The Role of Human Copper Transporter 1, hCTR1, in Copper and Cisplatin Entry in Mammalian Cells

BY

KRISTIN DIONNE IVY
B.S., Grambling State University, 2007

THESIS

Submitted as partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Biochemistry and Molecular Genetics
in the Graduate College of the
University of Illinois at Chicago, 2013

Chicago, Illinois

Defense Committee:

Jack H Kaplan, Chair and Advisor
Karen Colley
Yee Kin Ho
Sojin Shikano
Brad Merrill
William Walden, Microbiology and Immunology

ACKNOWLEDGEMENTS

I want to thank my mentor, Jack H. Kaplan, for all of his wonderful advise, guidance, wisdom, concern and patience. He has taught me how to scientifically approach a question and has helped me to accomplish my research goals. I thoroughly am appreciative for a very enjoyable environment to learn, explore and grow into a developing scientist.

I would like to express appreciation for my dissertation committee: Drs. Yee Kin Ho, Karen Colley, Brad Merrill and Sojin Shikano from the Department of Biochemistry and Molecular Genetics, and Dr. William Walden from the Microbiology and Immunology Department. Your words of wisdom and guidance are truly appreciated.

I also would like to thank the entire Kaplan lab: Dr. Edward Maryon, Dr. Adriana Zinmicka, Rebecca Clifford, Dr. Shannon Molloy, Dr. Huijun Yu, John Jellison, Leah Welsh, and undergraduates; Vinay Soni, Natasha Malles, Stephanie Humphries, and visiting students from University of Bath: Dave Bailie, Savroop Bhamra, as well as all the colleagues and fellow graduate students in the Department of Biochemistry and Molecular Genetics... I have enjoyed the great advice, friendly talks, and your kind support.

My family and friends have shown tremendous support and love over the past years that are most appreciated and valued. I am truly thankful for you.

I can do all things through Christ who strengthens me. Philippians 4:13

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LIST OF ABBREVIATIONS

AA	Ascorbate
Ag	Silver
AgNO3	Silver nitrate
Antisense	DMT1 scrambled DNA
A2780	Ovarian cisplatin-sensitive cells
A2780CP	Ovarian cisplatin-resistant cells
ATP7A	Menkes disease protein
ATP7B	Wilson disease protein
BCS	Bathocuproine disulfate
CBDC	Carboplatin
CCS	Copper chaperone for SOD1
Cis-Pt	Cisplatin
Cl	Chloride
Cox 17	Copper chaperone for cytochrome c oxidase
C-term	C-terminal
CTR1	Copper transporter 1
Cu	Copper
cDDP	Cisplatin
DIDS	4,4 – diisothiocynato -2,2- stilbenedisulfonic acid
DMT1	Divalent metal transporter 1
Dcytb	Duodenal cytochrome b
EGS	Ethylene glycolbis (succinimidylsuccinate)
ER	Endoplasmic reticulum

LIST OF ABBREVIATIONS (continued)

Fe	Iron
Flag	Epitope tag
G	Golgi
GFP	Green fluorescent protein
GI	Gastrointestinal tract
hCTR1	Human copper transporter 1
hCTR2	Human copper transporter 2
Hr	Hour
HEK	Human embryonic kidney cells
ICP-OES	Inductively coupled plasma optical emission spectroscopy
ICP-MS	Inductively coupled plasma mass spectrometry
K	Potassium
Kd	Knock-down
Ko	Knock-out
MDCK	Madin-Darby canine kidney
MEF	Mouse embryonic fibroblasts
MEM	Media
Min	Minute
Mn	Manganese
MT	Metallothionein
N-term	N- terminal
Neg cont	Negative control
Na	Sodium
Oxali	Oxaliplatin

LIST OF ABBREVIATIONS (continued)

Pmole	Picomole
Ppb	Parts per billion
PM	Plasma membrane
Pt	Platinum
Sense	DMT1 overexpressed cells
SLC26A	Solute carrier family 26A
SOD1	Superoxide dismutase 1
TB	Transport buffer
Tet	Tetracycline
TGN	Trans-golgi network
TM	Transmembrane domain
Trans	Transplatin
Tr179	C-terminal CTR1 mutant
WT	Wild-type
Y	Yeast
Zn	Zinc

SUMMARY

Human copper transporter 1 is a transmembrane protein responsible for high affinity copper uptake in human cells. Due to its redox activity, copper acts as cofactor for several proteins involved in various biological processes such as connective tissue formation, iron homeostasis and respiration to name a few. Copper is an essential trace metal and it's been shown that if copper acquisition is prevented, embryonic lethality results. Although copper is absolutely critical for proper cell function, an excess of copper is toxic to the cell. This delicate balance requires a highly regulated system of copper uptake, export and trafficking proteins responsible for managing the amount of copper in the cell. Cisplatin (cDDP) is an anti-cancer drug used to treat a number of malignancies including testicular, ovarian, lung, head, bladder, neck, and cervical cancers. Its use is limited by the development of resistance. Recent studies have rationalized the development of resistance via effects on cellular uptake. It has been claimed that hCTR1 is the major entry pathway for cDDP and related drugs and that the entry mechanism mimics that of copper. Clinical trials have been initiated that depend upon regulating membrane levels of hCTR1. As hCTR1 is a very selective Cu (I) transporter, a major role in cDDP uptake is surprising. In chapter II, we have compared in detail the uptake rates of copper with cDDP (and several analogs) into HEK293 cells over-expressing wild-type or mutant hCTR1, mouse embryonic fibroblasts (mefs) that do or do not express CTR1, and in chapter III we examined human ovarian carcinoma cells and cervical carcinoma cells that are sensitive or resistant to cDDP. We have also examined the effects of extracellular copper, which causes regulatory endocytosis of hCTR1, to that of cDDP. Our results show that the response of the transporter to the presence of extracellular drug is different from its response to copper, and that hCTR1 is not the major entry route of platinum drugs into human cells. Our data suggest that the entry pathway for platinum drugs is not saturable and may not be protein-mediated. If reduced drug uptake is a major factor in resistance, hCTR1 is unlikely to be a productive target.

SUMMARY (continued)

In chapter IV, we investigated hCTR1-independent copper uptake systems. First, we identified an anion uptake system in intestinal and kidney cells that is highly chloride dependent. This anion uptake system is located on the apical membrane in polarized cells thus having a position that allows for copper uptake into enterocytes. Secondly, we studied HEK 293 cells overexpressing divalent metal transporter 1. While ICP-OES analysis demonstrated that DMT1 transports Fe and Mn, Cu (I) and Cu (II) were not transported. Lastly, we also examined whether hCTR2, a protein with shared sequence similarity to hCTR1, could also transport copper. Fractionation and biotinylation assays of HEK293 overexpressing hCTR2 detected hCTR2 at the plasma membrane. However, when the rate of copper uptake was measured by radioactive copper uptake, hCTR2 transported copper at a much slower rate than hCTR1. This work is significant for the improved understanding of copper homeostasis particularly in knowing what proteins or uptake systems play critical roles in delivering dietary copper into cells.

I. INTRODUCTION TO COPPER HOMEOSTASIS

A. Copper an essential trace element

The importance of dietary copper was first documented in the 1930s by Australian veterinarians. Sheep born from mothers who grazed in copper-deficient pastures suffered from demyelination and porencephaly of the brain. One very noticeable trait seen with these sheep was the appearance of “kinky”, brittle wool. In 1962, a physician observed patients that also appeared to have “kinky” hair. He measured these patients’ copper and ceruloplasmin levels and both were below the normal range. The connection was soon made that the human patients suffered from Menkes disease, and that copper was an important metal essential for their development (Kaler 1998).

Copper is a trace metal found in various foods individuals eat on a daily basis. Rich sources are found in shellfish, organ meats, and seeds. Lesser amounts of copper are found in wheat bran, potatoes, grains, and legumes to name a few (Linder and Hazegh-Azam 1996) (Turnlund 1998). Approximately 1 mg of copper is taken up daily. Copper excretion via gastrointestinal tract and bile is 4.5mg/day. However, majority of copper excreted in the bile is reabsorbed by enterocytes (Linder and Hazegh-Azam 1996). After food is broken down in the stomach by digestive enzymes, it travels to the small intestine where nutrients are absorbed. Unlike iron, the majority of copper digested in the stomach is absorbed by the intestine (55-75%). Only about 10% of iron is absorbed (Linder and Hazegh-Azam 1996, Turnlund 1998). Following export from intestinal cells into the serum, copper binds to albumin and transcuprein allowing safe passage to the liver. The liver is the main site of copper maintenance. Under steady state conditions, copper is incorporated into ceruloplamsin (six copper ions for each ceruloplasmin). Ceruloplasmin plays an important role in iron metabolism and requires the redox activity of copper to function as a ferroxidase. Thus, copper bound to ceruloplasmin is not exchangeable with other copper binding proteins such as transcuprein or albumin.

Ceruloplasmin contains approximately 70- 80% of copper found in serum while albumin binds to the majority of the remaining copper. Free copper has the ability to bind to oxygen producing reactive oxygen species. Also copper can bind to many proteins non-specially through reactions with cysteines. Ceruloplasmin thus plays a crucial role in prevention of non-specific binding and passage of copper to peripheral tissues (Madsen and Gitlin 2007).

B. Copper transporters

Like iron and zinc, copper is only required in small amounts; however, all three metals are absolutely essential to sustain life. Every cell in the body utilizes copper for normal growth and development. Thus proteins which transport copper into the cell, traffic copper within the cell, and export copper out of the cell are required. The cell has a system of transporters which carry out these functions. Before copper is selectively transported into the cell, it must be in its reduced form. It is generally accepted that Cu (I) is the preferred state transported in cells by hCTR1 (Maryon, Molloy, and Kaplan 2007). The protein or proteins responsible for copper reduction have not yet been identified. It has been suggested that duodenal cytochrome b (Dcytb) or the steap family of metalloreductases possibly function to reduce Cu (II) to Cu (I). Dcytb is a transmembrane protein that has been shown to function in reducing both Fe and Cu (Wyman et al. 2008). hCTR1 is a high affinity uptake protein found in all cells. It is predominantly in the plasma membrane and functions to bind copper via the N-terminus which guides copper into its permeation pore. Copper then transverses the pore and enters the cell via the C-terminus of hCTR1 (Eisses and Kaplan 2002). Once copper is transported across the plasma membrane, it is immediately bound. Free copper has the potential to bind to many proteins nonspecifically and can catalyze the Fenton reaction producing superoxide anions. Together with hydrogen peroxide, they can act as a substrate for the production of damaging hydroxyl radicals (Valko, Morris, and Cronin 2005). Therefore after delivery into the cell, approximately 80% of copper is bound in the cytosol possibly to metallothionenins or glutathione

(Linder and Hazegh-Azam 1996). Glutathione is extremely abundant in the cell (1-10 mM) and is a candidate for binding copper. Prevention of non-specific binding and reactive oxygen species production is of high importance and is essential for the safety of the cell (Chen et al. 2008). Metallothionein is another important binding protein. It is a low-molecular weight protein which coordinates a variety of metals including copper, zinc, cadmium, and mercury via cysteine residues (Kelly et al. 1996).

Copper chaperones deliver copper to intracellular proteins where the metal is used as a cofactor. For example, Cox17 recruits copper to the mitochondria for the assembly of cyctochrome c oxidase which is the last enzyme of the respiratory chain (Culotta, Yang, and O'Halloran 2006). CCS is a second chaperone which traffics copper to superoxide dismutase (SOD) and functions in cellular antioxidant activity (Hamza et al. 2001). A third chaperone is Atox1 which binds copper via its metal binding domain and traffics copper to the trans-golgi network where ATP7A and ATP7B reside (Klomp et al. 1997). ATP7A and ATP7B are P-type ATPases which utilize ATP to pump copper across membranes. They have two functions: 1. incorporate copper with cuproproteins such as ceruloplasmin in iron metabolism; 2. export copper out the cell. Although they have shared sequence similarity, ATP7A and ATP7B have different properties. Both reside at the trans-golgi network and recycle back and forth to the plasma membrane in vesicles in response to copper. However, ATP7A is a ubiquitous protein whereas ATP7B is mostly found in liver cells. In liver cells, ATP7A relocates to the basolateral membrane whereas ATP7B relocates to the apical membrane (Linz and Lutsenko 2007).

Another protein thought to play a role in the delivery of copper across membranes is hCTR2. It shares homology to hCTR1 in the transmembrane regions; however, its function is less well understood. Sequence alignment suggests hCTR2 consists of 3 transmembrane domains like hCTR1 (Figure 26, p. 74). The MXXXM motif found in the second transmembrane domain of hCTR1 is also present in hCTR2. This domain is required for copper coordination. Amino acids shown to allow for oligomerization in hCTR1 are also present in hCTR2. However,

hCTR2 has several key differences. Histidine and methionine motifs located in the N-terminus essential for high affinity copper uptake by hCTR1 are lacking in hCTR2. Also the N15 required for N-linked glycosylation is not present. These observations suggest hCTR2 as a pseudo copper transporter. However the motifs essential for high affinity copper uptake are missing; therefore, it has been suggested that low affinity uptake is more likely (van den Berghe et al. 2007, Zhou and Gitschier 1997).

C. Diseases linked to copper misbalance

Copper levels within the body are regulated by well-conserved copper transporters. As mentioned, hCTR1 transports copper into cells, copper chaperones traffic copper to intracellular targets, and ATP7A and ATP7B export copper. Copper homeostasis is dependent upon all the players functioning properly. However, specific human genetic diseases have been characterized and are the direct result of copper misbalance. Several enzymes are affected by the inactivity of the ATPases. Some of these include tyrosinase, lysyl oxidase, and ceruloplasmin (Linz and Lutsenko 2007). Menkes disease is caused by defects in the X-chromosomal gene that encodes ATP7A. ATP7A is a ubiquitous protein, and it plays a major role in the export of copper from gastrointestinal cells into the portal blood stream during digestion. In Menkes disease patients, ATP7A is mutated resulting in intestinal copper accumulation and failure of copper to be transported into the blood for further distribution throughout the body. This results in high copper levels in the intestine and systemic copper deficiency in other organs. The brain is one of several organs profoundly affected. Patients with Menkes disease are born with this illness and suffer from axonal swelling and demyelination within the brain. As with all infants, the early years of life are very critical since babies develop very quickly after birth. However, copper deficiency particularly within the brain as the result of Menkes disease does not allow these infants to thrive, and they usually die by the age of three (Madsen and Gitlin 2007).

Whereas copper deficiency is seen with Menkes disease patients, copper overload is observed in Wilson's disease patients. Wilson's disease is caused by a loss of function mutation in the gene encoding for ATP7B. ATP7B is highly expressed in liver cells. ATP7B plays two important roles in copper homeostasis: 1. ATP7B incorporates copper into ceruloplasmin in the liver. Ceruloplasmin functions as ferroxidase in iron metabolism; 2. ATP7B exports excess copper into the bile. However in Wilson's disease patients, ATP7B is mutated and consequently, apoceruloplasmin (non-functioning ceruloplasmin) is generated. Also, mutated ATP7B loses its ability to export excess copper to the bile. Consequently, copper accumulates within liver cells resulting in necrosis of the liver. Cellular injury leads to a spilling out of copper into the blood and subsequently copper toxicity. This spilling out can be physically seen in patients with Kayser- Fleisher rings where copper accumulates in the cornea of the eye. Unlike Menkes disease, Wilson's disease is not fatal. Copper chelators have been shown to be clinically effective in lowering copper levels within the body (Madsen and Gitlin 2007).

Other complications are also evident when levels of copper within the body are misbalanced. Copper concentrations closely affect iron metabolism. Ceruloplasmin requires six copper ions for each Cp. Iron is trafficked within the cell and exported out of the cell in the ferrous state. Before iron can be bound to its serum protein, transferrin, it must first be oxidized. Ceruloplasmin oxidases Fe^{2+} to Fe^{3+} after iron is exported from cells. This redox activity is dependent on the presence of copper ions. Thus copper deficiency can also lead to iron accumulation in cells resulting in anemia, dementia, diabetes, dysarthria, and dystonia (Madsen and Gitlin 2007, Arredondo et al. 2004).

D. <u>Copper as a cofactor</u>

Copper is a trace metal that belongs to a sub-family of transition elements. Other metals included in this family are Fe, Cr, Mn, Co, Ni and Zn. Cu has two oxidation states: cuprous (Cu^{1+}) and cupric (Cu^{2+}). The ability of copper to convert between these two oxidation states

allows copper to catalyze redox reactions. Copper is mostly found in the Cu^{2+} state since it is readily oxidized while in the Cu^{1+} state. Copper is required to function as a cofactor with over 30 proteins and enzymes (Arredondo and Nunez 2005).

The following proteins depend upon the redox activity of copper. 1.) Superoxide dismutase is a ubiquitous, cytosolic protein which binds to copper and zinc. A single bound copper ion allows SOD to catalyze the disproportionation of superoxide into hydrogen peroxide and oxygen (Fridovich 1995). 2.) With two copper ions bound, tyrosinase functions in melanin pigment formation. Tyrosinse is involved in several steps in this process. The rate limiting step involving tyrosinase is the hydroxylation of tyrosine to dihydroxyphenylalanine (DOPA) (Lerner 1949). Tyrosinase also catalyzes the oxidation of DOPA to DOPAquinone (Korner and Pawelek 1982). 3.) Ceruloplasmin is a major copper-carrying protein that functions as an essential ferroxidase converting Fe^{2+} to Fe^{3+}. Only after the oxidation of iron can it be exported from cells and bound to its serum protein, transferrin (Hellman and Gitlin 2002). 4.) Lysyl oxidase is a copper-bound, amine oxidase allowing for inter and intra-strand crosslinking of elastin and collagen in the extracellular matrix (Kagan and Li 2003). 5.) Cyctochrome c oxidase is found in the mitochondria and is the last enzyme in an electron transport chain. It contains two copper centers (CuA and CuB). The net product is the reduction of oxygen into two water molecules (Bourens et al. 2012).6.) Dopamine-B-hydroxylase is a copper-containing enzyme that converts dopamine to norepinephrine. Norepinephrine functions as both a hormone and a neurotransmitter thus having very important roles in central nervous system and also the sympathetic nervous system (Rush and Geffen 1980). Copper is thus critical in many essential biochemical processes.

E. <u>Identification of hCTR1</u>

CTR1 proteins were discovered while examining mutants of *Saccharomyces cerevisiae* where the transcription of a ferric reductase, FRE1, was repressed (Dancis, Yuan, et al. 1994).

Ferrous uptake was reduced in these mutants but more interestingly, copper uptake was also significantly reduced. Further investigation resulted in the identification and characterization of the CTR1 gene in *Saccharomyces cerevisiae*. Yeast in which the CTR1 gene was repressed resulted in the loss of a high affinity copper uptake system (Km 1-4 μM). This system was specific for copper since other divalent metals (Fe, Mn, Ni, Co, Zn) did not compete for uptake. This gene encodes a multi-spanning plasma membrane protein consisting of 406 amino acids (Dancis, Yuan, et al. 1994). Dancis confirmed that CTR1 is required for high affinity copper uptake in yeast. Dancis demonstrated CTR1 is O-linked glycosylated, is present at the plasma membrane, and forms homodimers. Copper levels regulate the abundance of available CTR1. Yeast strains with deletions of genes (ctr1- and ctr3-) showed reduced high affinity copper uptake and thus showed growth defects due to copper deficiency. CTR3 was also identified consisting of 241 amino acids whereas CTR1 consisted of 406 (Dancis, Haile, et al. 1994). Identification of CTR1 in humans was performed by complementation in yeast. Sequencing of the homologs showed yCTR1 and hCTR1 to be 29% identical. A similar gene to hCTR1 was also identified: hCTR2. Both hCTR1 and hCTR2 were shown to be ubiquitously expressed (Zhou and Gitschier 1997). Isolation and characterization of murine CTR1 showed the protein sequence exhibited 92% identity to CTR1 in humans (Lee et al. 2000).

The CTR family of proteins all consists of three transmembrane domains. hCTR1 and yCTR1 are 29% identical; whereas hCTR1 and mCTR1 are 92% identical. hCTR1 (190 aa) is much smaller than yCTR1 (406 aa). hCTR1 lacks the majority of the C-terminus present in yCTR1. Also the N-terminus of hCTR1 is shorter. Based upon hydrophobicity analysis, hCTR1 was predicted to have 3 transmembrane domains (Zhou and Gitschier 1997). The N-terminus of hCTR1 is located on the extracellular surface of the plasma membrane, while the loop and C-terminus are located within the cytosol (Puig et al. 2002) (See Figure 1, p. 9). The loop is located between the first and second transmembrane domains and is poorly conserved. The N-terminus is N-linked glycosylated at N15 (Eisses and Kaplan 2002, Klomp et al. 2002) as well as

O-linked at Thr27. Maryon et al showed O-linked glycosylation protects the N-terminus from cellular proteolytic cleavage (Maryon, Molloy, and Kaplan 2007). Both yCTR1 and hCTR1 are rich in methionine and serine residues in the N-terminus. However, hCTR1 is also rich in histidine residues in the N-terminus (Zhou and Gitschier 1997). The methionine and histidine residues are potential metal binding sites that allow for high affinity copper uptake. The N-terminus of hCTR1 guides copper in the entry of the pore and methionine rings located within the second and third transmembrane domains coordinate copper allowing it to transverse the pore based upon size and charge (Eisses and Kaplan 2002). Whereas hCTR1 is rich in methionine and histidine residues, hCTR2 has far fewer of these potential metal binding residues. Zhou et al showed yeast deficient of CTR2 were more resistant to copper toxicity than control yeast cells. Therefore hCTR2 was thought to function in low affinity copper uptake whereas hCTR1 functions in high affinity copper uptake (Zhou and Gitschier 1997). The C-terminus of hCTR1 ends in His-Cys-His residues which is a putative metal binding site. Copper binds to the N-terminus of hCTR1 via methionine and histidine motifs which in turn guide copper into the permeation pore. A conformational change occurs within the loop resulting in an opening of the gate at the exit of the pore (Maryon et al. 2007).

F. <u>Structural elements of hCTR1</u>

CTR1 consists of three transmembrane domains; therefore, the N-terminal and C-terminal ends are located on opposite sides of the plasma membrane. Using flag-tagged CTR1 in yeast cells, protein shaving experiments using pronase indicated the C-terminus of CTR1 is located in the cytosol since the flag epitope was protected from degradation. Deletions of methionine residues in the amino terminus resulted in a shift of molecular weight indicating the N-terminus was O-linked glycosylated. Also immunofluorescence stainings detected c-Myc tagged human CTR1 at the cell surface. These results suggest the N-terminus of CTR1 is located in the extracellular space according to the hydropathy analysis, and CTR1 is located at

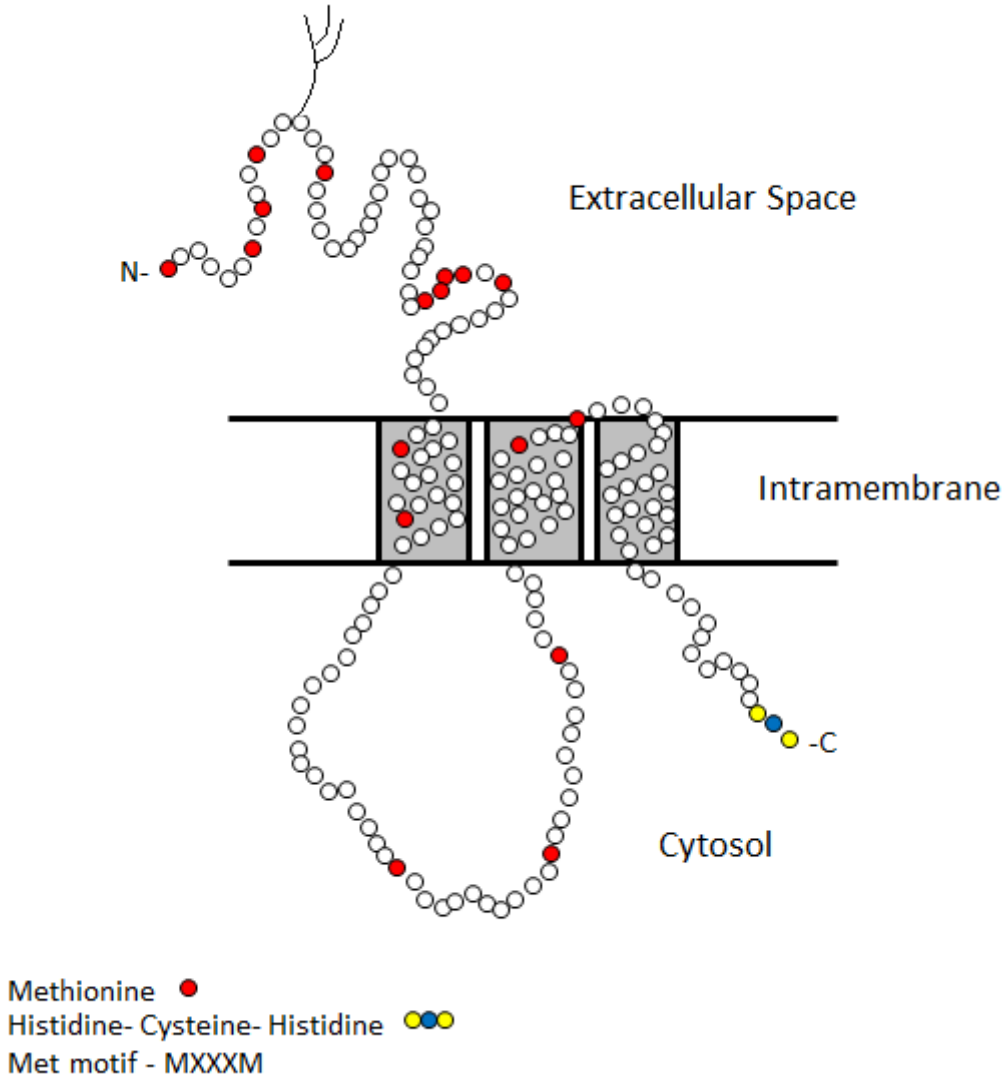

Fig. 1 Model of hCTR1

A topological model of hCTR1 at the plasma membrane is depicted. The N-llnked glycosylation site is shown as well as methionine residues (shown in red). His-Cys-His residues are shown (yellow-blue-yellow).

the plasma membrane. CTR1 alignment amongst several species showed several conserved sequences. Transmembrane domains 2 and 3 are more than 95% conserved in mammals. More specifically, yCTR1 and hCTR1 contain several methionine rich sequences in the first amino terminal domain. Deletion of methionine motifs resulted in the loss of copper transport as suggested by the inability of cells to grow on nonfermentable carbon sources due to defects in copper-requiring enzyme, cytochrome c oxidase and Fet 3 activity. Methionine 127 is the last methionine before the first transmembrane domain begins in yeast, and is located 20 amino acids from the first TMD. This location of a methionine is conserved in all CTR1 proteins. Conversion of Met127 in yeast to cysteine resulted in copper uptake similar to WT. However conversion to serine showed a reduction in copper transport. Serines, in contrast to methionines or cysteines, are not capable of binding copper or coordinating copper. These results suggest that Met 127 is required for transporting copper in yeast. Another conserved methionine motif (Met 256 and Met 260) lies in the second transmembrane domain but is closer to the extracellular space than to the cytosol and may play a role in coordinating extracellular copper into the pore. Conversion of each methionine to alanine resulted in the inability of yeast cells to grow and differentiate, indicative of a role in copper transport. However, GFP-tagged mutants were detected at the plasma membrane. Although these methionine motifs may play a role in copper transport, it did not appear they functioned in the localization of the transporter. Individual substitutions pinpointed that Met 260 but not Met 256 is essential for copper uptake in yeast cells (Puig et al. 2002).

Eisses et al (Eisses and Kaplan 2002) also addressed the question of orientation of CTR1 however in humans. Two substitution mutants were engineered consisting of potential N-linked glycosylation sites. The first mutant consisted of Asn15, whereas the second mutant consisted of Asn 112. The first model was utilized to determine if the N-terminus is located in the extracellular space and if so, Asn 15 would be glycosylated. On the other hand, the second model was used to investigate if the N-terminus is located in the cytosol. If so, Asn 112 would

be located in the extracellular and thus available for glycosylation. PNGase F treatment of the substitution mutants showed Asn 15 is the glycosylated site, and thus the N-terminus of hCTR1 is located in the extracellular with the C-terminus located in the cytosol. Both mutants had similar rates of uptake and Km values suggesting glycosylation of the uptake protein does not affect copper transport. hCTR1 only contains two cysteine residues within its protein sequence, and other copper transporting proteins have been shown to use sulfur to coordinate binding to copper ions. Substitution of the cysteines did not affect the rate of copper uptake; however, it did alter the ability of hCTR1 to form crosslinks with itself. Cysteines were found to play a role in the ability of hCTR1 to oligomerize into dimers and trimers. Based upon a trypsin treatment, it was shown that the loop of hCTR1 undergoes a conformational change upon binding to copper (Eisses and Kaplan 2002).

Electron crystallography of 2D protein crystals demonstrated hCTR1 oligomerizes into symmetrical trimers (Aller and Unger 2006). A 3D electron crystallography study showed hCTR1 forms a conical pore across the plasma membrane. At the extracellular end, the pore of hCTR1 is narrow, measuring ~8 angstroms in diameter; while the intracellular end is much larger, measuring ~22 angstroms across. The second transmembrane domain of hCTR1 is of key importance for the lining of the pore. It coordinates its methionine motifs (MXXXM) with copper ions transversing the pore. hCTR1 can stably bind 2 copper ions that are in their reduced oxidation state via copper-sulfur interactions (De Feo et al. 2009).

G. <u>Mechanics of copper uptake mediated by hCTR1</u>

Eisses et al (2002) investigated the mechanisms of copper uptake of this newly discovered transporter. Sf9 insect cells were infected with hCTR1 using the baculovirus-mediated system which is a very effective method to overexpress proteins. Also, very low levels of endogenous copper transporters have been reported in Sf9 cells. A subcellular fractionation showed hCTR1 localized in the endoplasmic reticulum, golgi, and the plasma membrane

(Eisses and Kaplan 2002). These results agree with the fluorescent staining of hCTR1 at the cell surface (Lee et al. 2002). hCTR1 ran on a SDS reducing gel as a smear around 25 kDa in the Sf9 cells. Another strong band was also seen at approximately 45 kDa, possibly a multimer of hCTR1. The kinetics of this protein as a copper uptake protein were next investigated. Uptake of copper in hCTR1-transfected cells was much higher than cells not transfected. Transfected cells showed high affinity, (Km of 3.2 µM) saturable copper uptake. Untransfected cells had very low rates of copper uptake (Eisses and Kaplan 2002).

Transfection of hCTR1 into yeast and human cells suggested hCTR1 indeed transported copper, and uptake seen was a high affinity, saturable system. Knockout of CTR1 in mice is embryonic lethal (Lee, Prohaska, and Thiele 2001). Therefore, Lee et al "isolated CTR1 homozygous and heterozygous knockout cells from embryos between E7 and E8 embryonic days" and characterized copper transport and copper-dependent proteins. Copper acts as a cofactor for several proteins. SOD, COX and tyrosinase were investigated in this study and all three proteins were reduced when measuring their protein levels. The rate of copper uptake in the heterozygous knockout cells was lower than control cells. However one key finding was that homozygous knockout cells of CTR1 still maintained 30% of uptake when compared to control cells. This suggested the presence of a non-CTR1 mediated copper uptake system. Further analysis showed the rate of copper uptake in CTR1-deficient cells was different than cells expressing CTR1. This non-CTR1 mediated uptake system had a Km for copper of ~10 µM whereas the Km measured for CTR1-mediatd uptake was ~1 µM. However, this system was also concentration-dependent and saturable. Studies in human cells overexpressing CTR1 showed copper uptake was specific and only inhibited by the presence of Ag (I) ions but not other metals: Zn, Fe, or Mn. Ag and Cu are isoelectric and approximately the same size. Mice deficient of CTR1 were incubated with a 50 molar excess of Cu, Zn Ag, Fe, Mn or Cd. Only Cu and Zn significantly reduced copper uptake through this non-CTR1 mediated system. Further investigation showed this system was stimulated in lower pH (5.5 and 6.5) in contrast to a

higher pH (7.5). It has been shown in HEK cells that components in media such as amino acids can chelate copper and limit available copper for uptake. Incubation of CTR1-deficient mouse cells with histidine and BSA hindered uptake. Cells deficient of CTR1 also seem to prefer oxidized Cu (II) ions in contrast to the CTR1-mediated pathway that is thought to prefer Cu (I) ions. These results show for the first time the presence of a non-CTR1 mediated pathway that transports copper in a time and concentration dependent manner that is saturable (Lee, Petris, and Thiele 2002). Chapter 4 is dedicated to examining possible transporters that may be responsible for the uptake seen in a system lacking CTR1.

