Copper accumulation and compartmentalization in mouse fibroblast lacking metallothionein and copper chaperone, Atox1

Takamitsu Miyayama a,b, Kazuo T. Suzuki a, Yasumitsu Ogra a,b,

a Graduate School of Pharmaceutical Sciences, Chiba University, Chuo, Chiba 260-8675, Japan
b Laboratory of Chemical Toxicology and Environmental Health, Showa Pharmaceutical University, Machida, Tokyo 194-8543, Japan

Abstract
Copper (Cu) is the active center of some enzymes because of its redox-active property, although that property could have harmful effects. Because of this, cells have strict regulation/detoxification systems for this metal. In this study, multi-disciplinary approaches, such as speciation and elemental imaging of Cu, were applied to reveal the detoxification mechanisms for Cu in cells bearing a defect in Cu-regulating genes. Although Cu concentration in metallothionein (MT)-knockout cells was increased by the knockdown of the Cu chaperone, Atox1, the concentrations of the Cu influx pump, Ctr1, and another Cu chaperone, Ccs, were paradoxically increased; namely, the cells responded to the Cu deficiency despite the fact that cellular Cu concentration was actually increased. Cu imaging showed that the elevated Cu was compartmentalized in cytoplasmic vesicles. Together, the results point to the novel roles of MT and cytoplasmic vesicles in the detoxification of Cu in mammalian cells.
Introduction

Copper (Cu) is an essential trace element in living organisms. It functions as the active center of cuproenzymes, such as cytochrome c oxidase (CCO), Cu,Zn-superoxide dismutase (Cu,Zn-SOD, SOD1), ceruloplasmin, lysyl oxidase, tyrosinase, and dopamine β-hydroxylase (Massaro, 2003). The mechanisms underlying Cu homeostasis are suggested as follows (Kim et al., 2008): Cu is mainly incorporated into cells as a monovalent Cu ion, i.e., cuprous ion, by Ctr1, a transporter expressed on the plasma membrane (Lee et al., 2000; Zhou and Giyschier, 1997). Incorporated Cu associates with one of three cytoplasmic Cu escort proteins, the so-called Cu chaperones, for it to be escorted to specific organelles or cuproenzymes. Atox1, a Cu chaperone for the Golgi apparatus, hands over Cu to Atp7a and Atp7b, which are ATP-dependent Cu transporters into the secretory pathway of Cu via the Golgi apparatus (Klomp et al., 1997; Hamza et al., 1999). In the Golgi apparatus, Cu is incorporated into cuproenzymes, such as ceruloplasmin and lysyl oxidase, due to the secretion of these cuproenzymes into extracellular fluid. Otherwise, Cu is directly excreted from cells by secretory vesicles that translocate to the plasma membrane to exocytose Cu. Cox17, a Cu chaperone for the mitochondria, is required to donate Cu to CCO and/or SCO1, which is a recipient protein of Cu on the mitochondrial membrane (Horng et al., 2005; Horng et al., 2004). CCS, a Cu chaperone for SOD1, transports Cu to SOD1 in cytosol by forming a heterodimer with SOD1. In addition to these Cu chaperones, a novel Cu-regulating protein, Commd1, was recently characterized (Maine and Burnstein, 2007). It does not have apparent Cu-binding motifs in its molecule but is proposed to participate in the Cu-efflux pathway by interacting with Atp7b (de Bie et al., 2005). Indeed, Bedlington terriers demonstrated inherited hepatic Cu toxicosis due to a defect in Commd1 (Forman et al., 2005). Metallothionein (MT) is suggested to be also a Cu-regulating protein. It actually binds Cu via Cu-thiolate clusters (Presta and Stillman, 1997). Since MT binding of Cu is thermodynamically and kinetically stable, excess Cu is sequestered by MT to mask Cu toxicity (Salgado and Stillman, 2004). On the other hand, an alleviative role of MT in Cu deficiency was also suggested (Ogra et al., 2006; Suzuki et al., 2002). Thus, MT may play a dual role in Cu homeostasis in mammalian cells. As mentioned above, Cu is regulated by various proteins, such as transporters across the membrane, chaperones in the cytoplasm, and MT that buffers Cu availability in cells. However, this rigid regulation may prevent us from experimentally disturbing intracellular Cu concentration and further analyzing the mechanisms of Cu regulation. In particular, MT is induced by either an excess or a deficiency of Cu to maintain Cu homeostasis. In some cases, it was suggested that MT
did not contribute to the detoxification of Cu. For instance, it is reported that Formosan squirrels abnormally and inheritably accumulated Cu in the liver (Suzuki et al., 2004). Although other inherited Cu toxicosis animals, such as toxic milk mice and Long–Evans Cinnamon (LEC) rats, accumulated Cu in the form bound to MT in their livers (Shim and Harris, 2003), it was shown that Cu accumulated in the liver of Formosan squirrels dominantly existed in the insoluble fraction of the liver and only a small amount was bound to MT (Suzuki et al., 2004). Alternatively, Cu that was rapidly administered to rats also existed in the form that was not bound to MT (Suzuki et al., 1989). However, these animals did not manifest Cu toxicity and thus, a detoxification mechanism that functions independently of MT is speculated. In this study, we intended to establish an experimental model in which Cu concentration was modified by gene knockdown (KD) in a Cu regulating protein, and to show that the contribution of MT to Cu homeostasis could be ignored. In this regard, fibroblasts established from an MT-null mutant mouse, i.e., MT-knockout cells (MT-KO) cells, are frail against the Cu toxicity, and those cells are more useful to reveal Cu homeostasis than MT-wild type (MT-WT) cells. Hence, Atox1-KD was introduced into MT-KO cells. In the present study, multi-disciplinary approaches were used to reveal the detoxification mechanisms for Cu in cells. First, speciation was used to determine the chemical species of Cu in the soluble fraction of cells. As the sample from cultured cells was too small to allow for analysis of Cu species by conventional HPLC coupled with an inductively coupled plasma mass spectrometer (ICP-MS), micro HPLC coupled with ICP-MS was adopted. Second, an elemental fluorescent probe for Cu, Cu sensor 1 (CS1), was used for Cu imaging. This probe enables visualization of intracellular Cu distribution in live cells. Thus, elemental imaging is a complementary technique to elemental speciation in metallochemical biology. The aim of this study is to reveal the MT-independent detoxification mechanisms for Cu using MT-KO cells bearing Atox1-KD with multi-disciplinary approaches.
Materials and methods

