SUPPLEMENTAL FIGURE LEGENDS

Figure S1: Effects of Atox1 siRNA on copper-induced cell proliferation (**a**) and cyclin D1 expression (**b**) in wild-type MEFs. Cells were transfected with Atox1 or control siRNAs (Circ Res 2005;96(7):723-9) in the presence of either $CuCl_2$ (10 uM) or BCS (200 uM). Copper-induced increase in cell number measured at 72 hr after transfection was expressed as % increase over that in BCS-treated cells. The data are shown as mean±SE for three separate experiments (*, $p<0.01$ vs. control siRNA treated cells). Lysates obtained at 72 hours after transfection were immunoblotted with anti-cyclin D1, -Atox1 or -tubulin antibodies. Tubulin blot serves as a loading control.

Figure S2: Effect of various metals on cell proliferation in wild-type MEFs. Cells were pretreated with either copper chelator BCS (200 μM) (for copper and silver), iron chelator DFO (deferoxamine) (100 μM), or zinc chelator TPEN (N,N,N',N'-tetrakis-(2-pyridylmethyl)-ethylenediamine) (5 μM), and then stimulated with $CuCl_2$, $Fe(NH_4)_2(SO_4)_2$, $Fe(SO_4)_36H_2O$, $ZnSO_4$, or $AgNO_3$, respectively, at the indicated concentrations for 72 hours. The increase in cell number was expressed as % increase over that in cells treated with the chelator for each metal. The data are shown as mean ± SEM for three separate experiments.

Figure S3: Protein expression (A) and specific activity (B) of secreted ceruloplasmin (Cp) in $Atox1^{+/+}$ (WT) and $Atox1^{-/-}$ (Atox1-KO) fibroblast cells in the presence of either $CuCl_2$ (10 μM)(+Cu) or BCS (200 μM)(-Cu). Cp secreted in the culture medium was collected at 72-hour after addition of copper and concentrated by Concanavalin-A sepharose chromatography. Protein expression of Cp in concentrated culture medium was examined by immunoblotting with antibody specific to Cp (1,2). Its activity was measured by p-phenylenediamine (PPD) oxidation as previously described (3). Specific activity of Cp was determined by the ratio of activity to relative amount of protein as previously described (4). The results are presented as mean ± SE from 3 separate experiments. $P < 0.01$ vs. BCS-treated cells. NS, not significant.

Figure S4: **a**, Effects of copper (Cu) or silver (Ag) on transactivation of the cyclin D1 gene promoter in wild-type MEFs. Cells were transiently transfected with cyclin D1 promoter luciferase reporter constructs (pGL3-cyclin D1 (-962/+134)) or empty reporter constructs (pGL3-Basic) in the presence of either $CuCl_2$ (10 μM), $AgNO_3$ (10 μM), or the copper chelator BCS (200 μM). Relative luciferase activity was assayed as described in Figure 3A. The data are shown as mean ± SEM for three separate experiments. **b**, Effects of copper or Ag on transcriptional activation of Atox1 in MEFs. GAL4-Atox1 hybrid constructs were cotransfected into MEFs along with the luciferase reporter vector containing GAL4 binding sites in the presence of either $CuCl_2$ (10 μM), $AgNO_3$ (10 μM), or the copper chelator BCS (200 μM). The data are shown as mean ± SEM for three separate experiments.

Figure S5: Purified GST, GST-Atox1, GST-Atox1-C12,15S, or GST-Atox1-K56,60E (10 ng of each) was incubated with the DNA probe in the presence of $CuCl_2$ (10 uM) at 25 ºC for 20 min, and DNA-protein complexes were visualized in an EMSA. The arrows indicate the positions of the complex between Atox1 and the cyclin D1 promoter fragments and of the free probe. Competition was performed in the presence of 100-fold molar excess of the unlabeled cyclin D1 promoter fragments (-545/-516), or mutated promoter fragments (-545/-516 mut (-535/-530)) as indicated (lanes 3 and 4).

