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Cellular sensing and transport of metal ions: implications in micronutrient homeostasis

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Abstract

Micronutrients include the transition metal ions zinc, copper, and iron. These metals are essential for life as they serve as cofactors for many different proteins. On the other hand, they can also be toxic to cell growth when in excess. As a consequence, all organisms require mechanisms to tightly regulate the levels of these metal ions. In eukaryotes, one of the primary ways in which metal levels are regulated is through changes in expression of genes required for metal uptake, compartmentalization, storage, and export. By tightly regulating the expression of these genes each organism is able to balance metal levels despite fluctuations in the diet or extracellular environment. The goal of this review is to provide an overview of how gene expression can be controlled at a transcriptional, post-transcriptional, and post-translational level in response to metal ions in lower and higher eukaryotes. Specifically, I review what is know about how these metallo-regulatory factors sense fluctuations in metal ion levels, and how changes in gene expression maintain nutrient homeostasis.

1. Introduction

A variety of transition metals including iron, copper, manganese, molybdenum, cobalt, and zinc, are essential for life [1, 2]. When bound to protein, these metals facilitate catalytic reactions and stabilize structural domains. Metals also have more specialized functions, including being intracellular secondary messengers and modulators of synaptic transmissions [3–6]. Although required for life, redox active metals such as iron and copper can catalyze the production of toxic free radicals [7]. Metal overload can also result in the wrong metal ion being incorporated into metalloproteins, which in turn can disrupt their function [1, 8–10]. To ensure that there are sufficient, but non toxic levels of metal ions for cellular metabolism, all organisms require mechanisms to tightly control metal levels and availability.

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To be able to maintain an optimal level of a metal requires that an organism is able to sense and adapt to fluctuating metal levels. The ability of an organism to sense metal ions, is largely dependent upon a class of metal-regulated factors that control the expression of genes involved in metal ion transport or storage. In eukaryotes, these types of factors control gene expression by regulating transcription, alternative splicing, translation, mRNA stability, protein activity, or protein stability (Figure 1). The goal of this review is to provide an overview of the mechanisms by which gene expression can be controlled at a transcriptional, post-transcriptional, and post-translational level in response to alterations in metal levels, and to discuss how changes in gene expression can allow cell to control metal ion distribution, levels, and expenditure.

2. Transcriptional control of metal-homeostasis

Transcription is the basic process by which an RNA copy is made from a gene sequence. Regulating transcription in response to metal deficiency or overload allows dynamic increases or decreases in gene expression. Additional advantages of transcriptional control include that a single transcription factor can regulate the expression of multiple genes allowing for the coordinate control of gene expression, while multiple regulatory factors can regulate the transcription of a single gene allowing for combinatorial control in response to different physiological conditions [11]. Although transcriptional regulatory mechanisms can affect the rates of transcriptional elongation and termination, the majority of studies in eukaryotic systems have so far focused on the regulation of transcriptional initiation by metal-responsive transcription factors.

2.1 Lessons from genetic model systems

Much of what we know about metal-dependent changes in transcription comes from studies of the unicellular organisms such as yeast and green algae. In these single-celled organisms, genes that are critical to metal ion homeostasis are robustly regulated at a transcriptional level in response to metal availability [9, 12–14]. The large transcriptional changes that are observed in these organisms have greatly facilitated the identification of genes important for metal homeostasis and have expedited further studies to determine how changes in the levels of these genes can affect metal uptake, storage, usage, and compartmentalization.

In lower eukaryotes, genes required for metal ion transport or metal ion storage are often tightly regulated at a transcriptional level (Figure 2). In general, as intracellular metal levels begin to drop below an ‘optimal’ concentration, most unicellular organisms increase the transcription of genes required for metal uptake and/or its release from intracellular stores. In contrast, when metal levels become too high, genes required for metal storage or export from the cytosol are transcribed. Through these coordinated changes in transcription, cells are able to continuously adjust cytosolic metal levels to maintain a concentration that is sufficient for normal cellular metabolism, but not inhibitory to cell growth.

Many unicellular organisms naturally live in ‘feast or famine’ environments. Studies of these organisms have therefore provided insight into how changes in gene expression can help cells to adapt and survive longer periods of metal ion starvation or exposure. Genes that are induced under these conditions include those that protect cells against the toxic

conditions that may arise from metal excess or deficiency [15, 16]. Transcriptional changes in response to metals can also remodel core metabolic pathways or metal-requiring processes to conserve or use metals. As an example, alcohol dehydrogenase 1 (Adh1) is one of the most abundant zinc-binding proteins in yeast. In both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, transcriptional mechanisms are present which reduce *adh1* gene expression when zinc is limiting [17, 18]. As these mechanisms lower the levels of the Adh1 protein, this strategy helps to conserve zinc for more essential functions. A different metal conservation mechanism is illustrated by the Cth1- and Cth2-mediated degradation of mRNAs in *S. cerevisiae*. Cth1 and Cth2 are RNA binding proteins that promote the degradation of mRNAs encoding iron-binding proteins or enzymes critical to iron-dependent processes [19, 20]. A critical regulatory aspect of this Cth-mediated RNA turnover is that *CTH1* and *CTH2* are induced in response to iron deficiency by the iron-responsive transcription factor Atf1 [20]. Thus, by increasing the expression of *CTH1* and *CTH2* in low iron, Aft1 is able to indirectly control the levels of a large number of mRNAs that are involved in iron metabolism thereby adjusting iron utilization according to the cells iron status.

An important aspect of metal homeostasis is determining how each of the metal-responsive transcription factors ‘senses’ an increase or decrease in a specific metal level. Although homologs of the metal-responsive transcription factors found in unicellular organisms are often not found in higher eukaryotes, studies of metal-responsive transcription factors from yeast and algae have significantly advanced our understanding of how metal ions are sensed, the types of domains that can be use to sense metal levels, where in a cell sensing occurs, whether additional proteins and metabolites facilitate the detection of metals, and what other cellular and environmental factors influence sensing.

In *S. cerevisiae*, two partially redundant transcription factors named Aft1 and Aft2, activate gene expression in response to iron limitation [21]. An important breakthrough in understanding how Aft1/2 sense iron was revealed in a genetic study examining the effect of mitochondrial iron-sulfur cluster synthesis on intracellular iron levels. The study demonstrated that in the presence of mutations disrupting mitochondrial iron-sulfur cluster synthesis, cytosolic iron levels could be increased, but Aft1 always sensed that the cell was iron limited [22]. These experiments suggested that Aft1 does not directly sense changes in cytosolic or nuclear iron levels, but instead senses an unknown signal that is dependent upon the mitochondrial iron-sulfur cluster machinery. Consistent with this observation, iron sensing is now known to be dependent upon a mitochondrial transport protein Atm1 [23], and a protein complex containing Grx3, Grx4, Fra1, and Fra2 [24, 25]. Of these proteins, Atm1 is thought to export a compound from the mitochondria that is either used to build cytosolic iron sulfur clusters or facilitate their insertion into proteins, while Grx3 and Grx4 are monothiol glutaredoxins - a class of proteins that are typically involved in the biogenesis or transfer of iron-sulfur clusters [26]. The requirement of these additional proteins for the regulation of Aft1 activity suggests a model in which the mitochondrial iron sulfur cluster machinery produces a compound under iron-replete conditions that is transported into the cytosol by Atm1. This compound then triggers an inhibitory signal to Aft1/2 via a signaling pathway involving Grx3, Grx4, Fra1, and Fra2. In support of this model, recent in vitro

analyses have found that the Fra2 and Grx3 proteins are able to facilitate the transfer of a [2Fe-2S] cluster to Atf1 and Aft2, promoting their dimerization and dissociation from DNA [27–29]. While these biochemical studies hint that the transfer of a [2Fe-2S] cluster to Aft1 and Aft2 leads to their inactivation, the identity of the compound that is exported by Atm1 is still an important unknown. Based on biochemical approaches, suggested candidate molecules include GSSG, GSSSG, and a GSH-coordinated [2Fe-2S] cluster [30–32]. However, no study has yet demonstrated that these molecules are the relevant signal in vivo (reviewed by [30]). Another important untested part of the model is that the unknown compound transported by Atm1 is generated/transported under iron-replete conditions. Thus, future studies to determine the identity and function of this elusive compound are required to fully understand the mechanisms by which *S. cerevisiae* senses iron, and the associated role of the mitochondrial iron sulfur cluster machinery.

