Three-dimensional structure of the human copper transporter hCTR1

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Copper uptake proteins (CTRs), mediate cellular acquisition of the essential metal copper in all eukaryotes. Here, we report the structure of the human copper transporter protein solved by electron crystallography to an in plane resolution of 7 Å. Reminiscent of the design of traditional ion channels, trimeric hCTR1 creates a pore that stretches across the membrane bilayer at the interface between the subunits. Alignment of the helices identifies the second transmembrane helix as the key element lining the pore, and reveals how functionally important residues on this helix could participate in Cu(I)-coordination during transport. Aligned with and sealing both ends of the pore, extracellular and intracellular domains of hCTR1 appear to provide additional metal binding sites. Consistent with the existence of distinct metal binding sites, we demonstrate that hCTR1 stably binds 2 Cu(I)-ions through 3-coordinate Cu-S bonds, and that mutations in one of these putative binding sites results in a change of coordination chemistry.

Results

hCTR1 Molecules Pack Tail-To-Tail in a Double-Layered Crystal Form. We reported the projection structure of hCTR1, obtained from a double-layered crystal form with p622 plane group symmetry (19). The projection revealed that hCTR1 is trimeric and suggested that the trimer contains a pore along the central 3-fold axis. Moreover, the shape of the putative membrane conduit for copper ions remains unknown, and no details could be derived about the arrangement of the transmembrane helices both within the monomer and the trimer. We therefore extended the analysis by recording images of crystals tilted up to ~46° (Table 1). The final reconstruction was calculated to an in plane resolution of ~7 Å in the plane of the membrane and ~15 Å in the vertical direction. The 3D-data confirmed the double-layered nature of the crystal form as it showed 2 well-resolved layers of hCTR1 trimers that were related by 2-fold axes (Fig. 1A). ImmunoGold labeling of the crystals revealed that the transporter molecules in the 2 layers were packed tail-to-tail with the intracellular C-terminal domains facing each other, because only antibodies recognizing extracellular parts of the transporter were able to bind to the surfaces of the crystals (Fig. 1E and F and Figs. S1–S3).

hCTR1 Contains a Cone-Shaped Pore at the Center of the Trimer. When seen from the direction parallel to the membrane, each hCTR1 trimer appeared as a compact molecule with dimensions of ~85 × 45 × 45 Å. Within the central region of the trimer, 9 well-resolved rods of density were observed that spanned a distance of at least 30 Å (Fig. 1A). At ~7 Å resolution, the spacing and dimensions of these densities were consistent with those of α-helices and therefore likely to represent the transmembrane helices (TM) of the transporter. The structure thus confirmed that each hCTR1 monomer spanned the bilayer 3 times (see Fig. S4A for topology diagram). A longitudinal section (Fig. 1B) revealed a cone-shaped internal vestibule that stretched across the entire membrane along the 3-fold axis of the trimer. At its narrow end toward the extracellular exit, the pore measured ~8 Å across, neglecting the contribution of sidechains, and only a single TM from each monomer contributed to the lining of the pore in this region, reminiscent of the structural design of classical channel proteins such as potassium or mechanosensitive channels (21–23). Toward the inner surface of the membrane, the cone expands into a cavity ~20 Å across.

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Data deposition: The structure reported in this paper has been deposited at the Electron Microscopy Data Bank, www.ebi.ac.uk/msdsmdb (accession no. EMD-1593).

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tracellular end, the pore widened into a large and presumably aqueous cavity, measuring ~22 Å across. This change in pore diameter was caused by a pronounced rearrangement of the helix packing, which morphed from a triskelion-shaped arrangement at the extracellular side into an even distribution of the helices around the pore at the intracellular side of the membrane (Fig. 1C and D).

