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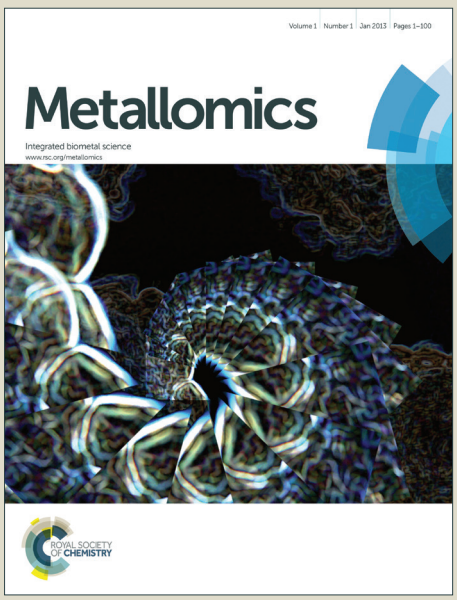

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Zinc'ing sensibly: Controlling zinc homeostasis at the transcriptional level

Sangyong Choi[1] and Amanda J. Bird[1,2,3]

[1]From the Department of Human Sciences, [2]the Department of Molecular Genetics, and the [3]Center for RNA Biology, The Ohio State University, Columbus, OH, 43210

To whom the correspondence should be addressed:

Amanda J. Bird, Department of Human Sciences and Department of Molecular Genetics, The Ohio State University, 1787 Neil Avenue, Columbus, OH, 43210, Tel.: (614) 247 1559; Fax (614) 292 8880; E-mail: bird.96@osu.edu

Abstract

Zinc-responsive transcription factors are found in all kingdoms of life and include the transcriptional activators ZntR, SczA, Zap1, bZip19, bZip23, and MTF-1, and transcriptional repressors Zur, AdcR, Loz1, and SmtB. These factors have two defining features; their activity is regulated by zinc and they all play a central role in zinc homeostasis by controlling the expression of genes that directly affect zinc levels or its availability. This review summarizes what is known about the mechanisms by which each of these factors sense changes in intracellular zinc levels and how they control zinc homeostasis through target gene regulation. Other factors that influence zinc ion sensing are also discussed.

1. Introduction

Zinc is an essential cofactor in a range of enzymes including carbonic anhydrases and alcohol dehydrogenases.[1] A large number of transcription factors and other regulatory proteins also contain smaller structural domains that are stabilized by zinc ions. These domains include the zinc finger, RING finger, and the LIM domain.[2, 3] More recent studies have revealed that zinc can also have a signaling role in vertebrates.[4, 5] Thus, zinc has many important biological functions and is vital for all life.

Given the importance of zinc for general cell metabolism, all organisms tightly control zinc levels and its availability. For example, as zinc is an important factor for the growth and survival of microbes, vertebrates have evolved strategies to sequester zinc from invading pathogens.[6] To counter this, some microbes have evolved systems that can obtain zinc from a range of environments, even those that are extremely limited in zinc.[7, 8] Thus, the tight control of zinc homeostasis is critical for survival of the host and pathogen. Imbalances in zinc levels can have important health consequences in humans. In children, zinc deficiency leads to an increased risk of diarrhea, pneumonia, and malaria.[9] Other symptoms that are associated with zinc deficiency include growth retardation, alopecia, immunodeficiency, and neuronal and sensory dysfunctions.[10, 11] In contrast, too much zinc in the diet can affect immune function, and in severe cases lead to widespread sensory and motor neuropathies through reduced copper absorption.[12, 13] In addition to nutritional problems associated with zinc, abnormal zinc levels or the aberrant expression of zinc transport genes, are commonly observed in a range of complex diseases, including prostate and pancreatic cancers, and Alzheimer's disease.[14-17] These observations suggest that imbalances in zinc levels or its distribution may be an important contributing factor to the onset or severity of specific diseases. Thus, the tight control of zinc levels is critical for the survival of all known organisms.

One of the primary means by which cells regulate zinc levels is through zinc-dependent changes in the expression of genes required for zinc transport and storage. This regulation in turn ensures that zinc levels are adjusted according to a cell's need for zinc. In the following article, we review the current understanding of the mechanisms by which genes are regulated at a transcriptional level in response to changes in zinc levels. In particular, we focus on the zinc-responsive regulatory factors and their target genes.

2. Zinc-responsive transcription factors

Zinc-responsive transcription factors are found in all kingdoms of life and include the prokaryotic factors ZntR, SczA, Zur, AdcR, and SmtB, and the eukaryotic factors Zap1, Loz1, bZip19, bZip23, and MTF-1 (Table 1). In general, these zinc-responsive factors can be divided into two classes: transcription factors that control zinc uptake and protect cells from zinc deficiency, and factors that control zinc efflux and/or storage, and protect cells from zinc excess.

Factors protecting cells from zinc deficiency include the transcription activators Zap1, bZip19, and bZip23, and the transcriptional repressors AdcR, Zur, and Loz1. The factors Zap1, bZip19, and bZip23 all activate the expression of genes required for zinc uptake when cytosolic zinc levels are limiting (Figure 1A, upper panel). The transcriptional repressors AdcR, Zur, and Loz1 also regulate the expression of zinc uptake genes. However, these factors repress gene expression when cytosolic zinc levels are in excess (Figure 1A, lower panel). As zinc-limitation leads to the inactivation of AdcR, Zur, and Loz1, and derepression of their target genes, these factors also ensure that genes required for zinc uptake are expressed when cytosolic levels are limiting. At the opposite end of the spectrum, SczA, ZntR, MTF-1, and SmtB all play a central role in protecting cells from zinc excess. In this class of zinc responsive factors, MTF-1, ZnTR, and SczA are activated by excess zinc and counteract increases in cytosolic zinc levels by inducing the expression of genes required for zinc efflux or zinc storage (Figure 1B, upper panel). SmtB and related family members also ensure that genes required for zinc storage

or zinc efflux are expressed when cytosolic zinc levels are high (Figure 1B, lower panel). However, SmtB family members are functional repressors in zinc-limited cells and are inactivated by zinc. Thus, zinc-responsive transcription factors include both transcription activators and repressors that maintain optimal cytosolic zinc levels by directly controlling the expression of zinc transport and zinc storage genes.

3. Zinc-regulated genes

Increased expression of genes required for zinc transport across the plasma membrane or the release of zinc from intracellular stores can lead to increased cytosolic zinc levels (Figure 2, Zinc deficiency). Transcriptional changes that decrease the use of abundant zinc binding proteins can also conserve zinc for more essential functions. In contrast, when zinc is in excess, increased expression of genes required for zinc efflux or zinc transport into organelle stores, can lead to reduced cytosolic zinc levels (Figure 2, Zinc excess). Increased expression of proteins that store zinc can also help protect the cytosol from the toxic effects of zinc. In the following section we review how these zinc-dependent changes in gene expression can impact zinc homeostasis.

3.1. Zinc transporters

In prokaryotes, proteins that transport zinc into or out of the cytosol include members of the ABC, ZIP, P-type ATPases, RND, and CDF families of transporters.[18] Whereas in eukaryotes, members of the ZIP and CDF families typically transport zinc into and out of the cytosol, respectively.[19, 20] A number of broad-spectrum transport systems also exist that transport zinc and other divalent metal ions and molecules.[21] As these transport proteins are typically the primary means by which zinc enters or exits a cell, changes in the expression of zinc transporter genes can be an important mechanism to precisely control intracellular zinc levels. As zinc can be compartmentalized into organelles in eukaryotes, the control of zinc entry or release from these stores can also be used as a method of balancing zinc levels

in these organisms. Zinc transport genes that are regulated by zinc-responsive factors have been summarized in Table 2.

In addition to regulating the levels of zinc entering or leaving a cell, in eukaryotes, transcriptional changes in the expression of zinc transport genes can be a mechanism of preferentially directing zinc to specific organelles when zinc is limiting. In *Saccharomyces cerevisiae* for example, the expression of *ZRG17*, a gene required for zinc transport into the endoplasmic reticulum (ER), is induced in response to zinc deficiency.[22] As many proteins bind or obtain zinc in the ER, zinc deficiency in this compartment can be detrimental to growth as it can lead to increased levels of unfolded proteins and increased ER stress.[23] The transcriptional regulation of *ZRG17* in response to zinc therefore serves as an important mechanism to help direct zinc into this compartment when cytosolic zinc levels are low. Recent studies suggest that a related mechanism may be important in higher eukaryotes. In humans, the zinc transporter Zip13 plays a critical role in delivering zinc to the ER and other organelles by controlling the release of labile pools of zinc that are located in vesicular stores.[24] Analysis of *ZIP13* transcript levels revealed that the expression of *ZIP13* increases with zinc deficiency.[24] Although, it is currently unknown if these increases in *ZIP13* expression are mediated by a transcriptional or post-transcriptional mechanism, this increase in gene expression potentially could help direct zinc to the ER under these conditions.

