Lipid-tuned Zinc Transport Activity of Human ZnT8 Protein Correlates with Risk for Type-2 Diabetes**

Chengfeng Merriman1†, Qiong Huang1,2,†, Guy A. Rutter5, and Dax Fu1,3

From the 1Department of Physiology, The Johns Hopkins School of Medicine, Baltimore, Maryland 21205 and the 2Section of Cell Biology and Functional Genomics, Department of Medicine, Imperial College London, London W1 2ONN, United Kingdom

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Zinc is a critical element for insulin storage in the secretory granules of pancreatic beta cells. The islet-specific zinc transporter ZnT8 mediates granular sequestration of zinc ions. A genetic variant of human ZnT8 arising from a single nonsynonymous nucleotide change contributes to increased susceptibility to type-2 diabetes (T2D), but it remains unclear how the high risk variant (Arg-325), which is also a higher frequency (>50%) allele, is correlated with zinc transport activity. Here, we compared the activity of Arg-325 with that of a low risk ZnT8 variant (Trp-325). The Arg-325 variant was found to be more active than the Trp-325 form following induced expression in HEK293 cells. We further examined the functional consequences of changing lipid conditions to mimic the impact of lipid remodeling on ZnT8 activity during insulin granule biogenesis. Purified ZnT8 variants in proteoliposomes exhibited more than 4-fold functional tunability by the anionic phospholipids, lysophosphatidylcholine and cholesterol. Over a broad range of permissive lipid compositions, the Arg-325 variant consistently exhibited accelerated zinc transport kinetics versus the Trp-form. In agreement with the human genetic finding that rare loss-of-function mutations in ZnT8 are associated with reduced T2D risk, our results suggested that the common high risk Arg-325 variant is hyperactive, and thus may be targeted for inhibition to reduce T2D risk in the general populations.

Zinc forms stable complexes with insulin hexamers, enabling crystalline insulin packing in secretory granules of pancreatic beta cells. Defective insulin secretion in the face of insulin resistance is a characteristic feature of T2D, a complex multifactorial polygenic disease with more than 80 T2D susceptibility loci genes identified so far by genome-wide association studies (GWASs). A nonsynonymous single nucleotide polymorphism in SLC30A8 (rs13266634 C→T), which causes an arginine to tryptophan change at position 325, is associated with increased risk of developing T2D (1). The risk allele is widespread in more than 50% of the population according to HapMap data (build 35). SLC30A8 encodes a granular zinc transporter known as ZnT8. In pancreatic beta cells, ZnT8 is highly expressed and responsible for transporting cytosolic zinc into insulin granules (2). However, the molecular mechanism underlying the genetic susceptibility of ZnT8 polymorphisms remains controversial. ZnT8 inactivation in various mouse models revealed large phenotypic variations ranging from decreased, unchanged to even enhanced insulin secretion (3). Functional characterization of overexpressed polymorphic alleles in pancreatic beta cells suggested an attenuated zinc transport activity associated with an increased T2D susceptibility (4, 5), whereas genotyping rare nonsense and frameshift mutations of ZnT8 in humans indicated an opposite causal relationship, suggesting that a reduction of ZnT8 expression actually decreased T2D risk (6). The conflicting results concerning the directional relationship between ZnT8 activity and T2D susceptibility prevent the identification of specific defects in risk variants for pharmacological interventions. At present, it is not clear whether activation or inhibition of ZnT8 might be a therapeutic option to reduce T2D risk in the general population.

