

Copper chaperone for superoxide dismutase-1 transfers copper to mitochondria but does not affect cytochrome c oxidase activity

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Abstract

Copper chaperone for superoxide dismutase-1 (CCS-1) is present in the cytosol and in the intermembrane space of mitochondria. It transfers copper ions to superoxide dismutase 1 in the cytosol, but its function in the mitochondria is not clear. The present study was undertaken to test the hypothesis that CCS-1 functions in mitochondrial copper homeostasis. Mitochondria were isolated from human umbilical vein endothelial cells and copper concentrations in the mitochondria were measured in the CCS-1 deficient cells made by siRNA targeting the protein. Copper concentrations in the mitochondria were about 10 fold higher than its total concentrations in the cell and the CCS-1 deficiency significantly reduced the copper level in the mitochondria. However, this decrease in the mitochondrial copper concentration did not affect cytochrome c oxidase (CCO) activity. On the other hand, siRNA targeting COX17, a copper chaperone for the CCO, significantly increased the mitochondrial copper concentration, but suppressed the CCO activity. This study thus demonstrates that CCS-1 facilitates copper trafficking to the mitochondria, but does not affect the transfer of copper to the CCO. In addition, the COX17 not only functions in the copper shuttling to the CCO, but also may participate in the copper efflux from the mitochondria.

Keywords: Copper, CCS-1, COX17, cytochrome c oxidase, mitochondria

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Introduction

The copper chaperone for superoxide dismutase-1 (CCS-1) is present in the cytosol and in the intermembrane space (IMS) of mitochondria.^{1,2} The CCS-1 transfers copper ions to superoxide dismutase 1 in the cytosol, but its function in the mitochondria is not clear. Although the presence of superoxide dismutase 1 in the mitochondria may predict the requirement for the CCS-1 to transfer copper ions to the enzyme in this location, the abundance and essential role of copper in mitochondrial metabolism and function suggest that the CCS-1 may contribute to the homeostasis of copper in the mitochondria.

One of the essential roles of copper in the mitochondria is its regulation of the assembly and function of cytochrome c oxidase (CCO), the last proton-pumping apparatus of the mitochondrial respiratory chain and catalysing the transfer of electron from reduced cytochrome c to molecular oxygen. This enzyme assemble is composed of 13 subunits, three of which (COX-I, -II, and -III) are encoded by the mitochondrial DNA and the rest are encoded by the nuclear DNA.^{3–5} In these subunits, COX-I and COX-II contain copper active sites and constitute the catalytic core of the CCO.⁶ Copper chaperones for CCO, including COX17, COX11, SCO1, and SCO2

are responsible for delivering copper ions to the copper active sites Cu_A on COX-II and Cu_B on COX-I in the CCO.^{7–10}

The COX17 transfers copper ions to SCO1, SCO2 and COX11. SCO1 and SCO2 facilitate copper insertion into the COX-II Cu_A, and COX11 transfers copper ions to the COX-I Cu_B active sites.^{8,10–14} These proteins are anchored to the mitochondrial inner membrane through a transmembrane α -helix and expose their copper binding sides in the IMS of mitochondria where the copper transfer takes place.¹⁵ Loss of function of COX17 attenuates the delivery of copper ions to SCO1 and COX11, and inhibits the CCO activity.^{16,17} Mutations in either SCO1 or SCO2 cause severe CCO assembly impairment and thus decrease the CCO activity,^{18,19} and mutations in COX11 also suppress the CCO activity.⁷

It was previously proposed that the COX17 might shuttle copper ions from the cytoplasm into the IMS of mitochondria.^{20,21} This hypothesis was later challenged by the observation that while apo-COX17 is predominantly a monomer with a simple hairpin structure, the protein-copper complex exists in a dimer/tetramer equilibrium,²² suggesting an unlikelihood for its free passage through the outer membrane protein channel.

Therefore, the key proteins responsible for the trafficking of copper ions from the cytoplasm to the mitochondria

remain to be identified, and the coordination between copper homeostasis and delivery to the CCO in the mitochondria is elusive. The present study was undertaken to test the hypothesis that the CCS-1 is involved in the trafficking of copper ions from the cytoplasm to the mitochondria. In addition, a relationship between the CCS-1 and the COX17 in the regulation of the CCO activity was examined. The data obtained suggest that the CCS-1 facilitates copper trafficking to the mitochondria, but does not participate in the transfer of copper ions to the CCO. Deficiency in the COX17 leads to the suppression of the CCO activity and to the increase in the mitochondrial copper concentration.

