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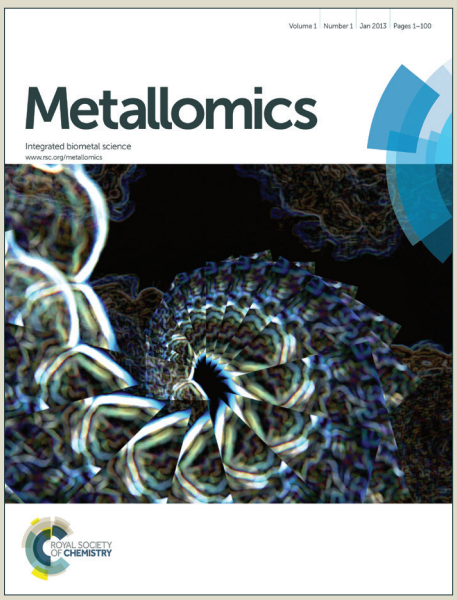





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# Iron regulatory proteins and their role in controlling iron metabolism

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***Summary***

Cellular iron homeostasis is regulated by post-transcriptional feedback mechanisms, which control the expression of proteins involved in iron uptake, release and storage. Two cytoplasmic proteins with mRNA-binding properties, iron regulatory proteins 1 and 2 (IRP1 and IRP2) play a central role in this regulation. Foremost, IRPs regulate ferritin H and ferritin L translation and thus iron storage, as well as transferrin receptor 1 (TfR1) mRNA stability, thereby adjusting receptor expression and iron uptake via receptor-mediated endocytosis of iron-loaded transferrin. In addition splice variants of iron transporters for import and export at the plasma-membrane, divalent metal transporter 1 (DMT1) and ferroportin are regulated by IRPs. These mechanisms have probably evolved to maintain the cytoplasmic labile iron pool (LIP) at an appropriate level. In certain tissues, the regulation exerted by IRPs influences iron homeostasis and utilization of the entire organism. In intestine, the control of ferritin expression limits intestinal iron absorption and, thus, whole body iron levels. In bone marrow, erythroid heme biosynthesis is coordinated with iron availability through IRP-mediated translational control of erythroid 5-aminolevulinate synthase mRNA. Moreover, the translational control of HIF2α mRNA in kidney by IRP1 coordinates erythropoietin synthesis with iron and oxygen supply. Besides IRPs, body iron absorption is negatively regulated by hepcidin. This peptide hormone, synthesized and secreted by the liver in response to high serum iron, downregulates ferroportin at the protein level and thereby limits iron absorption from the diet. Hepcidin will not be discussed in further detail here.

*Main text*

Iron is essential for all living organisms as iron-containing proteins play a central role in oxygen transport, electron transport, redox reactions, hydroxylations, and nucleotide biosynthesis, to mention just the most important ones. Most commonly, iron is incorporated into proteins after insertion into a porphyrin ring as a heme or together with sulfur in Fe-S clusters. Some proteins bind iron directly in pockets of their tertiary structure. Available iron in the cytoplasm is mainly divalent and potentially interacting with local counter-ions that are still poorly characterized. Citrate and thiol-containing compounds like glutathione and cysteine interact most likely with iron(III) and iron(II), respectively [1]. In addition iron(II) binds to iron chaperones poly(rC)-binding proteins (PCBP) 1 and 2 that facilitate its incorporation into ferritin [2, 3] and other non-heme iron-containing proteins [4]. By using calcein as a fluorescent probe, the "labile iron pool" (LIP) in the cytoplasm was estimated to have concentrations in the range of 1 μM [5]. This pool is still chelatable by compounds with iron chelating properties that cross the plasma membrane. It is thought to be at the crossroad between iron import and export, storage in ferritin, and transport into mitochondria or the nucleus where iron must be made available in sufficient quantity at sites of biosynthesis. The LIP is potentially harmful because of its ability to catalyze the formation of reactive oxygen species through Fenton chemistry. It is, therefore, essential for cells to control the homeostasis of the LIP, such as to ensure sufficient iron supply while limiting iron toxicity. In mammals, cellular LIP homeostasis is achieved by IRP1 and IRP2 by controlling iron uptake via transferrin receptor 1 (TfR1), iron export by ferroportin, and iron storage in ferritin.

