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Family reunion – The ZIP/prion gene family

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ABSTRACT

Prion diseases are fatal neurodegenerative diseases of humans and animals which, in addition to sporadic and familial modes of manifestation, can be acquired via an infectious route of propagation. In disease, the prion protein (PrP^{C}) undergoes a structural transition to its disease-causing form (PrP^{Sc}) with profoundly different physicochemical properties. Surprisingly, despite intense interest in the prion protein, its function in the context of other cellular activities has largely remained elusive. We recently employed quantitative mass spectrometry to characterize the interactome of the prion protein in a murine neuroblastoma cell line (N2a), an established cell model for prion replication. Extensive bioinformatic analyses subsequently established an evolutionary link between the prion gene family and the family of ZIP (Zrt-, Irt-like protein) metal ion transporters. More specifically, sequence alignments, structural threading data and multiple additional pieces of evidence placed a ZIP5/ZIP6/ZIP10-like ancestor gene at the root of the PrP gene family. In this review we examine the biology of prion proteins and ZIP transporters from the viewpoint of a shared phylogenetic origin. We summarize and compare available data that shed light on genetics, function, expression, signaling, post-translational modifications and metal binding preferences of PrP and ZIP family members. Finally, we explore data indicative of retropositional origins of the prion gene founder and discuss a possible function for the prion-like (PL) domain within ZIP transporters. While throughout the article emphasis is placed on ZIP proteins, the intent is to highlight connections between PrP and ZIP transporters and uncover promising directions for future research.

Contents

Abbreviations: AE, acrodermatitis enteropathica; CDF, cation diffusion facilitator; CFC, cysteine-flanked core; CTD, carboxy-terminal domain; Dm, *Drosophila melanogaster*; Dpl, Doppel; Dr, *Danio rerio*; EMT, epithelial-to-mesenchymal transition; Esg, Escargot; GPI, glycosylphosphatidylinositol; LZT, LIV-1 subfamily of ZIP zinc transporters; Mm, *Mus musculus*; ORF, open reading frame; PL, prion-like; PrP^{C}, cellular prion protein; Sho, Shadoo; SLC, solute carrier; Ta, *Trichoplax adhaerens*; TM, transmembrane; Tr, *Takifugu rubripes*; ZIP, Zrt-, Irt-like protein.

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1. Introduction

We recently proposed an evolutionary link between prion genes and ZIP metal ion transporters (Schmitt-Ulms et al., 2009), thereby ending the 'orphan' status of vertebrate prion genes and merging two protein families that until then had no explicit connection to each other. Due to its significance for a range of fatal human and livestock diseases, the prion protein has been under intense scrutiny for more than twenty-five years, during which more than 10,000 articles have been published on the subject. In contrast, very few reports on ZIP proteins appeared before 1996 (Eide et al., 1996; Manning et al., 1994; Masiakowski et al., 1982; Zhao and Eide, 1996a) and to this day the literature on ZIP proteins has remained comparably small, with approximately 100 articles published. Many updates on the *status quo* of prion science have been disseminated throughout the years and some of these reviews provide a wealth of information on the topic (Aguzzi et al., 2008; Prusiner, 1998). A number of insightful review articles have also covered the gene family of ZIP transporters and their proposed connections to cancer biology (Taylor et al., 2007; Taylor and Nicholson, 2003). Particular attention has been paid to ZIP4, a ZIP paralog genetically linked to acrodermatitis enteropathica (AE) (Kury et al., 2002; Wang et al., 2002), an inherited childhood zinc-deficiency disorder (Moynahan, 1974).

The intent of this review is to provide a fresh look at the biology of prion proteins and ZIP transporters from the perspective of their phylogenetic relatedness. Thus, the emphasis of this article will be on a comparison of aspects of genetics, biochemistry and protein biology not covered, or only touched upon, in our initial report (Schmitt-Ulms et al., 2009). In light of both the sheer volume of published work on the prion protein and the considerable diversity of ZIP transporters, we decided to highlight in particular ZIPs 5, 6 and 10. This restricts the literature review to topics that, in our mind, warrant particular scrutiny for ongoing efforts aimed at shedding light on the significance of the relationship between prion and ZIP genes in health and disease. These topics are: the (1) diversification, (2) function, (3) spatial distribution and (4) regulation of protein expression of ZIP/prion gene family members, a review of (5) post-translational modifications and (6) N-terminal repeat sequences and their metal binding specificities, and, finally, (7) a discussion of the origins of a cysteine-flanked core (CFC) subdomain and the prion founder gene and (8) a hypothesis on a function for the prion-like (PL) ectodomain that is present in a subset of ZIP transporters.

2. Ancestral ties and diversification

Solute carrier (SLC) proteins comprise the second largest group of membrane proteins in humans after G protein-coupled receptors (GPCRs) (Gloriam et al., 2007). The Gene Nomenclature Committee of the Human Genome Organization (HUGO) (Povey et al., 2001) and other investigators have assigned almost 400 human genes to 47 SLC families based on extensive sequence comparisons and data that suggest these proteins serve as exchangers, coupled transporters or passive transporters (Fredriksson et al., 2008; Hediger et al., 2004; Schlessinger et al., 2010). Members of a given family have minimally 20–25% amino acid sequence identity to at least one other member within that family and typically share a similar substrate. It has been suggested that some SLC families may share a common evolutionary origin (Fredriksson et al., 2008). Two distinct SLC families, SLC30 and SLC39, comprise multi-spanning transmembrane proteins, which contribute to cellular zinc homeostasis by transporting zinc ions across cellular membranes out of and into the cytosol, respectively. The genes encoding members of the ancient SLC39 (solute carrier 39) protein family, historically referred to as the Zrt-, Irt-like (ZIP) family (Guerinot, 2000), can be found in all organisms investigated, from archaea and eubacteria to eukaryotes (Gaither and Eide, 2001). The human SLC39 gene family is comprised of at least fourteen members that have been grouped into four subfamilies (Taylor and Nicholson, 2003). Based on multiple alignments of the membrane-spanning domain of ZIP transporters, however, an argument can be made for members of this protein family to fall into two main groups: ZIP transporters that (i) harbor a putative intramembrane metalloproteinase signature sequence, also referred to as the LIV-1 subfamily of ZIP zinc transporters (LZT) (Taylor and Nicholson, 2003), and (ii) those which do not. Whereas archaea and plantae genomes appear to code for a disproportionate number of non-LIV-1 subfamily members of ZIP transporters, a striking expansion of the LZT branch seems to have occurred in most metazoan lineages and, in particular, during early Chordata speciation, with the result that the genomic complement of LZT genes in tetrapods and teleosts is most pronounced and largely identical (Feeney et al., 2005). The majority of LZT sequences are equipped with N-terminal domains which are predicted to protrude into the extracellular/luminal space. For a subset of LZT paralogs this ectodomain contains a prion-like (PL) domain harboring a cysteine-flanked core (CFC), based on sequence similarities and structural predictions (Schmitt-Ulms et al., 2009) (Fig. 1). More specifically, prion gene sequences are most closely related in sequence to one phylogenetic branch within this subset of LZTs comprised of ZIPs 5, 6 and 10 (Table 1). Based on the apparent restriction of prion genes to the genomes of chordates, the emergence of the prion gene family from ZIP transporters is likely to have taken place early during Chordata radiation and prior to the divergence of teleost and tetrapod lineages. This conclusion is supported by sequence comparisons of fish and tetrapod prion gene sequences (Cotto et al., 2005; Premzl et al., 2003; Rivera-Milla et al., 2003, 2006), comparative genomics and synteny analyses (Premzl and Gamulin, 2007; Premzl et al., 2004; Kim et al., 2008; Rivera-Milla et al., 2006) (Fig. 2).

3. Function – novel links to EMT and cancer

In light of the wealth of biochemical, genetic, transgenic and structural data available for the prion protein, its elusive function is not only surprising but represents a formidable challenge to the field (Aguzzi et al., 2008; Flechsig and Weissmann, 2004; Hu et al., 2007). Over the past twenty years, several *Prnp*-knockout mouse lines have been generated and subjected to intense scrutiny for phenotypes. The persistence with these studies has led to multiple interesting observations (Brandner et al., 2000; Sakudo et al., 2006) and revealed, as recently as this year, a highly penetrant myelin maintenance defect in the peripheral nerves of PrP-deficient mice

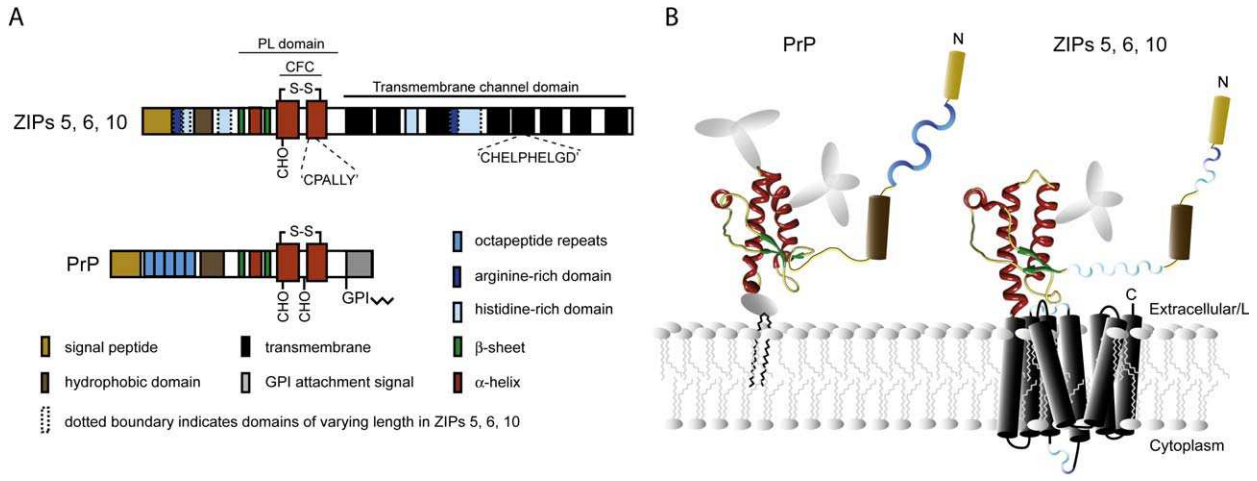

Fig. 1. Comparison of PrP and ZIPs 5, 6 and 10. (A) Domain organization and post-translational modifications. (B) Topology models.

