Structural basis for the Mg\(^{2+}\) recognition and regulation of the CorC Mg\(^{2+}\) transporter

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The CNNM/CorC family proteins are Mg\(^{2+}\) transporters that are widely distributed in all domains of life. In bacteria, CorC has been implicated in the survival of pathogenic microorganisms. In humans, CNNM proteins are involved in various biological events, such as body absorption/reabsorption of Mg\(^{2+}\) and genetic disorders. Here, we determined the crystal structure of the Mg\(^{2+}\)-bound CorC TM domain dimer. Each protomer has a single Mg\(^{2+}\) binding site with a fully dehydrated Mg\(^{2+}\) ion. The residues at the Mg\(^{2+}\) binding site are strictly conserved in both human CNNM2 and CNNM4, and many of these residues are associated with genetic diseases. Furthermore, we determined the structures of the CorC cytoplasmic region containing its regulatory ATP-binding domain. A combination of structural and functional analyses not only revealed the potential interface between the TM and cytoplasmic domains but also showed that ATP binding is important for the Mg\(^{2+}\) export activity of CorC.

INTRODUCTION

The magnesium ion (Mg\(^{2+}\)) is the most abundant biological divalent cation and is essential for all living organisms (1). Mg\(^{2+}\) plays a vital role in various physiological processes, such as maintaining genome stability and acting as enzymatic cofactors for adenosine 5'-triphosphate (ATP) hydrolysis and DNA replication (2, 3). In humans, Mg\(^{2+}\) is involved in the maintenance of heart rhythm, blood pressure, neuromuscular conduction, bone integrity, and glucose metabolism (4). Accordingly, cellular imbalance of Mg\(^{2+}\) homeostasis is associated with multiple diseases (4), and Mg\(^{2+}\) transport proteins play a primary role in homeostasis (5, 6).

The CNNM (cyclin M)/CorC family of Mg\(^{2+}\) transporters is widely distributed in all domains of life, from prokaryotes to eukaryotes, including humans (7). CorC, a prokaryotic member of the CNNM/CorC family of proteins, is implicated in Mg\(^{2+}\) transport in several microbial organisms (8–12). In Staphylococcus aureus, a pathogenic bacteria, CorC proteins confer resistance to high Mg\(^{2+}\) concentrations in their infected host environment, enhancing their pathogenicity (10, 12). In Lactobacillus plantarum, an interaction between CorC and an antimicrobial peptide of plantaricin EF is associated with divalent cation stress (13). Furthermore, CorC expression is up-regulated in the L22\(^{+}\) strain of Bacillus subtilis to increase Mg\(^{2+}\) flux, which is associated with resilience to ribosome-targeting antibiotics in the L22\(^{+}\) strain (11).

In mammals, including humans, CNNM proteins (CNNM1 to CNNM4) are widely expressed throughout the body (14). Among them, CNNM2 and CNNM4 mediate Mg\(^{2+}\) efflux, which is responsible for the absorption/reabsorption of Mg\(^{2+}\) from the intestines and kidney, respectively (15–17). Mutations of CNNM2 and CNNM4 are responsible for human congenital diseases, dominant hypomagnesemia (17) and Jalili syndrome (18, 19), respectively. Knockout mouse studies confirmed the occurrence of some disease-related phenotypes (15, 16) and revealed the importance of CNNM2 in blood pressure regulation (16). Furthermore, abnormal functions of CNNM3 and CNNM4 have been reported to cause tumor progression (20–22). Accordingly, the CorC and CNNM proteins are potential targets for the development of previously unknown antibiotics and cancer therapeutics, respectively.

Both CorC and CNNM proteins share a DUF21 transmembrane (TM) domain and cystathionine β-synthase (CBS) domain (8, 10, 14, 23). The DUF21 domain is responsible for the TM transport of Mg\(^{2+}\), and many disease-associated mutations of CNNMs are localized to the DUF21 domain (18, 19, 24). The CBS domain is an evolutionarily conserved domain found in a wide range of proteins and typically has binding activity to adenosine and its analogs, such as ATP and adenosine 5'-monophosphate, to regulate the functions of the associated enzymes and transporters (25–27). Mutations in the CBS domain of MpFA, a CorC ortholog from S. aureus, reportedly affect its transporter functions (10). ATP binding to the CBS domain of CNNM2 and CNNM4 is required for their Mg\(^{2+}\) efflux activities (28, 29). Furthermore, there have been multiple structures containing the CBS domain of the CNNM/CorC family proteins reported to date (29–36). Despite the physiological importance of the CNNM/CorC family, little is known about the Mg\(^{2+}\) transport mechanism of the CNNM/CorC family proteins, mainly because the structure of the CNNM/CorC proteins containing the DUF21 TM domain has not yet been reported.

In this work, we determined the high-resolution structure of the CorC TM domain in complex with Mg\(^{2+}\) ions, illuminating the Mg\(^{2+}\) recognition mechanism, which highly contrasts with that of the known Mg\(^{2+}\) channel structures. Notably, all residues at the Mg\(^{2+}\) binding site are strictly conserved in both human CNNM2 and CNNM4 Mg\(^{2+}\) exporters, and many of the corresponding residues in human CNNM2 and CNNM4 were associated with congenital diseases, providing a structural interpretation of these disease-associated
mutations. Further structural analyses of the CorC cytoplasmic domain containing the ATP-binding site and associated functional analyses not only revealed the potential interface between the TM and cytoplasmic domains but also showed that ATP binding is important for the Mg^{2+} export activity of CorC. Overall, our work provides structural insights into the Mg^{2+} recognition and regulation of the CorC Mg^{2+} transporter.

