The trafficking of metal ion transporters of the Zrt- and Irt-like protein (ZIP) family

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Running title
Trafficking of ZIP transporters

Synopsis
Metal ion transporters are important in the regulation of essential ion uptake. Intracellular protein trafficking provides a rapid way of modulating ion uptake. Here, we review the emerging mechanisms for trafficking the ZIP family of metal ion transporters in cells.

Abstract

Metal ion transporters of the Zrt- and Irt-like protein (ZIP, or SLC39A) family transport zinc, iron, manganese and/ or cadmium across cellular membranes and into the cytosol. The 14 human ZIP family proteins are expressed in a wide variety of tissues and function in many different cellular processes. Many of these proteins (including ZIP1, 2, 3, 4, 5, 6/10, 8, 9, 11, 12, 14) are situated, at least some of the time, on the plasma membrane, where they mediate metal ion uptake into cells. Their level on the cell surface can be controlled rapidly via protein trafficking in response to the ions they transport. For example, the cell surface level of many ZIPs (including ZIP1, 3, 4, 8, and 12) is mediated by the available concentration of zinc. Zinc depletion causes a decrease in endocytosis and degradation, resulting in more ZIP on the surface to take up the essential ion. ZIP levels on the cell surface are a balance between endocytosis, recycling and degradation. We review the trafficking mechanisms of human ZIP proteins, highlighting possible targeting motifs and suggesting a model of zinc-mediated endocytic trafficking. We also provide two possible models for ZIP14 trafficking and degradation.

Zinc uptake

Zinc is an essential trace metal. Estimates suggest that around 10% of all human proteins bind zinc,[1] with its importance confirmed by the consequences of zinc-deficiency, which are poor: growth, development, immune response, neurological and endocrine functions.[2] Excess zinc is toxic. Thus, the cellular levels of zinc are very tightly regulated. Two protein families, the zinc transporters (ZNT/ solute carrier 30 family [SLC30A]) and the Zrt- and Irt-like proteins (ZIP/ solute carrier 39 family [SLC39A]) play vital roles in cellular zinc homeostasis. ZIPs control the transport of zinc into the cytosol, either across the plasma membrane or from intracellular organelles, while ZNTs are believed to work in the opposite direction, transporting zinc away from the cytosol. Our focus here is on the ZIP family of metal ion transporters.

ZIP gene expression is tightly controlled, often in response to differing levels of zinc or other metal ions. However, it is also apparent that ZIP protein localization is controlled rapidly via post-translational trafficking within the cell. These trafficking mechanisms are essential in the regulation of transporter protein localization and are likely to impact dramatically on the tightly regulated processes of metal ion uptake into cells. The function of ZIPs in various cell types has been covered by others.[2-5] In general, the ZIPs are synthesized on ribosomes attached to the endoplasmic reticulum (ER) and then trafficked to different intracellular compartments. Unstable mutant proteins are often recognized in the ER, retrotranslocated and degraded by cytosolic proteasomes in a ubiquitin-dependent manner (as seen, for example, for ZIP13 mutants[6]). ZIP proteins often have several isoforms generated via alternative splicing (e.g. ZIP14 has 3 predicted isoforms: a, b and c[7,8]). However, as we are unaware of any isoform-specific differences in trafficking, we will make no distinction between them. Although detailed analysis of post-ER ZIP trafficking mechanisms is lacking, we will summarize the information available with a view to uncovering specific areas for future research. We discuss potential endocytosis signals for a subset of human ZIPs and suggest a model for zinc-mediated endocytosis and degradation.

The ZIP family of metal ion transporters

The ZIP family of transporters is named after the first three identified members: Zrt1 and Zrt2 in *S. cerevisiae*[9,10] and Irt1 in *A. thaliana*.[11] There are 14 identified members of this family in humans, the majority specific for zinc transport, but some are able to transport other metal ions. For example, in addition to zinc: ZIP7, 8 and 14 can transport manganese; and ZIP8 and 14 can transport iron and cadmium [reviewed in [4]; [12]]. ZIP proteins are expressed in a variety of cell types and in different intracellular locations.[13]

The ZIP family can be divided into four subfamilies using amino acid sequence similiarities: subfamily I contains ZIP9; subfamily II ZIP1, 2 and 3; the LIV-1 subfamily ZIP4, 5, 6, 7, 8, 10, 12, 13 and 14; and the gufA subfamily ZIP11.[4] All ZIP proteins are proposed to have a similar topology, with eight transmembrane (TM) domains and C- and N-termini extracellular when at the cell surface, or inside the lumen of intracellular organelles (see Fig. 1). Many membrane proteins, including multi-spanning membrane proteins, have cytosolic termini that contain trafficking information. For example, the insulin-responsive glucose transporter GLUT4 has amino acid sequences in its cytosolic C-terminus involved in binding to intracellular machinery and directing the trafficking of the protein within the cell.[14] However, in the case of the ZIPs, with neither the N- nor C-terminal domains located in the cytosol, we must look elsewhere in the protein for signals that may interact with the trafficking machinery. Perhaps the obvious place to look for such signals is in the relatively large intracellular loop between TM domains 3 and 4, L2 (see Fig. 1), and ZIP1 does indeed contain a trafficking signal in this region (see below and Fig. 2). The L2 loop is not conserved among ZIP proteins and trafficking signals may differ for each family member to take account of their divergent cell type expression and response to metal ions. It follows that ZIP proteins may have as yet uncharacterized trafficking signals in their L2 loops, but it is also possible that ZIPs may either have signals within other regions of the protein, or bind accessory proteins that direct their intracellular trafficking.

It may seem that the steady state localization of ZIP proteins in various cell types is well known. However, when interpreting localization data the following four important points should be noted: 1) Many proteins cycle in the cell and the steady state localization (i.e. the concentration of the protein at any one time) is not necessarily a reflection either of its site of action or its trafficking itinerary; 2) Intracellular organelles sit in very close proximity to one another and can be extremely difficult to resolve by light microscopy; 3) Epitope tags may affect the structure and hence the trafficking of proteins; and 4) Overexpression of exogenous proteins may saturate the protein trafficking machinery and result in aberrant localisations.

