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Glutaredoxin 1 is a major player in copper metabolism in neuroblastoma cells

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ABSTRACT

Background: Glutaredoxin 1 (Grx1), a small protein belonging to the thioredoxin family, is involved in redox-regulation since it catalyzes the reduction of protein disulfides and that of mixed disulfides. It was reported to modulate active copper extrusion from cells, by affecting the function of the pumps ATP7A and B. These are components of the network of protein chaperones involved in the control of the homeostasis of copper, an essential, though harmful, metal. However, the effect of Grx1 on copper levels, copper chaperones and copper-elicited cell toxicity was never investigated.
Methods: In order to investigate the effect of Grx1 on copper metabolism, we constitutively overexpressed Grx1 in human neuroblastoma SH-SY5Y cells (SH-Grx1 cells) and assessed a number of copper-related parameters.
Results: SH-Grx1 cells show a basal intracellular copper level higher than control cells, accumulate more copper upon $CuSO_4$ treatment, but are more resistant to copper-induced toxicity. Grx1 shows copper-binding properties and copper overload produces a decrease of Grx1 enzyme activity in SH-Grx1 cells. Finally, Grx1 overexpression decreases copper accumulation in mitochondria upon copper overload and modulates the expression of copper transporter 1 (Ctr1).
Conclusion: Altogether, these data demonstrate that Grx1 is a major player in copper metabolism in neuronal cells.

1. Introduction

Copper is an essential micronutrient for all aerobic organisms because it serves as the catalytic cofactor for many enzymes, including Cu,Zn-superoxide dismutase (SOD1), cytochrome c oxidase (COX), ceruloplasmin and dopamine β-monooxygenase [1]. All cells have evolved mechanisms strictly regulating intracellular copper level to support physiological requirements, while preventing copper accumulation that would lead to oxidative damage. In fact, when in excess or not properly bound to proteins, copper may catalyze the formation of reactive oxygen species that can adversely modify proteins, lipid and nucleic acids thus affecting the overall cell physiology including the function of organelles like mitochondria [2,3].

Copper homeostasis is controlled by protein chaperones, showing one or more copper-binding domains [4]. For the most part, copper enters cells through the high affinity transporter Ctr1, which upon multimerization forms a pore thus allowing the entrance of copper in an ATP-independent fashion, because of its methionine-rich copper-binding domain [5,6]. Ctr1 is regulated by copper concentrations, with the zinc-finger transcription factor Sp1 (Specific protein 1) mediating this response [7]. In the cytosol, copper can be distributed among three different routes, each specific for a cell district; the cytosolic compartment is mainly governed by CCS (copper chaperone for SOD1), which is responsible for the correct insertion of copper in the most abundant cytosolic copper-enzyme [4,6]. The mechanisms by which copper is delivered to mitochondria are not clear; a hypothesis is that it can pass through the mitochondrial membrane bound to a small non-proteinaceous complex. Then, it is transferred by several mitochondrial copper chaperones (COX17, COX11, SCO1 and SCO2) to the active site of COX [8–10]. Cytosolic chaperone Atox1 (also called HAH1) delivers copper in the *trans* Golgi network to the P-type ATPases ATP7A or ATP7B, possessing six CXXC copper-binding domains at the N-terminus. These ATPases are selectively expressed in different tissues and cell types, with ATP7B mainly expressed in liver. By cell trafficking and ATP-dependent catalytic activity, they achieve both the metallation of copper-dependent enzymes of the secretory pathway and efflux of excess copper from the cell [11,12].

Glutaredoxin 1 (Grx1) is a small (12 kDa) protein belonging to the thioredoxin family of proteins, that is abundant in the cytosol, but also

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present in the intermembrane space of mitochondria (IMS). It catalyzes both the reduction of protein disulfides and that of mixed disulfides formed between its substrate glutathione (GSH) and protein thiols (deglutathionylation). One or both redox-active cysteines located in the active site of Grx1 are involved in the catalytic mechanisms [13,14]. In both cases, oxidized GSH (GSSG) is produced, which is regenerated at the expense of NADPH. The reversible formation of mixed disulfides between protein thiols and GSH is a key mechanism in redox-regulation and signaling, comparable to reversible protein phosphorylation.

It was demonstrated that Grx1 interacts with the N-terminus of both ATP7A and ATP7B, and that this interaction requires copper and involves the CXXC motifs of the pumps [15,16]. Furthermore, Grx1 and GSH seem to be involved in the regulation of Atox1 [17]. However, no additional studies were performed to investigate the role played by Grx1 in copper homeostasis.

Here we report the effect of Grx1 on copper metabolism in an experimental model represented by human neuroblastoma SH-SY5Y cells; overexpression of Grx1 induces a number of modifications that suggest a major role for this protein in copper homeostasis.

