Crystal structures of a ZIP zinc transporter reveal a binuclear metal center in the transport pathway

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Zrt/Irt-like proteins (ZIPs) play fundamental roles in metal metabolism/homeostasis and are broadly involved in numerous physiological and pathological processes. The lack of high-resolution structure of the ZIPs hinders understanding of the metal transport mechanism. We report two crystal structures of a prokaryotic ZIP in lipidic cubic phase with bound metal substrates (Cd2+ at 2.7 Å and Zn2+ at 2.4 Å). The structures revealed a novel 3+2+3TM architecture and an inward-open conformation occluded at the extracellular side. Two metal ions were trapped halfway through the membrane, unexpectedly forming a binuclear metal center. The Zn2+-substituted structure suggested asymmetric functions of the two metal-binding sites and also revealed a route for zinc release. Mapping of disease-causing mutations, structure-guided mutagenesis, and cell-based zinc transport assay demonstrated the crucial role of the binuclear metal center for human ZIP4. A metal transport mechanism for the ZIP from Bordetella bronchiseptica was proposed, which is likely applicable to other ZIPs.

INTRODUCTION

Zinc is an essential trace element for life. It has been estimated that 10% of proteins (~3000 proteins) encoded by the human genome are zinc-binding proteins (1). Zinc is broadly involved in a variety of biological processes and plays particularly important roles in enzyme catalysis, gene regulation, and macromolecular stability. Zinc is also an emerging signaling molecule regulating physiological and pathological events (2). Accordingly, intracellular zinc levels need to be tightly and precisely controlled. In mammalian cells, the Zrt/Irt-like protein (ZIP) family [solute carrier family 39A (SLC39A)] and the ZnT family are responsible for zinc influx and efflux, respectively (3–5). In humans, a total of 14 ZIPs have been identified with diverse tissue/cellular distribution and distinct physiological functions (6). In particular, ZIP4 is essential for embryonic development and exclusively responsible for zinc uptake from dietary food. Dysfunctional mutations of ZIP4 lead to a lethal genetic disorder, acrodermatitis enteropathica (AE) (7, 8). Overexpression of ZIP4 has been associated with pancreatic cancer (9). To understand the structural basis of ZIP4 dysfunction caused by the AE-causing mutations and to facilitate drug discovery of ZIP4 inhibitors, we previously solved the crystal structure of the extracellular domain (ECD) of a mammalian ZIP4 (10), demonstrating that ZIP4-ECD is a crucial regulatory domain for optimal zinc transport. Here, we solved the crystal structures of a prokaryotic ZIP, which provides the first structural framework for understanding the metal transport mechanism of the ZIPs.

RESULTS

Crystallization and structure determination of a prokaryotic ZIP

The ZIP family consists of thousands of integral membrane proteins throughout the three kingdoms of life, and all of its members share a conserved transmembrane domain (TMD) with eight transmembrane helices (TMαs). In a thorough screening of candidates suitable for crystallographic study, a ZIP from Bordetella bronchiseptica (BbZIP) showed ideal solution behavior in detergents. BbZIP was purified in the presence of cadmium ion (Cd2+), which was reported to be a substrate of BbZIP (11). However, the purified protein in the apo form was prone to form aggregates, suggesting that substrate binding is required for protein stability in detergents. Because Cd2+-bound protein appeared to be more stable than Zn2+-bound protein, we chose Cd2+ in the following structural study. Although the detergent-solubilized protein was crystallized under multiple conditions, the crystals poorly diffracted. We then switched to crystallization in lipidic cubic phase (LCP), which provides a near-native membrane environment for integral membrane proteins. The crystals obtained in LCP with 100 mM CdCl2 at neutral pH diffracted better than 3.1 Å, and a mild dehydration treatment further improved the resolution. We eventually solved the structure by single-wavelength anomalous dispersion (SAD) using a selenomethionine (SeMet)-substituted crystal at 2.7 Å (table S1 and fig. S1).

Overall structure of BbZIP

One BbZIP molecule was observed in one asymmetric unit, and crystal packing analysis does not support strong interactions with the neighboring molecules, which are all in an opposite orientation (fig. S2). It is very unlikely that BbZIP forms an antiparallel dimer in the cell membrane because it has more TMs than the rarely reported antiparallel (or dual) transporters, which are usually very small in size and have only four TMs (12). Note that the freshly purified BbZIP often ran as two species on size exclusion columns, with apparent molecular weights of ~160,000 and ~110,000, respectively (fig. S3). The larger species (160 kDa) matches the reported BbZIP dimer in n-dodecyl-β-D-maltopyranoside (DDM) (11), and the smaller species (110 kDa) most likely corresponds to a monomeric form, given that the theoretical molecular weight of BbZIP is 31,000. Notably, both species can be crystallized under the same condition, and the current structure represents a conformation in the monomeric state.