Many unicellular organisms live in a feast or famine environment, and therefore have to survive rapid transitions from severe zinc deficiency to zinc excess. Studies in yeast and bacteria have shown that transcriptional changes in the expression of zinc transport genes can be critical for survival during these transitions. In *S. cerevisiae*, Zap1 regulates the expression of *ZRC1*, a gene required for import of zinc into the vacuolar storage compartment.[25] Since Zap1 target genes are induced in zinc-limited cells, at first it seems surprising that yeast would express higher levels of a gene required for zinc storage under zinc-limiting conditions. An explanation for the Zap1 dependent regulation of *ZRC1* was revealed in a zinc shock experiment.[26] In zinc shock, cells are grown under zinc-limiting conditions, which lead to the

expression of genes required for high affinity zinc uptake. If these cells are then exposed to a short dose of zinc, this leads to the rapid influx of zinc into a cell (i.e. zinc shock). Under these conditions, increased expression of *ZRC1* ensures that zinc can be rapidly removed into vacuolar stores. Thus, the regulation of *ZRC1* by Zap1 serves as a proactive mechanism to protect zinc-limited cells from a sudden exposure to high zinc. Studies in prokaryotes have revealed that they also use mechanisms to survive zinc shock.[27] In *Escherichia coli*, ZitB and ZntA both facilitate zinc efflux.[28-30] ZntA is regulated at a transcriptional level by the zinc-responsive factor ZntR, and is therefore primarily expressed in zinc-replete cells.[31] In contrast, ZitB is expressed in zinc-limited and zinc-replete cells.[27] While increased expression of ZntA in response to high zinc is critical for the survival of *E. coli* when zinc is in excess, the rationale for expressing a zinc efflux transporter under zinc-limiting conditions becomes apparent during zinc shock. Under these conditions, ZitB plays a critical role in the initial rapid efflux of zinc from a cell.[27] Thus, the precise expression level of zinc transporter genes can directly influence the levels of zinc in a cell and its distribution, and have protective functions.

3.2 Zinc-binding and non zinc-binding protein isoforms

In prokaryotes, one commonly used mechanism to mobilize or conserve zinc when it is limiting, is a shift from using a zinc-dependent enzyme to an equivalent non-zinc requiring enzyme. An elegant example of this type of switch is the alternative use of ribosomal protein isoforms.[32] Many bacterial genomes contain duplicate copies of specific ribosomal subunits (L36, L33, L31, and S14).[33] One copy of these subunits is constitutively expressed and contains a zinc-binding motif. The other copy lacks the zinc-binding motif and is often under the control of Zur.[33] As Zur target genes are preferentially expressed in zinc-deficient cells, the Zur-mediated regulation of these paralogs results in the increased expression of the non zinc-requiring subunits when zinc is limiting. Depending upon the location of the subunit within the ribosome, the Zur-mediated regulation of the paralogs can help mobilize zinc or help cells survive longer periods of zinc deficiency. The L31 subunit is surface exposed and is loosely associated with the ribosome. The non-zinc requiring L31 isoform is therefore able to displace the zinc-

requiring subunit from the ribosome.[34, 35] Although the molecular fate of the L31 zinc bound subunit has yet to be examined *in vivo*, its turnover presumably releases zinc for other functions. In contrast, the S14 subunit differs in that it is buried deep within the ribosome. The replacement of the zinc binding subunit with its non-zinc binding counterpart therefore requires *de novo* protein synthesis.[36] The Zur-dependent regulation of this subunit is thought therefore to serve as a 'fail-safe' mechanism to ensure that the function of the 30S ribosome is maintained under zinc-limiting conditions. Thus, the zinc-dependent changes in the L31 and S14 isoforms illustrate how changes in gene expression can mobilize zinc for other more essential functions or provide a means of reducing the zinc proteome to allow survival during conditions of severe zinc deficiency.

A number of other examples of zinc-regulated switches in protein isoform are found in prokaryotes, suggesting that this is a common mechanism for mobilizing or conserving zinc.[37-40] However, further analysis of other zinc-regulated protein isoforms suggests that not all gene switches lead to the replacement of a zinc-binding protein with a non-zinc binding protein. In *Anabaena* PCC 7120, Zur regulates an operon that contains 9 genes, two of which encode paralogs of the important housekeeping zinc binding proteins, ThrS, and FolE.[39] ThrS is a zinc-binding threonyl-tRNA synthetase, while FolE is the zinc-dependent enzyme GTP cyclohydrolase I. In contrast to non-zinc binding paralogs that are typically under the control of Zur, in *Anabaena* the Zur-regulated *thrS* and *folE* genes are atypical in that they retain their zinc-binding motif. Although it has yet to be determined whether these Zur-regulated paralogs bind zinc, their placement in a Zur-regulated operon suggests that they have an important function in a zinc-limited cell. In yeast, a number of iron-sulphur cluster proteins are expressed at extremely high levels. This high expression ensures that if iron becomes limiting, and only a small subset of these proteins obtain their metal cofactor, there are still sufficient levels of the iron-bound protein for a cell to function without any deleterious effects to cell growth.[41] Thus, the Zur-dependent regulation of the *thrS* and *folE* paralogs could be a mechanism to increase the level of these proteins when zinc is limiting, ensuring that at least a small subset of these proteins

obtain zinc. Alternately, the Zur-regulated isoforms might obtain zinc more readily than their counterpart, bind a different metal cofactor, or function more efficiently in zinc-limited cells.[39] While the precise reason for their regulation is unknown, an understanding of these atypical zinc-dependent gene switches will establish whether specialized forms of these enzymes have evolved to have an optimal activity in a zinc-limited cell, or if increased gene expression is a mechanism to guarantee that at least a subset of these proteins obtain zinc when it is limiting. Interestingly, studies with ribosomal L33 paralogs have revealed that expression of the non-zinc containing form under zinc-limiting conditions does not confer any major growth advantage.[34] While these results suggest that incorporation of the non-zinc binding L33 paralog does not lead to any significant change in the mobilizable pool of zinc, ribosomal subunits can have important regulatory functions outside of the ribosome, and differences in ribosomal composition can affect which subsets of mRNAs are translated.[42-45] These results raise the possibility that zinc-dependent switches in ribosomal protein isoforms may have alternative regulatory roles that affect a different aspect of zinc homeostasis.

Although the majority of these zinc-dependent changes in protein isoforms have been reported in prokaryotic systems, a notable exception is the zinc-dependent switch in alcohol dehydrogenase gene expression in yeast. In budding and fission yeast, *adh1* (alcohol dehydrogenase 1) expression is repressed in zinc-limited cells, while the expression of *adh4* (alcohol dehydrogenase 4) is induced.[46, 47] Both Adh1 and Adh4 are able to catalyze the conversion of acetaldehyde to ethanol, however they are structurally distinct enzymes; Adh1 is an abundant zinc-binding alcohol dehydrogenase, while Adh4 resembles the iron-activated *ADHII* from *Zymononas mobilis.*[48] As up to 5 % of all zinc is bound to Adh1 under normal growth conditions, a reduction in *ADH1* gene expression under zinc-limiting conditions helps to conserve zinc for other functions.[46] Consistent with this idea, *ADH1* gene expression is also increased by zinc excess in a range of bacterial species, suggesting that the tight control of this abundant enzyme in response to zinc is an important homeostasis mechanism.[49, 50] As Adh4 shares sequence homology with an iron-requiring alcohol dehydrogenase, the shift from using

Adh1 to Adh4 at first appears to be a straightforward mechanism of zinc conservation, where a zinc enzyme is replaced with an iron-binding enzyme. However, *in vitro*, Adh4 from *S. cerevisiae* is only active when bound by zinc and not by ferrous ions [51], raising the question: why replace one zinc binding protein with another? In yeast, Adh1 exists as a tetramer in which each monomer binds 2 zinc ions, while Adh4 is predicted to exist as a dimer, in which each monomer binds one zinc ion.[48] Thus, the switch from Adh1 to Adh4 could potentially save zinc. As Adh4 is strictly localized to the mitochondria,[52] and Adh1 is located within the cytosol, zinc-dependent isoform switches in eukaryotic cells may also be more complex and have other regulatory purposes. For example, the increased expression of *adh4* under zinc-limiting conditions may ensure that zinc for alcohol dehydrogenase function is preferentially taken from a labile mitochondrial zinc pool.[53] Alternatively, as the conversion of acetaldehyde to ethanol results in the regeneration of NAD^+ from NADH and the inner mitochondrial membrane is impermeable to these molecules, the switch to Adh4 would affect the balance the NAD^+/NADH ratio in the mitochondria and cytosol. Thus, the tight regulation of *adh4* gene expression in yeast may be due to differences in mitochondrial metabolism or cytosolic/mitochondrial zinc distributions, when zinc is limiting.

3.3 Metallothioneins

In a number of organisms, including cyanobacteria, *Schizosaccharomyces pombe*, and mammals, zinc-responsive transcription factors regulate the expression of metallothionein genes.[54-56] Metallothioneins are small, cysteine rich proteins that bind zinc, copper, and other heavy metal ions. As metallothionein gene expression increases when zinc is in excess, one of its functions is to bind excess zinc and protect cells from zinc toxicity. In addition to this protective function, metallothioneins may play a much more significant role in zinc homeostasis as the zinc bound to metallothionein is kinetically labile.[57] Metallothioneins are therefore able to donate zinc to apo-proteins or other ligands, and thus provide a labile pool of zinc that can be used for other functions as needed.[58] Notably, numerous prokaryotes rely on the increased expression of zinc efflux proteins as a primary mechanism of protecting against

zinc toxicity. However, many marine strains of cyanobacteria instead rely on increased expression of zinc-binding metallothioneins and thus zinc sequestration when zinc is in excess.[54] As zinc is likely limiting in most marine environments, this strategy of preferentially upregulating metallothionein expression could have a dual role in protection against zinc excess, and providing a source of zinc that could be used during periods of zinc-limitation.[54] While metallothioneins clearly play an important role in zinc homeostasis, it is noteworthy that they are not essential for life,[59, 60] and some organisms express copper-binding metallothioneins that are regulated by copper levels.[61, 62] Thus, metallothioneins likely play critical roles in zinc buffering, storage, and delivery in some organisms; however, there must also be other ligands or proteins that have similar functions (see section 5.5, Other factors that affect zinc ion sensing).