Materials and methods

Cell culture and treatment

Human umbilical vein endothelial cells (HUVECs) obtained from American Tissue Culture Collection (ATCC) were maintained at 37°C in L-DMEM (GIBCO, USA) media supplemented with 10% foetal bovine serum (FBS, Hyclone) and 1% penicillin/streptomycin (GIBCO, USA) in 5% CO₂ incubator. Stock cultures were maintained at 80% confluence and passaged by 0.25% Trypsin (GIBCO, USA) and 1% EDTA in Ca²⁺- and Mg²⁺-free phosphate-buffered saline (PBS). Experimental cells were subcultured in 25 cm² or 75 cm² flasks overnight.

Deficiency in CCS-1 and COX17

To produce cells deficient in CCS-1 or COX17, siRNA targeting CCS-1 or COX17 were designed and applied to cells cultured in 75 cm² flasks at 5×10^5 cells/flask. Briefly, three different siRNAs targeting each hCCS-1 or hCOX17 and negative mismatched siRNA were purchased from RiboBio (Ribo-Bio, China). The optimal transfection efficiency was determined from our preliminary studies testing the range from 5 to 50 nM, and we selected the condition and the siRNA sequence (from three different sequences) for each protein that can cause about 60–70% silencing effect with minimal cytotoxicity. HUVECs were transfected with 30 nM of the selected annealed siRNAs targeting hCCS-1, hCOX17 or negative-mismatched siRNA in antibiotic-free media. Lipofectamine 2000 (Invitrogen, USA) was used as the transfection reagent following the manufacturer's instruction. After 48-h transfection, cells were trypsinized and collected for further analysis as described in the experimental procedure.

Isolation of mitochondria

The monolayer cells, after removal of culture media, were washed with PBS and trypsinized for harvesting from the 75 cm² flasks. The cell suspension was transferred to 15-mL polypropylene tubes followed by centrifugation at 600 g for 5 min at 4°C. The collected cells were resuspended in 2 mL PBS followed by centrifugation. The final cell pellet was resuspended in 1 mL of ice-cold MSTE buffer (0.21 M D,L-Mannitol, 0.07 M sucrose, 10 mM Tris base, 1 mM EDTA, 0.5 mM EGTA, pH 7.4) and homogenized (at about 200 times) using a Dounce homogenizer. The homogenate was transferred to a 2 mL polypropylene centrifugation

tube and centrifuged at 600 g for 5 min at 4°C. The supernatant was collected and centrifuged at 1000 g for 5 min at 4°C. The supernatant was collected and centrifuged again at 7000 g for 10 min at 4°C. The mitochondrial pellet was then resuspended in 1.5 mL of MSTE buffer and centrifuged again, followed by resuspension in 1 mL of ice-cold MSTE buffer and centrifugation at 10,000 g for 10 min at 4°C, and the final pellet was mitochondrial fraction.

Determination of CCO activity

Isolated mitochondria described above were used and total mitochondrial protein concentrations were determined by Bio-Rad protein assay. An aliquot of 0.25 mg mitochondrial protein was dissolved in 0.2 mL of isosmotic medium containing 10 mM KH₂PO₄, pH 6.5, 50 mM KCl, 0.25 M sucrose, 1 mg/mL BSA, and 2.5 mM n-dodecylmaltoide. The CCO activity was determined by addition of 0.2 mM ferrocytochrome c to the reaction system to measure its reduction. The enzyme activity was calculated from the rate of decrease in the absorbance of reduced cytochrome c at 550 nm ($\epsilon = 19.1 \text{ mmol}^{-1} \text{ cm}^{-1}$). Because accurate estimation of the CCO activity could be compromised by variations in the mitochondrial yield and integrity, the CCO activity in the present study was normalized using an invariant marker of mitochondrial enzyme activity, citrate synthase. Citrate synthase was assayed by following the reduction of 0.1 mM 5,5'-dithio-bis (2-nitrobenzoic acid) in the presence of 0.25 mg mitochondrial protein, 0.25 mM acetyl-CoA, and 0.5 mM oxaloacetic acid in buffer containing 40 mM PBS, pH 8.0, 2 mM EDTA, 1 mg/mL BSA, and 0.1% Triton X-10. The change in the absorbance at 412 nm ($\epsilon = 13.6 \text{ mmol}^{-1} \text{ cm}^{-1}$) was monitored for 30 min (reflecting the formation of thionitrobenzoate).

