Zinc is essential for all organisms and yet detrimental at elevated levels. Hence, homeostasis of this metal is tightly regulated. The Zrt/Irt-like proteins (ZIPs) represent the only zinc importers in metazoans. Mutations in human ZIPs cause serious disorders, but the mechanism by which ZIPs transfer zinc remains elusive. Hitherto, structural information is only available for a model member, BbZIP, and as a single, ion-bound conformation, precluding mechanistic insights. Here, we elucidate an inward-open metal-free BbZIP structure, differing substantially in the relative positions of the two separate domains of ZIPs. With accompanying coevolutionary analyses, mutagenesis, and uptake assays, the data point to an elevator-type transport mechanism, likely shared within the ZIP family, unifying earlier functional data. Moreover, the structure reveals a previously unknown ninth transmembrane segment that is important for activity in vivo. Our findings outline the mechanistic principles governing ZIP-protein transport and enhance the molecular understanding of ZIP-related disorders.

INTRODUCTION
Zinc (Zn\(^{2+}\)) is essential for all domains of life. It is the second most abundant trace metal in humans (1), required for the function of more than 300 enzymes and 1000 transcription factors (1, 2). Consequently, zinc dyshomeostasis is linked to a broad palette of diseases, including diabetes, immune disorders, and progression of several forms of cancer (1, 3). The Zrt/Irt-like protein (ZIP) family of solute carrier 39A (SLC39A) membrane transporters is critical in maintaining appropriate cellular levels of zinc. ZIPs represent the only identified zinc importers in mammals, providing metal transfer from the extracellular space or from internal storage vesicles to the cytoplasm (3). As expected, malfunction of the human ZIPs, hZIPs, is associated with development of different disorders, e.g., hZIP4 and hZIP13 are directly linked to the severe acrodermatitis enteropathica by highly conserved hydrophobic residues. Consequently, the operational principles of ZIPs represent an outstanding question; even the overall classification as transporters or channels remains elusive. The high sequence homology among members suggests a shared overall shape and maintained mechanistic features throughout the entire ZIP family. Nevertheless, a multitude of transport modes is still permitted.

However, the structure and function of ZIPs remain elusive, preventing applied efforts to combat Zn\(^{2+}\) imbalance or directly related disorders as well. Both Michaelis-Menten kinetics, typical for secondary active transporters, and channel-like mechanisms have been proposed for ZIPs. Zn\(^{2+}/\text{HCO}_3^-\) (6–10) and Zn\(^{2+}/\text{H}^+\) symport (11) or even passive transport (12) have been detected for human members. Moreover, the ZIP from Bordetella bronchiseptica, BbZIP, has been suggested to display properties of nonsaturable electrogenic diffusion, operating as a channel (13). Similarly, although ZIPs are known to assemble as homodimers, the monomer–monomer arrangement is debated (14–16). Architectural information for ZIPs is limited to data on BbZIP and an isolated soluble domain of hZIP4 (16–18). The identified ZIP fold is unique and consists primarily of a transmembrane domain of eight pseudo-symmetrically arranged transmembrane segments (TMs) 1 to 8. Together, they form two separate helix bundles encompassing TM1, TM4, TM5, and TM6 and TM2, TM3, TM7, and TM8, respectively. Furthermore, a central ion-binding region was identified between the two bundles, with the metal coordinated by residues of TM4 and TM5 (16). However, the available BbZIP structures represent a single inward-open, outward-closed, and metal-bound state (with interacting Zn\(^{2+}\) and/or Cd\(^{2+}\)), in agreement with properties of transporters and, possibly, gated channels. In this configuration, a substantial cavity between the two bundles provides access to the metal-binding region from the intracellular side. In contrast, the putative entry pathway is blocked by highly conserved hydrophobic residues. Consequently, the operational principles of ZIPs represent an outstanding question; even the overall classification as transporters or channels remains elusive. The high sequence homology among members suggests a shared overall shape and maintained mechanistic features throughout the entire ZIP family. Nevertheless, a multitude of transport modes is still permitted.

Here, aiming to elucidate how Zn\(^{2+}\) traverses cellular membranes across ZIP proteins, we determined the structure of BbZIP in a previously uncharacterized conformation together with in silico and in vivo analyses. The findings point to a shared transport mechanism for the entire family of ZIP proteins.
the buffer. With such treated sample, we crystallized BbZIP in the lipidic cubic phase (LCP) using metal-free conditions at pH 4.5. The resulting crystals diffracted anisotropically to 2.6 to 3.6 Å, and the structure was readily solved by molecular replacement (Fig. 1A, table S1, and fig. S1). Notably, even initial analysis disclosed that the new structure is metal-free, as the metal-binding region is void of density or anomalous signal from bound cation. In addition, no difference peaks corresponding to an unmodeled ion appeared in the final model (fig. S1). In contrast, separate reproduction of the previously reported Cd²⁺-bound form (henceforth referred to as metal-bound), here determined at 2.5 Å resolution, pH 7.4, reveals metal presence using the same criteria (Fig. 1B, table S1, and fig. S2). Overall, the metal-bound and metal-free structures are reminiscent (Fig. 1D), each displaying an inward-open configuration of TM1 to TM8. However, the latter unveils an additional, approximately 30-residue-long and tilted transmembrane segment (here termed TMa) in the N terminus (Fig. 1, A and C). This region was missing in previously reported structures. Our crystallographic data and accompanying sequence analyses now clearly demonstrate that the ZIP fold can accommodate an additional helix, with potentially important consequences for cellular Zn²⁺ import (see more below and fig. S3).