*Initial discoveries*

Historically the discovery of IRPs started with the observation that ferritin protein levels are increased under conditions of high iron supply. Munro and coworkers found that rat liver induced ferritin biosynthesis within 2h after an injection of iron, independent of transcription and without any change of $^{14}$C-leucine incorporation into other liver proteins [6, 7]. They isolated liver polyribosomal and post-ribosomal fractions from normal and iron-loaded rats for mRNA *in vitro* translation in a wheat germ extract. They found that in normal rats 56% percent of the ferritin mRNA was on polyribosomes, whereas in iron-treated rats this fraction rose to 91% [7]. They concluded that iron stimulates the recruitment of ferritin mRNA to polyribosomes. With the advent of molecular gene cloning techniques they noticed that the translational regulation of both ferritin H and L mRNA depends on the presence of a conserved sequence with a hairpin structure in the 5'-untranslated region (UTR) [8]. This was independently confirmed by others, who coined the term "iron responsive element" (IRE) for this hairpin [9]. Leibold and Munro (1988) found next in gel-retardation assays that the IRE binds to a cytoplasmic protein that carries today the name of IRP (called IRE-BP or IRF in early literature)[10], a discovery again rapidly confirmed [11]. As shown later, IRE-bound IRP on the 5'-UTR of ferritin mRNA prevents the small 43 S ribosomal subunit from binding to the translation initiation complex and scanning to the initiation codon [12]. As will be discussed further, iron inactivates the IRE-IRP interaction and thus liberates blocked mRNA for translation, a finding that explains entirely the early results in rat liver.

About at the same time, Owen and Kühn (1987) showed that TfR1 protein expression changed inversely with iron levels in cell cultures, independent of transcription [13]. This

effect requires the presence of a strongly conserved structured region in the TfR1 mRNA 3'-UTR and is due to changes in mRNA stability [14]. Iron deprivation induces mRNA levels 10-fold within 15h, while adding iron salts back to culture medium provokes TfR1 mRNA decay with a 2h half-life. Grafting the 3'-UTR instability region to another mRNA renders this mRNA iron-dependently unstable. Subsequent work identified 5 IREs in the instability region, each of which binds IRPs with a similarly strong affinity as the ferritin IREs [15, 16]. The IRP binding activity is induced by iron chelators within 12h and rapidly inactivated by iron addition to the medium. It led to the conclusion that IRP binding is responsible of preventing of TfR1 mRNA degradation. Upon UV-crosslinking with a $^{32}$P-labelled IRE two proteins of about 100 and 110 kDa, today known as IRP1 (gene ACO1) and IRP2 (gene IREB2), were identified [15].

*IREs in various mRNAs*

Several mRNAs with IREs either in their 5'- or 3'-UTRs are now identified, strikingly all one way or the other connected to iron metabolism (Fig. 1). Besides in ferritin H and L mRNA, IREs are also present in the 5'-UTR of erythroid 5-aminolevulinate synthase mRNA [17, 18], mitochondrial aconitase mRNA [19], succinate dehydrogenase subunit b mRNA of Drosophila [20], ferroportin mRNA [21], and HIF2α mRNA [22, 23]. For all 5'-IREs translational regulation by iron was directly shown. However, in the case of ferroportin mRNA, a splice variant without the IRE, expressed in duodenum and erythroid cells, escapes this control [24]. Besides TfR1 mRNA, the divalent metal transporter DMT1 (SLC11A2) mRNA has a single IRE in the 3'-UTR of certain splice variants [25, 26]. This IRE like the ones of TfR1 mRNA confers increased mRNA expression after iron

deprivation [25], and mRNA expression decreases in intestine-specific IRP1 knock-out mice [27]. The IRE-IRP interaction probably results in mRNA stabilization as for TfR1 mRNA, but this was not directly shown. The effect on protein levels of DMT1 may also depend on the tissue-specific prevalence of splice variants without the IRE. Another IRE in a 3'-UTR was identified in human CDC42-binding kinase α (MRCKα, CDC42BPA) mRNA [28]. mRNA stabilization at low iron levels is thought to increase the kinase activity for myosin phosphorylation and thereby modulate transferrin endocytosis [29]. However, unlike TfR1 and DMT1 IREs that are present in all mammalian species and even chicken in the case of TfR1, the MRCKα IRE is only present in the human and absent in all others including chimpanzee. It suggests a very recent evolutionary acquisition, the functional importance of which does not seem to be general. Recent studies using microarrays for IRP-bound mRNAs identified additional 5'- and 3'-IREs in CDC14A mRNA [30] and numerous other potential target mRNAs [31], but their importance needs to be characterized further. Some *in vitro* IRE mutants identified by SELEX procedures showed high-affinity binding properties for IRP1 similar to native IREs, but were not found in natural mRNAs [32]. These mutants revealed a strong binding preference for loops in which residues 1 and 5 are base-paired, a feature confirmed by the 3-dimensional NMR structure of the ferritin IRE [33]. The NMR structure revealed further that conserved unpaired purines and pyrimidines at loop-positions 2 to 4 and the cytosine between the upper and lower stem are turned outwards providing specific contact sites for conserved amino acids on the surface of IRP1 as shown in the co-crystal structure [34, 35]. In view of these stringent features it is surprising that in amyloid precursor protein (APP) mRNA a 5'-IRE with an entirely different sequence and potentially very different folding was shown to have

strong *in vitro* binding properties for IRP1, but not IRP2, and was postulated to function as a translational regulatory element *in vivo* [36]. Another "odd" IRE was identified in the 3'-UTR of α-hemoglobin stabilizing protein (AHSP) mRNA and postulated to function in mRNA stabilization when IRPs are bound [37]. However, this IRE showed a poor *in vitro* binding to IRPs and evolutionary conservation restricted to simian primates. An IRE-like structure was also found upon *in vitro* selection in glycolate oxidase (HAO1) mRNA, but subsequently shown to be non-functional in 5'-UTR translational regulation [38]. Thus, defining physiologically relevant IREs requires both structural and functional criteria, among which evolutionary conservation helps to convince us of their pertinence.

