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## Molecular modulation of the copper and cisplatin transport function of CTR1 and its interaction with IRS-4

Cheng-Yu Tsai<sup>a</sup>, Christopher A. Larson<sup>a,1</sup>, Roohangiz Safaei<sup>a,2</sup>, and Stephen B. Howell<sup>a,b</sup>

Cheng-Yu Tsai: cht026@ucsd.edu; Christopher A. Larson: clarson@illumina.com; Stephen B. Howell: showell@ucsd.edu

<sup>a</sup>Moore's Cancer Center, University of California, San Diego, 3855 Health Sciences Drive, Mail Code 0819, La Jolla, CA 92093-0819, USA

<sup>b</sup>Department of Medicine, University of California, San Diego, 3855 Health Sciences Drive, Mail Code 0819, La Jolla, CA 92093-0819, USA

### Abstract

The copper influx transporter CTR1 is also a major influx transporter for cisplatin (cDDP) in tumor cells. It influences the cytotoxicity of cDDP both *in vivo* and *in vitro*. Whereas Cu triggers internalization of CTR1 from the plasma membrane, cDDP does not. To investigate the mechanisms of these effects, myc-tagged forms of wild type hCTR1 and variants in which Y103 was converted to alanine, C189 was converted to serine, or the K178/K179 dilysine motif was converted to alanines were re-expressed in mouse embryo cells in which both alleles of CTR1 had been knocked out and also in HEK293T cells. The Y103A mutation and to a lesser extent the C189S mutation reduced internalization of CTR1 induced by Cu while the K178A/K179A had little effect. Both Y103 and C189 were required for Cu and cDDP transport whereas the K178/K179 motif was not. While Y103 lies in an YXXM motif that, when phosphorylated, is a potential docking site for phosphatidylinositol 3-kinase and other proteins involved in endocytosis, Western blot analysis of immunoprecipitated myc-CTR1, and proteomic analysis of peptides derived from CTR1, failed to identify any basal or Cu-induced phosphorylation. However, proteomic analysis did identify an interaction of CTR1 with IRS-4 and this was confirmed by co-immunoprecipitation from HEK cells expressing either FLAG-CTR1 or myc-CTR1. The interaction was greater in the Y103A-expressing cells. We conclude that Y103 is required for the internalization of hCTR1 in response to Cu, that this occurs by a mechanism other than phosphorylation and that mutation of Y103 modulates the interaction with IRS-4.

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Correspondence to: Stephen B. Howell, showell@ucsd.edu.

<sup>1</sup>Present address: Illumina Inc, 5200 Illumina Way, San Diego, CA 92122, USA.

<sup>2</sup>Present address: 7556 Charmant Drive #1738, San Diego, CA 92122, USA.

### Competing interests

The authors declare that they have no competing interests.

### Authors' information

Participated in research design: Tsai, Larson, Safaei, and Howell. Conducted experiments: Tsai, Larson, and Safaei. Performed data analysis: Tsai, Larson, Safaei and Howell. Wrote or contributed to the writing of the manuscript: Tsai, Larson and Howell.

## Keywords

Cisplatin; Copper transporter 1; Insulin receptor substrate 4

## 1. Introduction

Copper transporter 1 (CTR1) is a high-affinity Cu influx transporter in mammalian cells. It is of interest to oncologists because it also mediates the uptake of the platinum-containing chemotherapeutic agents in tumor cells [1–7]. It regulates sensitivity to the cytotoxic effect of cDDP both *in vitro* and *in vivo*. Knockout of both alleles of CTR1 reduces initial cDDP influx [6], enhances efflux [8] and renders cells resistant to Cu, cDDP, carboplatin and oxaliplatin [1,2,5,6,9]. Re-expression of hCTR1 in cells lacking endogenous CTR1 restores cDDP uptake and sensitivity [6], as does forced over-expression in cells containing endogenous CTR1 [9].

As shown in Fig. 1, hCTR1 contains 190 amino acids and consists of an extracellular N-terminal domain, three transmembrane domains and two intracellular domains, the first being a loop connecting the first and second transmembrane domains and the second being the 15 amino acid C-terminal tail region. CTR1 exists as a homotrimer on the cell surface and electron crystallography and molecular modeling suggest that it forms a pore consisting of 4 stacked rings of 3 methionines each with a narrow entrance at the extracellular end and a vestibule at the intracellular end [10–12]. Both  $\text{Cu}^{1+}$  and cDDP are soft Lewis acids that readily form weak bonds with methionine, and it has been suggested that CTR1 transports  $\text{Cu}^{1+}$  through a series of transchelation reactions that pass the  $\text{Cu}^{1+}$  from one ring of methionines to the next and eventually to the ring of cysteines at the bottom of the pore [10,11,13]. The ability of CTR1 to transport  $\text{Cu}^{1+}$  but not  $\text{Cu}^{2+}$  may result from the tighter binding of  $\text{Cu}^{2+}$  to the methionines such that it cannot be handed through the pore by transchelation [13]. While the C-terminal C189 amino acids are quite far away from the methionine rings above them, biochemical studies indicate that the cysteines bind  $\text{Cu}^{1+}$  and do influence Cu transport [14]. It has been proposed that the cysteines function as a gate that controls movement through the other parts of the pore [11,15–17].

High concentrations of Cu trigger the internalization of CTR1, and in some types of cells this is accompanied by degradation [18–20] whereas in others it is not [7,21]. It has been hypothesized that degradation may serve to limit accumulation of toxic levels of the metal [9,21–23]. In yeast the degradation of CTR1 requires the E3 ubiquitin ligase Rsp5 [23].

There are three sites within hCTR1 that are of interest with respect to control of its internalization. The first of these is the tyrosine at position 103 that lies in the intracellular loop between trans-membrane domains one and two. Tyrosines in the cytosolic domains of proteins are often involved in endocytosis [24]. The Y103 site is of particular interest because the YXXM motif within which it resides is a potential phosphorylation site that, once phosphorylated, is a candidate for binding to the SH2 domain of the p85 subunit of PI3K or other proteins involved in the endocytosis of PDGFR and other surface proteins [25]. A second site of interest is the next to last amino acid which is a cysteine at position 189 that resides in an HCH motif at the C-terminal end of hCTR1. In the case of hCTR1,

C189 appears to be important for the proper assembly of the homotrimer in the membrane [26]. In yeast cysteines in the C-terminal tail appear to function as a switch that prevents further Cu transport through the pore [17]. A third site of interest is the pair of lysines at positions 178 and 179. Prior studies have demonstrated that yCTR1 and mCTR1 become ubiquitinated in response to Cu or cDDP exposure [23,27] and the K178/K179 is a potential ubiquitination site that is solvent exposed in the all-atom model of CTR1 [12].