RESULTS

Structural determination and functional characterization

We screened the expression of over 140 CNNM/CorC homologs by fluorescence-detection size exclusion chromatography (FSEC; table S1) (37). In the green fluorescent protein (GFP)–based FSEC method, we expressed targeted membrane proteins fused to the GFP tag, and whole-cell extracts were solubilized with detergents and subjected to size exclusion chromatography attached to a fluorescence detector to analyze the expression level and monodispersity of targeted membrane proteins (37). Among the screened CNNM/CorC orthologs, we identified the CorC protein from *Thermus parvatiensis* (TpCorC) as a suitable candidate for structural studies, with a high level of expression and sharp peak profile (Fig. 1A). The domain organization of TpCorC consists of a DUF21 TM domain, a CBS domain, and a CorC/HlyC domain, which is typical of CorC family proteins (Fig. 1B). Among these domains, the DUF21 and CBS domains are also shared by CNNM family proteins and have many disease-associated mutations (17–19, 24), indicating the possible functional importance of these core regions in CNNM/CorC family proteins.

TpCorC shares approximately 30% sequence identity with the *S. aureus* CorC orthologs Mpfa and Mpfb, which are known Mg^{2+} export CorC proteins (fig. S1) (10, 12). Thus, we tested the Mg^{2+} export activity of TpCorC using the Mg^{2+} efflux assay in human embryonic kidney (HEK) 293 cells (Fig. 1, C to E), which have been used to characterize the Mg^{2+} export activity of human CNNM2 and CNNM4 (28). To facilitate the cell surface expression of TpCorC, we fused the membrane targeting sequence from human CNNM4 (residues 1 to 178) at the N terminus (14), established a HEK293 cell line stably expressing TpCorC with the targeting sequence, and confirmed the cell surface expression (Fig. 1C). Using this cell line, we performed imaging analyses using Magnesium Green, a fluorescent indicator dye for Mg^{2+}, which has been successful for membrane protein crystallization (39). We applied the lipidic cubic phase (LCP) method, for the crystallization of the TpCorC TM domain constructs (38) and identified two mutants (V101A and V101A/G115A) with increased melting temperatures (*T*ₘ values) of 8.4°C and 8.9°C, respectively (Fig. 1F). Mutation at Val101 did not affect Mg^{2+} depletion (at 5 min, means ± SEM; empty, n = 30; wild type (WT), n = 30; WT (−Na^{+}), n = 20; TM-CBS, n = 20; and V101A, n = 20). (F) Thermostability assay of the WT and TpCorC TM domain mutants by GFP-based FSEC-TS.
crystallization with other divalent cations such as Ca$^{2+}$, Co$^{2+}$, Ni$^{2+}$, and Mn$^{2+}$. The initial phase was determined by the single-wavelength anomalous diffraction (SAD) method with the selenomethionine (SeMet)–labeled TpCorC V101A/G115A mutant protein and was further refined to 2.0 Å resolution using the native datasets of the TpCorC TM domain constructs (wild type and V101A) (fig. S2, A and B, and table S2). The two TpCorC TM domain structures (wild type and V101A) are essentially identical with a root mean square deviation (RMSD) of 0.2 Å. Thus, we mainly describe the (wild type and V101A) structure unless otherwise noted, as it was determined at higher resolution. The location of Val$^{101}$ in the TpCorC TM domain V101A structure is likely to be conserved among CNNM/CorC family proteins.

**TM domain architecture**

The crystallographic asymmetric unit of the TpCorC TM domain contains one protomer, which forms a dimer, related by crystallographic twofold symmetry (Fig. 2 and fig. S2, A and B). Each TpCorC protomer is composed of three TM helices with three cytoplasmic helices, termed cytoplasmic helix 1 (CH1) and CH2, located between the TM1 and TM2 helices, and the belt helix following the TM3 helix (Fig. 2, D and E). The N- and C-terminal ends of TpCorC extend into the periplasm and cytoplasm, respectively, because the C-terminal region of TpCorC has a cytoplasmic region including the CBS domain (Fig. 1B).

From the cytoplasmic view, there are close interactions within the TM1, TM2, and TM3 helices in each protomer but not within the TM helices of the neighboring subunit (Fig. 2C). In contrast, from the periplasmic view, the TM2 and TM3 helices interact closely with their counterparts in the neighboring subunit (Fig. 2B). Consequently, the TpCorC transporter structure seemingly adopts an inward-opening conformation, where the cytoplasmic side is solvent accessible but the periplasmic side is closed (Fig. 3A).

The belt helix after the TM3 helix has three unique features (Fig. 2 and fig. S4). First, it is nearly parallel to the plane of the membrane (Fig. 2A). Second, it is amphipathic so that the hydrophobic side of the belt helix is embedded in the membrane bilayer, whereas the hydrophilic side faces the solvent (Fig. 2A and fig. S4, A and B). Last, the belt helix is quite long (approximately 35 residues) and highly curved to interact with the TM1 and TM2 helices within the protomer and with the TM3 helix in the neighboring subunit (Fig. 2, B and C). Molecular dynamics (MD) simulations of the TpCorC TM domain dimer embedded in a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine membrane showed that the overall conformation of the TpCorC TM domain structure, including the unique belt helix, was mostly stable throughout the simulations (Fig. 2, F and G). The structural deviations from the original crystal structure were within 2.5 Å during 300-ns MD simulations, and the root mean square fluctuation values as a function of residue number were also within 2.5 Å after the 300-ns MD simulations. Overall, the protein fold of TpCorC is distinct from those of any previously reported membrane protein structures that are likely to be conserved among CNNM/CorC family proteins.

