



# Structure of the Ctr1 copper trans'PORE'ter reveals novel architecture

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**Copper is essential for biological processes such as free radical detoxification, mitochondrial respiration and iron metabolism. A central player in copper homeostasis is the high-affinity integral plasma membrane copper transporter Ctr1. However, the precise mechanisms by which Ctr1 functions are not known. Here, we highlight an important breakthrough in our understanding of how Ctr1 facilitates Cu(I) movement across membranes: the publication of structural details for human Ctr1 obtained from 2D crystallography and electron microscopy.**

## Eukaryotic copper transport

Copper is an essential nutrient for almost all eukaryotic organisms to carry out biological processes such as free radical detoxification, mitochondrial respiration, iron metabolism, neuropeptide maturation, connective-tissue formation and pigmentation. The redox potential of copper ions makes this metal a useful biological cofactor, but also enables it to have potential for toxicity. If allowed to react with oxidizing agents and undergo Fenton chemistry, copper can irreversibly damage cellular components including proteins, lipids and DNA. Fortunately, organisms have evolved mechanisms to carry out and tightly regulate the movement of copper across membranes and between proteins (for review, see Refs [1,2]). A central player in copper homeostasis is the high-affinity integral membrane copper transporter Ctr1 [3,4]. Recent work from Aller and Unger [5] represents an important breakthrough in our understanding of how Ctr1 facilitates Cu(I) movement across membranes, with the publication of structural details for human Ctr1 obtained from 2D crystallography and electron microscopy. Their data clearly demonstrate that human Ctr1 forms a symmetrical homotrimer with a putative pore between the subunit interfaces (Figure 1), suggesting that this family of copper transporters is structurally similar to channel proteins. Furthermore, the data address fundamentally crucial aspects of the mechanisms by which Ctr1 transports Cu(I) across the plasma membrane to the intracellular delivery pathway [5].

## Biological and physiological relevance of Ctr1

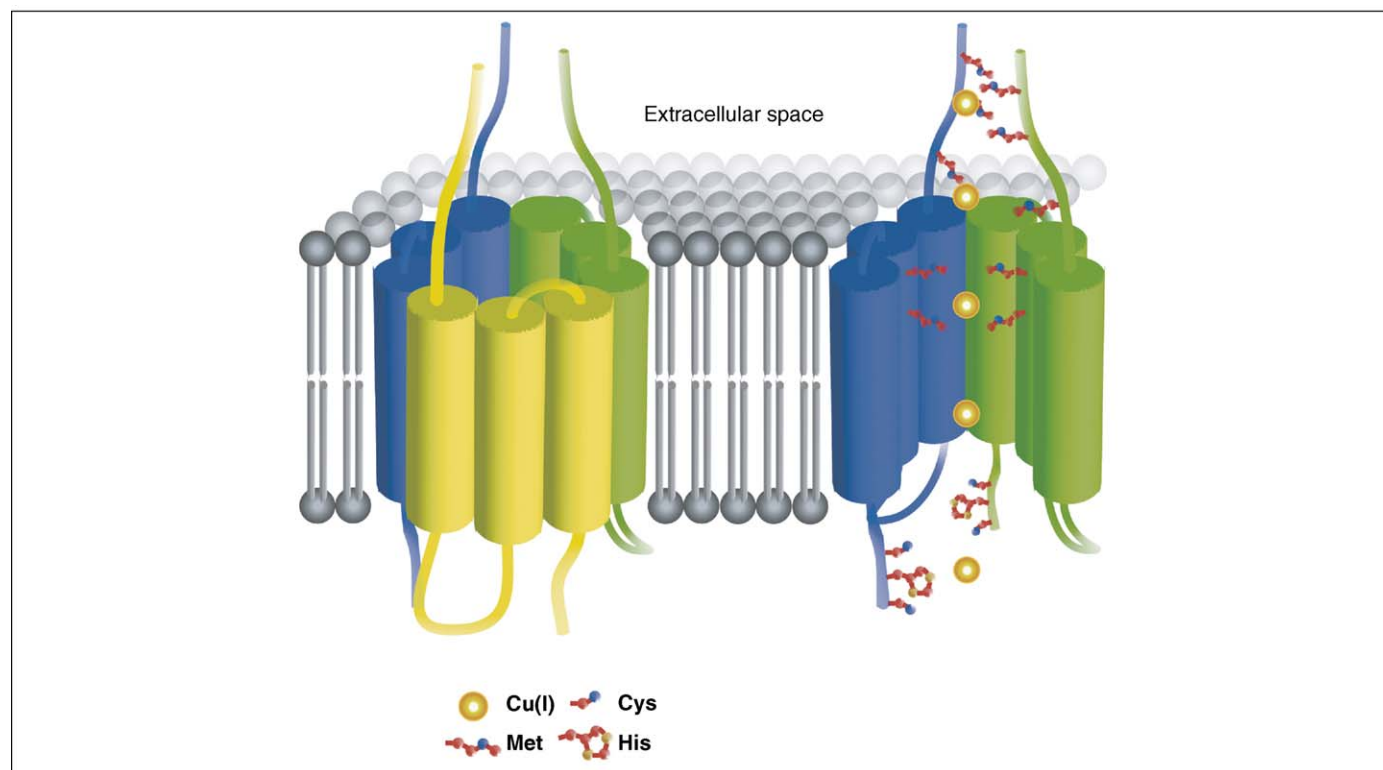
Ctr1 is a member of a family of proteins that provide copper to the copper chaperones, which then traffic copper ions to specific intracellular proteins or compartments such as the secretory machinery, mitochondria or Cu, Zn superoxide