*Regulation of IRP1*

The purification and subsequent cloning revealed that IRP1 is a cytoplasmic aconitase with strong similarities to mitochondrial aconitase [39, 40]. These enzymes insert a [4Fe-4S] cluster as part of their active site, which catalyses the transformation of citrate to isocitrate [41]. It immediately sparked the idea that IRP1 is a bifunctional protein with enzymatic activity when iron levels are sufficient to form the [4Fe-4S] cluster, but with an RNA-binding activity under conditions of iron deprivation. The idea proved to be correct as protein made *in vivo* in the presence or absence of iron had the expected properties and could be converted *in vitro* from the enzymatic form to the RNA-binding apo-protein [42] and from the apo-protein to the enzymatic form [43]. Moreover, mutants of all three cysteines that coordinate the [4Fe-4S] cluster resulted in a constitutively active RNA-binding protein independent of iron levels [44]. The fact that the aconitase form of IRP1 was inactive in RNA-binding was attributed to a closed conformation that would

prevent the IRE to find access to specific RNA-binding surfaces on the protein [42]. This idea is now entirely confirmed by the crystal structure of IRP1 first in its aconitase form [45] and subsequently complexed to the ferritin IRE [34]. Compared to the aconitase form, the RNA-binding form of IRP1 shows an opened structure with domains 3 and 4 rotated outwards giving rise to a large pocket that interacts with multiple sites on the IRE, most importantly with the conserved unpaired C and loop-nucleotides AGU at positions 2 to 4 [34].

*Feedback regulation of iron homeostasis*

As discussed so far, there exists a coordinate feedback control between the LIP level and proteins involved in iron storage, export and uptake (Fig. 2). This let me propose that "iron controls iron" [46]. Whenever the LIP drops below a certain level, iron becomes limiting to form the [4Fe-4S] cluster and IRP1 gets active in RNA-binding. Upon binding to IREs in various mRNAs repression of ferritin and ferroportin translation reduces the potential of iron storage and export while the repression of TfR1 and DMT1 mRNA degradation increases the potential of iron uptake. These effects are physiologically cumulative and tend to increase the LIP. As the LIP increases, it will reach a level at which the [4Fe-4S] cluster will be formed. IRP1 will be inactivated and the physiological effects inverted. In the end, without other outside perturbations, the LIP should reach a steady state that corresponds to the concentration needed to form the [4Fe-4S] cluster of cytoplasmic aconitase/IRP1. IRP1 can be considered as a natural sensor of the LIP and its activities control the LIP.

Measuring proteins that are influenced by IRP activity is a valid approach to obtain a view on tissue iron availability. It has found wide application in the clinics since about 1995 [47]. As a small fraction of newly synthesized ferritin is secreted and a fraction of TfR1 cleaved from cell surfaces, the assay consists in measuring the ratio of ferritin versus soluble TfR1 in the serum. A high ratio indicates iron overload and a low ratio iron deficiency. The other assays to assess IRP activity and the iron status are either gel retardation assays [10] or the use of IRE-reporter constructs that can be introduced into cell lines to assess translational repression.

The simplified model of feedback control introduced in Fig. 2 is largely incomplete without taking into account all participants. Firstly, as discussed below, there exists a second protein, IRP2, with similar RNA-binding properties as IRP1 but differently regulated, which largely contributes to controlling the LIP steady state. Secondly, the recently discovered iron chaperones may represent intermediate steps of iron incorporation into proteins with Kd values near the LIP concentration [2, 4]. Thirdly, based on research in yeast, it was proposed that Fe-S cluster biosynthesis takes mainly place in mitochondria and that cytoplasmic iron-sulfur cluster formation may depend on certain enzymes of the mitochondrial iron-sulfur cluster (ISCU) assembly machinery, notably the cysteine desulfurase complex Nfs1/Isd11 and a scaffold protein Isu1 [48]. It is thought that an intermediate sulfur-containing compound is exported from mitochondria by the ABCb7 transporter (equivalent of Atm1p in yeast). Then, final [4Fe-4S] cluster formation requires a certain number of cytoplasmic iron-sulfur assembly (CIA) factors [49]. It remains, however, debated whether in mammals cytoplasmic iron-sulfur cluster assembly depends on the mitochondrial pathway [50], as isoforms of critical ISCU enzymes were

found in the cytoplasm as a result of alternatively spliced transcripts. Needless to say that "sensing" the LIP during [4Fe-4S] cluster formation of cytoplasmic aconitase/IRP1 denotes a complex cascade to which both the mitochondrial and cytoplasmic iron availability may contribute. Finally, the scheme needs to take into account the effects of various oxygen species and signaling pathways on the formation and stability of the IRP1 [4Fe-4S] cluster [51].

