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Review

Zinc transporters and the cellular trafficking of zinc

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Abstract

Zinc is an essential nutrient for all organisms because this metal serves as a catalytic or structural cofactor for many different proteins. Zinc-dependent proteins are found in the cytoplasm and within many organelles of the eukaryotic cell including the nucleus, the endoplasmic reticulum, Golgi, secretory vesicles, and mitochondria. Thus, cells require zinc transport mechanisms to allow cells to efficiently accumulate the metal ion and distribute it within the cell. Our current knowledge of these transport systems in eukaryotes is the focus of this review.

Keywords: Zinc; Cell biology; Transporters; ZIP; CDF

Zinc is an essential cofactor required for the structure and function of many proteins. Studies of the cell biology of zinc date back perhaps to the discovery of the zinc-binding cytosolic metallothionein (MT) protein by Margoshes and Vallee in 1957 [1]. For over 30 years thereafter, the study of MT by many labs represented the frontier of the field. That changed dramatically in the mid-1990s with the discovery of the first zinc transporters responsible for the movement of zinc across cellular membranes. Subsequent studies over the past 10 years have told us much about these transporters and the molecular aspects of the cell biology of zinc. In this review, I will examine many of these new insights. I will first consider the level and forms of zinc within cells and then go on to discuss the mechanisms by which cells transport zinc across their plasma membranes and how intracellular free zinc is distributed among the various intracellular organelles.

1. The zinc quota, the zinc proteome, and intracellular free zinc

How much zinc do cells require to thrive? The concept of the "zinc quota" was first proposed by T. O'Halloran [2]. The zinc quota is defined as the total zinc content of a cell required for its optimum growth. In *Escherichia coli*, the zinc quota is approximately 10^5 atoms of zinc per cell [2]. The zinc quota of yeast is estimated to be about 10^7 atoms per cell while many mammalian cell types grown in culture require approximately 10^8 atoms per cell [3–5]. At face value, these numbers seem quite different as they vary over three orders of magnitude. However, when adjusted for the volume of these different types of cells, there is remarkable similarity in the *concentration* of zinc; each corresponds to a total cellular zinc concentration of 0.1–0.5 mM. These calculations treat cells bounded by their plasma membranes as homogeneous "bags of buffer"; obviously, this is not true and zinc is distributed to varying degrees among subcellular organelles as will be discussed below. Nonetheless, these calculations do provide an approximation of intracellular zinc concentrations.

There are certainly cell types that do not fit this rule. For example, certain types of neurons and cells of the prostate accumulate higher levels of zinc than other tissues, and this reflects the specialized roles of zinc within these particular types of cells [6,7]. For most types of cells, however, the concentration of total zinc appears to be very similar. I propose that this similarity likely reflects the conserved roles of zinc in cellular biochemistry. These data also make two other important points about the cell biology of zinc. First, while often referred to as a "trace element", zinc is obviously not a trace element from the perspective of the cell [2]. Second, these data highlight the ability of cells to accumulate and concentrate extracellular metal ions. For example, *E. coli* and yeast can accumulate zinc

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as much as 2000-fold over the available extracellular levels [2,8].

In most cell types grown under normal conditions, the vast majority of intracellular zinc is bound to proteins and serves in essential structural or catalytic roles. I refer to this population of zinc metalloproteins as the "zinc proteome". While the zinc proteome has not yet been considered in genome-wide detail, there is sufficient information available from model organisms such that we can get a sense of where this zinc is associated and to what level. In yeast, for example, major zinc binding proteins include Cu/Zn superoxide dismutase (SOD) and the predominant isozyme of alcohol dehydrogenase (Adh1). Cu/Zn SOD accumulates to about 5×10^5 molecules per cell while Adh1 accumulates to around 7.5×10^5 molecules per cell (accounting for 1.5×10^6 atoms of zinc) [9,10]. Similarly, six atoms of zinc are bound to each ribosome accounting for a total of 6×10^5 atoms of ribosomal zinc per cell [11]. These three examples alone account for almost 20% of the total zinc quota of the yeast cell. In addition to these high affinity sites, low affinity zinc binding sites abound in the cell. These include weaker binding sites on proteins, lipids, and DNA as well as small molecular weight compounds such as organic anions (e.g., citrate), amino acids (e.g., histidine), and glutathione.

Given these factors, it is likely that free zinc is present at very low levels in cells. The term "free" here is used to specify labile zinc that is freely available for binding by newly synthesized zinc metalloproteins. Attempts to measure free zinc in cells have produced a wide range of concentrations (10^{-5} to 10^{-12} M) depending on the approach used [4,12–17]. One source of this variability is that cytosolic extracts used for direct measurements can be contaminated with organellar zinc and zinc released from protein sites during the sample preparation due to oxidation or proteolysis. In addition, studies using zinc-responsive fluorophores are difficult to interpret because of the problems associated with using high levels of fluorescent probes to measure small pools of zinc [18]. Therefore, we still have no clear idea of what intracellular free zinc levels really are. Many zinc metalloproteins have metal binding affinities in the nM to pM range, arguing that free zinc is also in that range [19–22]. One nM corresponds to about 30 zinc atoms per yeast cell and about 300 atoms in a mammalian cell. If these predictions are correct, it is remarkable how low free zinc is when total zinc is so high. For example, 1 nM free zinc corresponds to less than 0.001% of the total zinc concentration of the cell. This comparison highlights the precise control of free zinc within cells and the delicate balance between zinc transport (i.e., uptake, efflux, and organellar sequestration) and the binding of zinc by newly synthesized metalloproteins.

