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Copper Transporter 2 Regulates Intracellular Copper and Sensitivity to Cisplatin

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Abstract

Mammalian cells express two copper (Cu) influx transporters, CTR1 and CTR2. CTR1 serves as an influx transporter for both Cu and cisplatin (cDDP). In mouse embryo fibroblasts, reduction of CTR1 expression renders cells resistant to cDDP whereas reduction of CTR2 makes them hypersensitive both *in vitro* and *in vivo*. To investigate the role of CTR2 on intracellular Cu and cDDP sensitivity its expression was molecularly altered in the human epithelial 2008 cancer cell model. Intracellular exchangeable Cu^+ was measured with the fluorescent probe Coppersensor-3 (CS3). The ability of CS3 to report on changes in intracellular Cu^+ was validated by showing that Cu chelators reduced its signal, and that changes in signal accompanied alterations in expression the major Cu influx transporter CTR1 and the two Cu efflux transporters, ATP7A and ATP7B. Constitutive knock down of CTR2 mRNA by ~50% reduced steady-state exchangeable Cu by 22–23% and increased the sensitivity of 2008 cells by a factor of 2.6 – 2.9 in two separate clones. Over-expression of CTR2 increased exchangeable Cu^+ by 150% and rendered the 2008 cells 2.5-fold resistant to cDDP. The results provide evidence that CS3 can quantitatively assess changes in exchangeable Cu^+, and that CTR2 regulates both the level of exchangeable Cu^+ and sensitivity to cDDP in a model of human epithelial cancer. This study introduces CS3 and related sensors as novel tools for probing and assaying Cu-dependent sensitivity to anticancer therapeutics.

Keywords

Cisplatin; copper transporter; CTR2; coppersensor-3

Introduction

The Pt-containing chemotherapeutic agent cisplatin (cDDP) is a highly polar molecule that does not diffuse easily across lipid membranes. Mammalian cells express two Cu influx transporters. The observation that knockout of the high affinity Cu transporter CTR1 in yeast resulted in resistance to cDDP [1, 2] led to the discovery that CTR1 is an important influx transporter for cDDP in mammalian cells (reviewed in [3]). Previous work has established that CTR1 is a major determinant of the cellular pharmacology of all three clinically used Pt-containing chemotherapeutic agents [4], and its ability to modulate sensitivity to the cytotoxic effect of cDDP has now been documented in many

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laboratories [5–7]. Knockout of CTR1 in a murine tumor model eliminates responsiveness to cDDP [4], and CTR1 expression is reduced in human ovarian cancer cell lines selected for resistance to cDDP [8]. Recent studies indicate that patients whose tumors express high levels of CTR1 and who are treated with Pt drug-containing regimens for non-small cell lung or ovarian cancer have an increase in both progression-free and overall survival [9, 10].

The second human Cu transporter, hCTR2, is structurally similar to hCTR1 with which it shares 41% amino acid sequence homology to human CTR1. While its N-terminal domain is shorter, hCTR2 has a similar set of 3 transmembrane domains and a large cytosolic loop [11, 12]. CTR2 was identified as a Cu transporter on the basis of its homology to yCTR2, and its ability to rescue the Cu deficient phenotype of *ctr1Δctr3Δ* mutants [11]. In yeast, Ctr2 and its *S. pombe* ortholog Ctr6 are localized in vacuoles with the C-terminal tail oriented toward the cytosol. It has been shown that yCtr2 releases Cu from intercellular stores and delivers Cu to various chaperones under conditions of Cu starvation [11, 13, 14]. However, the Ctr2-1 mutant of yeast Ctr2, that partially mislocalizes to the plasma membrane, mediates plasma membrane Cu transport in a manner similar to that of yCtr1 [15].

Less is known about the function of human CTR2. Based on its role in yeast, it has been presumed that hCTR2 is responsible for release of Cu from intracellular vesicle stores. CTR2 is primarily localized to late endosomes and lysosomes, although it has been reported to be on the plasma membrane in some cells [16, 17]. Like CTR1, CTR2 forms multimers, some of which co-localize with CTR1 [16]. Mammalian CTR2 has been shown to increase Cu influx in cells in which it localizes to the plasma membrane [17], although its affinity for Cu is less than that of CTR1 [16, 17]. Like CTR1, CTR2 is able to bind silver; but not zinc, iron or manganese [16]. Changes in CTR2 expression do not affect Cu efflux suggesting that it functions primarily as a regulator of influx perhaps via control of intracellular sequestration and Cu storage [16, 18, 19]. It has also been shown that CTR2 acts as an inhibitor of SOD protein expression, suggesting that CTR2 may be integral to the regulation of other Cu proteins involved in Cu homeostasis [16].

Given that CTR1 transports cDDP and that CTR2 has substantial structural similarity to CTR1, it appears likely that CTR2 can also act as a cDDP transporter perhaps regulating its flux into or out of intracellular compartments. However, previous work from this laboratory has shown that knock down of CTR2 in mouse embryo fibroblasts increases rather than decreases cDDP influx and enhances both cDDP DNA adduct formation and cytotoxicity [20]. The increased cDDP uptake was not due to either enhanced accumulation in intracellular vesicles or reduced export. Knock down of CTR2 increased the Pt accumulation by 9.1-fold when the cells were grown as xenografts in nu/nu mice and markedly increased tumor responsiveness [21]. These results hint that CTR2 plays an important role in controlling both Cu levels and sensitivity to cDDP in human malignancies, and that these may be linked.

In the current study we determined the effect of knocking down or over-expressing CTR2 in a the human epithelial cancer cell line 2008 on sensitivity to the cytotoxic effect of cDDP and on the availability of intracellular Cu using Coppersensor-3 (CS3), a novel fluorescent sensor for intracellular Cu^{+}[22–25]. We report that CS3 effectively reports on changes in intracellular Cu^{+} produced by established Cu chelators and alterations in the Cu influx transporter CTR1. Even modest knock down of hCTR2 was found to enhance sensitivity to cDDP and reduce exchangeable intracellular Cu. Over-expression of CTR2 produced the opposite effect consisting of a reduction in sensitivity to cDDP and an increase in exchangeable Cu. These results further establish CTR2 as a determinant of sensitivity to cDDP and a regulator of intracellular Cu, and demonstrate a reciprocal relationship between these two parameters. CS3 fluorescence may be useful for assessment of cDDP sensitivity.

