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Abstract:	Glutaredoxin1 (GRX1) is a glutathione (GSH)-dependent thiol oxidoreductase. The GRX1/GSH system is important for the protection of proteins from oxidative damage and in the regulation of protein function. Previously we demonstrated that GRX1/GSH regulates the activity of the essential copper-transporting P1B-Type ATPases (ATP7A, ATP7B) in a copper-responsive manner. It has also been established that GRX1 binds copper with high affinity and regulates the redox chemistry of the metallochaperone ATOX1, which delivers copper to the copper-ATPases. In this study, to further define the role of GRX1 in copper homeostasis, we examined the effects of manipulating GRX1 expression on copper homeostasis and cell survival in mouse embryonic fibroblasts and in human neuroblastoma cells (SH-SY5Y). GRX1 knockout led to cellular copper retention (especially at elevated copper levels) and reduced copper tolerance, while in GRX1-overexpressing cells challenged with elevated copper, there was a reduction in both intracellular copper levels and copper-induced reactive oxygen species (ROS), coupled with enhanced cell proliferation. These effects are consistent with a role for GRX1 in regulating ATP7A-mediated copper export, and further support a new function for GRX1 in neuronal copper homeostasis and in protection from copper-mediated oxidative injury.

Glutaredoxin1 protects neuronal cells from copper-induced toxicity

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The authors declare no conflict of interest.

Key Words: Glutaredoxin1; Metal; Copper; Oxidative Stress; Thiol Oxidoreductase; Glutathione.

Abbreviations used: GRX, glutaredoxin1; GSH, glutathione; ROS, reactive oxygen species; AD, Alzheimer's disease; PD, Parkinson's disease; ALS, Amyotrophic lateral sclerosis; SOD1, superoxide dismutase 1; MEF, mouse embryonic fibroblast; MAP2, microtubule-associated protein 2; DBH, dopamine- β -hydroxylase;

Abstract

Glutaredoxin1 (GRX1) is a glutathione (GSH)-dependent thiol oxidoreductase. The GRX1/GSH system is important for the protection of proteins from oxidative damage and in the regulation of protein function. Previously we demonstrated that GRX1/GSH regulates the activity of the essential copper-transporting P_{1B}-Type ATPases (ATP7A, ATP7B) in a copper-responsive manner. It has also been established that GRX1 binds copper with high affinity and regulates the redox chemistry of the metallochaperone ATOX1, which delivers copper to the copper-ATPases. In this study, to further define the role of GRX1 in copper homeostasis, we examined the effects of manipulating *GRX1* expression on copper homeostasis and cell survival in mouse embryonic fibroblasts and in human neuroblastoma cells (SH-SY5Y). *GRX1* knockout led to cellular copper retention (especially at elevated copper levels) and reduced copper tolerance, while in GRX1-overexpressing cells challenged with elevated copper, there was a reduction in both intracellular copper levels and copper-induced reactive oxygen species (ROS), coupled with enhanced cell proliferation. These effects are consistent with a role for GRX1 in regulating ATP7A-mediated copper export, and further support a new function for GRX1 in neuronal copper homeostasis and in protection from copper-mediated oxidative injury.

Introduction

Glutaredoxin1 (GRX1) is a glutathione (GSH)-dependent thiol oxidoreductase that is proposed to catalyze the reduction of protein-SSG mixed disulfides to their respective sulfhydryls (deglutathionylation) (reviewed in (Lillig and Berndt, 2013)). Protein glutathionylation (protein-SSG formation) is the reversible, post-translational addition of GSH to the cysteine residues of proteins via disulfide bond formation. Mechanisms of protein glutathionylation remain to be established and for most proteins is dependent on cellular redox status, typically occurring only following substantial disruption to the GSH:GSSG ratio (normally ~100:1) (Allen and Mielay, 2012). Thus, protein glutathionylation is increased under oxidative stress and protects thiols from oxidative modifications that can irreversibly alter protein function and/or stability (Mielay et al., 2008). Glutathionylation can thus alter protein function, either activating or temporarily inactivating proteins. GRX1-mediated reduction of protein-SSG or protein disulfides (P-SS) liberates the sulfhydryl groups, allowing recovery of normal protein function following removal of the oxidative challenge, and thus also regulates the redox status of protein

thiols (Lillig and Berndt, 2013). In addition to this protective role, modification of protein thiols through thiol disulfide exchange with GSH is emerging as a central mechanism of redox regulation of protein function and signalling, comparable to protein phosphorylation (reviewed in (Mieyal et al., 2008)). The GRX1/GSH system regulates thiol/disulfide exchange for over 200 mammalian proteins, including those involved in redox signalling, antioxidant defence, energy production, cell proliferation and apoptosis (Sabens Liedhegner et al., 2012).

