I, __________________ Jodie Michelle Reed _______________________, hereby submit this work as part of the requirements for the degree of:

Master of Science

in:

Environmental Genetics and Molecular Toxicology

It is entitled:

Characterization of the 8-Transmembrane ZIP8 Transporter: Evidence of Intracellular Trafficking in Response to Extracellular Metal Concentrations

This work and its defense approved by:

Chair: Timothy P. Dalton, Ph.D.
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Characterization of the 8-Transmembrane ZIP8 Transporter: Evidence of Intracellular Trafficking in Response to Extracellular Metal Concentrations

Submitted to the
Division of Research and Advanced Studies
of the University of Cincinnati

in partial fulfillment of the
requirements for the degree of

MASTER OF SCIENCE

in the Department of Environmental Health
of the College of Medicine

2007

by

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Abstract:

Cadmium (Cd\textsuperscript{2+}, Cd) is a non-essential toxic metal, ubiquitous in the environment. The two most common sources in humans include cigarette smoke and Cd-contaminated foods such as shellfish. Cd uptake occurs principally via the intestine and lung, after which the metal accumulates in the liver, reproductive organs and kidney; kidney is ultimately the main target organ. The primary target of Cd in the kidney is the proximal convoluted tubule. The mechanism(s) of Cd toxicity and carcinogenicity are not well understood, although it is recognized that Cd exerts its effects inside the cell. Previous studies in our lab have shown that the mouse \textit{Slc39a8} gene encodes ZIP8, a ubiquitous transporter responsible for Cd-induced testicular necrosis and renal failure. Using mouse fetal fibroblasts (MFFs) engineered to express ZIP8, our lab has found that essential metal substrates appear to include Mn\textsuperscript{2+} and Zn\textsuperscript{2+}; movement into the cell requires HCO\textsubscript{3}– ion, indicating that ZIP8 is a divalent ion/bicarbonate symporter. Cd is a rogue hitchhiker able to displace Mn or Zn and gain cell entry.

My studies extended this work to Madin-Darby canine kidney (MDCK) cells, a polarized epithelial cell line. Using stable retrovirally infected MDCK cells, ZIP8 was shown to localize to the apical rather than basolateral surface; some ZIP8 was also seen inside the cell but outside the nucleus. We found that ZIP8 trafficking is regulated by metal ion availability: ZIP8 is predominantly on the cell surface under conditions of Zn depletion and predominantly within intracellular membranes under conditions of Zn homeostasis. Metal ion regulation of ZIP8 could be critically important in controlling the uptake of Mn\textsuperscript{2+} and Zn\textsuperscript{2+}, as well as Cd and perhaps other non-essential metals—from the environment into the animal by way of this symporter on the apical surface of cells.
Acknowledgments:

- NIH Grant R01-ES10416
- NIH Grant P30-ES06096

I would like to thank my advisors, Dr. Timothy Dalton and Dr. Daniel Nebert for allowing me to work in their laboratories. I have learned a great deal in my time with the lab and appreciate their wisdom and encouragement. I thank my Mom and Dad for their love and support. I also want to thank my friends for their continued support, friendship, humor, and their ability to help maintain my sanity. Thank you to Chris Curran, Scott Schneider, Eric Kendig, Lei He, Ying Chen, and Hongbin Dong. Last, but certainly not least, I would like to thank my future husband, Scott Ozanne. Without his love, support, sense of humor, and unfaltering faith in me, I would not have made it to where I am today – I love you.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Figures</td>
<td>6</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>7</td>
</tr>
<tr>
<td>Introduction</td>
<td>9</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>12</td>
</tr>
<tr>
<td>Results</td>
<td>17</td>
</tr>
<tr>
<td>Discussion</td>
<td>24</td>
</tr>
<tr>
<td>Figures and Figure Legends</td>
<td>29</td>
</tr>
<tr>
<td>References</td>
<td>37</td>
</tr>
</tbody>
</table>
List of Figures

Figure 1. Phylogenetic dendrogram of the 14 mouse and 14 human SLC39 genes.

Figure 2. Predicted molecular structure of the ZIP8 metal transporter protein and localization of ZIP8 antibodies.

Figure 3A. Western immunoblot analysis of anti-ha antibody.
Figure 3B. Western immunoblot analysis of ZIP8 antibody, aZ-1.
Figure 3C. Western immunoblot analysis of ZIP8 antibody, aZ-3.
Figure 3D. Western immunoblot analysis of ZIP8 antibody, aZ-4.

Figure 4. Expression of ZIP8ha and ZIP4ha in MDCK cells.

Figure 5. PNGase F treatment and Western immunoblot of MDCK-ZIP8ha cells.

Figure 6. Cadmium toxicity in MDCK-ZIP8ha cells.

Figure 7. ZIP8 membrane localization in polarized renal epithelial cells.