The structure of BbZIP shows eight TMs forming a tightly associated helix bundle (fig. 1A), where TM2, TM4, TM5, and TM7 create an inner bundle surrounded by the other four TMs (fig. 1B), representing a novel fold for integral membrane proteins according to the three-dimensional protein structure similarity search on the Dali server (13). TM2 is unusually long (36 amino acid residues) with a kink where the helix is partially unwound because of a conserved proline residue (P110) (fig. S4). TM4 and TM5 are also kinked by the invariant proline residues in the metal-binding motifs (17)HN/PEG182 (h refers to hydrophobic residue, respectively. Further structural inspection

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reveals symmetrical relationships in the structure. The first three TMs (TM1 to TM3) are symmetrically related with the last three TMs (TM6 to TM8) by a pseudo-twofold axis, which is roughly parallel to the putative membrane plane (fig. S5). TM4 and TM5, which are also symmetrically related by the same axis, are sandwiched by the two 3-TM repeats. Therefore, the BbZIP structure shows an unusual 3+2+3TM architecture, which is unprecedented for transporters.

To avoid unfavorably exposing hydrophobic residues to the aqueous environment, the whole helix bundle of BbZIP has to be significantly tilted when it is embedded in the membrane (Figs. 1, A and C, and fig. S6), which is also supported by crystal packing of BbZIP in the lipid bilayer of monoolein (fig. S2). According to the established topology where both the N and the C termini of the ZIPs are exposed to the extracellular space (6), the putative transport pathway at the extracellular side is blocked by the highly conserved hydrophobic residues from the TMs of the inner helix bundle (M99 and A102 from TM2, L200 and I204 from TM5, and M269 from TM7) (Fig. 1, B and C, and fig. S4). The invariant S106 on TM2 is situated at the very bottom of the shallow and negatively charged entrance cavity. At the cytoplasmic side, the opening of the inner helix bundle results in a wide-opened exit cavity with high negative electrostatic potential (Fig. 1D). Therefore, the structure of BbZIP represents an inward-open conformation.

**Identification of a binuclear metal center in the transport pathway**

In the structure of BbZIP crystallized in the presence of CdCl₂, four heavy metal–binding sites (M1 to M4) were identified (Fig. 2, A and B). The anomalous signals derived from the data set collected at 1.81 Å are much stronger than those collected at 0.98 Å, ruling out the presence of zinc, copper, cobalt, iron, and nickel, but consistent with Cd²⁺. To further confirm the identity of the bound metals, we conducted inductively coupled plasma mass spectroscopy (ICP-MS) experiments on highly purified BbZIP eluted from two successive desalting columns for removal of free metal ions in the sample, and the results showed that Cd²⁺ is the most abundant transition metal (fig. S7). Compared with the hydrated Cd²⁺ ions at M3 and M4 on membrane-water interface, dehydrated Cd²⁺ ions at M1 and M2 are trapped about halfway through the membrane by the highly conserved residues primarily from H177, E181, Q207, E211, N178, E181, D208, E211, and E239 (from TM6), and one water molecule. In the native crystal with bound Cd²⁺, the sulfur atom of M99 also coordinates with Cd²⁺ at M1 (fig. S8). The kinks at TM4 and TM5 produced by P180 and P210 appear to facilitate metal binding by allowing the chelating residues to approach and bind metal in a synergic manner.

To investigate zinc binding on BbZIP, we soaked the Cd²⁺-loaded crystals with 100 mM ZnCl₂ at 21°C for 40 hours and collected diffraction data sets at two distinct wavelengths, λ₁ (1.2812 Å, 9.68 keV) and λ₂ (1.3225 Å, 9.37 keV), which are slightly shorter and significantly longer than the K-edge of zinc (1.2835 Å, 9.66 keV), respectively. As expected, the fully exposed Cd²⁺ ions at M3 and M4 were completely replaced by high concentration of Zn²⁺ ions, which is evidenced by the strong anomalous signals at λ₁, whereas no detectable anomalous

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**Fig. 1. Overall structure of BbZIP.** (A) Side views of BbZIP in the putative membrane with labeled TMs (α1 to α8). The protein is shown in cartoon mode and colored in rainbow with the N terminus in blue and the C terminus in red. The bound Cd²⁺ ions are shown as yellow-brown spheres. The dotted lines indicate the disordered interhelical loops (the loop between TM3 and TM4 and the loop between TM7 and TM8). (B) Top view of BbZIP in the direction indicated by the black arrow in (A). The two Cd²⁺-binding sites in the transport pathway are labeled as M1 and M2, respectively. (C) Cross-sectional view of the electrostatic potential map of BbZIP. The two highly negatively charged cavities are indicated by arrows. Note that the pathway from the entrance cavity to the metal-binding sites is blocked. (D) Electrostatic potential map of the exit cavity.
As in (C). The dashed lines indicate the bonding of Zn\(^{2+}\) and Cd\(^{2+}\) ions with the metal-chelating residues are labeled and shown in stick mode with the same colors collected at 1.2782 and 1.3190 Å, respectively. The K-edge of zinc is 1.2835 Å. The meshes (\(s = 5\)) of the metals at M1 and M2. The blue meshes (\(s = 5\)) and pink meshes (\(s = 3\)) show the anomalous different maps calculated from the data sets collected at 1.7969 Å, where Cd generates much stronger anomalous signals than at 0.9792 Å. The metal-chelating residues are labeled and shown in stick mode with the same colors as in (A). The dashed lines indicate the bonding of Cd\(^{2+}\) ions with the coordinating residues in the range of 2.6 to 2.9 Å. The ordered water molecules (W) are shown as small red spheres. (C) Metal binding to Zn\(^{2+}\)-substituted BbZIP. The seven metal-binding sites are labeled (M1 to M7). The bound Zn\(^{2+}\) and Cd\(^{2+}\) ions are shown as gray and yellow-brown spheres, respectively. The region in the dotted frame is shown in (B). (D) Zoomed-in view of the binuclear metal center in the direction indicated by the arrow in (A). The green meshes show the anomalous difference map of Cd\(^{2+}\) ions at M1 and M2 (\(s = 5\)). The data set was collected at 1.2792 Å, where Cd generates much stronger anomalous signals than at 1.3190 Å, respectively. The K-edge of zinc is 1.2835 Å. The metal-chelating residues are labeled and shown in stick mode with the same colors as in (C). The dashed lines indicate the bonding of Zn\(^{2+}\) and Cd\(^{2+}\) ions with the coordinating residues within the range of 2.0 to 2.2 Å (for Zn\(^{2+}\)) and 2.6 to 2.8 Å (for Cd\(^{2+}\)). The ordered water molecules are shown as small red spheres.