In order to further elucidate how Cu and cDDP differ with respect to their interaction with CTR1, we constructed vectors expressing N-terminally myc-tagged variant forms of hCTR1 in which either Y103 was converted to alanine, C189 was converted to a serine or both lysines in the K178/K179 motif were converted to alanine. These variants were expressed in mouse embryo fibroblast cells in which both alleles of *mCTR1* had been knocked out, and in human HEK293T cells. We report here that whereas Y103 and C189 are required for Cu and cDDP transport, the K178/K179 motif is not. We found that Y103 is required for the Cu-induced internalization of CTR1 from the plasma membrane, but that neither Y103 nor serines or threonines are phosphorylated under either basal conditions or after exposure to Cu. To identify partners that interact with CTR1 during Cu-induced internalization we conducted a proteomic analysis of proteins that immunoprecipitated with CTR1 and report here that the identification of a new interacting protein, insulin receptor substrate 4 (IRS-4).

## 2. Materials and methods

### 2.1. Drugs and reagents

cDDP was purchased as Platino<sup>TM</sup> from the pharmacy at the Moores Cancer Center; it contains 3.33 mM cDDP in 0.9% NaCl. The cDDP was diluted into DMEM-RS Reduced Serum Media (HyClone, Logan, UT). Bradford reagent and detergent-compatible protein assay kit, DC<sup>TM</sup> Protein Assay were purchased from BioRad Laboratories, Inc. (Hercules, CA). Anti-myc primary antibody 9B11, anti-Na<sup>+</sup>/K<sup>+</sup> ATPase antibody, anti-phosphotyrosine monoclonal antibody and anti-IRS-2 antibody were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Anti-FLAG antibody was purchased from Sigma Aldrich (St. Louis, MO). Anti-CTR1 primary antibody was purchased from Abcam (Cambridge, UK), anti-IRS-4 antibody and anti-phosphoserine/threonine/tyrosine antibody from Abcam (Cambridge, UK), and IR fluorophore-conjugated secondary antibodies were obtained from Li-Cor Biosciences (Lincoln, NE). DSP (dithiobis[succinimidylpropionate]) was purchased from Thermo Scientific (Pittsburg, PA).

### 2.2. Cell types, culture and engineering

Mouse embryonic fibroblasts containing wild type alleles of CTR1 (CTR1<sup>+/+</sup>) and a line in which both copies of CTR1 had been somatically knocked out (CTR1<sup>-/-</sup>) were graciously provided by Thiele [28]. The absence of CTR1 mRNA in the CTR1<sup>-/-</sup> cells has been repeatedly documented by qRT-PCR and the DDP-resistant phenotype by testing for cytotoxicity [6,8,29–32]. HEK293T cells were obtained from the American Type Tissue Culture Collection. The myc-CTR1<sup>-/-/WT</sup> and HEK293/myc-CTR1<sup>WT</sup> sublines were constructed by infecting the CTR1<sup>-/-</sup> and HEK293T cells with a lentivirus expressing wild type human CTR1 cDNA, N-terminally tagged with the myc epitope, using the ViraPower

Lentiviral Induction kit (Invitrogen, Carlsbad, CA). Point mutations to the myc-tagged wild type CTR1 molecule were generated with the GeneTailor Site-Directed Mutagenesis Kit (Invitrogen, Carlsbad, CA) using the following primers: for the myc-CTR1<sup>-/-</sup>/Y103A mutation (gcgtaagtcacaagtcagcattcgcgccaattccatgcctgtcccaggacca, tggctctgggacagggcatggaattggcgcgaatgctgactgtgacttacgc), for the myc-CTR1<sup>-/-</sup>/C189S mutation (atggaacaaaaactatttc, tcaatggctatgctctgtgatc). The Forward and reverse PCR primers for this production of the K178A/K179A mutation were, respectively, 5'-cctatgaaggagaagtcgaccctgctgctcaccatcacctatagtgtctcgtaacg-3' and 5'-cgttacgagacactataggtgatggtgacgacgacgggtcactctcctcatagg-3'. Sublines expressing these mutant forms of CTR1 were generated by infecting the CTR1<sup>-/-</sup> and HEK293T cells with lentivirus expressing the mutants using the ViraPower system, as described for the myc-CTR1<sup>-/-</sup>/WT and HEK293/myc-CTR<sup>WT</sup> cells above. HEK293 containing tetracycline-regulated wild type hCTR1 gene with N-terminally tagged with the FLAG epitope (HEK293 FLP-In T-Rex cell) was provided by Dr. Jack Kaplan, 1 µg/mL tetracycline was used to induce the over-expression on wild-type hCTR1.

### 2.3. Biotinylation and immunoblotting

Mouse embryo fibroblasts and HEK293 cells were surface biotinylated with EZ-LINK sulfo-NHS-SS-biotin (Thermo Scientific, Pittsburg, PA) for 30 min at 4 °C and quenched in PBS containing 200 mM glycine PBS for 25 min. Cells were harvested by scraping, washed twice in PBS and then solubilized in 1% Triton-X-100 for 60 min, and insoluble material was removed by centrifugation at 14,000 × g for 10 min. Protein concentrations were determined by detergent-compatible protein assay kit, DC<sup>TM</sup> Protein Assay (Bio-Rad, Hercules, CA) and equal amount of proteins were incubated overnight at 4 °C with 100 µL streptavidin-coated beads (Thermo Scientific, Pittsburg, PA). The beads were collected and washed, after which the proteins bound to the beads were cleaved with 5% β-mercaptoethanol in 2 × SDS electrophoresis sample buffer and subjected to Western blot analysis using an antibody to the myc tag or to CTR1 [33] and an antibody to the Na/K ATPase that was used as a lane loading control. Western blot analysis was performed as previously described [31]. Protein expression on the Western blots was quantified using an Odyssey Imaging System (Li-Cor Biosciences, Lincoln, NE) utilizing software version 3.0. Expression of CTR1 in the experimental cells was expressed as a percent of that in the control cells after normalization to the level of Na/K ATPase.

### 2.4. Reversible cross-link immunoprecipitation procedure

Cells were washed twice with PBS at room temperature to remove all traces of media and cell pellets were resuspended in PBS and incubated with freshly prepared dithiobis[succinimidyl propionate] (DSP) (Thermo Scientific, Pittsburg, PA) at a final concentration of 2.5 mM for 30 min at room temperature. The cross-linking reaction was quenched with 25 mM Tris-HCl pH 7.4 for 15 min at room temperature. The pelleted cells were lysed with RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100) plus protease and phosphatase inhibitors (Thermo Scientific, Pittsburg, PA). Equal amount of proteins were incubated with either anti-FLAG or anti-myc antibody and protein A/G beads (Thermo Scientific, Pittsburg, PA) overnight at 4 °C with end-over-end rotation. The beads were then washed 5 times with RIPA buffer and

proteins were eluted by incubating the beads with Laemmli's 2× sample buffer (125 mM Tris-HCl, pH 6.8, 2 mM EDTA, 6% SDS, 20% glycerol, 5% β-mercaptoethanol and 0.25% bromophenol blue) at 95 °C for 5 min and subjected to Western blot analysis.