Iron sulfur clusters have also been implicated in the regulation of other iron-responsive factors. In *S. cerevisiae*, the transcription factor Yap5 induces the expression of genes required for vacuolar iron storage in response to high iron [33]. The ability of Yap5 to activate gene expression is dependent upon Yap5 binding multiple iron sulfur clusters [34, 35]. Thus, in budding yeast iron sulfur cluster synthesis is necessary to signal high and low iron status to a cell. In *S. pombe*, a different pair of transcription factors protects cells from iron deficiency and iron toxicity. When iron is in excess, Fep1 represses gene expression, and a regulatory complex containing Php4 lowers gene expression in response to iron limitation [36]. While Fep1 and Php4 share little sequence similarity to Aft1/2 and Yap5, inactivation of Fep1 in response to low iron in *S. pombe* is dependent upon Grx4 and Fra2, and inactivation of Php4 in response to high iron requires Grx4 [37–39]. The requirement of Grx4 in the regulation of Fep1 and Php4 highlights that iron-sulfur clusters likely play a central role in sensing high and low iron in fission yeast. Although, it is not yet known if mutations to the mitochondrial iron-sulfur cluster machinery or Atm1 affect iron sensing in fission yeast, cells lacking Yta12, a chaperone-protease involved in the maturation and turnover of mitochondrial proteins, accumulates iron but transcriptionally resembles an iron-starved cell [40]. The impaired iron homeostasis response in the *yta12* mutant suggests that mitochondria may also be critical to sensing iron in this organism. Iron sulfur clusters have also been implicated in sensing in higher eukaryotes. For example, in humans the ability of Iron Regulatory Protein 1 (IRP1) to sense iron is dependent upon the binding of an iron sulfur cluster under iron-replete conditions (see section 5). Even though the mechanism by which iron sulfur clusters inactivate or activate each iron sensor differs, the common requirement of iron sulfur clusters in relaying iron status to a cell suggests that at least some aspects of iron sensing are evolutionarily conserved in eukaryotes.

Studies of Zap1, a zinc responsive transcriptional activator found in *S. cerevisiae*, have provided insight into how a transcription factor can sense cellular zinc deficiency. Zap1 contains two transactivation domains designated AD1 and AD2 that are independently regulated by zinc [41]. Of these domains, AD2 is the most widely studied. A number of lines of evidence suggest that zinc binding directly to AD2 allow its activity to be regulated by intracellular zinc levels. AD2 contains two C_2H_2-type zinc fingers that fold together to form a zinc finger pair [42, 43]. In a wild-type cell, AD2 is active when zinc levels are low, and is

inactive when zinc levels are sufficient. However, when mutations are present that interfere with zinc binding or the formation of the zinc finger pair, AD2 is always active. These results indicate that functional zinc finger domains, and the ability to form a zinc finger pair, are both necessary for the inactivation of AD2 by zinc. An additional unique feature of the AD2 zinc fingers is that they readily exchange zinc with metal ion chelators in vitro, indicating that the zinc bound to the AD2 zinc fingers is kinetically labile [42, 44]. Together these observations are consistent with a model in which zinc will rapidly exchange from the AD2 zinc fingers to other ligands when zinc is limiting in vivo. As this would lead to a largely unstructured conformation, acidic amino acid residues critical to activation domain function would be accessible to the transcriptional machinery allowing gene activation. In contrast, when zinc is in excess, the zinc occupied domains would fold together to form the zinc finger pair. In this more structured conformation, acidic residues critical for activation would be masked, lowering gene expression. In support of this model, zinc-dependent conformation changes of AD2 have been observed in vivo using the technique of FRET. When constructs expressing AD2 flanked by CFP and YFP are expressed in yeast, an increased FRET is observed in zinc-replete cells in a manner that is dependent upon zinc binding to the AD2 zinc fingers and formation of the zinc finger pair [45]. Similarly when AD2-based FRET constructs are expressed in human cells, an increase in FRET is observed when zinc is in excess [46, 47]. As humans lack a homolog of Zap1, the strong zinc-dependent FRET in human cell lines expressing AD2-based sensors also demonstrates that the conformation of AD2 is controlled by zinc in the absence of any additional yeast proteins. While it is plausible that a highly conserved protein or molecule is required for the regulation of AD2, the robust zinc-dependent FRET in humans cell lines support a model in which the zinc fingers within AD2 act as direct sensors of zinc.

In *S. cerevisiae*, Zap1 target genes include *ZRT1* and *ADH4*, which encode a high affinity zinc uptake system and alcohol dehydrogenase 4, respectively. In the fission yeast *S. pombe*, the homologs of these genes are tightly regulated at a transcriptional level in response to zinc [48, 49]. However, the *S. pombe* genome lacks a homolog of Zap1, suggesting that it must use a different factor(s) to control gene expression in response to zinc. Insight into the identity of the zinc-responsive factor in *S. pombe* was recently uncovered in a study examining *adh1* (alcohol dehydrogenase 1) expression [49]. In yeast, Adh1 is the primary alcohol dehydrogenase used for fermentation. When *adh1* was deleted from the genome in *S. pombe* it was noted that *adh1* Δ cells grew very slowly, yet rapidly obtained spontaneous second site mutations restoring normal growth. Further characterization of these mutations revealed that they led to *adh4* and *zrt1* being expressed under zinc-limiting and zinc-replete conditions. A straightforward explanation for occurrence of the spontaneous mutations occurring within *adh1* Δ cells is that they all resulted in increased expression of *adh4*, an alternative alcohol dehydrogenase. While this hypothesis has yet to be tested, the impaired *adh4* and *zrt1* expression in this mutant provided a unique means of identifying the factor involved in zinc-dependent transcriptional control. Additional mapping and sequencing analysis revealed that the spontaneous mutation within the *adh1* Δ genome caused an Arg510Gly substitution within a protein named Loz1 for Loss Of Zinc sensing 1 [49].

Loz1 is a nuclear localized protein that contains two C_2H_2-type zinc fingers at its extreme C-terminus. Deletion of *loz1* leads to constitutive expression of *adh4* and *zrt1*, suggesting that Loz1 is required for the repression of these genes when zinc is in excess. Additional dissection of the *adh4* promoter revealed that a GN(A/C)GATC promoter element is necessary for repression in response to zinc. A number of lines of evidence now suggest that Loz1 binds in a site-specific manner to this element. Gel shift analysis indicates that a small region of Loz1, consisting of the two zinc finger domains and an adjacent accessory domain, is sufficient to bind to the GN(A/C)GATC element in vitro. In addition, mutations that target zinc-binding residues within the zinc finger domains disrupt DNA binding in vitro and prevent gene repression in vivo [50]. Important questions remaining include what additional genes Loz1 regulates and how its activity is regulated by zinc. Studies so far suggest that Loz1 is regulated at multiple levels. At a transcriptional level, Loz1 can bind to its own promoter and auto-regulate its expression [49] Loz1 activity is also regulated at a post-translational level by zinc [50]. Deletion studies to map a minimal zinc-responsive domain revealed that a construct expressing the two zinc fingers and adjacent accessory domain was able to partially restore zinc-dependent changes in gene expression to *loz1* Δ cells. Chimeric proteins containing this minimal Loz1 domain fused to the N-terminal domain of a different transcription factor were also able to fully complement *loz1* Δ, consistent with this domain being necessary for DNA binding and zinc-dependent repression [50]. As this minimal domain contains 2 zinc finger domains, a simple model of how Loz1 activity is regulated by zinc is that the Loz1 zinc fingers only bind zinc when it is in excess, allowing Loz1 to bind to DNA and repress gene expression under this condition. However, it is not yet known if the GN(A/C)GATC promoter element is sufficient for zinc-dependent repression and whether Loz1 DNA binding activity is regulated by zinc. It is therefore possible that a larger pathway is present where other proteins/molecules sense zinc and relay this information to Loz1. As zinc fingers play a role in the zinc-dependent regulation of the mammalian transcription factor MTF-1, and potentially other human zinc-responsive factors (see Section 2.2), further studies with Loz1 will likely provide important insight into the roles of zinc fingers and zinc sensing in eukaryotes.

Copper-responsive transcription factors play a critical role in copper homeostasis in a variety of species including fungi, green algae, plants, and flies [51–53]. Biochemical analyses of a number of the factors have revealed that they contain unique copper-binding domains that allow them to sense copper ions. As one example, in *S. cerevisiae* the transcription factor Mac1 is active in copper-limited cells where it induces the expression of genes required for copper uptake [51]. Copper-dependent changes in Mac1 activity are thought to result from copper binding to a cysteine-rich domain that is located within an N-terminal transactivation domain [54]. Copper binding to this domain promotes an interaction between the transactivation domain and DNA binding domain, which in turn prevents Mac1 from binding to DNA and activating gene expression [55]. While this copper-induced allosteric switch explains how Mac1 activity can be regulated by copper, new studies have revealed that activation of gene expression under copper-limiting conditions requires a functional Cu-Zn superoxide dismutase (Sod1), and that Mac1 activity can be affected by the DNA damaging agent MMS [56, 57]. MMS is thought to trigger changes in the redox status of the regulatory cysteine residues within Mac1, which in turn correlates with

alterations in Mac1 activity [57]. These newer studies suggest that other environmental factors alter, or may be critical to, copper sensing. They also raise many new questions, such as why is Sod1 required for Mac1 activation in response to low copper, does Sod1 have a role in copper sensing in other organisms, and whether additional proteins and metabolites affect copper sensing and homeostasis.