(Bremer et al., 2010). Other functions ascribed to PrP^C include a role in olfactory behavior (Le Pichon et al., 2009), a positive influence on neuronal progenitor cell proliferation (Steele et al., 2006) and participation in the self-renewal of hematopoietic stem cells (Zhang et al., 2006). Thus, at this time there is no lack of biological systems to which PrP^C has been tied; the still-missing facet, however, is information about how precisely PrP^C exerts its role in these diverse biological systems at the molecular level. A parallel and orthogonal quest for insights into possible functions has been based on a similarly intense search for interacting proteins. Because this body of work has been reviewed before (Fasano et al., 2006; Flechsig and Weissmann, 2004; Lasmezas, 2003; Vana et al., 2007; Watts and Westaway, 2007), we will limit our discussion to aspects of the biology of PrP that may be derived from its connection to ZIP proteins.

The mere observation that ZIP genes appear to exist in the genomes of all organisms is indicative of an indispensable role for cell survival. A close inspection of amino acid sequences of individual ZIP transporters reveals a series of hydrophobic stretches of approximately 20–25 amino acids and the presence of multiple HX-repeat motifs. These features suggested early on that the ZIP proteins acquire a type III membrane topology and may be equipped with the ability to bind zinc or other divalent cations, respectively. Although investigations have often been inconclusive in the case of the prion gene knockout, gene targeting approaches remain the gold standard for the functional assignment of genes (Gondo et al., 2009). With *S. cerevisiae* being the most systematically investigated eukaryotic model organism, a look at its ZIP genes and their presumed functions may be informative. The *S. cerevisiae* genome codes for five ZIP genes, namely ZRT1, ZRT2, ZRT3, ATX2 and YKE4 (Kambe et al., 2006), with YKE4 being the only yeast paralog that can be classified as belonging to the LZT subfamily. Individual yeast ZIP transporter genes can be deleted without affecting viability in standard media (Lin and Culotta, 1996; MacDiarmid et al., 2000; Zhao and Eide, 1996b) and even a ZRT1/ZRT2 double knockout has been reported to be viable (Zhao and Eide, 1996b), suggesting that some functional redundancy may exist amongst yeast ZIP paralogs. Alternatively, an essential quality of individual ZIP members may only be teased out if cells are subjected to the right kind of stress. Indeed, slow growth can be observed when ZRT1- or YKE4-knockout cells are grown in media containing limiting or excessive amounts of zinc, respectively (Kumanovics et al., 2006; Zhao and Eide, 1996a). Taken together, the above data strongly hinted at a role of these proteins in cellular zinc homeostasis, a functional assignment which was further consolidated by the subsequent demonstration that mutations in human ZIP4 can cause the hereditary zinc uptake deficiency AE (Box 1). Only a few mammalian ZIP genes have been subjected to gene targeting investigations to date. The fourteen human *SLC39* paralogs have been mapped to 12 different chromosomes, with chromosome 8 coding for *SLC39A4* and *SLC39A14*, and chromosome 14 coding for the distantly related *SLC39A2* and *SLC39A9* genes. *SLC39A5*, *6* and *10* are encoded on the long arms of chromosomes 12, 18 and 2, respectively. No ZIP paralog is found in the vicinity of the human *PRNP-PRND* gene complex (Mastrangelo and Westaway, 2001) on chromosomal arm 20p or *SPRN* encoded by its gene locus on 10q (Table 1). ZIPs 1, 2 and 3 have been demonstrated to be nonessential in mice when zinc is replete but the knockout of these genes dramatically compromised development when zinc was limiting (Dufner-Beattie et al., 2006; Kambe et al., 2008). Homozygous ZIP4-knockout mice, generated by crossing ZIP4-heterozygous mice, display severe malformations and die during embryogenesis on day 10, a phenotype that cannot be rescued by feeding pregnant mothers a zinc-enriched diet (Dufner-Beattie et al., 2007). Haploinsufficiency of ZIP4 creates pleiotropic phenotypes that may range from retarded growth to domed head shapes or hydrocephali to missing eyes. Whereas the underlying molecular mechanisms that mediate these gross morphological abnormalities are not currently understood, the severity of these phenotypes correlates inversely with dietary zinc levels during pregnancy, consistent with the proposed role of ZIP4 in intestinal zinc uptake (Dufner-Beattie et al., 2007). Cumulatively, the above studies corroborated the conclusion that the primary cellular function of ZIP transporters might be the influx of zinc into the cytosol. What drives this import activity? Because zinc exists primarily in a protein-bound state within the cell, with cytosolic levels of free zinc estimated in the nanomolar range, a gradient of unbound zinc exists across the plasma membrane which might be sufficient to drive passive zinc uptake by facilitated diffusion. Indeed, at least for human ZIP2, available data suggest that its zinc import activity is neither energy-dependent, nor requires existing K^+ or Na^+ gradients across the plasma membrane (Gaither and Eide, 2001). Interestingly, however, the authors observed an increase in zinc uptake in response to treatment with HCO_3^-, arguing that this process may be facilitated by a Zn^{2+}–HCO_3^- symport mechanism. These data were reminiscent of an earlier report which documented a profound bicarbonate-dependent increase in zinc uptake in fibroblasts harvested from normal but not AE-afflicted individuals (Vazquez and Grider, 1995) but conflict with a recent report on a functionally reconstituted proteobacterial ZIP ortholog that argues against a symport mechanism (Lin et al., 2010).

Table 1
Characteristics of human PrP and ZIP paralogs.

Gene	Locus	Protein	Membrane attachment	CFC	MP in TM5	AA	Metal specif	Tissue distribution	Localization	Diseases	References
PRNP	20p13	PRNP/hPrP	GPI	Yes	No	253	Cu, Zn	Widespread	PM	CJD, vCJD, GSS, FFI, kuru	(Brown, 2009; Caughey et al., 1988; Collinge, 2001; Collinge and Clarke, 2007; Hu et al., 2008; Jeong et al., 2005; Makrinou et al., 2002; Mead et al., 2000; Peoc'h et al., 2000; Premzl et al., 2004; Uboldi et al., 2006; Watts and Westaway, 2007)
SPRN	10q26.3	SPRN/hSho	GPI	No	No	151	ND	CNS	PM	Unknown	(Collinge and Clarke, 2007; Hu et al., 2008; Jeong et al., 2005; Premzl et al., 2004; Uboldi et al., 2006; Watts and Westaway, 2007)
PRND	20p13	PRND/hDpl	GPI	Yes	No	176	Cu	Testis	PM	Unknown	(Collinge, 2001; Collinge and Clarke, 2007; Hu et al., 2008; Jeong et al., 2005; Makrinou et al., 2002; Premzl et al., 2004; Uboldi et al., 2006; Watts and Westaway, 2007)
SLC39A1	1q21.3	hZIP1	Type III TM	No	No	324	Zn	Widespread	PM	Prostate cancer	(Cousins et al., 2006; Eide, 2004, 2006; Franklin et al., 2005; Kambe et al., 2004; Liuzzi and Cousins, 2004; Mocchegiani et al., 2008)
SLC39A2	14q11.2	hZIP2	Type III TM	No	No	309	Zn	Liver, prostate, uterus	PM	Prostate cancer	(Cousins et al., 2006; Desouki et al., 2007; Eide, 2004, 2006; Kambe et al., 2004; Liuzzi and Cousins, 2004; Mocchegiani et al., 2008; Peters et al., 2007)
SLC39A3	19p13.3	hZIP3	Type III TM	No	No	314	Zn	Blood, breast, prostate	PM	Prostate cancer	(Cousins et al., 2006; Desouki et al., 2007; Eide, 2004, 2006; Liuzzi and Cousins, 2004; Mocchegiani et al., 2008)
SLC39A4	8q24.3	hZIP4	Type III TM	Yes	Yes	622	Zn	Cecum, colon, kidney, liver, small intestine, stomach	PM	Acrodermatitis enteropathica	(Cousins et al., 2006; Eide, 2004, 2006; Kambe et al., 2004; Mathews et al., 2005; Mocchegiani et al., 2008; Taylor et al., 2007; Wang et al., 2002; Weaver et al., 2010)
SLC39A5	12q13.3	hZIP5	Type III TM	Yes	Yes	540	Zn	Colon, kidney, liver, pancreas, spleen, stomach	PM	Unknown	(Cousins et al., 2006; Eide, 2004, 2006; Liuzzi and Cousins, 2004; Mocchegiani et al., 2008; Taylor et al., 2007)
SLC39A6	18q12.2	hZIP6/LIV-1	Type III TM	Yes	Yes	755	Zn	Widespread	PM	Breast cancer	(Chowanadisai et al., 2005; Cousins et al., 2006; Eide, 2004, 2006; Kambe et al., 2004; Mocchegiani et al., 2008; Taylor, 2008; Taylor et al., 2007)
SLC39A7	6p21.32	hZIP7/HKE4	Type III TM	No	Yes	469	Zn, Mn	Widespread	ER, Golgi	Breast cancer (Tamoxifen resistance)	(Cousins et al., 2006; Eide, 2004, 2006; Hogstrand et al., 2009; Liuzzi and Cousins, 2004; Mocchegiani et al., 2008; Taylor, 2008; Taylor et al., 2007)
SLC39A8	4q24	hZIP8/BIGM103	Type III TM	Yes	Yes	460	Cd, Fe, Mn, Zn	Widespread	PM, vesicles, mitochondria	Breast cancer (Fulvestrant resistance), coronary artery disease	(Besecker et al., 2008; Cousins et al., 2006; Dalton et al., 2005; Eide, 2004; Mocchegiani et al., 2008; Taylor et al., 2007; Waterworth et al., 2010)
SLC39A9	14q24.1	hZIP9	Type III TM	No	No	307	Zn	Widespread	ND	Unknown	(Cousins et al., 2006; Eide, 2004; Matsuura et al., 2009; Mocchegiani et al., 2008)
SLC39A10	2q32.3	hZIP10	Type III TM	Yes	Yes	831	Zn	Widespread	PM	Breast cancer	(Eide, 2004; Kagara et al., 2007; Kaler and Prasad, 2007; Mocchegiani et al., 2008; Taylor et al., 2007)
SLC39A11	17q24.3	hZIP11	Type III TM	No	No	335	Zn	Widespread	ND	Unknown	(Cousins et al., 2006; Eide, 2004; Mocchegiani et al., 2008)
SLC39A12	10p12.33	hZIP12	Type III TM	Yes	Yes	691	Zn	Widespread	PM	Asthma	(Eide, 2004; Mocchegiani et al., 2008; Taylor et al., 2007)
SLC39A13	11p11.2	hZIP13	Type III TM	Unclear	Yes	371	Zn	Widespread	Golgi	Connective tissue disorders	(Eide, 2004; Fukada et al., 2008; Mocchegiani et al., 2008; Taylor et al., 2007)
SLC39A14	8p21.3	hZIP14	Type III TM	Yes	Yes	492	Zn, Cd, Fe	Widespread	PM	Asthma, colorectal cancer, inflammatory hypozincemia	(Eide, 2004; He et al., 2009; Liuzzi et al., 2005; Mocchegiani et al., 2008; Taylor et al., 2007; Thorsen et al., 2010; Zhao et al., 2010)