## 2.5. Proteomic analysis

Proteomic analysis was performed in UC San Diego Biomolecular/Proteomics Mass Spectrometry Facility. The beads containing the immunoprecipitated protein were diluted in TNE (50 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA) buffer. RapiGest SF reagent (Waters Corp., Milford, MA) was added to the mixture to a final concentration of 0.1% and samples were boiled for 5 min. TCEP (Tris (2-carboxyethyl) phosphine) was added to a final concentration of 1 mM and the samples were incubated at 37 °C for 30 min. Subsequently, the samples were carboxymethylated with 0.5 mg/mL of iodoacetamide for 30 min at 37 °C followed by neutralization with TCEP at a final concentration of 2 mM. Proteins samples prepared as above were digested with trypsin (trypsin:protein ratio—1:50) overnight at 37 °C. RapiGest was degraded and removed by treating the samples with 250 mM HCl at 37 °C for 1 h followed by centrifugation at 14,000 rpm for 30 min at 4 °C. The soluble fraction was then added to a new tube and the peptides were extracted and desalted using C18 desalting columns (Thermo Scientific, Pittsburg, PA). LC-MS/MS analysis of the trypsin-digested peptides was carried out using ultra high pressure liquid chromatography coupled with tandem mass spectroscopy combined with nano-spray ionization. The nano-spray ionization experiments were performed using a Triple T of 5600 hybrid mass spectrometer (ABSCIEX, Framingham, MA) interfaced with nano-scale reverse-phase ultra high pressure liquid chromatography (Waters Corporation nano ACQUITY) using a 20 cm-75 micron ID glass capillary packed with 2.5-μm C18 (130) CSH™ beads (Waters Corp., Milford, MA). Peptides were eluted from the C18 column into the mass spectrometer using a linear gradient (5–80%) of acetonitrile at a flow rate of 250 μL/min for 1 h. The buffers used to create the acetonitrile gradient were: Buffer A (98% H<sub>2</sub>O, 2% acetonitrile, 0.1% formic acid, and 0.005% trifluoroacetic acid) and Buffer B (100% acetonitrile, 0.1% formic acid, and 0.005% trifluoroacetic acid). MS/MS data were acquired in a data-dependent manner in which the MS1 data was acquired for 250 ms at *m/z* of 400 to 1250 Da and the MS/MS data was acquired from *m/z* of 50 to 2000 Da. The independent data acquisition parameters were as follows: MS1-TOF acquisition time of 250 ms, followed by 50 MS2 events of 48 ms acquisition time for each event. The threshold to trigger MS2 event was set to 150 counts when the ion had the charge state +2, +3 and +4. The ion exclusion time was set to 4 s. Finally, the collected data were analyzed using Protein Pilot 4.5 (ABSCIEX, Framingham, MA) for peptide identifications.

## 2.6. Measurement of cellular drug accumulation

Drug accumulation was measured by inductively-coupled plasma mass spectroscopy using sulfur as a surrogate for total protein as described previously [6]; the only change made was the utilization of 1 mL volume of medium and the use of DMEM-RS in place of OptiMEM media.

## 2.7. Statistical analysis

All two-group comparisons utilized Student's *t*-test with the assumption of unequal variance. Data are presented as mean  $\pm$  SEM of a minimum of 3 independent experiments.

## 3. Results

### 3.1. Expression of CTR1 variants in CTR1<sup>-/-</sup> mouse embryo fibroblasts and HEK293T cells

Prior studies of the importance of various components of hCTR1 for Cu homeostasis have sometimes been confounded by the presence of endogenous CTR1. To avoid this problem, wild type and variant forms of hCTR1 were re-expressed in mouse embryo fibroblasts in which both alleles of CTR1 had been knocked out (CTR1<sup>-/-</sup> cells), and in human HEK293T cells that express low levels of endogenous CTR1. Lentiviral vectors containing a blastocidin resistance marker were constructed to express either an N-terminal myc-tagged form of wild type hCTR1, a variant in which the Y103 was converted to alanine, a variant in which C189 was converted to serine, or one in which the dilysine motif K178/K179 was replaced with alanines. CTR1<sup>-/-</sup> cells were infected and selected with blastocidin to generate the CTR1<sup>-/-</sup>/WT, CTR1<sup>-/-</sup>/Y103A, CTR1<sup>-/-</sup>/C189S and CTR1<sup>-/-</sup>/K178/K179A sublines that were then characterized with respect to the expression of each form of exogenous CTR1. HEK293T cells were infected with the same vectors to produce the HEK293/myc-CTR1<sup>WT</sup>, HEK293/myc-CTR1<sup>Y103A</sup>, HEK293/myc-CTR1<sup>C189S</sup> and HEK293/myc-CTR1<sup>K178A/K179A</sup> cell lines. To assess the level of CTR1 protein expression at the plasma membrane, the cell surface proteins were biotinylated by exposure to sulfo-NHS-SS-biotin before lysis, then recovered on streptavidin-coated beads and subjected to Western blot analysis using an antibody to the myc tag or to CTR1. The wild type and both variant forms of hCTR1 were present in the plasma membrane at nearly equivalent levels as determined by quantitative imaging and normalized for Na/K ATPase content. The CTR1<sup>-/-</sup>/Y103A cells expressed plasma membrane CTR1 at a mean of  $96 \pm 4\%$  of that in the CTR1<sup>-/-</sup>/WT cells ( $n = 3$ ), the CTR1<sup>-/-</sup>/C189S cells at  $91 \pm 5\%$  ( $n = 3$ ) and the CTR1<sup>-/-</sup>/K178A/K179A at  $87 \pm 8\%$  ( $n = 3$ ) of that in the CTR1<sup>-/-</sup>/WT cells. Western blot analyses of the expression of each of the variants in the plasma membrane of the HEK293T cells showed nearly equivalent expression of the wild type and Y103A and C189S variants but lower expression of the K178A/K179A variant. To document that the sulfo-NHS-SS-biotin reacted with only cell surface proteins, biotinylated proteins from each of the cell lines were analyzed by Western blot for the presence of the cytosolic protein glyceraldehyde 3-phosphate dehydrogenase and none was found (data not shown).

### 3.2. Effect of mutations on Cu and cDDP influx

To determine the effect of the Y103A, C189S and K178A/K179A mutations on the steady-state levels of cellular Cu we used the mouse embryo cells since they do not express any endogenous CTR1. The CTR1<sup>-/-</sup>/WT, CTR1<sup>-/-</sup>/Y103A, CTR1<sup>-/-</sup>/C189S and CTR1<sup>-/-</sup>/K178/K179A cells were grown in complete DMEM medium containing  $\sim 0.3 \mu\text{M}$  Cu. Fig. 2A shows that basal Cu was not significantly altered by re-expression of wild type CTR1 in the CTR1<sup>-/-</sup>/WT cells or by re-expression of any of the variant forms of CTR1.