Figure 8. Measurement of ZIP8ha surface level protein in MDCK cells.
**Abbreviations**

AE – Acrodermatitis enteropathica  
ATSDR – Agency for Toxic Substances and Disease Registry  
Ca$^{2+}$ - Calcium  
Cd – Cadmium  
CdCl$_2$ – Cadmium chloride  
Cdm – Cadmium locus  
DMEM – Dulbecco’s modified Eagle’s medium  
FBS – Fetal bovine serum  
GFP-NBC1 - Green fluorescent protein fused with the sodium bicarbonate cotransporter-1 protein  
ha – Hemagglutinin tag  
IARC – International Agency for Research on Cancer  
IRT – Iron-related transporter  
K$^+$ - Potassium  
MDCK – Madin-Darby canine kidney (cell line)  
MDCK-Luc - Retrovirally-transformed MDCK cells expressing luciferase  
MDCK-ZIP4ha – Retrovirally transformed MDCK cells expressing ZIP4 (plus a C-terminal ha tag)  
MDCK-ZIP8ha - Retrovirally transformed MDCK cells expressing ZIP8 (plus a C-terminal ha tag)  
MFFs – Mouse fetal fibroblasts  
Mn$^{2+}$ - Manganese
Na\textsuperscript{+} - Sodium

PBS – Phosphate buffered saline

PNA – Peanut agglutinin

SLC – Solute carrier gene superfamily

Slc39 – Solute carrier gene family 39 in mouse (SLC39A8 in human)

ZIP – Zrt-like, Irt-like Protein

ZIP4 – ZIP protein encoded by the gene Slc39a4

ZIP8 – ZIP protein encoded by the gene Slc39a8

Zn\textsuperscript{2+} - Zinc

ZnCl\textsubscript{2} – Zinc chloride

ZRT – Zinc-related transporter
Introduction

Cadmium (Cd$^{2+}$; Cd) is a naturally-occurring element in the earth’s crust. In terms of living organisms, Cd is a non-essential, highly toxic metal. Cd has become an environmental pollutant since the time of industrialization and increasingly poses a health threat to humans. Cd enters the air from mining, industry, burning coal and household wastes; the divalent cation enters water and soil from waste disposal and spills or leaks at hazardous waste sites. Cd does not break down in the environment, but can change forms. Fish, plants, and animals take up Cd from the environment (1).

Sources of Cd exposure include cigarette smoke, contaminated soil (around metal-smelting operations), and contaminated foods such as shellfish. Cd is taken up principally via the intestine and lung; accumulation occurs in the liver and reproductive organs, but mainly in the kidney. In the kidney, Cd primarily affects the proximal convoluted tubule, impairing reabsorption of numerous substances (2). Acute exposures to Cd can result in damage to the central nervous system, gastrointestinal tract, lung, liver, bone, placenta, ovary and testis (3, 4). High doses of Cd exposure lead to itai-itai disease, which occurred in Japan as the result of mining pollution. Itai-itai in Japanese translates to “ouch-ouch”, and the name is due to the pain experienced—due to osteomalacia and osteoporosis (5). These patients also experience renal dysfunction; it is believed by some that the progression in renal disease is what leads to the observed bone disorders (6). Cd stays in the body a long time and can build up—after many years of exposure to low environmental levels. Chronic exposure to low levels can lead to renal Fanconi syndrome, a disorder first described by Guido Fanconi in 1936. The disorder is marked by excessive urinary loss
of water, solutes (Na\(^+\), K\(^+\), Ca\(^{2+}\), Mg\(^{2+}\), carnitine), and low-molecular-weight proteins (enzymes, peptide hormones, light-chain immunoglobulins). Clinical features of this syndrome were identified in workers exposed to Cd and include polyuria, dehydration, hypokalemia, metabolic acidosis, impaired growth, and rickets (7).

The IARC has classified Cd as a “Category I” human carcinogen. The mechanisms by which Cd causes lung cancer or lung or kidney toxicity are not well understood, although it is known that Cd exerts its effects inside the cell. The populations with the highest risk for Cd-associated lung cancer are smokers, people eating excessive amounts of contaminated shellfish and high-fiber foods, women with low iron stores, and malnourished persons (8, 9). The mechanisms of Cd influx and efflux in mammalian cells are still elusive (10-12). Understanding these mechanisms could be instrumental in the prevention of environmental disease due to the accumulation of Cd and perhaps other non-essential metals.

Cadmium has no beneficial purpose in living organisms; because of this, it is believed that Cd “uses” transporters of one or more essential metal ions to enter the cell and intracellular organelles. In lower eukaryotic cells (plants, yeast) and prokaryotes, there have been many studies about Cd transport. Little was known, however, about the mechanisms of Cd transport in vertebrates—until the very recent publications from this laboratory. Several decades ago, it was observed that certain inbred strains of mice were resistant to Cd-induced testicular necrosis (13). This trait was found to be autosomal recessive, was assigned to mouse chromosome 3, and was termed the Cdm locus. The *Slc39a8* gene, which encodes the ZIP8 mRNA and protein, has been demonstrated by our group to be the Cdm locus (14).
The *Slc39a8* gene is a member of the solute-carrier (*SLC*) gene superfamily; this superfamily represents a large number of membrane-bound passive transporters, ion-coupled transporters, and exchangers (15). Different families of SLC transporters do not share close ancestry. A protein is assigned to a specific SLC family if it has at least 15-25% amino acid sequence identity in a “signature sequence” to other members of that family. At this time, there are 46 families and 360 purportedly functional *SLC* genes.