Subfamily I

ZIP9 is the only member of the ZIP subfamily I, and is an androgen receptor, coupled to G proteins.[15] Human ZIP9 has been localized to the trans Golgi network (TGN) in transfected HeLa cells in a zinc independent manner.[16] A more recent study suggests that ZIP9 may be present on the cell surface, as well as nuclear and mitochondrial membranes, in human breast and prostate cancer cell lines, where it mediates testosterone induction of apoptosis.[17] As yet, nothing is known about how ZIP9 may achieve these divergent localizations, or indeed how it is trafficked in the cell. The L2 loop of ZIP9 is very short, compared to other ZIP proteins (Fig. 2) and contains no predicted endocytosis motifs.

Subfamily II

This subfamily comprises ZIPs 1, 2 and 3. Human ZIP1 mRNA is ubiquitously expressed, while ZIP2 and ZIP3 are widely expressed at low levels with ZIP3 expressed most highly in mammary cells and testes.[3,4] ZIP2 mRNA levels are dramatically increased in response to zinc depletion, while levels of ZIP1 and ZIP3 mRNA are not.[18] This suggests that post-translational regulation plays an important role in regulating zinc uptake by ZIP1 and ZIP3. ZIP2 may also be subject to regulation by protein trafficking, but this has yet to be studied.

ZIP1

Reports state that the localization of ZIP1 protein is cell type specific, with ZIP1 largely at the cell surface in K562 human erythroleukaemia cells[19] and found in intracellular vesicles assigned to various compartments

including endosomes, Golgi and ER in adherent cell lines of human, monkey and hamster origin.[20-22]

The key to unraveling the story for ZIP1 came when it was observed that stably-transfected murine ZIP1 (mZIP1) tagged with a C-terminal HA tag was seen in intracellular compartments in human kidney HEK293 cells, but undergoes constitutive endocytosis from the plasma membrane.[22] This suggests that ZIP1 is in fact constantly cycling between intracellular compartments and the cell surface, see Fig. 3. Interestingly, this trafficking of mZIP1 is modulated in response to zinc ions as under conditions of zinc depletion more mZIP1 was found at the plasma membrane. This increased cell surface mZIP1 was attributed to a decreased rate of endocytosis following zinc depletion as less mZIP1 was detected intracellularly after 10 min in zinc-depleted cells compared to untreated cells, Fig. 3. Further studies, using a stably-transfected C-terminally Myc-tagged ZIP1 construct in Chinese hamster ovary (CHO) cells, identified a dileucine-type endocytosis motif within the intracellular L2 loop of human ZIP1, between TM3 and TM4 (see Fig. 2). This sequence, ETRALL, from amino acids 144-149, fits the consensus [DE]XXXL[LI] where X is any amino acid. This sequence is conserved amongst ZIP1 proteins: the sequence is ETRALL in mammalian ZIP1s and ETDALL in *X. laevis* ZIP1, suggesting that it is important for protein function. Such consensus motifs are known to interact with clathrin adaptor complexes and direct protein trafficking.[14] Mutation of L148 and L149 to A resulted in an increase of cell surface ZIP1, suggesting that this dileucine signal acts as an endocytosis signal for the protein. [21] Furthermore, the L2 loop of ZIP1 when fused to the ecto and transmembrane domains of the interleukin-2 receptor alpha resulted in a chimeric protein that was intracellular, suggesting that the signal is sufficient for endocytosis.[21]

Given the role of the dileucine motif in ZIP1 endocytosis, it is tempting to speculate that ZIP1, like other proteins with similar motifs, interacts with the heterotetrameric AP2 adaptor complex at the cell surface, a protein complex that provides a link between membrane proteins and the machinery of clathrin-mediated endocytosis.[14] The [DE]XXXL[LI] signal binds to sites on the α and σ_2 subunits of AP2.[23] It is also interesting to note that human ZIP1-myc, expressed in CHO cells, is degraded in lysosomes in a dileucine signal-dependent manner.[21] The current data suggest that ZIP1 possesses an adaptor-binding motif in the L2 loop for endocytosis and trafficking to lysosomes. Although all these experiments were done with tagged protein in exogenous cell lines, there is some evidence that the tag may not affect function or localisation of the protein or localization: Firstly, C-terminally tagged ZIP1 and ZIP3 were shown to be functional for zinc uptake, suggesting that the tag did not interfere with this function.[24] Secondly, endogenous human ZIP1 proteins are down-regulated in human prostate epithelial cells in response to zinc.[25]

In polarized cells, the [DE]XXXL[LI] type of dileucine signal is a recognized basolateral targeting signal[26] due to its ability to bind the AP1 adaptor complex. This is supported by data showing ZIP1 at the basolateral surface of prostate cells.[27] Other signals that mediate basolateral targeting of proteins include the tyrosine-based signals YXXØ or NPXY, where X is any amino acid and Ø is a bulky hydrophobic residue. No such

motifs can be seen in the L2 loop of ZIP1 (Fig. 2). Another report suggests that ZIP1 is found apically in mouse mammary epithelial cells.[28] Apical targeting signals are less well defined than those for basolateral targeting, but include N- or O-linked glycosylation or specific transmembrane domain signals.[29] Further analysis will be required to determine how additional signals influence the trafficking of ZIP1, and how trafficking is controlled in different cell types.

For ZIP1, a targeting signal has been identified and this may act at several trafficking steps in the cell to provide control over the levels of the transporter on the surface. ZIP1 cell surface levels/ endocytosis are sensitive to zinc, and so it follows that the accessibility of the dileucine signal to AP2 is regulated by zinc binding. How might this mechanism work? Huang and Kirschke[21] sensibly point out that the dileucine signal is very close to a putative zinc binding region in L2: residues 158-161, HWHD. Zinc binding here, under conditions of sufficient zinc, may allow (directly or indirectly) a structural change in the protein in order for [D/E]XXX[L/I] to interact with AP2 and promote endocytosis.

Similarly to ZIP1, ZIP3 has been shown to traffic in a zinc-dependent manner, please see the model presented in Fig. 3.[22,30] ZIP3 endocytosis is inhibited in conditions of zinc depletion, providing a mechanism to take up more zinc under these conditions. However, unlike ZIP1, ZIP3 has no identifiable trafficking signals in the L2 loop (Fig. 2).

