Metalloproteins and metal sensing

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Almost half of all enzymes must associate with a particular metal to function. An ambition is to understand why each metal–protein partnership arose and how it is maintained. Metal availability provides part of the explanation, and has changed over geological time and varies between habitats but is held within vital limits in cells. Such homeostasis needs metal sensors, and there is an ongoing search to discover the metal-sensing mechanisms. For metalloproteins to acquire the right metals, metal sensors must correctly distinguish between the inorganic elements.

The physical and chemical properties of a selected metal satisfy a protein's need to form structure, as for zinc-fingers, or to drive catalysis (Box 1). Proteins have also evolved to use those metals that are, or at least once were, most accessible[1–3]. A tenet of the cell biology of metals is that some metals tend to bind organic molecules more avidly than others. The natural order of stability for divalent metals, often called the Irving–Williams series[4], sets out a resulting trend with copper and zinc forming the tightest complexes, then nickel and cobalt, followed by ferrous iron and manganese and finally, forming the weakest complexes, calcium and magnesium (Box 1). Biology must overcome this trend for some proteins to bind uncompetitive metals and for other proteins in the same cell to bind competitive metals. Cells are not ideal solutions and kinetic factors can dominate the distribution of metals, for example where metals are delivered by metallochaperones. Nonetheless, excluding the wrong metals from proteins may be more challenging than acquiring the right ones.

A truism is that there has only been selection for most metalloproteins to obtain the correct element in the context of a cellular environment, with implications for the metal status of proteins expressed in heterologous cells and *in vitro*. Two proteins, one copper protein and one manganese protein in the periplasm of a cyanobacterium, illustrate this observation[5]. The folding compartment for the manganese protein MncA overrides its binding preference to control its metal content. The two proteins share similar cupin folds and the same protein ligands. Both prefer copper to manganese *in vitro*, consistent with the Irving–Williams series, but the manganese protein folds before export by means of the Tat translocase. It therefore traps manganese in the cytosol rather than the periplasm. A keystone of this article will be that metal availability is controlled in the cytoplasm[5–8]. Metal importers, metal exporters and metal stores maintain a limited supply of competitive metals such as copper. Thus, proteins compete with other molecules for these metals rather than metals competing with other metals for proteins. To achieve this state, the amount of each metal is somehow sensed to adjust the actions of transporters (at plasma membranes or internal compartments) and storage proteins for each element[9]. Sensors also adjust metabolism to minimize demand for scarce metals or to exploit abundant ones. These sensors are thus pivotal to metal selectivity because their capacity to distinguish the correct metal affects metal occupancy of other metalloproteins.

Here we summarize the use and acquisition of metals by proteins, and then give an overview of how cells sense metals. Multiple bacterial metal-binding, metal-sensing transcriptional regulators are known and have recently been reviewed elsewhere[9]. Metal specificity of bacterial metal sensors is determined by affinity, allostery and access[9]. Here these three concepts are used to question where metal discrimination lies in eukaryotic metal sensing. In yeast, some metal-responsive transcriptional regulators have been discovered, but their molecular mechanisms may harbour surprises. In mammals, one metal-binding, metal-sensing transcriptional regulator and a post-transcriptional regulator are known[10,11], but innumerable metal-responsive events, including elaborate switches in protein trafficking, are controlled by largely uncharted metal sensors.

An appraisal of metals used in proteins

The time is ripe to survey the use of metals in proteins, by exploiting bioinformatics and proteomics. Non-denaturing conditions must be used in proteomics if metal cofactors are to be retained[12], and this can be technically challenging, there being a potential for metal exchange, acquisition or loss during fractionation. A commonly cited approximation is that one-third of proteins require metals. A systematic bioinformatics survey of 1,371 different enzymes for which three-dimensional structures are known estimated that 47% required metals, with 41% containing metals at their catalytic centres[13]. It is noteworthy that complicated metal centres[14] can remain poorly defined even after structure determination. Metalloenzymes occur in all six Enzyme Commission classes, accounting for 44% of oxidoreductases, 40% of transferases, 39% of hydrolases, 36% of lyases, 36% of isomerases and 59% of ligases[13]. Magnesium is the most prevalent metal in metalloenzymes, although it is often involved in loose partnerships with phosphate-containing substrates such as ATP and is sometimes interchangeable with manganese. The frequency of manganese in protein structures may overestimate its use *in vivo*, where magnesium is truly the cofactor (Fig. 1a).

A catalogue of the principal type of enzyme that uses each metal reveals that iron (81%), copper (93%) and molybdenum plus tungsten (81%) are most commonly used as conduits for electrons in oxidoreductases[13] (Fig. 1b). Cobalt and molybdenum are found almost exclusively in association with cofactors in vitamin-B_{12}-dependent and molybdopterin-dependent enzymes (see page 839). The proportion of all proteins, not just enzymes, using metals is expected to be less than 47% and the relative contributions of the different metals may alter as the metals that perform structural roles, such as zinc in zinc-fingers, are more fully accounted for.

Evolving metal availability and use

The metals available to organisms have altered during evolution as species have occupied new niches and in response to massive environmental changes, for example the liberation of dioxygen by photosynthesis, which significantly changed metal solubility[1–3]. Crucial enzymes, which

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Box 1 | The selection of metals

At catalytic centres, metals increase acidity, electrophilicity and/or nucleophilicity of reacting species, promote heterolysis, or receive and donate electrons[13]. The protein's primary and secondary metal-coordination spheres tune the properties of the metal to optimize reactivity and influence metal selection. Donor ligands (S, O or N) can impart bias in favour of the correct metal. The metal-binding pocket can exclude ions with the wrong charge. Coordination geometry (octahedral, tetrahedral, square pyramidal, trigonal bipyramidal, square planar, trigonal or linear) can impart bias either in folded apoproteins if the preformed site is rigid or during folding if favourable energetics is coupled to the correct geometry. The figure shows the dominant geometries of four divalent metals in proteins[98].