Metal-dependent changes in transcription play an important role in multicellular organisms. Studies with these systems have increased our knowledge of genes involved in metal transport, sensing, and homeostasis. As an example, the nematode *Caenorhabditis elegans* is atypical in that it lacks many of the enzymes required for synthesizing heme, and therefore relies upon obtaining heme from its diet [58]. Nutritional studies investigating how *C. elegans* was able to survive in low heme medium revealed that heme deficiency led to the increased expression of 117 genes [59]. Further analysis of two of these genes, named *hrg-1* and *hrg-4* for heme-regulated gene 1 and 4, revealed that they encoded transporters that are required for the absorption of dietary heme from the intestine [59, 60]. Notably, homologs of these genes have similar roles in heme transport in mammals [59]. Thus, gene expression studies in worms facilitated the discovery of proteins critical for heme transport in humans.

Metal-dependent changes in transcription also play a central role in zinc and iron homeostasis in *C. elegans*. In worms, genes required for zinc excretion and storage are highly expressed when zinc levels are high [61]. While the metallo-regulatory factor that mediates this regulation has yet to be identified, new studies have mapped these changes in gene expression to a high zinc activation (HZA) DNA element within target gene promoters [62]. Further studies with worms may therefore lead to the discovery of new zinc-responsive factors and will further our knowledge of zinc-dependent tissue specific changes in gene expression. Genetic screens to identify regulatory factors involved in iron homeostasis revealed that hypoxia-inducible factor 1 (HIF-1) is required for transcriptional activation of genes required for iron uptake and transcriptional repression of genes required for iron storage in response to iron deficiency [63–66]. The involvement of a hypoxia-inducible factor in iron homeostasis is significant, as studies in mammals have also found that the activity of hypoxia-inducible factors is dependent upon iron status. Hypoxia-inducible factors consist of an oxygen-regulated alpha subunit and a constitutively expressed beta subunit [67]. In the presence of oxygen, the β subunit is hydroxylated by an iron and oxygen requiring enzyme prolyl hydroxylase. When hydroxylated, the HIFα subunits are recognized by the protein von Hippel-Lindau (pVHL) E3 ligase which targets them for proteasomal degradation [68–70]. In contrast, when iron is low or under hypoxic conditions, the prolyl hydroxylase is inactive allowing the HIFα subunit to translocate into the nucleus. This results in a transcriptional complex that activates target gene expression through the dimerization of HIFα with HIFβ [71]. The tight regulation of the HIFα subunits by an iron- and oxygen-requiring enzyme raises the possibility that the activity of these oxygen sensors is also potentially affected by changes in iron status [72, 73]. In mammals HIF-2α is also subject to an additional level of regulation in response to iron. The HIF-2α mRNA contains an IRE element within its 5’ UTR which results in reduced translation in iron-limited cells (see Section 4) [74, 75]. As HIF-2α plays a major role in inducing the expression of genes required for erythropoiesis in response to hypoxia, this second level of regulation potentially

serves as a feedback control mechanism to ensure that red blood cell synthesis is lowered when iron becomes too limiting (reviewed by [76]). Future studies in mammals examining the relationship between HIF-2α and IRP1, and in worms which have a single hypoxia-inducible factor, will thus further our knowledge of the close connections between oxygen and iron metabolism.

In *Drosophila melanogaster*, copper homeostasis is controlled at a transcriptional level by a homolog of the mammalian zinc-responsive factor MTF-1 [77]. In flies, MTF-1 is required for copper- and cadmium-dependent increases in transcription of a number of metallothionein genes [78]. Paradoxically, *Drosophila* MTF-1 also induces the expression of genes required for copper efflux in response to copper excess, and copper uptake in response to copper limitation [79, 80]. A number of new studies have begun to address how the *Drosophila* MTF-1 can regulate unique subsets of genes in response to different metal ion stresses. One important feature of the fly MTF-1 is that it uses different domains to discriminate between copper and cadmium. A copper-binding domain, which is absent from mammalian MTF-1, is critical for copper-dependent changes in transcription [81]. In contrast, cadmium-dependent changes in gene expression are dependent on a cysteine rich C-terminal domain [82]. In addition to separate metal-responsive domains, genome wide mapping of MTF-1 binding has revealed that in response to different metal stresses MTF-1 binds to slightly different DNA regulatory sequences, allowing for differential regulation of target gene expression [83]. Studies with MTF-1 from *Drosophila* therefore provide insight into how a single factor can fine tune metal homeostasis according the type of metal ion exposure.

2.2. Transcriptional control in mammals

In mammals, metal-dependent changes in transcription play a particularly important role in the regulation of zinc and iron levels.

Zinc transport genes that are regulated at a transcriptional level in response to zinc include *Zip10*, *ZnT-1*, *ZnT-2*, and *ZnT-5B* [84, 85]. Zip10 is a member of the ZIP family of zinc transporters that typically transport zinc into the cytosol. In contrast, ZnT-1, ZnT-2, and ZnT-5B belong to the CDF family of zinc transporters that usually transport zinc out of the cytosol. Consistent with these roles, *Zip10* is highly expressed when zinc is limiting [86] while *ZnT-1* and *ZnT-2* are expressed when intracellular zinc levels are high [80]. *ZnT-5B* encodes a bidirectional zinc transporter that localizes to the plasma membrane in human intestinal Caco-2 cells [87]. Unlike other zinc-regulated CDF family members, *ZnT-5B* is transcriptionally repressed when zinc levels are high [88].

A transcriptional activator named MTF-1 is responsible for many of the zinc-dependent changes in transcription in mammals. When zinc levels are high, MTF-1 induces the expression of the *ZnT-1* and *ZnT-2* zinc transport genes and the *MT-1* and *MT-2* metallothionein genes [77]. MTF-1 also inhibits *Zip10* expression in high zinc by binding immediately downstream of the TATA box and inhibiting the progression of RNA polymerase II [86]. Thus, when zinc is in excess, MTF-1-dependent changes in transcription enhance zinc storage and zinc export, and inhibit zinc uptake.

The ability of MTF-1 to activate gene expression in response to high zinc is complex and potentially results from zinc ions affecting MTF-1's DNA binding activity, transactivation domain function, and its cellular localization [77]. Under normal cellular conditions, MTF-1 is found in the nucleus and in the cytosol in an inactive form. However in response to high zinc and heavy metals ions, MTF-1 preferentially accumulates in the nucleus [89]. When zinc levels are high, MTF-1 binds to metal responsive elements (MREs) that are located in target gene promoters and activates gene expression. The MTF-1 DNA binding domain consists of 6 C_2H_2-type zinc fingers, of which four are essential for high affinity binding to MREs [90]. As a number of these zinc fingers have a lowered binding affinity for zinc, it was initially hypothesized that the regulation of MTF-1 DNA binding activity by zinc was a result of the differential occupancy of these low affinity fingers with zinc [90–92]. However, additional studies revealed that the specific linker region between zinc fingers 1 and 2 was critical to zinc-dependent binding [93], suggesting that the regulation of MTF-1 DNA binding function by zinc is more complex than initially hypothesized. In addition to this regulation, MTF-1 transactivation domain function is regulated by zinc in some cell types, suggesting that this may allow its activity to be further adjusted in a cell specific manner [94]. It is also noteworthy that copper, heavy metal ions, and other stresses also lead to an increase in MTF-1 activity in vivo. In vitro, the ability of MTF-1 to activate gene expression in response to copper and cadmium required the presence of a zinc-loaded metallothionein [95]. Thus, the ability of MTF-1 to sense other metals and stresses, could be an indirect result of these stresses displacing zinc from metallothionein and other zinc-containing proteins.

Not all zinc-dependent changes in gene expression in mammals are dependent upon MTF-1. For example, repression of *ZnT-5B* expression in response to zinc requires a unique promoter element called a Zinc Transcriptional regulatory element (ZTRE) [96]. Additional insight into the regulatory factor that binds to this element was recently revealed in a screen to identify factors that were associated with the ZTRE. This screen revealed that ZNF658, a transcription factor containing 21 zinc finger domains, is able to bind to the ZTRE in vitro [97]. siRNA-mediated knockdown of ZNF658 also revealed that a 60% reduction in *ZNF658* transcript levels was sufficient to abrogate the zinc-dependent repression of *ZnT-5B* in vivo. While these studies are consistent with ZNF658 playing a role in gene repression, important future studies include determining whether ZNF658 is sufficient to mediate zinc-dependent repression and whether the activity of ZNF658 is directly regulated at a post-translational level by zinc.

