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Sp1 Oscillation Is Involved in Copper Homeostasis Maintenance by Regulating hCtr1 Expression

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Running Title: Regulation of Copper Homeostasis in Human Cells

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Abbreviations : human copper transporter 1 (hCtr1); chromatin immunoprecipitation (ChIP); DNA-binding domain (DBD); divalent metal transporter-1 (DMT1); dominant negative (DN); tetrathiomolybdate (TM); small cell lung cancer (SCLC); RNase protection assay (RPA); zinc finger (ZF)

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Abstract

Copper (Cu) is an essential micronutrient for cell growth but is toxic when in excess. Copper transporter (Ctr1) plays an important role in regulating adequate Cu levels in mammalian cells. We previously showed that expression of the human high-affinity Cu transporter (hCtr1) was transcriptionally upregulated under Cu-depleted conditions and downregulated under replete conditions; moreover, elevated hCtr1 levels feedback to suppress hCtr1 expression (Song IS, et al. Mol Pharmocol 74:705-713, 2008). Expression of hCtr1 under Cu-stressed conditions is regulated by Sp1. In this study, we made the following important observations: (i) Sp1 expression is downregulated under Cu-replete conditions, but upregulated under Cu-depleted conditions. These up- and down-regulations of Sp1 in turn regulate hCtr1 expression to control Cu homeostasis. (ii) Cu-regulated Sp1 expression involved Sp1 binding to its own promoter as demonstrated by the chromatin immunoprecipitation assay; therefore, Sp1 is also transcriptionally self-regulated via hCtr1/Cu intermediation. (iii) Both zinc finger and glutamine-rich transactivation domains of Sp1 are involved in the Sp1-mediated hCtr1 and Sp1 regulation by Cu stresses. (iv) While Sp3 expression is also regulated by Cu availability, Sp3 does not regulate hCtr1 homeostasis. Collectively, our results demonstrated that mammalian cells use Sp1 oscillation in response to Cu availability to regulate Cu homeostasis through hCtr1 expression in a tripartite interregulatory relationship. These findings have important implications in mammalian Cu physiology regulation.

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Introduction

Copper (Cu) is a micronutrient that plays an essential role in normal human physiology. Cu deficiency in mammals causes embryonic and neonatal abnormalities in many systems including hematopoietic, cardiovascular, CNS, and liver functions. Moreover, Cu is a redox-active element, existing in both the Cu^{+} and Cu^{2+} states; and excess Cu ions cause damages to many vital organs as manifested in the Wilson disease (Collins et al. 2010). An evolutionarily conserved mechanism for maintaining cellular Cu homeostasis in response to stresses induced by Cu deficiency or excess in eukaryotic cells relies on the interplay among Cu transporters (Ctrs) for absorption and storage, chaperones for delivering Cu to various compartments, and exporters (ATP7A and ATP7B) for the elimination of excess Cu (for review, see Kim et al., 2008). However, the mechanisms that regulate Cu homeostasis in mammalian cells are largely unknown.

Many studies have demonstrated that regulation of the Cu homeostasis initial response to Cu stresses resides on Ctr1 (for reviews, see ref. Kim et al., 2008; Kuo et al., 2007)). The human high-affinity Ctr1 (hCtr1) is a single polypeptide of 190 amino acids consisting of three transmembrane motifs—a methionine-rich N terminus, a cystein-histidine cluster in the C-terminus and an MXXXM in the second transmembrane domain— that are important for the Cu transport function (for reviews, see ref. Kim et al., 2008; Kuo et al., 2007). In addition to its important role in regulating Cu physiology, hCtr1 is also known as a transporter for platinum drugs (Ishida et al., 2002); and reduced expression of hCtr1 is often associated with platinum drug resistance in cultured cells (Howell et al. 2010; Kuo et al., 2007) and in clinical settings (Chen et al. 2011; Ishida et al. 2010)

Soon after cells are treated with excess Cu, Ctr1 protein is internalized and degraded following an endocytic process (Ooi et al., 1996; Petris et al., 2003). Upon removal of Cu treatment, the internalized Cu recycles back to the membrane (Molloy and Kaplan, 2009),

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supporting a posttranslational mechanism of Ctr1 expression in response to Cu stress. Post-translational regulation of Ctr1 in response to Cu stress was observed in mice fed Cu-deficient diets (Nose et al. 2011).

Transcriptional regulation of hCtr1 in response to Cu stresses has also been reported. We previously demonstrated that in cultured cells overexpressing physiological Cu chelator glutathione (GSH) by transfecting the recombinant DNA encoding its rate-limiting enzyme, γ-glutamylcystine synthetase, hCtr1 mRNA and protein levels were increased (Chen et al., 2008). We also demonstrated that expression of hCtr1 mRNA can be induced by treating cells with the Cu chelator bathophenanthroline disulfonic acid and downregulated by treating with $CuS0_4$ (Song et al., 2008). The endogenous hCtr1 (endo-hCtr1) expression levels can be modulated by transfection with recombinant DNA encoding hCtr1 constructs (refer to here as exo-hCtr1), i.e., downregulation of endo-hCtr1 was also observed in cells transfected with the wild-type *hCtr1* (*hCtr1*-wt) plasmid (Song et al., 2008) to increase Cu transport activities; and upregulation of endo-hCtr1 mRNA was found in cells overexpressing dominant-negative hCtr1 (*hCtr1*-DN) by interfering with Cu transport activities. These *hCtr1*-DN recombinants were constructed by site-directed mutagenesis of several critical M residues located at the ecto- or the transmembrane MXXXM domains that are important for Cu transport (Liang et al., 2009). Regulation of hCtr1 expression is mediated by the transcription factor Sp1, which interacts with the three GC-boxes located at the *hCtr1* promoter (Song et al., 2008).

The missing piece of this transcriptional regulation mechanism of mammalian Cu homeostasis is how Sp1 itself behaves in response to Cu stresses. The current study was initiated to fill in this gap. We observed that Sp1 expression is also regulated by Cu stresses, either by changing Cu concentrations in cultured cells or by manipulating hCtr1 expression levels by transfection. Our results demonstrated that human cells use Sp1 oscillation in response to Cu stresses in regulating hCtr1 expression to maintain Cu homeostasis. These results, together with our previous findings (Liang et al., 2009; Song et al., 2008), which were

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further confirmed here, allowed us to construct a tripartite inter-regulatory model consisting of Sp1, hCtr1 and Cu to regulate mammalian Cu homeostasis. Our findings thus provide important new insights into mammalian Cu physiology regulation. They also have translational implication for the use of Cu modulators in regulating the treatment efficacy of platinum-based cancer chemotherapy.

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Materials and Methods

Cell Lines, Recombinant DNA, and Reagents. Small cell lung cancer (SCLC) cells were obtained from Niramol Savaraj, (Univ. Miami) and HEK293 cells from ATCC (Manassas, VA). Establishment of SCLC-*hCtr1*-wt and SCLC-*hCtr1*-DN stable cell lines has been described previously (Liang et al., 2009). Expression-recombinant DNA for *Sp1*-Wt, and *Sp2*, *Sp3* and *Sp4* were described previously (Tsai et al., 2009). Recombinant LexA-VP16 and reporter pLG-$(Gal4)_5$-$(Lex4)_2$-E1B-Luc (LexA-Luc) were obtained from M. Ptashne (Memorial Sloan-Kettering Cancer Center, New York, NY). Tetrathiomolybdate (TM) was obtained from Sigma-Aldrich (St Louis, MO).

Recombinant DNA Constructs and Transient Transfection Assays. To construct recombinant DNA encoding different domains of Sp1, double-stranded cDNA fragments were synthesized by PCR using appropriate primer sets each tagged with a *Not*I recognition sequence (available upon request), and full-length Sp1 cDNA as the template. The synthesized DNA was digested by *Not*I and cloned into the *Not*I site of the CIN-HA-pcDNA3 vector (Song et al., 2004), a neomycin-resistance marker was used for transfection selection.

The *Sp1*-Luc reporter construct was obtained from Dr. C. J. Ciudad (Univ. Barcelona, Spain) (Nicolas et al., 2001). Site-directed mutations in the *Sp1* promoter sequences in the reporter constructs (Sp1-M1 to Sp1-M10) were performed using the QuickChange™ site-directed mutagenesis kit (Agilent Tech. La Jolla, CA). Mutations in the Q-rich2 domain of the Sp1-M3 recombinant were constructed using the same procedure.

For constructing chimerical recombinants encoding the DNA-binding domain (DBD) of LexA or Sp1 fused to transactivating domain (TAD) from VP16 or Sp1, we used the two-step recombinant PCR method using recombinant LexA(1-102)-VP16(410-490) and Sp1 cDNA as templates and appropriate primer sets (available upon request) in the PCR reactions. The resulting DNA fragments were cloned into the *Not*I site of the CIN-HA-pcDNA3 vector. All

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plasmids were confirmed by DNA sequencing. Transient transfection assays were performed using lipofectamine (Invitrogene, San Diego, CA) according to the manufacturer's protocol. Luciferase expression was measured by illuminometer.