Fig. 2. Metal-binding sites in BbZIP. (A) Cd\(^{2+}\) binding to BbZIP. The protein is in cartoon mode and colored in rainbow. The four Cd\(^{2+}\)-binding sites are labeled (M1 to M4). The bound Cd\(^{2+}\) ions are shown as yellow-brown spheres. The region in the dotted frame is shown in (B). (B) Zoomed-in view of the binuclear metal center in the direction indicated by the arrow in (A). The blue meshes show the anomalous difference map of Cd\(^{2+}\) ions at M1 and M2 (\(s = 5\)). The data set was collected at 1.7969 Å, where Cd generates much stronger anomalous signals than at 0.9792 Å. The metal-chelating residues are labeled and shown in stick mode with the same colors as in (A). The dashed lines indicate the bonding of Cd\(^{2+}\) ions with the coordinating residues in the range of 2.6 to 2.9 Å. The ordered water molecules (W) are shown as small red spheres. (C) Metal binding to Zn\(^{2+}\)-substituted BbZIP. The seven metal-binding sites are labeled (M1 to M7). The bound Zn\(^{2+}\) and Cd\(^{2+}\) ions are shown as gray and yellow-brown spheres, respectively. The region in the dotted frame is shown in (D). (D) Zoomed-in view of the binuclear metal center in the direction indicated by the arrow in (C). The green meshes show the anomalous difference map of Cd\(^{2+}\) ions at M1 and M2. The blue meshes (\(s = 5\)) and pink meshes (\(s = 3\)) show the anomalous different maps calculated from the data sets collected at 1.7969 Å and 1.3190 Å, respectively. The K-edge of zinc is 1.2835 Å. The metal-chelating residues are labeled and shown in stick mode with the same colors as in (C). The dashed lines indicate the bonding of Zn\(^{2+}\) and Cd\(^{2+}\) ions with the coordinating residues within the range of 2.0 to 2.2 Å (for Zn\(^{2+}\)) and 2.6 to 2.8 Å (for Cd\(^{2+}\)).

Signals were shown at \(\lambda_2\) (fig. S9). Similarly, the Cd\(^{2+}\) at M1 was replaced by Zn\(^{2+}\), accompanied with significant changes in coordination environment: The side chains of M99 and H177 moved away from the cytoplasmic side. Notably, M5 is right below M1 and coordinated with E276, an absolutely conserved residue in the bacterial ZIPs, and H177 (Fig. 2D), a residue previously chelating Cd\(^{2+}\) at M1 (Fig. 2B), implying that side-chain flipping of H177 may be involved in the metal release from M1. Remarkably, the multiple conserved zinc-binding sites (M1, M5, M6, and M3; fig. S4) appear to constitute a route of zinc release to the cytoplasm.

Mapping disease-causing mutations on human ZIP4 model structure

A pairwise sequence alignment shows considerable homology between BbZIP and human ZIP4 (hZIP4) throughout the TMD, particularly in TM2 and the last five TMs (TM4 to TM8), where the two proteins share 21% identity and 61% similarity (fig. S10). Therefore, the major structural features of BbZIP should be preserved in hZIP4. We then constructed a structural model of the TMD of hZIP4 using the zinc-bound structure of BbZIP as a template in homology modeling (Fig. 3A). The overall model structure of hZIP4-TMD is very similar to a computational model based on coevolution of predicted contacting residues (fig. S11) (14), in particular, for four tightly associated TMs (TM1, TM4, TM5, and TM6).

To understand the structural basis of ZIP4 dysfunction caused by the AE mutations, the eight involved residues, which are generally conserved between hZIP4 and BbZIP (fig. S10), are mapped on the hZIP4 model structure. As shown in Fig. 3A, except for L372, the other seven residues are at or in close proximity to the TM-TM interface, suggestive of roles in mediating TM packing. For G330D, G374R, and G630R, the corresponding mutations in the mouse ZIP4 (mZIP4) led to markedly reduced zinc transport activity accompanied with diminished surface expression and defects on glycosylation, strongly indicating that these mutations severely affected protein folding and trafficking (15). Although the counterpart of G526R in mZIP4 appeared to be normal in cell surface expression and glycosylation (15), the substantially reduced activity may be caused by the disrupted interaction between G526 on TM5 and the highly conserved F519 on TM4. Remarkably, L372, which is topologically equivalent to M99 facing the binuclear metal center in BbZIP, is replaced by either a proline (16) or an arginine (17) in the AE patients. The proline substitution of the same residue in mZIP4 resulted in protein folding defects (15), probably due to an imposed kink on TM2. Differently, the arginine mutation most likely affects metal binding of the binuclear metal center and/or blocks the zinc transport pathway.