The effect of the CTR1 variants on the accumulation of Cu was analyzed by exposing the cells to media containing 100  $\mu\text{M}$  Cu for 1 h. As shown in Fig. 2B, re-expression of wild type hCTR1 resulted in a 2-fold increase in the amount of Cu accumulation when compared to uptake in the CTR1<sup>-/-</sup> cells ( $p = 0.003$ ). While the K178A/K179A variant was nearly as effective in increasing Cu uptake as the wild type, neither of the Y103A or the C189S variant was able to significantly increase Cu uptake. Thus, the integrity of both Y103 and C189 was required for Cu transport to occur in this system.

To analyze the effect of the Y103A and C189S substitutions on the initial influx of cDDP, the 4 types of cells were exposed to 30  $\mu\text{M}$  cDDP for 5 min, washed thoroughly and the Pt content measured by ICP-MS. A short duration of drug exposure was used because prior studies have shown that the greatest effect of CTR1 is on initial cDDP influx. As shown in Fig. 2C, re-expression of wild type CTR1 resulted in a 4.2-fold increase in cDDP accumulation ( $p = 9 \times 10^{-5}$ ). Expression of the Y103A variant resulted in only a 1.4-fold increase in cDDP accumulation, which was only 33% of the increase produced by re-expression of wild type CTR1 ( $p = 0.0009$ ). Expression of the C189S variant failed to increase cDDP uptake at all, and in fact reduced it by a small amount to 78% of that observed for the CTR1<sup>-/-</sup> cells. In contrast, the K178A/K179A variant increased cDDP uptake to 83% of that produced by the wild type CTR1. Thus, while the K178A/K179A variant retained almost normal transport activity, mutation of Y103 severely disabled cDDP transport and the C189S variant appeared to be transport dead.

### 3.3. Effect of mutations on Cu-induced internalization of CTR1

Exposure to Cu causes the rapid internalization of CTR1, whereas in HEK293T and small cell lung cancer cells, exposure to cDDP has been noted to increase the relative amount of higher molecular weight forms interpreted as being dimers and trimers [7,34]. To determine how the mutations we made in CTR1 affected these responses we used the HEK293T cells since CTR1 levels were higher and could be quantified more precisely. Fig. 3A shows the effect of a 30 min exposure to 100  $\mu\text{M}$  Cu on plasma membrane CTR1 in the wild type and the Y103A, C189S and K178A/K179A variants. Using a rabbit monoclonal antibody that we have previously extensively characterized [33], CTR1 was detected as bands of 33–35 and 62–64 kDa. The Cu exposure reduced CTR1 in the plasma membrane of the HEK293/myc-CTR1<sup>WT</sup> cells to  $15.9 \pm 2.2\%$  of that in the untreated cells ( $p = 0.0007$ ), whereas it reduced it to  $67.0 \pm 6.2\%$  ( $p = 0.07$ ) of that in the untreated HEK293/myc-CTR1<sup>Y103A</sup> cells, to  $52.6 \pm 3.6\%$  ( $p = 0.004$ ) of that in the untreated HEK293/myc-CTR1<sup>C189S</sup> cells and to  $34.0 \pm 9.0\%$  ( $p = 0.051$ ) of that in the untreated HEK293/myc-CTR1<sup>K178A/K179A</sup> cells as determined by quantitative imaging of 5 independent Western blot analyses normalized for Na/K ATPase content (Fig. 3B). Thus, both the Y103A and C189S mutations impaired the ability of Cu to trigger internalization of CTR1 while the K178A/K179A mutation had less effect. Exposure to cDDP did not cause loss of CTR1 from the plasma membrane in any of the 4 cell types indicating that it does not trigger the same internalization mechanism as Cu (data not shown).

### 3.4. Analysis of CTR1 for phosphorylation

CTR1 contains 6 serines, 3 threonines but only a single tyrosine in the solvent exposed intracellular portion of the molecule. Given that phosphorylation of plasma membrane transporters is one mechanism by which internalization is triggered, and given that conversion of the one tyrosine (Y103) to alanine blocked internalization, we were curious as to whether Cu triggered the phosphorylation of CTR1. We first sought to determine whether CTR1 was phosphorylated under basal conditions using the HEK293 FLP-In T-Rex cells in which FLAG-tagged CTR1 was induced by exposure to tetracycline. Cells were pretreated with the phosphatase inhibitor orthovanadate or calf intestinal alkaline phosphatase, or the combination of both orthovanadate and the alkaline phosphatase. CTR1 was immunoprecipitated using an antibody to the FLAG tag and subjected to Western blot analysis with an antibody capable of detecting phosphorylation on serine, threonine or tyrosine, and with an antibody specific for phosphotyrosine. No change in the signal detected by Western blot analysis was observed in response to either the phosphatase inhibitor or alkaline phosphatase (Fig. 4A). CTR1 was also immunoprecipitated from HEK293 FLP-In T-Rex cell cells before and after a 30 min exposure to 100  $\mu$ M Cu using the same technique. As shown in Fig. 4B, no phosphorylation of the tyrosine was detected. To refine the search further, CTR1 was immunoprecipitated before and 5 min after the start of exposure to 100  $\mu$ M Cu and then digested with trypsin and examined by mass spectroscopy of the presence of phosphopeptides. Peptides containing the possible phosphorylation sites from the internal portion of CTR1 were detected using this approach (<sup>103</sup>YNSMPVPGPNGTILMETHK and <sup>180</sup>AVVVDI-TEHCH); however, no phosphorylation of these was consistently demonstrated by this proteomic analysis. Based on these results, it appears that Cu triggers removal of CTR1 from the plasma membrane by some other mechanism.

### 3.5. Proteomic analysis of CTR1 interacting proteins

After successfully detecting CTR1 peptides using mass spectrometry, we carried out reversible cross-link immunoprecipitation and mass spectrometry experiments to identify potential CTR1 binding partners [35]. Labile interactions were stabilized prior to cell lysis by treating the cells with the cell-permeable, thiol-cleavable cross-linker DSP. After immunoprecipitation of CTR1 from the HEK293 FLP-In T-Rex cells with anti-FLAG antibody, crosslinked binding partners were selectively released by reduction with  $\beta$ -mercaptoethanol which resulted in an extremely efficient recovery with exceptionally low background. Proteomic analysis of the released trypsin-digested proteins identified insulin receptor substrate 4 (IRS-4) and this was considered of interest because IRS proteins mediate a variety of biological effects downstream of the insulin and insulin-like growth factor 1 (IGF-1) receptors, and we had previously demonstrated that CTR1 plays an important role in regulating signaling from receptor tyrosine kinases [36]. The IRS-4 peptides identified by mass spectrometry are presented in Table 1. To confirm the interaction between CTR1 and IRS-4, FLAG-CTR1 was immunoprecipitated from the HEK293 FLP-In T-Rex cells after tetracycline induction and subjected to Western blot analysis for the presence of IRS-4. As shown in Fig. 5, there was a  $21.5 \pm 4.0$ -fold increase in the association of CTR1 with IRS-4 after tetracycline induction ( $p < 0.05$ ,  $n = 4$ ). The