Studies of the functional effects of GWAS-identified risk variants in humans are often challenged by small effect sizes that do not provide a clinically useful predictor for disease risk. Although protective effects on T2D risk were observed in ZnT8 LOF mutants at an occurrence rate of about 350 carriers out of ~150,000 genotyped individuals (6), the extrapolation of the causal relationship obtained from rare penetrant mutants to common and yet mild ZnT8 polymorphic variants is complicated by a possible bell-shaped relationship between ZnT8 activity and diabetes risk in humans (7). To determine the functional effects of common ZnT8 polymorphic variants on zinc transport activity, we established induced expression of human ZnT8 variants in HEK293 cells, purified both Arg- and Trp-forms in a native state, and developed reconstitution in biomimetic membranes with defined lipid compositions. A direct...
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functional comparison of human ZnT8 polymorphic variants at the molecular level sidestepped inherent limitations to the use of rodent models for human metabolic syndrome. Our experiments demonstrated a clear correlation between elevated zinc transport activity and increased T2D risk.

Results

Induced Expression and Characterization of Human ZnT8 Variants—To produce recombinant human ZnT8 variants, we generated stable HEK293 cell lines, each with an isogenic integration of a His-tagged ZnT8 variant under a tetracycline-inducible promoter. Western blotting using an antibody to the His tag showed that induction of the variant encoding arginine at position 325 (Arg-form) reached a similar level of expression to the tryptophan 325 (Trp-form) variant, whereas the uninduced level was below the detection limit (Fig. 1a). Induced expression of ZnT8 variants was also monitored in cells by immunofluorescence labeling and confocal microscopy. Post-induction immunoreactivities toward the His tag were confined intracellularly (Fig. 1b), consistent with intracellular localization of ZnT8 in pancreatic beta cells (2). Additional strong immunostaining of the nuclear envelope was observed due to nonspecific antibody binding as shown by the same staining pattern in HEK293 cells with no genomic ZnT8 integration as a negative control. This cell line is referred to hereafter as blank (Blk). Cells labeled with a membrane-permeable zinc-selective fluorescent indicator, Zinpyr-1, revealed bright punctate staining in the juxtanuclear region (Fig. 1c), consistent with the trapping of Zinpyr-1 in the Golgi and acidic subcellular compartments (8). The intensity rank order of Zinpyr-1 staining was Arg > Trp > Blk following induced expression (Fig. 1c). Flow cytometry quantification over ~13,000 live cells showed a mean fluorescence intensity of 2860.32 ± 9.63 counts for the Arg-form and 2135.42 ± 7.64 counts for the Trp-form, respectively. This result validated a higher level of vesicular zinc accumulation by induced expression of the Arg-form. Moreover, ZnT8-mediated vesicular zinc accumulations were monitored in real time in stable expression cells or Blk loaded with Zinpyr-1. When the surface membrane was permeabilized using a zinc ionophore (1 μM pyrithione), zinc exposure triggered a linear rise of vesicular Zinpyr-1 fluorescence within 10 min (Fig. 1d). The net fluorescence difference between induced and uninduced cells reflected ZnT8-mediated vesicular zinc accumulation. At lower zinc concentrations (5 and 19 nM) that were slightly above the physiological zinc level in the cytosol (1–2 nM) (9), the activities of two ZnT8 variants were at the Blk level (Fig. 1d). However, at 283 nM, both the Arg- and Trp-forms exhibited robust Zinpyr-1 fluorescence increases above the Blk level. The rates of zinc transport mediated by the Arg-325 and Trp-325 forms were 0.16 ± 0.02 and 0.08 ± 0.01 min⁻¹, respectively. The Arg-form exhibited a significantly faster rate (p < 0.001, n = 4), indicating that the Arg-form was hyperactive with respect to the Trp-form.

