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# Mammalian Zinc Transport, Trafficking, and Signals*[S]

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Structural, catalytic, and regulatory functions of zinc in biology continue to be defined. The number of genes coding for proteins with zinc-binding domains is conservatively estimated at >3% of the human genome but possibly is as much as 10% (1, 2). Zinc utilization in abundant, yet diverse, applications illustrates why organisms have evolved specific pathways to homeostatically regulate availability of this essential micronutrient at specific cellular sites through an array of transporters. Animals regulate zinc gain and loss efficiently. In humans, about 1% of the total body zinc content is replenished daily by the diet (3). This is accomplished principally by tight control of two systems, absorption from the intestine and endogenous loss via pancreatic and other intestinal secretions.

Loss-of-function studies have not provided a delineation of how zinc participates in physiologic activities. Nevertheless, immune function and resistance to infection and control of nitrosative and/or oxidative stress of inflammation are two areas of particular contemporary interest. Unfolding knowledge of cell type-specific expression of zinc transporter genes that respond differentially to hormonal and cytokine stimulation should aid in understanding the role of zinc in these physiologic systems. Coordination of intracellular zinc trafficking has focused on the cysteine-rich protein metallothionein (MT).[2] Zinc-dependent expression of *Mt* and its well documented response to multiple mediators, including oxidants, cytokines, hormones, and nitric oxide, have been extensively investigated. Here we review recent findings of how zinc transporters and MT influence mammalian cellular zinc metabolism and signaling pathways.

## Zinc Transporters

The first mammalian zinc transporter gene, *ZnT1*, was identified in 1995 (4). Before then, zinc transport in animals was viewed as occurring via co-transport as an anionic complex, as an amino acid (cysteine or histidine) chelate, or via the transferrin receptor route (5, 6). Two protein families have now been implicated in zinc transport. The ZnT (solute-linked carrier 30 (SLC30A)) proteins lower intracellular zinc by mediating zinc efflux from cells or influx into intracellular vesicles. The Zip (Zrt- and Irt-like proteins (SLC39A)) proteins promote zinc transport from the extracellular fluid or from intracellular vesicles into the cytoplasm (7–9).

The mammalian ZnT family consists of 10 members (ZnT1–10). Zinc transporter activity for most ZnT proteins (ZnT1,2,4–8) has been confirmed indirectly by survival of cells in medium of high zinc content or directly through measuring zinc uptake/efflux or zinc accumulation in transfected or mutated mammalian cells, zinc-sensitive yeast strains, and *Xenopus* oocytes (4, 10–16). The ZnT-mediated transport mechanism is unknown. Cellular extrusion of zinc and zinc vesicular deposition occur against a zinc concentration gradient; therefore, it is likely that ZnT zinc transporters function as secondary active transporters or perhaps as antiporters. Homologous proteins function as antiporters exchanging $Zn^{2+}$ for $H^+$ or $K^+$ (17, 18) or are able to transport zinc without an energy source or proton gradient when reconstituted in proteoliposomes (19).

There is considerable sequence homology among human ZnT proteins (20). These sequences vary in size, and most of them are predicted to have 6 transmembrane domains (TMD) with the exception of ZnT5, which has 12. These proteins have both N and C termini on the cytoplasmic side of the membrane. In addition, most ZnT proteins have a long intracellular loop with a variable number of histidine residues (20). These loops should bind zinc and have been implicated as ion-binding domains (21). Hydropathy plots of SLC30 proteins from different species show that four amphipathic TMDs likely form the channel through which zinc is translocated (22, 23). Some ZnT proteins function as homo- or hetero-oligomers (24). These proteins are purportedly involved in the incorporation of zinc into enzymes, *e.g.* alkaline phosphatase, by mediating $Zn^{2+}$ transport into the lumen of the Golgi apparatus (24). Motifs have been found in ZnT sequences that may allow for protein-protein interactions (21, 25).

