Chapter 3
Zinc Transporter Proteins: A Review and a New View from Biochemistry

Taiho Kambe, Eisuke Suzuki, and Taiki Komori

Abstract Zinc is an essential biological metal found in approximately 10% of the human proteome. Zinc regulates a large number of proteins and their functions, including transcription factors, enzymes, adapters, receptors, and growth factors, acting as a structural or catalytic cofactor or as a signaling mediator. Increasing evidence indicates that the transport of zinc across biological membranes plays a pivotal role in its biological functions. Zinc transport is mostly mediated by two zinc transporter proteins, ZNT and ZIP. Members of both transporter families are involved in a variety of biological events, which in humans are often associated with health and disease. In this chapter, we review the current understanding of the biochemical functions of both transporter protein families with a particular focus on their biological subgroupings.

Keywords Zrt, Irt-like protein (ZIP) · Zn transporter (ZNT) · Solute carrier family (SLC) · Membrane transport · Cation diffusion facilitator (CDF)

3.1 Introduction

Under physiological conditions, zinc is present as a divalent cation that does not have a redox potential. In contrast to copper and iron, it therefore does not require a redox reaction during membrane transport (Kambe 2013). Thus, precise spatiotemporal control of the expression of zinc transport proteins is critically important for net zinc transport across biological membranes. Increasing evidence suggests that the cellular concentration and distribution of zinc, which are mediated through zinc mobilization across cellular membranes, are highly involved in a large number of cellular responses, such as activation/repression of transcription and translation, post-translational controls including membrane trafficking and protein stability, and

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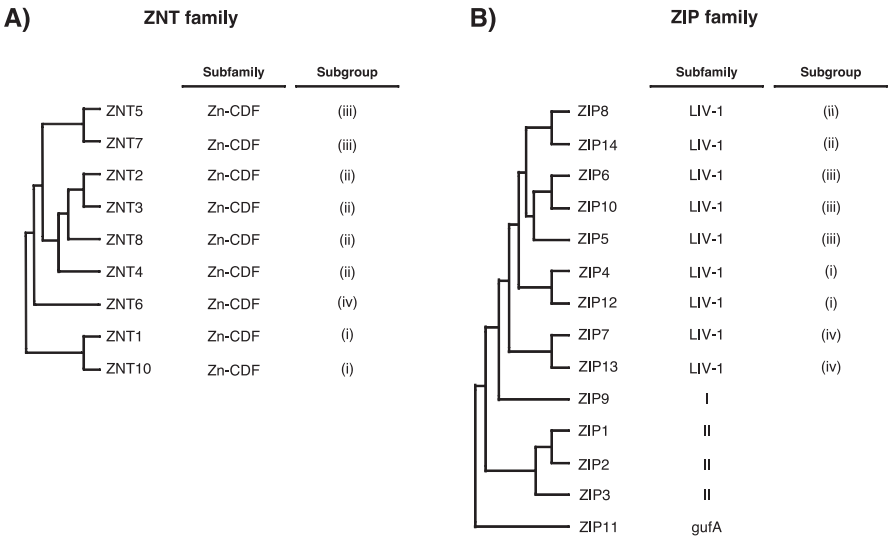

Fig. 3.1 Phylogeny of ZNT and ZIP family proteins. A neighbor-joining phylogenetic tree was generated with ClustalW, based on protein sequence alignments. (**a**) ZNT family members. (**b**) ZIP family members. In (**A**) and (**B**), the subfamily and subgroup classification assigned in this chapter are shown on the right

activation/inactivation of many enzymes related to cellular signaling (Fukada and Kambe 2011; Maret and Li 2009). Therefore, understanding the physiological/pathological regulations and biochemical properties of zinc transport is essential for understanding the biological roles of zinc. In metazoans, cellular zinc transport is primarily mediated by two zinc transporter proteins, the Zn transporter (ZnT)/solute carrier family 30 (ZNT/SLC30A) and Zrt, Irt-like protein (ZIP)/SLC39A (Fig. 3.1), that function as exporters and importers, respectively. In this chapter, the biochemical properties of these zinc transporter proteins are discussed. Their physiological and pathological roles are referred to in other chapters of this book.

3.2 History of ZNT and ZIP Family Proteins

In 1995, the first mammalian zinc transporter was identified by expression cloning using zinc-sensitive mutant Baby hamster kidney cells (Palmiter and Findley 1995). Since then, more than 20 zinc transporters, including ZNTs and ZIPs, have been identified (Gaither and Eide 2001; Kambe et al. 2004), all of which can function as secondary active transporters. The importance of zinc transporters is unquestionable, with a growing number of studies showing their involvement in both physiological and pathological processes. Before outlining their biochemical properties, we briefly discuss their historical backgrounds, which may give us important clues that would enable us to discover new functions of zinc transporter protein.

The first zinc transporter protein of the ZNT family, named Zrc1p, was identified in *Saccharomyces cerevisiae* and confers resistance to high zinc concentrations (Kamizono et al. 1989). Subsequently, Cot1p and CzcD were identified in *S. cerevisiae* and the *Cupriavidus metallidurans* (formerly *Alcaligenes eutrophus*) strain CH34, both of which were also shown to confer resistance to high levels of zinc and other metals such as cobalt (Conklin et al. 1992; Nies 1992). Due to their sequence homology, these three proteins were grouped together and named cation diffusion facilitator (CDF) proteins (Gaither and Eide 2001). However, it was later discovered that, despite their name, CDF proteins do not serve as diffusion facilitators but function as secondary active transporters. Proteins within this family are conserved at all phylogenetic levels (Gaither and Eide 2001). The first mammalian ZNT family member, rat Znt1, was identified as a member of the CDF family (Palmiter and Findley 1995). The second ZNT transporter, Znt2, was identified by expression cloning (Palmiter et al. 1996a) with other ZNT family proteins identified by other methods, including EST and genome database searches (Chimienti et al. 2004; Huang et al. 2002; Kambe et al. 2002; Kirschke and Huang 2003; Palmiter et al. 1996b). To date, there are ten members of the ZNT family, ZNT1-ZNT10; however, ZNT9 has been shown to act as a nuclear receptor coactivator (GRIP1-associated coactivator 63 (GAC 63)) (Chen et al. 2007), although there are studies reporting its involvement in zinc metabolism (Perez et al. 2017). Since the zinc transport ability of ZNT9 is yet to be fully elucidated, we have excluded it from our ZNT family discussion (Figs. 3.2 and 3.3).

Homologs of the human ZNT family proteins have been found in the genome sequences of rats, mice, chickens, zebrafish, fruit flies, nematodes, yeast, and plants (*Arabidopsis thaliana*) and are shown in Table 3.1. This table highlights that ZNT family proteins are highly conserved in vertebrates, and in nematodes, yeast, and plants. However, species such as nematodes, yeast, and plants have family members that produce ZNT family proteins, which are not homologous to vertebrate members. CDF family members are generally classified into three subfamilies, Zn-CDF, Zn/Fe-CDF, or Mn-CDF, based on their phylogenetic relationships and metal substrate specificities (Kambe 2012). All vertebrate ZNT family members belong to the Zn-CDF subgroup (Fig. 3.2). CDF members with low homology to vertebrates (Table 3.1) belong to Zn/Fe-CDF or Mn-CDF subfamilies and have been shown to be involved in other divalent cation transport in addition to zinc, which suggests that these proteins play diverse roles in metal transport.