Measurement of Endo-mRNA and Exo-mRNA Levels Using the RNase Protection Assay. Plasmid DNA was transfected into cultured cells using lipofectamine. Expression of exo- and endo-mRNA was determined by the RNA protection assay (RPA) using RNase RPA III™ Assay Kit (Applied Biosystems/Ambion, Austin, TX). The design of RNA probes that can simultaneously detect both endo- and exo-mRNA transcripts in the transfected cells were described previously (Liang et al., 2009; Song et al., 2008).

Chromatin Immunoprecipitation (ChIP) Assay. The ChIP assay was performed with a kit (Upstate Biotechnology, Waltham, MA) following the manufacturer's instructions. The *hCtr1* and *Sp1* promoter DNA sequences were determined by PCR using appropriate primer sets (available upon request) according to procedures described previously (Tsai et al., 2009).

Western Blotting and Immunohistochemical Staining. Procedures for the Western blotting using anti-hCtr1 antibody (Kuo et al., 2001) and anti-Sp1 antibody (Santa Cruz Biotechnology. Santa Cruz, CA) (Liang et al., 2009; Song et al., 2008), and immunocytochemical staining following confocal microscopy (Chen et al., 2008) were described previously.

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Results

Mammalian Cu Homeostasis Is Regulated within the Cu-hCtr1-Sp1 Interregulatory Loop. Regulation of mammalian Cu homeostasis is a complex mechanism involving interplay among Cu, hCtr1, and Sp1. For clarity, we will present experimental results in the sequential order (Fig. 1A to 1H) that lead to the development of the Cu-hCtr1-Sp1 tripartite interregulatory model as depicted in Fig. 1I. To present this model in its entirety, some results presented in this figure were confirmation of our previous studies and will be so indicated.

Figure 1A shows that treating SCLC cells with $CuSO_4$ resulted in time-dependent downregulation of Sp1 mRNA and hCtr1 mRNA as determined by RPA. Quantitative analyses review that no apparent changes in Sp1 mRNA and hCtr1 mRNA were observed within the first 30 min. of treatment. Reduction of Sp1 mRNA and hCtr1 mRNA levels began to decrease thereafter and about 40% and 70% reduction, respectively, were observed 24 hr after the treatment. Likewise, overexpression of HA-hCtr1-wt by transfection of recombinant plasmid DNA in five independently established clones resulted in downregulation of Sp1 expression (Fig. 1B), because increased hCtr1-wt expression increases the Cu transport activities and thus cellular Cu contents. These latter results together with those shown in Fig. 1A indicate that Sp1 expression is downregulated under Cu-replete conditions. Fig. 1C shows that knockdown Sp1 by siRNA approach reduces the expression of hCtr1. This result is consistent with our previous report showing that Sp1 functions as a positive regulator for hCtr1 expression (Song et al., 2008). In contrast, Fig. 1D shows that treating SCLC cells with the Cu chelator, TM, upregulates Sp1 expression as determined by Western blotting. Moreover, knockdown hCtr1 by siRNA resulted in up-regulation of Sp1 (Fig. 1E). These results demonstrated that expression of Sp1 is upregulated under Cu-depleted conditions. Fig. 1F shows a concentration-dependent induction of endo-hCtr1 expression by transfection with *Sp1*-wt recombinant (exo-Sp1), consistent with our previous results showing that Sp1 is a positive regulator for hCtr1 (Song et al., 2008). Here, we show that in these *Sp*1-wt-transfected cells, the expression of

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endogenous Sp1 (endo-Sp1) is suppressed, as determined by the RPA using a probe that can differentiate between exo-Sp1 and endo-Sp1 mRNA species (see below Fig. 4A for probe design) (Fig. 1F). These results demonstrated that Sp1 expression, like hCtr1, is self-regulated, and that the self-regulation mechanism is mediated by hCtr1 upregulation, because knockdown of hCtr1 by siRNA inhibits Sp1 self-regulation (Fig. 1G). We also demonstrated that suppression of hCtr1 by Cu treatment is mediated by downregulation of Sp1, because replenishment of Sp1 by transfection with *Sp1*-wt expression recombinant upregulates hCtr1 (Fig. 1H), instead of downregulating hCtr1 as when cells were treated with Cu alone (Fig. 1A). These results not only support the inter-regulatory relationships among Cu, hCtr1, and Sp1 but also demonstrate that each of them is self-regulated within the context of overall mammalian Cu homeostasis regulation as depicted in Fig. 1I.

Sp1 Is the Sensor of Cu Stresses. The results shown above strongly suggest that mammalian cells use Sp1 oscillation in response to Cu imbalance to drive Cu homeostatic regulation. This scenario underscores the role of Sp1 as the sensor of Cu stresses. To strengthen these observations, we first determined whether Cu-sensing Sp1 expression is mediated through the Sp1-binding sites located at the *Sp1* promoter. We used a reporter construct that contains 1615 nucleotides upstream from the transcription start site of *Sp1* linked to a bacterial *luciferase* gene. Ten putative Sp1-binding sites are located within this promoter region. We mutated each of these sites (Fig. 2A). These reporter plasmids were then transfected into HEK293 cells followed by treating the cells with $CuSO_4$ or TM. Fig. 2B shows that expression of the reporter is downregulated by Cu treatment but upregulated by TM treatment in the *Sp1-wt*-reporter-transfected cells. Mutations at sites 2, 3, 7, 8, 9, and 10 abolished the responses of reporter expression to both Cu and TM treatments, suggesting that these sites are important for Cu-mediated Sp1 regulation. These sites contain the consensus 5'-GGGCGG- or 5'-CCCGCC sequence which can be recognized by Sp1 transcription factor (Wierstra, 2008). These results

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demonstrate that regulation of Sp1 by Cu stresses, like hCtr1 that have been demonstrated previously (Song et al., 2008), is mediated by the Sp1 bindings to their respective promoters. The finding that these Cu-responsive sites are located in two clusters (sites 2 and 3 in one cluster and sites 7-10 in the other) suggests that regulation of Sp1 expression may involve the simultaneous binding of Sp1 to these two clusters *via* Sp1-Sp1 interactions, resulting in DNA looping at the *Sp1* promoter, as suggested from previous study of modified *thymidine kinase* promoter (Su et al., 1991).

To demonstrate that regulation of hCtr1 and endo-Sp1 by Sp1 in response to Cu concentration variations is mediated by promoter binding, we next performed a ChIP assay. HEK293 cells transfected with recombinant plasmid DNA were treated with either $CuSO_4$ or TM. Protein-genomic DNA cross-linking was performed by using formaldehyde. After sonication, the chromatin fragments were precipitated with anti-Sp1 antibody using non-immuned IgG as a control. The *hCtr1* and *Sp1* promoter sequences in the precipitates were determined by polymerase chain reaction (PCR) using primer sets specific to their promoters. Fig. 3A shows that $CuSO_4$ treatment reduces the bindings of Sp1 to *hCtr1* and *Sp1* promoters in both the empty vector- and the *hCtr1*-DN-transfected cells. These results demonstrated that downregulation of Sp1 and hCtr1 expression by Cu treatment as shown in Fig. 1A is due to reduced Sp1 interactions with the *hCtr1* and *Sp1* promoters. We used two cell lines, instead of one, to strengthen the results because regulation of hCtr1 expression by Cu stresses should occur in all cell lines. Given the complexity of the assay system, we could not be certain as whether there are differences of Sp1 binding capacities to the *Sp1* and *hCtr1* promoters between these two cell lines. TM treatment enhanced the bindings of Sp1 to the *hCtr1* and *Sp1* promoters in the empty vector- and *hCtr1*-wt-transfected cells (Fig. 3B), again demonstrating that upregulation of hCtr1 and Sp1 expression under Cu-depleted conditions is transcriptionally regulated.

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To substantiate the transcriptional involvement of Sp1 in the regulation of both Sp1 and hCtr1, we next investigated the effects of Sp1 and hCtr1 overexpression on the binding of Sp1 to their promoters in HEK293 cells grown in the regular medium using a transfection approach. We found that Sp1 preferentially bound to the *hCtr1* promoter as compared with the *Sp1* promoter in the *Sp*1-wt-transfected cells (Fig 3C). These results are consistent with our previous observation that elevated Sp1 expression upregulated hCtr1 but downregulated itself (Fig. 1F). In addition, overexpression of hCtr1 diminished Sp1 binding to both the *hCtr1* and *Sp1* promoters (Fig. 3C), which is also consistent with the results showing that overexpression of hCtr1 down-regulates both endo-hCtr1 and Sp1 expression (Fig. 1). These results, as summarized in Fig. 3D, not only support the many observations depicted in Fig. 1I, but also indicate the transcriptional regulation of Cu homeostasis by Sp1 transcriptional regulator.

