Supporting Information
for
Cuprous binding promotes interaction of copper transport protein hCTR1 with cell membranes

Experimental details

Materials

Bicinchoninic acid (BCA) and Hoechst (HOE) 33258 were purchased from Sigma-Aldrich. Ni-NTA resin was purchased from Qiagen. Dipalmitoyl-phosphatidylcholine (DPPC) and cholesterol were purchased from Avanti Polar Lipids. FITC was purchased from Aladdin Biotech.

A549, LO2 cells (ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Atlanta Biologicals). The cells were maintained in an incubator at 37 °C and 5% CO_2.

The protein expression and purification

The N-terminal domain of human copper transporter (aa 1-46, $hCTR1_{1-46}$) was expressed as a GB1 fusion protein GB1-$hCTR1_{1-46}$ using the pGBTNH vector.[1] The plasmid containing GB1-$hCTR1_{1-46}$ gene was transformed into *E. coli* BL21 (DE3) cells, and the cells were cultured in LB medium containing 100 μg/ml ampicillin at 37 °C. Protein expression was induced by 0.4 mM IPTG at 37 °C for 5 h when OD_{600} ~0.8 was reached. Cells were harvested by centrifugation (4000 rpm, 20 min, 20 °C), resuspended in binding buffer (20 mM HEPES, 200 mM NaCl, pH 8.0), and lysed by sonication. The lysate was centrifuged at 16000 rpm at 4 °C for 30 min to remove the cell debris. The fusion protein in the supernatant was loaded on Ni-NTA affinity column pre-equilibrated with binding buffer. After washing with 5 column volume buffer (binding buffer supplied with 5 mM imidazole), the protein was eluted by elution buffer (binding buffer supplied with 250 mM imidazole). The $hCTR1_{1-46}$ protein was obtained by removal of the N-terminal GB1-tag with TEV protease digestion. The protein was further purified by Ni-NTA affinity chromatography, gel filtration and HPLC chromatography. The final product was obtained by lyophilization, and was verified by 15% SDS-PAGE and ESI-MS (Figure S2).

Electrospray ionization mass spectrometry (ESI-MS)

Mass spectrometric measurements were performed on Exactive Plus mass spectrometer equipped with a standard ESI source (Thermo Fisher Scientific, CA, U.S.A.). $hCTR1_{1-46}$ (10 μM) were incubated with different molar equivalents of $[Cu(MeCN)_4]ClO_4$ and $CuSO_4$ in 5 mM ammonium acetate buffer at room temperature for 5 min. Samples were directly injected into the mass spectrometer. Data were processed by Xcalibur software (version 2.0, Thermo Finnigan).

Cu K-Edge X-ray absorption spectroscopy

Solutions of Cu(I) with $hCTR1_{1-46}$ (0.5 mM protein, 0.4 mM $[Cu(MeCN)_4]ClO_4$; 100 mM HEPES, 50% v/v glycerol) were prepared under an N_2 atmosphere, injected into Teflon sample holders with a Kapton tape window, and rapidly frozen. Prior to data collection, the samples were inspected with no obvious ice

crystals.

The prepared samples were subjected to x-ray absorption spectroscopy (XAS) analysis to characterize the local structure around Cu binding site. XAS experiments were employed at beamline BL14W1 in Shanghai Synchrotron Radiation Facility (SSRF). The storage ring was operated at 3.5 GeV with the currents of ~240 mA in "top-up" mode. The white light was monochromatized with a Si(111) double-crystal monochromator and the energy was calibrated with a Cu foil (8979 eV). The Cu K-edge data were collected in fluorescence mode with a 32-element Ge solid state detector.

In order to extract geometrical information around Cu(I) binding site, a quantitative analysis of XANES spectra were performed by MXAN package. The x-ray absorption cross sections were calculated using the full multiple scattering approach in the framework of the muffin-tin approximation. The convergent atomic cluster contains 37 atoms from 4 amino acid residues within 7 Å around Cu(I), and the best fit was achieved via minimum difference between experimental data and theoretical calculations obtained by various structure parameters.

The EXAFS data were analyzed using the software packages Demeter. The spectra were normalized using Athena firstly, and then shell fittings were performed with Artemis. The $\chi(k)$ function was Fourier transformed (FT) using k^3 weighting, and all fittings were done in R-space. The backscattering amplitude F(k) and phase shift $\Phi(k)$ were calculated by FEFF7.0 code. While the curve-fitting, the amplitude reduction factor (S_0^2) was estimated to be 0.916, according to the fitting results of the copper foil. In order to fit the curves in the R-range of 1.0-2.4 Å, we considered Cu-N and Cu-S paths as the central-peripheral. For each path, the structural parameters, like coordination number (CN), interatomic distance (R), Debye-Waller factors (σ^2) and inner potential correction edge-energy shift (ΔE_0) were opened to be varied. For the two Cu-N and two Cu-S coordination, the common adjustable parameters of ΔE_0 and σ^2 were employed to reduce the number of free parameters. The coordination parameters were obtained by fitting the experimental peaks with theoretical amplitude.

Fluorescence measurements

The fluorescence measurements were performed on a HITACHI F-4600 fluorescence spectrophotometer with 274 nm excitation wavelength. The $[Cu(MeCN)_4]ClO_4$ and $CuSO_4$ titration was carried out by adding 0-200 μM Cu(I) and Cu(II) into 20 μM $hCTR1_{1-46}$ in 50 mM potassium phosphate buffer. All samples were incubated at 25 °C for 5 min to ensure that equilibrium was reached.