Mitochondrial membrane potential assay

The reagent, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazoly-carbocyanine iodide (JC-1), was used to assess mitochondrial potential changes using a confocal microscope. Briefly, cells in 24-well plates at a density of 1×10^5 cells/mL cultured at 37°C overnight were treated with the selected siRNA targeting CCS-1 or COX17 for 24 h before addition of 1 µg/mL JC-1 in culture media for 30 min. The cells were examined by the confocal microscope (Nikon, Japan). Changes in the fluorescence from red to green indicate the changes in the mitochondrial membrane potential; the red/green ratio was calculated from the changes in the intensity of red and green fluorescence assessed using a computer program.

Co-immunoprecipitation assay

To determine whether CCS-1 interacts with COX17, HUVECs cultured in 75 cm² flasks were harvested and lysed with Tris-HCl (20 mM, pH 7.6), NaCl (150 mM), KCl (20 mM), MgCl₂ (15 mM), and NP40 (0.2%) with the addition of protease inhibitor cocktail (Roche). Cell lysates were incubated overnight with CCS-1 or COX17 antibody at 4°C, followed by the addition of protein A/G sepharose (Santa Cruz, USA) at 4°C for 3 h. After three washings with PBS,

Western blotting was used to determine the possible interaction (co-immunoprecipitation) between CCS-1 and COX17.

Western blotting

The protein contents of CCS-1 and COX17 were determined by Western blotting. Cells scraped in PBS were washed three times and the cell lysate was collected by using 1% SDS solution. Protein samples were mixed with $5 \times$ loading buffer, boiled for 10 min at 100°C and cooled. Equal amounts of protein ($50\text{--}100\mu\text{g}$) from each sample were separated by 12% SDS-PAGE. Proteins were then electrophoretically transferred to a polyvinylidene fluoride membrane (Bio-Rad, USA). Transferred proteins were blocked with 5% non-fat dry milk in Tris-HCl buffer solution containing Tris-HCl (50 mM), NaCl (150 mM), and Tween-20 (0.1%) (TBS-T) for 1 h at room temperature. The blots were then incubated with respective primary antibodies (anti-CCS-1 and anti-COX17, Santa Cruz, USA) in blocking solution according to the vendor's recommendations. After incubation, the blots were washed with TBS-T six times for 5 min each. The blots were incubated for 2 h with appropriate secondary antibody. After washing six times (5 min each), target proteins were visualized using chemiluminescence (Bio-rad, USA) and analysed by densitometry using a Quantity One Software.

Determination of copper concentrations

Copper concentrations in the isolated mitochondria and in the cell lysate were determined with graphite furnace

atomic absorption spectrophotometer (AAS). Briefly, the freeze-dried mitochondrial or cytosol samples were dissolved in $50\mu\text{L}$ concentrated HNO_3 for three days. Before the analysis, 0.5 mL de-ionized water was added and then copper concentrations were determined by the AAS.

Statistics

Data were obtained from three separate experiments and presented as mean values $\pm$ SEM. The significance of differences was determined using Student's *t*-test, and a *P* value of <0.05 was considered statistically significant.

Results

The isolated mitochondria were tested for their purity using β -tubuline and histone H1 as cytoplasmic and nucleus markers, respectively. These tests verified that the isolated mitochondria were essentially free of cytoplasmic and nuclear contaminations (Figure 1(a)). In addition, COX4, a nuclear-encoded subunit of CCO enzyme, was also tested. As expected, COX4 was highly concentrated in the isolated mitochondria and diluted in the whole cell lysate (Figure 1(a)). Copper concentrations in the isolated mitochondria were $0.338 \pm 0.034\mu\text{g}/\text{mg}$ protein, which were about 10 folds higher than the copper concentrations in the cell lysate ($0.031 \pm 0.005\mu\text{g}/\text{mg}$ protein), as shown in Figure 1(b).