Crystal packing in metal-free BbZIP crystals, coupled with coevolution analysis, uncover the BbZIP dimer interface

It is well established that ZIPs form dimers, as is also valid for detergent-purified BbZIP (16). The significance of this assembly is underscored by the fact that severe disease-causing mutations of hZIP4 have been suggested to influence dimerization (18). Nevertheless, the molecular details of the dimer assembly remain elusive. This is partly because the available metal-bound structures exhibit an antiparallel (i.e., inside-in/inside-out) crystal packing. Thus, these structures do not provide insight into the arrangement of ZIP dimers in native membranes. By investigating the crystal packing in our metal-free BbZIP structure, we identify two separate dimers displaying a parallel (i.e., inside-in/inside-in) crystal packing. One of these occurs within the asymmetric unit, while the other dimer is present in between two asymmetric units (fig. S4). To further investigate the native dimer architecture, we performed analysis of coevolved residues among ZIPs using the EVcouplings server (EVCSS) (19). Assuming that structurally and/or functionally important interfaces are more coevolved than other surfaces, such evaluation can identify oligomeric interactions (Fig. 2). The generated contact map is rich in information for the ZIP family, most consistent with the BbZIP structures, with the general trend of intrahelix-bundle interactions being dominating (Fig. 2A and fig. S5). Red boxes/red lines indicate examples. However, the calculated map illustrates some contacts not explained by the BbZIP monomer. In particular, there is a clear interaction between TM3 and TM8 (Fig. 2, A and B; black box/lines), two helices that, in the monomer, are more than 20 Å apart. This interface is only possible through integration of helices of a second monomer, resulting in a convincing dimer with a dimer-interface in-between two asymmetric units in the crystal packing (Fig. 2B and fig. S4). With this arrangement, all predicted contacts between TM3 and TM8 are fulfilled, strengthening the notion of a TM3/TM8-induced oligomerization of ZIPs (fig. S4).

Furthermore, surface conservation analysis of ZIP proteins is congruent with the identified dimer (fig. S6A), and the ColabFold software (20) predicts a highly similar dimer arrangement but with a tighter packing (fig. S7). Moreover, because of the latter arrangement, TM7 and TM8 from opposite protomers come in close proximity. Such an arrangement explains the evolutionary coupled residues between TM7 and TM8 in the coevolution analysis (Fig. 2A).

To further confirm the validity of this dimer interface, we conducted computational docking of BbZIP monomers. Using the prediction analysis of microarrays method (21), 100 docking poses were identified as six clusters (fig. S6B). The cluster with the highest population of docked monomers (fig. S6C) is congruent with the proposed dimer interface with a backbone root mean square deviation (RMSD) of 3.3 ± 0.8 Å (fig. S6D). Notably, these data also agree with what has been earlier proposed for hZIP2 and hZIP4, respectively (12, 22), and thus suggest validity for the entire ZIP family.

ZIP helix bundles rearrange as rigid bodies compatible with an alternating-access mechanism

Detailed structural comparisons of the metal-free and metal-bound inward-open structures expose notable differences for one of the two protomers (chain B), with an overall RMSD of 1.75 Å (hereafter referred to as the ion-free structure; Fig. 1E). To further and objectively dissect the observed variance, we generated a Cα-to-Cα distance difference matrix. Here, the calculated Cα-to-Cα distances for each of the states were subtracted and then averaged by TM helix (Fig. 1F) (23). Notably, helix bundle-dependent shifts are detected by our analysis. Helices within the two bundles (i.e., TM1, TM4, TM5, and TM6 and TM2, TM3, TM7, and TM8, respectively) are generally not displaced relative to each other. Conversely, the helices display larger movements between the bundles, particularly in between TM4, TM5, and TM6 and TM2 and TM8, respectively. Collectively, these observations indicate that the bundles move as rigid bodies relative to each other in a Zn²⁺-dependent manner. This suggests that we have captured a previously unidentified structural state of the ZIP proteins. Moreover, this is of significance, as the rocking bundle and elevator types of alternating-access mechanisms both achieve cargo transport through rigid body–like movements (24, 25). Hence, our observation may hint at the modus operandi of ZIP proteins. Our BbZIP structure displays many features that are congruent with an alternating-access mechanism. These include the two clearly distinct helix bundles with inverted repeat symmetry, a central metal-binding region, and, last, an inward-open state, with no obvious transport path or uptake region on the opposite side, as also reported earlier (16).

ZIPS exhibit hallmarks of two-domain elevator transporters

Evolutionary coupling may also hint at the mechanism type of membrane protein transporters. Specifically, the interface between the two bundles in well-established elevator-type transporters (UraA, UapA, GltPh, and BicA) are either lacking or have few residues that coevolve (fig. S8). In contrast, the domain interface of “rocker switch” proteins, such as the glucose transporters, have many amino acids that coevolve (26). Similarly, the bundle interface of “rocking-bundle” transporters, such as LeuT, also include multiple residues that coevolve. Because the bundle interface of BbZIP proteins has few residues that coevolve (Fig. 2), it is likely that ZIP proteins transport metals using an elevator-type mechanism.