*Slc39a8* is in the mouse *Slc39* gene family, of which there are 14 members. The human *SLC39* family also has 14 members and, when compared to the mouse *Slc39* family, all members are orthologous and high conserved. The *SLC39* genes encode ZIPs, meaning Zrt-like, Irt-like Proteins, which had been characterized first in *Saccharomyces cerevisiae* (*Zrt*) and *Arabidopsis thaliana* (*Irt*); ZRT stands for zinc-related transporter, whereas IRT stands for iron-related transporter (16). In mouse fetal fibroblasts stably infected with ZIP8, a 10-fold increase in the rate of Cd uptake and accumulation and a 30-fold increase in sensitivity to Cd-induced cell death was observed (17, 18).

In the work for this thesis, Madin-Darby canine kidney (MDCK) cells were used to characterize ZIP8 and to study trafficking properties of this 8-transmembrane (8TM) transporter. The use of MDCK cells increases the physiological relevance of the study, because they are a renal epithelial cell line. In 1958, the cell line was established by S. H. Madin and N. B. Darby from the kidney tissue of an adult female cocker spaniel. The MDCK cells demonstrate typical epithelial morphology, maintaining their apical and basolateral surfaces, and are widely used as a model for polarized epithelial cells.
Materials and Methods

**Chemicals** - CdCl₂ and ZnCl₂ were bought from Fisher Scientific (Pittsburgh, PA).

Bovine serum albumin and the remainder of the chemicals—including Chelex 100—were purchased from Sigma (St. Louis, MO).

**Cell culture and transfection methods** - MDCK cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen; Carlsbad, CA) plus 10% fetal bovine serum (FBS) from Hyclone (Logan, UT) at 37°C in 5% CO₂. All culture medium contained 100 units/ml of penicillin and 100 µg/ml streptomycin. The ZIP8-adenovirally-infected cells were maintained in selection medium (17) supplemented with hygromycin (600 µg/ml) and puromycin (5 µg/ml). For Z-stack analysis, transfection was carried out according to the manufacturer’s protocol for Lipofectamine 2000 (Invitrogen).

**Cloning of the ZIP8 cDNA and Delivery into MDCK Tet-Off Cells** - Oligo-dT-primed reverse transcription was carried out with C57BL/6J mouse testis total RNA. Primers for amplification began at the start codon and ended at the stop codon. A consensus Kozak sequence at the start-site was included for efficient expression. Restriction sites for ZIP8 were added at the 5’ (Bam HI) and 3’ (Cla I) ends of the coding sequence for cloning into the pRevTRE vector (Invitrogen); for ZIP4, the sites are 5’ (Bam HI) and 3’ (Sal I). The ZIP4-expressing vector was a gift from Glen Andrews (University of Kansas, Kansas City). In each 3’ primer, the coding sequence of a hemagglutinin (ha) tag was also inserted in-frame, before the termination codon of that protein-coding sequence. The MDCK cell line was a generous gift from the laboratory of Dr.
Manoocher Soleimani. These cells were infected with a retrovirus encoding the Tet-off receptor (19), and selection was carried out using puromycin (5 µg/ml) resistance. These cells were infected with retrovirus-encoding control luciferase (Luc), ZIP8ha, or ZIP4ha cDNAs, to generate, respectively, the stable MDCK-Luc, MDCK-ZIP8ha, and MDCK-ZIP4ha cell groups that were selected for hygromycin (600 µg/ml) resistance. The transporters are constitutively expressed in these cells via retroviruses expressed from pRevTRE.

**Measurement of Metal-Induced Cell Death** - The viability of cells was determined after exposure to increasing concentrations of CdCl$_2$. Cell viability was determined as the cleavage of 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT), as described by the manufacturer's protocol (Promega; Madison, WI).

**Western Immunoblot Analysis** - Cells cultured in 100-cm dishes were washed three times in ice-cold phosphate-buffered saline (PBS), and then collected (by scraping) into ice-cold PBS and pelleted by centrifugation. After three washes in ice-cold PBS, the cells were lysed by sonication in lysis buffer containing 62 mM Tris-Cl, pH 6.8, 2% sodium dodecylsulfate (SDS), 5 mM 2-mercaptoethanol, and protease inhibitors. The cellular proteins were then ready for polyacrylamide gel electrophoresis (PAGE). Proteins were quantified by using the BCA protein assay (Pierce; Rockford, IL). Proteins were denatured, run on SDS/PAGE gels, transferred to nitrocellulose, and detected by chemiluminescence using anti-ha antibodies or anti-ZIP8 antibodies (aZ-1, aZ-2, aZ-3, or aZ-4) at a 1:10,000 dilution (Bethyl Laboratories; Montgomery, TX),
followed by horseradish peroxidase-conjugated secondary antibodies at a dilution of 1:1,000 (Dako; Carpinteria, CA).

**Western Immunoblot Analysis of ZIP8 Protein Glycosylation** – Cells grown in 100-cm dishes were washed, collected, and pelleted in ice-cold PBS. PBS was removed, the pellets were dissolved in denaturing buffer (0.5% SDS plus 1% 2-mercaptoethanol), and protein concentrations of each cell lysate was determined by the BCA protein assay. Lysates (10 µg protein) were denatured at 37°C for 30 min. Nonidet P-40 and sodium phosphate buffer (pH 7.5, 25°C) were added to make final concentrations of 1% and 50 mM, respectively. PNGase F (1000 U; New England Biolabs, Beverly, MA) was added, and the mixture incubated for at 37°C for 2 h. Samples were denatured with SDS-PAGE loading buffer (37°C for 30 min), then run on a 10% SDS-PAGE gel, transferred to nitrocellulose, and detected by chemiluminescence using anti-ha antibodies at a 1:5,000 dilution, followed by horseradish peroxidase-conjugated secondary antibodies at a dilution of 1:1,000 (Dako).