A recent analysis of the affinity of zinc-responsive transcription factors of *E. coli* has often been cited as evidence for the absence of free zinc in cells. These factors, Zur and ZntR, respond to zinc in the femtomolar (10^{-15} M) range which is many orders of magnitude lower than the concentration of a single atom of zinc in an *E. coli* cell [2]. This result suggested that free Zn^{2+} is not utilized in cellular zinc transactions. Rather, these authors suggested that zinc may be trafficking within the cell bound to metallochaperones, as is the case for copper [23–25], that deliver zinc directly to metalloproteins via specific protein–protein interactions. While formally possible, I believe that this interpretation is unlikely for two reasons. First, it is difficult to imagine a population of metallochaperone proteins serving to deliver zinc to the hundreds of different zinc proteins found within a cell. This would constitute a major investment of an organism's genome and such an investment has not yet been discovered. It is conceivable that a relatively few zinc metallochaperones each deliver the metal to many different zinc metalloproteins. If so, one would predict that common mechanisms of protein–protein interaction would be conserved among the target proteins of a particular metallochaperone. The sequence conservation likely required to allow for these protein–protein interactions has not been observed among large groups of zinc proteins, e.g., zinc finger proteins. Thus, while some zinc proteins may be of high priority and have specific metallochaperones to maintain metallation under low zinc conditions, it seems unlikely that metallochaperones are a general mechanism of zinc trafficking.

In addition, the interpretation that there is no free zinc in cells based on these data assumes that the cell is at equilibrium with its environment. This is clearly not the case for living cells that are subject to fluctuations in environmental conditions, intracellular metabolism, and regulatory responses. Rather, what seems more likely is that individual *E. coli* cells (and probably other types of cells) rapidly cycle between periods of intracellular zinc deficiency and zinc repletion. For example, a zinc-deficient *E. coli* cell responds by increased expression of zinc uptake transporters and decreased expression of zinc efflux transporters. Zinc entering the cell would then shut off expression of the uptake transporters and induce activity of the efflux transporters. Because these responses are not instantaneous, the cell would accumulate zinc in excess of its demand level before the zinc-responsive changes in transporter activity can occur. After uptake ceases, the accumulated excess of zinc is then used by newly made zinc metalloproteins or is effluxed from the cell. Once the bolus of intracellular zinc is depleted, the cycle can begin anew. Thus, the high affinity of transcription factors like ZntR and Zur for zinc would still allow their function as "toggle switches" alternating between zinc deficient and replete states. This scenario provides one simple alternative explanation for how transcription factors with femtomolar affinity can maintain zinc homeostasis.

2. Families of eukaryotic zinc transporters

The uptake of zinc into cells and its transport into and out of intracellular organelles requires transporter proteins that span these membranes to facilitate the movement of zinc. Zinc transporters belong to at least 6 different transporter families. Three of these, the ABC transporters, the RND transporters, and the CorA proteins have been implicated in zinc transport in bacteria but not in eukaryotes [26] and will not be considered here. Similarly, few P-type ATPases have been found to play roles in eukaryotic zinc transport. Known eukaryotic zinc transporters come largely from two families, the ZIP (SLC39) and CDF/ZnT (SLC30) proteins. These two families will be

described briefly here and the reader is referred to other recent reviews for additional information [27–31].

2.1. The ZIP family of zinc transporters

The ZIP (Zrt-, Irt-like Protein) family is named after the yeast Zrt1 protein and the *Arabidopsis* Irt1 protein. These were the first identified members of this family. Mammalian members of this family have been given the systematic designation "SLC39" [30]. A key feature of the ZIP family is that, without any yet known exceptions, these proteins transport zinc and/or other metal ion substrates from the extracellular space or organellar lumen into the cytoplasm.

ZIP transporters are found at all phylogenetic levels including bacteria, fungi, plants, and mammals [32]. Most ZIP proteins have eight predicted transmembrane domains and similar predicted topologies with the N- and C-termini of the protein located on the extracytoplasmic face of the membrane (Fig. 1). Many members also have a long loop region located between transmembrane domains 3 and 4, and a histidine-rich sequence is frequently found in this TM3–4 loop. The function of this domain is unclear, but its potential metal binding ability suggests some function in zinc transport or its regulation. However, mutations in these residues in the yeast Zrt1 transporter had no apparent effect on transporter function but did alter the protein's subcellular localization [33]. The mechanism of transport used by ZIPs is not clear. Zinc transport by the yeast Zrt1 protein is energy dependent while the human Zip2 (SLC39A2) transporter is not [34,35]. Zinc uptake by this mammalian transporter may be driven by the gradient of HCO_3^- that exists across the membrane of cells [35].

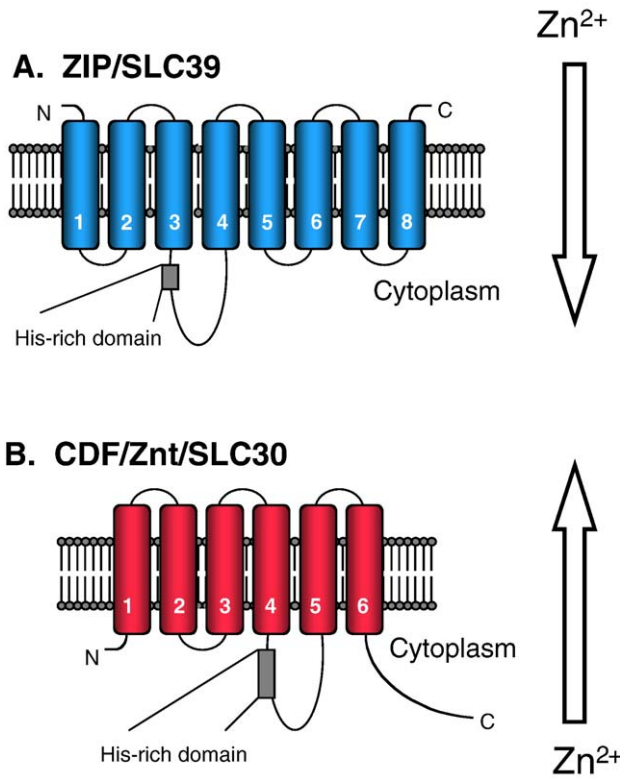

Fig. 1. The predicted membrane topologies of the ZIP/SLC39 and CDF/Znt/SLC30 families of metal ion transporters. The transmembrane domains are numbered 1, 2, etc.

