

Copper Transporter 2 (CTR2) regulates endocytosis and controls tumor growth and sensitivity to cisplatin *in vivo*

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The abbreviations used are: BCS, bathocuproine disulphonate; CTR1, copper transporter 1, SLC31A1; CTR2, copper transporter 2, SLC31A2; cDDP, cisplatin; ICP-MS, inductively coupled plasma mass spectrometry; ICP-OES, inductively coupled plasma optical emission spectroscopy; PI3K, phosphoinositide 3-kinase.

ABSTRACT

Copper transporter 2 (CTR2) is one of the four copper transporters in mammalian cells that influence the cellular pharmacology of cisplatin and carboplatin. CTR2 was knocked down using an shRNAi. Robust expression of CTR2 was observed in parental tumors grown *in vivo* whereas no staining was found in the tumors formed from cells in which CTR2 had been knocked down. Knockdown of CTR2 reduced growth rate by 5.8-fold, increased the frequency of apoptotic cells and decreased the vascular density, but did not change copper content. Knockdown of CTR2 increased the tumor accumulation of cisplatin (cDDP) by 9.1-fold and greatly increased its therapeutic efficacy. As altered endocytosis has been implicated in cDDP resistance, uptake of dextran was used to quantify the rate of macropinocytosis. Knockdown of CTR2 increased dextran uptake by 2.5-fold without reducing exocytosis. Inhibition of macropinocytosis with either amiloride or wortmannin blocked the increase in macropinocytosis mediated by CTR2 knockdown. Stimulation of macropinocytosis by PDGF coordinately increased dextran and cDDP uptake. Knockdown of CTR2 was associated with activation of the Rac1 and cdc42 GTPases that control macropinocytosis but not activation of the phosphoinositide-3 kinase pathway. We conclude that CTR2 is required for optimal tumor growth and that it is an unusually strong regulator of cisplatin accumulation and cytotoxicity. CTR2 regulates the transport of cDDP in part through control of the rate of macropinocytosis via activation of Rac1 and cdc42. Selective knockdown of CTR2 in tumors offers a strategy for enhancing the efficacy of cDDP.

Introduction

The platinum-based chemotherapeutic agents cisplatin (cDDP), carboplatin and oxaliplatin are widely used for the treatment of many types of cancer (Rabik and Dolan, 2007). Unfortunately, patients commonly develop resistance during sequential cycles of treatment with all of these agents. Changes in drug influx and efflux, deficiencies in the mismatch repair pathway and down-regulation of the apoptotic cascade have all been linked to the mechanism of resistance (Chu, 1994; Crul et al., 1997; Kuo et al., 2007; Manic et al., 2003; Sedletska et al., 2005). The platinum-based drugs are believed to act through the formation of adducts on the purine bases of nuclear DNA (Johnson et al., 1997; Sedletska et al., 2005; Zorbas and Keppler, 2005); however, it is still unclear as to how these drugs traffic through the cell to reach the nucleus. Tumors that acquire resistance accumulate less drug than those that remain sensitive (Gately and Howell, 1993; Kelland et al., 1992; Metcalfe et al., 1986; Oldenburg et al., 1994; Song et al., 2004; Teicher et al., 1991; Twentyman et al., 1992; Waud, 1987).

There is now strong evidence that the transporters and chaperones that manage Cu homeostasis also transport the Pt-containing drugs (Katano et al., 2002; Safaei and Howell, 2005; Safaei et al., 2004b). Specifically the Cu efflux transporters ATP7A and ATP7B are involved in the sequestration and export of the Pt-containing drugs whereas the major Cu influx transporter CTR1 controls their uptake (Holzer et al., 2006; Larson et al., 2008; Safaei and Howell, 2005). In addition the Cu chaperone ATOX1 modulates sensitivity to cDDP by a less well characterized mechanism (Safaei et al., 2009).

In addition to the high affinity Cu influx transporter CTR1, mammalian cells express a second structurally-related transporter, Copper Transporter 2 (CTR2), whose function is less well defined. CTR2 is found predominantly in association with vacuolar membranes in yeast (Portnoy

et al., 2001; Rees et al., 2004) and to be localized to late endosomes and lysosomes in mammalian cells (Bertinato et al., 2007; van den Berghe et al., 2007). However, when expressed at the cell surface CTR2 is capable of mediating Cu uptake (Bertinato et al., 2008). Although CTR1 functions to influx both Cu and cDDP into the cell, a recent study from this laboratory demonstrated that CTR2 has the opposite effect and serves to limit the accumulation of cDDP in cell line models (Blair et al., 2009). Cells in which the expression of CTR2 was knocked down (CTR2^{kd}) accumulated 2-3-fold more cDDP than wild type parental cells; the finding that there was no effect of knocking down CTR2 on cDPP efflux suggested that the enhanced uptake was a result of increased influx (Blair et al., 2009). Additionally, these CTR2 knockdown cells exhibited a greatly increased sensitivity to cDDP and carboplatin when compared to wild type cells (Blair et al., 2009). The effect of the loss of CTR2 on both the uptake and sensitivity to cDDP was shown to be independent of CTR1 expression (Blair et al., 2009). The expression of CTR2 has recently been shown to be regulated by the Cu level in the cell (Blair et al., 2010). In Cu starved cells, CTR2 is degraded whereas increased Cu causes up regulation. Further confirmation of the importance of CTR2 as a regulator of the cellular pharmacology and cytotoxicity of cDDP was provided by the fact that down-regulation of CTR2 expression by Cu starvation increased cDDP uptake and cytotoxicity whereas up-regulation by exposure to excess Cu has the opposite effect (Blair et al., 2010).

One potential means by which CTR2 may limit cDDP accumulation and cytotoxicity is through the regulation of intracellular sequestration. In yeast, CTR2 is expressed in the vacuolar membrane (Bellemare et al., 2002; Rees et al., 2004), and in mammalian cells it is found in the mammalian equivalent of the yeast vacuole, which consists of the late endosomal and lysosomal compartments (Bertinato et al., 2007; Rees et al., 2004; van den Berghe et al., 2007). Available

evidence suggests that CTR2 functions primarily to efflux Cu from these structures under conditions of low environmental Cu (van den Berghe et al., 2007). If CTR2 mobilizes cDDP in a similar manner, then the loss of CTR2 would be expected to lead to an increased amount of drug trapped in vesicles and an increase in total cellular cDDP. However, previous studies have provided evidence against this hypothesis (Blair et al., 2009). Knockdown of CTR2 had no effect on the absolute amount of cDDP that accumulated in intracellular vesicles (Blair et al., 2009). Additionally, knockdown of CTR2 enhanced the accumulation of cDDP within 5 min suggesting that CTR2 restrains initial influx, perhaps through regulation of the uptake at the plasma membrane or of trafficking to intracellular sites (Blair et al., 2009). Knockdown of CTR2 also had no effect on cDDP efflux suggesting that the major role of CTR2 is to mediate cDDP influx rather than to restrain efflux.