**Second Transmembrane Helix Is the Principal Pore Lining Helix of hCTR1.** To obtain further insights into the structure and possible mechanism of copper transport, we assigned the helices in the density map to the helices in the amino acid sequence. Based on the constraints given by our ImmunoGold-labeling results, biochemical data and clues in the density map itself, the assignment shown in Fig. 2 emerged as the most likely out of 12 possible models (six for each of the 2 possible orientations of the trimer with respect to the bilayer). In this assignment, TM1 was pinched between TM2 and TM3 from the same monomer, which resulted in a topology shared with the “3-helix bundle” architecture that is observed within the larger bundles of transporters in the major facilitator family (24, 25). Toward the intracellular side, contacts between subunits were mediated by interactions between TM2 and TM3 from adjacent monomers, whereas close to the extracellular side of the membrane all interactions between monomers were mediated by TM2. This design placed TM2 at the center of the trimer in the narrowest part of the pore, making TM2 the singly most important TM-segment to define the pore itself. Notably, the density map suggested that functionally important methionine residues within a MxxxM-motif at the extracellular end of TM2 (26–28) resided in close apposition to each other. To test this prediction, we generated a M154C mutant in a cysless background of hCTR1 (Fig. 2F and Fig. S5). As shown in Fig. 2G, this mutant was able to form a disulfide bond, indicating that symmetry related positions of M154 approached each other very closely within the trimer (Fig. S5D). In contrast, a TM2 H139C mutant did not form disulfide-linked monomers, which was consistent with the structure as observed. Interestingly, the H139C mutant also was less efficient at forming trimers than the other mutants used for this study (Fig. S5A and B). This suggested that H139 might be involved in interactions between monomers, rather than acting as potential ligand for copper ions (26).

### Table 1. Summary of structural statistics

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*Measured from the point spread function of the fitted data set (20).
†For fitted lattice lines, including data to IQ8 and to a nominal in-plane resolution of 6.0 Å.

Fig. 1. Molecular organization of hCTR1. (A) Side view of a CTR1 dimer-of-trimers that constitutes the basic building block of the 2D-lattice (contoured at 2σ above mean). Solid black lines indicate the approximate boundaries of the hydrophobic core of the membrane, assuming a thickness of 30 Å. The orientation (topology) of the trimers in the 2 layers was derived from gold labeling of hCTR1 2D crystals (E and F) and immuno-precipitation experiments using WT and cysless hCTR1 (Fig. S3). (B) Slab of a longitudinal section through the middle of one trimer molecule illustrating the shape of the membrane-spanning pore. Red double-headed arrows mark the narrowest (~8 Å, side chains neglected) and widest part (~23 Å, side chains neglected) of the pore. Areas marked red correspond to extramembrane densities that cap the pore at its ends. (C and D) 10 Å wide cross-sectional slabs taken from the extracellular (C), and intracellular (D) end of the membrane domain illustrate how the 9 TMs (three from each subunit) form the pore. (E and F) Representative examples of ImmunoGold labeling experiments demonstrating that the extracellular hemagglutinin epitope (HA) of the recombinant HA-hCTR1<sup>N15Q</sup> is accessible on the surface of the 2D lattice (E) whereas an epitope contained in the intracellular C terminus of hCTR1 is accessible only along the edges of crystals (F).
Fig. 2. Helix assignment. (A) Side view of a HA-hCTR1N15Q trimer (contoured at 3σ above mean) reveals a direct connectivity (red asterisks) between 2 TM-segments on the extracellular side of the membrane. TM segments are labeled with the number of the corresponding TM segments in the sequence (1–TM1, 2–TM2, 3–TM3). Assignments are shown for 2 subunits (TM123 and TM123’, respectively). (B) Top view looking down the 3-fold axis as seen from the extracellular side. The putative assignment of the subunit boundary (red triangle) and the short loop (red asterisk) connecting TM2 and 3 are shown. (C–E) Cross-sections through the membrane embedded domain. (F) Extracellular side. (D) Middle of the bilayer. (E) Intracellular side. Numbering of helices is as described for A. (F) Helical wheel plot of TM2. Nonpolar residues are colored gray. Black coloring indicates residues that are polar or possess metal binding properties. Residues His-139 and Met-154 are explicitly labeled because they were individually replaced by cysteine for disulfide cross-linking experiments. (G) Disulfide cross-linking of recombinant, purified HA-hCTR1N15Q(WT), HA-hCTR1N15Q,C161A,C189A (Cysless), HA-hCTR1N15Q,C161A,C189A,M154C (M154C), and HA-hCTR1N15Q,C161A,C189A,H139C (H139C). Proteins were incubated in the presence of copper phenanthroline (Cu-P) to facilitate disulfide bridge formation, separated and blotted. Ovals to the right indicate the mobility of hCTR1 oligomeric species in SDS. Similar results were obtained without use of Cu-P. Dimer formation of WT is through disulfide bridge formation between cysteines in the C-terminal HCH-sequence of 2 subunits of the hCTR1 trimer (19, 37). White separation between lanes indicates that some data were taken from separate gels.