Purification and Reconstitution of ZnT8 Variants—Significant levels of vesicular zinc accumulation were observed in the absence of induced ZnT8 expression (Fig. 1c). The high background vesicular zinc accumulation is attributable to endogenous zinc transport activities (10), precluding a quantitative comparison of ZnT8 variants in living cells. To directly compare zinc transport activities and determine which step(s) in the transport reaction cycle might be affected by the risk allele, we set out to purify ZnT8 variants for direct biochemical analysis. Homology modeling of human ZnT8 based on the X-ray structure of a bacterial zinc transporter YiiP (11) suggested that the Y-shaped ZnT8 homodimer is stabilized by lipid molecules filling the void space between two transmembrane domains (Fig. 2a). To retain the protein stability, we partially purified His-tagged ZnT8 variants with minimal delipidation. Most contaminants co-eluting with ZnT8 (Fig. 2b) were soluble proteins and thus readily separated from ZnT8 by reconstituting ZnT8 into proteoliposomes. Re-solubilization of washed proteoliposomes yielded largely purified ZnT8, which was further polished by size-exclusion HPLC (Fig. 2b). The molecular identity of the purified ZnT8 was confirmed by Western blotting using antibodies to an N-terminal epitope in ZnT8 and the C-terminal His tag, respectively (Fig. 2b). Analytical size-exclusion HPLC analysis of the purified ZnT8 revealed a single mono-dispersed peak by both fluorescent and UV detections (Fig. 2c). The apparent molecular mass of the purified ZnT8 was estimated to be ~120 kDa, in agreement with a dimeric assembly of two 36.5-kDa monomers in complex with lipids and detergents. Compared with GFP-tagged ZnT8 in the detergent crude...
extract, the purified ZnT8 without a GFP tag was slightly right-shifted as a result of protein size difference (Fig. 2c). The peak profile, however, remained essentially unchanged between the purified ZnT8 and unpurified ZnT8-GFP, indicating that ZnT8 retained a native fold after reconstitution and re-solubilization. For clarity, only the chromatogram of the Arg-form is shown, as the profile of the Trp-form was identical.

Kinetic Differences between ZnT8 Variants in Reconstituted Proteoliposomes—Zinc transport by ZnT8 is thought to be a two-step process, initiated by zinc binding to a transport site followed by a protein conformational change that moves the bound zinc ion across the membrane barrier (12). This kinetic process was characterized for bacterial zinc transporters in reconstituted proteoliposomes (13, 14). However, reconstitution of human ZnT8 variants caused either large vesicle leakage when *Escherichia coli* polar lipid extract was used or yielded no detectable transport activity when bovine liver polar lipid extract was used. We found that a mixture of synthetic DOPC, DOPE, and DOPG at a 2:1:1 ratio yielded rapid zinc influx into proteoliposomes encapsulated with Fluozin-3, a membrane-impermeant fluorescent zinc indicator (Fig. 3a). The initial rate of zinc influx increased linearly with a ZnT8-to-lipid ratio up to 1:25 (w/w) where zinc influx was ~12-fold faster than the background leakage (Blk) measured in liposomes that were prepared without protein incorporation (Fig. 3, a and c). The negligible background leakage and a linear influx relationship with ZnT8 input validated the functional assay. The reconstituted Arg- or Trp-forms responded to an increasing extravesicular zinc concentration with a progressive increase of Fluozin-3 fluorescence over 10 s (Fig. 3c). Least squares fitting of the steady-state kinetics to the Michaelis-Menten equation indicated that the rate of zinc transport ($V_{\text{max}}$) for the high risk Arg-form was 57% faster, accompanied by a small decease of $K_m$ (Fig. 3d and Table 1). The $V_{\text{max}}/K_m$ value of Arg-form, which is a measure of zinc transport efficacy, was about 2-fold higher as compared with that of the Trp-form.