2. Materials and methods

2.1. Plasmids

Mouse Glutaredoxin 1 (*glrx1*, accession no. NM053108) was cloned in plasmid pcDNA3 by RT-PCR from mouse brain cDNA as previously described [18].

2.2. Cell cultures and treatments

Human neuroblastoma SH-SY5Y cells were purchased from the European Collection of Cell Cultures (Salisbury, UK) and grown in Dulbecco's modified Eagle's F12 medium (Lonza, Milano, Italy) supplemented with 15% fetal calf serum at 37% in an atmosphere of 5% CO_2 in air. Cells were routinely seeded at a density of 2×10^5 cells/ml.

SH-SY5Y cells constitutively expressing Grx1 were obtained by transfection with Lipofectamine Plus (Life Technologies Italia, Monza, Italy) followed by selection with 400 μg/ml G418 (Gibco, Paisley, UK) as described in Ref. [18].

Treatment with copper was performed 24 h after plating by adding $CuSO_4$ to a final concentration of 50 μM or 150 μM for 24 h. Cells were detached in trypsin/EDTA, collected by centrifugation and washed with phosphate-buffered saline (PBS: 10 mM phosphate buffer, 2.7 mM KCl, and 137 mM NaCl, pH 7.4).

2.3. Mitochondrial isolation

Cells were homogenized in mitochondrial buffer (0.2 mM EDTA, 0.25 M sucrose, and 10 mM Tris/HCl, pH 7.4) and Protease Inhibitor Cocktail (Roche, Basel, CH) (1:1000, v:v) in a Potter glass homogenizer with a Teflon pestle. The samples were centrifuged at 600 ×*g* for 10 min at 4 °C and supernatants were then centrifuged at 7,000 ×*g* for 10 min at 4 °C to obtain the crude mitochondrial fraction.

2.4. Cell viability assessment

Cell viability was evaluated in a hemocytometer chamber under a phase contrast microscope by their capacity to exclude Trypan blue (0.2%, w:v). Viability was also estimated by measuring the capability of mitochondrial dehydrogenases to reduce MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethylphenyl]-2-[4-sulphophenyl]-2H-tetrazolium inner salt) (Promega, Madison, WI, USA) to formazan. For this assay, cells were grown and treated with $CuSO_4$ in 96-well plates and the formation of formazan was followed at 490 nm by a microplate reader (Benchmark, Bio-Rad, Hercules, CA, USA).

2.5. Cell lysis and Western blot analysis

Total cell lysates were obtained by resuspending pelleted cells in lysis buffer (10 mM Tris–HCl, 150 mM NaCl, 1% Triton X-100) containing Protease Inhibitor Cocktail (Roche, Basel, CH), for 30 min on ice, followed by centrifugation at 14,000 ×*g* for 30 min. Protein content was assayed according to Ref. [19].

After standard reducing, denaturing SDS–PAGE, Western blot was performed on nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Filters were incubated with the appropriate antibody at the following dilutions: Grx1, goat polyclonal antibody (R&D Systems, Minneapolis, MN, USA), 1:1000; GAPDH, mouse monoclonal antibody (Santa Cruz Biotechnology, CA, USA), 1:1000; CCS, rabbit polyclonal antibody (Santa Cruz Biotechnology, CA, USA), 1:500; ATP7A, mouse monoclonal antibody (BD Biosciences, Franklin Lakes, NJ, USA), 1:2500; Nrf2, rabbit polyclonal antibody (Santa Cruz Biotechnology, CA, USA), 1:500; Ctr1, goat polyclonal antibody (Santa Cruz Biotechnology, CA, USA) 1:500.

Filters were then incubated with the proper peroxidase conjugated secondary antibodies (Sigma-Aldrich, St. Louis, MO, USA) and developed using the ECL Advance Western Blotting Detection Kit (GE Healthcare, Buckinghamshire, UK).

2.6. Copper, iron and zinc content measurement

Cells in hypotonic PBS (1:2, v:v) were sonicated for 20 s. For copper, iron and zinc content assays, cell lysates were diluted 1:2 (v:v) with 65% nitric acid. After at least 1 week at room temperature, copper, iron and zinc contents were assayed by atomic absorption spectrometry using an AAnalyst 300 instrument equipped with a graphite furnace with platform (HGA800) and an AS-72 autosampler (Perkin-Elmer, Waltham, MA, USA).

2.7. SOD1 activity

SOD1 activity was determined by in-gel inhibition of the staining with nitro blue tetrazolium, as described [20].