LIV-1 subfamily
The LIV-1 subfamily includes 9 human proteins: ZIP4, 5, 6, 7, 8, 10, 12, 13 and 14. This subfamily differs from other ZIPs with the presence of a conserved metalloprotease motif in predicted TM5 (HEXPHEXGD), see Fig. 1. Of the LIV-1 subfamily, perhaps the best studied proteins with regard to cellular trafficking are ZIP4 and ZIP14.

ZIP4
ZIP4 is expressed most highly in the gastrointestinal tract, kidney, hippocampal neurons and keratinocytes.[4,31] ZIP4 mRNA levels are tightly regulated in response to zinc.[32,33] In addition, like ZIP1 and 3, ZIP4 shows increased plasma membrane levels in response to zinc deficiency.[34] This increase in cell surface levels was attributed to a decrease in endocytosis under conditions of zinc limitation as fewer antibodies to an extracellular epitope tag were taken up over time in cells grown in zinc-depleted medium compared to those grown in basal medium.[34] The initial experiments were done with tagged murine ZIP4 transfected into HEK293 cells, but later experiments showed similar trafficking of C-terminally HA-tagged and transfected human protein.[35] Under normal growth conditions (with zinc in the medium), ZIP4 was colocalised with the transferrin receptor, suggesting that the protein in found in recycling endosomes and may continuously cycle over the cell surface. This is supported, as the general endocytosis inhibitor methyl-β cyclodextrin (MCD) caused an accumulation of ZIP4 at the cell surface in the presence of zinc.

Does ZIP4 contain trafficking signals? Looking at the L2 region of ZIP4, it is interesting to note the presence of a [DE]XXXL[LI] consensus sequence (Fig. 2). This sequence is conserved across mammalian ZIP4

proteins and may be involved in binding to AP2 for endocytosis. Further analysis will be required to determine whether this sequence is important for the trafficking of ZIP4 in response to zinc.

ZIP4 is also degraded in response to zinc.[35] Thus, under conditions of zinc-depletion, endocytosis and degradation of ZIP4 are inhibited, leaving more ZIP4 on the cell surface where it can take up zinc (Fig. 3). The degradation of ZIP4 in response to zinc requires endocytosis and is blocked by both lysosomal and proteasomal proteases. The well-established route for the degradation of multi-spanning membrane proteins is via ubiquitination of the protein, and recognition of the ubiquitin by the endosomal sorting complexes required for transport (ESCRTs).[36] The ESCRTs act at the late endosome or multivesicular body (MVB) to sort the protein into lumenal vesicles. Protein degradation then follows upon MVB-lysosome fusion. Here, the effect of proteasome inhibitors on ZIP4 degradation may be due to the disruption of endosome to lysosome trafficking and/or the depletion of the cellular pool of free ubiquitin [37]. As well as experiments in transfected cell lines, endogenous ZIP4 is degraded in mouse intestinal enterocytes in response to zinc.[32,33,38] In these cells, and in conditions of zinc deficiency, ZIP4 is located on the apical plasma membrane. The determinants for its polarized sorting remain to be elucidated.

If ZIP4 is degraded in the lysosome (or proteasome), then ubiquitination of the protein would play a role in its trafficking. Indeed, the protein is ubiquitinated and this is dependent on the His-rich, putative zinc-binding region in L2 (HSSHSHGGHSH, from amino acids 438-448, see Fig. 2), leading Mao *et al.* to suggest that this region senses zinc levels and controls degradation via ubiquitination.[35] Interestingly though, the deletion of the His-rich region in L2 did not affect zinc-stimulated endocytosis suggesting that this mechanism requires a different sensing system.

Mutations in ZIP4 are known to cause acrodermatitis enteropathica (AE), a disease of zinc deficiency caused by the inability to take up dietary zinc. Of the known point mutations in ZIP4, several cause the protein to be retained in the ER after synthesis.[39] However, the P200L and G539R mutant murine proteins, when expressed in CHO cells were able to reach the cell surface, but their endocytosis was not stimulated by zinc. The same proteins in HEK293 cells showed an inhibition of zinc uptake. Neither P200 nor G539 are within the L2 loop of ZIP4: P200 is in the extracellular N-terminus and G539 within TM5. Thus it seems unlikely that these residues are part of endocytosis signals *per se*. However, these results suggest that transporter activity is linked to zinc sensing and ZIP4 endocytosis. Many other point mutations leading to AE have been identified[40], with many mutations in the N-terminal domain and others in the TM domains, but none in L2 or in fact any other predicted intracellular loop. Interestingly, a recent structure of the N-terminal domain of ZIP4 suggests that the protein may form dimers, as may other LIV-1 subfamily members.[41] Dimerization may have implications for the trafficking of these proteins, as it may mask a trafficking signal or create a signal that is not present in the monomer.

ZIP5
Another LIV-1 family member, ZIP5 has a similar tissue expression pattern to ZIP4.[32,42] However, in contrast to

ZIP4, ZIP5 is sorted to the basolateral surface of polarized cells. Cell surface levels of murine ZIP5-HA exogenously expressed in HEK293 cells were insensitive to zinc depletion, also in contrast to mZIP4 expressed under the same conditions.[42] However, in mice fed a zinc-deficient diet, endogenous ZIP5 was removed from the basolateral plasma membrane of enterocytes, endoderm cells, and pancreas.[32,43] Thus, under conditions of zinc deficiency, ZIP4 endocytosis from the apical surface is inhibited while ZIP5 endocytosis from the basolateral surface of the same cells is not. Little is know about the intracellular itinerary of ZIP5, but when expressed in neuroblastoma cells, ZIP5 is seen on the cell surface and on Rab5 positive endosomes.[44] This suggests that, like other cell surface ZIP proteins, ZIP5 continuously cycles on and off the cell surface via endosomes. Analysis of the predicted L2 loop of ZIP5 does not reveal any known trafficking motifs (Fig. 2), and the mechanisms of ZIP5 trafficking remain unclear. However, a recent study suggests that ZIP5 mutations lead to high myopia, with one identified mutation in the L2 loop (R319T; Fig. 2).[45] It is not yet known what effect this mutation has on the localization or trafficking of the protein, although it is not within a known endocytosis motif. Interestingly, the ZIP5, 6 and 10 sub-branch of the LIV-1 family are thought to share a common ancestral gene with the prion protein.[46]