Studies suggested hCTR1 and yCTR1 complemented each other. However further investigations were necessary to demonstrate hCTR1 indeed functioned as a copper transporter. Lee et al (2002) transiently transfected HEK 293 cells with hCTR1 and studied mechanistic properties of copper uptake. Overexpression of hCTR1 resulted in elevated copper transport when compared to vector cells. Components of DMEM such as amino acids and albumin were thought to possibly bind copper and inhibit uptake. When measuring copper uptake, transport was concentration-dependent and saturable. Km measured in the HEK cells was 2.56 ±1.04 μM with a Vmax of 1.45 ±0.17 pmol of Cu/min/mg of protein while HEK cells overexpressing hCTR1 had a copper uptake Km of 1.71 ±0.39 μM with a Vmax of 6.76 ±0.39 pmol of Cu/min/mg of protein. These properties indicated hCTR1 operated as a high affinity copper transporter. The specificity of this transporter was next investigated. Cells were incubated with a variety of metals (iron, zinc, manganese, silver, and cadmium) and tested to see if any of these metals could compete or inhibit uptake of copper. Besides excess copper, Ag (I) was the only metal to inhibit copper uptake. Since Ag was in the monovalent state, it was then suggested that the oxidation state of copper that is transported by hCTR1 is also in the monovalent state, Cu (I). Addition of ascorbic acid, a reducing agent that would convert Cu (II) to Cu (I), resulted in higher copper uptake. When determining the localization of hCTR1, HEK293 cells transfected with a c-Myc epitope-tagged hCTR1 were examined using

immunofluorescence. Strong staining was seen at the plasma membrane. This location agrees well with the function of hCTR1 as a copper uptake transporter. Since hCTR1 is a small protein (190 amino acids), it was likely that hCTR1 might form oligomers. Incubation of hCTR1-myc tagged cells with a crosslinker, EGS, resulted in multiple bands migrating on SDS gel. hCTR1-myc tagged proteins were measured as migrating at 29, 58, and 90 kDa in the possible forms of monomeric, homodimers and homotrimers (Lee et al. 2002).

H. CTR1 essential role in development

The importance of CTR1 in development was established upon the generation of homozygous and heterozygous knockout of CTR1 in mice. Homozygous CTR1 knockout in mice resulted in embryonic lethality. No living embryos were detected pass embryonic day 9.5. Heterozygous mice suffered from copper deficiency in the brain. Copper levels in the brain of heterozygous mice were reduced by 50% compared to WT littermates. Other tissues examined (intestine, kidney, liver) showed no significant reduction in copper content in the CTR1 knockout mice (Kuo et al. 2001). Lee et al (2001) also studied CTR1 in knockout mice. Homozygous knockout of CTR1 resulted in mice with growth and development defects and eventually died in utero in mid gestation. Heterozygous mice showed tissue specific defects. Cuproenzymes, such as SOD and COX, showed reduced activity. Four tissues were analyzed: liver, kidney, spleen and brain. Copper accumulation was reduced in the spleen but more significantly in the brain. This observation showed CTR1 played an integral role in the delivery of copper to the brain. Since copper is a key component of iron metabolism and homeostasis due to its ability to bind to ceruloplasmin allowing it to act as a ferroxidase, iron content was also measured. Levels of iron in the kidney, spleen and brain were slightly reduced in the CTR1 heterozygous mice when compared to control mice. Iron levels in the liver were significantly lower. The liver is the site of iron homeostasis when ceruloplasmin is generated and also where copper is first incorporated with Cp. Thus CTR1 plays an essential role in copper accumulation for mice development with a

specific importance for the delivery of copper to the brain. Also, iron homeostasis in the liver relies on the presence of CTR1 (Lee, Prohaska, and Thiele 2001).

After food is passed through the esophagus and broken down in the stomach, the first site of absorption of nutrients and ions is in the intestine. In order for the entire body to receive copper, it first must be taken up in the intestine and delivered into the portal circulation system. To study a possible role of mCTR1 in the gut, immunohistochemistry stainings of duodenal cross sections were collected. The presence of mCTR1 in the apical membrane of intestinal cells and in internal compartments was detected using a CTR1 antibody with increased staining in crypt cells. Intestinal epithelial specific mCTR1 knockout-mice were generated. These mice exhibited normal growth and development for the first 6-8 days. However, by day 10, mice began to show poor growth, hypopigmentation, ataxia, kinky hair, and skin laxity. These mice showed similar symptoms of mice suffering from Menkes disease. Shortly thereafter, the mice began to die. Activity of a cuproenzyme, cytochrome c oxidase, was measured in a few organs of the intestinal-knockout mice. Activity in the brain, heart and liver were all significantly reduced when compared to control mice. Copper levels within the brain, heart, liver, kidney, spleen, skeletal muscle, and serum were all reduced. Iron levels in the liver were nearly 4-fold higher in comparison to control mice. The mice showed cardiac hypertrophy. One very important finding was noted when measuring copper uptake in purified intestinal epithelial cells (IEC) from mCTR1 intestinal-knockout mice. Purified IEC accumulated 8 times more copper than control cells. These results indicated mCTR1 is essential for survival and development of the mice and implied to the possibility of an unknown copper uptake protein that functioned to take up dietary copper (Nose, Kim, and Thiele 2006). Due to the lack of high specificity antibodies, no systematic studies have been performed to accurately measure physiologically relevant CTR1 levels. One cannot assume that message levels correlate with protein levels.

I. Copper regulation of hCTR1

hCTR1 is a high-affinity copper transporter, essential for copper uptake in several tissues. Using immunofluorescence, the location of hCTR1 in Chinese hamster ovarian cells and human embryonic kidney cells overexpressing hCTR1 were analyzed (Petris et al. 2003). In basal media, hCTR1 resided mostly at the cell surface. However, in the presence of elevated extracellular copper, punctate vesicular staining within the cell was detected using a hCTR1 antibody. Immunoblots also detected reduced protein expression of hCTR1 in response to elevated copper. Next investigators aimed to clarify what pathway or mechanism was responsible for the internalization of hCTR1. Colocalization of hCTR1 with transferrin, an early endosome marker, suggested endocytosis was responsible for the internalization of hCTR1 (Petris et al. 2003). Previous studies indicated a methionine motif (MXXXM) in the first transmembrane domain was essential for high affinity copper uptake while another methionine motif located in the second transmembrane domain was essential for copper uptake. Investigators questioned whether these methionine motifs as well as other potential copper binding motifs, histidine motifs, could play a role in regulating copper-dependent internalization. Alanine substitutions were generated on two histidine motifs and 2 methionine motifs located in the first transmembrane domain and also a methionine motif (150 MXXXM 154) in the second transmembrane domain. Immunofluorescence stainings detected WT hCTR1 at the cell surface in basal media. In the presence of elevated extracellular copper, hCTR1 protein was internalized into cytoplasmic vesicles. All mutants detected similar findings of hCTR1 localization except mutants substituted for Methionine residues 150 and 154. hCTR1 was detected at the plasma membrane in the absence and presence of elevated copper. These results suggest the methionine motif 150MXXXM154 is required not only for copper uptake but also for regulating copper induced internalization (Guo et al. 2004).

Molloy further investigated the ability of hCTR1 to undergo internalization in HEK 293 cells overexpressing hCTR1. Utilizing biotinylation assays that detect cell surface proteins,

elevated extracellular copper induced hCTR1 internalization. About 30% of cell surface hCTR1 was lost from the membrane, and this same amount of internalized protein could be detected inside the cell. Since internalized hCTR1 could be detected inside the cell and therefore not degraded, further investigation studied the ability of the cell to internalize and recycle hCTR1. Cells incubated in media with excess copper showed a reduction of cell surface hCTR1, an indication of internalization. However the removal of copper resulted in recycling of hCTR1 back to the membrane. These results suggested for the first time copper-dependent internalization and recycling of hCTR1 in human cells. The endocytosis of hCTR1 in the presence of high copper limits the amount of copper entering cells. When extracellular copper levels return to steady state levels, hCTR1 returns to the membrane allowing again for copper entry and maintenance of copper homeostasis. Internalization of hCTR1 was also observed in other cell types including MDCK (Madin-Darby canine kidney), HeLa (human cervical carcinoma) and HepG2 (Human hepatocellular liver carcinoma) (Molloy and Kaplan 2009).

J. Cisplatin, the penicillin of cancer

In 1845, Michel Peyrone first described cisplatin (cDDP) and thus it was known as Peyrone's salt. Barnett Rosenberg, in 1965, recognized cDDP stimulated cell division arrest in E.coli (Rosenberg, Vancamp, and Krigas 1965) and by 1971, clinical trials began examining the ability of cDDP to be a cancer therapeutic agent. In 1978, the U.S. Food and Drug Administration approved the use of the cDDP for testicular and ovarian cancers (Hill and Speer 1982). cDDP is a very successful therapeutic drug used in the treatment of testicular cancer as evident by the 80% cure rate in patients (Kuo et al. 2007). Due to its widespread use and success as a cancer therapeutic agent, it has been termed the "penicillin of cancer". Over the years, it has been found useful for other types of cancers including ovarian, neck, head, lung, cervical, and bladder (Loehrer and Einhorn 1984, Gonzalez et al. 2001). cDDP can be used as a single therapeutic agent or in combination with a cocktail of drugs (Fu et al. 2012). Since its

discovery, other cancer drugs have been produced which are successful in treating cancers. However, cDDP has remained a cornerstone drug and still is widely used throughout the world.

K. Mechanism of action by platinum drugs

cDDP is an alkylating agent and its major target is DNA (Cohen and Lippard 2001). cDDP consists of a central platinum atom surrounded by two ammonia ligands and two chloride ligands in a cis position. During aquation, one of the chloride ligands is replaced by water. The water ligand is easily displaced and cDDP binds to DNA guanine bases within the nucleus. The other chloride ligand follows suit resulting in cDDP- DNA crosslinking. Crosslinking of DNA stops cell division, and the DNA repair system attempts to remove the cDDP-DNA crosslinking. When repair is unsuccessful, cell apoptosis is activated and kills the cell (Hall et al. 2008).

cDDP is very effective in killing cancer cells; however over time resistance develops and the drug is no longer as effective. The cytotoxity of the drug is dependent on how much enters and successfully reaches the nucleus to react with DNA (Andrews and Howell 1990). The development of cDDP resistance is multifactorial. Possible causes for resistance include: 1. reduced cellular drug import; 2. increased cellular drug export; 3. cytoplasmic detoxification of cDDP before interacting with DNA; or 4. successfully removal of cDDP by the DNA repair system (Kelland 2007). In chapters II and III, we investigate if reduced drug uptake by hCTR1 is responsible for cDDP resistance in human embryonic kidney cells, mouse embryonic fibroblasts, human ovarian carcinoma cells and human cervical carcinoma cells.

L. Analogs of cisplatin

Although cDDP is the most widely used cytotoxic drug, cDDP treatment has severe side effects. Due to its inability to distinguish normal cells from tumor cells, this cancer drug is taken up and is very toxic to both cells. Nephrotoxicity (kidney damage), neurotoxicity (nerve damage), ototoxity (hearing loss) and electrolyte disturbance are a few of the side effects

patients undergoing cDDP treatment encounter (McWhinney, Goldberg, and McLeod 2009). In attempts to overcome both the issue of resistance as well as reduce side effects, analogs to cDDP were generated. New drugs were created in hopes that they would be as effective in killing tumor cells but would not develop resistance like cDDP. Over the years, more than 30 analogs have been synthesized with only a few being approved for cancer therapy (Burger et al. 2011). I will discuss 3 cDDP analogs I have examined in my studies. All platinum drugs consist of a central platinum atom while their side chains are different or in different positions. (See Figure 2, p. 21)

1. Carboplatin (CBDCA) was FDA approved in 1989 for the effective treatment of ovarian, lung, head and neck cancers. Although carboplatin has far fewer side effects associated with patient treatment, it is also less effective than cDDP. It takes about 4 times as much carboplatin to be equally effective as cDDP. Carboplatin is often used as a substitute for cDDP in chemotherapy treatment (Burger et al. 2011).

2. Oxaliplatin (L-OHP) was FDA approved in 2002 for the effective treatment of advanced colon cancer. It is used in a combination therapy called FOLFOX: FOL (folinic acid), F(fluorouracil), and OX(oxaliplatin). It contains a large1,2-diaminocyclohexane carrier ligand and an oxalate-leaving group (Burger, 2011). Oxaliplatin is thought to operate differently than cisplatin and carboplatin. It has been suggested that platinum-DNA crosslinks by oxaliplatin are not recognized by the mismatch DNA repair system. Therefore oxaliplatin has been successful in mediating cell death in cisplatin and carboplatin resistant cells (Burger et al. 2011, Vaisman et al. 1998).

3. Transplatin is the trans steroisomer of cDDP. The chloride ligands are positioned on opposite sides (trans) instead of on the same side (cis). Due to this positioning, transplatin is unable to form DNA crosslinks and thus has no anticancer activity (Burger et al. 2011).

M. Summary of work

In chapters II and III, our goal was to investigate the role of hCTR1 in mediating cellular transport of platinum drugs. We examined several platinum drugs (carboplatin, oxaliplatin, transplatin, with emphasis on cisplatin) in human embryonic kidney cells overexpressing hCTR1, mouse embryonic fibroblasts that do or do not express hCTR1, human ovarian carcinoma cells and human cervical carcinoma cells which exhibit sensitivity or resistance to cDDP. We conclude that hCTR1 is not responsible for platinum drug uptake. In chapter IV, we identified an hCTR1-independent copper uptake system that is highly dependent on the presence of chloride ions located on the apical membrane in polarized intestinal cells. Additionally, we examined potential copper transporting proteins: divalent metal transporter 1 and human copper transporter 2. Our findings show DMT1 is a mediator of iron and manganese transport, but not of copper while hCTR2 may function as a low-affinity copper uptake protein.

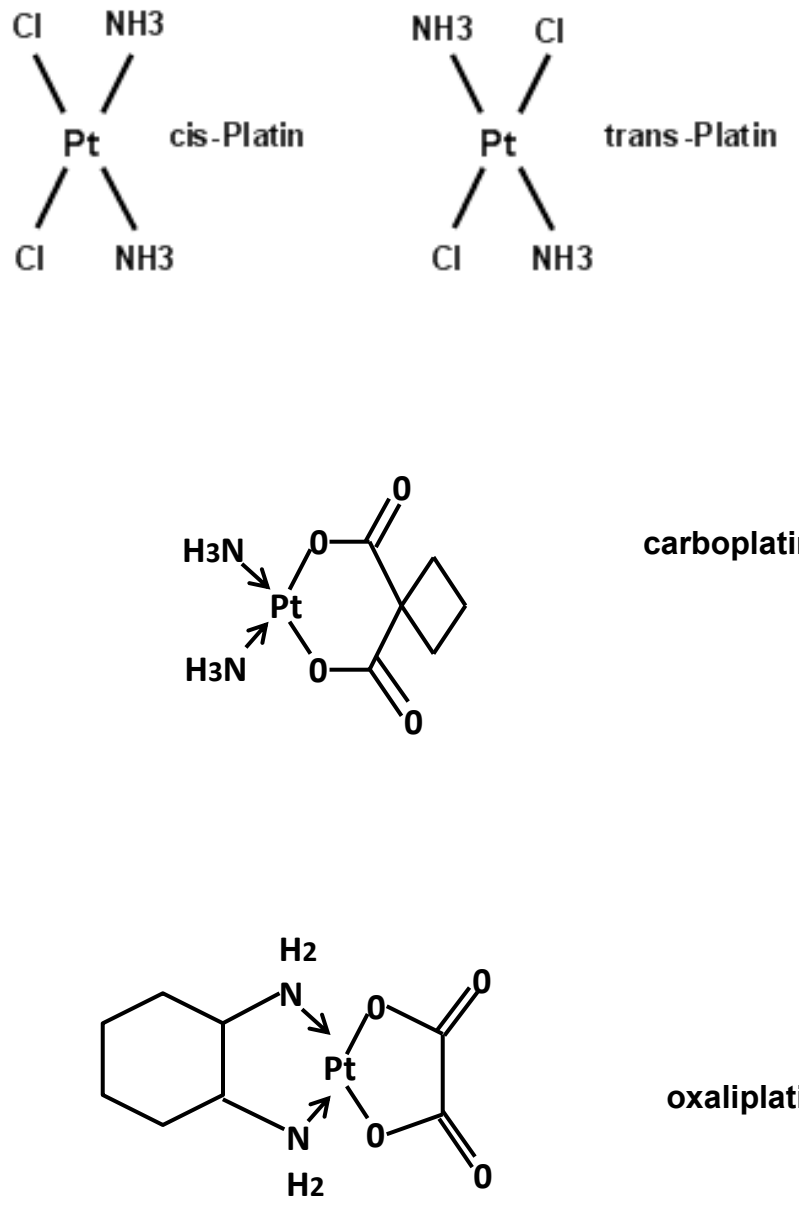

Fig. 2 Platinum drugs

The chemical structures of cisplatin, transplatin, carboplatin and oxaliplatin are shown.

II. Cisplatin Uptake in Model Cells, Human Embryonic Kidney Cells and Mouse Embryonic Fibroblasts

A. Introduction

Platinum drugs, including cisplatin (dichloro-diamino-platinum, or cDDP), have been used since the 1970's as chemotherapeutic agents for a variety of malignancies, including testicular, ovarian, cervical, bladder, lung, head, and neck cancers (Loehrer and Einhorn 1984, Zumaeta et al. 2001) . One major limitation of cDDP usage is the rapid development of resistance (Giaccone 2000). cDDP, along with its later developed analogs, act by forming DNA crosslinks. Platinum crosslinks inhibit DNA replication and halt cell division. The DNA repair system is activated and once repair is unsuccessful, apoptosis is activated (Kelland 2007, Andrews and Howell 1990). There has been considerable interest in understanding the cellular entry mechanism of platinum drugs, as their effectiveness depends on the amount delivered to the tumor cell.

Copper is an essential micronutrient playing a critical role as a co-factor in a number of cellular enzymes, including SOD, cytochrome c oxidase, among others. Initial studies on the entry of cDDP into yeast cells indicated that entry correlated with the expression of CTR1, the copper transporter in the yeast plasma membrane (Ishida et al. 2002, Lin et al. 2002) . The same correlation was reported with hCTR1 in some tumor cells (Song et al. 2004, Zisowsky et al. 2007) Several subsequent studies confirmed this correlation and led to the suggestion that hCTR1, the major high affinity copper-transporter, also mediates platinum-drug uptake (Pabla et al. 2009, Larson et al. 2009, Larson, Adams, Jandial, et al. 2010, Chen et al. 2012, Kalayda, Wagner, and Jaehde 2012)

Elevated copper in the extracellular media causes an internalization of the transporter, with an associated decrease in copper entry, a process that is reversed when copper loads return to normal. This acute regulatory response protects against excessive copper

accumulation (Molloy and Kaplan 2009). It had earlier been reported that copper-induced internalization of hCTR1 results in degradation of the transporter (Petris et al. 2003). Such internalization and degradation has also been reported following cDDP addition to human ovarian carcinoma cells (Holzer and Howell 2006). Copper homeostasis in mammalian cells utilizes hCTR1 for entry and ATP7A and ATP7B (copper-activated ATPases) for mediating its exit (Kaplan and Lutsenko 2009). These ATPases are also responsible for delivering copper to proteins in the secretory pathway. Samimi et al showed in Me32a human fibroblasts that expressed neither ATP7A nor ATP7B were more sensitive to cDDP, copper, and carboplatin than cells transfected with vectors expressing ATP7A or ATP7B (Samimi et al 2004). Thus transporters involved in copper homeostasis were again associated with cDDP efficacy. There has recently been a clinical trial assuming that increases in membrane levels of hCTR1 would lead to enhanced platinum drug entry and increased drug effectiveness (Fu et al. 2012).

Although there is experimental evidence that the proteins involved in copper homeostasis, the uptake and efflux transporters and cellular copper chaperones, may interact with platinum-containing drugs, most of the evidence that identifies hCTR1 as the major cDDP entry pathway relies upon correlative relationships rather than demonstrated causality. It is surprising that a highly selective transporter, such as hCTR1, that has been shown to transport only Ag and Cu (I) ions would also be capable of mediating cDDP transport. cDDP has a diameter of 9.57Å (Hilder and James 2007) while Cu (I) in aqueous solution has a diameter of about 3.6Å (Blumberger et al 2004). cDDP is over two times larger than copper and therefore highly unlikely to fit within the narrow pore of hCTR1, which has an estimated diameter at the entrance of less than 8Å (De Feo, Aller, and Unger 2007).

We have carried out a detailed analysis of the relationship between hCTR1, copper uptake, and cDDP (and several analogs) uptake in HEK cells that over-express hCTR1, in mefs that do or do not express CTR1, and in human ovarian tumor cells that are sensitive or resistant to cDDP. We have compared the responses of hCTR1 in these cells to cDDP with their

responses to copper, and have examined the effects of varied hCTR1 levels on copper and cDDP entry rates and also the consequences of altering hCTR1 expression on the rates of copper and platinum-drug entry. We provide evidence that the major entry pathway into human cells for platinum-containing anti-cancer drugs is not hCTR1, and is most likely not protein-mediated. Our data have considerable significance for future approaches to improve platinum-drug efficacy.

B. <u>Experimental Procedures</u>

Cell Culture. Cells were maintained in a humidified incubator at 37ºC under 5% CO_2 atmosphere and passaged every 3-5 days. HEK293 Flp-In ™ T-Rex ™ (Invitrogen) and Mefs +/+ and -/- cells (a generous gift from Dr Dennis Thiele, Duke University) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Atlanta Biologicals), and 25mM Hepes.

HEK 293 Flp-In cells containing tetracycline-regulated N-terminal FLAG –tagged hCTR1 were created as previous described (Maryon, Molloy, and Kaplan 2007). These cells were maintained with the following selective antibiotics: 100ug/ml zeocin/and 10µg/ml blasticidin. The cells were transfected with hCTR1 construct using Lipofectamine 2000 (Invitrogen). Transfected cells were selected in 12 µg/ml blastocidin S (RPI Corp.) and 400 µg/ml hygromycin (Invitrogen). Resistant colonies were pooled and tested for tetracycline-regulated expression. Cells were cultured in media containing 1 µg/ml tetracycline for 48 hrs before harvesting. Mutants of hCTR1 were created as previously described.

Biotinylation of Surface hCTR1. Biotinylation of cells were carried out using the cell-impermeable, thiol-cleavable Sulfo-NHS-SS-biotin (Pierce) reagent to label cell surface proteins. All biotinylation procedures were carried out at 4ºC. Before biotinylation, cells were grown to

confluence in petriplates. Plates were placed on ice, media was removed and cells were washed once in pre-chilled media and then twice with pre-chilled PBS supplemented with 0.1mM $CaCl_2$ and 1.0mM $MgCl_2$. Freshly prepared EZ-Link Sulfo-NHS-biotin at 0.5mg/ml was diluted in biotinylation buffer (10mM trithanolamine, pH 7.5, 2 mM CaCl2, and 150mM NaCl) and added to the cells. For a 10-cm plate, a volume of 5 ml was added. Cells were incubated on ice at 4ºC for 25 min with a slow rocking motion. The cells were then rinsed with quench buffer (PBS with 0.1mM CaCl2, 1.0 mM MgCl2, and 100mM glycine) for 20 min, and this step was repeated. The quench buffer was removed and the cells were lysed in lysis buffer (1%Triton-X-100, 150 mM NaCl, 5 mM EDTA, and 50 mM Tris-HCl, pH 7.5) and incubated for 1 hr rotating end-over-end. Cells were centrifuged at 10,000 x g for 10 min, and a 5% aliquot was taken for Western blot analysis to determine protein concentration. The remaining supernatant was incubated with streptavidin-agarose beads (Pierce) 100 µL/ml of supernatant in a centrifuge spin-column (Pierce) overnight at 4ºC with end-over-end rotation. Buffer was removed from the column by centrifugation at 500 X g for 3 min at 4ºC. The beads were then washed 3X with lysis buffer, twice with salt wash buffer (0.1% Triton-X-100, 500 mM EDTA, and 50mM Tris, pH 7.5) and once with no salt wash buffer (10mM Tris, pH 7.5). After the final wash, the column was centrifuged at 10,000 x g for 10 min at 4ºC. The protein was eluted by adding 100 uL of 2X-SDS PAGE sample buffer supplemented with 150mM dithiothreitol overnight. To elute the biotinylated protein from the beads, the sample was centrifuged at 3,000 X g for 5 min. Samples were separated by SDS-PAGE, and hCTR1 was detected using either an anti-hCTR1 or anti-FLAG antibody by Western blot analysis as described below.

SDS-PAGE and Western Blot Analysis. Protein samples were separated by adding 2X sample loading buffer (5% SDS, 1.6M urea, and 240mM Tris-HCl, pH 6.8). Samples were resolved using 10% Laemmli gels and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% powered milk and 0.1% Tween 20 (Fisher) and then treated with

antibodies diluted in 1% milk. For the detection of hCTR1 protein, the following primary antibodies were used: rabbit anti-C terminus hCTR1 antibody at 1:4000 dilution or mouse anti-Flag antibody (GenScript) followed by anti-rabbit IgG horseradish peroxidase conjugate (GE Healthcare) or anti-mouse HRP (Thermo Scientific). Western blot signals were obtained using SuperSignal West reagents (Pierce) and the intensity of the Western signals was measured using chemiluminescent imaging with the ChemiDoc XRS (BioRad) and quantitated using QUAN-TITY ONE Version 2.6.2 software (Bio-Rad).

^{64}Cu Uptake. The day before the assay, cells were plated at 0.6 x 10^6cells/ml into each well of 3 X 4 tissue culture plate in DMEM containing 10% fetal bovine serum and incubated at 37 ºC. The following day, fresh media containing ^{64}Cu (Isotrace Technologies Inc.) was added to each well and was allowed to incubate for 1hr at 37 ºC. Copper uptake was stopped by the addition of cold stop buffer (150mM NaCl, 5mM KCl, 25mM Hepes, pH 7.4, 2.5mM $MgCl_2$, and 10mM EDTA). The cells were washed an additional 2 times with ice-cold stop buffer. The cells were dislodged with PBS and an aliquot was collected and added to Eco-Lume scintillation liquid for scintillation counting (Beckman-Coulter LS6500). All transport determinations were carried out in triplicate. The amount of protein per well was determined after radioisotope decay, and the copper uptake/well was calculated as pmol of Cu/mg of protein, and the average of triplicate wells was determined for each treatment.

Pt-drug Uptake. One day prior to the assay, cells were plated at 10.8 x 10^6 cells/ml in 10-cm tissue culture plates in DMEM containing 10% fetal bovine serum and incubated at 37 ºC. The day of the assay, the media was aspirated and cells were incubated in cisplatin, carboplatin, transplatin or oxaliplatin dissolved in DMEM containing 10% fetal bovine serum for 1hr at 37ºC. The uptake was stopped by the addition of stop buffer. The cells were washed an additional 2 times with stop buffer. Cells were collected and resuspended in 70% nitric acid for 3 hrs.

Afterwards, the cells were diluted to 14% nitric acid by the addition of 1ml of water, mixed, centrifuged and the supernatant was submitted for platinum content by inductively coupled plasma mass spectrometry (ICP-MS) at the Chemical Analysis Laboratory in Athens, Ga. The results were expressed as parts per billion per milligram of protein.

Drugs. Cis-Platin (Enzo), transplatin (Sigma-Aldrich), carboplatin (TCI), oxaliplatin (Enzo) solutions were freshly prepared for each experiment. Cisplatin and transplatin were dissolved in 100% dimethyl sulfoxide (DMSO); carboplatin and oxaliplatin were dissolved in water.

Cell Fractionation. Fractionation of cells was carried out by a five-step sucrose gradient (Zimnicka, Maryon, and Kaplan 2007) with a total volume of 5 ml consisting of the following sucrose concentrations layered from the bottom to top: 0.417ml of 2.0M sucrose, 0.833ml of 1.6M sucrose + homogenate, 1.67ml of 1.4M sucrose, 1.67ml of 1.2M sucrose, 0.417ml of 0.8M sucrose. Cells were washed twice with PBS and harvested from 10-cm Petriplates in PBS using a cell scraper. The cells were pelleted @1000 x g for 10mins at 4ºC. The pellet was frozen for at least 2-3 hrs. The pellet was placed on ice and resuspended in 1.2ml of homogenization buffer (0.25M sucrose, 2mM EDTA, 10mM Tris, pH 7.4) containing protease inhibitor mixture (Roche Applied Science). The cells were homogenized in a glass dounce homogenizer on ice (20 strokes) and then passed through a 27.5 gauge needle (20 times). The lyzed cells were transferred to a tube and pelleted at 500 x g for 10 min at 4ºC. The supernatant was collected and adjusted to 2.55M sucrose to a final concentration of 1.4M sucrose and loaded onto the gradient. Tubes were spun in a Beckman SW28 rotor at 25,000 rpm for 2.5 hr at 4ºC. Three fractions enriched in plasma membrane, Golgi, and endoplasmic reticulum were collected from the top to the bottom. Plasma membrane fraction was removed and diluted in HB buffer and spun at 30,000 rpm for 30 min in Beckman SW28 rotor. The pellets were re-suspended in an appropriate volume of HB buffer.

Statistical analysis: Data are shown as means ± SD. Statistical analysis was performed using Student's *t*-test, and $P < 0.05$ was considered statically significant. The data are representative of means of at least three independent experiments.

C. Results

1. Copper and cisplatin uptake by hCTR1 in HEK cells

We have previously developed a line of HEK cells that express hCTR1, the human high affinity copper transporter at the flip-in recombinase site, under the influence of a tetracycline-sensitive promoter (Maryon, Molloy, and Kaplan 2007). When the initial rate of ^{64}Cu uptake into these cells, grown in the absence or presence of tetracycline, is measured, cells grown in the presence of the antibiotic show a higher (about 8-10-fold, see Figure 3, p. 29) rate. However, when the rate of cDDP entry is determined in these two sets of cells, there is no difference in the rate of uptake of the platinum drug (Figure 4, p. 30). These data suggest that elevated rates of copper entry correlate with an increase in the expression level of hCTR1 while the rate of cDDP entry is not altered by over-expression of hCTR1. The increase in hCTR1 expression has been previously documented (Zimnicka, Ivy, and Kaplan 2011).

2. hCTR1 localization in response to cisplatin

It has previously been shown that when cells are exposed to elevated copper levels, one mechanism to protect the cells against copper toxicity is a lowering of the entry rate. This is accomplished by a copper-dependent endocytosis of hCTR1 (Molloy and Kaplan 2009, Petris et al. 2003) and associated decrease in copper entry (Molloy and Kaplan 2009). This phenomenon is illustrated in the data of Figure 5, (p. 32) where cell-surface biotinylation was employed to determine the plasma membrane level of hCTR1. Clearly the amount of hCTR1 in the HEK plasma membranes falls when the cells are incubated with excess copper; and when copper is

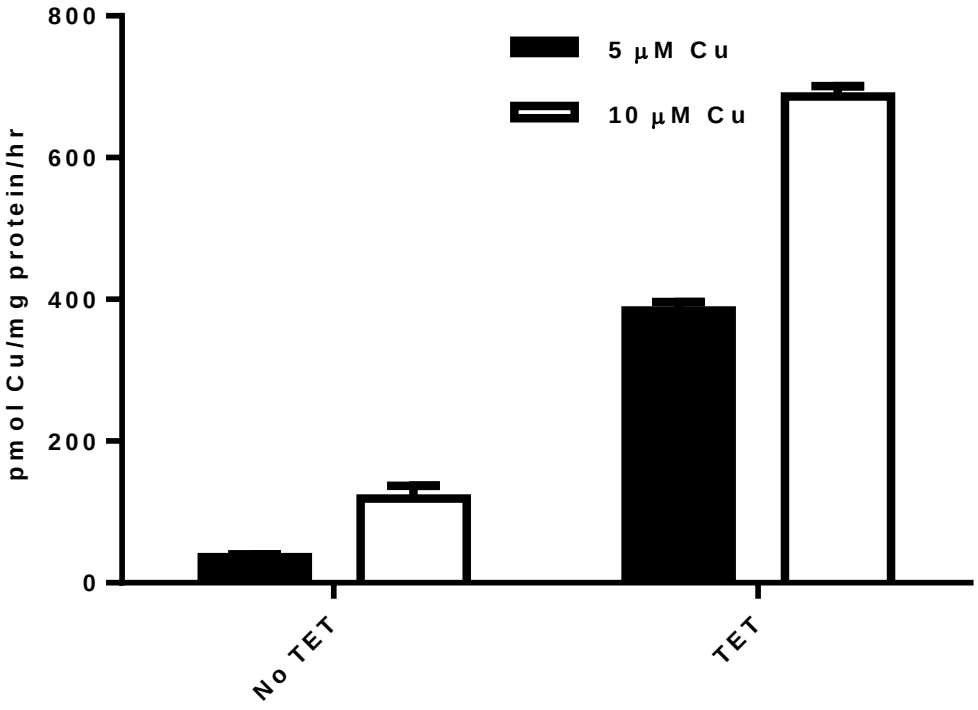

Fig. 3 Copper uptake in HEK 293 cells

HEK 293 cells over-expressing N-terminal flag-tagged hCTR1 were induced with tetracycline 48 hrs prior to experiment. ^{64}Cu transport assays were measured in cells incubated with 5 µM or 10 µM Cu for 30 minutes in media.