A hallmark of elevator proteins is that they share a dynamic transport domain that exclusively interacts with the cargo and undergoes displacement across the membrane, in addition to a rotational movement during transport. Conversely, a more static scaffold domain serves as the sole interface for obligate oligomerization.
Fig. 1. Structure of BbZIP in an inward-open metal-free state reveals bundle movements. (A) Side view of the inward-open metal-free structure without the presence of ions in metal-binding sites (M1, M2, M3, and M4 shown as purple spheres throughout). The structure reveals an additional N-terminal transmembrane segment (TMα in cyan). TM1, TM4, TM5, and TM6 constitute the transport domain (purple), while TM2, TM3, TM7, and TM8 form the scaffold domain (orange). (B) Side view of the inward-open metal-bound structure. Bound Cd$^{2+}$ ions are shown as purple spheres. (C) Topology of ZIPs including the previously unidentified helix (TMα) present in certain ZIP members. Purple triangles indicate symmetry. (D) Overall alignment of Cα atoms in the metal-bound and metal-free states (chain B), revealing overall similarity and yet prominent displacements. (E) Comparison of pairwise root mean square deviation (RMSD) values for the overall alignment of Cα reveals areas with substantial differences between the two conformations, particularly the cytoplasmic parts of TM1, TM4, TM5, and TM6. (F) Distance difference matrix demonstrating unbiased helix movements, calculated using a previously reported script (https://github.com/GaudetLab/archived-DDMP/) (50). The matrix was generated through calculation of pairwise Cα-Cα distances for each structure (TMα was not included) and, subsequently, by applying matrix subtraction to compare distances. A helix pair that remains fixed relative to each other will have lower RMSD value and is represented with dark color in the heatmap. Conversely, helix pairs having relatively high mobility are characterized by higher RMSD values and lighter colors. The scale bar indicates the RMSD differences (in Å) by TMs.
Notably, the scaffold domains of the four elevator-type transporters included in our analysis all align and superimpose well with the aforementioned TM2, TM3, TM7, and TM8 helix bundle of BbZIP, from now on termed the scaffold domain (fig. S9). In contrast, larger differences are found between the transport domains of all the proteins. However, the TM1, TM4, TM5, and TM6 bundle of BbZIP displays key characteristics of elevator transporter domains, as the metal-binding region is formed almost entirely by residues of TM4, TM5, and TM6. Hence, we designate it as the transport domain. In agreement with the notion that ZIPs operate as elevator transporters, the two domains have other properties in common with known elevator-type domains (25, 28). The scaffold domain is generally electrostatically neutral or hydrophobic, as it is also the case for BbZIP (Fig. 3A). In contrast, the transport domain is typically considerably polar, and this is valid also for BbZIP, for which this bundle is negatively charged (Fig. 3B). Furthermore, the scaffold domain of ZIPs shares common topological features exclusive to elevator-type transporters. These characteristics include the symmetrically inverted V-repeats of the TM helices and the presence of long, antiparallel, and tilted central transmembrane segments (consisting of TM2 and TM7 in BbZIP). Together, they reduce the distance (membrane height) that the ions need to travel across the membrane, from approximately 26 to 16 Å through formation of membrane invaginations (Fig. 3A) (24). Collectively, these data suggest that ZIPs exploit a two-domain elevator-type mechanism for cellular uptake of Zn$^{2+}$.

**Elevator conformational changes permit Zn$^{2+}$ uptake and release**

To investigate zinc-dependent movement of the transport domain, we aligned the metal-free and metal-bound structures using the
static scaffold domains (Fig. 4A). The analysis reveals a considerable displacement of the transport domain, with the ion-free form being shifted around 3 Å toward the intracellular inside, combined with a substantial twist that tilts the domain 11°. Thereby, the Zn$^{2+}$-binding region is directly exposed to the cytoplasm, in agreement with completion of ion release during the course of a transport cycle (Figs. 3, C and D; 4B; and 5).

Conversely, extracellular access is provided through electronegatively charged residues of TM2, TM3, and TM7. It is even possible that a common bowl covers the two scaffold domains, thereby orchestrating efficient recruitment of metal ions. Notably, evaluation of the few identified coevolved interdomain contacts also hints at a possible outward-facing configuration, as a subset of these are not fulfilled in the determined inward-facing states (Figs. 3, C and D; 4B; and 5).

**A transport mechanism involving conserved residues**

We propose that ZIPs form scaffold domain–based dimers and exploit an oscillating elevator-type mechanism. In this way, the metal-binding region would be alternately exposed to symmetrically related negatively charged cavities, which maybe even comprise common entry and exit areas for Zn$^{2+}$ within the dimer (Fig. 5). Considering this, it is
tempting to speculate that ZIP oligomerization serves a stabilizing role, rather than partaking in cooperativity and/or (allosteric) regulation. This is also supported by the observation that BbZIP dimerization appears reversible during purification in detergent environment. Regarding uptake, in the Na+/H+ antiporting elevators NapA and MjNhaP1, the outer surface of the scaffold domain is negatively charged, which has been proposed to enable selection and concentration of the transported cargo (23, 24). In BbZIP, cargo recruitment from the extracellular side may be achieved through the TM2/8 opening in an outward-facing state. This is possibly stimulated by the well-preserved S106 and D305, and the more surface-exposed D113 of the cavity, providing access to E181, E211, and Q207 of the ion-binding region (fig. S10). Next, metal-binding permits a modest rotation and a ~11-Å sliding of the transport domain toward the intracellular side along the inert scaffold domain. Here, Zn²⁺ becomes occluded, bound almost exclusive through side chains of the transport domain. Release is facilitated by the TM2, TM3, and TM7 bowl, lined by the invariant D144 and H275, and E276, as also demonstrated by our metal-bound and metal-free structures. Last, the movement of the entire transport domain reverts to allow a new transport cycle.