2.2. The CDF/Znt family of zinc transporters

The CDF designation stands for "cation diffusion facilitator" [36]. Mammalian members have been named "ZnT" and given the systematic name of SLC30 [31]. The key feature of this family is that they transport zinc and/or other metal ions from the cytoplasm into the lumen of intracellular organelles or to the outside of the cell. Thus, CDF proteins work in opposition to the ZIP transporters.

CDF transporters are also found at all phylogenetic levels [32]. Most members of this family have six predicted transmembrane domains (Fig. 1). Notable exceptions to this rule are the Msc2 and Znt5 proteins that form heteromeric complexes to transport zinc into compartments of the secretory pathway. These two proteins have the canonical six transmembrane domain motif of the CDF family at their C-termini with several (6–9) additional transmembrane domains at their N-termini. The function of these additional transmembrane domains is unknown but may be related to how these proteins interact with their partners in the heteromeric complexes. Like the ZIP proteins, many CDF family members have histidine-rich motifs, in this case usually in the cytoplasmic loop between transmembrane domains 4 and 5. These domains may also bind metal during transport. Intriguingly, a splice variant of a plant CDF protein (TgMTP1) that lacks this His-rich domain was found to have altered substrate specificity relative to the full-length protein [37].

Many CDF proteins have been implicated to function as homomeric dimers or higher order complexes. These include the Znt1, PtdMtp1, Mtp1, FieF, and Znt7 proteins [4,38–42]. As mentioned above, two heteromeric CDF complexes have also been identified, Msc2 in complex with Zrg17 and Znt5 bound to Znt6 [40,43]. The mechanism of transport for many CDF proteins appears to be via zinc/H^+ or K^+ antiport. The Zrc1 protein of yeast functions as a Zn/H^+ antiporter while the CzcD protein of *Ralstonia* may function as a H^+ or a K^+ antiporter [44–46]. Therefore, despite their name, CDF proteins do not serve as diffusion facilitators but rather as secondary active transporters, using the gradient of other ions to drive the transport of zinc.

3. Zinc transport across the plasma membrane in yeast

The first step in the cell biology of zinc is the transport of the ion across the plasma membrane into the cell. Over the past few years, many different transporters have been identified that play this role in different organisms and cell types. Most of these are members of the ZIP family of zinc transporters with only a few exceptions. Among eukaryotes, the identification of plasma membrane zinc transporters is most advanced in the yeast *Saccharomyces cerevisiae* where at least four different transporters are involved in zinc uptake by this single-celled organism (Fig. 2). The primary uptake system for zinc is the

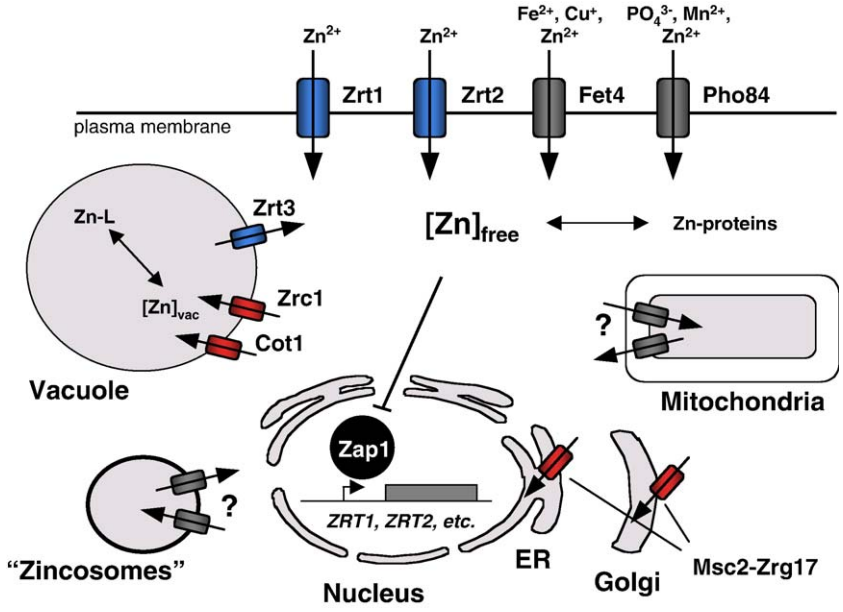

Fig. 2. An overview of zinc transport and trafficking in the yeast *S. cerevisiae*. ZIP family transporters are shown in blue and CDF family transporters are shown in red. Hypothetical transporters or known proteins from other families of transporters are shown in gray. The Zap1 transcriptional activator, shown in black, is responsible for the up-regulation of many target genes in zinc-limited cells. Zn^{2+} in the vacuole is likely bound by some ligand (L) to facilitate storage.