*Influence of oxygen, NO and signaling pathways on IRP1 activity*

As previously reviewed in further detail [52] foremost oxygen, nitric oxide, maybe counter-ions, the redox potential and signaling pathways influence IRP1 activity and hence its capacity to influence iron metabolism. The effects are summarized in Fig. 3. The *in vitro* formation of a complete [4Fe-4S] cluster requires a reducing, oxygen-free environment [53]. Also, *in vivo*, its formation is favored by low oxygen pressure, as IRP1 RNA-binding activity is reduced in hypoxia, but increased in cells exposed to high oxygen pressure at various LIP levels [54, 55]. Moreover, the induction of NO-synthase activity in macrophages, or direct exposure of cells to NO and compounds that liberate NO, favor the RNA-binding form on expense of the aconitase form [56]. NO has the capacity of dissociating existing [4Fe-4S] clusters and revert the enzyme to the form with RNA-binding activity [57]. IRP1 in absence of the [4Fe-4S] cluster can form intramolecular disulfide bonds that inhibit RNA-binding [44, 58]. After exposure to NO, therefore, the natural reducing agents glutathione and thioredoxin are required to recover full RNA-binding activity [59]. In iron-deprivation, it remains debated whether the [4Fe-4S] cluster can reversibly dissociate and convert IRP1 to its RNA-binding form [60], or whether there is just a partial inactivation of

the aconitase by the loss of one Fe atom [57]. In our hands, the protein synthesis inhibitor cycloheximide retarded the appearance of IRP1 RNA-binding activity after addition of an iron chelator [15], suggesting that de novo synthesis of the apo-protein is required. Iron chelation reversed some of the enzymatic form to the RNA-binding form only after many hours. Similarly, cycloheximide had no influence on the [4Fe-4S] cluster formation in hypoxia, but inhibited the reappearance of the RNA-binding form during subsequent reoxygenation [54]. As a consequence, under normal physiological conditions, the regulation of IRP1 may take 12 to 24 h to adapt to changed iron supply. This does not exclude the possibility that under certain physiological conditions [4Fe-4S] cluster dissociation might be facilitated by NO or IRP1 phosphorylation [61]. IRP1 was reported to be phosphorylated by protein kinase C facilitating the [4Fe-4S] cluster dissociation [62]. It remains to be investigated under which natural conditions this signaling plays a role *in vivo* in IRP1 activation. Exposure of cells even for only 15 min to external but not internal $H_2O_2$, a somewhat artificial stimulus, activates IRP1 probably through intracellular signaling [63]. The precise mode of activation and the physiological importance of this observation remain to be established. In contrast to external application of $H_2O_2$, natural intracellular reactive oxygen species, as they appear in post-ischemic reperfusion, inactivate IRP1 [64].

*IRP2 and its regulation*

The existence of IRP2 was suspected on the basis of a related cDNA [40], the existence of double bands in IRP-IRE cross-linking assays and double bands in gel retardation assays with rodent extracts [15]. IRP2 was isolated and characterized as a biochemically distinct

protein [65, 66]. Human IRP2 shows a 57% sequence identity with IRP1, binds ferritin and TfR1 IREs with similar affinity as IRP1 and exerts similar regulatory effects (Fig. 2). However in high iron conditions, unlike IRP1, IRP2 is not transformed into an aconitase. A mandatory residue acting as catalytic base is missing, and it is uncertain that IRP2 inserts a [4Fe-4S] cluster. Instead, IRP2 is rapidly degraded in high iron conditions [66]. In addition, IRP2, unlike IRP1, is activated by hypoxia [55, 67], because low oxygen pressure prevents IRP2 degradation [68]. At high iron conditions and increased oxygen pressure, IRP2 is rapidly ubiquitinated and degraded by proteasomes [69]. Initial studies suggested that a 73-amino acid region of IRP2, which is absent in IRP1, was iron-dependently oxidized and targeted by a specific HOIL-1 ubiquitin ligase [70, 71]. Oxidation of specific residues including cysteines in this region correlated with increased intracellular heme-levels [72] and a direct interaction of heme with a heme regulatory motif in IRP2 was thought to be the cause of IRP2 degradation [71, 73]. However, the importance of these findings was put into question, as a deletion of the 73-amino acid region, mutation of specific cysteines, or RNA-interference against HOIL-1 did not abrogate iron-dependent IRP2 degradation [68, 74]. Instead a short portion in the C-terminal domain was required for iron-dependant proteasomal degradation [75]. Moreover, artificial IRP1 cysteine-mutants that are permanently unable to insert the [4Fe-4S] cluster were also found to be sensitive to iron-dependent proteasomal degradation suggesting that a shared structural feature of IRP2 and mutant IRP1 triggers degradation [76]. For clarity, it should be noted, that normal apo-IRP1 escapes degradation, presumably because apo-IRP is only prevalent in iron-depleted conditions that are highly unfavorable for this degradation pathway.