Reagents.
Milli-Q water, 18.3 MΩ/cm, (Millipore) was used throughout. Tris(hydroxymethyl)aminomethan (Trizma®Base) was purchased from Sigma (St. Louis, MO, USA). Ammonium acetate, acetic acid, 28% ammonia solution, hydrochloric acid, and other reagents of the highest grade were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Cell culture and gene knockdown with siRNAs.
MT-wild type (MTWT) and MT-knockout (MT-KO) cells were established from embryonic fibroblasts of 129Sv MT-WT and MT-KO mice transformed with SV40 large T antigen by Kondo et al. (Kondo et al., 1999) and kindly provided by Professor Seiichiro Himeno (Tokushima Bunri University, Japan). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) of the high glucose type (4500 mg/l), and supplemented with 10% heat inactivated fetal bovine serum, 10 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C under 5% CO2 atmosphere. Double-stranded RNAs (dsRNAs) were used as siRNAs (Stealth RNAi, Invitrogen, Tokyo, Japan). The targeted sequence of Atox1 was as follows: siRNA_Atox1, 5′-ACAAGCUGGGGAGGAGUGGAGUUCAA-3′. MT-WT and MT-KO cellswere seeded on a six-well plastic plate at 1.0×105 cells/well, and were pre-incubated for 24 h. The preincubated cells were either transfected or not transfected with 100 nM siRNA targeting Atox1 in the medium optimized for siRNA transfection (Opti-MEM I, Invitrogen), containing 0.6% Lipofectamine 2000 (Invitrogen) for 24 h. The cells were harvested 24 h after the transfection and subjected to analysis as mentioned below.

Isolation of total RNA and determination of Cu-regulating gene mRNA expression by real-time reverse transcription polymerase chain reaction.
Total RNA was isolated from the cells with an RNAqueous®-Micro Kit (Ambion, Austin, TX, USA) according to the protocol provided by the manufacturer. Reverse transcription reaction, the first step of real-time PCR (qPCR), was performed with a QuantiTect Reverse Transcription Kit (Qiagen, Tokyo, Japan). PCR was performed with ABsolute QPCR SYBR Green ROX Mix (ABgene, New York, NY, USA). Gene-specific primers used for amplification of mouse MT-I (Genbank accession no. NM_013602), MT-II (NM_008630), Atox1 (NM_009720), Ctr1 (NM_175090), Ccs (NM_016892), Cox17 (NM_001017429), Atp7a (NM_009726), Atp7b (NM_007511) and β-actin cDNAs were as
follows: MT-I-forward (F), 5′-CACCAGATCTCGGAATGGAC-3′; MT-I-reverse (R), 5′-CGCTCCTAGAACTCTTCAAACC-3′; MT-II-F, 5′-CCGTCCTCAGAATCTCAGGAC-3′; MT-II-R, 5′-AGGAGCAGCAGCTCTTCTTG-3′; MT-I-F, 5′-CGCTCCTAGAACTCTTCAAACC-3′; MT-I-R, 5′-AGGAGCAGCAGCTCTTCTTG-3′; Atox1-F, 5′-AGGAGCAGCAGCTCTTCTTG-3′; Atox1-R, 5′-GACAAAGGATGGAAACCGG-3′; Ctr1-F, 5′-CGCTCCTAGAACTCTTCAAACC-3′; Ctr1-R, 5′-AGGAGCAGCAGCTCTTCTTG-3′; Ccs-F, 5′-TGATGATGATGGAGCCTCCTCAAACC-3′; Ccs-R, 5′-AGGAGCAGCAGCTCTTCTTG-3′; Cox17-F, 5′-TGATGATGATGGAGCCTCCTCAAACC-3′; Cox17-R, 5′-AGGAGCAGCAGCTCTTCTTG-3′; Atp7a-F, 5′-AGGAGCAGCAGCTCTTCTTG-3′; Atp7a-R, 5′-GACAAAGGATGGAAACCGG-3′; Atp7b-F, 5′-TGATGATGATGGAGCCTCCTCAAACC-3′; Atp7b-R, 5′-AGGAGCAGCAGCTCTTCTTG-3′; β-actin-F, 5′-TCGCCATGGATGACGATA-3′; β-actin-R, 5′-GAGTCCTTCTGACCCATTCC-3′.