Experimental Procedures

Cell Culture and Reagents

The wild type mouse embryo fibroblast cell line ($CTR^{+/+}$) and a line established from mice in which both alleles of CTR1 were knocked out ($CTR1^{-/-}$) were gifts from Dr. Dennis Thiele (Duke University). These cells were grown in DMEM Hi-glucose media with 20% FBS. (Invitrogen, Carlsbad, CA). The human epithelial tumor cell line 2008 was grown in RPMI-1640 media supplemented with 10% FBS. All cells were grown at 37°C and 5% CO_2. The 2008-based CTR2 knock down, mCherry, and mCherry-hCTR2 cell lines were grown continuously in 10 μg/mL puromycin supplemented media. cDDP was purchased from Teva Parenteral Medicines, Inc. (Irvine, CA). Mouse monoclonal antibody to hCTR2 was obtained from the National Cancer Institute. Tetrathiomolybdate and Tetrakis(acetonitrile)copper(I)hexafluorophosphate was purchased from Sigma-Aldrich, St. Louis, MO.

Knock down of hCTR2

The 2008 cells were infected with a lentiviral vector constitutively expressing an shRNA targeted to hCTR2 (Sigma Aldrich, cat #NM001860). The shRNAi hairpin sequence was: CCGGCCAGTCTCTAATCCATGTCATCTCGAGATGACATGGATTAGAGACTGGTT TTTG. Knock down cells were selected in 10 μg/mL puromycin containing media and hCTR2 mRNA level was quantified by qRT-PCR. mRNA was extracted with Trizol reagent (Invitrogen, Carlsbad) and converted to cDNA with an iScript cDNA synthesis kit (Biorad, Hercules, CA). qRT-PCR was performed on a Biorad MyiQ(™) system.

Western Blotting

Cells were dissolved in 2x RIPA lysis buffer with protease inhibitor for 1 h. The lysates were subjected to a 10 second sonication and centrifuges at 14,000x g for 10 min at 4°C. Protein concentration was measured with a Bio-Rad DC™ Protein Assay (Bio-Rad, Hercules, CA) and were then subjected to electrophoresis on 4% to 20% gels using 30 μg of protein per lane. A Bio-Rad Trans-Blot system was used to transfer the proteins to a PVDF Immobilon membrane (Millipore). Blots were incubated overnight at 4°C in Li-cor blocking buffer (Li-Cor Biosciences, Lincoln, NE). Blots were incubated overnight at 4°C in hCTR2 antibody at 1:1000 dilution. The blot was then rinsed 3 times in TBS-T (0.1% Tween) and incubated with the appropriate Li-Cor secondary antibodies conjugated with 680LT or 800CW infrared dyes (Li-Cor Biosciences #926-32210, 926-32211, 926-68020, 926-68021).

Cytotoxicity Assays

Cell viability following a 96 h exposure to cDDP, Cu or tetrathiomolybdate was determined with the CCK8 assay kit (Clonetech, Inc, Mountain View, CA). Each drug concentration was tested in triplicate wells and each experiment was conducted with 3 separate independent plates for each cell line and drug concentration.

Generation of 2008/mC-hCTR2 cells

A GFP-hCTR2-expressing vector was a generous gift Dr. Jessie Bertinato [17]. hCTR2 was cloned and placed into a pLVX-mCherry expression vector (Clonetech, Inc, Mountain View, CA.). This was then transfected into 293T cells with a Lenti-X HTX packaging system (Clonetech, Inc.) to generate lentivirus which was used to infect 2008 cells. After 48 hours of infection, the lentiviral supernatant was removed and replaced with fresh media. Cells were selected with 10 μg/mL puromycin and sorted by FACS based on mCherry fluorescence.

Coppersensor-3 (CS3) staining and image analysis

CS3 was synthesized according to literature methods [22] and dissolved in DMSO to produce a stock concentration of 2 mM. A total of 10^5 cells were cultured on a glass bottom Petri dishes (MatTek, Part no. P35GCOL-0-14-C, Ashland, MA) and grown overnight at 37°C. The cells were briefly rinsed in PBS twice and incubated at 37°C for 15 minutes with 5 μM CS3 in PBS containing Hoechst 33342 at a dilution of 1:5,000. The cells were rinsed once in PBS, placed in PBS, and then imaged. Confocal images were obtained on an Olympus FV-1000 confocal microscope with a 60X Acroplan oil objective.

Each experiment utilized one culture of control cells and one each for the different types of experimental cells. To minimize variation in time from staining to imaging, each culture was stained, rinsed and imaging immediately. After staining, 3 randomly chosen fields were imaged from each culture using 32 Z-slices with a step size of 0.8 μm in Z direction. Image J software was then used in the average intensity projection mode to exclude the Hoechst 33342 fluorescence and determine the mean CS3 fluorescence in an area of interest drawn around each of 10 cells from each of the 3 fields in a given culture. The mean fluorescence in the CS3 channel per unit area from 4 areas of interest drawn on background parts of the image containing no cells was subtracted from this value. Thus, from each culture a mean value was obtained for the total of 30 cells analyzed. Each experiment was repeated 3 times on separate days, and the mean values for each type of culture were averaged. Thus, the data is reported as the mean ± SEM (N = 3) of the average obtained from 30 cells in each of 3 repeats.

Statistics

Values are reported as mean ± SEM. Comparisons between values were made using Student's t-test with assumption of unequal variance.