Circular dichroism spectroscopy (CD)

CD spectra were recorded on a Jasco-720 Circular Dichroism Spectropolarimeter in a 0.1 cm path length quartz cuvette at room temperature. 20 μM apo- and Cu-bound $hCTR1_{1-46}$ was incubated with DPPC liposomes for 30 min and SDS micelles for 1 h to ensure that equilibrium was reached. Spectra were recorded from 180 – 300 nm at a scan speed 50 $nm \cdot min^{-1}$. All measurements were repeated three times and the signal was smoothed using Fast Fourier transform (FFT) to improve the signal to noise ratio.

Liposomes preparation

Liposomes were prepared as previously described.[2] Synthetic lipids dipalmitoyl-phosphatidylcholine (DPPC, 95%) or dimyristoyl-phosphoethanolamine (DMPE, 95%) with cholesterol (5%) were dissolved in chloroform

and transferred to a 10 ml round-bottom flask. Then the chloroform was removed by evaporation. The dried lipid film deposited on the flask was hydrated by adding 50 mM potassium phosphate buffer (pH 7.0) containing 100 mM NaCl and was ultrasonicated at 50 °C. The stock liposomes was prepared in a concentration of 40 mM.

Liposomes pull-down assay

20 μM $hCTR1_{1-46}$ was incubated with 5 molar equivalents of liposomes in phosphate potassium buffer (50 mM KH_2PO_4, 100 mM NaCl, pH 7.0) at room temperature for 30 min. Different molar ratios of Cu(I) ions were added to the mixture of protein and liposome sample and mixed for 10 min at room temperature. Control experiments were also performed with KCl (10 mM), $CaCl_2$ (1 mM), or $MgSO_4$ (1 mM). Samples were spun at 100,000 rpm (541,000 g) for 45 min at 4 °C in the TLA-100.3 rotor (Beckman) in L-100XP ultracentrifuge (Beckman). After centrifugation, the supernatant was removed and the pellet was analyzed with SDS-PAGE.

Isothermal titration calorimetry measurements

Measurements were performed on a MicroCal iTC_{200} (GE), which has a cell volume of 260 μl and the syringe volume is 60 μl. $hCTR1_{1-46}$ was dialyzed against 50 mM potassium phosphate buffer containing 200 mM NaCl and 200 μM protein was used for the titration. 20 μM DPPC liposomes were placed in the cell, and $hCTR1_{1-46}$ was added with the first injection of 1 μl and followed by 15 injections of 2 μl with a spacing time of 120 sec between injections. Measurements were performed at 25 °C. For the titrations of Cu-$hCTR1_{1-46}$ with DPPC liposomes, the buffer containing 5 mM β-mercaptoethanol was used in order to maintain the cuprous state of copper ions. Data were fitted with a single site binding model using the MicroCal Origin 7.0 software.

Fluorescein (FITC) labeling of $hCTR1_{1-46}$

Twenty to fifty milligrams of $hCTR1_{1-46}$ was mixed with 10~15 molar ratios of FITC in 50 mM phosphate buffer (pH 9.0) at 4 °C for overnight. The excess FITC was removed from $hCTR1_{1-46}$ by gel filtration chromatography. The FITC-$hCTR1_{1-46}$ was dialyzed in PBS buffer for cell experiments.

Fluorescence microscopy

The fluorescence of cells was detected using an Olympus IX71/IX51 fluorescence microscope. Cells were seeded in 6-well tissue culture plates at a density of 5×10^4 cells per well in DMEM containing 10% fetal bovine serum and incubated overnight at 37 °C in a 5% CO_2 incubator. The cells were incubated with fresh DMEM containing 10 μM FITC-$hCTR1_{1-46}$ at 37 °C for 30 min. Different concentrations of Cu(I) ions were added to the growth medium with 100 μM ascorbic acid. After incubation at 37 °C for 10 min, cells were washed three times with PBS to remove the residual protein. Then the cells were incubated with HOE33258 for 10 min for staining cell nucleus. Samples were washed twice with PBS and visualized in a confocal microscope (LSM 710 CLSM, Carl Zeiss, Jena, Germany). The FITC-$hCTR1_{1-46}$ can be detected under excitation at 488 nm and the fluorescence of HOE33258 can be detected under excitation at 346 nm.

UV-visible spectroscopy

UV-vis spectra were recorded on Agilent 8453 UV-visible spectrophotometer with a 1 cm cuvette at room

temperature. BCA titration experiment was carried out under anaerobic condition. Briefly, 20 μM apo-hCTR1$_{1\text{-}46}$ were mixed with an equal amount of [Cu(MeCN)$_4$]ClO$_4$ in 5 mM HEPES buffer (pH 7.4) in the presence of 10 mM ascorbic acid (V_C). The sample was transferred into the cuvette sealed with a septum cap. BCA was titrated into the cuvette using an airtight syringe. Spectra were recorded from 200 to 800 nm.