**Mg$^{2+}$ ion binding site**

The TpCorC TM domain dimer structure revealed a strong residual electron density peak surrounded by the side chain or main chain atoms of five amino acid residues of each protomer within the TM domain (fig. S5). We obtained crystals of the TpCorC TM domain by cocrystallization specifically in the presence of Mg$^{2+}$ ions. In addition to Mg$^{2+}$ ions, our crystallization conditions include Zn$^{2+}$ ions as divalent cations. Therefore, these two types of divalent cations, Mg$^{2+}$ and Zn$^{2+}$, would be potential origins for the strong electron density in our structure.
To clarify the origin of the strong electron density in the TM domain structure, we initially attempted to use Co²⁺, Ni²⁺, and Mn²⁺, heavy divalent cations, which can occupy Mg²⁺ binding sites based on their octahedral coordination geometry, similar to that of Mg²⁺ ions (40, 41). However, neither cocrySTALLization nor soaking with these heavy atoms yielded well-diffracting crystals. We also collected the diffraction data at the wavelength for the Zn²⁺ anomalous peak (40), which is consistent with the canonical distance of 2.1 Å between the densities as Mg²⁺ ions. This distance is crucial for the stable formation of the inward-facing conformation (40, 41).

We hypothesized, the removal of Mg²⁺ from the T106C/C282A mutant with EDTA in the presence of Na⁺ caused the loss of the cross-linked dimer (Fig. 4D), indicating disruption of the inward-facing conformation of the transporter. In contrast, there was a strong band for the TpCorC dimer in the presence of Mg²⁺ (Fig. 4D). The combination of Mg²⁺ removal with EDTA and the replacement of Na⁺ with K⁺ resulted in bands for both the TpCorC monomer and dimer (Fig. 4D), potentially suggesting a conformational equilibrium in the absence of both Mg²⁺ and Na⁺.

We then introduced mutations to the Mg²⁺ binding site of TpCorC (N90A and E130A) into the TpCorC T106C/C282A mutant. Notably, each mutant exhibited both bands for the TpCorC monomer and dimer and was not sensitive to either the addition of Mg²⁺ or EDTA (Fig. 4D). These results suggest that the Mg²⁺ binding site is crucial for the stable formation of the inward-facing conformation.
and suggest that adding Na\(^+\) in the absence of Mg\(^{2+}\) may further destabilize the inward-facing conformation.

**Mutational analysis of putative Na\(^+\) binding site**

Our Mg\(^{2+}\) efflux assay for TpCorC in HEK293 cells suggested Na\(^+\) dependency of the transport activity (Fig. 1, D and E). Upon close inspection of the TpCorC TM domain structure, we speculated that the dimer interface, located near the Mg\(^{2+}\) binding site, might be a potential candidate for Na\(^+\) binding sites (Fig. 5A) because this region includes multiple Asn residues (Asn\(^91\) and Asn\(^94\)), which are often involved in monovalent cation binding in transport proteins (43–46). Among them, Asn\(^94\) is conserved in both CNNM2 and CNNM4 (fig. S1), and CNNM4 is shown to have Na\(^+\)-dependent transport activity (15). Furthermore, the existence of a Na\(^+\) binding site proximal to the Mg\(^{2+}\) binding site would be consistent with Na\(^+\)-coupled Mg\(^{2+}\) transport, as often observed in other Na\(^+\)-driven secondary active transporter structures (43–46).

To test our notion, we introduced alanine-substituted mutations (N91A and N94A) into the T106C/C282A mutant of TpCorC and performed chemical cross-linking experiments (Fig. 5B). Whereas the addition of Na\(^+\) to the T106C/C282A mutant in the absence of Mg\(^{2+}\) caused the loss of the cross-linked dimer, the N94A/T106C/C282A exhibited bands for both the TpCorC monomer and dimer upon the addition of Na\(^+\) in the absence of Mg\(^{2+}\) (Fig. 5B), suggesting that the Asn\(^94\) residue may be involved in Na\(^+\) sensitivity of TpCorC. In contrast, mutation of the nonconserved Asn\(^91\) had little effect on Na\(^+\) sensitivity (Fig. 5B).

In the N94A/T106C/C282A mutant, Mg\(^{2+}\) had a weaker effect on the formation of the cross-linking dimer (Fig. 5B). This may be because Asn\(^94\) is indirectly involved in the formation of the Mg\(^{2+}\) binding site by forming a hydrogen bond with Ser\(^93\), which is directly involved in Mg\(^{2+}\) binding (Fig. 5A). In addition, the fractions of the chemical cross-linking dimer were reduced in the Na\(^+\)-free N91A/T106C/C282A mutant in the presence and absence of Mg\(^{2+}\).
compared to those in the T106C/C282A mutant. The reason is unknown, and further characterizations would be necessary but are beyond the scope of our study.

Furthermore, the \(Na^+\)-nonsensitive mutation at Asn\(^94\) and the corresponding mutation in \(Na^+\)-driven CNNM4 reduced the \(Mg^{2+}\) transport activities of both constructs (Fig. 5, C to E). Overall, these results suggest that Asn\(^94\) is involved in \(Na^+\) sensitivity in TpCorC and is important in its \(Mg^{2+}\) transport activity.

**\(Mg^{2+}\) binding site and disease-associated mutations of human CNNM2/4**

To gain mechanistic insights and to interpret the structure-function relationship of the \(Mg^{2+}\) binding site in human CNNM proteins, we performed structure-based mutational analysis of human CNNM2 and CNNM4 by the \(Mg^{2+}\) export assay in HEK293 cells (Fig. 6). We chose to characterize CNNM2 and CNNM4 among the CNNM transporters for the following three reasons: First, all of the amino acid residues involved in the \(Mg^{2+}\) binding site of TpCorC (Ser\(^{269}\), Ser\(^{273}\), Asn\(^{323}\), Gly\(^{356}\), and Glu\(^{357}\)) are strictly conserved in both human CNNM2 (Ser\(^{269}\), Ser\(^{273}\), Asn\(^{323}\), Gly\(^{356}\), and Glu\(^{357}\)) and CNNM4 (Ser\(^{196}\), Ser\(^{200}\), Asn\(^{250}\), Gly\(^{283}\), and Glu\(^{284}\)) (Fig. 6A). Second, both CNNM2 and CNNM4 also function as \(Mg^{2+}\) exporters (15, 28), as observed with TpCorC in our study. Last, on the basis of the sequence alignment, we noticed that many known disease-associated mutations in CNNM2 and CNNM4 are located at the \(Mg^{2+}\) binding site (Fig. 6A) (18, 24).