dismutase. The importance of Ctr1, and by inference its role in copper uptake, in mammalian growth and development was demonstrated in two independent gene-targeting studies in mice [6,7]. Indeed, homozygous deletion of Ctr1 resulted in embryonic lethality and a range of developmental phenotypes, with *Ctr1*<sup>-/-</sup> embryos ultimately resorbed into the uterus mid-way through gestation. Importantly, whereas *Ctr1*<sup>+/-</sup> mice exhibited no outward growth or developmental defects, they displayed copper accumulation defects in a subset of tissues including brain and spleen. Mouse embryonic fibroblasts lacking Ctr1 are largely defective in high-affinity copper uptake, demonstrating a prominent physiological role for Ctr1 in copper accumulation [8]. Furthermore, studies have suggested a role for Ctr1 in the accumulation of cisplatin, a potent and effective anti-cancer agent [9].

## Ctr1 mechanism of action

Although the precise mechanisms by which Ctr1 enhances copper movement across the plasma membrane are not known, recent studies have provided a basis for a working model. First, Ctr1 acts on reduced copper [Cu(I)] rather than the oxidized metal ion, Cu(II). This preference for Cu(I) is supported by the requirement of yeast cell-surface metalloreductase activity for high-affinity copper uptake [10], the stimulation of copper uptake in yeast and mammals by external reductant [10], and the ability of silver, a Cu(I) mimetic, to strongly compete for copper uptake stimulated by Ctr1 [11]. Second, studies by transfection into mammalian cells and assays of endogenous copper uptake activity in wild type and Ctr1 knock-out yeast and mammalian cells indicate that Ctr1 has a Michaelis–Menten  $K_m$  for copper in the low micromolar (1–5  $\mu$ M) range [3,11]. Third, mutagenesis experiments demonstrate that a lone conserved methionine residue in the N-terminal extracellular domain and two methionine residues in the second transmembrane domain (Met-Xaa-Xaa-Xaa-Met) are essential for both yeast and mammalian Ctr1 function [12]. Although serine or alanine residues at these positions render Ctr1 incapable of accelerating the rate of copper uptake *in vivo*, the partial functional replacement of methionine with either histidine or cysteine supports the idea that the three crucial methionine residues might function in the transport process via direct coordination to Cu(I) [12]. Fourth, the lack of an identifiable ATP-hydrolysis domain coupled with studies of energetic requirements for copper accumulation in cell culture indicate that Ctr1 facilitates copper movement across