3.4 Other zinc-regulated genes

In addition to genes that help maintain zinc homeostasis, zinc-responsive factors also control the expression of genes that can be critical for an organism to survive in their environmental niche. For example, in pathogenic fungi, zinc-responsive transcription factors can control the expression of additional genes that are important for virulence and invasion of host tissues.[63-65] Increased expression of zinc transport genes in pathogenic fungi and bacteria also is a contributing factor to survival and virulence on infection.[66-68] Other zinc-regulated genes can help cells to adapt and survive longer periods of zinc starvation. For example, zinc-limitation leads to increased levels of oxidative stress.[69] To counteract oxidative stress during zinc deficiency, Zap1 in *S. cerevisiae* increases the expression of *CTT1,* which encodes a cytosolic catalase,[70] and *TSA1*, which encodes a peroxiredoxin chaperone which helps to protect unfolded proteins from aggregating.[71, 72] Zap1 also suppresses sulphate assimilation by regulating the expression of *MET30*, a negative regulator of the sulphur gene network.[73] This suppression helps to conserve NADPH for antioxidant pathways that heavily rely on it. In addition to the above, zinc-dependent transcription factors regulate the expression of genes involved in a wide range of metabolic processes including copper homeostasis,[74] iron homeostasis,[75, 76] and phospholipid

biosynthesis.[77] While the reason for the change in gene expression is not always known, the direct regulation of a gene by zinc-responsive factors suggests that increased or decreased expression of the gene is likely to be beneficial to a zinc-limited or zinc-replete cell. Thus, a greater understanding of why these genes are regulated by zinc will likely provide important insight into other processes that require zinc, or are affected by alterations in zinc levels.

Transcriptome analyses in prokaryotes and eukaryotes have revealed that there is a hierarchy in which genes are induced and repressed in response to changes in zinc levels.[70, 78] In *S. cerevisiae*, Zap1 target genes can be divided into two groups; those that play a critical role in zinc homeostasis and those that are necessary for a yeast cell to adapt to longer periods of zinc starvation.[70] Genes that are rapidly induced under conditions of mild zinc deficiency are typically necessary for zinc uptake or release of zinc from intracellular stores. However, if cells become more severely zinc limited, additional genes are induced that help cells to survive and adapt to prolonged periods of zinc starvation. Thus, in addition to understanding which genes are regulated by zinc responsive factors, the temporal manner in which they are regulated could provide additional information on whether they have a primary role in zinc homeostasis or survival. So far, studies of specific target genes have revealed that a graded response in gene expression can arise from a number of mechanisms including differences in affinities and the number of Zap1 binding sites,[79] and the specific regulatory mechanisms controlling transcription and translation.[80] Studies in *Streptomyces coelicolor*, suggest that a graded response of target gene repression to zinc is also observed in prokaryotes. In *S. coelicolor*, mild zinc deficiency leads to the derepression of Zur-target genes required for zinc uptake, while more severe zinc deficiency leads to the derepression genes required for coelibactin synthesis.[78] Coelibactin is a non-ribosomal synthesized peptide that might act as a zincophore.[81] However, similar responses were not observed in *Bacillus subtilis,*[82] suggesting that this differential regulation of target gene expression in response to zinc is not common to all Zur family members.

4. Balancing Zinc Uptake and Efflux

All cells need to protect themselves from zinc limitation and zinc excess. In prokaryotes this precise balance is maintained using pairs of zinc-responsive factors, one to sense zinc deficiency and one to sense zinc excess. For example, *E. coli* expresses both Zur and ZntR to protect against zinc deficiency and zinc excess, respectively. *In vitro* analysis of the Zur and ZntR pair revealed that the levels of zinc required to repress Zur function are very close to the levels of zinc required to activate ZntR.[83] These results suggest that there is a very narrow range in which a cell has an optimal level of zinc, and that even a small deviation from the 'optimal norm' for zinc will trigger changes in gene expression to immediately counter the change in cytosolic zinc levels.

While the majority of prokaryotes rely on two zinc-responsive factors to control zinc homeostasis, a possible exception is found in the phytopathogen *Xanthomonas campestris*. In this bacterium, Zur functions as a repressor of a high affinity zinc uptake gene, and an activator of a zinc efflux system.[84] In both cases, Zur mediates the regulation by directly binding to target gene promoters. However, the precise DNA recognition element differs between the induced and repressed target genes. Currently, the mechanism of why Zur is an activator at one site and a repressor at another is unclear. The inverse regulation of zinc uptake and efflux systems by the same factor will also result in the extremely tight control of zinc homeostasis. As other bacterial zinc-responsive factors can have dual functions in gene activation and repression,[49] this might represent a common alternative strategy to coordinate zinc uptake with zinc efflux.

Eukaryotes have also evolved a variety of strategies that result in their zinc-responsive factors functioning as both activators and repressors of gene expression. In *S. cerevisiae*, Zap1 is a transcriptional activator that induces target gene expression when zinc is limiting. At most target genes, Zap1 binds to zinc-responsive elements (ZREs) in the promoter region, which in turn leads to gene activation (Figure 3A, Normal).[70] However, at the *ADH1* and *ADH3* promoters, Zap1 binds to a single ZRE that is located upstream of the binding sites for the transcriptional activators Gcr1 and Rap1.[46] Recruitment of Zap1 to this site, leads to the expression of intergenic non-protein coding RNA

(ncRNA) transcripts. Increased expression of these ncRNA transcripts in turn likely induces nucleosome deposition over the core promoter and Rap1/Gcr1 binding sites resulting in a reduction in expression of *ADH1* and *ADH3* (Figure 3A, Intergenic ncRNA).[46, 80] Thus, through the regulation of an ncRNA transcript, the transcriptional activator Zap1 is able to function as a transcriptional repressor. Recent studies have also shown that Loz1 regulates the expression of an intergenic transcript at the *zym1* promoter in *S. pombe*, and that *zym1* mRNA levels are inverse to those of the intergenic transcript.[55] Thus, a related ncRNA mechanism may also control *zym1* expression in fission yeast.

In *S. pombe*, Loz1 typically represses target gene expression when zinc is in excess (Figure 3B, Normal).[55] However, Loz1 is also required for the repression of *adh1* expression in zinc-limited cells. The regulation of *adh1* gene expression by zinc in *S. pombe* requires increased expression of an antisense transcript under zinc-limiting conditions (Figure 3B, Antisense ncRNA).[47] As deletion of *loz1* leads to the constitutive expression of the *adh1AS* transcript and repression of *adh1* gene expression, it is likely that Loz1 controls *adh1* gene expression by binding downstream of the *adh1* open reading frame and regulating the expression of the *adh1AS* transcript.[47, 55] Thus, by controlling the expression of an antisense ncRNA, Loz1 can indirectly function as a transcriptional repressor in zinc-limited cells.

A final strategy that can switch the regulatory action of zinc-responsive factors in eukaryotes is through the direct inhibition of RNA polymerase II progression. Examples of this regulatory switch include the regulation of *ZRT2* levels by Zap1,[79] and the regulation of *Zip10* expression by MTF-1.[85, 86] In mammals MTF-1 typically binds to metal responsive elements (MREs) in target gene promoters and activates gene expression when zinc is in excess (Figure 3C, Normal).[56] However, at the *Zip10* promoter, MTF1 binds to a MRE element that is located immediately downstream of the transcriptional start site.[85] Binding at this site blocks the progression of RNA polymerase II, thereby reducing *Zip10* expression in zinc-replete cells (Figure 3C, Inhibition of RNA pol II progression). As Zip10 plays a primary role in zinc uptake in hepatocytes these results illustrate how MTF-1 can also play an important role in protecting cell from zinc limitation.

Thus, through the regulation of ncRNAs, or by inhibiting the progression of RNA polymerase II, the regulation of zinc-responsive factors in eukaryotes can be flipped and zinc homeostasis can be precisely coordinated without the need for a different regulatory factor. In addition, these factors have opposing roles in the regulation of zinc uptake and zinc efflux/storage genes. This suggests that as with the bacterial systems, in eukaryotes there might also be an extremely narrow range in which a cell has optimal levels of zinc before homeostasis mechanisms start to protect cells from zinc deficiency or zinc excess.

5. Mechanisms of Zinc sensing

A critical part of maintaining zinc levels is the rapid activation or inactivation of a zinc-responsive factor by zinc. In the following section we review some of the recent advances that have been made in our understanding of how these factors sense zinc ions.