The identification of ZIP family members is somewhat confusing. Among the ZIP family, ZIP6 was the first to be identified. However, it was originally identified as a highly expressed gene in breast cancer and named LIV-1 (Manning et al. 1994), with its zinc transport activity not evident at that time (Taylor 2000). Numerous studies have since shown that members of the ZIP family, including ZIP6, are associated with cancer development and metastasis (Bafaro et al. 2017) (see Chap. 16), which is consistent with the initial identification of ZIP6 in breast cancer. Zrt1p and Zrt2p zinc transporters were subsequently identified in *S. cerevisiae* (Zhao and Eide 1996a, b) simultaneous to the identification of IRT1 iron transporter in *A. thaliana* (Eide et al. 1996), all of which were identified from excellent genetic studies in

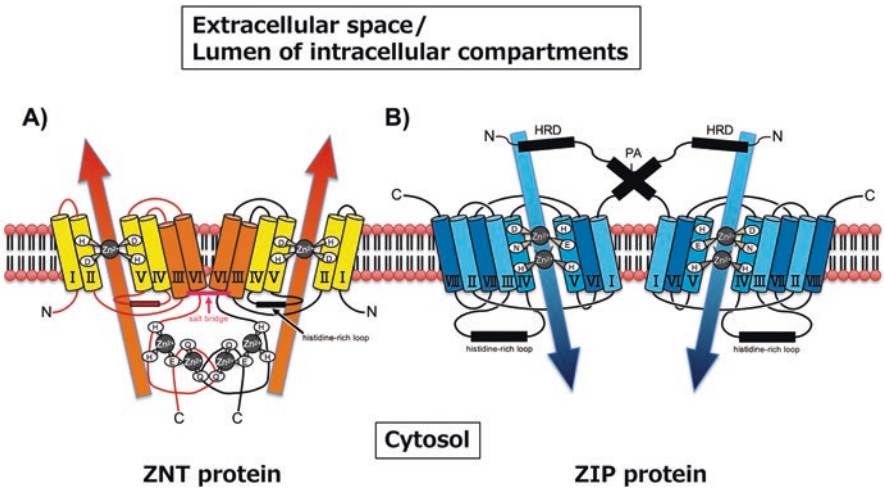

Fig. 3.2 Cartoon of predicted structures of ZNT and ZIP family proteins. (**A**). Predicted topology of the ZNT protein is shown. ZNT protomers most likely have six transmembrane domains (TMDs), in which TMDs, I, II, IV, and V form a compact four-helical bundle and the remaining TMDs, III and VI, form a two-helical pair outside the bundle. TMD II and V of the compact four-helical bundle create a transmembranous zinc binding site, which is formed by conserved aspartic acid (D) and histidine (H) residues. Predictions based on the structural properties of the bacterial homolog, YiiP (Coudray et al. 2013; Gupta et al. 2014; Lopez-Redondo et al. 2018; Lu et al. 2009; Lu and Fu 2007). ZNT proteins transport zinc from the cytosol into the extracellular space or intracellular compartments. (**B**) Predicted topology of the ZIP protein is shown. ZIP protomers are likely to have eight transmembrane domains (TMDs) consisting of a novel 3+2+3 TMD architecture, in which the first three TMDs (I to III) are symmetrically related to the last three TMDs (VI to VIII) by a pseudo-twofold axis. The conserved amphipathic amino acid residues in TMD IV (histidine, asparagine (N), and aspartic acid) and in TMD V (two histidines and one glutamic acid (E) in the potential metalloprotease motif (**HE**XP**H**EXGD) in LIV-1 subfamily, which are indicated in bold) form a binuclear metal center within the TMDs. However, the amphipathic amino acid residues in TMD V are only partially conserved in other subfamilies. Prediction based on the structure of the bacterial ZIP protein homolog, BbZIP (Zhang et al. 2017). ZIP4's long extracellular N-terminal region is divided into two structural domains, the helix-rich domain (HRD), and the PAL motif-domain (see text) (Zhang et al. 2016). ZIP family members transport zinc in the opposite direction to the ZNT family

yeast. These findings lead to the name "ZIP" (ZRT, IRT-like protein) (Eng et al. 1998), with the name suggesting that ZIP members can transport both zinc and iron, and possibly other divalent cations through a broader specificity. In fact, many recent studies show that mammalian ZIP family members can also transport manganese and cadmium (see Sect. 3.4.4.2) (Aydemir and Cousins 2018; Jenkitkasemwong et al. 2012). After the identification of ZIP1, ZIP2, and ZIP3 from EST database searches, an important discovery for the ZIP family in mammals was the identification of the *SLC39A4/ZIP4* gene, whose mutated form is responsible for *acrodermatitis enteropathica*, an inherited zinc deficiency disorder in humans. This finding

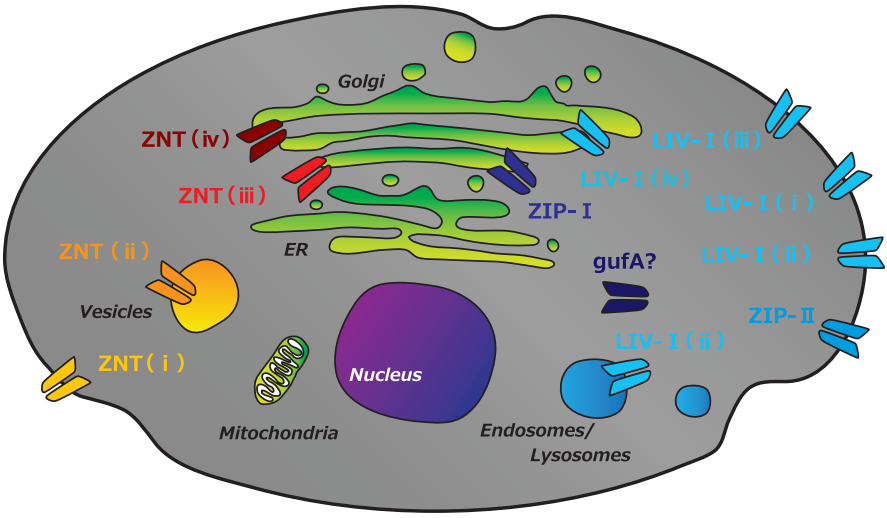

Fig. 3.3 Subcellular localization of ZNT and ZIP proteins from the view of subfamily and subgroup. The subcellular localizations of ZNT and ZIP protein members are shown according to their subfamily and subgroup assignments described in the main text and Fig. 3.1. ZNT members are shown according to their subgroups: subgroup (i) ZNT1 and ZNT10; subgroup (ii) ZNT2, ZNT3, ZNT4, and ZNT8; subgroup (iii) ZNT5 and ZNT7; and subgroup (iv) ZNT6. ZIP family members, except for LIV-1, are shown according to their subfamily: subfamily (ZIP-I) ZIP9; subfamily (ZIP-II) ZIP1, ZIP2, and ZIP3; and subfamily (gufA) ZIP11. LIV-1 subfamily members are assigned into subgroups: subgroup (i) ZIP4 and ZIP12; subgroup (ii) ZIP8 and ZIP14; subgroup (iii) ZIP5, ZIP6, and ZIP10; and subgroup (iv) ZIP7 and ZIP13

highlights the importance of the ZIP family for human and vertebrate physiopathology. Other ZIP family proteins have been discovered in genome databases, with 14 members now identified (Fig. 3.1) (Eide 2004; Jeong and Eide 2013).

Homologs of the human ZIP family have also been found in the genome sequences of rats, mice, chickens, zebrafish, fruit flies, nematodes, yeast, and plants (*A. thaliana*) and are shown in Table 3.2. This table shows that all ZIP family members are highly conserved in mammals, as in the case of the ZNT family. ZIP family members are generally classified into four subfamilies, ZIP-I, ZIP-II, LIV-1, and gufA (Fig. 3.1) (see Sect. 3.4.2) (Dempski 2012; Gaither and Eide 2001; Kambe et al. 2004; Taylor and Nicholson 2003), based on their phylogenetic relationships. ZIP4 is essential for zinc absorption in mammals, but a homologous gene is not present in chickens.

A number of ZNT and ZIP transporters have been shown to be involved in human genetic disorders. Moreover, numerous mutant and knockout animals have been generated for most of their orthologues. The data in Tables 3.1 and 3.2 may prove to be useful for reorganizing the orthologue functions of both the protein families.

Table 3.1 Amino acid sequence similarity among ZNT family members found in representative model organisms