Cu(I) Binding Constants

BCA, a competitive chelator for Cu(I), can form a magenta Cu(BCA)$_2$ complex which shows a characteristic absorbance at 562 nm. Therefore, BCA was titrated into the complex of hCTR1$_{1\text{-}46}$ and Cu(I) to detect the transfer of Cu(I) by monitoring the UV-Vis absorbance of Cu(BCA)$_2$ at 562 nm. Experiments of competition reaction between hCTR1$_{1\text{-}46}$ and BCA were carried out in HEPES buffer at pH 7.4 under anaerobic conditions. The binding constants of Cu(I) and BCA was 2×10^{17} according to previous literature.[3] The binding affinity of hCTR1$_{1\text{-}46}$ to Cu(I) was calculated according to the following equations.

$$\mathrm{P} + \mathrm{Cu}^{+} \rightleftharpoons \mathrm{CuP}$$

$$\mathrm{K_{CuP}} = \frac{[\mathrm{CuP}]}{[\mathrm{Cu}][\mathrm{P}]}$$

$$\mathrm{BCA} + \mathrm{Cu}^{+} \rightleftharpoons \mathrm{CuBCA}$$

$$\mathrm{K_1} = \frac{[\mathrm{CuBCA}]}{[\mathrm{Cu}][\mathrm{BCA}]}$$

$$2\mathrm{BCA} + \mathrm{Cu}^{+} \rightleftharpoons \mathrm{CuBCA_2}$$

$$\mathrm{K_{CuBCA_2}} = \mathrm{K_1K_2} = \frac{[\mathrm{CuBCA}]}{[\mathrm{Cu}][\mathrm{BCA}]^2}$$

$$\mathrm{CuP} + 2\mathrm{BCA} \rightleftharpoons \mathrm{CuBCA_2} + \mathrm{P}$$

$$\mathrm{K_{ex}} = \frac{[\mathrm{CuBCA}][\mathrm{P}]}{[\mathrm{CuP}][\mathrm{BCA}]^2} = \frac{\mathrm{K_{CuBCA_2}}}{\mathrm{K_{CuP}}}$$

$$[\mathrm{Cu}]_{\mathrm{Total}} = [\mathrm{Cu}^{+}]+[\mathrm{CuP}]+[\mathrm{CuBCA}]+[\mathrm{CuBCA_2}]$$

$$[\mathrm{P}]_{\mathrm{Total}} = [\mathrm{P}] + [\mathrm{CuP}]$$

$$[\mathrm{BCA}]_{\mathrm{Total}} = [\mathrm{BCA}]+[\mathrm{CuBCA}]+2[\mathrm{CuBCA_2}]$$

Where P denotes hCTR1$_{1\text{-}46}$.

References

[1] W. Bao, Y. Gao, Y. Chang, T. Zhang, X. Lin, X. Yan, H. Hu, *Protein Expression Purif.* **2006**, *47*, 599-606.

[2] L. D. Mayer, M. J. Hope, P. R. Cullis, *Biochim. Biophys. Acta* **1986**, *858*, 161-168.

[3] Z. Xiao, P. S. Donnelly, M. Zimmermann, A. G. Wedd, *Inorg. Chem.* **2008**, *47*, 4338-4347.

.

A

```
COPT1_HUMAN          1   --------MDHSHHMGMSYMDSNSTMQPSHHHPTTSASHSHGGGDSSMMMMPMTFYFGFK   52
COPT1_RAT            1   ---MRMNHMEM-HHMGMNHTDDNITM-PPHQHPTTSASHSH------EMMMPMTFYFGFK   49
COPT1_MOUSE          1   MNHMGMNHMEMHHHMGMNHTDDNITM-PPHHHPTTSASHSHGGGDS-MMMMPMTFYFDFK   58
```

B

10 20 30 40 46
S MDHSHHMGMS YMDSNSTMQP SHHHPTTSAS **HS**HGGGDSSM MMMPMT

Scheme S1. (A) Sequence alignment of the N-Terminal domain of CTR1 (residues 1−52 shown, human numbering). (B) The protein sequence of hCTR1$_{1-46}$ used in this work. An additional amino acid (serine) is left at N-terminus after TEV enzyme digestion.

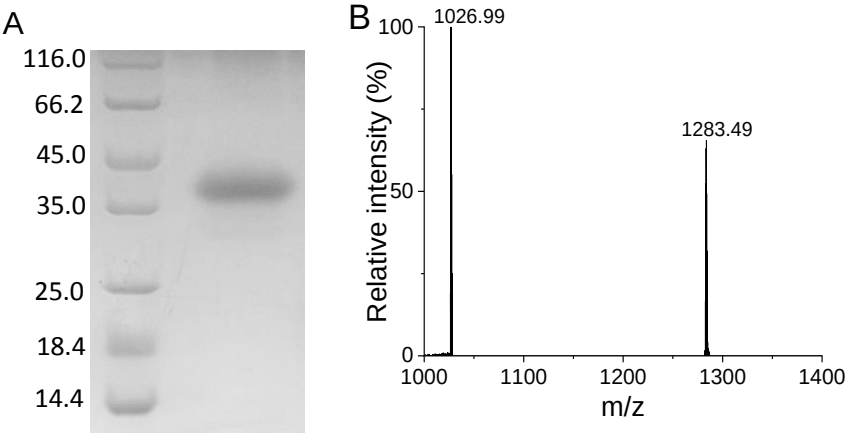

Figure S1. Identification of the hCTR1$_{1-46}$ protein. (A) 15% SDS PAGE analysis of the purified hCTR1$_{1-46}$; (B) ESI-MS analysis of hCTR1$_{1-46}$. The spectrum revealed two major peaks at m/z 1027 and 1283.7 respectively, corresponding to +4 and +5 charged signals of the protein (calculated values: 1027.13 and 1283.66).