ZIP6 and 10
ZIP6 and 10 can form a functional heteromer, and are located at the cell surface in a variety of cell types.[47,48] The ZIP6/ ZIP10 heteromer is particularly important in the epithelial to mesenchymal transition during development. The L2 loop of ZIP6 is particularly H-rich and contains both a [DE]XXXL[LI] consensus (QSDDLI in ZIP6) and a YXXØ consensus adaptor-binding motif (YHHI in ZIP6), both of which are conserved amongst mammalian ZIP6 sequences. The first of these has the initial acidic residue substituted for Q, as is seen in other proteins such as CD4 (this has a QIKRLL motif that binds the σ2 and α subunits of AP2[23]). However, CD4 requires a phospho-S residue preceding the Q to achieve a negative charge for binding. There is no S in this position in ZIP6, but a S (within a consensus casein kinase II site) follows the Q along with two D residues and these may provide the negative charge. The YXXØ motif follows shortly after the dileucine in the protein sequence and is a potential binding site for the µ subunit of AP2. Whether either, or possibly both, of these signals act in ZIP6/ ZIP10 endocytosis will require further study.

ZIP7
ZIP7 does not appear to localize to the cell surface and is instead on ER membranes[49,50] and/ or Golgi membranes.[28,51,52] The ER localization of ZIP7 does not alter in response to zinc.[53] A role for ZIP7 in ER and Golgi function is supported by studies of the *D. melanogaster* ZIP7 orthologue *Catsup*, which is involved in the trafficking of membrane proteins within the secretory pathway.[54] The *S. cerevisiae* orthologue of ZIP7, Yke4p, is localized to the ER, but unlike mammalian ZIP proteins, it is proposed to transport zinc in either direction across the membrane dependent on cellular ion concentrations.[52] The mouse ZIP7 gene can suppress the zinc-sensitive phenotype of yeast with the *YKE4* gene deleted, suggesting that the function of the transporter is conserved from yeast to mammals.[52]

Nothing is currently known about the trafficking or localization signals within ZIP7.

ZIP13

In addition to ZIP7, another LIV-1 protein also localizes to an intracellular location: ZIP13 is found mainly in the Golgi, where epitope-tagged ZIP13 and endogenous protein colocalize with the Golgi marker GM130.[55,56] Like ZIP7, its localization does not appear to alter in response to zinc and both ZIP7 and 13 form homodimers.[55,56] ZIP13 is highly expressed in connective tissue, and mutations in ZIP13 cause spondylocheirodysplastic Ehlers-Danlos syndrome, a disease affecting connective tissue, bone and teeth.[6] The mutations identified lead to instability of the protein (via rapid proteasomal degradation).[6] Studies of the *D. melanogaster* orthologue of ZIP13 (dZIP13) suggest that dZIP13 may transport essential iron into (rather than out of) ER and Golgi compartments, in order to provide iron for iron-requiring enzymes.[57] The mechanisms for trafficking ZIP13 to ER and Golgi are presently unknown.

ZIP12

ZIP12 is highly expressed in brain tissues, and like many other ZIP proteins, is found on the plasma membrane.[58] Similar to ZIP1, ZIP3 and ZIP4, plasma membrane levels of ZIP12 are increased in conditions of zinc deficiency (Fig. 3). ZIP12 does not contain any recognized targeting motifs within its L2 loop (Fig. 2).

ZIP8 and 14

The remaining two LIV-1 proteins, ZIP8 and ZIP14 are 50% identical at the amino acid level and have a broader metal ion substrate range than other ZIPs (ZIP14 transports zinc, iron, manganese and cadmium; ZIP8 zinc, manganese and cadmium), which may possibly be attributed to the metalloprotease motif in predicted TM5 being altered from HEXPHEXGD to EEXPHEXGD.[59] Both ZIP8 and ZIP14 are ubiquitously-expressed, but highest levels of ZIP8 are expressed in lung, and ZIP14 in liver.[12,59] Mutations in ZIP8 and ZIP14 cause disease: congenital disorder of glycosylation type II (CDGII) and Leigh disease in the case of ZIP8 mutations, and childhood-onset parkinsonism dystonia for ZIP14 mutations.[7,60-62] A ZIP8 single nucleotide polymorphism (A391T) has also been linked to schizophrenia.[63,64] Information regarding these diseases suggests that both ZIP8 and ZIP14 play essential roles in maintaining manganese homeostasis, and may be primarily manganese transporters. Importantly, the clinical symptoms of patients with either CDGII or parkinsonism dystonia linked to ZIP8 and ZIP14, respectively, can be improved significantly by manganese chelation therapy. This does not preclude the possibility that ZIP8 and ZIP14 may transport other ions, in different cell types or under different conditions. Indeed, many studies have shown the importance of ZIP14 in iron and zinc uptake into cells.[65-68]

ZIP8

Little is known about ZIP8 trafficking, but it has been localized to the plasma membrane and intracellular vesicles including early endosomes and lysosomes in transfected cells, and in polarized cells to the apical surface.[12,69-74] Like ZIP1, 3 and 4, ZIP8 localization at the cell surface can be regulated by zinc: zinc depletion resulted in higher levels of ZIP8 on the apical cell surface of MDCK cells,[75] see Fig. 3. In addition, ZIP8 cell surface levels are increased under conditions of iron overload,[12] similarly to ZIP14, see below. No linear endocytosis motifs are present within the L2 loop of ZIP8, but a [phospho-Y]ANP sequence is present (Fig. 2), for possible interaction with the SH2 domains of Src-family kinases. This may be significant as Src stimulates receptor endocytosis by phosphorylating dynamin 2, cortactin and Eps8.[76,77] Zinc also induces Src-dependent activation of epidermal growth factor receptor signaling in some cell types.[78]

