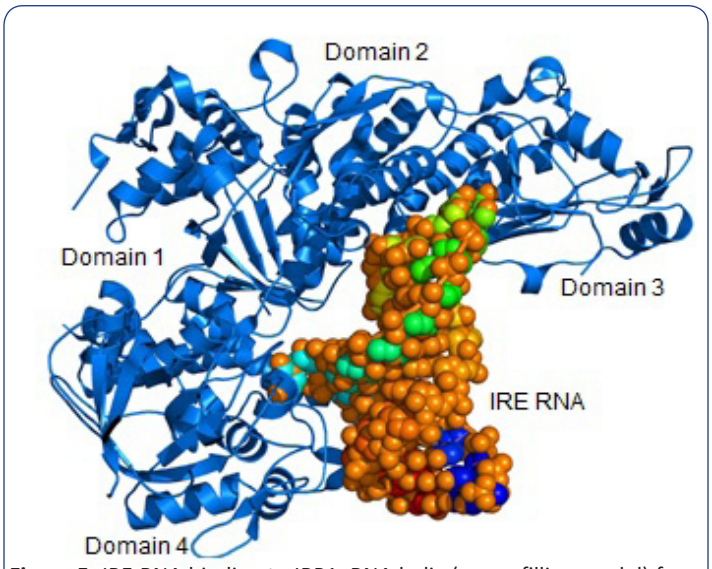

Figure 5: IRE-RNA binding to IRP1. RNA helix (space-filling model) from the Crystal structure ofIRP1:IRE RNA complex (Modified from Ref 14).

RNA backbone is distorted by a sharp midhelix turn, unpaired U19 in the hexaloop is flipped out of the IRE RNA helix, and unpaired U6 is tucked into the minor groove. The differences between the solution structure of free IRE-RNA and IRP protein tethered IRE-RNA require conformational changes in the RNA, and likely of the unliganded protein; while the structure of apo-IRP is not yet known, it is more disordered than in either the RNA or [4Fe-4S] complexes [64]. Crystal structure of the IRE RNA/IRP complex [16,63], a large surface of the IRE-RNA is exposed free for the interactions with other metal ions, RNA and proteins.

The conserved structure of the IRP contact sites in IRE-RNAs, the terminal loop and bulge C, means that differences in IRE-RNA/IRP complex stability must depend on structural differences in helices. IRP binding affinity is altered by increasing the length of the upper stem or by disrupting the helices above and below the C bulge [65]. Natural variations, which occur in helix base pairs of IRE-containing mRNAs coding for different proteins, coincide with quantitative differences in IRP binding affinity and the magnitude of the iron response *in vivo*. It is as if nature has created a set of graded dimmer switches in the IRE family, using helix sequences

that vary as much as 36%, rather than an on/off switch, so that IRE-mRNAs can have a range of sensitivities to iron. Ferritin and mitochondrial-aconitase IRE-mRNA differ by at least an order of magnitude *in vivo* response to iron levels and show the largest differences in IRP1 binding in solution [22]. The relative affinity of ferritin IRE-RNA and aconitaseIRE-RNA with IRP1 differ by 9-fold whether by mobility shift in gel electrophoresis or fluorescence quenching in solution; the picomolar binding constants from gel shifts contrast with nanomolar binding constants for solution fluorescence; this difference may be caused by an adsorptive component in the gels. Ferritin IRE-RNA with the additional U6 bulge [22] binds IRP the most tightly, and may reflect faster kinetics of helix bending during protein binding. Ferritin IRE-RNA conformation changes when IRP1 binds, based on comparison of solution nuclear magnetic resonance of the free IRE-RNA, the crystal structure of the ferritin IRE-RNA/IRP complex [16,21] and fluorescence of 2-aminopurine IRE-RNA [1]. In the IRE-RNA terminal loop, for example, conserved tri-loop bases A15 and G16 and helix bulge base C8 are flipped out and a large surface of the IRE-RNA remains exposed in the RNA protein complex (Figure 5) [16], even though IRP foot-prints indicate protection of the entire IRE-RNA [20]; IRE-RNA folding must create protected/solvent inaccessible regions of the IRE-RNA structure. Important as IRE RNA/protein binding equilibrium are, cell must respond rapidly to changes in metabolism and the environment, which makes IRE-RNA/IRP1 turnover kinetics likely to be a more sensitive regulatory target. Recently [7] we have shown the kinetics of IRE-RNA binding to IRP. Ferritin IRE RNA showed faster association than the aconitase IRE RNA for the binding of IRP1. At low metal ion concentration, IRE-RNA/IRP1 binding is fast and turnover of the complex is rapid.