AA: amino acids; CFC: cysteine-flanked core; CJD: Creutzfeldt-Jakob disease; CNS: central nervous system; ER: endoplasmic reticulum; FFI: fatal familial insomnia; GPI: glycophosphatidylinositol; GSS: Gerstmann-Sträussler-Scheinker syndrome; MP: metalloprotease; ND: not determined; PM: plasma membrane; TM: transmembrane; vCJD: variant Creutzfeldt-Jakob disease.

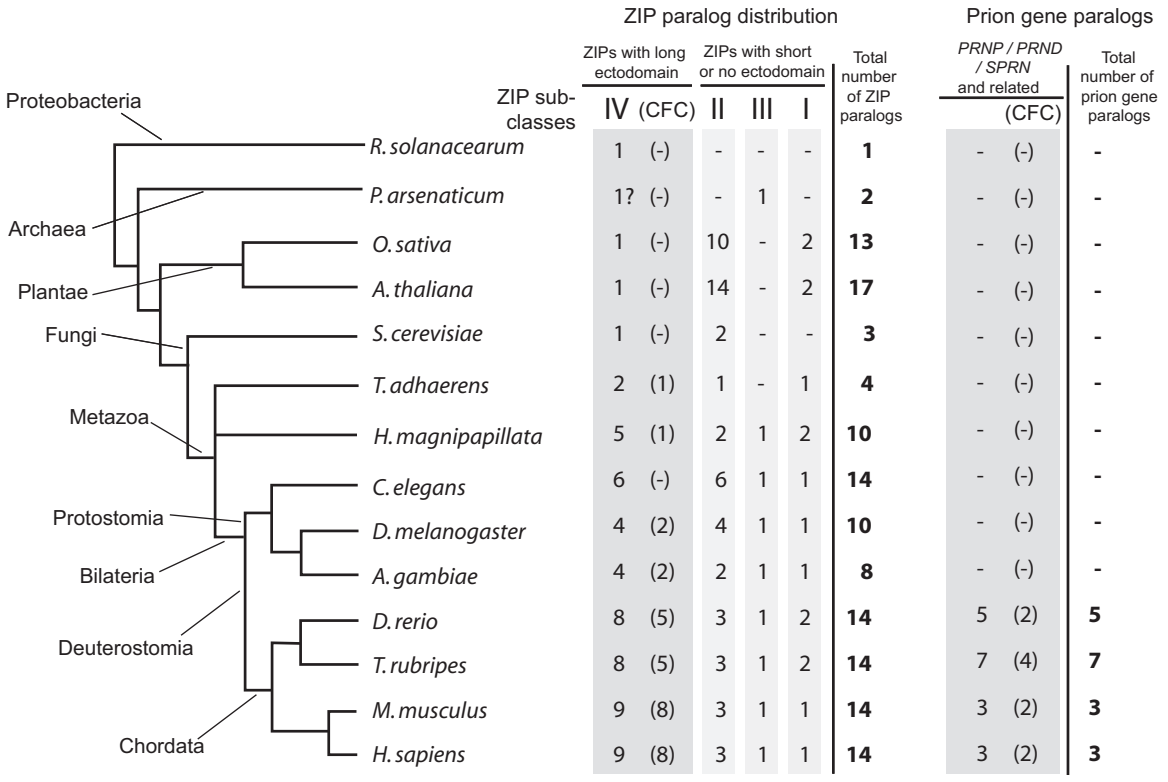

Fig. 2. Simplified phylogenetic tree and table depicting both the wide distribution of ZIP sequences in most organisms and the restricted distribution of prion genes to the Chordata lineage. The paralog distribution was deduced from alignments published by the Wellcome Trust Sanger Institute (TreeFam, http://www.treefam.org) or was determined by aligning representative ZIP and prion protein sequences to genomic sequences. The number of paralogs containing a cysteine-flanked core (CFC) domain is indicated in brackets.

Box 1. Glossary of technical terms.

Acrodermatitis enteropathica (AE): Autosomal recessive childhood zinc-deficiency disorder linked to mutations in the SLC39A4 (ZIP4) gene that presents initially in the form of skin lesions. Untreated AE may progress to severe phenotypes including neurological disturbances, growth retardation and death.

CHELPHELGD motif: A conserved motif within the fifth transmembrane domain of LZT ZIP proteins with similarities to the active center of metzincin and deformylase groups of zinc metalloproteinases (MPs). It is not known whether observed variations of this motif (C/L)(H/E)E(L/I/V/F)PHE(L/I/V/M)GD and surrounding residues assemble into an MP active center.

CPALLY motif: A conserved six-amino-acid stretch within the cysteine-flanked core (CFC) of LZT ZIP and teleost prion proteins of unknown functional significance. Variations of this motif match the consensus sequence (C/S/Y)P(A/T)(L/I/V)(L/I/V)(Q/Y/N/E)Q.

Cysteine-flanked core (CFC): Cysteine-flanked subdomain found in members of the prion protein family and within the prion-like domain of LZT ZIP proteins. The domain is set apart from surrounding sequences by a higher level of sequence conservation. A pair of highly conserved introns is flanking the CFC coding region in LZT ZIP genes.

Epithelial-to-mesenchymal transition (EMT): Developmental program characterized by detachment and migration of cells following suppression of E-cadherin-based cell-to-cell contacts. Similar molecular events underlie diverse morphogenetic programs including mesoderm, trachea and gonad formations, as well as cancer metastasis.

Homologs: Genes that are evolutionarily related by descent from a common ancestor. Whereas orthologs are separated in evolution through speciation, and typically retain the same function, paralogs evolve through gene duplication within a single genome, and frequently display relatively rapid divergence.

LZT: Protein members of the LIV-1 subfamily of ZIP zinc transporters, which were first cloned in breast cancer cell lines. LZT proteins are equipped with ectodomains and a conserved CHELPHELGD motif in their transmembrane domain V.

Retroposition: A genetic mechanism whereby RNA is reverse-transcribed and inserted into chromosomes creating a retrocopy of the RNA. In instances of mRNA retroposition, this process can, dependent on the chromosomal insertion site, give rise to dysfunctional pseudogenes or functional retrogenes.

Synteny: Colocalization of genes or genetic elements on the same chromosome. Synteny analyses are frequently employed to distinguish alternative mechanisms of gene duplication. Thus, whereas gene pairs related through genomic duplication often share a genomic neighborhood, retroposition gives rise to paralogs in novel genomic neighborhoods.

Zrt-, Irt-like protein (ZIP) (SLC39): Ancient family of proteins that assemble into an eight-transmembrane-spanning channel domain and import divalent cations into the cytosol by facilitated diffusion. A subset of ZIP metal ion transporters are equipped with an ectodomain which may act as a negative regulator/buffer of zinc influx.

