Supporting Information

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SI Materials and Methods

Cell Culture, Transfection, Stable Cell Lines, and Reagents. HEK 293 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) with 4.5 g/L glucose, 4 mM L-glutamine, 1 mM sodium pyruvate, and 10% FBS (Atlanta Biologicals). HepG2 cells were maintained in Minimum Essential Medium (Sigma) with 1× nonessential amino acids (NEAA) and 10% FBS. All cells were maintained in an incubator at 37 °C and 5% CO_2. Effectene transfection reagent (Qiagen) was used for transient transfection. Briefly, cells were seeded at 40% confluency in six-well plates or 100-mm culture dishes. Transfection began 24 h after seeding with 0.4 or 2 μg of plasmid DNA, 3.2 or 16 μL of enhancer, and 10 or 50 μL of Effectene reagent. Transfection was carried out for 48 h before further analysis. For establishing stable cell lines, HEK293 cells were transfected on day 1 by Effectene reagent, and on day 3 cells were selected with DMEM containing 800 μg/mL G418 (Geneticin). Selected clones were screened by immunoblotting analysis with M2 anti-FLAG-HRP antibody. Positive cell clones were maintained in DMEM supplemented with 10% FBS, 1× NEAA, and 500 μg/mL G418. Desferrioxamine (Hospira) and salicylaldehyde isonicotinyl hydrazone (SIH) (a kind gift from Prem Ponka of McGill University, Montreal) were used to deplete cellular iron. Ferric ammonium citrate (ICN Biomedicals) was used to load cells with iron. Cycloheximide (Sigma) was used to block protein synthesis. Brefeldin A (Sigma) and dynasore (Sigma) were used to disrupt the Golgi apparatus and inhibit endocytosis, respectively. Proteolytic degradation inhibitors used include MG132 (Calbiochem), epoxomicin (Calbiochem), pepstatin A (Sigma), E64 (Sigma), and leupeptin (Sigma).

Quantitative RT-PCR. RNA was extracted by NucleoSpin RNA II kit (Clontech) according to the manual; cDNA was synthesized by Moloney Murine Leukemia Virus Reverse Transcriptase (Promega). The quantitative RT-PCR (qRT-PCR) was performed by Applied Biosystems ViiA 7 system; Thermocycling conditions were the following: 50 °C for 2 min; 95 °C for 10 min; 95 °C for 15 s; and 60 °C for 1 min for 40 cycles. Each 15-μL reaction contained 1× SyberGreen Master Mix, 1 μL forward and reverse primer (5 μM each, Table S2), and 2 μL of cDNA template.

Plasmids and Site-Directed Mutagenesis. Plasmid-encoding WT human-myc-FLAG-ZIP14 (pCMV-Entry-hZIP14) was purchased from Origene. Plasmid-encoding ZIP14s with asparagines replaced by alanines or with His, L2 deletions were constructed by site-directed mutagenesis (QuikChange Lightning Kit, Stratagene). Briefly, 100 ng of double-stranded DNA template (pCMV-Entry-hZIP14, Origene) was mixed with 125 ng of forward and reverse primers (Table S3), 10 mM dNTPs, 1× reaction buffer, and Pfu DNA polymerase. The mixture was amplified by PCR. Initially, the reaction mix was incubated at 95 °C for 30 s. Cycles were as follows: denaturation for 30 s at 95 °C, annealing for 1 min at 55 °C, and extension synthesis at 68 °C for 6 min for 18 cycles. PCR products were digested with DpnI enzyme to remove the parental strands. The digested DNA mixture was transformed into *Escherichia coli* XL1-blue cells by heat shock at 42 °C. Mutagenesis products were all verified by DNA sequencing.