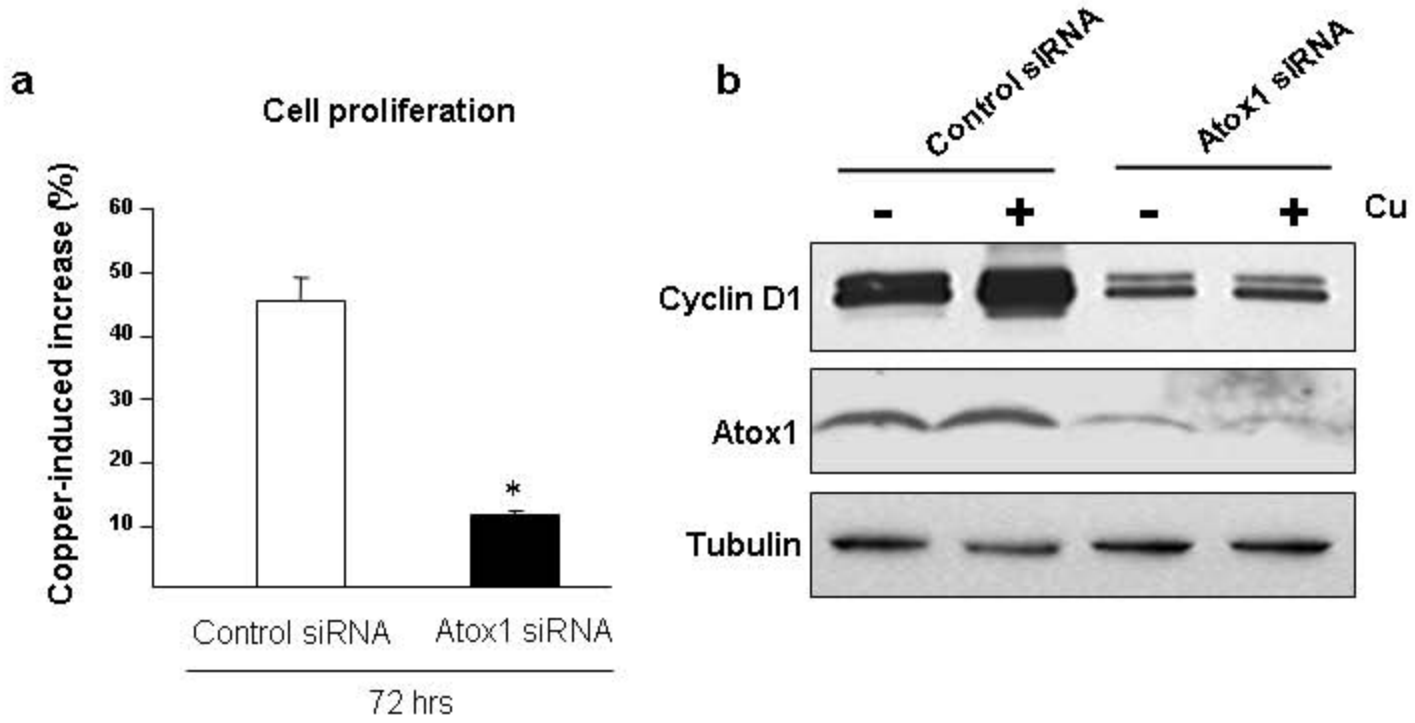
Figure S6: a, Time course of Atox1 expression in the nuclear or non-nuclear fraction in response to copper in MEFs. MEFs were stimulated with copper (10 uM) at 37 °C for 30 to 360 min. The nuclear extract (left panel) and non-nuclear fraction (right panel) were subjected to immunoblotting with anti-Atox1, anti-histone H3 (a marker for nuclear fraction), or anti-tubulin (a marker for cytosolic fraction) antibodies. The fold change in Atox1 protein levels is normalized to histone-H3 or tubulin as a loading control for each fraction. The bottom panel shows mean ± SE for three separate experiments (*, $p<0.01$ vs. control cells). **b**, Atox1 expression in the nuclear, non-nuclear, or total fraction in response to copper in MEFs. MEFs were incubated with or without copper (10 uM) at 37 °C for 60 min. The nuclear extract (left), non-nuclear fraction (center), and total fraction (right) were subjected to Western blotting with anti-Atox1, -histone H3 (a marker for nuclear fraction), or -tubulin (a marker for cytosolic fraction) antibodies. **c** and **d**, Effect of copper on Atox1 expression in each fraction in $Atox1^{-/-}$ MEFs transiently transfected with Flag-tagged Atox1-WT cDNA. **c**, After transfection, cells were cultured for 12 hours in the presence of either $CuCl_2$ (10 μM)(+Cu) or BCS (200 μM)(-Cu). The nuclear or non-nuclear fractions were subjected to Western blotting with anti-Atox1, anti-histone H3 (a marker for nuclear fraction), or anti-tubulin (a marker for cytosolic fraction) antibodies. In right panel, the data are expressed as fold change in Atox1 protein levels normalized to histone-H3 or tubulin in Cu treated cells as compared to BCS treated cells. *, $p<0.01$ vs. BCS treated cells. **d**, Immunofluorescence was performed using a Flag-M2 antibody followed by a FITC-conjugated goat anti-mouse IgG. *N>C*, cells showing higher nuclear staining; *N = C*, cells showing nearly equal staining intensities in the nucleus and the cytoplasm; *N<C*, cells showing lower nuclear staining. The data were obtained from three independent experiments. Approximately 100 cells were scored in each condition. *, $p<0.01$ vs. cells showing N>C.