H. sapiens	*R. norvegicus*	*M. musculus*	*G. gallus*	*D. rerio*	*D. melanogaster*	*C. elegans*	*S. cerevisiae*	*A. thaliana*
ZNT1 (SLC30A1) 507 A.A.	455/511 (89%)	455/508 (89%)	379/532 (71%)	351/495 (70%)	252/474 (53%)	61/100 (61%)	–	–
NP_067017.2	NP_074044.1	NP_033605.1	BBM28213.1	NP_957173.1	NP_647801.1	NP_509095.1 (Cdf1)	–	–
ZNT2 (SLC30A2) 372 A.A.	334/376 (88%)	333/373 (89%)	300/374 (80%)	260/341 (76%)	120/167 (71%)	229/331 (69%)	176/315 (55%)	180/315 (57%)
NP_001004434.1	NP_001076591	NP_001034766.1	XP_003642614.2	NP_001038485.1	NP_609741.3	NP_510091 (Cdf2)	NP_014961 (Cot1p)	NP_191753.1 (AtMTP2)
ZNT3 (SLC30A3) 388 A.A.	355/388 (91%)	359/388 (92%)	236/321 (73%)	–	201/404 (49%)	–	-	181/359 (50%)
NP_003450.2	NP_001013261.1	NP_035903.2	XP_025004924.1	–	NP_723901.1	–	–	NP_850459.1 (AtMTP1)
ZNT4 (SLC30A4) 429 A.A.	407/430 (94%)	408/430 (94%)	362/437 (82%)	222/310 (71%)	210/371 (57%)	173/274 (63%)	159/353 (45%)	183/312 (58%)
NP_037441.2	NP_742063.1	NP_035904.2	NP_001280212.1	NP_956937.1	NP_610185.1	NP_499691.1 (ttm-1)[a]	NP_013970.1 (Zrc1p)	NP_191440.2 (AtMTP3)
ZNT5 (SLC30A5) 765 A.A.	715/744 (96%)	736/765 (96%)	714/775 (92%)	675/781 (86%)	–	424/730 (58%)	159/291 (54%)	215/399 (53%)
NP_075053.2	NP_001099874.1	NP_075023.2	NP_001026590.2	NP_001002322.1	–	NP_740931 (CDF5)	NP_010491 (Msc2p)	NP_178539.2 (AtMTP12)
ZNT6 (SLC30A6) 501 A.A	437/501 (87%)	439/501 (87%)	433/501 (86%)	375/511 (73%)	–	157/330 (47%)	27/80 (33%)	112/247 (45%)
NP_001180442.1	NP_001264208.1	NP_659047.2	NP_001006402.1	NP_991214.1	–	NP_001033385.1 (Toc1)[b]	NP_014437 (Zrg17p)[b]	NP_187817.2 (AtMTP5)[b]
ZNT7 (SLC30A7) 376 A.A	364/378 (96%)	365/378 (96%)	339/378 (89%)	316/387 (81%)	270/374 (72%)	–	–	169/269 (63%)
NP_598003.2	NP_001178644.1	NP_075703.1	NP_001008788.1	NP_001093556.1	NP_650049.1	–	–	NP_178539.2

ZNT8 (SLC30A8) 369 A.A	288/322 (89%)	337/369 (91%)	285/378 (75%)	243/351 (69%)	205/351 (58%)	–	–	173/339 (51%)
NP_7762 50.2	NP_001124010.1	NP_766404.1	XP_025003910.1	XP_021323959.1	NP_723732.2[c]	–	–	NP_180502.2 (AtMTP4)
ZNT10 (SLC30A10) 485 A.A	407/486 (83%)	410/485 (84%)	329/490 (67%)	259/422 (61%)	194/455 (42%)	–	–	–
NP_061183.2	NP_001099455.1	NP_001028458.1	XP_015139382.1	NP_001121706.2	NP_649233.2[d]	–	–	–
Not classified[b]						NP_509164[e]	NP_013902 (Mft1p)[f]	OAP07976 (ZAT1)
						NP_498611[e]	NP_015100 (Mft2p)[f]	NP_182304.2 (AtMTP6)
						NP_492028[e]		NP_564594.1 (AtMTP7)
						NP_001024066		NP_191365.2 (AtMTP8)[g]
						NP_509279		NP_178070.2 (AtMTP9)
								NP_173081.2 (AtMTP10)[g]
								NP_181477.1 (AtMTP11)[g]

ZNT protein members and their homologs in humans (*Homo sapiens*), rats (*Rattus norvegicus*), mice (*Mus musculus*), chickens (*Gallus gallus*), zebrafish (*Danio rerio*), fruit flies (*Drosophila melanogaster*), nematodes (*Caenorhabditis elegans*), yeast (*Saccharomyces cerevisiae*), and plants (*Arabidopsis thaliana*) are arranged according to the sequence homology using NCBI BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST/). The numbers indicate the similarity (positives, %) between the human sequence and the corresponding sequence, whose accessions are described below

[a]Located to the plasma membrane (Roh et al. 2013)

[b]Not found by sequence alignment but are assigned according to their functional homology

[c]Has a long extra N-terminal portion

[d]Has HNED motif instead of NDHD motif in the intramembranous metal binding site

[e]Shows weak similarity to yeast Mft1p and Mft2p

[f]Known to transport iron

[g]Known to transport manganese

Table 3.2 Amino acid sequence similarity among ZIP family members found in representative model organisms

H. sapiens	*R. norvegicus*	*M. musculus*	*G. gallus*	*D. rerio*	*D. melanogaster*	*C. elegans*	*S. cerevisiae*	*A. thaliana*
ZIP1 (SLC39A1) 324A.A.	313/324 (96%)	311/324 (95%)	141/246 (57%)	172/271 (63%)	149/295 (50%)	165/338 (48%)	133/393 (33%)	98/239 (41%)
NP_001258886.1	NP_001128049.1	NP_038929.2	XP_015154092.2	NP_997748.2	NP_610231.2	NP_493626.1 (ZIPT-1)	NP_011259.1 (Zrt1p)[a]	NP_567590.3 (IRT1)
								103/249 (41%) NP_180786.1
								111/267 (41%) NP_055252.2 (ZIP5)
								101/241 (41%) NP_178488.1
								104/245 (42%) OAO90686.1 (ZIP8)
								103/285 (36%) OAO97412.1 (ZIP9)
								82/176 (46%) NP_564766.1 (IRT3)
ZIP2 (SLC39A2) 309A.A.	262/309 (84%)	265/309 (85%)	–	–	148/334 (44%)	147/385 (38%)	137/429 (31%)	134/327 (40%)
NP_055394.2	NP_001100730.1	NP_001034765.2			NP_525107.1	NP_001026796.1 (ZIPT-2.1)	NP_013231.1 (Zrt2p)[a]	NP_001031670.1 (IRT2)
						150/370 (40%) NP_500517.1 (ZIPT-2.2)		135/359 (37%) NP_187881.1
						148/339 (43%) NP_496876.1 (ZIPT-2.3)		
						164/498 (32%) NP_001249972.1 (ZIPT-2.4)		

ZIP3 (SLC39A3) 314A.A.	292/317 (92%)	292/317 (92%)	277/315 (87%)	–	147/297 (49%)	146/377 (38%)	–	122/310 (39%)
NP_653165	NP_001008357.1	NP_598896.2	XP_015155452.1		NP_525107.1	NP_495126.1 (ZIPT-3)		OAP13793.1 (ZIP11)
ZIP4 (SLC39A4) 647A.A.	527/657 (80%)	524/661 (79%)	–	367/659 (55%)	–	–	–	–
NP_570901.2	NP_001071137.1	NP_082340.1		XP_017207743.1				
ZIP5 (SLC39A5) 540A.A.	468/539 (86%)	471/539 (87%)	300/525 (57%)	286/520 (55%)	–	–	–	–
NP_775867.2	NP_001102198.1	NP_082368.1	XP_025001341.1	XP_690258.3				
ZIP6 (SLC39A6) 755A.A.	694/760 (91%)	709/766 (92%)	580/762 (76%)	443/751 (58%)	305/757 (40%)	–	–	–
NP_036451.4	NP_001019916.1	NP_631882.2	XP_419071.2	NP_001001591.1	NP_001303364			
ZIP7 (SLC39A7) 469A.A.	416/475 (87%)	427/478 (89%)	–	271/405 (66%)	225/354 (63%)	191/348 (54%)	193/485 (39%)	179/373 (47%)
NP_008910.2	NP_001008885.1	NP_001071177.1		NP_571006.3	NP_524931.1 (Catsup)	NP_503070.2 (ZipT-7.1) 289/512 (56%) NP_510563.2 (ZIPT-7.2)	NP_012241.1 (yKE4p)	OAP17350.1 (IAR1)
ZIP8 (SLC39A8) 460A.A.	426/462 (92%)	431/462 (93%)	355/436 (81%)	289/452 (63%)	–	–	–	–
NP_001128618.1	NP_001011952.1	NP_080504.3	XP_427970.3	XP_009305480.1				
ZIP9 (SLC39A9) 307A.A.	260/266 (97%)	297/308 (96%)	292/307 (95%)	280/309 (90%)	201/310 (64%)	210/389 (53%)	142/361 (39%)	71/179 (39%)
NP_060845.2	NP_001030101.1	NP_080520.2	NP_001007934.1	NP_001013558.1	NP_651919.3	NP_506393.1 (ZIPT-9)	NP_014722.1 (Atx2p)	NP_187881.1
ZIP10 (SLC39A10) 831A.A.	764/834 (91%)	769/833 (92%)	669/863 (77%)	501/896 (55%)	272/526 (51%)	–	–	176/842 (20%)
NP_065075.1	NP_001102266.2	NP_766241.2	XP_426562.2	NP_956965.1	NP_523974.3 (FOI)			NP_174411.2 (ZIP10)

(continued)

Table 3.2 (continued)