Functional Modulation of Human ZnT8 Variants by Lipids—During replenishment of insulin granules following insulin secretion, ZnT8 is trafficked along the insulin secretory pathway en route from the endoplasmic reticulum through Golgi networks to insulin granules (15). The intracellular trafficking process exposes ZnT8 to a dynamic lipid composition as subcellular membrane compartments undergo enormous lipid remodeling, resulting in enrichments of anionic phosphatidylinositol (PI), phosphatidylserine (PS), non-bilayer lysophosphatidylcholine (LPC), and cholesterol in insulin granules (16) (Fig. 4a). We further investigated the functional difference between ZnT8 variants under the influence of the following three major classes of lipids in insulin granules: anionic phospholipids, non-bilayer phospholipids, and cholesterol. The efficiencies of ZnT8 reconstitution for both variants as well as Fluozin-3 encapsulation were monitored to ensure equal amounts of ZnT8 reconstitution and dye loading for all the experiments. Compared with two-lipid proteoliposomes composed of DOPC/DOPE, three-lipid proteoliposomes with an added anionic phospholipid, DOPG, DOPS, or soyPI, all exhibited significantly elevated zinc transport activity, regardless of the exact chemical nature of the headgroup (Fig. 4b and Table 1). Of note, $V_{\text{max}}$ values of Arg-form were consistently higher than those of Trp-form under anionic lipid stimulations in proteoliposomes composed of DOPC/DOPE/DOPG, DOPC/DOPE/DOPS, or DOPC/DOPE/soyPI (Fig. 4b). A similar stim-
The non-cylindrical phospholipids alone do not form lipid bilayers but can be stabilized in the bilayer structure of cylindrical phospholipids (DOPC, DOPS, and DOPG) (17). Adding LPC to any form of proteoliposomes containing three to six different lipid types invariably reduced \( V_{\text{max}} \) to a basal level around 0.02 s\(^{-1}\) (Fig. 4b and Table 1). LPC is highly enriched in insulin granules, accounting for 20% of total granule lipids (16). When inserted into the lipid bilayer, the cone-shaped LPC introduces positive curvature. In contrast, DOPE in the lipid bilayer increases negative curvature (18). The positive curvature introduced by LPC is further augmented in bilayers of anionic lipids (19). Therefore, DPPE, which is highly enriched in insulin granules and has a similar effect on 

ululatory effect of anionic lipids was observed in four-lipid proteoliposomes composed of DOPC/DOPE/DOPS/soyPI (Fig. 4b).

Next, we examined the effects of two non-bilayer phospholipids, namely the inverted conical LPC and conical DOPE. These non-cylindrical phospholipids alone do not form lipid bilayers but can be stabilized in the bilayer structure of cylindrical phospholipids (DOPC, DOPS, and DOPG) (17). Adding LPC to any form of proteoliposomes containing three to six different lipid types invariably reduced \( V_{\text{max}} \) to a basal level around 0.02 s\(^{-1}\) (Fig. 4b and Table 1). LPC is highly enriched in insulin granules, accounting for 20% of total granule lipids (16). When inserted into the lipid bilayer, the cone-shaped LPC introduces positive curvature. In contrast, DOPE in the lipid bilayer increases negative curvature (18). The positive curvature introduced by LPC is further augmented in bilayers of anionic lipids (19). Therefore, DPPE, which is highly enriched in insulin granules and has a similar effect on

![TABLE 1](image)