Previous studies have reported a link between endocytosis and cDDP sensitivity (Safaei et al., 2004a; Shen et al., 2004a; Shen et al., 2004b). The GTPases Rac1, RhoA and cdc42, which control clathrin-independent endocytosis, are down-regulated in several cDDP-resistant cell lines (Shen et al., 2004b) as are cytoskeletal proteins necessary for endocytosis (Shen et al., 2004a). Furthermore CTR1, which also regulates cellular accumulation of cDDP, has been closely linked to macropinocytosis; CTR1 degradation triggered by exposure to Cu or cDDP exposure occurs by this process (Holzer and Howell, 2006). In the current study, we sought to determine whether CTR2 is an important determinant of the responsiveness to cDDP *in vivo* and further define the mechanism by which CTR2 controls the cellular pharmacology of cDDP. We examined the effect of knocking down the expression of CTR2 in malignant mouse embryo fibroblasts on the accumulation of cDDP and the ability of cDDP to slow tumor growth, and examined the effect on macropinocytosis. We report here that tumors derived from cells in which CTR2 had been

knocked down grew more slowly than those derived from wild type cells, and that this was associated with an increased frequency of apoptotic cells and decreased vascular density. However, the tumors in which CTR2 was knocked down accumulated much more platinum following injection of cDDP and exhibited a much greater response to treatment. We found that CTR2 regulates the rate of macropinocytosis through effects on endocytotic factors Rac1 and cdc42. These observations suggest that selective inhibition of CTR2 expression or function may be a useful strategy for enhancing the effectiveness of cDDP chemotherapy.

Materials and Methods

Drugs and Reagents. Platinol AQ was a gift from Bristol-Myers Squibb (Princeton, NJ); it contains cDDP at a concentration of 3.33 mM in 0.9 percent NaCl. The anti-CTR2 antibody was a gift from Dr. Jessie Bertinato (Health Canada, Ottawa, ON). Transferrin-Alexa Fluor 546 and 70-kDa dextran-Texas red were purchased from Invitrogen (Carlsbad, CA). Amiloride and wortmannin were purchased from Tocris Biologicals (Ellisville, Mo). Bradford reagent was purchased from BioRad Laboratories, Inc. (Hercules, CA). Sulforhodamine B was obtained from Sigma-Aldrich and 0.4% sulforhodamine B (w/v) was solubilized in 1% (v/v) acetic acid solution.

Cell Types. Parental mouse embryonic fibroblasts in which both copies of CTR1 had been somatically knocked out (CTR1^{-/-}) were a gift from Dr. Dennis Thiele (Lee et al., 2002). The CTR2^{kd} sublines were constructed by infecting the CTR1^{-/-} cells with lentivirus expressing a shRNAi targeting mouse CTR2 mRNA purchased from Sigma-Aldrich (St. Louis, MO) as previously described (Blair et al., 2009).

Western Blotting. Cell lysates were prepared and subsequent Western blots were conducted as previously described (Blair et al., 2009). The primary antibodies used for Western blot analysis were: anti-CTR2 (provided by Dr Jesse Bertinato), anti-CTR2 (Novus Biologicals, Littleton, CO), anti-tubulin (Santa Cruz Biotechnology, Inc. Santa Cruz, CA), anti-Rac1, anti-cdc42, anti-Akt, and anti pAkt S473 (Cell Signaling Technology, Inc. Boston, MA).

Immunohistochemistry and Chemiluminescent Immunoblotting. Upon harvesting, tumors were either imbedded in paraffin or frozen in O.C.T. compound as previously described (Holzer et al., 2006). Sections were stained according to the protocol outlined in the Catalyzed Signal Amplification System (cat. #K1500; DAKO, Carpinteria, CA). Endogenous biotin was blocked by first overlaying the slides with 0.1% avidin for 15 min and washing 3 times with 0.1% Triton-X in PBS. Slides were then overlaid with 0.01% biotin for 15 min followed by another 3 washes with 0.1% Triton-X in PBS. Nonspecific protein binding was blocked by immersion of the slides in 1% BSA in PBS for 20 min. Slides were incubated with anti-hCTR2 antibody at a dilution of 1:200 in 1% BSA in PBS overnight at 4 °C. As a negative control, parallel sections were incubated with non-immune rabbit IgG₁ sera (prediluted; DAKO). Slides were also stained with anti-Ki67 to determine the fraction of S phase nuclei and anti-CD31 antibody (BD Pharmingen, San Jose, CA) to quantify vascular density and apoptotic nuclei were detect by TUNEL assay (Latimer et al., 2009).

Quantification of Vascular Density. The mean vascular density (vessels per square millimeter) for each tumor was calculated as previously described (Lucidarme et al., 2006). Five light microscope pictures at $\times 40$ were taken at different locations in each tumor sample. The total count of CD31 stained vessels was divided by the area of the five fields to obtain the mean

vascular density. Eight to 10 tumors of each cell type were scored by two blinded observers; the values reported are the mean of the ratio for the two types of tumors.

Measurement of Drug Accumulation in Tumors. One h following intraperitoneal injection of 10 mg/kg cDDP tumors were harvested and digested in 70% nitric acid overnight, diluted to a final 5% nitric acid (0.1 percent Triton X-100, 1.4 percent nitric acid, 1 ppb In in ddH₂O). Pt concentration was measured using a Perkin-Elmer Element 2 ICP-OES located at the Analytical Facility at Scripps Institute of Oceanography at the University of California, San Diego. As a method of normalization, total sulfur was also measured by ICP-OES in each sample. Cu levels were measured using the same instrument. All data presented are the means of 40 samples of each type of tumor.

Determination of Drug Sensitivity *in Vivo*. All animal experiments were carried out in accordance with guidelines of the University of California San Diego Institutional Animal Care and Use Committee. To grow the various types of cells as xenografts, 3×10^6 cells in 100 μ l were inoculated at 4 subcutaneous sites into female nu/nu mice; 19 mice were included in the cDDP-treated group and 18 in the untreated group. Cell types were randomized between shoulder and hip, left and right, ensuring that there were always tumors of the same type on left and right. Tumors were allowed to grow until they were ~2 mm in diameter, at which point each mouse received either a single dose of cDDP 10 mg/kg by intraperitoneal injection or vehicle only. Tumor size was monitored 3 times per week for 7 weeks. Tumor volume was estimated using the equation $(\text{length} \times \text{width}^2)/2$.