Isolation of Plasma Membrane Proteins by Cell-Surface Biotinylation. Cells were first incubated with ice-cold DMEM for 5 min and then washed twice with ice-cold PBS. Plasma membrane proteins were labeled by using the membrane-impermeant thiol-cleavable biotinylation reagent biotin disulfide N-hydroxysulfosuccinimide ester (NHS-SS-biotin) (Thermo Scientific). The culture dishes were kept on ice, and all solutions were ice-cold. Each dish of cells was incubated with 0.25 mg/mL NHS-SS-biotin in PBS for 30 min with very gentle shaking. After biotinylation, 500 μL of quenching solution was added to each dish to block the unreacted NHS-SS-biotin. Cells were collected and solubilized in 500 μL 150 mM NaCl, 5 mM EDTA, 10 mM Tris, 1% Triton X-100, and 1× Complete Mini protease inhibitor Mixture (Roche), pH 7.4 (NETT) lysis buffer with 1× protease inhibitors followed by centrifugation at 10,000 × *g* for 10 min at 4 °C. The clarified supernatant was added to a spin column containing prewashed immobilized streptavidin gel and incubated for 60 min at 4 °C. After four washes, biotinylated samples were incubated with 50 mM DTT in 1× SDS/PAGE sample buffer for 60 min at 4 °C to cleave the disulfide bond and release biotinylated proteins. Immunoblotting analysis was used to detect cell-surface ZIP14 and plasma-type Na^+, K^+ ATPase.

Endocytosis Analysis with Mercaptoethanesulfonic Acid Treatment. Biotinylation was performed as described above. Biotinylated cell cultures were warmed to 37 °C for 15 min to induce endocytosis. Cells were then rinsed two times with PBS^{++} (1 mM $MgCl_2$, 0.5 mM $CaCl_2$) and rinsed one time with NT buffer (0.2% BSA, 0.15 M NaCl, 1 mM EDTA, 20 mM Tris·HCl, pH 8.0), followed by treatment with the membrane-impermeant reducing agent sodium-2 mercaptoethanesulfonic acid (500 mM; Sigma) as previously described (1, 2) to remove cell-surface biotinylation. All cells were rinsed two times with NT buffer and then incubated with 120 mM iodoacetamide (Sigma) in NT buffer for 10 min at 4 °C before cell lysis. Internalized cell-surface proteins were analyzed by immunoblotting as described below.

Separation of Cytosol and Membrane Fractions. To separate the cytosol and membrane fractions, cells were first homogenized in HEM buffer (20 mM Hepes, 1 mM EDTA, and 300 mM mannitol) by using a stainless steel cell douncer (Wheaton). Then the cell lysates were centrifuged at 10,000 × *g* for 10 min at 4 °C to remove nuclei and cell debris. The cleared cell lysates were centrifuged at 100,000 × *g* for 1 h at 4 °C to pellet-down the membrane fraction. The supernatant was collected as the cytosol fraction. The membrane pellet was resuspended in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, and 10 mM EDTA) for further analysis.

Immunoblotting Analysis. Cells were washed with cold PBS twice and lysed in NETT buffer. Protein concentrations of the cell lysates were measured by using the RC DC Protein Assay (Bio-Rad). PNGase F and Endo H (New England Biolabs) treatments were performed according to the manufacturer's protocol. Samples were mixed with 1× Laemmli buffer and incubated for 30 min at 37 °C. Proteins were separated electrophoretically on an SDS/10% polyacrylamide gel, transferred to nitrocellulose, and incubated for 1 h in blocking buffer [5% nonfat dry milk in Tris-buffered saline-Tween 20 (TBST)]. Blots were incubated for 1 h at room temperature in blocking buffer containing mouse anti-FLAG, anti-FLAG-horseradish peroxidase (HRP), M2 (1:10,000; Sigma), or mouse anti-TfR1 (1:5,000; Thermo Scientific). After four washes with TBST, blots were incubated with a 1:5,000 goat anti-mouse secondary antibody conjugated to HRP (Millipore). To confirm equivalent loading, blots were stripped for 15 min in Restore PLUS Western Blot Stripping Buffer (Thermo Scientific), blocked for 1 h in blocking buffer, and reprobed with

mouse anti-actin (1:10,000; Millipore) or rabbit anti-tubulin (1:5,000; Rockland) followed by HRP-conjugated goat anti-mouse (Millipore) or donkey anti-rabbit (GE Healthcare) secondary antibody. For loading control of plasma membrane proteins, mouse anti-Na^+, K^+ ATPase antibody (1:2,000; Santa Cruz) followed by HRP-conjugated secondary antibodies were used. After two washes with TBST and TBS, bands were visualized by using enhanced chemiluminescence (SuperSignal West Pico, Thermo Scientific) and X-ray film.