**Z-Stack Confocal Microscopy for Detecting Cell Surface-Bound ZIP Proteins** - Zinc depletion of FBS was carried out using Chelex 100. Briefly described, a solution of 5% (w/v) Chelex 100 resin in FBS was incubated overnight, with constant stirring, followed by filtration through a 0.2-µm filter. This FBS was then added into DMEM to make Chelex 100 medium. MDCK cells were seeded onto (non-coated) cover slips in a 24-well plate. The next day, each well of cells was transfected with the combination of the following plasmids—pRevTet-Off and pRevTRE-ZIP8—according to the manufacturer’s
transfection protocol for Lipofectamine 2000 (Invitrogen). Some wells containing cells were transfected with the plasmid encoding green fluorescent protein (GFP) fused with the \( \text{Na}^+/\text{HCO}_3^- \) cotransporter-1 protein GFP-NBC1 (20). Two days after transfection, the cells were incubated in Chelex 100 medium for 1 h and then fixed with 3% formaldehyde for 20 min. Next, the cells were permeabilized with 0.1% Triton X-100 for 4 min and blocked with 10% FBS-containing PBS medium for 1 h. Fixation, permeabilization, and blocking were all done at room temperature. The anti-ha antibody, at 1:500 dilution, was incubated overnight at 4°C with cells in 1% bovine serum albumin containing PBS. The next day, the primary antibody solution was removed, and the cells were washed three times with PBS (5 min each time). The secondary antibody, Alex488-\( \alpha \)-rabbit or Alex568-\( \alpha \)-rabbit (Invitrogen), was incubated with the cells at room temperature for 1 h. Next, cells transfected with GFP-NBC1 were mounted for confocal analysis; cells that were not transfected with GFP-NBC1 were co-stained with an apical membrane marker, peanut agglutinin (PNA)-lectin (Invitrogen), at 1:200 dilution for 1 h at room temperature and then mounted for confocal analysis. Images were taken on a Zeiss LSM510 confocal microscope (Carl Zeiss, Oberkochen, Germany). Both Z-line and Z-stack images were obtained, using the LSM5 Image software to analyze specific cellular membrane targeting.

Detection of ZIP8ha Protein Levels at the Plasma Membrane – The pool of ZIP4ha and ZIP8ha at the plasma membrane was assessed by measuring the levels of anti-ha antibodies bound to the surface of MDCK-ZIP4ha and MDCK-ZIP8ha cells. MDCK-ZIP4ha and MDCK-ZIP8ha cells were cultured in 6-well plates, and treated with Chelex-
treated medium. Briefly described, 3% (w/v) Chelex 100 resin in DMEM plus 10% FBS was incubated overnight with constant stirring, followed by filtration through a 0.2-µm filter. After the treatment period, cells were washed twice with ice-cold PBS, and fixed for 30 min at 4°C in 4% paraformaldehyde without subsequent permeabilization steps. Cells were then blocked for 1 h at room temperature, using 5% goat serum and 1% milk in PBS, and incubated with 5 µg/ml anti-ha antibody for 30 min at room temperature. Cells were washed five times in PBS to remove unbound antibodies and then lysed by sonication in lysis buffer, as described above for immunoblotting. Cell lysates containing the solubilized anti-ha antibodies—that were bound to the ZIP4ha or ZIP8ha protein at the plasma membrane—were separated using SDS-PAGE, transferred to nitrocellulose membranes, and the anti-ha antibodies were then detected using horseradish peroxidase-conjugated antibodies (Dako; 1:1,000) by chemiluminescence (Pierce).
Results

Development of Mouse ZIP8 Antibodies. The development of four separate ZIP8 antibodies was a combined effort by this lab and Bethyl Laboratories using the ZIP8 *mus musculus* protein sequence from NCBI, NP_080504. The four antibodies produced were: aZ-1, positioned at the N-terminus; aZ-2 and aZ-3 in the loop between transmembrane domains 3 and 4; and aZ-4, at the C-terminus (Fig. 2). Production of ZIP8 antibodies will alleviate a need for hemagglutinin-tagged proteins. Until now, the only way of detecting these transporters was by inserting a coding sequence for a hemagglutinin (ha) tag in-frame, at the 3’ primer (before the stop codon of the coding sequence). This would produce transporter protein with a short ha tag at the C-terminus to which ha antibodies could recognize and henceforth, be detected with western immunoblotting techniques. Previous studies by this lab have shown that addition of the ha tag does not influence the ability of ZIP8 to transport metals into the cell (17, 18). Albeit useful, the ha tag can only be employed in *in vitro* experiments. To investigate the role of ZIP8 in *vivo*, it is necessary to develop antibodies against ZIP8. Western immunoblotting techniques were used to determine binding efficiency of each of the four antibodies to ZIP8 protein in MFF-infected cells, using ha antibodies as control (Fig. 3A). ZIP8 antibody aZ-2 produced many bands in all cell types tested (data not shown), therefore, aZ-2 was deemed inadequate for identifying ZIP8 protein. Antibodies aZ-1 and aZ-3 (Fig. 3B and 3C) showed analogous banding patterns in both MFF-ZIP8 and MFF-ZIP8ha cells, with large bands appearing at ~150 kDa and also at ~55 kDa. A similar band pattern is seen with the anti-ha antibodies shown in Fig. 3A. The calculated molecular mass of the ZIP8 protein is 49.5 kDa without ha signal.
peptide, and 51.3 kDa with ha signal peptide. The probable explanation for the large upper band (~150 kDa) is glycosylation of the protein. Numerous secretory and plasma membrane proteins contain carbohydrate chains linked through serine or threonine (O-linked) or through asparagine (N-linked). The banding pattern of antibody aZ-4 showed a band at ~150 kDa, and a lower band at ~80 kDa. Interestingly, this band pattern was only observed in the MFF-ZIP8 cell line. The MFF-ZIP8ha lane was identical to control, due to interference from the signal peptide. Antibody aZ-4 is located at the C-terminal end of the protein where the signal peptide is positioned in the MFF-ZIP8ha cells. As a result, the signal peptide is hindering binding of the antibody to the protein.