H. sapiens	*R. norvegicus*	*M. musculus*	*G. gallus*	*D. rerio*	*D. melanogaster*	*C. elegans*	*S. cerevisiae*	*A. thaliana*
ZIP11 (SLC39A11) 342A.A.	306/342 (89%)	315/343 (91%)	276/342 (80%)	86/102 (84%)	230/346 (66%)	237/345 (68%)	151/526 (28%)	129/354 (36%)
NP_001153242.1	NP_001013060.1	NP_001159975.1	XP_024997602.1	XP_021335877.1	NP_610712.1	NP_491614.1 (ZIPT11)	NP_012746.1 (Zrt3p)	NP_001326769.1 (ZTP29)
ZIP12 (SLC39A12) 691A.A.	481/558 (86%)	583/693 (84%)	541/704 (76%)	–	–	200/743 (26%)	–	–
NP_001138667.1	NP_001099594.1	NP_001012305.1	XP_015137490.1			NP_503096.2 (ZIPT-17)		
ZIP13 (SLC39A13) 371A.A.	321/348 (92%)	346/371 (93%)	270/346 (78%)	233/327 (71%)	198/339 (58%)	180/412 (43%)	–	–
NP_509719.2 (ZIPT-13)	NP_001034285.1	NP_080997.1	NP_001008471.1	NP_001005306.3	NP_001189315	NP_001121697.2		
ZIP14 (SLC39A14) 492A.A.	441/487 (90%)	455/487 (93%)	417/457 (91%)	360/469 (76%)	189/406 (46%)	–	–	–
NP_001121903.1	NP_001100745.1	NP_659057.2	XP_024998709.1	NP_001313628.1	NP_001097608.1			

ZIP protein members and their homologs in humans (*Homo sapiens*), rats (*Rattus norvegicus*), mice (*Mus musculus*), chickens (*Gallus gallus*), zebrafish (*Danio rerio*), fruit flies (*Drosophila melanogaster*), nematodes (*Caenorhabditis elegans*), yeast (*Saccharomyces cerevisiae*), and plants (*Arabidopsis thaliana*) are arranged as in Table 3.1. The numbers indicate the similarity (positives, %) between the human sequence and the corresponding sequence, whose accessions are described below

[a]Zrt1p is a high-affinity while Zrp2p is a low-affinity zinc transporter

3.3 ZNT Transporters

3.3.1 Biochemical and Structural Properties of Bacterial ZNT Homologs

ZNT transporters function as zinc efflux proteins, transporting zinc from the cytosol into intracellular compartments or into the extracellular space. Based on high resolution structures of the bacterial homolog YiiP (*E. coli* and *S. oneidensis*), which belongs to Zn/Fe-CDF subfamily (Coudray et al. 2013; Gupta et al. 2014; Lopez-Redondo et al. 2018; Lu et al. 2009; Lu and Fu 2007), ZNT transporters are thought to form homodimers, enabling them to transport zinc across biological membranes. The structure of YiiP reveals a topology in which each protomer most likely has six transmembrane domains (TMDs) with cytosolic N- and C-termini, as predicted by hydrophobicity plots (Gaither and Eide 2001; Paulsen and Saier 1997). The TMDs are grouped into a compact four-helical bundle consisting of TMDs, I, II, IV, and V, and a two-helical pair outside the bundle, consisting of TMDs III and VI. The compact four-helical bundle creates a channel in which the intramembranous tetrahedral zinc-binding site of TMDs II and V is located. This zinc-binding site consists of one histidine (H) and three aspartic acid (D) residues (DDHD core motif). Homodimerization of YiiP is stabilized by intermolecular salt-bridges which ensure the correct orientation of TMDs III and VI by interlocking the TMDs at the dimer interface (Lu et al. 2009). Another zinc-binding site is formed in the cytosolic C-terminal region, which exhibits a binuclear zinc-coordination site. YiiP has a highly conserved metallochaperone-like structure, with a characteristic αββα structure despite a high degree of sequence variability (Cherezov et al. 2008; Higuchi et al. 2009; Uebe et al. 2018). Recently, a ZNT homolog lacking the C-terminal region was reported in a marine bacterium (Kolaj-Robin et al. 2015), suggesting that this region may not be required for zinc transport activity.

YiiP is functional as a proton-zinc exchanger, in which an alternative access mechanism is in operation. TMDs of YiiP can adopt cytosolic-facing (inward-facing) and periplasmic-facing (outward-facing) conformations, both of which can bind zinc or protons (Gupta et al. 2014; Lopez-Redondo et al. 2018). Zinc binding in the cytosolic C-terminal region may induce conformational changes in the TMDs, facilitating zinc transport by the alternative access mechanism (Coudray et al. 2013; Gupta et al. 2014; Lopez-Redondo et al. 2018) in which the extracellular proton provides a driving force for exporting zinc from the cytosol. This information provides the framework for exploring the biochemical and structural properties of ZNT transporters.

3.3.2 Properties of ZNT Transporter Proteins

ZNT family proteins are predicted to have a similar topology to YiiP and, therefore, are thought to form homodimers (Fukunaka et al. 2009; Golan et al. 2016; Itsumura et al., 2013; Lasry et al. 2014; Murgia et al. 2008). The conserved motif of (F/Y) G(W/Y/F)XRXE, which is positioned on the first cytosolic loop between TMDs II

and III, is thought to be involved in homodimer formation (Fig. 3.4) (Lasry et al. 2012). The conserved arginine (R) in the motif is thought to be involved in the formation of intermolecular salt-bridges on the cytoplasmic membrane surface which are important for zinc transport (Figs. 3.2 and 3.4) (Fukue et al. 2018). In addition to homodimer formation, ZNT5 and ZNT6 form heterodimers (Fukunaka et al. 2009; Golan et al. 2015; Lasry et al. 2014; Suzuki et al. 2005b). Other ZNT members could also form heterodimers (Golan et al. 2015; Zhao et al. 2016), the significance of which is unclear. Similar to YiiP, ZNT proteins are functional as zinc-proton exchangers (Ohana et al. 2009; Shusterman et al. 2014). This mechanism of zinc transport is reasonable, particularly in one subgroup (ZNT2, ZNT3, ZNT4, and ZNT8) (see Sect. 3.3.3.2) which are localized to acidic compartments, such as endosomes, lysosomes, or intracellular vesicles. The activity of ZNT proteins may be tunable and could be controlled by the lipid composition of the vesicles where they are localized (Merriman et al. 2016). Recent computational simulations, based on energy calculations, of the ZNT zinc permeation pathway shows a favorable zinc translocation via the alternative access mechanism, consistent with the model of YiiP (Golan et al. 2018)

Interestingly, the DDHD core motif of YiiP changes to HDHD within TMDs II and V in most of the ZNT members (Fig. 3.3). Numerous biochemical studies reveal that this core motif is essential for zinc transport, as substitution of histidine or aspartic acid residues to alanine (A) abolishes zinc transport (Fujimoto et al. 2013; Ohana et al. 2009; Tsuji et al. 2017). In addition, ZNT10 has an altered motif, NDHD, within TMDs II and V (Fig. 3.3), that enables ZNT10 to transport manganese (Leyva-Illades et al. 2014; Nishito et al. 2016) (see Sect. 3.3.3.1). Moreover, replacing the histidine residue in TMD II with aspartic acid (DDHD, *i.e.*, the YiiP motif) in ZNT5 and ZNT8 allows it to transport cadmium as well as zinc (Hoch et al. 2012). Thus, this position in TMD II, which constitutes the intramembranous zinc-binding site, is critical for regulating metal substrate specificity. ZNT6 has no zinc transport activity, with two amino acids of the HDHD motif replaced by hydrophobic residues (Fig. 3.4). Instead, ZNT6 is functional as an auxiliary protomer of

Fig. 3.4 (continued) external loops (EL), and C-terminal cytosolic α-helices and β-sheets based on the crystal structure of YiiP are indicated in yellow, pink, turquoise, and lavender, respectively. Residues highlighted in red indicate the histidine (H) and aspartic acid (D) residues constituting the intramembranous zinc-binding site. Residues highlighted in black and gray are highly conserved and semi-conserved, respectively. The asparagine residue (N) in TMD II of ZNT10, which has been speculated to be involved in the recognition of manganese, is indicated in green. Residues highlighted in light blue indicate the amino acid residues forming the cytosolic binuclear zinc-coordination site. Residues highlighted in blue indicate the highly conserved arginine (R) and well-conserved glutamic acid (E) or glutamine (Q) likely involved in the formation of salt-bridges on the cytosolic side. The amino acid sequences at the C-terminal end of ZNT6 (30 aa) and ZNT10 (4 aa) are not displayed in the alignment. The TMDs indicated in ZNT5 correspond to TMDs between X and XV. "-" denotes a gap in the alignment. Blue circles indicate the amino acid residues involved in zinc binding. The (F/Y)G(W/Y/F)XRXE sequence, which is proposed to be involved in dimerization, is indicated in lavender. Blue, green, red, or honey circles below the sequences indicate the amino acid residues involved in zinc binding (different color means the coordination of different zinc ions, and honey color means the coordination of zinc ion in the neighboring subunit). This figure is used and modified from Kambe et al. 2014 with permission