Only recently, the E3 ubiquitin ligase complex required for IRP2 degradation [77, 78] was

identified. One group searched for the ubiquitinase needed for IRP2 degradation using a siRNA screen [77]. The other group had identified an ubiquitin ligase with unknown function and searched to trap the interacting substrates with an appropriate F-box domain mutant [78]. Both groups identified a protein complex that consists of FBXL5, SKP1, cullin1 (CUL1) and RBX1. FBXL5 interacts directly with IRP2 and apo-IRP1 mutants in an iron-dependent fashion and increases IRP2 ubiquitination *in vitro* and *in vivo*. Most interestingly, FBXL5 is unstable and degraded by the proteasome when cellular free iron or oxygen concentrations are low. This involves the ubiquitin ligase HERC2 [79]. The iron- and oxygen-dependent stability of FBXL5 requires the presence of its N-terminal 199 amino acids. This region was predicted to fold into a hemerythrin-like domain that was previously not observed in vertebrates. Hemerythrin plays an important role in oxygen-sensing in certain bacteria by coordinating a diiron core that reversibly binds oxygen [80]. Mutation of predicted iron-binding histidine and carboxylate-containing residues showed that iron and oxygen are necessary for correct folding of the hemerythrin-like domain of FBXL5 [77, 81]. However, no direct interaction between the diiron center and oxygen could be demonstrated and the sensing of oxygen may involve a distinct mechanism that remains to be discovered [81, 82]. Iron and oxygen binding is not reversible but occurs early after biogenesis [82], which implies, as for IRP1 inactivation, a delay in the response to changes of iron levels. The regulation of IRP2 by FBXL5 is essential for the correct control of iron homeostasis. FBXL5$^{-/-}$ mice die *in utero* due to excessive iron accumulation, and iron-loaded mice with a conditional deletion of FBXL5 in the liver die from acute liver failure [83]. Additional deletion of IRP2 reversed the phenotype [83]. Since apo-IRP1 also interacts with FBXL5 [78], one wonders why IRP1 escapes protein

degradation. Maybe a swift [4Fe-4S] cluster insertion alters its structure rapidly, such that it is no longer recognized once FBXL5 becomes active.

In conclusion, FBXL5 appears to be yet another cellular sensor for free iron levels besides IRP1 (Fig. 2). However, the influence of oxygen on IRP1 inactivation and FBXL5 activity that results in IRP2 degradation is different. FBXL5 requires iron and oxygen for its stability due to a mechanism that remains to be elucidated. Only when both are at sufficient levels, will IRP2 be degraded [55, 67, 68]. This shows that iron sensing by this second iron center permits cells to respond to LIP changes over a wide range of physiological conditions. It may provide an explanation why two IRPs have appeared in evolution. At low oxygen conditions, IRP1 activity is more easily inactivated, even at low iron conditions, because the [4Fe-4S] cluster forms readily, while FBXL5 remains unstable and IRP2 undegraded (Fig. 3) [55]. At high oxygen concentrations, however, IRP1 stays more active at the same low iron condition, while IRP2 is more readily degraded. In splenic lymphocyte cell cultures, IRP1 reacts quite poorly to changes in iron levels at 3% oxygen concentrations, while IRP2 reacts readily. In contrast, at oxygen concentrations of 21% both IRPs react, but IRP2 is the less present [55].

Concerning the response to other cellular signals, IRP2 seems also to differ from IRP1 (Fig. 3). Unlike IRP1, IRP2 is rather inactivated by NO [84-86]. Possibly this is due to S-nitrosylation at specific cysteines [85] or internal disulfide bridge formation that was also demonstrated after induction of ROS [87]. The induction of protein kinase C by the phorbolester PMA has also an activating effect on IRP2 [88]. In contrast, a cell cycle-dependent phosphorylation event inhibits IRP2 activity transiently during the $G_2$/M phase

[89]. Exposure of cells to external $H_2O_2$ inhibits the degradation of IRP2 [90]. Again, the physiological significance remains to be established.