2.8. Grx1 activity

Grx1 enzyme activity was determined using β-hydroxyethylene disulfide (HED) as a substrate according to Ref. [21]. The decrease in absorbance at 340 nm was followed using a Perkin Elmer lambda-25 spectrophotometer. Activity was expressed as μmol of NADPH oxidized/min/mg protein (Units/mg protein) using an extinction coefficient of 6,200 M^{-1} cm^{-1}.

2.9. Immobilized metal-affinity chromatography (IMAC)

Copper binding proteins in SH-Grx1 cells were separated using a copper-loaded IMAC column according to [22]. This method is routinely applied for isolation of copper-binding proteins [23]. Both the loading and elution buffer contain 1 M NaCl, avoiding the possibility of unspecific binding of Grx1 to Cu-IMAC. Control runs were performed in the presence of 2 mM EDTA. Nrf2 protein, which is not known to bind copper, and CCS protein, a copper-binding protein, were used as a negative and positive control, respectively.

2.10. RNA preparation and qRT-PCR

Total RNA from SH-SY5Y and SH-Grx1 cells, either untreated or treated with 150 μM $CuSO_4$, was extracted using TRIzol Reagent (Life Technologies Italia, Monza, Italy), according to the manufacturer's instructions. RNA was then reverse transcribed using ImProm-II™ Reverse Transcription System (Promega, Madison, WI, USA).

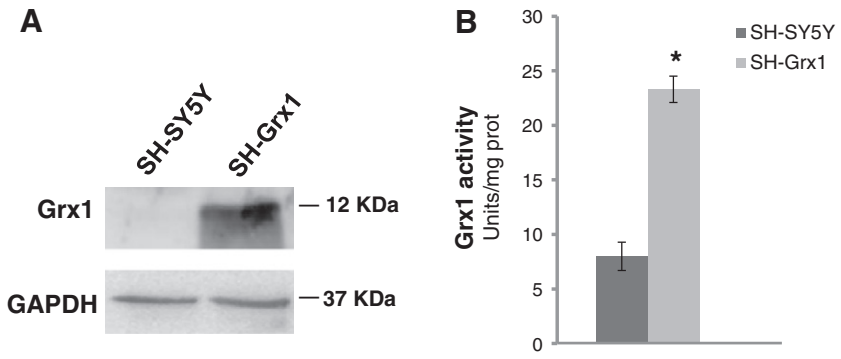


Fig. 1. Grx1 protein content and enzyme activity in SH-SY5Y cells overexpressing Grx1 (SH-Grx1). A. Cell lysates were applied to SDS–PAGE followed by Western blot with an anti-Grx1 polyclonal antibody. Thirty micrograms of protein was applied to each lane. GAPDH was used as loading control. One representative blot is shown, from three independent experiments giving comparable results. B. Enzyme activity of Grx1, expressed as μmol of NADPH oxidized/min/mg protein (Units/mg protein), was evaluated in cell total extracts. $n = 3$, $^{*}p < 0.05$.

qRT-PCR was performed on Light Cycler® 480 System with LightCycler® 480 SYBR Green I Master Mix (Roche Applied Science) in triplicate from reverse transcribed cDNAs.

Human CTR1 forward primer TGATGATGATGCCTATGACCT and reverse primer GGCATGGAATTGTAGCGAAT were used. Sp1 CTA TAG CAA ATG CCC CAG GT and reverse primer TCTGGGCTGTTTTCTCCTTC were used. qRT-PCR conditions were 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing/extension at 60 °C for 30 s, in a 20 μl reaction volume. Gene expression was normalized using β-actin levels.

2.11. Statistical analysis

The Shapiro–Wilk test was used to check the normal distribution of the analyzed variables. All tested variables were normally distributed. Results are then presented as mean ± standard deviation and analyzed by Student's t test. A value of $p < 0.05$ was accepted as the level of significance.

3. Results

3.1. Glutaredoxin 1 protects human neuroblastoma cells from copper-induced toxicity

To investigate the putative role of Grx1 in copper metabolism, we used SH-SY5Y cells constitutively overexpressing Grx1 (SH-Grx1 cells) (Fig. 1A and B). The increase in activity is significant, but moderate if compared to the pronounced increase of the protein level. This may be due to the intrinsically different sensitivity of the two methods employed for detection.

In order to evaluate whether Grx1 overexpression may modulate the cell response to copper overload, we treated the cells with $CuSO_4$ (50 or 150 μM) for 24 h and we analyzed its effect on cell viability by two independent methods (Fig. 2A and B). Only a slight decrease of viability is observed by the Trypan blue permeability assay in cells treated with 150 μM $CuSO_4$ (Fig. 2A); conversely, as evaluated by the MTS test, a dose-dependent decrease in mitochondrial function is observed, with SH-Grx1 cells partially protected from $CuSO_4$ toxicity (Fig. 2B).