The mammalian Zip family (SLC39) consists of 14 members. Zinc transport activity for Zip1–8 and 14 has been confirmed (26–34) by using transfection of DNA into mammalian (human embryonic kidney, K562, and Chinese hamster ovary) cells. Transport activity has been measured by $^{65}Zn$ uptake or by using probes that produce emission fluorescence upon binding intracellular labile zinc. Cell-permeable fluorophores include Zinquin, Zin-naphthopyr 1, Newport Green, and FluoZin-3AM. Ion specificity and advances in fluorescence output and photostability of these fluorophores have improved their use for zinc trafficking and imaging studies. The mechanism of Zip-mediated transport is not well understood. Zinc uptake could be a facilitated process driven by a concentration gradient. hZip1 and hZip2 transporter activities do not require ATP (26, 27). Zip-dependent transport activity is induced by $HCO_3^-$, which suggests a symport mechanism (27). Metal competition studies indicate some mammalian Zip proteins are specific for zinc (28–30); however, zinc uptake activity for h/mZip1, mZip3, and hZip2 was found to be inhibited by multiple cations

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[2] The abbreviations used are: MT, metallothionein; ZnT, zinc transporter; TMD, transmembrane domain; IL, interleukin; LZT, LIV-1 subfamily of Zip zinc transporters; STAT, signal transducer and activator of transcription; iNOS, inducible nitric oxide synthase; CDF, cation diffusion facilitator; EMT, epithelial-mesenchymal transition.

(26–28). Recently, the transport activity of Zip14 for both zinc and non-transferrin-bound iron, when overexpressed in HEK cells and Sf9 insect cells, has been demonstrated.[3]

A dendogram shows sequence similarity among mouse Zip proteins (supplemental Fig. 1). The Zip family can be divided into four subfamilies: Zip I, Zip II, gufA, and LZT (35). Most mammalian Zip proteins, including Zip4–8, Zip10, and Zip12–14, belong to the LZT subfamily. Zip1–3 are from the Zip II subfamily; Zip9 is from the Zip I subfamily; and Zip11 clusters within the gufA subfamily. The LZT transporter family was named after LIV-1 (Zip6), the first Zip member (36). Most Zip proteins are predicted to have eight TMDs, but LIV-1 has only six. TMDs IV and V are highly conserved and could form the pore through which metals pass (37, 38). Zip proteins are predicted to have extracellular N and C termini and a long intracellular loop with a histidine-rich repeat (35). In contrast, immunolocalization studies suggest mouse Zip14 has an extracellular histidine-rich loop (34). The presence of specific motifs may confer to Zip proteins the option of other functions separate from zinc transport or for protein-protein interactions involving zinc. For example, the novel metalloprotease motif (HEXPHEXGD) of LZT proteins may allow them to function as matrix metalloproteases or participate in the catalytic properties of these enzymes. Zip10 has putative $C_2H_2$ zinc finger and cytochrome *c* motifs in its first TMD, suggesting novel roles for targeted metal transport.

Most ZnT proteins have been found in intracellular compartments, usually associated with endosomes, Golgi, or endoplasmic reticulum. ZnT1 appears to be the only ZnT transporter located at the plasma membrane, congruent with its role as the primary regulator of cellular zinc efflux (4). ZnT2 has a vesicular localization in pancreatic acinar cells, whereas ZnT1 has a vesicular localization but is also at the plasma membrane (39). ZnT9 is located in the nucleus during mitosis (25). ZnT5 localizes with secretory vesicles of pancreatic $\beta$ cells and at the apical membrane of enterocytes. ZnT10 could be located at the plasma membrane according to software calculations. Most Zip proteins have been observed at the plasma membrane; however, Zip7 was located at the Golgi apparatus (32). The localization of some Zip transporters may change according to zinc availability or physiologic conditions. Zip5 has a basolateral plasma membrane orientation in polarized cells during dietary zinc sufficiency, but its regulation by zinc is not defined (30, 40). Similarly, Zip14 is mobilized to the sinusoidal membrane of the mouse hepatocyte during acute inflammation and, therefore, increases zinc uptake as a component of the acute phase response.