*Importance of IRP1 and IRP2 in vivo*

Several studies have analyzed the importance of IRPs in general iron physiology by mouse knock-out models [91]. Initial straight deletion of IRP2 showed increased iron staining and overexpression of ferritin, DMT1, and ferroportin in villus epithelial cells of the duodenum [92]. The mice also showed accumulation of iron in the brain with signs of neurodegeneration, including ataxia, tremor and mild locomotor dysfunction, particularly with advanced age [92]. This phenotype was less evident in a tissue-specific knock-out model [93] and provoked a discussion in which the initial authors demonstrated that the neurodegeneration was further accentuated by the additional loss of an IRP1 allele [94]. Neurons might become iron-depleted due to ferritin over-expression and increased iron storage [95]. Moreover, young IRP2$^{-/-}$ mice showed a mild microcytic anemia phenotype with a pronounced disregulation of ferritin, erythroid 5-amino-levulinate synthase and TfR1 levels in the bone marrow, accentuated by the additional loss of an IRP1 allele [95, 96]. In contrast, straight IRP1$^{-/-}$ mice showed a much less pronounced phenotype with a normal iron metabolism in most tissues except kidney and brown fat [97]. Thus under normal physiological conditions, IPR2 appeared to be more important than IRP1 for the response to changes in iron supply [97]. This is also highlighted by the fact that the regulator of IRP2 levels, FBXL5 is essential for iron homeostasis, but that its deletion can be compensated by the IRP2 deletion [83]. On the other hand, the response to NO mainly depends on the presence of IRP1, rather than IRP2 [86]. Moreover, the importance of IRP1

is highlighted by the fact that phenotypic changes were severely increased in IRP2$^{-/-}$ IRP1$^{+/-}$ compared to IRP2$^{-/-}$ IRP1$^{+/+}$ mice [98]. The presence of at least one functional copy of either IRP gene is essential, as a double deletion of IRP1 and IRP2 in the entire mouse is embryonic lethal [98]. A conditional deletion of IRP1 and IRP2 only in the liver provokes a severe phenotype of insufficient iron supply to mitochondria resulting in a lethal reduction of Fe-S and heme biosynthesis and an accordingly strong reduction of mitochondrial enzyme activity [99].

With the refinement of tissue-specific knock-out models and careful analysis of new target mRNAs it has become evident that the IRP network has not only local cell-specific effects, but influences also the total body iron status and hematological parameters. Notably, the specific deletion of both IRPs in intestine of mice by villin-Cre provoked high mortality in the first weeks of life [27]. IRE-containing mRNAs were as expected strongly deregulated showing strongly reduced TfR1 mRNA levels and increased ferritin H and L, as well as ferroportin protein levels. DMT1 protein levels were clearly reduced, with a 2-fold reduction at the mRNA level. Conditional deletion of the IRPs in intestine of adult animals showed a qualitatively similar change in these targets, with an additional transcriptional reduction of the DMT1 mRNA possibly via increased HIF2α translation [100]. Overall, the loss of intestinal IRPs provokes a failure to absorb adequate amounts of iron in spite of sufficient DMT1 and ferroportin expression [100]. This phenotype is due to a mucosal block presumably exerted by excessive expression of mucosal ferritin, which captures some iron on its way through the intestinal cell. The phenotype is opposite to the one observed in intestinal ferritin deletion, which increases iron absorption [101].

In erythroid cells, there is a coordinate control of protoporphyrin synthesis with iron availability through inhibition of erythroid 5-aminolevulinate synthase mRNA translation by active IRPs [17, 102]. The importance of this control is probably best demonstrated in the IRP2$^{-/-}$ mice that show a pronounced protoporphyria [95, 96]. The same mice also have a mild microcytotic anemia probably due to insufficient TfR1 expression [95, 96].

Finally, the translational regulation of the transcription factor HIF2α in kidney by IRP1 has recently revealed a remarkable IRP-dependent coordination between iron and oxygen metabolism. Discovered quite recently, the 5'-UTR of HIF2α mRNA comprises an IRE with an unusual sequence in the upper stem (Fig. 1) that is entirely functional and shows binding properties to IRPs similar to the ferritin H IRE with a preference for IRP1 [22]. HIF2α mRNA translation was found to be regulated by iron levels in cell lines [22]. The authors postulated that under hypoxia conditions when HIF2α is stabilized and active in erythropoietin (EPO) gene transcription, iron deficiency might attenuate HIF2α mRNA translation and thereby diminish EPO synthesis, such as to adjust erythropoiesis to reduced iron availability. This hypothesis is now established. It is confirmed that the HIF2α IRE binds preferentially to IRP1 rather than IRP2 [23]. Under normal iron conditions, this IRE-IRP1 interaction is diminished by hypoxia as compared to normoxia [23], presumably because the [4Fe-4S] cluster forms more readily at low oxygen concentrations and inactivates IRP1 [55]. Most importantly, in IRP1$^{-/-}$ mice, HIF2α mRNA translation is entirely derepressed and this provokes excessive EPO synthesis, which then induces excessive red blood cell counts with hematocrits rising up to 65% [103, 104]. The viscosity of the blood can endanger the cardiovascular system and contribute to sudden death from abdominal hemorrhage [103]. The mice suffer in addition from pulmonary

hypertension due to increased endothelin-1, another transcription target of HIF2α [103]. The phenotype is particularly pronounced in iron-deficient [103] and juvenile mice [104]. It demonstrates that IRP1 is essential to coordinate the mechanism of oxygen-sensing which induces HIF2α expression with an iron-sensing mechanism to control erythropoiesis.