3.2. Grx1 overexpression affects copper chaperones and hinders copper accumulation in mitochondria

In order to understand whether Grx1 overexpression affects intracellular copper content, we analyzed total cell lysates from SH-Grx1 cells by atomic absorption spectrometry; unexpectedly, under basal conditions, copper content in these cells is 3 times higher than in control, untransfected SH-SY5Y cells (Fig. 3A). Furthermore, when SH-Grx1 cells were exposed for 24 h to $CuSO_4$ (50 or 150 μM), they accumulate more copper than untransfected cells (Fig. 3A). This effect is specific for copper, since no difference in the levels of iron (Fig. 3B) and zinc (Fig. 3C) is observed both in basal conditions and after copper overload.

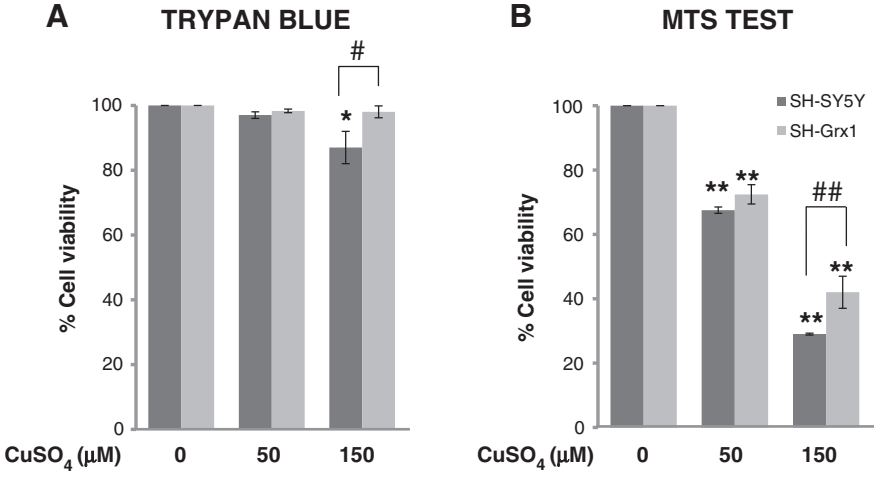


Fig. 2. Grx1 overexpression protects from copper toxicity. Cells were incubated with 50 or 150 μM $CuSO_4$ for 24 h. A. Cell viability was measured by Trypan blue exclusion. $n = 3$, $^{*}p < 0.05$ with respect to the untreated cells; $^{\#}p < 0.05$ with respect to untransfected cells. B. Cell viability was measured by the MTS reduction test. $n = 6$, $^{**}p < 0.001$ with respect to the untreated cells; $^{\#\#}p < 0.001$ with respect to the untransfected cells.

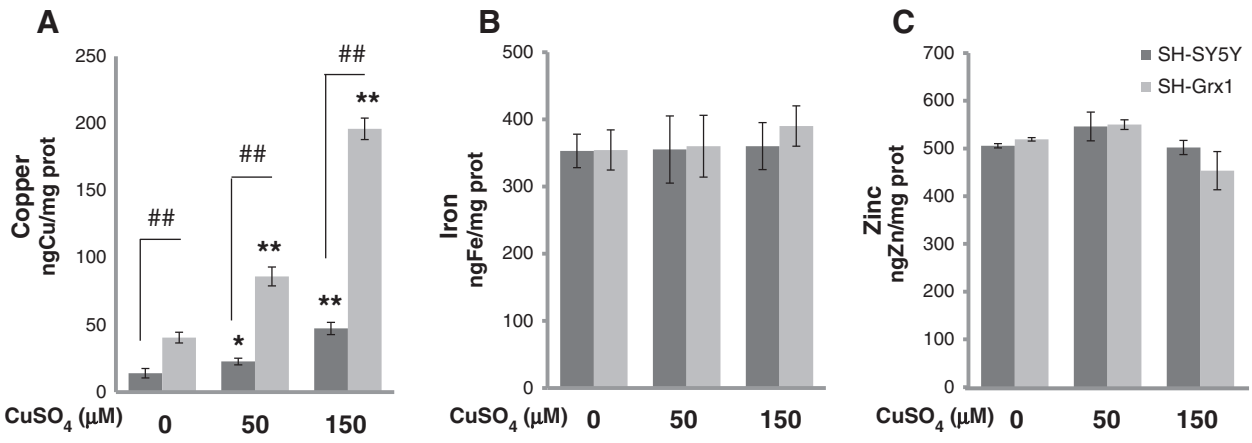


Fig. 3. Grx1 overexpression in SH-SY5Y cells causes a higher intracellular copper accumulation. Cells were incubated for 24 h in the presence of 50 or 150 μM $CuSO_4$. Copper, iron and zinc were measured by atomic absorption spectrometry on total cell lysates and expressed as ng/mg protein. $n = 3$, $^{*}p < 0.05$ and $^{**}p < 0.001$ with respect to the untreated cells only; $^{\#\#}p < 0.001$ with respect to untransfected SH-SY5Y cells.