Iron-dependent changes in transcription play a critical role in controlling iron absorption and in the systemic regulation of iron homeostasis in mammals. Some iron-dependent changes in transcription are potentially dependent upon hypoxia inducible factors (see section 2.1). Studies with *HAMP*, which encodes a peptide hormone called hepcidin, indicate that other factors also influence iron-dependent changes in transcription in humans. In a healthy individual, *HAMP* is expressed in the liver when blood iron levels are high [98, 99]. *HAMP* gene expression is also tightly regulated by a number of additional factors including inflammation, erythropoiesis, anemia, and hypoxia [100–103]. After being secreted into bloodstream hepcidin is processed to an active form, which promotes endocytosis and

degradation of ferroportin, an iron export protein that is located on the surface of duodenal enterocytes, iron-recycling macrophages, and hepatocytes [104]. As hepcidin directly affects ferroportin levels, increased transcription of *HAMP* in response to high iron effectively lowers blood iron levels by blocking iron absorption in the duodenum, and iron release from macrophages and the liver [98, 99]. In support of hepcidin playing a central role in the control of systemic iron levels, mutations in *HAMP* lead to the juvenile-onset form of the genetic iron overload disorder hereditary hemochromatosis [105].

Following the discovery that hepcidin plays a central role in systemic iron homeostasis, many studies have investigated the mechanism by which *HAMP* gene expression is regulated by iron [106, 107]. Increased transcription of *HAMP* in response to high iron is largely dependent upon a BMP6 signaling pathway [108, 109]. In this pathway, binding of Bmp6 to cell surface receptors triggers a phosphorylation cascade, which ultimately leads to the formation of an active transcriptional complex containing SMAD4 that can translocate into the nucleus and activate *HAMP* gene expression [110–112]. What is less clear is how alterations in extracellular iron status trigger signaling through this pathway. A number of important regulatory factors that affect *HAMP* expression have been identified from studies of human genetic disorders and transgenic mouse models that cause iron overload. For example, genetic mutations in *HJV*, *HFE*, and *TfR2*, lead to reduced *HAMP* gene expression [113–115], and various forms of hereditary hemochromatosis [116]. In contrast, mutations in *TMPRSS6*, lead to increased *HAMP* expression and cause iron-refractory iron-deficiency anemia [117]. Analyses of the above mutations have shown that the regulation of *HAMP* expression by iron is complex, and that multiple mechanisms likely signal iron status to the BMP6 signaling pathway. More details of the regulation of hepcidin levels by iron can be found in the following review articles [99, 103, 107, 118].

3. Post-transcriptional control mechanisms – Alternative Splicing

Following transcription post-transcriptional processing of RNAs can include 5’ and 3’ end maturation, pre-mRNA splicing, transport, translation, and degradation [119]. The majority of studies examining metal-dependent post-transcriptional control have so far focused on the regulation of alternative splicing, translation, and mRNA degradation.

Splicing is the basic process by which introns are removed from a precursor mRNA and the remaining exons are joined together to form a mature mRNA [120]. In alternative splicing, different patterns of introns are removed to generate different mRNAs that have unique properties or encode distinct protein products. Alternative splicing is therefore a powerful process that increases the coding potential of the genome and allows versatile regulation of gene expression [121].

Cell and tissue specific alternative splicing can result in related RNA transcripts that are subject to different metal-dependent regulatory mechanisms and/or proteins with unique properties. As an example, alternative splicing of the *DMT1* (for Divalent Metal Transporter 1) transcript results in a splice variant containing an IRE within its 3’UTR and is therefore subject to iron-dependent regulation (see section 5), and a splice variant that lacks this element and encodes a protein with a distinct C- terminus [122, 123]. Alternative splicing

can also occur in a manner that is dependent upon cellular metals levels, suggesting the presence of metallo-regulatory alternative splicing factors [124]. New studies investigating alternative splicing of exon 6 from the Fas/CD95 transcript have provided mechanistic insight into how such metal-dependent splicing events can occur (Figure 3).

Fas/CD95 is a cell surface death receptor that upon binding the Fas ligand promotes apoptosis and cell death [125, 126]. Alternative splicing of exon 6 from Fas/CD95 results in a membrane bound form of the Fas receptor that triggers apoptosis, and a secreted soluble form of Fas/CD95 lacking the transmembrane domain which inhibits antibody-mediated apoptosis [127]. Using a genomic wide siRNA based screen to identify factors affecting alternative splicing of Fas/CD95 exon 6, Valcárcel and colleagues noted that silencing events that lowered iron levels promoted exon 6 skipping, while silencing events that increased iron levels promoted exon 6 inclusion [128]. Further analysis revealed that this iron-dependent splicing switch was dependent upon SRSF7 (for Zinc-finger-Containing Splicing Regulator 7), a SR family member that binds to target RNAs using a zinc knuckle motif. As iron had no effect on the stability or localization of SRSF7, but could inhibit RNA binding in vitro and in vivo, the authors propose a model in which SRSF7 RNA binding activity is directly regulated by cellular iron status. In this model, in low iron SRSF7 binds to the Fas/CD95 transcript and promotes exon 6 skipping. However, in high iron binding of SRSF7 is inhibited which promotes exon 6 inclusion (Figure 3). Important questions remaining include what is the significance of this iron-dependent apoptotic switch, whether other transcripts are subject to iron-dependent SRSF7 alternative splicing, and whether this switch is affected by zinc levels.

4. Post-transcriptional control mechanisms – Translation

The process of translation includes initiation, elongation, termination, and ribosomal recycling [129]. As translation control mechanisms act on existing mRNAs, a major advantage of this type of regulation is that it allows very rapid changes in protein production in response to changes in cellular and environmental conditions.

One of the most widely studied examples of how translation can be regulated by a metal-responsive regulatory protein is the iron-dependent regulation of the ferritin mRNA transcripts by IRP1 and IRP2 (Figure 4). IRP1 and IRP2 are cytosolic proteins whose activity is dependent upon cellular iron levels. In low iron, IRP1 and IRP2 bind to RNA stem loop structures called iron-responsive elements (IREs) that are found in the 5’ or 3’UTRs of many RNAs involved in iron homeostasis [130, 131].

RNA transcripts that contain an IRE within their 5’UTR include mRNAs encoding the heavy and light chains of the intracellular iron storage protein ferritin [132]. When the IRE element is located within the 5’UTR, binding of the IRP proteins in low iron blocks translation [133, 134]. The ferritin mRNAs are therefore only translated when iron is in excess, a condition when cells need to store iron. Other mRNA transcripts that contain an IRE in their 5’UTR include transcripts encoding proteins involved in iron efflux, that bind iron, or that are part of iron-requiring metabolic pathways [134–136]. The *HIF-2*αmRNA also contains an IRE within its 5’ UTR (see section 2.1). Thus, when iron is in excess, the

IRP regulatory system maintains iron homeostasis by enhancing iron storage, iron export, and iron utilization. In addition to controlling translational initiation in response to iron, IRPs also regulate the stability of mRNAs involved in iron acquisition (see section 5).

Recent studies with Zip5 suggest the presence of other metal-dependent translational control mechanisms in humans. Zip5 is a member of the ZIP family of zinc transporters that is localized to the basolateral membrane of enterocytes and pancreatic acinar cells when zinc levels are high [137, 138]. Although the *ZIP5* mRNA transcript accumulates under all conditions, the Zip5 protein only accumulates in high zinc, suggesting a translation control mechanism [139]. Additional analysis to determine the underlying mechanism behind this regulation revealed that the presence of a conserved stem loop structure within the 3' UTR of the Zip5 transcript is critical to translational stalling during zinc deficiency and that this loop forms the binding site for multiple miRNAs [140]. While the precise mechanism of how this miRNA bound stem loop enhances translation of the Zip5 mRNA in high zinc is unclear, it suggests that other metallo-regulatory proteins involved in translation control have yet to be identified, and that translational control plays an important role in zinc homeostasis.

5. Post-transcriptional control mechanisms - mRNA degradation

As the levels of an mRNA within a cell affect the window of time in which it can be translated, metal-dependent changes in the half-life of an mRNA can contribute significantly to the regulation of gene expression.

In the cytosol, mRNAs are typically protected from degradation by a 5' cap structure and a 3' poly A tail. At the end of an mRNAs life, shortening of the poly A tail can lead to the removal of the 5' cap allowing 5'-3' exonucleolytic decay, or removal of the poly A tail allowing 3'-5' exonucleolytic decay [141]. Studies of the RNA binding proteins, Cth1 and Cth2, in budding yeast have revealed how this decay process can be enhanced in response to iron deficiency.

Cth1 and Cth2 each contain two tandem CCCH-type zinc fingers that facilitate binding to adenosine/uridine rich elements (AREs) within the 3' UTR of their target mRNAs [20]. On binding to their target RNA transcripts, Cth1 and Cth2 can enhance degradation in the nucleus by interfering with polyadenylation, which in turn leads to the generation of run-on transcripts that are rapidly degraded [142, 143]. In the cytosol, Cth1 and Cth2 can also interact with specific components of the mRNA degradation machinery enhancing the rate of mRNA decay [144]. In *S. cerevisiae*, higher levels of the Cth proteins are observed in iron-limited cells resulting in increased degradation of target transcripts under this condition. Consistent with this iron-dependent regulation, Cth2 targets include mRNAs encoding proteins involved in iron-dependent processes (e.g. the TCA cycle, respiration, heme biosynthesis and amino acid biosynthesis), as well as vacuolar iron transport [20]. The target transcripts of Cth1 are more limited and partially overlap with those of Cth2. Cth1 targets mRNAs typically encode proteins found in pathways with a high iron demand [19]. Thus, Cth1 and Cth2, play a critical role in protecting cells from iron deficiency, by lowering the flux of iron into non-essential iron requiring processes.