Results

Validation of the ability of CS3 to detect changes in exchangeable intracellular Cu^+

CS3 is a fluorescent-responsive Cu chelator that enters cells readily and selectively increases its fluorescence upon binding Cu^+ [22]. Since the intracellular concentration of free Cu^+ is thought to be $<10^{-18}$ M [26], CS3 must compete for Cu^+ with Cu^+ binding proteins. CS3 has a apparent Kd of 8.9×10^{-14} M [22], and thus its fluorescence once inside the cell likely reflects the Cu^+ that is available for exchange from proteins and small molecules with comparable or lower affinity. To provide evidence of the ability of CS3 to report on the availability of exchangeable intracellular Cu^+, we confirmed the concentration dependence of CS3 fluorescence and examined the effect of altering cellular Cu levels by using Cu^+ chelators or changing the expression of Cu transporters. Figure 1A shows that the fluorescence produced by adding 4 μM Cu^+ in the form of tetrakis(acetonitrile) copper(I) hexafluorophosphate to a 2.5 μM solution of CS3 in HEPES buffer was progressively reduced by the addition of increasing concentrations of the Cu^+ chelator neocuproine. Figure 1B shows that another Cu^+ chelator, tetrathiomolybdate, also effectively competed with CS3 for Cu and reduced the fluorescent signal. To validate the ability of CS3 to detect exchangeable Cu in living cells, human epithelial carcinoma 2008 cells were incubated with 5 μM CS3 for 15 min in the absence or presence of 300 μM neocuproine or tetrathiomolybdate (TTM). As shown in Figures 2A and 2B, neocuproine reduced CS3 fluorescence to 57.99 ± 0.03 % of control ($p = 0.011$), and tetrathiomolybdate reduced it to 31.36 ± 0.02 % of that in the cells exposed to CS3 alone ($p = 0.007$). Thus, CS3 accurately reported on the activity of two chelators with known ability to limit the availability of intracellular Cu and reduce the activity of Cu-dependent enzymes.

Changes in the availability of intracellular Cu that occur as a result of altered expression of Cu transporters are more subtle. To determine whether CS3 was sensitive to these perturbations it was used to examine the effect of knocking out CTR1 in mouse embryo fibroblasts. We previously reported that the whole cell Cu content of the parental $CTR1^{+/+}$ fibroblasts was 1.10 ± 0.02 ng of Cu/ug S, and that knockout of both alleles of CTR1 reduced steady-state Cu to 0.90 ± 0.10 ng Cu/ug S [21]. The data presented in Figures 3A and 3B show that knockout of CTR1 reduced CS3 fluorescence to 83.2 ± 2.8 % of that in the parental control cells ($p = 0.023$). Thus, in these cells the reduction in exchangeable Cu was of a similar magnitude as the reduction in whole cell Cu.

ATP7A and ATP7B are responsible for the export of Cu from the cell. The ability of the these exports to modulate whole cell Cu was previously studied by re-expressing them in a human fibroblast cell line established from a Menkes disease patient that does not express either protein (Me32a cells) [27]. Re-expression of ATP7A to produce the MeMNK cells reduced whole cell Cu by 80.8% from 56.7 ± 4.1 ng Cu/mg protein to 10.9 ± 0.9 ng Cu/mg protein [28]. Re-expression of ATP7B to produce the MeWND line reduced whole cell Cu by 76.3% to 13.5 ± 1.7 ng Cu/mg protein. Figures 4A and 4B show that CS3 staining intensity in the MeMNK cells was reduced to 68.5 ± 4.3% of that in the Me32a cells ($p = 0.006$), and that it was reduced to 74.0 ± 4.4% in the MeWND cells ($p = 0.012$). Thus, similar to the situation for CTR1, the reduction in CS3 fluorescence reflected the change in whole cell Cu content produced by these transporters as well, although in this case the magnitude of the change in CS3 staining was substantially less than that of whole cell Cu content.

Knock down of CTR2 in human epithelial cancer cells enhances sensitivity to cDDP

Knock down of CTR2 in mouse embryo fibroblasts was previously reported to result in increased sensitivity to cDDP both *in vitro* and *in vivo* [20]. To determine whether the effect of CTR2 knock down on cDDP sensitivity was specific to mouse embryo fibroblasts or could also be observed in epithelial cells, a lentiviral vector constitutively expressing an shRNA targeting to CTR2 mRNA was used to knock down CTR2 expression in the human epithelial cell line 2008. Multiple clones were isolated from the lentiviral-infected population and tested for their level of CTR2 expression by qRT-PCR and their sensitivity to cDDP using the CCK8 assay. Figure 5A shows the results for the two clones with the greatest degree of CTR2 knock down. Even in these clones, the CTR2 mRNA was not reduced to <50% of that in the 2008-SCR expressing a scrambled shRNAi that did not target any human mRNA (Figure 5A). Reduction of CTR2 expression at the protein level could not be assessed due to limited sensitivity of the available anti-CTR2 antibody. However, this degree of knock down was sufficient to significantly enhance sensitivity to cDDP by a factor of 2.7–2.9 fold ($p < 0.001$ for both clones) (Figure 5B). The cDDP IC_{50} value for the 2008/WT cells was 3.02 ± 0.30 μM while that for the 2008/CTR2KD-E2 and 2008/CTR2KD-F5 clones had IC_{50} values of 1.71 ± 0.07 μM ($p = 0.02$) and 1.38 ± 0.07 μM ($p = 0.01$), respectively. As shown in Figure 5B, infection of 2008 cells with a vector expressing a scrambled shRNAi (2008-SCR) had little effect on cDDP sensitivity. No difference was observed in sensitivity to Cu (data not shown). However, as shown in Figure 5C, knockdown of CTR2 reduced the IC_{50} of the Cu chelator tetrathiomolybdate from 15.4 ± 2.2 to 7.6 ± 2.0 in the E2 clone ($p = 0.023$) and 8.5 ± 1.5 in the F5 clone ($p = 0.026$).

To determine whether the change in cDDP sensitivity induced by knock down of CTR2 was accompanied by change in exchangeable intracellular Cu, the 2008-SCR, 2008/CTR2KD-E2 and 2008/CTR2KD-F5 cells were stained with CS3. Figures 6A and 6B show that CS3 fluorescence in the 2008/CTR2KD-E2 cells was reduced to 78.4 ± 0.1% of than in the 2008-SCR cells ($p = 0.011$) and in the 2008/CTR2KD-F5 cells it was reduced to 77.0 ± 0.1 % of

control ($p = 0.04$). Thus, enhanced sensitivity to cDDP mediated by reduction in CTR2 was accompanied by evidence of reduced exchangeable Cu.

