Generation of polyclonal antibody specific for hCtr1

Polyclonal antibody specific for hCtr1 was prepared using a recombinant hCtr1 protein derived from the first 67 amino acid residues at N-terminus of hCtr1 (*17, 20*). A DNA fragment encompassing the first 67 amino acid residues at N-terminus of the hCtr1 gene was amplified from a pCDNA-3.1 vector encoding hCtr1 by polymerase chain reaction (PCR), using sense primer 5' CAC CAT GGA TCA TTC CCA CCA TAT GGG 3' and antisense primer 5' TCC AGC TGT ATT GAT CAC CAA ACC 3', and an AccuTaq LA core PCR kit from Sigma (St. Louis, MO). The PCR-amplified DNA fragment was cloned into the pET-10 expression vector (Invitrogen, Carlsbad, CA) to express recombinant hCtr1 containing a his-tag at the C-terminus. A 25 kD recombinant hCtr1 protein encompassing the first 67 amino acid residues at N-terminus of hCtr1 was isolated by affinity chromatography using ProBond™ Nickel-Chelating Resin, in a method from Invitrogen (Carlsbad, CA). Antiserum specific for hCtr1 was obtained after injection of rabbits with the purified recombinant hCtr1 protein and polyclonal antibodies specific for hCtr1 were further purified by affinity chromatography based on the manufacturer's instruction (Alpha Diagnostic, San Antonio, TX).

Construction and characterization of hCtr1 shRNA plasmid

Plasmid vectors encoding hCtr1short hairpin RNA (hCtr1 shRNA plasmid) were constructed by inserting hCtr1 shRNA sequence (Table 1) into a pGeneClip puromycin vector (Promega (Madison, WI) and control plasmid containing scrambled shRNA sequence (SCR shRNA plasmid) was constructed based on the manufacturer's protocol from the vendor (SuperArray Biosciences, Frederick, MD). Using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), PC-3 cells were transfected with hCtr1 shRNA plasmid or control SCR shRNA plasmid DNA, and puromycin-resistant cells were harvested after culture of the transfected PC-3 cells in medium containing puromycin (2.0 μg/mL) for 3 days. Total cellular RNA was extracted using a Trizol

RNA extraction kit from Qiagen (Valencia, CA) and the quantity of hCtr1 mRNA was determined by mRNA quantitative real-time reversed transcription PCR (qRT-PCR) as described previously (*21*). The primers specific for hCtr1 were 5'-CACCATGGATCATTCCCACCATATGGG-3' and 5'-TCCAGCTGTATTGATCACCAAACC-3', and 18s RNA primers (5'-GGAATTGACGGAAGGGCACCACC-3' and 5'- GTGCAGCCCCGGACATCTAAGG-3') were used as quantitative control.

Western blot

Cell lysates were prepared using cell lysis buffer from Cell signaling (Danvers, MA). Total protein concentration of supernatant was determined using BCA protein Assay Kit (Bio-Rad, Hercules, CA). Following SDS-PAGE (12%) separation of total cellular proteins (60 μg/lane) and transfer of separated proteins onto PVDF membrane, the protein blots were blocked at 4°C overnight in 5% defatted milk in PBST (130mM NaCl, 10mM NaH_2PO_4, pH=7.4, 0.1% Tween-20). A 3-color prestained protein molecular marker from Pierce Biotechnology (Rockford, IL) was used for estimation of molecular weight of SDS-PAGE separated protein molecules. The blots were incubated with purified polyclonal anti-hCtr1 antibody (1:1000 dilution) at 37°C for 1h. The blots were washed 3 times with PBST and incubated with Horse Radish Peroxidase (HRP) conjugated goat anti-rabbit antibody from Chemicon (Temecula, CA) in PBST containing 5% milk for 1h at room temperature. The signal of immunoreactivity was visualized with Super Signal Western Blot Enhancer kit from Pierce Biotechnology (Rockford, IL). Subsequently, membrane was stripped with Blot Stripping buffer from Thermo Scientific (Rockford, IL), and re-incubated with anti-β-actin antibody from Novus (Littleton, CO) at a dilution of 1:10,000 followed by incubation with HRP conjugated secondary rabbit-anti-mouse antibody from Novus for detecting β-actin signal as a loading control. Semi-quantitative analyses of the intensity of each band on Western blot were conducted by densitometry using Alpha Ease FC™ software (Alpha Innotech, Santa Clara, CA) as described previously (*22*).

Cell Proliferation Assay

Cell proliferation assay was conducted using a CCK-8 Cell Proliferation Assay kit (Dojindo, Rockville, MD) as described previously (*23*). Briefly, cells were inoculated into a 96-well plate (1×10^3 cells/well) and subjected to serum starvation for 24h by culture of the cells in a medium without FBS. Subsequently, cells were incubated with medium with or without $CuCl_2$ supplement (10 μM) at 37°C up to 72h. CCK-8 assay reagent was added at different times (24, 48, or 72 hr after incubation) and optical density (OD) was measured at a wavelength of 460 nm with a reference wave length of 630nm, using a Microplate Multiskan spectrophotometer (Thermo Scientific, Hudson, NH). Data for the cell number relative to the number of cells initially inoculated were calculated in triplicate at each time point and were repeated 3 times.

Supplemental Table 1. Plasmid encoding short hairpin RNA (shRNA) specific for hCtr1

Plasmid	Sequence of shRNA
hCtr1 -shRNA plasmid #1 *	GTTCTATGAAGGACTCAAGAT
hCtr1-shRNA plasmid #2	GGAGTACACTTTCATGTGATT
hCtr1-shRNA plasmid #3	AAGACAGAATGCTATGACTTT
hCtr1-shRNA plasmid #4	CAAGGCCAGTTAAGACAGAAT
SCR-shRNA plasmid **	GGAATCTCATTCGATGCATAC

* hCtr1, human copper transporter 1, ** SCR-shRNA, nonspecific scrambled shRNA