Functional characterization of the putative binuclear metal center of hZIP4

Because the residues coordinating metal ions at M1 and M2 are conserved (Fig. 3B), we modeled two Zn\(^{2+}\) ions in the hZIP4 structure and optimized the geometry of coordination sphere by manually adjusting the \(\chi\) angles of the involved residues (Fig. 3C). The modeled Zn\(^{2+}\) ions can be comfortably accommodated at both sites without significantly affecting the backbone structure. The Zn\(^{2+}\) ion at M1 is pentacoordinated with H507, D511, H536, and H540 (corresponding to H177, E181, Q207, and E211 in BbZIP, respectively), and the Zn\(^{2+}\) ion at M2 is tetrahedrally coordinated with N508, D511, and E537 (corresponding to N178, E181, and D208 in BbZIP, respectively). D511, which is topologically equivalent to the bridging residue E181 in BbZIP, connects the two Zn\(^{2+}\) ions, which are 4.2 Å apart.

To examine the importance of the metal-chelating residues in the putative binuclear metal center of hZIP4, we conducted an alanine
scanning by substituting an involved residue with an alanine residue. As shown in Fig. 3D, the D511A variant exhibited a markedly reduced zinc transport activity (~20% of the wild-type hZIP4), whereas the other variants (H507A, N508A, H536A, and E537A) only showed moderately reduced activity, suggesting that the integrity of the binuclear metal center is most crucial for metal transport. Unexpectedly, H540A mutation significantly increased the transport activity by more than twofold but also reduced the apparent affinity toward zinc (fig. S12). Because H540 is the closest to the exit cavity among the metal-chelating residues in the binuclear metal center, we postulate that H540 may play a role in controlling the rate of metal release and/or the rate of conformational switch of the transporter during the transport cycle.

To further explore the roles of metal binding at the binuclear metal center, we conducted an arginine scanning by replacing the individual metal-chelating residue with an arginine residue (Fig. 3E). D511 was not included because the alanine mutation already abolished the activity. The bulky and positively charged side chain of Arg is expected to eliminate metal binding and block the transport pathway. It turned out that H540R mutation led to completely abolished zinc transport activity, indicating that disrupting metal binding at M1 and
M2 severely impaired the function of hZIP4. N508R mutation reduced the activity by approximately 60%, and the residual activity suggests that the bulky side chain of the introduced Arg does not completely block the transport pathway. Similarly, the H540R variant showed a comparable (or even slightly higher) activity to the wild-type protein, but its activity and apparent affinity to zinc were substantially reduced when compared with the H540A variant (fig. S12). Given that N508 and H540 are both close to the widely opened exit cavity, it is not unexpected that the Arg residues at these two sites may be oriented in such a way that the transport pathway is still at least partially open.

**Implications on metal transport mechanism of BbZIP**

A proposed metal transport pathway for BbZIP is illustrated in Fig. 4. The inward-open conformation of BbZIP is stabilized by substrate binding at the conserved binuclear metal center in the middle of the transport pathway, and the bound metals will be released to the cytoplasm through a chain of metal-chelating residues (H177, E276, H275, and D144), with an aid of the histidine-rich loop connecting TM3 and TM4. The multiple weak zinc-binding sites at the exit cavity constitute a “metal sink” facilitating metal release from the binuclear metal center. Likely driven by repulsive electrostatic forces between the metal-chelating residues and/or the removal of geometric constraints, metal release may lead to a rearrangement of the TMs to generate an open channel at the extracellular side blocked by conserved hydrophobic residues (M99 and A102 on TM2, L200 and I204 on TM5, and M269 on TM7) in the inward-open conformation. In the entrance cavity, the two invariant metal-chelating residues (D113 and D305; fig. S4) may be crucial for recruiting metal substrates. The absolutely conserved S106 is located at the very bottom of the entrance cavity, implying a function in guiding metal substrate into the transport pathway. Alanine mutation of H379 in hZIP4, which is equivalent to S106 in BbZIP (fig. S10), severely diminished zinc transport (14). This result is further confirmed by an H379A/D375A double mutation, which resulted in a loss of 75% activity (Fig. 3E). According to the proposed transport pathway (Fig. 4), D375 on TM2 of hZIP4, which is topologically equivalent to A102 of BbZIP (fig. S10), is likely to be a pore-lining residue at the extracellular side.