Intriguingly, overexpression of Grx1 also affects the level of Ctr1, the high affinity copper transporter. The level of Ctr1 protein increases in SH-Grx1 cells, in basal conditions with respect to parental cells (Fig. 4A). After copper overload, Ctr1 decreases in both cell lines, but its level is still higher in SH-Grx1 cells. mRNA for Ctr1 follows the same trend (Fig. 4B). This effect is possibly mediated by transcription factor Sp1, which is upregulated as well in SH-Grx1 cells (Fig. 4C).

We also analyzed the cytosolic copper chaperone CCS, which decreases upon treatment with 150 μM $CuSO_4$, in both cell lines (Fig. 5A). This reduction is significantly higher in SH-Grx1 cells with respect to the control cells. We also determined SOD1 activity in the two cell lines upon treatment with $CuSO_4$ by an in-gel assay, in order to evaluate whether CCS reduction in SH-Grx1 cells affects SOD1 activity. As shown in Fig. 5B, this is not the case.

Interestingly, the protein level of ATP7A does not change between the two cell lines upon $CuSO_4$ treatment (Fig. 5C).

Copper treatment is known to compromise cell viability, possibly because of copper accumulation in mitochondria and mitochondrial damage [2]. We treated the two cell lines with 150 μM $CuSO_4$ for 24 h and we quantified mitochondrial copper content, in isolated mitochondria (Fig. 6). The two cell lines show the same mitochondrial copper content under basal conditions (despite their different total cell copper

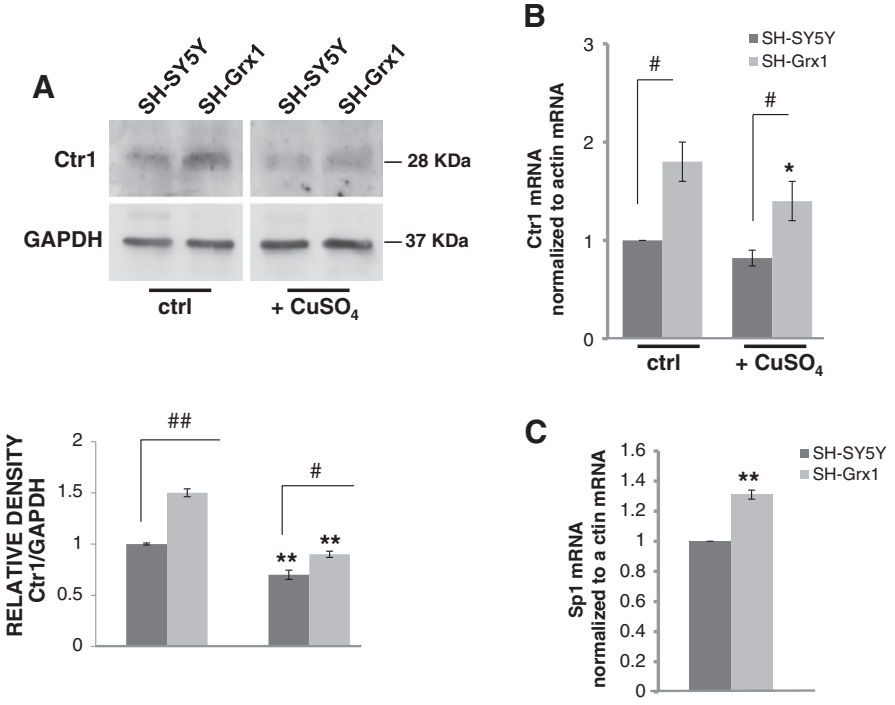
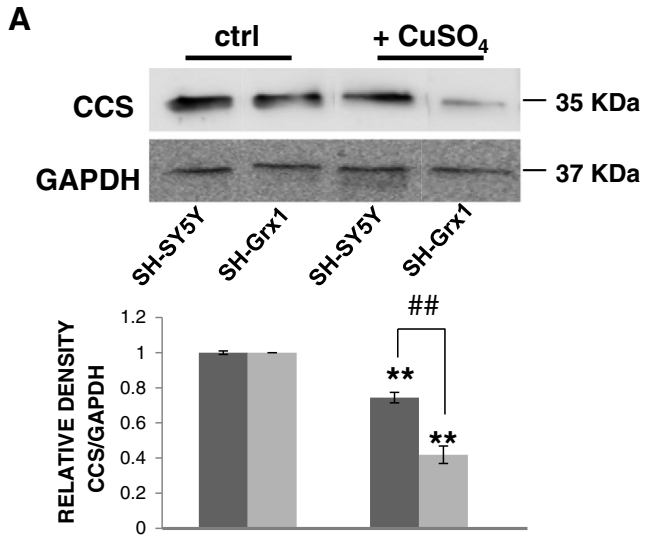