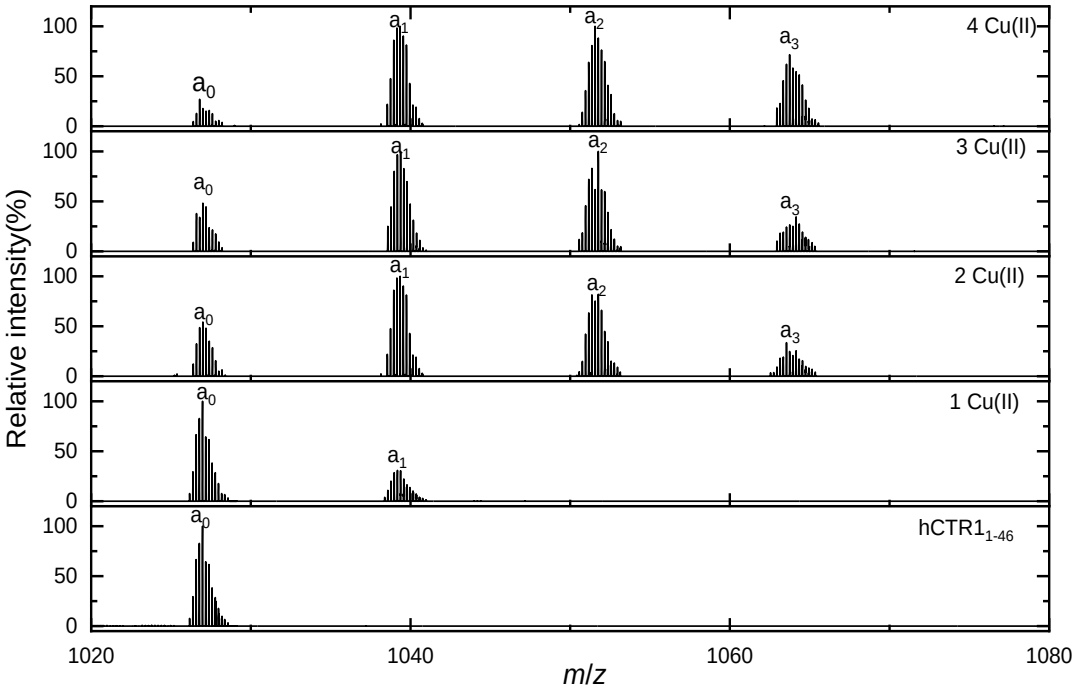

Figure S2. ESI-MS spectra of the binding of Cu(II) to hCTR1$_{1-46}$. Samples were prepared by incubation of hCTR1$_{1-46}$ with different molar equivalents of $CuSO_4$. Spectra were recorded in positively charged mode, and the +5 charged region was plotted. Peaks were assigned as apo-hCTR1$_{1-46}$ (a_0); Cu-hCTR1$_{1-46}$ (a_1); Cu_2-hCTR1$_{1-46}$ (a_2); Cu_3-hCTR1$_{1-46}$ (a_3). See following Table S1 for the detailed assignment.

Table S1. ESI-MS spectra analysis of the major products in the reactions of $hCTR1_{1-46}$ with Cu(II).

Peaks	Composition	Formula	Observed m/z	Calculated m/z
a_1	$[hCTR1_{1-46} + Cu(II) + 3H]^{5+}$	$C_{203}H_{305}N_{65}O_{72}S_{10}Cu$	1039.35	1039.38
a_2	$[hCTR1_{1-46} + 2Cu(II) + H]^{5+}$	$C_{203}H_{303}N_{65}O_{72}S_{10}Cu_2$	1051.54	1051.56
a_3	$[hCTR1_{1-46} + 3Cu(II) - H]^{5+}$	$C_{203}H_{301}N_{65}O_{72}S_{10}Cu_3$	1063.92	1063.95

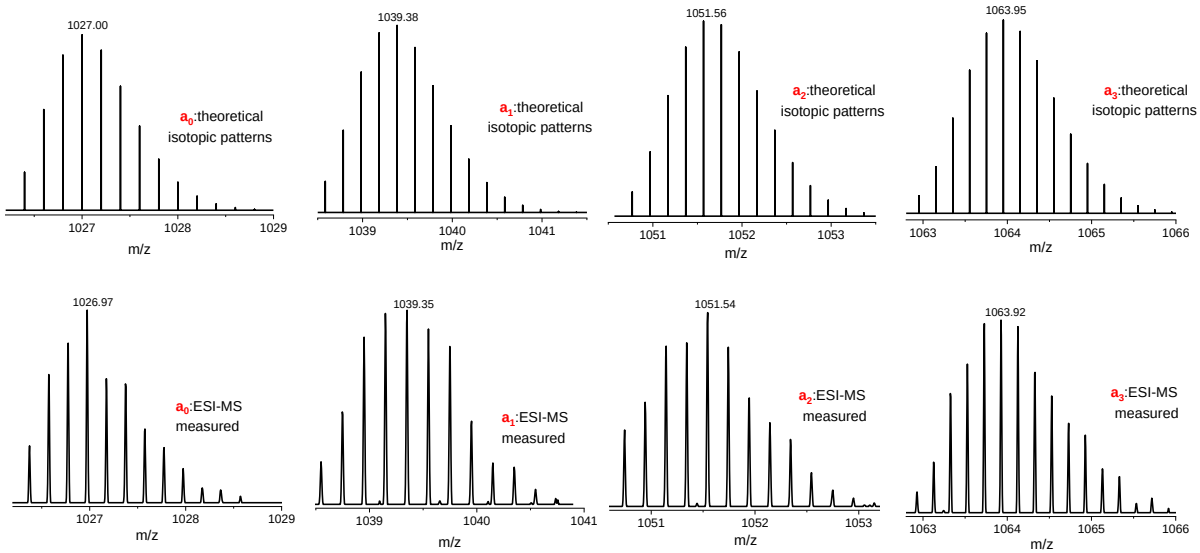

Figure S3. Comparison of the observed ESI-MS signals with the theoretical isotopic patterns of apo-$hCTR1_{1-46}$ and Cu(II)-bound $hCTR1_{1-46}$.