On the basis of the sequence similarity between TpCorC and human CNNM2/4, we constructed homology models of the TM domain of CNNM2 and CNNM4 using our TpCorC TM domain structure. Because all amino acid residues in the \(Mg^{2+}\) binding site are strictly conserved, the \(Mg^{2+}\) binding pockets and their coordination to \(Mg^{2+}\) ions in the homology models are almost identical to those of TpCorC (Figs. 3A and 6, B and C).

We first generated alanine-substituted mutants of human CNNM2 at (Ser\(^{269}\), Ser\(^{273}\), Asn\(^{323}\), Gly\(^{356}\), and Glu\(^{357}\)) and near (Gly\(^{322}\) and Pro\(^{360}\)) the \(Mg^{2+}\) binding site (Fig. 6A). Pro\(^{360}\) is strictly conserved among the CNNM/CorC family proteins, and Gly\(^{322}\) is also highly conserved in the family (fig. S1). Among the seven mutants at or near the \(Mg^{2+}\) binding site, the S269A and N323A mutants were not properly expressed at the cell surface, and S273A was not expressed at all (Fig. 6D and fig. S6). While the expression of wild-type CNNM2 induced a clear decrease in the fluorescence intensity after the depletion of \(Mg^{2+}\), G356A and E357A, both \(Mg^{2+}\) binding site mutants that were properly expressed at the cell surface, produced little change in the fluorescence signal compared to the level of the control (Fig. 6E). The mutation at the strictly conserved Pro\(^{360}\) located near the \(Mg^{2+}\) binding site also abolished the \(Mg^{2+}\) export activity of CNNM2 (Fig. 6E), whereas G322A maintained \(Mg^{2+}\) export activity (Fig. 6E).

Because we were not able to measure the \(Mg^{2+}\) export activity of three of the \(Mg^{2+}\) binding site mutants of CNNM2 (S269A, S273A, and N323A) because of technical problems with cell surface expression, we then generated three mutants of CNNM4 at the corresponding positions (S196P, S200Y, and N250A). For S196P and S200Y, we chose Pro and Tyr residues, respectively, because S196P and S200Y are reportedly associated with Jalili syndrome (18). All three mutants were successfully expressed at the cell membrane surface and exhibited the loss of \(Mg^{2+}\) export activity (Fig. 6, D and F).

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**Fig. 6. **\(Mg^{2+}\) binding sites of human CNNM2 and CNNM4. (A) Sequence alignment of TpCorC and human CNNM2 and CNNM4 at the \(Mg^{2+}\) binding site. Purple and green circles indicate disease-associated mutation sites and \(Mg^{2+}\) binding sites, respectively. (B and C) Close-up view of the \(Mg^{2+}\) binding sites in human CNNM2 (B) and CNNM4 (C) based on the homology model. Amino acid residues involved with \(Mg^{2+}\) ions are shown in stick representation. \(Mg^{2+}\) ions are shown as spheres. Dashed lines indicate hydrogen bonds. (D) Cell surface expression of CNNM \(Mg^{2+}\) binding site mutants. (E and F) \(Mg^{2+}\) export assay. Bar graph: Relative fluorescence intensities after \(Mg^{2+}\) depletion (at 5 min, means ± SEM, n = 10).

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Overall, our results showed that these \(Mg^{2+}\) binding site mutants (CNNM2: G356A and E357A; CNNM4: S196P, S200Y, and N250A) abolished \(Mg^{2+}\) export activity (Fig. 6, E and F), indicating that both CNNM2 and CNNM4 would likely recognize \(Mg^{2+}\) ions in essentially the same manner as TpCorC (Figs. 3A and 6, B and C). Since many disease-associated mutations of CNNM2 and CNNM4 are located in the \(Mg^{2+}\) binding site, these results indicate structure-function relationships between the \(Mg^{2+}\) binding site and disease-associated mutations of CNNM2 and CNNM4.

**CBS domain structure and TM-CBS interface**

We further determined a nearly complete structure of the TpCorC CBS domain, including the region between the TM and CBS domains (residues 184 to 352) (fig. S2C). Thus, the structures that we obtained (TM, residues 28 to 182; CBS, residues 184 to 352) almost completely covered the entire region of the TM and CBS domains.
The linker loop region between the TM and CBS domains consists of 11 amino acid residues (Gly\(^{178}\)–Ser\(^{188}\)), and with this loop length constrained, the loop connection between the C-terminal end of the TM domain structure and the N-terminal end of the CBS domain structure yielded a nearly unique TM-CBS interface, as shown in Fig. 7C. In this model, CH3 of the CBS domain interacts with CH1 of the TM domain in the neighboring subunit (Fig. 7C).

To verify this model, we generated the double cysteine mutant of the TpCorC Cys-less mutant (C282A), having cysteine substitutions at Lys\(^{60}\) in CH1 and Glu\(^{190}\) in CH3, where the \(d\) distance between Lys\(^{60}\) in one subunit and Glu\(^{190}\) in the other subunit is 9.4 Å. Consistent with our model, the K60C/E190C mutant of TpCorC showed a strong band for the TpCorC dimer in the presence of Cu\(^{2+}\) phenanthroline (Fig. 7D). FSEC analysis showed that the Cys-less mutant and the K60C/E190C double mutant treated with Cu\(^{2+}\) phenanthroline eluted in similar positions, indicating that Cu\(^{2+}\) phenanthroline–dependent dimer formation likely occurs within two protomers in a single TpCorC dimer and is not caused by non-specific disulfide bond formation between the two adjacent TpCorC dimers (Fig. 7E). Furthermore, MD simulations of the TM-CBS model showed that the overall structure and TM-CBS interface (plotted by \(d\) distances between Lys\(^{60}\) in one subunit and Glu\(^{190}\) in the other subunit) are mostly stable throughout the simulation (Fig. 7, F and G), further verifying our TM-CBS structure model.