Immunofluorescence. Cells seeded on poly-L-lysine–coated coverslips (Sigma) were washed two times with $PBS^{+/+}$ (PBS with 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$) and fixed with 4% paraformaldehyde for 15 min at room temperature. To compare the internalization of holo-Tf, cells were incubated with 30 μg/mL Alexa Fluor-594–labeled holo-Tf or PBS for 30 min before fixation. After fixation, cells were permeabilized with 0.1% saponin for 10 min, and washed three times with PBS before blocking in 1% BSA for 30 min. Cells were washed three times with PBS and blocked with 1% BSA for 30 min. For Golgi 97 labeling, cells were incubated with mouse anti-Golgi 97 (1:500; CDF4, Molecular Probes) primary antibody for 30 min at room temperature. The secondary antibody was donkey anti-mouse IgG Alexa Fluor-594 (1:500; Invitrogen). To stain actin and nuclei, cells were incubated with two drops of ActinGreen and NucBlueReadyProbes reagents (Invitrogen) in 1 mL PBS for 15 min. After three washes of PBS, coverslips were mounted on microscope slides with antifade mounting medium (Invitrogen) and sealed with nail polish. Images were captured by a Zeiss LSM 710 confocal microscope with a 63× oil objective (Oregon Health and Science University Jungers Core Facility).

Statistics. Data were analyzed by one-way ANOVA with Graphpad Prism software, version 5. Tukey's post hoc comparisons tests were performed in all statistical analyses. P values <0.05 were considered to be statistically significant.

1. Schmidt A, Hannah MJ, Huttner WB (1997) Synaptic-like microvesicles of neuroendocrine cells originate from a novel compartment that is continuous with the plasma membrane and devoid of transferrin receptor. *J Cell Biol* 137(2):445–458.

2. VanSlyke JK, Musil LS (2005) Cytosolic stress reduces degradation of connexin43 internalized from the cell surface and enhances gap junction formation and function. *Mol Biol Cell* 16(11):5247–5257.

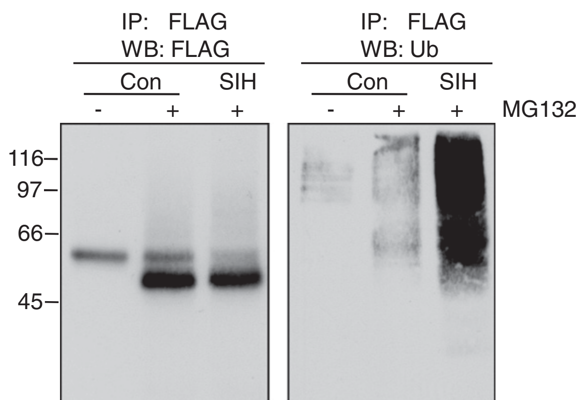

Fig. S1. HepG2-ZIP14-FLAG cells were used. Cells were treated with 100 μM SIH and 10 μM MG132 for 24 h before the immunoprecipitation procedure. Half of the eluted fraction was analyzed by immunoblotting for ZIP14 and the other half was probed with anti-ubiquitin antibody.

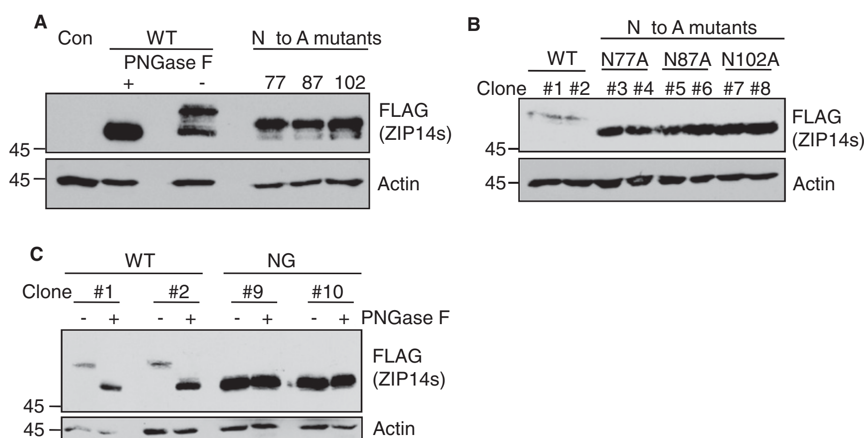

Fig. S2. (*A*) Immunoblotting analysis of lysates from HEK293 cells transiently transfected with empty vector (Con) or ZIP14-FLAG expression vectors encoding either wild-type (WT) or asparagine mutants (N77A, N87A, N102A). Cell lysates from WT ZIP14-transfected cells were treated with or without PNGase F. (*B*) Immunoblotting of lysates from stable transfected HEK293 cells, expressing WT or single N-to-A mutant ZIP14. (*C*) Lysates from HEK293 stable cells expressing WT or the triple asparagine mutant ZIP14 (NG ZIP14, N77/87/102A) were digested with PNGase F. Numbers with "#" indicate different stable cell clones (Table S1).