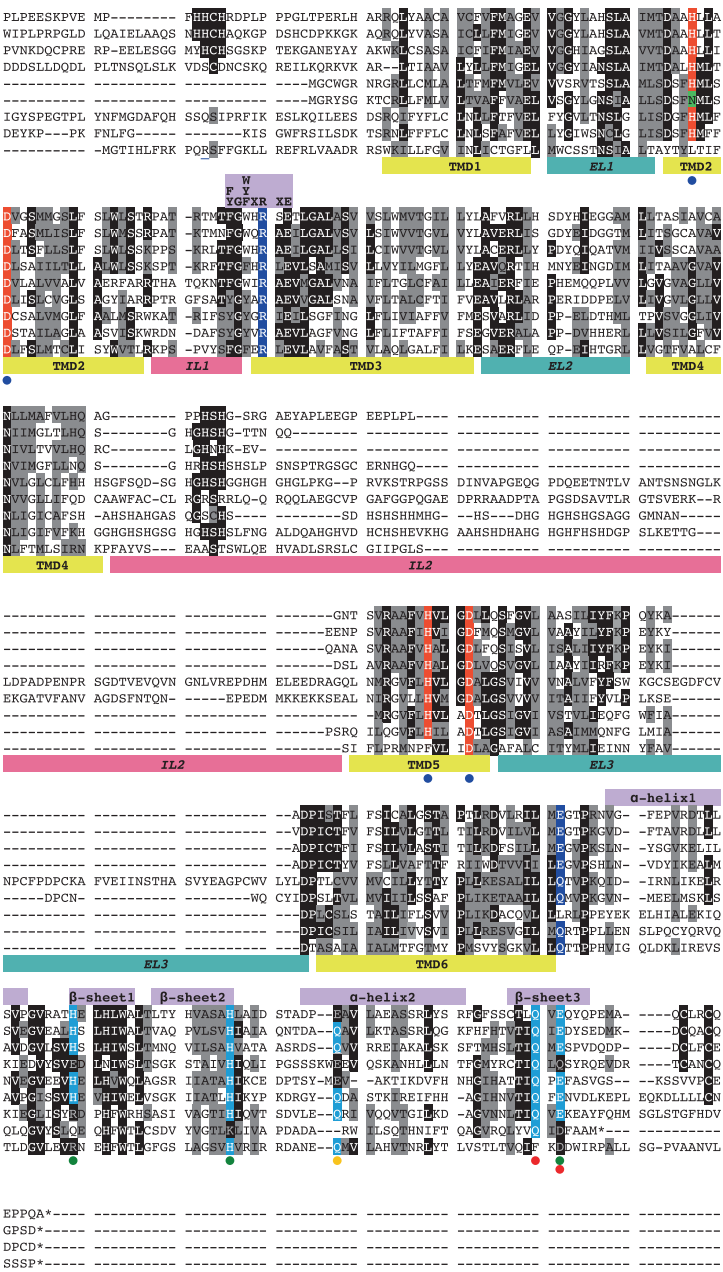

Fig. 3.4 Sequence alignment of ZNT family proteins. The alignment is ordered according to similarities among subfamilies. The putative transmembrane domains (TMD), intracel lular loops (IL),

the ZNT5 and ZNT6 heterodimer (Fukunaka et al. 2009) (see Sect. 3.3.3.4). Interestingly, several plant ZNT homologs (*e.g.*, MTP8, MTP9, and MTP11), which are known to be manganese transporters and are grouped within the Mn-CDF subfamily of CDF proteins (Gustin et al. 2011; Pedas et al. 2014; Tsunemitsu et al. 2018; Ueno et al. 2015), have a DDDD core motif. No orthologues have been found in vertebrate genomes (see Table 3.1).

Due to a lack of sequence similarity with YiiP, several unique features of ZNT transporter proteins have been determined in a number of biochemical studies. One such feature is the unique cytosolic histidine-rich loop with variable lengths between TMDs IV and V, which is also found in the plant ZNT homologs (Blindauer and Schmid 2010; Kambe et al. 2014). The histidine-rich loop was thought to be important for zinc transport or as a sensor for cytosolic zinc levels by coordinating cytosolic zinc through its histidine residues (Arus et al. 2013; Kawachi et al. 2008; Suzuki et al. 2005b; Tanaka et al. 2015; Tanaka et al. 2013). However, a ZNT mutant, in which all histidine residues in the loop are mutated to alanine, still possesses zinc transport activity, although this activity is decreased, indicating that the histidine residues are not essential for zinc transport (Fukue et al. 2018). Another unique feature of the ZNT proteins, in comparison to YiiP, is the role of the cytosolic N-terminal region, which, in YiiP, is too short to study. A recent study indicates that ZNT members are likely to be functional even when the N-terminal is absent, although this region could regulate zinc transport via an interaction with the cytosolic histidine-rich loop (Fukue et al. 2018). Thus far, the functions of the N-terminal region are reported as a mitochondrial sorting motif (Seo et al. 2011), a zinc binding (sensor) motif (Arus et al. 2013), and a potential protein–protein interaction motif resembling the leucine zipper motif (Murgia et al. 1999), in addition to participating in the regulation of zinc transport (Kawachi et al. 2012). Interestingly, ZNT5 has a uniquely long N-terminal sequence containing nine potential TMDs (Kambe et al. 2002), however its functional importance is not yet known.

The nine ZNT members described above all belong to the Zn-CDF subfamily of CDF proteins (Kambe 2012; Montanini et al. 2007). Further groupings, based on sequence similarity, subdivide them into four groups: (i) ZNT1 and ZNT10, (ii) ZNT2, ZNT3, ZNT4, and ZNT8, (iii) ZNT5 and ZNT7, and (iv) ZNT6 (Gustin et al. 2011; Kambe 2012; Kambe et al. 2006) (Figs. 3.1 and 3.3). Subgroup (i) contains the cell surface localized ZNTs, although, despite it belonging to the Zn-CDF subfamily, ZNT10 transports manganese. Subgroup (ii) contains transporters involved in intracellular compartments and vesicles, while subgroup (iii) contains transporters involved in zinc transport in the early secretory pathway. Subgroup (iv) contains only ZNT6, which is functional as an auxiliary protomer without zinc transport activity, as described above.

3.3.3 Biochemical Characterization of the ZNT Subgroups

Here, we provide a brief summary of each of the ZNT subgroups. For more detailed information about their physiopathological functions, we refer the reader to the following reviews, Bowers and Srai (2018), Hara et al. (2017), and Kambe et al. (2015). Additionally, the details of mice phenotypes have been described in other chapters of this book.

3.3.3.1 ZNT1 and ZNT10 Subgroup

Both ZNT1 and ZNT10 are known to be functional at the plasma membrane as efflux transporters of cytosolic zinc and manganese, respectively (see above) (Palmiter and Findley 1995; Leyva-Illades et al. 2014; Nishito and Kambe 2019), although their intracellular localization is also reported. As mentioned above, their difference in metal substrate specificity is due to differences in their metal binding motifs (HDHD in ZNT1, NDHD in ZNT10). ZNT1 expression increases in response to excess zinc through binding of metal-response element-binding transcription factor-1 (MTF-1) to the metal response element (MRE) in its promoter, in a fashion similar to metallothionein (Langmade et al. 2000). This is consistent with its cellular function, which is to reduce the toxicity of excess zinc. In contrast, there are no reports describing manganese-induced expression of ZNT10, although such regulation would be important as mutations of *SL C30A10/ZNT10* gene result in parkinsonism with hypermanganesemia (Quadri et al. 2012; Tuschl et al. 2012). ZNT1 is thought to be mostly localized to the basolateral membrane in polarized cells (McMahon and Cousins 1998), while ZNT10 is reported to be localized to the apical membrane (Taylor et al. 2019). Overall, they have a sequence similarity of 37% (Nishito et al. 2016), therefore, the differing amino acids may be responsible for their unique subcellular localizations, although the regulatory mechanisms for their trafficking to the cell surface have not yet been elucidated. Both have a very short cytosolic N-terminal region (Fukue et al. 2018; Kambe et al. 2014) (Fig. 3.3). Recently, ZNT10 was reported to be functional as a manganese-calcium exchanger (Levy et al. 2019), the mode of which needs to be further investigated.