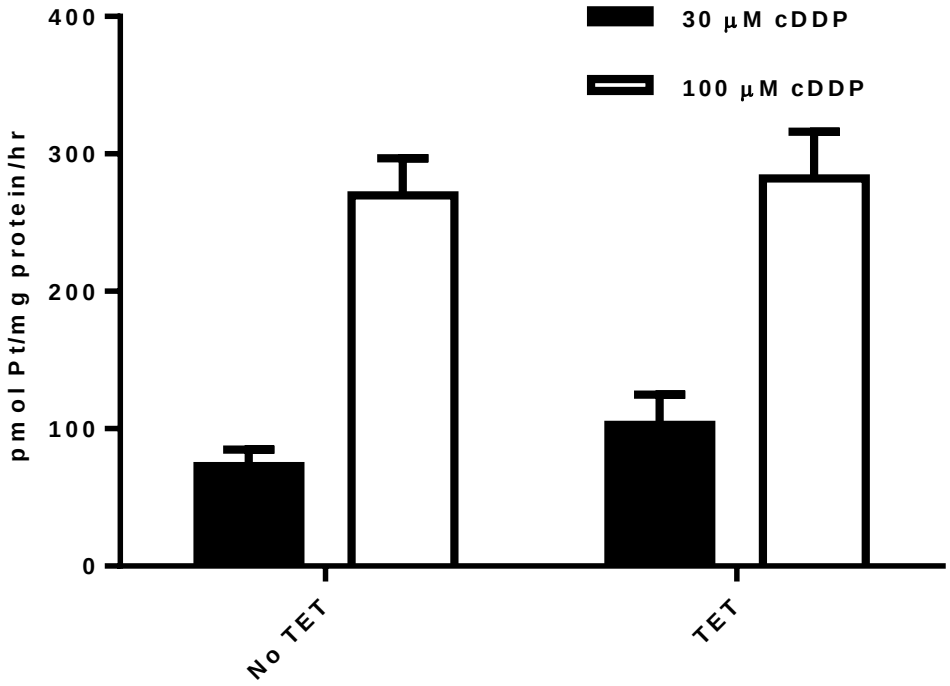

Fig. 4 cDDP accumulation in HEK 293 cells

HEK 293 cells over-expressing N-terminal flag-tagged hCTR1 were induced with tetracycline 48 hrs prior to experiment. cDDP accumulation was measured in cells incubated with 30 µM or 100 µM cDDP for 5 hrs.

removed, the transporter returns to the surface (Figure 5, p. 32). In these same cells, there is no internalization of the transporter in response to high levels of extracellular cDDP (Figure 5, p. 32). This is in contrast to results reported in human ovarian carcinoma cells (Holzer and Howell 2006, Holzer, Katano, et al. 2004) and in agreement with previous studies in HEK293 cells (Guo, Smith, and Petris 2004, Pabla et al. 2009) and renal proximal tubules cells as well as studies performed in mice (Pabla et al. 2009). Previous studies have suggested that only a brief exposure (5-15 minutes) to cDDP causes rapid internalization and degradation of hCTR1 (Larson et al. 2009, Holzer and Howell 2006, Holzer, Katano, et al. 2004). We exposed tetracycline-induced HEK cells to 50 µM cDDP for 1, 5, 10, 30, and 180 minutes. No reduction of surface hCTR1 (ie internalization) was evident (Figure 6, p. 33). We also exposed cells to 3 µM cDDP for the same times, with the same results (data not shown). No hCTR1 protein expression is seen in cells not tetracycline-induced (See Figure 7, p. 34). These results suggest cDDP does not cause internalization of hCTR1, and thus cellular entry of cDDP is unlikely to be via the endocytosis of hCTR1.

3. ATP7A localization in response to cisplatin

Another important copper transporter that is involved in the copper exit pathway is ATP7A, the ubiquitous copper-activated ATPase. Exposure of cells expressing ATP7A to excess copper causes trafficking of the ATPase from the trans-golgi compartment to the plasma membrane to reduce intracellular copper levels (Petris et al. 1996, Petris and Mercer 1999). This phenomenon can be seen in the HEK cells where ATP7A levels at the plasma membrane are increased in response to exposure to high copper (See Figure 7, p.34). It has been reported

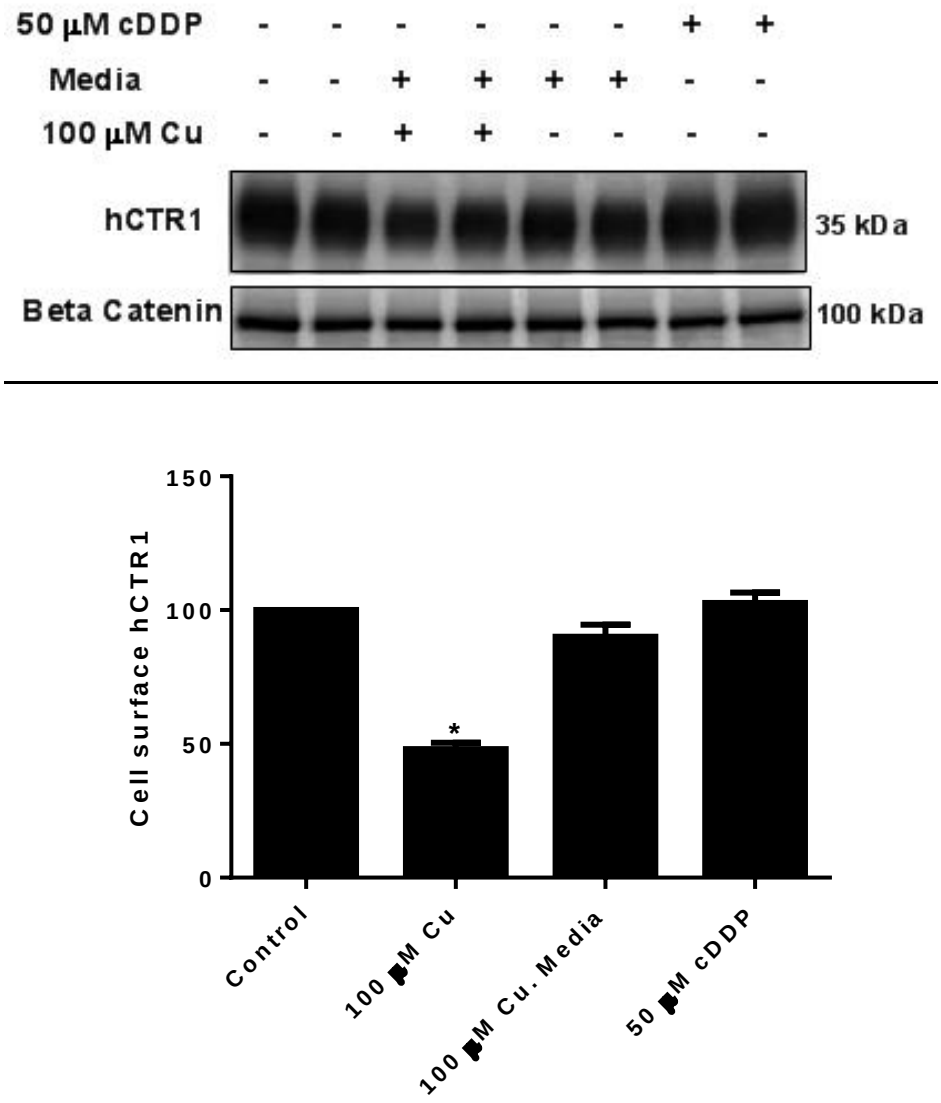

Fig. 5 Biotinylation of hCTR1 in HEK 293 cells

HEK 293 cells over-expressing N-terminal flag-tagged hCTR1 were induced with tetracycline 48 hrs prior to experiment. Cells were incubated with 100 µM Cu for 30 min, 100 µM Cu and then washed 3X with media and placed back in media for 30 min, or 50 µM cDDP for 30 min. Cells were biotinylated and the biotinylated protein was analyzed on western blots. hCTR1 protein was detected using an anti-Flag antibody and the protein levels were normalized to Beta catenin, a loading control.

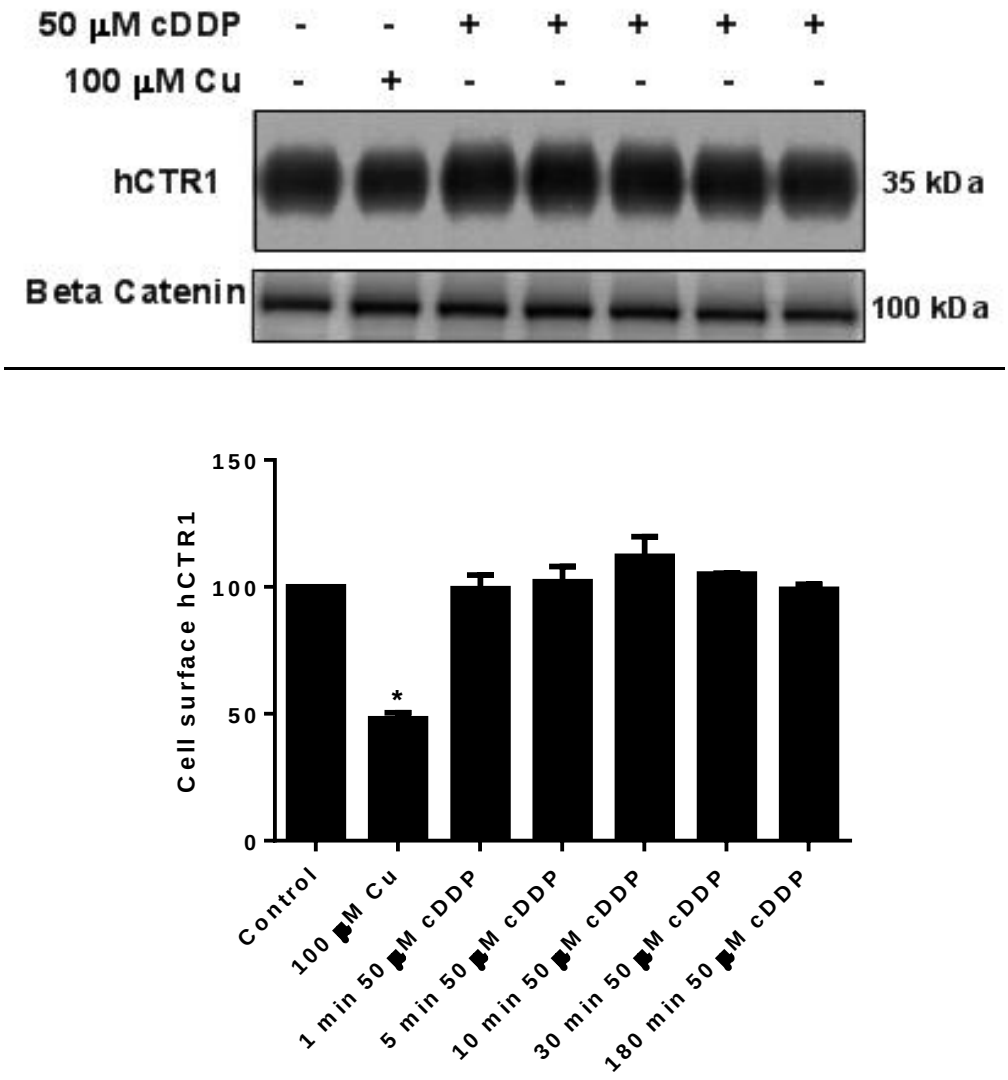

Fig. 6 Biotinylation of hCTR1 in HEK 293 cells

HEK 293 cells over-expressing N-terminal flag-tagged hCTR1 were induced with tetracycline 48 hrs prior to experiment. Cells were incubated with 100 µM Cu for 1 hr, or 50 µM cDDP for 1, 5, 10, 30, or 180 mins. Cells were biotinylated and the biotinylated protein was analyzed on western blots. hCTR1 protein was detected using an anti-Flag antibody and the protein levels were normalized to Beta catenin, a loading control.

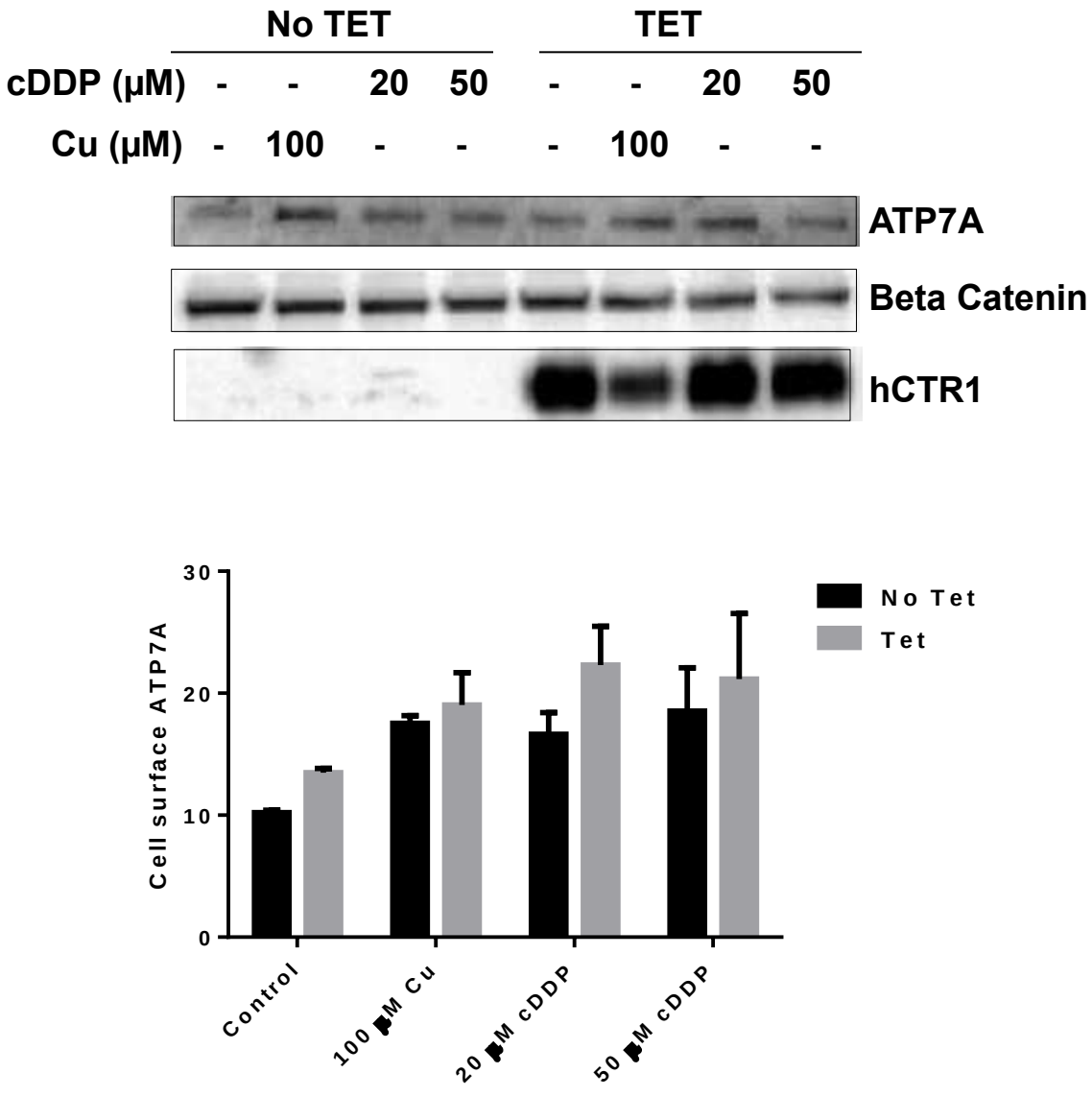

Fig. 7 Biotinylation of ATP7A in HEK 293 cells

HEK 293 cells over-expressing N-terminal flag-tagged hCTR1 were induced with tetracycline 48 hrs prior to experiment. Cells were incubated with 100 μM Cu, 20 or 50 μM cDDP for 1 hr. Cells were biotinylated and the biotinylated protein was analyzed on western blots. hCTR1 protein was detected using an anti-Flag antibody and ATP7A was detected with a rabbit ATP7A antibody. Protein levels were normalized to Beta catenin, a loading control.

that cDDP interacts with ATP7A via its metal-binding domains (Cater et al. 2004, Guo et al. 2005, Strausak et al. 1999, Voskoboinik, Camakaris, and Mercer 2002). However, as can be seen in Figure 7 (p. 34), cDDP does not cause the same increase in plasma membrane ATP7A as is seen with copper. It appears then that neither hCTR1 nor ATP7A respond to cDDP in the same way as they do to elevated copper in HEK 293 cells. A strict relationship between hCTR1 expression and ATP7A expression has not been systemically demonstrated.

4. Copper and cisplatin uptake in hCTR1 mutant

If hCTR1 is important for the entry of cDDP, and copper and cDDP interact in a similar fashion with hCTR1, it would be expected that cells expressing mutants of hCTR1 that show elevated rates of copper entry would also show enhanced cDDP entry rates. We have recently discovered that truncations at the intracellular C-terminus of hCTR1 produce hCTR1 mutants with greatly enhanced (about 10-fold) Vmax values for copper entry (Maryon et al. 2013). The entry of copper into cells expressing the tr179 mutant that lacks the final 11 amino acid residues of hCTR1 has a much higher rate (about 15-fold) than cells expressing wild-type hCTR1 (Figure 8B, p. 36). However, the rates of cDDP entry of these two sets of cells are identical and the same as cells not over-expressing hCTR1 (Figure 8C, p. 36). Thus, it is likely that cDDP is not transported by hCTR1 in the same manner as copper in these cells.

C-terminal hCTR1 mutants generated by the Kaplan lab have very different uptake kinetics than wild-type hCTR1. We suggest the C-terminus acts as a plug of the copper uptake pore limiting the amount of copper entering the cell. Removal or mutation of the plug results in a surge of copper influx. As mentioned in the introduction, free copper has the potential to bind to several proteins nonspecifically. Also free copper can bind to oxygen, generating reactive oxygen species. Thus the levels of cellular copper must be highly regulated and maintained for the safety of the cell. Membrane transporters have been shown to operate in only a few ways.

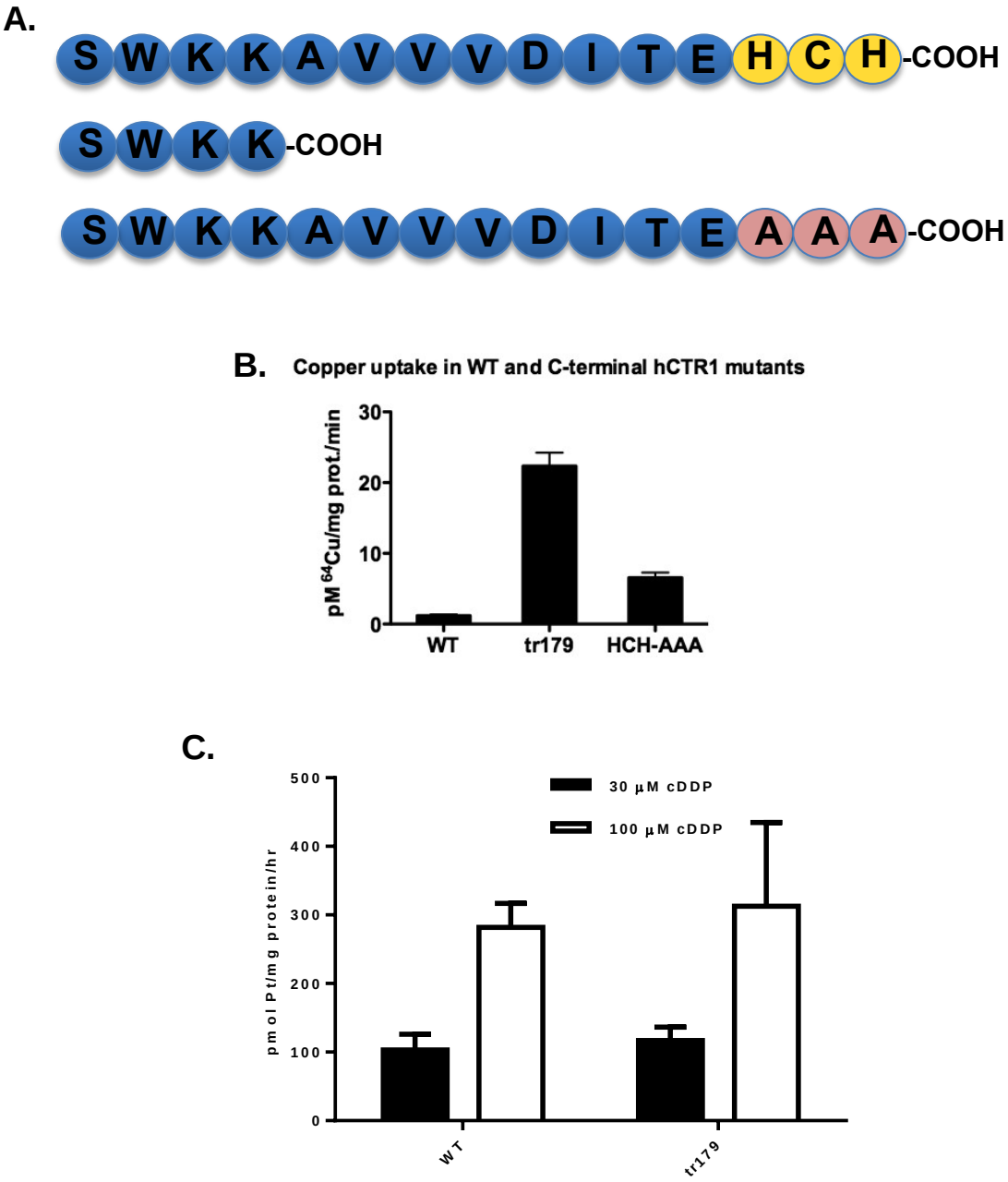

Fig. 8 cDDP accumulation in C-terminal hCTR1 mutant

HEK 293 cells over-expressing N-terminal flag-tagged hCTR1, tr179 mutant or AAA mutant were induced with tetracycline 48 hrs prior to experiment. A) Protein structure of the C-terminus of hCTR1 WT, tr179 mutant, and HCH-AAA mutant. B) **(E. Maryon)** 64-Cu uptake in WT, tr179 mutant, and HCH-AAA mutant. C) **(K. Ivy)** cDDP accumulation was measured in cells incubated with 30 µM or 100 µM cDDP for 5hrs.

Carrier proteins bind to specific solutes with high affinity and undergo a series of conformational changes transferring the solute across the membrane (Alberts et al. 2002). In contrast to carrier proteins, channel proteins form a pore across the membrane allowing only solutes of a specific size and charge to cross. Channel proteins form a pore across the membrane allowing only solutes of a specific size and charge to cross. Other proteins undergo the process of endocytosis. One example of a protein entering the cell via this mechanism is iron's serum carrier, transferrin. Transferrin bound with iron binds to a transferrin receptor present at the cell surface. The entire transferrin-Fe-transferrin receptor complex undergoes receptor mediated endocytosis. Due to low pH in early endosomes, Fe is released from transferrin while apotransferrin (iron-free transferrin) and the transferrin receptor remained attached. They continue through the tubular extensions of the early endosomes but are recycled back to the membrane before they are degraded in the lysosomes (Alberts et al. 2002). Work in the Kaplan lab suggests hCTR1 undergoes copper stimulated internalization. However upon the removal of extracelluar copper, hCTR1 recycles back to steady state levels at the cell surface (Molloy and Kaplan 2009). The removal of the transporter from the cell surface in response to elevated copper limits the amount of cellular copper and thus protects the cell from copper toxicity. When elevated copper levels are removed, hCTR1 returns to the plasma membrane to maintain copper homeostasis. This protective mechanism is essential for the integrity of the cell.

Removal of the majority of the C-terminus (tr179) or mutating a putative metal binding domain (HCH-AAA) results in elevated rates of copper transport in comparison to wildtype hCTR1 (Figure 8B, p. 36). Biotinylation of the mutants in the absence or presence of copper had no effect on the steady state level of hCTR1 present at the cell surface, whereas wild-type hCTR1 protein expression was reduced by ~50% in the presence of copper (Figure 9, p. 38). Petris et al have suggested extracellular copper is transported into cells by endocytosis of the entire hCTR1 protein complex. hCTR1 is next degraded and copper ions are released (Petris et al. 2003). This would suggest hCTR1 is constantly recycling to and from the membrane. Work in

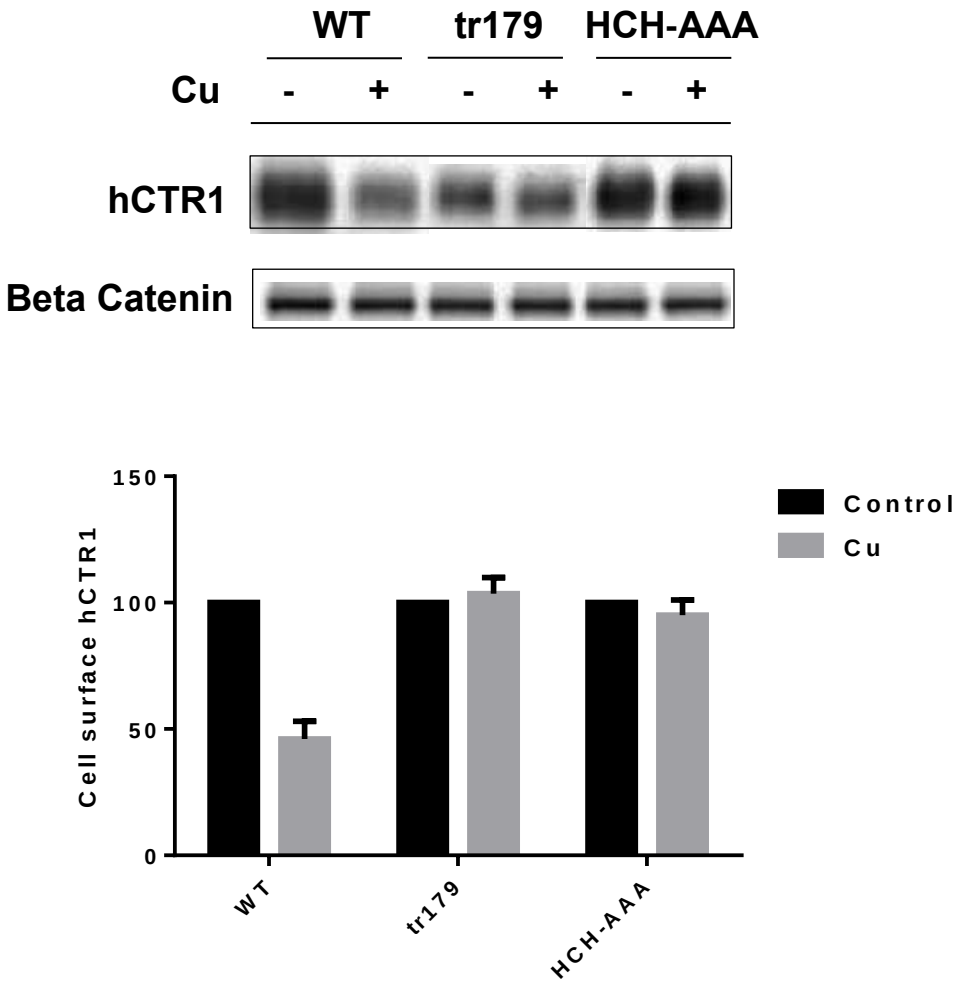

Fig. 9 Biotinylation of hCTR1 in HEK 293 wild-type and mutant cells

HEK 293 cells over-expressing N-terminal flag-tagged hCTR1 or C-terminal hCTR1 mutant (tr179 and HCH-AAA) cells were induced with tetracycline 48 hrs prior to experiment. Cells were incubated with 100 µM Cu for 1 hr. Cells were biotinylated and the biotinylated protein was analyzed on western blots. hCTR1 protein was detected using an anti-Flag antibody and the protein levels were normalized to Beta catenin, a loading control.

the Kaplan lab shows the turnover rate of hCTR1 is not fast enough to maintain copper homeostasis if this were the case (Maryon et al. 2013). Recycling only occurs in absence of extracellular high copper. While we do agree copper alters surface protein expression of hCTR1, our work shows copper does not enter cells by endocytosis of its transporter, hCTR1, since the cell surface localization of the hCTR1 mutants was unchanged in response to copper, although the uptake rates of copper were much faster than wildtype hCTR1. Copper stimulated internalization of hCTR1 is an acute regulatory mechanism the cell has to protect against copper overload.

As mentioned earlier, polar solutes can cross cell membranes a few ways: via a carrier protein, a channel protein, or endocytosis. It is mostly accepted that hCTR1 acts as a channel protein, forming a pore extending across the cell membrane allowing specific solutes to transverse the pore based upon size and charge. We demonstrated hCTR1 does not transport copper by endocytosis of the complex as shown by hCTR1 mutants that transport copper at elevated rates (Figure 8B, p. 36) but do not undergo copper-stimulated internalization (Figure 9, p. 38). If hCTR1 is responsible for transporting cDDP, it is possible cDDP is transported in a different manner than copper. We reasoned since cDDP is 2 times the size of copper, it is unlikely hCTR1 operates as a pore allowing cDDP to transverse the membrane. hCTR1 is very specific. Only silver has been shown to inhibit copper uptake. Silver and copper have the same charge and are very similar in size. To examine whether endocytosis of hCTR1 is the pathway in which cDDP crosses the membrane, we employed biotinylation assays to measure cell surface levels of hCTR1. In wildtype cells, copper stimulates a reduction in plasma membrane levels of hCTR1. However cDDP does not (Figure 10, p. 40). These results eliminate endocytosis of hCTR1 as the pathway for cellular cDDP entry.

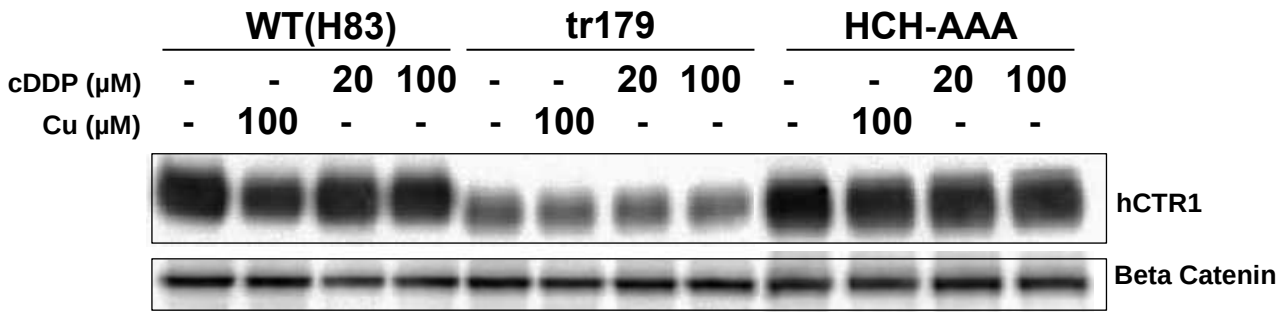

Fig. 10 Biotinylation of hCTR1 in HEK 293 wild-type and mutant cells

HEK 293 cells over-expressing N-terminal flag-tagged hCTR1 or C-terminal hCTR1 mutants (tr179 and HCH-AAA) cells were induced with tetracycline 48 hrs prior to experiment. Cells were incubated with 100 μM Cu, 20 μM or 100 μM cDDP for 1 hr. Cells were biotinylated and the biotinylated protein was analyzed on western blots. hCTR1 protein was detected using an anti-Flag antibody and the protein levels were normalized to beta catenin, a loading control.