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**Figure 1.** Interpretation of the structural and functional model of Ctr1. The radial symmetrical homotrimer of Ctr1 is shown (left). Nine transmembrane domains form a pore in the lipid bilayer. One subunit of Ctr1 is removed to reveal the interior of the pore (right). Ctr1 might initially bind to Cu(I) via the conserved methionine residues. Copper might sequentially bind to the methionine residues near the first transmembrane domain, the Met-Xaa-Xaa-Met motifs in second transmembrane domain, and C-terminal His-Cys-His residues. Each Ctr1 monomer is represented in a different color. Copper atoms (orange spheres) and the sidechains of methionine, histidine and cysteine are indicated.

membranes in an ATP-independent manner [11]. Fifth, in contrast to transporters such as the divalent metal transporter 1 (DMT1), which transports Fe(II), Mn(II), Zn and other divalent metal ions [13], Ctr1 is specific for Cu(I) [11]. Whereas genetic and biochemical experiments have provided an initial glimpse into the importance of conserved structural features and reveal clues as to how Ctr1 might function, the data provided by Aller and Unger [5], together with future detailed structural studies, are essential for deciphering the mechanistic details of Ctr1-mediated copper and cisplatin accumulation.

### Structural details of Ctr1

Although the functional features of Ctr1 strongly support its role as a metal ion permease, the presence of only three putative transmembrane (TM) domains presented a puzzling question of how it could move Cu(I) across the plasma membrane. Other characterized transporters typically either possess a sufficient number of TM domains within a single polypeptide to form a pore, or form transport pores at the interface of two distinct transport subunits. Aller and Unger [5] describe the unique structure of human Ctr1 protein crystals in a native phospholipid bilayer. The data clearly demonstrate that human Ctr1, with a membrane topology based on previous differential epitope accessibility experiments [12], forms a symmetrical homotrimer with a putative pore between the subunit interfaces (Figure 1). Whereas the region of low electron density in the center of the trimer is estimated to be ~9 Å in diameter, the pore is likely to be smaller given that amino acid

sidechains are not resolved. The channel architecture of Ctr1 proteins suggests the potential need for a gating mechanism to control the import of Cu(I), which could be achieved either extracellularly by a metalloredox protein or the N terminus of Ctr1, or intracellularly through the cysteine or histidine residues in the cytosolic tail of Ctr1 [14] or via the interaction with an intracellular copper chaperone protein.

This structural elucidation is exciting in light of previous genetic trans-complementation experiments and biochemical purification or crosslinking studies that suggested that higher-order structures, perhaps homodimers or homotrimers, formed by Ctr1 from yeast and human cells are crucial features of Ctr1 function in copper uptake. Initial reports for yeast and human Ctr transporters demonstrated that these proteins form stable species on SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) that are compatible with the expected sizes of homodimers and homotrimers; however, hetero-oligomerization could not be distinguished [15,16]. More definitive evidence for homo-oligomerization came from *in vivo* trans-complementation experiments. Two distinct non-functional mutant alleles of yeast or human Ctr1 were shown to re-constitute a functional Cu(I)-uptake activity when co-expressed in the same yeast cell, providing strong evidence for Ctr1 functioning at least as a homodimeric complex [12].

How does Ctr1 form a homotrimer? Several recent reports have identified potential amino acid residues or motifs that are important for Ctr1 oligomerization.