RT-PCR was conducted under the following conditions: reverse transcription reaction of cDNA at 42 °C for 15 min, denaturation with reverse transcriptase at 95 °C for 3 min, followed by 40 cycles of PCR, i.e., denaturation of cDNA at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. PCR product sizes were MT-I (120 bp), MT-II (165 bp), Atox1 (148 bp), Ctr1 (160 bp), Ccs (168 bp), Cox17 (143 bp), Atp7a (168 bp), Atp7b (61 bp) and β-actin (160 bp).

Fluorescence intensity of the amplified genes was determined by ABI PRISM 7000 SDS (Applied Biosystems, Foster, CA, USA).

Western blotting.
Cells were lysed in phosphate buffered saline (PBS) containing 1% Triton X-100, 0.1% SDS, 1 mM EDTA, and a cocktail of protease inhibitors (F. Hoffmann-La Roche Ltd., Basel, Switzerland) for 20 min on ice. The supernatant for Western blotting was obtained by centrifugation of the lysate for 20 min at 16,000 g at 4 °C (Payne et al., 1998). The protein samples (50 μg) were electrophoresed through polyacrylamide gels (Ready Gel 12% Tris–HCl, 4–20% Tris–HCl, and 10–20% Tris–Tricine/Peptide, Bio-Rad Laboratories, Hercules, CA, USA) and transferred to a nitrocellulose membrane at 20 V for 60 min. The membrane was blocked overnight with 5% skim milk in 25 mM Tris–HCl containing 0.9% NaCl, 0.05% Tween 20, pH 7.5 (TBS-T) at 4 °C, incubated with each antibody, i.e., anti-Ctr1 (1:2000 diluted), anti-Css (1:1500), anti-Cox17 (1:1000), anti-Atox1 (1:500), anti-Atp7a (1:2000), anti-Atp7b (1:2500), anti-SOD1 (1:2500) or anti-β-actin (1:5000) in TBS-T for 1 h, and washed three times with TBS-T. A synthetic peptide of the Ctr1 sequence H2N-YSIRYNSMPVPGPNGTILCCOOH, which corresponds to the cytosolic loop between transmembrane domains 1 and 2 of mouse and
human Ctr1, was used as antigen for generation and affinity purification of rabbit polyclonal antiserum by Bethyl Laboratories, Inc. (Montgomery, TX, USA). Rabbit polyclonal antisera against Atox1, Atp7a, and Atp7b were kindly provided by Professor Jonathan D. Gitlin (Washington University School of Medicine, Seattle, WA, USA). Rabbit anti-Cox17 and anti-SOD1 polyclonal antibodies were purchased from Proteintech Group, Inc. (Chicago, IL, USA) and Assay Designs, Inc. (Ann Arbor, MI, USA), respectively. The secondary antibody (1:5000) was incubated with the membrane in TBS-T containing 5% skim milk, and washed three times with TBS-T. This rabbit secondary antibody was purchased from GE Healthcare Bio-Sciences KK (Tokyo, Japan). The blots were detected with Super Signal Pico Chemiluminescent Reagent (Pierce Biotechnology, Inc. Rockford, IL, USA) on an X-ray film (HyBlot CL™ 203×254 mm, Denville Scientific, Inc. Plainfield, NJ, USA) according to the manufacturer's instructions.

Determination and speciation of metals in cultured cells.

MT-WT and MT-KO cells were seeded and treated with siRNA according to the same protocol as that mentioned above. After the treatment, the cells were collected and counted to determine Cu concentration. The harvested cells were wet-ashed with nitric acid for 24 h on a hot plate and then diluted with deionized water. Cu concentration in the samples was determined by an inductively coupled plasma mass spectrometer (ICP-MS, Agilent7500ce, Agilent Technologies, Hachiouji, Japan) at m/z 65. For the determination of Cu concentration in cultured cells, the sample was pneumatically introduced into an ICP-MS through an interface consisting of a micro flow nebulizer (PFA-20, Glass Expansion, Pty. Ltd., West Melbourne, Australia) and a cyclone chamber (Glass Expansion). The cells were collected and suspended to a concentration of 2.0×10^4 cells/μl with 10 mM Tris–HCl, pH 7.2, for metal speciation. The suspended cells were disrupted with an ultrasonic homogenizer (Bioruptors UCD-200, Cosmo Bio Ltd., Tokyo, Japan) on ice at 200 W, 20 kHz for 30 s three times at 30 s intervals. The cytosolic fraction was obtained by ultracentrifugation of the homogenate at 105,000 g for 60 min at 4 °C. The HPLC system (Prominence, Shimadzu, Kyoto, Japan) equipped with a narrow bore column was coupled with an ICP-MS (HPLC–ICP-MS) and was used for metal speciation in cultured cells. A narrowbore multi-mode gel-filtration column (Shodex Protein KW802.5-2E, 2.0 mm i.d.×250 mm) was kindly provided by Showa Denko (Tokyo, Japan). A 5.0 μl aliquot of cytosol was applied to the column and the column was directly eluted with 100 mM ammonium acetate, pH 7.2, at a flow rate of 40 μl/min. The eluate was introduced into a micro concentric nebulizer (Ari Mist HP
Nebulizer, Burgener Research Inc., Ontario, Canada) connected to the cyclone chamber of an ICP-MS. Cu and Zn in the eluate were monitored at m/z 65 and 66, respectively.