5. Cisplatin uptake in mouse embryonic fibroblasts (Mefs)

Thus far we have analyzed the rates of uptake of copper and cDDP by hCTR1 in HEK293 cells as well as the response of cell surface hCTR1 to copper or cDDP via biotinylation and have found no evidence to support the notion that cDDP is transported by hCTR1 by either endocytosis or via a protein-mediated uptake by hCTR1. When the rate of cDDP entry into cells that do not express CTR1 (mefs -/-) are compared with similar cells that endogenously express the copper transporter (mefs +/+), it is apparent that the presence or absence of CTR1 has minimal influence on the rate of cDDP entry (see Figure 11, p. 42). Furthermore, the presence of copper in the medium did not inhibit the rate of cDDP entry (Figure 11, p. 42). It has previously been shown that the copper entry rate into the -/- mefs is about 25% -30% of the +/+ cells via an unidentified pathway (Lee, Petris, and Thiele 2002, Zimnicka, Ivy, and Kaplan 2011, Eisses and Kaplan 2002). Thus it seems unlikely that CTR1 in these cells plays a significant role in cDDP entry. We have also examined the entry rates of several analogs of cDDP that are in clinical use. The absence or presence of CTR1 does not alter their rates of uptake (Figure 12, p. 43). To determine if cDDP can stimulate endocytosis of hCTR1 in mef (+/+) cells, we exposed cells to cDDP for 15 and 60 minutes with 3 or 30 μM cDDP. Whereas copper induced internalization of CTR1, cDDP did not cause a significant decrease of CTR1 (Figure 13, p. 44).

It is apparent from these studies that CTR1 does not play a major role in mediating platinum-drug entry into these model cells. However, the most relevant issue is whether or not hCTR1 plays a role in cDDP entry into tumor cells. We have extended our studies on cDDP entry to tumor cells of relevance to these issues.

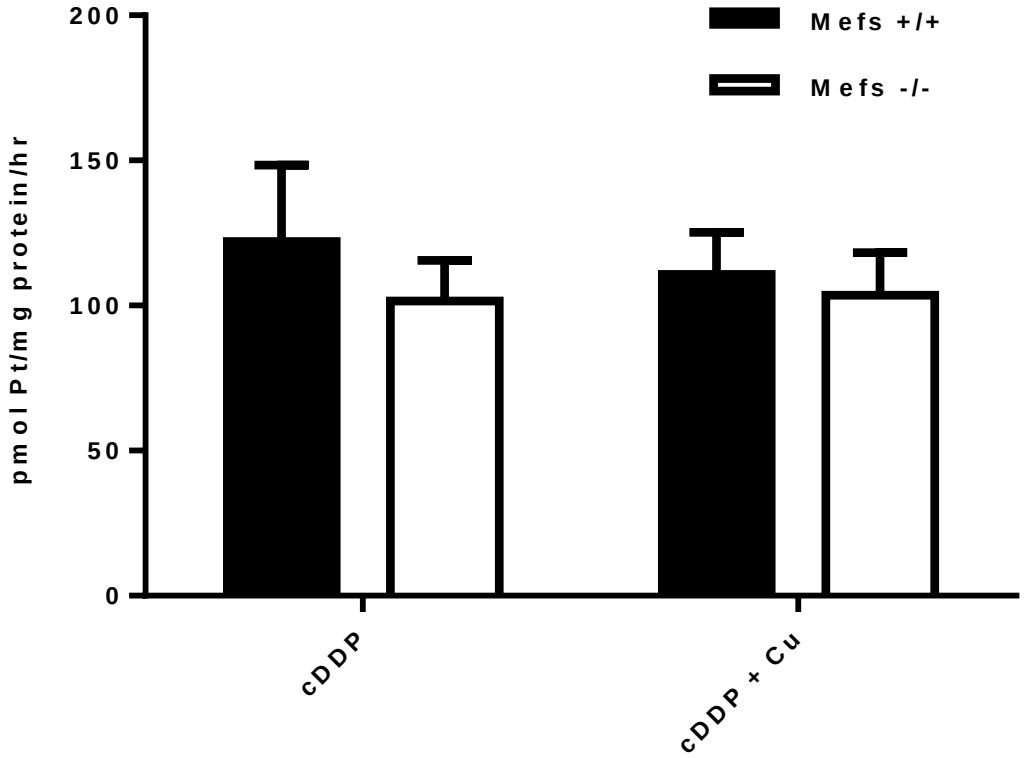

Fig. 11 Cisplatin accumulation in Mefs

Mefs (+/+) and (-/-) were incubated with 30 µM cDDP or 30 µM cDDP and 100 µM Cu for 5 hrs in media.

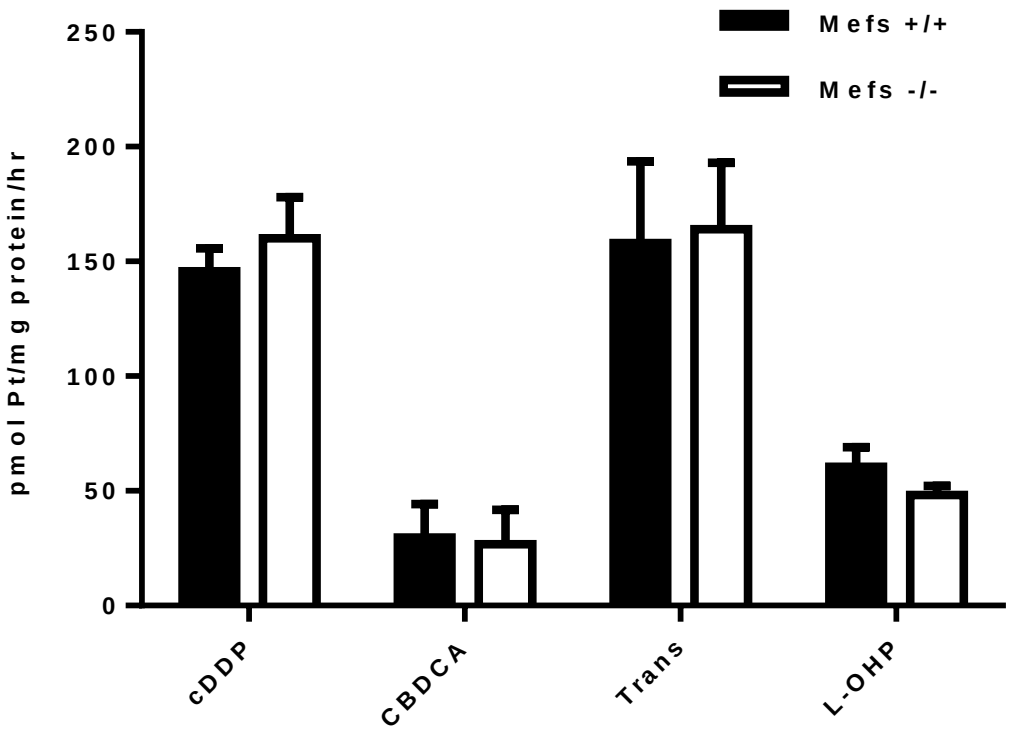

Fig. 12 Platinum accumulation in Mefs

Mefs (+/+) and (-/-) were incubated with 30 μM cDDP, carboplatin, transplatin or oxaliplatin for 5 hrs.

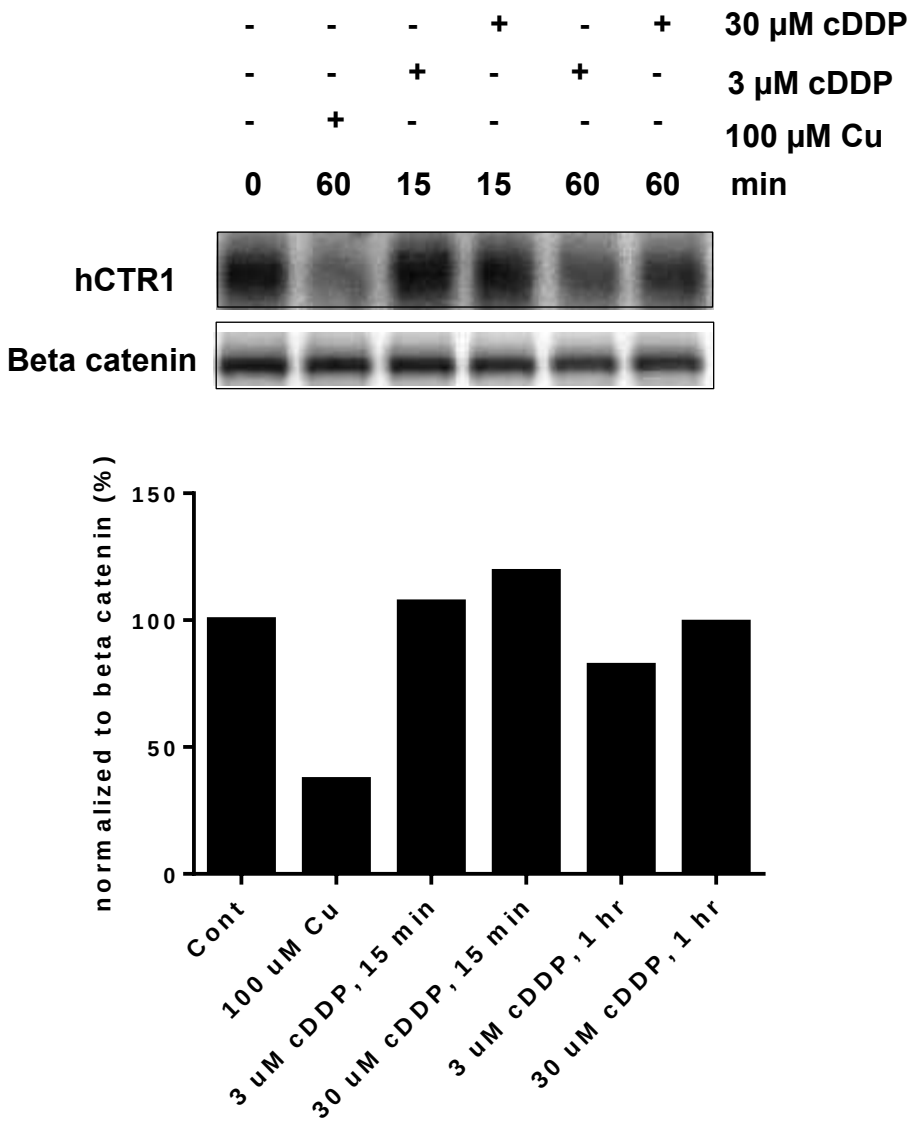

Fig. 13 Biotinylation of CTR1 in Mefs +/+

Mefs +/+ were incubated with Cu or cDDP for indicated times. Cells were biotinylated and the biotinylated protein was analyzed on western blots. hCTR1 protein was detected using an C-terminal CTR1 antibody and the protein levels were normalized to Beta catenin, a loading control.

D. Discussion

Correlations between hCTR1 expression and drug entry in yeast and tumor cells (Ishida et al. 2002, Lin et al. 2002, Song et al. 2004) led to the idea that the copper transporter mediated cDDP entry. The basis for clinical trials (Fu et al. 2012) of agents that might influence responsiveness to cDDP is that cDDP is transported like copper, via a pore formed by the transporter (Larson, Adams, Jandial, et al. 2010). It was also suggested that extracellular cDDP, like copper, causes internalization of hCTR1 (Holzer and Howell 2006, Holzer, Katano, et al. 2004). Thus, hCTR1 would be down-regulated in response to cDDP and further uptake would be reduced, leading to resistance. Our studies provide strong evidence that, although the proteins involved in copper homeostasis influence cellular cDDP levels, hCTR1 does not play a major role in cDDP entry. The most probable entry mechanism is via a non-protein-mediated pathway.

cDDP uptake in normal eukaryotic cells.

hCTR1 does not play a major role in cDDP uptake in HEK and mef cells based on the following: (a) over-expression of hCTR1 increases copper entry, but not cDDP (Figures 3, 4 p.29, 30); (b) cells lacking CTR1 (-/- mefs) show the same rates of cDDP entry as cells (+/+ mefs) with hCTR1 (Figures 11, 12, p. 42, 43); (c) cells overexpressing hyperactive hCTR1 mutants have identical rates of cDDP uptake to cells not over-expressing hCTR1 (Figure 8, p. 36).

Previous studies in mefs, HEK cells and yeast yielded varied conclusions. In HEK cells no increase in cytotoxicity to cDDP was seen when hCTR1 was over-expressed (Rabik et al. 2009). Other studies in HEK and other renal cells claimed that siRNA-induced decrease in hCTR1 resulted in a decrease in Pt uptake (Pabla et al. 2009). However, questions remain on the antibody used to quantitate hCTR1 (see internalization, below). In previous studies using mef's, in contrast to our results, higher uptake of Pt was seen in cells expressing hCTR1 than in

-/- cells (Larson, Adams, Jandial, et al. 2010, Larson et al. 2009, Larson, Adams, Blair, et al. 2010). These studies make several interesting points: most of the over-expressed hCTR1 protein was not delivered to the plasma membrane; there is a lack of correlation between cDDP uptake and cytotoxicity; truncation of the first 45 amino acids of hCTR1 do not inhibit cDDP uptake (Larson, Adams, Jandial, et al. 2010), and replacement of Met residues 150, 154 (Larson, Adams, Blair, et al. 2010) increase cDDP uptake and decrease Cu uptake. The authors conclude that if hCTR1 is responsible for cDDP uptake, its interactions with cDDP are different from those with Cu. Cytotoxic effects seen in those studies are observed following very brief (15 seconds or 5 minutes) exposures to cDDP. It is difficult to conclude that surface binding phenomena are separated from uptake. The results of the present work suggest that if hCTR1 does interact with cDDP it can play only a minor role, if any in its cellular accumulation. Studies in yeast were the first to show the correlation between copper transporter and cDDP entry (Ishida et al. 2002). However, yeast ctr1 and hCTR1 have only 29% identity and yeast protein is considerably larger. cDDP transport was characterized in ctr1-expressing strains and Km values for copper and cDDP were reported of 129 µM and 140 µM respectively (Lin et al. 2002). The values for copper are considerably higher than the 3-5 µM reported for hCTR1 (Liang et al. 2009, Eisses and Kaplan 2002, Zimnicka, Maryon, and Kaplan 2007) and 5 µM for yeast ctr1 reported previously (Dancis, Yuan, et al. 1994).

Platinum-drugs and copper homeostasis.

cDDP interacts with several proteins in copper homeostasis. Increased expression of ATP7A and ATP7B has been associated with cDDP resistance (Komatsu et al. 2000, Katano et al. 2002, Rabik and Dolan 2007). cDDP also binds to the copper chaperone, Atox1, destabilizing Atox1, resulting in unfolding and aggregation. Atox1 was suggested as a potential candidate for cDDP resistance (Palm et al. 2011). Further work is needed to clarify the interactions between platinum drugs and proteins in copper homeostasis. Such interactions are

significant as lowering cell copper alters tumor cell sensitivity to platinum-drugs (Ishida et al. 2010). The observations of Holzer (Holzer, Samimi, et al. 2004) that separate cytotoxic effects of cDDP from uptake, suggest that a more complete understanding of the fates of cDDP following its initial interactions with the cell surface would be of great value. Inhibition of proteasomal activity enhances delivery of cDDP in ovarian carcinoma cells and promises significant clinical potential, but its basis is also not yet clear (Jandial et al. 2009). The recent observation that copper is involved in growth factor kinase cycling in tumor cells introduces a new complexity (Tsai et al. 2012), and recent studies suggest important interactions between ATP7A, hCTR1, Atox1 and the PDGF receptor in cell activation (Ashino et al. 2010).

The present studies focus attention on the proteins involved in copper homeostasis as important contributors to the complexity of the cellular pharmacology of the widely-used Pt-drugs. It seems likely that interactions among the proteins involved in copper homeostasis play a major role in deciding the fate and effectiveness of these drugs, following cellular entry through a pathway that is not related to the entry mechanisms utilized by copper.

Acknowledgements

We would like to thank Dennis Thiele (Duke University) for the mouse embryonic fibroblasts; Betty Eipper (University of Connecticut) for the ATP7A antibody; and Edward Maryon (University of Illinois at Chicago) for constructing the C-terminal hCTR1 mutants. These studies were supported by NIH Grant P01 GM 067166.

III. Cisplatin Uptake in Ovarian Tumor Cells

A. Introduction

In Chapter II, we investigated cDDP uptake in human embryonic kidney cells expressing wild-type hCTR1 or C-terminal hCTR1 mutants and mouse embryonic fibroblasts. We employed radioactive copper uptake assays, cDDP accumulation studies and biotinylation of hCTR1 in the presence of elevated extracellular copper or cDDP. It is apparent from these studies that hCTR1 does not play a major role in mediating platinum-drug entry into these model cells. However, the most relevant issue is whether or not hCTR1 plays a role in cDDP entry into tumor cells. We have extended our studies on cDDP entry to tumor cells of relevance to these issues. We have employed A2780 and A2780CP cells (ovarian carcinoma) and 2008 and C13*5.25 cells (cervical carcinoma) which exhibit sensitivity or resistance respectively to cDDP.

Several attempts have been made to understand the mechanism of resistance. A few possible explanations include: reduced cellular drug import, increased cellular drug export, cytoplasmic sequestration of the drug away from the nucleus, and successful DNA repair. As mentioned in chapter II, hCTR1 has been thought to play a role in mediating cDDP uptake. Early studies performed in yeast correlated the amount of CTR1 to cDDP entry. This same correlation was later seen in ovarian and cervical tumor (Ishida et al. 2002, Lin et al. 2002, Zisowsky et al. 2007, Song et al. 2004). Several subsequent studies confirmed this correlation and led to the suggestion that hCTR1, the major high affinity copper-transporter also mediates platinum uptake (Larson, Adams, Jandial, et al. 2010, Larson et al. 2009, Pabla et al. 2009, Chen et al. 2012, Kalayda, Wagner, and Jaehde 2012). At least one clinical trial has designed its study around targeting membrane levels of hCTR1. Increased cell surface levels of hCTR1 could potentially increase platinum drug uptake and therefore increase drug efficacy if hCTR1 is indeed responsible for platinum drug uptake (Fu et al. 2012).

As mentioned in chapter II, hCTR1 is a highly selective copper uptake protein and thus it is surprising an anti-cancer drug that is twice the size of copper could fit within the narrow pore of hCTR1 (Hilder and James 2007, Blumberger et al. 2004). We have carried out a detailed analysis of the relationship between cDDP uptake and hCTR1 in human ovarian tumor cells and human cervical tumor cells which exhibit sensitivity or resistance to cDDP. We have compared the rates of copper or cDDP uptake as well as compared the responses of hCTR1 in these cells to cDDP with their response to Cu. We also have examined the effects of the varied hCTR1 levels on Cu and cDDP entry rates and also the consequences of altering hCTR1 expression on the rates of Cu and Pt-drug entry. We conclude hCTR1 does not play a major role in cDDP entry into tumor cells and thus should not be a candidate for drug therapy.

B. <u>Experimental Procedures</u>

See protocols described in detail in chapter 2

Cell Culture. Cells were maintained in a humidified incubator at 37ºC under 5% CO_2 atmosphere and passaged every 3-5 days. Ovarian carcinoma A2780CP and A2780 cells (Fox chase cancer society) and 2008 and C13*5.25 cervical carcinoma cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Atlanta Biologicals), and 25 mM Hepes.

siRNA-mediated knock-down of hCTR1. A2780 cells were transiently transfected with siRNA plasmids from Ambion (Life Technologies) using Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to the manufacturer's instructions. The cells were cultured to 50% confluency and incubated with either siRNA duplexes against CTR1 or siRNA negative control in Opti-MEM medium (Invitrogen). The following day, fresh DMEM media was added to each well. Human CTR1 knockdown was a pre-designed sequence. Forward target sequence used

was as follows: 5'-GCCUAUGACCUUCUACUUUtt-3'; Reverse target sequence: 5'-AAAGUAGAAGGUCAUAGGCat-3'.

Statistical analysis: Data are shown as means ± SD. Statistical analysis was performed using Student's *t*-test, and $P < 0.05$ was considered statically significant. The data are representative of means of at least three independent experiments

C. Results

1. hCTR1 protein expression in human ovarian cells

Two ovarian cancer cell lines were examined, A2780 and A2780CP, that were sensitive or resistant to cDDP, respectively (see Methods). It is clear that there is a significant difference in the expression levels of hCTR1 in the plasma membrane of these cells. The sensitive cells have a higher level of hCTR1 than the resistant cells (about 2 fold, see Figures 14, p. 51). This agrees with previous correlations that led to the suggestion that hCTR1 played a major role in cDDP entry, as the resistant line would take up less cDDP than sensitive tumor cells. Rates of copper transport were measured and resistant tumor cells had a 2-3 fold lower rate of copper uptake when compared to sensitive tumor cells (Figure 15A, p. 52), in keeping with their lower expression of hCTR1. When cDDP entry rates are compared in the two cell lines, the resistant cells take up cDDP at a significantly slower rate (Figure 15B, p. 52), which correlates with the hCTR1 expression levels.

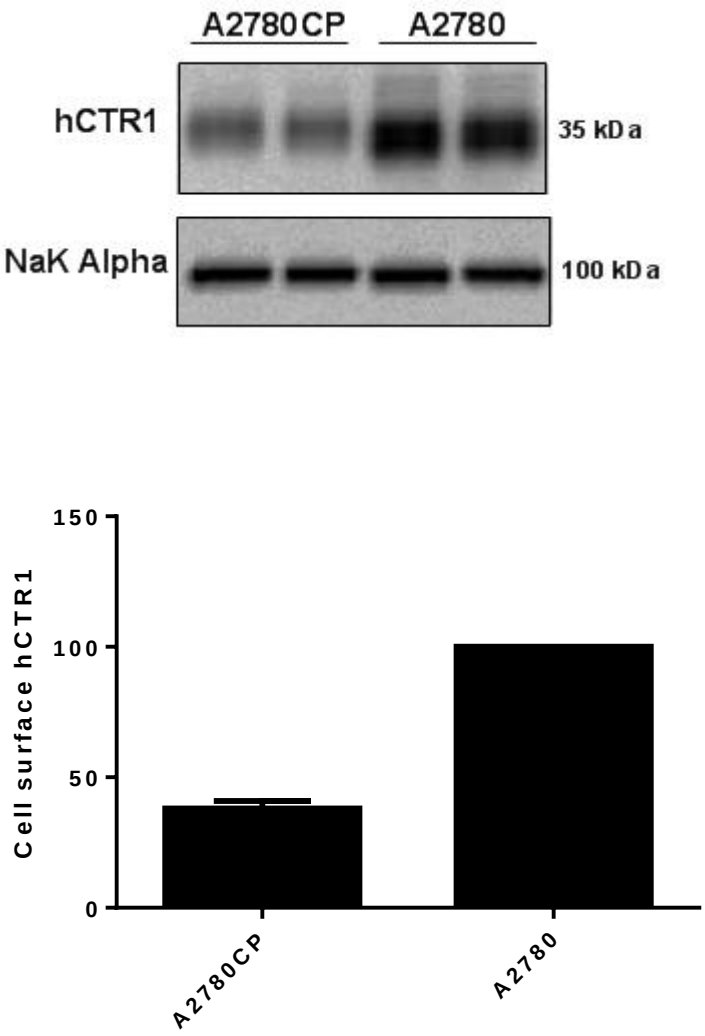

Fig. 14 hCTR1 protein expression in ovarian carcinoma cells

cDDP-resistant (A2780CP) and cDDP-sensitive (A2780) ovarian carcinoma cells were collected, fractionated and plasma membrane preps were obtained and analyzed on western blots. hCTR1 protein was detected by using an anti-CTR1 antibody (rabbit). Na^+ K^+ alpha was used as a loading control.

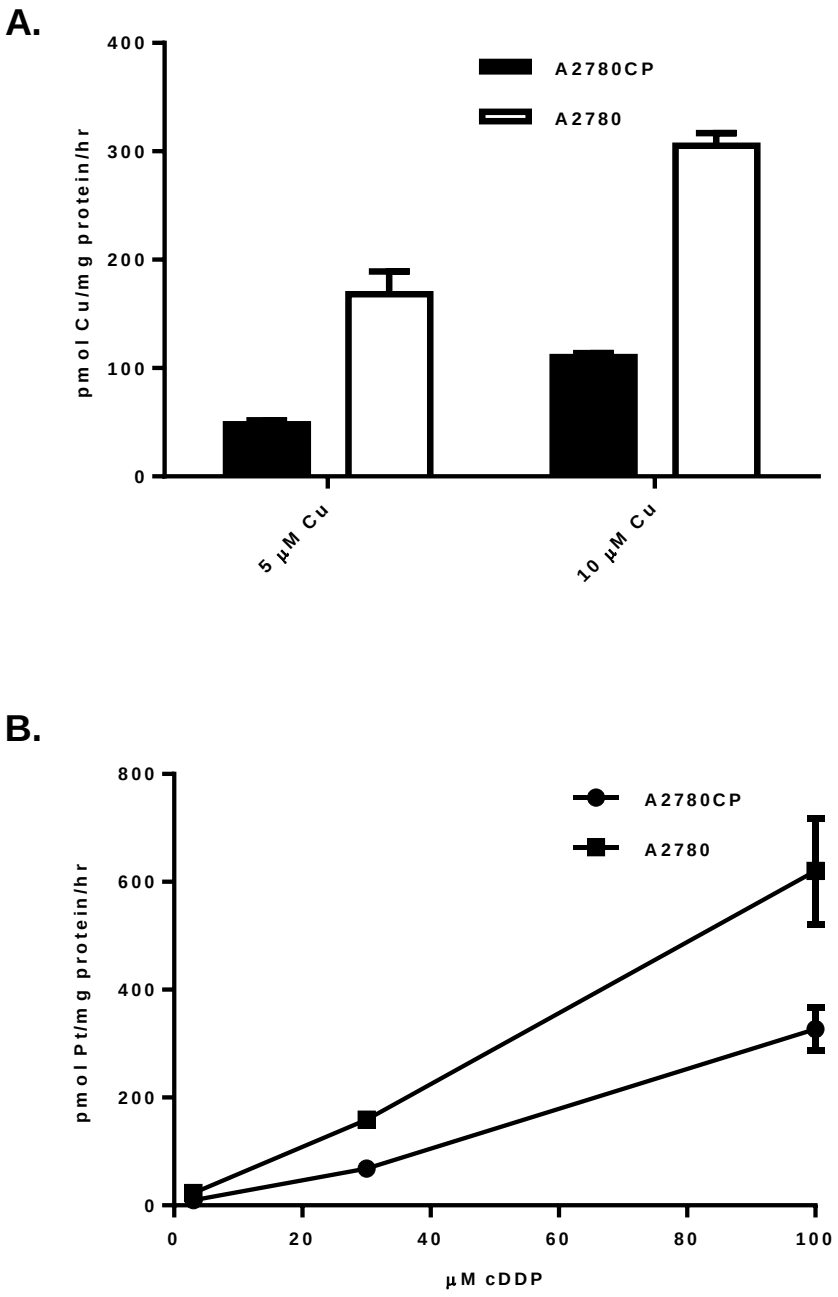

Fig. 15 Copper uptake and cDDP accumulation in human ovarian carcinoma cells

A) ^{64}Cu was measured in cells incubated with 5 µM or 10 µM Cu for 30 minutes in media. B) cDDP was measured in cells incubated with 3 µM, 30 µM, or 100 µM cDDP for 5 hrs in media.

2. Cell surface hCTR1 in response to copper and cisplatin

Exposure of the tumor cells to excess copper causes an internalization of plasma membrane hCTR1 (see Figure 16, p. 54) as in HEK 293 cells (Figures 5, 6, p. 32, 33). Exposure of the tumor cells to cDDP (20 μM or 50 μM) did not cause internalization of the transporter (Figure 16, p. 54), just as had been observed in the HEK cells, emphasizing that hCTR1 does not respond in tumor cells or in normal cells in the same manner to excess cDDP and to excess copper. However in contrast to the HEK cells, removal of copper from the external medium did not result in the return of hCTR1 to the plasma membrane of the ovarian tumor cells (Figure 17A, p. 55) as is observed in the acute regulatory mechanism seen in HEK cells. This enabled a further experiment to be performed in this cell system. If hCTR1 is internalized following exposure to elevated copper (~50%) and if hCTR1 is responsible for cDDP entry, then cDDP entry should be inhibited by a prior Cu treatment of the ovarian cells. This protocol was applied to the ovarian cells. It can be seen that although about 50% of the plasma membrane hCTR1 is internalized following copper treatment, cDDP uptake is unaffected (Figure 17B, p. 55).

3. hCTR1 siRNA in A2780 cells

In an experiment to directly alter hCTR1 expression in tumor cells we employed siRNA of hCTR1 in the A2780 ovarian carcinoma cells. Figure 18 shows we achieved ~80% knockdown of plasma membrane hCTR1 (p. 56). In the cells transiently transfected with hCTR1 siRNA, the rate of copper transport was reduced by ~50% (Figure 19A, p. 57). However, cDDP uptake was not altered (Figure 19B, p. 57). Mouse embryonic fibroblasts deficient of CTR1 maintained 20- 30% of copper uptake in comparison to wild-type (Lee, Petris, and Thiele 2002). The presence of a CTR1-independent uptake pathway explains why only a partial reduction of copper uptake in the CTR1 siRNA treated cells was seen.

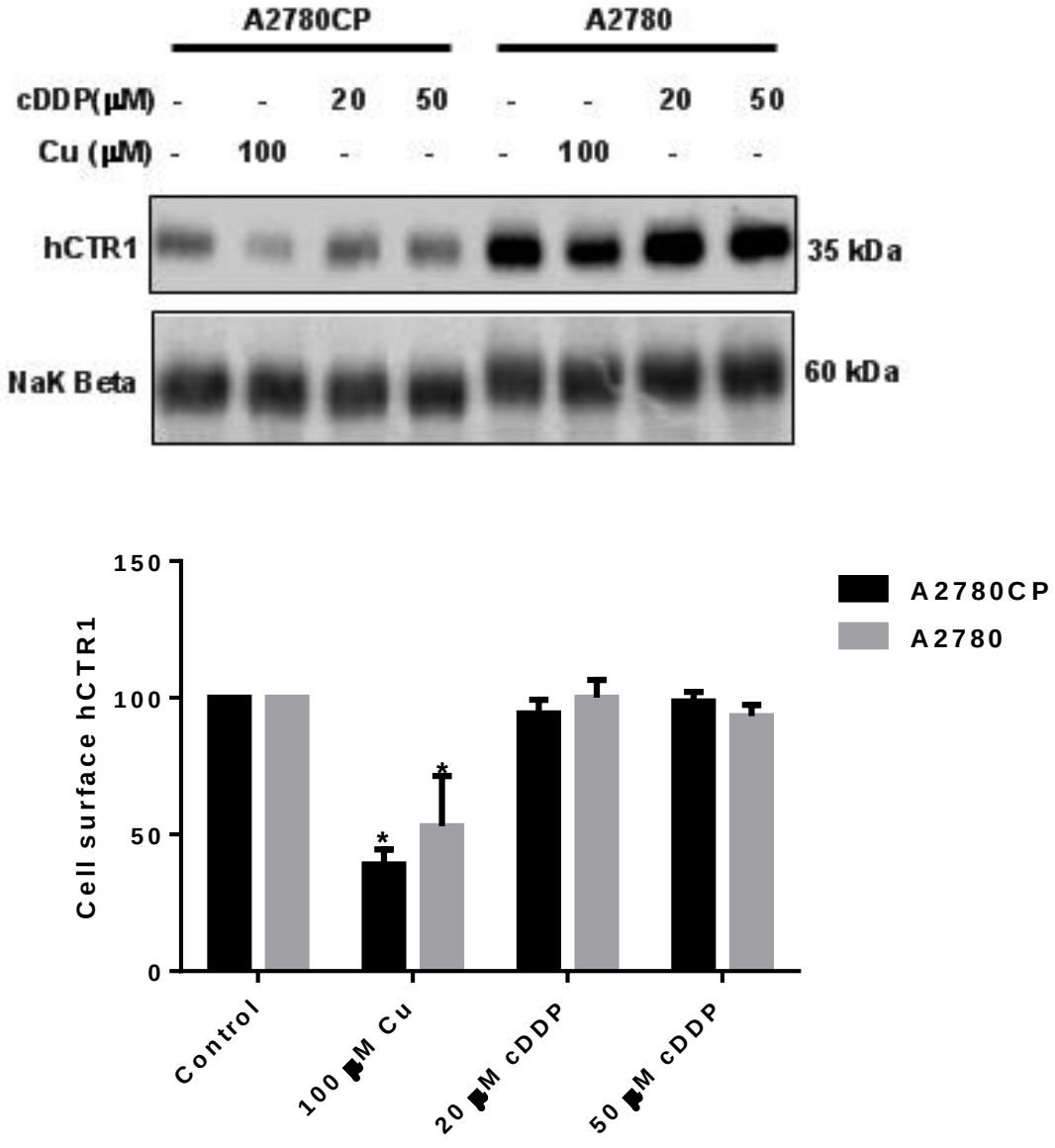

Fig. 16 Internalization of hCTR1 stimulated by copper

cDDP-resistant (A2780CP) and cDDP-sensitive (A2780) ovarian carcinoma cells were incubated with 100 μM Cu, 20 μM cDDP or 50 μM cDDP for 1 hr in media.