Structure inspection further suggests that TM1, TM4, TM5, and TM6 associate closely, forming a tight 4-TM bundle, which appears to be weakly associated with the other four TMs (fig. S13). Because the metals captured at the binuclear metal center are primarily held on this 4-TM bundle (TM2, through M99, only associates with Cd$^{2+}$, but not with Zn$^{2+}$), a rigid rocking-like movement of this helix bundle, together with the coordinative movement of the other four TMs (particularly the long and bent TM2), will alternately expose the metal substrate to the extracellular space and the cytoplasm (fig. S13). Although a structural characterization of an outward-open conformation is required to establish a full transport cycle, the absence of an open entrance at the extracellular side in the current structure already indicates that a significant conformational change is required for metal transport, which is consistent with the extremely slow zinc flux through BbZIP compared with typical ion channel (11).

**DISCUSSION**

Zinc homeostasis is crucial for any living organism. The ZIP family is a major player that emerged from the early stage of life evolution in regulating zinc import (and some other transition metals, such as iron and manganese) from the environment. The success of this ancient protein family in evolution is reflected by the fact that almost every single species, from bacteria and archaea to humans, keeps at least one zip gene in the genome. To understand the working mechanism of this conserved zinc transport machinery whose efficiency has been proven over billions of years of evolution, we crystallized a prokaryotic ZIP in LCP, which leads us to discover a novel transporter fold, identify an unexpected binuclear metal center in the transport pathway, and propose a metal transport mechanism likely applicable to other ZIPs.

A previous bioinformatics analysis suggested that the ZIP family belongs to the drug/metabolite transporter (DMT) superfamily with a predicted 3+5TM architecture (18), and a recent structural study of the first DMT member SnYddG has shown that the inverted 5-TM repeats are derived from the primitive 4-TM DMT members such as EmrE (19). Unexpectedly, BbZIP structure reveals an unusual 3+2+3TM arrangement, raising an issue of the origin of the ZIP family. Because the symmetrically related substrate-binding TMs (TM4 and TM5) are not included but sandwiched by the two 3-TM repeats in both primary sequence and tertiary structure, the BbZIP structure represents an unprecedented fold different from any known carrier structure. As an ancient transporter family present in almost every single species, the ZIPs may diverge very early in evolution and evolve from the primitive 3-TM segment observed in numerous transporters (20). Likely by gene duplication and acquisition of additional two TMs with metal-binding motifs, the ZIPs complete the 3+2+3TM architecture and evolve into metal transporters. Accordingly, different from the other families, the ZIP family represents a fairly distant branch within the DMT superfamily.

It is quite unexpected that a binuclear metal center is observed in the transport pathway of BbZIP. Binuclear metal centers are known for their catalytic roles in enzymes (16, 17), including membrane enzymes (21, 22), but it has not been observed within the transport pathway for any known carriers. The distinct coordination environment and markedly different exchangeability suggest that the roles of M1 and M2 are asymmetric. Because the Cd$^{2+}$ ion at M1 can be readily replaced by Zn$^{2+}$.
and M1 is closer to the putative entrance at the extracellular side (fig. S14), we postulate that M1 is exactly situated in the transport pathway to allow for rapid metal binding and release during transport. Because a computational study has shown that the second metal in a binuclear metal cluster attenuates binding affinity but increases metal selectivity of the first metal-binding site (23), we postulate that the metal bound at M2 may play a regulatory role in modulating the properties (affinity, selectivity, kinetics, etc.) of M1 by affecting the charge and the geometry of the metal-chelating residues at M1 through the bridging residue(s). Alternatively, both M1 and M2 are within the metal transport pathway, and the distinct exchangeability at M1 and M2 may reflect the order of metal release from the binuclear metal center; that is, the bound metal at M2 has to slowly pass M1 and then gets released to the cytoplasm. Nevertheless, the markedly reduced activity of D511A in hZIP4 indicates that bridging the two metal ions in the binuclear metal center is crucial for zinc transport.

Together, the first atomic structural framework of the ZIPs presented in this work, together with the unexpected binuclear metal center in the transport pathway, establishes a foundation for further investigation of the unique metal transport mechanism and substrate specificity of the ZIPs. Clarification of structure-function relationship of the ZIPs will not only facilitate drug discovery of the ZIP inhibitors against cancers (9, 24) and inflammatory diseases, such as osteoarthritis (25), but also aid protein engineering of plant ZIPs, which are the primary transport metal transporters in the roots of many plants, for nutrition fortification (26, 27) and phytoremediation (28).

**MATERIALS AND METHODS**

**Genes, plasmids, and reagents**

The gene encoding BbZIP (B. Bronchiseptica; National Center for Biotechnology Information reference code: WP_010926504) was synthesized (Integrated DNA Technologies) with optimized codons for *Escherichia coli* and subcloned into a plW01 vector (gift from L. Waskell of the University of Michigan), where a thrombin cleavage site was inserted between an N-terminal His6-tag and BbZIP. The complementary DNA of full-length hZIP4 from the Mammalian Gene Collection was obtained from GE Healthcare (GenBank code: BC062625). The gene of hZIP4 was subcloned into a modified pEGFP-N1 vector (Clontech), in which the downstream EGFP (enhanced green fluorescent protein) gene was replaced by a hemagglutinin (HA) tag. All the mutations were made using QuikChange Mutagenesis kit (Agilent). Genes, plasmids, and primers are listed in table S2. 1-Oleoyl-rac-glycerol (monoolein) was purchased from Sigma-Aldrich. L(+)-selenomethionine was purchased from Sigma-Aldrich. t(+)−selenomethionine was purchased from Acros Organics. The reagents and tools for protein crystallization were obtained from Hampton Research. Thrombin was purchased from Novagen.

