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Review

Overview of mammalian zinc transporters

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Abstract. In recent years, a number of mammalian zinc transporters have been identified, and candidate genes are rapidly growing. These transporters are classified into two families: ZIP (ZRT, IRT-like protein) and CDF (cation diffusion facilitator). ZIP members facilitate zinc influx into the cytosol, while CDF members facilitate its efflux from the cytosol. Molecular characterization of the transporters has brought about major advances in our understanding of their physiological functions. Zinc metabolism is regulated primarily through zinc-dependent control of transcription, translation, and intracellular trafficking of transporters. Analyses of mice whose zinc transporter genes have been genetically disrupted and of the naturally occurring mutant mice with symptoms related to abnormal zinc metabolism have provided compelling evidence that some zinc transporters play critical roles in zinc homeostasis. In this review, we review the literature of mammalian zinc transporters with emphasis on very recent findings and elicit integrative knowledge of zinc homeostasis.

Key words. Mammalian zinc transporter; ZIP; uptake; CDF; ZnT; efflux; zinc homeostasis.

Introduction

Zinc is a trace nutrient indispensable for life. More than 300 metalloenzymes of six major functional classes require zinc as a key structural component or as a cofactor [1–3]. Moreover, zinc is an element requisite for zinc-containing DNA binding proteins. Human genes encoding C2H2 zinc-finger proteins amount to more than 1% of the total [4]. Zinc can potentially modulate cellular signal recognition, second messenger metabolism and the function of protein kinase and protein phosphatase [5]. Consequently, the disturbance of zinc homeostasis causes a variety of severe detrimental effects on animals, including humans. For example, a deficiency of zinc impairs growth, immune activity and brain functions [1, 6]. On the other hand, excess zinc can be toxic to cells [7]. The toxic mechanism is not clear, but the toxicity is probably due to the binding of zinc to metalloproteins that require other metals for their biological activities. Therefore, sophisticated regulatory systems must exist to maintain zinc homeostasis.

In animals, zinc balance is primarily maintained through a regulated rate of intestinal uptake, fecal elimination of excess zinc, renal reabsorption and distribution to cells, including intracellular storage [8]. Most, if not all, of these zinc movement pathways are mediated by membrane proteins, zinc transporters, as described later. Dietary zinc is incorporated into the intestinal epithelium from its apical side and transferred through an unknown intracellular pathway to the basolateral side, from where zinc is released into the portal circulation. Excess zinc in the diet together with the endogenous zinc derived from the pancreatic exocrine and biliary secretions and from the sloughed mucosal cells is excreted into the feces

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[1, 8]. In nonpathological conditions, urinary loss of zinc is very low, indicative of extensive reabsorption of zinc along kidney proximal tubules [9]. Most of the circulating zinc binds with albumin and α2-macroglobulin [10]. Once the circulating zinc is taken up across the plasma membrane, it is thought to be available for four intracellular pools (fig. 1). First, zinc binds tightly to metalloproteins as a structural component or a cofactor [3]. Second, metallothioneins (MTs) capable of binding to zinc with a low affinity may provide an important labile pool that has been thought to be a reservoir and buffer of cytosolic zinc [11]. Third, zinc is compartmentalized into intracellular organelles to supply it to zinc-dependent proteins in situ. Sequestration of zinc in the organelles (particularly endosomes/lysosomes) may also contribute to zinc storage and/or detoxification. Such compartmentalization is found not only in conventional organelles such as mitochondria, the endoplasmic reticulum (ER), the Golgi apparatus and endosomes/lysosomes, but also in specialized organelles, such as synaptic vesicles and secretory granules [12–14]. As described later, zinc in these specialized organelles may play an important role in the unique biological processes, but its roles are poorly understood. Finally, the concentration of cytosolic free zinc is estimated to be well below a nanomolar level [15].

The transporter-mediated movement of zinc has been suggested by a number of classical kinetic studies of zinc transfer across the membrane, because its rate is time- and temperature-dependent and saturable [10, 16]. In this decade, a number of zinc transporters have been cloned from a variety of organisms, and the number of zinc-transporter candidates found from the sequence homology is rapidly growing. Some zinc transporters have been investigated at molecular levels, and there has been an increasing body of evidence demonstrating that functional analyses of zinc transporters, including their gene expression, are very important for understanding the mechanism by which zinc homeostasis is achieved. Zinc transporters are largely assigned to two metal-transporter families: ZIP (ZRT, IRT-like protein) and CDF (cation diffusion facilitator). ZIP-family transporters function in zinc influx into cytosol from the outside of cells or from the lumen of intracellular compartments, while CDF-family transporters mobilize zinc in the opposite direction; that is, CDF transporters facilitate zinc efflux from cytosol to the outside of cells and transport the cytosolic zinc into intracellular organelles (fig. 1, 2). Some members of both families appear to transport other metals such as iron, copper, nickel, cadmium and cobalt, but the molecular mechanism of broad substrate specificity and its physiological significance are unknown. It is noted that the inhibitory effect of other metals on zinc transport does not necessarily indicate the metal transport, because the inhibition may simply be derived from interference of

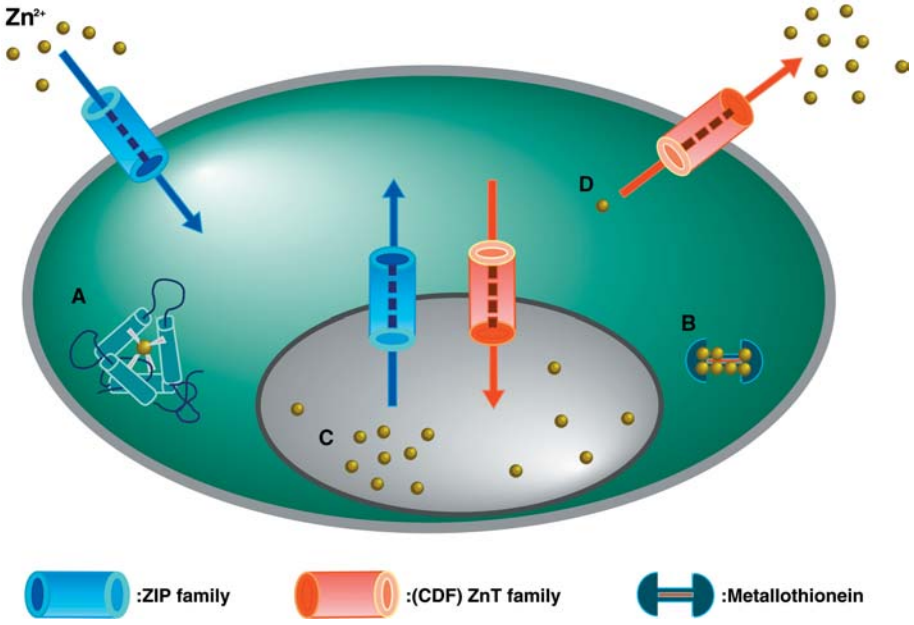

Figure 1. Zinc in mammalian cells exists in four distinct pools. The transport of zinc into or out of cytoplasm is directed by two zinc transporter families, the ZIP family and the CDF family. (*A*) Zinc in cytoplasm is tightly bound to metalloproteins as a structural component or as a cofactor or (*B*) loosely bound to MTs to reserve zinc. (*C*) Zinc is compartmentalized in intracellular organelles. (*D*) The free zinc concentration in the cytosol is estimated to be well below a nanomolar level. The zinc state (bound or free) in the organelles is not known except for zinc in the vesicles in some specialized cells (see ZnT-3 and *ZnT-3–/–* mouse, and ZnT-5 and *ZnT-5–/–* mouse). ZIP members transport zinc into the cytoplasm out of cells or from intracellular compartments, while CDF members move zinc from the cytoplasm into the extracellular space or into intracellular compartments.

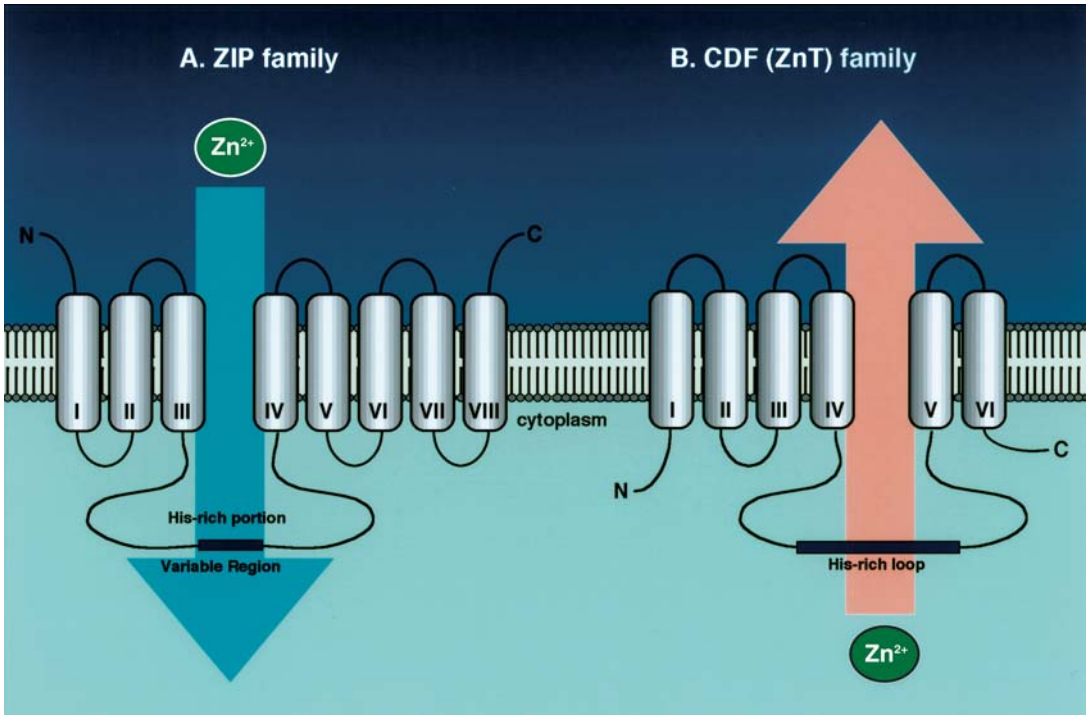

Figure 2. Predicted structures of ZIP and CDF (ZnT) members. (*A*) ZIP members are predicted to have eight transmembrane domains, with the exception of seven transmembrane domains in the LIV-1 subfamily. A variable region between transmembrane domains III and IV is cytoplasmic and contains the His-rich portion. ZIP members transport zinc into the cytoplasm out of cells or from intracellular compartments (blue arrow). (*B*) CDF (ZnT) members are predicted to have six transmembrane domains. As with ZIP members, the His-rich loop is in the cytoplasm. CDF (ZnT) members transport zinc from the cytoplasm into the extracellular space or into intracellular compartments (pink arrow). In both families, the His-rich motif appears to bind the zinc ion, and the transmembrane domains appear to form pores to pass the zinc ion.