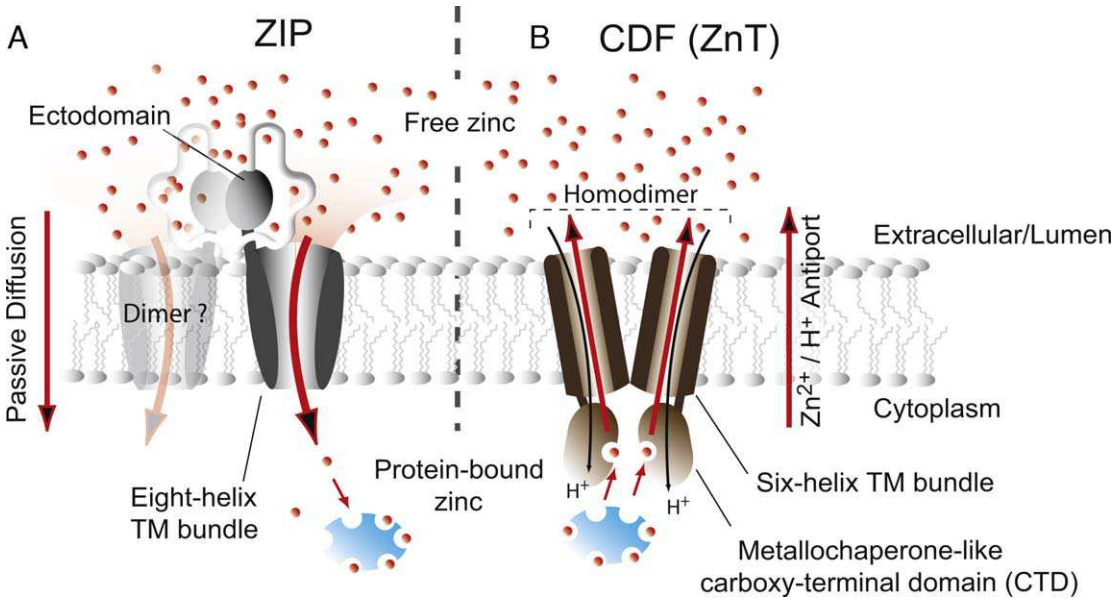

Fig. 3. Cartoon comparing properties of ZIP and CDF (ZnT) zinc ion transporters. (A) ZIP-mediated passive zinc import. (B) Antiport model of CDF (ZnT).

A high-resolution structure for members of the SLC39 family is needed to elucidate mechanistic details of their transport activity. In the absence of such data, a look at SLC30 transporters, also known as cation diffusion facilitators (CDFs) or ZnTs, zinc transporters which do not appear to be related in sequence but share with SLC39 family members the ability to transport zinc across cellular membranes, may offer some insights into possible implementations of such a transport function. Because CDFs transport zinc against the physiological zinc gradient out of the cytosol, their activity cannot (despite their name) be based on facilitated diffusion (Fig. 3). The arguably best-understood member of this family is a protein referred to as YiiP, one of two CDFs coded by the *E. coli* genome (Chao and Fu, 2004). YiiP exists as a homodimer of two 33kDa subunits, each anchored in the membrane by six TM domains and carrying a carboxy-terminal domain (CTD) that protrudes into the cytoplasm. YiiP has been shown to employ a mechanism that relies on the antiport of protons to drive the export of Zn^{2+} from native membrane vesicles (Grass et al., 2005). High resolution X-ray scattering and NMR data for YiiP (Lu and Fu, 2007) and its molecular cousin CzrB in *Thermus thermophilus* (Cherezov et al., 2008; Hofer et al., 2007) revealed that CTDs acquire a metallochaperone fold and contribute to homodimerization by coordinating the binding of multiple zinc ions at the protein–protein interface. Site-directed fluorescence energy transfer (FRET) measurements suggest that zinc binding triggers hinge movements of the CTDs relative to the six-helix TM bundle and leads to subtle rearrangements of the transmembrane helices that may alter zinc coordination in the active site and thus promote transport (Lu et al., 2009). It is likely that the ectodomain in the LZT subfamily of SLC39 members may mimic aspects of the CTD role in SLC30 members. However, it is to be expected that the influx activity of SLC39 family members may not recapitulate all facets of events proposed for SLC30 exporters because free versus protein-bound divalent cations, available in the extracellular and cytosolic environments, respectively, may pose different challenges for metal capture. Aside from conspicuous differences in the number of helices of transmembrane domains, protruding domains of the two protein families may have developed distinct folds as an adaptation to distinct metal availabilities and redox environments. Thus, the metallochaperone-like fold of the CTD may be an absolute requirement for wresting a divalent cation from a cytosolic metal-binding protein but may be of lesser relevance for a zinc importer which accesses the extracellular pool of free divalent cations. We will return to a discussion of possible functions of LZT ectodomains in Section 8.

Although it is currently unknown whether ZIP proteins, like SLC30 transporters, operate as functional dimers, evidence is accumulating that proteins harboring a prion-like domain are capable of engaging in direct interactions with each other. Genetically, a direct interaction between PrP^C and Doppel (Dpl) or Dpl-like N-terminally truncated mutants of PrP (ΔPrP) has been postulated based on the ability of PrP^C to counteract the toxicity associated with cerebellar expression of either Dpl or ΔPrP (Watts and Westaway, 2007). However, evidence of biochemical interactions between Dpl and PrP^C had been lacking until recently when two independent studies reported the co-immunoprecipitation of Dpl and PrP^C (Qin et al., 2006; Watts et al., 2009), suggesting that the two proteins either interact directly with each other or exist in close spatial proximity. Although PrP^C may not exist at the cell membrane as a constitutive dimer, dimeric forms of PrP^C, the formation of which appears to be mediated by the well-conserved hydrophobic tract region, have been observed in cell culture and shown to be required for PrP to exert its protective role (Priola et al., 1995; Rambold et al., 2008). Consistent with this observation, PrP deletion mutants lacking the hydrophobic domain (and therefore possibly impaired in dimerization) are highly neurotoxic *in vivo* (Baumann et al., 2007; Li et al., 2007). Whereas hydrophobic tract-mediated dimerization may also explain the ability of PrP^C to interact with Sho (Jiayu et al., 2009; Watts et al., 2009), additional elements within the prion-like domain may have an intrinsic ability to contribute to dimerization. This is also suggested by the aforementioned data documenting the ability of PrP^C to bind to Dpl, ZIP6 and ZIP10, proteins that do not contain a PrP/Sho-like hydrophobic tract.

With zinc being increasingly recognized as a second messenger (Yamasaki et al., 2007), research into cellular zinc homeostasis and zinc-related diseases has experienced a renaissance in recent years. Beyond their contribution to the overall health of a cell through maintaining stable zinc levels, proteins involved in zinc homeostasis are now thought to influence all major cell-fate decisions (Cousins et al., 2006; Devirgiliis et al., 2007; Frederickson et al., 2005). At the molecular level, this influence is primarily mediated by zinc providing structural stability (e.g., in zinc-finger-motif-containing transcriptional regulators) or acting as a catalytic

cofactor (e.g., in superoxide dismutase, carbonic anhydrase and metalloenzymes). From a prion research perspective, gene-targeting experiments that may shed light on the specific functions of ZIPs 5, 6 or 10 in rodents would seem to be most relevant but are currently lacking. At present, the most illuminating data in this regard may originate from knockout studies of ZIP orthologs conducted in fruitflies. The *D. melanogaster* genome has been reported to code for a total of nine ZIP orthologs (Taylor and Nicholson, 2003). While the exact orthologous relationships of fruitfly and human ZIPs remain to be determined, a relatively detailed functional characterization has been conducted for a gene referred to as *fear-of-intimacy* (*foi*) that exhibits the strongest sequence similarity to the branch of mammalian ZIPs 5, 6 and 10 (Taylor and Nicholson, 2006; Van Doren et al., 2003). The naming of this gene originally referred to an impairment seen in FOI-deficient fruitfly embryos which prevented a small group of specialized cells from coalescing following morphogenetic rearrangements and cell migration processes underlying gonad and trachea formations (Mathews et al., 2005, 2006; Moore et al., 1998; Van Doren et al., 2003). These developmental processes themselves are reminiscent of epithelial-to-mesenchymal transitions (EMTs) occurring during gastrulation. The connection to EMT is intriguing in this context as independent developmental investigations in zebrafish implicated both ZIP6 (Yamashita et al., 2004) and the prion protein (Malaga-Trillo et al., 2009) in morphogenetic cell movements during zebrafish gastrulation. Finally, it appears as if lessons learned from these model organisms also apply, at least partially, to their mammalian cousins. Aggressive forms of cancer with a propensity to turn malignant have long been proposed to hijack an already-in-place molecular EMT program for their transition to invasiveness (Thiery et al., 2009). ZIP6 and ZIP10 have not only been found to be upregulated in certain cancers (Schneider et al., 2006; Taylor et al., 2007; Tozlu et al., 2006), but they have also been linked repeatedly to a role during the EMT of a subset of human carcinomas in recent years (Kagara et al., 2007; Shen et al., 2009; Taylor et al., 2004; Unno et al., 2009; Zhau et al., 2008).