However, because proteins have flexibility, steric selection between metals is imperfect, especially in nascent polypeptides. Under these conditions, the relative affinities of metals for proteins are significantly governed by the ligand field stabilization energies of the metals themselves. This creates the universal orders of preference, which for divalent metals is the Irving–Williams series. There is ambiguity about the position of zinc, which is either at the top of the series or somewhere above cobalt. This ambiguity is attributed to the nephelauxetic effect[99]. Cuprous ions, expected to dominate in more reducing cell environments, are also competitive, and some exceptionally tight ferric complexes are known. Crucially, such affinity series underpin calculations that each metal's relative abundance in the biological locality is paramount in governing selective metal binding by proteins[100], highlighting the vital contribution of cell biology to the selection of metals by metalloproteins.

cannot be readily replaced, have created dependences on metal species that were once plentiful but are now scarce. For example, under anoxic conditions iron is soluble in ferrous form, but in the presence of dioxygen ferric iron is poorly soluble; hence, plants and microbes release siderophores and plants express root-surface metal reductases to scavenge meagre amounts of iron from the environment[15,16]. Despite these efforts, plants remain a poor dietary source of iron and, in consequence, iron deficiency is the most common human dietary deficiency worldwide and one of the ten most prevalent causes of disease[15].

In today's open oceans, the poor availability of iron, but the presence of some copper, has led organisms to replace the former and exploit the latter[17,18]. The oceanic diatom *Thalassiosira oceanica* has switched from using iron-containing cytochrome c_6 to using copper-containing plastocyanin[17,18], which was otherwise only known in some cyanobacteria and photosynthetic organisms containing chlorophyll *b*. The sulphur isotope record also implies that ancient oceans were dominated by sulphide and, hence, metal sulphides[2]. Under these conditions, copper, zinc and cadmium were poorly available, but cobalt and nickel more available[2]. Greater volcanic-nickel input also preceded this era[19,20]. Enzymes that use cobalt and nickel are considered to be metal relics from these times[1,2,19,20]. Some carbonic anhydrases are cambialistic, capable of substituting cobalt for zinc in ocean surface waters where zinc is in short supply[21]. Even more noteworthy are a subset of structurally modified carbonic anhydrases from marine diatoms that have abnormal loops near the active site to facilitate metal substitution with catalytically active cadmium[21–23], a rare example of enzymes that have evolved to use this normally solely toxic metal.

The exploitation of metals varies between species, and trends exist in the superkingdoms[3]. The slope of plots comparing the number of zinc-binding domains with the total number of protein domains encoded by each genome is greater in eukaryotes than in archaea or bacteria; the reverse trend is true for iron-, manganese- and cobalt-binding domains[3]. An implication is that zinc was increasingly recruited as eukaryotic genomes became more complex, and this is especially true for multicellular eukaryotes, with a point of inflection detected at the intersection between unicellular and multicellular species[3]. This reflects the eukaryotic diversification of structural zinc-binding domains, notably zinc-finger and RING-finger domains, which constitute ~3% and ~1% of the human proteome, respectively[24]. Zinc-fingers are prevalent in the control of gene expression associated with differentiation into multiple cell types. Archaea and bacteria have, as a proportion, more iron–sulphur proteins (see page 831) but fewer haem proteins than eukaryotes, and within the bacteria aerobic species have fewer iron–sulphur cluster proteins and more haem proteins than anaerobic bacteria[3].

Metal mis-population

It might be predicted that a consequence of proteins having tight affinities for incorrect metals is a potential for metals to mislocate, yet few such examples are documented. This rarity may reflect an exceptional efficiency of metal homeostasis or perhaps the experimental effort necessary to establish which metals are bound to proteins inside cells. Binding sites of proteins involved in metal homeostasis typically allow metal exchange, but other sites can be buried, with the metal being kinetically trapped and safe from replacement with an incorrect metal. Even so, the correct metal must somehow become trapped in the first place.

A subset of *Escherichia coli* manganese superoxide dismutase, MnSOD, is known *in vivo* to acquire iron[25], which is catalytically inactive. Recombinant MnSODs from other organisms, expressed in *E. coli*, mis-populate with iron, cobalt or nickel[25]. Eukaryotic MnSOD, in the mitochondrial matrix, acquires catalytically inactive iron when mitochondrial manganese and/or iron homeostasis is perturbed[25]. Cobalt mimics hypoxia in humans possibly because cobalt replaces iron in prolyl 4-hydroxylase, which in iron-form hydroxylates the hypoxia-responsive transcription factor HIF1 (also known as HIF1A)[26]. The wrong metals can also mislocate to metal prosthetic groups[27]. A lack of specificity in ferrochelatase, the enzyme that inserts iron in the synthesis of haem, can give rise to metal mis-incorporation *in vivo* such that high concentrations of zinc protoporphyrin in the blood are used in the clinical diagnosis of iron deficiency[28].

Metallochaperones

One way to ensure that the correct metal is acquired by a metalloenzyme is to exploit delivery proteins, that is, metallochaperones. For example, nickel is inserted into bacterial hydrogenase (see page 814) and urease by dedicated nickel metallochaperones. It is unclear where metallochaperones acquire metal. No single donor for either of two yeast copper metallochaperones emerged from a systematic analysis of mutants lacking transporters[29]. Metal transfer has nonetheless been observed *in vitro* between cytosolic regions of the copper importer Ctr1 and a copper chaperone[30], and a recent electron crystallographic structure of Ctr1 might be exploited to visualize how this could occur[31]. Metal is passed from metallochaperones to cognate apoproteins by means of ligand-exchange reactions[32,33]. Thus, the specificity of protein contact, and of the subsequent ligand-exchange reactions, determines which proteins gain access to those metals supplied by metallochaperones. However, rather few metallochaperones are known, so most proteins are assumed to obtain metal from exchangeable cellular pools, and the contributions of metal sensors thus become vital.