One study demonstrated that Cys189, located in the C terminal cytosolic domain, is important for oligomerization of human Ctr1 and thereby suggests that Ctr1 multimerization might be nucleated or stabilized via disulfide bonds [14]. Yeast two-hybrid experiments demonstrated that the Ctr1 N-terminal extracellular domain supports self-interaction that is not modulated by copper [17]. Indeed, Unger and colleagues demonstrated in a previous report that the conserved Gly-Xaa-Xaa-Xaa-Gly (GG4) motif in transmembrane domain three of Ctr family transporters is important for multimerization and for proper localization to the yeast plasma membrane [18]. The GG4 motif has also been implicated in intermolecular interactions in other families of transporters. The predicted  $\alpha$ -helical structure of the Ctr1 transmembrane domains places the two glycine residues on the same face of the helix and, therefore, in an appropriate position to engage in protein-protein interactions [18]. Given that 2D crystallography and electron microscopy at 6-Å resolution does not resolve amino acid sidechains, the contributions of the GG4 motif and other residues in intramolecular interhelical packing, intermolecular trimerization or additional roles such as in ion selectivity must await higher-resolution structural determination.

### Implications for Ctr1 trimerization

How might trimerization of Ctr1 be important for its secretion to the plasma membrane, Cu(I)-transport activity and specificity? Mutations in the third transmembrane domain of Ctr transporters that disrupt multimerization also result in mis-localization [18]. As such, it is possible that the homotrimer masks signals in the monomer that are required for retention and folding in the secretory compartment, thereby enabling plasma-membrane localization. Together with observations and speculations from reports by others, Aller and Unger [5] suggest a model for the function of homotrimeric Ctr1 in which Cu(I) first coordinates to the methionine-rich (Mets) motifs in the Ctr1 extracellular N terminus. Although these motifs are not absolutely essential for Cu(I) transport, their importance for Cu(I) uptake *in vivo* is more apparent at highly limiting extracellular Cu concentrations. Furthermore, a single conserved methionine residue ~20 residues upstream of the first transmembrane domain is essential for transport [12]. Moreover, recent *in vitro* experiments with model peptides mimicking the Mets motifs of copper-binding proteins demonstrate that these motifs are sufficient for selectively binding Cu(I) via methionine-only coordination with an affinity corresponding to a low micromolar dissociation constant ( $K_D$ ), which is similar to the  $K_m$  of Ctr1 for copper *in vivo* [19]. As a soft base, the sulfur atoms of cysteine or methionine ligands prefer to bind Cu(I), perhaps enhancing both the specificity and the lability of the bound copper, two features that are desirable for an uptake activity. Once Cu(I) is bound by the sulfur-rich extracellular domain, it might be mobilized down through the pore structure that is symmetrically placed between the subunits in the Ctr1 homotrimer, as illuminated in the Aller and Unger study [5] (Figure 1). Like the glycines of the GG4 motif in Ctr1 TM3, the

Met-Xaa-Xaa-Xaa-Met motif in TM2 would be predicted to position the methionines on the same face of the helix, perhaps lining the intra-membranous pore with thioether-binding ligands contributed by each member of the homotrimer, to carefully control Cu(I) reactivity as it traverses the membrane. More detailed structural information will determine whether the Mets motifs are in close proximity to the putative Cu(I) pore formed by the homotrimer, whether Cu(I) binding to the N terminus results in a conformational change that augments or regulates the movement of Cu(I) through this pore, and the precise structure of the transport pore. Paired cysteine residues in the cytoplasmic C terminus could serve as intracellular donors for Cu(I) for its mobilization to the Cu chaperones. Although it is currently unclear whether these chaperones directly dock with the Ctr1 homotrimer to engage in cargo transfer or whether there are other intermediates in the delivery system, one study indicates that Cu(I) bound to an isolated Ctr1 C-terminal domain can transfer Cu(I) to the Atx1 Cu chaperone *in vitro* [20]. How the trimerization might also influence potential direct interactions with Cu chaperones within cells, or with metalloredutases on the cell surface, is not yet clear.

### Concluding remarks

Should Ctr1 be thought of more as a Cu-specific channel than a metal-transport molecule? Aller and Unger [5] have taken a major step towards understanding the global structure of Ctr1. Further structural studies, including the 3D structure of apo- and copper-bound protein, and perhaps Ctr1-cisplatin and Ctr1-Cu chaperone complexes, will be required to more fully understand the biochemical mechanisms and regulatory interactions used by this family of copper-homeostasis proteins.

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