Both the Zinc Finger Domain and Transactivation Domains of Sp1 Are Important for Sp1 Sensing of Cu Stress. Sp1 consists of a transactivation domain (TAD) that contains two serine/threonine (S/T)-rich and glutamine (Q)-rich subdomains and a DNA-binding domain that contains three zinc fingers (ZF). We previously found that the ZF domain in Sp1 is important for Sp1-mediated hCtr1 expression under Cu-depleted conditions (Song et al., 2008. To elucidate whether other domains are also involved in Sp1-mediated hCtr1 expression and whether these domains are also important for the hCtr1-mediated Sp1 self-regulation, we constructed several Sp1 deletion mutants encompassing various subdomains (Fig. 4B). These mutants Sp1 were transfected into HEK293 cells and the transfected cells were treated with $CuSO_4$. Alternatively, these mutants were co-transfected with *hCtr1*-wt recombinant. We envisioned that co-transfection with *hCtr1*-wt would increase Cu uptake, much like cells treated with $CuSO_4$. We used a probe that can distinguish between exo-Sp1 and endo-Sp1 mRNA signals for the RPA (Fig. 4A).

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The following results were obtained from these two sets of experiments: (i) High Cu content suppresses the expression of endo-hCtr1 and endo-Sp1 (Figs. 4C and 4D, lanes 3), consistent with the results shown earlier (Song et al., 2008). (ii) Overexpression of Sp1-wt upregulates endo-hCtr1, but downregulates endo-Sp1 expression (Figs. 4C and 4D, lanes 4), confirming the results shown in Fig. 1F. (iii) Deletion of the three ZF in Sp1 (M3 mutant) suppresses the Sp1-mediated increased endo-hCtr1 expression but decreased endo-Sp1 expression (Fig. 4C and 4D, lanes 5). (iv) Expression of the intact TAD neutralizes the effects of endo-Sp1 and endo-hCtr1 expression by Cu treatment, *i.e.*, their expression levels are similar to those seen in the un-transfected or vector-transfected controls (Fig. 4C and 4D, lanes 6). Thus, Sp1-TAD has a dominant-negative function in Cu response. Deleting the Q-rich2 sub-domain (mutant M8) abolishes the dominant-negative function of TAD; however, the Q-rich2 domain together with Sp1-DBD (M7) can not confer the dominant negative function. These results suggest that other sub-domains in the TAD are required for the dominant-negative function. (v) Transfection of other mutants with truncated TAD (i.e., M8, M9, M10 and M13) failed to alter the expression of endo-hCtr1 and endo-Sp1 (Figs. 4C and 4D, lanes 8-11). We found that mutations that affected endo-hCtr1 expression also affected endo-Sp1 expression, whereas those that did not affect endo-Sp1 expression had no effect on hCtr1 expression. This striking concordance further confirms the interregulatory relationship among Sp1, hCtr1, and Cu in the maintenance of Cu homeostasis and supports the notion that Sp1-regulated hCtr1 and Sp1 expression may involve the same functional domains.

Previous studies have demonstrated that whereas the ZF domain is involved in promoter recognition and the Q-rich2 sub-domain of TAD is involved in Sp1-mediated transcription activation by cross-talk with basal transcription machinery (Gill et al., 1994). This Q-rich2 subdomain is also involved in Sp1 oligomerization (Pascal and Tjian, 1991). To further elucidate the amino acid residues in the Q-rich2 subdomain that are involved in Cu responsiveness, we introduced several mutations at the Q-rich2 subdomain (Fig. 4E).

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Transient transfection assay revealed that double-mutations (Q to L) at two amino acid clusters 384/385 (M2 mutant) and 413/416 (M5 mutant) (Fig. 4E circled) abolished the dominant-negative effects of TAD on the regulation of Sp1 and hCtr1 expression by Cu (Fig. 4F). These results identified the roles of the Q-rich2 subdomain in the Sp1-mediated transcriptional regulation of Sp1 and hCtr1 expression by Cu.

The Zinc Fingers of Sp1 are the Sensors of Cu Stress. To further characterize the Cu-sensing mechanism of Sp1, we performed domain-swapping experiments by constructing fusions composed of the Sp1-ZF and the TAD from herpes simplex virus VP16 (TAD_{vp16}), and DNA-binding domain from *Escherichia coli* LexA (DBD_{lexA}) and the TAD from Sp1 (TAD_{Sp1}). We avoided the commonly used GAL4 DBD because it contains a ZF sequence, whereas DBD_{lexA} does not contain any metal binding motif. DBD_{lexA}-TAD_{vp16} or D_{lexA}-TAD_{Sp1} was co-transfected with the Luc reporter recombinant containing eight copies of *LexA*-binding sequences (*LexA*-Luc); whereas Sp1-ZF-TAD_{vp16} recombinants were co-transfected with *hCtr1*-Luc or *Sp1*-Luc. Neither Cu nor TM treatments altered reporter expression levels when DBD_{lexA}-TAD_{vp16} and DBD_{lexA}-TAD_{Sp1} recombinants were used (Fig. 5). In contrast, $CuSO_4$ treatment down-regulated reporter expression in cells co-transfecting Sp1-ZF-VP16 with *hCtr1*-Luc or with *Sp1*-Luc; and TM treatment upregulated reporter expression in these similarly transfected cells. These results collectively demonstrated that Sp1-ZF is the sensor of Cu stresses in this surrogate assay system.

Cu Stress Does Not Appear to Induce Cytoplasmic Sequestration of Sp1. It has been demonstrated that the ZF domain is involved in nuclear targeting of Sp1 (Ito et al. 2010). Because we found that Sp1-ZF plays an important role in Cu sensing that regulate Sp1 and hCtr1 expression, we investigated whether Cu stresses may affect nuclear localization of Sp1. SCLC cells were treated with $CuSO_4$ or TM followed by immunocytochemical stainings with an anti-Sp1 antibody. We found that, although the overall staining intensities showing Sp1

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expression levels were reduced in Cu-treated cells but increased in TM-treated cells as consistent with the biochemical results shown in Fig. 1B and Fig. 1E, respectively, no apparent re-distribution of Sp1 between cytoplasmic and nuclear compartments was observed (Supplemental Figure 1).

Sp3 Expression Is Also Regulated by Cu Stresses, but It Does Not Regulate hCtr1 Expression. Sp1 was the first described member of the Sp1/Krűppel-like factor (KLF) family, which currently consists of eight Sp and 15 KLF members. Among the eight Sp proteins, Sp1-Sp4 form a subgroup and Sp5-Sp8 form another on the basis of their structural similarities (Kaczynski et al., 2003). This raises an important question about the roles of other Sp members in the regulation of hCtr1 expression, particularly Sp3 which is most closely related to Sp1 in terms of their structural similarities.

To determine whether Sp3 expression is also regulated by Cu and whether its expression also modulates hCtr1 expression, we treated empty vector-, *hCtr1*-wt- and *hCtr1*-DN-transfected cell lines with $CuSO_4$ or TM. As consistent with our previous report (Song et al., 2008) and those shown in Fig. 1, levels of Sp1 expression were increased in the TM-treated cells (Fig. 6A) but down-regulated in the $CuSO_4$-treated cells (Fig. 6B). Although the magnitudes of regulation here were small that reflected experimental variations, the results were very reproducible. In contrast to the expression patterns of Sp1, we found the expression of Sp3 was downregulated in these cell lines treated with TM (Fig. 6A), but was slightly upregulated in cells treated with $CuSO_4$ (Fig. 6B). Again, the magnitudes of Sp3 regulation by Cu stresses were not remarkable, but the results were very reproducible in all three cell lines, i.e., vector-, *hCtr1*-wt- and *hCtr1*-DN-transfected cell lines. Moreover, transfections of expression recombinants encoding Sp2, Sp3, and Sp4, failed to upregulate endo-hCtr1 expression in the *hCtr1*-wt-transfected cells, whereas transfection with Sp1 recombinant did (Fig. 6C). Transfections of recombinants encoding each of these Sp family members into *hCtr1*-DN-

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transfected cells did not alter expression levels of endo-hCtr1 levels, because these cells already express high levels of endo-hCtr1 (Fig. 6D). This is consistent with the interpretation that limited maximal capacity whereby hCtr1 expression can be regulated by Cu stresses. Finally, knockdown of Sp3 expression using siRNA did not alter endo-hCtr1 expression in vector- or *hCtr1*-wt-transfected cells treated with $CuSO_4$ (Fig. 6E). These results demonstrated that although Sp3 expression is regulated by Cu stresses in an opposite manner to that of Sp1, its expression does not affect hCtr1 expression under Cu stress conditions. These results highlight the importance of Sp1 in the KLF family in the regulation of Cu mammalian physiology.

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Discussion

The Sp1-hCtr1-Cu Tripartite Model in Regulating Cu Homeostasis in Mammalian Cells. Extracellular Cu exists in the oxidized form (Cu^{2+}) which is reduced to Cu^{+} by membrane-associated cupric reductases, similar to the FRE1 and FRE2 reductases found in yeast (Georgatsou et al., 1997), and the reduced Cu^{+} is primarily transported by the high-affinity Ctr1 to regulate cellular Cu bioavailability. Mechanisms for regulation of hCtr1 expression in response to Cu bioavailability are complex. In the present communication, we have constructed a tripartite regulation model whereby the maintenance of Cu homeostasis in human cells is regulated by an interregulatory loop consisting of Cu, hCtr1, and Sp1 elements. Within this loop, these elements are mutually regulated among themselves, but each is also self-regulated. The demonstration that mammalian cells use Sp1 oscillation in mammalian Cu homeostasis regulation is consistent with the multivariant homeostatic regulatory principle: a regulator that regulates a self-regulatory regulator should also be self-regulated. Thus, this study reveals a previously undiscovered homeostatic regulation mechanism for the maintenance of intracellular Cu budget in mammalian cells.