interaction was further confirmed by showing a  $4.8 \pm 0.4$ -fold increase when the CTR1 immunoprecipitated from in HEK293/myc-CTR1<sup>WT</sup> cells as compared to that precipitated from the empty vector control cells ( $p < 0.001$ ,  $n = 4$ ). No interaction between CTR1 and IRS-1 or IRS-2 was detected indicating that the interaction is specific to IRS-4 (data not shown). To determine whether either Y103 or C189 is important to the interaction with IRS-4, the HEK293/myc-CTR1<sup>Y103A</sup> and HEK293/myc-CTR1<sup>C189S</sup> cells were analyzed in the same way. As shown in Fig. 6 there was a  $2.7 \pm 0.2$ -fold increase in the CTR1-bound IRS-4 level in HEK293/myc-CTR1<sup>Y103A</sup> cells compared to that in the HEK293/myc-CTR1<sup>WT</sup> cells ( $p = 0.041$ ,  $n = 3$ ), but no significant interaction in the HEK293/myc-CTR1<sup>C189S</sup> cells. This suggests that not only is Y103 important to the ability of Cu to induce CTR1 internalization but it also participates in the interaction between CTR1 and IRS-4.

#### 4. Discussion

The re-expression of wild type and variant forms of hCTR1 in cells in which both alleles of CTR1 have been knocked out provides a powerful way of identifying structural components essential for function. The results of this study provide further insight into the domains of CTR1 that are required for the transport of Cu and cDDP, and those required for the internalization of CTR1 in response to exposure to Cu. Perhaps the most important new finding to emerge from this study is that Y103, in addition to being required for the transport of Cu and cDDP, is important to the ability of Cu to trigger CTR1 internalization. Conversion of Y103 to alanine eliminated the increase in whole cell Cu produced by a 1 h exposure to even a very high concentration of Cu. The intracellular loop that spans transmembrane domains 1 and 2 has not previously been thought to play an important role in the accumulation of either Cu or cDDP. However, this domain contains several motifs that are of interest to a potential scaffold role of CTR1 that facilitates the binding of other proteins. In *Xenopus* CTR1 is important for signaling during embryonic development [37] and recent studies have shown that it is essential for signaling from multiple receptor tyrosine kinases in mouse cells [36,38]. Molecular modeling suggests that the intracellular loop is a likely site for the docking of proteins that participate in this pathway [12]. The phosphorylation of tyrosines in the cytosolic domains of transmembrane proteins is known to be involved in both clathrin and non-clathrin mediated endocytosis [24]. The Y103 site in hCTR1 is of particular interest because of its location within a YXXM motif that, once phosphorylated, is a candidate for binding to the p85 subunit of PI3K which mediates the endocytosis of PDGF and many other surface proteins [25,39]. PI3K is involved in protein sorting in a variety of trafficking pathways such as agonist-induced endocytosis [40] and multi-vesicular body formation [41]. However, despite the fact that Y103 is solvent exposed in the all atom model of CTR1 [12], we found no evidence that Y103 became phosphorylated during Cu-induced internalization. Using PhosphoSitePlus, NetPhos predictor, Phospho. ELM database and other major online phosphorylation site prediction programs, we also did not find suggestion that the tyrosine on <sup>103</sup>YNSM of CTR1 can be phosphorylated. Nevertheless, we speculate that Y103 itself, or the conformation that it enforces on the cytoplasmic loop, is required for the engagement of CTR1 with the endosomal machinery and quite likely with proteins or peptides that can accept Cu from

CTR1. Since Cu is not free inside the cell, engagement with such Cu acceptors is probably essential for transport to occur [42]. Whether such an acceptor is functionally linked to the endocytic machinery is an interesting area for future investigation.

The results of this study confirm prior reports that C189 is required for the transport of Cu by human CTR1 [14,43] and indicate that it is also essential for the transport of cDDP. However, they are at odds with results obtained in a small cell lung cancer model in which Cu and cDDP transport were equivalent in cells forced to express either wild type CTR1 or a variant in which the last 3 amino acids  $^{188}\text{HCH}^{190}$  were deleted [4]. It is uncertain whether the difference in the two models is due to interaction of the variant with endogenous CTR1 in the small cell model or deletion of the terminal 3 amino acids rather than just substitution of C189 by serine. Electron crystallography studies of CTR1 indicate that it forms a pore with a narrow entrance at the extracellular end and a vestibule at the intracellular end [10,11]. The HCH motif in the C-terminal tail is one of the most highly conserved parts of CTR1, and the all atom molecular model of CTR1 suggests that the cysteines contributed by each CTR1 monomer participate in the formation of Cu binding sites at the exit of the vestibule [12]. In both human and yeast CTR1 the C-terminal domain appears to be involved in interactions between the monomers that affect the structural stability of the trimer [44], and this region may function as a Cu sensor that switches the pore between an open and closed configuration [17]. The HCH motif is also reported to be involved in interactions between CTR1 and ATOX1 [15,43,45]. In our study, conversion of C189 to serine reduced Cu and cDDP transport but did not ablate the ability of Cu to trigger internalization of CTR1 indicating that transport function is not essential to the internalization process. It is important to note it has been reported that the C189S variant does not assemble correctly as a trimer in the plasma membrane which may account for its failure to support normal Cu uptake [26]. It has also been reported that replacement of the entire HCH motif with AAA increases rather than decreases Cu transport [14] which suggests that the two histidines that flank C189 also serve a role in gating Cu influx, a concept consistent with their potential role in hosting a Cu binding site at the cytoplasmic end of the trimer [12].

Along with the  $^{188}\text{HCH}^{190}$  the  $^{178}\text{K/K}^{179}$  motif is conserved across species. Prior studies have demonstrated that yCTR1 and mCTR1 become ubiquitinated in response to Cu or cDDP exposure [23,27]. The  $^{178}\text{K/K}^{179}$  motif was selected for investigation because it is a potential site of ubiquitinylation. yCTR1 contains a typical ubiquitinylation signal in the form of a pair of lysines at  $^{340}\text{K/K}^{345}$  [23]. yCTR1 is ubiquitinated by the ubiquitin ligase Rsp5p that is attracted to CTR1 by an adaptor protein in response to Cu exposure. This occurs prior to endocytosis, and mutation of the  $^{340}\text{K/K}^{345}$  motif in yCTR1 or deletion of the adaptor protein results in failure of trafficking of CTR1 to the vacuole and inhibition of degradation [23]. Similar to the  $^{340}\text{K/K}^{345}$  residues in yeast, the  $^{178}\text{K/K}^{179}$  motif in hCTR1 is also located in the C-terminal end of the protein. However, the results of the current study indicated that conversion of both K178 and K179 to alanines had no significant effect on the transport of Cu, produced only a 20% decrease in cDDP uptake, and only insignificantly impaired the ability of Cu to trigger internalization of CTR1 from the plasma membrane. The lack of effect on transport is consistent with the finding that the  $^{340}\text{K/K}^{345}$  in yCTR1 is not essential for Cu transport [23], and that truncation of hCTR1 at K178 still permitted some Cu transport when expressed in Sf9 cells [43]. We concluded that, despite the role of a

similarly positioned KK motif in yCTR1, the <sup>178</sup>K/K<sup>179</sup> motif in hCTR1 either is either not a site of ubiquitinylation or ubiquitinylation is not essential for the Cu-induced internalization of CTR1.