Fig. 4. Grx1 modulates Ctr1 expression. A. (top) Cells were treated with 150 μM $CuSO_4$ for 24 h. Western blot analysis of Ctr1. Thirty micrograms of protein was loaded to each lane and GAPDH was used as loading control. One representative blot is shown out of three independent experiments. A. (bottom) Densitometric analysis of Ctr1 protein level after normalization with GAPDH. $n = 3$, $^{**}p < 0.001$ with respect to the untreated cells; $^{\#}p < 0.05$ and $^{\#\#}p < 0.001$ with respect to the untransfected cells. B. Cells were treated with 150 μM $CuSO_4$ for 24 h. Ctr1 mRNA was measured by quantitative RT-PCR and normalized to β-actin. $n = 3$, $^{*}p < 0.05$ with respect to the untreated cells; $^{\#}p < 0.05$ with respect to the untransfected cells. C. Sp1 mRNA was measured in basal conditions by quantitative RT-PCR and normalized to β-actin. $n = 3$, $^{**}p < 0.001$ with respect to the untransfected cells.

content, see Fig. 3A); however, upon $CuSO_4$ treatment, mitochondria of SH-Grx1 cells accumulate less copper than those of control cells (Fig. 6).



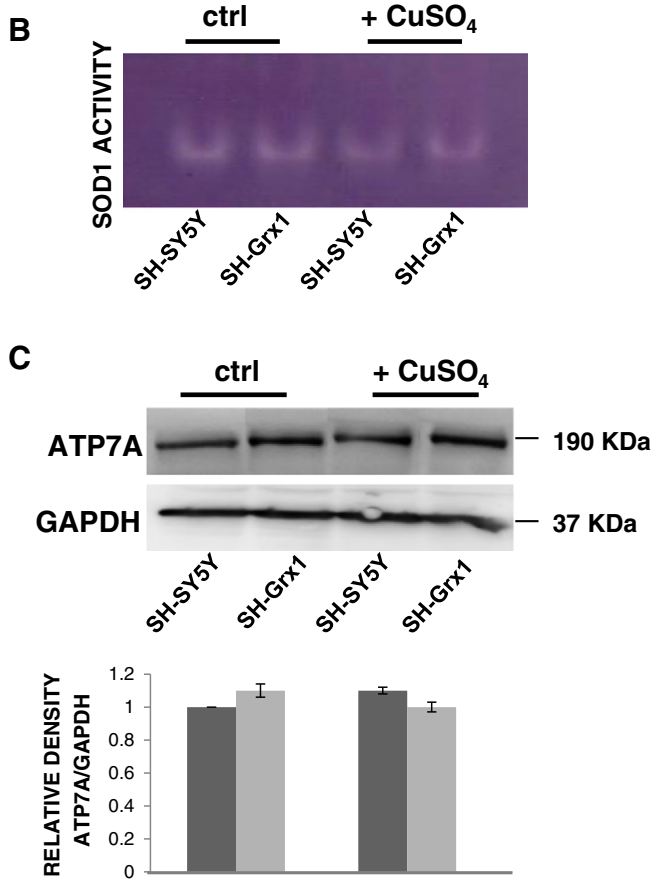


Fig. 5. Effect of Grx1 overexpression on copper-related proteins. Cells were treated with 150 μM $CuSO_4$ for 24 h. A. (top panel) Cell extracts were applied to SDS–PAGE followed by Western blot with monoclonal antibodies for CCS. Fifty micrograms of protein was loaded on each lane. GAPDH was used as the control of the loading. One representative blot is shown for each antigen, out of three independent experiments. A. (lower panel) Densitometric analysis of the CCS protein level after normalization with the GAPDH level. $n = 3$, $^{**}p < 0.001$ with respect to the untreated cells only; $^{\#\#}p < 0.001$ with respect to the control cells. B. SOD1 activity was assessed in cell lysates using an in-gel assay, under non-denaturing, SDS-free, conditions; fifty micrograms of protein was applied to each lane. One representative assay is shown, out of three different experiments. C. As in A, for ATP7A.