Tyrosinase Activity Assay. CTR1^{+/+}, CTR1^{+/+} CTR2^{kd}, CTR1^{-/-} and CTR1^{-/-} CTR2^{kd} cells were plated in 96 well tissue culture dishes (~20,000 cells per well, 15 wells per cell type) and 24 h later were treated L-DOPA-containing assay buffer (10 mM L-dopa (L-3,4-

dihydroxyphenylalanine), 500 mM phosphate buffer (pH 6.8)). Following a 72 h exposure the absorbance of each well at 400 nm was recorded using a Versamax Tunable Microplate Reader (Molecular Devices, Sunnyvale, CA). All experiments were repeated at least 3 times using 3 cultures for each drug concentration and the data is presented as the percent tyrosinase activity relative to wild type control cells. Parallel plates were stained using 100 μ l of 0.4% sulforhodamine B in 1% acetic acid at room temperature for 15 min. Following washing, the absorbance of each well at 515 nm was recorded using a Versamax Tunable Microplate Reader (Molecular Devices, Sunnyvale, CA) as a control for cell density.

Measurement of Macropinocytosis by Dextran Uptake. $CTR1^{+/+}$, $CTR1^{+/+} CTR2^{kd}$, $CTR1^{-/-}$ and $CTR1^{-/-} CTR2^{kd}$ cells were plated in 96 well tissue culture dishes (~20-50,000 cells per well, 15 wells per cell type) and 24 h later were treated with transferrin-Alexa Fluor 546 and 70-kDa dextran-Texas red for 30 min. The wells were immediately washed with ice cold PBS three times and fixed with 3.7% formalin in PBS for 30 min followed by three 10 min PBS washes. Fluorescence was measured using an Infinite M-200 micro-plate reader (Tecan US, Inc. San Jose, CA). The cells were then stained using 100 μ l of 0.4% sulforhodamine B in 1% acetic acid at room temperature for 15 min. Following washing, the absorbance of each well at 515 nm was recorded using a Versamax Tunable Microplate Reader (Molecular Devices, Sunnyvale, CA) as a control for cell density. All experiments were repeated at least three times using three cultures for each drug concentration. To assess the effect of copper starvation on macropinocytosis, $CTR1^{+/+}$ and $CTR1^{-/-}$ cells were pretreated with 100 μ M BCS for 1 h or left untreated. Cells were then washed with 37°C PBS three times and uptake of Texas-red dextran was measured.

Measurement of Dextran Exocytosis. CTR1^{+/+}, CTR1^{+/+} CTR2^{kd}, CTR1^{-/-} and CTR1^{-/-} CTR2^{kd} cells were plated in 96 well tissue culture dishes (~20-50,000 cells per well, 15 wells per cell type) and 24 h later cells were treated with transferrin-Alexa Fluor 546 and 70-kDa dextran-Texas red for 30 min. Cells were immediately washed with 37°C PBS three times and placed in normal media. At 0, 1, 2, 4 and 10 min following removal of dextran plates were fixed with 3.7% formalin in PBS for 30 min followed by three 10 min PBS washes. Fluorescence and absorbance were then measured. All conditions were repeated at least three times using three cultures for each drug concentration. Retention of Texas-red dextran was assayed as a marker for rate of dextran exocytosis.

Inhibition of Endocytosis. Cells were treated with 5 mM amiloride, 100 nM wortmannin or control medium for 30 min and then washed with 37°C PBS 3 times. The media was then removed and the cells were exposed to 500 µl of OptiMEM medium (Invitrogen, Carlsbad, CA) containing 30 µM cDDP at 37°C for either 0 or 15 minutes after which the drug-containing medium was removed, the plates were washed 3 times with ice-cold PBS and were processed for Pt measurement.

Measurement of Whole Cell Drug Accumulation Following PDGF Stimulation. Cells were grown to 90% confluence in T-150 tissue culture flasks. Cells were then harvested using trypsin, and 7.5×10^5 cells were placed into each well of 6-well tissue culture plates and allowed to grow overnight in 2.5 ml of reduced serum media at 37°C in 5% CO₂. The next day, medium was removed by aspiration and the cells either treated with either 5 ng/mL PDGF-BB or control media for 4 h. The media was then removed and the cells were exposed to 500 µl of cDDP-containing OptiMEM medium (Invitrogen) at 37°C for either 0 or 60 min. The drug-containing medium was removed, and the plates were washed 3 times with ice-cold PBS and then placed on

ice. In the case of the time zero samples, the drug-containing medium was aspirated within 15 sec of the start of drug exposure. Two-hundred and fifteen μ l of concentrated (50-70%) nitric acid was added to each well and the plate was incubated overnight at room temperature. The following day the acid was moved into Omni-vials (Wheaton, Millville, NJ) and incubated at room temperature overnight to thoroughly dissolve all cellular debris. The samples were then diluted with 3 ml of buffer (0.1% Triton X-100, 1.4% nitric acid, 1 ppb In in ddH₂O). Pt concentration was measured using a Perkin-Elmer Element 2 ICP-MS located at the Analytical Facility at Scripps Institute of Oceanography at UCSD. As a method of normalization, total sulfur was measured using a Perkin-Elmer ICP-OES also located at SIO at UCSD. Samples that were previously prepared for the ICP-MS were then introduced into the ICP-OES where total μ g of sulfur was measured. All data presented are the means of at least three independent experiments each performed with six wells per concentration tested.

Western Blot Analysis of Activated Rac1 and cdc42. Cells grown in serum free media for 24 h to ~80% confluence were washed 3 times with PBS and immediately lysed in 1 ml of lysis buffer (50mM Tris, 150 mM NaCl, 0.1 % SDS, 0.5 % sodium deoxycholate, 1% Triton X 100, protease inhibitor). The lysate was then incubated with 10 μ g of PAK-1 PBD agarose (Millipore, Lake Placid, NY) for 1 h at 4° C. Beads were isolated and washed 3 times with lysis buffer and then resuspended in Laemmli buffer and analyzed by Western blot using antibodies specific for total and phosphorylated forms of Rac1, cdc2 and Akt.

Statistical Analysis. All data shown were derived from at least 3 independent experiments each of which included triplicate cultures for each data point. Values are presented as mean \pm SEM. Statistical comparisons were performed using a two-tailed Student's t-test with

the assumption of unequal variance. A total of 23 mice were used for tumor experiments (12 CDDP-treated, 11 untreated) each with 4 tumors distributed as described above.

Results

Effect of CTR2 Knockdown on Tumor Growth Rate. To determine the dependence of tumor growth on CTR2 *in vivo* we utilized a malignant mouse embryo fibroblast cell line in which both alleles of CTR1 had already been deleted. The expression of CTR2 in these CTR1^{-/-} cells was constitutively knocked down using a lentiviral vector expressing an shRNAi directed to the CTR2 mRNA (Blair et al., 2009). Figure 1A shows a western blot analysis that documents that the level of expression of CTR2 protein in the cell line before tumor inoculation was reduced by 87.1 ± 4.6 (SEM) % below that in the parental CTR1^{-/-} cells. Both the parental CTR1^{-/-} and CTR1^{-/-} CTR2^{kd} cells were inoculated subcutaneously into nu/nu mice, and both types of cells formed tumors with equal frequency. Immunohistochemical analysis of sections from these tumors demonstrated robust expression of CTR2 in the CTR1^{-/-} tumors, but no detectable CTR2 expression in the CTR1^{-/-} CTR2^{kd} tumors (Figure 1B). As shown in Figure 1C, CTR1^{-/-} tumors grew 5.8-fold more rapidly than CTR1^{-/-} CTR2^{kd} tumors.