The results of this study provide additional evidence that, despite the fact that Cu<sup>1+</sup> and cDDP appear to be substrates for the CTR1 transporter, the molecular details of the mechanism by which CTR1 imports cDDP and Cu are different. This may be a reflection of the ability of Cu<sup>1+</sup> to bind 3 or 4 sulfur atoms, whereas cDDP and its variants are expected to only form bis-adducts with just 2 sulfur atoms. Other evidence comes from studies in yeast where FRET analysis of the interaction between the C-terminal tails of yCTR1 monomers showed that Cu but not cDDP brought them closer together. A variant of yCTR1 defective in Cu transport nevertheless still enhanced cDDP accumulation [44]. Some N-terminal methionine-rich motifs that are dispensable for Cu transport are required for cDDP uptake [44]. Refinement of the understanding of how cDDP is transported by hCTR1 is important because cellular uptake is a direct determinant of cytotoxicity, and insights into how transport can be manipulated have the potential to enhance overall efficacy of chemotherapy with this agent.

The only prior evidence that CTR1 interacts with any other protein was provided in a *Xenopus* model in which the Src-related kinase Laloo was reported to physically interact with CTR1 and the FGFR docking protein FRS2 $\alpha$  [37]. We and another group have reported that CTR1 plays a pivotal role in regulating receptor tyrosine kinase signaling [36,38] on the basis that knockout of CTR1 resulted in impaired insulin, EGF, FGF and PDGF-stimulated activation of Ras/MAPK signaling as evidenced by a reduction in ERK1/2 phosphorylation. We speculated that CTR1 is associated with other signaling proteins to mediate the Ras/MAPK signaling. In the current study reversible cross-link immunoprecipitation and mass spectrometric analysis identified IRS-4 as a potential CTR1 interacting protein. IRS-4 is the most recently identified member of the IRS family which contains IRS-1, IRS-2, IRS-3, and IRS-4 [46]. The IRS proteins are found associated with the insulin and IGF-1 receptors and upon receptor activation they become phosphorylated and provide docking sites for SH2 domain containing proteins such as PI3K and Grb2 [47,48]. The four IRS proteins have some redundant functions and some unique roles that are still being clarified. IRS-4 knockout mice showed almost no phenotype other than slightly reduced weight and slightly lower glucose levels [49]. IRS-4 was shown to compensate for IRS-1 in liver regeneration and brown adipocyte differentiation and for IRS-2 in preventing apoptosis of pancreatic  $\beta$ -cells [50,51]. Although IRS-4 has a very similar modular structure to other IRS members, it has only 27 and 29% sequence identity with IRS-1 and IRS-2, respectively. IRS-4 is also known to interact with Shp2 and WDR6 and all these proteins have been implicated in FGFR signaling [52,53]. No interaction was found between CTR1 and IRS-1 or IRS-2 indicating that CTR1 is specifically associated with IRS-4 only. We speculate that the interaction between CTR1 and IRS-4 is transient and labile because it was not detected in the absence of the cross-linker. The basis for the increased steady-state association of CTR1 and IRS-4 in the Y103A mutant is unknown but may be linked with the failure of the Y103A variant to internalize in response to Cu. How Cu exposure modulates the interaction of CTR1 and IRS-4 has yet to be investigated.

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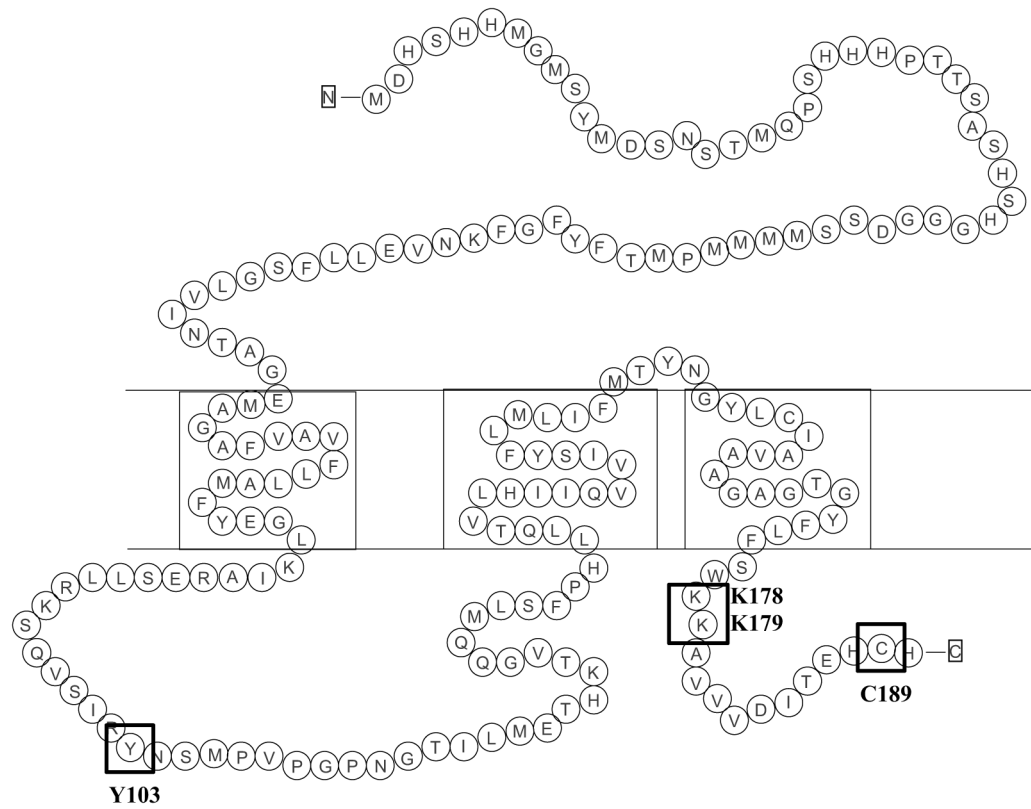
The authors would like to thank Dr. Dennis Thiele for providing the CTR1<sup>+/+</sup> and CTR1<sup>-/-</sup> mouse embryo fibroblasts, Dr. Jack Kaplan for providing HEK293 FLP-In T-Rex cells and Preston Adams and Gerald Manorek for technical assistance. Valuable advice was provided by Drs. Brian Blair, Paolo Abada, Jacob Quail, Michael Petris and Xinjian Lin. This work was supported by grants from the National Institutes of Health (CA152185 and T32CA121938), and the U.S. Department of Defense (W81XWH-08-1-0135). Additional support for core laboratories was from the UCSD Cancer Center Support Grant P30 CA23100.