## Transporter Regulation

Studies where zinc transporter regulation has been examined within an integrative context merge well with known zinc physiology (Fig. 1). The positive mode of *ZnT1* regulation by zinc supports regulation by MTF1 (metal-responsive transcription factor 1). *ZnT1* was identified as the gene responsible for zinc resistance in mutated baby hamster kidney cells and genomic

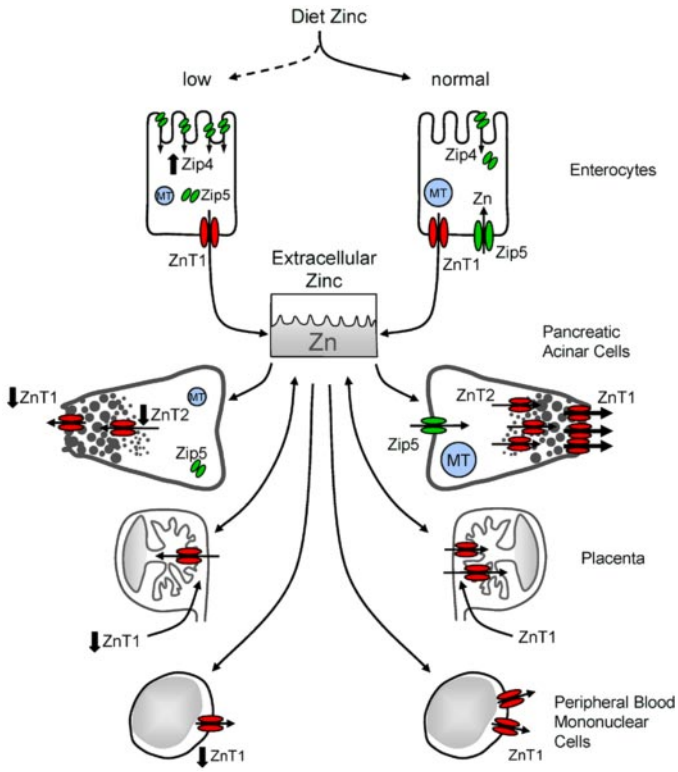


FIGURE 1. **Changes in murine zinc transporter expression in response to dietary zinc content.** Enterocytes increase *Zip4* expression when dietary zinc is low, with more Zip4 localized to the apical membrane. Presumably, this increases zinc acquisition from the diet. With an adequate zinc supply, enterocytes have a greater expression of MT, and Zip5 is localized to the basolateral membrane. Low dietary zinc decreases *ZnT1* and *ZnT2* expression in pancreatic acinar cells and causes internalization of Zip5 and a reduction in MT. Low zinc intake also decreases ZnT1 expression in placenta (visceral yolk sac) and peripheral blood mononuclear cells. These events are proposed to be among changes that reflect an attempt to restore zinc homeostasis during dietary zinc restriction through increased intestinal absorption, with concurrent reduction in zinc loss from pancreatic and intestinal secretions, coupled with zinc conservation by cells with high turnover such as those of the immune system.

*ZnT1* sequences (4). The behavior of *ZnT1* and a metal-responsive element-$\beta$ Geo reporter gene to zinc concentrations was similar. Homozygous knock-outs of either *ZnT1* or *MTF1* in mice are lethal to the embryo (41). Homozygous *MTF1*$^{-/-}$ embryos do not express any ZnT1 mRNA compared with those from wild-type or heterozygous mice, suggesting *ZnT1* is MTF1-regulated. In rodents, *ZnT1* is ubiquitously expressed, but mRNA abundance exhibits wide differences among tissues (7, 9). The response of ZnT1 to dietary zinc restriction or supplementation is also variable among different tissues (41–43). ZnT1 transcript levels are markedly reduced in peripheral blood mononuclear cells of zinc-deficient mice (39). In humans, ZnT1 mRNA levels in leukocytes increase markedly upon dietary zinc supplementation (44). Among human leukocyte subsets, ZnT1 transcripts are more abundant in monocytes than T-lymphocytes or neutrophils. Zinc responsiveness of *ZnT1* is not dependent upon MT expression as it is normal in MT$^{-/-}$ mice (45). Expression of neuronal ZnT1 is induced by forebrain ischemia (46), and considerable evidence shows ZnT1 is protective against $Zn^{2+}$-induced neuronal cell damage by promoting efflux (47, 48).