In the western immunoblot studies presented here of the four ZIP8 antibodies developed, aZ-1 and aZ-3 were better at identifying ZIP8 protein in the cell lines tested. These antibodies can be useful in other experiments also, as they have successfully identified ZIP8 in immunohistochemical studies by this lab (14).

Expression of ZIP Proteins in MDCK Cells. Previous studies by this lab have used MFF cells to characterize the ZIP8 protein. These cells have been useful in determining metal uptake properties, transport properties, and heavy metal toxicity (18). However, a main target of Cd toxicity is the epithelia of the proximal convoluted tubule in the kidney. The use of a renal epithelial cell line will serve as a better model for studying the transport and intracellular trafficking of the 8-TM protein. MDCK cells have been utilized since the late 1950’s and are widely used as a model for polarized epithelial cells. They exhibit typical polarized cell morphology, retaining their basolateral and apical surfaces. Here, we have infected MDCK cells with retrovirus-encoding control luciferase (Luc),
mouse ZIP4ha, or mouse ZIP8ha cDNAs, to generate the stable MDCK-Luc, MDCK-ZIP4ha, and MDCK-ZIP8ha cell lines. Figure 4 shows the total protein levels of the infected MDCK cells using anti-ha antibodies to detect the ZIP4ha and ZIP8ha proteins. Lane 2 contains the ZIP4ha protein and a large band pattern can be observed between ~70 to ~80 kDa. The calculated molecular mass of ZIP4 is 70.9 kDa. Lane 3 contains the ZIP8ha protein with a large upper band at ~150 kDa, a mid-range band pattern at ~65 kDa and a lower band around 50 kDa, which is close to the calculated molecular mass of the core ZIP8 protein. The mid-range bands are possibly due to the varying degrees of saturation of existing glycosylation sites on the protein.

**ZIP8 Protein Glycosylation.** Glycosylation of proteins is a common post-translational modification in eukaryotic cells. Many membrane proteins undergo glycosylation where carbohydrate chains are linked to the protein via serine or threonine, or via asparagine. Two potential glycosylation sites were predicted for the ZIP8 sequence at Asn-40 and Asn-88 (N-linked); no O-linked glycosylation sites were predicted. To verify this prediction, PNGase F treatment followed by Western immunoblot analysis was performed. Peptide:N-Glycosidase F, or PNGase F, is an amidase that cleaves between the innermost asparagines and N-acetylglucosamine residues of high mannose, hybrid, and complex oligosaccharides from N-linked glycoprotein (21). PNGase F will not cleave N-linked glycans containing the core α1,3-fucose. Figure 5 shows there is a significant shift in the apparent molecular mass of the ZIP8ha protein in infected MDCK cells after PNGase F treatment. The band shift occurs from ~75 kDa to ~50 kDa, which is the calculated molecular mass of the ZIP8ha protein (51.3 kDa).
Cd-Induced Cell Death. ZIP8 has been shown to transport Cd with high affinity and thereby sensitizing MFF-infected cells to Cd-induced cell death (17, 18). To determine if Cd toxicity would be as severe in renal epithelial cells as it is in fibroblasts, we studied MDCK-ZIP8ha cell viability in the presence of Cd. Figure 6 shows that MDCK-ZIP8ha cells are much more sensitive than MDCK-Luc cells to Cd toxicity. MDCK-ZIP4ha cells were slightly more sensitive than the control cells (Luc), but not nearly as sensitive as the ZIP8ha-infected MDCK cells. This suggests that it is not merely the presence of any ZIP family member that greatly sensitizes cells to Cd. It is however, the high affinity at which ZIP8 transports Cd, $K_m$ of 0.62 µM (18), that is responsible for the sensitization to Cd toxicity of infected-MDCK cells.

Apical Membrane Localization of ZIP8 in Polarized Cells. In Dalton et al., 2005 (17), it was shown that the ZIP8 protein is detected in the membrane fraction of MFF cells, and confocal images clearly showed that ZIP8 protein is mainly localized on the plasma membrane of MFF cells; however, expression of ZIP8 was seen in intracellular membranes as well. The intracellular expression is most likely due to the protein procession from the endoplasmic reticulum to the Golgi apparatus, and transport from vesicles to the plasma membrane via the endocytic pathway.