Our success in elucidating the interregulatory mechanism of mammalian Cu homeostasis regulation lies in the use of the RPA which is sensitive and reliable in measuring the hCtr1 mRNA expression levels. Importantly, by proper probe design it can simultaneously determine endo-transcripts vs exo-transcripts levels in the transfection assays. We showed that under Cu-adequate conditions, elevated expression of Sp1 by transfection promotes preferential binding of Sp1 to *hCtr1* promoter and upregulates hCtr1 expression. We observed that upregulation of hCtr1 and Sp1 expression under Cu-depleted conditions is mediated by enhanced Sp1 binding to these promoters. The feed-back suppression of *hCtr1* and *Sp1* expression under Cu-replete conditions is also mediated by the diminished Sp1 binding to the promoters of these genes. These results strongly suggest that the control mechanism of Cu

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homeostasis in the mammalian system is mediated by the differential promoter binding of Sp1 between *hCtr1* and *Sp1* promoters.

Copper homeostasis is evolutionarily conserved from yeasts to humans. Humans have one high-affinity Cu transporter (hCtr1), whereas yeast has two (yCtr1 and yCtr3). Like *hCtr1,* expression of *yCtr1* and *yCtr3* is transcriptionally upregulated under Cu-deplete conditions but is downregulated under Cu-replete conditions. When Cu is deficient, the transcription factor Mac1p binds to the metal-responsive elements located within the promoters of *yCtr1* and *yCtr3* and turns on the expression of these genes (Jensen et al., 1998; Labbe et al., 1997). Under Cu-sufficient conditions, Mac1p dissociates from the promoters, resulting in the shutting down of *yCtr1* and *yCtr3* expression. The transcription factor Ace1 is then activated to induce the expression of genes encoding Cu-chelating proteins (Cup1 and Crs5) and the antioxidant superoxide dismutase (Sod1) (Gralla et al., 1991; Gross et al., 2000) to protect cells from Cu toxicities. Moreover, expression of *Drosophila dCtr1B* under Cu-deficiency conditions is also regulated by promote binding of transcription factor MTF1 (Selvaraj et al., 2005). Regulation of plant Cu transporters *COPT1* and *COPT2* in *Arabidopsis* under Cu-limiting conditions also involves the interactions of the transcription factor *SQUAMOSA*-promoter like binding protein 7 (SPL-7) with the promoters of these genes (Yamasaki et al., 2009). In all cases, these transcription fractions bind to the promoters and upregulate the expression of their target genes under Cu-deficient conditions; whereas under Cu-replete conditions, these transcription factors dissociate from their promoters. Thus, the tripartite regulatory model may also apply to the regulation of Cu homeostasis in theseorganisms, however, whether these Cu-sensing transcription regulators are also regulated within the context of Cu homeostasis has not been investigated.

Sp1 is a ubiquitous transcription factor that was previously considered as a regulator for housekeeping genes. The present study reveals a new role of Sp1 in the regulation of Cu homeostasis. Of particular intrigue is the finding that Sp1 expression is self-regulated through

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the hCtr1 intermediate. Several studies have demonstrated that Sp1 may enhance its transcriptional activities through post-translational modifications and/or by cross-talking with coregulators (Wierstra, 2008), we cannot rule out the possible involvements of these post-translational mechanisms in fine-turning of Sp1 activity in regulating Cu homeostasis.

Mechanisms for Cu Sensing by Sp1. Many transcription factors regulate the expression of Cu transporters through their sensing of Cu availability. For Ace1, excess Cu promotes its DNA binding (Furst et al., 1988). By contrast, for Mac1p and SPL-7 (Yamasaki et al., 2009), excess Cu inhibits their DNA-binding activities whereas reduced Cu promote-binding activities. All these transcriptional regulators contain ZF modules that function as Cu sensors in regulating their target genes. These findings suggest that the sensing mechanisms by ZF in different transcription regulators are complex. In Sp1, each ZF domain consists of two cysteine and two histidine residues that are coordinated by one Zn molecule in a tetrahedral conformation. The finding that excess Cu prevents Sp1 from binding to *Sp1* and *hCtr1* promoters can be readily explained that excess Cu may poison the ZF domain of Sp1 by displacing the bound Zn, resulting in conformational changes that compromise its DNA-binding activities at both promoters, leading to downregulation of hCtr1 and Sp1 expression. This mechanism is consistent with that describing the effects of cadmium on the downregulation of cadmium transporter encoded by *Zip8* by Sp1 as we previously reported (Aiba et al., 2008). Strikingly, we observed that Sp1 and hCtr1 expression is elevated under Cu-depleted conditions through enhanced binding of Sp1 to their promoters. While these results constitute the important part in the tripartite inter-regulatory mechanism of Cu homeostasis regulation, given the complexity of the effects of Cu on ZF-DNA interactions as mentioned, the underlying mechanism is currently unknown and requires further in-depth investigation. Although it has been reported that Cu stress can affect Zn homeostasis and likewise, Zn can affect Cu homeostasis (Hoffman et al., 1988), our preliminary results showed that interactions of Sp1 with its recognition of GC box can

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not be simply explained by Zn contents, because modulation of Zn levels failed to alter Sp1 binding specificity (unpublished data). Currently, we favor the hypothesis that conformational change in the ZF may also occur under Cu-limiting conditions that enhance the stabilization of interactions between Sp1 and the *Sp1* and *hCtr1* promoters.

We observed that despite the fact that Sp3 also contains three ZFs and a TAD and is also regulated by Cu availability, but Sp3 expression does not regulate hCtr1 expression. These observations reflect promoter selectivity in gene regulation by the Sp1/KLF transcription factor family. How the bioavailable Cu affects the transcriptional selectivity of Sp1/hCtr1/Sp3 remains to be investigated.

Our finding that the Q-rich2 subdomain within the Sp1-TAD is involved in Sp1-regulated *Sp1* and *hCtr1* expression by Cu stress has added another aspect of complexity in the Sp1-hCtr1-Cu homeostatic regulation. This Q-rich2 domain interacts with $TAF_{II}110$ in the TFIID, a core promoter recognition multiple protein complex in the basal transcriptional machinery of RNA polymerase II (Gill et al., 1994; Liu et al., 2009). How Cu concentrations affect interactions between Sp1-TAD and the basal transcriptional assembly which as a whole contains more than 80 proteins, remain to be investigated. Taken together, our current study provides a new paradigm for the transcriptional regulation of Cu homeostasis in the mammalian system.

Implications of Cu metabolism regulation by the Sp1-hCtr1-Cu Tripartite Model. The Sp1-hCtr1-Cu inter-regulatory model described in this communication suggests that changes of any one component within this loop would result in either feedback or feed-forward to affect the expression of the other two. This model underscores the dynamic regulation mechanism for the maintenance of Cu homeostasis. Accordingly, the magnitudes of Sp1 and hCtr1 regulation by Cu imbalance depend upon the intrinsic (basal) expression levels of Sp1 and hCtr1 themselves and are not anticipated to be very high (ZDL manuscript submitted).

Another example of this Cu-related inter-regulatory relationship may be found in the human *prion* gene regulation. Prion is a Cu-binding protein that is intimately associated with transmissible spongiform encephalopathies. Not only does Cu regulate prion expression, but prion expression can also affect Cu metabolism (Kralovicova et al., 2009). Moreover, Bellingham et al (2009) reported that Sp1 functions as a Cu-sensing transcription factor that regulates *prion* gene expression. Recent bioinformatics from an analysis of 57 different species across the evolutionary tree estimated that the size of the copper proteome is generally less than 1% of the total proteome of an organism in both eukaryotes and prokaryotes (Andreini et al., 2008). It remains to be investigated whether many of these Cu-binding proteins, such as prion, Sp1, and hCtr1, may be regulated by Cu bioavailability.

Finally, the findings that Sp1 and Sp3 expression are regulated under Cu stressed conditions, although in an opposite manners, expand the gene regulation profiling by Cu homeostasis beyond the present study, because these transcriptional regulators are known to regulate a vast number of genes involved in cell growth, differentiation, apoptosis, and tumor development (Wierstra, 2008). The recently developed ChIP-sequencing approach, the entire repertoire of Sp1-regulated genes under various Cu stresses can be accessed. These studies eventually should provide a comprehensive picture for the global effect of gene regulation and resulting physiological consequences in mammalian Cu physiology.