Iron Responsive Element (IRE)-mRNA/IRP Regulatory System

As an important protein for cellular iron homeostasis, ferritin protein expression is controlled at the translational level by changes in iron concentration. A hairpin structure which is located in the 5' noncoding region of ferritin mRNA has been identified, responsible for iron regulation of ferritin mRNA translation, and named it as iron responsive elements (IRE) [31].The ferritin IRE has 30 nucleotides containing a 10 base pair stem with bulges and CAGUG terminal nucleotides. The unpaired C8 bulge interrupts the stem and divides the helix into upper stem and lower stem. In the CAGUG terminal nucleotides, C: G pair up and form the –AGU-loop [16]. Identification of the IRE structure leads to a protein binding to the IRE structure, the IRP [66]. IRP binding to the IRE in ferritin mRNA inhibits ferritin protein synthesis. IRP/IRE binding affinity changes with cellular iron level fluctuations, which leads to ferritin protein synthesis responding to iron levels. IRP binds to ferritin IRE with high affinity at lower iron level and ferritin protein synthesis is inhibited which answers cellular needs of less iron storage protein. Whereas IRP binds to ferritin IRE with low affinity at high iron level and ferritin protein synthesis starts which is required by iron concentration elevation. The coordination between IRP/IRE binding affinity change and ferritin protein synthesis stabilizes cellular iron.By using nuclease protection analysis, Beard, et al. [67] found IRP binding spans the entire 30 nucleotide ferritin IRE structure. The ferritin IRE RNA terminal loop and lower stem directly contact with the IRP protein. The A15, G16, U17 and C8 are the key nucleotides for IRP binding to ferritin IRE RNA [63]. NMR revealed that bound ferritin IRE RNA has significant conformational changes compared to free ferritin IRE RNA. In free ferritin IRE RNA, A15 stacks on top of C14:G18 pair whereas in the protein-RNA complex A15 and U16 stack together and stick out from the rest of the IRE RNA structure. Unlike the distorted C8 in unbound ferritin IRE RNA, C8 is also extended away from the stem (Figure 2 and 5) [7,16]. After identification of the IRE in ferritin mRNA, IRE structures were also found in other mRNA encoding proteins for cellular iron metabolism such as mitochondrial aconitase, ferroportin and succinate dehydrogenase. In addition to the 5' noncoding regions, IREs are also located in some mRNA's 3' noncoding region such as divalent metal transporter1and transferrin receptor protein 1. In contrast to 5' noncoding region IRE mRNA inhibition of translation by IRP binding to IRE, IRP binding to the 3' noncoding region IRE has positive effects on mRNA stability. By binding with 3' noncoding IRE, IRP can inhibit mRNA degradation from the exosome, which increases the life time of the mRNA. IREs located in the 3' noncoding region have opposite effects on mRNA translation after binding with IRP, comparing with the IREs located in the 5' noncoding. But the results of cellular iron level are identical. IREs are found in the3' noncoding region mRNA encoding proteins for iron acquisition and IREs are found in the 5' noncoding region mRNA encoding proteins for iron storage or utilization. At low iron concentration, cells need to transport extracellular iron into the cell which requires more iron acquisition protein (active translation by IRP/IRE binding). At the same time, there is no need to synthesize iron storage or utilization proteins which have 5' noncoding region IREs (repress translation by IRP/IRE binding). At high iron concentration, the requirement is reversed. What cells need now is iron storage or utilization proteins (translation activation) not iron acquisition proteins (translation repression) (Figure 6) [22,68]. By coordination of IREs, cellular iron concentration is stable at a constant level.