**Summary of kinetic parameters**

Data are means ± S.E. \( n = 2–4 \) independent experiments, each with nine independent measurements. \( V_{\text{max}} \) differences between ZnT8-R and ZnT8-W are as follows: **, \( p < 0.01 \), not statistically significant; *, \( p < 0.02 \), statistically significant; and ***, \( p < 0.01 \) very statistically significant.
bilayer promotes negative curvature (17). Increasing the concentration of DOPE in DOPC/DOPE/DOPG proteoliposomes from a ratio of 2:1:1 to 1:1:1 and 1:2:1 progressively reduced V_{max} of both variants but retained a V_{max} rank order of Arg/H11022Trp (Fig. 4b and Table 1). The third highly enriched lipid class in insulin granules is cholesterol. It is a compact and conical molecule that can fit into the void space between fatty acid chains, increasing packing density and bending rigidity of the lipid bilayer. A cholesterol content probably in the range of 20–25% is required for normal insulin secretion (18). Many residential proteins of insulin granules have a high affinity for cholesterol, likely lowering the actual cholesterol level in the granular membrane. Adding 5% cholesterol to proteoliposomes made with DOPC/DOPE/DOPG, DOPC/DOPE/DOPS, or DOPE/DOPC/LPC/DOPS reduced the transport activities of both ZnT8 variants to the basal level (Fig. 4b and Table 1). However, the inhibitory effect of cholesterol on higher order multiple lipid proteoliposomes was not as consistent as those of LPC, ranging from modest inhibition to weak stimulation for proteoliposomes made with DOPC/DOPE/soyPI/DOPS at a ratio of 45:20:23:12 (Fig. 4b and Table 1). Cholesterol may interact with sphingomyelin (SM) to form tightly packaged lipid rafts (19), but no obvious regulatory effect of SM was observed after adding 10% SM to modify the lipid compositions of proteoliposomes with or without cholesterol. Overall, a V_{max} rank order of Arg > Trp was consistently observed for cholesterol-containing proteoliposomes composed of 5–7 different types of lipids.
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**Correlation with T2D Risk**—The T2D odds ratio, which is a statistical measure of the association between the presence of a ZnT8 mutation/polymorphic variation and having T2D in a given population, is 1.18 for the high risk Arg-form and 1.00 for the low risk Trp-form, respectively (1). Strikingly, the measured $V_{max}$ values under 15 different lipid conditions are correlated with published T2D odds ratios in a linear fashion (Fig. 4c). The ZnT8 structural model shown in Fig. 2a predicts that the LOF ZnT8 mutants identified in human populations would be unable to fold properly in the membrane, thus giving a $V_{max}$ value of zero. Carriers of these mutants were found to have 65% reduced T2D risk with an overall odds ratio of 0.34 (6).

**Discussion**

A single high risk Arg-325 variant may be insufficient to produce a detectable phenotype in the polygenic diabetes model (20). Although it has generally been assumed that the common Arg variant associated with T2D risk is likely to encode a less active form of ZnT8, definitive biochemical assays of the activity of this and the protective Trp-form have been missing. Earlier studies have been reliant on the use of living beta cells expressing either the Arg- or Trp-forms in the presence of a high level of endogenous ZnT8 and of intracellularly trappable zinc probes with uncertain distribution between the cytosol and the secretory granule (4). This issue has become more critical recently with the discovery that rare LOF ZnT8 mutants appear to be protective toward diabetes (6). To better compare the activities of the Arg- and Trp-forms of ZnT8, we have developed here a highly sensitive functional assay in vitro. Integration of our kinetic measurements with human genetic data suggests a linear correlation between ZnT8 activity and T2D risk from extreme LOF mutants to mild polymorphic variants (Fig. 4c). The association of the human high risk Arg-325 variant with ZnT8 hyperactivity suggests that this common polymorphic variant may be targeted for inhibition to reduce T2D risk in the general population.

Kinetic analysis of reconstituted ZnT8 with defined lipid compositions provides insights into functional dynamics of human ZnT8 in pancreatic beta cells. Rapid turnover of the secretory pathway is a basic functional requirement for pancreatic beta cells to maintain homeostatic abundance of insulin granules. During this process, ZnT8 is trafficked with insulin granule biogenesis and thus encounters drastically different lipid compositions from the endoplasmic reticulum to matured insulin secretory granules (Fig. 4a). Our experiments showed that the transport activity of human ZnT8 is sensitive to functional regulations by three classes of lipids rich in insulin secretory granules. Anionic lipids activate while LPC strongly inhibits both ZnT8 variants. Cholesterol is likely another major modulator with mixed effects ranging from strong inhibition to slight stimulation. The regulatory LPC and cholesterol are both non-bilayer lipids. Their partitions between residential lipid-binding proteins and the granular membrane may strongly influence ZnT8 activity in the insulin granule membrane. Of note, the Arg variant is consistently more active than the Trp variant under various lipid conditions. The Arg-to-Trp polymorphic substitution is predicted to occur at the distal end of CTD at the dimeric interface (Fig. 2a). The effects of polymorphic variations at this position are likely independent of lipid modulation taking place within the membrane.

