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Cathepsin Protease Controls Copper and Cisplatin Accumulation via Cleavage of the Ctr1 Metal-binding Ectodomain*[S]◆

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Copper is an essential metal ion for embryonic development, iron acquisition, cardiac function, neuropeptide biogenesis, and other critical physiological processes. Ctr1 is a high affinity Cu^+ transporter on the plasma membrane and endosomes that exists as a full-length protein and a truncated form of Ctr1 lacking the methionine- and histidine-rich metal-binding ectodomain, and it exhibits reduced Cu^+ transport activity. Here, we identify the cathepsin L/B endolysosomal proteases functioning in a direct and rate-limiting step in the Ctr1 ectodomain cleavage. Cells and mice lacking cathepsin L accumulate full-length Ctr1 and hyper-accumulate copper. As Ctr1 also transports the chemotherapeutic drug cisplatin via direct binding to the ectodomain, we demonstrate that the combination of cisplatin with a cathepsin L/B inhibitor enhances cisplatin uptake and cell killing. These studies identify a new processing event and the key protease that cleaves the Ctr1 metal-binding ectodomain, which functions to regulate cellular Cu^+ and cisplatin acquisition.

Copper is essential for key biological processes, including electron transfer, iron acquisition, dopamine hydrolysis, and superoxide disproportionation, and defects in copper metabolism are associated with cardiomyopathy, anemia, peripheral neuropathy, and neutropenia (1–6). Although many proteins involved in the acquisition and intracellular distribution of copper have been identified, little is known about the regulation of copper import. The transport of Cu^+ from the extracellular environment is accomplished by the evolutionarily conserved homotrimeric integral membrane protein, copper transporter 1 (Ctr1) (7–13), which resides on the plasma membrane and in endosomal compartments (14–18). High affinity Cu^+ import via Ctr1 requires a methionine- and histidine-rich metal-binding extracellular domain (ectodomain) that is thought to concentrate extracellular Cu^+ near the ion trans-membrane pore (19–23). Additionally, Ctr1 binds the chemotherapeutic agent cisplatin via the methionine ligands in the ectodomain and imports cisplatin and other platinum-based chemotherapeutic agents via an endocytic mechanism (24–28). Despite a critical role for the Ctr1 ectodomain in both Cu^+ and cisplatin import, both a full-length form and a truncated form of Ctr1 (tCtr1)[4] are present in cultured cells and tissues (18, 29, 30). The latter lacks the ectodomain and drives ~50% of the Cu^+ uptake as compared with full-length Ctr1 (31).

Previously, an integral membrane protein similar to Ctr1, denoted Ctr2, was shown to both interact with and regulate the ratio of full-length Ctr1 to tCtr1 in mouse embryonic fibroblasts (MEFs) and in specific tissues in Ctr2 knock-out mice (32). In the absence of Ctr2, MEFs possess dramatically lower levels of tCtr1, while simultaneously expressing high levels of full-length Ctr1 and accumulating Cu^+ and cisplatin. A large fraction of the Cu^+ accumulated in $Ctr2^{-/-}$ MEFs and mouse tissues is found in endosomal compartments (32). The mechanism by which Ctr2 governs the abundance of tCtr1, thereby controlling Cu^+ and cisplatin accumulation, and the subcellular location in which this process occurs have not been elucidated.

Here, we report that Ctr2 stimulates the proteolytic processing of full-length Ctr1 to yield tCtr1 and that this process is due to direct Ctr1 ectodomain cleavage by the endolysosomal cysteine proteases, cathepsins L and B. Both proteases co-purify with endolysosomes harboring Ctr1 and Ctr2, consistent with

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[4] The abbreviations used are: tCtr1, truncated Ctr1; MEF, mouse embryonic fibroblast; E64d, (2*S*,3*S*)-*trans*-epoxy-succinyl-L-leucylamido-3-methylbutane ethyl ester; Tet-On, tetracycline-inducible expression; SUMO, small ubiquitin-related modifier; ICP-MS, inductively coupled plasma MS; MEM, minimal essential medium.

ectodomain processing occurring in the acidic endolysosomal compartment. Pharmacological or genetic inhibition of cathepsins L and B results in the accumulation of full-length Ctr1 and drives increased copper accumulation both in MEFs and in cathepsin knock-out mice. Similarly, treatment of cells with cisplatin, in combination with a cathepsin L/B inhibitor, enhances cisplatin accumulation and sensitizes cells to cisplatin toxicity. These findings identify a regulatory mechanism for modulating Ctr1-mediated Cu^{+} and cisplatin uptake and identify the cathepsin L and B proteases as new components of the metal homeostasis machinery that are amenable to pharmacological manipulation.

Experimental Procedures

Chemicals—Protease inhibitors, including TIMP-2 and -3 (R&D Systems), TAPI-2, cathepsin L inhibitor III (benzyloxycarbonyl FY(*t*-Bu)-DMK) (Calbiochem, EMD), BB-94 (Batimastat, Tocris Biosciences), E64d ((2*S*,3*S*)-*trans*-epoxy-succinyl-L-leucylamido-3-methylbutane ethyl ester), and CA074Me (Sigma), were purchased from the indicated vendors.

Animals and Ethical Statement—Wild type (WT) and cathepsin $L^{-/-}$ mice were euthanized by CO_2 and perfused with PBS, and the selected tissues were dissected, snap-frozen in liquid nitrogen, and stored at −80 °C until use. All procedures were approved by the Ethical Committee at the Albert-Ludwigs-Universität in Freiburg, Germany.

Cell Culture, Generation of Cell Lines, and Transfections—Wild type and cathepsin $L^{-/-}$, cathepsin $B^{-/-}$, and cathepsin $L^{-/-}B^{-/-}$ MEFs were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 1× MEM non-essential amino acids, and 1× antibiotic/antimycotic. Wild type ($Ctr2^{+/+}$), $Ctr2^{-/-}$, and doxycycline-inducible $Ctr2^{-/-}$ MEFs were cultured in DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 1× MEM non-essential amino acids, 2 mM HEPES, 1× antibiotic/antimycotic, 55 μM β-mercaptoethanol, and 100 μg/ml hygromycin B. Wild type, $Ctr1^{-/-}$, and $Ctr1^{-/-}$ $Ctr2^{-/-}$ MEFs and derivatives harboring a Tet-On-Ctr2 allele, were cultured in DMEM (Gibco) supplemented with 20% (v/v) heat-inactivated FBS, 1× MEM non-essential amino acids, 50 μg/ml uridine, 100 units/ml penicillin/streptomycin, and 55 μM β-mercaptoethanol. HEK293T cells were cultured in DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 100 units/ml penicillin/streptomycin. Chinese hamster ovary (CHO) cells were cultured in F-12K medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum. A doxycycline-regulated Ctr2 cell line was generated using the pTRIPZ vector (GE Healthcare). Briefly, the *RFP* gene in this plasmid was replaced with a mouse Ctr2 cDNA by standard cloning methods. Lentiviral particles were used to infect $Ctr2^{-/-}$ MEFs and stable clones selected via puromycin treatment and purified. MEFs lacking both Ctr1 and Ctr2 were generated by transfecting $Ctr1^{-/-}$ MEFs with a plasmid expressing Cas9 and a guide RNA specific for Ctr2. Clones were screened via PCR and a single clone lacking an intact Ctr2 reading frame was selected and cultured under 5% CO_2 at 37 °C. For cell viability assays $Ctr1^{+/+}$ and $Ctr1^{-/-}$ MEFs were seeded at a density of 20,000 cells/well in a transparent 96-well plate. Cells were pre-treated with E64d for 2 h before 50 μM cisplatin was added, and cells were incubated for 12 h. CellTiter Blue (Promega) was used to measure cell viability according to the instructions of the manufacturer, analyzing the metabolic capacity in living cells by recording the reduction of the dye resazurin into the fluorescent end product resorufin at 560_{Ex}/590_{Em} nm.