IRE RNA is not a conservative element; IREs have 36% to 85% sequence difference even in a single organism. However, all IREs have conserved structures: 9 to 10 base pair double stand RNA helix, terminal –CAGUGU/C- nucleotides and an unpaired C8 residue in the helix. Recently, IRE RNA structures are also been found in the amyloid precursor protein mRNA [67] and alpha synuclein mRNA [69], which implies iron plays a role in neurodegenerative diseases. Despite highly diversified IRE sequence, IRP can bind to all IREs. There are two types of IRP in the cell. IRP1 belongs to the cellular aconitase family which catalyzes the isomerization of β- hydroxyl-acid metabolites. Active aconitases require a Fe-S cluster as a coenzyme. The IRP1 is the apoenzyme of cellular

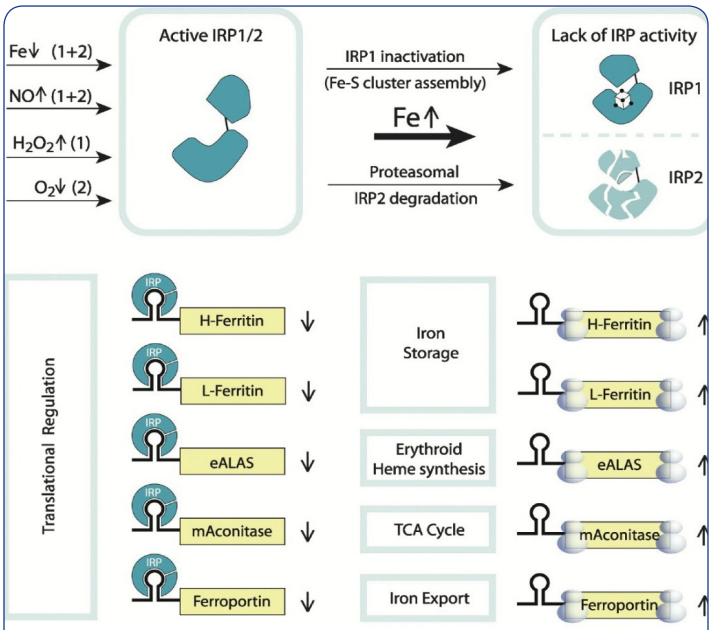

Figure 6: Iron Responsive Protein (IRP) and Iron Responsive Elements (IRE) RNA Regulatory System. A representative model of iron-induced translation of 5'-UTR IRE RNAs (modified from Ref. 20, 68).

aconitase which only binds to IRE without the Fe-S cluster. IRP1 is composed of 4 domains. Domain 1 and 2 are stable and form the core of IRP1 which is responsible for aconitase activity. When IRP binds to IRE conformation changes occur in domain 3 and 4. The domain 3 rotates 52° and translates ~ 13 A° and domain 4 rotes 32° and translates ~14 A° [16]. IRP2 has 60% sequence identity to IRP1 which doesn't bind Fe-S and doesn't have aconitase activity. The unbound IRP2 readily degraded. IRPs bind to IREs located in either the 5' or 3' noncoding region of IRE-mRNAs. At low iron level, IRPs bind to both 5' IRE and 3' IRE with high affinity. IRE-mRNA translation is repressed to inhibit iron storage and utilization protein synthesis such as ferritin. And 3' IRE-mRNA translation is activated to synthesize iron transporter protein such as TfR1. At high iron level IRPs do not bind to IREs. 5' IRE-mRNA translates into protein such as ferritin to lower iron level. The 3' IRE-mRNA is degraded to inhibit iron uptake (Figure 6).