4. Expression – crosstalk and division of labor

The five yeast ZIP transporters localize to the plasma membrane (ZRTs 1 and 2) (Zhao and Eide, 1996a), endoplasmic reticulum (ER) (YKE4) (Kumanovics et al., 2006), Golgi apparatus (ATX2) (Lin and Culotta, 1996) and vacuolar membrane (ZRT3) (MacDiarmid et al., 2000), suggesting that cells may gain a selective advantage by dispatching specialized ZIP transporters to distinct locales. However, the assignment of more than one ZIP transporter to the yeast plasma membrane requires a different explanation: it has been observed that ZRT1 and ZRT2 exhibit dramatically different zinc uptake affinities. Whereas ZRT1 is active in zinc-limited cells as a high-affinity zinc uptake transporter (Zhao and Eide, 1996a), ZRT2 is active in zinc-replete cells (Zhao and Eide, 1996b). Current insights into the tissue expression of human ZIP (hZIP) genes reflect a series of independent observations rather than systematic analyses. Available data suggest widespread expression of most hZIP paralogs except for hZIPs 2, 3, 4 and 5. Of the three ZIP paralogs most closely related to prion genes, ZIP5 is primarily expressed in visceral organs (kidney, liver, colon, pancreas), ZIP6 levels are highest in hormonal tissues (breast, prostate, pituitary gland) but can also be detected in the brain (Taylor et al., 2003; Taylor and Nicholson, 2003), and ZIP10 is predominantly expressed in the brain and spinal cord (Taylor et al., 2007). Reminiscent of the situation in yeast, the majority of hZIP transporters appear to be targeted to the plasma membrane, and only a few select ZIPs, for example hZIP7 and hZIP9, seem to localize predominantly to membranes surrounding the ER (Suzuki and Endo, 2002), Golgi (Huang et al., 2005), or the trans-Golgi network (Matsuura et al., 2009). Whereas all eukaryotic cells appear to express multiple ZIP paralogs, relatively little is known about the biology that determines the subset of ZIP paralogs expressed in a given cell or tissue. Observations of reciprocal expression levels and complementary targeting of apical and basolateral cell surfaces have been reported for ZIP4 and ZIP5, suggesting that an intricate balance and crosstalk may exist amongst individual ZIP transporters in response to extracellular stimuli (Dufner-Beattie et al., 2004; Wang et al., 2004c).

In light of their emerging role in the cellular homeostasis of zinc and other divalent cations, it is not surprising that the expression of individual ZIPs can be shown to respond to zinc levels. For example, in yeast the expression of ZRT transporters correlates inversely with zinc levels in the medium (Lyons et al., 2000). More specifically, the transcription of ZRT1 which exhibits higher affinity to zinc than its paralog ZRT2 (Zhao and Eide, 1996a) is increased when zinc levels in the medium are low, but is subject to rapid ubiquitin-mediated degradation once zinc is replete (Gitan and Eide, 2000; Gitan et al., 1998). However, responsiveness to zinc levels is not a generic characteristic of ZIP transporters, as no such correlation has been observed for the yeast paralog ATX2 (which appears to primarily function in cellular manganese homeostasis (Lin and Culotta, 1996)). A systematic analysis of zinc transporter mRNA levels in a range of tissues with varying levels of zinc in the aquatic environment has been reported for zebrafish (Feeney et al., 2005). The study uncovered complex relationships between aquatic zinc and mRNA expression levels for individual ZIPs, with some ZIP genes being unresponsive to varying zinc levels (e.g., ZIP6) and others responding profoundly but in an inconsistent manner in a diverse spectrum of tissues investigated (e.g., ZIPs 1, 3, 4, 7 and 10). Similar patterns of zinc responsiveness appear to exist for ZIP transporters expressed in mammalian cells (Ryu et al., 2008). For example, ZIP10 levels in rat renal brush border cell membrane have been shown to correlate with extracellular zinc levels and zinc uptake has been reported to be time-, temperature- and ZIP10-concentration-dependent in this cell model (Kaler and Prasad, 2007). For ZIP5, it has been proposed that cells might increase responsiveness to zinc levels by employing a translational stalling mechanism that attaches ZIP5 mRNA to polysomes when zinc is limiting (Weaver et al., 2007).

Although the highest levels of PrP^C expression are observed in the mammalian central nervous system, substantial levels are also observed in cardiac muscle and lung, and lower levels can be found in other tissues including the intestine, limb muscles and spleen, and on lymphocytes. As such, the expression profile of PrP may more closely resemble the expression of ZIP6 and ZIP10 than the expression profile of ZIP5; however, cell-specific characterizations of expression levels are needed to dissect whether levels of PrP and individual ZIP proteins are correlated. Like many ZIPs, the expression of PrP appears to respond to the presence of metals in complex ways (Choi et al., 2006; Rana et al., 2009). For example, copper in the medium was shown to decrease PrP^C expression in immortalized murine GN11 neurons (Toni et al., 2005), but translated into increases in PrP^C expression in human HeLa and mouse N2a cells (Qin et al., 2009), mouse fibroblast cells (Armendariz et al., 2004) and primary rat hippocampal and cortical neurons (Varela-Nallar et al., 2006). Based on the data reviewed here, it is likely that PrP may encounter distinct combinations of multiple ZIP transporters in the wide range of cell types in which it is known to be expressed. It will be of interest to observe whether individual ZIPs and PrP can reciprocally influence each other's expression levels. This scenario would not be surprising given the complementary nature of PrP and Sho expression observed in select areas of the mouse brain (Watts et al., 2007).

5. Signaling – from Tinman to Snail and beyond

What can insights into signaling pathways operating upstream or downstream of ZIPs 5, 6 or 10 tell us about PrP? Very little is known about the regulation of expression of ZIPs 5 or 10. Given the zinc-responsiveness of expression observed for many zinc transporters, it is not surprising that metal response elements (MREs) exist in their promoter regions (Wimmer et al., 2005; Zheng et al., 2008). This is similar to the prion gene for which it has been shown that copper-replete conditions can promote its expression in certain cell types, including human fibroblast cells, through the activity of metal-responsive transcription factor-1 (MTF-1) (Bellingham et al., 2009). Additional promoter elements may also come into play; in the case of PrP, for example, a well-known pathway that involves the ataxia telangiectasia mutated (ATM) protein kinase and components of the MAP kinase pathway culminates in the transcription factor SP1 occupying its corresponding binding sites on the PrP promoter and causing an upregulation of PrP expression in response to an increase in intracellular copper (Qin et al., 2009). The 5′-upstream region of the human *PRNP* gene contains putative binding sites for many additional transcription factors including AP-1 (FOS) and AP-2 (TFAP2A), p53 (TP53), MyoD (MYOD1) and NKX2-5 (Funke-Kaiser et al., 2001; Mahal et al., 2001). Comparably little is known about promoter elements within ZIP genes. While compelling data have established a role for STAT3 acting upstream of ZIP6 in zebrafish (Yamashita et al., 2004), it is currently not known whether STAT3 acts immediately upstream of ZIP6, for example as an activator of its transcription, or whether signaling mediators are positioned between STAT3 and ZIP6 (Fig. 4). In the fruitfly, FOI is transcriptionally activated by Tinman (Moore et al., 1998; Riechmann et al., 1998), the *D. melanogaster* ortholog to the mammalian NKX2-5 transcription factor (Fu et al., 1998) for which, as mentioned, consensus binding elements have also been described in the prion promoter.

The similarity of gastrulation phenotypes linked to PrP deficiency and ZIP6 (LIV-1) inactivation in zebrafish may be a particularly rewarding angle from which to explore overlaps in signaling pathways downstream of ZIP6 and/or PrP (Malaga-Trillo et al., 2009; Yamashita et al., 2004). It is noteworthy that both publications tied PrP or ZIP6 functions to the regulation of cell adherence and the E-cadherin protein. How does ZIP6 expression influence E-cadherin levels? A first indication of what might be occurring is the observation of a ZIP6-dependent nuclear translocation of the transcriptional repressor Snail (Yamashita et al., 2004), a master regulator of EMT (Cano et al., 2000). As a zinc finger protein, Snail requires zinc as a structural cofactor for binding to elements present within the E-cadherin promoter. Consequently, the possibility arises that a local increase in zinc levels, mediated by ZIP6-dependent zinc import, charges cytoplasmic Snail with zinc and thus triggers its translocation into the nucleus. Consistent with observations in zebrafish, the fruitfly ZIP ortholog FOI acts upstream of the Snail family member Escargot (Esg) to exert the aforementioned tracheal branch fusion process (Tanaka-Matakatsu et al., 1996). Surprisingly, whereas both the tracheal and gonadal FOI-mediated phenotypes in the fruitfly depend on E-cadherin expression, the gonad coalescence phenotype appears to be independent of Esg. In light of the data reviewed herein, the link between ZIP6 and cancer metastasis may be explained by a body of literature that ties the molecular biology revolving around STAT3, EMT and Snail (Nieto, 2002) to cancer malignancy (Groner et al., 2008; Inghirami et al., 2005; Micalizzi and Ford, 2009; Thiery et al., 2009; van Zijl et al., 2009). Cells with ectopic Snail expression adopt a fibroblastoid phenotype and acquire tumorigenic and invasive properties (Cano et al., 2000). In line with this model, recent reports document that inhibition of ZIP6 in both the cervical cancer-derived HeLa cell model and pancreatic cancer cells causes the deactivation of Snail and interferes with their metastatic features (Unno et al., 2009; Zhao et al., 2007). However, it is already emerging that the underlying phenomena are considerably more complex than we currently understand and likely differ amongst experimental paradigms. Thus, additional stimuli, such as the exposure of cells to bacterial lipopolysaccharides, have been shown to feed into the signaling pathways that control ZIP6 expression (Kitamura et al., 2006). Furthermore, E-cadherin levels correlate inversely with ZIP6 levels in some paradigms but in others appear to follow ZIP6 levels directly as, for example, in breast tumor cells (T47D or MCF-7) (Lopez and Kelleher, 2010; Shen et al., 2009) or the previously mentioned FOI-dependent gonad formation phenotype in the fruitfly. Interestingly, ZIP6 also appears to have opposite effects when compared to PrP on E-cadherin levels in the zebrafish gastrulation paradigm. Whereas the knockdown of PrP appears to cause a destabilization of the mature E-cadherin pool, transcriptional inactivation of ZIP6 is linked to E-cadherin stabilization. The observed influence of PrP on cadherin may well be independent of ZIP zinc transport activities and instead be based on PrP's ability to influence cellular copper and zinc homeostasis (Watt and Hooper, 2003). Alternatively, PrP may exert its influence on EMT through binding to NCAM (Schmitt-Ulms et al., 2001), an interaction known to facilitate the recruitment of NCAM into lipid rafts and to promote activation of Fyn kinase (Santuccione et al., 2005). Fyn has been shown to phosphorylate focal adhesion kinase and thus promote the assembly of integrin-mediated focal adhesions, cell