Earlier biochemical studies on the bacterial homolog YiiP showed that mutations at the CTD interface affected zinc transport activity via an allosteric mechanism (21), which might explain the hyperactivity of the Arg variant by introducing a positively charged residue to influence dimeric interactions. Nevertheless, this charge substitution had no detectable effect on ZnT8 expression, stability, and subcellular localization in HEK293 cells.

ZnT8 is commonly assumed to pump cytosolic zinc into insulin granules against a steep concentration gradient. However, the abundant presence of strong inhibitory lipids in the late insulin secretory pathway argues for a functional down-regulation of granular ZnT8 (Fig. 4a). Secretory granules store an exceptionally high level of zinc in a 10–20 mM range (22), whereas the free zinc concentration in the cytoplasm is kept around a nanomolar homeostatic set point (9). The large outward zinc concentration gradient may reverse ZnT8-mediated transport from granular zinc sequestration to backflow. Down-regulations of ZnT8 by LPC and probably by cholesterol seem functionally beneficial to leakage prevention (Fig. 4a). The lipid compositions in the early secretory pathway may be more permissive for ZnT8-mediated zinc sequestration. Functional studies of subcellular ZnT8 activities still await enabling technologies to track spatiotemporal dynamics of lipid compositions, subcellular ZnT8 localization, and zinc concentrations at the same time.

**Experimental Procedures**

**Expression Constructs**—The human ZnT8 isoform-2 cDNA (NM_001172814.1) housed in a pCMV6-entry vector (OriGene Technologies) was shuttled into a mammalian expression vector pCMV6-AC-GFP with a C-terminal green fluorescence protein (GFP) for transient expression by Lipofectamine transfection. The human ZnT8 cDNA insert was further subcloned into a pCDNA5/FRT/TO expression vector that used a tetracycline-inducible (CMV)/TetO2 promoter to control ZnT8 expression (Life Technologies, Inc.). A hexahistidine tag was added to the C terminus to facilitate affinity purification. The resulting His-tagged ZnT8 expression construct (Arg-form), pZnT8-His, was also mutated to generate the Trp-form using Q5 site-directed mutagenesis (New England Biolabs). All constructs were confirmed by double strand DNA sequencing.

**Stable Cell Lines**—Expression stable cell lines were generated by co-transfection of pZnT8-His and pOG44 at a 1:9 ratio into Flp-In, T-Rex-HEK293 host cells (Life Technologies, Inc.). Genomic integration of the pZnT8-His construct activated the expression of a hygromycin-resistant gene, allowing antibiotic selection of stable expression cells. Polyclonal stable cell lines were pooled and used for all experiments as described below. A separate cell line (Blk) with stable integration of the empty pCDNA5/FRT/TO vector was generated in parallel as a negative control.

**ZnT8 Expression**—Flp-In, T-Rex-HEK293 cells stably expressing His-tagged ZnT8 variants were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% tetracycline-reduced fetal bovine serum, 100 μg/ml hygromycin B, 10 μg/ml

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blasticidin in 95% air, 5% carbon dioxide at 37 °C. Early passage cells with a >95% viability were counted (typically at 1 × 10^7 cells/ml) and seeded appropriately for different experiments on poly-Lys-coated surfaces. When cells were grown exponentially to ~70% confluence in monolayers, doxycycline was added to 1 μg/ml to induce ZnT8 expression. Experiments were performed 18–24 h after induction.

**Immunofluorescence**—Stable expression cells grown on coverslips at 50% confluence were fixed with 4% paraformaldehyde, treated with a primary mouse monoclonal antibody recognizing the C-terminal His tag (Abcam, catalog no. ab5000, dilution 1:100), followed by a goat anti-mouse secondary antibody conjugated with Alexa Fluor-594 (Thermo Fisher, catalog no. A11005, dilution 1:200). Nuclei were counterstained with DAPI. Cells were then imaged using a Zeiss LSM 700 inverted confocal microscope with a ×63 oil objective. Fluorescence probes were excited by three separate laser lines (561, 405, and 488 nm) and monitored at respective emission wavelength ranges under the control of Zen software.