Transfection into MEFs was carried out by electroporation using Amaxa Nucleofection MEF2 kit (Lonza) according to the manufacturer's instructions. The vector pcDNA3.1(+) backbone was used when transfecting human CTR1 into $Ctr1^{-/-}$ MEFs. The SMARTpool siRNA for mouse Ctr2 (*SLC31A2*) (Dharmacon) was used to knock down Ctr2 expression in cathepsin $L^{-/-}$ MEFs. MEFs were seeded in 6-well plates at a density of 1×10^6 cells/well. The following day siRNA or scRNA (non-targeting RNA) were added and incubated for 72 h before collection for analysis.

Protein Extraction and Immunoblotting—For the isolation of protein extracts, mouse tissues or cell pellets were homogenized in ~10 volumes of ice-cold lysis buffer (phosphate-buffered saline (PBS, pH 7.4), 1% Triton X-100, 0.1% SDS, and 1 mM EDTA, proteinase inhibitors (Halt Protease Inhibitor Mixture, Thermo Scientific)); homogenates were incubated in ice for 30 min and centrifuged at 16,000 × *g* at 4 °C for 20 min, and supernatants were collected. Protein concentrations were measured with the BCA protein assay kit (Thermo Scientific). SDS-PAGE and immunoblotting were carried out by standard protocols. Anti-Ctr1 and anti-Ctr2 antibodies have been described previously. Antibodies used were anti-cytochrome *c* oxidase (CoxIV; Mitosciences, Eugene, OR), anti-cathepsin L (Santa Cruz Biotechnology, Santa Cruz, CA), anti-cathepsin B and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Abcam), anti-β-tubulin and anti-Lamp1 (Cell Signaling Technology, Danvers, MA), and anti-Cu/Zn superoxide dismutase (SOD1; StressGen, Ann Arbor, MI). Horseradish peroxidase (HRP)-conjugated anti-mouse or -rabbit IgG (GE Healthcare) were used as secondary antibody for immunoblotting.

Tissue and Cell Metal Measurements—Tissue and whole cell copper and zinc concentrations were measured by ICP-MS. Briefly, tissues were collected into acid-washed 1.5-ml microcentrifuge tubes and weighed. The cultured cells were rinsed once with PBS, harvested into ice-cold PBS, and divided into two tubes. One tube was used to measure protein content, and the other sample was collected by centrifugation at 400 × *g* for 5 min at 4 °C. Tissues or cell pellets were suspended in 10 times volume/weight (μl/mg wet weight) of trace analysis grade nitric acid (Sigma), heated at 85–95 °C for ~1 h, and subjected to ICP-MS analysis. For cisplatin accumulation, $Ctr1^{-/-}$ cells were transfected with either empty vector or human Ctr1 and pretreated with DMSO or 10 μM cathepsin L inhibitor prior to treatment with 200 μM cisplatin (American Pharmaceutical Partners, Inc., Los Angeles) in Opti-MEM for 2 h and digested in HNO_3/HCl (3:1) followed by ICP-MS. The analyses were performed by Environmental and Agricultural Testing Service, Department of Soil Science, North Carolina State University, Raleigh. Values were normalized by protein concentration or tissue wet weight.

Discontinuous Density Gradient Fractionation—$Ctr2^{+/+}$, $Ctr2^{-/-}$, and WT MEFs were cultured in 15-cm diameter dishes until confluent, rinsed twice with ice-cold PBS, scraped, and pelleted at 900 × *g* for 2 min at 4 °C. The 200–250-mg (wet weight) pellets were dissolved by gentle vortexing in 800 μl of lysis buffer with protease inhibitors, incubated in ice for 2 min, and subjected to sonication with 10 bursts in ice with a pre-cooled probe. Lysates were centrifuged at 500 × *g* for 10 min 4 °C to pellet the nuclear fraction, and supernatants were fractionated by discontinuous iodixanol density gradient centrifugation (Thermo Scientific) at 145,000 × *g* for 2 h at 4 °C in a swing rotor. Three fractions were collected, and each fraction was divided into 3 aliquots for protein quantitation, ICP-MS analysis, and immunoblotting.

Ctr1 Ectodomain Purification and Cleavage—Recombinant human CTR1 ectodomain (residues 1–55) was expressed as an amino-terminal SUMO fusion followed by a FLAG tag, CTR1 ectodomain, and a carboxyl-terminal Strep II tag. This coding region was subcloned into the pET-15b vector, transformed into *Escherichia coli* BL21 (DE3) cells, and expressed at 37 °C for ~4 h with 1 mM isopropyl 1-thio-β-D-galactopyranoside. Cells were lysed via sonication in 100 mM Tris, 150 mM NaCl, 1 mM EDTA and centrifuged to remove debris, and the lysate was loaded onto a 5-ml StrepTrap FPLC column (GE Healthcare), washed with lysis buffer, and eluted with lysis buffer supplemented with 5 mM *d*-desthiobiotin. Fractions containing the CTR1 ectodomain were pooled and treated with SUMO protease (Invitrogen), and the ectodomain was separated from the SUMO moiety by size fractionation over a Superdex 75 26/60 FPLC column (GE Healthcare). Fractions were pooled and concentrated to 40 μM. Purified recombinant cathepsin L prepared as described (33) was activated by a 30-min incubation in Activation Buffer (25 mM NaOAc, pH 5.5, 150 mM NaCl, 1 mM EDTA, 2 mM DTT), diluted to appropriate concentrations in Reaction Buffer (100 mM MES, pH 6.0, 150 mM NaCl, 1 mM EDTA), and mixed in a 1:1 (v/v) ratio with recombinant CTR1 ectodomain. Reactions were incubated at 37 °C for 1 h, terminated by the addition of SDS-PAGE loading buffer, and resolved by SDS-PAGE. Samples subjected to peptide analysis were treated as above with reactions terminated by a 5-min incubation at 95 °C prior to analysis by LC-MS/MS performed by the Duke University Proteomics Core.

Results

Ctr2 Stimulates Ctr1 Ectodomain Cleavage by Cysteine Proteases—In mouse and human cells Ctr1 and tCtr1 differ by the presence or absence of a histidine- and methionine-rich glycosylated ectodomain, in which tCtr1 initiates at a small cluster of sites previously identified by mass spectrometry (Fig. 1*A*) (32). Ctr2 forms a complex with Ctr1 *in vivo* and positively influences the abundance of the tCtr1 species. To ascertain the role of Ctr2 in regulating the biogenesis of tCtr1, the temporal effect of Ctr2 expression on the appearance of the two Ctr1 species was investigated in a $Ctr2^{-/-}$ MEF line in which Ctr2 expression is controlled by doxycycline. As shown in Fig. 1*B*, Ctr2 expression was paralleled by a time-dependent increase in the appearance of tCtr1. Notably, the formation of tCtr1 was accompanied by a corresponding reduction in the full-length Ctr1, suggesting that the formation of the tCtr1 may be due to proteolytic processing of full-length Ctr1, rather than stabilization of the tCtr1 protein by Ctr2. Although this precursor-product relationship between full-length and tCtr1 is often observed, in some experiments the reduction in tCtr1 is not accompanied by an increase in full-length Ctr1 and could reflect differences in stability. To determine the nature of this putative proteolytic process involved in tCtr1 generation, a collection of protease inhibitors was evaluated for their effect on the abundance of the tCtr1 species in wild type (WT) MEFs. A broad range of protease inhibitors was tested, including the matrix metalloprotease inhibitors TIMP-2, TIMP-3, BB-94 (Batimastat), and the ADAM17, -15, -8, -10, and -12 inhibitor TAPI-2. However, none of these or other inhibitors influenced the abundance of tCtr1 (Fig. 1*C*). In contrast, MEFs treated with the cysteine cathepsin/calpain protease inhibitor E64d displayed a striking reduction in the levels of tCtr1, suggesting that one or more E64d-inhibitable proteases are involved in the proteolytic processing of the Ctr1 ectodomain (Fig. 1*D*). A reduction in tCtr1 levels is also observed when Chinese hamster ovary cells (CHO) or human embryonic kidney cells (HEK293T) were treated with E64d, suggesting that Ctr1 truncation occurs via a proteolytic mechanism that is conserved among these species. The robust increase in tCtr1 levels caused by induced expression of Ctr2 can be abrogated by incubation with E64d (Fig. 1*E*). Together, these results suggest that the generation of tCtr1 is a conserved proteolytic process mediated by an E64d-inhibitable protease activity that acts downstream of Ctr2.