A bacterial model for metal sensing

Bacteria have sets of metal- and DNA-binding, metal-sensing transcriptional regulators classified into families of metal de-repressors (ArsR–SmtB, CsoR–RcnR and CopY), metal co-repressors (Fur, NikR and DtxR) and metal activators (MerR)[34,35]. A few sensors also detect metals external to the plasma membrane. The affinities of cytosolic sensors for the metals they detect have been used to make inferences about the concentrations of metals available to proteins[7,8]. On the assumption that the sensors undergo metal exchange with cytosolic metal pools, their affinities become the thresholds for homeostasis. In switching from apo to holo form, metal sensors alter production of proteins to

acquire, expel or sequester metal, so the concentrations at which the sensors bind metal may not be exceeded. The affinities for metal inducers increase as the sensors detect metals farther up the Irving–Williams series[9], implying that the most competitive metals are bound and buffered to exceedingly low concentrations[7,8]. For example, two zinc sensors of *E. coli*, Zur and ZntR, are estimated to have femtomolar zinc affinities[7], which is analogous to there being fewer than one metal ion per *E. coli* cell volume (formally nanomolar).

In addition to metal affinity, the following two other factors dictate which metals a sensor detects *in vivo*.

Access

Metal availability in the cytosol is not standardized across species[25,36,37]. The metals detected by a sensor can change when tested in a different bacterium, perhaps chosen because it was more tractable for experimentation[36,37]. Kinetic factors, perhaps mediated by protein interactions, also affect access and differ between species.

Allostery

A manganese sensor from *Bacillus subtilis*[38], a nickel sensor from *E. coli*[39] or *Helicobacter pylori* and a nickel/cobalt sensor from *Mycobacterium tuberculosis*[36] all bind metals with orders of affinity that match the Irving–Williams series. However, in these sensors only the correct metals bind in an optimal conformation to alter DNA binding allosterically.

Metal sensors in yeast

Studies of metal-regulated gene expression have focused on a set of DNA-binding transcription factors in *Saccharomyces cerevisiae* (Fig. 2a). Functionally equivalent factors occur in other yeasts such as *Schizosaccharomyces pombe*, although the complement and mechanisms vary[40,41]. Metal-responsive signal transduction pathways emanating from metal sensing at the plasma membrane are either absent or yet to be discovered in yeast. Metalloregulation of these yeast DNA-binding transcription factors can involve metal-dependent change in nuclear versus cytosolic localization, metal-dependent repression of activation-domain function and metal-mediated change in DNA binding[42]. In *S. cerevisiae*, Zap1 responds to zinc, Mac1 plus Ace1 (also known as Cup2) to copper, and Aft1 plus Aft2 to iron (Fig. 2a).

The metal-responsive transcription factors regulate expression of a repertoire of transporters that import or compartmentalize metals[42] (Fig. 2b). Mac1 has also been implicated in a pathway of metal-responsive transporter degradation[43]. Yeast cells store excess metal in vacuoles[44,45], with plasma-membrane exporters known only for cadmium and arsenic, and some yeasts have metal-sequestering metallothioneins. The factors also control metabolic switching; for example, the transcription factors Aft1 and Aft2 indirectly (through Cth1 and Cth2 (also known as Tis11)) reduce the stability of transcripts encoding iron-dependent enzymes when iron is in poor supply[46,47]. The iron regulon is also induced at the diauxic shift to support the enhanced iron requirements of respiration[48]. The extent to which the responses are triggered by metal binding to the *S. cerevisiae* transcription factors themselves has been explored.

Metal sensing by metal binding

Mac1 and Ace1 activate transcription in response to low and, respectively, high concentrations of copper in *S. cerevisiae*[49,50]. Cysteine-rich motifs in both Mac1 and Ace1 can bind copper *in vitro* to form polycopper clusters that are bridged by thiolates[51]. Structures of the clusters have been modelled from extended X-ray absorption fine structure (EXAFS) data[51] (Fig. 2c). As copper concentrations increase, there is a reciprocal relationship between the loss of activation of Mac1-regulated genes and the induction of Ace1-regulated genes[52]. A simple model is that at some critical intracellular copper concentration, clusters form in both proteins to repress Mac1 function while activating Ace1. DNA binding by Ace1 is enhanced by copper *in vitro*[49]. It is not yet known whether Mac1 and Ace1 compete for the same pool of copper or whether copper is delivered by a metallochaperone. Polycopper clusters are structurally distinct and so have the potential to provide allosteric metal specificity in favour of copper through the unique protein conformations they induce.

Activation (or repression on some promoters) of gene expression in response to zinc is controlled by the Zap1 transcription factor, a process that depends on its zinc-fingers. Zap1 has two transcriptional activation domains, one of which is located within an unusual pair of zinc-fingers[53]. The zinc in these atypical fingers is more labile than in the standard DNA-binding zinc-fingers[53]. Zap1 metal regulation by means of variable zinc occupancy of these atypical fingers is strongly supported by *in vivo* observations of fluorescence resonance energy transfer (FRET) between probes on adjacent fingers[54] (Fig. 2d). The fluorophores only become close enough to allow energy transfer when the holo-fingers form, and this occurs only when zinc concentrations increase. Thus, for elevated zinc concentrations these atypical zinc-fingers become occupied by metal, masking the activation domain and thereby inactivating gene expression. The zinc affinities of the labile Zap1 zinc-fingers, expressed without the rest of Zap1, are in the nanomolar range[53,55]. This might imply that intracellular available zinc accumulates to a higher concentration in yeast than in bacteria, but perhaps the zinc affinities are tighter in intact Zap1. There is cooperative binding of zinc to the two atypical fingers of Zap1 (ref. 55), offering the potential to confer allosteric metal specificity.