To verify the functional importance of these residues, we designed an in vivo metal toxicity assay, attributing decreased cell viability to increased zinc uptake. Cells with wild-type BbZIP were more intolerant to high Zn²⁺ or Cd²⁺ concentrations than control cells lacking the protein (Fig. 6A and fig. S11). To ensure an unbiased comparison of the mutant forms, the BbZIP protein levels in the cell membranes were also monitored (Fig. 6B).
The elevator transport mechanism of ZIPs

The proposed transport mechanism with functionally important residues marked in red. The dimer forms a stable scaffold that allows the transport domains to oscillate between the states. The ion-binding region of the transport domain is alternatively exposed to functional residues of the scaffold domain linked to the cellular outside and inside, respectively. Zinc ions (shown as gray spheres) are recruited to the negatively charged area around S106, Q207, E211, and D305. It is likely that in a hypothetical outward-facing state, the zinc-coordinating residues in the metal-binding site will move closer toward S106 and D305, as proposed in our outward-open model. Metal binding permits conformational changes, most notably a distinct elevator-like translocation across the membrane of the transport domain through the membrane. In the inward-open metal-bound state, release of the ion from the metal-binding region is mediated through zinc-binding residues, including D144, H275, and E276. Last, the structural rearrangement detected here has to occur to fully release bound metal.

extracellular side, alanine substitution of S106 supports a critical role of this amino acid residue for the transport function, while the D305A form indicates that the residue is too peripheral to have a critical impact (Fig. 6A and fig. S11). Similarly, a series of functionally important electronegatively charged residues have been identified in this region of hZIP2 (i.e., H63, E67, E70, and E71), and mutation of the corresponding residue of S106 in hZIP4 (i.e., H379) severely diminished zinc transport (16). Intracellularly, changes of D144 and E276 to alanine indicate they play key roles in zinc transport, while H275A exhibited marginal effect on the function. Likewise, alanine replacements of M99 and H177 located between the scaffold and transport domains support the notion of this surface being tolerant to certain amino acid substitutions, as also previously shown for other elevator proteins (31, 32).

The metal-free structure reveals an additional unique transmembrane segment of BbZIP, TMa

The additional transmembrane segment in BbZIP preceding TM1, TMa, is unexpected, not being visible in the previously determined structures. The resolved part represents 30 N-terminal amino acids and a highly tilted helix, interacting only with TM3 and TM6 and peripherally relative to the dimer assembly (Figs. 2B, 7, and 8A). As only approximately 20 residues are left unresolved, this feature is likely transforming previously proposed topology of the termini from an out/out to an in/out arrangement, with the N-terminal segment facing rather the intracellular side. To provide evidence that BbZIP exists with an in/out topology in vivo, we exploited a cysteine accessibility assay based on Escherichia coli cells expressing...
two different BbZIP forms, i.e., S6C in the N terminus or R53C following TMa (Figs. 7A and 8, A to C, and fig. S13) (39). When treating with the membrane-impermeable (2-[trimethylammonium]ethyl) methanethiosulfonate bromide (MTSET), only the R53C mutant displayed the electrophoretic shift associated with periplasmic localization, while the S6C form remained unaltered. However, when treating with the membrane-permeable 2-aminoethyl methanethiosulfonate hydrobromide (MTSEA), both S6C and R53C displayed an electrophoretic shift in agreement with S6C having a cytoplasmic localization. Thus, the in/out topology is present in vivo and does not reflect a crystallization artifact. On the basis of the metal-bound structures, it cannot be excluded that the position of the helix in the membrane is dynamic (snorkeling). However, it is more likely that this additional segment is simply flexible (perhaps detergent-induced) with maintained membrane position and, therefore, not visible in the earlier structures. It is tempting to speculate that many of the established difficulties in isolating ZIPs relate to the typical out/out topology, an arrangement known to be associated with complications.
with protein expression (40). In contrast, it is possible to recover acceptable quantities of BbZIP that exhibits an in/out setup. This may also hint at a general possible role of this additional helix in ZIPs beyond BbZIP, as a driver of expression to increase the accumulation of protein in the bacterial membranes, thereby increasing cellular Zn$^{2+}$ import.

The N terminus may modulate Zn$^{2+}$ import among ZIPs with nine TMs

To further investigate the biological role of TMa and the N-terminal tail, we assessed additional BbZIP forms using the in vivo metal toxicity assay. Notably, we observed that a truncation T1, lacking the first 20 residues ($\Delta$3 to $\Delta$20), appears hyperactive compared to the wild type. This suggests that the cytoplasmic N-terminal segment may be autoinhibitory or down-regulatory of Zn$^{2+}$ transport (Fig. 7B and figs. S11 and S14). From a structural point of view, this is certainly possible, as the beginning of TMa is immediately adjacent to the ion release cavity (Fig. 8A). Moreover, it is located in the proximity of conserved features of the second intracellular loop (ICL2), a stretch that has been shown to be important for Zn$^{2+}$ transport in hZIP4 (41). Thus, the tail may well interfere with metal release to the intracellular side. Conversely, both a more prominent truncation,
removing all the residues before TM1, i.e., T2 (∆3 to ∆52), and the H22A mutation, interfering with a putative Zn$^{2+}$-binding histidine present before TMs, decrease functionality. This agrees with the notion that H22 may partake in Zn$^{2+}$ release. Conversely, the H16A variant of another histidine in the N terminus only moderately alters the activity. Thus, while not omnipresent among ZIPs, our data suggest that the identified N-terminal tail-TMα feature may be directly involved in Zn$^{2+}$ transport and regulation in ZIPs with nine-TM topology. Mechanistically, it is hence possible that the N terminus of nine-TM ZIPs structurally and functionally replaces the distinctly longer and histidine-rich ICL2 of the human members. Noteworthy, all human ZIPs are predicted to share an eight-TM topology, and hence, they lack an intracellular tail, but the ICL2 instead contributes to zinc binding, thereby regulating Zn$^{2+}$ transport activity (42, 43).