Cathepsin Inhibition Decreases tCtr1 Levels and Increases Copper Accumulation—The cell-permeable and irreversible protease inhibitor E64d inhibits cysteine cathepsin proteases, with a preference for cathepsins L and B at the doses used in our experiments, as well as the calcium-dependent calpain proteases (34). To explore which of these protease families is relevant for the formation of tCtr1, proteases were evaluated for co-localization with Ctr1 and Ctr2. Ctr1 has been shown to continuously cycle between the plasma membrane and endocytic vesicles, where it localizes to endolysosomal compartments that also harbor Ctr2 (14, 30, 32, 35). Because Ctr1 cleavage has previously been suggested to occur at an endosomal compartment (30), subcellular fractionation was carried out in an attempt to localize Ctr1 with the appropriate protease. The Ctr1 ectodomain faces the intraluminal space, and thus any protease involved in Ctr1 cleavage must be present inside the vesicular lumen. Because calpain proteases are localized to the cytosol, our studies focused on cathepsins L and B as they are known to primarily localize to endolysosomal compartments. Analysis of subcellular fractions by immunoblotting revealed that Ctr1, Ctr2, and cathepsins L and B co-fractionated together with the lysosomal marker Lamp1 (Fig. 2*A*), indicating that they are all in endolysosomal compartments.

To test the involvement of cathepsin L/B in generating tCtr1, the extent of Ctr1 truncation was evaluated in WT MEFs and those lacking cathepsin L or B. Cells lacking cathepsin L exhibit low levels of tCtr1 and elevated levels of both the full-length and a potential intermediate form of Ctr1 as compared with wild type and cathepsin B-deficient cells (Fig. 2*B*). However, the

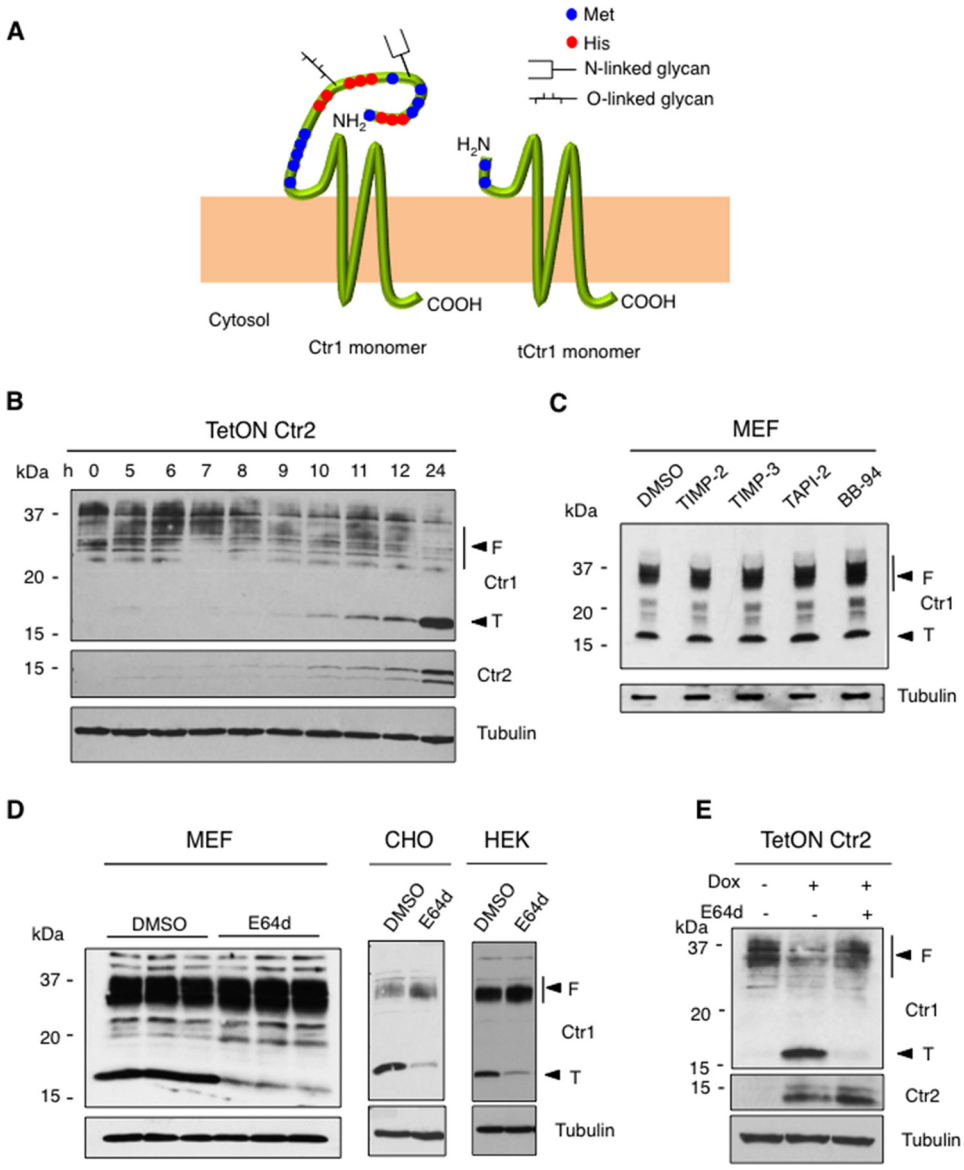

FIGURE 1. **Ctr1 ectodomain cleavage occurs in a Ctr2-stimulated, cysteine protease-dependent manner.** *A,* model showing monomeric full-length Ctr1 and tCtr1, with Met and His residues in the ectodomain indicated in *blue* and *red*, respectively, and glycans indicated with branches. *B,* cells containing a doxycycline-inducible Ctr2 (Tet-On Ctr2) were treated with 100 ng/ml doxycycline for the times indicated, harvested, and immunoblotted with anti-Ctr2, anti-Ctr1 (*T*, truncated; *F*, full-length), and anti-tubulin antibody. *C,* MEFs were treated with DMSO, 2 μg/ml TIMP-2, TIMP-3, and TAPI-2 or 5 μM BB-94 for 16 h, and protein extract was analyzed by immunoblotting with anti-Ctr1 (*T,* truncated; *F,* full-length) and anti-tubulin antibody. *D,* MEFs were treated with 10 μM E64d, and CHO and HEK cells were treated with 50 μM E64d for 16 h before immunoblot analysis as in *B*. *E,* Tet-On Ctr2 cells were cultured with or without 100 ng/ml doxycycline for 24 h before treatment with either DMSO or E64d (50 μM), and protein extracts were analyzed with immunoblotting as in *B*.

simultaneous absence of both cathepsins L and B caused a more severe reduction in Ctr1 ectodomain processing as compared with the absence of cathepsin L alone (Fig. 2*C*). The introduction of cathepsin L into cathepsin $L^{-/-}/B^{-/-}$ cells rescued the Ctr1 processing activity. In agreement with these genetic validation experiments, WT MEFs treated with a cathepsin L-selective inhibitor (cathepsin L inhibitor III) were compromised for the generation of tCtr1, whereas cells treated with the cathepsin B-selective inhibitor (CA074Me) or DMSO did not affect Ctr1 truncation (Fig. 2*D*). These results indicate that cathepsin L plays a prominent role in the processing of Ctr1 to form tCtr1 in MEFs, with a minor contribution by cathepsin B.