Elemental fluorescence imaging of Cu.

CS1, a specific imaging probe for monovalent Cu (cuprous) ion, was kindly provided by Professor Christopher J. Chang (University of California, Berkeley, California, USA). MT-WT and MT-KO cells were seeded and treated with siRNA according to the same protocol as that mentioned above. After the treatment, the cells were incubated with 5 μM CS1 dissolved in dimethyl sulfoxide for 120, 180, and 240 s at 37 °C, and fluorescence of the Cu–CS1 complex in the cells was detected by Axio Observer (Carl Zeiss, Oberkochen, Germany) at an excitation wavelength of 543 nm and an emission wavelength of 560 nm.

Immunofluorescence imaging of Ctr1.

siRNA transfected cells were washed twice with PBS, fixed with 4% formaldehyde in PBS for 30 min at room temperature, and permeabilized with 0.1% Triton X-100 for 30 min. The cells were washed twice with PBS, blocked in 5% skim milk in PBS at room temperature for 24 h, and incubated with the primary antibody against Ctr1 (1:100). The complex of Ctr1 and its primary antibody was detected with Alexa Fluor® 488 goat anti-rabbit IgG as the secondary antibody (Molecular Probes, Eugene, OR, USA). The nuclei were stained with 2-(4- amidinophenyl)-1H-indole-6-carboxamidine (DAPI, Molecular Probes) for 10 min. Coverslips were mounted in ProLong Antifade (Molecular Probes). Alexa Fluor® 488 and DAPI fluorescence was detected with a Zeiss Axio Imager wide widefield fluorescence microscope (Carl Zeiss) at excitation wavelengths of 485 and 387 nm and emission wavelengths of 624 and 447 nm, respectively. Digital fluorescence images were captured using MetaMorph 7.5 system (Molecular Devices, Downingtown, PA, USA).

Statistics.

The results are presented as means ± standard deviation (SD) of three independent samples. The significant difference was calculated by the Student's t-test, and a p value of less than 0.05 was considered to be statistically significant.
Results

Effects of Atox1 knockdown on Cu-regulating gene expression
Relative mRNA expressions of Atox1 in MT-WT and MT-KO cells decreased to 0.25 and 0.23, respectively, when cells were treated with Atox1-targeting siRNA (Figs. 1A and G). Atox1 protein expression in both MT-WT and MT-KO cells was also silenced (Figs. 2A and I). Relative mRNA expressions of Ctr1, Cox17, Ccs, Atp7a and Atp7b were not significantly changed by the Atox1 siRNA treatment (Figs. 1B–F), and their protein expressions coincided with mRNA expressions (data not shown) in MT-WT cells. Consequently, although Atox1 protein expression was suppressed by Atox1-KD, the expression of other Cu-regulating proteins was not changed in MT-WT cells. On the other hand, Atox1-KD significantly increased Ctr1 mRNA expression to 2.5 times that of control in MT-KO cells (Fig. 1H), and Ctr1 protein expression was increased as well (Fig. 2B). Although Cox17 mRNA expression showed a tendency to increase, the increase was not significant (Fig. 1I). Indeed, Cox17 protein expression was not changed by Atox1-KD (Fig. 2C). Ccs mRNA expression was not significantly changed (Fig. 1J) but its protein expression was apparently increased, suggesting that Ccs protein expression was post-translationally regulated (Fig. 2D). Although Atp7a mRNA and protein expressions were not changed (Figs. 1K and 2E), Atp7b mRNA and protein expressions were significantly decreased by the Atox1 siRNA treatment (Figs. 1L and 2F). In summary, Atox1-KD increased Ctr1 and Ccs expression and decreased Atp7b expression in MT-KO cells. These observations could reflect Cu deficiency in MT-KO/Atox1-KD cells.