Indirect responses to metals

Iron sensing in yeast is mediated by Aft1 (ref. 56) (Fig. 2a) or its paralogue, Aft2 (ref. 57). The more extensively studied Aft1 undergoes iron-responsive nucleocytoplasmic shuttling[58–60]. When the iron concentration is low, Aft1 accumulates in the nucleus. Nuclear Aft1 activates transcription of a regulon that includes genes encoding iron transporters[61] (Fig. 2b). Details of the iron-switch are being pieced together. The switch requires cysteine residues in either Aft1 or Aft2 (refs 56, 57), two monothiol glutaredoxins (Grx3 and Grx4)[60] and two cytosolic proteins (Fra1 and Fra2)[59]. *In vitro*, Grx3 and Grx4 homodimers associate with a [2Fe–2S] cluster[62] and Aft1 is known to be responsive to the degree of iron–sulphur-cluster biosynthesis in the mitochondria[63]. Aft1 might respond to iron indirectly, with the proteins of iron–sulphur-cluster

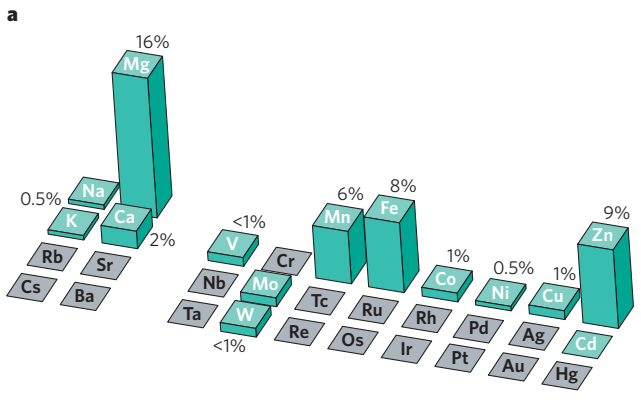

Figure 1 | Metals used in catalysis. **a**, The elements used as cofactors by enzymes are shown in blue. The height of each column represents the proportion of all enzymes with known structures using the respective metal. A single enzyme uses cadmium[22]. **b**, The proportion of proteins using the indicated metals that occur in each of the six enzyme classes: oxidoreductases (EC 1), blue; transferases (EC 2), yellow; hydrolases (EC 3), purple; lyases (EC 4), pink; isomerases (EC 5), green; ligases (EC 6), grey. EC, Enzyme Commission. Data obtained from ref. 13.

biosynthesis being the incipient discriminatory sensors.

Classical signal transduction pathways may also be involved in copper sensing, because the DNA-binding form of Mac1 is phosphorylated[64]. Furthermore, as hinted previously, no copper chaperone has been linked to copper delivery to the nucleus. In a search for such a donor, Mac1-responsive gene expression was systematically tested in mutants lacking each of the known copper metallochaperones[65]. A dependence on the copper donor for superoxide dismutase (Sod1) was discovered; however, it transpires that this is only indirect, because Mac1 needs catalytically active Sod1 in the nucleus to function[65]. The mere suppression of oxidative stress does not overcome the need of Mac1 for nuclear Sod1, suggesting a key role of redox changes in the nucleus for the copper response of Mac1 (ref. 65) (Fig. 2e). The activity of Sod1, itself an abundant copper enzyme, may be the indicator used to monitor copper sufficiency, raising many questions about where metal discrimination takes place.

Metal sensing in animals

The cells of multicellular eukaryotes can respond to the metal status of the whole organism as well as to their own status, and systemic signalling molecules have been discovered such as hepcidin in animals. Many responses to metals in plants and animals occur post-transcriptionally, by adjusting messenger-RNA stability or translation, or post-translationally, by adjusting intracellular protein trafficking or degradation. With a few significant exceptions, the metal-binding, metal-sensing sites that trigger these events remain to be discovered. Plant metal homeostasis is reviewed elsewhere[16,66], but the discovery of Spl7 (Crr in *Chlamydomonas*), which possibly binds (and senses) copper, should be noted.

Metal regulation of transcription

Just one metal-binding, metal-sensing transcriptional regulator is currently known in animals, the metal-regulatory transcription