The treatment of the cells with siRNA targeting CCS-1 significantly decreased the protein level of the CCS-1, as shown in Figure 2(a). The depression of the CCS-1 was

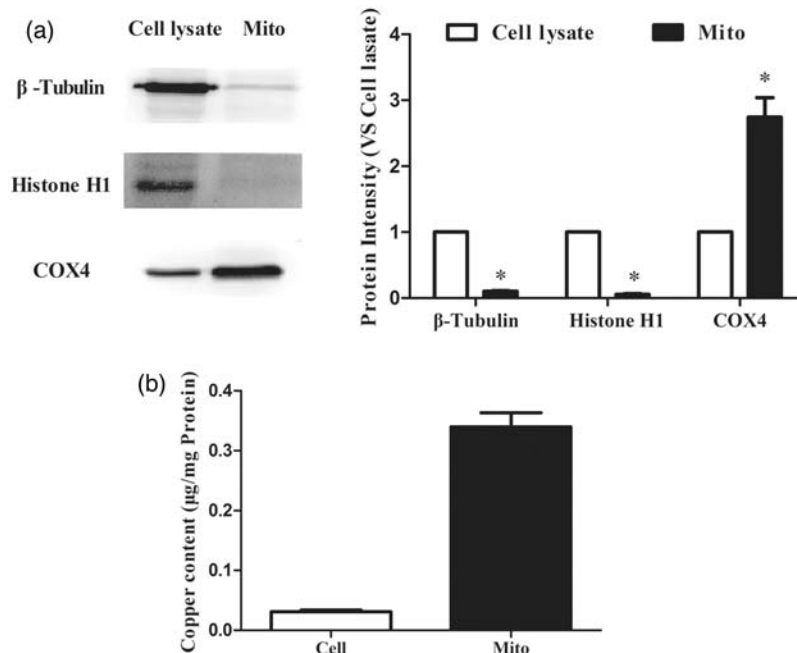


Figure 1 Determination of the purity of the isolated mitochondria by Western blot analysis of the cytoplasmic and nuclear markers. (a) The representative Western blot analysis displays the comparison in cytoplasmic marker β -tubuline, nuclear marker histone H1, and COX4 between the total cell lysate and the mitochondrial extract. The semiquantitative data presented in the bar graphs were obtained from three independent Western blotting experiments measuring the intensity changes by densitometry of each protein on the blot. (b) Copper concentrations in the total cell lysate and mitochondrial extract. The data expressed are means $\pm$ SEM. *, significantly different from the cell lysate ($P < 0.05$)