Effect of CTR2 on Proliferation and Apoptosis *in Vivo*. To examine the basis for the difference in growth rate, CTR1^{-/-} and CTR1^{-/-} CTR2^{kd} tumors were harvested and fixed in formalin. Ki67 is an antigen expressed in the S phase of the cell cycle that is widely used to quantify the fraction of proliferating cells in tumors (Iatropoulos and Williams, 1996). The tumors were stained with an antibody to Ki67 to determine the effect of knocking down CTR2 on proliferation rate. CTR2^{kd} tumors contained $24.3 \pm 10.3\%$ ($p < 0.02$) fewer Ki67-positive cells than CTR1^{-/-} tumors (Figure 2A). The tumors were sectioned and the frequency of apoptotic cells

measured by TUNEL assay as shown in Figure 2B. The average number of TUNEL positive nuclei per high power field was determined for each tumor type. CTR1^{-/-} tumors had an average 42.8 ± 6.2 TUNEL positive nuclei per high power field. In contrast CTR1^{-/-} CTR2^{kd} tumors had an average of 81.8 ± 10.8 TUNEL positive nuclei per high power field. Thus, the frequency of apoptotic cells in the CTR1^{-/-} CTR2^{kd} tumors was 1.9-fold higher than in the CTR1^{-/-} tumors suggesting that the death rate of tumor cells was increased by a large amount when CTR2 was knocked down.

Effect of CTR2 on Vessel Density *in Vivo*. Cu is essential for angiogenesis, and adequate vascularization is required for tumor growth. To determine whether knockdown of CTR2 altered the extent of angiogenesis in tumors, subcutaneously implanted CTR1^{-/-} and CTR1^{-/-} CTR2^{kd} tumors were harvested and frozen in O.C.T. compound. Tumors were sectioned and stained with an antibody to the endothelial cell marker CD31 which stains tumor capillaries. Figure 2C shows a reduced density of CD31-expressing cells in the CTR1^{-/-} CTR2^{kd} tumors. The mean (number of vessels per square mm) was 83.7 ± 7.0 in the CTR1^{-/-} tumors but was reduced to 57.3 ± 3.5 in the CTR1^{-/-} CTR2^{kd} tumors (Figure 3B). Thus, the vessel density was 1.5-fold higher in the CTR1^{-/-} tumors ($p=0.00003$) indicating that CTR2 has a substantial effect on tumor vessel formation.

Effect of CTR2 on Cu Content *In Vitro* and *In Vivo*. The exact role of CTR2 in Cu homeostasis remains poorly defined. Knockdown of CTR2 was found to increase the steady-state level of Cu in the CTR1^{-/-} CTR2^{kd} cells when grown *in vitro* in the absence of any added Cu. The level in CTR1^{+/+} cells was 1.10 ± 0.02 ng Cu/ug sulfur when grown in standard tissue culture medium. The level in the CTR1^{-/-} cells did not significantly differ being 0.90 ± 0.10 ng Cu/ μ g sulfur. Knockdown of CTR2 in the CTR1^{-/-} cells increased the steady-state Cu level by 2.1-fold

to 1.89 ± 0.01 ng Cu/ μ g sulfur. To determine whether similar differences were observed when the CTR1^{-/-} and CTR1^{-/-} CTR2^{kd} cells were grown *in vivo*, untreated CTR1^{-/-} and CTR1^{-/-} CTR2^{kd} subcutaneous tumors were harvested and dissolved in nitric acid and the Cu levels were assayed by ICP-MS. There was no significant difference in steady state Cu levels which were 552.2 ± 18.3 ng Cu/mg tumor in the CTR1^{-/-} tumors and 535.9 ± 36.0 ng Cu/mg tumor in the CTR1^{-/-} CTR2^{kd} tumors ($p = 0.7$). Thus, despite the clear effect of knocking down CTR2 on cellular Cu levels when grown *in vitro*, when grown *in vivo* the knockdown of CTR2 did not alter Cu levels when measured in the mixture of all cell types in the tumor.

Tyrosinase activity was measured as an additional assay of the affect of CTR2 loss on the ability of Cu to reach down-stream targets. The tyrosinase activity in the CTR1^{-/-} cells was $56.6 \pm 7.7\%$ ($p < 0.0001$), and in the CTR1^{-/-} CTR2^{kd} cells was $58.2 \pm 4.9\%$ ($p < 0.0002$), of that in the CTR1^{+/+} cells. Thus, loss of CTR1 reduced the amount of Cu reaching tyrosinase. In contrast, knockdown of CTR2 did not significantly affect tyrosinase activity in either the CTR1^{+/+} or CTR1^{-/-} background. The tyrosinase activity in the CTR1^{+/+} CTR2^{kd} cells was $113.5 \pm 12.7\%$ of that in the CTR1^{+/+} cells ($p = 0.15$), and that in the CTR1^{-/-} CTR2^{kd} cells was $97.2 \pm 6.0\%$ ($p = 0.48$) of that in the CTR1^{-/-} cells. Thus, despite similar whole cell Cu levels, the loss of CTR1 limited the availability of intracellular Cu, at least as measured by tyrosinase activity, whereas knockdown of CTR2 did not.

Effect of CTR2 Knockdown on cDDP Accumulation *In Vivo*. Nu/nu mice with subcutaneous CTR1^{-/-} and CTR1^{-/-} CTR2^{kd} tumors were injected intraperitoneally with 10 mg/kg cDDP and 1 h later the mice were sacrificed and tumors harvested. The Pt level in each tumor was determined by ICP-OES. The average Pt level in the CTR1^{-/-} tumors was 2.26 ± 0.36 ng Pt/mg tumor. The average Pt level in the CTR1^{-/-} CTR2^{kd} tumors was 20.62 ± 3.53 ng Pt/mg

tumor. Thus, the $CTR1^{-/-} CTR2^{kd}$ tumors accumulated 9.1-fold more Pt at 1 h after injection of CDDP than the $CTR1^{-/-}$ tumors ($p = 0.006$). This is a very large difference in Pt accumulation compared to the 3.5-fold difference in uptake observed for these cells when grown *in vitro* (Blair et al., 2009) and what is generally observed in cDDP-sensitive and cDDP-resistant cell lines.