the zinc transport process, including the competitive binding of other metals to the zinc binding site. Another metal transporter, DCT1/DMT1/Nramp2, is a member of the Nramp family of transporters and is structurally unrelated to either ZIP or CDF members. Gunshin et al. [17] showed that, in addition to the efficient transport of iron, DCT1/DMT1/Nramp2 has the potential for zinc transport activity, but recent reports have provided counterevidence to this activity [18, 19]. DCT1/DMT1/Nramp2 is responsible for iron uptake in the intestine [19, 20].

Although information on zinc transporters is amply documented, we are still far from a full understanding of the molecular mechanisms that control zinc metabolism. Thus far, many excellent reviews concerning isolation and characterization of ZIP and CDF family members have been published [21–32]. However, there has been rapid progress in mammalian zinc transporters since these reviews. This article focuses on the current knowledge of mammalian zinc transporters. First, we outline their molecular characteristics, including cellular and subcellular localization and control of gene expression, which allows us to delineate their possible physiological functions. Experimental results of zinc transporters that were obtained from other organisms are also included, because such information would be helpful for further studies of mammalian zinc transporters. Second, phenotypes of knockout or mutant mice of some zinc transporters will be described (summarized in table 1). It appears that some of the phenotypes suggest roles of zinc transporters not recognized in the previous studies.

Chelatable zinc in specialized cells

In addition to the biological functions for metalloenzymes and DNA-binding proteins in all types of cells, zinc has a unique feature in that it is accumulated in some specialized cells. This type of zinc is free or weakly bound and therefore detectable by several zinc-staining methods using its chelatable property. For example, chelatable zinc is found in the submandibular gland epithelial/myoepithelial cells [33], sperm cells [34, 35] and pigment epithelial cells in the retina [36]. Moreover, more intimate analyses revealed chelatable zinc in subcellular compartments such as synaptic vesicles in telencephalic neurons [12, 13], secretory granules in pancreatic β cells [14], pituitary secretory cells [37], submandibular gland granular convoluted tubule cells [38] and leukocytes [39], and apical secretory vesicles and lysosome-like structures in the epithelial cells of the lateral prostate [40]. Var-

Table 1. Phenotypes of genetically disrupted or mutant mice and AE patients.

Name	Type	Phenotype	Reference
ZnT-1	KO	embryonic lethal	[27, 99]
ZnT-3	KO	prone to seizure elicited by kainic acid treatment	[129]
ZnT-4	*lm*	production of zinc-deficient milk; zinc deficiency after 8 months of age	[137, 145]
ZnT-5	KO	poor growth; osteopenia; weak muscle; male-specific cardiac sudden death; lower fat	[152]
hZIP4	AE	severe zinc deficiency	[65]

KO, *lm* and AE indicate gene-targeting mouse, lethal milk mouse and acrodermatitis enteropathica patients, respectively.

ious physiological functions of chelatable zinc in these compartments have been proposed, but none have been established so far. Zinc transporters responsible for the accumulation of chelatable zinc are also obscure except that ZnT-3 transports zinc into synaptic vesicles [41]. Disruption of the mouse *ZnT-3* gene resulted in the disappearance of chelatable zinc in synaptic vesicles but failed to reveal its biological function (see ZnT-3 and *ZnT-3–/–* mouse).

ZIP transporter family

Characteristics of ZIP members

The ZIP family is named from the first identified members, ZRT1 and ZRT2 of *Saccharomyces cerevisiae* [42, 43] and IRT1 of *Arabidopsis thaliana* [44]. ZIP members widely span from prokaryotic to eukaryotic organisms including archeae, bacteria, fungi, plants and mammals [29]. At this time, 14 members have been found in humans (fig. 3). ZIP members mainly transport zinc, but they can also mobilize iron, manganese and cadmium, suggesting that not all human members necessarily engage in zinc transport alone.

Gaither and Eide recently reported that the ZIP family members can be divided into four subgroups based on their degree of sequence conservation [29]. According to their assignment, human ZIP members are classified to the subfamilies I, II, LIV-1 and gufA. Both hZIP1 and hZIP2, which were first characterized as human ZIP members, belong to subfamily II [29]. Members belonging to subfamilies I and II are predicted to have eight transmembrane domains and a membrane topology in which the N- and C-terminal ends are located outside the plasma membrane [28, 29] (fig. 2 A). Another characteristic of these subfamilies is the ‘variable region’ between transmembrane domain III and IV. This region shows little homology in the amino acid sequence and length among family members. [28, 29]. The variable region is predicted to be in the cytoplasm and generally contains a His-rich portion (for instance, HSHGHL in hZIP2) of potential zinc-binding domain [28, 29] (fig. 2). Immunochemical studies of some members confirmed these predictions of membrane topology; the N-terminus of hZIP1 and the C-terminus of hZIP2 were found in the extracytoplasmic side [45, 46], and the variable region of yeast ZRT1 was cytoplasmic [47]. The most conserved portion among the members is found in transmembrane domains IV and V, both of which are partially amphipathic and contain the conserved His residues [23, 29]. It has been proposed that these domains comprise an aqueous channel through which the zinc ion passes and that the conserved His residues and adjacent (semi)polar residues constitute an intramembranous heavy-metal binding site [23]. Consistent with this proposal, mutations of the conserved His or adjacent (semi)polar residues in transmembrane domains IV and V of IRT1 abolish its transport activity [48].

The LIV-1 subfamily is a newly classified subfamily, and its occurrence is currently restricted to eukaryotes [29, 49]. This subfamily is composed of proteins with sequences homologous to the LIV-1 protein. The function of the LIV-1 protein is as yet unknown, but interestingly, its expression is highly enhanced in metastatic breast cancer tissues [50, 51]. A large number of human ZIP members belong to this subfamily (fig. 3). LIV-1 members have some unique architectural characteristics. For example, they possess a conserved metalloprotease-like sequence (HEXPHEXGD) [49, 52]. This motif is functional in zinc metalloproteases as a catalytic zinc-binding sequence [53], which suggests that LIV-1 members may trap zinc via this motif [49]. Most members of the LIV-1 subfamily have a large N-terminal sequence in comparison to other ZIP subfamily members. The membrane topology of LIV-1 members is controversial. By analogy to other ZIP family members, LIV-1 members are thought to contain eight transmembrane domains [49, 54], but their hydropathy profiles indicate seven domains [52, 55]. It appears that the domain IV in other ZIP members is missing in LIV-1 members [52]. More detailed analysis is necessary to clear up the membrane topology of the LIV-1 subfamily.

The subfamily gufA is a new group that consists of proteins related to the gufA protein of *Myxococcus xanthus*