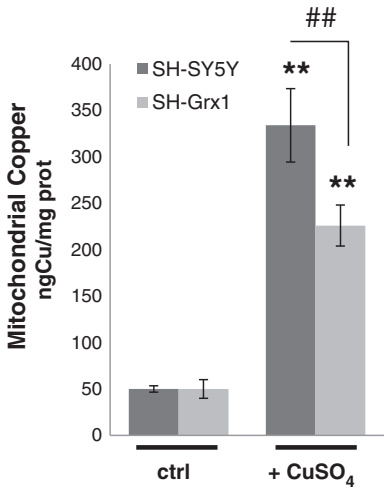


Fig. 6. Grx1 hinders copper accumulation in mitochondria. Cells were treated with 150 μM $CuSO_4$ for 24 h and crude mitochondrial fraction was isolated. Copper was measured by atomic absorption spectrometry on isolated mitochondria and is expressed as ng Cu/mg protein. $n = 3$, $^{**}p < 0.001$ with respect to the untreated cells; $^{\#\#}p < 0.001$ with respect to untransfected cells.

3.3. Grx1 has copper binding capabilities

We hypothesized that Grx1, which is mainly a cytosolic protein, may bind copper thus limiting its translocation to mitochondria and protecting cells from copper toxicity. To assess whether this is the case, we loaded SH-Grx1 cell lysates onto a copper-IMAC column and eluted proteins with increasing concentrations of imidazole. Western blot analysis performed on the collected fractions shows that Grx1 is retained and elutes from the column in 40 μM imidazole (Fig. 7A, top), together with CCS, indicating that Grx1 binds copper. The binding of Grx1 to copper-IMAC is resistant to elution with 2 mM EDTA (Fig. 7A, bottom).

$CuSO_4$ treatment impairs Grx1 activity in SH-Grx1 cells (Fig. 7B), suggesting that copper-binding affects enzyme function. This effect is not visible in SH-SY5Y cells treated with copper, possibly because basal Grx1 activity is already very low in those cells and any further decrease would be below the sensitivity of the method.

4. Discussion

It was demonstrated that some proteins involved in copper homeostasis, like ATP7A/B, or Atox1 are regulated by GSH and Grx1. Grx1 catalyzes ATP7A/B deglutathionylation by interacting with their copper-binding domains in the presence of the metal, thus promoting their function [15,16] and also facilitates the reduction of Atox1 disulfide when intracellular GSH is low [17]. Thus, GSH balance and copper homeostasis seem to be functionally linked.

In this work, we focused on the effect of Grx1 on copper homeostasis, in human neuroblastoma SH-SY5Y cells transfected for the constitutive overexpression of Grx1 (SH-Grx1 cells). We found that these cells show higher intracellular copper level than control cells and accumulate more metal when grown in overload conditions. These data are apparently in contrast with those obtained by Singleton et al. [16], who showed that Grx1 silencing increases intracellular copper content. This discrepancy may be explained by additional mechanisms involving Grx1 in copper metabolism, highlighted in conditions of Grx1 overexpression.

It is well known that copper has a cytotoxic effect on the SH-SY5Y cells, possibly because of copper accumulation in mitochondria and subsequent damage [2]. In this work we observed that the SH-Grx1 cells have a higher tolerance to $CuSO_4$ treatment and accumulate less copper in mitochondria with respect to the control cells. This suggests that high