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Authorship Contributions:

Participated in research design: Zheng, Kuo, Tsai, and Savaraj
Conducted experiments: Zheng, Tsai,and Lee
Performed data analysis: Zheng, Tsai, and Kuo
Wrote or contributed to the writing of the manuscript: Kuo and Zheng

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Legends for Figures

Fig. 1. Interregulatory relationships among Cu, hCtr1, and Sp1 in Cu homeostasis. All experiments were performed in SCLC cells except where otherwise indicated, in all cases where 18S RNA measurements were used as loading controls for the RPA, and β-actin or α-tubulin measurements were used as loading controls for the western blotting. (A) Autoradiograph of RNP showing the time-dependent inhibition of Sp1 and hCtr1 expression (left), and densitometric measurement of the autoradiographs as shown in the right panel. Error bars show standard deviations from three experiments. Note that the time was not drawn in scale. (B) Downregulation of Sp1 expression by transfection with HA-*hCtr1*-wt DNA as determined by Western blotting using the indicated antibodies. 1 - 5 denote that five independent cell lines were used. (C) Downregulation of hCtr1 expression by Sp1-siRNA. (D) Concentration-dependent upregulation of Sp1 by TM. (E) Time-dependent upregulation of Sp1 after transfection with hCtr1-siRNA. (F) Upregulation of endo-hCtr1 and downregulation of endo-Sp1 expression by transfecting exo-Sp1 recombinant analyzed by RNP using probe as described below (Fig. 4A). (G) Down-regulation of endo-Sp1 induced by transfection with exo-Sp1 was partially suppressed by co-transfection with hCtr1 siRNA. c, refers to control siRNA. (H) Increased hCtr1 expression in $CuSO_4$-treated cells by transfection with Sp1 recombinant. (I) Schematic diagram showing a summary of the results. The labels A–F refer to the results shown in panels A to F, respectively. The upward arrows denote increased levels. Downward arrows denote decreased levels. The thick curved arrows point to the results of the treatments. As an example for panel A, increased Cu concentration downregulates Sp1 is indicated by (hCtr1↑ ⟹ Sp1↓). Note that maintenance of Cu homeostasis in human cells is controlled by the cyclic regulatory mechanism as shown.

Fig. 2. Identification of Cu-responsive elements at the *Sp1* promoter

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(A) Schematic diagram showing the locations of 10 putative Sp1 binding sites and their sequences. Mutated sequences on these sites (M1 to M10) were indicated below. Numbers at the right refer to positions of these nucleotides upstream from the transcription start site (not drawn in scale). (B) Transient expression analyses of mutants Sp1 binding sites in response to Cu and TM. Each Sp1 mutant was transfected into HEK293 cells and treated with 100 μM $CuSO_4$ or 40 μM TM for 16 hr. Expression of luciferase reporter was determined by measuring the luciferase activity per μg of total cellular protein.

Fig. 3. Determinations of Sp1 Binding to the *hCtr1* and *Sp1* Promoters under Various Treatments by the ChIP Assay. (A) SCLC or *hCtr1*-DN-transfected cells were treated with 100 μM $CuSO_4$ for 16 hr followed by the ChIP assay of Sp1 binding to *hCtr1* and *Sp1* promoters as indicated. Input refers to total chromatin DNA before immunoprecipitation with anti-Sp1 antibody or non-immune immunoglobulin IgG. (B) The ChIP assay was performed in empty-vector- and *hCtr*1-wt transfected cells treated with or without 40 μM TM for 16 hr. (C) ChIP assay of Sp1 binding to the *Sp1* and *hCtr1* promoters in HEK293 cells transfected with the empty vector, *Sp1*-encoding and *hCtr1*-encoding recombinants for 16 hr as indicated. (D) Schematic diagram showing the summary of results presented in panels A – C. Details can be referred by Fig. 1I.

Fig. 4. The effects of various Sp1 domains and nucleotide residues on the Cu-mediated expression of endo-hCtr1 and endo-Sp1. (A) A schematic diagram showing the design of the hybridization probe used in RPA assay for measuring endo- and exo-Sp1 mRNA in transfection experiments. (B) A schematic diagram showing the recombinant Sp1 encoding the wild-type (wt) and deleted mutants (M3 – M13). Sp1-wt contains two serine/threonine (S/T rich1 and S/T rich2) and two glutamine (Q)-rich domains and three ZF domains. Numbers refer to the amino

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acid positions from the N-terminus. (C) Effects of various truncated Sp1 on endo-Sp1 and endo-hCtr1 mRNA expression under Cu-replete conditions. HEK293 cells were transfected with empty vector alone (V), *Sp1*-wt, or various *Sp1* mutants (M3-M13) as indicated. Cells were treated with 100 μM $CuSO_4$ for 16 hr. Endo-hCtr1 mRNA, exo-Sp1 mRNA, and endo-Sp1mRNA levels were determined by the RPA, using 18S RNA as the loading control. (D) Effects of various truncated Sp1 on endo-Sp1 and endo-hCtr1 mRNA expression under Cu-replete conditions. The experiment design was similar to that shown in panel (C) except that *hCtr1*-wt recombinant was used in co-transfection into the cells to increase the Cu contents. Note that lanes 7 (M7) and 11 (M13) did not show the exo-Sp1 signals because these mutants Sp1 do not contain the N-terminal sequence therefore cannot be detected by the RPA probe. Instead, their expression was verified by western blotting using anti-HA antibody (data not shown). (E) A schematic diagram showing the mutated amino acids (Q to L) in the Q-rich2 domain of Sp1-M3 recombinant. Numbers refer to the positions of amino acids. Circles refer to important amino acid residues for the Cu-regulated Sp1 effects. (F) Effects of Q-rich 2 mutations on the expression of endo-Sp1, endo-hCtr1 under Cu treatment. Recombinant constructs as shown in (E) were transfected into HEK293 cells. Cells were treated with 100 μM $CuSO_4$ for 16 hr and the expression levels of endo-Sp1, exo-Sp1, and endo-hCtr1 RNA levels were determined by RPA.

Fig. 5. Dissecting the roles of the Sp1 DBD and TAD in the regulation of Cu stress-induced hCtr1 reporter Expression using a chimeric fusion approach. Recombinants encoding the chimeric transcription regulators containing DBD either from LexA (DBD_{LexA}) or from Sp1-ZF were fused with either VP16 (TAD_{VP16}) or TAD of Sp1 (TAD_{Sp1}). These recombinant DNA were co-transfected into HEK293 cells with their cognate *luciferase* reporters

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as indicated and treated with either 100 μM $CuSO_4$ or 40 μM TM for 18 hrs. The luciferase activities were assayed and calculated with reference to that in the reference reporter

Fig. 6. Regulation of Sp1 family members by Cu and TM and the effects of these members on hCtr1 expression. (A) Regulation of Sp1 and Sp3 expression by the treatments of TM using the concentrations as indicated in the three SCLC cell lines. Densimetric presentations of the levels of expression with reference to the untreated controls (as 100%) are correspondingly below. (B) Regulation of Sp1 and Sp3 by the treatment of different concentrations of $CuSO_4$ in the three SCLC cell lines. Corresponding quantitative results are shown below. (C) Upregulation of endo-hCtr1 in *hCtr1*-wt-transfected cells by transfection with recombinant encoding Sp1, but not by those encoding Sp2, Sp3, or Sp4. Expression of endo-hCtr1 and exo-hCtr1 was determined by RPA using 18S RNA as a loading control, whereas expression of Sp1- Sp4 were determined by Western blotting. Probe design for differentiating between exo- and endo-hCtr1 mRNA was described previously (Liang et al., 2009) using the strategy similar to that described in Fig. 4A. (D) Overexpression of Sp1, Sp2, Sp3 and Sp4 by transfection did not alteration the expression of endo-hCtr1 mRNA levels in hCtr1-DN-transfected cells. (E) No effect on endo-Sp1 and endo-hCtr1 expression by knockdown of Sp3 expression with or without $CuSO_4$ treatments in the vector- and hCtr1-treated cells.