Multiple mechanisms lead to the transient production of Cth1 and Cth2 in response to low iron. *CTH1* and *CTH2* are both transcriptionally regulated by the iron-responsive factor Aft1 [19, 20]. On top of this control, Cth1 and Cth2 regulate their own levels [145]. This auto- and cross- regulation leads to the transient production of the Cth1 and Cth2 proteins. One advantage of the rapid depletion of Cth1 and Cth2 is that it allows cells to rapidly adapt to changes in iron bioavailability. As an example, when an iron-limited cell is rapidly exposed to iron, the rapid depletion of the Cth proteins, leads to the increased stability of target mRNAs allowing the cell to maximally use iron in metabolism.

An alternative mechanism that can lead to the rapid decay of an mRNA is a targeted endonucleolytic cleavage. Examples of metal-regulated endonucleolytic-dependent degradation include the Rnt1-mediated degradation of transcripts involved in iron acquisition in *S. cerevisiae*, and the IRP1 and IRP2-dependent degradation of the transferrin receptor 1 (TfR1) in mammals.

In the budding yeast, Rnt1 is a dsRNA specific ribonuclease that is involved a number of RNA processing events, including the maturation of ribosomal RNA and specific small RNAs, and polyadenylation-independent transcriptional termination [146]. Rnt1 facilitates processing by cleaving specific RNA hairpin tetraloop structures located within its target RNAs [147]. In addition to its general role in RNA processing, in high iron, Rnt1 binds to and cleaves mRNAs involved in iron uptake and iron acquisition [148]. Although the mechanism by which Rnt1 acts on these mRNAs in high iron is unknown, cells lacking Rnt1 are sensitive to high iron, suggesting that this mechanism is important for survival in high iron.

Much more is known about the iron-dependent degradation of the TfR1 mRNA. Transferrin is a glycoprotein that binds and transports Fe^{3+} within the bloodstream. When iron bound transferrin reaches a cell that requires iron, it binds to the TfR1 on the cell surface to form a TfR1/Transferrin/Fe complex. This complex is then internalized by endocytosis and following acidification of the endosome Fe^{3+} is released. Fe^{3+} is then reduced and transported across the endosomal membrane into the cytosol by DMT1 [149]. Consistent with TfR1 playing a critical role in attaining iron from the blood, cells accumulate higher levels of the TfR1 mRNA when they are limited for iron, in a manner that is dependent upon the RNA binding proteins IRP1 and IRP2 [131, 135]. In contrast to the IRP-dependent regulation of the ferritin mRNAs, the TfR1 mRNA contains multiple IREs within its 3'UTR. When the IRP proteins bind to the 3'UTR of the TfR1 transcript in low iron, they protect it from endonucleolytic cleavage. Thus, through the differential positioning of IREs within an mRNA transcript, the IRPs stabilize the TfR1 mRNA and inhibit the translation of ferritin mRNAs in low iron (Figure 4). Other transcripts that contain IREs in their 3'UTR include *DMT1*, which encodes a protein required for intestinal iron absorption [134, 135].

The mechanisms by which IRP1 and IRP2 are regulated by iron have been widely studied. In high iron, IRP1 acquires an iron-sulfur cluster and can function as a cytosolic aconitase. In low iron, IRP1 looses its iron-sulfur cluster and gains the ability to bind to IREs within target mRNAs [150]. Thus, iron sulfur clusters play a key role in the ability of IRP1 to sense iron. Although mutations that disrupt mitochondrial iron sulfur synthesis impair the ability

of IRP1 to sense iron, it is currently unclear if mitochondrial iron sulfur cluster synthesis is obligatory for iron sensing (reviewed by [26, 131]). In contrast to IRP1, IRP2 does not bind an iron sulfur cluster and has no aconitase activity. Instead IRP2 is ubiquitinated and targeted for proteasomal degradation in high iron [151, 152]. Ubiquitination of IRP2 in high iron is dependent upon an E3 ubiquitin ligase complex containing the F-box protein FBXL5 (F-box and leucine-rich repeat protein 5) [151, 152]. In low iron, FBXL5 is targeted for degradation by the large HECT-type ubiquitin ligase HERC2 [153]. However, when iron levels are high, iron binds to a hemerythrin domain within FBXL5 resulting in its stabilization [154]. Thus, the regulation of IRP1 by iron sulfur clusters and IRP2 via FBXL5 indicate that the mechanism of iron sensing differs between the two sensors. It is also noteworthy that as an E3 ligase, FBXL5 has the potential to target additional proteins for degradation. For example, FBXL5 promotes the ubiquitin dependent degradation of Snail, a transcription factor involved in the transition of epithelial cells to mesenchymal stem cells [155]. Thus, important future studies should include identifying additional protein targets of FBXL5 and understanding their connections to iron homeostasis.

Metal-dependent changes in mRNA stability also play an important role in zinc homeostasis in mammals. For example, *Zip4* encodes a zinc transporter that is highly expressed in the small intestine during zinc deficiency [156]. Mutations in the *Zip4* gene lead to *acrodermatitis enteropathica*, a rare recessive disorder characterized by a reduced ability to absorb zinc from the diet [157, 158]. Although *Zip4* mRNA abundance is tightly regulated by zinc, transcriptional run on assays using nuclei purified from mice fed a zinc deficient or zinc adequate diet revealed that there is no difference in the rate of transcription of *Zip4* in response to zinc [139]. These results suggest that *Zip4* is not an inducible gene and that zinc deficiency leads to the stabilization of *Zip4* mRNA transcripts. While the molecular mechanism behind this zinc-dependent stabilization remains elusive, the stabilization of *Zip4* mRNA in response to zinc deficiency explains why higher levels of zinc are absorbed from the diet under this condition. Other studies have also noted zinc-dependent changes in mRNA stability [159, 160], suggesting that zinc-dependent post-transcriptional control may be a common method of regulating gene expression in mammals.

6. Post-translational control mechanisms - phosphorylation

Once translated the activity of a protein can be altered by post-translational modifications. Many different types of these modifications have been identified including phosphorylation, glycosylation, ubiquitination, and SUMOylation [161]. As each of these events can affect protein function, metal-dependent regulation of these processes allows rapid changes in protein activity.

One of the most common protein modifications is the addition or removal of a phosphate group. Advantages of regulating protein function by phosphorylation and dephosphorylation, are that these events can rapidly alter the function or activity of a protein, and that they are typically reversible i.e. if the addition of a phosphate group activates an enzyme, the removal of the phosphate group usually has the opposite effect and inactivates it. Therefore, metal-dependent changes in phosphorylation of a protein can allow biologically reactions or processes to be rapidly turned on or off in response to metal status.

Metal-dependent alterations in phosphorylation can arise from changes in the expression of protein kinases. Alternately, metal ions can bind to kinases and directly control their activity. As an example, the Ras/mitogen-activated protein kinase (MAPK) signaling pathway regulates fundamental biological processes such as proliferation, differentiation, motility, stress response, and survival [162, 163]. In the MAPK pathway, a broad range of extracellular stimuli trigger a kinase cascade, which ultimately leads to the phosphorylation and activation of MAPK. Recent studies have shown that the MAP kinase kinase Mek1 binds two copper ions and that copper binding to Mek1 enhances the ability of it to phosphorylate the MAPK Erk in vitro [164]. In addition, in flies and mammals copper deficiency, or genetic mutations that lead to copper deficiency, reduce the ability of Mek1 to phosphorylate Erk [164]. While these studies are consistent with Mek1 activity being controlled by copper, they raise many new questions. When in excess, copper can drive the production of hydroxyl radicals via the Fenton reaction and has the potential to displace other metals from metalloproteins (see below). As a consequence, cells keep cytosolic copper levels low and utilize a class of proteins called copper chaperones to safely deliver copper to a respective partner protein [165, 166]. Thus, is there a specific copper chaperone for Mek1, or are copper levels buffered at a low level and small changes in this buffered pool modulate Mek1 activity? Alternatively, do other proteins/metabolites signal changes in copper to Mek1? While these questions remain to be answered, copper is essential for development and cell proliferation. The regulation of the MAPK signaling pathway by copper could therefore be a mechanism of fine tuning basic cellular processes according to copper availability. In nematodes and mammals, signaling through the MAPK pathway is also regulated by zinc availability [167–169]. Together, these results suggest that this fundamental signaling pathway is subject to multiple metal-dependent control steps, and could be a means by which a broad range of biological processes are be modified in response to metal levels.