Summary
Fold and mechanism represent fundamental aspects in the understanding of protein function, providing insights valuable for both basic and translational sciences. In this work, we unraveled through structural, functional, and in silico analyses that the ZIP proteins likely operate as two-domain elevator-type transporters. Our results provide an extended conformational landscape for ZIP transporters. Furthermore, they highlight that this transporter family harbors an immobile dimer-forming scaffold domain and a transport domain that displaces a central ion-binding region to transfer cargo across membranes. The transport relies on symmetrically related negatively charged invaginations of the rigid scaffold domain shared within the dimer, limiting the displacement distance across the membrane required for passage. Moreover, the elevator transport mechanism and charge compensation principle can unify the functional and structural data available for ZIPs, with several different detected modes for ion transfer and selectivity. These are ranging from channel- to transporter-like models and from coupled to uncoupled passage, as achieved by subtle local differences along the binding region. Hence, elevator-type proteins appear rather diverse, as also previously observed, for example, in the CLC family of proteins that operate both as channels and as transporters despite overall very similar folds (44). We also demonstrate that certain ZIPs have an unorthodox topology, with an additional transmembrane segment in the N terminus. This extra helix may affect both the available cellular levels of the protein and its function. Undoubtedly, the structural and mechanistic insights into ZIPs and related elevator proteins will continue to grow. To fully understand the transport mechanism, additional structures, especially outward-facing conformations, are essential because of the shortage of architectural data. Concomitantly, more in-depth studies, such as cross-linking experiments with selected cysteine mutations or using linkers of variable lengths, have the potential to further validate and refine the ZIP transporter elevator mechanism revealed here. All in all, our results will prime these downstream efforts and provide a conceptual framework for analyses at the molecular level of the many known ZIP-related disorders.

MATERIALS AND METHODS

Overproduction and purification of BbZIP
The pET15b(+) vector encoding wild-type ZIP from B. bronchiseptica (BbZIP) (UniProt ID: A0A0H3LM39) codon-optimized for expression in E. coli and fused N-terminally with His$_6$-tag, followed by the thrombin cleavage site was purchased from GenScript, USA. BbZIP mutants were prepared using the QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent, USA) according to the instructions of the manufacturer. Primers used for engineering of the mutants were purchased from TAG Copenhagen A/S, Denmark, and their sequences are shown in table S2. Identity of the mutants was verified by DNA sequencing.

Precultures of E. coli [strain C43(DE3)] (Thermo Fisher Scientific, USA) were inoculated from frozen stocks and grown for 16 hours at 37°C in 5 ml of Luria-Bertani (LB) medium supplemented with ampicillin (100 µg/ml). Subsequently, 1 ml of the preculture was transferred into 50 ml of LB medium (containing ampicillin) and grown for ~1 hour at 37°C before the entire bacterial culture was used to inoculate 2 liters of Terrific broth medium (containing ampicillin). The resulting culture was grown at 37°C until optical density at 600 nm (OD$_{600}$) = 0.9 before the protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. Following the induction, the expression was allowed for ~18 hours at 20°C before the cells were harvested by centrifugation at 6000g for 10 min at 4°C. Obtained cell pellets were resuspended with ice-cold lysis buffer [50 mM Hepes-NaOH (pH 7.3), 500 mM NaCl, and 20% glycerol] and kept at −80°C until use. Thawed cell pellets were supplemented with Complete protease inhibitor cocktail (Sigma-Aldrich, USA) and opened by purging twice through a cell disrupter (Constant Systems Limited, UK) operated at 26 kpsi. After cell disruption, 1 mM phenylmethylsulfonyl fluoride was added, and the cell lysate was centrifuged at 20,000g for 20 min at 4°C to remove cell debris. Subsequently, the membrane fraction was isolated by ultracentrifugation using Ti45 rotor (Beckman, USA) at 138,000g for 3 hours at 4°C. Obtained crude membranes were resuspended in solubilization buffer [10 ml/g; 50 mM Hepes-NaOH (pH 7.3), 20 mM NaCl, and 20% glycerol] and kept at −80°C until use.

Purification of BbZIP for crystallization
Membranes at the total protein concentration of 5 mg/ml were solubilized with 1.5 to 2% (w/v) n-dodecyl-β-D-maltopyranoside (DDM) (Glycon Biochemicals, Germany) for 4 hours in the presence of the 0.1 mM CdCl$_2$. Subsequently, solubilized membranes were diluted to reduce concentration of DDM to 0.33% (w/v), and the additional NaCl was added to increase the concentration to 500 mM before the material was loaded on a 5 ml of HisTrap HP column (Cytiva, Denmark). Bound protein was washed with immobilized-metal affinity chromatography (IMAC) buffer [50 mM Hepes-NaOH (pH 7.3), 200 mM NaCl, 20% glycerol, 50 mM imidazole, and 0.1% (w/v) DDM] and eluted in IMAC Buffer using a linear imidazole gradient (50 to 500 mM). Fractions containing protein were pooled and immediately dialyzed against IMAC buffer for 16 hours at 4°C. Subsequently, protein was concentrated in a Vivaspin 20 centrifugal concentrator (MWCO, 50 kDa; Sartorius, Germany) to ~4 mg/ml. The sample was loaded onto a Superdex Increase 200 10/300 GL column (Cytiva) equilibrated with size exclusion chromatography (SEC) buffer [20 mM NaOAc-HOAc (pH 4.5), 200 mM NaCl, 15% glycerol, and 0.03% (w/v) DDM].

Crystallization of BbZIP
SEC-pure BbZIP was concentrated to 16 to 36 mg/ml as described above and filtered, and 10 to 20 µl of the sample was loaded into a
of Cd\textsuperscript{2+}-bound BbZIP appeared after 10 days in 100 mM Hepes-NaOH.