Zrt1 transporter of the ZIP family that is required for growth in low zinc conditions [47]. Zrt1 expression levels are up-regulated by zinc deficiency at the transcriptional level by the Zap1 zinc-responsive activator protein [48]. Expression of Zrt1 in zinc-limited cells is induced approximately 100-fold over zinc-replete expression levels. Zrt1 has a remarkably high affinity for zinc with an estimated apparent K_m of 10 nM for free Zn^{2+} ions. A second ZIP protein, Zrt2, has a lower affinity for free zinc (apparent $K_m = \sim 100$ nM) and plays a role in zinc acquisition under less severe zinc-limiting conditions [34]. Zrt2 is induced by Zap1 under conditions of mild zinc limitation but then repressed by Zap1 under more severe zinc-limiting conditions [49]. This intriguing pattern of regulation involves the positioning of three Zap1 binding sites within the promoter. Two binding sites are located upstream of the TATA box and serve to activate gene expression. A third binding site is located downstream of TATA near the start site of transcription, and Zap1 binding at this site blocks Zrt2 transcription.

Mutational inactivation of Zrt1 and Zrt2 revealed the existence of additional zinc uptake systems with lower affinity. One such system was found to be the Fet4 transporter which is involved in the low affinity uptake of iron and copper as well as zinc [50]. Fet4 is not a ZIP protein and, surprisingly, Fet4-related proteins are only found in Ascomycete fungi such as *S. cerevisiae*, *Schizosaccharomyces pombe*, *Candida albicans* and *Aspergillus fumigatus*. A fourth system of zinc uptake in yeast is likely to be the Pho84 high affinity phosphate transporter [51]. Pho84 is a member of the major facilitator superfamily of transporters [52]. Mutation of the *PHO84* gene confers zinc tolerance and reduces the accumulation of zinc consistent with Pho84 being able to transport zinc in addition to phosphate. While likely to have a low affinity for zinc, this system is highly induced by phosphate limitation so it may play a more significant role in zinc homeostasis under low phosphate growth conditions [53].

4. Plasma membrane zinc uptake transporters in other organisms

Many zinc uptake transporters have been identified in other organisms over the past few years. Space limitations prevent a full accounting of these discoveries so I will focus here on recent advances in the field of mammalian zinc uptake transporters with apologies to those colleagues studying other types of organisms. There are 14 known members of the ZIP family encoded by the human genome [30]. The Zip1/SLC39A1 transporter is ubiquitously expressed in tissues of mice and humans [54,55]. This protein is likely to play a role in zinc uptake by many cell types. Zip1 has been shown to be the major zinc uptake system in K562 erythroleukemia cells and in prostate cells [54,56,57]. Zip1 is localized to the plasma membrane in some cell types but not in others perhaps owing to the zinc-responsive regulation of this protein's subcellular localization [54,58]. In zinc-deficient cells, Zip1 migrates to the plasma membrane while it is found associated with intracellular compartments in zinc-replete cells [59]. In addition to Zip1, the Zip2/SLC39A2 and Zip3/SLC39A3 proteins have also been implicated in zinc uptake by mammalian cells [35,60,61]. More specifically, Zip3 has been implicated in zinc uptake by mammary epithelial cells to supply zinc to milk [62,63].

The Zip4/SLC39A4 protein has been found to be an important transporter for the acquisition of zinc from the diet. This role of Zip4 in dietary zinc absorption was first discovered by two groups whose studies linked mutations in the *SLC39A4* gene to a human genetic disease, acrodermatitis enteropathica (AE) [64–66]. AE is a recessive disorder of dietary absorption.

Patients with AE were found to have mutations in the *SLC39A4* gene that disrupt the zinc transport function of the protein [67]. Consistent with its proposed action in dietary zinc absorption, the Zip4 protein localizes to the apical (gut lumenal) surface of intestinal enterocytes [60]. Moreover, expression of Zip4 in the mouse visceral yolk sac suggests that this protein may also deliver maternal zinc to the developing fetus [60]. It is clear that Zip4 is not the only zinc transporter responsible for dietary zinc uptake. Many AE mutations completely disrupt function of the transporter [67]. Nonetheless, AE generally affects infants and not adults indicating that other transporters play the primary role in zinc absorption after infancy. Moreover, zinc supplements readily suppress AE symptoms in infants suggesting the presence of other transporters on the apical face of the enterocytes. The Zip1 protein or another ZIP may also participate in dietary zinc absorption.

In contrast to Zip4, the Zip5/SLC39A5 protein is expressed on the basolateral surface of intestinal enterocytes [68,69]. Therefore, while Zip4 is responsible for zinc uptake from the gut, Zip5 may mediate zinc transport from the bloodstream into the enterocyte. This activity of Zip5 may serve two functions. First, Zip5 may deliver needed zinc from body stores to the enterocytes when dietary levels are low. Alternatively, Zip5 may transport zinc into the enterocyte as a means to communicate the levels of serum zinc to the enterocyte to control the level of zinc absorption from the diet.

As a final example, a recent study has indicated the role of yet another ZIP, Zip14/SLC39A14, in the uptake of zinc into liver in response to acute inflammation and infection [70–72]. Under these conditions, serum zinc levels drop and liver levels rise in a likely effort to withhold this essential metal nutrient from the invading pathogen. Zip14 expression in the liver is induced in response to interleukin-6 (IL-6) during the acute phase response. These results suggested that induction of Zip14 by IL-6 is responsible for the serum hypozincemia associated with infection.

5. Plasma membrane zinc efflux transporters

Plasma membrane efflux transporters are important components of cellular and organismal zinc homeostasis in many organisms. These transporters mediate the efflux of intracellular zinc to prevent the cellular overaccumulation of the metal ion thereby saving the cell from the toxic consequences of zinc overload. In addition, zinc efflux systems are critical for the movement of zinc across polarized epithelial cell layers. For example, during zinc absorption in the intestine, resorption of zinc in the kidney, and during embryonic development, delivery of zinc to the bloodstream or the embryo is dependent on the uptake of zinc at one face of polarized epithelial cells and the subsequent efflux of the metal on the other side of those cells.