Cadmium uptake into an active organism occurs through epithelial cells of the lung, small intestine, and renal reabsorption tubules. Since epithelia are polarized cells, their apical sides usually face the lumen, while the basolateral sides are attached to adjacent cells and the basement membrane of tissues. The apical and basolateral sides of epithelial cells have vastly different expression patterns of proteins; this
represents their distinct duties in handling different substrates. To further study localization of ZIP8 in a polarized cell, MDCK-ZIP8ha cells were employed.

Figure 7 shows that ZIP8ha protein is mainly localized on the cell surface of the polarized MDCK cells. NBC1 is a sodium-bicarbonate cotransporter known to be localized to the basolateral surface of MDCK cells (20). Z-stack analysis in Figure 7 (left panel) shows that GFP-NBC1 basolateral localization is separate from ZIP8ha localization. Also, ZIP8ha was localized to the surface of MDCK cells in contact with culture medium. In the right panel of Figure 7, ZIP8ha is localized to the same cell surface as PNA-lectin, which is known to bind only to the apical membrane (20). Taken together, these data strongly suggest that ZIP8 is localized to the apical membrane of polarized MDCK cells. Generally speaking, the apical-basolateral orientation of proteins in the polarized epithelia of one tissue type, are nearly always constant with the orientation pattern in the polarized epithelia of other tissues. Therefore, ZIP8 could be responsible for the uptake of endogenous and exogenous metals from the air (in lungs), food (in small intestine), blood (in endothelial cells), and glomerular filtrate (in kidney proximal or distal tubules); these metals then enter into tissues and organs of the body where they are distributed and/or accumulated.

**ZIP8 Distribution to the Plasma Membrane Is Altered by Zinc Availability.** Members of the ZIP family of transporters have mostly been found to transport metal cations into the cell, except for ZIP5 which is responsible for moving metals from inside the cell out to the bloodstream. The most widely studied ZIP family member to date is ZIP4. ZIP4 is a zinc transporter implicated in the uptake of zinc from diet in humans (22). SLC39A4
(which encodes hZIP4) is mutated in the disease acrodermatitis enteropathica (AE), where, if left untreated, the patient becomes zinc-deficient. Studies of the yeast Zrt1 and mammalian ZIP4 have demonstrated that zinc levels control the cellular distribution of these proteins. At higher levels of zinc, the protein is moved from the cell surface and uptake activity decreases. At lower levels of zinc, the protein accumulates at the cell surface to mediate the uptake of zinc (23).

ZIP8 has been shown to transport numerous metal ions, both essential and non-essential, such as Cd\(^{2+}\), Mn\(^{2+}\), Zn\(^{2+}\), and Hg\(^{2+}\) (18); yet no specific endogenous transporter has been unequivocally identified for ZIP8. Studies using metal cation competition and uptake kinetics show that ZIP8 has a high affinity for Mn\(^{2+}\) (\(K_m = 2.2 \mu M\)), suggesting one possible endogenous substrate (18). ZIP8 has also been shown to take up Zn\(^{2+}\); however due to the highly regulated control of zinc in mammalian cells, it has been difficult to obtain kinetic measures. Future studies using *Xenopus* oocytes will allow for a determination of zinc uptake kinetics. *Xenopus* oocytes are commonly used to characterize transporters because they have very low levels of endogenous transporters. Therefore, Zn\(^{2+}\) could also be a potential endogenous substrate for ZIP8.

To examine the effects of zinc status on ZIP8 protein localization, the surface level of ZIP8ha proteins in MDCK-ZIP8ha cells was assessed by Western immunoblotting. MDCK-Luc, MDCK-ZIP4ha, and MDCK-ZIP8ha cells were exposed to Chelex-treated culture medium overnight to remove labile zinc. Chelex 100 resin is a styrene divinylbenzene copolymer paired with iminodiacetate ions which act as chelating groups in binding polyvalent metal ions. The resin has a much higher selectivity for divalent ions than for monovalent ions (~5,000 to 1). After treatment,
cells were washed, fixed without being permeabilized, and probed with rabbit anti-ha antibody. As shown in Figure 8, incubation of MDCK-ZIP4ha and MDCK-ZIP8ha in Chelex-treated medium resulted in increased surface levels of these proteins. The addition of 10 μM ZnCl₂ (for 1 hour, prior to collection), to the cell incubation in Chelex-treated medium, blocked the surface accumulation of both ZIP4ha and ZIP8ha.
Discussion

Presently, there are 46 families within the solute carrier superfamily (SLC) with a total of 360 reputedly functional genes (www.bioparadigms.org/slc/menu.asp). The SLC39 family consists of ZIP transporter proteins which have 14 members; highly conserved orthologs exist between mouse and human (Fig. 1). A protein is assigned to a specific SLC family if it has at least 15-25% amino acid sequence identity in the “signature sequence” to other members of that family. Several plant ZIP proteins are involved in the transport of Zn$^{2+}$, Mn$^{2+}$, and Fe$^{2+}$ (24), and some have even demonstrated the ability to transport Cd, such as Irt1 and TcZNT1 (25). Currently, all mammalian ZIP transporters examined are able to transport Zn$^{2+}$ at micromolar concentrations. In 2005, Dalton et al, (17) was the first to demonstrate cadmium transport by a ZIP protein in vertebrates; the mouse Slc39a8 gene was proven to encode ZIP8.