Effects of Atox1-knockdown on Cu concentration in whole cells and distribution in cytoplasm
Although Cu concentration in MT-WT cells showed a tendency to increase with Atox1-KD, the increase was not significant (Fig. 3A). On the other hand, Cu concentration in MT-KO cells was significantly increased by Atox1-KD (Fig. 3B). In addition, our finding that Cu concentration in MT-KO cells (222 ± 22 pg/1.0×10^5 cells) was lower than that in MT-WT cells (367±51 pg/1.0×10^5 cells) without Atox1-KD coincided with a previous report (Ogra et al., 2006). Two major Cu peaks appearing at retention times of 11.6 and 13.0 min were detected in the chromatogram of MT-WT cells (Fig. 4A). The molecule assignable to the former peak bound both Cu and Zn in equal molar amounts and its chromatographic behavior was identical to that previously reported (Miyayama et al., 2008), suggesting that it could be assigned to SOD1. The
molecule assignable to the latter peak also bound Cu and Zn but the molar ratio of Zn to Cu was considerably high (Figs. 4A and B). As its chromatographic behavior was identical to that previously reported (Miyayama et al., 2008), this peak was assigned to MT. However, it was previously shown that this column was unable to separate the two major MT isoforms because of their highly homologous amino acid sequences, very similar molecular sizes, and metal-binding properties (Miyayama et al., 2008). Although Atox1-KD did not change the mRNA expressions of both MT isoforms, i.e., MT-I and MT-II (data not shown), the amount of Cu bound to MT was slightly increased by 1.37 times (Fig. 4A). No apparent changes in Zn distribution were noted between control and Atox1 siRNA treated cells (Fig. 4B). Although the peak corresponding to SOD1 in the cytoplasm of MT-KO cells was identical to that of MT-WT cells, the peak corresponding to MT was lacking in the chromatogram of MT-KO cells (Fig. 4D). Cu was eluted at the retention time corresponding to MT; however, it was not bound to MT (no MT proteins are expressed in MT-KO cells) and the identity of the Cu-binding protein remains unknown (Fig. 4C). There were no apparent changes in Cu and Zn distributions between control and Atox1 siRNA treated MT-KO cells (Figs. 4C and D). These results suggest that the increased Cu concentration in MT-KO cells due to Atox1-KD did not distribute to the cytoplasm, and other techniques to reveal Cu distribution are needed, in addition to speciation.

Effects of Atox1-knockdown on Cu distribution in whole cells detected by Cu-specific fluorescent probe

Speciation, which is conducted to analyze the distribution of Cu or other metals, is limited to the soluble fraction, i.e., the cytoplasm. Fluorescence imaging of Cu, which investigates intracellular Cu distribution in the whole cell, is a complementary technique to speciation. In this experiment, CS1 was used as the Cu-specific probe. Although CS1 has many advantages for Cu imaging in live cells, including solubility in water and a turn-on fluorescent sensor with a high Cu+ selectivity and sensitivity (Zeng et al., 2006), fluorescence bleaching is fast. Thus, we detected the fluorescence of the Cu–CS1 complex 120, 180, and 240 s after the addition of CS1 into the cells. The fluorescence of the Cu–CS1 complex was primarily localized in the cytoplasm of MT-WT cells, and as expected, it was bleached in a time dependent manner (Figs. 5A–C). The fluorescence intensity seemed to be slightly increased by Atox1-KD in MT-WT cells (Figs. 5D–F). This was consistent with the increasing tendency of Cu concentration with Atox1-KD in MT-WT cells. In MT-KO cells, the fluorescence of the Cu–CS1 complex was dominantly detected in the cytoplasm (Figs. 5G–I) and its intensity was apparently
increased by Atox1·KD (Figs. 5J–L). Moreover, large vesicles exhibiting the fluorescence of the Cu–CS1 complex were observed in cells bearing a double defect in MT and Atox1, suggesting that the Cu concentration increase with Atox1·KD was compartmentalized in these vesicles in MT·KO cells (arrowheads in Fig. 5L).

Effects of Atox1·knockdown on Ctr1 localization in cells
As mentioned above, Atox1·KD induced Ctr1 expression at both mRNA and protein levels in MT·KO cells (Figs. 1H and 2B). To investigate whether the elevated Ctr1 protein was expressed in its primary site of localization, i.e., plasma membrane, or other sites resulting from relocalization, Ctr1 was stained. Consistent with previous results, Ctr1 was primarily localized on the plasma membrane of both MT·WT and MT·KO cells with the control siRNA treatment (Figs. 6A and B). The amount and localization of Ctr1 in MT·WT cells were not changed by Atox1·KD (Fig. 6C). On the other hand, the induction of Ctr1 by Atox1·KD in MT·KO cells was also observed by immunocytochemistry, and the induced Ctr1 localized on the plasma membrane (Fig. 6D). This result also suggests that cells bearing a double defect in MT and Atox1 show paradoxical response to the Cu deficiency resulting in the increase in Ctr1 expression, despite the fact that Cu concentration in whole cells is actually increased by Atox1·KD in MT·KO cells.
Discussion