Zinc efflux is a critical component of zinc homeostasis in *E. coli* and many other bacteria [26]. Among eukaryotes, yeast may be unusual in not having any zinc efflux systems. These cells transport excess zinc into an intracellular organelle for zinc storage and detoxification rather than release that zinc from the cell (see below). In mammalian cells, the CDF family member Znt1/SLC30A1 has been implicated in effluxing zinc for both cellular zinc homeostasis and trans-epithelial zinc trafficking [4,73–75]. The role of zinc efflux transporters in cellular zinc homeostasis may be particularly important in the brain where high levels of zinc are accumulated by neurons following ischemia or epileptic seizures [73,76]. In plants, the TgMtp1 CDF protein of *Thlaspi goesingense* and the *Arabidopsis* Hma2 and Hma4 transporters of the P-type ATPase family have been implicated in the plasma membrane efflux of zinc from the vascular tissues of roots, stems, and leaves [77–79].

6. The transport of zinc into and out of intracellular organelles

6.1. The secretory pathway

Many zinc metalloproteins are secreted or are resident within the secretory pathway. Notable examples of secreted proteins that require zinc are the matrix metalloproteases (MMPs). The proteolytic activity of these secreted proteins are required for developmental processes such as organ branching, morphogenesis, angiogenesis, and extracellular matrix remodeling [80]. MMPs also play important roles in cancer progression and metastasis by allowing cell migration into tissues and by promoting angiogenesis and the vascularization of tumors. Secreted alkaline phosphatases are also zinc dependent [72]. In addition, the lumen of the ER is the site for the initial folding and post-translational modification of proteins destined for secretion or residency within secretory pathway, and many of these processes are zinc-dependent. For example, glycosylphosphatidylinositol phosphoethanolamine transferases (GPI-PETs) in the endoplasmic reticulum act in the biosynthesis of GPI anchors that attach many proteins to cell surfaces. GPI-PETs have a zinc-binding motif similar to that found in alkaline phosphatases [81] and, in both mammals and yeast, require zinc for their function [82,83]. The yeast DnaJ homolog Scj1 is a protein co-chaperone in the ER that contains two zinc fingers and requires zinc for its function [84]. Disrupting Scj1 function causes defects in protein folding and less effective degradation of aberrant proteins by ER quality control mechanisms [85]. Similarly, the ER chaperones calnexin and calreticulin require zinc for their interaction with the ERp57 thiol oxidoreductase [86]. These chaperones are needed for glycosylation of secreted and lumenal glycoproteins.

The Golgi also contains zinc-dependent resident proteins. For example, cleavage of the Alzheimer's disease Aβ protein from the amyloid precursor protein in the Golgi depends on resident zinc metalloproteases in that compartment [87]. The packaging of insulin in secretory granules relies on zinc. Insulin is packaged in a crystalline structure within these vesicles with a 2:1 Zn:insulin stoichiometry [88] (see below). The hexamerization of proinsulin that occurs in the Golgi, an early step in forming the Zn:insulin crystal, is zinc-dependent as well [89].

There is very little information regarding where in the secretory pathway zinc is delivered to specific resident and secreted apoproteins. The zinc requirement of lumenal ER proteins such as DnaJ-like chaperones and GPI-PETs indicates

that some metallation occurs in the ER. Studies of insulin processing indicates that some zinc is delivered to the *cis* compartment of the Golgi apparatus [89]. Matrix metalloproteases, which are inhibited by an intramolecular repression domain, can be activated in the Golgi by proteolytic removal of this inhibitory region [90,91]. Because MMP activity depends on zinc, these observations indirectly demonstrate that zinc is bound to these proteins by the time they reach the Golgi. Thus, these few examples indicate that zinc is delivered to the ER and Golgi compartments by zinc transporter proteins.

Zinc transporters responsible for delivery of zinc to the secretory pathway have been recently identified, and these proteins belong to the CDF family. For example, in yeast, the heteromeric Msc2/Zrg17 complex mediates zinc transport into the ER [43,92,93] (Fig. 2). Mutational inactivation of either or both subunits of this complex results in pleiotropic defects in ER function, including induction of the Unfolded Protein Response (UPR) and defects in ER-associated protein degradation. Cell wall biogenesis, a process highly dependent on secretory pathway function, is also likely to be disrupted in *msc2* or *zrg17* mutants.

The Msc2/Zrg17 transporter complex is important for transport of zinc into the ER under low zinc conditions. When higher zinc levels are provided in the medium, their mutant phenotypes are completely suppressed. This indicates that other transport mechanisms are present to supply zinc to the ER. The vacuolar CDF transporters Zrc1 and Cot1 (see below) were shown to contribute to ER zinc [92]. These proteins may be active in the early secretory pathway soon after their synthesis and before they transit to the vacuole. Surprisingly, in a mutant disrupted for Msc2, Zrg17, Zrc1 and Cot1, increased exogenous zinc could still suppress the defects in ER function. Thus, additional pathways of ER zinc transport exist in yeast.