Cd is a highly toxic, non-essential metal. In the environment, Cd levels have been on the rise since industrialization (past 150 years), posing a serious health risk to human populations. Since Cd holds no benefit whatsoever in living organisms, plants and animals have no specific “Cd transporter”; therefore, Cd must use existing transporters acting as a “hitchhiker”. ZIP8 has been shown to transport Cd very efficiently – the uptake process is optimum at a pH of 7.5, is energy-dependent, does not depend on a K$^+$, Na$^+$, or Cl$^-$ gradient, but does depend on HCO$_3^-$ being present in the transport medium (17). The $K_m$ of ZIP8 for Cd is 0.62 µM when measured in a mouse tissue culture system and is reported to be half that of SLC11A2 (DMT1) for Cd when expressed in Xenopus laevis oocytes (17, 26). SLC11A2 is located apically and
is believed to be important in Cd-uptake from the small intestine. ZIP8 mRNA levels were measured in various mouse tissues with the highest levels in the lung. Next highest mRNA levels were measured in the kidney and testis, with lower levels found in the liver, small intestine, and brain (14). Here, we have shown ZIP8 to be apically localized in polarized renal epithelial cells (Fig. 7). Taken together, this suggests that ZIP8 is a probable candidate for Cd uptake from the lung, glomerular filtrate, and small intestine. ZIP8 has already been shown to mediate Cd uptake from the plasma into the testis via the endothelial cells of the testis vasculature (17).

In the current study using polarized MDCK cells as a model system, it was determined that the expressed recombinant ZIP8 protein is localized to the apical membrane (Fig. 7). This finding is similar to other reports demonstrating ZIP1, ZIP2, ZIP3, and ZIP4 are also located on the apical membrane of murine enterocytes and visceral yolk sac epithelial cells. To date, ZIP5 is the only ZIP transporter localized to the basolateral membrane, as determined in acinar cells, enterocytes, visceral endoderm cells, and MDCK cells (23, 27, 28). ZIP8 also shows cytoplasmic distribution possibly meaning there is trafficking between the plasma membrane and intracellular compartments, via the endocytic pathway, during physiological and pathological states. Yeast Zrt1, and mouse ZIP1, ZIP3 and ZIP4 have all been shown to undergo Zn\(^{2+}\)-stimulated endocytosis. The status of Zn\(^{2+}\) controls the cellular distribution of these proteins – at low levels, these proteins accumulate at the surface of the cell to mediate uptake; at high levels the proteins are removed and uptake activity decreases (22, 23).

To examine the effects of Zn\(^{2+}\) status on ZIP8 in MDCK cells, we measured the levels of ZIP8ha at the surface of the cell in differing concentrations of Zn\(^{2+}\). Figure 8
shows surface levels of ZIP8 are increased in a state of Zn-deficiency. Upon addition of Zn\(^{2+}\) back to the system, the levels of ZIP8 at the surface of the cell were decreased to basal levels. This may suggest that the cell is somehow able to use these proteins as a way to determine Zn\(^{2+}\) status. The cell may be able to continuously track extracellular Zn\(^{2+}\) levels, allowing for fast accumulation of ZIP8 at the plasma membrane, should Zn\(^{2+}\) concentrations become diminished. On the other hand, it may be that ZIP8 trafficking is responding to intracellular Zn\(^{2+}\) levels. The ability to “sense” Zn\(^{2+}\) could be occurring through direct binding of Zn\(^{2+}\) to ZIP8 or via some intermediary protein that initiates the trafficking process. Kim et al., (2004) have hypothesized that extracellular Zn\(^{2+}\) could be sensed by the histidine-rich N-terminal end, prompting conformational changes in ZIP4 thereby stimulating interactions between a targeting motif of the endocytic pathway and the cell trafficking machinery. This mechanism is beneficial because - in pathological states of reduced Zn\(^{2+}\) - the cell can quickly respond by increasing the amount of ZIP transporter to the surface, thereby increasing the amount of Zn\(^{2+}\) uptake, returning the cell to normal physiological state. Another benefit of this phenomenon is that the cell would not have to waste time and energy on producing new ZIP proteins to be distributed throughout the cell. ZIP4 has been shown to undergo endocytosis in response to metals other than Zn\(^{2+}\). Mn, Co, and Cd were all shown to stimulate ZIP4 endocytosis in HEK293/mZIP4-HA cells; however much higher concentrations of these metals were required: 20 µM Mn, Co, or Cd versus 1 µM Zn (23). Future studies with MDCK-ZIP8ha cells will determine if ZIP8ha protein will be responsive to varying levels of Cd and Mn. These studies may also produce further evidence on identifying the endogenous substrate for ZIP8, if lower levels of Zn and/or Mn can stimulate the
endocytosis of ZIP8. Mn has already been implicated as one possible endogenous substrate for ZIP8. Previous studies in a MFF cell system by this lab have shown that ZIP8 has a high affinity for Mn with a $K_m = 2.2 \mu M$. This is close to physiological concentrations in mammalian tissues which range from 0.3 to 2.9 µg/g wet tissue weight (29).

Maintaining metal homeostasis is essential for the function of many enzymes, transcription factors, and other subcellular proteins. Disruption of metal homeostasis can result in disease and in extreme cases, death. Deletion of $Slc39a8$ at a whole animal level would be definitive in determining the physiological role of ZIP8. It is already known that the loss of functioning human ZIP4 causes Zn deficiency and the disease acrodermatitis enteropathica (AE). Future studies using ZIP8-knockout mice will be invaluable for examining the physiological importance of ZIP8.