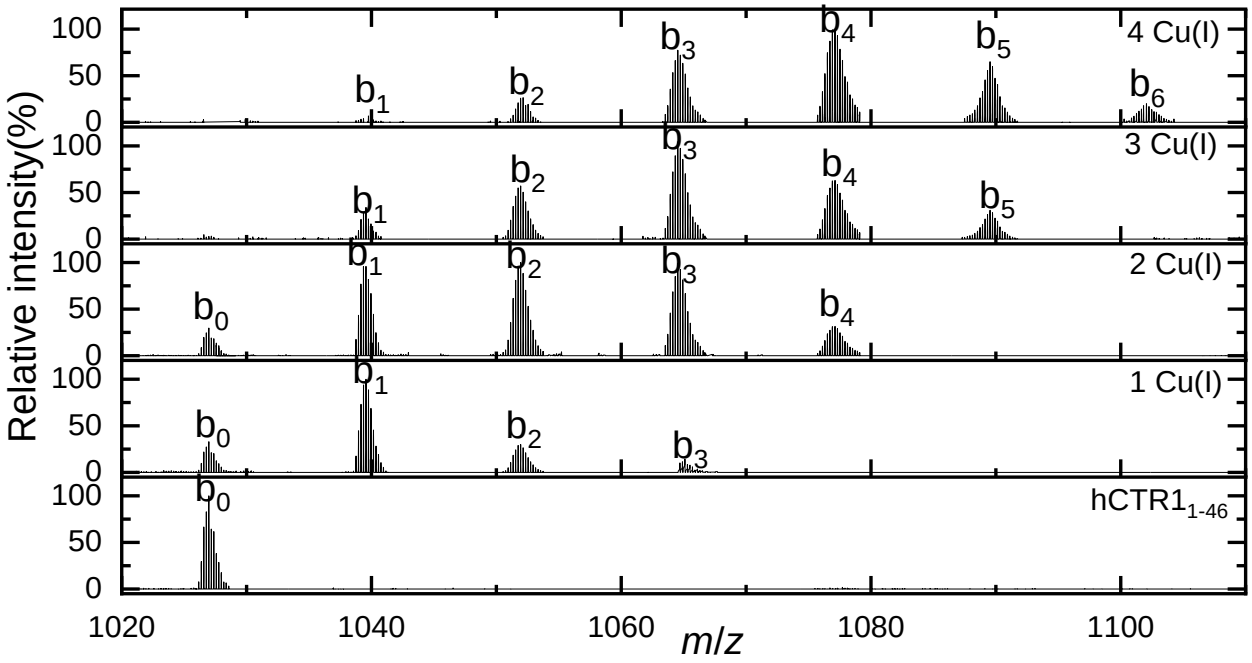

Figure S4. ESI-MS spectra of $hCTR1_{1-46}$ with the addition of different molar equivalents of $[Cu(MeCN)_4]ClO_4$ in the presence of 100 μM ascorbic acid. Spectra were recorded in positively charged mode, and the +5 charged region was plotted. Peaks were assigned as apo-$hCTR1_{1-46}$ (b_0); Cu-$hCTR1_{1-46}$ (b_1); Cu_2-$hCTR1_{1-46}$ (b_2); Cu_3-$hCTR1_{1-46}$ (b_3); Cu_4-$hCTR1_{1-46}$ (b_4); Cu_5-$hCTR1_{1-46}$ (b_5); Cu_6-$hCTR1_{1-46}$ (b_6). See following Table S2 for the detailed assignment.

Table S2. ESI-MS spectra analysis of the major products in the reactions of $hCTR1_{1-46}$ with Cu(I)

Peaks	Composition	Formula	Observed m/z	Calculated m/z
b_1	$[hCTR1_{1-46} + Cu(I) + 4H]^{5+}$	$C_{203}H_{306}N_{65}O_{72}S_{10}Cu$	1039.56	1039.58
b_2	$[hCTR1_{1-46} + 2Cu(I) + 3H]^{5+}$	$C_{203}H_{305}N_{65}O_{72}S_{10}Cu_2$	1051.94	1051.97
b_3	$[hCTR1_{1-46} + 3Cu(I) + 2H]^{5+}$	$C_{203}H_{304}N_{65}O_{72}S_{10}Cu_3$	1064.53	1064.56
b_4	$[hCTR1_{1-46} + 4Cu(I) + H]^{5+}$	$C_{203}H_{303}N_{65}O_{72}S_{10}Cu_4$	1077.11	1077.14
b_5	$[hCTR1_{1-46} + 5Cu(I)]^{5+}$	$C_{203}H_{302}N_{65}O_{72}S_{10}Cu_5$	1089.50	1089.53
b_6	$[hCTR1_{1-46} + 6Cu(I) - H]^{5+}$	$C_{203}H_{301}N_{65}O_{72}S_{10}Cu_6$	1102.08	1102.11