As expected, Atox1-KD increased cellular Cu concentration. Cu bound to Atox1 is transferred to Atp7a or Apt7b across the Golgi membrane and is excreted into extracellular fluid mediated by the trans-Golgi network (Forbes et al., 1999; Dierick et al., 1997). Thus, the manifestations of Atox1-KO mice resemble those of mice bearing a defect in Atp7a that resulted in a defect in Cu absorption in the intestine (Hamza et al., 2001). On the other hand, as Atp7b is specifically expressed in the liver, manifestations in the liver or cells of Atp7b-defective animals also resemble those of cells lacking Atox1, i.e., the accumulation of Cu in the liver or cells (Buiakova et al., 1999). In addition, a defect in Commd1, which is supposed to cooperate with Atp7b in excreting Cu, results in manifestations resembling those of Atp7b defect. The most symptomatic manifestation of the defect in these genes, i.e., the increase in Cu concentration, produces several cellular responses, such as the induction of MT (Sugawara et al., 1991; Mercer et al., 1992; Mulder et al., 1992), the relocalization of Ctr1 (Petris et al., 2003) and Cu-transporting ATPases (Krajacic et al., 2006), and the degradation of Cu chaperones (Lamb et al., 2000) in order to detoxify and normalize the elevated intracellular Cu concentration. Among the above cellular responses, the induction of MT is commonly and primarily detected in cells bearing a defect in Atp7a, Atp7b, Commd1 or Atox1. Indeed, the slight increase in Cu bound to MT was observed in MT-WT/Atox1-KD cells (Fig. 4A). In contrast to MT-WT cells, MT-KO cells exhibited unique responses to the elevation of intracellular Cu concentration by Atox1-KD. The speciation study showed that elevated Cu was not distributed in the cytoplasm, and elemental imaging revealed that Cu was localized in intracellular vesicles. Recently, it was reported that late endosomes and lysosomes act as a Cu pool for the maintenance of cellular Cu homeostasis in HEK-293T, HeLa, and U2OS cell lines (van den Berghe et al., 2007). Although it remains to be demonstrated that intracellular vesicles, i.e., late endosomes and lysosomes, contain Cu by direct methods, such as elemental imaging of Cu, the vesicles may be one of the candidates for explaining Cu-containing vesicles in MT-KO/Atox1-KD cells detected by the direct method. Based on our observations of the vesicles, we suppose that the vesicles isolate and detoxify elevated Cu in place of MT in MT-KO/Atox1-KD cells. Even in MT-WT cells, the vesicles might act when MT is not induced or the amount of elevated Cu exceeds the biosynthetic capacity of MT. Several responses of Cu-regulating proteins to Atox1-KD were detected at mRNA and protein levels in MT-KO cells, i.e., increases in Ctr1 and Ccs and the decrease in Atp7b were observed. Ctr1 functions in the high-affinity uptake of Cu on the plasma membrane of various mammalian cells (Lee et al., 2002; Puig and Thiele, 2002; Eisses et al., 2005),
and is rapidly liberated from the plasma membrane in response to the increase in the intracellular concentration of Cu but not other metals (Petris et al., 2003). These phenomena seem to be quite reasonable because the desorption of Ctr1, the high-affinity Cu influx transporter, from the plasma membrane decreases Cu incorporation. However, Ctr1 expression was increased in both mRNA and protein levels and the elevated Ctr1 was fixed on the plasma membrane by Atox1-KD in MT-KO cells. These observations apparently suggest that the cells perceive the Cu deficiency. It has been reported that Ccs is degraded by 26S proteosome depending on the intracellular Cu concentration (Bertinato and L'Abbé, 2003; West and Prohaska, 2004; Caruano-Yzermans et al., 2006). According to the literature, the amount of Cu bound to Ccs may be minimal as the exchange of Cu from Ccs to SOD1 is very rapid. When cellular Cu level increases, the amount of holo-Ccs (Cu-binding Ccs) also increases. Holo Ccs more efficiently undergoes ubiquitin-mediated proteosomal degradation than apo-Ccs. Thus, the elevation of cellular Cu results in the decrease in Ccs protein and Ccs protein expression inversely reflects cellular Cu concentration. In our observations, Ccs protein expression was apparently increased by Atox1-KD in MT-KO cells. Taken together, although cells bearing a double defect in MT and Atox1 were actually accumulated more copper, the cells showed Cu deficiency from the results of Ccs expression. Interestingly, it was reported that Ccs expression was increased in intestinal epithelial cells of mice whose Ctr1 was conditionally knocked out despite the increase in Cu concentration in the cells (Nose et al., 2006). Although the mechanisms of the increased Ccs expression are still unclear, the results well coincide with our observations. At present, there is no literature indicating that Atp7b expression is responsible for the cellular Cu concentration. However, as Atp7b can act as a Cu-efflux pump, it is straightforward to assume that the decrease in Atp7b expression is a response to the Cu deficiency. Further studies to reveal the regulation mechanisms for Atp7a and Atp7b expressions are needed to answer the question why Atp7a expression is not changed. Consequently, although cellular Cu concentration was actually increased, all responses of Cu-regulating proteins detected in this study suggested that cells bearing the double defect in MT and Atox1 were Cudeficient. This discrepancy can be explained as follows. Elevated Cu in MT-KO/Atox1-KD cells was compartmentalized by vesicles to mask Cu toxicity. Elevated Cu masked by MT may be promptly used when Cu concentration decreases. However, in MT-KO cells, elevated Cu due to Atox1-KD was masked by vesicles; thus, the once-compartmentalized Cu by vesicles may not be promptly utilized by cells when Cu concentration decreases. In other words, Cu compartmentalized by vesicles is less bioavailable than Cu sequestered by MT.
MT may play a suitable role in maintaining Cu homeostasis by immediately responding to Cu status ranging from deficiency to excess. In summary, intracellular Cu concentration was increased by Atox1-KD in MT-KO cells and elevated Cu was compartmentalized in cellular vesicles in the form of late endosomes and/or lysosomes. Although intracellular Cu concentration was actually elevated, several manifestations of Cu deficiency appeared in the cells. These suggest that Cu compartmentalized in the vesicles may be less bioavailable than Cu bound to MT. Our results provide new insights into the mechanisms involved in the maintenance of Cu homeostasis, namely, novel roles of MT and cellular vesicles in the detoxification of Cu in mammalian cells.