Effect of CTR2 Knockdown on Responsiveness to cDDP *In Vivo*. As shown in Figure 3A, a single intraperitoneal injection of the maximum tolerated dose of cDDP (10 mg/kg) produced little slowing of the growth of $CTR1^{-/-}$ tumors relative to the growth rate of the untreated control tumors ($p = 0.75$). However, the same dose of cDDP clearly slowed the growth of $CTR1^{-/-} CTR2^{kd}$ tumors ($p < 0.0009$) (Figure 3B). The average volume of the cDDP-treated $CTR1^{-/-} CTR2^{kd}$ tumors was 74% smaller than the untreated $CTR1^{-/-} CTR2^{kd}$ tumors by week 7. Many of these tumors shrank in size and remained smaller than they were before treatment. Six of the 16 tumors became undetectable and never regrew during the period of observation. The remaining tumors either stopped growing or grew at a much slower rate than the $CTR1^{-/-}$ tumors. Thus, consistent with the effect of CTR2 on cDDP accumulation, CTR2 is a major determinant of therapeutic efficacy of cDDP *in vivo*.

Loss of CTR2 Increases the Rate of Endocytosis. Changes in sensitivity to cDDP has been linked to perturbations in the rate of endocytosis in the past, and CTR1 is cleared from the plasma membrane by macropinocytosis. The accumulation of Texas red-labeled dextran is a well-validated measure of the rate of macropinocytosis (Chauhan et al., 2003; Holzer and Howell, 2006). The rate of macropinocytosis in the $CTR1^{+/+}$, $CTR1^{+/+} CTR2^{kd}$, $CTR1^{-/-}$ and $CTR1^{-/-} CTR2^{kd}$ cells was assessed by exposing them to the labeled dextran for 30 min. As shown in Figure 4A, loss of CTR1 did not significantly change the rate of macropinocytosis as measured by the uptake of dextran; there was no difference in its accumulation in $CTR1^{-/-}$ and $CTR1^{+/+}$

cells ($p = 0.45$). In contrast, loss of CTR2 significantly increased the uptake of dextran. The CTR1^{+/+} CTR2^{kd} cells took up 2.5-fold more dextran over a 30 min period than the CTR1^{+/+} cells ($p = 0.01$). This increase in macropinocytosis in CTR2^{kd} cells occurred independently of CTR1 status. CTR1^{-/-} CTR2^{kd} cells accumulated 2.1-fold more dextran over a 30 min period than CTR1^{-/-} cells ($p = 0.049$).

CTR2 Degradation Due to Copper Starvation Induces Endocytosis. Cu depletion by exposure to the high affinity Cu chelator BCS results in a rapid and near total degradation of CTR2 in these mouse embryo fibroblasts (Blair et al., 2010). To strengthen the link between CTR2 and macropinocytosis, cells were exposed to BCS for 1 h to reduce CTR2 levels by ~90-95%. As shown in Figure 4B, BCS-induced down-regulation of CTR2 expression enhanced dextran uptake by 1.9-fold in both the CTR1^{+/+} cells ($p = 0.039$) and CTR1^{-/-} cells ($p = 0.021$). Thus, macropinocytosis was enhanced when CTR2 expression was reduced by either of two independent means.

Enhanced Dextran Accumulation is not Due to Changes in Exocytotic Rate.

Enhanced accumulation of dextran may be due to either increased macropinocytosis or reduced exocytosis. Exocytosis of Texas-red dextran was measured in the CTR1^{+/+}, CTR1^{+/+} CTR2^{kd}, CTR1^{-/-} and CTR1^{-/-} CTR2^{kd} cells. As shown in Figure 4C, the majority (~55%) of Texas-red dextran exited the cells in the first 2 min and there was no significant difference in the rate of Texas-red dextran exocytosis in CTR1^{+/+}, CTR1^{+/+} CTR2^{kd}, CTR1^{-/-} and CTR1^{-/-} CTR2^{kd} cells during either the initial or late phases of efflux.

Inhibition of Macropinocytosis Blocks CTR2-Dependent cDDP Accumulation. As described in previous studies, knockdown of CTR2 mediated by either shRNAi or Cu starvation substantially increases cDDP uptake *in vitro* (Blair et al., 2009) as well as *in vivo*. To determine

whether this increase is due to enhanced macropinocytosis, cDDP accumulation was measured in the $CTR1^{+/+}$, $CTR1^{+/+} CTR2^{kd}$, $CTR1^{-/-}$ and $CTR1^{-/-} CTR2^{kd}$ cells with and without inhibition of macropinocytosis by pretreatment with either 5 mM amiloride or 100 nM wortmannin for 30 min. Both of these drugs have been widely used to inhibit macropinocytosis although neither is entirely specific for this process (Ivanov, 2008). As shown in Figure 5A, knockdown of CTR2 in untreated cells caused a 2.8-fold increase in cDDP accumulation in the $CTR1^{+/+}$ cells ($p = 0.01$) and a 2.7-fold increase in the $CTR1^{-/-}$ cells ($p = 0.03$). Treatment with amiloride did not significantly change cDDP uptake in either the $CTR1^{+/+}$ or $CTR1^{-/-}$ cells but it completely blocked the increased cDDP uptake in the $CTR2^{kd}$ cells. When compared to untreated cells, amiloride pre-treatment produced a 71.0% ($p = 0.001$) and 65.3% ($p = 0.002$) decrease in cDDP accumulation in $CTR1^{+/+} CTR2^{kd}$ and $CTR1^{-/-} CTR2^{kd}$ cells, respectively. As shown in Figure 5B, $CTR1^{+/+}$ cells pretreated with wortmannin accumulated 1.8-fold ($p = 0.02$) more cDDP than untreated $CTR1^{+/+}$ cells; wortmannin had no effect on the accumulation of cDDP in $CTR1^{-/-}$ cells, indicating that this effect was specific to CTR1-expressing cells. This effect may be related to the ability of wortmannin to interfere with the clearance of CTR1 from the plasma membrane in a manner similar to the effect of proteosomal inhibition (Jandial et al., 2009). As observed with amiloride, wortmannin completely blocked the increased accumulation of cDDP in untreated $CTR1^{+/+} CTR2^{kd}$ and $CTR1^{-/-} CTR2^{kd}$ cells, producing 65.7% ($p = 0.001$) and 79.4% ($p = 0.0001$) decreases in cDDP accumulation, respectively. These results provide further evidence that the enhanced cDDP uptake that accompanies knockdown of CTR2 is mediated by an increased rate of macropinocytosis.