Fig. 1

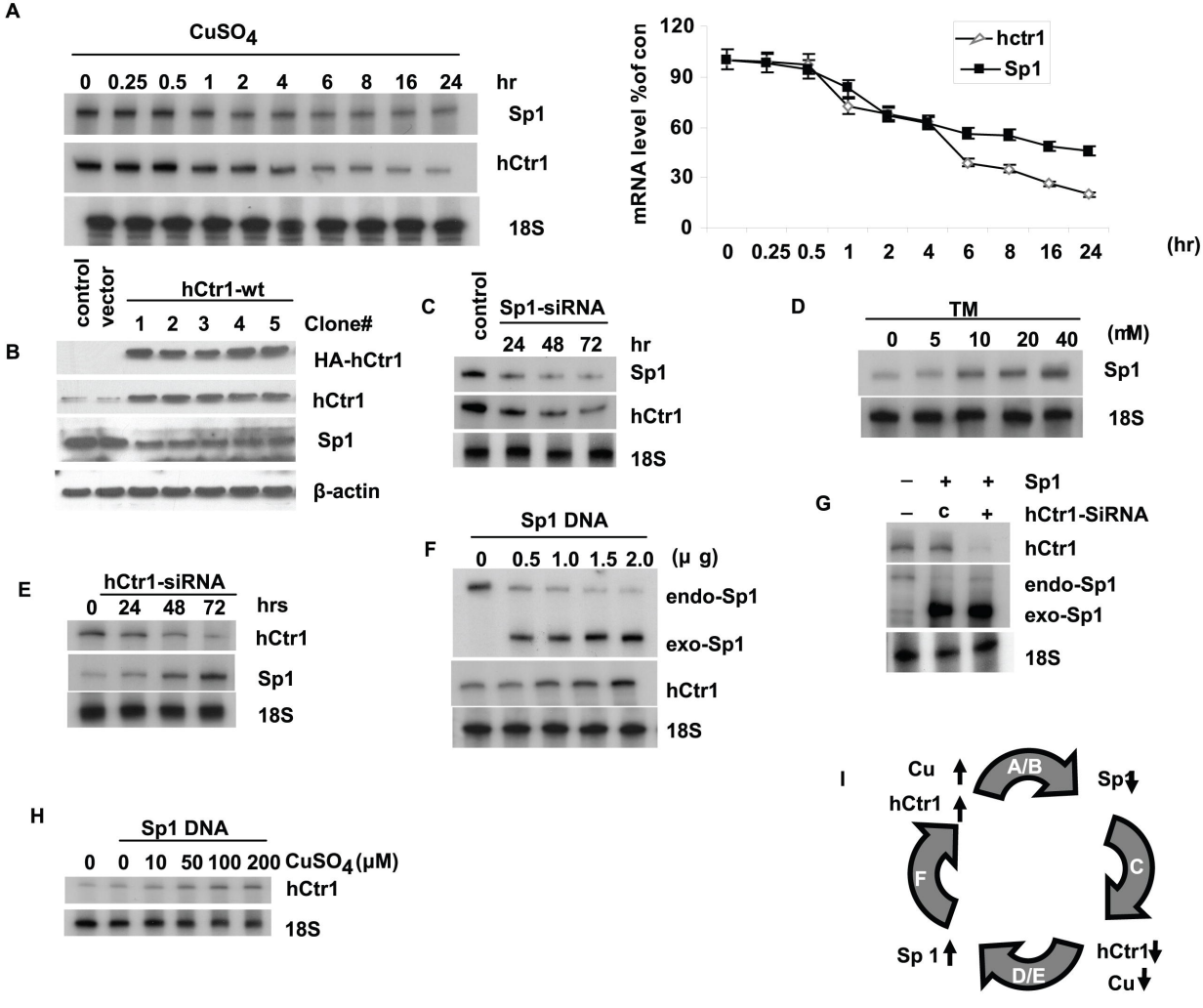
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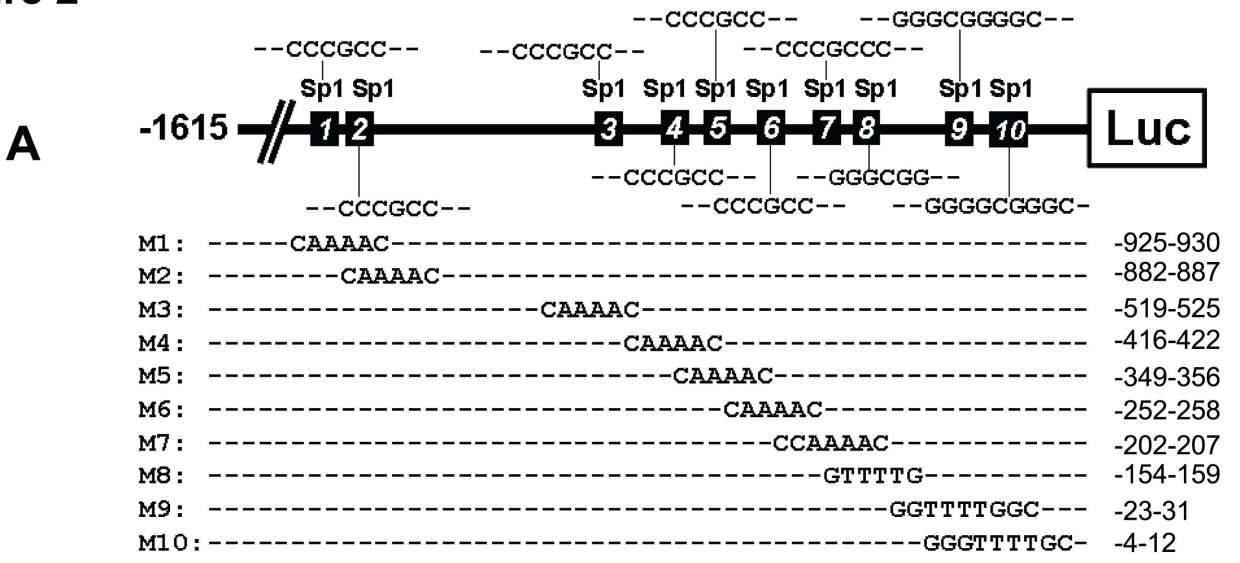
Figure 2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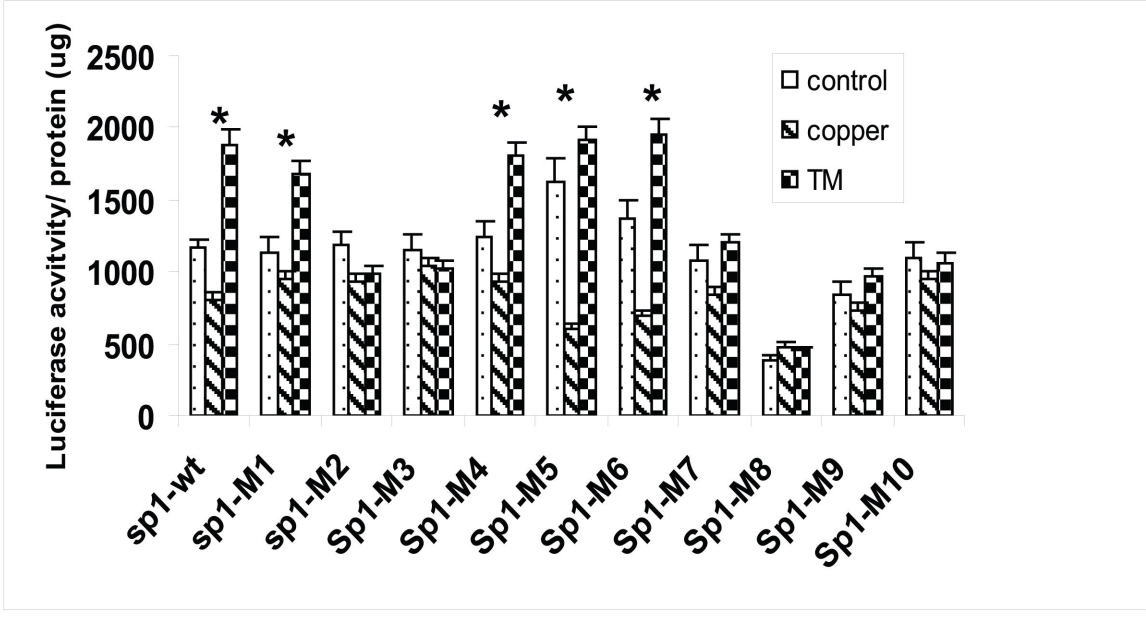
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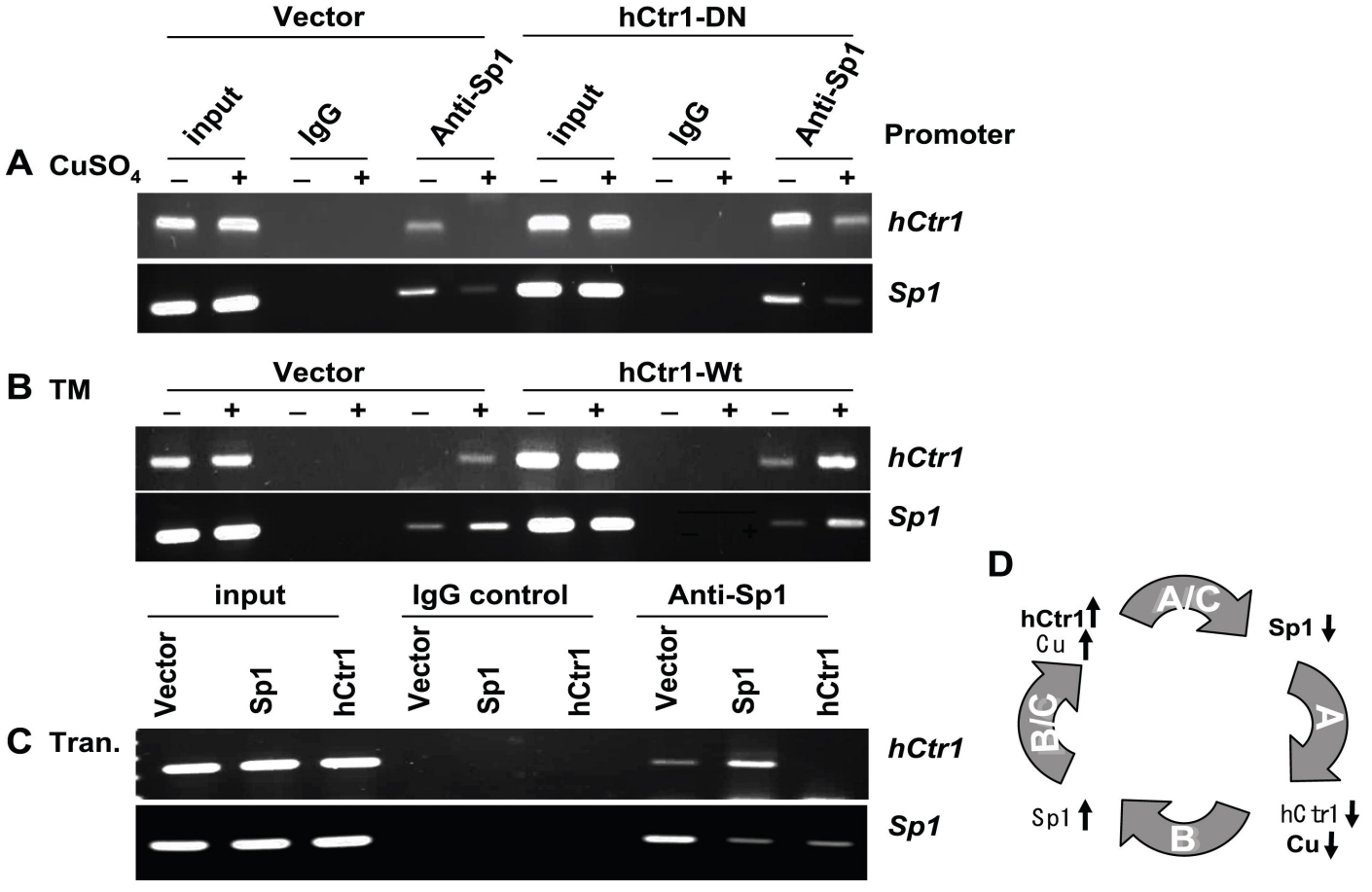
Figure 3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hCtr1
Sp1
B TM
Vector
hCtr1-Wt
– + – + – + – + – + – +
hCtr1
Sp1
input
IgG control
Anti-Sp1
Vector
Sp1
hCtr1
Vector
Sp1
hCtr1
Vector
Sp1
hCtr1
C Tran.
hCtr1
Sp1
D
hCtr1↑
Cu↑
A/C
Sp1↓
B/C
A
Sp1↑
B
hCtr1↓
Cu↓