Acknowledgments
The authors deeply appreciate Professor Dennis J. Thiele of Duke University Medical Center for valuable discussions. Part of this study was conducted in his laboratory. The authors wish to thank Showa Denko for providing the narrow bore column and the HPLC system. The authors would also like to acknowledge Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan (Nos. 16689005 and 19390033), and the financial support from Agilent Technologies Foundation, USA.
References


Nose, Y., Kim, B.E., Thiele, D.J., 2006. Ctr1 drives intestinal copper absorption and is essential for growth, iron metabolism, and neonatal cardiac function. Cell Metab. 4, 235–244.
Figures

Fig. 1. Effects of Atox1-KD on the mRNA expression of Cu-regulating genes in MT-WT and MT-KO cells. MT-WT (A–F) and MT-KO (G–L) cells were pre-incubated for 24 h and the cells were treated with control or Atox1 siRNA at 100 nM for 24 h. After the siRNA transfection, total RNA was isolated from the cells. The mRNA expressions of Atox1 (A and G) and other Cu-regulating genes, such as Ctr1 (B and H), Cox17 (C and I), and Ccs (D and J), were quantified by RT-PCR analysis and normalized to β-actin levels. Atox1 expression was significantly knocked down by the siRNA treatment in MT-WT and MT-KO cells (A and G). Ctr1 mRNA expression was significantly increased by Atox1-KD in MT-KO cells (H). Data are expressed as means ± SD of three independent determinations.

Fig. 2. Effects of Atox1-KD on the expressions of Cu-regulating proteins in MT-KO (A–H) and MT-WT cells (I and J). MT-KO and MT-WT cells were pre-incubated for 24 h and then treated with control or Atox1 siRNA at 100 nM for 24 h. After the siRNA transfection, cells were lysed and the supernatant for Western blotting was obtained by centrifugation. The protein samples (50 μg) were electrophoresed and transferred to a nitrocellulose membrane. After the membranes were blocked, they were incubated with antibodies against Atox1 (A and I), Ctr1 (B), Cox17 (C), Ccs (D), Atp7a (E), Atp7b (F), SOD1 (G) or β-actin (H and J). After the incubation with a secondary antibody, the blots were detected with a chemiluminescent reagent on an X-ray film. The amount of Atox1 protein was also reduced by Atox1-KD in MT-KO and MT-WT cells (A and I). Increases in Ctr1 (B) and Ccs (D) and the decrease in Atp7b (F) at the protein level were observed in MT-KO cells with Atox1-KD.

Fig. 3. Cu concentration in MT-WT and MT-KO cells treated with control or Atox1 siRNA. MT-WT (A) and MT-KO (B) cells were pre-incubated for 24 h and then treated with control or Atox1 siRNA at 100 nM for 24 h. After the siRNA transfection, the harvested cells were wet-ashed with nitric acid for 24 h on a hot plate and then diluted with deionized water. Cu concentration in the samples was determined by an ICP-MS equipped with a micro flow nebulizer (PFA-20) at m/z 65. Cu concentration in MT-KO cells was significantly increased by Atox1-KD (B), and that in MT-WT cells showed a tendency to increase (A). Data are expressed as means±SD of three independent determinations.
Fig. 4. Elution profiles of Cu and Zn in the cytosolic fraction of MT-WT and MT-KO cells treated with control or Atox1 siRNA on the gel-filtration column. MT-WT (A) and MT-KO (B) cells were pre-incubated for 24 h and the cells were treated with control or Atox1 siRNA at 100 nM for 24 h. After the siRNA transfection, the cells were collected and suspended to a concentration of 2.0×104 cells/μl with 10mMTris–HCl, pH 7.2. The suspended cells were disrupted with an ultrasonic homogenizer on ice and the cytosolic fraction for application to a narrow bore HPLC–ICP-MS was obtained by ultracentrifugation of the homogenate. The HPLC system equipped with a narrow bore multi-mode gel-filtration column (Shodex Protein KW802.5·2E, 2.0mmi.d.×250mm) was used. A 5.0 μl aliquot of cytosol was applied to the column and the column was eluted with 100mMammonium acetate, pH 7.2, at a flowrate of 40 μl/min. The eluate was introduced into a micro concentric nebulizer connected to a cyclone chamber of ICP-MS. Cu and Zn in the eluate were monitored at m/z 65 and 66, respectively. Two major Cu peaks appearing at retention times of 11.6 and 13.0 min were detected in the chromatogram of MT-WT cells (A). The former and latter peaks correspond to SOD1 and MT, respectively. Although there were no changes in the Zn elution profiles between control and Atox1 siRNA treated cells (B), the amount of Cu bound to MT was slightly increased by the Atox1 siRNA treatment (A). There were no apparent changes in the Cu and Zn distributions in MT-KO cells treated with control and Atox1 siRNA (C and D).