**Cell culture and transfection**

HEK293T cells (American Type Culture Collection) were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (Invitrogen) and 1 × Gibco Antibiotic-Antimycotic (Invitrogen). The plasmids were prepared by Qiagen Plasmid Maxi kit (Qiagen) and used to transiently transfect HEK293T cells. Cells were seeded on poly-d-lysine (Corning BioCoat)−coated 24-well plate at 1 × 10⁴ cells per well and were transfected with Lipofectamine 2000 (Invitrogen) after overnight culture. After transfection, the cells were cultured at 37°C and 5% CO₂ for 24 hours before the activity assay and Western blot.

**Western blot and ZIP4 surface expression assay**

The expression of hZIP4-HA (and other constructs and mutants) was detected by Western blot using an anti-HA antibody (catalog #26183, Thermo Scientific Pierce), as previously described (10). The expression level of hZIP4-HA protein (and other constructs and mutants) at cell surface was measured by the surface-bound anti-HA antibodies recognizing the C-terminal HA tag (15, 29). The cells from multiple subwells of the same 24-well plate for zinc uptake assay were used to determine the surface expression levels of the corresponding proteins. After washing twice with Dulbecco’s phosphate-buffered saline (DPBS; Sigma-Aldrich), cells were fixed for 10 min in 4% formaldehyde at room temperature. Cells were then washed three times with DPBS and incubated with anti-HA antibody (3 µg ml⁻¹) for 1 hour at room temperature. Cells were washed five times in DPBS to remove unbound antibodies and then lysed by sonication in SDS–polyacrylamide gel electrophoresis (PAGE) sample loading buffer. Cell lysates containing the solubilized anti-HA antibodies bound to the surface ZIP4 were separated using 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. As a loading control, β-actin levels were detected using anti–β-actin antibodies at 1:2500 dilution (catalog #4970, Cell Signaling Technology). Bound primary antibody was detected with horseradish peroxidase (HRP)–conjugated goat anti-mouse immunoglobulin G (IgG) (1:2000 dilution; catalog #32230, Thermo Scientific Pierce) or goat anti-rabbit IgG (1:5000 dilution; catalog #7074, Cell Signaling Technology) HRP by chemiluminescence. The band of the heavy chain of anti-HA antibody was used for quantitation of surface expression level of ZIP4. The surface expression levels quantitated using Image Lab (Bio-Rad) program were expressed as a ratio relative to the surface expression level of the wild-type hZIP4 and then used to calibrate the apparent zinc uptake activity (see below).

65Zn uptake assay and data analysis

The zinc uptake assay is slightly modified from the previously reported protocol (10). Twenty-four hours after transfection (cells were around 90% confluency), cells were washed by 300-µl zinc uptake buffer containing 10 mM Hepes, 142 mM NaCl, 5 mM KCl, and 10 mM glucose (pH 7.3) and then incubated with 300-µl zinc uptake buffer with indicated concentration of ZnCl₂ (containing 20% 65ZnCl₂) in the same buffer for 20 min at 37°C with occasional gentle shaking. Zinc uptake by HEK293T cells overexpressing wild-type hZIP4 was linear up to 30 min of incubation time (fig. S16A). Uptake was terminated by adding 300-µl stop buffer (ice-cold zinc uptake buffer with 1 mM EDTA). Resuspended cells in each well (24-well plate) were transferred to a 1.5-ml centrifuge tube and collected by centrifugation with 100g for 5 min at 4°C. Pelleted cells were gently washed twice with 300-µl zinc stop buffer, lysed in 400-µl uptake buffer with 0.5% Triton X-100, and then transferred into polystyrene tubes. Cell-associated radioactivity was measured with a Packard Cobra Auto-Gamma γ-counter, and the amount of zinc was calculated using a standard curve generated by plotting 65Zn radioactivity against the amount of 65ZnCl₂. The cells transfected with the empty vector were treated in the same manner and used as a control in the assay. The amount of zinc transported into the cells through ZIP4 was calculated by subtracting the zinc uptake in control cells from the zinc uptake in the cells transfected with ZIP4 gene.

In the alanine and arginine scanning experiments, 10 µM ZnCl₂ was used in the transport assay. At least three independent experiments were performed with three repeats for each alanine (or arginine) mutant in every single experiment. The amounts of zinc accumulated in the
cells were adjusted by protein concentration, which were measured by the Bradford method (Bio-Rad). Because of variations of transient expression among different batches of cells, we did not directly combine the values from independent experiments. Instead, after subtracting the background from control samples, the activities were normalized by setting the reading of the wild-type hZIP4 as 100%, and the activities of the mutants were expressed as a percentage of the activity of the wild-type hZIP4. Then, the relative activities were calibrated using the quantitated surface expression levels, which were finally used for statistical analysis using the Student’s t test approach. Each data point shown in Fig. 3 (D and E) represents a mean of a total of nine calibrated relative zinc uptake activities from three independent experiments. The data of each experiment are shown in fig. S15.