Supply of zinc to the Golgi may occur by vesicular trafficking of ER zinc to that compartment. Alternatively, zinc transporters may be active in the Golgi as well. In yeast, not all of the Msc2/Zrg17 complex localizes to the ER suggesting that some fraction may be present in the Golgi. In vertebrate cells, the Znt5/Znt6 complex localizes to the Golgi and is required for supplying zinc to alkaline phosphatase and probably to other secreted zinc proteins [94–96]. In contrast, the Znt7/SLC30A7 protein, which also localizes to the Golgi, forms homo-oligomers and also transports zinc into the Golgi [40,97]. To date, no CDF transporters have been localized specifically to the ER in vertebrates. This is in striking contrast to yeast where Msc2 and Zrg17 are predominantly localized to the ER. Supply of zinc to the ER in vertebrate cells may involve the transport activity of the Golgi-localized transporters as they transit through the ER as proposed above for the yeast vacuolar zinc transporters. Alternatively, retrograde vesicular trafficking from the Golgi to the ER may carry labile zinc back to the ER for the function of metalloproteins in that compartment. A similar retrograde trafficking of ions has been proposed for Ca^{2+} [98].

Zinc may also be exported from the Golgi. The mammalian Zip7/SLC39A7 protein localizes to the Golgi and appears to mediate transport of secretory pathway zinc back to the cytoplasm [99]. This may be a means to recover unused zinc in the secretory pathway prior to its loss by secretion. It is not clear if *S. cerevisiae* uses a similar strategy but a yeast ZIP transporter related to Zip7, called Yke4, could fulfill this function.

Finally, secretory vesicles of various types have also been shown to contain zinc transporters in their membranes. For example, the Znt4/SLC30A4 protein is required for the transfer of zinc to milk; mutational defects in Znt4 cause the *lethal milk* phenotype in mice [100]. This transporter is localized to vesicles in cells that are presumably involved in the secretion of zinc [101,102]. Similarly, the Znt3/SLC30A3 transporter is localized to synaptic vesicles in certain types of neurons that release high levels of zinc along with neurotransmitters such as glutamate [103,104]. Intriguingly, the pool of synaptic zinc may be a critical factor in the development of Alzheimer's disease as indicated using a mouse model of this disease [105]. Finally, the Znt8/SLC30A8 transporter is associated with the secretory granules of pancreatic β-cells [106,107]. Znt8 is likely to supply the zinc required for the packaging of insulin in these granules as described above.

6.2. The fungal and plant vacuole

The vacuole of yeast and plants serves as a major site of zinc sequestration and detoxification [108–110] (Fig. 3). For example, in *S. cerevisiae*, wild type cells can tolerate exogenous zinc concentrations as high as 5 mM. When the transport systems for vacuolar zinc sequestration are disrupted (see below), the maximum level of zinc tolerable by the cells drops by ~100-fold [3,111–113].

Chronic exposure to zinc at the millimolar levels that can be tolerated by wild type cells is not often encountered by yeast growing in their normal environment. Thus, vacuolar sequestration is likely to be more important to the cell for growth under other conditions. One such condition is "zinc shock." Zinc shock occurs when cells are grown under zinc-limiting conditions and then resupplied with even low levels of zinc. Zinc-deficient cells are poised to accumulate substantial amounts of zinc due to the up-regulation of the plasma membrane transporter Zrt1. When resupplied with zinc, these cells accumulate large amounts of the metal ion before transcriptional and post-translational mechanisms that regulate Zrt1 can shut off additional uptake. We have shown that mutant cells unable to sequester zinc in the vacuole during zinc shock are sensitive to zinc concentrations as low as 0.1 μM [114]. Given that microbial cells growing in the environment live an existence of sporadic nutrient availability, we believe the primary function of the vacuole in zinc detoxification is during zinc shock caused by fluctuations in zinc availability.

Zinc sequestered within the vacuole also serves as a storage pool of zinc that can be mobilized under zinc-deficient conditions for use by the cell. Our recent results indicate that zinc can accumulate in the vacuole to millimolar levels (C. Simm, A. LeFurgey, P. Ingram, and D. Eide, unpublished observation). This is a sufficient level of zinc to support many yeast cell generations in the absence of an exogenous supply. Zinc uptake into the yeast vacuole is mediated by two members

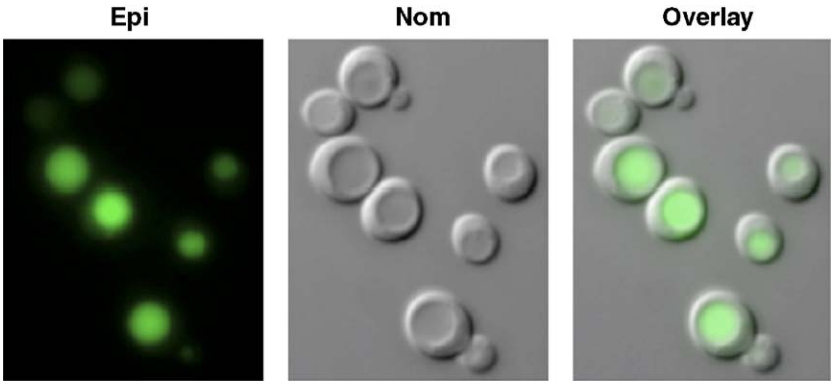

Fig. 3. Accumulation of zinc in the yeast vacuole. The zinc-responsive fluorophore FuraZin-1 acetoxymethyl ester was loaded into the vacuole of zinc-limited wild type cells. These cells were then treated with zinc and fluorophore signal was detected by epifluorescence (Epi). Nomarski optics (Nom) shows the large yeast vacuole present in many cells and the overlay of the images shows the localization of the fluorophore to the vacuole. FuraZin-1 is a useful probe for the qualitative detection of vacuolar zinc in yeast [114].

of the CDF family, Zrc1 and Cot1 (Fig. 2). Zrc1 has been shown to be a Zn^{2+}/H^{+} antiporter which allows zinc accumulation in the vacuole to be driven by the proton concentration gradient provided by the vacuolar H^{+}-ATPase [44]. The Cot1 protein may act in a similar fashion, although its biochemical properties have not yet been analyzed. Zinc release to the cytosol under conditions of zinc deficiency is mediated by Zrt3, a member of the ZIP family of transporters [3]. Zrt3 expression is up-regulated under low zinc conditions to facilitate this mobilization of vacuolar zinc.