5.1 Zinc sensing in prokaryotes

The zinc-responsive transcription factors found in prokaryotes all belong to well-characterized, larger families of structurally related transcription factors.[18, 87] For example, Zur belongs to the Fur family of transcription factors that typically bind to DNA and repress target gene expression when metals are in excess.[88] Zur family members are unique in that they detect changes in cytosolic zinc levels. Structural and mutagenesis analysis of Zur proteins, suggests that under normal conditions Zur is found as an inactive dimer that binds one structural zinc ion/monomer.[78, 82, 89] However, as zinc levels increase, depending on the organism, Zur binds 1-2 additional regulatory zinc ions/monomer leading to a fully active repressor with a high affinity for DNA. Thus, Zur proteins directly 'sense' intracellular zinc ions, which in turn influence their ability to bind to DNA and repress target gene expression.

While the large majority of prokaryotes rely on Zur proteins to control the expression of high affinity zinc uptake genes, *Streptococci* and *Lactococci* differ in that members of the MarR family of transcription factors, AdcR and ZitR, sense zinc limitation and control zinc uptake. Recent analyses with AdcR

indicated that in its zinc-bound form AdcR binds with a high affinity to operator regions and represses target gene expression.[90] *Streptococci* are also atypical in that they use SczA, a member of the TetR family of transcription factors, to sense zinc excess and control zinc efflux.[50] Interestingly, *in vitro* footprinting and EMSA analysis of SczA binding to the *czcD* promoter suggests that under zinc-limiting conditions, SczA binds downstream of consensus sequences for RNA polymerase recruitment and represses gene expression. However, when zinc is in excess, SczA binds to a different upstream site in the promoter and mediates activation of *czcD*.[50] Currently, the mechanism that leads to this switch in DNA binding site occupancy is unclear. SmtB, ZiaR, and CzrA all belong to the ArsR-SmtB family.[87] Members of this family bind to DNA and repress gene expression when metals are limiting. As metal levels increase, metal ion binding to each of the factors leads to loss of DNA binding function, and derepression of target gene expression.

While zinc-dependent changes in DNA binding function play a critical role in the regulation of most prokaryotic zinc sensors, ZntR differs from the other prokaryotic zinc responsive factors in that it is bound to DNA in both zinc-limiting and replete conditions. ZntR belongs to the MerR family of transcription factors.[18] In this family metal binding induces a conformational change that unwinds and distorts the DNA helix, which in turn aligns critical DNA elements for RNA polymerase recruitment and gene activation.[91] Thus, a variety of zinc-responsive factors are utilized in prokaryotic systems to control zinc homeostasis. Although the precise mechanism of gene regulation differs, in all known cases, zinc binding to each factor induces a conformational change that in turn directly affects function.

Since the prokaryotic factors all 'sense' zinc levels by binding zinc directly, studies of these sensors have provided important insight into the optimal 'set point' around which zinc levels fluctuate. *In vitro,* the prokaryotic factors Zur and ZntR respond to zinc in the femtomolar range,[83] suggesting that the large majority of zinc found in an *E. coli* cell is either bound or buffered, and that the levels of 'free' zinc ions which the metallosensors detect are extremely low (less than one atom per cell). Studies with different metal sensing systems have also revealed that the affinities of other metallo sensors for their

respective metal ion effector have evolved according to the natural order of stability for divalent metals, or the Irving-Williams series[87, 92]. Metals such as zinc and copper are placed at the top of the Irving-Williams series, as they tend to bind more strongly to organic molecules than other essential divalent metal ions. Thus, the extremely low set points for prokaryotic zinc sensors is consistent with 'free' zinc ions being kept at relatively low levels in the cytoplasm to ensure that zinc does not interfere with the homeostasis of weaker divalent metals ions.

While the above *in vitro* analyses provide important information of the levels of zinc that are necessary to trigger a conformational change in the zinc-responsive factors, it is noteworthy that *in vivo*, a number of the factors are subject to additional levels of regulation. For example, some AdcR and SmtB family members bind to their own promoter and auto-regulate their own expression.[93, 94] In *E. coli,* ZntR has a shorter protein half-life in zinc-limited cells due to increased degradation by the ClpXP and Lon proteases.[95] Zinc binding to ZntR and binding of ZntR to DNA both contribute to the enhanced stability of ZntR in zinc-replete cells. Thus, at least in some species the precise levels of zinc-responsive factors in a cell at any given time, will be influenced by zinc levels. Recent studies have also analyzed the ability of the zinc sensors to sense zinc *in vivo*, under zinc shock conditions.[27, 96] Using a zinc-responsive carbonic anhydrase FRET reporter to measure dynamic changes in intracellular zinc levels *in vivo,* Wang, *et al.* observed the expected rapid influx of zinc into a cell upon zinc shock.[27] However, the levels of total zinc remained significantly higher than the 'free' or 'readily exchangeable' zinc. In addition, by measuring changes in the expression of the target gene *zntA*, ZntR was found to sense zinc in the nanomolar range *in vivo*. The differences between the *in vitro* and *in vivo* analyses of ZntR suggest that other factors influence zinc ion sensing *in vivo.* For example, ligands in the cytosol may play an important role in buffering and providing a readily exchangeable pool of zinc that the sensors detect. Zinc shock in *E. coli* also leads to the transient increase in the activities of the iron-responsive factor Fur and the oxidative stress regulator SoxS.[96] This suggests that the large influx of zinc into a cell upon zinc shock, at least for a short time, affects other aspects of cell metabolism. Thus,

interesting future questions will be: which ligands buffer zinc in the cytosol, whether these ligands also buffer other metal ions, and whether their levels are altered by changes in cellular zinc levels.

5.2 Zinc Sensing in fungi

Much of what we know about how eukaryotic cells sense zinc deficiency comes from studies of Zap1, a transcriptional activator that was originally identified in the budding yeast *S. cerevisiae*. At least four different mechanisms ensure that Zap1 is only active in zinc-limited cells: auto-regulation, regulation of trans-activation domain 1 (AD1) by zinc, regulation of trans-activation domain 2 (AD2) by zinc, and the regulation of DNA binding activity.

The most widely studied zinc-regulated domain from Zap1 is AD2. AD2 contains two C_2H_2-type zinc finger domains, which fold together to form a zinc finger pair.[97] Both zinc finger domains and amino acid residues that are critical to pair formation, are necessary for the regulation of AD2 function by zinc *in vivo*.[98] In contrast to other zinc finger pair domains, the zinc bound to the AD2 zinc fingers is labile in nature, i.e. the bound zinc rapidly exchanges with other ligands.[98, 99] These requirements and properties have led to the hypothesis that in zinc-limited cells, the zinc fingers are not bound with zinc and AD2 is in an open active conformation. However, as zinc levels increase, binding of zinc to the zinc finger pair results in a conformational change masking amino acid residues critical for recruitment of co-activators. In strong support of this hypothesis, a FRET sensor containing AD2 flanked by enhanced yellow and cyan fluorescent protein is robustly regulated by zinc *in vivo*.[100] Moreover, when a related AD2-based FRET sensor was introduced into human cells, a similar zinc-dependent FRET was observed.[101] Since humans lack a Zap1 homolog, the strong zinc-dependent regulation of the AD2 FRET sensor in human cells indicates that the zinc-induced conformational changes of AD2 occur without the assistance of any additional yeast specific proteins, and therefore support the hypothesis that AD2 is a direct sensor of cytosolic zinc levels.

Less is known about the mechanisms by which zinc ions control the activity of AD1 and the Zap1 DNA binding domain. AD1 contains no known zinc binding motifs. However, it binds multiple zinc ions *in vitro,* and conserved cysteine and histidine residues that are located within AD1 are necessary for zinc sensing *in vivo.*[102] Regulation of AD1 by zinc also requires the presence of the Zap1 DNA binding domain.[102] These observations suggest that zinc binding to AD1 might induce a conformational change leading to an intramolecular interaction between it and the DNA binding domain inhibiting transactivation domain function. The Zap1 DNA binding domain contains 5 C_2H_2 zinc finger domains.[103, 104] As Zap1 is active in zinc-limited cells and all 5 of the zinc fingers that form the DNA binding domain are critical for binding, the regulation of Zap1 DNA binding activity is unlikely to be a result of changes in the zinc occupancy of any of the 5 zinc fingers. In addition, excess zinc does not inhibit Zap1 binding to ZREs *in vitro*,[104] suggesting that DNA binding control is not a result of zinc binding to an alternative 'regulatory' site in the DNA binding domain. In other transcription factors, phosphorylation of zinc finger linker regions can inhibit DNA binding function,[105, 106] while other zinc fingers have dual functions in mediating protein-protein interactions and DNA-protein interactions.[107] Thus, zinc-dependent regulation of the Zap1 DNA binding function could be indirect through a yet to be discovered post-translational mechanism.

In contrast to the prokaryotic sensors, in which a straightforward zinc-dependent allosteric switch can control their activity, Zap1 contains multiple zinc-responsive domains. Which raises the question, why would a single factor need multiple domains to sense zinc ions? *In vivo*, AD1 is a much stronger trans-activation domain than AD2, and therefore plays the primary role in activating gene expression during zinc limitation.[108] However, when zinc deficiency is combined with additional stresses such as heat shock, activation of a subset of Zap1 target genes requires the presence of AD1 and AD2.[108] A potential explanation for the dual requirement of two activation domains for the regulation of some target genes, would be that each activation domain recruits a different subset of coactivators. However, *in vivo* AD1 and AD2 interact with a similar set of coactivators under zinc-limiting

conditions.[109] Thus, it appears that the advantage of having two zinc-regulated trans-activation domains is that the additive effect of two domains enhances the recruitment of general co-activating proteins and thus ensures maximal gene activation under more extreme stress conditions.