**TM3 Is Involved in Tight Helix Packing.** Further evidence for the model came from the observation that TM2 showed a direct connectivity to one of the adjacent helices on the extracellular side of the membrane (labeled by red asterisks in Fig. 2A and B). Coincidentally, the loop that connects TM2 to TM3 on the extracellular side of the membrane is predicted to consist of only 3 residues (Fig. S4). This was consistent with the density map, and identified TM3 as the helix that was the most perpendicular to the membrane plane. Notably, the presence of an abundance of small amino acids—including the structurally important GxxxG-motif (29)—allowed TM3 to approach TM1 of the same monomer so closely that the 2 helices were not completely resolved at 7 Å in plane resolution (Fig. 2D). Supporting the idea that the GxxG-motif may contribute to tight helix packing between TM1 and 3, similar “merging” of helix cross-sections was observed in regions harboring GxxG-motifs in other membrane proteins when their known crystal structures were filtered to the lower resolution of our reconstruction (Fig. S6). Having assigned both TM2 and TM3 left only one choice for TM1, thus completing the assignment of helices in the density map.

**hCTR1 Stably Binds 2 Cu(I) Through 3-Coordinate Cu–S Bonds.** The model that emerged from assigning the TM-helices suggested that Met residues of the MxxxM-motif on TM2 could contribute directly to copper conduction along the central pore of hCTR1 by providing a closely spaced pair of 3-coordinate binding sites for the ion. Resonating with the idea that copper moves across the plasma membrane by exchange between binding sites, additional features, capping the pore at both ends (Figs. 1B and 2A, and Fig. S7), were positioned about the 3-fold axis of the trimer. Located outside the membrane, these additional densities must have been formed by the N-terminal and intracellular domains of hCTR1, suggesting that the extramembranous regions of CTR1 provided important structural elements that regulated copper access to and from the pore. Indeed, previous biochemical studies established the importance of an MPM-sequence motif in the N terminus of CTR-proteins (26–28) and, Cys-containing clusters of metal binding amino acids are found at the C termini of many CTR-proteins. Interestingly, the copper binding ability of these Cys-containing sequences has been implicated in playing important roles in CTR1 function (30–32) (Fig. S4). An attractive hypothesis was that the MPM-motif and HCH-sequence were located within the additional density features that guarded the ends of the copper permeable pore in the density map. This hypothesis implied that hCTR1 should contain distinct Cu(I)-binding sites. To test this idea, we determined the copper binding properties of WT hCTR1 and a cysteine mutant that lacked the potential sulfur-ligand in the C-terminal HCH-sequence of the hCTR1 sequence. Inductive plasma-organic emission spectroscopy (ICPOES) indicated that each of these proteins stably bound 2 Cu(1)-ions per trimer (See Fig. 3C). This result could have been fully accounted for by Cu(I)-coordination through the N-terminal MPM- and TM2 MxxxM-motifs, which would also explain why mutating the C-terminal HCH-sequence to HAH in the cysteic hCTR1 did not change the stoichiometry of Cu(I)-binding. However, ICPOES does not reflect the actual chemistry of metal coordination. Therefore, a contribution of the C-terminal HCH-/HAH-sequence could still have been
possible if in the cysless mutant Cu(I) ions were coordinated by His residues. To resolve this issue, we turned to extended X-ray absorption spectroscopy (XAS) to determine the coordination chemistry of the bound copper ions in both the WT and cysless hCTR1 transporters. For the WT protein, the data were best simulated by 3 Cu–S interactions per binding site at 2.25 Å (Fig. 3A and Table S1), consistent with the hypothesis that both Cu(I) ions were bound either by Cys, Met or both (e.g., MPM-motif, MxxxM-motif). In contrast, the cysless protein exhibited an altered EXAFS spectrum that showed the appearance of Cu-N(His) coordination (Fig. 3B). The easiest explanation for this observation was that the C-terminal HCH/HAH-motif contributed to Cu(I)-binding in both proteins because the appearance of Cu-N(His)-coordination and the lengthening of the Cu–S bond to 2.31 Å (which is more typical of Cu–S(Met) than Cu–S(Cys) (33, 34)) were a consistent with a replacement of the lost cysteine ligands by histidines (see Table S1 for details on fits of the EXAFS data). Although additional studies, will be required to fully establish the contribution of multiple binding sites to copper capture, transport and distribution, the data presented here are the first direct proof that hCTR1 can bind Cu(I) through distinct binding sites, and that the trimers are able to stably accommodate 2 copper ions.