Nevertheless, because we have tested only one disulfide pair in the chemical cross-linking experiments, the constraint is insufficient to verify our proposed model as the unique one. Thus, further biophysical and structural analyses will be required to draw a firm conclusion.

**ATP-binding site in the CBS domain**

To gain insights into the ATP binding and ATP-dependent modulation of TpCorC, we also determined the crystal structure of the TpCorC CBS domain in complex with ATP (Fig. 8A and fig. S2, D and E). ATP molecules are located at the interface between two tandem CBS repeats in each subunit, with their phosphate groups facing each other (Fig. 8A).

The adenine base of ATP is recognized by three hydrogen bonds with the main chain amino and carbonyl groups of Val\(^ {255}\) and the main chain carbonyl group of Arg\(^ {257}\) and by a stacking interaction with Tyr\(^ {255}\) (Fig. 8A). The ribose group forms two hydrogen bonds with the side chain of Asp\(^ {339}\) (Fig. 8A), whereas the phosphate groups of ATP interact with the side chain and main chain amino group of Ser\(^ {356}\) and the side chains of Arg\(^ {257}\) and Thr\(^ {336}\) (Fig. 8A). Furthermore, the conformation of the phosphate groups is seemingly stabilized by Mg\(^ {2+}\) ions, which are further bridged by Glu\(^ {338}\) via a water molecule (Fig. 8A). Notably, most of these residues at the ATP-binding site are highly conserved among the CNNM/CorC family proteins (fig. S1).

To test the ATP-binding mechanism, we generated three ATP-binding site mutants of TpCorC, Y255A (adenine ring), T336I (ribose), and Y255A/T336I and performed an ATP-binding assay using isothermal titration calorimetry (ITC). The disease-associated CNNM2 mutant T568I, corresponding to the T336I mutant of TpCorC, reportedly lost both ATP-binding activity and Mg\(^ {2+}\) export activity \( (17, 28)\). The Y255A mutant and T336I mutant of the TpCorC CBS domain exhibited dissociation constant \( (K_d)\) values of 2.8 and 9.4 \( \mu \)M for ATP, whereas the wild type showed a \(K_d\) of 0.46 \( \mu \)M for ATP, indicating that these mutations weakened the affinity of ATP for TpCorC \( (Fig. 8B)\). The Y255A/T336I double mutant lost ATP-binding activity (Fig. 8B). Notably, in addition to ATP binding, we performed an ATP hydrolysis assay with TpCorC (Fig. 8C) and did not detect hydrolysis activity compared to apyrase, an ATP diposphohydrolase (Fig. 8C).
Given the high affinity of the TpCorC CBS domain for ATP (~500 nM) and the typical concentration of cytoplasmic ATP at millimolar level (47), ATP may constitutively bind to CorC as a regulatory cofactor in vivo rather than functioning in the binding and release cycles. Overall, our results not only verify the ATP-binding mechanism of CorC but also show that ATP binding to the CBS domain is important for Mg^{2+} transport by CorC.

**DISCUSSION**

In this work, we determined the crystal structure of the TpCorC TM domain in complex with Mg^{2+} ions (Fig. 2), unveiling the novel fold of the DUF21 TM domain conserved in the CNNM/CorC family. Notably, Mg^{2+} ions are fully dehydrated by the surrounding amino acid residues in the structure (Fig. 3), and these residues are important for the Mg^{2+} efflux activities of TpCorC and human CNNM2 and CNNM4 (Figs. 3 and 6). This Mg^{2+} recognition contrasts with that of the known Mg^{2+} channel structures, such as the MgtE and CorA Mg^{2+} channels (48, 49). For instance, in the MgtE structure, Mg^{2+} ions are fully hydrated with all six water molecules in the first hydration shell (49). The dehydration energy for Mg^{2+} ions is much higher than that of monovalent cations and other biological divalent cations (50), and ion channels typically accomplish ion transport much faster than secondary active transporters. Therefore, this difference in Mg^{2+} recognition may account for the differences in transport kinetics between ion channels (MgtE and CorA) and transporters (CNNM/CorC).

Another unique feature in the TpCorC TM domain structure is the amphipathic belt helix (Fig. 2A and fig. S4). Since the belt helix is directly linked to the following regulatory CBS domain, it is tempting to speculate that the belt helix in the CorC TM domain might play an important role in transport regulation. Furthermore, the belt helix interacts closely with all three TM helices and may therefore affect the conformation of the TM domain (Fig. 2, B and C). Supporting our notion, mutation at the conserved Gly 178 of TpCorC fore affects the conformation of the TM domain (Fig. 2, B and C).

We then performed an Mg^{2+} export assay in HEK293 cells with the Y255A/T336I mutant, which lacks ATP-binding ability (Fig. 8, D and E). Consistent with the loss of affinity for ATP, the Y255A/T336I double mutant showed low Mg^{2+} export activity (Fig. 8, D and E).
In the Mg$^{2+}$-free inward-facing state, these local structural differences may make the binding pocket more solvent accessible (fig. S7A). In addition, since Glu$^{130}$ might face outward from the binding pocket in the Mg$^{2+}$-free inward-facing state (fig. S7A), Glu$^{130}$ would be positioned to interact more easily with Mg$^{2+}$ ion in the cytoplasm (fig. S7A). Thus, it is tempting to speculate that in the Mg$^{2+}$ binding process, Glu$^{130}$ may first interact with the fully hydrated Mg$^{2+}$ and then partially dehydrate the Mg$^{2+}$ ion (fig. S7B). Such interaction may cancel the interaction between Glu$^{130}$ and the positively charged Lys$^{134}$, and the following conformational change of Glu$^{130}$ might push Mg$^{2+}$ deeper into the Mg$^{2+}$ binding pocket, facilitating further dehydration by the other residues by mimicking the hexacoordination of Mg$^{2+}$ (fig. S7B). In this way, CorC might facilitate the dehydration of Mg$^{2+}$ (fig. S7, A and B), whereas the dehydration of Mg$^{2+}$ is usually energetically unfavorable and very slow, as we mentioned above. On the other hand, the details of the structural changes from the inward-facing conformation to the outward-facing conformation are not yet clear, but the chemical cross-linking experiment suggested that the addition of Na$^{+}$ seems to destabilize the inward-facing state (Fig. 4D). Therefore, it is possible that the addition of Na$^{+}$ may shift the structural equilibrium toward an outward-facing state (fig. S7, C to E), and such a structural change may promote the release of Mg$^{2+}$ ion to the periplasmic side (fig. S7E). However, further structural and functional analyses are required for future work to fully understand the transport cycle.