Previous results demonstrated that MEFs or mouse tissues lacking Ctr2 have increased levels of full-length Ctr1, decreased tCtr1, and a corresponding increase in intracellular Cu^+ levels (32). Hence, to assess whether the increase in intracellular Cu^+ observed in Ctr2-deficient cells and tissues is imposed by inhibition of cathepsin-mediated Ctr1 processing, MEFs were incubated with E64d and cell-associated copper was measured. Indeed, MEFs treated with E64d exhibited elevated cell-associated copper levels (Fig. 2*E*), and MEFs treated with a cathepsin L-specific inhibitor showed a similar increase in copper accumulation (Fig. 2*F*). Taken together, these data demonstrate that inhibition or loss of cathepsin L reduces Ctr1 ectodomain cleavage, which results in more full-length Ctr1 and elevated copper accumulation. Moreover, these results identify cathepsins L and B as critical components for Ctr1 ectodomain processing that play an important role in Cu^+ uptake in cultured cells.

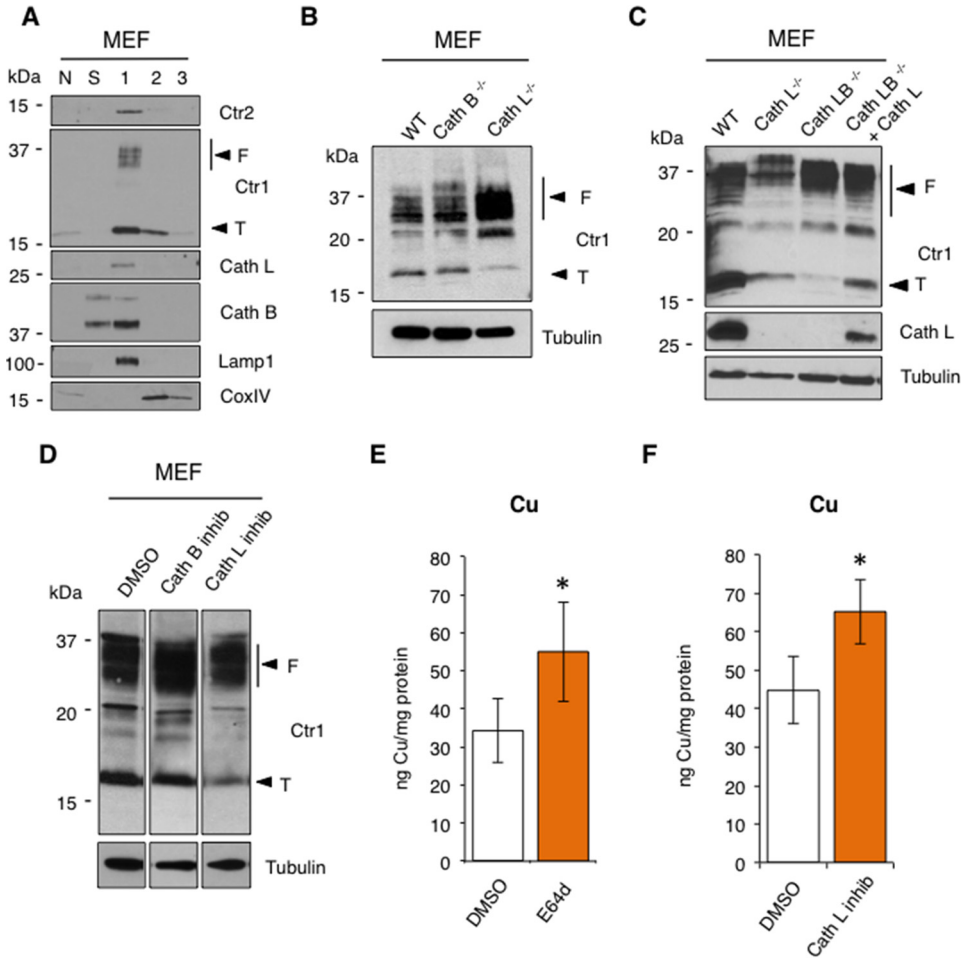

FIGURE 2. **Cathepsin L inhibition results in decreased Ctr1 ectodomain cleavage and increased copper accumulation.** *A,* MEFs were harvested and processed for endolysosomal isolation (see "Experimental Procedures"). Samples were resolved via SDS-PAGE and analyzed by immunoblotting with anti-Ctr2, anti-Ctr1 (*T*, truncated; *F*, full-length), anti-cathepsin L, anti-cathepsin B, anti-Lamp1, and anti-Cox IV. *N*, nuclear fraction; *S*, soluble fraction; *1*, *2*, and *3* represent fractions collected from the iodixanol gradient after ultracentrifugation. *B,* total protein extracts were isolated from wild type (*WT*); cathepsin $B^{-/-}$ and cathepsin (*Cath*) $L^{-/-}$ MEFs were analyzed by immunoblotting as in *A*. *C,* cathepsin $L^{-/-}B^{-/-}$ cells were transfected with a plasmid expressing cathepsin L and cultured for 48 h. Total protein extracts were resolved by SDS-PAGE and analyzed by immunoblotting alongside WT, cathepsin $L^{-/-}$, and cathepsin $L^{-/-}B^{-/-}$ cells. *D,* MEFs were treated with DMSO, 10 μM cathepsin B (*CA074Me*), or 10 μM cathepsin L inhibitors (*Inhib*) for 16 h and analyzed by immunoblotting as in *B*. Shown are lanes cut from the same membrane with the same x-ray film exposure time. *E* and *F,* cells were treated with 30 μM indicated inhibitor and 100 μM bathocuproinedisulfonic acid for 16 h before switching to media containing 5 μM copper and fresh inhibitor before harvesting and ICP-MS analysis of total cellular copper. Data are presented as mean ± S.D. from four biological replicates. *, $p \leq 0.05$.

Contribution of Cathepsin B and Ctr2 to the Processing of Ctr1—Data presented here demonstrate that the Ctr2 integral membrane protein stimulates Ctr1 ectodomain cleavage in a cysteine protease-dependent manner, and we have identified cathepsin L as having a major role in this process. To further decipher a potential role for cathepsin B, or other E64d-inhibitable proteases in the processing of Ctr1, WT and cathepsin $L^{-/-}$ MEFs were treated with E64d. Indeed, cathepsin $L^{-/-}$ MEFs treated with E64d showed higher levels of full-length Ctr1, suggesting that cathepsin B contributes to Ctr1 ectodomain processing (Fig. 3*A*). However, cathepsin L represents the majority of the proteolytic activity involved in biogenesis of tCtr1 in MEFs.

To gain insight into how Ctr2 regulates the processing of Ctr1 to tCtr1, in concert with cathepsins, Ctr2 expression was knocked down in both WT and cathepsin $L^{-/-}$ MEFs. As has been observed previously, silencing of Ctr2 in WT MEFs reduced the levels of tCtr1 and increased full-length Ctr1 (Fig. 3*B*). Notably, in cathepsin $L^{-/-}$ cells, where the steady state levels of tCtr1 levels are low, silencing of Ctr2 caused a further reduction in the levels of truncated Ctr1. This indicates that, although cathepsin L has a major role in Ctr1 processing, Ctr2 also stimulates formation of tCtr1 via means other than cathepsin L. This observation is supported by data in Fig. 2*A* showing that residual tCtr1 is observed in cells lacking cathepsin L. Interestingly, cells lacking cathepsin L show increased levels of cathepsin B (Fig. 3*C*). This suggests that cathepsin B might also contribute to Ctr1 processing, a notion supported by the observed lower levels of tCtr1 in cathepsin $L^{-/-}$, cathepsin $B^{-/-}$ double-deficient cells compared with cathepsin $L^{-/-}$ cells (Fig. 2*C*). This observation is in agreement with a previous study showing that cathepsin B levels increase in mice and cells lacking cathepsin L and by genetic studies showing that cathepsins B and L functionally compensate each other (36, 37).