Discussion
Recent years have seen much progress in elucidating the mechanistic details of copper trafficking within cells (18, 35). In contrast, the mechanism of copper movement across cellular membranes has been elusive, in part because no detailed structural information had been available for copper transport proteins of the CTR-family or the copper transport ATPases. Extending our previous projection structure, the 3D-structure of hCTR1 reveals that overall the transporter conforms with the design of a “traditional” ion channel in which a membrane spanning pore is created by symmetry related TM-segments from multiple subunits of a symmetric oligomer (19). Furthermore, the strictly conserved MxxxM-sequence motif on TM2 of CTR-proteins provides ligands that are likely to bind Cu(I) during passage, reminiscent of the selectivity filter in K-channels (22). Supporting this idea, the TM2 M154C-variant of hCTR1 can form disulfide-linked dimers, which places the symmetry related methionine residues in direct vicinity to the 3-fold axis. Moreover, the EXAFS data demonstrated 3-coordinate Cu(I)-binding, which to date is the most compelling evidence that copper moves at the interface between subunits of the CTR trimer.

Although the structure, EXAFS, and biochemical data all argue for a participation of the MxxxM-motif in Cu(I) transport, the exact mechanism of their contribution remains unknown. In the simplest case, the sulfur atoms of the 2 “triangles” of methionine residues form a “static slide” that allows copper to pass along. Alternatively, the 2 consecutive binding sites may be “out of register” or, even more extreme, only 1 of these 2 binding sites may adopt the proper geometry for Cu(I)-binding at any one time. Any mechanism involving these latter 2 cases implies that passage of copper is associated with conformational changes. Interestingly, 3 independent lines of biochemical evidence all point toward a mechanism that involves conformational changes during transport: (i) fluorescence resonance transfer studies suggest that the C termini move during copper transport in yeast CTR1 (36), (ii) high-extracellular copper concentrations result in a clearance of hCTR1 from the cell surface (which can only happen if the occupancy of extracellular Cu(I)-binding sites is communicated to the intracellular side of the membrane) (28) and (iii) trypsin digestion of hCTR1 in the absence or presence of copper ions results in different proteolytic cleavage patterns (37). In addition to these observations, our own studies are also suggestive of a mechanism that involves different conformational states. First, the EXAFS data demon-
strated that the 2 bound ions were spatially well separated because no copper clusters were detected in the spectra for either WT or cysteine hCTR1. Chemically, this separation makes sense because the presence of large distances between the potential metal binding sites at the N and C termi

and the sites furnished by the MxxxM-motif on TM2 may require copper release from and recapture by the protein as the ion travels through the membrane. Because this would generate solvated Cu(I), direct collision between copper ions must be avoided to prevent disproportionation to Cu(0) and Cu(II), suggesting that any movement of copper ions through CTR1 needs to be highly coordinated. Second, biochemical evidence shows that the Met residues in the MxxxM-motif can be replaced by Cys or His residues (27), and even abrogation of one of the sites through substitution by Ile residues is compatible with transport (26).

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52x352]solubilized in 10 mM Mops pH 7.4, 280 mM NaCl, 2 mM EDTA, and 60 mM n-octyl β-D-glucopyranoside (Anatrace). Gel-filtered hCTR1 (0.8–1 mg/mL) was then added to solubilized lipids to a protein/lipid ratio of 1:5:1 (final protein 37 μg), and mixed for 1 h at 26 °C. Detergent was removed by dialysis (24)

Images were collected on a Tecnai F20 field-emission microscope, operated at 200 keV, liquid nitrogen temperatures, magnification, and mean underfocusing ranging from 4,000 to 15,000 Å. Samples were mounted on GATAN 622 side-entry cryoholders. Images were collected at 10–12 e/Å2. Nominal sample tilts were in the range of 0–46°. Spectral imaging was carried out with the program EXCURVE 9.2 (Binsted N, Gurman SJ, Campbell JW (1998) EXCURVE 9.2 Warrington, England (44–46) as described in refs. 43, 47, and 48.