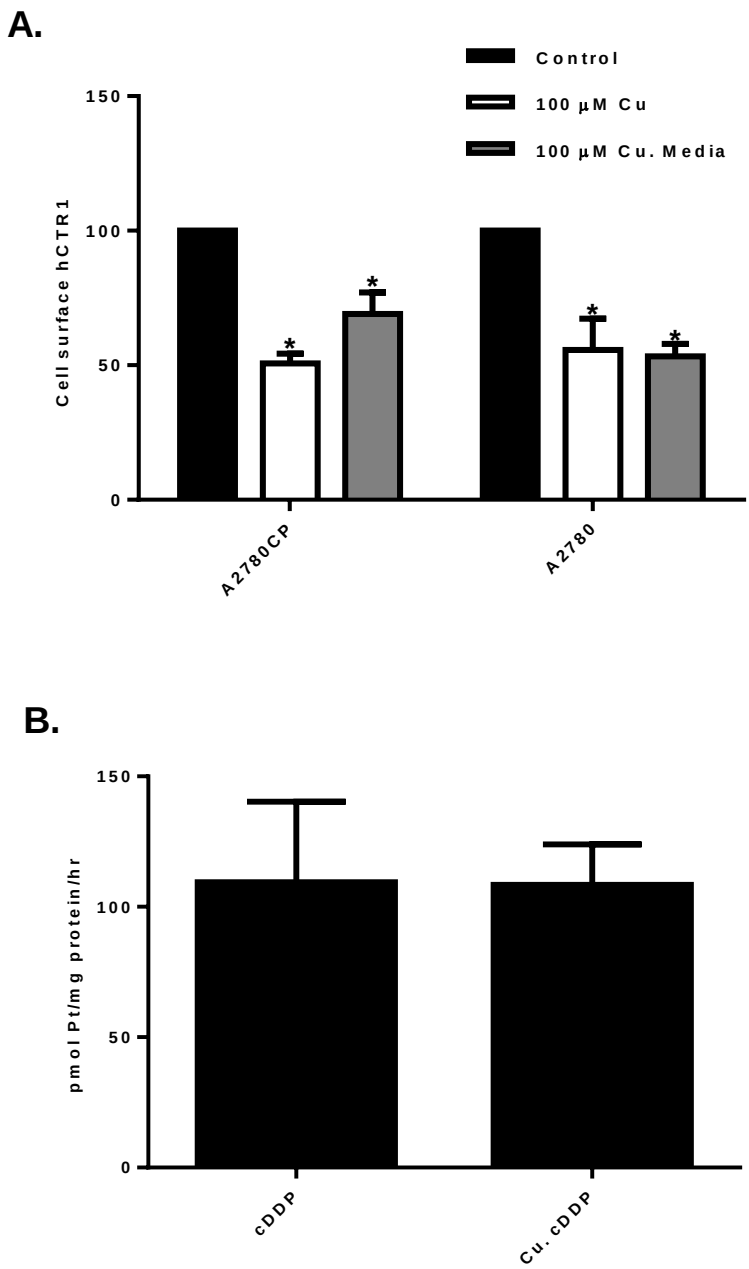

Fig. 17 Failure of hCTR1 to recycle back to cell surface after removal of copper

A) cDDP-resistant (A2780CP) and cDDP-sensitive (A2780). Cells were incubated with 100 µM Cu for 30 min or 100 µM Cu and then washed 3X with media and placed back in media for 30 min. Cells were biotinylated and the biotinylated protein was analyzed on western blots. hCTR1 protein was detected using an anti-hCTR1(rabbit) antibody. Beta catenin was used as a loading control. The results were normalized as a percentage to control (no copper or cDDP). B) A2780 cells were incubated with 100 µM Cu for 1 hr, rinsed 3X with media, and then placed back in fresh media with 30 µM cDDP for 3 hrs.

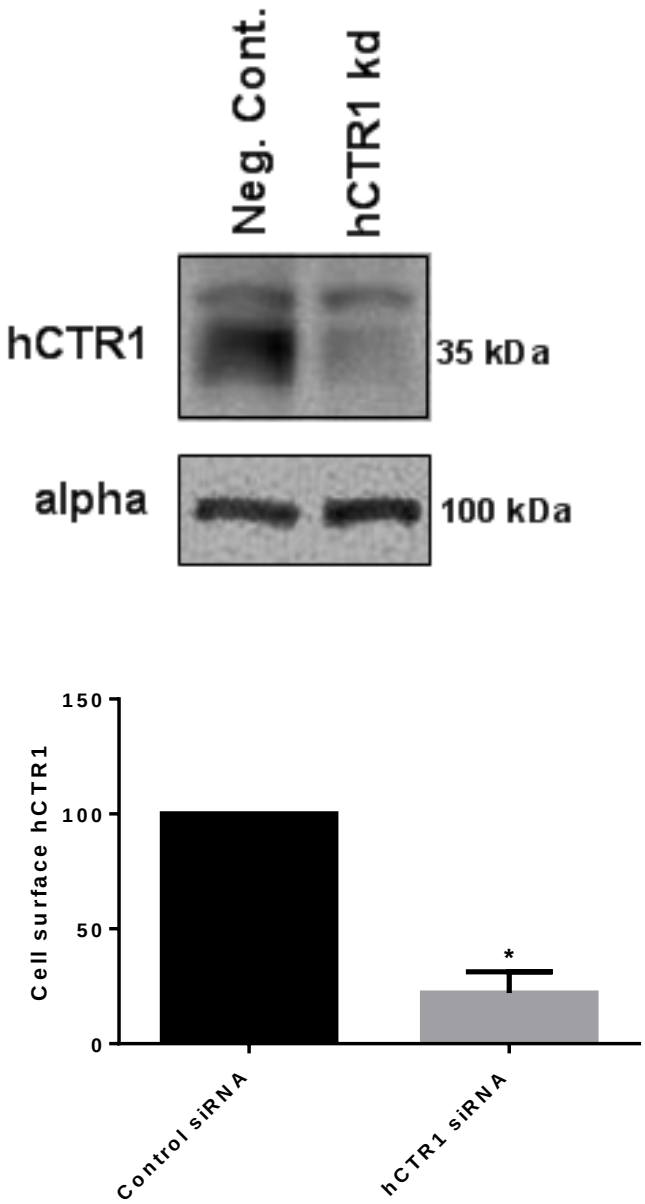

Fig. 18 hCTR1 knockdown in A2780 cells

cDDP-sensitive (A2780) ovarian carcinoma cells were transiently transfected with siRNA plasmids from using Lipofectamine RNAiMAX transfection reagent according to the manufacturer's instructions with either siRNA duplexes against CTR1 or siRNA negative control in Opti-MEM medium. Cells were biotinylated and the biotinylated protein analyzed on western blots. hCTR1 protein was detected by using an anti-CTR1 antibody (rabbit). Na^+ K^+ alpha was used as a loading control.

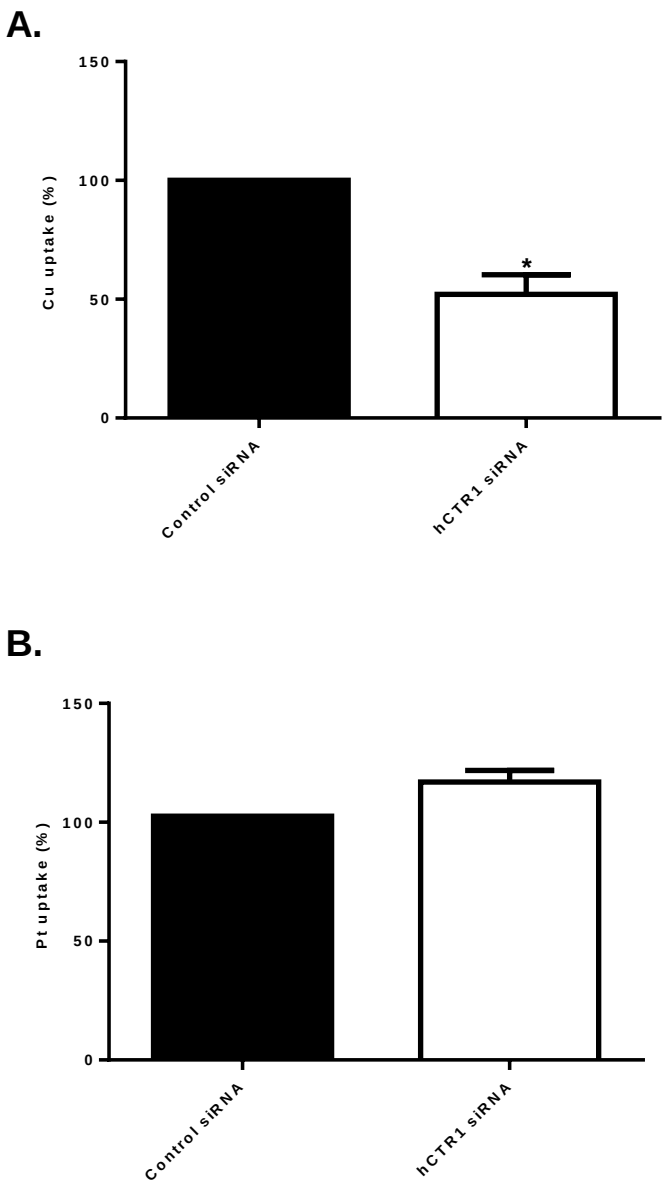

Fig. 19 Copper and cisplatin uptake in hCTR1 siRNA cells

cDDP-sensitive (A2780) ovarian carcinoma cells were transiently transfected with siRNA plasmids from using Lipofectamine RNAiMAX transfection reagent according to the manufacturer's instructions with either siRNA duplexes against CTR1 or siRNA negative control in Opti-MEM medium. Cells were biotinylated and the biotinylated protein analyzed on western blots. hCTR1 protein was detected by using an anti-CTR1 antibody (rabbit). Na^+ K^+ alpha was used as a loading control. A) After transient transfection, cells were incubated with 5 µM copper for 1 hr or B) 30 µM cDDP for 3 hrs.

4. Cis vs trans uptake

In earlier studies in Ehrlich ascites tumor cells, based on the use of non-specific chemical reagents it was suggested that cDDP entry might occur by a non-protein-mediated pathway (Gale et al. 1973). The most likely being a partition-diffusion mechanism, where an uncharged or lipophilic molecule partitions into the membrane and diffuses down its concentration gradient to emerge into the cytosol. One of the characteristics of such a mechanism is that it would be non-saturating (since a limited number of sites are not involved). Furthermore, interactions between proteins and substrates are expected to show some stereospecific discrimination. This is often seen where D but not L isomers are recognized by enzymes or transporters (Narawa, Tsuda, and Itoh 2007). We have tested both of these expectations.

In Figure 20B (p. 59), the concentration-dependence of cDDP uptake into ovarian tumor cells has been examined. Over a wide range of concentrations uptake was approximately linear and there was no sign of saturation. Although D and L isomers of cDDP are not available, the essential elements of such enantiomers are retained by comparison of cDDP (or cisplatin) and the non-therapeutic analog, transplatin (Figure 20A, p. 59). The concentration-dependence of the uptakes of these two analogs is identical (Figure 20B, p. 59).

5. hCTR1 protein expression in human cervical cells

We extended our studies to include a different tumor cell line. The 2008 (cDDP-sensitive) and C13*5.25 (cDDP-resistant) cells are derived from cervical tumors. We obtained these cells to examine if our findings in the ovarian tumor cells (A2780 and A2780CP) are cell line specific. The amount of hCTR1 present at the cell surface under basal conditions was measured utilizing a biotinylation assay. Figure 21 (p. 61) shows hCTR1 protein expression was much more abundant (about 2.5 fold higher) in the 2008 cells than the C13*5.25 cells. Higher hCTR1 protein expression was also seen in A2780 and A2780CP cells.This finding supports the

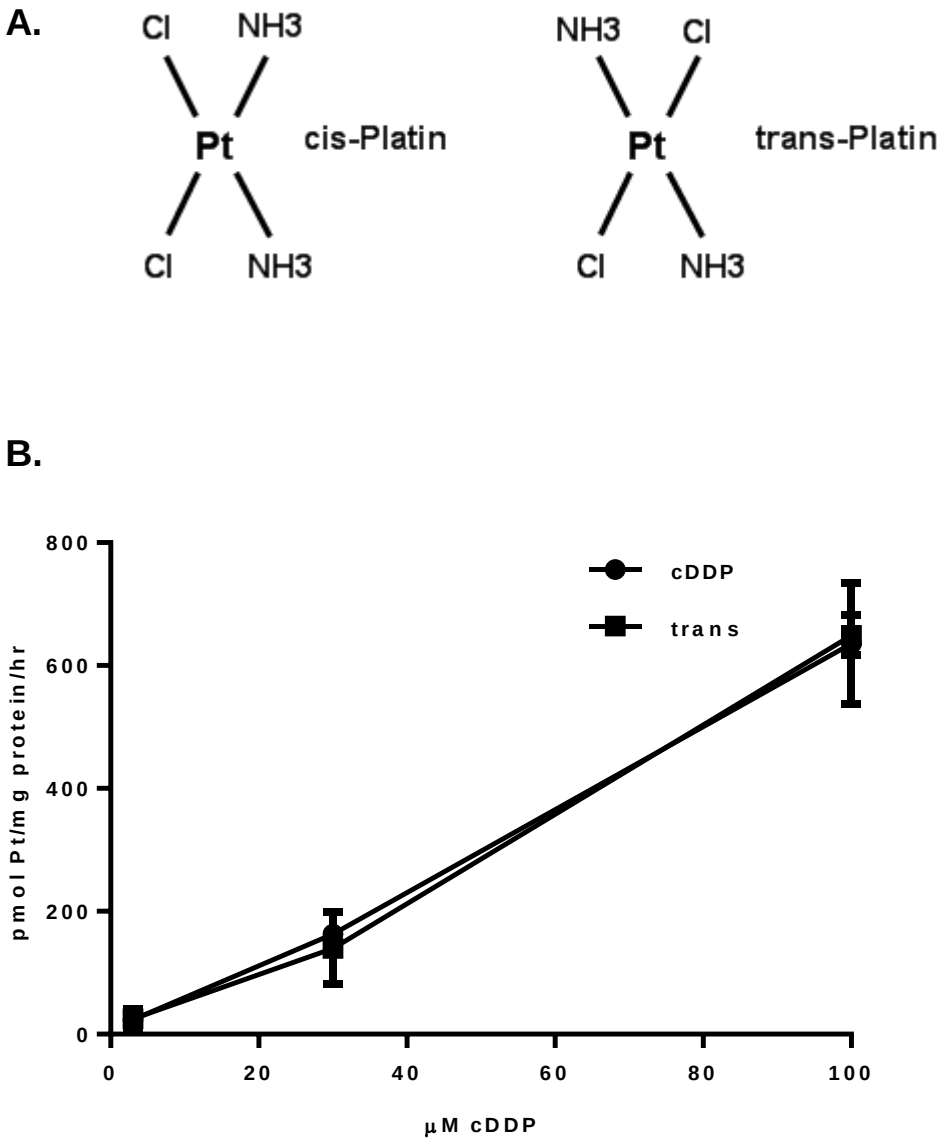

Fig. 20 Cis vs trans uptake

A) The chemical structure of cisplatin and transplatin are shown. B) cDDP -sensitive (A2780) ovarian carcinoma cells were incubated with 3 µM, 30 µM, or 100 µM cDDP or transplatin for 5 hrs in media. Platinum content was measured by ICP-MS and calculations are shown as ppb/mg of protein/hr.

correlation that reduced hCTR1 protein expression is a potential explanation for reduced cDDP accumulation and also cDDP resistance in the A2870 and A2780CP ovarian tumor cells and also the 2008 and C13*5.25 cervical tumor cells.

6. Localization of hCTR1 in response to cisplatin in 2008 and C13*5.25 cells

We investigated if the entry of cDDP is via endocytosis of a hCTR1-Pt drug complex. Exposure of the 2008 and C13*5.25 tumor cells to excess copper caused internalization of plasma membrane hCTR1 (see Figure 22A & 22B, p. 62) as was previously demonstrated in the HEK 293 cells (Figures 5, 6, p. 32, 33) and A2780 tumor cells (Figure 16, p. 54). Exposure of the tumor cells to cDDP (20 µM or 50 µM) did not cause internalization of the transporter (Figure 22A and 22B, p. 62). Time of exposure to the drug had no effect on surface levels of hCTR1 as evident by short and long time (15 and 60 min) incubations. These results confirm our findings in the HEK cells and A2780 cells and emphasize that hCTR1 does not respond in the same manner to cDDP and Cu in both tumor cells and in normal cells.

7. Cisplatin uptake in 2008 and C13*5.25 cells

While examining the A2780 and A2780CP cells, we discovered that unlike the HEK cells, the ovarian carcinoma cells did not recover their surface hCTR1 after elevated extracellular copper incubation. Upon elevated copper incubation, cell surface hCTR1 protein expression was dramatically reduced. However when copper was removed, cell surface hCTR1 levels did not return to pre-copper levels. We next examined the 2008 and C13*5.25 cervical carcinoma cells under the same conditions. Exposure to elevated copper stimulated internalization of hCTR1, however removal of copper did not result in cell surface hCTR1 recovery (Figure 23, p. 64). Approximately 50- 70% less hCTR1 was at the membrane in copper

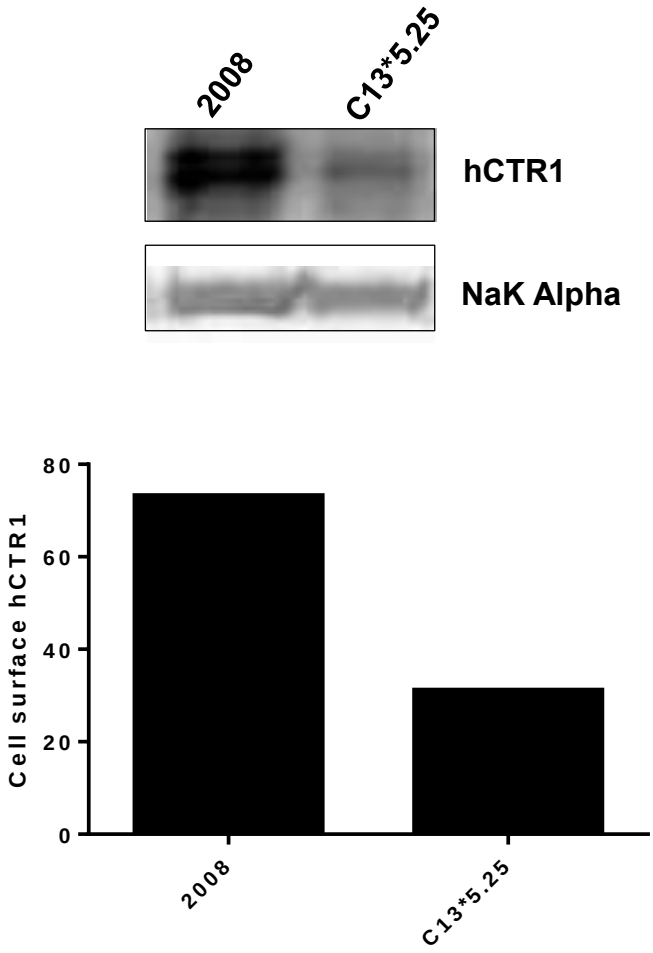

Fig. 21 Protein expression of hCTR1 in 2008 and C13*5.25 cervical tumor cells

2008 (cis-Pt-sensitive) and C13*5.25 (cis-Pt-resistant) cervical carcinoma cell pellets were collected. 5 step sucrose gradient fractionation procedure was performed and plasma membrane samples were collected. The fractionated protein was analyzed on Western blots, and a C-terminal hCTR1 antibody was used to detect hCTR1 protein. Alpha was used as a loading control.

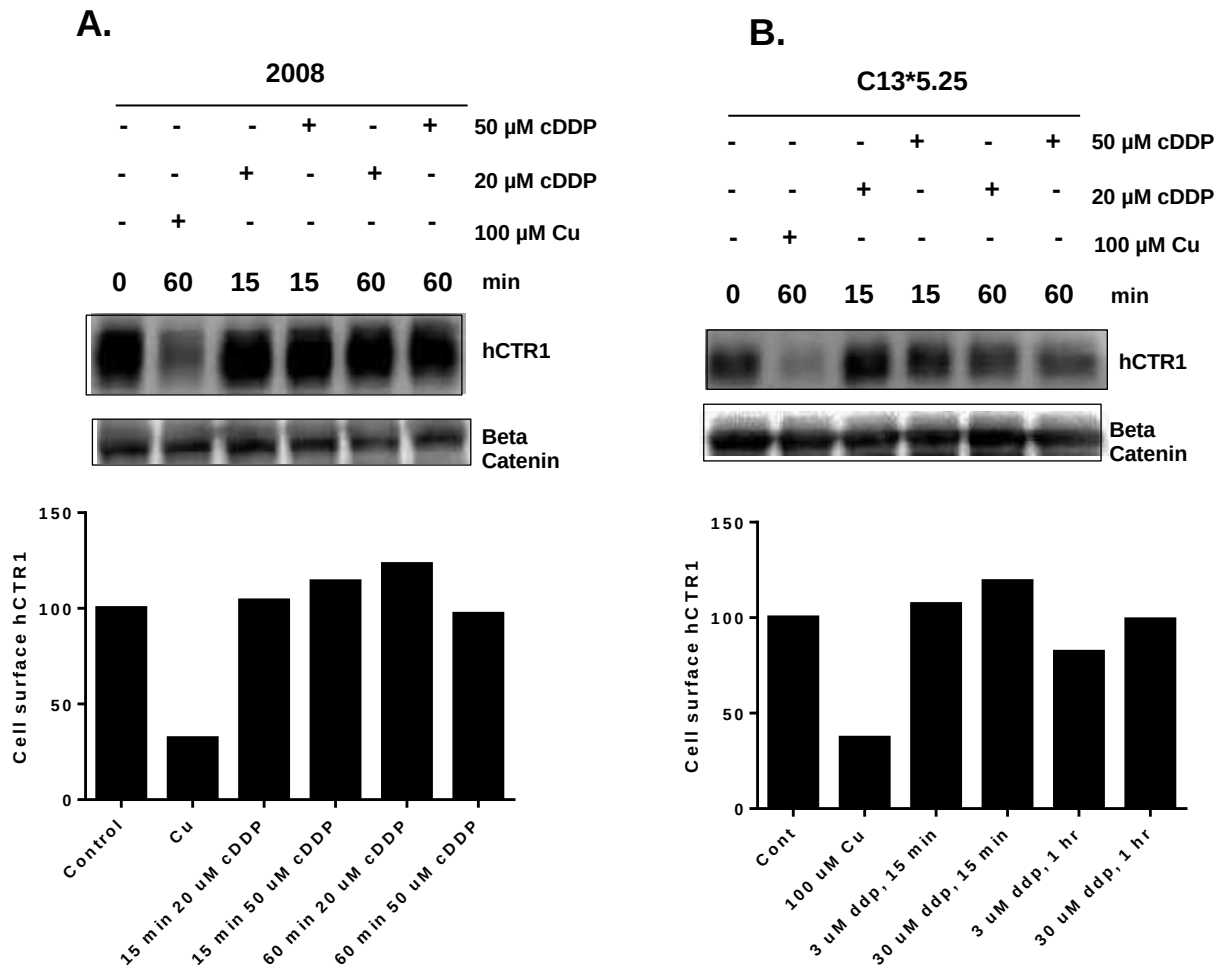

Fig. 22 Cell surface hCTR1 expression in response to cDDP

A) 2008 (cis-Pt-sensitive) and B) C13*5.25 (cis-Pt-resistant) cervical carcinoma cells were incubated with 100 µM copper for 1 hr, 20 µM ddp or 50 µM ddp for 15 minutes or 1 hr at 37° C. The cells were then placed on ice and biotinylated. The biotinylated protein was analyzed on Western blots, and a C-terminal hCTR1 antibody was used to detect hCTR1 protein. Beta catenin was used as a loading control.

free media. We incubated the cells with cDDP and measured accumulation. If cell surface hCTR1 is reduced by elevated copper and if hCTR1 is the major transporter for cDDP, then cDDP accumulation should be also reduced in cells pretreated with copper. cDDP accumulation was much higher in 2008 (cDDP-sensitive) cells in comparison to C13*5.25 (cDDP-resistant) cells. However, cDDP uptake was the same regardless of 50% less hCTR1 being present at the membrane due to copper-induced internalization (Figure 24, p. 65).

8. Copper stimulated internalization in 2008 cells

To ensure hCTR1 protein returning back to the membrane after copper exposure was not newly synthesized protein, we incubated the cells with a protein synthesis inhibitor, cyclohexamide, before copper treatment. The presence of cyclohexamide did not alter the amount of hCTR1 recovery. Incubation with a proteasomal inhibitor, MG132, also had no effect (Figure 25, p. 66). We conclude hCTR1 undergoes copper-induced internalization; however cDDP fails to stimulate hCTR1 relocalization in cervical tumor cells. We also show cDDP accumulation is independent of hCTR1, and therefore hCTR1 is not responsible for cDDP uptake in cervical tumor cells.

Discussion

Although understanding the role of hCTR1 in cDDP transport in non-tumor cells is important, the essential question is how cDDP is transported in tumor cells. It is in tumor cells where this anti-cancer drug is required to be taken up in order to function as a successful therapeutic agent.

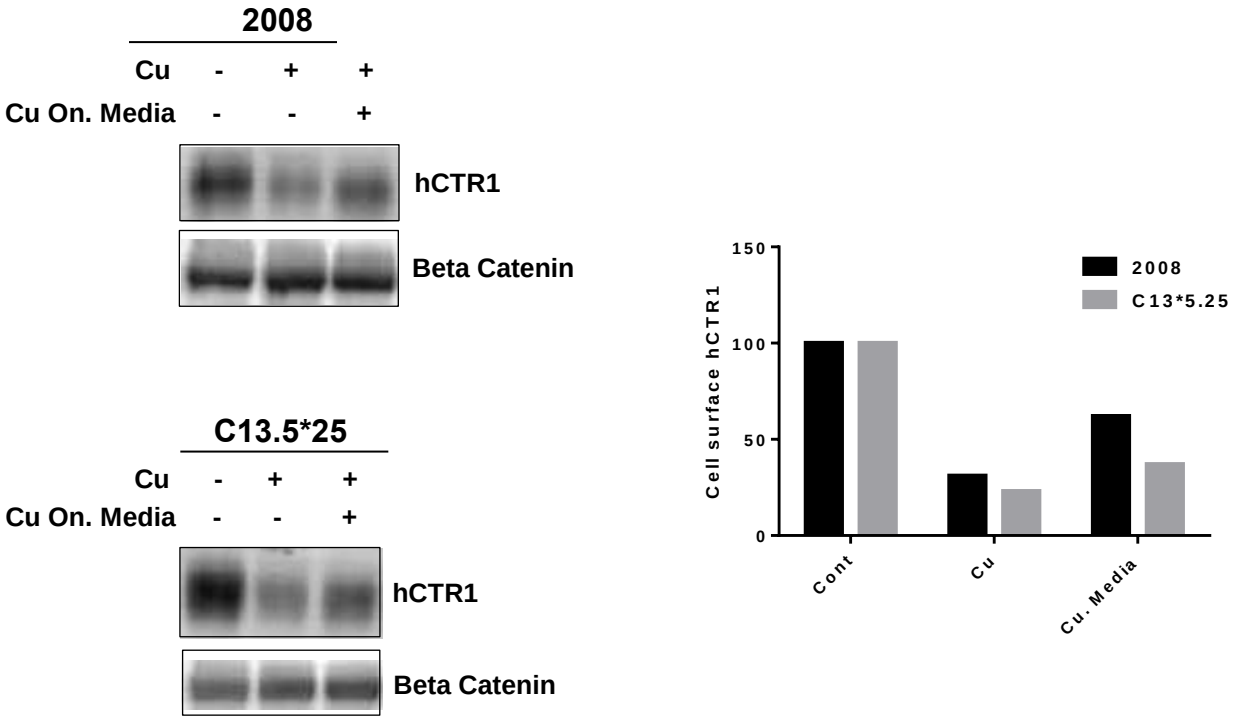

Fig. 23 Failure of hCTR1 to recycle back to membrane

2008 (cis-Pt-sensitive) and C13*5.25 (cis-Pt-resistant) cervical carcinoma cells were incubated with 100 µM Cu for 30 minutes, rinsed, and placed back into media for 30 minutes. The cells were then placed on ice and biotinylated. The biotinylated protein was analyzed on Western blots, and a C-terminal hCTR1 antibody was used to detect hCTR1 protein. Beta catenin was used as a loading control.

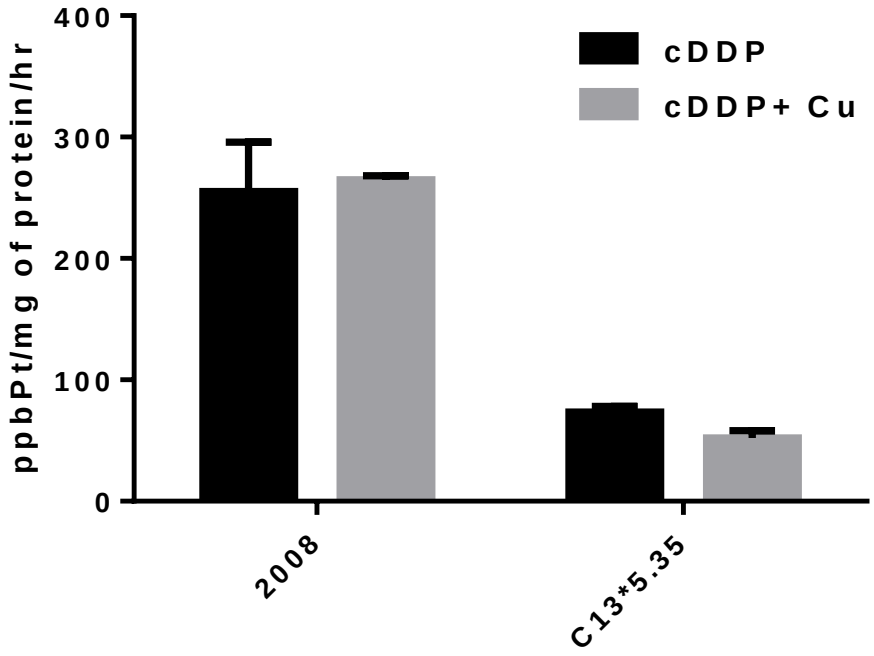

Fig. 24 cDDP uptake in presence of copper

2008 (cis-Pt-sensitive) and C13*5.25 (cis-Pt-resistant) cervical carcinoma cells were incubated with 100 µM Cu for 30 minutes, rinsed, and placed back into media for 30 minutes. Cells were then incubated with 30 µM ddp for 3 hrs, washed, pelleted and resuspended in 70% nitric acid for 3 hrs. Afterwards, the cells were diluted to 14% nitric acid with water, mixed, centrifuged and the supernatent was sent off for ICP-OES analysis at the Chemical Analysis Laboratory in Athens, Ga.