**Imaging of Vesicular Zinc Accumulation**—Induced cells in glass bottom microwell dishes at 50% confluence were labeled with 5 μM Zinpyr-1 that was added directly to the culture medium. After a 30-min incubation at 37 °C, cells were washed free of excess Zinpyr-1 with Dulbecco’s PBS, exposed to 100 μM extracellular zinc, and then imaged as described above.

**Flow Cytometry**—Stable expression cells were induced and labeled with 5 μM Zinpyr-1 as described above. The labeled cells were washed free of excess Zinpyr-1 with Hanks’ balanced salt solution with glucose and exposed to 100 μM extracellular zinc at room temperature for 30 min. Then, cells were trypsinized and resuspended in ice-chilled Hanks’ balanced salt solution at a density of 1 × 10^6 cells/ml. Flow cytometric analyses of vesicular zinc accumulation were performed on a MoFlo XDP cell sorter (Beckman Coulter) equipped with a 488-nm laser. Data were collected on forward scatter, side scatter, and a 525 nm fluorescence channel. More than 99% of scattering events belonged to a singlet cell population with 98% cell viability based on propidium iodide staining analysis. Zinpyr-1 fluorescence intensities are presented as mean ± S.E., n = ~13,000 live cells.

**Vesicular Zinc Uptake**—Uptake buffer with a free zinc concentration ranging from 5 to 283 μM was prepared by adding 0.3–1.7 mM ZnSO_4, 1 mM ADA (N-(2-Acetamido)iminodiacetic acid, N-(Carbamoylmethyl)iminodiacetic acid), 1 mM EGTA, and 1 μM pyrithione to assay buffer (100 mM NaCl, 20 mM HEPES, 1 mM tris(2-carboxyethyl)phosphine, pH 7.0). The free zinc concentration in the ADA/EGTA dual buffering system was calculated using MAXCHELATOR. Induced cells were loaded with Zinpyr-1, trypsinized, and then resuspended in an ice-chilled zinc-free uptake buffer. Cells densities were adjusted to 4 × 10^6 cells/ml and then dispersed in 50-μl aliquots to a clear-bottom 96-well microplate (Greiner Bio-One). Zinc uptakes were initiated by adding 50 μl of uptake buffers with 2× free zinc concentrations. The Zinpyr-1 fluorescence increases in response to various free zinc concentrations were recorded using a Flexstation-3 microplate reader (Molecular Dynamics) at 15 °C over a time course of 10 min, normalized to initial Zinpyr-1 fluorescence (F₀). The rates of zinc uptake were calculated by linear regression of Zinpyr-1 fluorescence increase as a function of time. All measurements were performed in 12 replicates.