*Outlook*

Our understanding of the cellular and systemic regulation of iron homeostasis has progressed tremendously over the past 15 years with the discoveries of hepcidin, iron transporters DMT1 and ferroportin, FBXL5, the IRP1-mediated control of HIF2α, and new knowledge on Fe-S protein biosynthesis. The role of IRP1 and IRP2 in mechanisms controlling iron homeostasis has been strongly extended by the use of knock-out mice to study their respective role in various tissues. As it turns out, there are good reasons for having two IRPs that are both necessary to fully control iron homeostasis under varying physiological conditions, low and high iron or oxygen supply, or inflammation among others. Iron being involved in such basic biochemical functions as oxygen supply, energy metabolism, DNA synthesis, detoxification of chemicals and many more, it is very rewarding to see that its regulation integrates with other basic regulatory pathways of metabolism. Most spectacular is probably the coordinate control of iron metabolism with oxygen metabolism. With the discovery of new potential IRE targets, maybe IRP1- or IRP2-specific ones, we can hope to find new unexpected connections of this kind. Numerous questions in the current models remain to be elucidated: the nature and control of basic assembly pathways to synthesize cytoplasmic and nuclear Fe-S proteins, the

nature of oxygen-dependent stability of FBXL5, and the potential role of signaling pathways, iron chaperons and other iron-interacting or oxygen compounds in the modulation of IRP activity. New knock-out models will further clarify the importance of each IRP in each tissue, and potentially elucidate the control of iron transport across epithelia other than intestine, like the blood-brain barrier or placenta. In terms of understanding iron metabolism, there is also a need to get a grip on transmembrane transporters of heme, be it in intestine, mitochondria or other membranes. This area shows promising new avenues [105] that will undoubtedly receive major attention in the coming years.

*Figure legends*

**Figure 1. Most studied IREs in mRNAs related to iron metabolism.** IREs present in different mRNAs show a conserved hairpin structure with an upper and a lower paired stem of nucleotides, at least one conserved unpaired C, and a 6-base loop sequence with conserved residues CAG(A,U)G(A,U,C), in which positions 1 and 5 are paired. The two IREs in DMT1 and HIF2α mRNA with an additional nucleotide in the upper stem bind better to IRP1 than IRP2 [22, 25]. All IREs are well conserved in vertebrates and for some genes found in insects, annelids [106] and snails [107]. They appeared first in metazoan ferritins indicating a long history of evolution [108]. Recently a number of potential new IRP-targets with IREs have been identified by mRNA co-immunoprecipitation with IRP1 or IRP2 and microarray analysis [31]. They are not listed here as they need further physiological characterization.

**Figure 2. Feedback mechanisms that control cellular iron homeostasis.** The scheme depicts IRP1 and IRP2, which are active as RNA-binding proteins at low LIP levels. By binding to IREs, they inhibit the translation or degradation of mRNAs encoding proteins required for cellular iron storage and import, thereby increasing the LIP. Once it has reached a sufficiently high concentration, labile free iron then contributes to the assembly of the [4Fe-4S] cluster that inactivates RNA-binding of IRP1. Concomitantly, insertion of a diiron center into a hemerythrin-like domain of FBXL5 renders this protein more stable such that it combines with additional subunits to form an E3 ubiquitine ligase complex, which then binds IRP2 and induces its degradation by the proteasomal pathway. The assembly of these two iron centers corresponds to an iron sensing mechanism, in which free iron acts on its own level through these elaborate feedback loops.

**Figure 3. Sensitivity of IRP1 and IRP2 to various compounds.** Schematic representation of the two cellular iron-containing clusters that sense iron in cells. The [4Fe-4S] cluster associates with IRP1 (gene ACO1) and inactivates its RNA-binding properties to generate a cytoplasmic aconitase. The diiron center of the E3 ubiquitinase subunit FBXL5, only in presence of sufficient dioxygen, stabilizes this protein such that the RNA-binding IRP2 is ubiquitinated and degraded in proteasomes. Various conditions promote or reduce the assembly of these clusters and accordingly the IRP1 and IRP2 activities indicated by upwards or downwards arrows.

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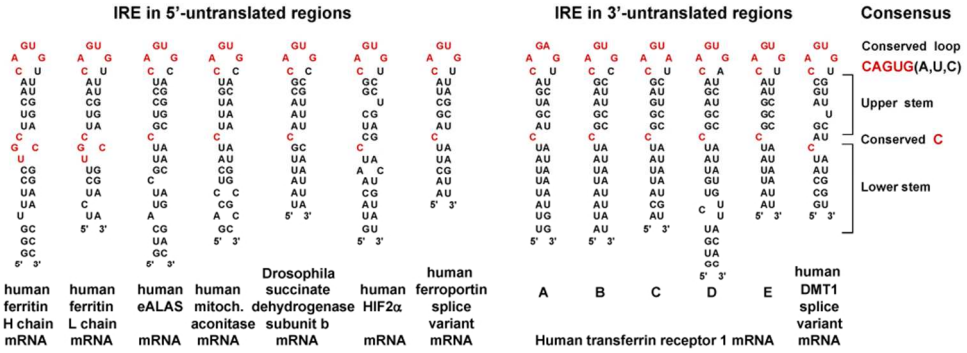