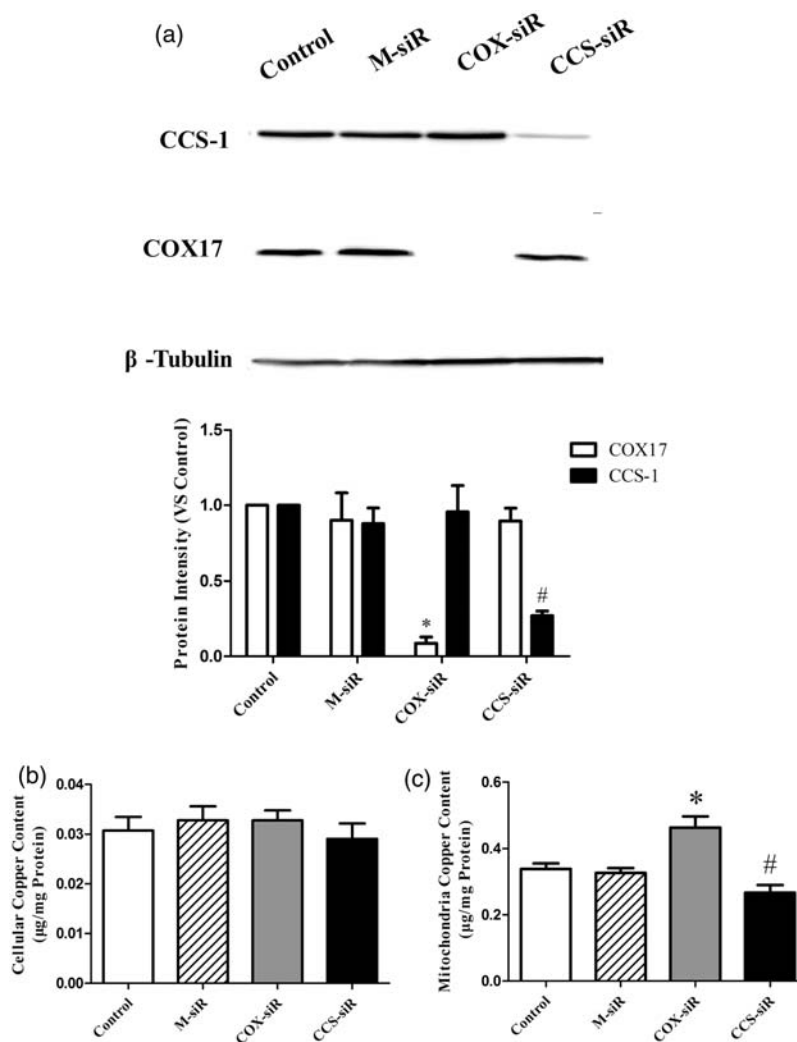


Figure 2 Quantitation of total cellular and mitochondrial copper concentration in the CCS-1 or COX17-deficient cells. (a) The effects of the siRNA targeting CCS-1 or the siRNA targeting COX17 on the respective protein levels in the cells, determined by Western blotting and semiquantitative analysis of the intensity changes by densitometry of the proteins on the blot. The bar graphs present the data obtained from three independent experiments. Control: cells were cultured for 48 h; M-siR: cells were exposed to Mismatch siRNA for 48 h; COX-siR: cells were exposed to COX17 siRNA for 48 h; CCS-siR, cells were exposed to CCS-1 siRNA for 48 h. (b) Copper concentrations in the cells treated with CCS-1 siRNA or COX17 siRNA. (c) Copper concentrations in the mitochondria isolated from the HUVECs treated with CCS-1 siRNA or COX17 siRNA. Both bar graphs (b and c) present the data obtained from four independent experiments. All of the data are expressed as means $\pm$ SEM. *, significantly different from control group ($P < 0.05$) and #, significantly different from control and COX-siRNA treatment groups ($P < 0.05$)

accompanied by a significant decrease in copper concentrations in the mitochondria, but did not change the total level of cellular copper. The treatment of the cells with siRNA targeting COX17 significantly decreased the protein level and significantly increased the copper concentration in the mitochondria, but did not change the total level of cellular copper (Figure 2).

To understand the effects of the CCS-1 or the COX17 deficiency on mitochondrial metabolism and function, we measured the mitochondrial CCO activity and changes in the mitochondrial membrane potential under the condition of the CCS-1 or the COX17 deficiency. As shown in Figure 3, CCO activities were significantly depressed in the COX17-deficient cells, but were not affected in the CCS-1 deficient cells. In association with the CCO activity changes, the mitochondrial membrane potential collapsed in the COX17-deficient cells as evidenced by fluorescence probe JC-1. This change in mitochondrial membrane potential

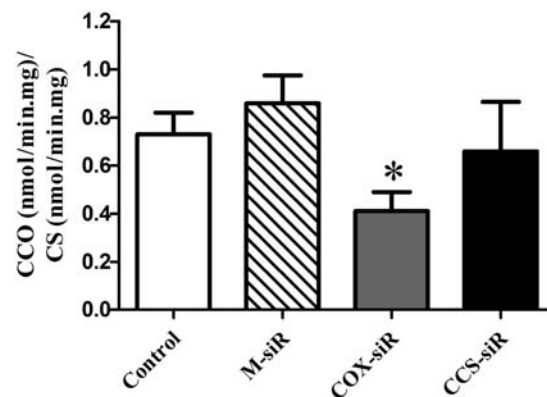


Figure 3 Enzymatic assay of CCO activity changes in the CCS-1 or COX17-deficient cells. The enzyme activity was calculated from the rate of decrease in the absorbance of reduced cytochrome c at 550 nm and normalized by citrate synthase activity. The treatment protocol was as the same as presented in Figure 2. Bar graphs show the means $\pm$ SEM from three independent experiments. *, significantly different from the control group ($P < 0.05$)