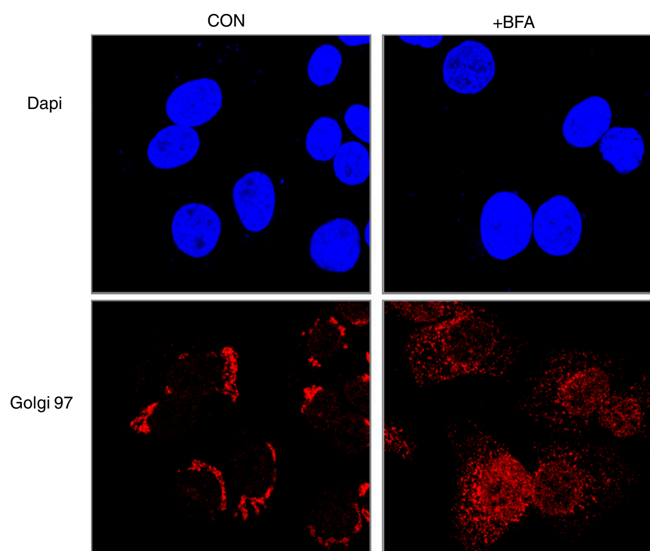

Fig. S3. Images from two channels in Fig. 4*D* are shown separately.

Table S1. Stable transgenic HEK293 cell clones used in this study

Clone nos.	Clone names	Gene expressed
#1, 2	HEK293-ZIP14	Wild-type *ZIP14*
#3, 4	HEK293-ZIP14^{N77A}	*ZIP14* with Asn-77 replaced by Ala
#5, 6	HEK293-ZIP14^{N87A}	*ZIP14* with Asn-87 replaced by Ala
#7, 8	HEK293-ZIP14^{N102A}	*ZIP14* with Asn-102 replaced by Ala
#9, 10	HEK293-NG-ZIP14	*ZIP14* with Asn-77, -87, and -102 replaced by Ala
#11, 12	HEK293-ΔHis-ZIP14	*ZIP14* with deletion of the histidine-rich region
#13, 14	HEK293-ΔL2-ZIP14	*ZIP14* with deletion of the second intracellular loop

Table S2. Primers used for qRT-PCR analysis

Protein	Primer	Primer sequence (5′–3′)
ZIP14	Forward	GTCTGGCCTTTGGCATCCT
	Reverse	AGGGAACATATCAGCCAGAGAAAT
FLAG	Forward	GGCCGCTCGAGCAGAAAC
	Reverse	TCGTCGTCATCCTTGTAATCCAGG

Table S3. Primers used for generating ZIP14 mutants

Mutation	Primer	Primer sequence (5′–3′)
N77A	Forward	GGAGTGGGCCGGGGTGCTGTCACCCAGCAC
	Reverse	GTGCTGGGTGACAGCACCCCGGCCCACTCC
N87A	Forward	CGTGCAAGGACACAGGGCCCTCTCCACGTGCTTT
	Reverse	AAAGCACGTGGAGAGGGCCCTGTGTCCTTGCACG
N102A	Forward	CCTCTTCACTGCCCACGCTTTCAGCGAGCAGTCG
	Reverse	CGACTGCTCGCTGAAAGCGTGGGCAGTGAAGAGG
ΔHis	Forward	CTTCTTAAGCAGAAAAATGAGTATGCCTCTGAGTCGCTTCC
	Reverse	GGAAGCGACTCAGAGGCATACTCATTTTTCTGCTTAAGAAG
ΔL2	Forward1	CTTTTATCTTTTCTTTTTCACAGAGTCTAGAAAGATCTTGAAG ATTCTTCTTAAGC
	Reverse1	GCTTAAGAAGAATCTTCAAGATCTTTCTAGACTCTGTGAAAAA GAAAAGATAAAAG
	Forward2	TGATATCGGCACTCTGTCTAGAGCCTGGATGATCACTC
	Reverse2	GAGTGATCATCCAGGCTCTAGACAGAGTGCCGATATCA