Multiple mutations in human *Zip4* produce the zinc malab-

[3] J. P. Liuzzi, F. Aydemir, H. Nam, M. D. Knutson, and R. J. Cousins, unpublished observations.

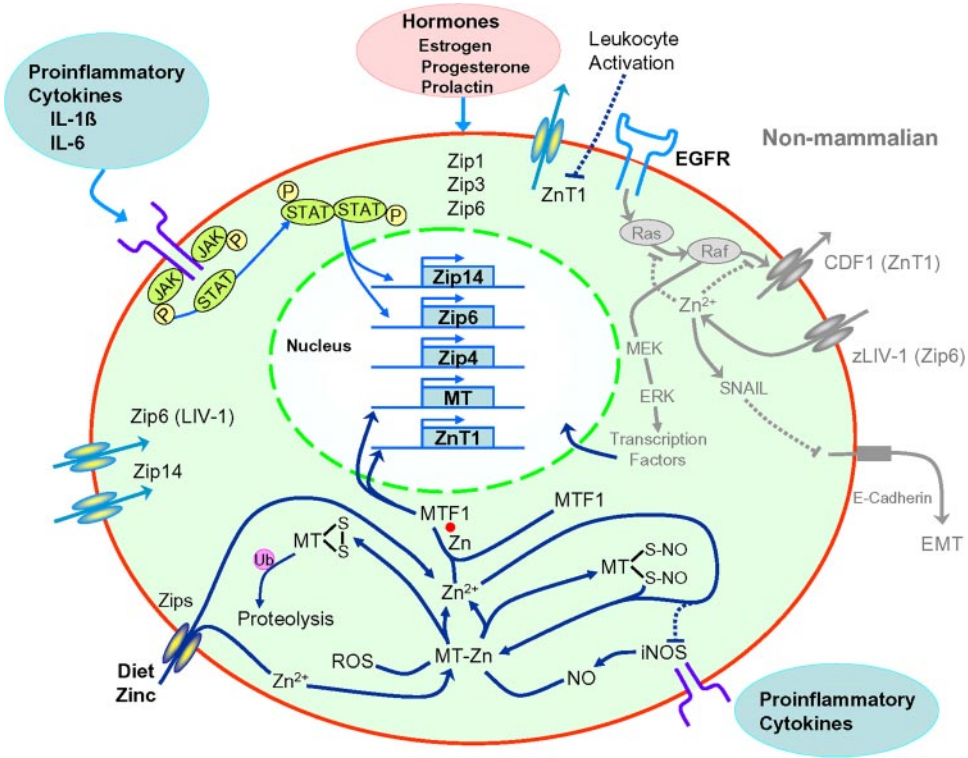


FIGURE 2. **Signaling pathways that regulate some ZnT and Zip genes and influence intracellular $Zn^{2+}$ trafficking.** Note: these events are cell-specific, but mechanisms are not well understood. Zinc-dependent changes in transporter localization are not shown. Changes in the dietary zinc supply influence *ZnT1*, *ZnT2*, *Zip4*, and *Mt* expression. MTF1 controls *Mt* and *ZnT1*. Nitrosative stress induced by proinflammatory cytokines increases NO and stimulation of $Zn^{2+}$ release from MT, which activates MTF1. Events that induce iNOS and increase NO lead to MT thionitrosation, which may be reversed by additional $Zn^{2+}$. In contrast, irreversible oxidation of MT by reactive oxygen species (*ROS*) leads to degradation, probably via ubiquitination. Proinflammatory cytokines, including IL1$\beta$ and IL6, stimulate STAT-mediated signaling, up-regulation of *Zip14* and *Zip6*, and cellular zinc influx. Prolactin, estrogen, and testosterone regulate cell-specific expression of some Zip transporter genes. Non-mammalian models have shown $Zn^{2+}$-mediated regulation of major signaling pathways by homologues of mammalian ZnT1, and Zip6 influences important components needed for development and wound healing (shown in *gray*). EMT may be a factor controlled in part by $Zn^{2+}$ via transporter regulation. *Solid lines* indicate positive modes of action, and *dashed lines* indicate an inhibitory process. *EGFR*, epidermal growth factor receptor.

sorption phenotype, acrodermatitis enteropathica (49). Dietary zinc restriction in mice up-regulates *Zip4* and causes an increase in Zip4 localized to the plasma membrane of enterocytes (29, 39). Repletion with zinc rapidly down-regulates Zip4 expression and lowers membrane localization to basal conditions (39). Presumably, individuals with acrodermatitis enteropathica do not synthesize a fully functional Zip4 and are not capable of transporting sufficient dietary zinc to prevent a systemic deficiency. The defect can be overridden with supplemental dietary zinc. Therefore, supplemental zinc is used as a therapeutic measure (3). The mechanism of the negative responsiveness of *Zip4* to zinc is not known. Up-regulation of *Zip4* in enterocytes and concurrent down-regulation of *ZnT1* and *ZnT2* in pancreatic acinar cells of mice when dietary zinc intake is low are key factors that may balance intestinal intake with endogenous loss via pancreatic secretions (homeostasis) (39). Additionally, it has been