Figure S7: **a**, Effect of re-expression of Atox1-WT or mutants on transactivation of the cyclin D1 gene promoter in $Atox1^{-/-}$ MEFs. Cyclin D1 promoter luciferase reporter constructs along with either pcDNA/Atox1-WT or mutants were transiently transfected in cells treated with either BCS (200 uM) or $CuCl_2$ at the dose indicated. **b**, Effect of re-expression of Atox1-WT or mutants on cell proliferation in $Atox1^{-/-}$ MEFs. 72 hours after transfection, cell numbers were counted. Bars are the mean ± SE from at least three independent transfection experiments, each performed in quadruplicate *, $p<0.01$.

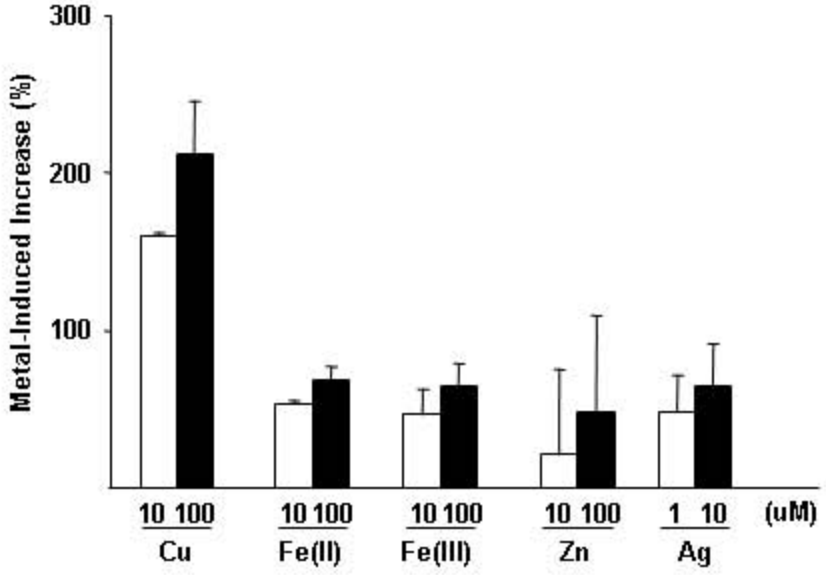
References

1. Chu, F. F., and Olden, K. (1985) *Biochem Biophys Res Commun* **126**(1), 15-24
2. Hellman, N. E., and Gitlin, J. D. (2002) *Annu Rev Nutr* **22**, 439-458
3. Sunderman, F. W., Jr., and Nomoto, S. (1970) *Clin Chem* **16**(11), 903-910
4. Jeney, V., Itoh, S., Wendt, M., Gradek, Q., Ushio-Fukai, M., Harrison, D. G., and Fukai, T. (2005) *Circ Res* **96**(7), 723-729