Our results show that ATP binding to the CorC CBS domain is important for its Mg$^{2+}$ transport activity (Fig. 8). A comparison of the apo and ATP-bound structures of the CBS domain suggests that in the ATP-bound structure, the helix region exterior of the ATP-binding site moves slightly outward from the pocket, mainly via its contact with the ribose moiety of ATP (Fig. 8F). Such structural changes in the CBS domain might be important for the regulation of transport activity through interaction with the TM-CBS interface region. Overall, our study presents a structural framework for understanding the Mg$^{2+}$ recognition and regulation of the CNNM/CorC family, whose eukaryotic and bacterial physiological functions are implicated in various diseases.

**METHODS**

**Expression and purification**

The expression screening of CNNM/CorC homologs was performed by the GFP-based FSEC method (37). Alanine scanning was carried out by the FSEC-TS assay (38). The TM domain (residues 26 to 182) of the CorC gene from TpCorC and its mutants were synthesized (GENEWIZ, China); subcloned into a pET vector containing a human rhinovirus (HRV) 3C protease cleavage site, GFPuv, and an octahistidine tag at the C terminus; and transformed into the _Escherichia coli_ Rosetta (DE3) strain. Cells were cultured in LB medium supplemented with ampicillin (50 μg/ml) and 150 mM NaCl and purified as described above. For cocrystallization of the TpCorC TM domain V101A mutant with CsCl, 150 mM NaCl in buffer C was replaced with 150 mM CsCl for size exclusion chromatography. To generate SeMet-substituted proteins, the TpCorC TM domain mutant of L38M/V101A/G115A/L173M was overexpressed in the _E. coli_ B834 (DE3) strain, cultured in LeMaster medium supplemented with SeMet (50 mg/liter), and purified as described above.

All CBS domain constructs of TpCorC (residues 183 to 361, Y255A, and residues 202 to 361) were subcloned into the pET vector containing an HRV 3C protease cleavage site and an octahistidine tag and transformed into the _E. coli_ Rosetta (DE3) strain. Cells were cultured in LB medium supplemented with ampicillin (50 μg/ml); residues 183 to 361, Y255A; residues 202 to 361) at 37°C and then induced by 0.5 mM IPTG at an OD$_{600}$ of 0.6. The cells were further cultured at 37°C for 3 hours, harvested by centrifugation (5000g, 15 min), and disrupted in buffer A. The cell lysate was centrifuged (20,000g, 1 hour), and the supernatant was mixed with TALON resin (Takara, Japan). After binding for 1 hour, the resin was washed with buffer B containing 10 mM imidazole and eluted with buffer B containing 300 mM imidazole. The eluate was mixed with a Histagged HRV 3C protease to cleave the GFPuv-octahistidine tag and dialyzed against buffer B overnight. The sample was reapplied to TALON resin equilibrated with buffer B, and the flowthrough fractions were concentrated using an Amicon Ultra 50K filter (Merck Millipore, USA) and applied to a Superdex 200 10/300 size exclusion chromatography column (GE Healthcare, USA) in buffer C [150 mM NaCl, 20 mM Heps (pH 7.5), 0.05% DDM, and 0.01% CHS]. The peak fractions were concentrated to 10 mg/ml for crystallization.

The V101A mutant of the TpCorC TM domain was expressed and purified as described above. For cocrystallization of the TpCorC TM domain V101A mutant with CsCl, 150 mM NaCl in buffer C was replaced with 150 mM CsCl for size exclusion chromatography. To generate SeMet-substituted proteins, the TpCorC TM domain mutant of L38M/V101A/G115A/L173M was overexpressed in the _E. coli_ B834 (DE3) strain, cultured in LeMaster medium supplemented with SeMet (50 mg/liter), and purified as described above.

All CBS domain constructs of TpCorC (residues 183 to 361, Y255A, and residues 202 to 361) were subcloned into the pET vector containing an HRV 3C protease cleavage site and an octahistidine tag and transformed into the _E. coli_ Rosetta (DE3) strain. Cells were cultured in LB medium supplemented with ampicillin (50 μg/ml); residues 183 to 361, Y255A; residues 202 to 361) at 37°C and then induced by 0.5 mM IPTG at an OD$_{600}$ of 0.6. The cells were further cultured at 37°C for 3 hours, harvested by centrifugation (5000g, 15 min), and disrupted in buffer A. The cell lysate was centrifuged (20,000g, 1 hour), and the supernatant was mixed with TALON resin (Takara, Japan). After binding for 1 hour, the resin was washed with buffer B containing 10 mM imidazole and eluted with buffer B containing 300 mM imidazole. The eluate was mixed with a Histagged HRV 3C protease to cleave the GFPuv-octahistidine tag and dialyzed against buffer B overnight. The sample was reapplied to TALON resin equilibrated with buffer B, and the flowthrough fractions were concentrated using an Amicon Ultra 50K filter (Merck Millipore, USA) and applied to a Superdex 75 Increase 10/300 size exclusion chromatography column (GE Healthcare, USA) in buffer D [100 mM NaCl and 20 mM Hepes (pH 7.5)]. The peak fractions were concentrated to 10 mg/ml for crystallization. The CBS domain construct of TpCorC (residues 202 to 361) was mixed with ATP and MgCl$_2$, at final concentrations of 1.5 and 10 mM, respectively, before crystallization.