Crystals of wild-type metal-free BbZIP (achieved for the protein concentration of 36 mg/ml) appeared after 2 weeks in 100 mM NaOAc-HOAc (pH 4.5), 100 to 400 mM LiSO\textsubscript{4}, and 25 to 40% PEG400 (polyethylene glycol, molecular weight 400). The best diffracting crystals were obtained in 300 mM LiSO\textsubscript{4} and 35% PEG400 and grew to a size of 10 to 30 µm with the shape of trapezoidal prisms. Crystals of Cd\textsuperscript{2+}-bound BbZIP appeared after 10 days in 100 mM Hepes-NaOH (pH 7.4), 100 mM CdCl\textsubscript{2}, and 30% PEG400 and grew to full size after 10 days. Crystals were allowed to grow for additional 2 weeks before being fished into 0.06-mm loops (Molecular Dimensions Limited, UK) and flash-frozen in liquid nitrogen.

**Data collection and structure determination**

Diffraction data were collected at the beamline PXIII X06SA (Swiss Light Source, Switzerland). The data were indexed, integrated, and scaled using the XDS package (45). The crystal structures of BbZIP were determined by molecular replacement in PHASER (46) using the metal-bound BbZIP structure [Protein Data Bank (PDB) ID: 5TSA] (16) as the search model. COOT (47) was used for model building, and refinement was performed in PHENIX (48). Assignment of registry for the TMa and further rebuilding of the model was performed using ISOLDE (49), followed by final refinement in PHENIX. Information about data collection and refinement statistics is shown in table S1. Metal-free BbZIP contains two molecules in the asymmetric unit. Electron densities were observed for residues 21 to 145, 165 to 279, and 285 to 309 in chain A and for residues 18 to 77, 84 to 145, 166 to 219, and 225 to 307 in chain B, respectively. Chain B was used for further analysis and generation of figures.

**RMSD calculations and distance difference matrices**

Calculations of RMSD values of residues were performed with the ColorByRMSD PyMOL script. Calculations of RMSD values of structures were performed with the super command in PyMOL. Distance difference matrices and plots were created with a previously reported Python-based script (https://github.com/GaudetLab/archived-DMMP/) (50). The TM definitions from the metal-bound BbZIP structure (PDB ID: 5TSA) (16) were used for the calculations.

**Analysis of evolutionary coupled amino acid residues**

The sequence of wild-type BbZIP was used as a query. Automatic identification of the sequences annotated with the Pfam PF02535 ZIP identifier was used to generate the multiple sequence alignment (MSA) used as input for the sequence coevolution analysis using the EVCS implementing the pIbDCA algorithm (https://evcouplings2.hms.harvard.edu/) (19). Analysis was performed with default parameters, and 22,433 effective sequences were exploited for an analysis with the overall high effective sequence-to-protein length ratio (i.e., 90.1 seq/liter). The N-terminal region of BbZIP was not covered in the alignment. Significance cutoff was set to 0.9 in the subsequent plot visualizations.

**Generation of the outward-facing model of BbZIP**

The outward-facing model of BbZIP was generated on the basis of serial translations of the transport domain of the inward-open metal-bound structure across the membrane toward the outside. The intention was to minimize the distance between evolutionary coupled residues in-between the two helix bundles that were unsatisfied in the inward-facing conformation, namely, Ser\textsuperscript{106}-Gln\textsuperscript{207}, Ser\textsuperscript{261}-Met\textsuperscript{183}, Val\textsuperscript{272}-Thr\textsuperscript{173}, Ala\textsuperscript{102}-Gln\textsuperscript{207}, Met\textsuperscript{269}-Glu\textsuperscript{211}, and Met\textsuperscript{269}-Asp\textsuperscript{208}. To minimize the distance between these residue pairs, a stepwise translation across the membrane in steps of 1 Å was performed. The shortest total distance between the residues was obtained through an 8 Å translation, thereby satisfying the abovementioned evolutionary coupled pairs.

**Cysteine accessibility assay**

Cell pellets resulting from cultures of *E. coli* [strain C43(DE3)] expressing single-cysteine BbZIP mutants were washed one time in labeling buffer [100 mM tris-HCl (pH 7), 60 mM NaCl, and 10 mM KCl] and subsequently resuspended in labeling buffer to the OD\textsubscript{600} = 4. Subsequently, 100 µl of the cell suspension was aliquoted per well in a 96-well plate and mixed with either 100 µl of 6 mM MTSET (Biotium, USA) or 100 µl of 6 mM MTSEA (Biotium, USA). The mixture was incubated for 30 min before the addition of 5 µl of 60 mM N-ethylmaleimide (NEM; Sigma-Aldrich, USA) and subsequently resuspended in labeling buffer to the OD\textsubscript{600} = 4. After two consecutive washes in labeling buffer, cells were resuspended in 60 µl of denaturing buffer (100 mM tris-HCl (pH 7), 6 M urea, 0.5% SDS, and 0.5 mM 1,4-dithiothreitol) and incubated for 1 hour. Samples were then centrifuged at 2700g for 15 min at 18°C. Subsequently, 20 µl of the supernatant was mixed with 7 µl of 6 M 5000 methoxypolyethylene glycol maleimide (Sigma-Aldrich, USA) and incubated for 1 hour at 37°C before 5.4 µl of 5× SDS sample buffer containing 700 mM β-mercaptoethanol was added to stop the reaction. As a negative control, 100 µl of the labeling buffer was applied instead of the thiol modifiers. Subsequently, 25 µl of the respective samples were resolved on 4 to 20% SDS–polyacrylamide gel electrophoresis gels (Thermo Fisher Scientific). Protein bands were then detected using immunoblotting, using an anti-His\textsubscript{6} monoclonal antibody–horseradish peroxidase conjugate (Takara Bio, USA) according to recommendations of the manufacturer. Immunoblots were visualized by chemiluminescence with SuperSignal West Femto Maximum Sensitivity substrate (Thermo Fisher Scientific) using an Alphalager (Alpha Innotech, USA) machine. Relative signal intensities of the bands were quantified using ImageJ software (https://imagej.nih.gov/ij/) (51). Each experiment was conducted using two biological replicates.