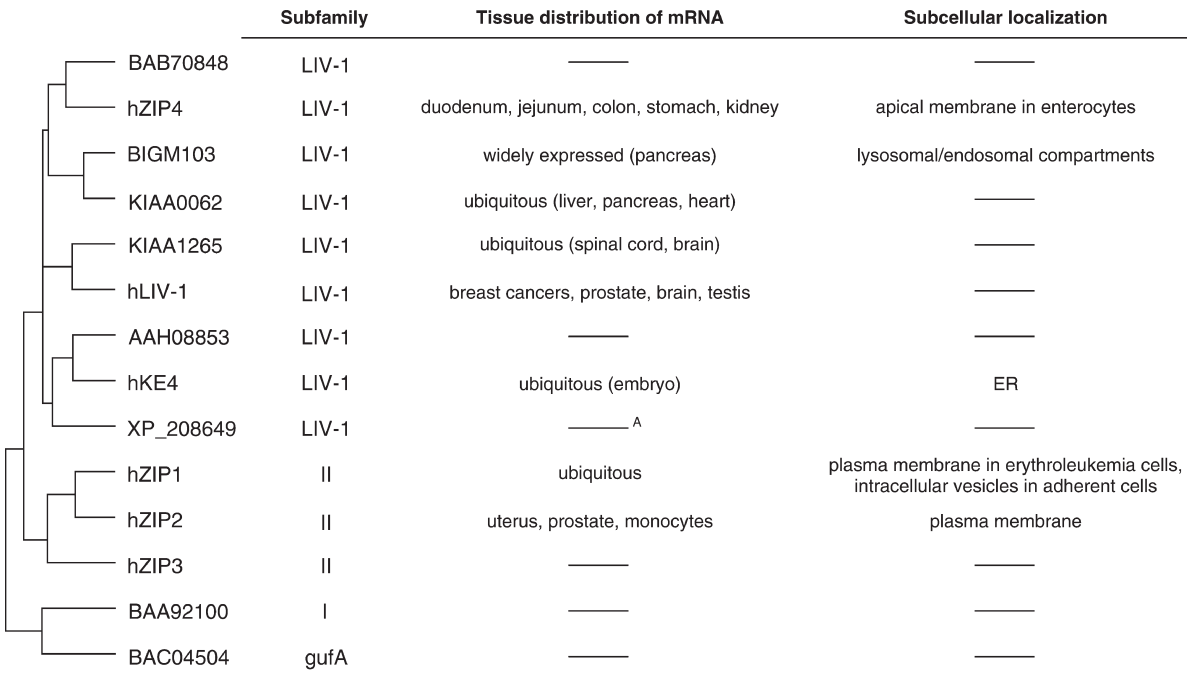

Figure 3. A dendrogram showing the sequence similarity among human ZIP members. The class of subfamily follows the assignment by Gaither and Eide [29]. Tissue distribution of mRNA and subcellular localization of these proteins are also shown. The dendrogram is generated using GENETYX-MAC software. The accession numbers for the sequences used are: NP_570901 for hZIP4, NP_071437 for BIGM103, BAA06685 for KIAA0062, BAA86579 for KIAA1265, NP_036451 for hLIV-1, CAA20238 for hKE4, NP_055252 for hZIP1, AAF35832 for hZIP2 and AAH05869 for hZIP3. The capital letter A indicates primary expression in the colon epithelium and in adenocarcinoma cells (SAGEmap database). The horizontal lines indicate no available information.

[29]. Although the function of the gufA protein is unknown, classification of the yeast zinc transporter ZRT3 into this subfamily suggests that gufA members may have zinc transport activity [29]. A human protein (BAC 04504) recently submitted to the GenBank is classified to the gufA subfamily.

Regulation of the expression of mammalian ZIP members is not well understood. The expression levels of some mammalian ZIP members are regulated by zinc; messenger RNA (mRNA) levels of *hZIP1, 2* and *4* increase in zinc-limited condition, but it is unknown whether the increase results from transcriptional activation or stabilization of mRNA [56–58]. *ZRT1* and *ZRT2* in yeast ZIP members are controlled at the transcription level [42, 59]. In addition, ZRT1, with a high affinity to zinc, but not ZRT2, with a low affinity, is regulated post-translationally. When cells are exposed to high levels of extracellular zinc, ZRT1 is rapidly inactivated through ubiquitin-mediated endocytosis and degraded in vacuoles [47, 60]. This type of regulation may be a cell-protective device that serves in response to extreme zinc excess. Similar post-translational regulations may operate in mammalian ZIP members.

The mechanism of zinc transport of mammalian ZIP members has not been studied extensively. Zinc transport activity of hZIP2 is shown to increase in the presence of HCO_3^- [46], but it is not known whether this holds true for other mammalian members. Neither hZIP1 nor hZIP2 requires ATP for transport activity [45, 46], although some ZIP members of yeast and plants mobilize zinc in an energy-dependent manner [42, 44]. Mammalian ZIP members may have unique properties in their transport activity.

A phylogenetic tree of hZIP members, including those yet to be characterized, is shown in figure 3. It also includes their tissue distribution and subcellular localization. Further features of each ZIP member are described below.

hZIP1

hZIP1 was identified as a transporter that can take up zinc into human erythroleukemia K562 cells [45]. Exogenously expressed hZIP1-mediated zinc uptake is concentration- and time-dependent, and saturable. The uptake was not reduced by the inhibitors of ATP production [45]. Some transition metals, such as nickel, copper, iron and cadmium, inhibit the zinc uptake activity of hZIP1. The results obtained by iron uptake studies, however, showed that iron was not transported by hZIP1 [45]. Interestingly, subcellular localization of hZIP1 varies depending on cell type. The tagged hZIP1 protein is localized to the plasma membrane of erythroleukemia cells, while in adherent cell lines it was found in the vesicular compartment and also partly in the ER [61]. Localization to intracellular or-

ganelles suggests that this transporter may also function in the mobilization of stored zinc into cytoplasm. The similar directional transfer of zinc has been shown in yeast ZIP member; ZRT3 mobilizes zinc from the lumen of vacuoles into the cytoplasm [62].

hZIP1 (mRNA) is ubiquitously expressed [45, 63], suggesting that the hZIP1 protein plays a housekeeping function in many types of cells. Expression of the mouse homologue is developmentally regulated and is detected in the relatively late developmental stage when most cell types are undergoing terminal differentiation [63]. The prostate gland is unique in that it accumulates zinc at very high levels, although the special functions of the accumulated zinc remain unknown. This accumulation is regulated by prolactin and testosterone. It has been shown that *hZIP1* mRNA in human malignant prostate cell lines is increased by treatment with prolactin and that zinc uptake into these cells is stimulated by treatment with physiological levels of prolactin and testosterone [56]. Expression of *hZIP1* mRNA was down-regulated when the cells were exposed to high levels of zinc.

hZIP2

hZIP2 was the first ZIP member characterized in mammals by virtue of its similarity to ZIP members in yeast and plants [46]. K562 cells manipulated to express the exogenous hZIP2 accumulate more zinc than parental cells in time-, temperature- and concentration-dependent and saturable manners, and the epitope-tagged hZIP2 is localized to the plasma membrane in K562 cells, indicating that hZIP2 functions as an importer of zinc out of the cells. This zinc uptake directed by hZIP2 is stimulated by HCO_3^- treatment but is not dependent on K^+ or Na^+ gradients, which suggests the Zn^{2+}/HCO_3^- symport mechanism. Several transition metals, such as iron, cobalt, cadmium, copper and manganese, inhibit the zinc uptake activity by hZIP2. This pattern of inhibitory metals is somewhat different from that of hZIP1 [45]. Studies on cellular transport have verified that iron cannot be a substrate of the metal transporter activity of hZIP2 [45]. Expression of *hZIP2* mRNA is very low, and a survey of the expressed sequence tags suggests expression of *hZIP2* only in the prostate and uterus [46]. Later, a relatively high expression of *hZIP2* was found in peripheral blood mononuclear cells (PBMCs) and monocytes [57]. Interestingly, expression of *hZIP2* mRNA is dramatically but transiently induced by contact inhibition and to a lesser extent by serum starvation in ectocervical epithelial cells and squamous carcinoma cell lines [64]. Moreover, *hZIP2* mRNA increases by zinc depletion in PBMCs and monocytes with inverse relation to MT mRNA expression [57]. The physiological relevance of these alterations of *hZIP2* expression needs to be studied.

hZIP4 and inherited disorder

Acrodermatitis enteropathica (AE) is a rare autosomal recessive disorder in humans characterized by the development of perioral dermatitis, acral erosion, intermittent diarrhea and growth failure in early infancy [65]. This disease results from insufficient absorption of zinc by the intestine [66, 67] and can be fatal unless the diet is supplemented with zinc [68]. Therefore, mutations of gene(s) associated with cellular zinc transport have been thought to be responsible for AE. Recently, the AE susceptibility locus was localized on chromosome 8q24.3 by homozygosity mapping [69], and subsequently, the *hZIP4* gene has been cloned as a zinc transporter responsible for AE [54, 70]. *hZIP4* mRNA is expressed in the small intestine (duodenum and jejunum), colon, stomach and kidney [54, 70]. The duodenum and jejunum are crucial sites of zinc absorption [1, 8]. *hZIP4* mRNA is detected mostly in the matured enterocytes of the intestinal villus, and hZIP4 protein resides on the apical surface of the enterocytes [54], consistent with its physiological function as a transporter for zinc uptake from the intestinal lumen. Many missense mutations that probably affect the function of hZIP4 were found by the sequence analysis of amplified genomic DNA from AE patients [54, 70]. Half of the mutations occur in amino acid residues specific to hZIP4, but the rest are found in amino acid residues highly conserved in the transmembrane domains of all ZIP members. These residues seem to be very important for ZIP members to exert their functions. It is noted that dietary supplementation of zinc cures this genetic disease [68]. This fact indicates that humans have a backup system that can take up dietary zinc with an efficiency lower than that of hZIP4, although in some AE patients mutated hZIP4 proteins might still be capable of transporting zinc at a reduced rate. Expression of other ZIP members, such as *hZIP1*, is found in the intestine.

Several His residues in a large N-terminal sequence of hZIP4 that are presumably located in the extracellular space may serve to take up zinc. The *hZIP4* mRNA level is dramatically elevated in the embryonic visceral yolk sac during maternal dietary zinc deficiency [58], which suggests that expression of hZIP4 is regulated by extracellular zinc levels. The observation that zinc absorption from the small intestine was depressed upon intraperitoneal injection of a large dose of zinc may be relevant to the zinc-dependent regulation of hZIP4 expression [71].

BIGM103

BIGM103 was identified as an inducible gene during immune activation, that is, in monocytes challenged by bacterial cell walls [52]. This activation is very interesting because zinc is essential for an intact immune system, and monocytes are the only cell populations that can be directly activated by zinc in the immune system [72]. Differentia-

tion of monocytes to dendritic cells and macrophages is accompanied by an elevation of *BIGM103* mRNA. Expression of *BIGM103* is also induced by inflammatory cytokines such as TNFα but is abolished by treatment with phorbol ester. *BIGM103* mRNA is widely expressed and abundant in the pancreas. The BIGM103 protein is unique in that in addition to the structural features of ZIP members, it has a leucine-zipper motif composed of four consecutive L-(X)6 repeats in the long N-terminal sequence. This motif is widely used for protein-protein interaction, but a protein interacting with BIGM103 has not been identified. The BIGM103 protein has been shown to be modified post-translationally, probably by glycosylation [52]. The epitope-tagged BIGM103 protein in several cell lines is localized to lysosomal/endosomal compartments and overexpresed BIGM103 protein in CHO cells increases intracellular zinc. Because it has not been examined whether the increased zinc is accumulated in lysosomal compartments or cytoplasm, the direction of zinc transport by BIGM103 remains unknown.