Other Methods. Mutation constructs, fermentation and purification descriptions, disulfide cross-linking experiment description, and more in depth image processing methods are included in the SI Materials and Methods.

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Supporting Information

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SI Materials and Methods

Mutants. Mutations were introduced into the HA-hCTR1^{N15Q} (WT) (1) background using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). First, the 2 endogenous cysteines were mutated to alanine, to generate a cysses hCTR1 variant. The cysses template was then used to introduce single Cys residues by replacing M154 (M154C) or H139 (H139C). All constructs were verified by nucleotide sequencing and transformed into the P. pastoris strain SMD1163, as described in ref. 1.

Fermentation and Purification. Recombinant HA-tagged hCTR1^{N15Q} was expressed in P. pastoris (strain SMD1163). The recombinant strains were fermented in a BioFlo 110 Fermentor (New Brunswick Scientific). Initial growth in minimal medium was followed by ~55-h glycerol batch feed (www.nbsc.com/TechPaperDetails.asp?paptype=Pichiapastoris&Section=Pichiapastoris), typically reaching an OD_{600} of >150. The culture was then starved, and the pH was raised from 5.0 to 6.0. Expression of hCTR1 was induced for 15–18 h by feeding methanol, supplemented with trace metals, and a lower than normal copper content (0.5 μM). Cells were harvested and flash-frozen in liquid N2. A fraction of the total harvest was used to purify the recombinant hCTR1 as described in ref. 1, except for the following modifications: 4 mM EDTA was added to the eluate of the metal-affinity step, fractions were concentrated to ~10 mg/mL, and incubated at 4 °C for at least 24 h before final gel filtration on a Superdex 200 10/300 GL column (GE Healthcare). Fractions from the gel filtration were stored in 10 mM Mops pH 7.4, 280 mM NaCl, 2 mM EDTA, with 4 mM α-decyl β-D-maltoside (DM) (Anatrace) at 4 °C until later use.

Disulfide Cross-linking. In addition to WT protein, the cysses, H139C and M154C mutants were femerated and purified as described above. For the cross-linking reaction, CuSO_{4} (20 mM stock in ddH_{2}O) and 1,10-phenantroline (67 mM stock in ethanol) were added to gel-filtration fractions of hCTR1 trimer to give final concentrations of 13 μM and 45 μM respectively. The oxidation reaction was allowed to proceed for 15 min before quenching by the addition of 1 mM N-ethylmaleimide (500 mM stock in ethanol; Sigma) and 25 mM EDTA. SDS/PAGE sample loading buffer with and without 5% 2-mercaptoethanol was added, and samples were run on 12% SDS/PAGE gel, followed by Western blot analysis.

Image Processing. A low-resolution 3D-model obtained from negatively stained crystals served as initial reference for determining the approximate tilt geometry, and handedness of the data from unstained samples. After merging data from several crystals with low tilts, the combined data contained sufficient 3D information to allow successive addition of images without need for the initial model. Image data (amplitude and phase) were merged in the 2-sided plane group p622, using ORIGITLTD. LATTILE was then used to fit lattice lines (c = 400, real space envelope = 150, 150) and a 3D model was calculated using data to ~15-Å resolution, where data were complete and not affected by errors in the assignment of the contrast transfer function (CTF). This first model calculated from data of unstained crystals then served as reference for an initial refinement of the tilt geometry of all crystals. Using the refined tilt geometries, the data were then merged to 6 Å, and inspection of the phase data identified images that needed adjustments in their defocus values. After CTF-corrections, a final refinement of the tilt geometries was carried out. In a final refinement step, images of large crystals (>4,000 × 4,000 pixel) with tilts >35° were reprocessed taking into account the effects of the tilt transfer function (TTMASK, TTBOX), and using the tilt geometries and defocus values that were determined through the previous refinements. Data were then remerged, lattice lines were fitted, and fitted structure factors with phase errors >60° were deleted from the fitted dataset (PREPMKMTZ).