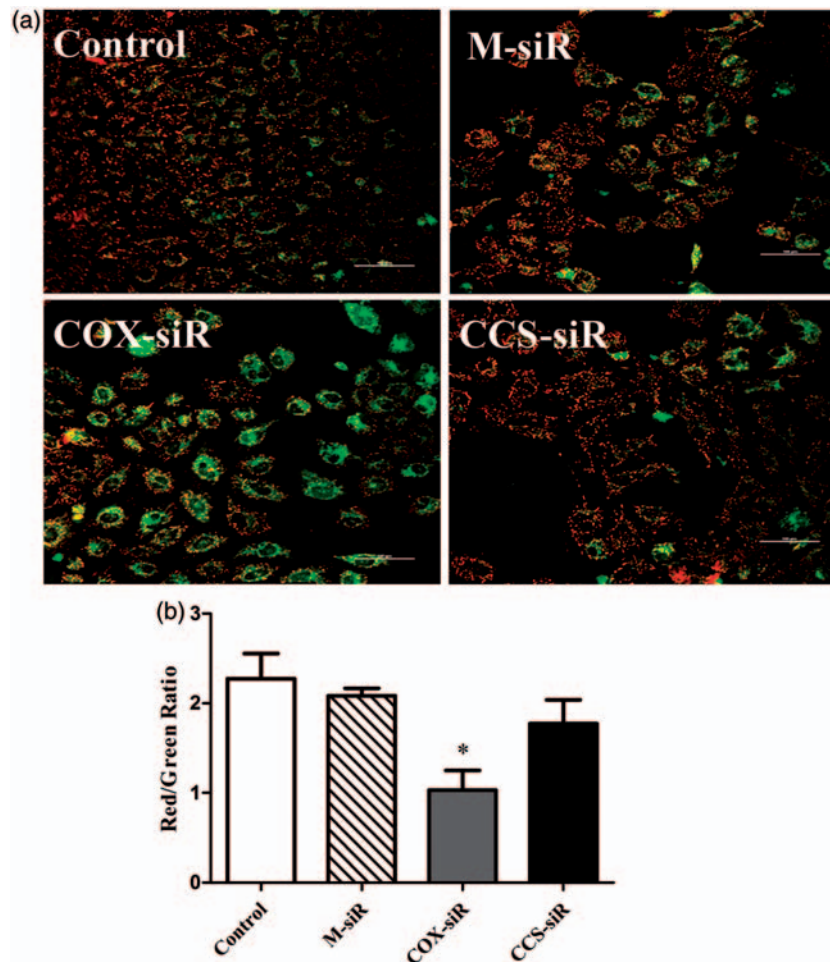


Figure 4 Changes in mitochondrial membrane potential measured by the JC-1 fluorescence in the CCS-1 or COX17-deficient cells. (a) Changes in the JC-1 fluorescence (red versus green) detected by confocal microscopy. (b) Semiquantitative analyses of the red/green ratio. The treatment protocol was as the same as presented in Figure 2. Bar graphs show the means $\pm$ SEM from three independent experiments. *, significantly different from the control group ($P < 0.05$). (A color version of this figure is available in the online journal)

was not observed in the CCS-1 deficient cells, as shown in Figure 4.

We performed a co-immunoprecipitation assay to define the relationship between CCS-1 and COX17. As shown in Figure 5, the immunoprecipitation with either anti-CCS-1 antibody or anti-COX17 antibody did not identify the interaction between CCS-1 and COX17.

Discussion

Mitochondrial copper homeostasis and delivery to CCO are fundamental features of the mitochondrial metabolism and function. The data obtained from the present study provided evidence showing that the CCS-1 deficiency induced by siRNA targeting the protein decreased the copper concentration in the mitochondria, suggesting the role of the CCS-1 in the transfer of copper to the mitochondria. However, this depression of the copper concentration did not affect the CCO activity in the mitochondria or disrupt the mitochondrial membrane potential. Therefore, the CCS-1 would not participate in the transfer of copper ions to the CCO, which was further confirmed by the result that CCS-1