Other LIV-1 subfamily members

No human ZIP members belonging to the LIV-1 subfamily other than hZIP4 and BIGM103 have been characterized functionally, but their structural similarities to hZIP4 and BIGM103 suggest that most of these ZIP members may possess zinc transport activity. In this section, we bring together fragmentary knowledge of the LIV-1 members, describing their features.

LIV-1 was identified as an estrogen-regulated gene in breast cancer cells [73] and is known to show a significant association with the spread of breast cancer to the lymph nodes [50]. Expression of *LIV-1* mRNA in breast cancer cells is positively regulated by steroid hormones, including estrogen, and by growth factors such as insulin with an additive effect to steroid hormones, while phorbol esters depress its expression [51, 74]. *LIV-1* mRNA was also detected in prostate carcinomas and benign prostatic hyperplasia [51]. Recently, the truncated form of the mouse *LIV-1* homologue, named ermelin, was cloned from skeletal cells [75]. *LIV-1/ermelin* mRNA was detected predominantly in the brain and testis (breast tissue was not tested), which is reminiscent of the expression pattern of *ZnT-3* mRNA as described below [76]. Intriguingly, *LIV-1/ermelin* mRNA is highly expressed in the hippocampus and cerebellum in the brain and in seminiferous tubules in the testes [75]. Moreover, the expression is induced during differentiation of neuroblastoma cells, while it is reduced during myogenic differentiation of skeletal muscle cells.

KE4 was first identified as a gene located in the H-2K region of mouse major histocompatibility complex (MHC) and is expressed in embryo [77]. Later, the human homologue was also identified in the MHC region [78]. *KE4* mRNA is constitutively expressed in all tissues but abundantly in embryonic carcinoma cells [77, 79], suggesting that KE4 may possess an important function(s) during embryogenesis. Recently, the tagged KE4 protein has been shown to reside on the ER [75]. The function of the KE4 protein has not been characterized thus far, but KE4 may transport zinc or copper out of the ER, because expression of *KE4* cDNA complements the defects of mutant of *IAR1*, an *Arabidopsis* homologue of *KE4,* and one of the predicted functions of IAR1 is to transport zinc or copper out of the ER [55]. *Drosophila* Catsup protein, a homologue of KE4, down-regulates tyrosine hydroxylase activity [80]. KE4 may potentially possess multi-physiological functions.

KIAA0062 cDNA was isolated from a human immature myeloid cell cDNA library [81]. This gene is ubiquitously expressed but more abundantly in the liver, pancreas and heart, in that order. *KIAA1265* cDNA is isolated from an adult brain cDNA library [82]. The KIAA1265 protein possesses the long His-rich N-terminal sequence like those in LIV-1 and KE4 [49, 79]. *KIAA1265* mRNA is ubiquitously expressed at a low level but predominantly in the central nervous system, especially the spinal cord [82].

There are patients with AE not mapped to chromosome 8q24.3 harboring hZIP4. The primary expression of another LIV-1 member (XP_208649) in the colon epithelium (according to the SAGEmap database) raises the possibility that a mutation of this gene may be responsible for this type of AE (XP_208649 is referred to as hORF1 in [54]). This gene is also highly expressed in adenocarcinoma cells [54].

CDF transporter family

Characteristics of CDF (ZnT) members

The CDF family was first proposed as a gene group encoding proteins that confer metal resistance to cells [83]. It contained *CzcD* of *Ralstonia* sp. (84), and *ZRC1* [85] and *COT1* (86) of yeast. Members belonging to this family have been shown to occur in diverse organisms from bacteria and fungi to plants and mammals [24, 29]. To date, seven mammalian CDF members have been characterized, and recently another member was submitted to the GenBank. They are named ZnT-1~8 (zinc transporter) (fig. 4). Based on sequence similarities, Gaither and Eide divided the CDF family into three subgroups: CDF subfamilies I, II and III [29]. Mammalian ZnT members are assigned to subfamilies II and III (fig. 4). Most of the CDF members are predicted to have similar topology, with six transmenbrane domains, an intracellular N-terminus and C-terminus and a His-rich loop between transmembrane domains IV and V [24, 29, 32] (fig. 2B). In only a few cases, this topology has been verified by the use of translationally fused reporters. The reporter-fused

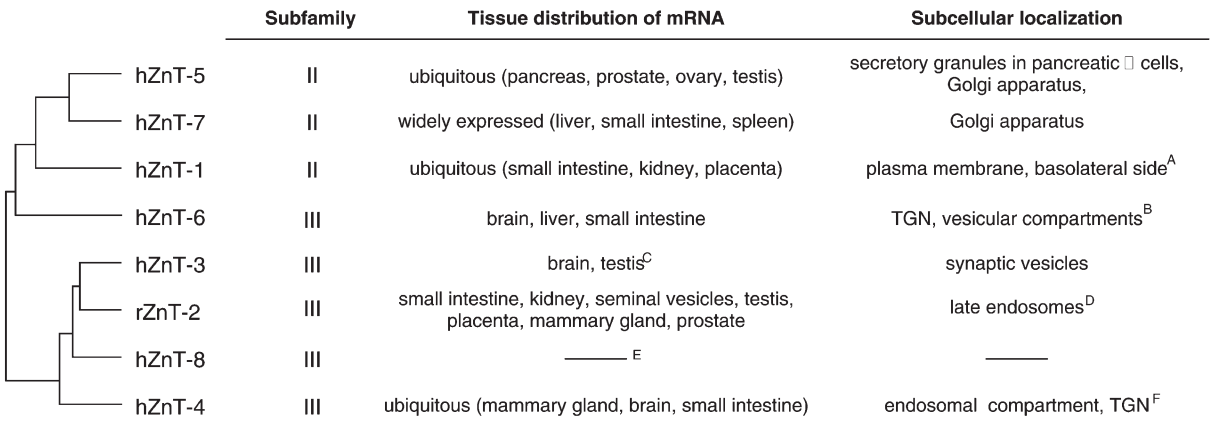

Figure 4. A dendrogram showing the sequence similarity among ZnT members. The class of subfamily follows the assignment by Gaither and Eide [29]. Tissue distribution of mRNA and subcellular localization of these proteins are also shown. The dendrogram is generated using GENETYX-MAC software. All sequences except for ZnT-2 (rat) are human sequences. The accession numbers for the sequences used are: AAM09099 for hZnT-5, AAM21969 for hZnT-7, Q9Y6M5 for hZnT-1, NP_060434 for hZnT-6, Q99726 for hZnT-3, AAB02775 for rZnT-2, AAM80562 for hZnT-8 and AAM80562 for hZnT-4. The capital letters indicate the following: A, endosomal structures in maternal small intestine at day 1 of lactation or villus yolk sac of the placenta [111]; B, more diffuse in response to zinc; C, not translated in the testis; D, apically located vesicles in the small intestine; E, specifically expressed in pancreatic β cells; F, more diffuse in response to zinc. The horizontal lines indicate no available information. TGN, trans-Golgi network.

C-terminus of the ZnT-1 protein is located in cytoplasm [87], and the N-terminus and C-terminus of the CzcD protein of *Ralstonia* sp. are present in the cytoplasmic side [88]. It has been postulated that the four amphipathic transmembrane domains I, II, V and VI comprise an inner core forming a channel, while the remaining hydrophobic transmembrane domains III and IV are located in the more lipid-exposed outer shell. The β-carboxyls of the highly conserved three Asp residues in transmembrane domains II, V and VI may form a zinc-binding site inside the channel [24]. Analyses of ZitB, a CDF member of *Escherichia coli*, with site-directed mutagenesis demonstrated the importance of two Asp residues within transmembrane domains V and VI in zinc transport [89]. Furthermore, Lee et al. showed that the two His residues within transmembrane domains II and V are very important for zinc transport, but that the Glu residue within transmembrane I is not [89]. These residues are highly conserved in mammalian ZnT members.

Like ZIP members, CDF members have a His-rich loop exposed to cytoplasm [24, 29] (fig. 2B). The length of the loop varies among the members, but the loop contains an (HX)n motif, where n ranges from 3–6 and X is often S or G [29]. This loop could function as a potential zinc binding site. In fact, the His-rich loop in ZnT-4 and ZAT1, a plant CDF member, has been shown to bind zinc [90, 91]. In addition to zinc, ZnT-4 binds other divalent cations such as copper, nickel and cobalt [90]. Intriguingly, alterations within the His-rich loop affect its metal specificity [92], indicating that this region is involved in metal recognition as well as metal binding. Existence of the second potential zinc-binding motif is also suggested in the cytoplasmic C-terminal tail [29].