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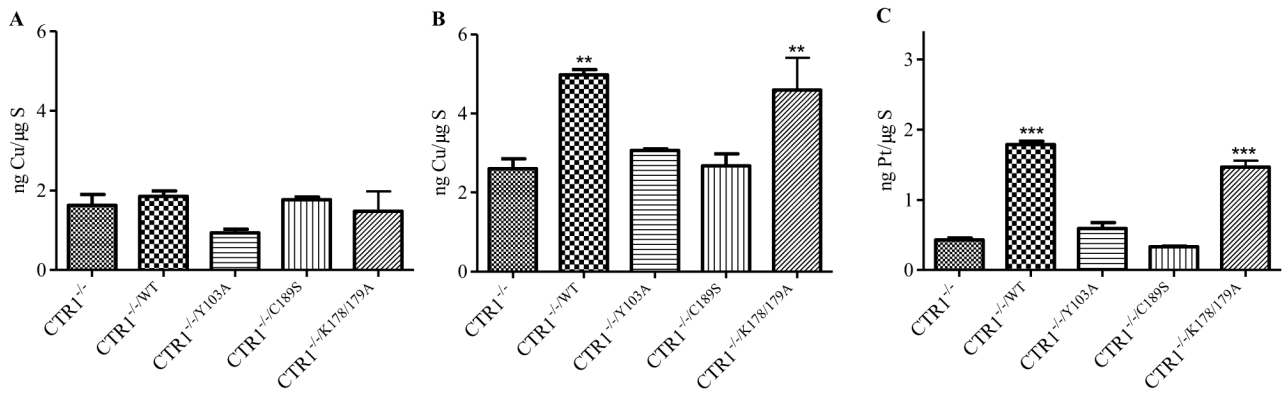
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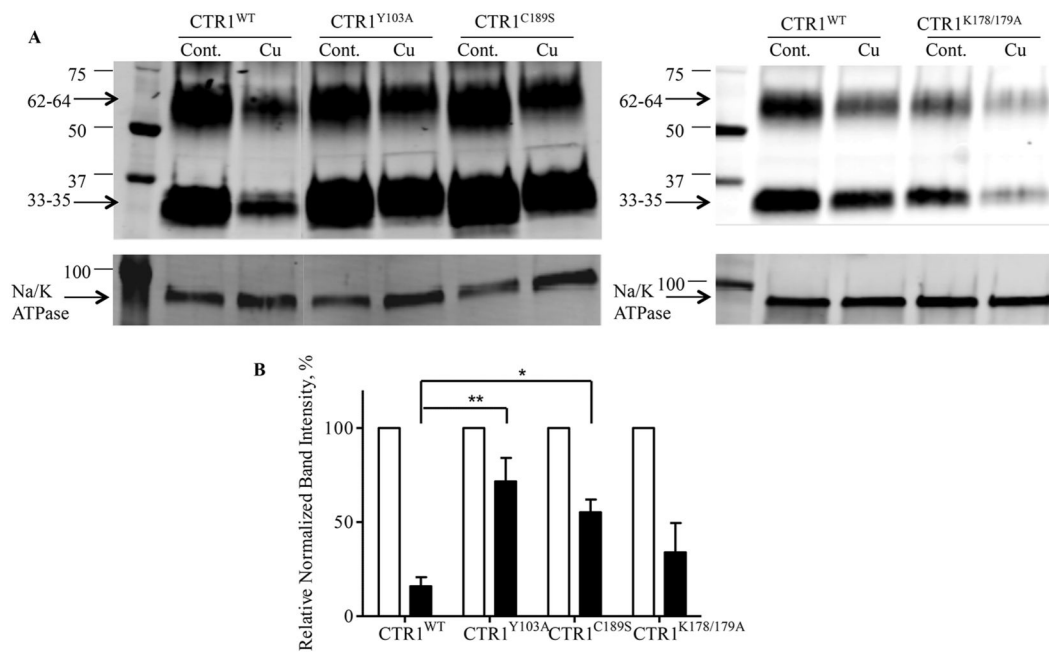


**Fig. 1.** Schematic diagram of the amino acid sequence of hCTR1. Boxes highlight the Y103, C189 and K178/K179 residues.

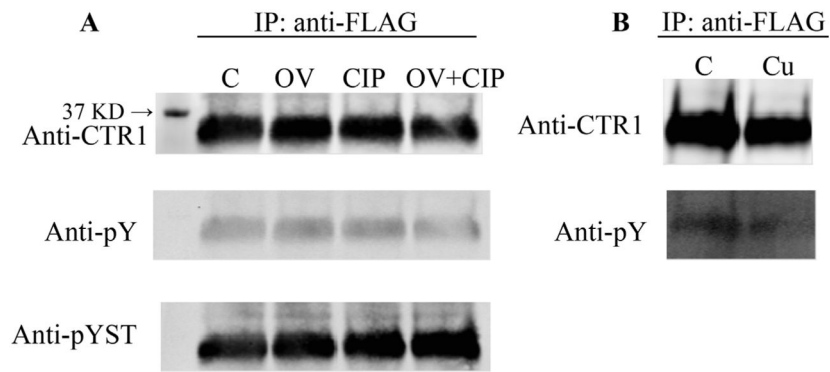
**Fig. 2.**

Effect of modifying Y103, C189 and K178/K179 on Cu and cDDP accumulation. (A) basal whole cell Cu content; (B) total Cu following 1 h exposure to 100  $\mu$ M Cu; (C) net accumulation of Pt following 5 min exposure to 30  $\mu$ M cDDP. All values represent means of at least 3 independent experiments each containing sextuplet replicates. Vertical bars,  $\pm$ SEM. \*  $p < 0.05$ ; \*\*\*  $p < 0.001$ .