To further characterize the transport properties of ZIP8, studies using *Xenopus laevis* oocytes is being utilized in this lab. These unique cells are often used for transporter studies because they have very low levels of endogenous transporters. ZIP8 mRNA can be expressed in these cells and transport properties measured, such as the kinetics of $Zn^{2+}$ transport. Therefore, using *Xenopus* oocytes removes possible confounding effects from other metal transporters (DMTs, calcium transporters) that are present in mammalian cells.

Examining the physiological role of all the ZIP transporters will be beneficial in understanding metal homeostasis. ZIP transporters have been found to be present throughout the body and are involved in a number of different functions. Hopefully,
future studies will provide a better understanding of how ZIPs function in relation to other metal-transporter families to maintain metal homeostasis.
Fig. 1. Phylogenetic dendrogram, generated from alignment of the amino acid sequences derived from the 14 mouse and 14 human SLC39 genes. Upper one of each pair, lower-case letters, denotes the mouse genes; lower one of each pair, all upper-case letters, denotes the human genes. The branch in red illustrates that the SLC39A14 and SLC39A8 genes are much more highly homologous to one another than to any of the other 12 members of the SLC39 family; the horizontal distances shown by the mouse-human split (for each of the 14 pairs) would represent about 80 million years ago (MYA), suggesting that the SLC39A14 and SLC39A8 genes arose from a duplication event between 250 and 500 MYA. The horizontal lines are not linear, and the extreme left of this tree represents genes that probably arose between 500 and 3000 MYA.
Fig. 2. Predicted molecular structure of the ZIP8 metal transporter protein. It is believed that all ZIP proteins contain 8-transmembrane segments. Yellow denotes the hemagglutinin (ha) tag on the C-terminal end. Red denotes the four approximate positions from which 11-mers of amino acids were selected for generating four independent antibodies (aZ-1, aZ-2, aZ-3 and aZ-4). The human SLC39A8 gene has nine exons, spanning 83 kb from the start of exon 1 to the end of exon 9; the hZIP8 mRNA contains 3,309 nucleotides and encodes a protein of 460 amino acids. The mouse Slc39a8 gene has nine exons, spanning 66 kb from the start of exon 1 to the end of exon 9; the mZIP8 mRNA contains 3,211 nucleotides and encodes a protein of 462 amino acids.
Fig. 3. Western immunoblot analysis of ZIP8 antibodies. Expression of ZIP8 protein levels were measured in MFF-infected cells containing luciferase (control), ZIP8 (no ha tag), and ZIP8ha (ha tagged) cDNAs. Four separate ZIP8 antibodies were developed: aZ-1 at the N-terminus (B), aZ-2 at the beginning of the loop between TM3 and TM4, aZ-3 at the end of the loop between TM3 and TM4 (C), and aZ-4 at the C-terminus (D). The anti-ha antibody was used as a control in these experiments (A).
**Fig. 4.** Expression of ZIP4ha and ZIP8ha proteins in the retroviral stably-infected MDCK cells. Western immunoblot analysis of total protein levels in cells grown in basal medium. The blot was probed with anti-ha antibodies to detect ZIP-ha proteins. Equal loading was confirmed by Coomassie staining (data not shown).
Fig. 5. Western immunoblot of control (Luc) and ZIP8ha stably-infected MDCK cells, with or without PNGase F treatment. PNGase F cleaves glycoproteins. Coomassie staining confirmed equal lane loading (not shown).
Fig. 6. Cd toxicity in ZIP8-expressing MDCK cells. Dose-response curves for Cd-induced cell death. Cells were treated for 24 h with the indicated concentration of CdCl$_2$, and cell death was monitored by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium (MTS) bromide assay. Asterisks denote significant differences between MDCK-ZIP8 and MDCK-Luc at the indicated concentration, $P < 0.05$ (Student’s $t$ test).
Fig. 7. Membrane localization of ZIP8 in MDCK cells. Z-stack confocal microscopy shows that the NBC1-GFP transporter, known to be basolateral (left panel), is located opposite to that of ZIP8ha (both panels) and PNA-lectin (right panel), known to be apical. Expression vectors carrying the indicated transporter proteins were transiently transfected into cells. After 2 days, the cells were fixed, blocked, and incubated with primary anti-ha antibody, then the secondary fluorescence antibody. Stained cells were examined under a Zeiss 510 laser-scanning confocal microscope. X-Y and Z denote the three axes studied. NBC1 – sodium bicarbonate-1 transporter; GFP – green fluorescent protein.
**Fig. 8.** Detection of the amounts of ZIP4ha and ZIP8ha protein on the surfaces of stably-transfected MDCK cells. Cells were treated with basal medium, Chelex-treated medium, or Chelex-treated medium plus 10 µM ZnCl$_2$. The cells were exposed to Chelex-treated medium overnight, and 10 µM ZnCl$_2$ for 1 h. Cells were washed, fixed (without being permeabilized) and probed with rabbit anti-ha antibody. The cells were washed to remove any unbound antibodies, collected, lysed, and then analyzed by immunoblotting with HRP-conjugated goat anti-rabbit antibody. Equal loading was confirmed by Coomassie staining (not shown).
References


- 37 -