Metal-dependent changes in phosphorylation may also result from metals directly regulating the activity of protein phosphatases [169]. As an example, protein tyrosine phosphatase 1B (PTP1B) is a negative regulator of the insulin and leptin receptor signaling pathways [170, 171]. In cell culture, zinc mimics the actions of insulin, suggesting that zinc may modulate this pathway. Zinc also strongly inhibits PTP1B phosphorylation in vivo [172]. In vitro, low nanomolar zinc concentrations inhibit PTP1B, suggesting that alterations in cytosolic zinc levels may modulate the activity of this enzyme in vivo [173]. A growing amount of evidence suggests that at least in some types of human cells, zinc is rapidly exported from the endoplasmic reticulum in a transient wave in response to specific extracellular stimuli [174]. Within cells, PTP1B is attached to the endoplasmic reticulum (ER) with its catalytic domain facing the cytosol [175]. Thus, the rapid release of zinc from the ER could potentially modulate PTP1B activity and insulin signaling [176]. Interesting future studies could be to determine if the zinc-dependent modulation of PTP1B activity is dependent on its localization to the ER and whether the activity of related tyrosine phosphatases are modified by zinc in vivo.

7. Post-translational control mechanisms - degradation

A different type of post-translational modification is the addition of ubiquitin. Ubiquitination of a protein can affect its activity or target it for degradation [177, 178]. An advantage of degrading a protein under a given condition is that it is irreversible, and therefore can rapidly terminate a reaction or process allowing cells to adapt to a new physiological condition.

The addition of a ubiquitin group to a protein occurs in a three-step reaction requiring a ubiquitin-activating enzyme (E1), a ubiquitin conjugating enzyme (E2), and a ubiquitin ligase enzyme (E3). In mammals, a number of metallo-regulated E3 ligases have been identified that play important roles in metal homeostasis. FBXL5 is an E3 ligase that is stabilized by iron binding. As FBXL5 targets IRP2 for degradation in high iron, this regulatory switch plays a key role in cellular iron homeostasis (see section 5). XIAP (for X-linked inhibitor of apoptosis) is a multifunctional protein with E3 ligase activity that suppresses apoptotic cell death and regulates NF-kB activation. XIAP also binds copper and regulates a number of proteins important for copper homeostasis [179]. In contrast to the iron-stabilized FBXL5, copper-bound XIAP is less stable [180].

In mammals, COMMD1 is involved in the regulation of the copper-transporting ATPases ATP7A and ATP7B. Due to differences in tissue specific expression, ATP7A and ATP7B play critical roles in copper absorption and copper excretion into the bile, respectively [181, 182]. When copper levels are low, ATP7A and ATP7B are both localized to the trans Golgi network (TGN) where they transport copper to copper-requiring enzymes located within the TGN. However, when copper levels are elevated both transporters re-localize to the plasma membrane or vesicles at the cell periphery and facilitate copper export. In enterocytes, relocalization of ATP7A facilitates the transport of copper across the basolateral membrane into the portal system. In the liver, the copper-dependent relocalization of ATP7B leads to the removal of excess copper into the bile. Thus, the copper-dependent movement of ATP7A and ATP7B results in the tight regulation of copper absorption and excretion, respectively [181, 182]. Notably, mutations that disrupt ATP7A function lead to Menkes disease, a genetic disorder characterized by copper deficiency, while mutations in ATP7B lead to Wilson’s disease, a genetic disorder characterized by copper overload [183].

Although the precise mechanism remains a subject of debate, multiple studies suggest that COMMD1 plays an important role in copper homeostasis by facilitating the ATP7A and ATP7B dependent export of copper from cells [184–187]. The regulation of COMMD1 protein levels by XIAP therefore has important consequence on copper homeostasis. XIAP-dependent degradation of COMMD1 reduces copper export, which in turn leads to an increase in intracellular copper levels. As XIAP is less stable in high copper, this in turn creates a feedback mechanism for restoring COMMD1 levels, and thus copper export [188]. Recent studies have shown that the copper chaperone CCS delivers copper to XIAP [189]. CCS also delivers copper to superoxide dismutase 1 (Sod1) [190]. Unexpectedly, CCS is also a target of XIAP. However, under normal conditions XIAP-dependent ubiquitination of CCS does not lead to degradation, but instead enhances the CCS-dependent activation of Sod1 [189]. An interesting twist to the regulation is that when copper levels are high,

ubiquitination of CCS leads to its targeted degradation [191]. While it is unknown if XIAP mediates the degradation of CCS in high copper, the copper-dependent turnover of CCS is dependent upon a CXC motif that is critical for the transfer of copper to Sod1. This result suggests that this regulatory mechanism may help to prevent the unused copper-bound chaperone from accumulating to high levels [192].

A number of metal transport proteins are also subject to metal-dependent degradation. Studies in yeast and mammals have provided insight into the mechanisms by which this can occur. In response to high zinc, Zrt1 from *S. cerevisiae*, and Zip4 from mammals are ubiquitinated, endocytosed, and degraded [193–195]. As both proteins play a critical role in zinc uptake, their targeted degradation prevents over-accumulation of zinc. Mutagenesis of *Zip4* revealed that its ubiquitin-dependent degradation requires a conserved histidine-rich cluster that is located within a cytosolic loop [193]. A potential mechanism to explain zinc-dependent turnover is therefore that zinc binding to this histidine-rich cluster promotes a conformation change that allows ubiquitination of key lysine residues. Metal binding residues are also required for the copper-dependent degradation of the high affinity copper uptake protein Ctr1 in mammals [196]. The metal binding motif required for degradation of Ctr1 is located within a transmembrane domain, and is critical for Cu^{+} transport. While the involvement of this metal binding motif hints that Ctr1 could be a copper sensor, Ctr1-dependent degradation has not been observed in all studies, suggesting that other factors may influence this regulatory process [197]. New studies have also revealed that cleavage of Ctr1 leads to a truncated form that plays an active role in mobilizing copper from endosomal stores, and that cleavage is dependent upon a structurally related protein Ctr2 [198]. While the mechanism by which Ctr2 regulates the proteolytic processing of Ctr1 is not yet known, it is notable that a number of proteins which resemble glucose transporters act as sensors of glucose [199, 200]. Future studies are therefore needed to determine if copper regulates cleavage of Ctr1, and whether Ctr2 represents another class of metallo-sensing protein.

8. Putting it all together

Metallo-regulatory factors play a central role in metal homeostasis by controlling the expression of genes, mRNAs, or proteins required for metal transport, metal acquisition, metal storage, or metal-dependent reactions or cellular processes. Studies of these metallo-regulatory factors have shown that they can control gene expression at a transcriptional, post-transcriptional, or post-translational level by regulating transcriptional initiation, alternative splicing, translation, mRNA stability, protein activity, or protein stability.

Understanding metal-dependent changes in gene expression is of medical importance as alterations in metal levels can have significant health consequences. A number of genetic diseases result from mutations in metal transport proteins or other genes critical to metal homeostasis [201–203]. Altered metal ion levels are also commonly observed in many complex diseases, suggesting that imbalances in their levels may interfere with normal cellular metabolism [204–206]. While knowledge of metal-dependent changes in gene expression is critical to determine why metal levels are altered in the complex diseases, it is noteworthy that many metal homeostasis genes are subject to multiple levels of regulation, and that there is often redundancy in how a metal can be transported into or out of the

cytosol. As an example, *Zip4* is frequently over-expressed in pancreatic adenocarcinomas [207]. As Zip4 is required for zinc uptake, it has been suggested that the increased expression of *Zip4* leads to increased levels of zinc within these cells, which in turn may enhance tumor growth. While this model is straight forward, other studies have shown that zinc levels are reduced in pancreatic adenocarcinomas, and that the expression of other zinc uptake genes was reduced [208, 209]. Thus, to understand the roles of metals in disease, it is not only important to identify each of the individual metallo-regulatory factors and their direct targets, but also to appreciate the presence of additional metal-regulated pathways within cells and the overlapping role that they play in controlling metal levels.