proposed that Zip 5, although constitutively expressed in intestine and pancreatic acinar cells, is differentially internalized in response to dietary zinc intake and thus contributes to integrative homeostasis through producing less plasma to cell zinc transport in intestine (40) and kidney (30). *Mtf1*$^{-/-}$ liver cells show up-regulation of *Zip10*, suggesting MTF1 represses expression under basal conditions (50). The up-regulation of *Zip4* during zinc deficiency may be another example of MTF1 negative regulation (29, 39). Similarly, monocytic human cell lines made zinc deficient using a cell-permeant $Zn^{2+}$ chelator show increased *Zip2* expression (51). A corresponding increase in expression of a putative, uncharacterized, zinc-responsive transcription factor MTF2 was observed. Of note is that in zinc-supplemented human subjects, *ZnT1* and *Mt* expression, both of which are MTF1 mediated, are increased, although *Zip3* expression is decreased under the same conditions (44). Findings that some Zip genes are down-regulated by zinc is of considerable interest, as it is indicative of another potential mode of regulation by zinc.

A number of ZnT/Zip transporter genes appear to be regulated by hormones or cytokines (Fig. 2). These relationships closely follow established roles of these mediators in zinc physiology. Developmentally regulated changes in expression of some ZnT genes have been reported (9). Zip1 in prostate is regulated by prolactin and testosterone and may contribute to the atypically high zinc in the prostatic epithelium (52). Zip1 is markedly reduced in the malignant prostate and correlates with concomitantly lower zinc concentration in the malignant cells. Zip3 expression is very high in mammary epithelial cells, is inducible with prolactin, and is correlated with $^{65}Zn$ transport as shown by knockdown with small interfering RNA (53). LIV1 (Zip6) was identified as a novel estrogen-responsive gene in breast cancer cells that sequence data revealed was a member of the Zip transporter family (36). Zip6 expression is related to metastasis to lymph nodes. cDNA transfection and Newport Green ($Zn^{2+}$ fluorophore) were used to demonstrate Zip6 transporter function. *Zip8* (*BIGM103*) was identified as a TNF$\alpha$- and endotoxin-induced gene expressed in monocytes (33). Expression is very low in unstimulated monocytes. DNA microarray analysis of the response of humans to acute systemic inflammation induced by endotoxin administration revealed a marked transient increase in *Zip8* expression in total leukocytic RNA.[4] Among human leukocytes, *Zip8* exhibits extremely high transcript abundance in T-lymphocytes (44).