Induction of Macropinocytosis by PDGF Increases cDDP Accumulation. Inhibition of macropinocytosis blocked the increased cDDP accumulation produced by knocking down

CTR2. To further explore the role of macropinocytosis in the cellular accumulation of cDDP, PDGF was used to up-regulate macropinocytosis in the wild type CTR1^{+/+} cells. As previously reported {Dharmawardhane, 2000 #14621, pretreatment with 5 ng/mL PDGF-BB for 4 h increased dextran uptake by 1.8-fold ($p < 0.02$) (Figure 5A). As demonstrated in Figure 5B, CTR1^{+/+} cells pretreated with PDGF accumulated 2.1-fold ($p < 0.001$) more cDDP following a 1 h exposure to 30 μ M cDDP, consistent with the conclusion that cDDP can enter cells through the macropinocytotic pathway.

Knockdown of CTR2 Activates Rac1 and cdc42. The active form of the Rho GTPases Rac1 and cdc42 are necessary for macropinocytosis. To determine whether CTR2 can regulate the activation of these proteins, the levels of active Rac1 and cdc42 were quantified by Western blot analysis using antibodies to the phosphorylated forms of these proteins in the CTR1^{+/+}, CTR1^{+/+} CTR2^{kd}, CTR1^{-/-} and CTR1^{-/-} CTR2^{kd} cells. As shown in Figure 6A, under basal conditions, neither the CTR1^{+/+} nor CTR1^{-/-} cells contained detectable active Rac1 or cdc42. However, knockdown of CTR2 resulted in a very strong activation of both Rac1 and cdc42 regardless of CTR1 status. The total amount of Rac1 and cdc42 protein was similar in all four cell lines, indicating that loss of CTR2 constitutively activates these GTPases without changing their level.

Loss of CTR2 Does Not Affect Akt Activation. PI3K has been shown to activate Rac1 and cdc42. To determine whether the loss of CTR2 activates Rac1 and cdc42 via the PI3K pathway, the extent of Akt phosphorylation was measured in the CTR1^{+/+}, CTR1^{+/+} CTR2^{kd}, CTR1^{-/-} and CTR1^{-/-} CTR2^{kd} cells since Akt becomes phosphorylated upon PI3K activation. As shown in Figure 6B, Western blot analysis using an antibody specific for Akt phosphorylated at S473 demonstrated that the loss of either CTR1 or CTR2 did not change Akt phosphorylation,

thus suggesting that loss of CTR2 regulates Rac1 and cdc42 phosphorylation downstream of PI3K.

Discussion

In this study we sought to investigate the effect of CTR2 on tumor growth rate, cDDP accumulation and responsiveness *in vivo*, and to further elucidate the mechanism by which CTR2 controls the cellular pharmacology of cDDP. The first finding of importance was that the growth rate of the CTR1^{-/-} CTR2^{kd} tumors was substantially slower than that of the CTR1^{-/-} tumors. The finding of a higher frequency of apoptotic cells, lower proliferation index and a lower density of CD31-positive capillaries in the CTR1^{-/-} CTR2^{kd} tumors provides a reasonable explanation for why the growth rate was impaired, but how the loss of CTR2 produces these effects remains unknown. There was a 2.1-fold higher level of Cu in the CTR1^{-/-} CTR2^{kd} cells when grown *in vitro* that was not observed when they were grown *in vivo*. Since we harvested the whole tumor, which contains mouse derived endothelial cells, mesenchymal and inflammatory cells in addition to tumor cells, it is conceivable that the difference in the Cu content of the actual tumor cells was missed. However, the fact that there was such a large difference in Pt levels suggests that the extent of contamination of the tumor cells by normal mouse cells was not large, and the fact that knockdown of CTR2 did not impair tyrosinase activity suggests no major limitation of intracellular Cu availability. There is a substantial body of data indicating that Cu is required for angiogenesis (reviewed in (Finney et al., 2009)) but the fact that no differences were detected in steady-state Cu levels between the two types of tumors leaves open the possibility that, despite its important role in Cu homeostasis, CTR2 modulates the angiogenic response via effects on other pathways. It is of interest that CTR1 has been shown to regulate signaling via the FGF

receptor pathway during embryonic development of neurectoderm in *Xenopus* (Haremakei et al., 2007). Given the structural similarity of CTR1 and CTR2, it is conceivable that CTR2 regulates the synthesis or release of angiogenic factors from tumor cells.

CTR2 is an important determinant of the cytotoxicity of cDDP when cells are grown in tissue culture (Blair et al., 2009); however, results obtained using cultured cells do not always extrapolate to the *in vivo* setting. Gratifyingly, the results of the current study show that CTR2 is a major determinant of both cDDP accumulation and its therapeutic effectiveness *in vivo* as well as *in vitro*. Given the fact that CTR2 also regulates sensitivity to carboplatin *in vitro*, it is likely that CTR2 expression is also important to the tumor pharmacology and responsiveness of this drug *in vivo* as well. The magnitude of the effect of knocking down CTR2 on cDDP accumulation *in vivo* (9.1-fold) was quite a bit larger than the difference in uptake observed when the cells were grown *in vitro* (3.5-fold), and is very large relative to the ~50% difference in cDDP uptake typically observed in isogenic pairs of cDDP-sensitive and resistant cell lines (Andrews and Howell, 1990; Muggia and Los, 1993). This suggests that CTR2 is particularly important to cDDP accumulation in the complex *in vivo* environment in which rates of drug delivery, protein binding and aquation are different from those obtained when cells are exposed to CDDP *in vitro*. Although the cellular level of Cu does modulate the uptake of cDDP, the similarity of the Cu levels in the CTR1^{-/-} and CTR1^{-/-} CTR2^{kd} tumors makes it unlikely that intracellular Cu availability alone accounts for the difference in cDDP accumulation.

Prior studies from this laboratory demonstrated that CTR2 has a large effect on the cellular accumulation of cDDP, and the results reported here provide insight into the mechanism of this effect. We have previously reported that the increased cDDP uptake in CTR2^{kd} cells is not due to a change in drug efflux or microsomal storage of cDDP (Blair et al., 2009). The results

reported here indicate that CTR2 regulates cDDP accumulation at least in part through an effect on macropinocytosis. Knockdown or BCS-induced degradation of CTR2 was accompanied by a large increase in macropinocytosis, and inhibition of macropinocytosis with either amiloride or wortmannin blocked this effect. Stimulation of macropinocytosis with PDGF mimicked the effect of knocking down CTR2 on dextran and cDDP uptake, and knockdown of CTR2 was associated with activation of two Rho GTPases that regulate various endocytotic pathways. Taken together, these data suggest that the increased uptake of cDDP in CTR2^{kd} cells is due to the observed up-regulation of macropinocytosis.