GRX1/GSH-mediated protection is associated with several neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS) (reviewed in (Sabens Liedhegner et al., 2012)). GRX1 is expressed throughout the brain with the highest activity in hippocampus, cortex and midbrain regions (Aon-Bertolino et al., 2011; Balijepalli et al., 1999). In affected regions of the AD brain, GSH levels are reduced and protein glutathionylation is increased (Sabens Liedhegner et al., 2012), and these changes may occur prior to the onset of AD symptoms (Zhang et al., 2012). Furthermore, GRX1 was upregulated in regions of the AD brain that were protected from degeneration, and *in vitro* protected SH-SY5Y cells from the toxic effects of the A β (1-42) peptide (Akterin et al., 2005), the major pathological agent of senile plaques in AD brains. In neuronal and mouse models of PD and neurolathyrism, GRX1 was essential for mitochondrial integrity and function and protected neurons from cell death (Diwakar et al., 2007; Kenchappa et al., 2004; Kenchappa et al., 2002; Rodriguez-Rocha et al., 2012; Saeed et al., 2008). In studies of ALS, GRX1 prevented the pathogenic aggregation of mutant SOD1 proteins in the NSC-34 motor neuron cell line (Bouldin et al., 2012; Cozzolino et al., 2008). Despite compelling evidence for a fundamental role for GRX1/GSH in protection against oxidative stress implicated in pathophysiological processes, functional studies of GRX1 in the brain, or in neuronal cell models, have been limited.

Homeostasis of the transition metals copper, zinc and iron is perturbed in the major neurodegenerative disorders, including AD, PD and ALS, with dysregulation and miscompartmentalization of these metals implicated in disease pathogenesis and progression (reviewed in (Ayton et al., 2012)). The imbalance in these metals is likely to contribute to the oxidative stress present in these disorders and is being targeted for therapeutic purposes (Mot et al., 2011). Hence tight homeostatic control of the redox active copper and iron is essential to prevent oxidative injury. Copper is imported into

cells by CTR1, and then bound by cytosolic metallo-chaperones (such as ATOX1, CCS) for delivery to specific subcellular destinations. CCS is the chaperone for copper insertion into SOD1, whereas ATOX1 delivers copper to the P-type ATPases (copper-ATPases) ATP7A and ATP7B at the *trans*Golgi network (TGN). Copper transport into the mitochondria for metallation of cytochrome c oxidase (Cco) is via mitochondrial chaperones, COX11, COX17, SCO1 and SCO2 (Kim et al., 2008). GSH and metallothioneins are involved in copper sequestration and detoxification (Freedman et al., 1989; Freedman and Peisach, 1989). GSH, typically at millimolar concentrations within the cell, can buffer free copper(I)_{aq} concentrations towards femtomolar levels (Xiao et al., 2011), and recent evidence suggested that GSH mediates copper(I) transfer between CTR1 and the copper chaperones (Maryon et al., 2013). ATP7A and ATP7B catalyse ATP-dependent copper transport across cellular membranes. ATP7A is essential for supplying copper to most cells of the body, whereas ATP7B regulates copper excretion from the liver into bile. Both copper-ATPases transport copper for metallation of copper-dependent enzymes in the TGN (eg ceruloplasmin, Cp) (Lutsenko et al., 2007). With elevated intracellular copper, ATP7A and ATP7B traffic towards the cell periphery to export the excess copper (La Fontaine and Mercer, 2007). These proteins have a cysteine-rich N-terminus with six metal-binding domains (MBDs), each with a copper-binding site (GMXCXXC) to which copper is coordinated via the cysteinyl thiol groups (Lutsenko et al., 2007).