Exogenously expressed ZIP8-dsRed has also been shown to localize to mitochondria, in the human lung BEAS-2B cell line.[70] Taken together with recent studies showing that mutation of ZIP8 causes Leigh disease (a disease of mitochondrial function), it seems possible that endogenous ZIP8 may also be localized at least in part to mitochondria. The mechanisms that may traffic the protein to the cell surface/ endosomes/ lysosomes and to mitochondria are unclear, but make an interesting case for further study. Targeting of proteins into the ER, in this case into the ER membrane, for further trafficking to the cell surface and other intracellular compartments of the biosynthetic and endocytic pathways typically requires a hydrophobic region or "signal sequence" at the N-terminus. This is bound by the signal recognition particle (SRP) as the nascent chain emerges from the ribosome and directs translocation of the protein into the ER membrane. Targeting to the mitochondrion requires a mitochondrial targeting sequence (MTS), which is usually an N-terminal amphipathic alpha helix. The signal sequence and MTS are considered highly specific for ER and mitochondrial-targeting, respectively. However, in cases where SRP is deleted in yeast, some proteins targeted normally to the ER are instead found in the mitochondria.[79] This suggests some functional similarity in the ER signal sequences and MTS, but does not explain the ability of a protein such as ZIP8 to be targeted to both locations. Also of note is the situation of the yeast fumarate reductase Osm1p, which is in fact targeted to both ER and mitochondria.[80] In this case, the *OSM1* encodes two in-frame start codons, encoding either a protein with an ER signal sequence or a protein with an MTS, explaining the dual localization. Several isoforms of human ZIP8 can be found in protein sequence databases, and one of these, ZIP8 isoform c has a truncated N-terminus. Also, ZIP8 isoforms a and b have a second methionine residue at position 55, possibly constituting a second translation start site (M^{55}). However, while isoforms a and b have predicted ER signal sequences in their full-length versions, neither isoform c nor a possible isoform a/b with the M^{55} start site uncovers a typical MTS sequence. The localization of ZIP8 to mitochondria requires further study, as it is perhaps more likely that ZIP8 mediates manganese transport across the plasma membrane to provide manganese ions for mitochondrial functions.[60]

In addition to its mitochondrial role, ZIP8 is required for protein glycosylation in the Golgi, by providing the manganese required for the function of β 1, 4-galactosyltransferase.[60-62] This is proposed to occur via ZIP8 taking up manganese at the cell surface providing it, via the cytosol, to the Golgi.[60,74] In this way, ZIP8 is essential for the glycosylation and trafficking of other proteins in the Golgi complex.

ZIP14

ZIP14 is expressed on the cell surface and in intracellular compartments.[81,82] Endogenous, tagged,

intracellular ZIP14 partially colocalizes with the transferrin receptor, EEA1 and LAMP1 (recycling endosomes, sorting endosomes and lysosomes, respectively) in HepG2 liver cells.[83] Like ZIP8 and ZIP4, ZIP14 is targeted to the apical surface of polarized cells.[8] ZIP14 cell surface levels are decreased under conditions of iron-depletion and the ZIP14 protein is degraded.[84] Internalisation from the cell surface under these conditions is believed to occur via endocytosis, as it was inhibited by Dynasore. Dynasore is a small molecular inhibitor of the GTPase dynamin, which is required for the pinching off of endocytic vesicles from the plasma membrane.[85] Dynasore also disrupts lipid raft organization in a dynamin-independent manner, so the specific role of dynamin in ZIP14 endocytosis remains unclear.[86] ZIP14 contains one YXXØ endocytosis motif in the L2 loop (Fig. 2), though it is very close to predicted transmembrane domain 4 and may not be accessible as an AP2 binding site. Further study will be required to determine whether this motif does promote endocytosis and if not, what does. Interestingly, use of proteasomal and lysosomal inhibitors suggested that ZIP14 is degraded in a proteasome-dependent manner and is not degraded in lysosomes. The degradation of ZIP14 relies on de-glycosylation of the protein in the N-terminal domain, and this de-glycosylation (rather than any sequence in L2) determines the iron sensitivity of ZIP14 trafficking.[84] The degradation of ZIP14 is also dependent on ubiquitination, and while the hypothesis that ZIP14 is degraded in the proteasome remains based on the use of inhibitors, a more classical lysosomal degradation pathway cannot be ruled out. If the proteasomal degradation of ZIP14 stands up to further testing, this would constitute a novel degradation pathway for a multiple-spanning membrane protein Such a pathway would require membrane extraction at some point in the endocytic pathway, involving a membrane protease.[87] The two contrasting models for ZIP14 degradation are shown in Fig. 4.

GufA subfamily

ZIP11 is the only human member of the gufA ZIP subfamily. The murine protein is expressed highly in stomach, other digestive tissues and testis.[88] In contrast to many other ZIP proteins, zinc depletion causes the downregulation of ZIP11 protein expression in the stomach.[88,89] ZIP11 is thought to localize to the nucleus, intracellular vesicles and the plasma membrane of mouse stomach and colon.[89] Another report localizes ZIP11 to the Golgi in mouse mammary epithelial cells.[28] How the protein may traffic to these various locations remains unclear, although an endocytosis motif of the [DE]XXXL[LI] type is seen within the predicted L2 loop of the human sequence (Fig. 2). However, the EGPALL sequence is not conserved in ZIP11 sequences from other species suggesting that perhaps this sequence may not be important for trafficking. Also in the gufA subfamily and therefore related to ZIP11 is *S. cerevisiae* Zrt3p. Zrt3p mediates efflux of zinc from the yeast vacuole (the equivalent of the lysosome) to the cytosol. Zrt3p is localized to the vacuole membrane, and protein expression is upregulated in response to zinc depletion.[90,91] Zrt3p is degraded in response to excess zinc, by a mechanism that requires polyubiquitination involving the ubiquitin ligases Rsp5p and Tul1p, and the ESCRTs.[91]

Conclusions and future perspectives

Most of the ZIP proteins are found at the cell surface at least some of the time (with the exception of ZIP7 and ZIP13). For those ZIPs that have been studied in some detail, it appears that endocytosis is a key feature of their trafficking from the surface and that endocytosis rates are modulated in response to ion levels. Thus, rates of endocytosis are key to regulating the cell surface levels of many of these transporters. In addition to this, cell surface levels will be regulated by the rate of return to the cell surface (recycling) from endosomes and/ or Golgi compartments and the rate of degradation (see Fig. 2). It will require a detailed analysis of the kinetics of endocytosis, recycling and degradation to fully understand the regulation of individual ZIP proteins under different conditions.