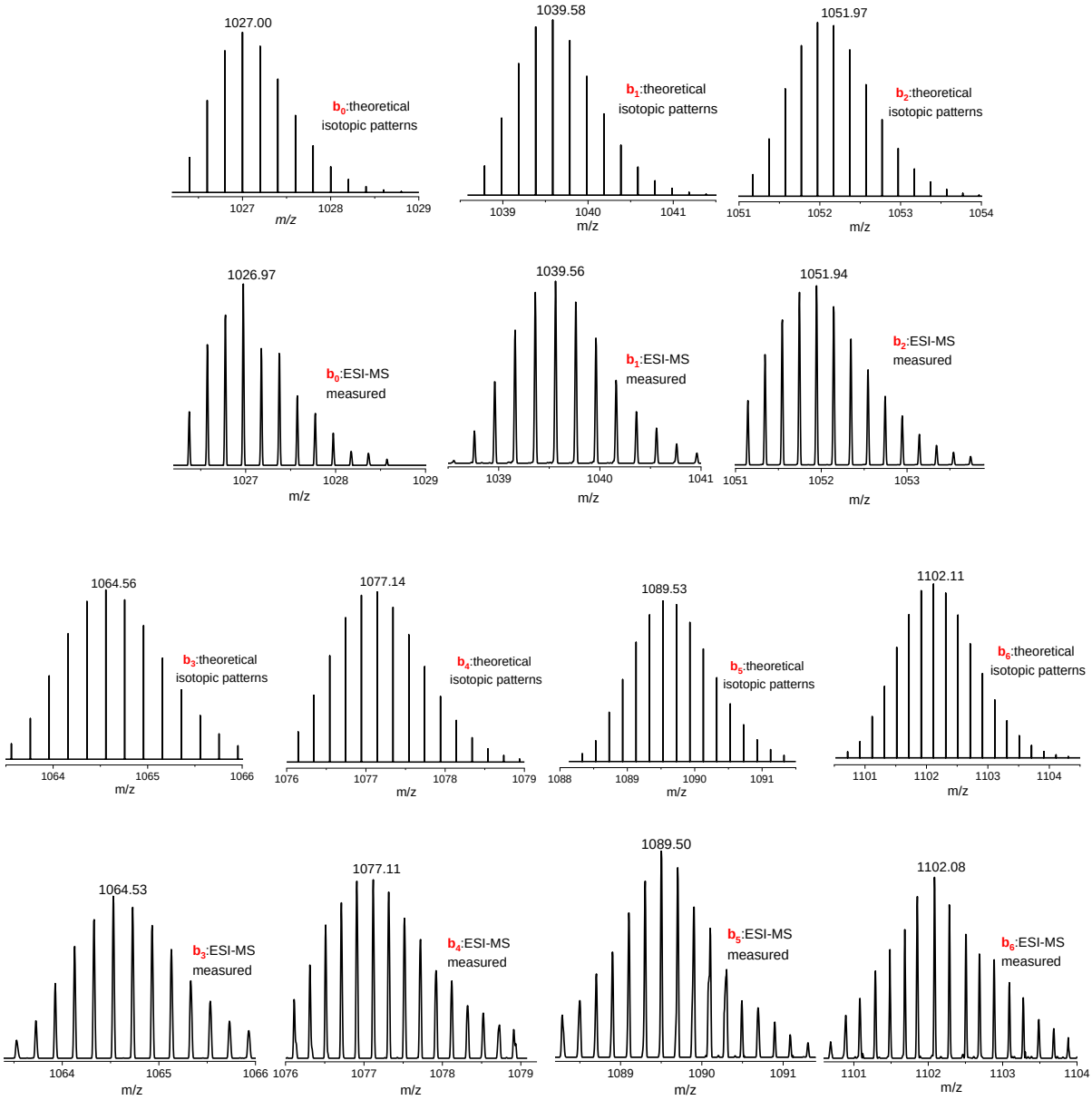

Figure S5. Comparison of the observed ESI-MS signals with the theoretical isotopic patterns of apo-$hCTR1_{1-46}$ and Cu(I)-bound $hCTR1_{1-46}$.

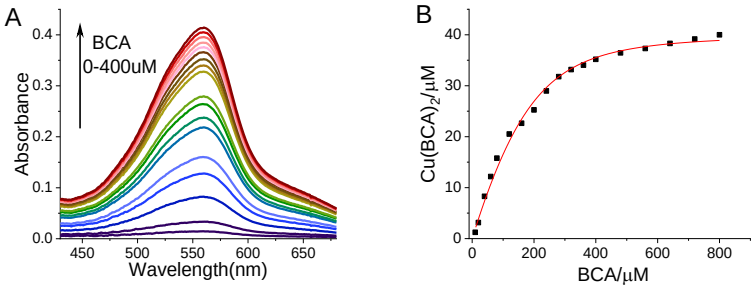

Figure S6. Titration of Cu-hCTR1$_{1-46}$ with BCA. (A) UV-visible spectra of 20 μM Cu-hCTR1$_{1-46}$ with the addition of BCA. Cu-hCTR1$_{1-46}$ was prepared by incubation of 20 μM $[Cu(MeCN)_4]ClO_4$ and 20 μM hCTR1$_{1-46}$ in the presence of 100 μM ascorbic acid. (B) Fitting the UV absorbance at 562 nm.