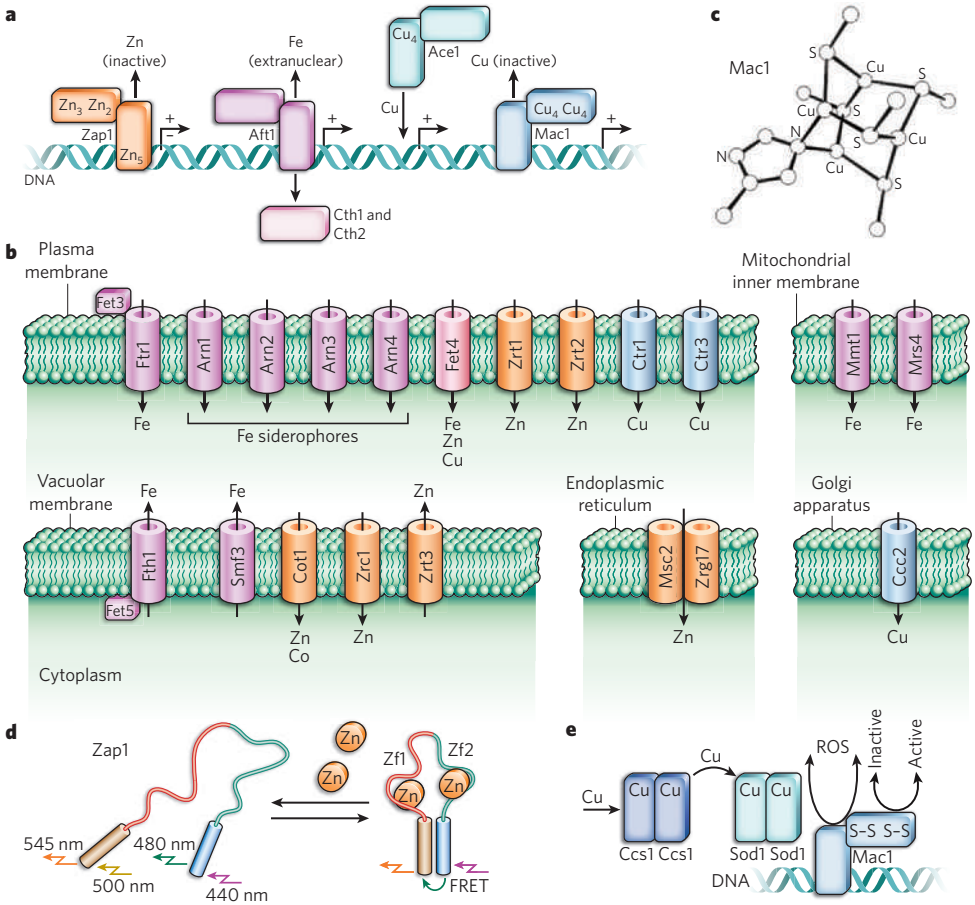

Figure 2 | Metal sensing in *Saccharomyces cerevisiae*. **a**, Each metal-responsive transcription factor has a DNA-binding domain and an activation domain (shown as two blocks). Zap1 (orange) has multiple sets of zinc-fingers (subscripts indicate the number of zinc atoms in each set). When zinc binds to zinc-fingers 1 and 2 (Zf1 and Zf2) in the activation domain (bound zinc indicated by Zn_2), activation is repressed. Copper-cluster formation (Cu_4) in Ace1 (blue-green) encourages DNA binding. By contrast, in Mac1 (blue), copper clusters inhibit activation-domain function and weaken DNA binding. Under high-iron conditions, Aft1 (pink) becomes extranuclear. These factors generally activate transcription when bound to DNA (+), but Zap1 can also act as a repressor (–). The mRNA-binding proteins Cth1 and Cth2 (pink) downregulate transcripts encoding iron-requiring proteins, and the genes encoding Cth1 and Cth2 are controlled by Aft1 and Aft2. **b**, The metal-responsive transcription factors control the expression of genes encoding a subset of metal transporters (tubes), which allow the passage of metals through various membranes in *S. cerevisiae* (pink, iron transporters; orange, zinc transporters; and blue, copper transporters). The colour of the transporter also corresponds to the specific transcription factor involved in regulation: Aft1 and/or Aft2 (pink), Zap1 (orange), and/or Mac1 (blue), with several exceptions (Fet4 is regulated by Zap1 and Aft1; Cot1 is regulated by Aft1; Zrg17 is regulated by Zap1 but Msc2 is not; and Ccc2 is regulated by Aft1). Arrows show the direction of metal transport. Fet3 and Fet5 are ferroxidases. Fet4 is a different shade of pink because it can transport iron, zinc and copper. **c**, Copper clusters of Mac1 (or Ace1, not shown) may allow allosteric metal specificity. Model of the metal site based on X-ray absorption fine structure (EXAFS) data. Spheres represent carbon atoms unless labelled otherwise. (Panel reproduced, with permission, from ref. 51.) **d**, The binding of zinc to the zinc-fingers in Zap1 (Zf1 shown in red, and Zf2 in cyan) has been followed *in vivo* using FRET: the energy transfer between enhanced cyan fluorescent protein and enhanced yellow fluorescent protein attached to Zf2 and Zf1 was measured, providing evidence that zinc occupancy of these fingers is modulated *in vivo*. Zigzag arrows indicate the light (wavelength shown) either absorbed or emitted by the fluorescent proteins. (Panel modified, with permission, from ref. 54.) **e**, Redox chemistry is involved in regulating the activity of Mac1. Mac1 function depends on the presence of active Sod1 in the nucleus, which depends on copper being delivered to Sod1 by the metallochaperone Ccs1. The consumption or generation of a reactive oxygen species (ROS) by Sod1 might modify regulatory cysteine residues on Mac1 (ref. 65).

factor MTF1, although other DNA-binding zinc-finger proteins, such as SP1, do act downstream of unknown metal sensors[67]. Zinc stabilizes a complex between MTF1 and its DNA-binding site, with at least some of the sensing mechanism residing in its DNA-binding Cys_2His_2 zinc-fingers[10]. MTF1 is commonly, although not exclusively, a transcriptional activator[68,69], acting with other factors[70]. In the special case of the *Drosophila* MTF1 homologue, some of these interactions are linked to metal specificity[71].

Transcription of mammalian MTF1 target genes is induced in response to metals such as copper and cadmium, not merely zinc[71,72]. Oddly, in a cell-free system copper or cadmium have the opposite effect, inhibiting mammalian MTF1-dependent transcription, but this is reversed simply by adding one further component, zinc metallothionein, to the reaction mixture[73]. Addition of apothionein, by contrast, is inhibitory[73]. An explanation is that cadmium and copper displace zinc from binding sites such as those of zinc metallothionein; the liberated zinc then binds to the zinc-fingers of mammalian MTF1 (Fig. 3a). Here, metal specificity is achieved through a combination of metal access and allostery.