While clear homologs of *ZAP1* can be found in the genomes of many fungal species, a notable exception was that no *ZAP1* homolog was present in the fission yeast *S. pombe* genome. This observation was surprising as the expression of genes necessary for zinc uptake is robustly regulated by zinc at a transcriptional level in fission yeast.[110] Recent studies have now revealed that *S. pombe* uses an entirely different factor to sense zinc, the zinc-responsive repressor Loz1.

Loz1 was discovered during a study examining the zinc-dependent regulation of *adh1* and *adh4* gene expression.[55] During a transformation to delete *adh1* from the genome, an *adh1*Δ mutant was isolated that contained a partial loss of function mutation in *loz1*. This loss of function allele (named *loz1-1*) conferred a growth advantageous to *adh1*Δ cells and led to the increased expression of genes that were typically not expressed in zinc-replete cells, including *zrt1* and *adh4*. As one consequence of the *loz1-1* allele was a large increase in *adh4* expression, it was hypothesized that the spontaneous occurrence of the *loz1-1* mutation in the *adh1*Δ background was due to increased expression of *adh4,* which in turn compensated for the absence of *adh1*. In support of this hypothesis, over expression of *adh4* in zinc-replete cells rescues all of the growth defects that are associated with *adh1*Δ cells.[55]

The discovery of Loz1 has raised many new questions, with one of the most significant being: does Loz1 directly sense zinc or is it part of a larger complex or signaling pathway? Loz1 contains 522 amino acids with only one known structural domain, a double zinc finger domain located at its extreme C-terminus. In other transcription factors, double zinc finger domains can mediate interactions with DNA.[111, 112] Consistent with the Loz1 double zinc finger domain being required for DNA binding activity, it is necessary for site specific binding to a GNNGATC element *in vitro*, and the *loz1-1* allele contained a C-G mutation leading to an arginine to glycine substitution at position 1 of the alpha helix of zinc

finger 2.[55] Amino acid residues at positions -1, 3 and 6 of the alpha helix typically make hydrogen bond contacts with three consecutive nucleotides in DNA.[2] Thus, a substitution at position 1 could potentially interfere with DNA binding.

Outside of the double zinc finger domain, only a few regions of Loz1 are conserved in closely related species. For example, in the N-terminus, a cluster of cysteine and histidine residues is conserved in *Schizosaccharomyces japonicus* and *Schizosaccharomyces octosporus* (Figure 4). However, this cluster is absent from *Schizosaccharomyces cryophilus*, a species that grows at lower temperatures relative to the other fission yeast species. Whether these conserved residues form a novel zinc-regulated domain that is advantageous under specific stress conditions, e.g. heat shock, remains to be determined. The high conservation of the Loz1 double zinc finger in *Schizosaccharomyces* species, and studies with Zap1 which demonstrate that zinc finger domains can act as cellular sensors of zinc, make the double zinc finger domain from Loz1 an attractive candidate for being involved in zinc sensing. Interestingly, this domain shares significant conservation with zinc finger domains from other fungal transcription factors. For example, the Loz1 double zinc finger domain shares 67% identity with the double zinc finger domain from MtfA in *Aspergillus nidulans*. MtfA is a transcriptional activator that regulates sexual and asexual development, and the synthesis of a number of secondary metabolites including penicillin and the mycotoxin sterigmatocystin.[113] Thus, if the Loz1 zinc finger domains are critical for zinc sensing, it raises the possibility that the activity of these other factors might also be responsive to zinc. Thus future studies with Loz1 are likely to provide important new insight into the mechanisms of zinc sensing.

5.3 Zinc sensing in plants and green algae

Zinc-dependent changes in the expression of genes involved in zinc transport has been observed in a wide variety of plants including rice, beans, and barley.[114-116] However, the majority of what is known comes from studies with the plant model system *Arabidopsis thaliana*. Here we focus on what is known

about the regulatory factors that mediate zinc-dependent changes in plants. More detailed information concerning tissue specific expression patterns of zinc transporters and other proteins/ligands involved in zinc homeostasis in plants can be found in the following reviews.[117, 118]

In *A. thaliana*, the basic-leucine zipper (bZIP) transcription factors, bZIP19 and bZIP23, play a central role in zinc homeostasis. The factors were identified using a one-hybrid based approach to identify genes that were required for the zinc-dependent regulation of the *ZIP4* zinc uptake transporter.[119] *In vivo,* single *bzip19* and *bzip23* mutants have no major growth defects. However, *bzip19 bzip23* double mutants are hypersensitive to zinc deficiency, suggesting that the factors have a redundant role in protecting *Arabidopsis* from zinc deficiency. Consistent with this hypothesis, transcriptome profiling revealed that the expression of 23 genes, including 5 additional zinc transport genes, was dependent upon bZIP19 and bZIP23.[119]

Currently, it is unclear how zinc modulates the activity of bZIP19 and bZIP23. Analysis of *bZIP19* and *bZIP23* transcript levels revealed a modest increase in 3 week old *Arabidopsis* seedlings grown in zinc-limited medium vs. zinc-replete.[119] As transcript abundance is largely unaffected by cellular zinc levels, these results suggest that zinc might directly or indirectly affect protein stability or other aspects of protein function (e.g. subcellular localization, DNA binding activity, or transactivation function). In contrast to the other zinc-responsive factors identified in eukaryotes, bZIP19 and bZIP23 do not contain any known zinc-binding motif. Both of these factors do contain a region that is rich in cysteine and histidine residues located at their N-terminus,[120] however, a highly related domain is present in bZIP24, a different bZIP family member that is a regulator of salt stress in *Arabidopsis*. Thus, future studies are required to determine if the activity of these factors is directly regulated by zinc and whether the N-terminal cysteine/histidine rich region is critical for this regulation.

Additional insight into zinc homeostasis in photosynthesizing organisms comes from studies with the green alga, *Chlamydomonas reinhardtii*. In this organism, zinc sensing and zinc homeostasis are

tightly linked to copper homeostasis. In C. *reinhardtii*, the Copper Responsive Regulator 1 (CRR1) is required for the activation of gene expression under conditions of copper deficiency.[121] CRR1 contains two metal-responsive domains: an SBP domain that contains two adjacent zinc finger like domains which fold together to form a single globular domain, and a C-terminal cysteine rich domain resembling the copper-sensing metallothionein like domain found in the *drosophila* MTF-1. While the SBP domain has a dual role in DNA binding and copper sensing, deletion of the C-terminal cysteine rich region resulted in an increase in expression of genes required for zinc uptake, and a 5-fold increase in cellular zinc levels.[121] Although it is not yet clear why deletion of this domain leads to aberrant zinc homeostasis, additional studies indicate that there is a tight connection between copper and zinc homeostasis in this organism. Zinc deficient C. *reinhardtii* cells hyperaccumulate copper, but are copper deficient from a metallo-sensing perspective.[122] Interestingly, this copper-zinc connection is reminiscent of the crosstalk between metal set points in prokaryotic cells, where intracellular copper levels are kept lower than zinc to avoid incorporation of copper into zinc-binding sites. One explanation for this regulation could therefore be that green alga compartmentalizes copper, or keeps copper in a bio-unavailable form, to ensure that it is not deleterious to growth when zinc is limiting. Thus, future studies with green alga will likely provide new insights into the crosstalk that exists between copper and zinc homeostasis in photosynthesizing organisms.

5.4 Zinc sensing in animals

Much of what is known about zinc sensing in the animal kingdom comes from studies with MTF-1 (for Metal-responsive transcription factor-1). MTF-1 is a transcriptional activator that is found in insects, fish, reptiles, and mammals.[56] In fish, reptiles, and mammals, MTF-1 plays a central role in zinc homeostasis by activating the expression of zinc efflux and metallothionein genes when zinc is in excess. In flies, MTF-1 activity is tightly regulated by copper availability, and it has a primary role in maintaining copper homeostasis. In this review we have focused on the role of MTF-1 in zinc

homeostasis. More details of the role that MTF-1 plays in copper homeostasis can be found in other reviews.[56, 123]

The regulation of MTF-1 activity by zinc is complex, in that zinc affects DNA binding activity, subcellular localization, and trans-activation function. Under normal conditions, MTF-1 is located within the nucleus and cytoplasm.[124] However, when a cell is exposed to high zinc or other stressors, MTF-1 accumulates in the nucleus. While the ability of a transcription factor to shuttle between the nucleus and cytosol can play a critical role in metal ion sensing,[125] when MTF-1 activity was examined in the presence of an inhibitor of nuclear export, it was still inducible by zinc.[124] These results suggest that the changes in the cellular localization of MTF-1 are not critical to zinc sensing, and possibly serve as a mechanism to enrich it in the nucleus under conditions of stress.