Immunoprecipitations. The following samples were used for these experiments: The leading edge of the dimer-of-trimer peak during gel filtration of the HA-hCTR1^{N15Q} (WT-Hexamer) species, the middle of the peak representing trimeric HA-hCTR1^{N15Q} (WT-Trimer), and the leading edge of the peak for the trimeric HA-hCTR1^{N15Q,C161A,C189A} (Cysless) (Fig. S3). To carry out the precipitations, ~15 μL of ProteinA/G beads (Calbiochem) were washed 4 times in crystallization buffer that contained 4 mM DM and preincubated with ~500 μL of primary antibody [anti-C-terminal (1:1,000), anti-loop (1:70) and anti-N-terminal (1:40)] for 1 h while rotating slowly. After washing the beads 4 times in buffer, 6 ng of hCTR1 (in ~500 μL of buffer) was added to the antibody-bound beads and incubated for 1 h while slowly rotating. At the end of the incubation, beads were allowed to settle, the supernatant was removed (and stored), and the beads were washed 4 times in protein-free buffer as above. Beads were then resuspended in a volume of buffer equal to volume of supernatant that was removed (10 mM Mops pH7.4, 280 mM NaCl, 2 mM EDTA, with 4 mM DM) and 2× SDS/PAGE loading buffer was added to all samples. Out of this final volume, equal amounts of the samples (typically ~25 μL, containing ~120 pg of protein) were separated on 12% SDS/PAGE gels, blotted, and blots were probed with anti-HA antibody. ImageJ (2) was used to quantitate the amount of hCTR1 in the supernatant and precipitated lanes. The percentage of pulled-down to total protein in both supernatant and bead lanes were plotted in a histogram (Fig. S3).


Fig. S1. Low magnification images from gold-labeled hCTR1 2D crystals. Two antibodies were used for extracellular epitopes: HA-tag (A) and an N-terminal epitope (B). Similarly, 2 antibodies were used to probe for intracellular epitopes located in the large intracellular loop (C) and the C terminus (D). (Scale Bar: 0.5 μm.)
Fig. S2. High magnification images from gold-labeled hCTR1 2D crystals. Same as Fig. S1, but images were taken at higher magnification to see the gold labeling in relation to the lattice. (Scale bar: 50 nm.)
Fig. S3. Antibody accessibility experiment. The “dimer-of-trimer” species of hCTR1 was used for crystallization. Gold labeling experiments of the crystals showed that only N-terminal epitopes were accessible on the crystal surfaces. To further analyze epitope accessibility we carried out immunoprecipitation (IP) experiments on 3 samples: the dimer-of-trimer used for crystallization (WT-Hexamer) (A), a trimeric form of WT hCTR1 that was obtained as a separate fraction during the final gel-filtration step (WT-trimer) (B), and trimers formed by the cysless hCTR1 variant (C). Gel-filtration fractions whose protein was used in the experiments are indicated in A–C, and pictograms are used to depict whether the species is a trimer (single cylinder) or a “dimer-of-trimers” (double cylinder). The solid red lines demarcate the gel-filtration fraction used in the experiment. (D) Histogram representing the fraction of hCTR1 that was immunoprecipitated relative to the sum of the IP and supernatant lanes on a SDS/PAGE gel. Error bars indicate the standard deviation from 3 to 4 separate experiments. These experiments indicated that the C terminus is the most sterically hindered epitope, but is easily accessible in the obligate trimer formed by the cysless mutant. Note that the intracellular loop epitope was recognized equally well in all 3 protein variants, suggesting that the C terminus was nested within a space that is bounded by the intracellular loops.
CTR topology. (A) CTR proteins are known to possess 3 transmembrane helices, an extracellular N terminus, intracellular C terminus, a large intracellular loop, and a very short extracellular loop connecting TM2 and 3 (red star). Although most of the conserved amino acids are contained within the TM segments, there are conserved regions with metal binding capabilities immediately proximal to the membrane-spanning region (B). The residues highlighted in B are the most conserved metal binding domains just proximal to the entrance and exit to the membrane domain.