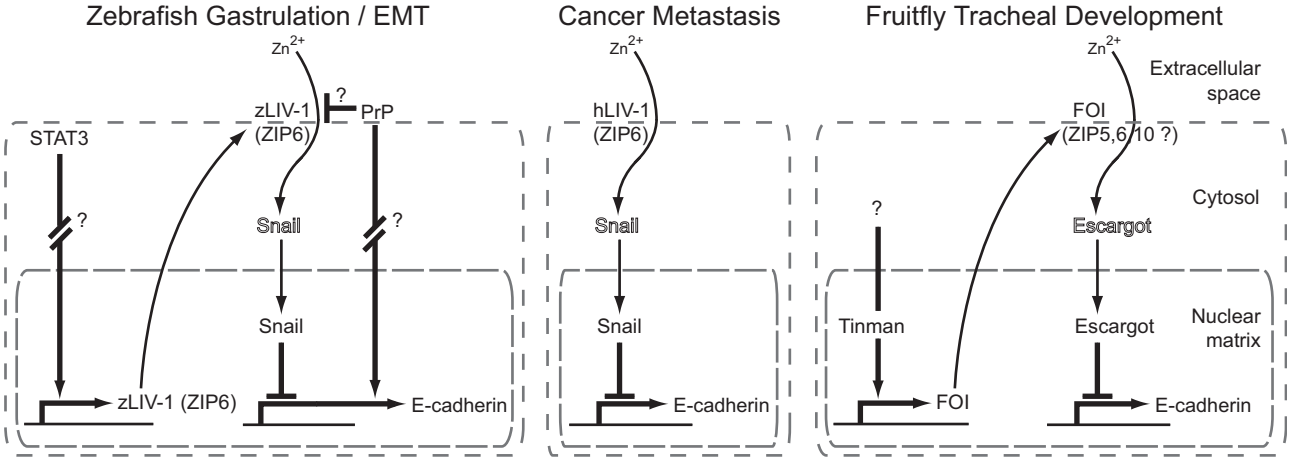

Fig. 4. ZIP-dependent signaling upstream of E-cadherin expression in three biological paradigms.

spreading and EMT (Lehembre et al., 2008). Ablation of NCAM inhibits EMT and overexpression of NCAM correlates with tumor invasion (Frame and Inman, 2008). Finally, an intriguing possibility is that PrP may directly influence the signaling events outlined above based on its capacity to bind to ZIP6, consistent with our data documenting co-affinity purification of PrP and ZIP6 following *in vivo* crosslinking with formaldehyde (Watts et al., 2009). The latter explanation would suggest that the influence of PrP on ZIP6 is that of a negative regulator.

6. Post-translational modifications

The existence of a highly conserved disulfide bridge spanning alpha helices B and C within the globular domain of the mammalian prion protein is well-documented (Wuthrich and Riek, 2001). Not surprisingly, Sho, devoid of the corresponding domain and of cysteine residues in general, lacks a disulfide bridge. Dpl, on the other hand, has been shown to harbor a second disulfide bridge serving to further stabilize its globular domain (Silverman et al., 2000; Zahn et al., 2003). The existence of disulfide bridges within ZIP proteins has not been rigorously documented to date. However, except for human ZIP11, whose amino acid sequence is devoid of cysteine residues, all human ZIP paralogs contain multiple cysteines. Most of these are scattered throughout the respective primary structures with little conservation across ZIP paralogs. However, two pairs of cysteines conserved in a subset of paralogs within the LIV-1 branch of ZIP transporters are the exception. One of the pairs (flanking the CFC), with regard to its position within the predicted globular domain in these proteins, matches the location of the disulfide bridge in PrP (Schmitt-Ulms et al., 2009). The remaining two conserved cysteines are found within the CPALLY and CHELPHELGD motifs (Box 1) in the predicted extracellular globular domain and the putative transmembrane domain V, respectively, and have been hypothesized to interact in regulating the movement of zinc across the membrane (Taylor et al., 2007).

Members of the mammalian prion gene family are post-translationally modified on up to two N-glycan acceptor sites (Silverman et al., 2000; Watts et al., 2007). However, only one of the two N-glycosylation motifs found in each of PrP and Dpl is conserved between these proteins. This site is positioned two residues C-terminal to the first cysteine residue (which participates in the formation of the aforementioned conserved disulfide bridge). Whereas a subset of human ZIP paralogs contains 'NX(T/S)' consensus acceptor site motifs for N-glycosylation primarily on their N-terminal ectodomains, the occupancy of these sites has not been experimentally determined. However, indirect evidence for N-glycosylation of these transporters has been obtained for ZIP4 (Wang et al., 2004b), ZIP6 (Taylor et al., 2003), ZIP8 (Besecker et al., 2008; He et al., 2006) and the closely related transporter ZIP14 (Girijashanker et al., 2008; Taylor et al., 2005) by documenting faster migration of denatured proteins via SDS-PAGE following the global removal of N-linked sugars by treatment of proteins with N-glycosidase F (PNGase F) or after culturing cells in the presence of tunicamycin. A similar gel shift could not be observed for ZIP1 and ZIP3 following N-glycosidase F treatment (Wang et al., 2004a), adding weight to the impression that N-glycosylation may be restricted to transporters with extended N-terminal domains. It will be of interest to determine whether ZIPs 5, 6 and 10, which contain a conserved NX(T/S) motif in the same relative position as the conserved N-glycosylation site shared between PrP and Dpl, are in fact N-glycosylated at this acceptor site (Fig. 1).

It has repeatedly been shown that PrP can be endoproteolytically processed through a so-called alpha-site cleavage of the peptide bond connecting amino acids 110 and 111 (corresponding to human PrP), causing the release of an N-terminal fragment (N1) into the medium (Chen et al., 1995; Guillot-Sestier et al., 2009; Haigh et al., 2009; Jimenez-Huete et al., 1998; Pan et al., 1992; Shyng et al., 1993; Vincent et al., 2000). ADAM10 and ADAM17, the latter also known as TACE, have been proposed to catalyze this cleavage in response to phorbol ester stimulation (Alfa Cisse et al., 2008; Cisse et al., 2005; Vincent et al., 2001) in a late compartment of the secretory pathway (Walmsley et al., 2009). Interestingly, the same stimuli and metalloendoproteinases have also been implicated (Taylor et al., 2009) in a second PrP^C cleavage activity (between amino acids 228 and 229), leaving behind a very short three-amino-acid stub and causing the shedding of a soluble ectodomain form of PrP^C from the cell surface (Borchelt et al., 1993; Harris et al., 1993). The entire ectodomain has been observed to be released when PrP^C is subjected to a distinct phospholipase activity which can cleave the glycosylphosphatidylinositol (GPI) anchor directly (Parkin et al., 2004). Finally, a scissile bond within PrP, termed the beta-cleavage site (Mange et al., 2004), has been identified in proximity to the end of the octapeptide repeat region (Jimenez-Huete et al., 1998; Pan et al., 1992). At least in a subset of experimental paradigms, cleavage at this site, leading to the secretion of a shorter N-terminal fragment (N2) into the medium, appears to be directly mediated by free reactive oxygen radicals (McMahon et al., 2001; Watt et al., 2005). Cleavage at or near this site may further be mediated by endolysosomal cathepsin-like proteases (Dron et al., 2010). Little is known about proteolytic cleavages within ZIP transporters. However, it has recently been shown that the N-terminal domain of ZIP4 is shed through an endoproteolytic event when cells are grown for prolonged durations in zinc-depleted cell culture media (Kambe and Andrews, 2009). Levels of cleaved ZIP4 were reduced in the presence of endocytosis inhibitors, possibly indicating that the N-terminally truncated ZIP4 protein can be recycled to the plasma membrane. An additional regulatory sequence has been mapped to a histidine-rich motif within the intracellular loop connecting transmembrane domains III and IV of ZIP4. Ubiquitination of this domain has been proposed to regulate ZIP4 endocytosis and its priming for proteasomal degradation (Mao et al., 2007).

No systematic endoproteolytic analyses have been undertaken for other ZIP proteins. Unpublished results from our laboratory suggest, however, that ZIPs 5, 6 and 10 are subject to distinct and complex internal cleavages. As mentioned above, members of the LIV-1 subfamily of ZIP transporters contain a HEXXH amino acid sequence motif ('CHELPHELGD motif') within their predicted transmembrane domain V (Fig. 1) (Taylor, 2000). The presence of this motif, best known for its occurrence in the active center of metalloproteinases (MPs), has provoked the tantalizing hypothesis that these proteins may not only bind and import zinc and other divalent cations into the cytosol, but possibly serve a second proteolytic function (Taylor et al., 2003; Taylor and Nicholson, 2003). When present in MPs, this motif is typically embedded in extended active site signature sequence patterns shared amongst closely related proteins (Jongeneel et al., 1989; Rawlings and Barrett, 1995). Thus, in addition to the two ion-coordinating histidine residues and the catalytic acid embedded in the pentapeptide core motif HEXXH, a glutamine, asparagine or histidine residue contributed by different structural elements work together with the help of a tetrahedrally coordinated zinc (or rarely an alternative metal) to polarize the metal-bound water molecule for its nucleophilic attack (Kinch et al., 2006) on a nearby peptide bond. Detailed scrutiny of the sequence environment of the consensus HEXXH motif within the LIV-1 branch of ZIP proteins revealed additional similarities to metzincin and deformylase groups of zinc MPs (Taylor, 2000; Taylor et al., 2003) but did not meet discriminative criteria for assignment to any known MP subfamily. This observation is perhaps not surprising given the topological restraints of intramembrane proteolysis, also exempli-

fied by X-ray crystallographic data of a Site-2 protease (S2P) (Feng et al., 2007). This family of intramembrane MPs appears to coordinate zinc based on an alpha-helical membrane-embedded HEXXH pentapeptide and a highly conserved aspartic acid that is distant in sequence but spatially close in the tertiary structure. Thus, whether members of the LIV-1 branch of the ZIP protein family (which, except for the shared HEXXH motif, bear no apparent resemblance to S2P proteases) contain a novel MP active site awaits further investigation.