Zinc uptakes of the variants at different zinc concentrations were measured, and the kinetic parameters of H540A were determined (fig. S12A) (10). Curve fitting to the Hill model was conducted by using the concatenate mode with the instrumental weighting method in Origin. We did not conduct curve fitting for D511A, H507R, H536R, E537R, and H379A/D375A because of very low or completely abolished activity, whereas the $K_m$ of the other three alanine and arginine variants were determined (fig. S12B). We noticed that the linear range of zinc uptake by the H540A variant shrank to about (or less than) 20 min (fig. S16B), probably because of its much higher zinc transport activity than the wild-type protein. Therefore, zinc uptake assay of H540A was also conducted with an incubation time of 15 min, and the measured $K_m$ was slightly larger than that obtained at 20 min (fig. S12B).

**Protein expression and purification**

The expression of BbZIP was induced in the strain of C41 (DE3) pLysS (Lucigen) in LBE-5052 medium (30) and grew for 24 hours at room temperature before harvest. To generate SeMet-substituted BbZIP, cells were cultured at 37°C in M9 minimal media supplemented with SeMet and then induced by 0.2 mM isopropyl-β-D-thiogalactopyranoside at OD$_600$ (optical density at 600 nm) = 0.6. The cells were cultured at room temperature for additional 16 hours before harvesting. Spheroplasts were prepared (31) and suspended in a buffer containing 20 mM Heps (pH 7.3), 300 mM NaCl, 0.25 mM CdCl$_2$, and complete protease inhibitors (Sigma-Aldrich). DDM (Anatrace) was added to solubilize the membrane fraction with a final concentration of 1.5% (w/v). The His$_6$-tagged protein was purified using HisPur Cobalt Resin (Thermo Fisher Scientific) in 20 mM Heps (pH 7.3), 300 mM NaCl, 5% glycerol, 0.25 mM CdCl$_2$, and 0.1% DDM. After removing imidazole using an Amicon centrifugal filter device, the protein sample was treated with thrombin overnight at 4°C and then loaded onto a Superdex Increase 200 column (GE Healthcare) equilibrated with 10 mM Heps (pH 7.3), 300 mM NaCl, 5% glycerol, 0.25 mM CdCl$_2$, and 0.05% DDM. Purification and crystallization of SeMet-substituted protein were the same as the native protein, except for addition of 1 mM tris(2-carboxyethyl)phosphine in all solutions.

**Crystallization in LCP**

Purified BbZIP protein was concentrated to 15 mg/ml with a 30,000– molecular weight cutoff Amicon centrifugal filter device. The protein solution was then mixed with the molten monoolein with two coupled syringes at a ratio of 2:3 (protein/monoolein; v/v). All crystallization trials were set up using a Gryphon crystallization robot (Art Robbins Instruments). Fifty nanoliters of BbZIP-monoolein mixture covered with 800 nl of well solution was sandwiched with LCP sandwich set (Hampton Research). Stick-shaped crystals appeared about 2 weeks under a condition containing 33% (w/v) polyethylene glycol 400 (PEG 400), 100 mM NaCl, 100 mM CdCl$_2$, and 100 mM tris-HCl (pH 7.5) at 21°C and grew to full size in 4 weeks. Crystals were dehydrated with a slightly increased concentration of PEG 400, 100 mM NaCl, 100 mM CdCl$_2$, and 100 mM tris-HCl (pH 7.5) for 24 hours before they were harvested with a MiTeGen micromesh and flash-frozen in liquid nitrogen. After removing the Cd$^{2+}$-containing solution from crystallization drops, native BbZIP crystals were soaked with 35% PEG 400, 100 mM NaCl, 100 mM ZnCl$_2$, and 100 mM tris-HCl for 48 hours before harvesting.

**Diffraction data collection and structure determination**

X-ray diffraction data were collected at the Life Sciences Collaborative Access Team (LS-CAT) (21-ID-D) and General Medicine and Cancer Institutes Collaborative Access Team (GM/CAT-CAT) (23-ID-B and 23-ID-D) at Advanced Photon Source (APS) using a 20- or 10-μm beam on Eiger 9M or Pilatus 6M detectors and beamline 5.0.2 at the Berkeley Center for Structural Biology (BCSB) using a charge-coupled device detector at the Advanced Light Source (ALS) facility. LCP crystals were located and centered using the rasting strategy. The diffraction data set of SeMet crystals was collected at 0.9792 Å for experimental phasing. The data sets of SeMet crystals at 1.8066 Å were used to detect the anomalous signals of Cd$^{2+}$. To unambiguously establish the identity of the bound metals in the Zn$^{2+}$-soaked crystals, two data sets were collected on the same native crystal at 1.2812 and 1.3225 Å, respectively.

The diffraction data of SeMet BbZIP crystal were indexed, integrated, and scaled by HKL2000 (32). The experimental phase was determined using SAD at 2.7 Å by AutoSol in Phenix (33), and all 12 selenium atoms in one BbZIP were identified. The experimental electron density map was clear enough to recognize the eight TMs and trace the backbone of the polypeptide chain. Iterative model building and refinement were conducted in COOT (34) and reinaC5 in CCP4 suite (35) or phenix.refine. The Zn$^{2+}$-substituted structure was solved at 2.4 Å through molecular replacement using the Cd$^{2+}$-bound structure as search template. Except for the data set of SeMet collected at 0.9792 Å, the other data sets were indexed and integrated by xsnap (36) and scaled by Aimless in CCP4 with a changed space group name from C2 to I2 (both space group no. 5), which did not affect structure determination, model building, refinement, and crystallographic statistics. All figures of protein structures were generated by PyMOL v1.3 (Schrödinger LLC).