Over-expression of CTR2 in human epithelial cancer cells reduces sensitivity to cDDP

Knock down of CTR2 in 2008 cells resulted in enhanced sensitivity to a cDDP. To determine whether an increase in expression of CTR2 would produce the opposite effect, wild type 2008 cells were infected with lentiviral vectors expressing either mCherry (mC) alone or mC fused to the N-terminal end of CTR2. The western blot shown in Figure 7A was probed with anti-mCherry antibody and demonstrates the expression of mC alone in the 2008/mC cells, which migrates at 33 kDa, and mC-CTR2 in the 2008/mC-CTR2 cells, which migrates at 42 kDa. As shown in Figure 7B the anti-CTR2 antibody detected the same bands in the 2008/mC-CTR2 cells, but in addition it detected a less intense band at 34.0 kDa which is likely a cleavage product that has lost the mC tag. The subcellular distribution of CTR1 in the 2008/mC and 2008/mC-2008 cells was assessed by immunocytochemical analysis of cells stained with anti-CTR2 antibody. As shown in the panels of Figure 8, when stained with the anti-mC antibody the mC-CTR2 protein was distributed in a finely granular pattern throughout the cytoplasm. There was no consistent co-localization with the Golgi marker syntaxin-6 upon examination of more than 300 cells. Thus, the mC-CTR2 fusion protein appeared to have the same non-Golgi distribution as what has previously been reported for the endogenous CTR2 [16].

The concentration-survival curves in Figure 9A indicate that the 2008/mC-CTR2 cells were 2.5-fold resistant to the cytotoxic effect of cDDP. The 2008/mC control cells had an IC_{50} of 0.93 ± 0.03 μM whereas the 2008/mC-CTR2 cells had an IC_{50} of 2.37 ± 0.16 μM ($p < 0.001$). The 2008/mC-CTR2 cells were also 1.8-fold resistant to Cu with an IC_{50} value of 49.2 ± 7.0 μM compared to that of 26.9 ± 1.5 μM for the 2008/mC cells ($p = 0.0056$) (Figure 9B). Thus, in the human epithelial cells, lowering the level of CTR2 enhanced sensitivity to cDDP and increasing it reduced sensitivity. In the CTR2 over-expressing cells there was concordance between the change in sensitivity to cDDP and Cu; the effect on sensitivity to Cu was less than that on sensitivity to cDDP consistent with prior observations that small changes in sensitivity to Cu are accompanied by larger changes in sensitivity to cDDP [29].

CS3 staining was used to determine how over-expression of CTR2 altered the level of exchangeable Cu. Figures 10A and 10B show that CS3 staining in the 2008/mC-CTR2 cells was $150.0 \pm 0.1\%$ of that in the CTR2/EV-mC cells ($p = 0.003$). Thus, decreasing CTR2 expression reduced exchangeable Cu, and increasing it augmented steady-state Cu levels thus providing evidence that CTR2 participates actively in the control of whole cell exchangeable Cu, and that these changes mirror the changes in cDDP sensitivity. It is noteworthy that neither knock down of CTR2 nor its over-expression produced any clear differences in the distribution of CS3 fluorescence within the cell.

Discussion

The ability of increased or decreased expression of CTR1 to modulate sensitivity to cDDP has been demonstrated in multiple different murine and human cell types [3]. Changing the expression of CTR2 appears to have the opposite effect on cDDP sensitivity, but this has only previously been demonstrated in mouse embryo fibroblasts [20, 21]. Given the very large effect that knocking down mCTR2 had on cDDP uptake *in vivo*, and on the responsiveness of the mouse embryo fibroblast tumors, it was of particular interest to determine whether this phenomenon was also observable in fully malignant human cells. We report here that the effects of knocking down CTR2 with respect to cDDP sensitivity are concordant in both cell systems, and that CTR1 and CTR2 have opposite effects on cDDP sensitivity. In the 2008 human epithelial cell line even a modest ~50% reduction in CTR2 expression at the

mRNA level was sufficient to render the cells 2.6 – 2.9-fold more sensitive to cDDP *in vitro*, and over-expression of CTR2 reduced sensitivity by a factor of 2.5-fold. While these changes are modest in degree, for the Pt-containing class of drugs even small changes in sensitivity detected *in vitro* are sufficient to account for clinical failure of treatment [30].

There is now some clinical evidence that CTR2 expression is linked to outcome in patients with ovarian cancer treated with Pt-containing drugs. Patients whose tumors express low levels of CTR1 mRNA, particularly in combination with high levels of CTR2 mRNA, have a particularly poor outcome [10]. CTR2 appears to be regulated by Cu and cDDP in a manner opposite to that of CTR1. Whereas Cu depletion increases the expression of CTR1 it reduces that of CTR2 [31]. Recent reports have highlighted the role of the Sp1 transcription factor in the regulation of transcription from the CTR1 promoter [32], but it remains unknown whether this transcription factor is reciprocally involved in controlling the CTR2 promoter.

What role CTR2 plays in the transport of Cu in mammalian cells is largely unknown. In COS7 cells some CTR2 is found in the plasma membrane and has been reported to increase Cu uptake [17]. However, immunocytochemical analysis suggests that CTR2 is primarily located in intracellular membranes where it has been reported to be exclusively associated with late endosomes and lysosomes [16]. In the current study, altering the expression of CTR2 clearly modulated the steady-state level of exchangeable Cu establishing that this transporter is an active participant in Cu homeostasis. However, the data do not speak to the issue of whether the effect of CTR2 on the Cu pool is the result of altered influx or efflux at the plasma membrane, or whether CTR2 influences the release of Cu from sites where it cannot be accessed by CS3. If the latter is true, there may not be tight correspondence between the pool of exchangeable Cu and total cellular Cu content. Whether CS3 staining closely reflects the availability of Cu to Cu-dependent enzymes whose affinities may differ has yet to be established. The mechanism by which over-expression of CTR2 renders cells resistant to the cytotoxic effect of exogenous Cu remains to be defined but may be the result of a reduction in CTR1 expression.

Changes in the expression of CTR2 produced parallel but opposite changes in exchangeable Cu and sensitivity to cDDP and tetrathiomolybdate. Reducing CTR2 produced a decrease in exchangeable Cu but an increase in sensitivity to cDDP and tetrathiomolybdate and *vice versa*. This argues strongly that CTR2 does not function as an influx transporter, but leaves open the possibility that it modifies the function of CTR1, either directly or indirectly. cDDP has now been shown to bind to the Cu^{+}-binding proteins ATOX1 [33–35] and ATP7B [36, 37] raising the possibility that the effect of lower CTR2 expression on cDDP sensitivity is secondary to reduction in the pool of exchangeable Cu which allows more facile access of cDDP to these targets where, once bound, it likely interferes with their function.