Whereas PrP has been shown to be post-translationally modified by the addition of a GPI anchor, ZIP transporters are multi-spanning transmembrane proteins. The orientation and distance of the CFC domain to the respective membrane attachment sites are similar in PrP and ZIP proteins. Furthermore, the amino acid sequences of the first transmembrane domain in ZIPs and the GPI anchor attachment signal in PrP are similar. These observations led to the inference that the signal peptide for the attachment of a GPI anchor present in PrP may have evolutionarily originated from the first transmembrane domain of a ZIP ancestral molecule (Schmitt-Ulms et al., 2009). This conclusion was supported by previous reports documenting that small changes within or in proximity to the first transmembrane domain can be sufficient to cause a shift to a GPI-anchored mode of membrane attachment (Bell et al., 1994; Lanier et al., 1989; Naghibalhossaini and Stanners, 2004).

7. Binding of divalent cations – a common denominator

Although the predominant preference of ZIP proteins for zinc transport has been well-established, the metal binding characteristics of these proteins are complex. Thus, whereas good agreement amongst available studies exists to suggest that ZIPs 5, 6 and 10 are primarily responsible for the cytosolic import of zinc, a few select ZIP transporters have long been known to contribute to the transport of different metals (Eide et al., 1996). In particular, ZIP8 and ZIP14 have been shown to transport, in addition to zinc (Taylor et al., 2005; Tominaga et al., 2005), cadmium, manganese and iron (Table 1) (Dalton et al., 2005; Girijashanker et al., 2008; Himeno et al., 2009; Liuzzi et al., 2006). These observations suggest that the core molecular architecture of ZIP transporters is intrinsically adaptable toward the transport of a range of divalent metals. It further suggests that around the time in evolution when the most pronounced radiation of the LZT branch of ZIP genes occurred, i.e., prior to the divergence of teleosts and tetrapods (Kambe et al., 2006; Schmitt-Ulms et al., 2009), the metal transport selectivity of ancient LZT proteins may have been less well-developed. The structural components which contribute to metal-binding and specificity of individual ZIP transporters are currently poorly understood. Various authors have noted the presence of histidine-rich repeat motifs found within N-terminal extracellular domains and in loop sequences connecting putative TM domains 2–3 and 3–4 of many ZIP transporters (Eng et al., 1998; Taylor, 2000). Most often, these histidine-rich motifs are of the type $[HX]_n$ (or derivatives of this motif), whereby 'X' represents any amino acid and '*n*' may range from 2 to ~30. Consistent with a possible role in metal binding, similar motifs can also be found in many members of the SLC30 family of zinc exporters (Taylor, 2000). However, the absence of some or all of these histidine-rich stretches in individual ZIP transporter paralogs suggests that these sequences are not essential for transport but instead may serve other functions, for example, in the sensing or initial capture of metal ions and/or interaction with other proteins. In the IRT1 ZIP transporter from thale cress (*Arabidopsis thaliana*), a specific histidine residue and two nearby acidic residues were identified by alanine scanning mutagenesis to play a role in the metal specificity of this transporter (Rogers et al., 2000). Two highly conserved histidine residues within putative transmembrane domains IV and V have been proposed to line the metal transport pathway and serve as parts of an intramembraneous metal binding site (Eng et al., 1998). Consistent with the notion that the histidine residue in TM domain V may contribute to metal transport specificity, this residue is replaced with glutamic acid in ZIPs 8 and 14, two mammalian paralogs shown to exhibit relatively broad metal ion transport specificities.

The metal binding characteristics of mammalian prion proteins have been under intense scrutiny ever since the protein sequence of PrP^C was shown to harbor four or five copies of a histidine-containing octapeptide motif (PHGGGWGQ) in its N-terminal domain (reviewed in Choi et al., 2006; Millhauser, 2007; Rana et al., 2009). Although considerable discord exists in the literature with regard to which metals bind to which sites within PrP^C, the predominant view has been that mammalian octapeptide repeat sequences preferentially bind copper and have lesser affinities to zinc, manganese, iron and other divalent metals. However, it has repeatedly been pointed out that binding of PrP to zinc, albeit shown to occur with lower affinity *in vitro*, may become significant in an *in vivo* environment where zinc levels may greatly exceed local copper concentrations (reviewed in Watt and Hooper, 2003). Consistent with this notion, PrP^C appears to respond equally well to zinc or copper but not to other divalent cations in both a well-known endocytosis paradigm (Pauly and Harris, 1998; Perera and Hooper, 2001) and an assay which can monitor the ability of unilamellar-embedded N-terminal fragments of PrP to engage in direct interactions (Kenward et al., 2007). A more detailed understanding of the metal-binding characteristics of the octapeptide repeat domain and an adjacent metal binding site involving human PrP histidine residues 96 and 111 has emerged in recent years, in particular from structural characterizations of bacterially expressed prion proteins or synthetic peptides (Morante et al., 2004; Zahn, 2003). A refined model which takes into account physiological zinc and copper levels posits that both zinc and copper may bind to the octarepeat domain when copper levels are low. As copper levels increase, copper may displace residual PrP-bound zinc, which in turn may continue to influence the copper coordination mode of the prion protein (Walter et al., 2007).

Like PrP^C, Dpl can also bind copper ions, albeit in the conspicuous absence of histidine-containing octarepeat motifs (Cereghetti et al., 2004; Qin et al., 2003). While Dpl appears to form complexes with zinc transport proteins (Watts et al., 2009), it remains to be established whether zinc plays a role in these interactions or whether Dpl can capture zinc ions independently. Investigations into a possible involvement of Dpl in cellular zinc homeostasis may shed light on a male sterility phenotype linked to Dpl function (Behrens et al., 2002; Paisley et al., 2004), a research direction supported by a literature that ties cellular zinc biology to sperm cell proliferation and motility (Yamaguchi et al., 2009).

Much less is known about the metal binding specificities of prion protein orthologs found in teleosts. It has been noted before that little sequence conservation exists in N-terminal repeat sequences between tetrapod and teleost prion proteins (Rivera-Milla et al., 2006). Significantly, the tandem repeat region in zebrafish PrP-related2 (annotated as PrP-related3 in some databases) has been shown to harbor histidine clusters of the type $[HX]_n$ seen in ZIP proteins that can efficiently bind to zinc (Camponeschi et al., 2009; Szyrwiel et al., 2008). Thus, in both ZIP transporters and prion genes there is evidence for a parallel divergent evolution of N-terminal sequences that may have created distinct specializations in metal binding preferences.

8. Origins of the CFC and prion founder gene

PrP consists of a structurally disordered N-terminal domain and a globular C-terminal domain formed by two short beta-strands

and three alpha-helices (Wuthrich and Riek, 2001). The latter domain is highly conserved in PrP sequences found in avian and mammalian classes but is much less conserved in PrP sequences identified in fish genomes (Rivera-Milla et al., 2003). Structural threading predicts that the corresponding prion-like (PL) domain of ZIPs 5, 6 and 10 may acquire a fold similar to PrP and its mammalian paralog Dpl (Fig. 1B) (Schmitt-Ulms et al., 2009). A broader comparison of all human ZIP transporters revealed that a relatively well-conserved cysteine-flanked core (CFC) is present in most paralogs of the LZT subfamily (Schmitt-Ulms et al., 2009). Reminiscent of observations made with teleost and tetrapod PrP sequences, there is considerable divergence in the length and molecular organization of segments within the ZIP domains that are N-terminal to the CFC and predicted to acquire a largely disordered fold. Furthermore, multiple alignments of prion and ZIP sequences from a diverse selection of organisms reveal a dichotomy in the degree of sequence conservation within the globular PL domain itself, i.e. sequences N-terminal to the CFC domain, including PrP sequence stretches comprising helix A, are conspicuously enriched in charged residues but, in contrast to sequences within the CFC, show relatively little positional conservation (Ehsani et al., 2011). This observation is interesting in light of structural data (Wille et al., 2002) and a subset of emerging models for the disease-associated scrapie (PrP^{Sc}) conformer of the prion protein (Govaerts et al., 2004) which posit that helices B and C, comprising the CFC, may be refractory to the extensive structural rearrangements within PrP that constitute the hallmark biochemical event underlying prion diseases (Prusiner, 1998). What could be the basis for this dichotomy in the degree of positional conservation? ZIP sequences containing CFC domains can be identified in the genomes of metazoa with relatively primitive body plans, including cnidarians and the amoeba-like organism *Trichoplax adhaerens* (Ta) (unpublished observation), but these domains seem to be absent in ZIP genes of all other branches of life. Thus, approximately a billion years ago, around the time when the metazoa lineage emerged, the CFC domain may have either gradually evolved or became inserted as a module into a preexisting ZIP gene. This gene rearrangement event therefore preceded the split of PrP genes from ZIP transporter genes by hundreds of millions of years. Indicative of the modular insertion scenario, possibly involving an exon-shuffling event, conserved exon/intron boundaries can be found immediately N- and C-terminal to the CFC domain not only in all human LZT genes but also in distant LZT sequences found in the genomes of species that range from *Trichoplax* to fruitflies to pufferfish. Consistent with their ancient origins, the lengths of these positionally conserved introns vary widely from a few nucleotides to thousands of base pairs.