**Inductively coupled plasma mass spectroscopy**

The metal contents in BbZIP sample were measured using Thermo Scientific iCAP Q ICP-MS. Free Cd$^{2+}$ in the highly purified BbZIP was removed by using two successive PD-10 desalting columns (GE Healthcare). The control sample (gel filtration buffer plus 1% DDM) was treated in the same manner. The protein sample, control sample, and buffer solution were heated at 65°C in 2 M HNO$_3$ for 20 min, kept at room temperature overnight, and centrifuged for 20 min at 14,000g. The metal concentrations (in parts per billion) of the common transition metals (Cr, Mn, Fe, Co, Ni, Cu, Zn, Ag, Cd, Sn, and Pb) in the supernatant were analyzed by ICP-MS. The protein concentration was determined by using the Bradford method.

**Homology modeling**

Homology modeling was conducted on the SWISS-MODEL server (https://swissmodel.expasy.org/interactive) (37). The structure of the TMD of hZIP4 (residues 327 to 647) was modeled using the Zn$^{2+}$-substituted structure at 2.4 Å as the structural template. The residues within the
predicted unstructured loops (between TM3 and TM4 and between TM7 and TM8) were removed from the sequence of hZIP4 for better modeling, and the resulting model with a GMQE (global model quality estimation) score of 0.65 is shown in Fig. 3A. The local quality assessment, which is based on the QMEAN scoring function (38), showed that the residues within TM2 and TM4 to TM7 have the highest scores (about or higher than 0.7) than the other regions of the model structure, indicative of higher reliability for these regions, which include the putative transport pathway and the binuclear metal center. Zn$^{2+}$ ions were initially added to the model at the same positions as in the structure of Zn$^{2+}$-substituted structure. To fulfill the geometric constraints for Zn$^{2+}$ coordination, the $\chi$ angles of the side chain of the potential metal-chelating residues were manually tuned in COOT, with a slight adjustment in the metal position. In the final model, there is no obvious steric clash due to side-chain adjustment and Zn$^{2+}$ modeling.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/3/8/e1700344/DC1

- fig. S1. Crystal structure of BbZIP.
- fig. S2. Crystal packing of zinc-substituted BbZIP in two perpendicular directions.
- fig. S3. Size exclusion chromatography of BbZIP in DDM.
- fig. S4. Multiple sequence alignment of bacterial ZIPs.
- fig. S5. A novel fold of BbZIP.
- fig. S6. Tilted structure of BbZIP in the putative membrane.
- fig. S7. Determination of the zinc uptake kinetic parameters.
- fig. S8. The yeast ZRT1 gene encodes the zinc transporter protein of a high-affinity uptake system induced by zinc limitation. *Proc. Natl. Acad. Sci. U.S.A.*, 93, 2454–2458 (1996).
- fig. S9. Structural comparison of BbZIP. Zn$^{2+}$-substitution structure.
- fig. S10. Sequence alignment of BbZIP with hZIP4 TM4.
- fig. S11. Comparison of hZIP4 structure models.
- fig. S12. Determination of the zinc uptake kinetic parameters.
- fig. S13. Proposed conformational change facilitated by metal release from the binuclear metal center.
- fig. S14. The distances between the two metal-binding sites (M1 and M2) and M10. Zn$^{2+}$-substitution structure.
- fig. S15. Three independent zinc uptake assays.
- fig. S16. Time-dependent zinc uptake by HEK293T cells overexpressing hZIP4.
Acknowledgments: We thank S. Ferguson-Miller of the Department of Biochemistry and Molecular Biology at the Michigan State University for the critical reading of the manuscript. We thank beamline scientists at GM/CA-CAT, LS-CAT at APS, and BCSB at ALS for the instructions of data collections. We thank G. Girard of the Department of Earth and Environmental Sciences at the Michigan State University for the assistance in the ICP-MS experiment. Funding: This work is supported by the NIH (R01GM115373 to J.H.) and a startup fund of Michigan State University (to J.H.). Author contributions: J.H. conceived and designed the project. T.Z., J.L., C.Z., and D.S. conducted the experiments. T.Z., J.L., M.F., and J.H. collected the diffraction data and solved the crystal structures. T.Z., M.F., C.Z., and J.H. analyzed the data and wrote the manuscript. Competing interests: The authors declare that they have no competing interests. Data and materials availability: The atomic coordinates and structure factors have been deposited in the Protein Data Bank with access codes of STSB (Cd\(^{2+}\)-bound SeMet BbZIP) and STSA (Zn\(^{2+}\)-substituted native BbZIP). All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

Citation: T. Zhang, J. Liu, M. Fellner, C. Zhang, D. Sui, J. Hu, Crystal structures of a ZIP zinc transporter reveal a binuclear metal center in the transport pathway. Sci. Adv. 3, e1700344 (2017).
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Sci Adv 3 (8), e1700344.
DOI: 10.1126/sciadv.1700344