**In vivo metal toxicity assay**

*E. coli* [strain C43(DE3)] cultures were allowed to express wild-type BbZIP and the respective mutants for 4 hours at 37°C before being spotted (1 µl at OD\textsubscript{600} = 0.001) on LB agar plates containing the corresponding concentrations of metal. Plates were then incubated for 16 hours at 37°C before the colonies were imaged using an Alphalager (Alpha Innotech) scanner. Contrast between cell colonies and the background agar was increased by systematically
Possible dimerization sites of BbZIP were evaluated by a protein-protein docking strategy. Identical monomeric chains were docked blindly into all possible interfaces to the monomer itself. The docking was performed using the protein-protein docking module in the MOE-2016.0802 software (52). The affinity binding energies for 100 dimers were calculated on the basis of Amber94 force field parameters (53) and were ranked according to the binding score from lowest to the highest. The affinity binding score or docking energy was estimated from the enthalpic contribution to the binding free energy between two binding monomers. In addition, each docking pose was refined and resulted in refinement energy. The docking poses were clustered on the basis of the docking and refinement energy parameters by the prediction analysis of microarrays docking poses were clustered on the basis of the docking and refinement energy between two binding monomers. In addition, each docking pose was refined and resulted in refinement energy. The docking poses were clustered on the basis of the docking and refinement energy parameters by the prediction analysis of microarrays docking poses were clustered on the basis of the docking and refinement energy between two binding monomers. In addition, each docking pose was refined and resulted in refinement energy.

REFERENCES AND NOTES

1. T. Har, T.-A. Takeda, T. Takagishi, K. Fukue, T. Kambe, T. Fukuda, Physiological roles of zinc transporters: Molecular and genetic importance in zinc homeostasis. J. Physiol. Sci. 67, 283–301 (2017).
2. C. Andrew, L. Bänz, I. Bertini, A. Rosato, Counting the zinc-proteins encoded in the human genome. J. Proteome Res. 5, 196–201 (2006).
3. B. H. Bin, J. Seo, S. T. Kim, Function, structure, and transport aspects of Zn and ZnT zinc transporters in immune cells. J. Immunol. Res. 2018, 9365747 (2018).
4. S. Kúr, B. Drén, S. Bézieu, S. Giraudet, M. Kharfi, R. Kamoun, J. P. Moisan, Identification of SCL39A4, a gene involved in acrodermatitis enteropathica. Nat. Genet. 31, 239–240 (2002).
5. T. Fukuda, N. Civc, T. Furuchi, S. Shimoda, K. Mishima, H. Higashiyama, Y. Iida, Y. Asada, H. Kitamura, S. Yamasaki, S. Hojo, M. Nakayama, O. Obara, H. Koseki, H. G. dos Santos, L. Bonafe, R. Ha-Vinh, A. Zankl, S. Unger, M. E. Kraenzlin, J. S. Beckmann, I. Saito, C. Rivolta, S. Ikeyawa, A. Superti-Furga, T. Hirano, The zinc transporter SLC39A13/ZIP13 is required for connective tissue development; its involvement in BMP/TGF-β signaling pathways. PLOS ONE 3, e3664 (2008).
6. L. A. Gaither, D. J. Eide, Functional expression of the human H2Zn2 zinc transporter. J. Biol. Chem. 275, 5560–5564 (2000).
7. L. He, K. Girijashanker, T. P. Dalton, J. Reed, H. Li, M. Soleimani, D. W. Nebert, ZIP8, member of the solute-carrier-39 (SLC39) metal-transporter family: Characterization of transporter properties. Mol. Pharmacol. 70, 171–180 (2006).
8. G. Gyimesi, G. Albano, D. G. Fuster, M. A. Hediger, J. Pujol-Giménez, Unraveling the structural elements of pH sensitivity and substrate binding in the human zinc transporter SLC39A2 (ZIP2). J. Biol. Chem. 294, 8046–8063 (2009).
9. L. Lin, J. Chai, J. Love, D. Fu, Selective electrodiffusion of zinc ions in a Zrt-, Ir-like protein, ZIP5. J. Biol. Chem. 285, 39019–39020 (2010).
10. B. H. Bin, T. Fukuda, T. Hosaka, S. Yamamoto, W. Ohashi, S. Hojo, T. Miyaj, K. Nishida, S. Yokoyama, T. Hirano, Biochemical characterization of human ZIP13 protein.

A homo-dimerized zinc transporter involved in the spondylohecho dysplastic Ehlers-Danlos syndrome. J. Biol. Chem. 286, 40253–40265 (2011).
11. T. P. Ajesh, S. Ignacimuthu, S. Antony Chezar, Structure, function, regulation and phylogenetic relationship of ZIP family transporters of plants. Front. Plant. Sci. 11, 662 (2020).
12. T. Zhang, J. Liu, M. Fellner, C. Zhang, D. Sui, J. Hu, Crystal structures of a zinc transporter reveal a biruinular metal center in the transport pathway. Sci. Adv. 3, e1700344 (2017).
13. T. Zhang, D. Sui, C. Zhang, L. Cole, J. Hu, Asymmetric functions of a biruinular metal center within the transport pathway of a human zinc transporter ZIP4. FASEB J. 34, 237–247 (2020).
14. T. Zhang, D. Sui, J. Hu, Structural insights of ZIP4 extracellular domain critical for optimal zinc transport. Nat. Commun. 7, 11979 (2016).
15. T. A. Hop, A. G. Green, B. Schubert, S. Mersmann, C. P. I. Scharfe, J. B. Ingham, A. Toth-Petroczy, K. Brock, A. J. Rieselmen, P. Palmedo, C. Kaag, R. Sheridan, E. J. Draizen, C. Dallago, C. Sander, D. S. Marks, The EVCoupling Python framework for coevolutionary sequence analysis. Bioinformatics 33, 1582–1584 (2019).
16. M. Mirdita, S. Ovchinnikov, M. Steinegger, COLabFold—Making protein folding accessible to all. bioRxiv 10.1101/2021.08.15.456425 [Preprint] 2021.
of membrane proteins with diverse topologies: Application to investigation of bacterial homologues of human divalent metal ion and nucleoside transporters. PLOS ONE 10, e0143010 (2015).