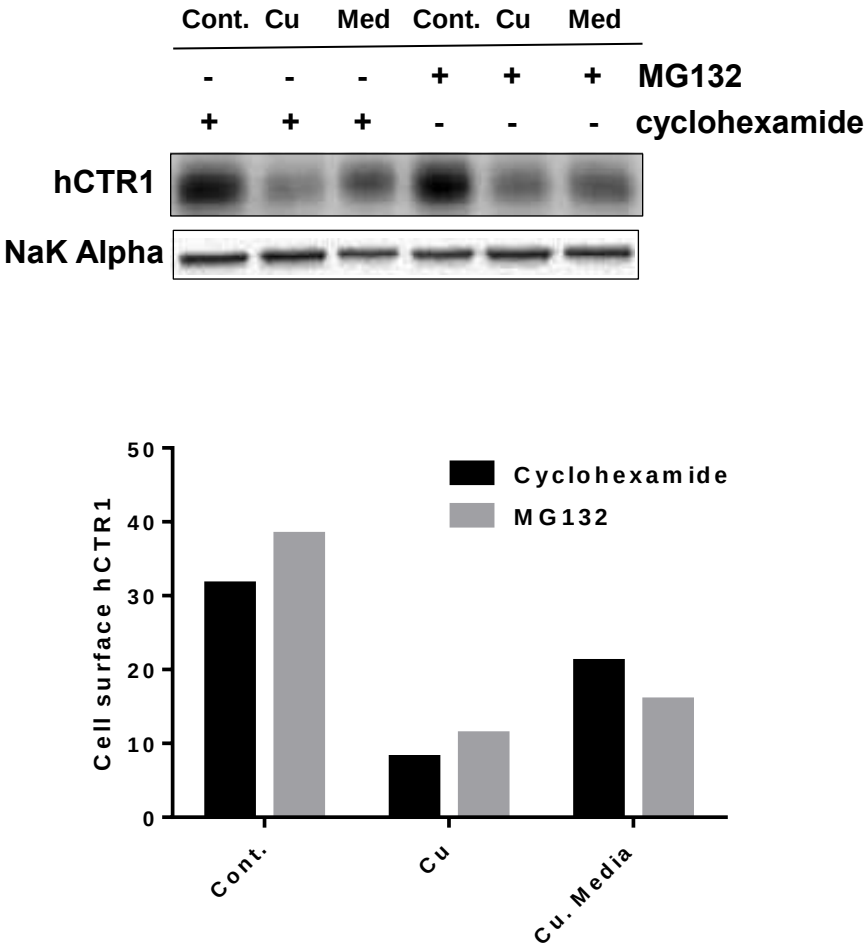

Fig. 25 Copper-stimulated internalization in 2008 cells

2008 (cis-Pt sensitive) cervical carcinoma cells were pretreated with 100 mg/ml of cyclohexamide or 10 µM MG132 for 30 min. The cells were then incubated with 100 µM Cu for 30 minutes, rinsed, and placed back into media for 30 minutes. The cells were then placed on ice and biotinylated. The biotinylated protein was analyzed on Western blots, and a C-terminal hCTR1 antibody was used to detect hCTR1 protein. Beta catenin was used as a loading control.

cDDP uptake in tumor cells.

Our observations on cDDP entry into tumor cells replicate findings in normal cells: (a) copper treatment which lowers surface hCTR1 does not affect cDDP entry (Figures 17A, 17B, p. 55); (b) lowering hCTR1, by 80% using siRNA (Figures 18, p. 56), reduces copper entry (Figure 19A, p. 57), but not cDDP uptake (Figure 19B, p. 57); (c) exposure to cDDP does not cause internalization of hCTR1 (Figures 16, p. 54).

In ovarian carcinoma cells (A2780), Holzer et al achieved twenty-fold over-expression of hCTR1 and only 30-50% increase in cDDP accumulation (Holzer, Samimi, et al. 2004). They also reported cDDP treatment of cells decreased hCTR1 and copper uptake. Subsequently, they confirmed these observations, and interestingly that increased cDDP accumulation was not associated with increased cytotoxicity (Holzer, Samimi, et al. 2004). In contrast, it was recently reported on the same cells, in agreement with our findings, that increased membrane levels of hCTR1 had no effect on cDDP accumulation and cDDP treatment did not decrease hCTR1 levels (Kalayda, Wagner, and Jaehde 2012). Similarly, in cervix squamous carcinoma cells (A431), hCTR1 overexpression (3-4 fold) had no effect on cDDP accumulation (Beretta et al. 2004). In tumor cells, the concentration-dependence of cDDP uptake has been reported. In agreement with our results in A2780 cells, uptake is linear up to 100 µM cDDP in 2008 cells (Andrews et al. 1988), in Ehrlich ascites cells (Gale et al. 1973) and in A2780 and HeLa cells (Zisowsky et al. 2007). In small cell lung carcinoma cells a Km of about 15 µM for cDDP uptake was reported, (Liang et al. 2009) a value that is considerably lower (at least an order of magnitude) than reported in other cells.

The entry mechanism of cDDP.

It seems likely that most of cDDP entry into tumor cells is mediated via a non-protein-mediated pathway. This is supported by our observations that over a wide range of concentrations no saturation of uptake is seen (Figure 15B, p.52), and that the cis and trans

isomers of cDDP have identical uptake rates (Figure 20B, p. 59). Our data support entry being predominantly via solubility-diffusion through the membrane (Gately and Howell 1993). Other candidates have been proposed (OCT2, Na^+ K^+ ATPase, and SLC family members) (Ahmed et al. 2009, Koepsell and Endou 2004, Filipski et al. 2009). Our observations suggest they are not primary players.

Internalization of hCTR1 in response to cDDP.

Study of hCTR1 has been hampered by limitations of many of the available antibodies. For example, a very interesting study of the effects of mutations on cDDP uptake in small cell lung cancer cells (Liang et al. 2009) shows an apparent mass of 25 kDa for both wild-type and the N15Q mutant. It has been shown in insect cells that N15Q has a reduced mass of endogenous protein by about 10 kDa in comparison to wild-type (Eisses and Kaplan 2002). hCTR1 can appear as multiple bands above 35 kDa, due to the presence of stable monomers and dimers (Eisses and Kaplan 2002, Maryon, Molloy, and Kaplan 2007) which complicates analysis. These issues make it important that antibodies used to show the appropriate behavior in Western blots: i.e. shift to lower mass on enzymatic deglycosylation (or with a non-glycosylated mutant), do not appear in cells that do not express hCTR1 or disappear on down-regulation with siRNA. In HEK cells, we do not observe any hCTR1 internalization with cDDP, although extracellular copper causes internalization. This confirms previous findings in HEK cells with cDDP using epitope-tagged hCTR1 and an anti-myc Ab (Guo, Smith, and Petris 2004). cDDP has been claimed to cause internalization and degradation in A2780 cells (Holzer, Samimi, et al. 2004) but has been recently disputed (Kalayda, Wagner, and Jaehde 2012), in agreement with the present work. Similarly, in yeast, relocalization of CTR1 following cDDP treatment was not observed (Sinani et al. 2007).

One potentially significant difference in the response of hCTR1 to copper in normal cells and tumor cells was that the tumor cells do not seem to show the recovery (recycling) of hCTR1

at the plasma membrane when the extracellular copper load is returned to low levels (Figure 17A, p. 55). It is unclear why this occurs, but it suggests that tumor cells may respond to copper load differently than normal cells. Little is known about the endocytosis and recycling mechanisms acting on hCTR1 and experiments are underway to characterize these pathways. Although hCTR1 is generally expressed at very low levels, there has not been a systematic study of comparative hCTR1 levels in tumor cells and the normal cells from which they are derived.

In order for cDDP to be effective it must enter the cell. One of the major limitations in drug action is entering cells by either passive diffusion or protein-mediated uptake. Uptake of cDDP resembles more closely a solubility diffusion mechanism rather a protein mediated uptake system. In a solubility diffusion system, solutes partition into the membrane based upon their hydrophobicity. This type of uptake is usually characterized by non-saturating uptake where specific inhibitors are ineffective. Early studies reported uptake of cDDP was mainly through passive diffusion (Alberts et al. 2002). Evidence supporting this theory included a non-saturating uptake system in Ehrlich ascites tumor cells, indicating the rate-limiting factor was concentration of drug and not a specific protein transporter (Gale et al. 1973). Additional reports showed uptake of cDDP in human ovarian carcinoma 2008 cells was not inhibited by analogs of cDDP: transplatin and carboplatin (Andrews et al. 1987). We also observed that cDDP uptake is non-saturating and not inhibited by copper in normal and tumor cells. The partition coefficient reflects the hydrophobicity of a drug based on how well it distributes in an organic phase compared with an aqueous phase. cDDP has a partition coefficient of log p_{oct} = -2.53 whereas carboplatin is -2.3 and oxaliplatin is -1.65 (Screnci et al. 2000). While we would predict cDDP and carboplatin to have similar rates of transport since their partition coefficients are comparable, carboplatin rate of uptake is typically one fourth that of cDDP. Based upon the partition coefficient of oxaliplatin, this drug should have a higher rate of uptake than cDDP. However, cDDP enters the

cell faster than both oxaliplatin and carboplatin. Our results point to a solubility diffusion pathway.

We have examined the role of hCTR1 in cDDP accumulation in ovarian carcinoma cells as well as cervical tumor cells and have found no evidence that it plays a major role in the uptake of cDDP, and therefore is not the cause of the development of resistance. If reduced drug uptake is a major factor in resistance, hCTR1 is unlikely to be a productive target.

Acknowledgements

We would like to thank Fox chase cancer society for the A2780 and A2780CP ovarian tumor cells and Stephen Howell (University of California, San Diego) for the 2008 and C13*5.25 ovarian tumor cells. These studies were supported by NIH Grant P01 GM 067166.

IV. hCTR1-Independent Copper Uptake

A. Introduction

Although hCTR1 is the major high affinity copper transporter, other systems may contribute to copper uptake. In this chapter, several non-hCTR1 candidates have been examined. After food is broken down by digestive enzymes in the stomach, it travels to the small intestine which is the first site of copper acquisition. hCTR1 is responsible for high affinity copper uptake and thus was thought to be located on the apical (lumen) membrane in intestinal cells. However, further investigation demonstrated hCTR1 resides on the basolateral membrane in epithelial intestinal cells (Zimnicka, Maryon, and Kaplan 2007). Cell surface biotinylation and immunofluorescence assays in intestinal and renal epithelial cells detected hCTR1 on the basolateral membrane in these polarized cells. Radioactive copper uptake assays measured a 7-fold increase of copper uptake on the basolateral membrane (blood side) of polarized cells in comparison to copper uptake on the apical membrane (lumen side). The role of hCTR1 on the basolateral membrane allows for copper uptake from the blood into the intestine (Zimnicka, Maryon, and Kaplan 2007). However, this location of hCTR1 does not allow for uptake of copper from the intestine of newly processed nutrients taken up from an individual's diet. Intestine-specific knockout mice accumulated high levels of copper despite the absence CTR1 (Nose, Kim, and Thiele 2006). Also mouse embryonic fibroblasts deficient of CTR1 were able to transport copper ~ 30% of control cells (Lee, Petris, and Thiele 2002). These results suggest the presence of a system other than CTR1 present at the apical (lumen side) membrane functioning in the acquisition of copper from the diet.

One of the candidates suggested to play a role in the uptake of copper is DMT1, divalent metal transporter 1. DMT1 is expressed in all tissues and is a member of the highly conserved Nramp (natural resistance associated macrophage protein) family of proteins. DMT1 has mostly been documented as an iron uptake protein (Bannon et al. 2003). Like copper, iron is a trace

metal essential to sustain life. Iron plays an integral role with several proteins such as hemoglobin, myoglobin, NADH dehydrogenase, cytochromes, and phosphatases. Iron has two oxidation states: ferrous (Fe^{2+}) and ferric (Fe^{3+}). The oxidation of iron by enzymes is critical for several processes like oxygen transport and storage, energy generation, free radial detoxification, and synthesis of DNA (Fox, 1997, Comprehensive biological catalysis). Humans absorb iron from their dietary intake from foods like oysters, beef, poultry, fish, and dried fruit to name a few. Approximately 1 mg of iron is taken up daily (Linder et al. 2003). Iron is absorbed in the intestine which is the major site of iron absorption and regulation. Before entry into cells, Fe^{3+} must be reduced by a cell surface protein, DcytB, to Fe^{2+}. Residing at the plasma membrane, DMT1 functions to allow passage of Fe into the cell (Linder et al. 2003).

Early studies demonstrated DMT1 functions in the uptake of iron ions. The ferrous state (Fe^{2+}) was preferred by DMT1. Further investigation suggested DMT1 could transport a wide array of divalent metals including Mn, Cu, Co, Ni, Pb, Zn, and Cd. Two microelectrode voltage clamp system was utilized to measure currents of oocytes injected with DMT1 mRNA while in the presence of varying metals. The addition of Fe^{2+} stimulated a large, positive inward current at a pH of 5.5. Similar large inward currents were seen when Zn^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+}, or Cd^{2+}. Less significant currents were seen with Pb^{2+} and Ni^{2+} (Gunshin et al. 1997). Further studies investigated DMT1 as a potential uptake protein for different metals. Intestinal Caco2 cells were transfected with antisense oligonucleotides to DMT1 resulting in an 80% reduction in iron uptake in comparison to control cells. Not only was iron uptake decreased, copper uptake was also reduced by 47%. Iron uptake was inhibited by the addition of copper and vice versa. Copper uptake was more significantly inhibited when ascorbate was present reducing Cu^{2+} to Cu^{1+}. These results suggested not only is DMT1 an iron transporter, but also DMT1 possibly could play a role in delivering copper across the brush border of intestinal cells (Arredondo et al. 2003).

Another potential candidate for transporting copper is CTR2. CTR2 shares homology to CTR1; however, its function is less well understood. Like CTR1, sequence alignment suggests CTR2 consists of 3 transmembrane domains (See Figure 26, p. 74). CTR2 contains the metal binding motif, MXXXM, located in the second transmembrane domain which has been shown to be essential for copper uptake in CTR1. CTR2 also contains amino acids in the first transmembrane domain that allow for oligomerization in CTR1. Although CTR2 and CTR1 have several similar features, CTR2 also has key differences that possibly could determine if and how CTR2 functions as a copper transporter. Histidine and methionine motifs located in the N-terminus essential for high affinity copper uptake by CTR1 are not present in CTR2 (Zhou and Gitschier 1997, van den Berghe et al. 2007). Also N15, which is required for N-linked glycosylation, as well as Thr27, which is required for O-linked glycosylation, are absent (van den Berghe et al. 2007, Eisses and Kaplan 2002). Yeast cells lacking CTR1 showed growth defects as a result of copper deficiency. Overexpression of CTR2 did not improve high affinity copper uptake; however, yeast cells were more resistant to copper toxicity. The localization of CTR2 in yeast cells were detected in the vacuolar membrane (Kampfenkel et al. 1995). No studies have examined the localization of hCTR2 in polarized intestinal cells. Based upon these observations, CTR2 might function as a copper transport protein. However since the motifs essential for high affinity copper uptake are missing, it has been suggested low affinity uptake is more likely (van den Berghe et al. 2007).

We examined Caco2 enterocytes to investigate the mechanism of dietary copper uptake and cell lines expressing DMT1 to examine its role in copper transport. In addition we reinvestigated the copper transport properties of hCTR2.

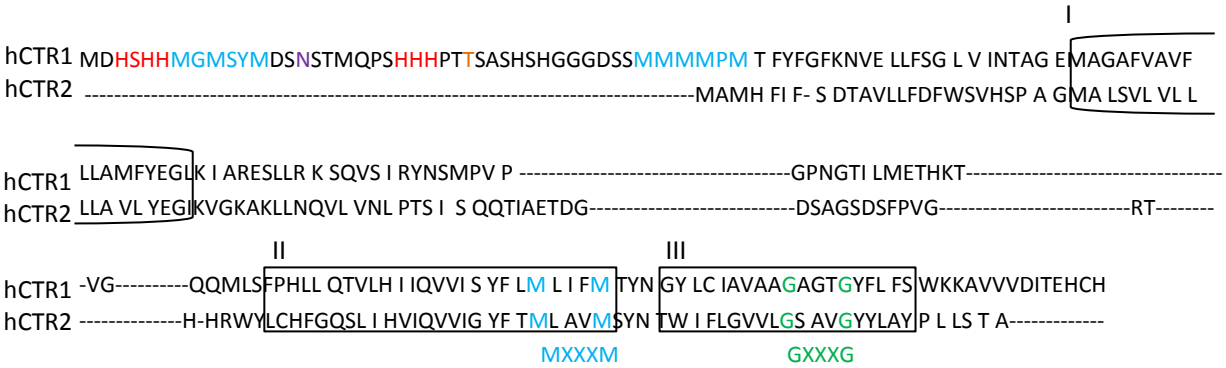

Fig. 26 Sequence alignment of hCTR1 and hCTR2

Sequence alignment of hCTR1 and hCTR2. Transmembrane regions are enclosed in boxes and indicated by roman numerals. Histidine motifs are indicated in red, methionine motifs in blue, and glycine motifs in green. Asn^{15} is indicated in purple and Thr^{27} in orange.

B. Experimental Procedures

See protocols described in detail in chapter 2

Cell culture. All cells were maintained in a humidified incubator at 37°C under a 5% CO_2 atmosphere. Caco2 cells were grown in growth media consisted of DMEM (no. 11995, GIBCO) supplemented with 20% fetal bovine serum (FBS; no. s11550, Atlanta Biologicals.) For polarization studies, Caco2 cells (HTB-37 purchased from American Type Culture Collection) were grown on 24-mm Polyester membrane Transwells with 0.4 µm pores (no. 3450, Corning) until confluent, after which cells were grown for additional 2 wks until differentiated, with the growth medium exchanged every 1–2 days. Formation of tight junctions was monitored by the measurement of transepithelial electrical resistance (TEER) using an EVOM meter and STX2 electrodes (World Precision Instruments). Caco2 cells were judged to be differentiated when TEER value was ~250 Ω·cm2, which usually developed ~14 days postconfluence.

DMT1 sense, DMT1 antisense, and HEK293T parental cells were obtained from Marianne Wessling-Resnick (2009, Buckett, Am J Physio) and were grown in alpha MEM (CellGro) with 10% FBS and 50 U/ml penicillin and 50 g/ml streptomycin. DMT1 antibody (rabbit) used was received from Michael Garrick.

HEK 293 Flp-In cells containing tetracycline-regulated N-terminal FLAG–tagged, C-terminal FLAG-tagged, and N-terminal GFP-tagged hCTR2 were generated by ligating hCTR2 cDNA (received from Vinzenz Unger, Yale) into the FLp-InTM vector pcDNA5/FRT/TO© (Invitrogen) as previous described (Maryon, Molloy, and Kaplan 2007). These cells were maintained with the following selective antibiotics: 100ug/ml zeocin/and 10µg/ml blasticidin. The cells were transfected with hCTR2 construct using Lipofectamine 2000 (Invitrogen). Transfected cells were selected in 12 µg/ml blastocidin S (RPI Corp.) and 400 µg/ml hygromycin (Invitrogen). Resistant colonies were pooled and tested for tetracycline-regulated expression. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal

bovine serum (Atlanta Biologicals), and 25mM Hepes containing 1 µg/ml tetracycline for 48 hrs before harvesting. hCTR2 antibody (rabbit) used was a gift from Jesse Bertinato (Sir Frederick G. Banting Research Centre).

Reagents and buffers. Choline chloride, sodium-gluconate, potassium- gluconate, magnesium gluconate, bumetanide, glybenclamide, niflumic acid, 4-acetamido-4'-isothiocyanato-2,2'-stilbenedisulfonic acid disodium salt hydrate (SITS), 4,4'-diisothiocyanato-2,2'-stilbenedisulfonic acid disodium salt hydrate (DIDS), L-histidine, and D-histidine, and other amino acids were all purchased from Sigma. The composition of the transport buffers used was as follows: salt buffer or buffered saline medium (chloride buffer) containing (in mM) 150 NaCl, 5 KCl, 2.5 $MgCl_2$, and 25 HEPES, pH 7.4; gluconate buffer (chloride-free buffer) containing (in mM) 150 sodium gluconate, 5 potassium gluconate, 2.5 magnesium gluconate, and 25 HEPES, pH 7.4; sulfate buffer (chloride-free buffer) containing (in mM) 100 Na2SO4, 3.3 K2SO4, 3.75 $MgSO_4$, and 25 HEPES, pH 7.4; and choline buffer (sodium-free buffer) containing (in mM) 150 choline chloride, 5 KCl, 2.5 $MgCl_2$, and 25 HEPES, pH 7.4. In cases where pH of buffers was lowered from 7.4 to 6.4 or 6.0, 25 mM HEPES was substituted with 25 mM PIPES. The bicarbonate-buffered saline medium (bicarbonate buffer) contained (in mM) 150 NaCl, 5 KCl, 2.5 $MgCl_2$, and 25 $NaHCO_3$, pH 7.4.

Effects of DMEM and serum on copper fluxes were tested using the following transport buffers: *1*) DMEM, 10% FBS, and 25 mM HEPES, pH 7.4; *2*) salt buffer with 10% FBS (150 mM NaCl, 5 mM KCl, 2.5 mM MgCl2, 10% FBS, and 25 mM HEPES, pH 7.4); and *3*) salt buffer supplemented with selected DMEM components (in mg/l: 84 arginine, 62.6 cysteine, 584 glutamine, 30 glycine, 42 histidine, 104.8 isoleucine, 104.8 leucine, 146.2 lysine, 30 methionine, 66 phenylalanine, 42 serine, 95.2 threonine, 16 tryptophan, 103.8 tyrosine, 94 valine, 4 calcium pantothenate, 4 choline chloride, 4 folic acid, 7.2 inositol, 4 nicotinamide, 4 pyridoxine, 0.4

riboflavin, 4 thiamine, 4,500 glucose, and 110 sodium pyruvate, in saline buffer of 150 mM NaCl, 5 mM KCl, 2.5 mM MgCl2, and 25 mM HEPES, pH 7.4). Hypotonic saline medium was prepared by addition of extra 10, 25, or 50 mM sodium gluconate to the standard salt buffer, as follows: 150 mM NaCl, 5 mM KCl, 2.5 mM $MgCl_2$, and 25 mM HEPES, pH 7.4, supplemented with either 10, 25, or 50 mM Na-gluconate.

^{64}-Cu uptake. Assays for copper uptake in Transwells employed Caco2 cells plated on 4.67-cm^2 permeable membrane supports in Transwells (Corning) and grown as described above, until polarized. Before the assay, the cells were washed twice in transport medium and fresh transport medium was then added to the apical (1.35 ml) and to the basal (1.8 ml) compartments. Cells were equilibrated with the medium for 30 min at 37°C. Cu uptake was initiated by addition of 10 μM copper solution containing trace levels of 64Cu (Division of Radiological Sciences, Mallinckrodt Institute of Radiology, Washington University School of Medicine, St. Louis, MO) and the desired concentration of $CuCl_2$. Assays were run for 1 h at 37°C, and copper transport was terminated by addition of ice-cold stop buffer (150 mM NaCl, 5 mM KCl, 2.5 mM $MgCl_2$, 25 mM HEPES, pH 7.4, and 10 mM Na_2EDTA), after which cells were washed three additional times\with ice-cold stop buffer. The wash media were then aspirated, and membrane supports were excised from the Transwells with a scalpel and placed in a container with Eco-Lume scintillation liquid (no. 882470, ICN Biomedicals) for scintillation counting (model LS6500, Beckman-Coulter). Experiments done on impermeable surfaces were carried out as described above, except that after the last wash, cells were resuspended in 0.1 N NaOH and removed for scintillation counting, with a small aliquot being retained for determination of protein concentration. All transport determinations were carried out intriplicate. ^{64}Cu content of the initial tracer-containing buffer was determined for the calculation of specific activity. Cu uptake was then expressed as pmoles of Cu taken up by the cells per milligram protein per hour, following determination of the protein content of the cell monolayer by the Bio-

Rad protein assay (model 500-0006, Bio-Rad). Effects of drugs, histidine, ascorbate, or competition by Fe and Mn ions with 64Cu uptake were determined by inclusion of a 50-fold molar excess of $FeSO_4$ or $MnNO_3$, or the indicated concentration of drug or amino acid, in the transport medium for 30 min before the assay, for equilibration. The assay was begun by addition of 10 μM $CuCl_2$ labeled with trace amounts of ^{64}Cu, was run for 1 h at 37°C, and was then terminated with addition of ice-cold stop buffer.

ICP-OES-To determine the amount of various accumulated metal content, cells were incubated in a metal solution, washed, pelleted and resuspended in 70% nitric acid for 3 hrs. Afterwards, the cells were diluted to 14% nitric acid with water, mixed, centrifuged and the supernatent was sent off for ICP-OES analysis at the Chemical Analysis Laboratory in Athens, Ga.

C. Results

1. Anion-dependent copper uptake

a. Copper uptake in FBS-supplemented growth medium (DMEM) or in HEPES-buffered salt medium.

Previous work examined the ability of polarized enterocytes to transport copper across either the apical or basolateral plasma membrane. These studies were performed in the presence of normal growth media supplemented with 10% fetal bovine serum. Radioisotopic copper measurements demonstrated much higher uptake rates on the basolateral membrane than the apical membrane which correlated well with higher cell surface protein expression of hCTR1 as examined by biotinylation assay and confocal images (Zimnicka, Maryon, and Kaplan 2007).

In the present work, we examined radioisotopic copper uptake rates in polarized Caco2 intestinal cells in the presence of salt buffer. We observed 3 very interesting results: 1) The asymmetry in the rate of copper uptake across the basolateral side versus the apical side was

not as pronounced in salt buffer as was seen in DMEM 10% FBS media; 2) Total copper uptake values were much higher in salt buffer; 3) In salt buffer, silver did not inhibit copper uptake across the apical membrane, although about 50% copper uptake inhibition was evident on the basolateral membrane. On the other hand, silver inhibited copper uptake by 90% on the basolateral membrane in DMEM 10% FBS. Silver is a well-known inhibitor of hCTR1- mediated copper uptake (Figure 27A, p. 81).

As Figure 27A demonstrates (p. 81), the absolute copper uptake values are significantly higher in salt buffer than in DMEM supplemented with 10% FBS. DMEM is composed of amino acids, vitamins, and inorganic salts which act as nutrients for cell growth. To investigate the effect of DMEM on the rate of copper entry, all the amino acids of DMEM were added to the salt buffer. This addition dramatically inhibited copper transport. The addition of FBS furthered reduced copper uptake (Figure 27B, p. 81).

b. Dependence of copper uptake rates on ionic composition of the medium.

The two major components of the salt buffer are sodium and chloride. To evaluate the importance of each component individually, chloride ions were replaced with gluconate or sulfate, keeping the ionic strength constant. Gluconate buffer reduced copper uptake on the apical membrane by 80% and the basolateral membrane by 30% (Figure 28A, p. 82). Replacement of chloride ions with sulfate had similar results. Substitution of the sodium ions with choline had no effect on copper uptake. These results very strongly demonstrated the rate of copper entry on the apical membrane in salt buffer is highly dependent on the presence of chloride ions, but not sodium ions. Removal of chloride ions may cause cell shrinkage. It is possible that a reduction in cell volume could affect the rate of copper uptake. We think it is unlikely that a volume-sensitive mechanism would have such a dramatic effect on copper uptake (~80 reduction). To ensure the reduction in the rate of copper entry was not a consequence of chloride ions exiting (and therefore cell shrinkage), we placed the cells in a

hyperosmotic environment which would cause cells shrinkage before measuring copper uptake. The osmolarity of blood serum is ~300 mosM while the salt buffer and growth media is ~360 mosM. We decided to lower the amount of NaCl in salt buffer from 150 mM to 125 mM resulting in a final osmolarity of ~310 mosM (which is comparable to blood serum). The rate of copper uptake was decreased by 30% with the addition of 10 mM Na gluconate (from 1,030 ±33 pmol cu/mg protein/hr to 728 ±133 pmol cu/ mg protein/hr) while the addition of 25 mM Na gluconate produced a similar reduction. The rate of copper uptake in salt buffer with osmolarity closely matching blood serum was very similar to the uptake rate of copper in growth media. Also increasing levels of Na gluconate produced similar effects on copper uptake. Thus the rate of copper uptake is dependent on chloride ions and not a volume-dependent regulation of copper transport.

c. Inhibition of copper uptake by histidine.

These results demonstrate the complex mixture of DMEM components hinder the entry rate of copper uptake. We reasoned that components which have the ability to bind to copper would inhibit copper uptake. Histidine is an amino acid that is well known to bind to copper, and thus we investigated its potential to inhibit copper uptake. Figure 29A (p. 83) demonstrates the presence of 100 mM histidine reduced copper uptake 80% on the apical membrane and 30% on the basolateral membrane. Substitution of the naturally occurring L-histidine with D-histidine had no major differences in the rate of copper entry, suggesting a specific interaction of a protein site is not involved. No effect on the rate of copper entry was seen in the presence of glycine.

Large quantities of bicarbonate are secreted in the lumen of the intestine. We investigated what effect bicarbonate would have on copper uptake by replacing Hepes with sodium bicarbonate. A 25% reduction was seen (Figure 29A, p. 83). While examining the effect of bicarbonate, the rate of entry in salt buffer was 50% less of what had more frequently been

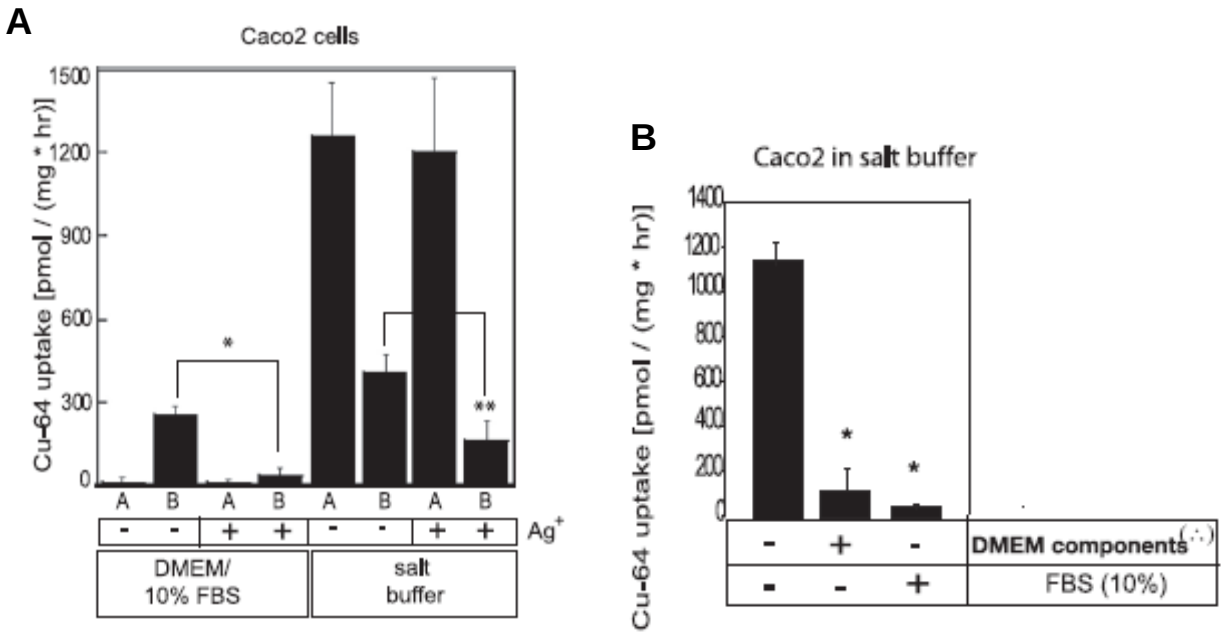

Fig. 27 Copper uptake in growth media and transport buffer

*A) (**A. Zimnicka**)*: polarized Caco2 cells. Caco2 cells were grown in Transwells until polarized, Uptake was initiated by addition of 5 μM $CuCl_2$ labeled with trace amounts of ^{64}Cu to either the apical (A) or basolateral (B) compartments of each Transwell at 37°C. The radioactivity retained by the cells after 1 h was determined by scintillation counting. Effect of silver on copper uptake was measured following the addition of 50 μM $AgNO_3$ to the transport buffer on both sides of the Transwells. Additions to the apical side of Transwell are indicated with letter A, and additions to the basolateral side are indicated with letter B. B) **(K. Ivy)** DMEM components and 10% FBS inhibit apical copper uptake. Caco2 cells were grown in Transwells until polarized. Apical copper uptake was assayed by addition of 5 μM radioactively labeled $CuCl_2$. Experiments were done in salt buffer alone, in salt buffer with the added components of DMEM or in salt buffer with 10% FBS. Assays were run for 1 h at 37°C, terminated with cold stop buffer, and rinsed 2× more with cold stop buffer. Filters were excised and radioactivity was determined using a scintillation counter.