We recently revealed a new key role for the GRX1/GSH system in maintaining cellular copper homeostasis by regulating the redox state of the essential ATP7A and ATP7B proteins, turning them 'on' in a mechanism that responds to elevated intracellular copper levels (Singleton et al., 2010). We reported a copper-dependent interaction between GRX1 and the copper-ATPases that required the ATP7A/7B copper-binding sites (Lim et al., 2006; Singleton et al., 2010). We further demonstrated reduced binding of GSH to ATP7A as copper levels increased, which corresponded with increased GRX1/ATP7A interaction (Singleton et al., 2010). Based on these data, a model was proposed whereby in the presence of excess copper, GRX1 interaction increases to deglutathionylate ATP7A/7B-SSG mixed disulphides releasing reduced (and activated) ATP7A/7B-SH protein that can then bind copper(I) ions for subsequent transport (Singleton et al., 2010). However, the redox state of the copper-ATPases, and in particular the N-terminal copper-binding cysteine residues, is likely to be dependent on the local protein environment and cellular redox status, and new data suggest that under

normal cellular conditions, the disulfide form (P-SS) of the copper-ATPase MBDs is likely to be dominant, with the glutathionylated species likely to occur only transiently (Brose et al., 2014). The GRX1/GSH system recently was shown to reduce an intramolecular disulfide bond within the copper chaperone, ATOX1 (Hatori et al., 2012) and SOD1 (Bouldin et al., 2012). We demonstrated that GRX1 binds copper with high (femtomolar) affinity and catalyses the redox sulfur chemistry of ATOX1, regulated by both the reduction potential of the GSSG/2GSH couple and the availability of copper(I) (Brose et al., 2014). Therefore, GRX1 also may regulate ATOX1-mediated copper delivery to ATP7A/7B. The GRX1/GSH system clearly plays a role in regulating key proteins involved in maintaining copper homeostasis.

In this study we sought to determine the role of GRX1 in neuronal copper homeostasis and in protection from copper-induced toxicity. We demonstrated that modulation of GRX1 levels leads to alterations in intracellular copper levels, copper-induced ROS production and cell proliferation. Our data are consistent with a role for GRX1 in protection from copper toxicity and in regulating ATP7A-mediated cellular copper export.

Materials and Methods

Cell lines and reagents

Mouse embryonic fibroblasts (MEFs) (wt and GRX-KO) were kindly provided by Prof. Ye-Shih Ho (Wayne State University) (Ho et al., 2007) and cultured in DMEM (Trace BioSciences) supplemented with 10% (v/v) foetal calf serum (FCS), 2 mM L-glutamine, 10 mM HEPES, 1 mM Na-pyruvate and 1 % (v/v) Non-essential Amino Acids (MEM). Human neuroblastoma SH-SY5Y cells were cultured in RPMI medium as previously described (Cater et al., 2008). Human wtGRX1 cDNA was a kind gift from Prof. Caryn Outten (University of South Carolina, USA). To generate the pcDNA3-based mammalian expression construct encoding wtGRX1, pGEX-4T-2/GRX1 (pSLB247) was digested with *EcoRI* and *BamHI* to excise the region encoding wtGRX1, which was then ligated into the pcDNA3 vector using the same sites (pSLB369). SH-SY5Y cells were stably transfected with either pcDNA3 alone, or pcDNA3 encoding wtGRX1, using FuGENE[®]HD (Promega) and by following the manufacturer's recommendations. Transfected SH-SY5Y cells were cultured in RPMI medium supplemented with 500 µg/ml G418 (Life Technologies). SH-SY5Y cells were differentiated by culturing in growth medium with reduced serum (3%

(v/v) FCS) and 10 mM retinoic acid (RA) (Sigma) for seven days. This treatment medium was replaced every second day and the differentiated cultures used for experimentation on day eight.