The MTF-1 DNA binding domain contains 6 C_2H_2-type zinc fingers. In contrast to the majority of C_2H_2-type zinc finger domains that bind zinc with an extremely high affinity, *in vitro* studies with MTF-1 suggested that zinc finger domains 5 and 6 bind zinc with a lower affinity.[126-128] As MTF-1 binds to DNA in zinc-replete cells, these results suggested that zinc-dependent changes in the occupancy of these low affinity zinc finger domains could serve as a mechanism to control DNA binding function. In contrast, other studies found that there is relatively little difference in the zinc binding affinities of the MTF-1 zinc finger *in vitro*,[129] suggesting that other properties of the zinc fingers may be critical to zinc sensing. *In vivo*, zinc finger 1-4 are sufficient for zinc-dependent regulation of an MRE reporter.[130] However, all 6 zinc fingers are necessary for full zinc-dependent induction of *MT1* gene expression *in vivo*, suggesting that zinc fingers 5 and 6 are necessary for gene regulation on endogenous chromatin templates.[131] While the mechanism of DNA binding control remains controversial, in mice but not humans, mutations that target the linker region between zinc fingers 1 and 2 impair the ability of MTF-1 to sense zinc, suggesting that additional mechanisms may also contribute to the zinc-dependent regulation of MTF-1 function in some organisms.[56, 132]

More recent studies have focused on how MTF-1 transactivation domain function is regulated by zinc. MTF-1 contains three transactivation domains. Of these, the strongest is an acidic rich domain that is adjacent to the DNA binding domain.[56] When this domain was fused to a heterologous DNA binding domain, its activity was regulated by zinc in some mammalian cell lines, but not others.[124] These results suggest that zinc affects MTF-1 transactivation domain function. As this regulation is only observed in some cells types, it is also possible that other factors that are only found or expressed in those cells may be critical for the zinc-dependent regulation of this domain. While this hypothesis has yet to be tested, if other factors are required, this could provide a means of fine tuning MTF-1 activity in response to other developmental or cellular signals.

In addition to zinc, other cellular stressors including heavy metal ions, hypoxia, and oxidative stress, lead to an increase in MTF-1 activity.[56] *In vitro* MTF-1 is robustly regulated by zinc, but not by cadmium or copper ions. However, when zinc-bound metallothionein was added to the *in vitro* system, cadmium and copper ions were able to regulate MTF-1 activity.[133] Thus, the regulation of MTF-1 function by other metals and stressors could be a direct result of displacement of zinc from MT-1, or other zinc-containing proteins, which in turn regulates MTF-1 activity.

While the majority of studies in mammals have so far focused on MTF-1, other factors (or alternative regulatory mechanisms) must exist to coordinate gene expression with changes in cellular zinc levels in higher eukaryotes. For example, ZnT5 expression is induced by zinc excess.[68] This regulation is independent of MTF-1 and requires the presence of zinc transcriptional regulatory element (ZTRE) in the ZnT5 promoter.[134] Transcriptional profiling also has shown that the expression of a large number of genes is affected by zinc deficiency or zinc excess in human cells.[135, 136] For the most part, the regulatory mechanisms of these changes are unknown.

In addition to the direct regulation of genes responsible for zinc homeostasis by zinc-responsive factors, new studies suggest that other zinc-binding proteins may have important roles in sensing changes in

cellular zinc levels. Copper-zinc Superoxide Dismutase I (Sod1), is an abundant enzyme that destroys superoxide radicals. In humans, mutations in *SOD1* can lead to amyotrophic lateral sclerosis (ALS), a neurodegenerative disorder that leads to a loss of motor neurons in the central nervous system.[137] Although many different mutations in *SOD1* can lead to ALS, a significant number of these mutations lead to mutant Sod1 proteins that gain the ability to bind to the cytosolic C-terminal domain of Derlin-1, a component of the ER-associated degradation (ERAD) machinery.[138] The interaction of the mutant Sod1 proteins with Derlin-1 triggers the ER stress response, leading to apoptosis and ultimately motor neuron death. Under severely zinc-limiting conditions, Sod1 adopts a similar conformation to the mutant Sod1 proteins observed in ALS patients, and as a consequence interacts with Derlin-1 and triggers the ER stress response.[139] Intriguingly, one consequence of the ER stress response is an increased expression of the *ZIP14* zinc uptake system.[139] The direct regulation of *ZIP14* through this pathway, suggests that in addition to its known role in destroying superoxide radicals, Sod1 may have an additional function by acting as a cytosolic sensor of zinc levels. As the ER stress response has many other global affects on cell metabolism, including attenuating translation, this Sod1-dependent regulation could represent a survival mechanism that protects cells from severe zinc deficiency.

5.5 Other factors that affect zinc ion sensing

Studies of other metallo-regulatory systems have identified specialized protein chaperones, which deliver metal ions to their respective protein partner.[140, 141] As the number of individual proteins that require a zinc cofactor is large, it is unlikely that specialized zinc chaperones are present that deliver zinc to a specific protein. A more likely scenario is that zinc-binding ligands and/or proteins buffer zinc in the cytosol and provide a readily exchangeable pool of zinc for cellular metabolism. In addition to zinc-binding metallothioneins (see above), transcriptome profiling has revealed a number of additional proteins that may play an important role in buffering/trafficking zinc. In prokaryotes and eukaryotes, COG0523 domain proteins are often highly upregulated in response to zinc-limitation,[142] suggesting that these proteins may have an important role in zinc homeostasis. In bacteria, other genes that are

commonly found in operons with zinc transporter genes, encode periplasmic zinc-binding proteins. Recent studies have shown that such proteins are able to scavenge zinc in the periplasm, for later delivery to zinc uptake systems.[143-145]

Small molecules that potentially buffer zinc include glutathione, and the amino acids cysteine and histidine.[146-149] In *E. coli*, ZntR target genes include 9 genes required for the synthesis of cysteine.[147] As ZntR target genes are expressed when zinc is high, the increased production of cysteine could serve as a mechanism to buffer zinc, or potentially retain zinc in the cytosol. Genetic screens performed in *Caenorhabditis elegans* revealed that mutations in *haly-1* conferred a significant tolerance to zinc.[148] *haly-1* encodes histidine ammonia lyase, the first enzyme that is required for the breakdown of the amino acid histidine. As *haly-1* mutants display elevated levels of histidine, these results are consistent with increased levels of histidine helping to buffer zinc. In mammals, increased levels of histidine in the diet can also lead to elevated excretion of histidine and zinc in the urine.[150, 151] Thus, changes in the levels of these small molecules can protect against zinc toxicity, and can potentially influence cellular and systemic zinc ion distribution.

6. Conclusions

In summary, zinc-responsive transcription factors are found in all kingdoms of life, and play a central role in zinc homeostasis by regulating the expression of genes required for zinc uptake and zinc efflux/storage. These factors also control the expression of additional genes that help cells to survive and adapt to conditions of zinc starvation or zinc overload. Although zinc-responsive factors from different species greatly differ in structure, some aspects of their function are conserved. In prokaryotes for example, pairs of transcription factors typically ensure that zinc levels tightly fluctuate around an 'optimal set point'. In yeast and mammals, individual zinc-responsive factors can have reciprocal roles in regulating the expression of genes that protect against zinc deficiency and zinc excess, suggesting that these cells might also rapidly swing from being zinc-limiting to zinc-replete.

Other commonly used strategies include the tight regulation of metallothionein gene expression by zinc, and changes in gene expression that result in the decreased use of abundant zinc binding proteins when zinc is limiting.

Although a number of new zinc-responsive factors have been recently identified, and studies of known factors and target genes have provided important advances in understanding the basic mechanisms of zinc sensing and zinc homeostasis, many questions still remain. In all cells, it is largely unknown how zinc is buffered in the cytosol and whether the zinc buffering capacity changes with cellular zinc status. In eukaryotes, the mechanisms by which zinc-responsive factors sense zinc are largely unknown. In addition, it is generally unclear if the activity of zinc-regulated domains is through direct zinc ion binding, or if the regulation is indirect and requires additional proteins or metabolites. Transcriptomic profiling has suggested that many genes are regulated by zinc in a manner that is independent of known zinc-responsive factors. Thus, other factors or regulatory mechanisms have yet to be identified. Zinc can also act as a signaling molecule in some cell types.[4, 5] As zinc signaling leads to dynamic changes in cytosolic zinc levels, in these cells it will be important to determine what effects this has on the zinc proteome, and how zinc homeostasis is restored following signaling. Finally, in addition to zinc-dependent changes in gene expression, most organisms contain additional pathways that allow the tight control of zinc homeostasis. For example, in prokaryotes, two-component regulatory systems can play an important role in zinc sensing in the periplasm.[152] In eukaryotes, mRNA stability, and protein translation and stability, can be regulated by zinc.[153-158] Thus, a complete understanding of zinc homeostasis will require knowledge of how these mechanisms work together to ensure that zinc levels are precisely balanced.