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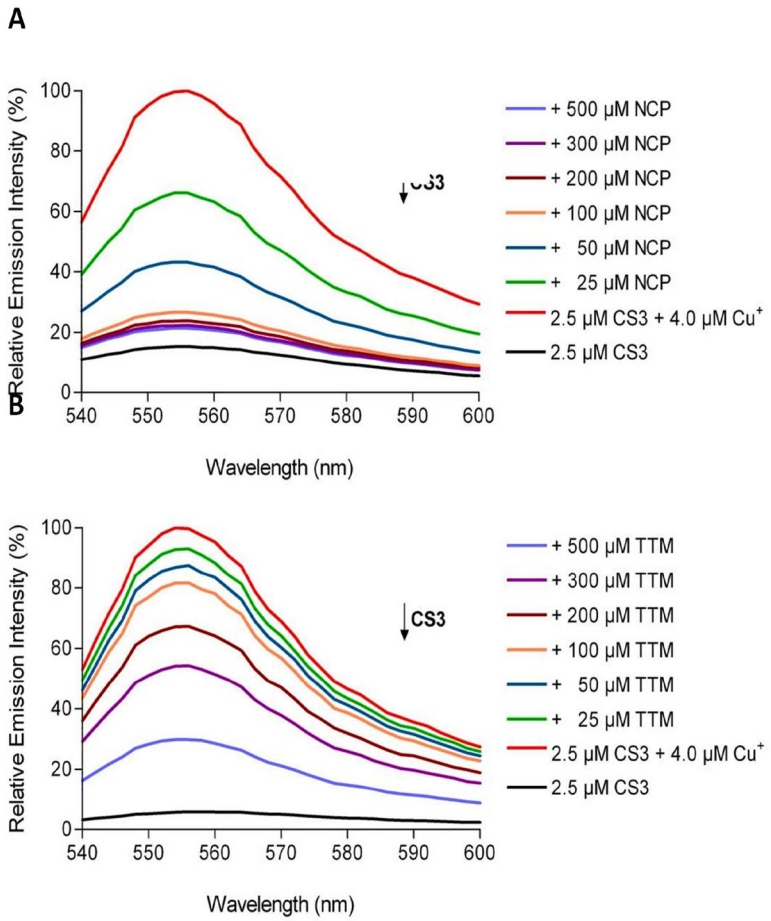

Figure 1.

Effect of decreasing Cu availability on Cs3 fluorescence. A, Decreasing CS3 signal as a function of the concentration of neocuproine (NCP) added to a solution of 2.5 μM CS3 and 4.0 μM Cu^{+} in the form of tetrakis(acetonitrile)copper(I)hexafluorophosphate. B, Decreasing CS3 signal as a function of tetrathiomolybdate (TTM) concentration. CS3 was dissolved at 2.5 μM in 20 mM HEPES buffer (pH 7.4) containing 4 μM Cu^{+}.

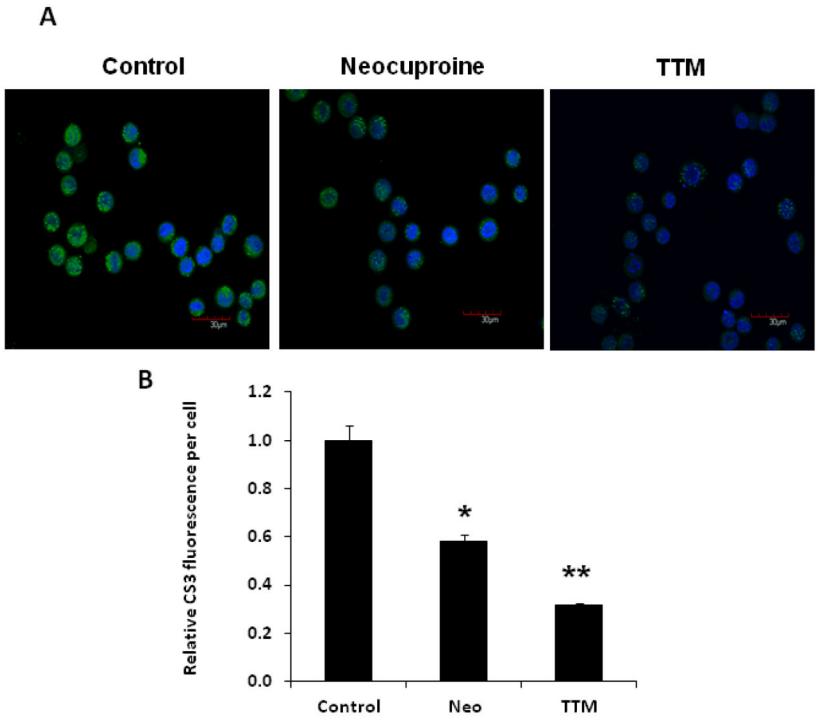

Figure 2.

Effect of exposure neocuproine and tetrathiomolybdate (TTM) on the ability of CS3 to detect Cu^{+} in human epithelial 2008 cells. A, Images of cells that were incubated with 5 μM CS3 for 15 min in the presence or absence of 300 μM neocuproine or TTM. Green, CS3; blue, nuclei stained with Hoechst 33342. B, Histogram of mean changes determined from 3 independent experiments. Vertical bars, ± SEM, N = 3. *p = 0.012; **p = 0.008.

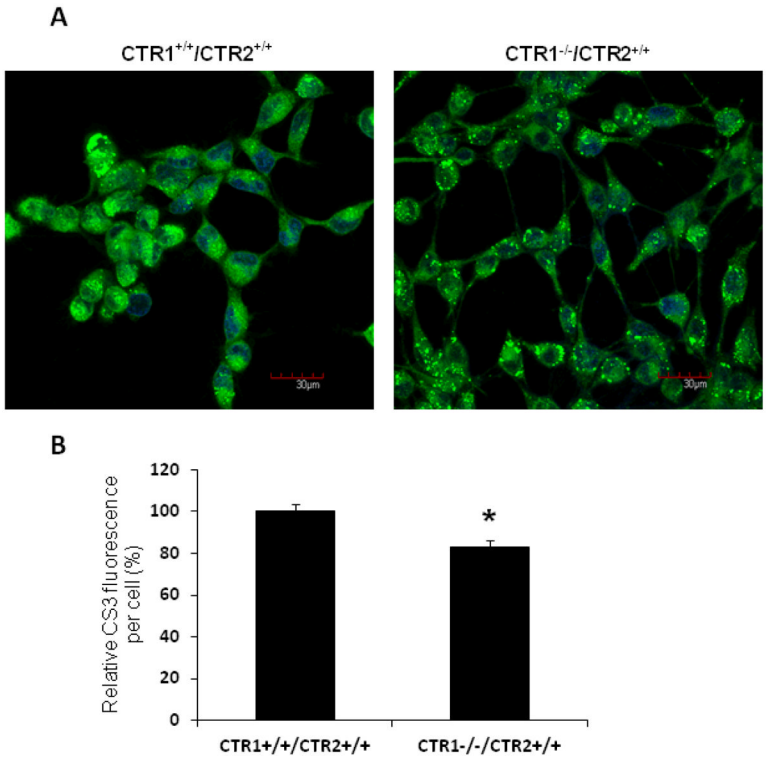

Figure 3.