The *Drosophila* protein MTF1 is distinct in having a copper cluster and zinc-fingers[74]. Flies in which the cysteine residues that coordinate the cluster are converted to alanine fail to upregulate transcription of a copper-metallothionein gene in response to a high copper concentration. Hypothetical co-regulators are proposed to interact with *Drosophila* MTF1 dependent on cluster formation. Alternative co-regulators are thought to enable *Drosophila* MTF1 to upregulate some genes in response to copper excess while upregulating others, such as that encoding the CTR1B copper importer, in response to copper limitation. Zinc can bind to the cysteine thiolate ligands of the copper cluster *in vitro* without impairing subsequent copper binding[74]. As for yeast copper-responsive transcription factors, structurally distinct polycopper clusters have the potential to confer allosteric metal specificity by means of the unique protein conformations they induce.

Metal regulation of mRNA stability and translation

In a highly parsimonious way, the iron-responsive proteins IRP1 (also known as ACO1) and IRP2 (IREB2) control translation or mRNA stability reciprocally for different gene products[75]. A cleft in IRP1 and, by inference, IRP2 binds to iron-responsive elements (IREs) that form stem–loops in mRNAs[11] (Fig. 3b). IREs occur in the 5′ untranslated region of mRNA encoding the ferritin iron store and in the 3′ untranslated region of the mRNA encoding the transferrin receptor for iron uptake. Ferritin is needed when iron is in surplus, and transferrin receptor is needed when iron is lacking. IRP binding to the 5′ region blocks translation whereas binding to the 3′ region stabilizes the mRNA. An iron–sulphur cluster binds within, and hence blocks, the cleft of holo-IRP1. Thus, holo-IRP1 dissociates from mRNAs to allow translation of ferritin but degradation of transferrin-receptor mRNA when iron–sulphur clusters are sufficiently abundant to fill its cleft[11]. As for polycopper clusters, binding of iron–sulphur clusters to a sensor may confer allosteric metal specificity, but because iron–sulphur clusters are themselves products of a biochemical pathway, perhaps — as noted for yeast Aft1 — metal discrimination really takes place in the cluster-assembly proteins.

Post-translational metal regulation

Metal-responsive degradation of metal-homeostasis proteins is a general strategy that applies, for example, to IRP2 (ref. 76), to the copper chaperone CCS[77], to the copper importer CTR1 (also known as SLC31A1)[78], to the iron-efflux protein ferroportin[79] and to the zinc-uptake transporter ZIP4 (SLC39A4)[80] (Fig. 4). ZIP4 has three levels of post-translational regulation in response to zinc. The extracellular amino-terminal half of ZIP4 is proteolytically processed during prolonged zinc deficiency[81], ZIP4 is removed from the plasma membrane to a perinuclear location when zinc becomes replete[82] and ZIP4 is degraded when zinc is highly elevated[80]. The third process involves perception of zinc by a cytoplasmic histidine-rich motif that somehow exposes a site for ubiquitination and, hence, proteasomal degradation. The same histidine-rich motif triggers ZIP4 degradation in response to cadmium, but degradation does not occur in response to iron, manganese, nickel, copper or cobalt[80]. The question of which elements the motif distinguishes through affinity, allostery or access awaits exploration.

As is the case for ZIP4 during the transition between low-zinc and zinc-replete conditions, other metal transporters also undergo metal-dependent trafficking (not solely for degradation). This was first observed for copper-transporting ATPase ATP7A, which redistributed from internal *trans*-Golgi membranes to the plasma membrane in cell cultures exposed to high copper concentrations[83] (Fig. 4). Under low-copper conditions ATP7A supplies metal to copper proteins in the *trans*-Golgi network, whereas under high-copper conditions it aids efflux. An analogous protein (ATP7B) is expressed in the liver, where it relocates to apical hepatocyte membranes under high-copper conditions to cause efflux of the surplus into bile canaliculi[84] (Fig. 4). Several mutations in ATP7A or ATP7B cause the transporters to remain within internal membranes, and increased trafficking to the plasma membrane rather than reduced re-internalization by endocytosis is triggered by high copper concentrations[85]. A nine-residue stretch of ATP7B is speculated to bind a partner in response to copper[84]. In this model, the copper-sensing sites are responsible for exposing the nine-residue signal but the sensor remains a mystery. One suggestion is that the acyl phosphate intermediate state of the ATPase itself exposes the nine-residue motif[84]. Trafficking of CTR1 in response to copper is also linked to its transport activity, and to a methionine-rich motif[78]. Here, the levels of catalytic activity of CTR1 and ATP7B become coupled to trafficking, and selectivity of sensing becomes a function of the selectivity of transport.

Systemic signals of metal status

Hepcidin is a disulphide-rich peptide consisting of 25 amino acids that is synthesized and released by the liver in response to increased iron[79]. Hepcidin binds to ferroportin to induce internalization and lysosomal