To assess whether Ctr2 regulates Ctr1 ectodomain cleavage by recruiting Ctr1 or cathepsin L to endolysosomes, subcellular fractionation was performed to compare the localization of these proteins in WT and $Ctr2^{-/-}$ MEFs. Immunoblot analysis

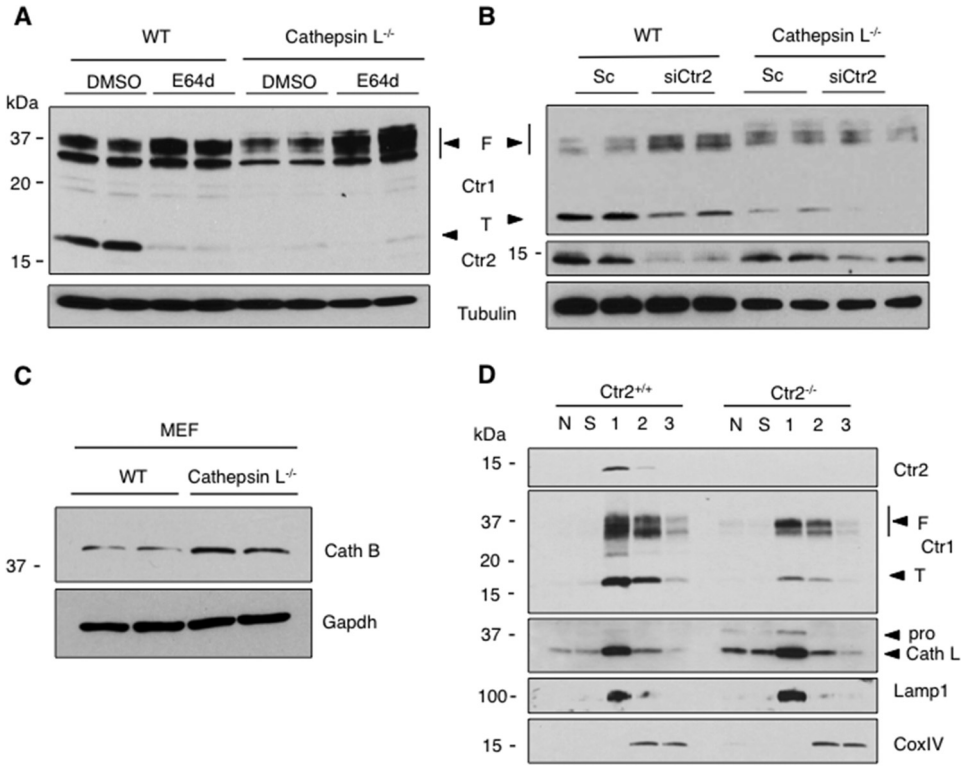

FIGURE 3. **Contribution of cathepsin B and Ctr2 to the processing of Ctr1.** *A,* wild type (cathepsin $L^{+/+}$) and cathepsin $L^{-/-}$ MEFs were treated with DMSO or 10 μM E64d inhibitor for 16 h. Total protein extract was resolved by SDS-PAGE and immunoblotted with anti-Ctr1 and anti-tubulin antibody. *B,* wild type (cathepsin $L^{+/+}$) and cathepsin $L^{-/-}$ MEFs were treated with 25 nM scrambled (*Sc*) RNA or siRNA directed against Ctr2 for 72 h. Total protein extracts were immunoblotted with anti-Ctr1, anti-Ctr2, and anti-tubulin antibody. *C,* total protein extracts from wild type (cathepsin $L^{+/+}$) and cathepsin $L^{-/-}$ MEFs, resolved by SDS-PAGE, and analyzed by immunoblotting with anti-cathepsin B antibody. *Cath,* cathepsin. GAPDH as loading control. *D,* immunoblotting of wild type ($Ctr2^{+/+}$) and $Ctr2^{-/-}$ MEF protein extracts derived from endolysosomal enrichment (see "Experimental Procedures"). Blots were analyzed by probing with anti-Ctr2, anti-Ctr1 (*T*, truncated; *F*, full-length), anti-cathepsin L, anti-cathepsin B, anti-Lamp1, and anti-CoxIV. *N*, nuclear fraction; *S*, soluble fraction; *1*, *2*, and *3* represent fractions collected from the iodixanol gradient after ultracentrifugation.

revealed that Ctr1, Ctr2, and cathepsin L were all found in the same fraction, together with the lysosomal marker Lamp1, in both WT and $Ctr2^{-/-}$ cells (Fig. 3*D*). We noted that the levels of pro-cathepsin L were modestly increased in MEFs lacking Ctr2, suggesting that Ctr2 could promote cathepsin L processing of the Ctr1 ectodomain by mechanisms other than affecting its subcellular localization or expression. Taken together, these data suggest that Ctr1 ectodomain processing occurs in endolysosomal compartments containing both Ctr2 and the cysteine cathepsins, but Ctr2 is not necessary for their recruitment to this compartment.

Cathepsin L Cleaves the Ctr1 Ectodomain in Vitro and Primes Further Processing—Although cathepsin L functions in Ctr1 ectodomain cleavage and in the regulation of Cu^{+} uptake in MEFs, it is not known whether cathepsin L plays a direct or indirect role in this process. To address this question, an *in vitro* substrate processing assay was developed using the recombinant cathepsin L and recombinant CTR1 ectodomains (Fig. 4*A*). Incubation of the purified CTR1 ectodomain with cathepsin L at a range of concentrations, followed by SDS-PAGE and Ponceau S staining revealed the formation of two lower molecular weight protein species in a cathepsin L dose-dependent manner (Fig. 4*B*). Mass spectrometry analysis identified the species as CTR1 ectodomain fragments generated as the result of cleavage between residues 8 and 9 from the amino terminus (Fig. 4*C*, indicated by *red arrowhead*) (see supplemental Fig. S1 for high resolution MS/MS spectrum (A) and deconvoluted MS spectrum (B)).

The cathepsin L-mediated Ctr1 ectodomain cleavage site is conserved among mammals (Fig. 4*C*) raising the possibility that Ctr1 cleavage by this enzyme may be a common feature among mammals. However, the amino terminus generated by cathepsin L cleavage *in vitro* is not synonymous with the amino terminus of the tCtr1 generated *in vivo*, previously identified to begin within the methionine-rich region of both the mouse and human Ctr1 proteins (32) (Fig. 4*C,* indicated by *blue arrowheads*). This suggests that post-cathepsin L processing of Ctr1 occurs to yield the fully processed tCtr1. To assess this possibility and to ascertain a potential role for Ctr2 in this mechanism, a $Ctr1^{-/-}$ $Ctr2^{-/-}$ MEF cell line was generated in which Ctr2 expression is induced by doxycycline. In the absence of Ctr2, the abundance of tCtr1 is low and is further diminished by incubation with E64d (Fig. 4*D*, compare *lanes 2* and *3*). In agreement with previous results, cells lacking Ctr2 show increased full-length Ctr1 levels and decreased tCtr1 levels (Fig. 4*D*, compare *lanes 2* and *7*), and treatment with E64d decreased tCtr1 levels (Fig. 4*D*, compare *lane 2 versus lane 3* and *lane 7 versus lane 8*). Expression of a Ctr1 variant (Ctr1Δ1–8), to mimic the post-cathepsin L cleavage form, was further processed to yield a Ctr1 species that electrophoretically co-migrates with tCtr1 (Fig. 4*D*, *lane 4*). Interestingly, processing of the Ctr1Δ1–8 variant proceeded in the absence of Ctr2, although expression of