Fig. 5. Cu imaging in MT-WT and MT-KO cells treated with control or Atox1 siRNA. MT-WT (A–F) and MT-KO (G–L) cells were pre-incubated for 24 h and then treated with control or Atox1 siRNA at 100 nM for 24 h. After the siRNA transfection, the cells were incubated with 5 μM CS1 dissolved in dimethyl sulfoxide for 120, 180, and 240 s at 37 °C. Then, the fluorescence of the Cu–CS1 complex in the cells was detected. The fluorescence of the Cu–CS1 complex was primarily localized in the cytoplasm of MT-WT cells, and as expected, it was bleached in a time-dependent manner (A–C). The fluorescence intensity seemed to be slightly increased by the Atox1-KD in MT-WT cells (D–F). In MT-KO cells, the fluorescence of the Cu–CS1 complex was dominantly detected in the cytoplasm (G–I), and the fluorescence intensity was apparently increased by the Atox1-KD (J–L). Cu elevated by the Atox1-KD was compartmentalized in these vesicles in MT-KO cells (arrowheads in panel L).

Fig. 6. Localization of Ctr1 in MT-WT and MT-KO cells treated with control or Atox1 siRNA. MT-WT (A and C) and MT-KO (B and D) cells were pre-incubated for 24 h and then treated with control or Atox1 siRNA at 100 nM for 24 h. After the siRNA
transfection, the cells were washed twice with PBS, fixed with 4% formaldehyde in PBS for 30 min at room temperature, and permeabilized with 0.1% Triton X-100 for 30 min. After blocking the fixed cells, the cells were incubated with the primary antibody against Ctr1. The complex of Ctr1 and its primary antibody was detected with Alexa Fluor® 488 goat anti-rabbit IgG as the secondary antibody. The nuclei were stained with DAPI for 10 min. Alexa Fluor® 488 and DAPI fluorescence was detected with a fluorescence microscope and their digital images were captured with the MetaMorph 7.5 system. Ctr1 was primarily localized on the plasma membrane of both MT-WT and MT-KO cells treated with control siRNA (A and B). Although the amount and localization of Ctr1 in MT-WT cells were not changed by Atox1-KD (C), Ctr1 was induced by Atox1-KD in MT-KO cells, and the induced Ctr1 localized on the plasma membrane (D).
Fig. 1
Fig. 2

MT-KO

<table>
<thead>
<tr>
<th>siRNA</th>
<th>control</th>
<th>Atox1</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A)</td>
<td>Atox1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.9</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>37</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>siRNA</th>
<th>control</th>
<th>Atox1</th>
</tr>
</thead>
<tbody>
<tr>
<td>(B)</td>
<td>Ctr1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>150</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>siRNA</th>
<th>control</th>
<th>Atox1</th>
</tr>
</thead>
<tbody>
<tr>
<td>(C)</td>
<td>Cox17</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.9</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>37</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>siRNA</th>
<th>control</th>
<th>Atox1</th>
</tr>
</thead>
<tbody>
<tr>
<td>(D)</td>
<td>Ccs</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>37</td>
</tr>
</tbody>
</table>

MT-WT

<table>
<thead>
<tr>
<th>siRNA</th>
<th>control</th>
<th>Atox1</th>
</tr>
</thead>
<tbody>
<tr>
<td>(E)</td>
<td>Atp7a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>150</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>siRNA</th>
<th>control</th>
<th>Atox1</th>
</tr>
</thead>
<tbody>
<tr>
<td>(F)</td>
<td>Atp7b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>150</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>siRNA</th>
<th>control</th>
<th>Atox1</th>
</tr>
</thead>
<tbody>
<tr>
<td>(G)</td>
<td>SOD1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.9</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>37</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>siRNA</th>
<th>control</th>
<th>Atox1</th>
</tr>
</thead>
<tbody>
<tr>
<td>(I)</td>
<td>Atox1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.9</td>
<td>3.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>siRNA</th>
<th>control</th>
<th>Atox1</th>
</tr>
</thead>
<tbody>
<tr>
<td>(J)</td>
<td>β-actin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>37</td>
</tr>
</tbody>
</table>
Fig. 3

**MT-WT**

(A)

Cu concentration (pg/1.0 x 10^6 cell)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Atox1</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT-WT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**MT-KO**

(B)

Cu concentration (pg/1.0 x 10^6 cell)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Atox1</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT-KO</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* indicates a significant difference between control and Atox1 conditions.
Fig. 4
Fig. 5

MT-WT

(A) control 120 s  (B) siRNA 180 s  (C) siRNA 240 s

MT-KO

(G) control 120 s  (H) siRNA 180 s  (I) siRNA 240 s

Atox1

(D) control  (E) siRNA  (F) siRNA

Atox1

(J) control  (K) siRNA  (L) siRNA