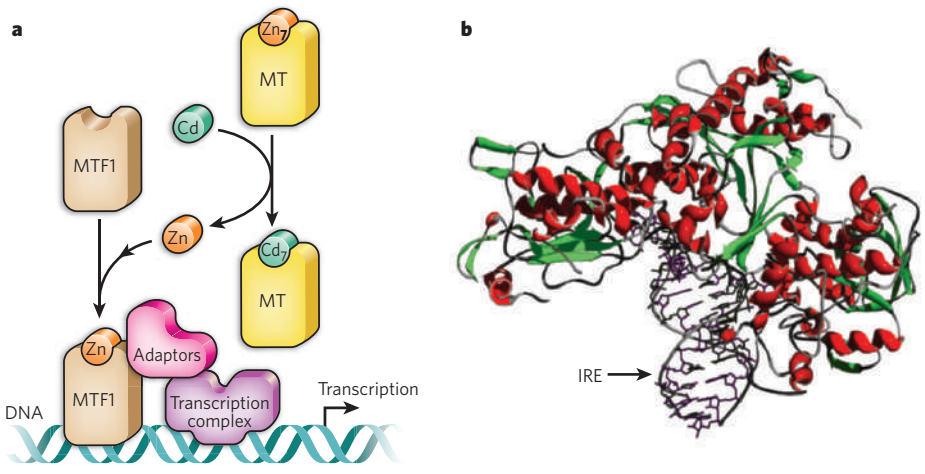

Figure 3 | Metal sensors in animals. a, MTF1 responds to cadmium *in vivo* (but not *in vitro*) indirectly, as a function of access to zinc. Cadmium binds to metallothionein (MT) more tightly than does zinc. Zinc released from metallothionein is taken up by zinc-fingers of MTF1, which in turn interacts with metal-response elements on DNA to effect the transcription of target genes through adaptor proteins[73]. **b**, Iron-responsive protein IRP1 (α-helices, red; β-strands, green) in complex with an iron-regulatory element (IRE) from the 5′ untranslated region of ferritin mRNA (purple, in stick format) (Protein Data Bank code 2IPY)[11]. Binding of IRPs to this mRNA region inhibits the translation of the iron-storage protein ferritin.

degradation[79]. Ferroportin is otherwise responsible for iron export into the circulation across the basolateral membrane of enterocytes[86] (Fig. 4). Thus, hepcidin inhibits dietary iron absorption. The mechanism by which hepcidin synthesis and release respond to iron is independent of IRPs because there are no IREs in the hepcidin mRNA. The iron signal is triggered by competition between transferrin receptors one and two for the hereditary haemochromatosis protein HFE, which is influenced by occupancy of receptor one with holo-transferrin[87]. Metal discrimination could be said to take place in the formation of diferric transferrin, in which the valence state of iron plays a major part in selectivity.

Outlook

Bias in favour of the correct metal can occur during folding of a metalloenzyme if coupled to favourable energetics[88] (Box 1). Perhaps some metal-coordination spheres have evolved to leave patches exposed if an incorrect metal binds, to attract folding chaperones. Such a mechanism might check that metalloproteins have enfolded the correct metals *in vivo*, a form of proofreading of metal acquisition. To understand metal occupancy, methods used decades ago to fractionate metalloproteins should be revisited, but equipped today with improved multiple-metal detection by inductively coupled plasma mass spectrometry and inordinately improved protein identification by mass fingerprinting. Mathematical methods such as principal-component analysis circumvent the need to purify holoproteins to homogeneity before identification[5]. Metallochaperones can influence the intracellular distribution of metals but other kinetic factors, such as interactions with metal importers (or exporters from storage compartments) or proximity to sites of metalloprotein degradation, may also create metal niches.

Metal sensors influence metal availability in cells, which in turn influences metal occupancy of metalloproteins. The contribution of allostery to metal specificity in bacterial metal sensors is predicted to be especially important for metal co-repressors and metal activators, in which metal binding to the protein must organize an active protein–DNA adduct. Another prediction is that extra ligands may have been selected adjacent to some sensory metal sites to lure the wrong metals into non-productive binding configurations. A further untested prediction is that access to metal is partly a function of the relative affinities of a set of metal sensors[9]. For example, provided that a zinc sensor has the tightest affinity for zinc in the set, other sensors will never gain access to zinc even if they could otherwise bind zinc more tightly than their bona fide metal effectors.

An important question in relation to metal sensing in *S. cerevisiae* is that of what redox chemistry is associated with Aft1 and Mac1. X-ray crystal structures, plus NMR solution studies, of bacterial DNA-binding metal sensors in apo form, in holo forms with different metals, as multimers and as DNA adducts, have helped visualize metal sensing. Equivalent insight is largely missing from eukaryotic metal sensing, with one exception being IRP1 in complex with stem–loops of a ferritin transcript[11] (Fig. 3b). Recombinant metal-binding domains have been examined in isolation, including the zinc-fingers of Zap1 (ref. 55), those of Ace1 homologue Amt1 (also known as Mep1)[89] and of MTF1 (ref. 90), and the copper clusters of Ace1 (ref. 51), Mac1 (ref. 51) and *Drosophila* MTF1 (ref. 74). Technical advances in determining larger solution and crystal structures hold promise for visualizing the apo and holo forms of intact eukaryotic metal-sensing transcriptional regulators, particularly in complex with co-regulators and with DNA.

In animals, there are plenty of metal-mediated changes in the abundance of transcripts that neither contain IRP-binding sites nor are encoded by genes with promoters containing the MTF1-recognition element. For instance, ZNT5 (also known as SLC30A5) transcripts are subject to both zinc-responsive synthesis and stability through unknown factors[91]. Repositories of transcriptomic data catalogue a multitude of responses to altered levels of different metals in mammalian tissues or cells (see, for example, ArrayExpress (European Bioinformatics Institute; http://www.ebi.ac.uk/microarray-as/ae/) and the Gene Expression Omnibus (US National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/geo/)). To discover unknown DNA- or RNA-binding factors, it should be possible to interrogate the sequences of metal-responsive genes to identify common nucleotide elements in those with similar responses to a metal, thereby identifying bait sequences with which to search for cognate metal regulators. Some iron-responsive genes contain the nucleotide-binding site for HIF1 (ref. 92), and one unproven