**Purification and Reconstitution**—About 24 h after induction, early passage cells (passage number <10) at 90% confluence were scraped and collected in assay buffer supplemented with an EDTA-free protease mixture tablet (Sigma). Membrane vesicles were prepared by 20 passages through a high shear fluid processor at 120 p.s.i. (Microfluidics) and then pelleted by ultracentrifugation at 258,000 × g for 60 min. The membrane pellet was solubilized by n-dodecyl β-D-maltoside (DDM) at a DDM-to-membrane ratio of 1.5 (w/w). The membrane crude extract was cleared of debris by ultracentrifugation at 258,000 × g for 15 min, then mixed with Talon affinity resins (GE Healthcare), and incubated at 8 °C on a rotary shaker for 45 min. The immobilized ZnT8 was minimally washed by assay buffer supplemented with 25 mM imidazole and then eluted by 250 mM imidazole. The eluted ZnT8 was validated by Western blotting using two antibodies to the C-terminal His tag (Cell Signaling Technology, catalog no. 2365S, 1:1000 dilution) and to an N-terminal linear epitope of human ZnT8 (Proteinotech, catalog no.16169-1-AP, 1:500 dilution), respectively. Preformed liposomes with a defined lipid composition as indicated were prepared by mixing stock solutions of lipids in chloroform. The lipid mixture was dried under a stream of nitrogen gas, rehydrated with assay buffer to a lipid concentration of 50 mg/ml, and then sonicated in a cup-horn sonicator at 100 watts for 2 min (cycles of 10 s on and 10 s off) in an ice-chilled water bath. The resulting liposome suspension was diluted with assay buffer to a final lipid concentration of 7.5 mg/ml with DDM added at a DDM/lipid ratio of 1:1 (w/w). Reconstitution of ZnT8 took place immediately after eluting from Talon resins by mixing a ZnT8 variant or an equal volume of elution buffer with DDM-destabilized preformed liposomes at a ZnT8-to-lipid w/w ratio of 1:25. The reconstitution mixture was incubated on a rotary shaker at 8 °C for 1 h, and then polystyrene beads (BioBeads, SM-2, Bio-Rad) were added to the reconstitution mixture in a 60:1 w/w ratio to DDM. After incubation overnight at 8 °C, the resulting proteoliposomes or liposomes (Blk) were pelleted by ultracentrifugation at 258,000 × g (2 h), then resuspended in 0.2 ml of assay buffer with 200 μM Fluoizin-3, subjected to three cycles of freeze-thaw, followed by a 10-s sonication to complete dye encapsulation. The extravesicular Fluoizin-3 was removed by washing vesicles three times with 25 ml of assay buffer.

**Stopped-flow Kinetics**—Experiments were performed at 8 °C on an SFM-3000 stopped-flow apparatus (Bio-logic). Proteoliposome or liposome samples and an assay buffer containing varying concentrations of ZnSO_4 as indicated were loaded into two separate mixing syringes. Zinc influx reactions were initiated by pushing 101 μl of fresh reactants at a 1:1 ratio into a mixing chamber at a flow rate of 10 ml/s. The reactants were excited at 490 nm, and emissions were monitored at 525 nm using a 10-nm bandpass cutoff filter. All kinetic traces were cumulative averages of nine successive recordings. Liposome traces were collected as baselines and subtracted from proteoliposome traces to yield net fluorescence changes ΔF. ΔF/ΔF_max was obtained by normalizing ΔF to the maximum proteolipo-
some response elicited by an assay buffer containing 3 mM ZnSO$_4$ plus 2% octyl-$\beta$-glucoside. The initial rate of zinc influx was obtained by linear regression of data points ($t < 1$ s) in the quasi-linear phase of the initial fluorescence rise. Concentration dependence data were analyzed by least squares fits of the initial transport rate to a hyperbola defined by $v = V_{\text{max}} M / (M + K_m)$, where $M$ represents the zinc ion concentration; $V_{\text{max}}$ is the maximum transport rate and the rate of transport approaches a quasi-stationary state, and $K_m$ is the Michaelis-Menten constant. Fits of experimental data were performed using the data analysis software SIGMAPLOT (SPSS Inc.).

Homology Modeling—Protein sequences of the Arg allele of human ZnT8 isoform-2 and E. coli zinc transporter YiiP were aligned using MODELLER version 9.16. The alignment was imported into Swiss model to generate a homolog model of ZnT8 using the crystal structure of YiiP at 2.9-Å resolution as a template (Protein Data Bank code 3H90). The graphic representation of the resulted model was prepared using PyMOL (Delano Scientific).

Statistics—The difference between two groups was analyzed using unpaired $t$ test.

Author Contributions—D. F. and G. A. R. conceived the idea. D. F. developed the assays. C. M. performed the biochemistry experiments. Q. H. performed the cell biology experiments. All authors contributed to data analysis. D. F. wrote the paper with C. M. and G. A. R.

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