Systemic responses to sepsis and inflammation include hepatic zinc uptake, which produces a transient hypozincemia

[4] R. J. Cousins and T. Beker Aydemir, unpublished results.

(3). Zip14 was identified as highly induced in hepatocytes of mice during the acute phase response (34). This novel Zip protein was demonstrated to transport zinc (31, 34). Wild-type mice produce robust amounts of Zip14 mRNA in response to acute inflammation and exhibit hypozincemia, whereas *IL6*$^{-/-}$ mice produce no Zip14 and do not experience hypozincemia. Inflammation and IL6 *in vitro* increase Zip14 at the plasma membrane of hepatocytes. A probable signal pathway for this IL6-mediated response is via STAT regulation. *Zip14* regulation by lipopolysaccharide during the acute phase is more complex, including NO-induced activation of AP-1.[5] Zip14 regulation by IL6 and NO has implications for a role of zinc in resistance to toxin-induced liver injury and cancer progression.

## Integrative Interactions of Metallothionein-bound Zinc

Physiologic stimuli that regulate metallothionein synthesis merge with those that regulate specific ZnT and Zip genes (Fig. 2). Expression of MT is ubiquitous but is particularly high in parenchymal cells of the intestine, pancreas, kidney, and liver. Spatial arrangement of the 20 cysteines of the MT molecule accounts for metal binding that has high thermodynamic and high kinetic lability (54). The $\beta$ domain binds three $Zn^{2+}$ atoms via thiolate ligands from nine cysteines. The $\alpha$ domain binds four $Zn^{2+}$ atoms via eleven cysteines. The metabolic pool from which the apo-MT (thionein) molecule acquires these seven $Zn^{2+}$ atoms has not been identified. Structural studies using $^{1}H$- and $^{113}Cd$-NMR spectroscopy reveal that $Zn^{2+}$ atoms bound to thiolates of the $\beta$ domain are more labile than those of the $\alpha$ domain (54). This suggests the $\beta$ domain is physiologically relevant, whereas the $\alpha$ domain may be related to metal detoxification. Such dual properties for the same molecule complicate interpretation of studies aimed at function. MT binds $Zn^{2+}$ strongly (up to $10^{13}$ $M^{-1}$). However, experiments using model systems suggest MT may assume a donor/acceptor role for zinc-binding motifs and could activate or deactivate apo-zinc finger proteins or other zinc metalloproteins (55). Viability of *MT*$^{-/-}$ mice suggests essential zinc-dependent functions, such as formation of zinc finger proteins, acquire zinc from sources other than MT.