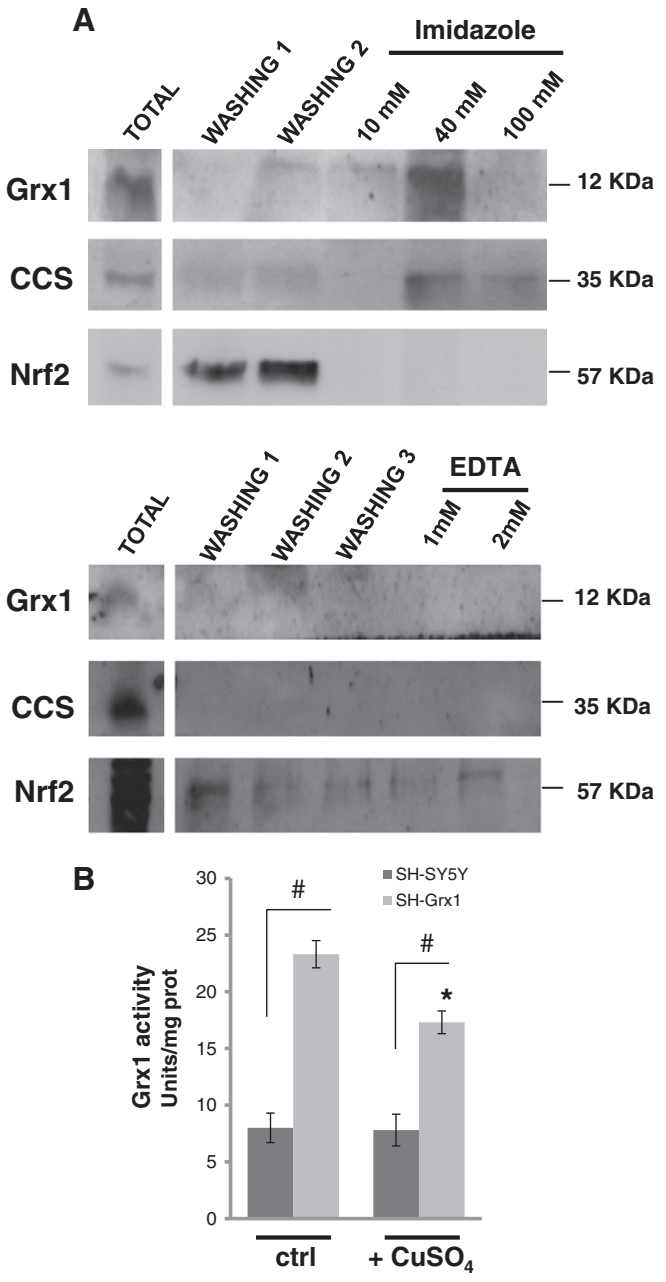


Fig. 7. Grx1 has copper binding capability and its activity in cells decreases upon copper overload. A. (top) Lysates from SH-Grx1 cells were applied to a copper-IMAC column. After elution with increasing imidazole concentrations (10, 40, 100 mM), proteins in the different fractions were separated by SDS–PAGE followed by Western blot analysis. A positive (CCS) and a negative (Nrf2) control were used to validate this data. One representative blot is shown, out of three different experiments. A. (bottom) The same experiment was repeated eluting the proteins by 1 or 2 mM EDTA. B. Cells were incubated with $CuSO_4$ for 24 h and the Grx1 enzyme activity was evaluated in cell lysates by a spectrophotometric assay. Activity is expressed as μmol of NADPH oxidized/min/mg protein (Units/mg protein). $n = 3$, $^{*}p < 0.05$ with respect to the untreated cells only; $^{\#}p < 0.05$ with respect to the untransfected cells.

levels of Grx1 might hinder copper transfer to mitochondria, thus protecting the cells from copper-induced toxicity. This seems to be the case in our model, possibly because, as we demonstrated, Grx1 binds copper and thus may sequester the metal in the cytosol, partially preventing its translocation into the mitochondria. However, other mechanisms cannot be excluded, since Grx1 may also modulate factors involved in copper translocation into mitochondria. We are currently investigating this aspect and characterizing the copper-binding properties of Grx1 in *in vitro* models.

A mechanism for copper-buffering properties of Grx1 can be envisaged, in the light of the observed impairment of Grx1 activity by copper treatment of cells. Indeed, Grx1 catalytic activity relies on the presence of two cysteine residues localized in its active site. During the catalysis, the most N-terminal cysteine carries out a nucleophilic attack on the disulfide of the protein target promoting the formation of a mixed disulfide intermediate. This reacts with the second cysteine in the active site forming a disulfide in turn reduced by two GSH molecules [13]. The finding that copper overload decreases Grx1 enzyme activity suggests that copper binding to Grx1 involves the catalytic cysteines.

Copper sequestering by Grx1 may provide an explanation for the upregulation of Ctr1 observed under Grx1 overexpression, which in turn may be responsible for the increase in intracellular copper. It is known that adequate or excess levels of copper induce Ctr1 degradation and downregulation [6,7], while copper-deficiency induces Ctr1 gene transcription [7]. In our experimental model, Grx1 trapping of copper may alter this signaling. This hypothesis is reinforced by the observation that Sp1 expression is increased in SH-Grx1 cells.

On the other hand, ATP7A protein, which should extrude excess copper, is not increased in our model. ATP7A activity or trafficking might not be enhanced, either because copper may not be available, being sequestered by Grx1, or because ATP7A is glutathionylated [16], due to lower activity of Grx1. Altogether, this results in intracellular copper accumulation.

Despite Grx1 copper-buffering capacity, we observed a decrease of CCS in SH-Grx1 cells upon $CuSO_4$ treatment. It is well known that copper induces degradation of CCS [24]. Most probably, the capacity of Grx1 to sequester copper is not sufficient to abolish the signal that triggers CCS proteolysis in this model. However, SOD1 activity does not decrease; these results confirm that molecules other than CCS may act as donors of the metal to SOD1 [25–27]. The hypothesis that Grx1 itself might be involved in SOD1 activation has to be kept in mind, in the light than the two proteins were shown to interact [26].

In conclusion, results from this study provide evidence that Grx1 overexpression affects copper homeostasis directly, by sequestering the metal and thus protecting the cells from copper-induced toxicity, and indirectly, through the modulation of proteins involved in copper homeostasis. They also reinforce the concept that copper toxicity occurs when mitochondrial copper level is altered.

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