In contrast to *S. cerevisiae*, the ER may be a major site of zinc detoxification and storage in the fission yeast, *Schizosaccharomyces pombe*. The *S. pombe* Zhf protein of the CDF family localizes to the ER and mutations in its gene results in reduced cellular zinc accumulation and zinc sensitivity [115]. These effects are similar to what is seen for *zrc1* and *cot1* mutants of *S. cerevisiae* and are not observed for *msc2* or *zrg17* mutants.

In plants, members of the CDF family of transporters have been implicated in the vacuolar accumulation of zinc. For example, the *Arabidopsis* Mtp1 protein has been localized to the vacuolar membranes of shoots and leaf cells and can transport zinc when expressed in *Xenopus laevis* oocytes. Reduced expression of Mtp1 results in zinc sensitivity and lower zinc accumulation in plant tissues [116–118]. Plant proteins responsible for mobilizing vacuolar zinc have not yet been identified. However, there are several members of the ZIP family encoded by plant genomes. For example, *Arabidopsis* has 11 ZIP genes, and one or more of these may be playing roles analogous to yeast Zrt3 [29].

Zinc stored within vacuoles may not simply accumulate as free Zn^{2+} ions. X-ray absorption spectroscopy analysis of vacuolar zinc in plants indicates that Zn^{2+} may instead accumulate bound to organic anions. For example, in *Arabidopsis halleri*, a plant species capable of hyperaccumulating large amounts of zinc, the metal was found complexed primarily with phosphate and/or organic acids such as malate and citrate depending on the growth conditions and the plant tissues examined [119]. Similar results were obtained with *Thlaspi caerulescens*, another zinc hyperaccumulating plant species [120]. Binding of vacuolar zinc by such compounds may provide a means of accumulating large amounts of zinc within the vacuolar compartment for storage and detoxification. Similarly, the glutamate levels in synaptic vesicles are proportional to their zinc accumulation, suggesting that the anion influences the zinc loading capacity of the vesicle [121]. It is unclear at this time if vacuolar zinc in yeast is similarly bound to anions within the vacuole, but it seems a likely possibility.

6.3. Zincosomes

Vesicular storage sites for zinc may also exist in mammalian cells. Several studies have used zinc-responsive fluorophores (e.g., TSQ, zinquin, Zinpyr-1) to examine the distribution of labile intracellular zinc [93,102,122–127]. These studies have consistently indicated that labile zinc can be detected within membrane-bound vesicles in a large variety of mammalian cell types. These vesicles have been designated “zincosomes” [128,129], a term I will use here for convenience. However, the relationship of zincosomes to established organelles is not clear. Palmiter and colleagues showed that, at least in one cell type, these zinc-containing vesicles are late endosomes [122,130]. Whether zincosomes correspond to late endosomes in other cells remains to be tested.

Zincosomes have also been observed in yeast [93,131]. As shown in Fig. 4, zincosomes accumulate in zinc-treated yeast cells. This accumulation is dependent on the Zrt1 zinc uptake transporter but not on the Zrc1 or Cot1 vacuolar zinc transporters nor is it dependent on endocytosis [131]. Co-localization studies indicate that yeast zincosomes do not correspond to the vacuole, Golgi or the prevacuolar compartment (C. MacDiarmid and D. E., unpublished results). Thus, the identity and function of these compartments are still unknown.

What is the function of zincosomes in zinc metabolism and homeostasis? Some studies suggested a role in detoxifying excess zinc [122]. For example, treating baby hamster kidney cells with high zinc greatly increased the number and fluorescence intensity of the vesicles, indicating an increase in vesicular zinc levels. Moreover, the zinquin-staining vesicles

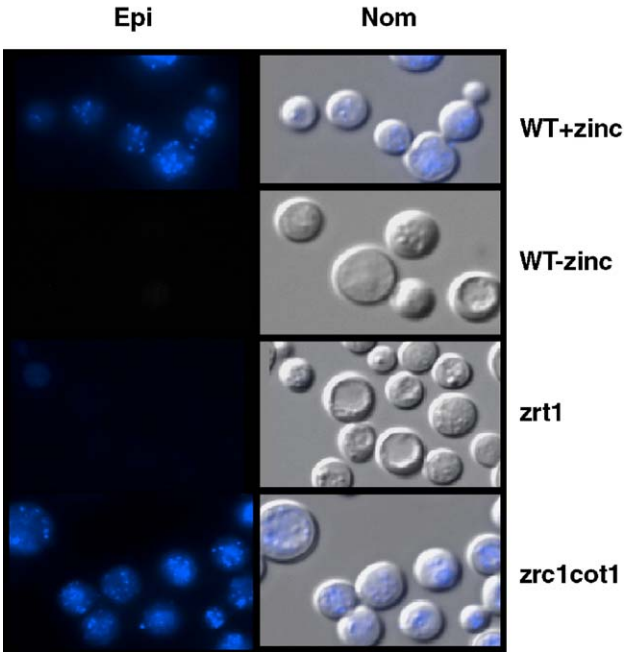

Fig. 4. Detection of yeast zincosomes. The zinc-responsive fluorophore zinquin was loaded into zinc-limited wild type or mutant yeast cells. These cells were then treated with zinc (100 μM for 10 min) and the zinc-responsive fluorophore signal was detected by epifluorescence (Epi). Nomarski optics (Nom) shows the large yeast vacuole present in many cells. Zinquin staining does not co-localize with the vacuole. Untreated wild type cells (designated WT -zinc) are shown for comparison. Zinquin was kindly provided by T. O'Halloran.