Figure 4

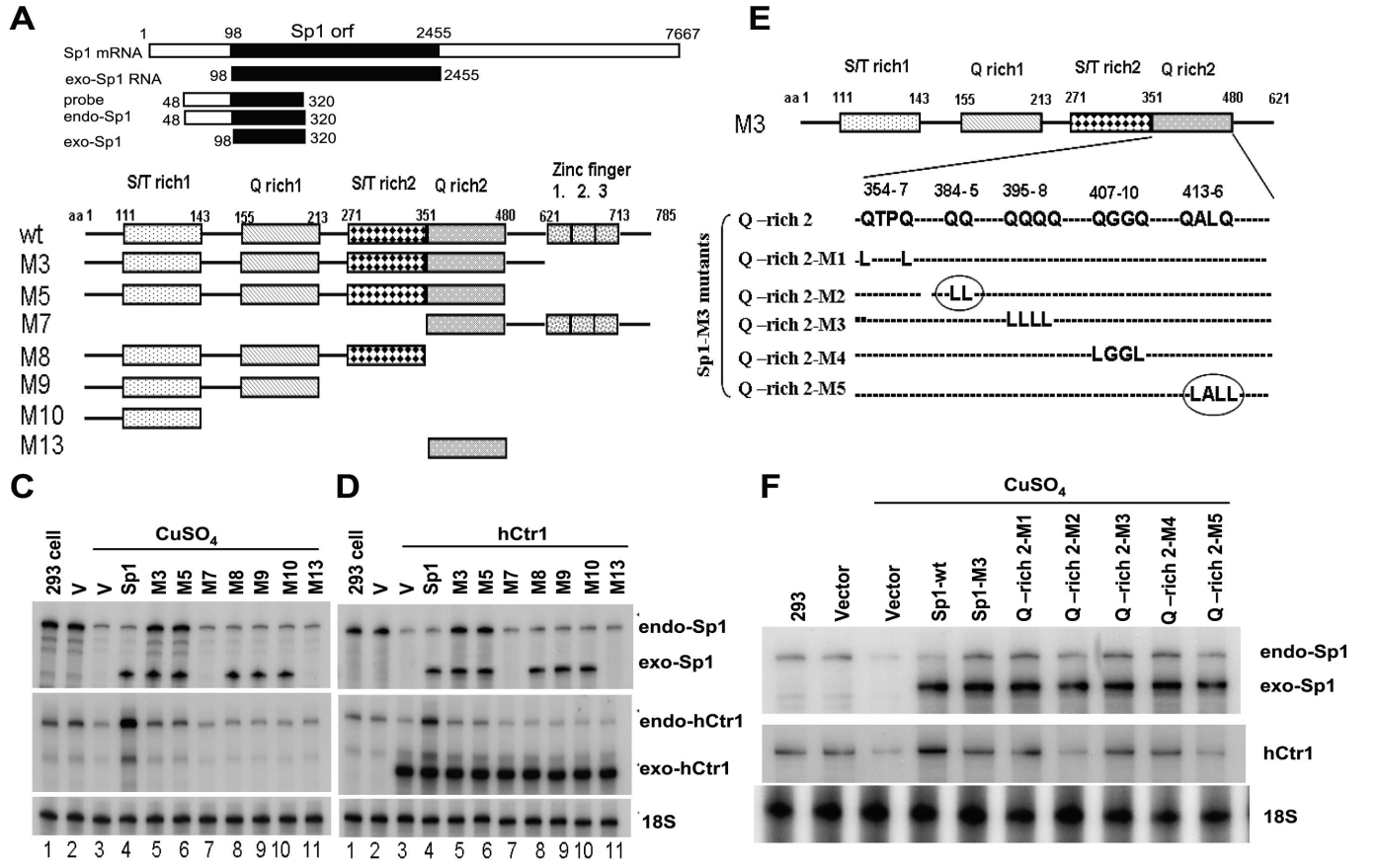

A
Sp1 mRNA
exo-Sp1 RNA
probe
endo-Sp1
exo-Sp1
Sp1 orf
S/T rich1
Q rich1
S/T rich2
Q rich2
Zinc finger
wt
M3
M5
M7
M8
M9
M10
M13
E
M3
354-7
384-5
395-8
407-10
413-6
Q –rich 2
Q –rich 2-M1
Q –rich 2-M2
Q –rich 2-M3
Q –rich 2-M4
Q –rich 2-M5
Sp1-M3 mutants
C
D
293 cell
V
Sp1
M3
M5
M7
M8
M9
M10
M13
CuSO4
hCtr1
endo-Sp1
exo-Sp1
endo-hCtr1
exo-hCtr1
18S
F
293
Vector
Sp1-wt
Sp1-M3
Q –rich 2-M1
Q –rich 2-M2
Q –rich 2-M3
Q –rich 2-M4
Q –rich 2-M5
hCtr1