The mechanism of zinc transport by mammalian ZnT members has not yet been examined extensively, but recent studies of CDF members from microorganisms provide some insight. CzcD of *Bacillus subtilis* transports zinc by an antiport mechanism in which zinc is exchanged with K^+ and/or H^+ [93]. Likewise, the *E. coli* zinc transporter ZitB functions as a Zn^{2+}/K^+ antiporter [89]. ZRC1 of yeast transports zinc into vacuoles by an antiport mechanism coupled to the vacuole H^+, but not the K^+ gradient generated by V-type H^+-ATPase [94]. Sequestering zinc into vacuoles detoxifies it, and, therefore, the yeast strain lacking *ZRC1* ($\Delta zrc1$) is susceptible to zinc toxicity. Because vacuoles in yeast are similar to mammalian endosomes/lysosomes in terms of acidic pH, some ZnT members (ZnT-2, 3 and 4) localized to the endosomal/lysosomal compartments might transport zinc by the Zn^+/H^+ antiport mechanism. The result that overexpression of the ZnT-4 protein in the $\Delta zrc1$ yeast strain confers resistance to a high zinc condition may imply a zinc transport process by ZnT-4 [95]. However, there is a report that inhibition of acidification does not affect zinc transport into endosomes by ZnT-2 [96].

ZnT members are transcriptionally and post-translationally regulated by zinc. Expression of *ZnT-1* and *2* is induced by high zinc conditions [71, 97–100], consistent with a possible role of ZnT members, that is, detoxification of zinc [87, 96, 97, 101]. Intriguingly, high zinc conditions redistribute ZnT-4 and ZnT-6 protein from the perinuclear region to the cell periphery [102]. Such regulated protein trafficking would also be an important post-translational regulation to maintain intracellular zinc homeostasis.

A phylogenetic tree of mammalian ZnT members, including their tissue distribution and subcellular localiza-

tion, is described in figure 4. Each mammalian ZnT member is reviewed below in detail.

ZnT-1 and *ZnT-1–/–* mouse

ZnT-1 is the first mammalian zinc transporter isolated by using complementation assay of a mutated zinc-sensitive BHK cell line [87]. Expression of the *ZnT-1* gene allowed the cell to grow in the presence of high levels of extracellular zinc by promoting zinc efflux activity. Furthermore, ZnT-1 could confer zinc resistance to some wild-type cell lines [87, 97, 101]. Taken together with the observation that the tagged ZnT-1 is localized to the plasma membrane, ZnT-1 has been proposed as a zinc exporter out of the cells. To date, ZnT-1 is the only zinc transporter protein that localizes to the plasma membrane among mammalian ZnT members. Yeast does not possess this type of zinc efflux protein; the transport of zinc into vacuoles appears to be a major route to avoid zinc toxicity in yeast. Western blot analysis detects ZnT-1 protein with different sizes depending on the tissues [98, 101], suggesting that this protein is subject to post-translational modifications in a tissue-dependent manner. As the mutant ZnT-1 lacking the first transmembrane domain shows a dominant negative effect on some cell lines [87, 101], ZnT-1 appears to function as a multimer complex [87]. Rat ZnT-1 consists of 507 amino acid residues [87]. A large deletion of the C-terminal tail (Δ156 aa from the C-terminus) is rather toxic to the wild-type BHK cells for an unknown reason, while a small deletion (Δ72 aa from the C-terminus) and C-terminally tagged ZnT-1 confers zinc resistance to the mutant BHK cells, suggesting that the cytoplasmic C-terminal portion (84 aa) near the last transmembrane domain is essential for ZnT-1 function, but that the tail portion is not.

Recently, it was shown that the *Caenorhabditis elegans* ZnT-1 homologue, CDF-1, positively regulates Ras-mediated signaling by promoting zinc efflux out of cells; accumulation of cytosolic zinc negatively regulates the Ras signaling cascade [103–105]. *CDF-1* mutant worms show a weak, vulvaless phenotype presumably caused by reduced Ras-mediated signaling during vulval induction. The mutant worms are hypersensitive to zinc but not to cobalt, copper and cadmium. Introduction of the *ZnT-1* gene into the *CDF-1* mutant worms rescues these defects [104]. Furthermore, expression of *CDF-1* or *ZnT-1* in *Xenopus* oocytes stimulates mitogen-activated protein kinase (MAPK) activation during oocyte maturation [104], indicating that the physiological concentration of zinc reduces the Ras signaling rate in the oocytes. These results make it plausible that ZnT-1 plays an important role in tuning Ras-mediated signaling in mammals. However, many reports indicate that extracellular zinc at a subtoxic concentration positively regulates MAPK signaling in some mammalian cells [5]. An intriguing hypothesis that the mammalian Ras-MAPK signaling pathway is regulated by zinc awaits further investigation. Mutations that constitutively activate Ras signaling are frequently found in human tumors. Such mutations may include those that occur in zinc-response proteins involved in Ras signaling.

ZnT-1 mRNA is expressed ubiquitously, but abundantly in the small intestine, kidney and placenta [76, 100]. In the small intestine, the ZnT-1 protein is abundant in the duodeum and jejunum and is dominantly localized to the basolateral surface of enterocytes lining the villus [26, 98], which suggests that intestinal ZnT-1 plays an important role in exporting zinc out of enterocytes and into the bloodstream. The ZnT-1 protein is also found mainly on the basolateral surface of renal tubular cells [26, 27]. This renal localization implicates another function of ZnT-1: the renal ZnT-1 recovers zinc from glomerulus filtrate by exporting it into the blood-stream from renal tubular cells that take up zinc in the filtrate.

ZnT-1 mRNA expression is rapidly and dramatically induced in cultured cells by zinc treatment [97, 99] and in the small intestine, kidney and liver by oral administration of zinc [71, 98, 100]. Conversely, zinc deficiency reduces *ZnT-1* expression [99, 100]. Transcription of *ZnT-1* is under the control of the metal response element (MRE)-binding transcription factor-1 (MTF-1). The *ZnT-1* promoter contains two MRE consensus sequences (–87 bp and –116 bp relative to the transcription start site), and MTF-1 binds to both sites, resulting in transcriptional activation [99]. Expression of MT-1 is also regulated in a similar fashion [99, 106].

It has been proposed that in addition to modulating neuronal transmission (see ZnT-3), zinc may contribute to neuron death in pathological conditions; accumulation of cytosolic zinc correlates well with selective neuron death in ischemia, seizures and trauma [7, 107]. Transient forebrain ischemia in rats increased chelatable zinc in degenerating neurons in the hippocampal CA1, cerebral cortex and other regions [7]. A brief ischemic insult in gerbil brain caused a marked increase in both chelatable zinc and *ZnT-1* mRNA in the hippocampal CA1 pyramidal neurons that are sensitive to ischemia [97]. Furthermore, when primary hippocampal neurons were exposed to a high dose of zinc, *ZnT-1* mRNA was induced [97]. These results suggest that the ischemic induction of *ZnT-1* mRNA is due to zinc-induced transcriptional activation of the *ZnT-1* gene. A high expression of the ZnT-1 protein is detected in the cerebral cortex, which is also vulnerable to ischemia [108]. Taken together, ZnT-1 in the brain appears to fight hard against zinc influx into the neurons, which occurs in pathological conditions of brain, and thereby reduces neuronal death as much as possible.

The mortality of *ZnT-1* null mice in embryos confirms that ZnT-1-mediated zinc transport is critical for normal development [27, 99].

ZnT-2

ZnT-2 was identified by the same strategy used to isolate the *ZnT-1* cDNA that conferred zinc resistance to the mutant BHK cells [96]. The characteristics and physiological significance of ZnT-2 have not yet been studied extensively. The epitope-tagged ZnT-2 is localized on acidic vesicles, and overexpression of *ZnT-2* facilitates the accumulation of zinc into the vesicles in high zinc conditions [96]. Later, the vesicles with a highly vacuolated appearance were shown to be late endosomes [109]. Taken together, ZnT-2 functions as a zinc transporter to sequester zinc into endosomes. Unlike ZRC1, the yeast vacuolar zinc transporter, acidic conditions in endosomes are not required for ZnT-2 to transport zinc into the lumen [96].

ZnT-2 mRNA is detectable in the small intestine, kidney, seminal vesicles, testis [76], placenta [100], mammary gland [110, 111] and prostate [112]. In the small intestine, the ZnT-2 protein is concentrated to the apical side directly adjacent to the microvilli [111]. Interestingly, *ZnT-2* expression in the liver is undetected in the normal condition but induced in response to oral zinc load [100]. *ZnT-2* expression in the small intestine and kidney is enhanced by supplemental zinc intake [100], suggesting that *ZnT-2* expression is regulated by a mechanism similar to that of *ZnT-1* and *MT-1*. *ZnT-2* expression in the small intestine of neonatal pups is developmentally up-regulated, and high expression is observed until weaning [111]. This enhanced expression may be caused by higher levels of zinc in milk compared with maternal blood plasma [111, 113]. A recent report that *ZnT-2* mRNA is present abundantly in the lateral and dorsal lobes of the prostate but not in the ventral lobe is intriguing because these two portions contain high amounts of chelatable zinc excreted into the seminal fluid [112]. Chelatable zinc in the epithelial cells of the lateral lobe is contained in apically located vesicles and lysosome-like structures [40], which suggested that ZnT-2 transports zinc into these compartments. Compared with none levels, zinc levels in the prostate gland are significantly lower in patients with prostatic cancer and higher in those with benign prostatic hypertrophy than those in the normals [114], but the causality between these diseases and zinc levels is unclear.