**Crystallization**

Purified CorC TM domain proteins were mixed with MgCl$_2$ at a final concentration of 50 mM, incubated for 30 min, and then mixed with monoolein (Nu-Chek, USA) at a ratio of 2:3 (w/w) with a coupled syringe mixer to generate LCP. The robotic LCP crystallization trial was performed by dispensing 50 nl of LCP drops onto 96-well sandwich plates and overlaid with 700 nl of reservoir solution using a Gryphon LCP crystallization robot (Art Robbins Instruments, USA). All LCP crystals were obtained in a solution containing 10 mM ZnCl$_2$, 100 mM sodium acetate (pH 4.0), and 40% polyethylene glycol 200 (PEG 200) at 18°C. Crystals typically appeared in 3 days and grew to.

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their maximum size within 1 week. Crystals were then harvested in reservoir solution supplemented with 40% PEG 200 and 50 mM MgCl$_2$ and flash-frozen with liquid nitrogen for x-ray diffraction experiments.

For crystallization by the vapor diffusion method, 1 µl of the TpCorC CBS domain proteins (residues 183 to 361, Y255A, and residues 202 to 361) was mixed with 1 µl of reservoir solution [0.4 M ammonium thiocyanate, 0.1 M sodium acetate (pH 4.5), and 15% PEG 4000 and 0.1 M CaCl$_2$, 0.1 M Hepes (pH 7.5), and 5% PEG 8000] and stored at 18°C. Before flash freezing in liquid nitrogen, crystals were harvested with the corresponding cryoprotectant solutions: for the crystals of the TpCorC CBS domain protein (residues 183 to 361, Y255A), 30% glycerol, 0.4 M ammonium thiocyanate, 0.1 M sodium acetate (pH 4.5), and 15% PEG 4000 and for the crystals of the TpCorC CBS domain protein (residues 202 to 361), 30% glycerol, 0.1 M CaCl$_2$, 0.1 M Hepes (pH 7.5), and 5% PEG 8000.

Data collection and structure determination
X-ray diffraction datasets were collected at the SPring-8 beamlines of BL32XU and BL41XU with the automated data collection system ZOOL (51) and processed with KAMO (52) for automatic data processing using XDS (53). For the SeMet-substituted TM domain of TpCorC, selenium sites were identified from the SAD data with SHELXD (54, 55). The initial phase was calculated and improved with SHELXE (54, 55), followed by automatic model building. The model was then further manually rebuilt with Coot (56) and refined with PHENIX (57). The structures of the wild type and the V101A mutant of the TpCorC TM domain were determined by molecular replacement with Phaser using the initial model of the SeMet-substituted TM domain of TpCorC (58) and further refined through multiple rounds of manual model building by Coot (56) and refinement by PHENIX (57).

The structure of the TpCorC CBS domain constructs of TpCorC (residues 183 to 361, Y255A, and residues 202 to 361) were initially determined by molecular replacement with Phaser using the homology model from SWISS-MODEL (59). The structure was further manually rebuilt using Coot (56) and refined with PHENIX (57).

The Ramachandran plots were calculated with MolProbity (60). Data collection and refinement statistics are summarized in table S2. All figures showing structures were generated using PyMOL (https://pymol.org/).

Isothermal titration calorimetry
All measurements were performed using a MicroCal iTC200 (GE Healthcare, USA) at 25°C. The TpCorC CBS domain and its mutants were purified by a similar method to that described above, and buffer E [100 mM KCl, 5 mM MgCl$_2$, and 20 mM Hepes (pH 7.5)] was used for size exclusion chromatography. The ATP solutions were prepared by adding ATP to buffer E for size exclusion chromatography at a final concentration of 0.5 mM. A total of 250 µl of TpCorC CBS domain proteins (50 to 100 µM) was applied to the thermally equilibrated ITC cell, and the ligand syringe was filled with 40 µl of ATP solution. The molar ratio of proteins to ligands was 1:10. The ligands were injected 20 times (0.5 µl for injection 1 and 2 µl for injections 2 to 20), with 120-s intervals between injections. The background data obtained from buffer E (for ATP) were subtracted before data analysis. The data were analyzed with Microcal Origin software. Measurements were performed at least twice, and similar results were obtained.

Biochemical cross-linking
The wild-type, Cys-less mutant C282A and its mutants with cysteine-substitutions of TpCorC (residues 23 to 441) were purified by a similar method to that described above, except that buffer F [150 mM NaCl, 20 mM Hepes (pH 7.5), and 0.03% DDM] and buffer G [150 mM KCl, 20 mM Hepes (pH 7.5), and 0.03% DDM] were used for size exclusion chromatography. A total of 4.0 µl of 20 µM TpCorC protein was mixed with 0.5 µl of 2 mM EDTA, 100 mM MgCl$_2$, or Milli-Q water at the respective concentrations and then incubated for 1 hour at 4°C. Then, 0.5 µl of the reaction solution (10 mM Cu$^{2+}$ bis-1,10-phenanthroline in a 1:3 molar ratio), Milli-Q water, or 20 mM tris(2-carboxyethyl)phosphine solution was added, followed by incubation for 30 min at 4°C. The samples were analyzed by nonreducing SDS–polyacrylamide gel electrophoresis and FSEC. Experiments were performed at least twice, and similar results were obtained.