Abbreviations

ABC ATP binding cassette

CDF	Cation Diffusion Facilitator
EMSA	Electrophoretic Mobility Shift Assay
FRET	Fluorescence Resonance Energy Transfer
LIM	Lin11 Isl-1 Mec-3
RING	Really Interesting New gene
RND	Resistance Nodulation Division
ZIP	ZRT1 IRT1 like protein

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Figure legends

Figure 1. Zinc responsive factors directly regulate the expression of genes required for zinc uptake, zinc efflux, and/or zinc storage. (a) The transcription factors Zap1, bZip19, bZip13, Zur, Loz1, and AdcR protect cells from zinc deficiency by regulating the expression of genes required for zinc uptake. Zap1, bZip19, and bZip13 activate gene expression under zinc-limiting conditions, while Zur, Loz1, and AdcR are derepressed under these conditions. (b) The factors MTF-1, ZntR, SczA, and ZiaR protect cells from zinc excess by regulating the expression of genes required for zinc efflux and/or storage. MTF-1, ZntR, and SczA, activate gene expression when zinc is in excess, while SmtB represses gene expression when zinc is limiting. In the diagram, grey circles with 'A' represent activators, grey circles with 'R' represent repressors, rectangles represent the target genes, and the red hexagon represents the zinc storage protein metallothionein.

Figure 2. Changes in gene expression that affect zinc homeostasis. When zinc is limiting, transcriptional changes that lead to increased cytosolic zinc levels include the up-regulation of genes required for zinc uptake (1) and in eukaryotes release of zinc from intracellular stores (2). Changes in gene expression that reduce the levels of zinc-binding proteins also help to conserve zinc for more essential functions (3). When zinc is in excess, transcriptional mechanisms that help to decrease cytosolic zinc levels include the increased expression of genes required for zinc efflux (4), storage (5), and in eukaryotes compartmentalization (6). In eukaryotes, zinc can be compartmentalized in organelles or in specialized zinc storage vesicles called zincosomes.

Figure 3. Mechanisms of gene regulation in eukaryotes that reverse the regulatory action of a zinc-responsive transcription factor. (a) In *S. cerevisiae*, Zap1 normally activates gene expression in zinc-limited cells. At the *ADH1* locus, Rap1 and Gcr1 activate gene expression when zinc is in excess. When zinc is limiting, Zap1 binds to an upstream ZRE and induces the expression of an intergenic transcript. This transcript inhibits *ADH1* expression by possibly increasing nucleosome deposition over the promoter. (b) In *S. pombe*, Loz1 normally represses gene expression when zinc is in excess. At the *adh1* locus, in zinc-replete cells *adh1* is expressed at high levels. When zinc is limiting, Loz1 derepression results in the increased expression of an antisense transcript inhibiting *adh1* expression. (c) In humans, MTF-1 is normally active in zinc-replete cells. At the *ZIP10* locus, MTF-1 binds to an MRE that is located downstream of the transcriptional start site. Binding at this site inhibits progression of RNA polymerase II.

Figure 4. Conserved domains in Loz1 homologs. An alignment of the conserved C-terminal double zinc finger domain of Loz1 from *Schizosaccharomyces pombe*, *Schizosaccharomyces japonicus*, *Schizosaccharomyces octosporus,* and *Schizosaccharomyces cryophilus*. An alignment of an N-terminal cysteine/histidine domain is also shown. This domain is not present in the Loz1 homolog from *S. cryophilus.*

Table 1. Zinc-responsive transcription factors

Founding zinc-responsive member	Transcription Factor	Host organism	Reference
Zur	Zur	*Bacillus subtilis*	159
		Escherichia coli	160
		Listeria monocytogenes	161
		Staphylcoccus aureus	162
		Salmonella enterica serovar typhimurium	163
		Xanthomonas campestris	164
	FurB	*Mycobacterium tuberculosis*	165
	Zur	*Xanthomonas oryzae pv. oryzae*	166
		Streptomyces coelicolor	167
		Streptococcus suis	168
		Corynebacterium diphtheriae	169
		Yersinia pestis	170
		Corynebacterium glutamicum ATCC 13032	171
		Anabaena sp. PCC 7120	39
		Pseudomonas protegens Pf-5	74
		Neisseria meningitidis	172
		Nostoc punctiforme	173
	Np20	*Pseudomonas aeruginosa*	174
AdcR	AdcR	*Streptococcus pneumoniae*	175
	ZitR	*Lactococcus lactis*	176
ZntR	ZntR	*Escherichia coli*	31
		Comamonas testosteroni S44	177
SczA	SczA	*Streptococcus pneumoniae*	50
SmtB[1]	SmtB	*Synechococcus* PCC 7942	178
	ZiaR	*Synechocystis* PCC 6803	179
	CzrA[2]	*Staphylococcus aureus*	180, 181
Zap1	Zap1	*Saccharomyces cerevisiae*	182
	ZafA	*Aspergillus fumigatus*	183
	Csr1	*Candida albicans*	65
	Zap1	*Cryptococcus gattii*	63
Loz1	Loz1	*Schizosaccharomyces pombe*	55
bZip19/bZip23	bZip19/bZip23	*Arabidopsis thaliana*	119
MTF-1	MTF-1	*Mus musculus* (mouse)	184
		Homo sapiens (human)	185
		Fugu rubripes (puffer fish)	186
		Danio rerio (zebrafish)	187, 188
		Hydrochoerus hydrochaeris (capybara)	189
		Oreochromis aureus (tilapia)	190
		Cyprinus carpio (common carp)	191
		Crassostrea gigas (pacific oyster)	192
		Anguis fragilis (slow worm)	193

[1] Other members of the ArsR-SmtB family sense multiple divalent cations including zinc

[2] CzrA has also be named ZntR in the literature

Table 2. Major zinc transporters that are regulated at a transcriptional level by zinc-responsive factors

Transcription Factor	Transporter Family	Zinc transporter	Primary function	Reference
Zur	ABC transporter	ZnuA (*E. coli*) YceA (*B. subtilus*)	High affinity zinc uptake	159, 160
AdcR	ABC uptake system	AdcC	High affinity zinc uptake	175
ZntR	P-type ATPase	ZntA	Zinc Efflux	31
SczA	RND transenvelope family	CzcD	Zinc Efflux	50
ZiaR	P-type ATPase	ZiaA	Zinc Efflux	179
Zap1	ZIP family	Zrt1	High affinity zinc uptake	182
	ZIP family	Zrt2	Low affinity zinc uptake	182
	cl12096: Iron permease superfamily	Fet4	Low affinity zinc/iron uptake	194
	ZIP family	Zrt3	Vacuolar zinc efflux	195
	CDF family	Zrg17	ER zinc influx	22
	CDF family	Zrc1	Vacuolar zinc influx	26
Loz1	ZIP family	zrt1	High affinity zinc uptake	55
bZip19, bZip23	ZIP family	Zip1, Zip3, Zip4, Zip5, Zip9, Zip10	Zinc uptake/organelle zinc influx	119
MTF-1	CDF family	ZnT1	Zinc Efflux	196
	CDF family	ZnT2	Intracellular vesicular influx	197
	ZIP family	Zip10	Zinc uptake	85, 86
	ZIP family	Zip11	Zinc uptake	198
	Solute carrier family 40	Fpn1	Iron uptake	76