ZnT-3 and *ZnT-3 –/–* mouse

The *ZnT-3* gene was cloned by virtue of its homology to *ZnT-2* [76]. *ZnT-3* is exclusively expressed in the brain and testis, which possess high amounts of vesicular zinc [35]. Chelatable zinc in the brain is accumulated in synaptic vesicles of a subset of glutamatergic neurons at over 1 mM [39]. This chelatable zinc and glutamate are presumably concurrently released into the synaptic cleft upon excitation [115]. The in situ hybridization and immunohistochemical analysis showed that ZnT-3 is abundant in the hippocampus, amygdala and cerebral cortex [76], where chelatable zinc is found at high concentrations [116]. The ZnT-3 protein is most abundantly detected in zinc-enriched mossy fibers that project from the dentate granule cells to hilar and CA3 pyramidal neurons [76], and the localization of the ZnT-3 protein is coincident with clear, small, round synaptic vesicles accumulating zinc [117]. The *ZnT-3–/–* mouse generated by gene-targeting provided conclusive evidence that the ZnT-3 protein is required to load zinc into synaptic vesicles; the *ZnT-3* null mouse failed to accumulate zinc in the cerebral cortex, amygdala, olfactory bulb and cochlear nucleus as well as in the mossy fibers in the hippocampus [41]. The *ZnT-3+/–* brain has an intermediate level of both the ZnT-3 protein and chelatable zinc in synaptic vesicles compared with *ZnT-3+/+* and *ZnT-3–/–* mice [41, 118], indicating that the vesicular zinc content is determined by the abundance of ZnT-3 protein in synaptic vesicle membranes.

In addition to glutamatergic neurons, there is increasing histological evidence that some of the inhibitory neurons in the dorsal spinal cord and cerebellum also express the ZnT-3 protein and accumulate zinc [119–122].

Zinc released into the synaptic cleft upon excitation is estimated to reach nearly 300 μM [123]. Vesicular zinc has been proposed to have neuromodulatory functions [39, 124] through regulation of multiple ligand- and voltage-gated ion channels, including inhibition of *N*-methyl-D-aspartate (NMDA) receptors, potentiation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor responses, inhibition of γ-aminobutyric acid ($GABA_A$) receptors and antagonism of voltage-gated calcium channels [125, 126]. Therefore, the *ZnT-3–/–* mouse was expected to show neuronal defects. Although vesicular zinc in the hippocampus and cortex almost completely disappeared [41, 118], the *ZnT-3–/–* mouse showed normal phenotypes so far as examined [127, 128], with the exception that the *ZnT-3–/–* mouse is more susceptible than the wild-type to seizures elicited by kainic acid [129]. Further studies need to be done to reveal the function(s) of vesicular zinc in neurons.

Zinc may also contribute to neuronal injury in certain pathological conditions such as ischemia, seizure and trauma [107]. The release of vesicular zinc from presynaptic neurons into the neural cleft and the subsequent uptake by postsynaptic neurons via some calcium ion channels was thought to induce neuron death [107]. However, experimental results with the *ZnT-3–/–* mouse showed that zinc accumulation in degenerating neurons robustly occurs in the *ZnT-3* null mouse, as it does in the wild type [130]. Zinc would derive from histochemically invisible, yet unidentified, nonsynaptic stores.

Endogenous metals have been implicated in β-amyloid (Aβ) deposits in Alzheimer disease [131, 132]. Zinc is the only physiologically available metal to precipitate Aβ at

pH 7.4 [131, 133]. Transgenic mice manipulated to express the human Swedish mutant of β-amyloid precursor protein (APP) show age-dependent cerebral amyloid plaque pathology (134). Recently, interesting results were obtained by breeding *hAPP+* mice with *ZnT-3–/–* mice. In comparison with *hAPP+*:*ZnT-3+/+* mice, *hAPP+*:*ZnT-3–/–* mice of both sexes had a markedly reduced plaque number and less insoluble amyloid β, indicating that synaptic vesicular zinc contributes to amyloid deposition. Moreover, female *hAPP+*:*ZnT-3+/+* mice had higher levels of synaptic zinc, insoluble amyloid β and plaques than their male counterparts [118]. These sex differences disappeared in *hAPP+*:*ZnT-3–/–* mice. Although the mechanism by which zinc is accumulated more in synaptic vesicles of females than in those of males is unknown, this result suggests that synaptic vesicular zinc is involved in the sex difference in the incidence of Alzheimer disease. The age-adjusted incidence for Alzheimer disease is substantially higher in females than in males [135]. The β-amyloid accumulation in the *hAPP+* transgenic mouse is markedly reduced by oral treatment with clioquinol, a retired USP antibiotic and bioavailable Cu/Zn chelator [136], which supports the pathological importance of vesicular zinc.

Sequestration of zinc into synaptic vesicles by ZnT-3 appears to commence in the maturation processes of synaptic vesicles. Zinc in vesicles in the cell body or Golgi apparatus of neurons cannot be stained, but the vesicular zinc in axonal transit can be [39]. This result indicates that ZnT-3 loads zinc into the newly formed vesicles leaving the trans-Golgi network (TGN) and into maturing synaptic vesicles. It appears that ZnT-3 becomes functional in the maturation processes of synaptic vesicles. This may explain why ZnT-3 failed to confer zinc-resistance to zinc-sensitive BHK cells: because BHK cells would lack the maturation process into synaptic vesicles [76].

In the testis, which also contains vesicular zinc, *ZnT-3* mRNA is abundantly expressed in germ cells, particularly in pachytene spermatocytes and round spermatids. ZnT-3 protein, however, was undetectable by the immunohistochemical analysis [76]. The relevance of this observation was supported by the observation that disruption of the *ZnT-3* gene did not affect the chelatable zinc level in spermatids [41]. The transporter that accumulates the chelatable zinc in the testis is unknown.

ZnT-4 and mutant mouse

In a search for the gene responsible for a mouse mutant (*pallid*) by the positional cloning method, *ZnT-4* was serendipitously isolated as a gene causing lethal milk (*lm*) syndrome [95]. The *lm* gene is so named because pups suckling on *lm/lm* dams die before weaning due to zinc deficiency. The zinc content in *lm/lm* milk is approximately 34% less than that in the wild type [137]. Symptoms can be reduced upon administration of zinc to either the *lm/lm* mothers or the pups, or by fostering the pups on normal dams. Mouse ZnT-4 is a 430-amino-acid protein, and the *lm* mutant has a premature translational termination codon at Arg codon 297 by C$\rightarrow$T substitution, resulting in the production of a truncated ZnT-4. This finding indicates an important role of ZnT-4 in zinc transfer in the mammary epithelium. In fact, *ZnT-4* mRNA is expressed ubiquitously, but abundantly in the mammary gland [100] and mammary gland-derived cell lines [95]. Rat milk contains 10 times as much zinc as blood plasma [113], and the lactating human breast secrets about 0.5 L of milk containing approximately 50 µM zinc in one day [138]. Therefore, the expression of ZnT-4 was expected to increase during the lactation period. However, ZnT-4 protein levels increased only slightly (about twofold) in rat mammary gland during the lactation period [111], and no significant difference was found in ZnT-4 protein levels between resting and lactating human tissues [139]. ZnT-4 has been localized to intracellular vesicles in human luminal epithelial cells of both the alveolus and the duct [139]. Similar localization occurs in rat mammary epithelial cells [110] and in a human breast epithelial cell line, PMC42 [139]. However, overlapping between the vesicular zinc and ZnT-4 could not be found in PMC42 cells [139], which made it unlikely for ZnT-4 to be directly involved in the transport of zinc into these intracellular vesicles. Further studies are needed to know whether this finding is restricted to the cell line or whether it holds true for lactating tissues.

The toxic milk mouse (*tx*) produces copper-deficient milk [140], which is presumably attributed to the mislocalization of the Wilson protein, a copper transporter [141]. The wild-type Wilson protein redistributes from the perinuclear region (presumably TGN) to the diffuse vesicular compartment during lactation, but the mutated Wilson protein of the *tx* mouse predominantly remains in the perinuclear region [141]. Similar trafficking of this protein is found in the elevated copper condition [142]. Likewise, trafficking control of ZnT-4 in response to zinc demand appears to operate in the mammary gland; the localization of ZnT-4 in the lactating breast is more extensive in cytoplasm compared with the resting breast [139]. This notion is also supported by the observation that ZnT-4 protein redistributes from the perinuclear location to the cytoplasm in response to zinc concentration [102].

ZnT-4 mRNA is also abundantly expressed in the brain and small intestine [90, 95, 100]. In the small intestine, expression of *ZnT-4* mRNA is abundant in the villus epithelium and is up-regulated in differentiating epithelium during gut development [100, 111, 143]. ZnT-4 protein is located in vesicles, and the majority of the vesicles are concentrated in the basal side of the polarized enterocytes, as in the mammary epithelial cells. A similar local-

ization of ZnT-4 protein was found in the polarized kidney epithelial MDCK cells [144]. It appears from these results that ZnT-4 protein is located in vesicles that mainly reside in the basal side of epithelial cells. The tagged ZnT-4 protein partially overlapped with the endosomal proteins [90], and later, endogenous ZnT-4 protein was shown to reside in TGN in addition to endosomes [102].

Unlike *ZnT-1* and *ZnT-2*, the expression of *ZnT-4* mRNA is not induced by supplemental zinc intake in the small intestine, liver and kidney [100] and by zinc treatment in breast epithelial cells [139]. Moreover, a deficiency of zinc intake (1mg Zn/kg) does not affect, or only slightly reduces, the expression in the small intestine, liver, kidney [100], testis and brain [90]. Thus, the expression of *ZnT-4* in these organs is not zinc-responsive. On the other hand, marginal zinc intake (10 mg Zn/kg) during the lactation period increases ZnT-4 mRNA expression and protein levels in the mammary glands [110]. This enhancement of ZnT-4 may have physiological relevance because it enables the effective transfer of zinc into milk.

The ZnT-4 protein is unique in that it contains a leucine-zipper motif composed of four consecutive L-(X)6 repeats in the N-terminal cytoplasmic regions. Interaction of the leucine-zipper motif of ZnT-4 with 80-kDa and 100-kDa proteins was shown by the use of extracts from metabolically labeled cells [90]. Thus far, these proteins have not been identified, but they may possess important roles in the regulation of zinc transport activity of ZnT-4 by forming a multisubunit complex or by transferring ZnT-4 to the correct sites.