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Further description of HA-hCTR1 mutants used in the disulfide cross-linking experiment. (A and B) The gel filtration profiles of the purified hCTR1 constructs are presented in (A&B). (A) Profiles of HA-hCTR1N15Q (WT) and the HA-hCTR1N15Q,C161A,C189A (Cysless) mutant. (B) WT, HA-hCTR1N15Q,C161A,C189A,M154C (M154C), and the HA-hCTR1N15Q,C161A,C189A,H139C (H139C) mutant. To facilitate comparison between traces, the y axis was normalized, and all traces were aligned along the x axis starting at 0 mL. Arrows highlight the elution volume of the trimeric species that was used for the disulfide cross-linking experiments of each of the protein variants. Individual traces are identified to the right of the chromatograms. Note, the chromatogram for the H139C indicates a mix of defined oligomeric states. (C) Sequence of the hCTR1 construct used in this study and the positions of each mutant. Of the 4 different mutants mentioned in A and B, all, including the WT construct, contain the N15Q mutation indicated in gray. The 2 additional mutations in the cysless mutant are indicated in the 2 blue boxes. The 2 final mutants contain all of the previous 3 mutations in addition to either the M154C mutation, or the H139C mutation, highlighted in the red and yellow boxes respectively. (D) The relative location of these mutations is indicated in the topological diagram, with the same color scheme as in C. The arrows pointing from the topology diagram to the 2 cross-sections of hCTR1’s membrane domain (top-extracellular and bottom-intracellular; see Fig. 2C and E) help to illustrate our interpretation of the disulfide experiment presented in the text. The top cross-section illustrates that the disulfide identified when the M154C mutant is oxidized (see Fig. 2G), could be explained by an intermolecular disulfide (line connecting 2 adjacent monomeric subunits; division of monomers indicated by ovals in both cross-sections) because of the close apposition of TM2 to itself at the extracellular side of the membrane domain. This would leave 1 remaining free cysteine (indicated by the red X), and thus explain why both a monomer and a dimer oligomeric species is observed when separated on an SDS/PAGE gel without reducing agent (see Fig. 2G). The cysteines in the H139C mutant, however, given their putative location on the intracellular side of TM2, would be 2 far away from its symmetrically related position to form a disulfide under oxidizing conditions (bottom cross-section; red Xs). Given our helix assignment and crystal topology, this interpretation would explain why the H139C mutant could only migrate as a monomer when separated under similar conditions.
GxxxG (GG4)-motifs at low resolution. TM3 in hCTR1 contains a highly conserved and essential GG4 motif. (A) When cross-sectioned (Upper Right) near the middle of the membrane (black solid line, Upper Left), where the GG4-motif was located, the TM-segments revealed a “budding-yeast” packing morphology between TM3 and TM1 (black stars, Upper Right). This morphology resembled the envelope of GG4 packing contacts (red stars, Right) in other membrane proteins (B and C) if their crystal structures were filtered to ~7Å resolution (Using EMAN [Ludtke SJ, Baldwin PR, Chiu W (1999) J Struct Biol 128: 82–97]). Examples shown here are the structures of aquaporin (B, 2F2B) and the ammonia channel (D, 1U77). Cross-sections were taken along the planes demarcated by the solid black line (in Left), and are shown to the right of their corresponding high-resolution structures. The GG4 residues from the PDB models for each protein are highlighted in red.
Fig. S7. Pore blocking density is significant. View down the 6-fold symmetry axis from the extracellular end (A) and the intracellular end (B). The map only contains 1 layer of the crystals for clarity and is contoured at 3σ. The surface area of the densities blocking the pore at both sides of the membrane is highlighted red.
Fig. S8. Comparison of fits of XAS-data. (A) Simulations of XAS data for 2 coordination schemes (cysless Fits A and C in Table S1): black line 2 Cu-S and 1 Cu-N(His), red line 1.5 Cu-S and 1.5 Cu-N(His). (B) Overlay of simulated data with experimental data.
Table S1. Fits obtained to the EXAFS of WT CTR1 and its cysless variant by curve-fitting using the program EXCURVE 9.2

<table>
<thead>
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<th>F†</th>
<th>C-N(His)*</th>
<th>Cu-S</th>
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<td>No.‡</td>
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*Fits modeled histidine coordination by an imidazole ring, which included single and multiple scattering contributions from the second shell (C2/C5) and third shell (C3/N4) atoms respectively. The Cu-N-Cα angles were as follows: Cu-N-C2 126°, Cu-N-C3 126°, Cu-N-C4 163°, Cu-N-C5 163°.

†F is a least-squares fitting parameter defined as \( F^2 = \frac{1}{N} \sum_{i=1}^{N} \frac{k^6}{(\text{Data} - \text{Model})^2} \).

‡Coordination numbers are generally considered accurate to ±25%.

§In any one fit, the statistical error in bond-lengths is ±0.005 Å. However, when errors due to imperfect background subtraction, phase-shift calculations, and noise in the data are compounded, the actual error is probably closer to ±0.02 Å.