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Figure 1

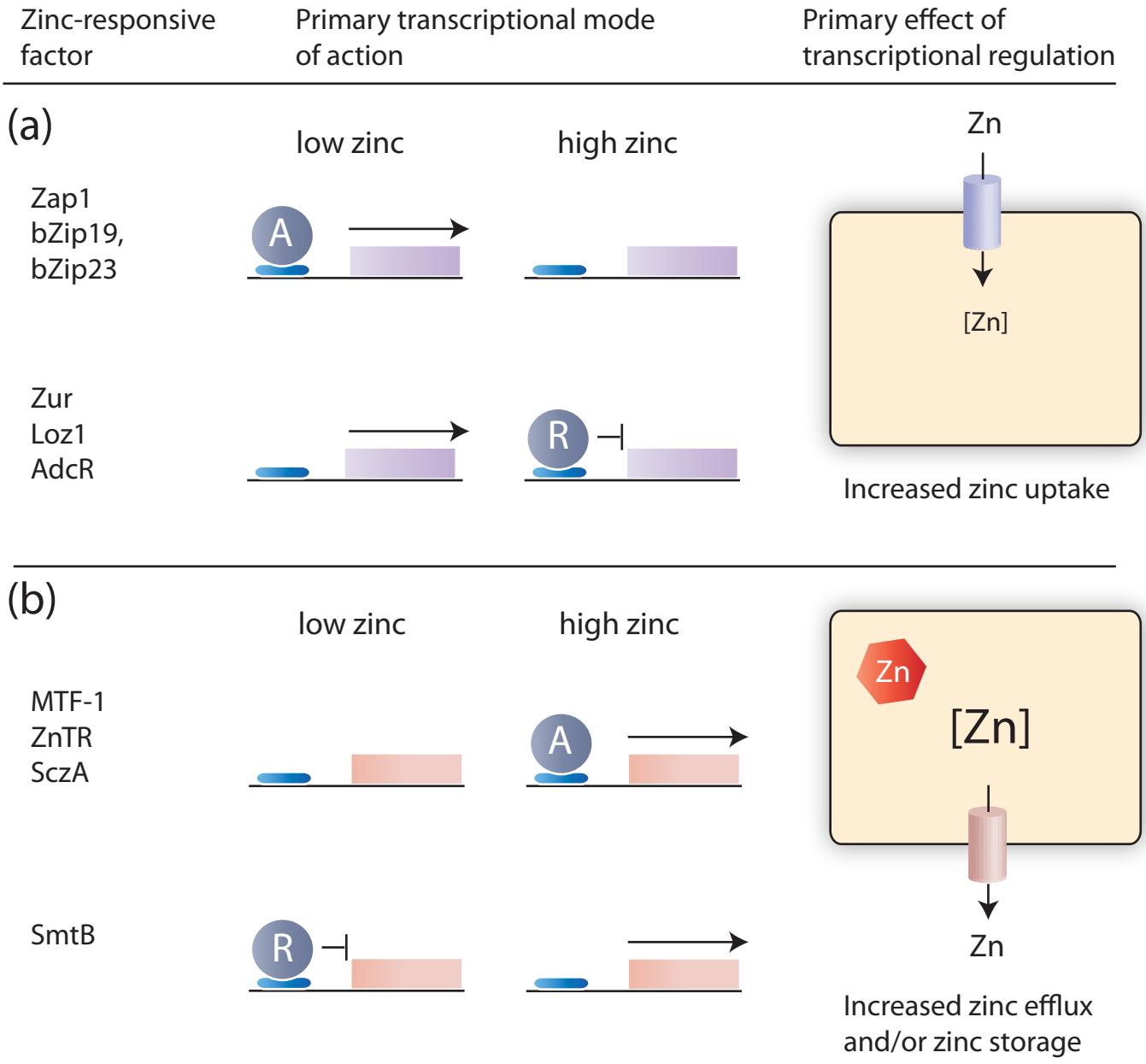


Figure 2

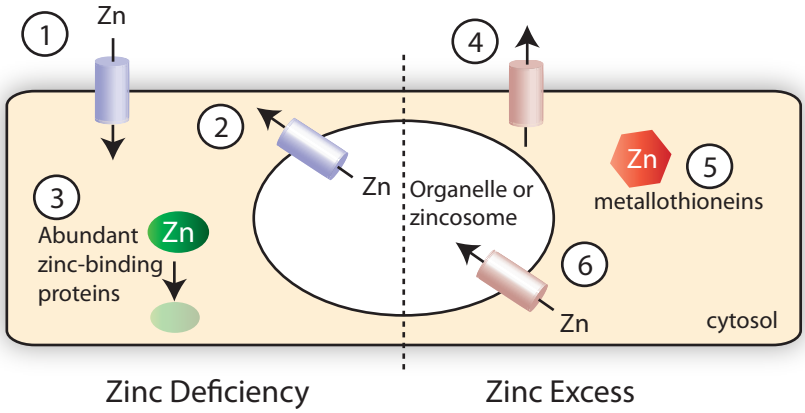


Figure 3

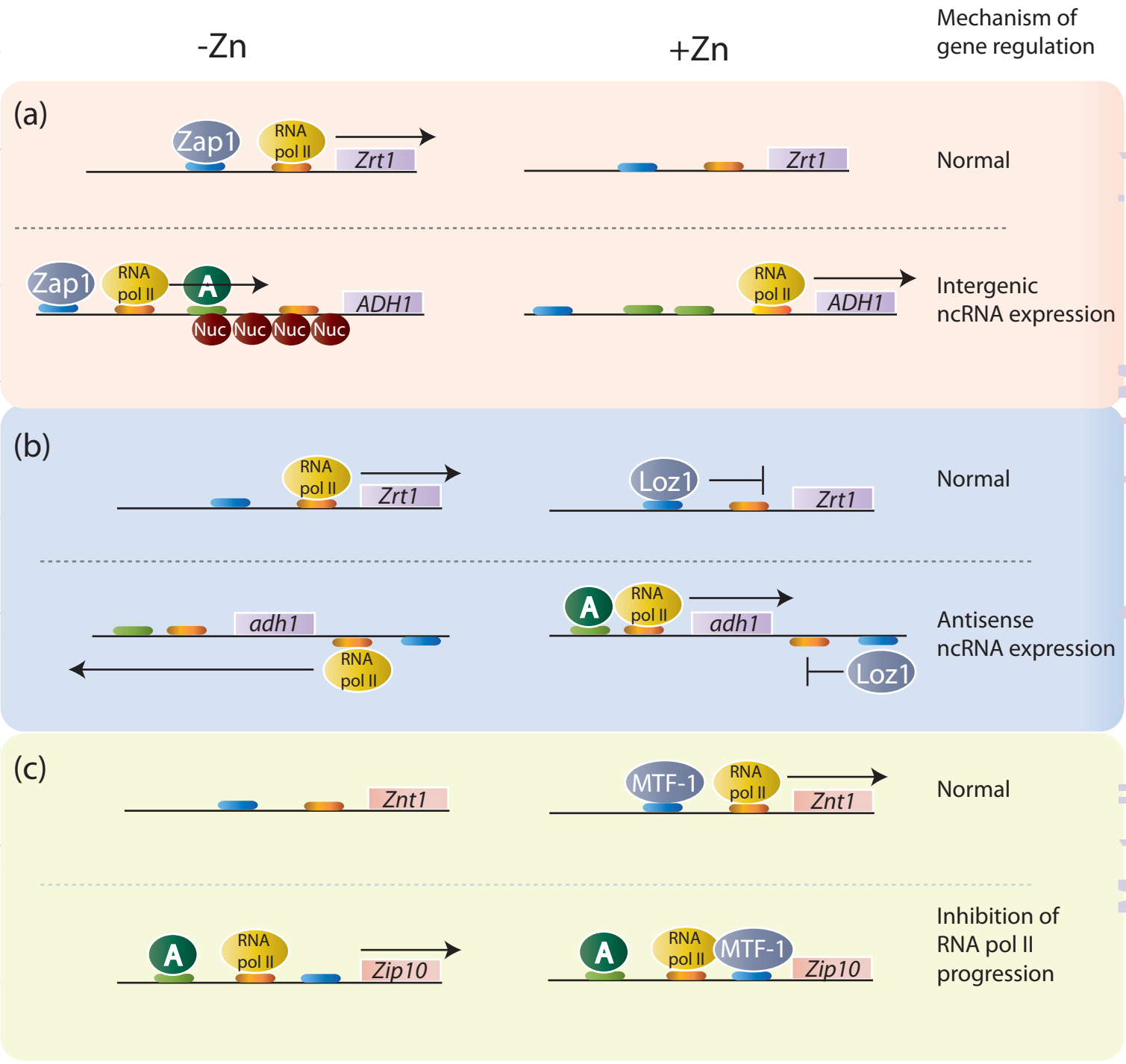
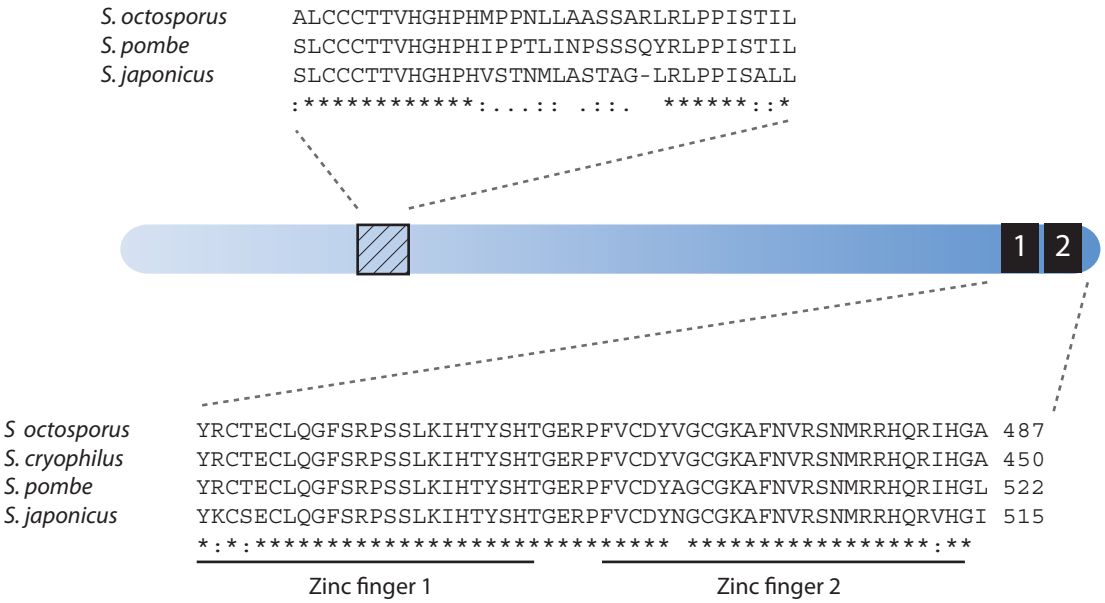
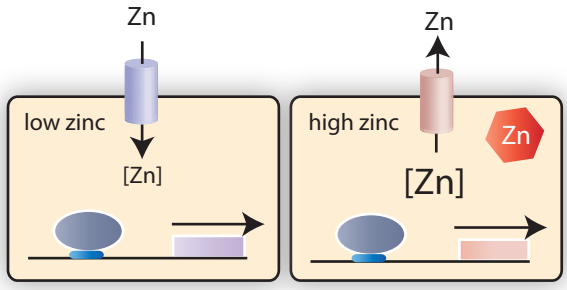


Figure 4





Zinc-responsive transcription factors play a central role in zinc homeostasis by regulating zinc transporter and metallothionein gene expression