Phenotypes of the *lm* mouse have suggested another interesting function of ZnT-4 in other tissues. *lm/lm* mice nursed on normal dams or on *lm* dams who received zinc administration survive and become reproductively mature [137, 145], but over 8 months of age they develop symptoms characteristic of zinc deficiency such as dermatitis, alopecia and skin lesions [145], which suggests that ZnT-4 has an important role in intestinal zinc absorption from dietary sources. Based on its similarity to AE disease [65], the *lm* mouse has been thought to be a model mouse of this disease, but the involvement of ZnT-4 in AE disease has been excluded [146–148]. In addition to the similarity of symptoms between *lm* and AE, the fact that ZnT-4 protein is concentrated in vesicles mainly on the basal side within the intestinal epithelial cells may implicate the involvement of ZnT-4 in the intracellular transfer of zinc from the apical side to the basolateral one from where zinc is released into the portal blood.

In addition to producing of zinc-deficient milk, the *lm/lm* mouse shows the congenital defect of otolith (calcium carbonate crystals in the inner ear), which causes difficulty in righting and unstable swimming. The development of otolith requires carbonic anhydrase, a zinc-containing enzyme that produces carbonic ions [149]. Zinc supplementation to the *lm* dam does not prevent a defect of utricular otolith but improves the development of saccular otolith [145]. Thus, ZnT-4 appears to be essential for zinc delivery to the utricle.

ZnT-5 and *ZnT-5–/–* mouse

ZnT-5 was identified by the use of its homology to ZRC1 of yeast [150] or ZnT-1 [151] and was found as a gene whose expression is transiently induced in response to intimal denudation of rabbit aorta [152]. Among CDF members that have six transmembrane domains, ZnT-5 is unique in that it has 15 putative transmembrane domains. The C-terminal portion of ZnT-5 (six transmembrane domains with ~365 amino-acid residues) is homologous to other ZnT family members, but the long N-terminal portion (nine transmembrane domains with ~400 amino-acid residues) has no homology to any characterized transporters. Thus far, MSC2 of yeast is the only CDF member possessing the long N-terminal portion (a total of 12 transmembrane domains) [153], but no homology is found in the N-terminal portions between ZnT-5 and MSC2. *ZnT-5* mRNA is ubiquitously expressed and abundantly expressed in the pancreas [150], prostate, ovary and testis [152]. Immunofluorescence analysis indicates that ZnT-5 protein in the pancreas is abundantly expressed in insulin-secreting β cells, but not in glucagon-secreting α cells and most acinar cells [150]. Electron microscopy showed that its localization in β cells is associated with insulin granules [150], suggesting that ZnT-5 transports zinc into secretory granules. This result is interesting because pancreatic β cells possess zinc at the highest level in the body, as insulin is stored in granules as zinc/insulin hexamer complex (crystals) [154]. mRNAs of *ZnT-1–4* are also detected by reverse-transcription polymerase chain reaction (RT-PCR) analysis in pancreatic islets [155], but neither the cellular nor the subcellular distribution of these transporters in the pancreas is known. The overexpressed ZnT-5 protein is localized in the Golgi apparatus [150, 152], and the ZnT-5-enriched vesicles prepared from Golgi membranes show increased zinc uptake [150], suggesting that ubiquitously expressed ZnT-5 may transport zinc into the Golgi apparatus. *ZnT-5* mRNA in differentiated intestinal cells increased more than 2-fold in response to extracellular zinc, but that in placental cells did not [151].

During disruption of a gene whose expression is induced upon intimal denudation of rabbit aorta, this gene turned out to be *ZnT-5* [150, 152]. The *ZnT-5–/–* mouse shows several intriguing features including poor growth, lean phenotype, decreased bone density and weak muscle [152]. Moreover, more than 60% of *ZnT-5–/–* male mice die suddenly because of bradyarrhythmias [152]. In pathohistologic examinations, most of the organs show

no abnormalities related to this sudden death. The localization of the ZnT-5 protein in the perimeter of insulin granules led us to hypothesize that glucose homeostasis of the *ZnT-5* null mouse might be perturbed through unregulated insulin secretion [150]. However, the serum concentrations of glucose as well as zinc are normal in the *ZnT-5–/–* mouse [152]. ZnT-8 (the accession number AAM80562) is expressed in pancreatic β cells and may complement the disrupted *ZnT-5*.

The mechanism that confers the interesting features to the *ZnT-5–/–* mouse has not been well understood but has been partially investigated. The osteopenia of the *ZnT-5–/–* mouse was not caused by the failure of proliferation and differentiation from precursor cells to osteoblasts but by the impairment of osteoblast maturation to osteocytes, thereby resulting in poor bone mineralization [152]. Tissue-nonspecific alkaline phosphatase (ALP) requires zinc as an essential element [156], and ALP activity is crucial for postnatal bone mineralization [157, 158]. ALP activity is reduced in the *ZnT5–/–* mouse [152]. Sudden cardiac death was seen only in male mice, but female mice also showed impairment of the cardiac conduction system. This sex difference is not attributable to sex hormones [152]. In the heart of the *ZnT-5–/–* mouse, mRNAs coding immediate-early response factors and heat shock proteins were down-regulated [152], suggesting that zinc mobilized by ZnT-5 may be involved in the expression of these proteins. These various phenotypes of the *ZnT-5–/–* mouse suggest complicated functions of zinc not expected previously.

ZnT-6

ZnT-6 was identified by virtue of its homology to ZnT-4 from the expressed sequence tag (EST) databases [102]. The zinc transport activity of ZnT-6 was detected by growth suppression analysis using both the wild-type yeast and mutants (Δ*zrt1*, Δ*zrt3* and Δ*msc2*). The results suggested that ZnT-6 transports the cytosolic zinc either to an intracellular pool or out of the cells. The intracellular localization of the ZnT-6 protein overlapping with TGN38 and the transferrin receptor in normal rat kidney cells indicated that this transporter may function in transporting the cytosolic zinc into the Golgi apparatus as well as the vesicular compartment.

A structural feature of the ZnT-6 protein is that it lacks the His-rich region in the cytoplasmic loop between transmembrane domains IV and V (see fig. 2B), while Ser residues are retained. Moreover, two His residues within transmembrane domains II and V that are highly conserved among all ZnT members and thought to be important for transporter function [89] are altered to Leu and Phe, respectively [102]. The physiological or functional significance of these characteristics in ZnT-6 is totally unknown.

ZnT-6 mRNA is expressed abundantly in the brain, liver and small intestine, but ZnT-6 protein is detectable only in the brain and lung [102]. ZnT-6 protein in the liver was undetectable despite a high-level expression of *ZnT-6* mRNA, while in the lung it is abundantly present despite a very low level of its mRNA. It appears that ZnT-6 expression is controlled at the translational level in a tissue-specific manner.

The ZnT-6 protein is normally localized to TGN and vesicular compartments. Interestingly, the intracellular distribution of ZnT-6 is regulated by zinc; ZnT-6 relocates from TGN toward the periphery of the cells in response to the extracellular-elevated zinc, which is reminiscent of copper transporters whose localization is regulated by copper. Under an elevated copper concentration, the Menkes protein moves from TGN to the plasma membrane [159] and the Wilson protein to the vesicular compartment [142]. As the expression of *ZnT-6* mRNA is not regulated by extracellular zinc concentrations [102], zinc-dependent intracellular localization may be critical for the function of ZnT-6. Similar regulation of trafficking is found in ZnT-4, but the trafficking of ZnT-6 is more sensitive to zinc than ZnT-4 [102].

ZnT-7

ZnT-7 was cloned very recently by searching the EST databases based on the amino-acid sequence of ZnT-1 [160]. *ZnT-7* is widely transcribed with abundant expression in the liver, small intestine and spleen and moderate expression in the lung, but the ZnT-7 protein is detected mainly in the small intestine and lung. The abundant expression of the ZnT-7 protein in the duodenum and jejunum of the small intestine suggests that this protein may play a role in zinc absorption. The ZnT-7 protein is detected as several bands by Western blot analysis [160], which indicates that it may be post-translationally modified. The ZnT-7 protein resides in the Golgi apparatus, and the myc-tagged ZnT-7 protein expressed in CHO cells transported zinc into the Golgi lumen when the cells were exposed to a high concentration of zinc. ZnT-7 may have an important role for loading zinc to secretory or membrane-bound proteins in the Golgi lumen.

Zinc-related disease

Hermansky-Pudluk syndrome and model mice

The Hermansky-Pudluk syndrome (HPS) defines a group of genetic disorders characterized by defective lysosome-related organelles such as melanosomes and platelet-dense granules, which cause hypopigmentation, hemorrhage and death due to lung abnormalities [161]. In humans, four responsible genes (HPS1–4) have been characterized, while 14 HPS-like disorders have been re-

ported in mice [161]. Some of the mutant mice show interesting phenotypes related to zinc homeostasis. The *Mocha* mouse, lacking the δ-subunit of AP-3 complex, shows an absence of vesicular zinc in the hippocampus and neocortex, and the absence of zinc is due to the lack of ZnT-3 in the synaptic vesicles by mislocalization of ZnT-3 [162]. The *Mocha* mouse displays a defect of otolith formation in the inner ear that is prevented or reduced by supplementing the diet of pregnant dams with zinc [163]. ZnT-4 appears to be involved in otolith formation (see ZnT-4 and mutant mouse). The *Pearl* mouse, one of the HPS model mice, has mutations in the gene encoding the β3A-subunit of AP-3 complex [164]. The fibroblasts from *pearl* cannot accumulate vesicular zinc [165], which is probably the result of the mislocalization of zinc transporter(s), as in the *mocha* mouse. Other mutants of the HPS model mice, *pallid* and *muted* mice, display an otolith defect similar to that of the *mocha* mouse [161]. Unlike *mocha* and *pearl* mice, however, *pallid* and *muted* mice show normal intracellular zinc storage [166]. The gene products of these mutants are all involved in vesicle traffic, docking and fusion: subunits of AP-3 complex in *mocha* and *pearl* mice; pallidin interacting with a t-SNARE protein, syntaxin 13, in *pallid* mice [167]; and muted protein interacting with pallidin in BLOC-1 complex in *muted* mice [166]. Thus, zinc-related diseases include disabled intracellular trafficking of zinc transporters.