Regarding the mechanism of post-ER trafficking of ZIP proteins, signals found in one protein (e.g. the dileucine signal in ZIP1) are not conserved amongst other members of the family and so it seems likely that the signals and mechanisms are unique for each protein. This perhaps reflects the varying expression patterns and functions of the ZIP proteins. The situation is further complicated in polarized cells, where some ZIP proteins have been shown to traffic to the apical surface and some to the basolateral, sometimes in the same cells.

Whilst the signals and mechanisms may be different, we can learn some lessons from the current data. It seems likely that, as is already known for ZIP1, trafficking signals that bind cytosolic factors (such as endocytic adaptor proteins) may reside in the cytosolic L2 loop between TM3 and TM4, or in other cytosolic regions of the protein. Similarly, as is the case for ZIP14, posttranslational modifications such as glycosylation of intralumenal residues and ubiquitination of cytosolic residues may influence trafficking.[84] While many of the ZIP mutations identified in human disease result in an accumulation of the mutant protein in the ER (presumably due to protein instability, leading to retrotranslocation and degradation), and others have mutations in ion translocation, some mutations may affect protein trafficking and thus the ability for the transporter to be in the right place under particular conditions. Finally, evidence is emerging that ZIP proteins themselves (e.g. ZIP7, ZIP9, ZIP13) may be important in the regulation of protein trafficking, by controlling the levels of ions within intracellular organelles.[5]

Acknowledgements

Figure legends

Figure 1. Predicted topology of ZIP family metal ion transporter proteins. ZIP proteins are believed to have 8 transmembrane domains (TM, labeled 1-8) and N- and C-termini that are extracellular or within the lumen of intracellular organelles. The variable loop region, L2, between TM3 and TM4 contains a H-rich region in many ZIP proteins. The metalloprotease motif found in the LIV-1 subfamily (HEXPHEXGD, with the first H changed to E in ZIP8 and ZIP14) is shown. ZIP family proteins have regions in TM4 and TM5 that are partially amphipathic and contain conserved H

residues. This may constitute an aqueous channel through the membrane with a heavy metal binding site.[92,93]

Fig. 2. L2 loops of cell surface ZIP proteins. The protein sequences of the proposed cytosolic loops between TM3 and TM4 (L2) are shown for ZIP proteins thought to be at the cell surface at least some of the time. These sequences bear little similarity to each other. Histidine residues that are thought to be a key feature of these sequences are highlighted in grey. The histidine-rich motifs of ZIP1 and 4 believed to be important in zinc binding is shown in green. The characterized dileucine-based endocytosis signal in ZIP1 is show in blue. Possible further endocytosis signals that fit the same consensus sequence ([DE]XXXL[LI]) are shown in yellow. The acidic residue [DE] may be replaced by Q e.g. in ZIP6, as discussed in the text. Predicted tyrosine-based endocytosis signals (YXXØ) are highlighted in pink. The potential SH2-binding sequence for Src-family kinases in ZIP8 is highlighted in brown. The R319 amino acid that is mutated to T in high myopia is shown in orange.[45] Linear motifs were predicted using the Eukaryotic Linear Motif Resource,[94] and the probability of tyrosine phosphorylation (in relation to predicted SH2-binding motifs) using NetPhos3.1.[95] The L2 loop sequences are based on protein topology predictions.[24,33,42,55,59,89,96,97] The L2 region of ZIP9 was predicted using TMpred.

Fig. 3. Model for the trafficking of ZIP proteins in response to zinc ions. a) ZIPs 1, 3, 4, 8 and 12 (ZIP on the diagram) are transported after synthesis from the ER, though the Golgi and to the cell surface. They are predominantly seen on the cell surface and in intracellular compartments, where their steady state localization is maintained by a balance of endocytosis, recycling and degradation. b) Under conditions of zinc-depletion, ZIP endocytosis is inhibited. This allows the ZIP cell surface levels to increase rapidly. It should be noted that ZIP recycling from endosomes to the cell surface has not been measured. ZIP degradation is likely to be dependent on ubiquitination and involve ESCRT-dependent sorting at multivesicular bodies (MVBs) for subsequent degradation in lysosomes. Trafficking processes that are either known, or predicted, to be inhibited during zinc depletion are shown in dashed lines with crosses through them. Other possible trafficking routes for ZIPs are shown in grey. EE, early endosome. RE, recycling endosome.

Fig. 4. Two contrasting models for the trafficking of ZIP14. ZIP14 is endocytosed from the plasma membrane via a mechanism likely to involve clathrin-mediated endocytosis. ZIP14 is seen in recycling endosomes, where it colocalises with the transferrin receptor. Therefore, under conditions of iron overload we suggest the predominant pathway is recycling via the recycling endosome to the cell surface to maintain cell surface levels of the protein. Recycling of ZIP14 to the cell surface has not been measured and may be inhibited under conditions of iron depletion. **Model a)** Upon iron depletion, it has been suggested that ZIP14 is endocytosed (possibly at a higher rate) and degraded via proteasomes, a mechanism involving extraction of the protein from the membrane at some point in the endocytic pathway.[84] This mechanism involves ubiquitination of the protein. **Model b)** A second model for ZIP14 degradation on iron depletion involves transport of the protein to multivesicular bodies, where

it is sorted onto the inner membranes in an ESCRT-dependent manner. This sorting would involve the recognition of ubiquitin by ESCRT components and the degradation of ZIP14 will be ubiquitination-dependent. In this model, ZIP14 will be degraded in lysosomes. EE, early endosome. RE, recycling endosome.