A widely studied role of MT is its ability to protect *in vivo* and *in vitro* against cellular stressors such as carbon-centered radicals and reactive oxygen and nitrogen species. The mechanism of cytoprotection remains unclear. *In vitro* studies have shown that the metal thiolate clusters of MT possess the unique ability to function as a redox unit; therefore, the protein has the potential to be involved in redox-sensitive signaling pathways (56). Comparative studies with nitric oxide (NO), $H_2O_2$, singlet oxygen, peroxyl radicals, and peroxynitrite suggest only stress from NO causing *S*-nitrosylation of MT cysteines and $Zn^{2+}$ release is sufficiently mild to allow reconstitution of MT through $Zn^{2+}$ rebinding (57). Fluorescence resonance energy transfer experiments have confirmed that in cultured endothelial cells MT undergoes conformational changes in the presence of NO donors, *e.g. S*-nitrocysteine (58). These alterations in cellular zinc homeostasis suggest protective effects of MT against nitric oxide toxicity (54). During inflammation or endotoxemia, hepatocytes respond to cytokines by up-regulating inducible nitric oxide synthase (iNOS), which generates large amounts of NO from arginine. Increased MT expression parallels the rise in cellular NO (59). NO promotes zinc release from MT, which in turn may repress iNOS (54). In zinc-deficient rodents, iNOS expression increases (60), perhaps because MT-bound zinc is not available. Another link between NO and MT may be through MTF1 activation. MTF1 nuclear translocation occurs under a variety of stress conditions. Hypoxia and oxidants such as $H_2O_2$ and *tert*-butylhydroquinone have been shown to increase MTF1 binding to the metal-responsive elements of the *MT* promoter. Release of labile zinc from MT or other thiolate ligands by NO may provide the means, via zinc binding to MTF1 and other zinc finger proteins, for regulation of other metal-responsive genes that are involved in cellular protection, including those of the *ZnT* and *Zip* families.

## Non-mammalian Zinc Transporters and Signaling

Homology between mammalian and non-mammalian zinc transporters suggests conserved functions. Here we present two examples, drawn from the non-mammalian literature, that integrate zinc transport into cell signaling mechanisms common to divergent species, including mammals (Fig. 2). Zinc has been shown to inhibit Ras signaling in numerous *in vitro* experiments. In *Caenorhabditis elegans*, the cation diffusion facilitator (CDF) protein family confers resistance to many heavy metals. CDF-1, which is a homologue of mammalian ZnT1, responds to $Zn^{2+}$ in a mode consistent with activity as a zinc exporter (61). CDF-1 activity reduces cytosolic $Zn^{2+}$ concentrations and concomitantly increases Ras-mediated signaling. $Zn^{2+}$ may function to maintain Ras in an inactive state. Because *CDF-1* is expressed in intestinal cells of *C. elegans*, there is a potential for ZnT1 involvement in the abnormal regulation of Ras associated with colon cancer in humans. Similarly, CDF-1 and ZnT1 bind to Raf-1 and may be responsible for full activation of this downstream component of transduction (62) initiated through ligand binding by receptors for growth factors, mitogens, and hormones.

Zip6 (LIV1) may be involved in control of epithelial-mesenchymal transition (EMT). EMT is essential for embryonic development, tissue regeneration, and metastasis of neoplastic cells. During gastrulation in zebrafish, STAT3-dependent zLIV-1 (Zip6) expression regulates the nuclear translocation of the zinc finger protein Snail, which regulates EMT in organizer cells, and their invasive behavior (63). Through comparable mechanisms, Zip6 and Snail may be involved in metastasis of breast cancer cells, possibly through constitutive activation by STAT3 (64). Similarly, the LIV1 (Zip6) homologue FOI, the fear of intimacy gene, is required for proper gonad formation in *Drosophila* (65). A common downstream target for LIV1 (Zip6) and its homologues could be to inactivate cadherin expression, which has important roles in development, tissue remodeling, wound healing, and tumor promotion and metastasis.

## Conclusions

Within the past decade, tremendous strides have been made toward our understanding of zinc transport. Evolving descriptions of tissue-specific expression and regulation by zinc

[5] L. A. Lichten and R. J. Cousins, unpublished results.

through metal-responsive transcription factor, MTF1, or through cytokines, growth factors, and hormones merge well with known zinc physiology. The emerging role of $Zn^{2+}$ as another secondary signaling mediator and control of that signaling through cellular and vesicular transport is providing exciting new insights into functional outcomes of zinc trafficking. Pathways of $Zn^{2+}$ transport and their dysregulation have been linked to specific diseases. The labile nature of $Zn^{2+}$ bound to specific zinc finger motifs and in cluster domains, as found in metallothionein, has shown how nitric oxide and reactive oxygen species mobilize bound $Zn^{2+}$ with subsequent reentry into cellular $Zn^{2+}$ pools.

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