Conclusions and perspectives

An extensive collection of information, including that yet to be established, sometimes makes it difficult to extract what we have understood based on strong evidence at this moment. fig. 5 may reduce this difficulty (see also figs. 3 and 4). Analyses of genes responsible for AE have verified that hZIP4 is the primary importer of dietary zinc in the intestine. The localization in the apical plasma membrane of the enterocytes defines the function of this transporter. hZIP1 may function as a backup system in the zinc uptake from diet, but it may have a housekeeping function

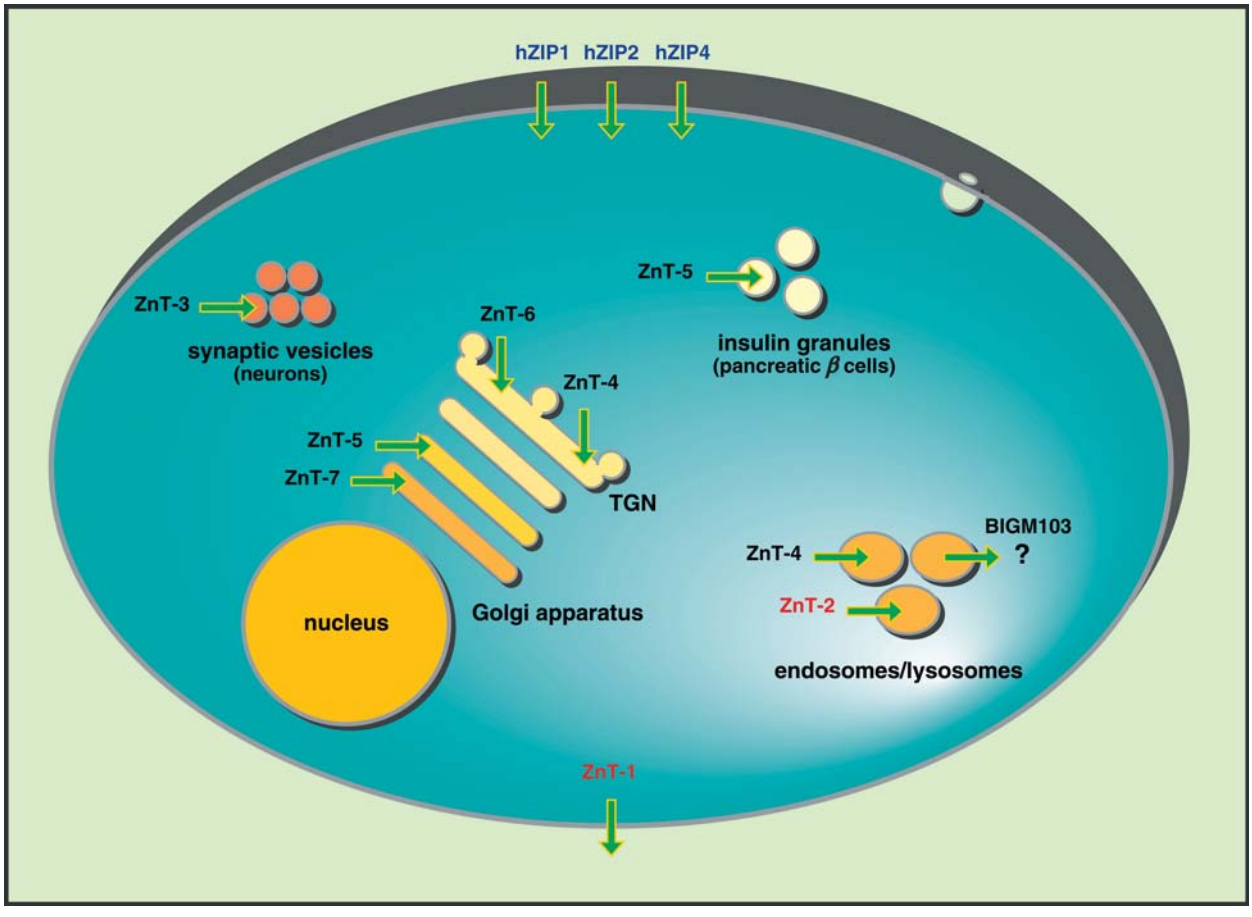

Figure 5. Localization and functions of zinc transporters. Localization and possible functions of ZIP and CDF members are shown according to information available to date (see figs. 3 and 4). Arrows indicate the direction of zinc mobilization. ZIP members indicated by blue letters are induced in zinc-limited condition (hZIP1, 2 and 4), while ZnT members indicated by red letters are induced by zinc administration (ZnT-1 and 2). TGN, trans-Golgi network.

in many other types of cells. Tissue distribution (uterus, prostate and monocytes) of *hZIP2* mRNA suggests its unique mission. An increase of mRNA levels of these ZIP transporters in response to zinc deficiency is consistent with the notion that these transporters are important in the cellular uptake of zinc. A new member of the ZIP family, BIGM103, may play a critical role for mobilizing zinc stored in endosomal/lysosomal compartments.

ZnT-1 is the only member of the ZnT family that is localized in the plasma membrane. Basolateral distribution in the enterocytes and the renal tubular cells indicates that ZnT-1 is responsible for the efflux of zinc in these cells into the blood-stream. ZnT-1 in neurons and possibly in other cells contributes to cell survival from zinc toxicity by exporting cytosolic zinc. Consistent with this, the transcription of *ZnT-1* is markedly activated by zinc. Other ZnT members (ZnT-2–7) distribute the cytosolic zinc into intracellular organelles and vesicles. The entry of zinc into these compartments supplies zinc to proteins and other components in situ, accumulates zinc in specialized compartments and stores and/or detoxicates excess cytosolic zinc. Of these members, a role of ZnT-3 in neurons has been established; it is responsible for the accumulation of chelatable zinc in synaptic vesicles. So far, the physiological function(s) of the vesicular zinc is unknown, but analyses of *hAPP+:ZnT-3–/–* mice yielded a result profoundly influential in seeking the etiology of Alzheimer disease. ZnT-4 accumulates zinc into the vesicles that are destined to secrete their contents into milk, where zinc is present at a high level. ZnT-5–7 are thought to import zinc into the Golgi apparatus, but a role specialized to each transporter has not been elucidated. ZnT-5 is highly associated with insulin granules of pancreatic β cells.

The mass of literature published on zinc transporters and candidate genes over the past few years clearly indicates the rapid progress in our understanding of zinc homeostasis and also raises many questions to be studied. Why do mammals have so many zinc transporters? An enigma to be solved is whether all of these are engaged in transporting zinc. The expression of candidate genes at both mRNA and protein levels should be examined. Some of the expressed genes may play more important roles in zinc transport than transporters already found, some may function as backup systems for the principal transporters, and some may function as essential subunits in the transporters whose zinc transport activity is expressed through formation of hetero-oligomers. The strategies that we have at this time to answer these questions are not sufficient and require hard work. Although classical complementation assays through the use of eukaryotes, including yeasts, and prokaryotic cells with mutated defects of zinc transport, are still promising, the development of mutant animal cells, such as BHK cells that played a critical role in the identification of *ZnT-1* and *ZnT-2*, would be more important. Primary cultured cells or cell lines derived from mice whose zinc-related genes have been disrupted may be useful. Regrettably, so far only a few zinc transporter genes have been knocked out. Reconstituted proteoliposomes will be a fruitful strategy that can provide strong evidence in some cases [91], although when hetero-oligomers are functional, this strategy may yield misleading conclusions. Direct transport assays with proteoliposomes will give us insight into the transport mechanism, including direction of zinc movement; energy-dependency, including symport and antiport; a true substrate (free ion or bound form); and specificity of metals. Therefore, this assay also needs to be done for the already identified transporters. Analyses of site-directed mutants using proteoliposomes reveal structural requirements of transport activity. The expression of a large quantity of membrane protein and its isolation before reconstitution of proteoliposomes is painstaking work, but it may open the door to X-ray crystallographic analysis of zinc transporters.

Disruption of the *ZnT-3* and *ZnT-5* genes provided conclusive evidence for the function of the former in limited tissues and yielded unexpected phenotypes in both, raising further interesting questions. The disruption of *ZnT-1* caused embryonal death; therefore, developmental stage-dependent conditional disruption of the gene is necessary. The further production and analyses of zinc-transporter null mice and also the breeding of mice with double and triple knockout genes will greatly contribute to revealing the regulatory mechanism of zinc metabolism. We can find prominent efficacy of this type of experiment in producing *hAPP+:ZnT-3–/–* mice, as described earlier.

Information on the mechanisms regulating the expression of zinc transporters, including transcription, translation, trafficking and turnover of transporter proteins, is far from complete. Zinc may be one critical regulator. Then, the effects of dietary zinc deficiency and orally or intravenously given zinc on these processes must be investigated more extensively to understand zinc homeostasis in the entire body. Age-dependent changes of these processes are interesting questions in relation to lowered immune activity, brain dysfunction, and poor taste etc. in elders.

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