Although much progress has been made in characterizing how metallo-regulatory factors control metal homeostasis, in many cases it is unclear how many genes, mRNAs, or proteins they directly regulate, and whether additional factors influence sensing. There are also many examples of metal-dependent changes in gene expression that are not yet linked to a known factor, suggesting the presence of other metal-regulated factors or processes [210, 211]. Finally, in some biological systems multiple metallo-regulatory factors have been identified that sense the same metal. In these cases it will be important to determine whether the factors have redundant, overlapping, or distinct roles in metal homeostasis. In addition to identifying and characterizing metal-regulated factors, a number of significant questions regarding metal ion sensing have yet to be answered. Many studies have shown that the activities of specific metallo-regulatory factors is altered by metal levels in vitro and in vivo, suggesting that they are sensing changes in a ‘labile’ pool of a specific metal ion. What is less clear is what is this labile pool? Does it consist of buffered metals ions, and if so what molecules and/or metabolites act as buffers? In most cases, it is also not known whether metallo-regulatory proteins directly bind metal ions, or if they are part of a larger signaling pathway where other proteins/molecules relay metal ion status. A related question concerns how metalloproteins obtain the correct metal ion cofactor. Metals have a tendency to associate with a metal-binding site in a protein according to the Irving-Williams series. In this series, the relative stability of complexes formed with essential transition metals is: Mn(II) < Fe(II) < Ni(II) < Co(II) < Cu(II) > Zn(II). Metallo-proteins therefore preferentially bind Cu(II) and Zn(II) over less competitive metal ions such Mn(II). If metal binding is based on this series, how do metalloproteins that require less competitive metals obtain their correct cofactor in vivo? Elegant studies in bacterial systems have shown that the metal binding affinities of the sensors of competitive metal ions are significantly higher than those for the sensors of less competitive metal ions [212, 213]. As a consequence, the buffered level of a specific metal within a prokaryotic cell, and therefore its availability for incorporation into a metalloprotein, is largely governed by the relative affinity of the sensor for its respective metal. In eukaryotes, metal sensing is more complicated in that all of the sensors are not in the same cellular compartment, many sensors contain multiple metal sensing domains that act independently of one another, and some metal-sensing domains can be dependent upon cell or tissue type. Important questions to resolve therefore include whether metallosensors and the buffered metal levels within the cytosol and nucleus are set according to the Irving-Williams series, or whether other mechanisms exist to ensure that the correct metal is incorporated in a metalloprotein. As many metalloproteins are located within distinct organelle compartments in eukaryotes, it will also be necessary to investigate

whether compartmentalization plays a role in correct metal occupancy, and how compartmentalized metallo-proteins obtain their correct metal cofactor. Thus, in future studies it will be important to characterize metallo-sensing proteins and their target genes, and determine how these factors work in unison with other metallo-regulatory systems. Together this information will provide the key to understanding the complexity of balancing metal levels according to cellular demand and dietary availability, and how defects in metal homeostasis can lead to, or increase, the risk for specific diseases.

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Abbreviations

BMP	Bone Morphogenetic Protein
CDF	Cation Diffusion Facilitator
COMMD	COMM domain-containing protein
dsRNA	double stranded RNA
FRET	Fluorescence resonance energy transfer
HAMP	Hepcidin AntiMicrobial Peptide
HFE	high Iron (Fe)
MMS	Methyl Methane Sulfonate
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
siRNA	Small Interfering RNA
TCA	Tricarboxylic Acid
TfR	Transferrin Receptor
ZIP	Zrt1 Irt1-like Protein

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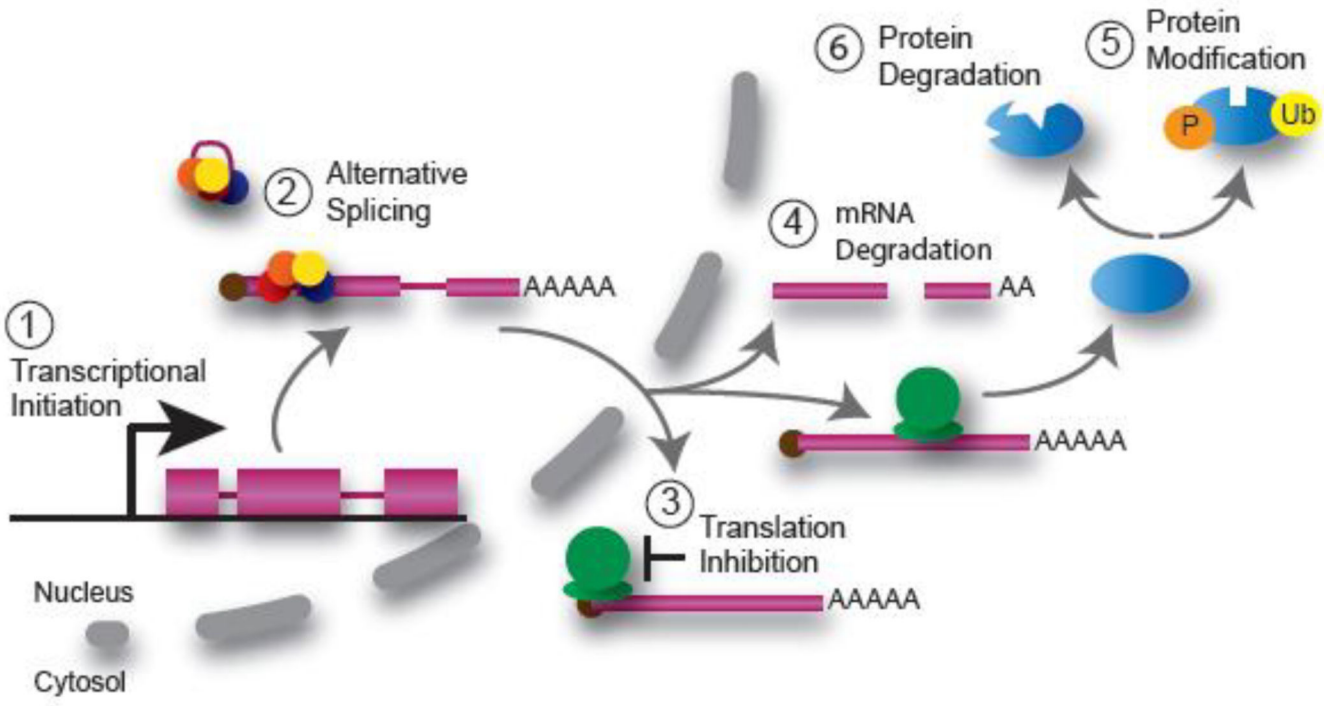

Figure 1.

Metal-dependent changes in gene expression in eukaryotes. In eukaryotes, metallo-regulatory factors have been identified that control transcriptional initiation (1), alternative splicing (2), translation (3), mRNA stability (4), protein modifications (5), and protein stability (6).

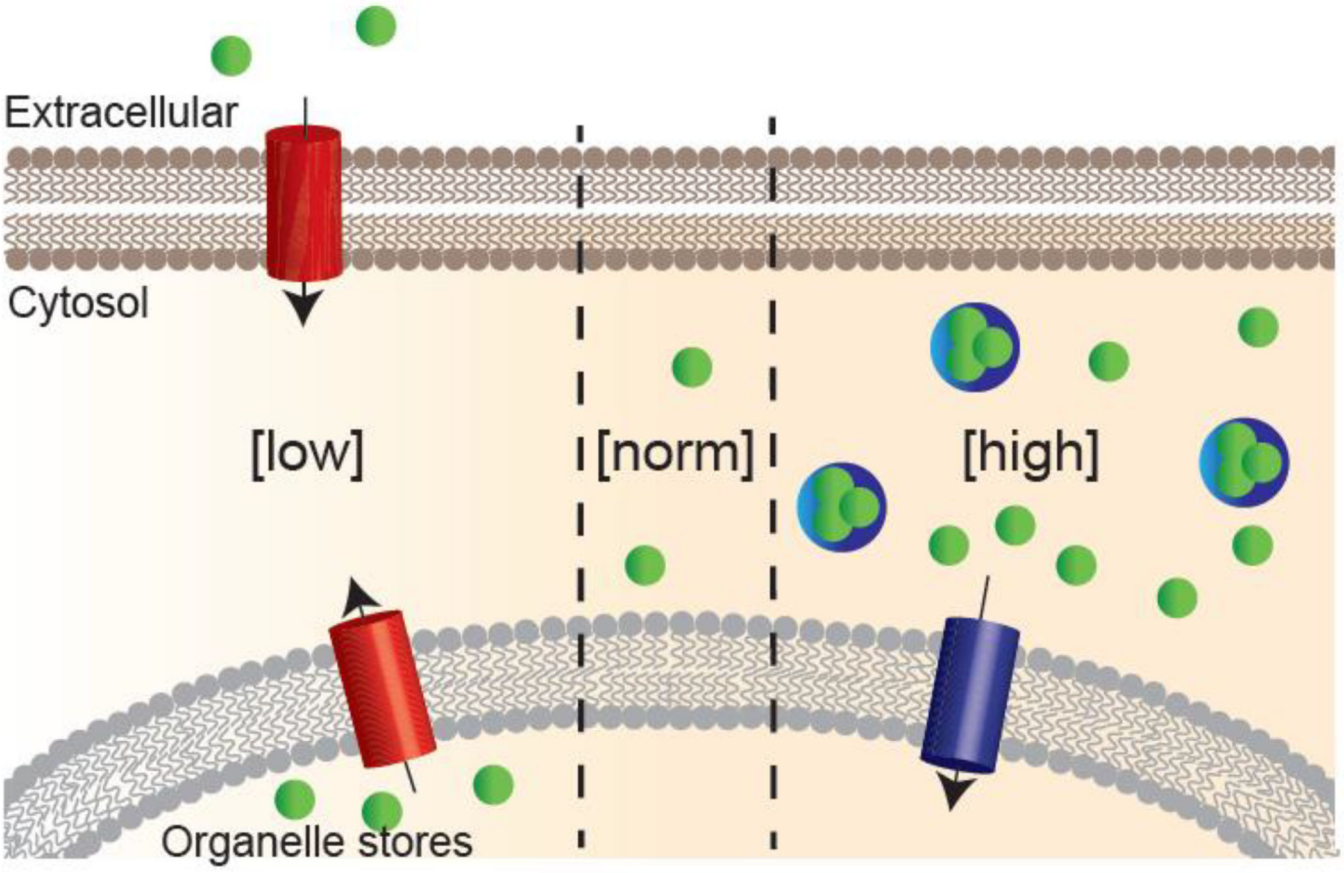

Figure 2.