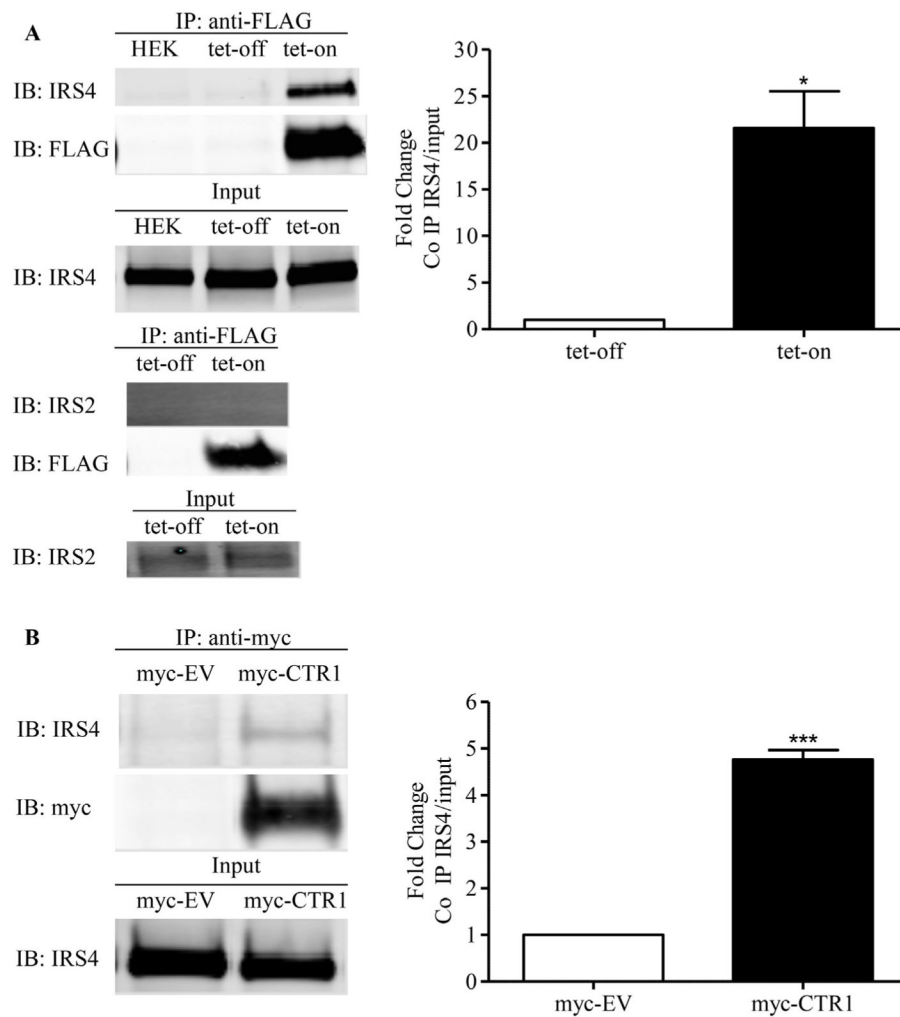




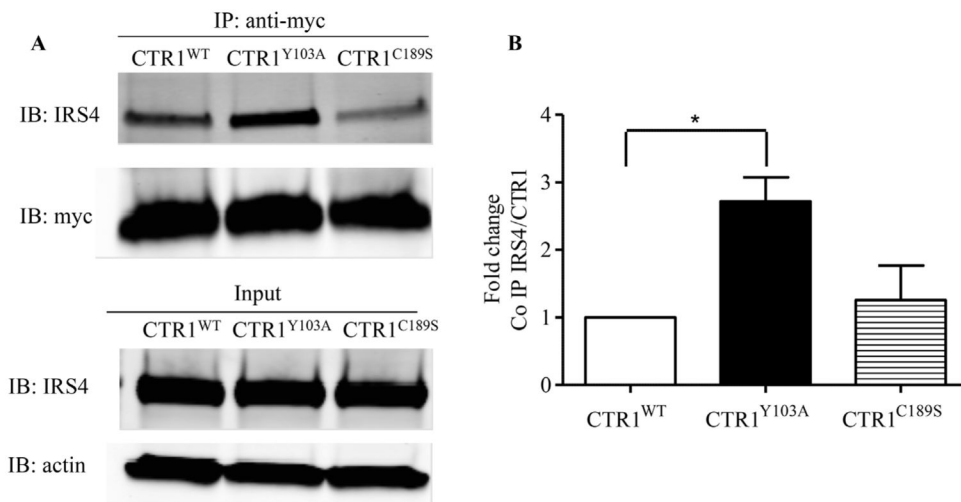
**Fig. 3.** Effect of modifying Y103, C189 and K178/K179 on the ability of Cu to trigger CTR1 internalization. HEK293/myc-CTR1<sup>WT</sup> and variant cells were exposed to 100  $\mu$ M Cu for 30 min prior to being biotinylated. (A) Western blots of biotinylated proteins were probed with anti-CTR1 antibody. Na/K ATPase served as a loading control. (B) Quantification of Western blots. Relative amount CTR1 in cells treated with Cu (black bars) is shown as a percentage of that in untreated control cells. Values are the mean of 5 independent experiments  $\pm$  SEM. \*  $p < 0.05$ ; \*\*  $p < 0.01$ .



**Fig. 4.** Analysis of CTR1 for phosphorylation following exposure to Cu. (A) FLAG-CTR1-expressing HEK293 FLP-In T-Rex cells were treated with 1 mM orthovanadate for 30 min and CTR1 was immunoprecipitated with anti-FLAG antibody and A/G beads. The beads were treated with calf intestinal alkaline phosphatase for 1 h at 37 °C; C, control; OV, orthovanadate; CIP, calf intestinal alkaline phosphatase. (B) CTR1 was immunoprecipitated from HEK293 FLP-In T-Rex cells before and after a 30 min exposure to 100 μM Cu.



**Fig. 5.** CTR1 interacts with IRS-4 in HEK 293 cells. (A) FLAG-CTR1 was immunoprecipitated from DSP-treated HEK293 FLP-In T-Rex cells before and after induction with tetracycline and subjected to Western blot analysis for the presence of IRS-2 and IRS-4. (B) myc-CTR1 was immunoprecipitated from DSP-treated HEK293/myc-EV and HEK293/myc-CTR1<sup>WT</sup> cells and subjected to Western blot analysis for the presence of IRS-4. Histograms show mean  $\pm$  SEM determined from 4 experiments. \*  $p < 0.05$ ; \*\*\*  $p < 0.001$ .



**Fig. 6.** CTR1 mutants interact with IRS-4 in HEK 293 cells. (A) myc-CTR1 was immunoprecipitated from DSP-treated HEK293/myc-CTR<sup>WT</sup>, HEK293/myc-CTR<sup>Y103A</sup>, HEK293/myc-CTR<sup>C189S</sup> and subjected to Western blot analysis for the presence of IRS-4. (B) Mean  $\pm$  SEM determined from 3 independent experiments. \*  $p < 0.05$ .

**Table 1**

IRS-4 fragments identified by mass spectrometry from immunoprecipitated FLAG-CTR1.

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AAAAAAAAAASGAAIPPLIPR
AAVSAPFTDSLRL
AIGDGEDEMLFTR
DAASKPSGEGSFSKPGDGGSPSKPSDHEPPK
DLSPPSAPAVASAAEPTLALSQVVAAASALAAAPGIGAAAAAAGFDSASAR
EVSYNWDPK
FVTPSEPVAHSR
GAQDVAGGSNPGAHNPSANLAR
GDNQAGGAAAAAAPEPPPR
GYMMMFPR
HLGLVPLEPGGWLR
LNTEVASVVVQLLSIR
LSSEVSGSGSGNFGGEEGNPQ GK
LNTEVASVVVQLLSIRR
VSPPPAPSPPK
LSQVVAAASALAAAPGI
ESNTPAPSTQGLPDSWGIIAEPR
AIGDGEDEMLFTR
DAASKPSGEGSFSKPGDGGSPSKPSDHEPPK
GAQDVAGGSNPGAHNPSANLAR
GDNQAGGAAAAAAPEPPPR

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