co-localized with the Znt2/SLC30A2 zinc transporter, a CDF transporter, whose expression is known to confer zinc tolerance. It has also been noted that stress agents, such as nitric oxide (NO), increase labile zinc levels probably due to the release of zinc from proteins [16,127–129,132]. This newly released labile zinc subsequently accumulated in zincosomes. Thus, the zincosome may serve to buffer cytosolic zinc from transient perturbations of zinc homeostasis much like the vacuole in yeast. Other studies have implicated zincosomes in the storage of zinc that can be later mobilized under zinc deficiency. For example, the total zinc content of cells, and the fluorescence intensity of the zinquin-staining vesicles all decreased when HL60 cells [133] or rat hepatocytes [16] were transferred from zinc-replete to low zinc culture media. These results suggest that zinc has been stored in the vesicles and is mobilized for use during this transition.

6.4. Mitochondria

Zinc is required in the matrix of the mitochondria for function of proteins within that compartment. In yeast, these include alcohol dehydrogenase 3 (Adh3) which converts acetaldehyde to ethanol as a means to shuttle NADH across the inner membrane [134]. The Leu4 protein, alpha-isopropylmalate synthase, mediates the first step of leucine synthesis. Leu4 is found in the matrix and is a zinc metalloenzyme [135]. Zim17 is a zinc finger matrix protein required for protein import [136]. Finally, labile zinc can be detected in the mitochondria of mammalian neuronal cells using a zinc-responsive fluorophore, RhodZin-3 [137]. Further studies by these authors indicate that mitochondrial zinc in neurons is held in that compartment by the mitochondrial membrane potential [138]. Disruption of that membrane potential results in the release of zinc to the cytosol. This release of mitochondrial labile zinc may be a contributing cause of neuronal cell death during ischemia or epilepsy [139].

These examples indicate that mechanisms must exist to get zinc into the mitochondria. Zinc must first traverse the outer membrane, most likely through the porin channels. Then, the zinc must cross the inner membrane via transporter proteins. Previous studies suggested that zinc may enter the mitochondria matrix via the Ca^{2+} uniporter system. For example, rhuthenium red, a Ca^{2+} uniporter blocker, protects mitochondria from the adverse effects of high zinc [140–142]. However, these studies did not provide direct evidence for mitochondrial Zn^{2+} uptake.

A recent study of rat brain mitochondria by Reynolds and colleagues using methods to directly visualize mitochondrial zinc accumulation indicated both Ca^{2+} uniporter-dependent and uniporter-independent pathways [143]. Zinc uptake was partially dependent on the mitochondrial membrane potential, and the transporter activities were found to be of high affinity with apparent K_m's of ~1 μM. This value is likely be an overestimate of their true apparent K_m's because the binding of substrate by organic anions in the uptake assay buffer (glutamate, malate) was not considered. Thus, the affinity of these transporters is likely to be even higher and therefore in a physiologically relevant range. These studies also detected zinc efflux from mitochondria.

What is the substrate for mitochondrial zinc influx transporters? Is the substrate free Zn^{2+}, or can zinc-chelate complexes serve as substrates? An intriguing study addressing this question was recently published by Costello and colleagues [144]. In vitro studies of zinc uptake into mitochondria isolated from rat prostate cells revealed the activity of a mitochondrial zinc transporter with an apparent K_m of approximately 60 μM when assayed using ^{65}Zn supplied as $ZnCl_2$. Such a high K_m argues that this system is unlikely to be physiologically relevant. Surprisingly, however, it was found that several zinc-chelates including zinc-citrate, zinc-aspartate, and zinc-histidine also had similar apparent K_m's for transport as was observed with $ZnCl_2$. These compounds can bind zinc relatively tightly and thereby reduce the free Zn^{2+} available for transport. The fact that these chelators did not inhibit zinc uptake despite their ability to reduce free zinc suggests that transport may occur via ligand exchange reactions between zinc–chelate complexes and the transporter. This hypothesis was supported by the observation that the zinc traverses the membrane while the chelator does not [144]. Alternatively, it has been proposed that metallothionein may serve as one source of zinc for uptake to the mitochondrial matrix. MT can be detected in the intermembrane space of mitochondria [145], and MT-supplied zinc can be taken up into isolated mitochondria in vitro [146].

7. Future directions for studies of the cell biology of zinc

In this review, I have attempted to provide an overview of our current knowledge of the cell biology of zinc. What may be most obvious from this discussion is all that we do not yet know. Clearly, several questions of great importance still need to be addressed. First, what are the intracellular forms of labile zinc, and how is that zinc delivered to zinc metalloproteins? Do any zinc metalloproteins obtain their zinc from metallochaperones? In addition, what are the biochemical mechanisms used by ZIP and CDF proteins to mediate zinc transport across cellular membranes? While we have some indication of how CDF proteins work, we still have no idea for the ZIPs. What are the various functions of the many members of these families that are found in organisms? For example, the human genome encodes 14 ZIPs and 9 CDFs. What do all of these proteins do? Even in the relatively simple yeast, we still do not know the function of all the ZIP and CDF proteins. In addition, we are still learning of new roles for previously characterized transporter proteins. How does zinc get into and out of mitochondria and other organelles? We have an evolving picture, but it is still very incomplete. What are "zincosomes" and how do they participate in zinc homeostasis and trafficking? Finally, although it was beyond the scope of this review, we need to consider how the various transport and trafficking systems are regulated by zinc availability, metabolic demands for zinc, and other factors to maintain zinc homeostasis and optimal cell function. Thus, while we have learned much over the past few years, it is exciting to consider the new horizons that still await researchers in this field.

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