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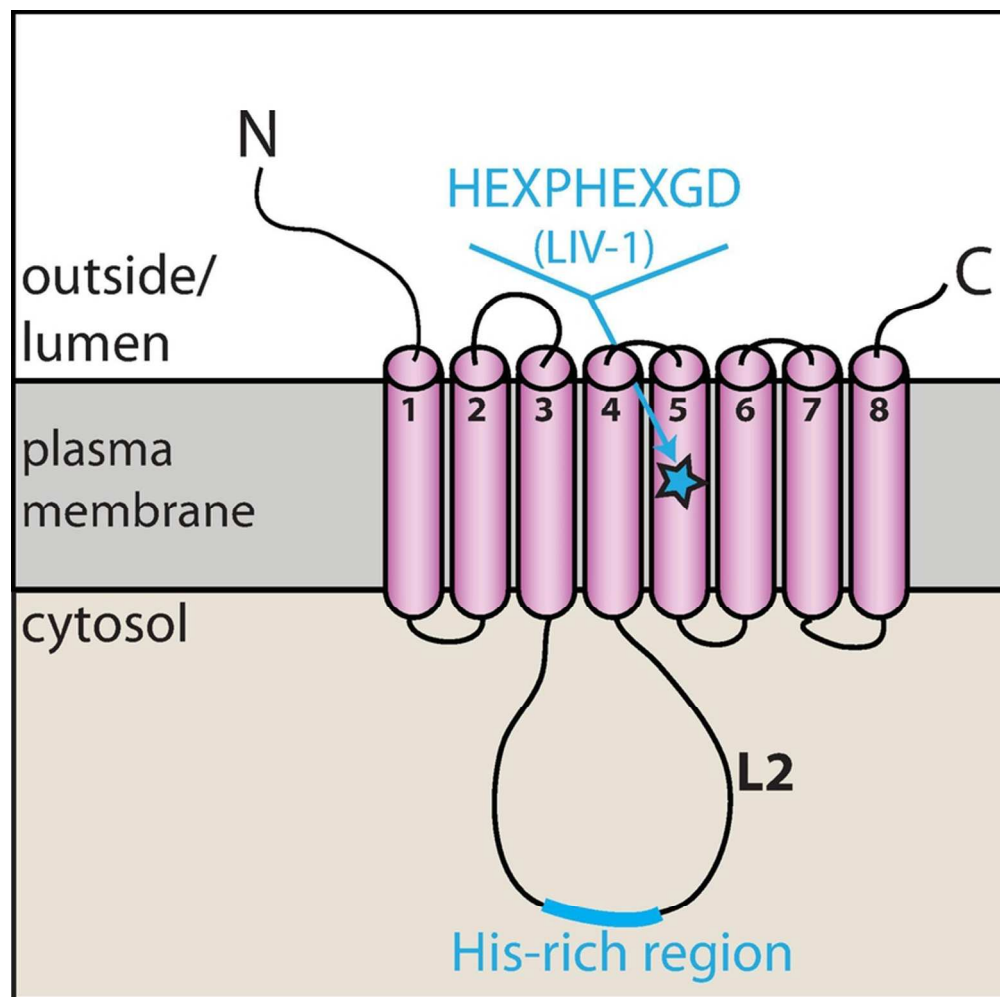
Figure 1. Predicted topology of ZIP family metal ion transporter proteins. ZIP proteins are believed to have 8 transmembrane domains (TM, labeled 1-8) and N- and C-termini that are extracellular or within the lumen of intracellular organelles. The variable loop region, L2, between TM3 and TM4 contains a H-rich region in many ZIP proteins. The metalloprotease motif found in the LIV-1 subfamily (HEXPHEXGD, with the first H changed to E in ZIP8 and ZIP14) is shown. ZIP family proteins have regions in TM4 and TM5 that are partially amphipathic and contain conserved H residues. This may constitute an aqueous channel through the membrane with a heavy metal binding site.[92,93]

```
ZIP1   ---YKEQSGPSPLEETRALLGTVNGGPQHWHDGPGVPQASGAPATPSALR---
ZIP2   ---CPGAAGGSTVQDEEWGGAHIFELHSHGHLPSPSKGPLR---
ZIP3   ---KEKPSFIDLETFNAGSDVGSDSEYESPFMGGARGHALYVEPHGHGPSLSVQGLSRASPVRL---
ZIP4   ---LPRDPEDLEDGPCGHSSHSHGGHSHGVSLQLAPSELRQPKPPHEGSRADLVAEESPELLNPEPRRLSPELRLLPY---
ZIP5   ---LRHRGLRPRCCRRKRRNLETRNLDPENGSGMALQPLQAAPEPGAQGQREKNSQHPPALAPPGHQGHSHGHQGGTD---
ZIP6   ---KQFKDKKKKNQKKPENDDDVEIKKQLSKYESQLSTNEEKVDTDDRTEGYLRADSQEPSHFDSQQPAVLEEEEVMIAHAH
          PQEVYNEYVPRGCKNKCHSHFHDTLGQSDDLIHHHHDYHHILHHHHHQNHHPHSHSQRYSREELKDAGVAT---
ZIP8   ---KTYGQNGHTHFGNDNFGPQEKTHQPKALPAINGVTCYANPAVTEANGHIHFDNVSVVSLQDGKKEPSSCTCLKGPKLSEIGT---
ZIP9   ---DQIGNSHVHSTDDPEAARSSNSKITTTL---
ZIP10  ---HYKQQRGKQKWFMKQNTEESTIGRKLSDHKLNNTPDSDWLQLKPLAGTDDSVVSEDRLNETELTDLEGQQESPPKNYLCIE
          EEKIIDHSHSDGLHTIHEHDLHAAAHNHHGENKTVLRKHNHQWHHKHSHHSHGPCHSGSDLKETGIANIA--
ZIP11  ---FVYLADLLMPHLGAAEDPQTTLALNFGSTLMKKKSDPEGPALLFPESELSIRIGRAGLLSDKSENGEAYQRKKAAATGLPE
          GPAVPVPSRGNLAQPGGSSWRR
ZIP12  ---SPNDKQGLSLVNGHVGHSHHLALNSELSDQAGRGKSASTIQLKSPEDSQAAEMPIGSMTASNRKCKAISLLA---
ZIP14  ---KQKNEHHHGHSHYASESLPSKKDQEEGVMEKLQNGDLDHMIPQHCSSELDGKAPMVDEKVIVGSLSVQDLQASQSACYWLKGVRYSDIGT
```