Endocytosis, and specifically macropinocytosis, is a tightly controlled process requiring activation of the GTPases Rac1 and cdc42 (Conner and Schmid, 2003). Once activated, these GTPases stabilize the formation of actin and filamin projections into the extracellular space allowing the formation of endocytotic vesicles. Previous studies have shown that Rac1, cdc42, actin and filamin are all down-regulated in many cDDP resistant cell lines (Shen et al., 2004a; Shen et al., 2004b). Endocytosis itself has also been reported to be reduced in many cDDP-resistant cells (Chauhan et al., 2003; Liang et al., 2006). CTR2^{kd} cells were found to have constitutively active Rac1 and cdc42, inferring that this mechanism may underlie up-regulated rate of macropinocytosis in these cells.

The concordance of the *in vitro* and *in vivo* results with respect to cDDP accumulation and cytotoxicity further validates CTR2 as a target against which one could develop drugs capable of sensitizing tumors to cDDP. Gene silencing via targeted siRNA therapy is being explored in clinical trials (Castanotto and Rossi, 2009; Siomi, 2009; Soutschek et al., 2004), and our success in knocking down CTR2 suggests it is a good target for this approach. Furthermore, CTR2 is quickly down-regulated upon Cu starvation (Blair et al., 2010). Many Cu chelating

drugs such as d-penicillamine, tetrathiomolybdate, clioquinol, and trientine either have been or are currently being investigated in clinical trials for their ability to slow tumor growth (Gupte and Mumper, 2008; Pan et al., 2002; Yoshii et al., 2001; Yu et al., 2006). If these drugs effectively reduce Cu levels in tumor cells, we would predict a concurrent down-regulation of CTR2 and enhanced cDDP uptake and tumor cell kill that would be expected to result in clinical synergy between these agents and the Pt-containing drugs.

Conflict of interest statement

None of the authors has a conflict of interest with respect to the work presented in this paper.

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Authorship Contributions

Participated in research design: Blair, Safaei, Howell

Conducted experiments: Blair, Larson, Adams, Abada, Pesce

Performed data analysis: Blair, Larson, Howell

Wrote or contributed to writing of manuscript: Blair, Howell

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Footnotes

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Figure Legends

Figure 1. Expression of CTR2 and growth rate of CTR1^{-/-} and CTR2kd tumors. A) Western blot analysis of CTR2 levels in CTR1^{+/+}, CTR1^{-/-}, CTR^{+/+} CTR2kd and CTR^{-/-} CTR2kd cells. B) Immunohistochemical staining of CTR1^{-/-} and CTR^{-/-} CTR2kd tumors for expression of CTR2 (brown). C) Tumor volume as a function of time; (■), CTR1^{-/-} tumors; (□), CTR1^{-/-} CTR2kd tumors. Vertical bars, SEM.

Figure 2. Immunohistochemical characterization of proliferation, apoptosis and vessel density in CTR1^{-/-} and CTR^{-/-} CTR2kd tumors. (A) Ki67 staining for proliferation; numerical quantification of Ki67 positive cells per 5 high power fields. (B) TUNEL staining for apoptotic nuclei; numerical quantification of TUNEL-positive nucleus per 5 high power fields. Immunohistochemical staining for CD31 (red); numerical quantification of vessel density. Vertical bars, ± SEM. * p < 0.02.

Figure 3. Effect of knocking down CTR2 on responsiveness to cDDP *in vivo*. Tumor volume as a function of time with (□) or without (◆) IP injection of 10 mg/kg cDDP. A) CTR1^{-/-} tumors; B) CTR1^{-/-} CTR2kd tumors. Vertical bars, ± SEM.

Figure 4. Effect of CTR2 on whole cell accumulation and efflux of Texas red- labeled dextran. (A) Dextran accumulation CTR1^{+/+}, CTR1^{+/+} CTR2^{kd}, CTR1^{-/-} and CTR1^{-/-} CTR2^{kd} cells. (B) Dextran accumulation in cells following a 1 h exposure to 100 μM BCS. (C) Dextran content as a function of efflux time in (◇), CTR1^{+/+}; (□), CTR1^{+/+} CTR2^{kd}; (▲), CTR1^{-/-}; and (■), CTR1^{-/-} CTR2^{kd} cells. Vertical bars, ± SEM. * p < 0.04

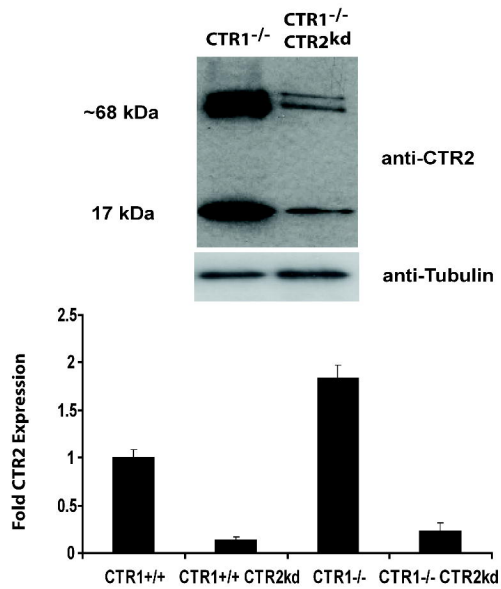
Figure 5. Effect of amiloride, wortmannin and PDGF on the CTR2 regulation of cDDP

accumulation. (A) Accumulation of Pt following a 30 min exposure to cDDP without (filled bar) or with (open bar) inhibition of macropinocytosis by amiloride. (B) Accumulation of Pt following a 1 h exposure to cDDP without (filled bar) or with (open bar) inhibition of macropinocytosis by wortmannin. (C) 30 min dextran accumulation with and without PDGF pretreatment. (D) Effect of PDGF pre-treatment on uptake of cDDP following an exposure to 30 μ M cDDP for 1 h. Vertical bars, SEM. * $p < 0.001$

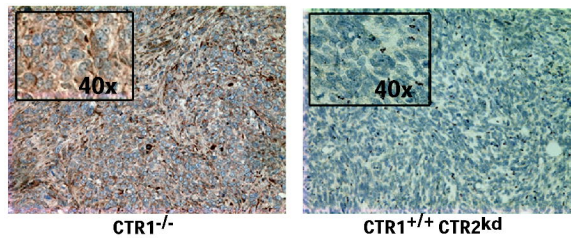
Figure 6. CTR2 activates the GTPases that control macropinocytosis. Panel A, relative levels of total and phosphorylated Rac1 and cdc42 in CTR1^{+/+}, CTR1^{+/+} CTR2^{kd}, CTR1^{-/-} and CTR1^{-/-} CTR2^{kd} cells. Panel B, relative levels of phosphorylated Akt.