Effect of altering the expression of Cu transporters on exchangeable Cu^{+}. A, Effect of knocking down the expression of CTR1 in mouse embryo fibroblasts. Green, CS3; blue, nuclei stained with Hoechst 33342. B, histogram of mean changes determined from 3 independent experiments. Vertical bars, ± SEM, N = 3. *p = 0.023.

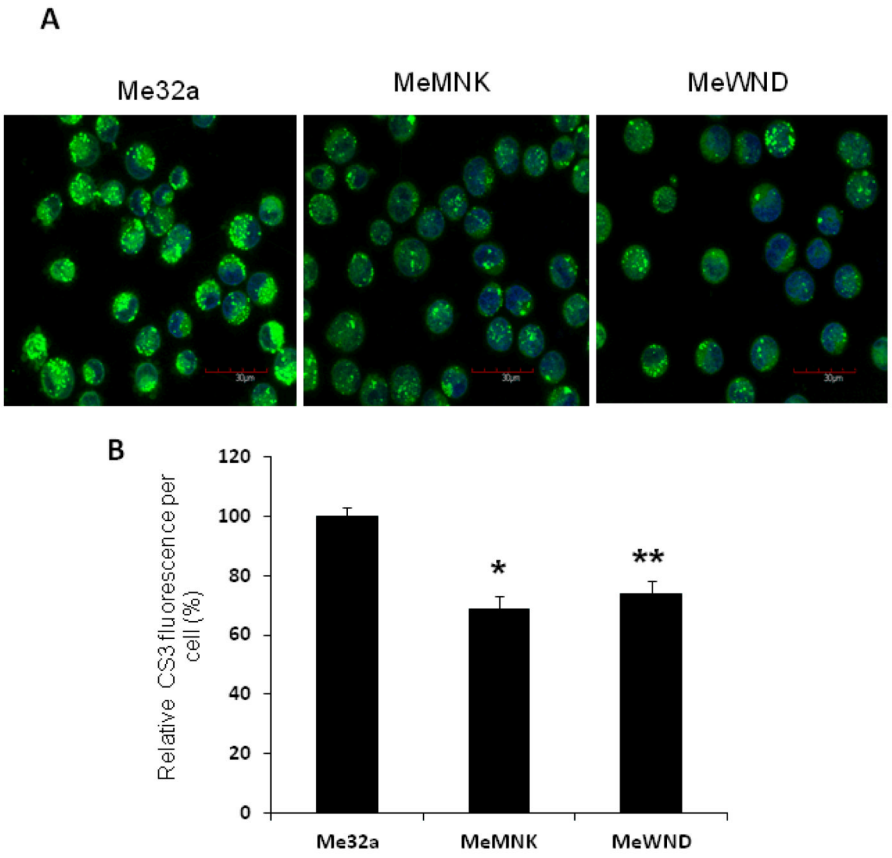

Figure 4.

Exchangeable Cu^{+} levels in MeMNK cells expressing ATP7A and MeWND cells expressing ATP7B. A, confocal fluorescent image; green, CS3; blue, nuclei stained with Hoechst 33342. B, histogram of mean changes determined from 3 independent experiments. Vertical bars, ± SEM, N = 3. *p = 0.006; **p = 0.012.

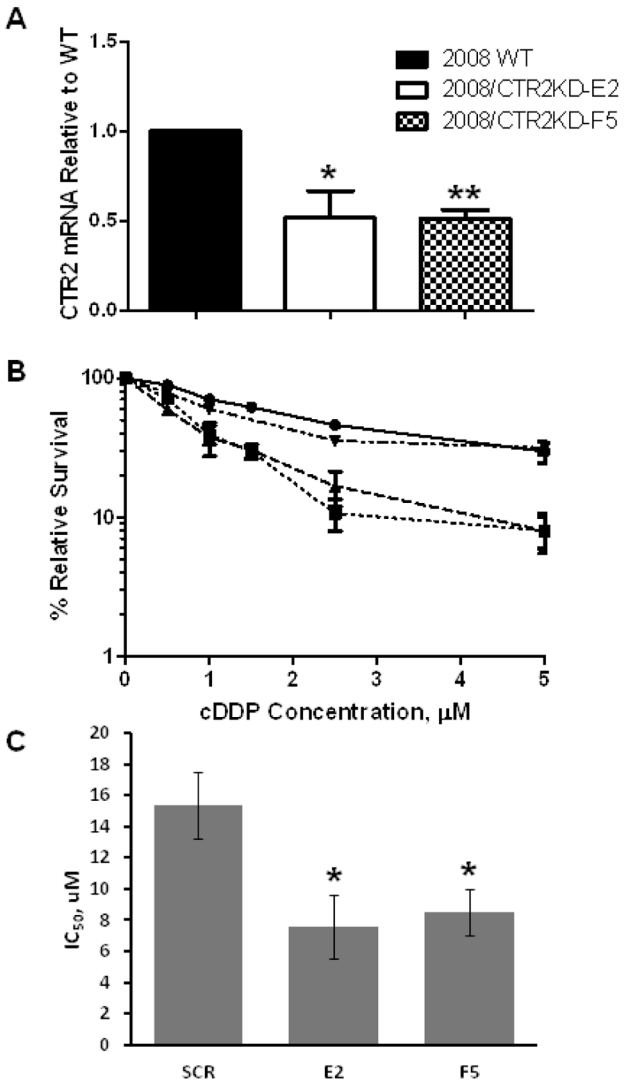

Figure 5.