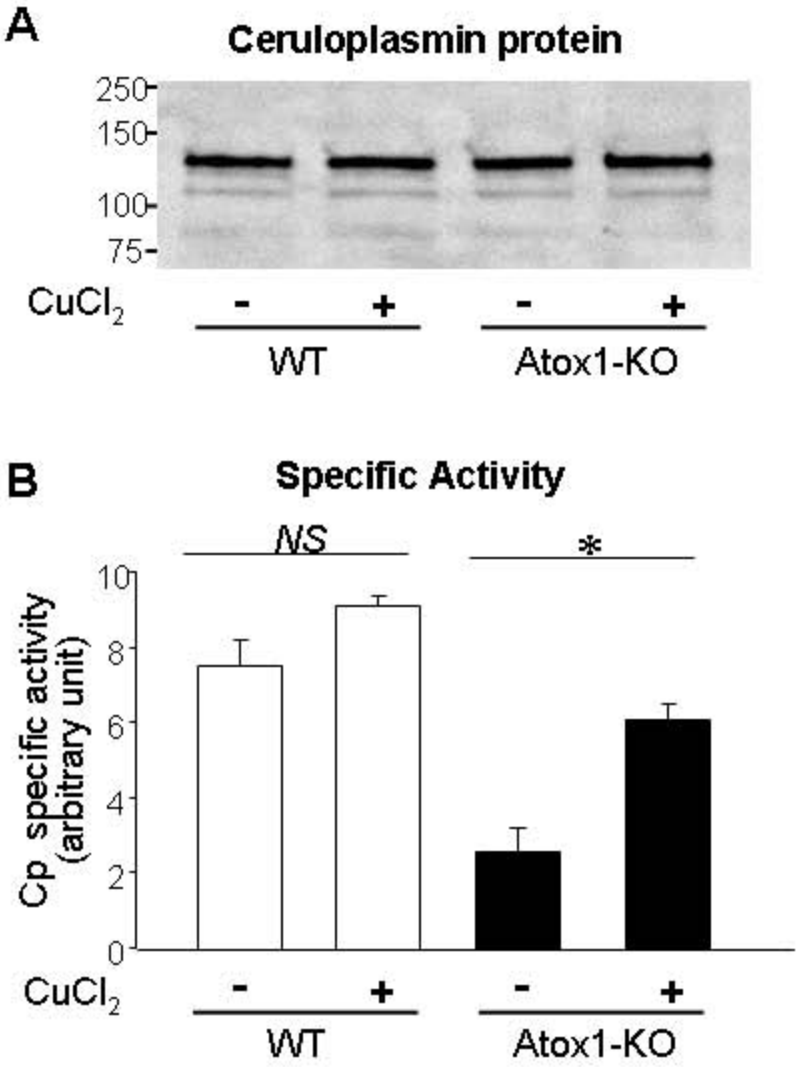
Suppl. Fig. S1


a
Cell proliferation
Copper-induced increase (%)
60
50
40
30
20
10
*
Control siRNA
Atox1 siRNA
72 hrs
b
Control siRNA
Atox1 siRNA
-
+
-
+
Cu
Cyclin D1
Atox1
Tubulin