3.3.3.2 ZNT2, ZNT3, ZNT4, and ZNT8 Subgroup

These ZNT subgroup members are localized to the membranes of cytosolic secretory vesicles and play essential roles in transporting zinc into their lumens (Hennigar and Kelleher 2012; Kambe 2011). This subgroup functions as zinc-proton exchangers and is able to do so due to the acidic environment of the vesicular lumen they transport to. Apart from ZNT4, these transporters are expressed in a tissue-

specific manner. Zinc transport into cytosolic secretory vesicles is crucial as a high concentration of zinc, which is required for many physiological responses. Examples include the insulin granules in pancreatic β cells, which accumulate high levels of zinc mediated by ZNT8, and the presynaptic vesicles in a subset of glutamatergic neurons, which also accumulate high levels of zinc mediated by ZNT3. This zinc accumulation is essential for good health as single nucleotide polymorphisms in *SLC30A8/ZNT8* are shown to be associated with an increased susceptibility to type 2 diabetes (see Chap. 12), while ZNT3 is crucial for neuronal activity (see Chaps. 9 and 11). High levels of zinc in breast milk are secreted through the vesicles of the secretory mammary epithelial cells (Lee and Kelleher 2016), which in humans is mediated by ZNT2. Thus, mutations of *SLC30A2/ZNT2* gene cause *transient neonatal zinc deficiency*, another inherited zinc deficiency in humans (Golan et al. 2017). A similar phenotype (*i.e.*, low levels of zinc secretion) was observed in mice with a loss of function Znt4 mutant (McCormick et al. 2016). ZNT2 plays a role in zinc transport into the cell granules of Paneth cells in the crypts of Lieberkühn of the small intestine (Podany et al. 2016). There are other vesicles in the body which accumulate high levels of zinc, such as cytosolic vesicles in epithelial cells of the lateral prostate, pigment epithelial cells in the retina, and mast cells (Kambe et al. 2014), whose zinc transport may also be controlled by ZNT family members within this subgroup. ZNT4 has been shown to localize to endosomes, lysosomes, and the *trans*-Golgi network, in addition to cytosolic vesicles. Consistent with this, ZNT4 is likely to play other roles, including in the regulation of several enzymes (McCormick and Kelleher 2012; Tsuji et al. 2017).

3.3.3.3 ZNT5 and ZNT7 Subgroup

ZNT5 and ZNT7 are localized to the early secretory pathway, including the endoplasmic reticulum (ER) and the Golgi apparatus, both of which require efficient zinc transport. Impairment of zinc transport by both ZNT5 and ZNT7 most likely results in the misfolding or incomplete assembly of some zinc-containing proteins. This leads to a decrease in the activity of zinc-dependent ectoenzymes and zinc-dependent chaperone proteins that monitor and assist protein folding, which can trigger the unfolded protein response (UPR) (Ellis et al. 2004; Fukunaka et al. 2011; Ishihara et al. 2006; Takeda et al. 2018). Thus, both ZNTs are important for homeostasis in the early secretory pathway by mediating the zinc supply to the nascent proteins (Kambe et al. 2016, 2017). Consistent with this, the expression of *ZNT5* mRNA is transcriptionally upregulated by the UPR (Ishihara et al. 2006). Both ZNT5 and ZNT7 have a unique di-proline motif (PP-motif) in luminal loop 2, which is essential for the activation of ectoenzymes, such as alkaline phosphatases (Fujimoto et al. 2016). This unique motif is highly conserved in most orthologues suggesting an important role in enzyme activation.

3.3.3.4 ZNT6 Subgroup

Co-immunoprecipitation, bimolecular fluorescence complementation, and genetic experiments show the heterodimerization of ZNT6 and ZNT5 (Fukunaka et al. 2009; Lasry et al. 2014; Suzuki et al. 2005a). The heterodimer formation of ZNT5 and ZNT6 is a unique feature among the ZNT family and is highly conserved among ZNT5 and ZNT6 functional orthologues, such as those in nematode (Cdf5 and Toc1) (Fujimoto et al. 2016), yeast (Msc2p and Zrg17p in *S. cerevisiae*, and Cis4 and Zrg17 in *Schizosaccharomyces pombe*) (Choi et al. 2018; Ellis et al. 2005; Ellis et al. 2004), and plant (MTP12 and MTP5 in *A. thaliana*) (Fujiwara et al., 2015). Each of these functional orthologues is also localized to the early secretory pathway. Considering the conservation of heterodimer formation, it is interesting to note that the fruit fly does not possess orthologues of ZNT5 and ZNT6 (see Table 3.1), which may suggest a unique regulatory mechanism of zinc transport and physiology in the fruit fly.

3.4 ZIP Transporters

3.4.1 Biochemical and Structural Properties of Bacterial ZIP Homologs

As with ZNT proteins, recent structural studies reveal that ZIP homologs (*Bordetella bronchiseptica* ZIP (BbZIP)) form homodimers. These studies reveal that each protomer has eight TMDs with extracellular N- and C-termini (Zhang et al. 2017), as predicted in ZIP family proteins (Gaither and Eide 2001; Kambe et al. 2004). BbZIP has a novel 3+2+3 transmembrane architecture, in which the first three TMDs (TMDs I to III) are symmetrically related to the last three TMDs (TMDs VI to VIII) by a pseudo-twofold axis. The TMDs IV and V, which are symmetrically related by the same axis, are sandwiched between the three TMD repeats (Zhang et al. 2017). BbZIP has a binuclear metal center, which coordinates zinc and is formed by several conserved amphipathic amino acid residues, including histidine, asparagine (N), and aspartic acid, within TMD IV, and two histidine residues and one glutamic acid (E) in TMD V (Figs. 3.2 and 3.5). The latter amino acids correspond to those in the potential metalloprotease motif (HEXPHEXGD) of LIV-1 subfamily (see 3.4.4). An in vitro study using reconstituted proteoliposomes proposes that the zinc transport of BbZIP (reported as ZIPB (Lin et al. 2010)) may be by a selective electrodiffusional channel. However, a definitive zinc transport mechanism for ZIP family proteins has not yet been clarified.