Fig. 2. L2 loops of cell surface ZIP proteins. The protein sequences of the proposed cytosolic loops between TM3 and TM4 (L2) are shown for ZIP proteins thought to be at the cell surface at least some of the time. These sequences bear little similarity to each other. Histidine residues that are thought to be a key feature of these sequences are highlighted in grey. The histidine-rich motifs of ZIP1 and 4 believed to be important in zinc binding is shown in green. The characterized dileucine-based endocytosis signal in ZIP1 is show in blue. Possible further endocytosis signals that fit the same consensus sequence ([DE]XXXL[LI]) are shown in yellow. The acidic residue [DE] may be replaced by Q e.g. in ZIP6, as discussed in the text. Predicted tyrosine-based endocytosis signals (YXXØ) are highlighted in pink. The potential SH2-binding sequence for Src-family kinases in ZIP8 is highlighted in brown. The R319 amino acid that is mutated to T in high myopia is shown in orange.45 Linear motifs were predicted using the Eukaryotic Linear Motif Resource,94 and the probability of tyrosine phosphorylation (in relation to predicted SH2-binding motifs) using NetPhos3.1.95 The L2 loop sequences are based on protein topology predictions.24,33,42,55,59,89,96,97 The L2 region of ZIP9 was predicted using TMpred.
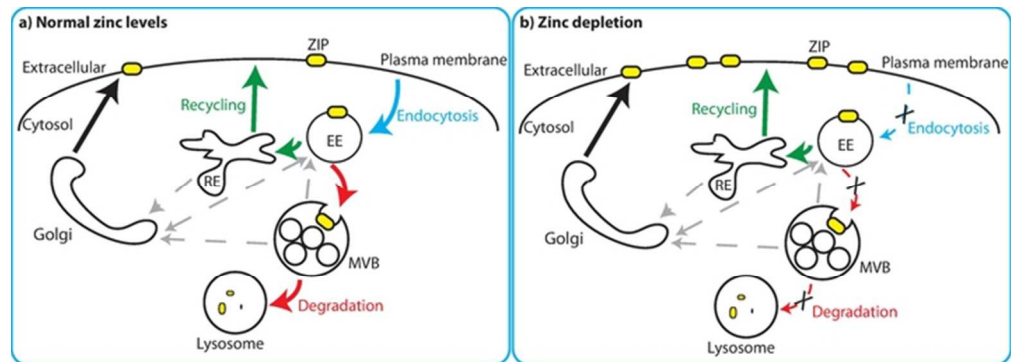
Fig. 3. Model for the trafficking of ZIP proteins in response to zinc ions. a) ZIPs 1, 3, 4, 8 and 12 (ZIP on the diagram) are transported after synthesis from the ER, though the Golgi and to the cell surface. They are predominantly seen on the cell surface and in intracellular compartments, where their steady state localization is maintained by a balance of endocytosis, recycling and degradation. b) Under conditions of zinc-depletion, ZIP endocytosis is inhibited. This allows the ZIP cell surface levels to increase rapidly. It should be noted that ZIP recycling from endosomes to the cell surface has not been measured. ZIP degradation is likely to be dependent on ubiquitination and involve ESCRT-dependent sorting at multivesicular bodies (MVBs) for subsequent degradation in lysosomes. Trafficking processes that are either known, or predicted, to be inhibited during zinc depletion are shown in dashed lines with crosses through them. Other possible trafficking routes for ZIPs are shown in grey. EE, early endosome. RE, recycling endosome.
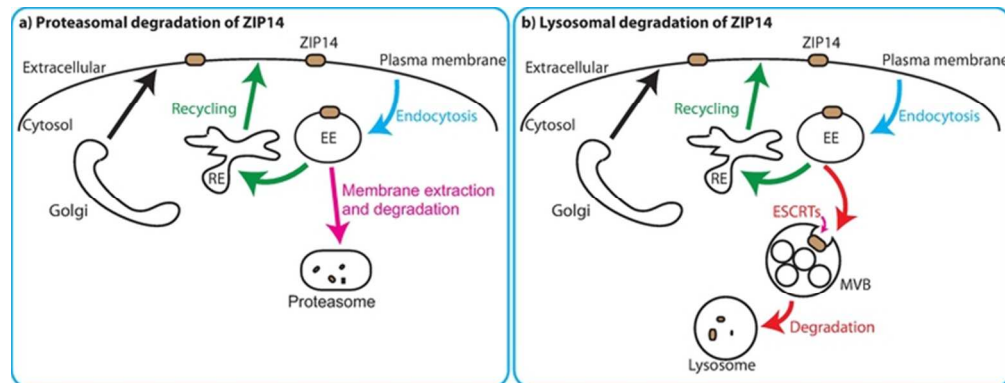
Fig. 4. Two contrasting models for the trafficking of ZIP14. ZIP14 is endocytosed from the plasma membrane via a mechanism likely to involve clathrin-mediated endocytosis. ZIP14 is seen in recycling endosomes, where it colocalises with the transferrin receptor. Therefore, under conditions of iron overload we suggest the predominant pathway is recycling via the recycling endosome to the cell surface to maintain cell surface levels of the protein. Recycling of ZIP14 to the cell surface has not been measured and may be inhibited under conditions of iron depletion. Model a) Upon iron depletion, it has been suggested that ZIP14 is endocytosed (possibly at a higher rate) and degraded via proteasomes, a mechanism involving extraction of the protein from the membrane at some point in the endocytic pathway.84 This mechanism involves ubiquitination of the protein. Model b) A second model for ZIP14 degradation on iron depletion involves transport of the protein to multivesicular bodies, where it is sorted onto the inner membranes in an ESCRT-dependent manner. This sorting would involve the recognition of ubiquitin by ESCRT components and the degradation of ZIP14 will be ubiquitination-dependent. In this model, ZIP14 will be degraded in lysosomes. EE, early endosome. RE, recycling endosome.