Changes in the expression of genes encoding metal transporters affects intracellular metal ion levels. Metal transporters are shown as red and blue cylinders. Metal storage proteins and metal ions are represented by blue and green circles, respectively.

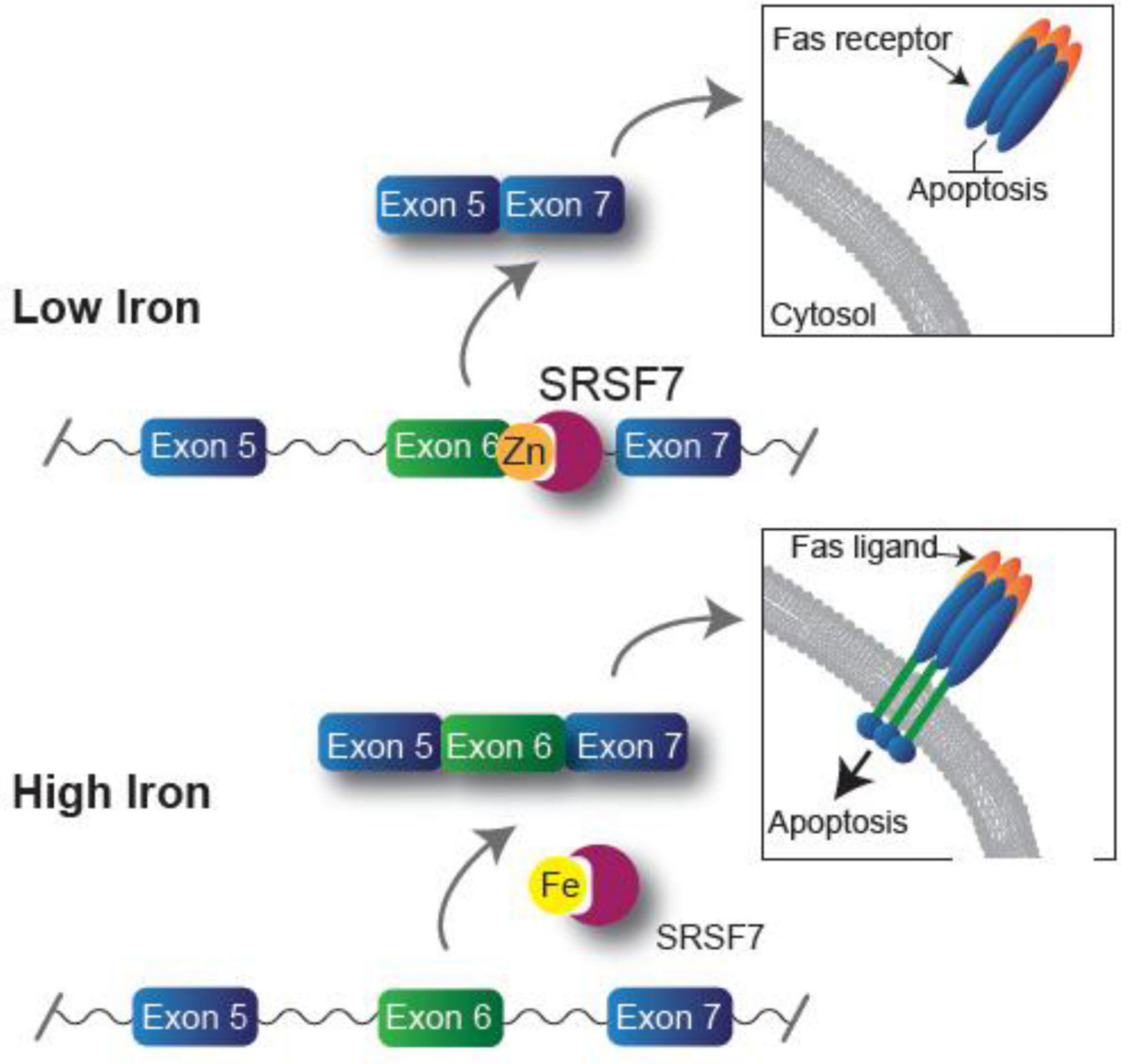

Figure 3.

Iron-dependent alternative splicing of the Fas/CD95 pre-mRNA. When iron levels are low, SRSF7 binds to the Fas/CD95 pre-mRNA using a zinc knuckle motif and promotes exon 6 skipping. The mRNA product generated from this alternative splicing event encodes a soluble secreted receptor that binds the Fas ligand inhibiting apoptosis. In high iron, SRSF7 binding is compromised promoting exon 6 inclusion. The mRNA product generated encodes a membrane bound receptor that upon binding of the Fas ligand triggers apoptosis.

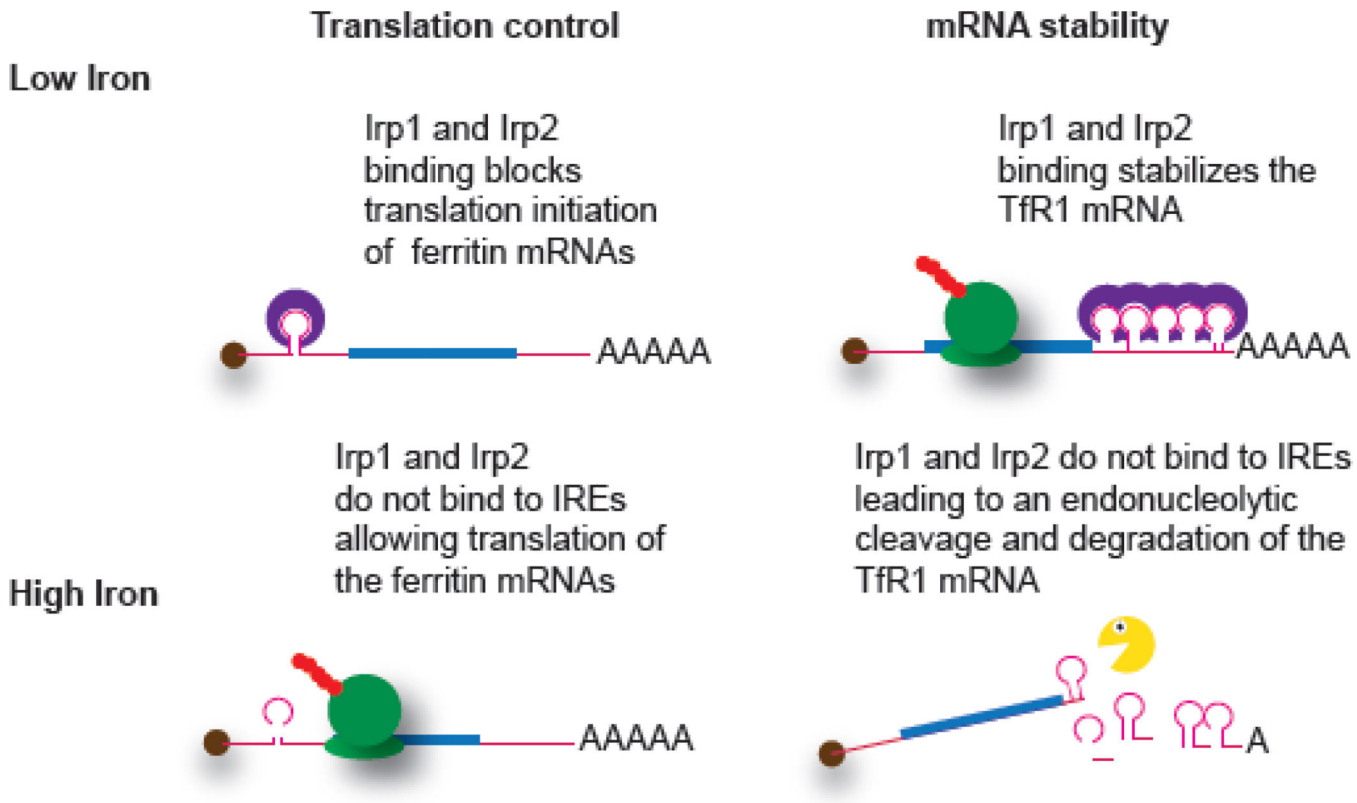

Figure 4.
Regulation of translational initiation and mRNA stability by IRP1 and IRP2.