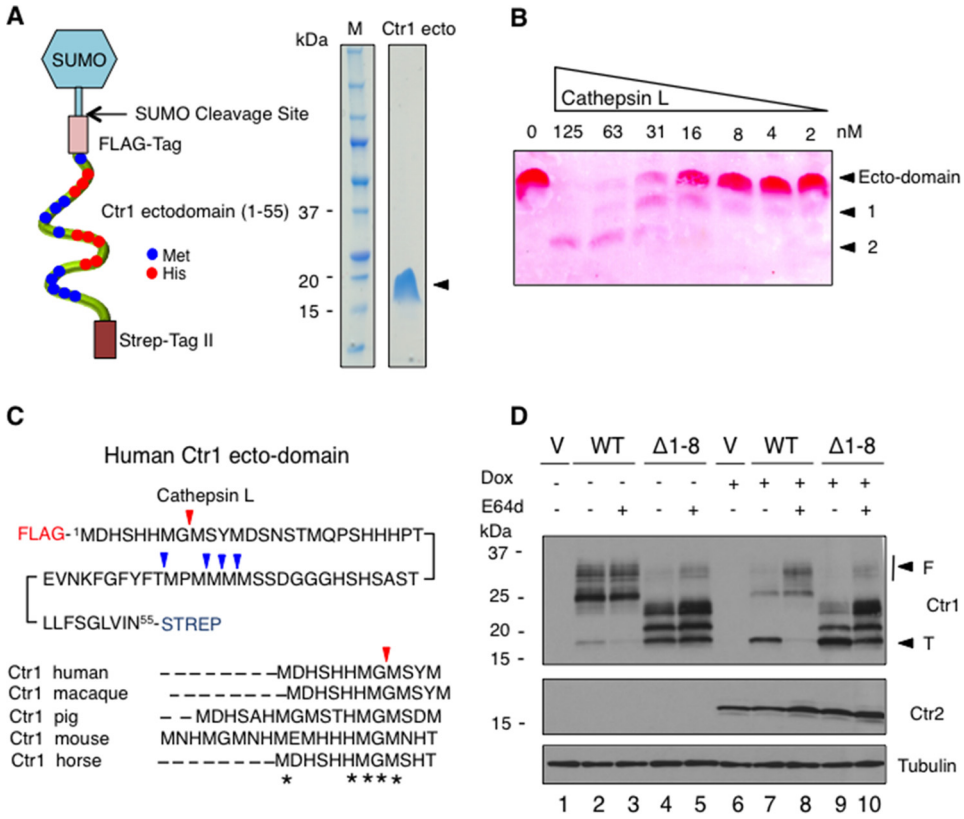

FIGURE 4. **Cathepsin L cleaves the CTR1 ectodomain *in vitro* and primes further ectodomain processing.** *A,* diagram of the CTR1 ectodomain purified for *in vitro* cathepsin cleavage studies and SDS-PAGE of purified ectodomain (*Ctr1 ecto*). *Lane M*, molecular mass marker. *B,* CTR1 ectodomain was incubated with recombinant cathepsin L at the indicated enzyme concentration, and products were resolved by SDS-PAGE and Ponceau S staining. The products generated by cathepsin L cleavage are indicated as *1* or *2*. *C,* site of cathepsin L cleavage (*red arrowhead*) determined by mass spectrometry and the previously identified amino termini of tCtr1 (*blue arrowhead*). *Asterisks* indicate residues within the cathepsin L cleavage site found in several mammalian Ctr1 proteins. *D,* $Ctr1^{-/-}Ctr2^{-/-}$ cells containing a Dox-inducible *Ctr2* gene were transfected with either an empty vector (*V*), a vector containing wild type Ctr1 (*WT*), or Ctr1 lacking the first 8 residues (Ctr1Δ1–8) before being treated with 100 ng/ml doxycycline for 48 h. Cells were then harvested and processed for immunoblotting with the indicated antibodies.

Ctr2 further enhanced the production of tCtr1 (Fig. 4*D*, compare *lanes 4* and *9*). Although treatment with E64d gave rise to slightly higher levels of uncleaved Ctr1Δ1–8, tCtr1 was still present (Fig. 4*D*, compare *lanes 4* and *5*). Notably, the contribution of Ctr2 to the processing of Ctr1Δ1–8 is abrogated by addition of E64d (Fig. 4*D*, compare *lanes 4* and *5* to *lanes 9* and *10*), suggesting that the formation of tCtr1 is initiated by cathepsin L and proceeds via both E64d-sensitive and E64d-resistant proteases, with Ctr2 stimulating the E64d-sensitive proteases. Taken together, these results suggest that proteases other than cathepsin L participate in Ctr1 ectodomain processing, but they function downstream of the rate-limiting cathepsin L cleavage.

Cathepsin $L^{-/-}$ Mice Are Defective in Ctr1 Processing and Accumulate Copper—Data presented here demonstrate a direct and rate-limiting role for cathepsin L in Ctr1 ectodomain cleavage and in the modulation of cellular copper accumulation. To investigate a potential physiological role for cathepsin L in Ctr1 ectodomain cleavage and copper accumulation in animals, cathepsin $L^{-/-}$ mice were evaluated. Cathepsin $L^{-/-}$ mice exhibit increased copper accumulation in brain, lymph nodes, and testis in comparison with wild type littermates (Fig. 5*A*). However, cathepsin L deficiency did not affect copper levels in liver, a finding that is in agreement with the lack of impact of Ctr2-deficiency on liver copper levels (32). Zinc levels were not different between wild type and cathepsin $L^{-/-}$ mice (Fig. 5*B*). Moreover, lower levels of tCtr1 were observed in lymph nodes and kidneys from cathepsin $L^{-/-}$ mice as compared with wild type mice, along with elevated levels of full-length Ctr1 in brain, spleen, lymph nodes, and testis (Fig. 5*C*). Indeed, the tissue types that exhibited increased copper accumulation and decreased Ctr1 cleavage in cathepsin $L^{-/-}$ mice paralleled the tissues demonstrating similar defects in $Ctr2^{-/-}$ mice (32). The considerable amount of tCtr1 in cathepsin $L^{-/-}$ mouse tissues suggests a discrepancy between the Ctr1 processing in mice compared with MEFs. Hence, in the cathepsin $L^{-/-}$ mice, cathepsin L or B may be more dominant in some tissues, whereas in MEFs cathepsin L may be the primary protease responsible for Ctr1 ectodomain cleavage. Taken together, these observations support a physiological role for cathepsin L, working in concert with Ctr2, in Ctr1 ectodomain cleavage. Additionally, these studies establish cathepsin L as a novel physiological regulator of mammalian copper homeostasis.

Cathepsin L Inhibition Enhances Cisplatin Accumulation and Cell Death—Cisplatin and related derivatives are platinum-based chemotherapeutic agents widely used for cancer types, including ovarian, colon, lung, testicular, and head and neck (38). Previous studies demonstrated that mammalian Ctr1 functions in cisplatin uptake, that cisplatin binds directly to methionine residues within the Ctr1 ectodomain, and that the