Effect of Metal Ions on the Binding of IRE-RNA/IRP Regulation

Metal ions play important role in the regulation of IRE RNA-IRP. Iron effects on protein biosynthesis *in vitro* mimic the effects of increasing cellular iron concentrations *in vivo* or in cultured mammalian cell models. Studies suggested that the direct iron-IRE RNA binding as the biological mechanisms of iron dependent regulation of IRE RNAs. Messenger RNAs encoding proteins of iron traffic and oxidative metabolism are more active when cellular iron increases. Metal ions effects the function of many RNA such as, rRNA, tRNA, ribozymes [23,25,70], riboswitches in bacterial mRNAs, where metals contribute both to RNA function and to metal sensing [71,72], and possibly hammerhead, mammalian mRNAs [29]. Changes in translation of the iron responsive messenger RNAs, dependent on noncoding structures (IRE), are currently attributed entirely to iron effects on the protein repressors, IRP1 and IRP2, that bind IRE-RNAs and which are degraded or modified by increases in cell iron [9, 11]. IRE-RNA binds metal ions at specific sites [17] (Figure 7) [22] as do rRNAs, tRNAs, riboswitches and ribozymes. Metals alter messenger IRE-RNA/IRP complexes directly. Khan et al (2009) [22] had choose two IRE-RNAs to compare, ferritin and mt-aconitase, because they varied in structure at site of metal binding to ferritin IRE-RNA and because they have quantitatively different responses to the same iron signal *in vivo* (rats) [73]; the ferritin-IRE evolved before the mitochondrial- aconitase IRE [8]. It has been reported that IRP1 binding distinguishes among different IRE structures and that IRE RNA /IRP complexes of the two messenger RNAs are selectively destabilized by Fe (II), Mn (II) and Mg (II) [22]. Ironbinds to RNA and changes interactions with the repressor protein, IRP, and RNA conformation [7]. Iron activates ferritin mRNA in cell free protein biosynthesis studies [1,22]. Direct binding of iron to IRE RNA was measured in solution as changes in the fluorescence of IRE-RNA ethidium bromide complexes, the conformational changes in IRE RNA are independent of the conformation changes in IRP. The addition of iron chelators, exemplified by recent study of the Alzheimer amyloid precursor protein IRE, illustrates the effects of changing iron concentrations: cells exposed to the iron chelator desferrioxamine increased binding of IRP1 to the IRE-RNA [74], as observed for many IRE-RNAs in a variety of cultured cell types [75]. We have observed that ferrous ions weakened IRE-RNA/IRP1 complexes [22]. The magnitude of the metal ion effects varies with individual IRE-RNA structures. Such as, mitochondrial aconitase IRE-RNA/IRP1 binding decreased approximately 5-fold while ferritin IRE-RNA/IRP1 binding affinity decreased approximately 10-fold over the concentration ranges used (Figure8) [22], indicating the impact of nature's modulation of riboregulation among IRE-RNAs. Metal ions bind directly to the IRE-RNA based on ethidium bromide displacement effects on NMR spectra, binding of metal complexes [17] and the absence of predicted metal ion binding sites on IRP beyond the [4Fe-4S] cluster insertion site. Khan et al shown that metal ion effects were reduced when the ferritin IRE-RNA bulge U6 was deleted [22] and the earlier studies of Cu-1,10-phenanthroline and modelling of Co(III) hexamine binding [17] support the idea of the IRE-RNA midhelix bulge as the binding site of regulatory metal ions. Bulge bases are metal ion binding sites in other RNAs [29]. The metal selectivity of iron on IRP binding to IRE-RNAs indicates that iron is the physiological signal that targets the IRE-RNA riboregulator to increase translation of the IRE-mRNA and decrease IRP binding [19].

Metal ions weakened the IRE-RNA/IRP1 interactions for both ferritin and mitochondrial aconitase mRNAs. The ferritin IRE-RNA/IRP1 complex was more stable than the mitochondrial aconitase IRE-RNA complex in solution, illustrating the effects of phylogenetically conserved differences [8] between the two