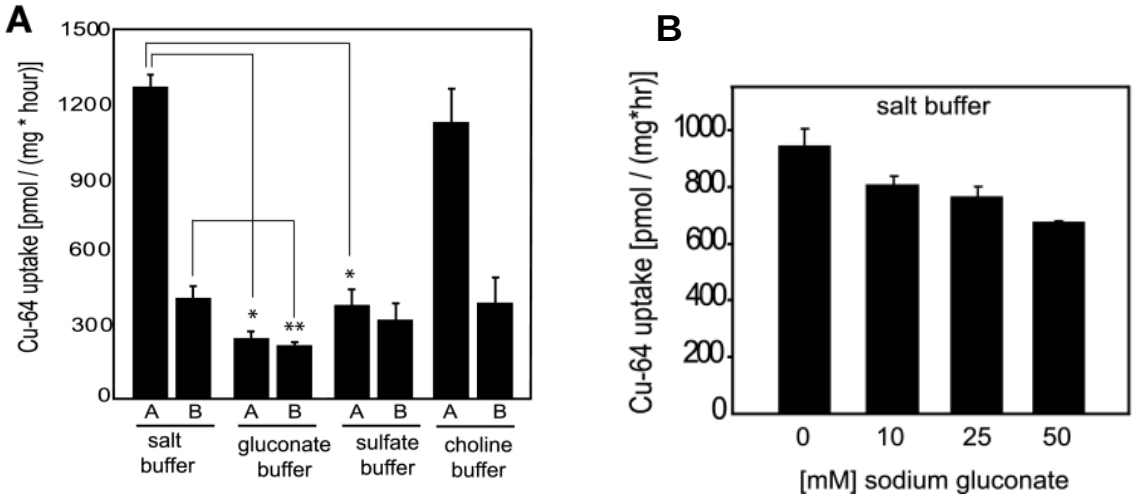

Fig. 28 Copper uptake and ionic composition of transport buffer

A) **(A. Zimnicka)** replacement of chloride and sodium ions in transport buffer. Caco2 cells were grown in Transwells and processed for the assay as described in Fig. 1. Transport buffer consisted of either standard salt buffer (150 mM NaCl, 5 mM KCl, 2.5 mM $MgCl_2$, 25 mM HEPES, pH 7.4), or chloride-free gluconate buffer (150 mM Na-gluconate, 5 mM K-gluconate, 25 mM HEPES, pH 7.4), or chloride-free sulfate buffer (100 mM Na_2SO_4, 3.3 mM K_2SO_4, 3.75 mM $MgSO_4$, 25 mM HEPES, pH 7.4). In addition, a sodium-free choline buffer was prepared, consisting of 150 mM choline chloride, 5 mM KCl, 2.5 mM $MgCl_2$, and 25 mM HEPES, pH 7.4. All the assays were begun by addition of 5 µM radioactive $CuCl_2$ and were carried out for 1 h at 37°C. B) **(K. Ivy)** copper uptake in hypotonic saline medium. Caco2 cells were grown in Transwells until polarized and copper uptakes were assayed from the apical side using 5 µM radioactively labeled $CuCl_2$. Transport buffer consisted of either standard saline medium (150 mM NaCl, 5 mM KCl, 2.5 mM MgCl2, 25 mM HEPES, pH 7.4), or hypotonic saline medium prepared by supplementing salt buffer with additional 10, 25, or 50 mM sodium gluconate (150 mM NaCl, 5 mM KCl, 2.5 mM $MgCl_2$, 25 mM HEPES, pH 7.4 plus 10 mM, 25 mM, or 50 mM sodium gluconate). Assays were run for 1 h at 37°C, terminated with cold stop buffer, rinsed twice with cold stop buffer. Filters were excised, and radioactivity was determined using a scintillation counter.

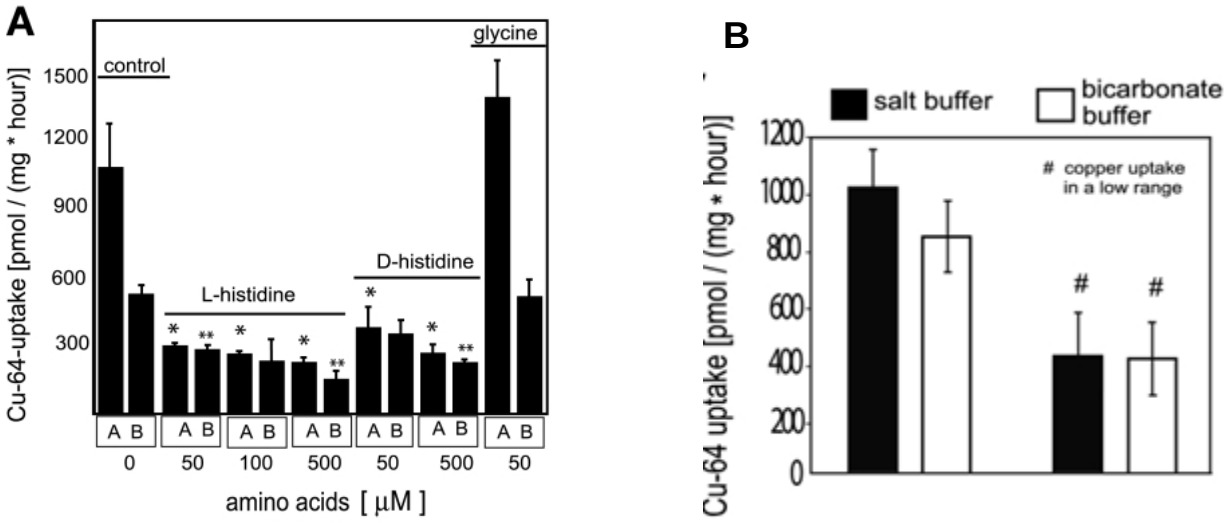

Fig. 29 Effects of amino acids and carbonate on copper uptake

A) **(A. Zimnicka)** inhibition of copper uptake by histidine. Cells were grown on filters until polarized. Assays were carried out in transport salt buffer consisting of 150 mM NaCl, 5 mM KCl, 2.5 mM $MgCl_2$, and 25 mM HEPES, pH 7.4. Uptake was initiated by addition of 5 µM $CuCl_2$ labeled with trace amounts of ^{64}Cu to either the apical or the basolateral compartments of each Transwell. Where indicated, l-histidine, d-histidine, or glycine was included in either the apical (A) or basolateral (B) compartment of Transwells. Assays were run for 1 h at 37°C, filters with cell monolayers were excised, and the amount of radioactivity in the cells was determined by scintillation counting. B) **(K. Ivy)** Effect of bicarbonate on copper uptake. Caco2 cells were grown in Transwells until polarized and assayed for ^{64}Cu uptake from the apical side as described in materials and methods. Transport buffer consisted of standard HEPES-buffered saline medium (150 mM NaCl, 5 mM KCl, 2.5 mM $MgCl_2$, 25 mM HEPES, pH 7.4) or bicarbonate buffer (150 mM NaCl, 5 mM KCl, 2.5 mM $MgCl_2$, and 25 mM $NaHCO_3$, pH 7.4). The mean ± SD obtained from triplicate measurements for 10 independent experiments is shown for each point (#-three independent experiments that fell into low range of copper uptake).

seen (400 pmol Cu/mg of protein/hr vs 100 pmol Cu/ mg of protein/hr). The addition of bicarbonate had less of an effect. From these studies, we conclude the presence of sodium bicarbonate, an abundant compound naturally found in the lumen of the intestine, does not significantly inhibit copper transport (Figure 29B, p. 83).

d. Bioavailability of copper for ATP7A.

A copper uptake system present in intestine-specific CTR1 knock mice transported copper to 8-fold higher levels than control mice. In these mice however, copper was not bioavailable as demonstrated by a reduction in activity of copper chaperones and copper-requiring proteins (Nose, Kim, and Thiele 2006). To determine whether copper taken up via the chloride-dependent anion system is bioavailable, polarized Caco2 intestinal cells were incubated with high levels of extracellular copper in salt buffer. When intestinal cells are polarized, tight junctions are formed dividing the cell into two sides. The apical side is the lumen side and is in contact with newly digested nutrients and ions from the diet. The basolateral side is the blood side where ions are transported out of the intestine into the portal circulation. As a indication of polarization, Na^+ K^+ ATPase beta subunit is located on the basolateral membrane in fully polarized intestinal cells. In Figure 30A (p. 86), Na^+ K^+ beta is detected solely on the basolateral membrane indicating tight junctions have been formed. Under steady state conditions, ATP7A resides at the trans golgi network incorporating cuproenzymes with copper. ATP7A relocates in vesicles to the plasma membrane when copper levels become elevated and thus functions to export copper. If copper is not available, ATP7A will remain at the trans golgi network. Figure 30A (p. 86) demonstrates an increase of cell surface ATP7A at the basolateral membrane in the presence of high copper load, whereas less hCTR1 is detected at the basolateral membrane in comparison to the apical membrane. These results show ATP7A and hCTR1 relocalize in response to extracelluar copper in transport buffer and thus copper taken up via the chloride-dependent anion system is bioavailable.

To further evaluate the bioavailability of copper for cuproenzymes and to eliminate the possibility of CTR1-mediated copper uptake, mouse embryonic fibroblasts expressing CTR1 (Mefs +/+) or not expressing CTR1 (Mefs -/-) were incubated with copper or bathocuproine disulfonic acid (Figure 30B, p. 86). BCS is a copper chelator and thus will limit the amount of copper available in growth media for uptake into cells. Biotinylation assay was performed to detect cell surface proteins that respond to extracellular copper. In the presence of BCS, low levels of ATP7A were detected in Mefs +/+ cells (Figure 30B, p. 86). However when elevated extracellular copper was added, there was a significant increase in cell surface ATP7A. Similar results were illustrated in Mefs -/- cells. In Mefs +/+ cells, CTR1 was more highly expressed at the membrane in the absence of copper than in the presence of copper. No detection of CTR1 was found in Mefs -/- cells. Na^+K^+ ATPase alpha subunit was used as a loading control. This experiment was performed in growth media. Substitution of salt buffer for growth media had similar findings (Data not shown). Together, these data suggest copper uptake via a non-CTR1 pathway is available to ATP7A as indicated by the relocalization of this export protein in response to copper from the trans golgi network to the plasma membrane in Mefs -/- cells. Furthermore, the presence of CTR1 is not required for such relocalization of ATP7A in Mefs +/+ cells.

We identified an hCTR1-independent copper uptake system that is highly chloride dependent and present in human intestinal epithelial cells as well as human embryonic kidney cells (Zimnicka, Ivy, and Kaplan 2011). hCTR1 is found on the basolateral membrane in intestinal cells allowing for entry of copper from the blood (Zimnicka, Maryon, and Kaplan 2007). However the system or systems involved in uptake of copper from the apical membrane are still unclear. Thiele documented a copper uptake system that did not rely on CTR1 while studying mouse embryonic fibroblasts. Mefs -/- had 30% of copper uptake to that of mefs +/+. These data indicated that CTR1 is not the only protein or uptake system capable of transporting copper. We questioned if other metal transporting proteins have the ability to transport copper.

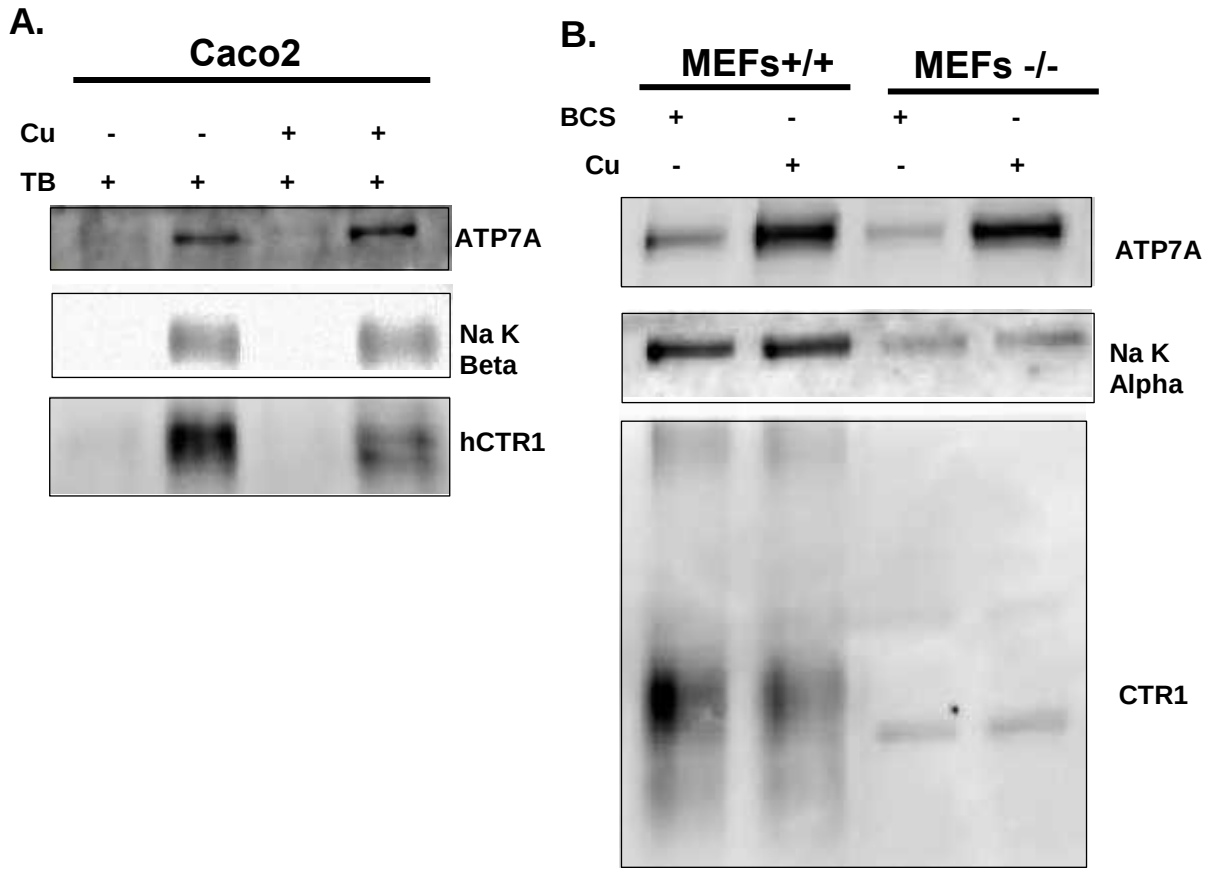

Fig. 30 Biotinylated ATP7A and hCTR1 in polarized Caco2 cells and MEFs

A) Caco2 intestinal cells were grown for 2 weeks on permeable membrane supports in Transwells until polarized. Before the assay, the cells were washed twice in transport medium and fresh transport medium was then added to the apical and to the basal compartments. Cells were incubated with 5 µM Cu for 1 hr. Cells were biotinylated and the biotinylated protein was analyzed on western blots. Protein levels were normalized to Beta catenin, a loading control.
B) Mefs +/+ and Mefs -/- cells were incubated with 200 µM BCS or 20 µM Cu in media for 2 hrs. Cells were biotinylated and the biotinylated protein was analyzed on western blots. Protein levels were normalized to Na^+ K^+ Alpha, a loading control.

2. DMT1, Divalent Metal Transporter 1

a. Protein expression and copper uptake

As previously mentioned, Gunshin et al suggested DMT1 plays a role in mediating copper transport (Gunshin et al. 1997). We reinvestigated the ability of DMT1 to transport copper utilizing HEK 293 cells over-expressing DMT1. Western blot shows DMT1 protein expression in DMT1-overexpressing cells (sense) but not in cells transfected with scrambled cDNA (control) or HEK cells (Figure 31, p. 88). Na^+ K^+ ATPase alpha subunit was used as a loading control. Next we examined if DMT1 mediates copper transport. Cells overexpressing DMT1 were incubated with 5 µM $CuCl_2$ with trace elements of radioactive ^{64}Cu for 1 hr (Figure 32. p.89). The assay was terminated with the addition of cold stop buffer, and the radioactivity retained in the cells was measured using a scintillation counter. The amount of copper taken up in the cells overexpressing DMT1 (sense cells) was the same as control cells. In the presence of a copper reducing agent, ascorbate, uptake in both cell lines was enhanced by equal amounts such that no difference was evident. These results suggest Cu (I) is the preferred oxidation state taken up by these cells; however DMT1 is not the protein responsible for the uptake.

b. Effect of low pH on copper uptake

Previous studies demonstrated metal transport by DMT1 is enhanced in environments of lower pH. Iron and lead uptake was measured in intestinal Caco2 cells as a function of pH and concentration. Iron uptake was saturable and over 2-fold greater at lower pH (5.5) in comparison to higher pH (7.4). Similar results were seen in cells incubated with lead such that uptake was saturable and enhanced at lower pH (Bannon et al. 2003). We incubated our cells with copper at pH 7.4 and pH 6.0 and measured radioactive ^{64}Cu uptake. At pH 7.4, no difference was detected between sense and control cells. Lower pH (6.0) produced an increase

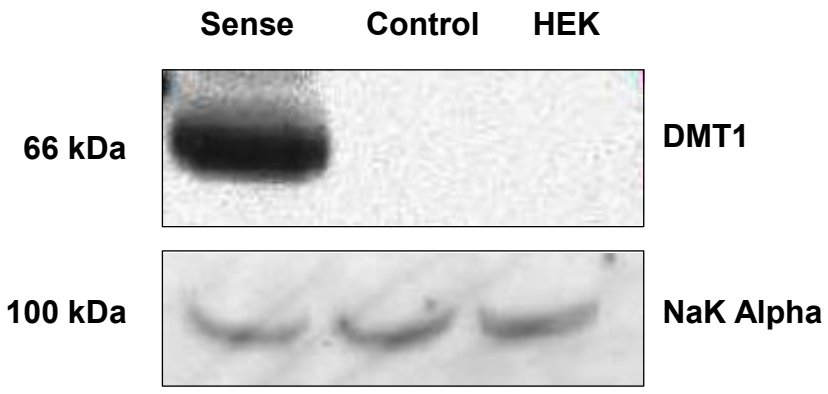

Fig. 31 Protein expression of DMT1

Human embryonic kidney cells overexpressing DMT1, control cells transfected with scrambled DMT1 cDNA, and untransfected cells were fractionated using a 5 step sucrose gradient. Plasma membrane preps were examined using Western blot analysis. Blots were probed with an anti-DMT1 antibody or anti-Na/K ATPase α subunit antibody which was used as a loading control.

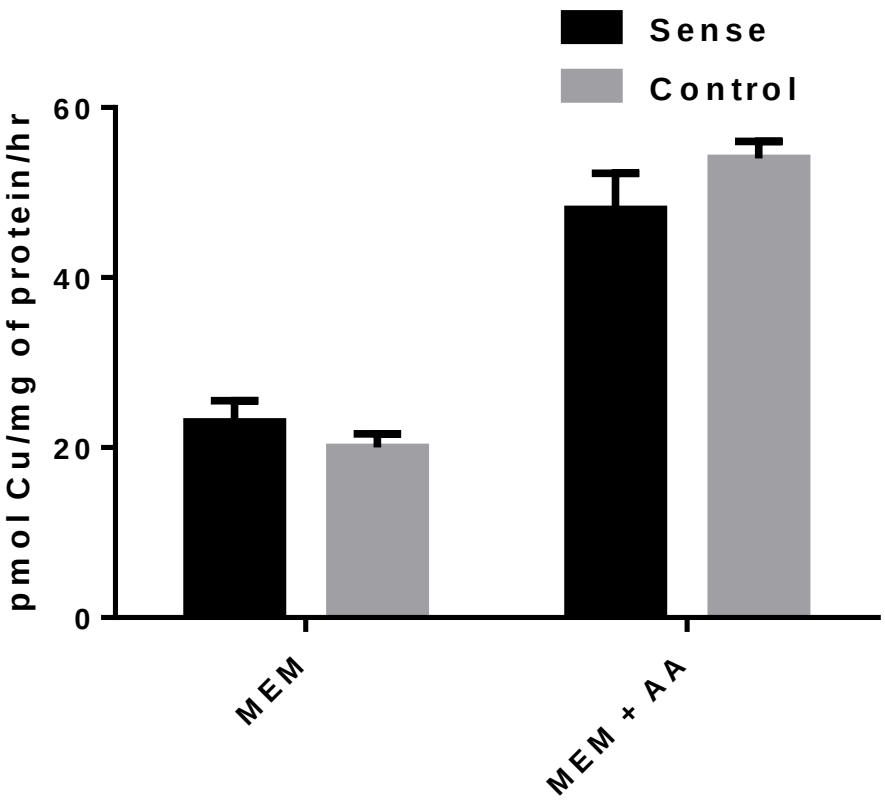

Fig. 32 Copper uptake in HEK293 cells overexpressing DMT1

Human embryonic kidney cells overexpressing DMT1 and control cells were incubated in media supplemented with 10% FBS. Uptake was initiated by addition of 5 µM $CuCl_2$ labeled with trace amounts of ^{64}Cu. Where indicated, ascorbate (1 mM) was added to investigate Cu^{1+} transport. Assays were run for 1 hour at 37º C and then terminated by the addition of ice-cold stop buffer. Radioactivity retained by the cells was determined by a scintillation counter. Uptake values were normalized by dividing by the amount of total protein.

in both cell lines but the copper uptake rates were comparable (Figure 33, p.91). The addition of ascorbate also enhanced uptake with the largest increase at pH 6. However, no significant difference in copper uptake was detected amongst the two cell lines (Figure 33, p. 91). These results suggest DMT1 is not the major protein responsible for copper uptake.

c. Effect of inhibitors on copper and iron uptake

hCTR1 functions as a high affinity copper transporter, and it is strongly suggested Cu (I) is the preferred oxidation state taken up by cells (Kim, Nevitt, and Thiele 2008). Lower pH increases the rate of copper transport by hCTR1 (Lee et al. 2002). To investigate if hCTR1 is responsible for the uptake seen in these two cell lines, we incubated the cells with silver, a known inhibitor of hCTR1 mediated-copper uptake. As shown in Figure 34 (p. 92), ascorbate produced an increase in copper uptake in comparison to cells with just media. The addition of silver reduced copper uptake by ~70%. It has previously been shown that the copper entry rate into the -/- mefs is about 25% -30% of the +/+ mefs via an unidentified pathway (Lee, Petris, and Thiele 2002).

Iron and manganese are two well-established metals transported by DMT1 (Garrick et al. 2006). To confirm that the cells overexpressing DMT1 were functioning in metal uptake, we incubated the cells with iron, manganese, or copper at pH 6.5. After metal incubation overnight, the samples were collected and sent for ICP-OES analysis. Whereas no difference in the amount of copper uptake was apparent in sense cells and control cells, iron and manganese were taken up significantly more in the sense than the control cells. However, copper ions did inhibit iron and manganese uptake (Figure 35, p. 93). To confirm iron and manganese uptake is via DMT1, we incubated the cells with an inhibitor, NTA. NTA is a chelating agent which forms tight compounds with metals such as Fe^{3+}. As Figure 36 (p. 94) shows, NTA was successful in binding to available iron and thus limiting iron uptake in both transport buffer and growth media. These results strongly show DMT1 is not a copper transporter in HEK 293 cells.

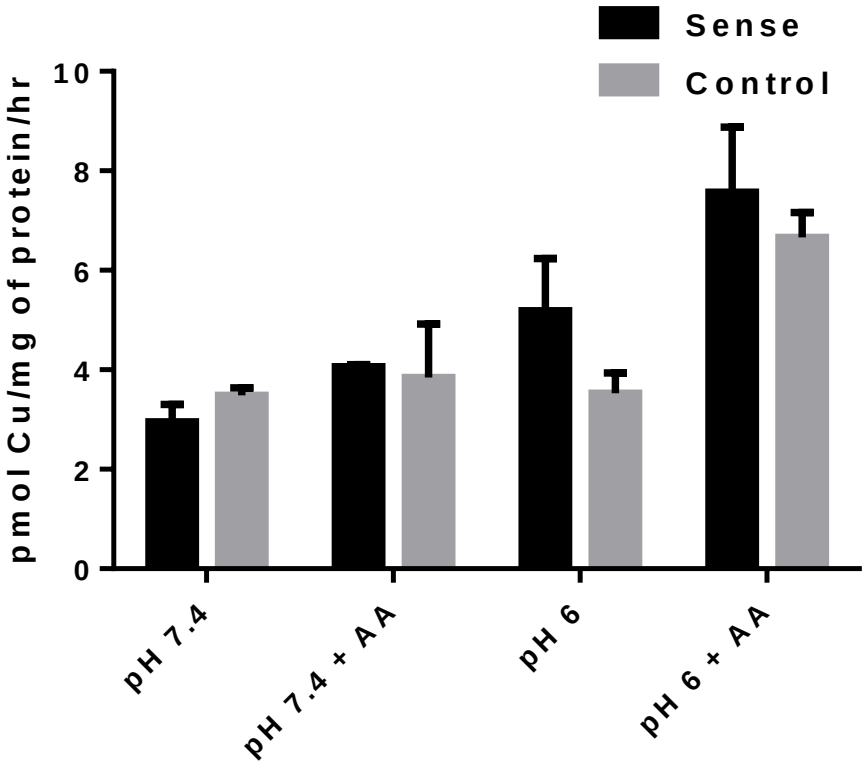

Fig. 33 ^{64}Cu uptake in low and high pH

Human embryonic kidney cells overexpressing DMT1 and control cells were incubated in transport buffer consisting of 150 mM NaCl, 5 mM KCl, 2.5 mM $MgCl_2$, and 25 mM Hepes, pH 7.4 and 6.0 supplemented with 10 % FBS. Uptake was initiated by addition of 5 µM $CuCl_2$ labeled with trace amounts of ^{64}Cu. Where indicated, ascorbate (1 mM) was added to investigate Cu^{1+} transport. Assays were run for 1 hour at 37º C and then terminated by the addition of ice-cold stop buffer. Radioactivity retained by the cells was determined by a scintillation counter. Uptake values were normalized by dividing by the amount of total protein.

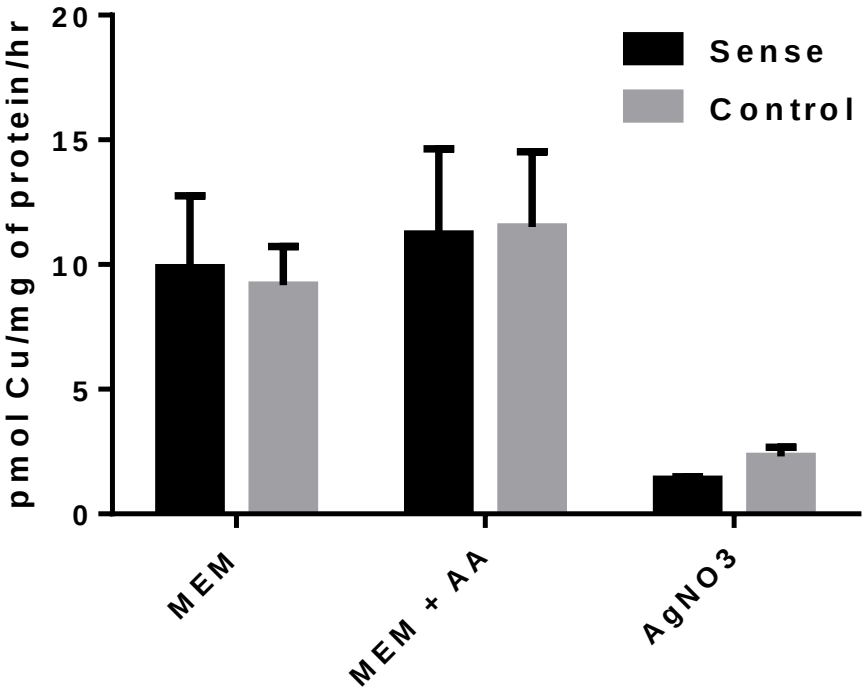

Fig. 34 ^{64}Cu uptake in the presence of an inhibitor, $AgNO_3$.

Human embryonic kidney cells overexpressing DMT1 and control cells were incubated in media supplemented with 10% FBS and 5 µM $CuCl_2$ labeled with trace amounts of ^{64}Cu. Ascorbate (1 mM) was added to investigate Cu^{1+} transport. $AgNO_3$ (250 µM) was added since it is an inhibitor of copper uptake through Ctr1. Assays were run for 1 hour at 37º C and then terminated by the addition of ice-cold stop buffer. Radioactivity retained by the cells was determined by a scintillation counter. Uptake values were adjusted by dividing by the amount of total protein.

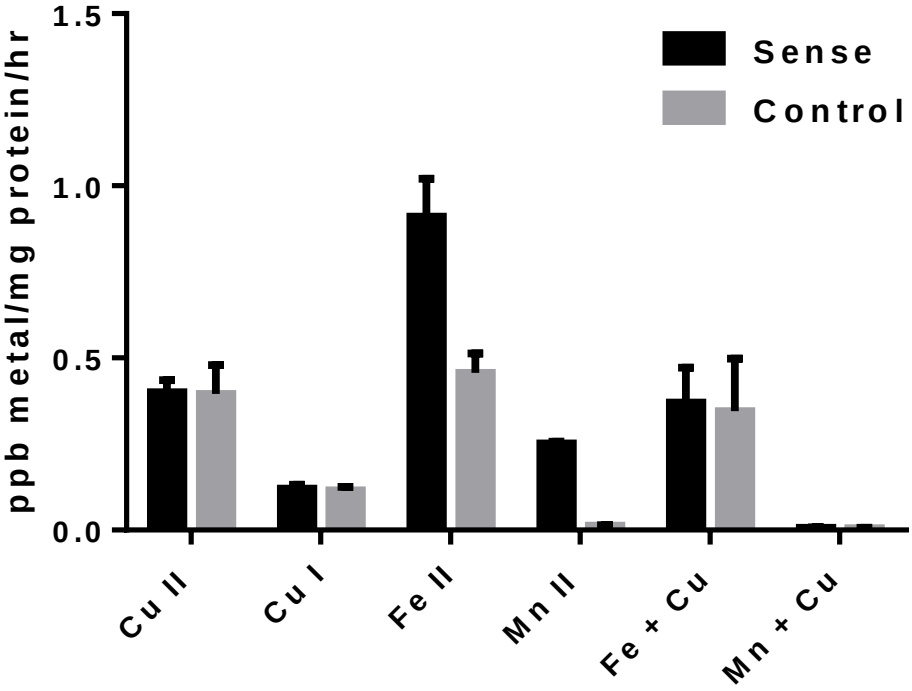

Fig. 35 Metal uptake via DMT1

Human embryonic kidney cells overexpressing DMT1 and control cells were incubated in transport buffer consisting of 150 mM NaCl, 5 mM KCl, 2.5 mM $MgCl_2$, and 25 mM Hepes, pH 6.5. CuCl (50 µM), $FeSO_4$ (50 µM), or $MnCl_2$ (50 µM) was added as indicated. Ascorbate (1 mM) was added to investigate Cu^{1+} transport. Cells were incubated in metal solution overnight at 37º C. The following day, uptake was terminated by the addition of ice-cold stop buffer. Cells were washed, pelleted and resuspended in 70% nitric acid for 3 hrs. Afterwards, the cells were diluted to 14% nitric acid with the addition of water, mixed, centrifuged and the supernatent was sent off for ICP-OES analysis.