ATP hydrolysis activity assay
The ATP hydrolysis assay of TpCorC and its mutant was performed by the malachite green method (61). Briefly, malachite green dye solution was freshly prepared on the day of the experiment by mixing 0.045% malachite green, 4.2% ammonium molybdate, and 1% Triton X-100 at a volume ratio of 36:12:1. Then, 1 µM TpCorC or 1 nM apyrase (Sigma-Aldrich, USA) was prepared in buffer H [20 mM Hepes (pH 7.5), 150 mM NaCl, and 0.05% DDM]. ATP was dissolved in buffer H containing 4 mM MgCl$_2$ at a final concentration of 4 mM. To initiate the reaction, the ATP solution was added to the protein solution at an equal volume to obtain a final reaction mixture consisting of 20 mM Hepes (pH 7.5), 2 mM MgCl$_2$, 150 mM NaCl, 0.05% DDM, and 2 mM ATP. The samples were then incubated at room temperature, and aliquots were taken at multiple time points (5, 30, 60, 90, and 120 min). Fifty microliters of each aliquot at each time point was mixed with 850 µl of the malachite green dye solution and 100 µl of 34% citric acid, and the absorbance was measured at 660 nm. The absorbance standard curve for inorganic phosphate was established with standard H$_3$PO$_4$ solutions.

MD simulations of the TpCorC TM and TM-CBS domains
MD simulations were carried out using Desmond (62). The CorC TM domain structure and TM-CBS structure model were embedded into a pre-equilibrated 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine lipid bilayer membrane. The protein/lipid system was placed at the center of a 10 × 10 × 10 box and then solvated with the simple point charge water model. The system was neutralized by adding sodium ions as counterions. NaCl (150 mM) was added to mimic the physiological conditions. The OPLS-2005 force field was used (63, 64). All simulations were carried out for 300 ns under an NPT (isothermal–isobaric) ensemble using a K40c Nvidia graphics processing unit, with the temperature kept at 300 K using a Nose-Hoover chain thermostat and the pressure at 1 atm using a Martyna-Tobias-Klein barostat. The long-range electrostatic interactions were calculated using the particle mesh Ewald method. The Coulomb interactions were analyzed with a cutoff of 9.0 Å. The reversible reference system propagator algorithm integrator was used with a time step of 2 fs, and coordinate trajectories were saved every 200 ps during the sampling process. The trajectory analysis was carried out using the Simulation Event Analysis and the Simulation Interactions Diagram modules of Desmond.
Expression constructs for Mg\textsuperscript{2+} export experiments

The cDNAs for human CNNM4 and mouse CNNM2 were generated in previous studies (15). The cDNAs for TpCorC containing the membrane targeting sequence from human CNNM4 (residues 1 to 178) were synthesized (GENEWIZ, China). Amino acid–substituted mutants were generated by GENEWIZ. Each cDNA was inserted into pCMV tag 4A (Agilent) for expression in HEK293 cells.

Cell culture and transfection

HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium (Nissu, Japan) supplemented with 10% fetal bovine serum and antibiotics. Expression plasmids were transfected using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer’s instructions. For the analyses of CNNM2 and CNNM4, cells were directly subjected to immunofluorescence microscopy or the Mg\textsuperscript{2+} export assay. For the analyses of TpCorC, HEK293-derived cell lines stably expressing TpCorC mutants were established by selecting the transfected cells with growth medium supplemented with G418 (800 ng/ml) and 40 mM Mg\textsuperscript{2+} (Mg\textsuperscript{2+} was added to avoid potential Mg\textsuperscript{2+} export assay). The expression of each cell lines stably expressing TpCorC were cultured in growth medium.

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Acknowledgments: We thank F. Cai, Z. Zhang, and K. Hirata for technical support and C.-H. Lee and H. Kato for critical comments on the manuscript. We also thank the staff from BL32XU and BL41XU beamlines at Spring-8 and from BL17U1 at Shanghai Synchrotron Radiation Facility (SSRF) for assistance during data collection. The diffraction experiments were performed at Spring-8 (BL32XU and BL41XU) (proposal nos. 2017A2523, 2018A2507, and 2019A2514) and at SSRF BL17U1 (proposal no. 2018-SSRF-PT-004257). Funding: This work was supported by funding provided by the Ministry of Science and Technology of China (National Key R&D Program of China: 2016YFA0502800) to M.H. and by funding provided by the National Natural Science Foundation of China (31850410466 and 32071234). The opening Project of State Key Laboratory of Genetic Engineering (SKLGE-1813), the Innovative Research Team of High-Level Local University in Shanghai, and a key laboratory program of the Education Commission of Shanghai Municipal (ZDSYS14005). This work was also supported by funding provided by the Japan Society for the Promotion of Science (JP26111007, JP17H04041, and JP20H03515) to H.M. and (JP20K07312, JP20H05508, and JP17K19396). Y.F. authored contributions: Y.H. and F.J. expressed and purified CorA and its mutants for the structural and functional studies and determined the structures with assistance from M.S., Y.Z., and M.H. Y.H. performed the ITC and biochemical cross-linking experiments. Y.F. performed the Mg$^{2+}$ export assay. Z.X. and W.Z. performed the computational experiments at the early stage of the project. J.W. and Y.Y. performed MD simulations. Y.H., F.J., F.Y., H.M., and M.H. wrote the manuscript. H.M. and M.H. supervised the...
research. All authors discussed the manuscript. **Competing interests:** The authors declare that they have no competing interests. **Data and materials availability:** All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. The atomic coordinates and structural factors for the structures of the wild-type CorC TM domain, its V101A mutant, and the CorC CBS domain in the apo and ATP-bound forms have been deposited in the Protein Data Bank under the accession codes 7CFG, 7CFF, 7CFH, and 7CFI, respectively. Additional data related to this paper may be requested from the authors.

**Submitted 2 September 2020**
**Accepted 23 December 2020**
**Published 10 February 2021**
10.1126/sciadv.abe6140

**Citation:** Y. Huang, F. Jin, Y. Funato, Z. Xu, W. Zhu, J. Wang, M. Sun, Y. Zhao, Y. Yu, H. Miki, M. Hattori, Structural basis for the Mg$^{2+}$ recognition and regulation of the CorC Mg$^{2+}$ transporter. Sci. Adv. 7, eabe6140 (2021).