Table S3. The distance between Cu(I) and four amino acid residues calculated from X-ray absorption spectroscopy (Figure 1)

Atom pairs	Cu-S (Met)	Cu-N (His)	Cu-N (His)	Cu-S (Met)
distance (Å)	2.17±0.02	1.89±0.02	2.21±0.02	2.94±0.03

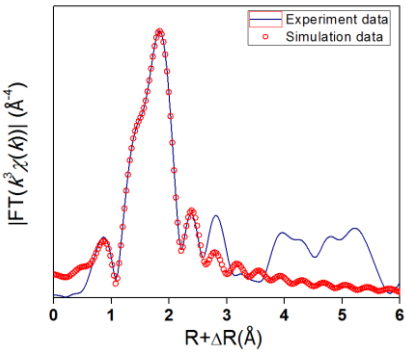

Figure S7. The experiment data and simulation result of Cu(I)-hCTR1 in R space.

Table S4. EXAFS fitting parameters at the Cu K-edge various samples (S_0^2=0.916)

Sample	Shell	N^a	R(Å)^b	σ^2(Å^2)^c	ΔE_0(eV)^d	R factor
Cu foil	Cu-Cu	12	2.55	0.0088	5.7	0.0006
hCTR1	Cu-N	0.9	1.90	0.0027	-3.3	0.0002
	Cu-S	1.1	2.17	0.0025		
	Cu-N	1.0	2.19	0.0027		
	Cu-S	0.7	2.94	0.0025		

[a]N: coordination numbers; [b]R: bond distance; [c]σ^2: Debye-Waller factors; [d] ΔE_0: the inner potential correction. R factor: goodness of fit. S_0^2 was set to 0.916, according to the experimental EXAFS fit of Cu foil by fixing CN as the known crystallographic value.

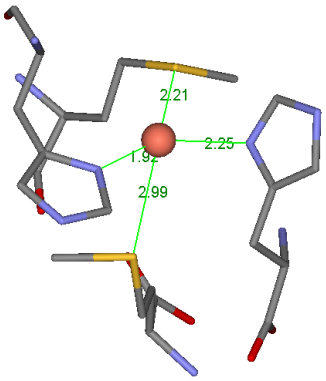

Figure S8. DFT geometry-optimized structure of $[CuI(Met)_2(His)_2]$ model complex.

Table S5. The distance between Cu(I) and four amino acid residues calculated from density function theory

Atom pairs	Cu-S (Met)	Cu-N (His)	Cu-N (His)	Cu-S (Met)
distance (Å)	2.21	1.92	2.25	2.99

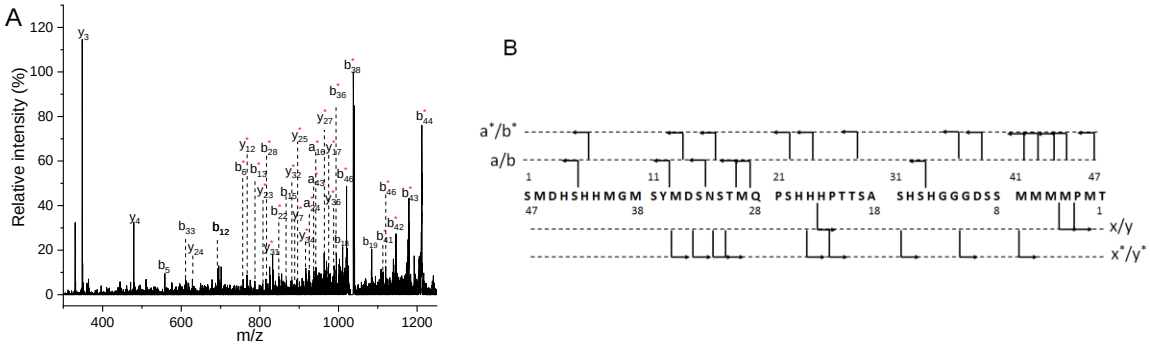

Figure S9. ESI-MS/MS analysis of Cu-hCTR1$_{1-46}$. (A) MS/MS spectra from CID experiments on the +5 charged peaks at m/z 1039. The Cu(I)-bound hCTR1$_{1-46}$ was generated by incubation of 10 µM hCTR1$_{1-46}$ with 20 µM $[Cu(MeCN)_4]ClO_4$ for 30 min in 5 mM NH_4OAc buffer with 100 µM ascorbic acid (pH 6.8) at room temperature. (B) Fragmentation schemes of the MS/MS spectra of hCTR1$_{1-16}$. Asterisks (*) indicate the fragments containing Cu(I).

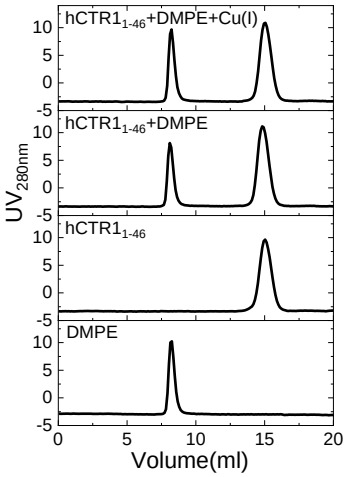

Figure S10. Gel filtration detection of the interactions of hCTR1$_{1-46}$ with DMPE liposomes in the absence and presence of $[Cu(MeCN)_4]ClO_4$ in 50 mM phosphate potassium solution (pH 7.4).