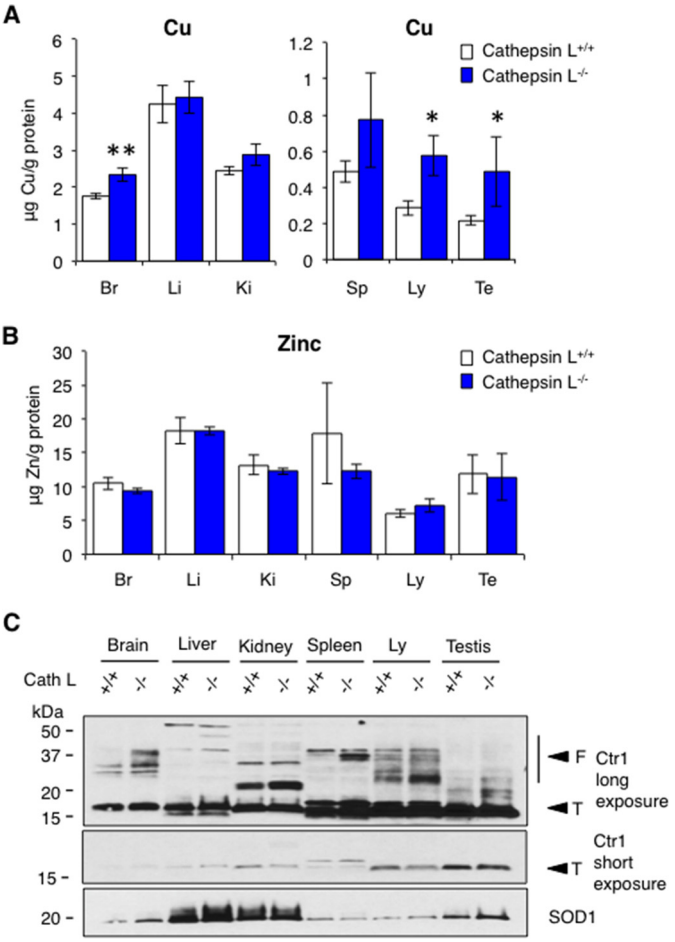

FIGURE 5. **Cathepsin L$^{-/-}$ mice exhibit defects in Ctr1 ectodomain cleavage and accumulate copper.** *A,* 2-month-old wild type (cathepsin L$^{+/+}$) and cathepsin L$^{-/-}$ mice were analyzed for copper levels by ICP-MS in brain (*Br*), liver (*Li*), kidney (*Ki*), spleen (*Sp*), lymph nodes (*Ly*), and testis (*Te*). Note different scales for copper levels in different tissues. *B,* tissue zinc analysis as in *A*. Data are presented as means ± S.D. from three to four mice, *, $p \leq 0.05$; **, $p \leq 0.01$. *C,* protein extracts from the indicated tissues from WT ($^{+/+}$) and cathepsin L$^{-/-}$ (*Cath L*$^{-/-}$) littermates were immunoblotted with anti-Ctr1 and anti-SOD1 antibody, with cathepsin L genotypes indicated. Shown are the full-length (*F*) and truncated (*T*) forms of Ctr1. A short exposure shows the abundance of tCtr1 and a longer exposure reveals the expression of both full-length and truncated Ctr1.

ectodomain and its methionine residues play a critical role in cisplatin uptake (24–26, 29, 39, 40). Consistent with these observations, Ctr2$^{-/-}$ MEFs, which express more full-length Ctr1, accumulate significantly higher levels of cisplatin compared with WT MEFs (32). To test whether preservation of the Ctr1 cisplatin-binding ectodomain by inhibiting the rate-limiting step in cleavage augments cisplatin accumulation, Ctr1$^{-/-}$ MEFs were transfected with an empty vector or a vector expressing human Ctr1 and treated with either vehicle or cathepsin L inhibitor, in combination with cisplatin. Ctr1$^{-/-}$ MEFs transfected with the Ctr1 expression plasmid accumulated more platinum as compared with vector-transfected cells (Fig. 6*A*). Moreover, when Ctr1-expressing cells were treated with the cathepsin L inhibitor, platinum accumulation strongly increased over untreated cells. These results demonstrate that cisplatin uptake is stimulated by co-administration of a cathepsin L inhibitor and, furthermore, that the stimulation of cisplatin accumulation is dependent on Ctr1. Given the results presented here, this likely occurs via inhibition of Ctr1 ectodomain cleavage mediated by cathepsin L. To investigate whether the increased cisplatin accumulation provoked by inhibition of cysteine cathepsins enhances cisplatin-mediated toxicity, cell viability was measured after addition of E64d, in combination with cisplatin, to WT and Ctr1$^{-/-}$ MEFs. Cisplatin treatment alone reduced cell viability by 28%, whereas a combination of cisplatin and E64d reduced cell viability further to 43% (Fig. 6*B*). MEFs lacking Ctr1 displayed no additional reduction in cell viability when cells were treated with E64d (Fig. 6*B*), demonstrating that the decreased cell survival following treatment with cisplatin in combination with E64d is dependent on Ctr1-mediated cisplatin uptake.

Discussion

The Ctr1 high affinity copper transporter exists as two species, full-length and tCtr1, at varying levels in different cell lines and tissues (18, 31, 32). We previously demonstrated that MEFs and specific mouse tissues lacking the Ctr2 integral membrane protein have lower levels of tCtr1, increased levels of full-length Ctr1, and a corresponding increase in copper accumulation (32). Here, we demonstrate that tCtr1 is generated by proteolytic cleavage of full-length Ctr1, which is initiated by the cysteine cathepsins L and B, and that this cleavage occurs in a Ctr2-dependent manner. We also demonstrate that truncation of Ctr1 by these cathepsins has a functional impact on cellular accumulation of both Cu^+ and cisplatin, both of which bind directly to the metal ligand-rich ectodomain (24, 26, 27, 29). Our results establish cathepsin L/B cleavage as a direct and rate-limiting step in the generation of tCtr1. Moreover, these observations establish a new mechanism for the regulation of Ctr1-mediated Cu^+ accumulation and identify cathepsin L, and to a lesser extent cathepsin B, as an important regulator of the mammalian copper homeostasis machinery. In line with our findings, proteolytic ectodomain processing of Zip4 has been proposed to be a regulatory mechanisms controlling zinc uptake by Zip4 (41).

We previously demonstrated that Ctr1 and Ctr2 form a complex *in vivo* and co-purify in the same endolysosomal enrichment fraction (32). Cathepsins L and B are classical proteases of the endolysosome that also co-purify with the Ctr1- and Ctr2-containing vesicular fraction, potentially placing these components in proximity for their involvement in Ctr1 ectodomain cleavage. We envision distinct mechanisms whereby Ctr2 and cathepsins L/B could function in concert to initiate Ctr1 ectodomain removal (Fig. 6*C*). Upon endocytosis from the plasma membrane into an endolysosomal compartment, Ctr1 could interact with Ctr2 in a manner that stimulates cleavage by cathepsin L/B. This could occur either by Ctr2-mediated recruitment of cathepsin L/B [*1*] or by a Ctr2-induced conformational change in the Ctr1 ectodomain such that the cleavage site for cathepsin L/B becomes accessible [*2*] (see Fig. 6*C*). Because Ctr1 undergoes constitutive endocytosis and recycling to the plasma membrane (14, 15, 35, 42), ectodomain cleavage within the endolysosomal compartment would result in population of the plasma membrane with tCtr1 molecules, thereby reducing Cu^+ import relative to a Ctr1-enriched plasma membrane. Interestingly, blocking the Ctr1 ectodomain *O*-linked