S. Antala, R. E. Dempski, The human ZIP4 transporter has two distinct binding affinities and mediates transport of multiple transition metals. Biochemistry 51, 963–973 (2012).

K. M. Taylor, R. I. Nicholson, The LZT proteins; the Liv1− subfamily of zinc transporters. Biochem. Biophys. Acta 1611, 16–30 (2003).

E. M. Bafaro, M. W. Maciejewski, J. C. Hoch, R. E. Dempski, Concomitant disorder and high-affinity zinc binding in the human zinc- and iron-regulated transport protein 4 intracellular loop. Protein Sci. 28, 868–880 (2019).

E. Park, R. Mackinnon, Structure of the CLC-1 chloride channel from Homo Sapiens. eLife 7, e36629 (2018).

W. Kabsch, XDS. Acta Crystallogr. D Biol. Crystallogr. 66, 125–132 (2010).

A. J. McCoy, R. W. Grosse-Kunstleve, P. D. Adams, M. D. Winn, L. C. Storoni, R. J. Read, Phaser crystallographic software. J. Appl. Cryst. 40, 658–674 (2007).

P. Emsley, K. Cowtan, Coot: Model-building tools for molecular graphics. Acta Crystallogr. D Biol. Crystallogr. 60, 2126–2132 (2004).

P. D. Adams, P. V. Afonine, G. Bunkóczi, V. B. Chen, I. W. Davis, N. Echols, J. J. Headd, L. W. Hung, G. J. Kapral, R. W. Grosse-Kunstleve, A. J. McCoy, N. W. Moriarty, R. Oeffner, R. J. Read, D. C. Richardson, J. S. Richardson, T. C. Terwilliger, P. H. Zwart, PHENIX: A comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr. D Biol. Crystallogr. 66, 213–220 (2010).

T. I. Croll, ISOLDE: A physically realistic environment for model building into low-resolution electron-density maps. Acta Crystallogr. D Struct. Biol. 74, 519–530 (2018).

A. T. Bozzi, C. M. ZimanJ, J. M. Nicolaides, B. K. Lee, C. H. Zhang, R. Gaudet, Structures in multiple conformations reveal distinct transition metal and proton pathways in an nmr trapper. eLife 8, e41124 (2019).

C. A. Schneider, W. S. Rasband, K. W. Eliceiri, NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671–675 (2012).

S. Villar, G. Cozza, S. Moro, Medicinal chemistry and the molecular operating environment (MOE): Application of QSAR and molecular docking to drug discovery. Curr. Top. Med. Chem. 8, 1555–1572 (2008).

C. I. Bayly, K. M. Merz, D. M. Ferguson, W. D. Cornell, T. Fox, J. W. Caldwell, P. A. Kollman, P. E. Bourne, The Protein Data Bank. Nucleic Acids Res. 34, W344–W350 (2016).

J. Mistry, S. Chuguransky, L. Williams, M. Qureshi, G. A. Salazar, E. L. L. Sohnhammer, S. C. E. Tosatto, L. Paladini, S. Raj, L. J. Richardson, R. D. Finn, A. Bateman, Pfam: The protein families database in 2021. Nucleic Acids Res. 49, D412–D419 (2021).

K. D. Tsirigos, C. Peters, N. Shu, L. Käll, A. Elofsson, The TOPCONS web server for consensus prediction of membrane protein topology and signal peptides. Nucleic Acids Res. 43, W401–W407 (2015).

J. D. Thompson, D. G. Higgins, CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680 (1994).

I. Letunic, P. Bork, Interactive Tree of Life (ITOL) v4: Recent updates and new developments. Nucleic Acids Res. 47, W256–W259 (2019).

E. Krissinel, K. Henrick, Inference of macromolecular assemblies from crystalline state. J. Mol. Biol. 372, 774–797 (2007).

X. Yu, G. Yang, C. Yan, J. L. Baylon, J. Jiang, H. Fan, G. Lu, K. Hasegawa, H. Okumura, T. Wang, E. Tajkhorshid, S. Li, N. Yan, Dimeric structure of the ura3: Proton symporter UraP provides mechanistic insights into the SLC4/23/26 transporters. Cell Res. 27, 1020–1033 (2017).

E. R. Geertzma, Y. N. Chang, F. R. Shaik, Y. Neldner, E. Pardon, J. Steyaert, R. Dutzler, Structure of a prokaryotic fumarate transporter reveals the architecture of the SLC26 family. Nat. Struct. Mol. Biol. 22, 803–808 (2015).

Y. Alguel, S. Amillis, J. Leung, G. Lambrindis, S. Capaldi, N. J. Scull, C. Graven, S. Iwata, A. Armstrong, E. Mikos, G. Díazlínas, A. D. Cameron, B. Byrne, Structure of eukaryotic purine/H+ symporter UapA suggests a role for homodimerization in transport activity. Nat. Commun. 7, 11336 (2016).

H. M. Berman, J. Westbrook, Z. Feng, G. Illgilland, T. N. Bhat, H. Weissig, I. N. Shindyalov, P. E. Bourne, The Protein Data Bank. Nucleic Acids Res. 28, 235–242 (2000).

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