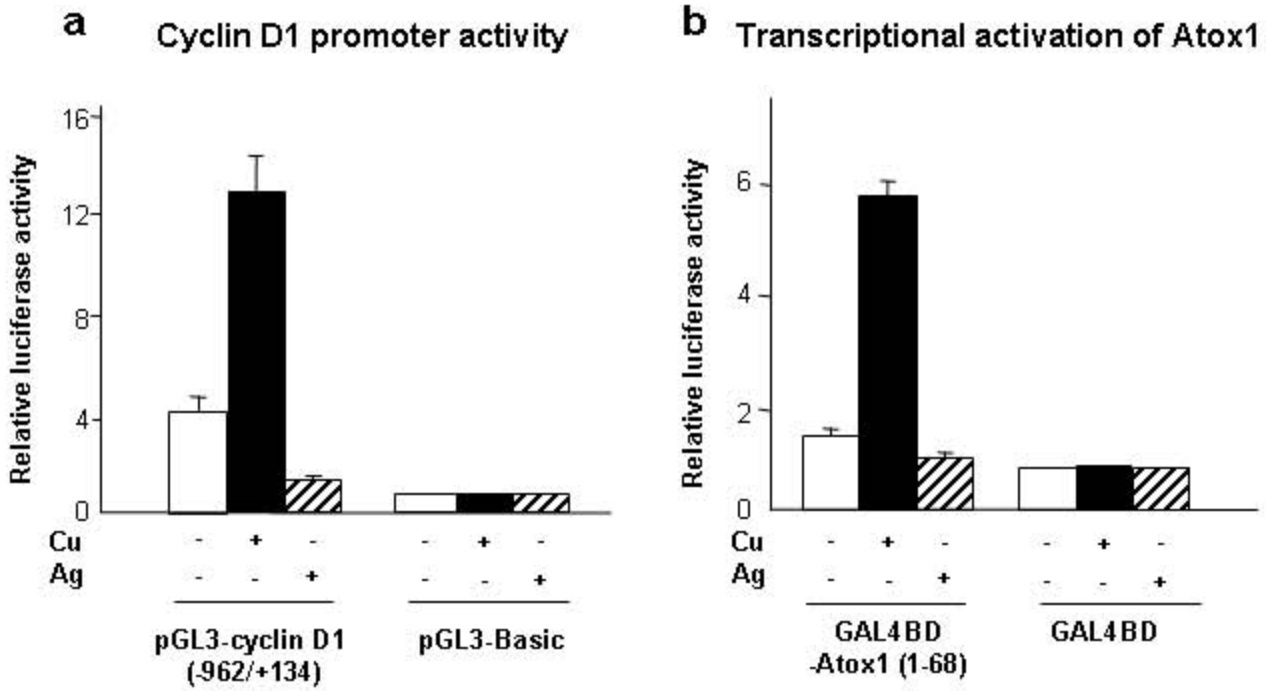
Suppl. Fig. S2


Metal-Induced Increase (%)
300
200
100
10 100
Cu
10 100
Fe(II)
10 100
Fe(III)
10 100
Zn
1 10
Ag
(uM)

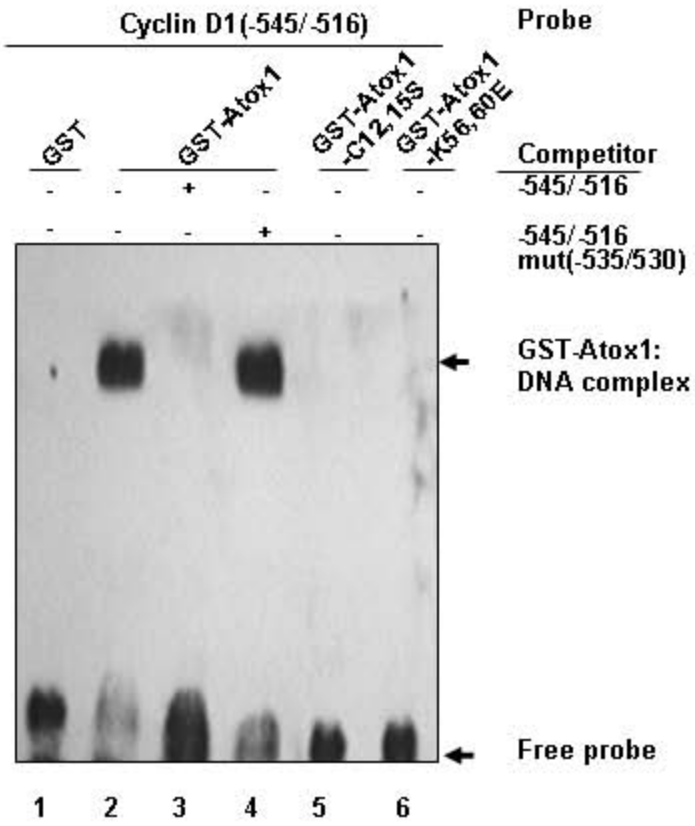
Suppl. Fig. S3


A
Ceruloplasmin protein
250
150
100
75
CuCl2
-
+
-
+
WT
Atox1-KO
B
Specific Activity
NS
*
Cp specific activity (arbitrary unit)
10
8
6
4
2
0
CuCl2
-
+
-
+
WT
Atox1-KO