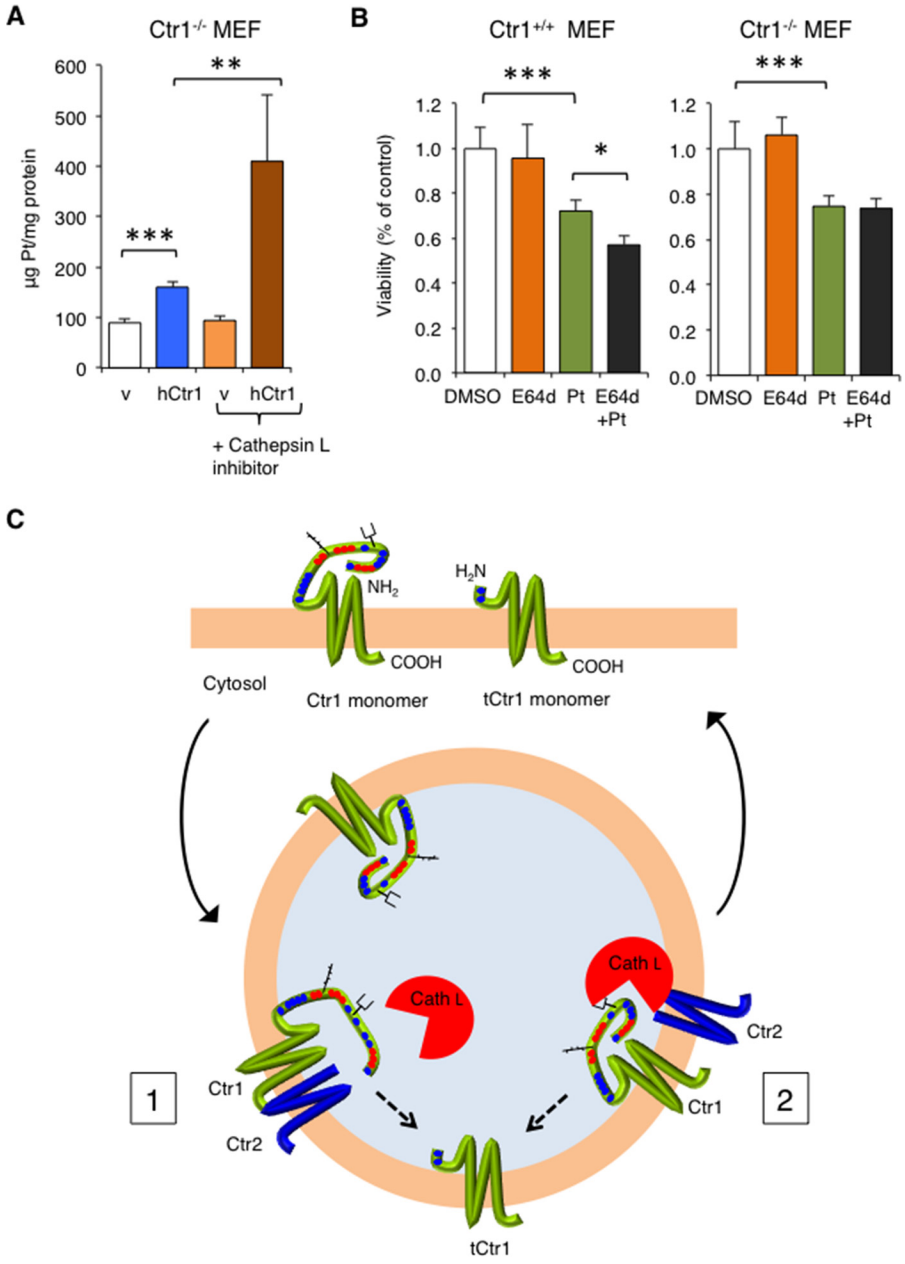

FIGURE 6. **Cathepsin L inhibition of Ctr1 ectodomain cleavage enhances cisplatin accumulation and efficacy *in vitro*.** *A*, Ctr1$^{-/-}$ MEFs were transfected with vector control (*v*) or vector containing human Ctr1 (*hCtr1*) and treated either with DMSO or 10 μM cathepsin L inhibitor for 16 h followed by a pulse of 200 μM cisplatin for 2 h. Cells were harvested and total cellular platinum (*Pt*) levels measured by ICP-MS and normalized to protein concentrations. *B*, wild type or Ctr1$^{-/-}$ MEFs were pre-treated with DMSO or 10 μM E64d for 2 h followed by DMSO or 50 μM cisplatin for 12 h, and cell viability was assessed by recording the formation of the fluorescent product resorufin, with normalization against vehicle-treated cells. Data are presented as mean $\pm$ S.D. from three to four biological replicates. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$. *C*, model for the Ctr2 and cathepsin L (*Cath L*)-mediated cleavage of the Ctr1 ectodomain to generate tCtr1, within an endolysosomal compartment. In this model, we envision two mechanisms by which Ctr2 and cathepsin L/B cooperate in Ctr1 ectodomain cleavage; [*1*] binding of Ctr2 to Ctr1 may provoke a conformational switch in the Ctr1 ectodomain, providing access by cathepsin L/B, or [*2*] Ctr2 may recruit cathepsin L/B and deliver the protease to the ectodomain cleavage site.

glycosylation by mutating Thr-27 increased the abundance of tCtr1 (31). Although Thr-27 is distal to the cathepsin L cleavage site within the ectodomain, glycosylation could sterically hinder cleavage at position 8 within the ectodomain. This raises the possibility that ectodomain cleavage, and therefore Cu^+ transport, could also be regulated by cellular glycosylation/deglycosylation enzymes. As copper concentrations near or above the apparent K_m value stimulate Ctr1 endocytosis (15), it is possible that this trafficking event serves as a regulatory step to reduce Cu^+ uptake via enhancing cleavage of the Ctr1 ectodomain cleavage, thereby locating a lower efficiency Cu^+ transporter at the cell surface, without the wholesale removal of Ctr1 from the plasma membrane. Additional studies will be required to decipher whether and how Ctr1 cleavage is regulated as a function of cellular copper status. It is also intriguing that in both Ctr2$^{-/-}$ and cathepsin L$^{-/-}$ mice, the same tissue-specific patterns of diminished Ctr1 ectodomain cleavage and increased copper accumulation are apparent (32). This could be due to differences in the expression or activity of Ctr2, cathepsin L/B, or other as yet unknown components of this pathway to

Ctr2/cathepsin-independent Ctr1 ectodomain cleavage mechanisms or to differences in Ctr1 trafficking, as has been observed in distinct mouse tissues (43–45).

The cysteine cathepsins have historically been thought of as quite nonspecific proteases involved in bulk degradation of lysosomal content (46, 47). However, recent evidence has shown that several cathepsins have specific biological functions by cleaving substrates at specific sites, thereby acting as convertases to modulate the production or activity of peptide hormones, histones, complement factors, proteases, vesicular transporters, and even as sheddases by cleaving ectodomains of several membrane-anchored adhesion proteins and receptors (48–58). Cathepsin L cleavage specificity is primarily guided by P2 preference for aromatic, and to lesser extent aliphatic, amino acids downstream of the cleavage site (42, 59, 60). The cleavage site in Ctr1 identified by mass spectrometry revealed that there is a conserved Met at the P2 site in Ctr1, offering a good substrate-protease fit. Interestingly, the cathepsin L cleavage site within the Ctr1 ectodomain is conserved across a variety of mammalian genomes that also encode a Ctr2 homologue. Given the difference between the location of the cathepsin L cleavage site *in vitro*, compared with the amino terminus of tCtr1 *in vivo* (32), it is possible that Ctr2 or other factors could influence the cathepsin L cleavage site choice *in vivo*.

Ctr1 plays a significant role in cisplatin import in mammalian cells (25, 28, 61). Similar to Cu^+, platinum binds to Met-rich motifs within the Ctr1 ectodomain (27), but studies using Ctr1 mutants that are defective in Cu^+ transport, but competent for cisplatin acquisition, suggest that Ctr1 delivers platinum into cells via receptor-mediated endocytosis rather than acting as a membrane channel as it does for Cu^+ (15, 26). Cancer patient survival after treatment with platinum-based drugs is associated with high levels of CTR1 expression and lower CTR1 levels associated with decreased survival to cisplatin (62, 63). Intriguingly, and in contrast to CTR1, low levels of CTR2 positively associate with patient survival after cisplatin therapy (63, 64). In agreement with this observation, cultured cells lacking Ctr2 exhibit increased whole-cell platinum accumulation (32, 65). Also, low Ctr2 levels correlate with an increased sensitivity to platinum treatment in several cancer cell lines (66). Low Ctr2 levels would be expected to give rise to increased Ctr1 harboring the platinum-binding ectodomain, thereby increasing uptake. Interestingly, high cathepsin B and L expression levels have been associated with poor prognosis for multiple cancer entities, although knock-out or inhibition of cathepsins attenuates cancer progression and enhances the efficacy of chemotherapies (67, 68). Taken together, these results support a concept in which cathepsin L/B together with Ctr2 modulate both Cu^+ and platinum accumulation via a Ctr1-dependent mechanism involving Ctr1 ectodomain cleavage. The discovery that cathepsin L/B function in the modulation of Ctr1-dependent Cu^+ transport identifies these proteases as new components of the copper homeostasis machinery and potentially provides a new point for the regulation of copper transport. Perhaps the pharmacological modulation of this pathway could enhance cisplatin efficacy in resistant tumors or decrease the therapeutic dose needed for clinical efficacy.

Author Contributions—H. O., B. L., and D. J. T. designed the experiments. H. O. and B. L. prepared samples and performed the experiments. T. R. housed, genotyped, and prepared samples from cathepsin L mice knock-out strain. B. T. purified cathepsin L. H. O., B. L., and D. J. T. analyzed the data and prepared the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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