Figure 1

A



B



C

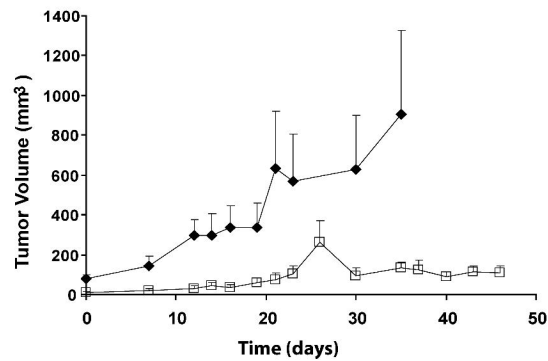
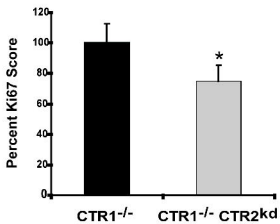
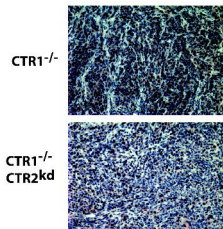
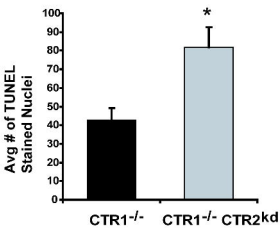
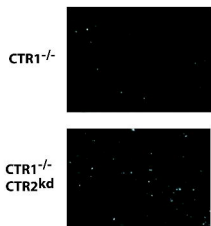


Figure 2

A



B



C

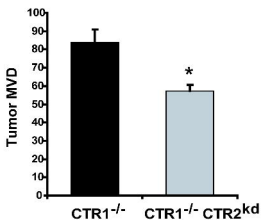
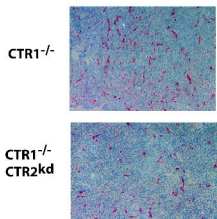
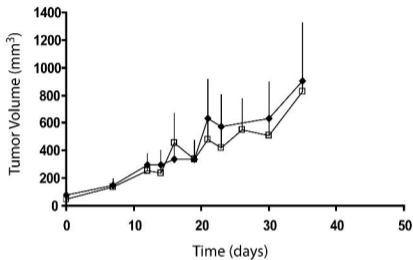


Figure 3

A



B

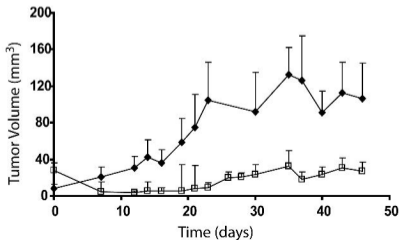
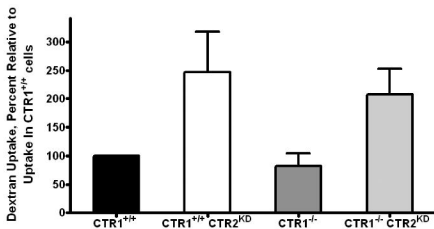
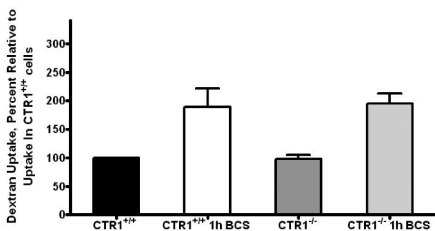


Figure 4

A



B



C

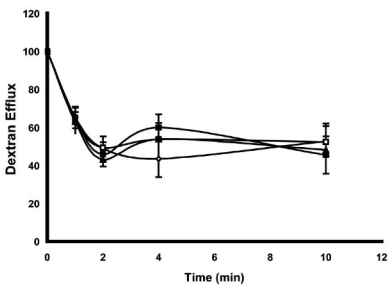


Figure 5

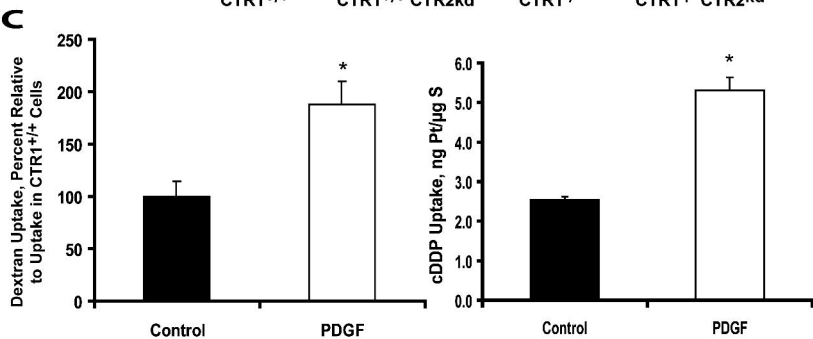
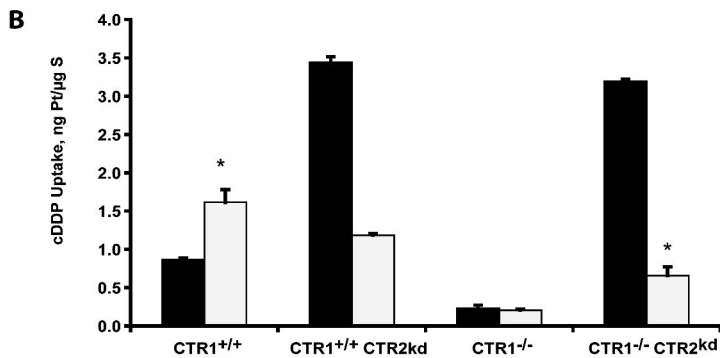
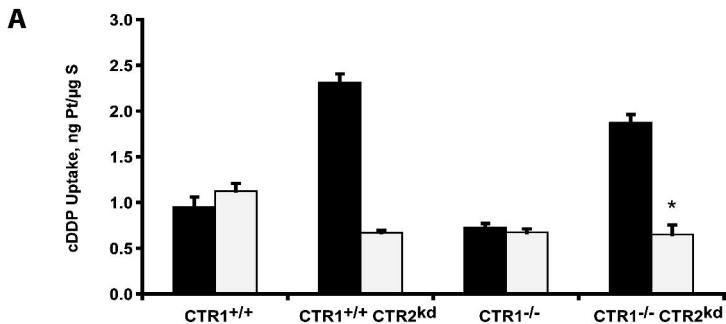
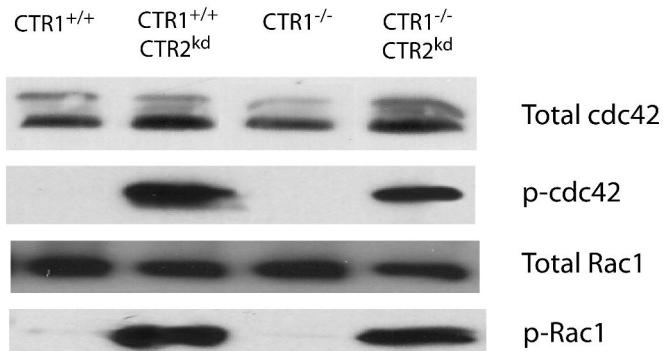


Figure 6

A



B