Figure 5

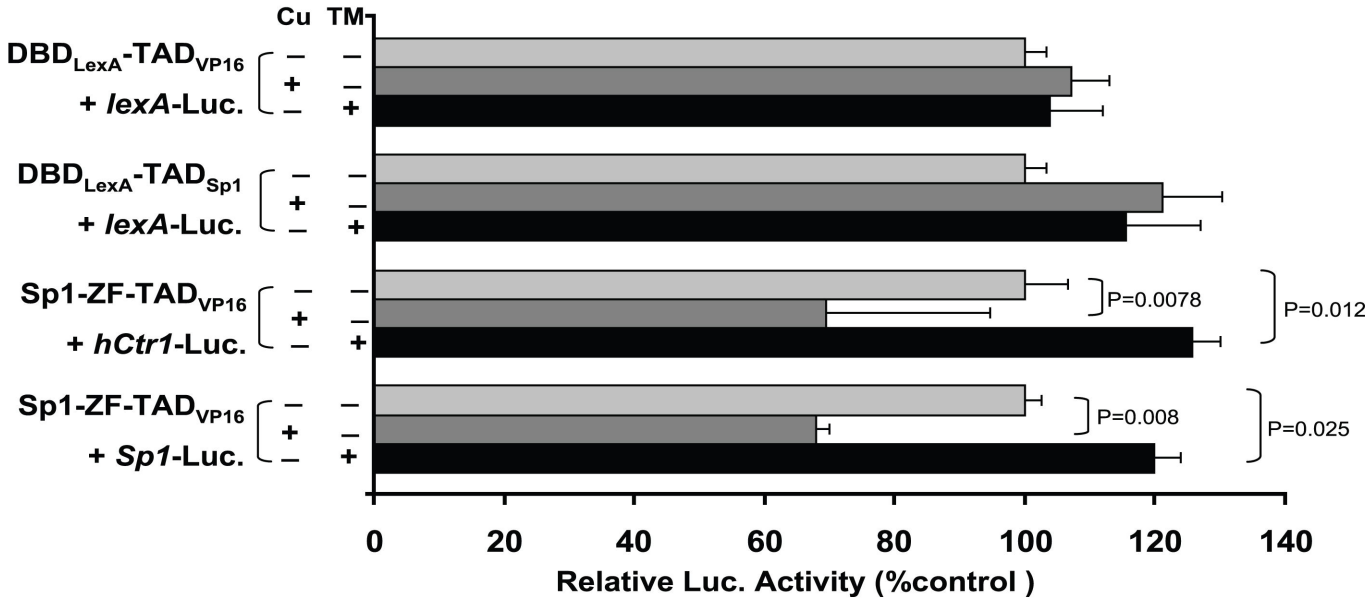
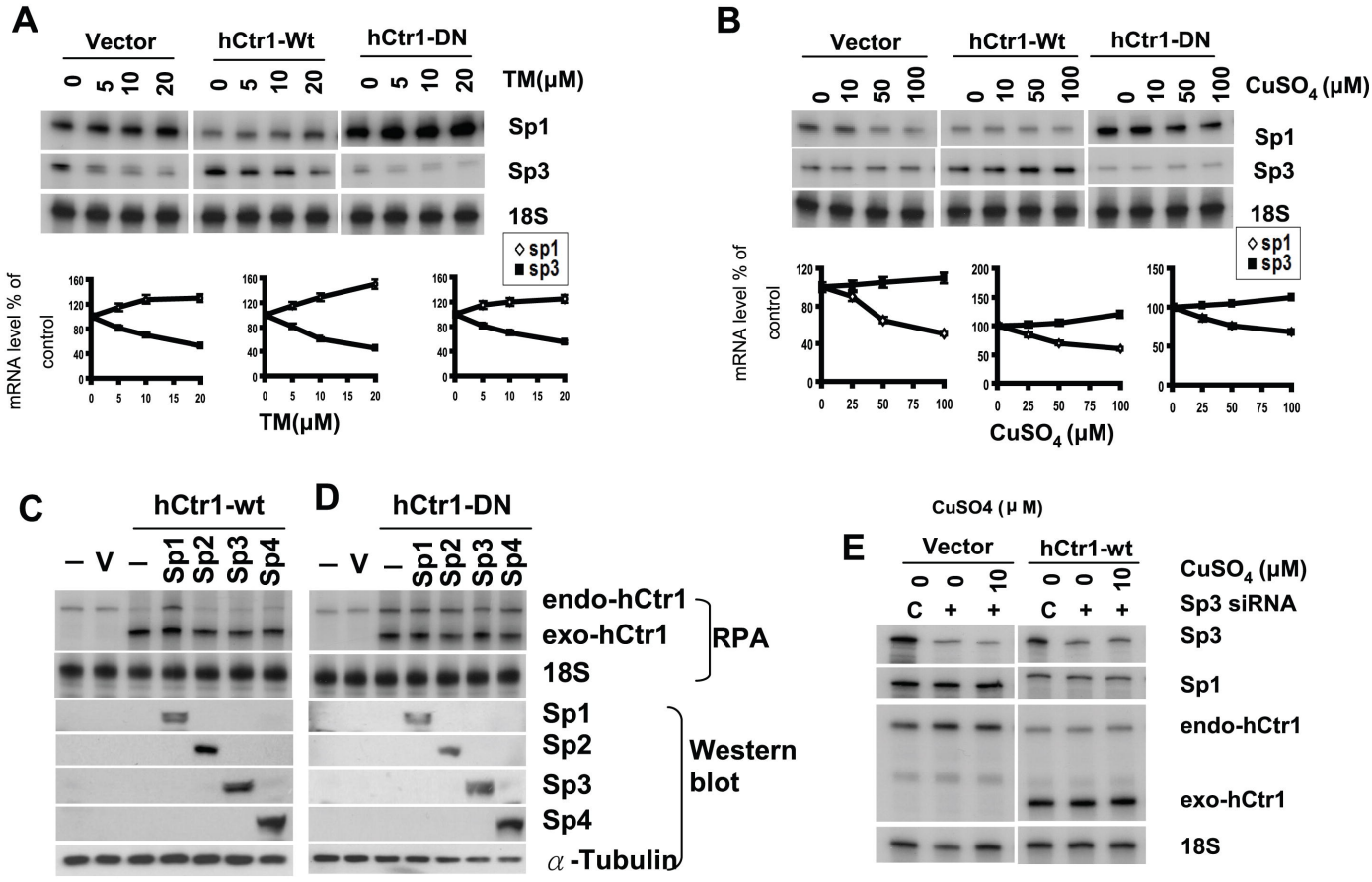

Cu
TM
DBD$_{LexA}$-TAD$_{VP16}$
+ *lexA*-Luc.
DBD$_{LexA}$-TAD$_{Sp1}$
+ *lexA*-Luc.
Sp1-ZF-TAD$_{VP16}$
+ *hCtr1*-Luc.
Sp1-ZF-TAD$_{VP16}$
+ *Sp1*-Luc.
P=0.0078
P=0.012
P=0.008
P=0.025
0
20
40
60
80
100
120
140
Relative Luc. Activity (%control)

Figure 6


A
Vector
hCtr1-Wt
hCtr1-DN
0 5 10 20
TM(μM)
Sp1
Sp3
18S
◇ sp1
■ sp3
mRNA level % of control
TM(μM)
B
Vector
hCtr1-Wt
hCtr1-DN
0 10 50 100
CuSO4 (μM)
Sp1
Sp3
18S
◇ sp1
■ sp3
mRNA level % of control
CuSO4 (μM)
C
hCtr1-wt
– V – Sp1 Sp2 Sp3 Sp4
endo-hCtr1
exo-hCtr1
18S
RPA
Sp1
Sp2
Sp3
Sp4
α-Tubulin
Western blot
D
hCtr1-DN
– V – Sp1 Sp2 Sp3 Sp4
E
CuSO4 (μ M)
Vector
hCtr1-wt
0 0 10 0 0 10
C + + C + +
CuSO4 (μM)
Sp3 siRNA
Sp3
Sp1
endo-hCtr1
exo-hCtr1
18S

Sp1 Oscillation Is Involved in Copper Homeostasis Maintenance by Regulating hCtr1 Expression

Zheng D. Liang, Wen-Bin Tsai, Mei-Yi Lee, Niramol Savaraj, and Macus Tien Kuo

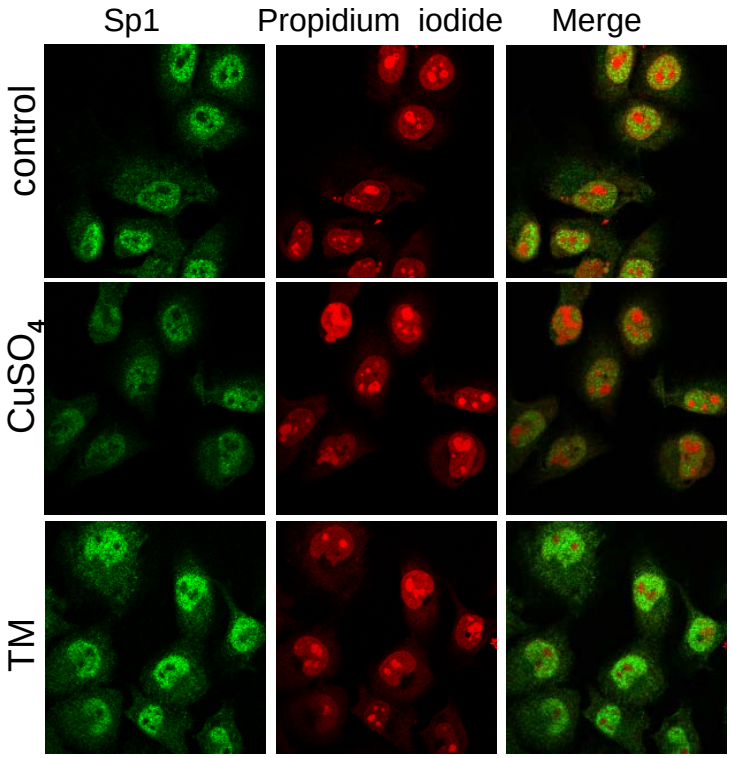


Figure 1. **Immunostaining of Sp1 in SCLC cells treated with $CuSO_4$ or TM.**
Cells were plated on cover slides and treated with 100 μM $CuSO_4$ or 40 μM TM for 16 hr. Immunostaining was performed with anti-Sp1 antibody and countered with propidium iodide. Images were viewed by confocal microscopy.