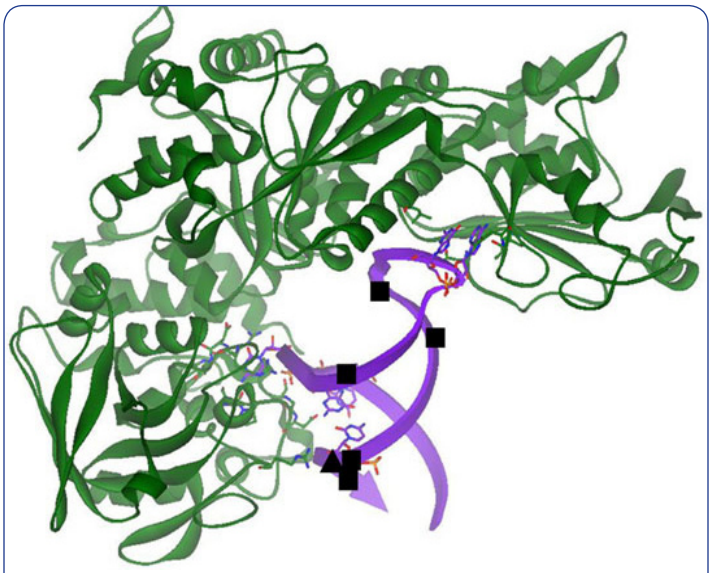

Figure 7: IRE RNA sites influenced by metal binding.The three dimensional crystal structure of the ferritin-IRE/IRP1-complex, modified to show metal sites.■-Mg(II) determined by solution by NMR;▼ 1.10-phenanthroline- cleavage sites; green-protein; purple-RNA. Figure of the RNA/protein complex was taken from ref. 20.

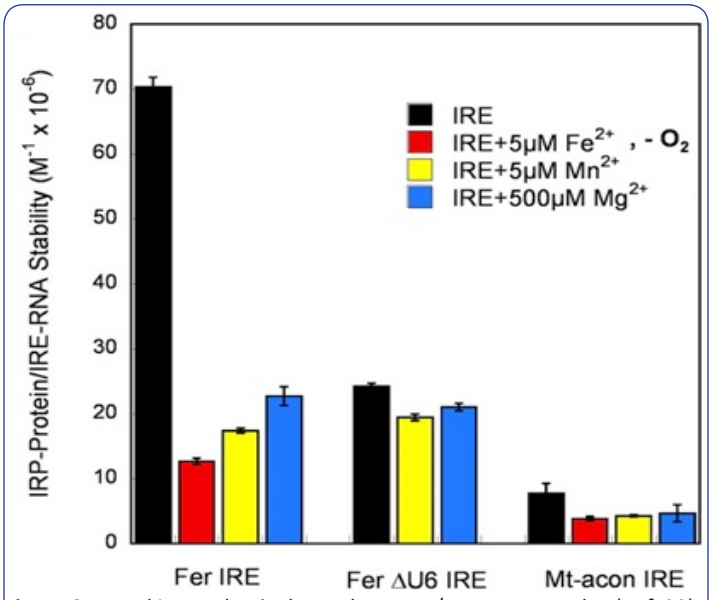

Figure 8: Metal ions selectively weaken IRP1/IRE RNA complex (Ref. 20).

IRE-RNAs, as well as quantitating qualitative binding differences observed as the amount of protein required to alter the mobility of IRE-RNA during gel electrophoresis EMSA [62,76] and can be related to stronger ferritin synthesis response iron *in vivo* [73]. The dissociation constant for the ferritin-IRE RNA/IRP1 complex was smaller than for the mitochondrial aconitase IRE RNA/IRP1 complex (Figure 8). The different dissociation constant indicate that a larger fraction of mitochondrial aconitase mRNA will be "free" of IRP, or derepressed, than ferritin mRNA even when iron concentrations are low, Thus, a relatively larger fraction of mitochondrial aconitase mRNA molecules will be translated constitutively, and the relative effect of increased iron on mitochondrial aconitase mRNA will be smaller than on ferritin mRNA, as observed in animal experiments [73]. The effects of metals were larger for the ferritin-IRE/RNA, indicating that metal ions recognize different IRE-RNA/IRP interactions. Metal ions induced destabilization of the RNA/protein complexes was significantly larger for wild type ferritin and mitochondrial aconitase IRE RNA structures than for the mutant ferritin U6 IRE-RNA [22]. Differences between the ancient ferritin-IRE and the more modern mitochondrial aconitase IRE-RNAs [8] in metal responses could include selective effects of metals. Metal ion destabilization of the IRE-RNA/IRP1 complexes competes with the stabilization conferred by the very large number of bonds between the protein and the RNA [16] and the stability of the IRE-RNA fold [77]. RNA/protein destabilization more likely reflects metal ion binding to the RNA or the RNA/protein complex rather than the protein for the following reasons. First, direct metal binding to selective IRE-RNA sites is known [17]. Second, the only direct metal binding to IRP protein known is [4Fe-4S] cluster binding in IRP1, which confers aconites activity [64,78], and Cd or Zn-induced, sites of protein aggregation in crude cell extracts [79]; the molecular signals for iron-induced IRP turnover have not been identified. Third, when Fe(II) is added to IRE-RNA, the electrophoretic mobility changes [22]. How metal ions weaken the IRE/IRP complex is not clear from "foot printing"/nuclease protection experiments where the IRE-RNA was completely protected by the protein [59]. However, in a recent crystal structure of the ferritin IRE-RNA/IRP1complex [16], there are several IRE-RNA sites without protein contacts that could be accessed by solvent or metal ions; the location of known IRE-RNA metal binding sites [18] is shown superimposed on the crystal structure of the RNA/protein complex. Many molecular conformations are sampled in nuclease protections experiments, while only few conformers are sampled in crystals. The different IRE RNA/protein interactions, observed by the two methods, suggest there is more than one IRE-RNA/IRP conformation. Thus, even when IRP is bound to an IRE-RNA, some RNA sites in a fraction of the complexes, possibly kinetically controlled, can be exposed to metal ions that destabilize the IRE-RNA/IRP complex. Selective iron binding to different IRE-RNAs in IRP complexes as well as other selective, metal-RNA interactions [72], have well-known parallels in the selectivity of metal- protein interactions and emphasize that three-dimensional structural specificity of folded RNAs and proteins are shared. The lower stability and smaller response to iron of mitochondrial aconitase IRE-RNA/IRP repressor complexes compared to ferritin IRE-RNA/IRP repressor complexes observed in solution and gel mobility, and the smaller response of mitochondrial aconitase to iron, compared to ferritin in whole body experiments, illustrate novel features of the IRE- messenger RNA family. First, phylogenetically conserved variations in IRE–RNA structure that occur among members of the messenger RNA family, such as the U6 bulge in ferritin and the AUG initiator codon in mitochondrial aconitase IRE-RNA, contribute to differences IRP binding in solution and iron responses *in vivo*. Finally, iron indicates that iron can have a direct regulatory