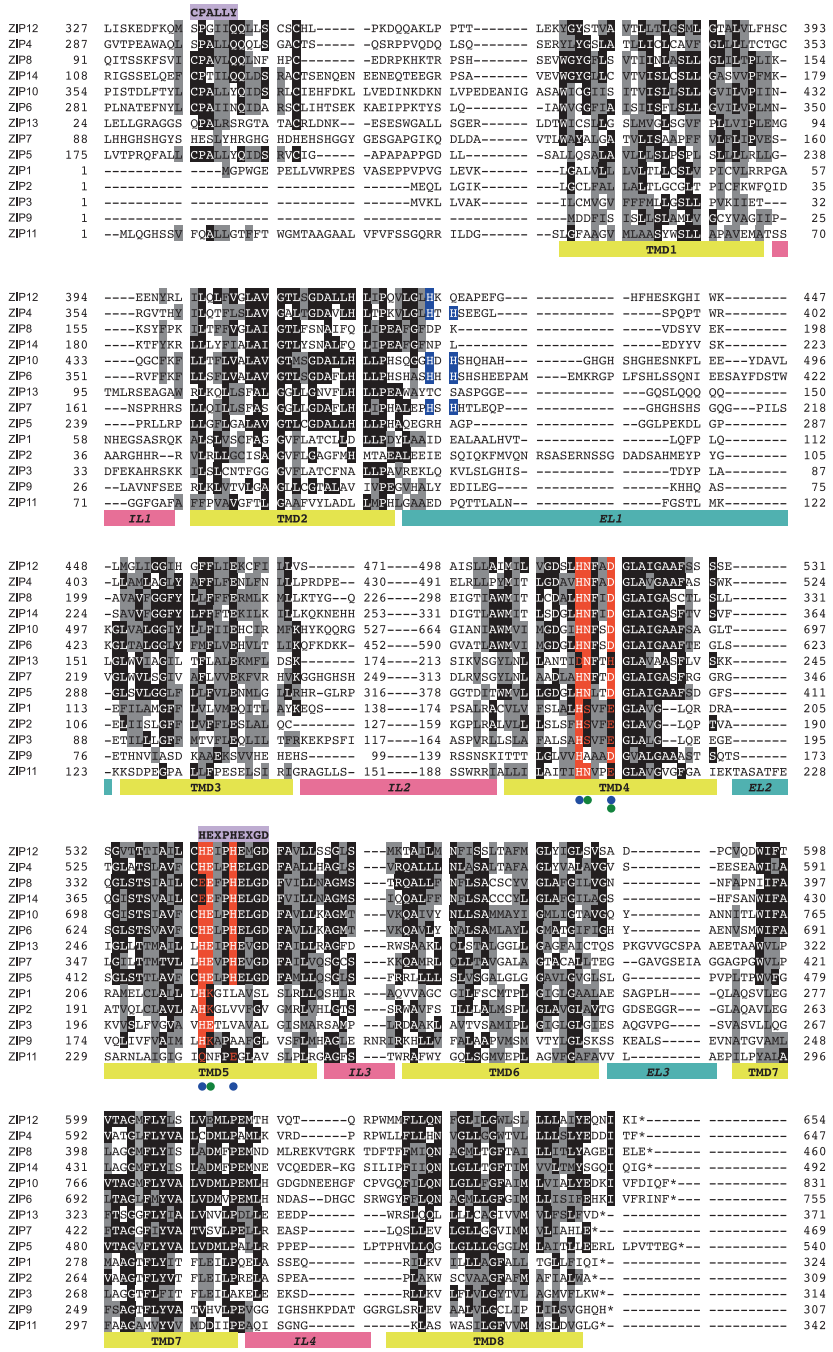

Fig. 3.5 Sequence alignment of ZIP family proteins. The alignment is ordered according to similarities among subfamilies. The putative transmembrane domains (TMD), intracellular loops (IL),

3.4.2 *Properties of ZIP Family Proteins*

Proteins of the ZIP family form homodimers which enable them to transport zinc across membranes (Bin et al. 2011), as shown in BbZIP (Lin et al. 2010; Zhang et al. 2017). Moreover, recent studies show that they can also form heterodimers (Taylor et al. 2016) (see Sect. 3.4.4.3). ZIP family proteins are thought to have eight TMDs, in which the N- and C-terminal regions are located outside the plasma membrane or in the lumen of intracellular compartments, as predicted by hydrophobicity plots (Taylor and Nicholson 2003) and computational modeling (Antala et al. 2015). This predicted topology is consistent with that of BbZIP (Zhang et al. 2017). The region, which is conserved the most among the ZIP family, is found in TMDs IV and V where numerous amphipathic amino acids, including a conserved histidine, asparagine, aspartic acid, and glutamic acid residues, are found (Fig. 3.2). These amino acid residues constitute an intramembranous binuclear zinc-binding site, and thus form a pore through which zinc passes, as shown in the BbZIP structure. A symport mechanism, whereby zinc is transported alongside bicarbonate ions, is suggested (Gaither and Eide 2000; Girijashanker et al. 2008) but has not been confirmed experimentally (Franz et al. 2018). Recently, a proton-mediated regulatory mechanism was proposed for regulating the velocity and directionality of zinc transport by the ZIP family (Franz et al. 2018; Franz et al. 2014). Most ZIP family proteins have a variable cytosolic loop that is rich in histidine residues between TMDs III and IV (Blindauer and Schmid 2010; Kambe et al. 2015). Its physiological function has not yet been elucidated but it may play a regulatory role in trafficking (Bowers and Srai 2018; Huang and Kirschke 2007) or contribute to binding and sensing of cytosolic zinc (Bafaro et al. 2015), as suggested for the ZNT family. Moreover, histidine residues within the loop may have a unique function, as complete substitution of all histidine residues to alanine in ZIP4 causes a loss of zinc-induced ubiquitination and degradation, although this has no effect on zinc-stimulated endocytosis (Mao et al. 2007). The histidine residues (HXH motif) in the extracellular loop between TMD II and III, which are conserved in a number of ZIP family members, are required for zinc sensing and zinc-induced endocytosis of ZIP4 (Chun et al. 2018) (Fig. 3.5). These results suggest that endocytosis of ZIP proteins, in

Fig. 3.5 (continued) and external loops (EL) are shown below the alignment in yellow, pink, and turquoise, respectively. Residues highlighted in black and gray are highly conserved and semi-conserved, respectively. Residues highlighted in red indicate the positions of residues important for zinc binding. Residues highlighted in blue indicate the positions of residues likely required for zinc sensing and zinc-induced endocytosis of a number of LIV-1 members. Conserved sequences in ZIP family proteins (PAL and HEXPHEXGD motifs) are indicated in lavender. The amino acid sequences of the N-terminal region of the LIV-1 subfamily members (the first nine proteins) are not displayed in the alignment, and the IL2 loop between TMD3 and TMD4 is omitted from the figure. "-" denotes a gap in the alignment. Blue or green circles below the sequences indicate the amino acid residues involved in zinc binding (different color means the coordination of different zinc ions). This figure is used and modified from Kambe et al. 2014 with permission

response to excess zinc, is mediated via zinc binding to conserved sensory sequences. The long extracellular region of some ZIP family members is known to be cleaved during zinc deficiency and in response to other stimuli (Ehsani et al. 2012; Hogstrand et al. 2013; Kambe and Andrews 2009), which may be important for the regulation of their zinc transport activity or their cellular trafficking.

As described above, ZIP family members are classified into four subfamilies based on their phylogenetic relationships (Gaither and Eide 2001; Kambe et al. 2004). The 14 mammalian members are classified as ZIP-I (ZIP9), ZIP-II (ZIP1-ZIP3), gufA (ZIP11), and LIV-1 (ZIP4–8, ZIP10, ZIP12-ZIP14) (Figs. 3.1 and 3.3) (Dempski 2012; Jeong and Eide 2013; Taylor and Nicholson 2003). Among them, the LIV-1 subfamily, whose name arises from its original identification in breast cancer, is the largest. LIV-1 subfamily members have a potential metalloprotease motif (HEXPHEXGD) in TMD V and an extracellular CPALLY motif (hereafter referred to as a PAL motif, see below) immediately preceding TMD I (Taylor et al., 2007) (Figs. 3.2 and 3.5). LIV-1 subfamily proteins are further divided into four subgroups: (i) ZIP4 and ZIP12; (ii) ZIP8 and ZIP14; (iii) ZIP5, ZIP6, and ZIP10; and (iv) ZIP7 and ZIP13 (Kambe et al. 2006; Zhang et al. 2016) (Figs. 3.1 and 3.3), each of which has unique sequence similarities. ZIP4 and ZIP12 of subgroup (i) possess a helix rich domain (HRD) in addition to the PAL motif-domain in the extracellular N-terminal region (Zhang et al. 2016). Subgroup (ii) members have a unique ability to transport manganese and iron, in addition to zinc. Subgroup (iii) contains ZIPs whose extracellular region, proximal to the membrane, has a unique domain called a prion fold. Subgroup (iv) contains members which are localized to the early secretory pathway and transport zinc from the lumen to the cytosol.

3.4.3 Biochemical Characterization of the ZIP Subfamilies

Here, we provide a brief summary of each of the ZIP subfamilies with the exception of LIV-1 which is further discussed in Sect. 3.4.4. For a more detailed discussion about the physiopathological functions of ZIP families, we refer the reader to the following reviews, Bowers and Srai (2018), Hara et al. (2017), and Kambe et al. (2015). Additionally, the details of mice phenotypes have been described in other chapters of this book.

3.4.3.1 ZIP-I Subfamily

ZIP9 is the only member belonging to this subfamily in vertebrates (Matsuura et al. 2009). ZIP9 is described as a dual-functioning protein because in addition to transporting zinc across cellular membranes, it can also function as a high affinity membrane androgen receptor through which testosterone activates G proteins thereby inducing cell signaling (Thomas et al. 2014). Thus, steroid and zinc signaling pathways cooperate to regulate physiological functions in mammalian cells through ZIP9 (Thomas et al. 2018). ZIP9 is localized to the plasma membrane and

intracellular compartments, such as the Golgi (Berg et al. 2014; Matsuura et al. 2009). It may regulate its function by altering its subcellular localization.

3.4.3.2 ZIP–II Subfamily

ZIP-II subfamily includes ZIP1, ZIP2, and ZIP3. These proteins are homologous to each other, both in amino acid sequence and gene structure. In particular, regions of TMD IV are highly conserved (Dufner-Beattie et al. 2003; Kambe et al. 2014), with the aspartic acid residue observed in LIV-1 subfamily, which is expected to form a binuclear metal center, replaced by glutamic acid (Fig. 3.5). Moreover, the highly conserved histidine in TMD V, which is also expected to form a binuclear metal center, is replaced by a hydrophobic amino acid (valine (V) or leucine (L)) (Fig. 3.5), suggesting that the mechanism of zinc coordination in this subfamily may be different from other ZIP homologs including LIV-1 subfamily. Consistent with their homology, the ZIP-II subfamily do have similar zinc transport mechanisms (Dufner-Beattie et al. 2003). However, their expression is differentially regulated in a tissue-specific manner. ZIP1 has been extensively investigated and its endocytosis and degradation are shown to be mediated through a di-leucine motif (LL motif, not shown in Fig. 3.5) within the variable cytosolic loop between TMD III and IV (Huang and Kirschke 2007). This motif is not conserved in ZIP2 and ZIP3. Mice containing knockouts of each individual gene show zinc-sensitive phenotypes during pregnancy, similar to the phenotypes observed in triple knockout mice (Dufner-Beattie et al. 2006; Kambe et al. 2008; Peters et al. 2007). This suggests that each protein has a unique cell-specific function that is required for adaptation to a zinc deficiency during development.