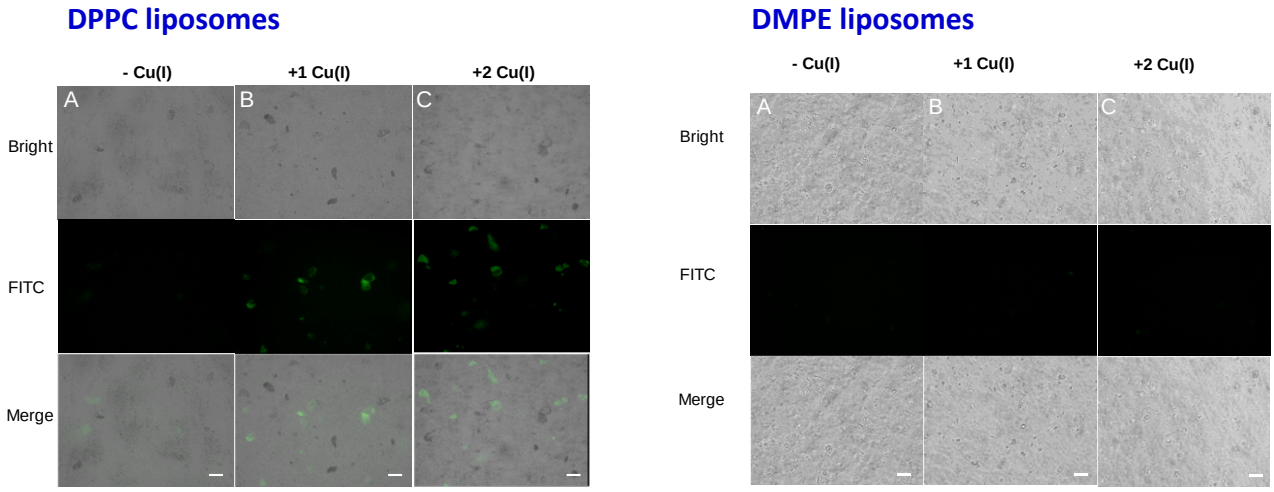

Figure S11. Fluorescence microscopic images of FITC labeled hCTR1$_{1\text{-}46}$ and liposomes in the absence and presence of $[Cu(MeCN)_4]ClO_4$. **Left portion**: DPPC liposomes; **right portion**: DMPE liposomes. (A) PBS, (B) 10 µM $[Cu(MeCN)_4]ClO_4$ and (C) 20 µM $[Cu(MeCN)_4]ClO_4$ were added to the growth medium in the presence of 100 µM ascorbic acid. DPPC liposomes were preincubated with 10 µM FITC-hCTR1$_{1\text{-}46}$ at 37 °C for 30 min, then cells were incubated with Cu(I) for 10 min at 37 °C. Both images were obtained with a 40X objective. λ_{em} = 488 nm.

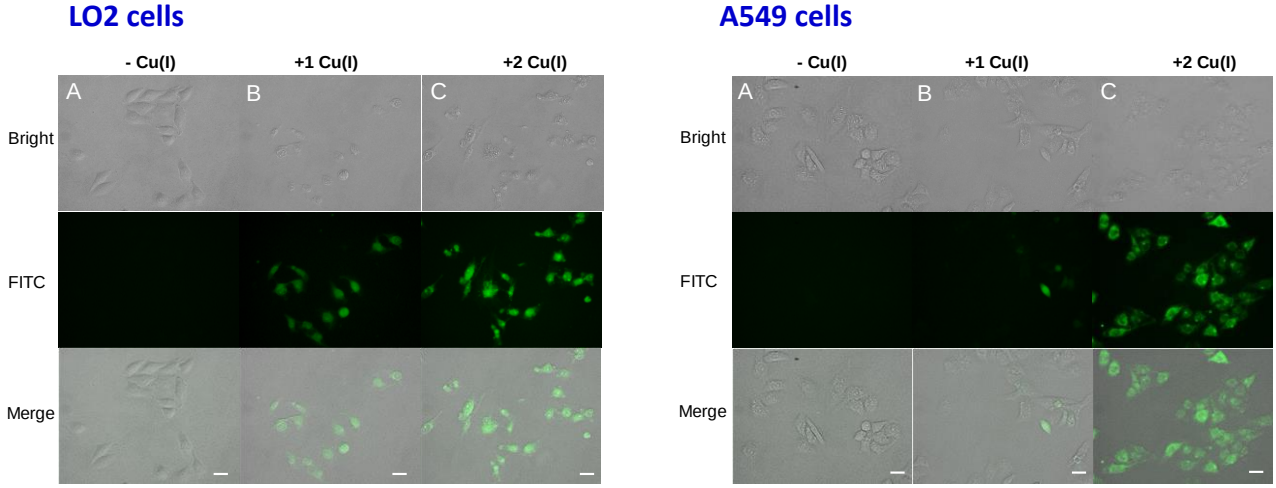

Figure S12. Fluorescence microscopic images of FITC labeled hCTR1$_{1\text{-}46}$ and cells in the absence and presence of $[Cu(MeCN)_4]ClO_4$. **Left portion**: LO2 cells, **right portion**: A549 cells. (A) PBS, (B) 10 µM $[Cu(MeCN)_4]ClO_4$ and (C) 20 µM $[Cu(MeCN)_4]ClO_4$ were added to the growth medium in the presence of 100 µM ascorbic acid. LO2 cells were preincubated with 10 µM FITC-hCTR1$_{1\text{-}46}$ at 37 °C for 30 min, then cells were incubated with Cu(I) for 10 min at 37 °C. The magnification is ×40. λ_{em} = 488 nm.