In-depth analyses of prion genes in diverse organisms have repeatedly revealed a common gene structure composed of one or two short 5′ noncoding exons and a relatively long exon that codes for a short 5′ untranslated region, the entire open reading frame (ORF) and a 3′ untranslated region (Basler et al., 1986; Lee et al., 1998; Saeki et al., 1996; Simonic et al., 2000; Suzuki et al., 2002). Significantly, the absence of introns flanking the CFC region in today's prion sequences suggests that these introns disappeared shortly after or during the emergence of the prion gene founder from its ZIP ancestor. Whenever intron loss is observed for two juxtaposed conserved introns, it appears to be the result of a reverse transcription of RNA intermediates (Roy and Penny, 2006). Mechanistically, RNA intermediates play a role in two types of intron loss events: gene conversions by recombination with spliced transcripts from the affected gene, or retroposon-mediated gene transfers (Coulombe-Huntington and Majewski, 2007; Kaessmann et al., 2009). Whereas the former mechanism converts the gene in its original genomic environment, the latter causes a transposition of a spliced copy of the original gene into a distant genomic acceptor site, generally assumed to represent transcriptionally active and open chromatin (Cereseto and Giacca, 2004; Sandmeyer et al., 1990). Consequently, criteria for the designation of retrocopies are the absence of synteny relationships with parent genes and the loss of at least two introns in regions that can be aligned to homologous parent genes (Marques et al., 2005; Vinckenbosch et al., 2006). In the context discussed here, the application of these criteria suggests the interesting possibility that the emergence of the prion gene founder may have been the result of an ancient germline retroposition event which occurred around the time of vertebrate speciation, a few hundred million years after the emergence of the CFC domain. Efforts to shed light on the genomic rearrangements which preceded the emergence of the prion founder are ongoing. Current results based on comparative gene structure, promoter and synteny analyses support a two-step model (Ehsani et al., 2011).

9. Function of the ZIP ectodomain

Regardless of the precise gene arrangements which occurred at the time, the presence of the disulfide bridge which defines the boundaries of the CFC would be expected to pose a spatial restraint that may limit the stretch of sequence within the CFC to evolve. A related restraint might also be at play if the CFC domain and the surrounding sequences were serving a distinct physiological function that places a strong negative natural selection on genomic alterations in this region. Currently, very little is known about the role of the PL domain in ZIP transporters and therefore one can only speculate about what such a function might be. The observation that both unicellular organisms and plant genomes appear to lack ZIP transporters containing a PL domain (Schmitt-Ulms et al., 2009) indicates that this domain is not part of the catalytic core required for metal import. Mutations located in the N-terminal domain of ZIP4, including a subset of mutations mapping to the PL domain, have been linked to AE, indicating that this domain may be critical for a ZIP transporter responsible for dietary zinc uptake (Wang et al., 2004b). Significantly, the latter mutations have been shown to interfere with the aforementioned ZIP4 ectodomain shedding observed during prolonged zinc deficiency, a cellular response that led to an increase in the rate of zinc import by the truncated ZIP transporter (Kambe and Andrews, 2009). Taken together, these data suggest that the entire N-terminal domain may serve a role as a negative regulator or buffer of metal import when zinc is replete, which can be shed when zinc is limiting (Kambe and Andrews, 2009). The PL domain itself may represent a molecular module within the N-terminal domain which can integrate cues of metal sensing and translate them into the protection (replete metal status) or exposure (limiting metal status) of a nearby proteolytic cleavage site. Mechanistically, such a switch could, for example, rely on the PL domain controlling access to a proteolytic cleavage site by engaging in protein–protein interactions or steering the spatial distribution of ZIP transporters. Alternatively, the PL domain may be able to undergo a metal-dependent conformational change that exposes a nearby scissile bond. Due to their limited mobility or outright sessile nature, unicellular organisms and plants are typically exposed to relatively stable metal levels in their environment. Metazoa, in contrast, are exposed to relatively large fluctuations of metal levels either through dietary exposure or on account of their mobility. Thus, the evolutionary success of the PL domain in this branch of life might be the result of a natural adaptation that enabled metazoa to cope with complex changes to metal levels. The development of a buffering ectodomain equipped with metal-dependent 'release' functionality might represent an economical solution to address this need (Fig. 5).

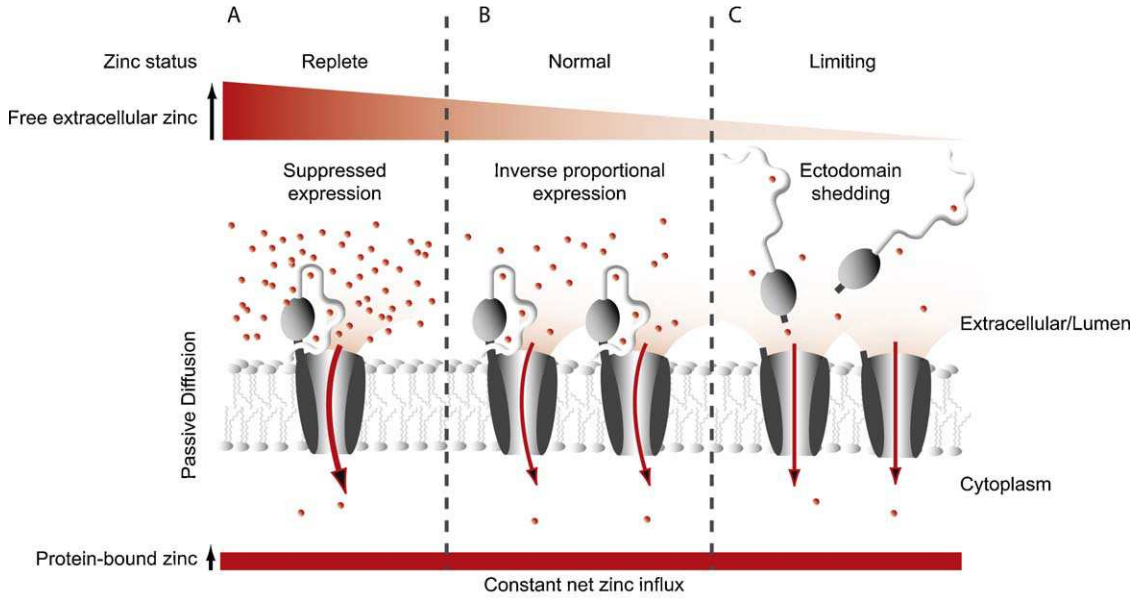

Fig. 5. Schematic depicting adaptive cellular responses to changes in extracellular divalent cation levels. The model is based on biochemical data reported for ZIP4 (Kambe and Andrews, 2009) and unpublished observations made for murine ZIPs 6 and 10. LZT ZIP exposure to (A) replete, (B) normal, and (C) limiting zinc levels in medium.

Naturally, it is currently unclear whether present-day PrP^C molecules have retained functional features of ZIP PL domains. It will, for example, be of interest to determine whether PrP recapitulates zinc- or copper-dependent endoproteolytic cleavages observed in ZIPs. A hint that endoproteolytic cleavages are critical for PrP to exert its biological role has come from the previously mentioned myelin-maintenance phenotype observed in PrP-knockout mice. Interestingly, this phenotype could be rescued by reintroduction of full-length PrP or PrP constructs that retained an amino-proximal cleavage site, but not by variants nonpermissive to cleavage (Bremer et al., 2010).

Assuming PrP had retained an ability to act as a negative regulator of metal import, is there a plausible way to reconcile the observed opposite effects of PrP and ZIP6 on E-cadherin expression in the zebrafish gastrulation paradigm using the above model? As commented on earlier, this phenotype has been proposed to depend on the ability of ZIP6 to import zinc and thereby charge the Snail transcriptional repressor with zinc (Yamashita et al., 2004). Thus, any cellular activity that quenches zinc levels outside the cell or inhibits ZIP6 directly may counteract this process. It is tempting to hypothesize that zebrafish PrP (or PrP-related sequences) may fulfill one or even both of these roles by acting as a physiological zinc buffer and/or by directly binding to zebrafish ZIP6 and therefore fortifying the zinc uptake barrier created by the ectodomain of the ZIP6 zinc transporter.

10. Conclusions and future directions

It is hoped that this review will help scientists who embark on research in this direction to sort through a large body of literature. Much remains to be discovered, in particular about the large and omnipresent family of ZIP transporters. Not only does the ZIP–PrP link introduce a fresh angle and ability to 'focus', but it also translates directly into novel experimental paradigms and testable hypotheses. Aside from the high-resolution structural characterization and comparison of ZIP transporters and prion proteins, it is to be anticipated that this finding will trigger investigations which (1) explore the possible functional involvement of prion proteins in ZIP-mediated metal ion transport, (2) determine the specificity and interface of the prion–ZIP interaction and whether binding occurs within the same cell or between neighboring cells, (3) aim at understanding the significance of a potential metalloproteinase catalytic center embedded within the C-terminal domain of ZIP proteins, (4) probe whether the N-terminal domains of ZIP proteins, as part of their physiological metal sensing function, undergo extensive conformational changes (such changes would be interesting as they may mimic changes in the physicochemical properties of prion proteins encountered in prion disease) and (5) study the possibility of a direct involvement of ZIP transporters in the manifestation and propagation of prion diseases.

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