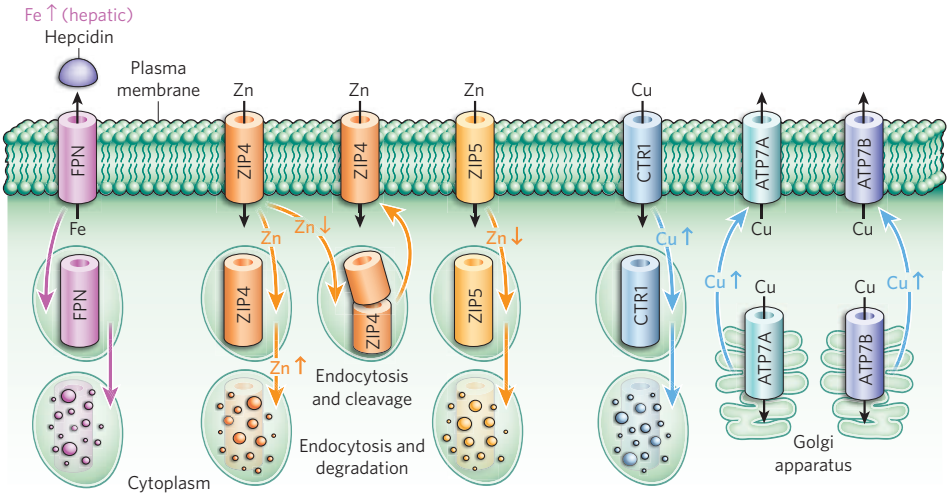

Figure 4 | Metal-responsive trafficking and processing of metal transporters. Mammalian metal transporters (coloured tubes) themselves move within the cell in response to metal ions. Depending on whether the metal concentrations are high (↑), low (↓) or optimal (no arrow) for a particular transporter, the transporter can be internalized and transported within the cell (curved coloured arrows) and then degraded or cleaved. Most of the sites at which metals bind to, and are sensed by, transporters, thereby effecting these changes, remain to be discovered. Within an organism, the distribution of metal transporters can be widespread (as is the case for CTR1 and ATP7A) or restricted to particular cell types (all other transporters). In addition, in polarized cells, transporters can be expressed on the apical or basolateral side. For example, in response to high concentrations of iron, the liver produces hepcidin (left), which binds to ferroportin (FPN) in the basolateral membranes of intestinal cells known as enterocytes, triggering the internalization and degradation of FPN. The amount of iron in the liver can thus influence how much iron is transported from the intestine into the circulation. Known distributions of metal transporters are as follows: FPN, intestine (basolateral), placental trophoblast (basolateral), and monocytes and macrophages; ZIP4, pancreatic β-cells, intestine (apical), embryonic visceral yolk sac (apical, in mouse) and kidney; ZIP5, pancreatic acinar cells (basolateral), embryonic visceral yolk sac (basolateral, in mouse), kidney, spleen, liver and intestine (basolateral); ATP7A, polarized cells (basolateral); ATP7B, liver (apical), mammary gland (apical), kidney (apical) and placental trophoblast (apical).

hypothesis is that HIF1 activity responds to iron through the iron dependence of its prolyl 4-hydroxylase[26,93]. Extracellular zinc also seems to trigger signal pathways through a G-protein-coupled mechanism[94], suggesting the presence of unknown plasma-membrane metal receptors. Metazoans also somehow modify the set points for metal sensing to sustain different metal concentrations in different tissues, and all cells somehow prioritize the expression of different metalloproteins under conditions of metal deficiency.

With so many metabolic processes requiring metals, dyshomeostasis is expected to feature widely in pathologies. Aberrant copper transport is the cause of Menkes disease (mutations in ATP7A) and Wilson's disease (mutations in ATP7B)[85], aberrant zinc transport is a cause of acrodermatitis enteropathica (mutations in ZIP4)[81], and aberrant iron transport is a cause of haemochromatosis (mutations in ferroportin, HFE, TFR2, hepcidin or haemojuvelin)[87], to name but a minuscule subset of examples. Type 2 diabetes mellitus results from interaction between environmental and genetic factors, and a linked polymorphism is found in a zinc transporter that supplies insulin granules in β-cells[95]. Perhaps progressive accumulations of competitive and/or redox-active metals in the wrong locations underlie some links between metals and multiple neurological disorders[96]. A more complete knowledge of metal homeostasis and, by implication, metal sensing is likely to precede an understanding of its aberrations. A knowledge of how sensors discriminate between metals has the potential to aid the development of therapies.

Changing metal availabilities over geological time fashioned the metal–protein partnerships. As dissolved CO_2 acidifies the modern oceans[97], metal availabilities may be changing yet again. The action of carbonic anhydrase in providing substrate for oceanic photosynthetic carbon fixation by phytoplankton is central to primary productivity and to the carbon cycle through its removal of acidifying bicarbonate. It is unclear how organisms with carbonic anhydrases of differing metal requirements will respond. There is pressing interest in coupling photosynthesis to the production of biofuels such as gaseous hydrogen. Hydrogenase will have to be supplied with nickel by means of its metallochaperone, but the metallochaperone might be unable to acquire enough metal in a heterologous photosynthetic bacterium. We already know that nickel is so poorly available in a cyanobacterial cytosol that a mycobacterial nickel/cobalt sensor fails to sense nickel[36]. To populate metalloproteins with the correct metals in heterologous cells, special strains should be made in which bioavailable metal is adjusted, for instance by changing the affinities of metal sensors to alter the threshold concentrations within which a desired metal is buffered. A robust understanding of how metal sensors discriminate between the elements is a prerequisite to understanding and, hence, to engineering optimal 'metallation'. ■

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From computational studies, it is inferred that in the absence of metallochaperones the specificity of a metal for a set of ligands in a protein depends mainly on the metal's abundance in the locality.

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