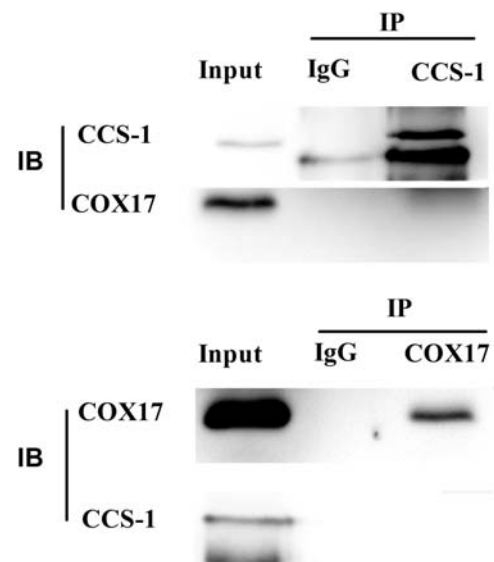


Figure 5 Co-immunoprecipitation between CCS-1 and COX17. The cell lysate was immunoprecipitated with anti-CCS-1 antibody or anti-COX17 antibody, and then the precipitated proteins were subjected to Western blotting analysis. The blotted proteins were detected by both CCS-1 and COX17 antibody to detect their co-immunoprecipitation

did not interact with copper chaperone for CCO, COX17, as revealed by the co-immunoprecipitation assay.

The deficiency in COX17 by siRNA targeting the protein significantly suppressed the CCO activity and caused a collapse of the mitochondrial membrane potential. This was expectable because it was well established that COX17 transfers copper ions to COX11, SCO1 and SCO2, which in turn insert copper ions to the copper active sites in the CCO. However, it was unexpected that the COX17 deficiency significantly increased the copper concentration in the mitochondria. This result demonstrates that (1) although the COX17 transfers copper ions to the CCO, it would not be responsible for the transfer of copper to the mitochondria, which has been a debating issue in the literature^{23–25}; and (2) the COX17 may be involved in the efflux of copper from the mitochondria, a topic that has not been addressed in the past.

It is thus possible that the transfer of copper ions to the mitochondria and the delivery of copper ions to the CCO take place in different mechanisms. While the present study identified that the CCS-1 may be a mechanism for copper transfer to the mitochondria, the question that remains to be answered is how copper ions are delivered to the CCO through COX17. Mitochondria contain a matrix pool of copper bound to a low-molecular weight ligand.²³ It was shown that this copper source contributes to the metallation of the CCO and mitochondrial superoxide dismutase 1.²⁴ This ligand has been found in the cytoplasm and it has been suggested that this ligand may recruit copper and function in place of a copper chaperone, facilitating copper translocation and storage in the mitochondrial matrix.²⁴

It has been known that the COX17 is responsible for the transfer of copper ions to COX11, SCO1 and SCO2, which in turn insert copper ions to the copper active sites in the CCO.¹⁶ It is possible that COX17 binds copper in the cytosol, but unlikely that the metallized COX17 can be transported to the mitochondria because the dimer/tetramer nature of the protein copper complex prevents its free passage through the outer membrane protein channel.²² The unlikelihood that the CCS-1 transfers copper ions to COX17 was suggested in the present study. The result obtained from co-immunoprecipitation identified that there was no interaction between CCS-1 and COX17. The low-molecular weight ligand²³ mentioned above would likely be a candidate for the upstream copper chaperone for COX17, as it has been demonstrated that this copper source is related to the metallation of the CCO.²⁴ This is an important question that needs to be addressed in the future studies.

An important novel finding in the present study was that the deficiency in COX17 led to an increase in the mitochondrial copper concentration. Until present, all of the studies related to mitochondrial copper homeostasis have focused on the transport of copper into the mitochondria. The export of copper from the mitochondria is an important topic in the understanding of the mitochondrial copper homeostasis. The reduction of COX17 resulted in the accumulation of the mitochondrial copper pool would suggest that the COX17 may function in the export of copper ions from the mitochondria. How does this take place and how

is this function of the COX17 regulated are critical questions that will be addressed in our future studies.

In summary, the present study identified that the CCS-1 is likely involved in the transfer of copper ions to the mitochondria, but not to the CCO. The suppression of the CCS-1 reduced the mitochondrial copper concentration, but did not affect the CCO activity or disturb the mitochondrial membrane potential. On the other hand, the COX17 was critical for the CCO activity and its deficiency suppressed the CCO activity and caused a collapse of the mitochondrial membrane potential. Furthermore, the COX17 deficiency resulted in an increase in the mitochondrial copper concentration, suggesting that this copper chaperone may also function in the copper export from the mitochondria.

Author contributions: Participated in research design: YJK, BW and DD; Conducted experiments: BW and DD; Performed data analysis: BW, DD and YJK; Wrote or contributed to the writing of the manuscript: YJK and BW.

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