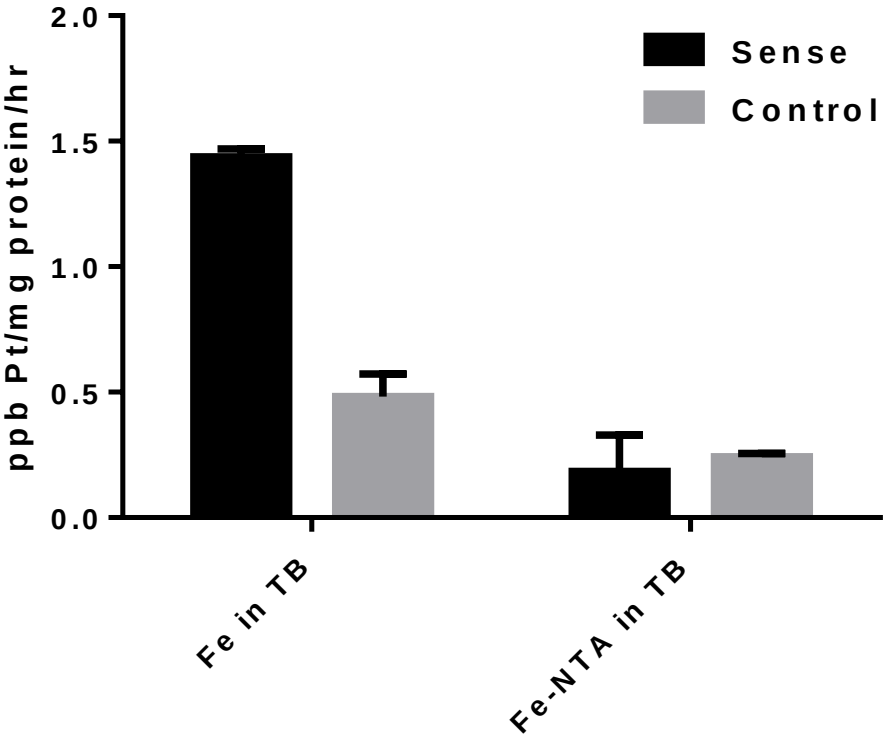

Fig. 36 Iron uptake via DMT1 in the presence of inhibitor, NTA

Human embryonic kidney cells overexpressing DMT1 and control cells were incubated in media or transport buffer consisting of 150 mM NaCl, 5 mM KCl, 2.5 mM $MgCl_2$, and 25 mM Hepes, pH 6.5. $FeSO_4$ (50 µM) added along with 200 µM NTA where indicated. Cells were incubated in metal solution overnight at 37º C. The following day, uptake was terminated by the addition of ice-cold stop buffer. Cells were washed, pelleted and resuspended in 70% nitric acid for 3 hrs. Afterwards, the cells were diluted to 14% nitric acid with the addition of water, mixed, centrifuged and the supernatent was sent off for ICP-OES analysis.

d. Cell surface DMT1 expression in response to Cu or BCS

Copper regulates the cell surface localization of hCTR1. As a protective mechanism, hCTR1 undergoes internalization in the presence of elevated extracellular copper (Petris et al. 2003, Molloy and Kaplan 2009). This limits the amount of copper entering the cell. Once copper levels return to basal levels, hCTR1 returns to the membrane and permits the entry of copper ions (Molloy and Kaplan 2009). We questioned if DMT1 is also responsive to elevated cellular copper levels. Cells were incubated in two different environments: 1. elevated copper or 2. no copper via a copper chelator. BCS binds to any copper present in the media preventing copper from binding to other proteins. Biotinylation is a very effective method to quantify the amount of proteins present at the cell surface. DMT1-overexpressing cells were incubated with copper or BCS (Figure 37, p. 96). The cell surface DMT1 levels were unchanged by the copper treatment. Excess copper did not cause internalization of DMT1. These results show cell surface DMT1 is not responsive to excess copper or low levels of copper.

3. hCTR2, Human Copper Transporter 2

a. hCTR2 cellular localization

To characterize the role of hCTR2 in copper homeostasis, we transfected HEK293 cells with epitope-tagged hCTR2 at the flip-in recombinase site, under the influence of a tetracycline-sensitive promoter (Maryon, Molloy, and Kaplan 2007). A C-terminal Flag tag, an N-terminal Flag tag, and an N-terminal GFP tagged hCTR2 were constructed and compared to N-terminal Flag-tagged hCTR1. To investigate the localization of hCTR2, a 5-step sucrose gradient fractionation was performed collecting three fractions enriched in plasma membrane, golgi, and endoplasmic reticulum. Following fractionation, protein was separated by SDS-PAGE, and hCTR2 and hCTR1 were detected using an anti-FLAG antibody by Western blot analysis. hCTR2 was detected migrating on a SDS gel at ~17 kDa which is in agreement with its predicted size based upon amino acid sequence (Figure 38, p. 98). hCTR2 was detected in all 3

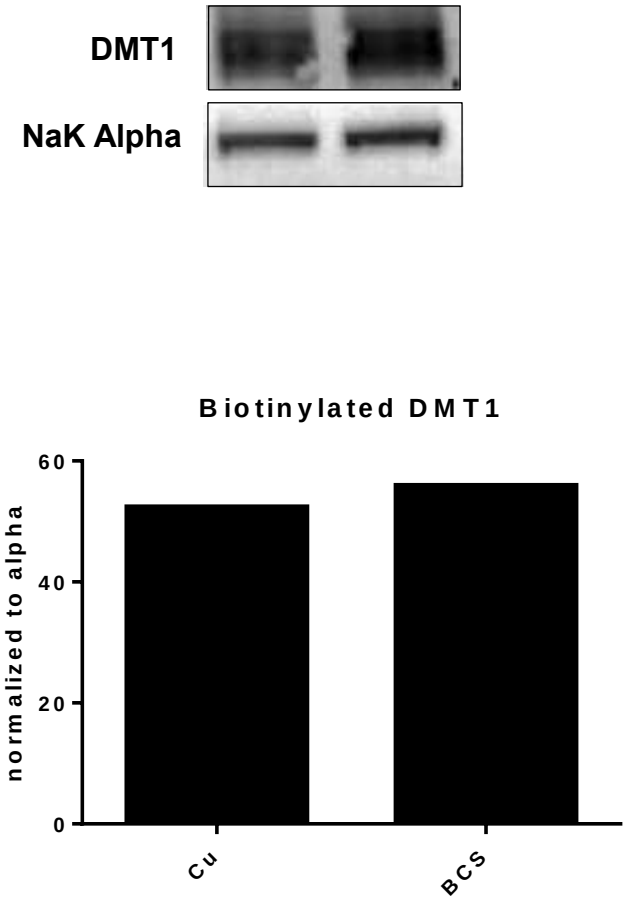

Fig. 37 Cell surface DMT1 after incubation with Cu or BCS

Human embryonic kidney cells overexpressing DMT1 were incubated with 20 µM Cu or 200 µM BCS in media for 1 hr. Cells were biotinylated and the biotinylated protein was analyzed on western blots. Protein levels were normalized to Na^+ K^+ Alpha, a loading control.

fractions collected, in both C-terminal and N-terminal tagged hCTR2 constructs. However, the majority of hCTR2 protein was collected in the plasma membrane fraction. hCTR1 protein was also found in all three fractions with the majority of protein in the plasma membrane fraction. These results suggest overexpressed-hCTR2, like hCTR1, is located partially at the cell surface.

To confirm our cell fractionation findings that hCTR2 is indeed localized at the plasma membrane, we employed biotinylation. Only proteins expressed at the cell surface are available for labeling. All three hCTR2 constructs were successfully biotinylated. The flag-tagged constructs were measured at ~17 kDa, whereas the GFP-tagged construct ran higher due to the GFP tag (Figure 39. p. 99). This is in agreement with findings performed in COS-7cells. Bertinato et al also performed biotinylation assays and showed hCTR2 as well as Na^+K^+ ATPase alpha were labeled. However, a cytoplasmic residing protein, SOD1, was not biotinylated (Bertinato et al. 2008). To investigate the cellular localization further, we employed immunofluorescence microscopy of tet- induced GFP-tagged hCTR2. Strong staining was detected using an anti-GFP antibody at the cell surface in tet-induced cells. Cells not tet-induced showed no staining (Figure 40, p. 100). These results confirm that over-expressed hCTR2 is at least partially located at the plasma membrane, and therefore could mediate copper uptake.

Berghe et al utilized confocal microscopy to detect hCTR2 in HeLa, U2OS and HEK293 cells. Strong staining was detected in large intracellular vesicles in all three cell types. Although hCTR1 staining was seen at the plasma membrane, hCTR1 was detected mainly in intracellular vesicles in HeLa cells. Further investigation identified hCTR2 colocalized with markers residing with late endosomes and lysosomes. These results suggest hCTR1 and hCTR2 reside mainly in intracellular vesicles. Using a MRE-luciferase reporter that measures cytoplasmic copper, in the presence of copper hCTR1 and hCTR2 stimulated high activity with hCTR1 activating the

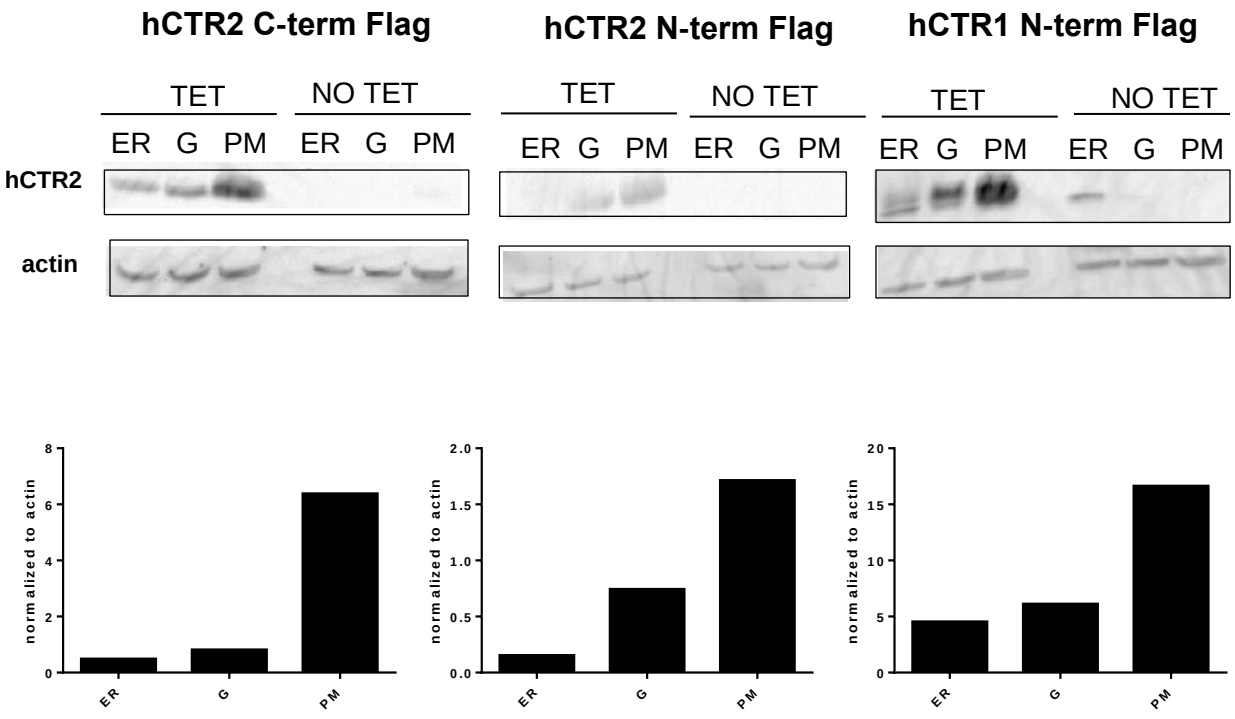

Fig. 38 Fractionation of hCTR2 and hCTR1

HEK 293 Flp-In cells containing tetracycline-regulated C-terminal FLAG–tagged hCTR2, N-terminal FLAG-tagged hCTR2, and N-terminal FLAG-tagged hCTR1 were fractionated using a 5 step sucrose gradient. Preps were examined using Western blot analysis. Blots were probed with an anti-Flag antibody or anti-actin antibody which was used as a loading control.

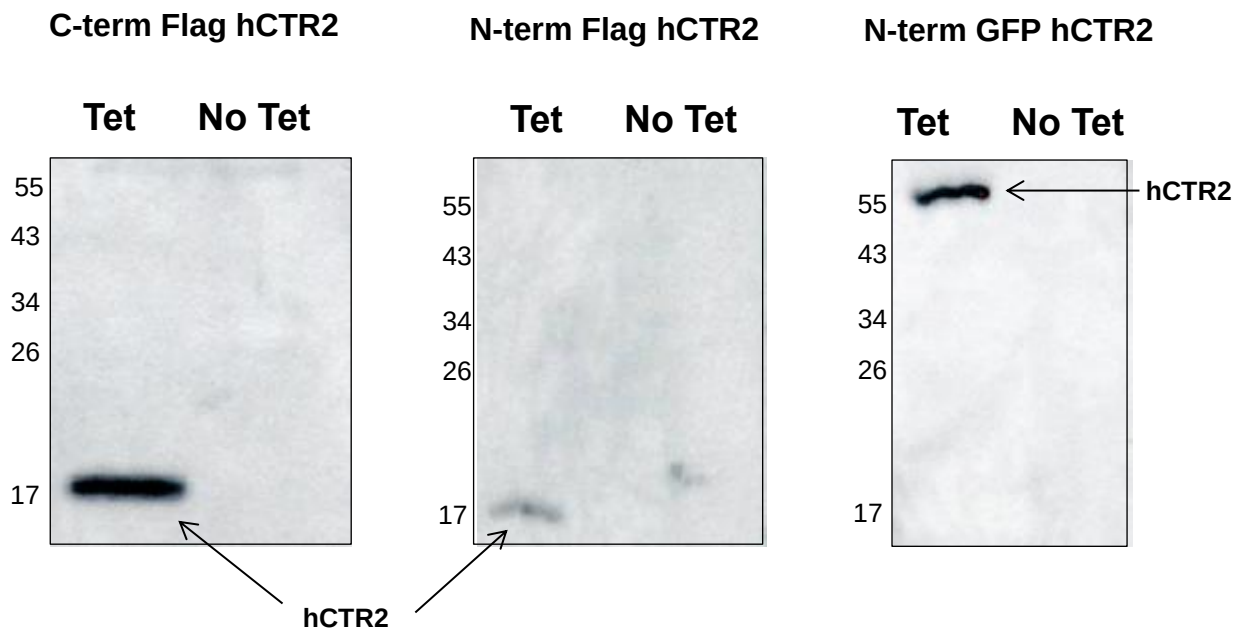

Fig. 39 Biotinylated hCTR2 constructs

HEK 293 Flp-In cells containing tetracycline-regulated C-terminal FLAG–tagged hCTR2, N-terminal FLAG-tagged hCTR2, and N-terminal GFP-tagged hCTR2 were tet-induced and biotinylated. The biotinylated protein was analyzed on western blots and probed with an anti-Flag antibody or anti-GFP antibody.

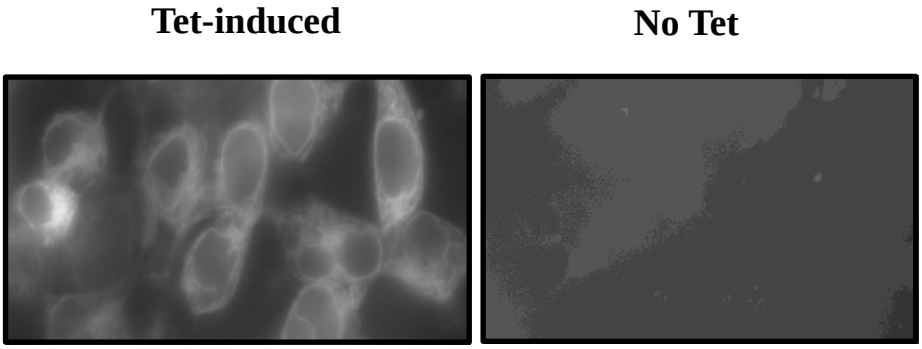

Fig. 40 Fluorescent imaging of hCTR2

N-terminal GFP-tagged hCTR2 were tet-induced and analyzed with confocal microscopy. Anti-GFP antibody was used to detect hCTR2.

reporter to a much greater extent than hCTR2 (van den Berghe et al. 2007). hCTR1 is a well-defined copper transporter. Its ability to function as such is dependent on it residing at the surface available to take up copper ions. These findings in HeLa cells suggest hCTR1 is mostly located in intracellular compartments; however, this location does not align well with the function of hCTR1 as a copper uptake protein.

b. Copper uptake via hCTR2

Thus far, we have shown overexpressed hCTR2 is located partially at the plasma membrane in HEK293 cells as demonstrated by sucrose gradient fractionation, biotinylation and immunofluorescence images. Next we investigated the ability of hCTR2 to function in copper uptake. The rate of copper uptake was very low in tet-induced hCTR2 flag-tagged cells at both 5 and 45 minute time points (Figure 41A, p. 102). However, transport was higher at 45 minutes than at 5 minutes. Uptake in cells not tet-induced was unchanged at the two time points. hCTR1 flag-tagged cells demonstrated a very high rate of copper uptake in tet-induced cells at 45 minutes. Cells not tet-induced showed very little uptake. Similar results were also seen in GFP-tagged constructs of hCTR1 and hCTR2 (Figure 41B, p. 102). Using a MRE-luciferase reporter that measures cytoplasmic copper, in the presence of copper hCTR1 and hCTR2 stimulated high activity with hCTR1 activating the reporter at much higher rate than hCTR2 (van den Berghe et al. 2007). Bertinato et al also reported a slower copper uptake seen with hCTR2. Copper uptake via hCTR2 was saturable with a Km of 11 ±2.5 μM and a Vmax of 6.9 ±0.7 μM. This uptake system was specific since only the presence of silver ions could inhibit copper uptake (Bertinato et al. 2008). Our results suggest that hCTR2 transports copper only very poorly, if at all, compared to hCTR1 when expression levels of both proteins are comparable.

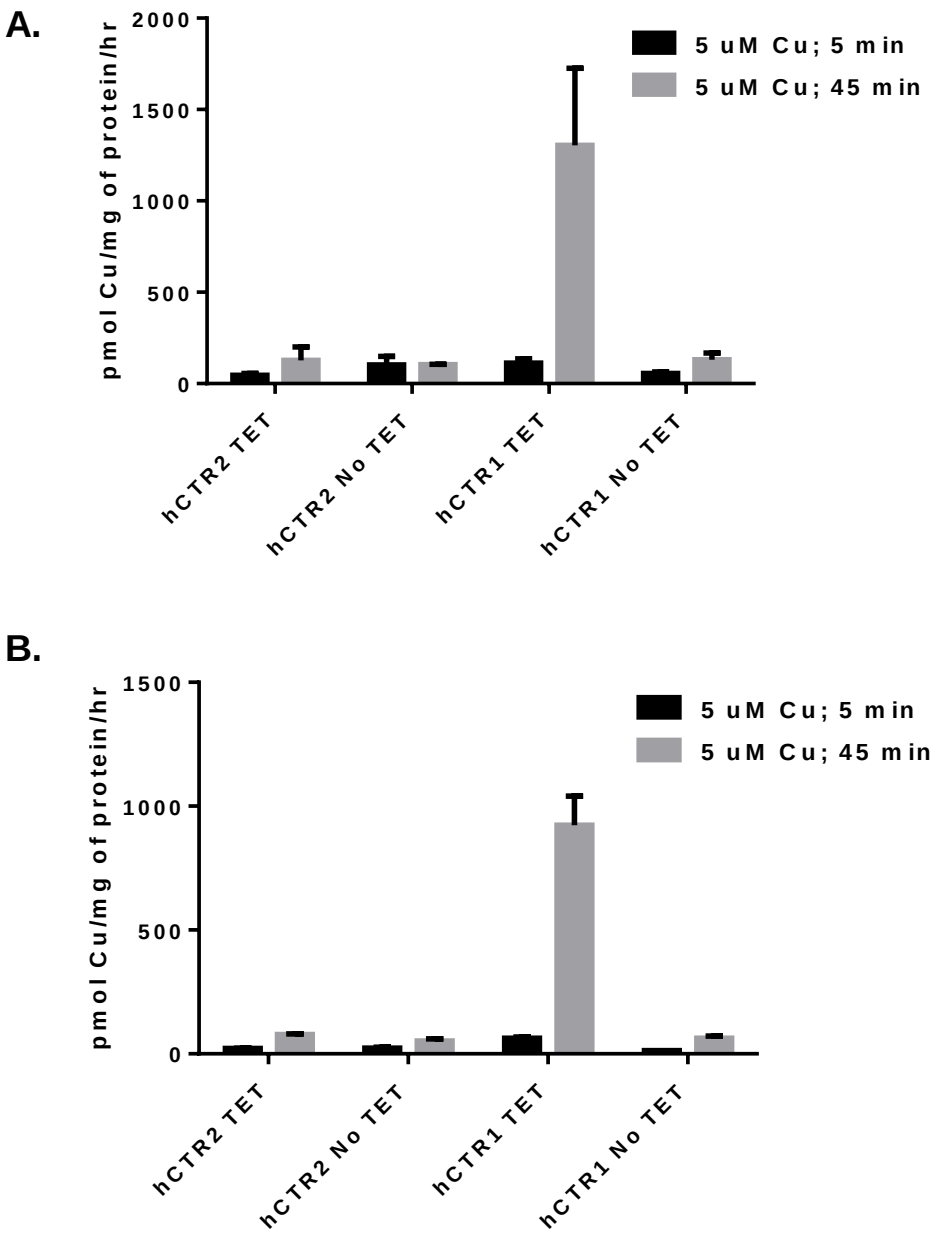

Fig. 41 ^{64}Cu uptake by Flag-tagged and GFP-tagged hCTR2 and hCTR1

HEK 293 Flp-In cells containing tetracycline-regulated A) N-terminal FLAG-tagged hCTR2 and N-terminal FLAG-tagged hCTR1 or B) N-terminal GFP-tagged hCTR2 and N-terminal GFP-tagged hCTR1 were incubated with 5 µM $CuCl_2$ labeled with trace amounts of ^{64}Cu for 5 or 45 mins. Uptake was terminated by the addition of ice-cold stop buffer. Radioactivity retained by the cells was determined by a scintillation counter. Uptake values were normalized by dividing by the amount of total protein.

Discussion

The mechanism which allows for the uptake of dietary copper into the intestine has drawn attention to the cellular localization of hCTR1. Since hCTR1 is the major high affinity copper uptake protein, it was assumed hCTR1 was present at the apical membrane (lumen side) in enterocytes and thus responsible for the acquisition of dietary copper (Nose et al. 2010). However, biotinylation assay and confocal imaging detect the majority of hCTR1 protein located at the basolateral membrane in polarized enterocytes with correlating copper transport rates to match (Zimnicka, Maryon, and Kaplan 2007). This data indicated hCTR1 is not present on the apical membrane and therefore is not responsible for mediating copper uptake from the diet. However immunohistochemistry studies in rat, mouse and pig intestinal cells suggests hCTR1 is present on the apical membrane and functions in uptake of dietary copper (Nose et al. 2010). The present work was aimed to elucidate the mechanism of copper acquisition by identifying other potential copper uptake proteins or mechanisms.

DMT1 transports a wide array of divalent metal ions. The intestine is the major site of iron uptake and regulation, and DMT1 is abundant on the apical (lumen side) membrane in enterocytes. Its cellular location and ability to transport varying metals present DMT1 as a candidate to mediate dietary copper uptake. Intestine-specific CTR1 ko mice hyperaccumulated copper. Also mouse embryonic fibroblasts deficient of CTR1 were able to take up copper at 30% of the rate of mefs expressing CTR1. Gunshin et al suggested that DMT1 is a promiscuous transporter of several metals including copper (Gunshin et al. 1997). Before we could investigate the role of DMT1 in copper uptake seen in systems where CTR1 was absent, we first examined its ability to simply transport copper. In summary, our work demonstrates DMT1 is not a copper transport protein as cells overexpressing DMT1 had the same rate of ^{64}Cu uptake as cells not expressing DMT1 as shown by radioactive copper assays (Figure 32, p. 89) although iron and manganese uptake was significantly increased in DMT1 overexpressing cells (Figure 35, p. 93). On the other hand, the uptake seen in these studies resembles more closely

hCTR1-mediated uptake as illustrated by: 1. ^{64}Cu rate of uptake doubled when a metal reducing agent was present (reducing Cu^{2+} to Cu^{1+}, the preferred oxidation state transported by hCTR1) in the uptake solution (Figure 32, p. 89); 2. lower pH stimulated copper uptake (Figure 33, p. 91); 3. a copper inhibitor, silver, significantly reduced copper uptake (Figure 34, p. 92). Recent findings confirm our results. Illing et al utilized Xenopus oocytes injected with DMT1 RNA to analyze transport of several metal ions via radiotracer transport assays. Cd^{2+}, Fe^{2+}, Co^{2+}, Mn^{2+} and to a lesser extent Zn^{2+}, Ni^{2+} and VO^{2+} all were transported (evoked currents at -70 mV) by DMT1. Neither Cu^{1+} nor Cu^{2+} were transported by DMT1 (Illing et al. 2012).

hCTR2 shares sequence similarity to hCTR1 and thus could potentially function in copper uptake similar to hCTR1. Although metal binding motifs essential for high affinity copper uptake are lacking in the protein structure of hCTR2, motifs responsible for coordinating copper ions are present. We examined the localization and ability of hCTR2 to transport copper utilizing human embryonic kidney cells overexpressing hCTR2 via a tetracycline promoter. We showed via sucrose fractionation, biotinylation and fluorescent imaging the localization of hCTR2 was mostly located at the plasma membrane. We conclude that if hCTR2 is a copper transporter, it is much less effective than hCTR1. Further studies are required to examine hCTR2 in polarized enterocytes in order to study if hCTR2 contributes to the uptake seen on the apical membrane and to establish a role for hCTR2 in copper homeostasis.

Although literature exists on the mechanisms and regulations of hCTR1-mediated copper uptake, the functional role of hCTR2 is less well understood. CTR6, the CTR2 yeast equivalent, is primarily located in vacuolar membranes (Bellemare et al. 2002) whereas in mammalian cells hCTR2 is located in lysosomes and late endosomes (van den Berghe et al. 2007). These cellular compartments share similar functions in that they provide the cell with a means to degrade/store unwanted material. It was suggested hCTR2 functioned to export copper out of these compartments for reuse by the cell (van den Berghe et al. 2007). CTR2 was also found to be partially located at the plasma membrane in COS-7 fibroblast cells and

functioned in low-affinity copper uptake (Bertinato et al. 2008). Our findings are in agreement with this study as we showed by biotinylation, fractionation, and immunofluorescent imaging the plasma membrane localization of hCTR2. The identification of hCTR1 on the blood side in polarized enterocytes inspired the question of what system is responsible for dietary copper entry. In the pursuit of analyzing potential copper uptake proteins, we identified the presence of an anion chloride-dependent copper uptake system present on the apical membrane in polarized enterocytes. We also examined DMT1 and hCTR2 and their ability to transport copper and thus potentially compensate for hCTR1. Whereas we found no evidence to support the notion that DMT1 is a copper uptake protein, we suggest over-expressed hCTR2 when localized at the plasma membrane is a much less effective copper transporter than hCTR1.

Acknowledgements

We would like to thank Marianne Wessling-Resnick (Harvard Univ) for the DMT1 sense, DMT1 antisense, and HEK293T parental cells. These studies were supported by NIH Grant P01 GM 067166.

V. Concluding Remarks

Research presented in this thesis examined the role of hCTR1 in transporting platinum anti-cancer drugs across the cell membrane. hCTR1 is a high affinity copper uptake protein, thus the possibility that this highly selective transporter could also mediate the uptake of a much larger platinum drug was surprising. Cisplatin and its analogs are highly effective anti-cancer drugs; however over time, patients develop resistance and the platinum containing drugs are no longer as effective in treating tumors. Understanding the mechanism of drug entry is of vital importance since clinical trials are presently taking place with intent to target and manipulate plasma membrane levels of hCTR1 in the pursuit of improved drug entry in patients suffering from various carcinomas. In chapters II and III, we demonstrate hCTR1 does not mediate the uptake for cisplatin or its analogs (carboplatin, oxaliplatin, and transplatin) in both model cells as well as tumor cells, and thus hCTR1 is unlikely to be an effective therapeutic target in attempts to improve platinum drug efficacy.

Several questions remain unanswered. How do cisplatin and platinum-containing anti-cancer drugs enter cells? What causes tumor cells to become resistant to drug therapy? Why do cisplatin-resistant cells fail to recovery to the plasma membrane after copper treatment? What system is responsible for copper uptake from the diet? Although we do not have all of the answers to these questions, we are able to exclude a few possibilities and also shed light on others. Our data show clearly that hCTR1 is not responsible for mediating the uptake of cisplatin or platinum analogs. Cisplatin-resistant cells have reduced protein expression of hCTR1, reduced copper uptake, and reduced cisplatin uptake. Consequently, it was proposed that reduced hCTR1 protein expression was the cause of cisplatin resistance. It is important to separate correlation from cause. Several proteins involved in copper homeostasis have been suggested to interact with platinum drugs including ATP7A, ATP7B and ATOX1. A cisplatin-resistant cell line is generated by treating cells with platinum drug for several days. Those cells that survive the treatment are picked, cloned and expanded. While this is a useful tool to study

drug resistance, a detailed understanding of what causes cells to become resistant and the identification of which systems are affected by this treatment have yet to be elucidated. A systematic and detailed analysis of cisplatin-resistance cells and cells from which they were derived from should be carried out. Key properties to investigate are protein and message levels of a wide array of membrane proteins that have been implicated to transport metals as well as cell surface proteins with no role in metal transport. In addition, our work suggests that an examination of the effects on proteins involved in copper homeostasis might be very informative. The mechanism of platinum drug uptake is unknown. Although other proteins (OCT2, Na^+ K^+ ATPase, and SLC family members) have been considered, our data supports a passive diffusion system since cis and trans isomers had identical rates of platinum uptakes and no saturation was seen. A wider cisplatin concentration range should be tested. Further study is needed to examine the rates of platinum drug entry in phospholiposomes which might shed light on the mechanism of entry of platinum-containing drugs.

Increasing evidence demonstrates hCTR1 is not the only mode of copper entry into the cell. In chapter IV we investigated a chloride-dependent anion system and found it is responsible for copper uptake in enterocytes on the apical membrane surface allowing for entry of copper ions from an individual's diet. This anion system is inhibited by histidine and components found in the complex mixture of components in DMEM. Copper ions complex with chloride ions to form an anionic species and are delivered into the cell. Little data now exists on the identification of the anion system responsible for this type of copper uptake. Future studies should include the knock-out of several anion transporters and examining the effects on the rate of copper uptake. It is not yet clear if one or several anion transporters transport copper in epithelial cells.

We also examined an iron uptake protein, DMT1, and its ability to mediate copper ions as well as a protein with sequence similarity to hCTR1: hCTR2. Our findings suggest that although DMT1 can transport a wide range of metals, Cu^{1+} and Cu^{2+} are not included. In human

embryonic kidney cells, overexpressed hCTR2 was primarily localized at the plasma membrane and demonstrated only a small effect (if any) on the rate of copper uptake in these cells compared with the effectiveness of hCTR1 as a copper transporter. The important physiological role of hCTR2 has yet to be identified.

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VITA

NAME: Kristin Dionne Ivy

EDUCATION: B.S., Biology, with Honors
Grambling State University, 2007

Ph.D., Biochemistry and Molecular Genetics
University of Illinois at Chicago, 2013

PROFESSIONAL MEMBERSHIP: Scientific Minority and Diversity Program

National Biotechnology Pharmaceutical Association

ABSTRACTS: **Ivy, K.D,** and Kaplan, J.H (2012) Mechanism of cellular uptake of Pt drugs: The role of hCTR1. AACR #813.

Ivy, K.D, and Kaplan, J.H (2010) Copper Homeostasis and the role of hCTR2. Trace Elements in Biology & Medicine.

PUBLICATIONS: Maryon E.B., Molloy. S.A., **Ivy. K.D.**, Yu.H., and Kaplan, J.H. (2013) Rate and Regulation of Copper Transport by Human Copper Transporter 1 (hCTR1). Journal of Biological Chemistry

Ivy, K.D., and Kaplan, J.H. (2013) A Re-evaluation of the Role of hCTR1, the Human High Affinity Cu Transporter in Pt-Drug Entry into Human Cells. *Molecular Pharmacology 83:1-10*

Zimnicka. A. M., **Ivy, K.D.,** and Kaplan. J.H. (2011) Acquisition of Dietary Copper: A Role for Anion Transporters in Intestinal Apical Copper Uptake. *American Journal of Physiology 300: C588-C599*