Effect of knocking down CTR2 in human epithelial 2008 tumor cells on sensitivity to the cytotoxic effect of cDDP and tetrathiomolybdate. A, CTR2 mRNA levels in 2008, 2008/CTR2KD-E2 and 2008/CTR2KD-F5 cells measured in 3 independent experiments each utilizing triplicate cultures. B, concentration-survival curves for 2008 (●), 2008-SCR (▼) 2008/CTR2KD-E2 (■), and 2008/CTR2KD-F5 (▲) cells. Each point in the concentration survival curves represents the mean of 3 independent experiments each performed with triplicate cultures for each drug concentration. C, IC_{50} values for tetrathiomolybdate. Vertical bars, ± SEM, N = 3. *p <0.05; **p <0.01.

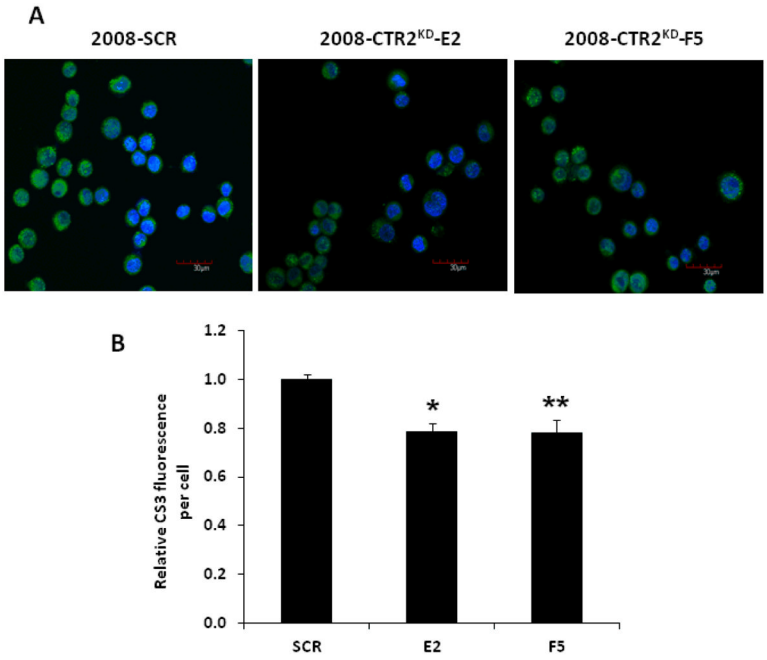

Figure 6.

Effect of knocking down CTR2 in human epithelial 2008 tumor cells on intracellular exchangeable Cu. A, CS3 staining intensity in CTR2 knock down cells. CS3; blue, nuclei stained with Hoechst 33342. B, Histogram of mean changes determined from 3 independent experiments. Vertical bars, ± SEM, N = 3. *p = 0.011; **p = 0.047.

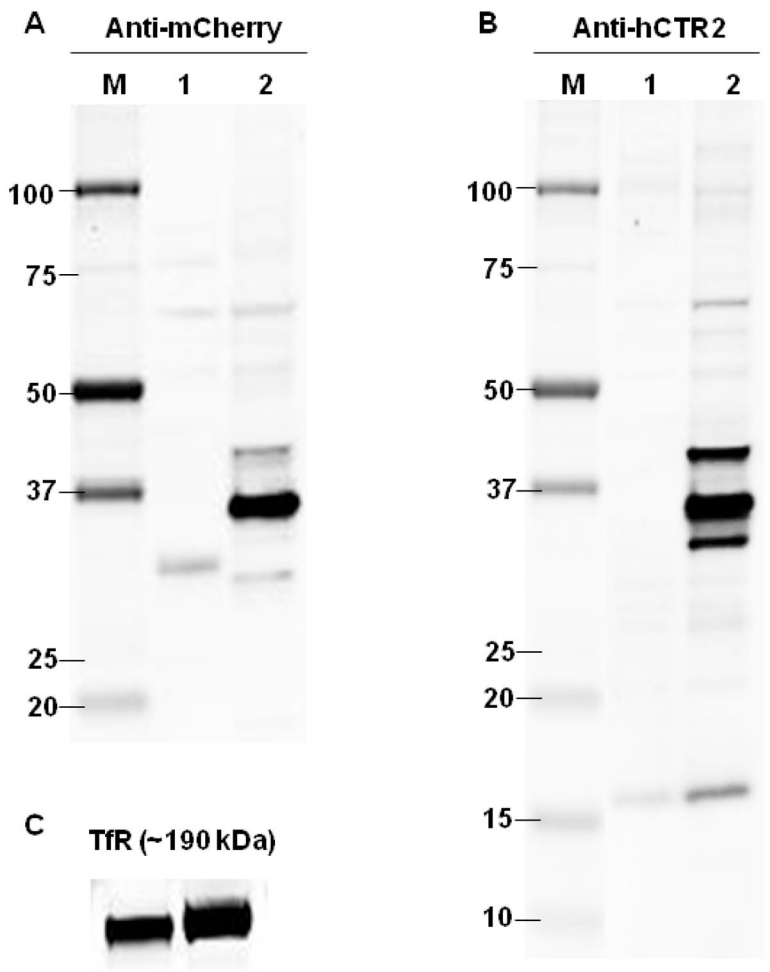

Figure 7.
Western blot documenting increased expression of mC-CTR2 in 2008 cells. Lane 1, 2008/mC cells expressing just mCherry; lane 2, 2008/mC-CTR2 cells expressing mCherry-tagged CTR2. A, blot probed with anti-mCherry antibody. B, Blot probed with anti-CTR2 antibody. C, blot probed with anti-transferrin antibody as a lane loading control.