Suppl. Fig. S4

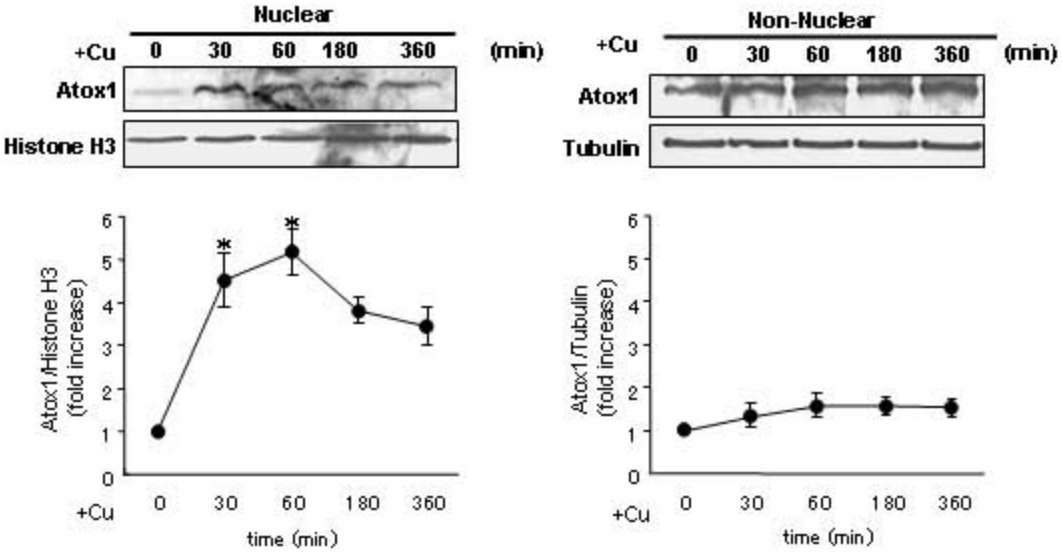

a
Cyclin D1 promoter activity
Relative luciferase activity
16
12
8
4
0
Cu
Ag
pGL3-cyclin D1
(-962/+134)
pGL3-Basic
b
Transcriptional activation of Atox1
Relative luciferase activity
6
4
2
0
Cu
Ag
GAL4BD
-Atox1 (1-68)
GAL4BD

Suppl. Fig. S5

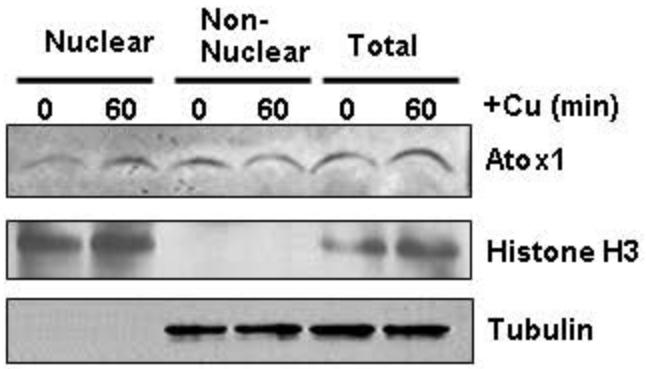

Cyclin D1(-545/-516)
Probe
GST
GST-Atox1
GST-Atox1 -C12,15S
GST-Atox1 -K56,60E
Competitor
-545/-516
-545/-516 mut(-535/530)
GST-Atox1: DNA complex
Free probe
1 2 3 4 5 6