effect on IRE-RNA/IRP complexes by weakening of IRE-RNA/IRP complex, complementary to iron effects on protein turnover or Fe-S binding and that IRE-mRNA senses iron ions and possibly other metal ions.

Perspective

Since the beginning of this century the IRP/IRE-RNA regulatory network emerged as the central system for the control of cellular iron homeostasis. This review highlighted the role of IRE-mRNA and IRP binding in the cellular iron homeostasis. Further, how the iron affects the IRE-mRNA/IRP binding and protein biosynthesis. The cellular and molecular mechanisms that regulate iron absorption, transport, storage and utilization are complex and interconnected. The regulation of iron homeostasis has revealed its increasing complexity and its interactions with a number of molecular pathways and processes. The work highlighted here has shown that significant advances have been made in elucidating the role of the IRE-mRNA/IRP in the post-transcriptional control of the key mRNA molecules involved in iron metabolism. IRE-mRNAs selectively bind iron and regulate the stability of the iron repressor protein, IRP, and protein biosynthesis. Iron selectively binds to IRE RNA and balance the iron in cellular system. Differences in the sequence of the each IRE-RNA helix base pairs modulate both IRP interaction and the iron binding, turning protein biosynthesis to *in vivo* environmental iron.

Iron directly bind RNA, and code a group of proteins that balance iron in animals.IRE RNA is a stem loop, which contains a trans-loop C-G base pair that creates a triloop, AUG. the bent RNA A helix contains an unpaired C bulge. Both the C bulge and the terminal triloop are contains contact points for the IRP and creates a different IRE-RNA-IRP binding stabilities. When free iron concentrations increase in cells, ferritin protein synthesis increases more than the housekeeping protein, because when iron concentrations were low, a larger fraction of ferritin IRE-mRNA were inactivated by IRP binding. As a result, ferritin protein lowers the free iron and increases IRP binding to ferritin mRNA RNA and decreases ferritin protein synthesis. IRE RNA regulation could alter in patients with iron overload from transfusion therapies of hemoglobinapathies, such as sickle cell anaemia, thalassemia, an increase in ferritin synthesis could be beneficial. The development of small molecules and/or drug that selectively disrupt ferritin IRE RNA/IRP interactions could be therapeutic potential. To better understand the iron regulation future experiments will need to characterize the mechanism of the involvement of the initiation factors in the translation process of protein biosynthesis.

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