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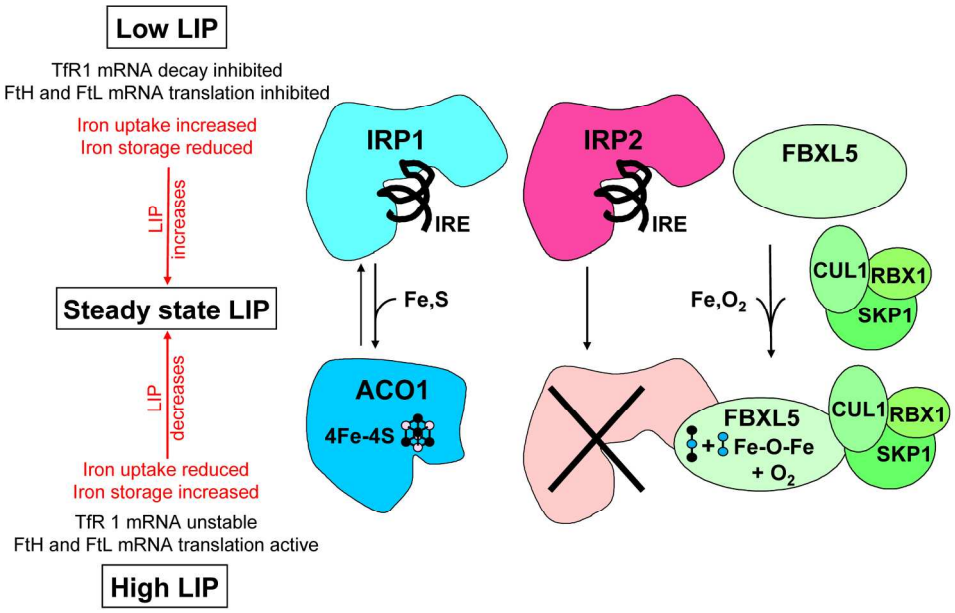


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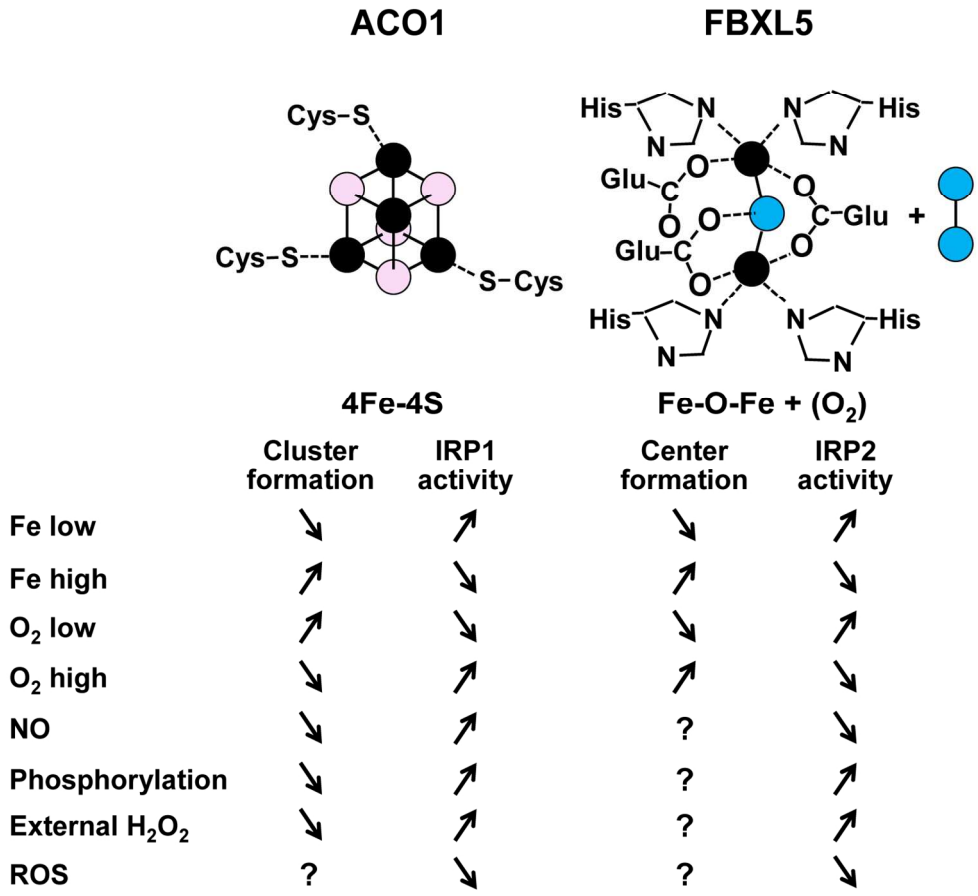


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