Suppl. Fig. S6

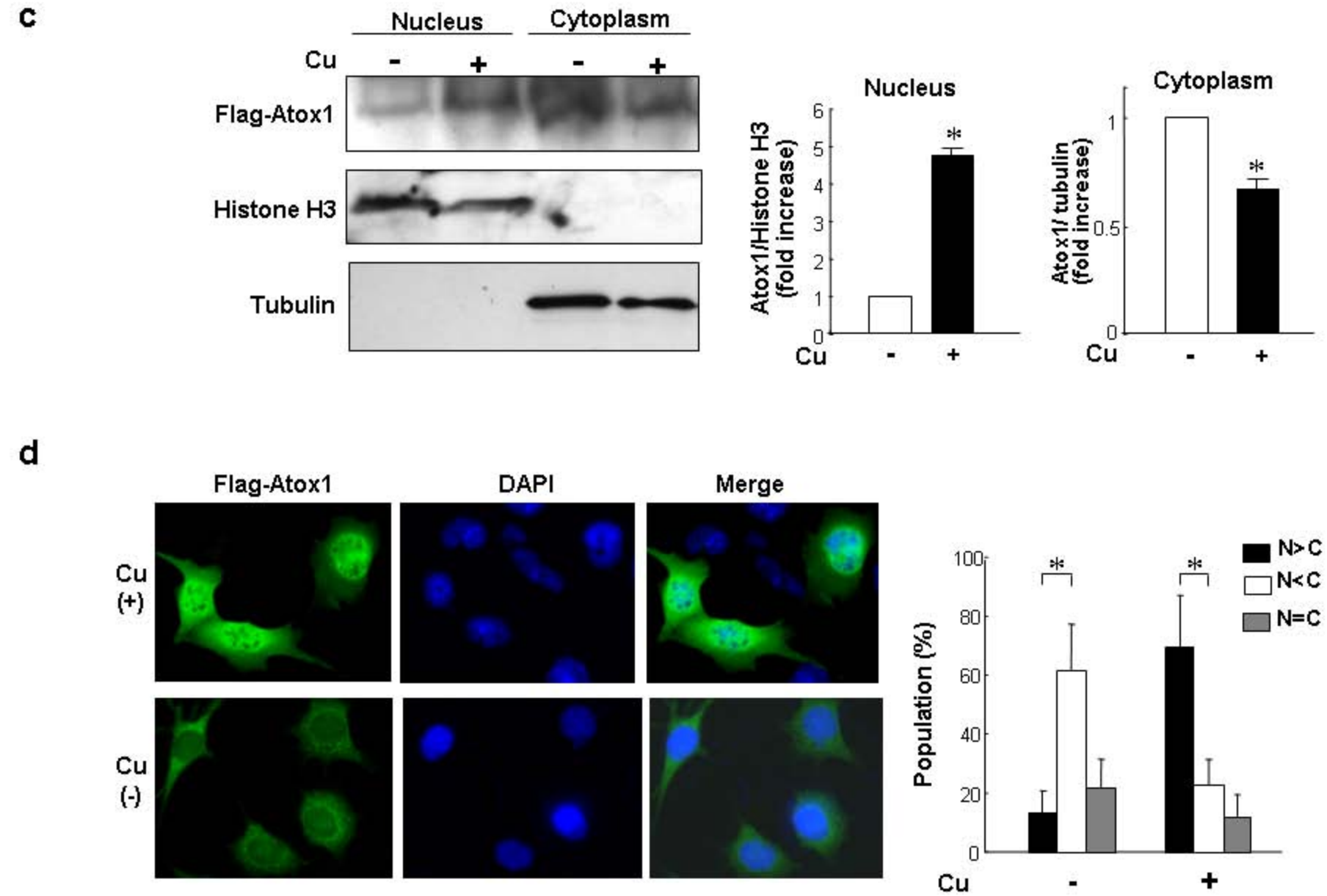
a



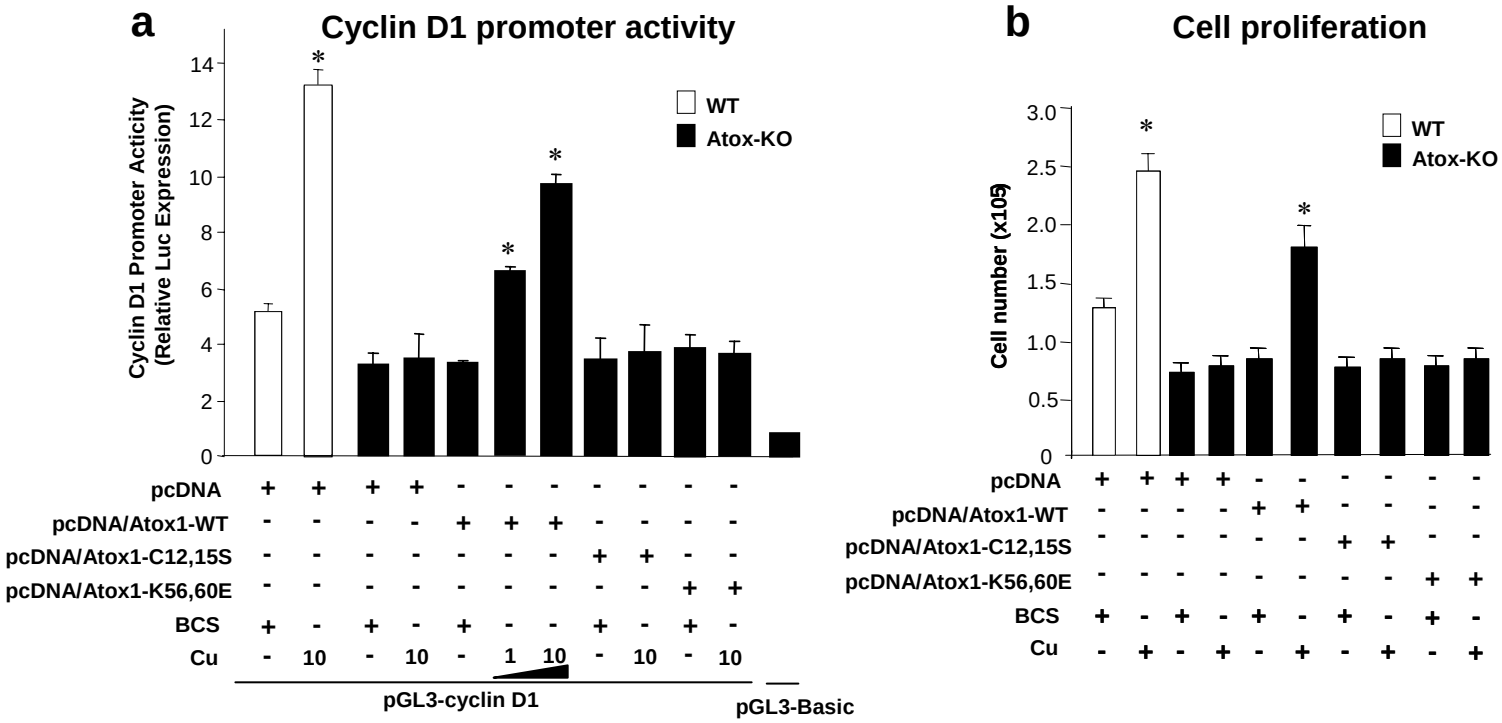
b



Suppl. Fig. S6


c
Nucleus
Cytoplasm
Cu
-
+
-
+
Flag-Atox1
Histone H3
Tubulin
Nucleus
Atox1/Histone H3 (fold increase)
Cytoplasm
Atox1/ tubulin (fold increase)
d
Flag-Atox1
DAPI
Merge
Cu (+)
Cu (-)
Population (%)
N>C
N<C
N=C

Suppl. Fig. S7


a
Cyclin D1 promoter activity
Cyclin D1 Promoter Acticity
(Relative Luc Expression)
WT
Atox-KO
pcDNA
pcDNA/Atox1-WT
pcDNA/Atox1-C12,15S
pcDNA/Atox1-K56,60E
BCS
Cu
pGL3-cyclin D1
pGL3-Basic
b
Cell proliferation
Cell number (x10⁵)
WT
Atox-KO
pcDNA
pcDNA/Atox1-WT
pcDNA/Atox1-C12,15S
pcDNA/Atox1-K56,60E
BCS
Cu

Supplemental Table S1

	Copper		Copper
Human normal tissue	22 ±7 μM(1)	Human plasma	About 20 μM(2)
Human malignant tissue	33±12 μM(1)	Mouse normal tissue	15-60 μM(3)
Human milk	1-5 μM(4)	Mouse plasma	12±4 μM(5)
Cerebrospinal fluid	70 μM(4)		

1. Margalioth, E. J., Schenker, J. G., and Chevion, M. (1983) *Cancer* **52**(5), 868-872
2. Linder, M. C. (1991)
3. Prohaska, J. R., Bailey, W. R., Gross, A. M., and Korte, J. J. (1990) *J Nutr Biochem* **1**(3), 149-154
4. Schumann, K., Classen, H. G., Dieter, H. H., Konig, J., Multhaup, G., Rukgauer, M., Summer, K. H., Bernhardt, J., and Biesalski, H. K. (2002) *Eur J Clin Nutr* **56**(6), 469-483
5. Percival, S. S. (1998) *Am J Clin Nutr* **67**(5 Suppl), 1064S-1068S