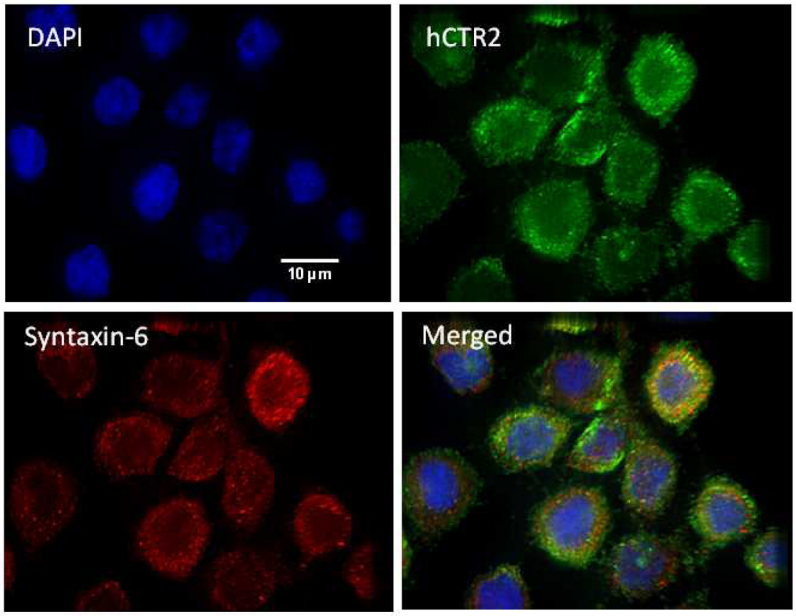

Figure 8.
Immunocytochemical analysis of co-localization between mC-CTR2 and syntaxin-6 in 2008/mC-CTR2 cells. Blue, DAPI stained nuclei; green, CTR2; red, syntaxin-6.

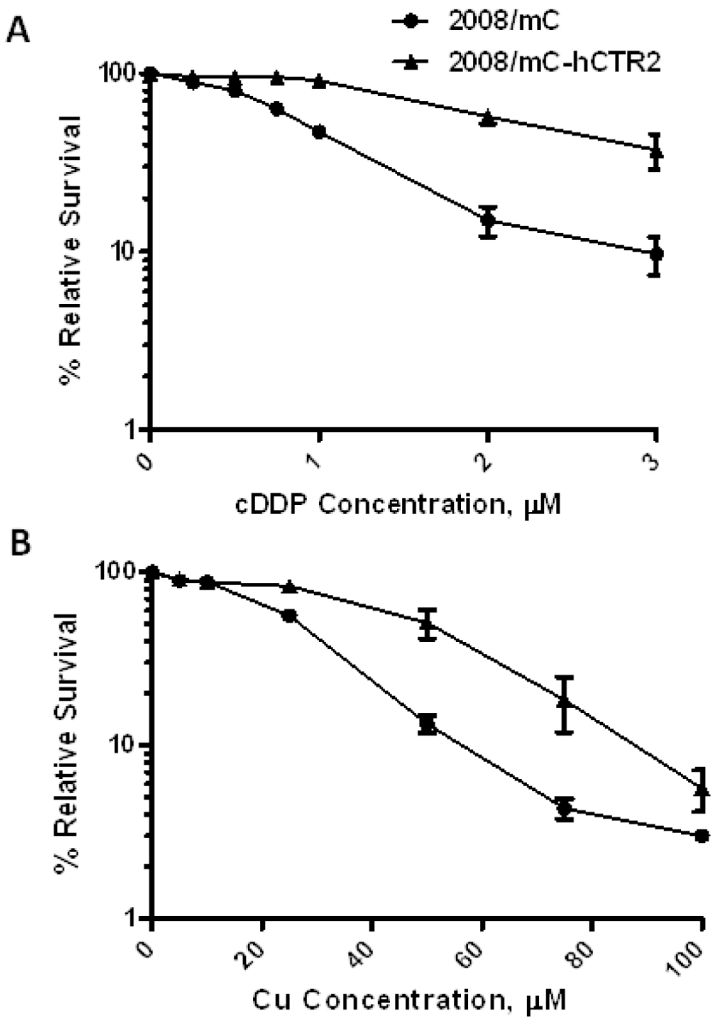

Figure 9.

Effect of over-expressing mC-CTR2 in 2008 cells on sensitivity to cDDP and Cu. A, Effect on sensitivity to cDDP. B, Effect on sensitivity to Cu. Each point in the concentration survival curves represents the mean of 3 independent experiments each performed with triplicate cultures for each drug concentration. Vertical bars, ± SEM, N = 3.

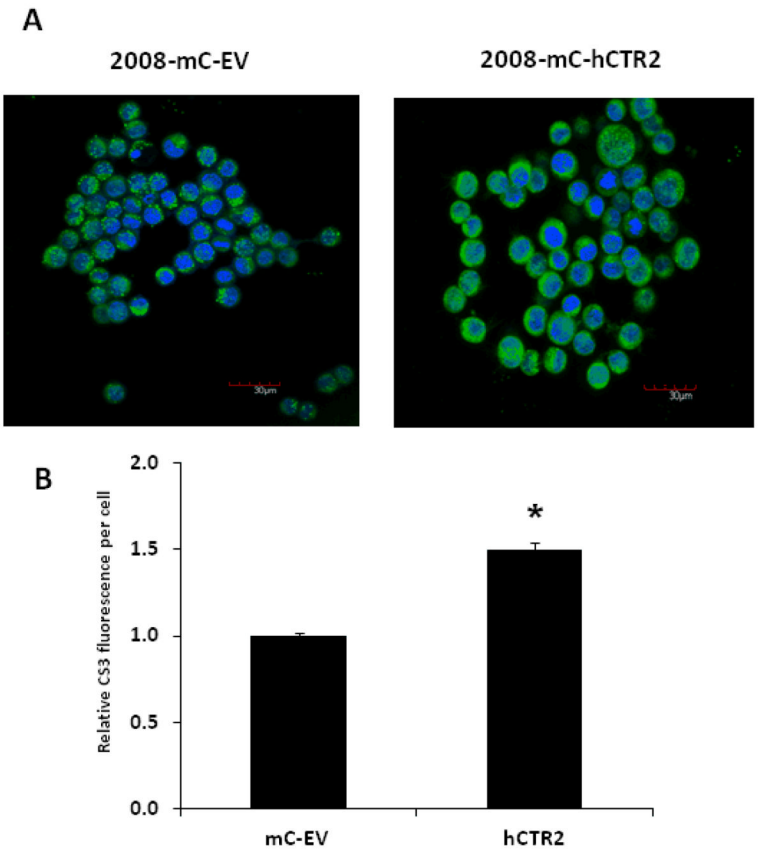

Figure 10.

Effect of over-expressing mC-CTR2 in 2008 cells on exchangeable Cu. A, Effect on CS3 staining intensity. CS3; blue, nuclei stained with Hoechst 33342. B, histogram of mean changes determined from 3 independent experiments. Vertical bars, ± SEM, N = 3. *p = 0.004.