3.4.3.3 gufA Subfamily

ZIP11 is the only vertebrate protein of the gufA subfamily. This subfamily includes the bacterial and archaeal ZupT (Yu et al. 2013) and *S. cerevisiae* Zrt3p, suggesting that the gufA subfamily proteins arose from an ancient zinc transporter. The biological functions or expression profiles of gufA subfamily members remains unclear, although its subcellular localization to the nucleus or Golgi apparatus is reported (Kelleher et al. 2012; Martin et al. 2013). Interestingly, ZIP11 almost lacks histidine and cysteine residues, which suggests that ZIP11 binds zinc in a different manner than other ZIP family members (Kambe et al. 2015; Yu et al. 2013).

3.4.4 Biochemical Characterization of LIV-1 Subfamily

As mentioned above, LIV-1 subfamily members are further divided into four subgroups, (i) ZIP4 and ZIP12, (ii) ZIP8 and ZIP14, (iii) ZIP5, ZIP6, and ZIP10, and (iv) ZIP7 and ZIP13 (Kambe et al. 2006; Zhang et al. 2016). Recent reports indicate

unique physiopathological roles for each of the members, this is discussed in detail in other reviews (Bowers and Srai 2018; Hara et al. 2017; Kambe et al. 2015) and in other chapters of this book. Here, we provide a brief summary of each of the LIV-1 subgroups.

3.4.4.1 ZIP4 and ZIP12 Subgroup

While both ZIP4 and ZIP12 contain HRD and PAL motif-domains in the extracellular N-terminal region (Zhang et al. 2016), their physiological functions are very different. The biochemistry, physiopathology, and structure of ZIP4 have been extensively studied, while much less is known about ZIP12. ZIP12 has been identified as an important molecule for neurulation and neurite extension (Chowanadisai et al. 2013), and as a major regulator of hypoxia-induced pulmonary vascular remodeling (Zhao et al. 2015). The long extracellular N-terminal region of ZIP4 forms a homodimer without the need for TMDs (Zhang et al. 2017), in which the PAL motif is crucial (Zhang et al. 2016). This region is cleaved during zinc deficiency, raising the question as to how the cleavage is regulated. ZIP4 protein expression is regulated in response to zinc levels, while the regulation of ZIP12 expression has not yet been elucidated. Overall sequence similarity between ZIP4 and ZIP12 is 46%.

3.4.4.2 ZIP8 and ZIP14 Subgroup

A prominent feature of this subgroup is that both proteins can transport iron and manganese as well as zinc (Aydemir and Cousins 2018; Jenkitkasemwong et al. 2012). Their involvement in iron and manganese transport is crucial as mutations within both genes result in disease, such as severe dysglycosylation due to a type II congenital disorder of glycosylation (CDG) (Boycott et al. 2015; Park et al. 2015) or childhood-onset parkinsonism-dystonia (Tuschl et al. 2016). Their unique metal-binding specificities are most likely related to their sequences; ZIP8 and ZIP14 have glutamic acid in place of histidine in the HEXPHEXGD motif of TMD V (i.e., EEXPHELGD in both) (Fig. 3.5). However, this has not yet been tested at the molecular level. Both proteins are primarily localized to the plasma membrane, with some reports suggesting that their subcellular localization includes endosomes and lysosomes (Aydemir et al. 2009; Guthrie et al. 2015). Their physiopathological importance for zinc physiology is reported in many articles (Hojyo et al. 2011; Kim et al. 2014; Liu et al. 2013; Liuzzi et al. 2005).

3.4.4.3 ZIP5, ZIP6, and ZIP10 Subgroup

These three ZIPs have a unique extracellular domain in the region proximal to the membrane, called a prion fold, which may indicate an evolutionary link between them and prion proteins (Ehsani et al. 2011; Ehsani et al. 2012; Pocanschi et al. 2013).

This may also indicate their involvement in the etiology of prion diseases. Among these three, ZIP6 and ZIP10 share the highest homology and have been shown to form functional heterodimers in an interactome analysis (Brethour et al. 2017; Taylor et al. 2016), which could explain the overlap in ZIP6- and ZIP10-dependent phenotypes, such as cancer development and metastasis (Brethour et al. 2017) or oocyte-to-embryo transitions (Kong et al. 2014). An important aspect is that *Zip10*-knockout mice show significant phenotypes (Bin et al. 2017b; Gao et al. 2017; Hojyo et al. 2014; Miyai et al. 2014), which seem to be independent of ZIP6. In contrast to their extended expression, ZIP5 is expressed in a tissue-specific manner and is uniquely localized to the basolateral membrane in polarized cells, such as intestinal epithelial cells (Dufner-Beattie et al. 2004; Wang et al. 2004).

3.4.4.4 ZIP7 and ZIP13 Subgroup

The N-terminal region of this subgroup is shorter than those of other LIV-1 subfamily members, therefore sequence alignment of their PAL motifs is difficult (Fig. 3.5). Compared with other LIV-1 subfamily members, both proteins are uniquely localized to the early secretory pathway, including the ER and Golgi apparatus. Zinc release from the early secretory pathway, mediated by these proteins, plays an important role in regulating cell signaling (Fukada et al. 2008; Fukunaka et al. 2017; Nimmanon et al. 2017; Taylor et al. 2012; Tuncay et al. 2017). Moreover, their zinc transport activity contributes to the homeostatic maintenance of the secretory pathway (Bin et al. 2017a; Jeong et al. 2012; Ohashi et al. 2016). Notably, ZIP13 and its orthologues have a unique zinc coordination motif in TMD IV, in which the conserved histidine is replaced by aspartic acid (Dxx**H**NFx**D** sequence changes to Nxx**D**NFx**H**) (Xiao et al. 2014) (Fig. 3.5). Considering the evidence for ZNT1 and ZNT10 subgroup (see Sect. 3.3.3.1) and ZIP8 and ZIP14 subgroup in the LIV-1 subfamily (see Sect. 3.4.4.2), this change may alter the metal substrate specificity. Interestingly, the ZIP13 orthologue in the fruit fly is reported as an iron transporter, which mediates iron transport from the cytosol to the lumen of the ER or that of the Golgi (Xiao et al. 2019; Xiao et al. 2014), which is the opposite direction assumed for mammalian ZIP13. These findings raise the necessity for further investigation in ZIP13.

Compared with ZIP13, ZIP7 is conserved within other species (Table 3.2). Their physiological functions are extremely diverse but are related to the early secretory pathway, in particular the ER, and have been shown in yeast (yKE4p), drosophila (Catsup), nematode (ZipT-7.1), and plant (IAR1) (Groth et al. 2013; Kumanovics et al. 2006; Lasswell et al. 2000; Zhao et al. 2018), indicating its biological significance. The histidine-rich sequence in the N-terminal region is highly conserved among ZIP7 orthologues (Adulcikas et al 2018), suggesting that a unique zinc-sensing mechanism may be used. In mammals, ZIP7 expression is upregulated by the UPR and its loss results in disruption of ER functions (Ohashi et al. 2016; Tuncay et al. 2017), similar to ZNT5 and ZNT7 (see Sect. 3.3.3.3) (Ishihara et al.

2006; Tuncay et al. 2017). Thus, luminal zinc homeostasis in the early secretory pathway is regulated by both ZIPs and ZNTs.

3.5 Concluding Remarks and Perspectives

A number of studies reveal that many ZIP and ZNT family proteins are involved in human genetic diseases. Moreover, various phenotypes of numerous knockout mice unveil the fundamental importance of ZNTs and ZIPs (see other chapters). This chapter summarized the biochemical features of both protein families, with a particular focus on their biological subgroupings. To our knowledge, this is the first time such a review has been attempted and is therefore useful in providing a comprehensive overview of both ZNT and ZIP families. Future studies should aim to elucidate the molecular mechanisms that enable both families to control their spatiotemporal zinc transport. These studies would be facilitated by a comprehensive resource in which their classification and subgroups are described. This is required in order to further understand zinc signaling in physiopathological processes.

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