Western blot analysis

Cell lysates were prepared as previously described (Singleton et al., 2010). Protein samples were fractionated using the Mini-PROTEAN® Tetra System (Bio-Rad Laboratories) and transferred to nitrocellulose membranes (Amersham Biosciences) using the XCellIII™ blot module and the Xcell Surelock™ mini-cell system (Invitrogen), according to the manufacturer's instructions. The nitrocellulose membrane was then blocked using 5% (w/v) skim milk powder in TBS (1 h at room temperature, 22°C) before being incubated with primary antibody diluted in TBS-T (TBS with 0.1% Tween 20) (1–2 h at room temperature). The following primary antibody dilutions were used: sheep anti-ATP7A (R17-BX, affinity purified) (diluted 1/1000) (Ke et al., 2006); goat polyclonal anti-glutaredoxin1 (GRX1) (R&D Systems #AF3399) (diluted 1/500); mouse monoclonal anti-glutaredoxin1 (GRX1) (Abcam #ab16877) (diluted 1/1000); rabbit anti-dopamine β hydroxylase (DBH) (Millipore #AB1585) (diluted 1/1000); rabbit anti-microtubule-associated protein 2 (MAP2) (Millipore #AB5622) (diluted 1/1000); rabbit anti-calnexin (H-70) (Santa Cruz # sc-11397) (diluted 1/500); mouse anti- β -actin (Sigma #A5441) (diluted 1/10,000). The following secondary antibody dilutions were used: horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Millipore #AP307P) (diluted 1/4000); HRP-conjugated rabbit anti-goat IgG (Sigma #A5420) (diluted 1/4000) and HRP-conjugated goat anti-mouse IgG (Dako #P0447) (diluted 1/10,000). For visualisation, Immobilon Western HRP Substrate Peroxide Solution (Millipore) was used and images captured using the Luminescent Image Analyser LAS-3000 (Fujifilm). Densitometric analysis was used to evaluate immunolabelled protein intensity. The pixel intensities (arbitrary units) were quantified using Multi Gauge software (Fuji) and levels normalized against a loading control of either β -actin or calnexin. At least three independent experiments were used for all comparisons.

Intracellular metal analysis

Total intracellular metal content was measured in the MEFs (wt and GRX-KO) and SH-SY5Y cell lines subsequent to the treatments outlined in the Results. Cells were cultured and treated in 25cm² flasks. Treatment medium was discarded and the cells washed with

2 ml of trypsin solution (0.025% trypsin and 0.02% EDTA) before being harvested with 1.6 ml of trypsin solution. A 0.1 ml aliquot of the cell suspension was used for cell counting using the Countess[®] Automated Cell Counter (Invitrogen). The remaining cell suspension (1.5 ml) was centrifuged at 1000 *g* for 5 min at room temperature to pellet the cells, after which the supernatant was removed. Intracellular metal concentration was measured using inductively-coupled plasma mass spectrometry (ICP-MS, Agilent 7700, Varian). The means of quadruplicate determinations for each test condition with standard deviations ($\pm$ S.D.) were used for comparison.

Intracellular ROS analysis

SH-SY5Y cells were seeded into 12-well plates and cultured for 24 h to allow adhesion. Cells were then treated with either basal medium, or medium supplemented with 200 μ M CuCl₂ for 16 h. Where differentiated SH-SY5Y cells were analysed, seeded cells were initially cultured in a combination of reduced serum (3% (v/v) FCS) and 10 mM retinoic acid (RA) (Sigma) for 7 days. Intracellular ROS levels were measured as previously described (Wolyniec et al., 2013). Briefly, cells were incubated for 15 min in the dark at 37°C with 10 μ M of the cell permeable fluorogenic probe, 2',7'-Dichlorofluorescein Diacetate (DCF-DA) (Calbiochem). DCF-DA fluorescence was measured using flow cytometry (BD FACSCanto 2) with excitation at 488nm and emission at 530 nm. Data was analysed using FlowJo 8.7 software. The means of quadruplicate determinations for each test condition with standard deviations ($\pm$ S.D.) were used for comparison.

Statistical Analyses

Statistical analyses were performed using two-tailed unpaired *t* tests. The means of at least triplicate determinations for each test condition with standard deviations ($\pm$ S.D.) were used for comparisons.

Results

GRX1 promotes cellular tolerance to copper.

Exposure of cells to elevated copper increases ROS levels, leading to oxidative stress (Arciello et al., 2005; Watt and Hooper, 2001). Based on our previous observations of an interaction between GRX1 and the copper-ATPases, and that knockdown of *GRX1* in HEK293T cells resulted in a 30% increase in intracellular copper levels (Singleton et al.,

2010), we postulated that GRX1 plays a significant role in regulating cellular copper homeostasis and in preventing copper-mediated ROS production in cells. To test this hypothesis, we compared the intracellular metal levels between wild-type (wt) and *GRX1*-knockout (*GRX1*-KO) mouse embryonic fibroblasts (MEFs) (Fig. 1). Immunoblotting verified the absence of GRX1 in *GRX1*-KO cells (Fig. 1a) and this had a negligible effect on the level of mATP7A, the primary copper-ATPase in MEFs (Fig. 1b(i)+(ii)). There was no significant difference in intracellular copper levels between wt and *GRX1*-KO MEFs cultured in basal medium (Fig. 1c(i), Lanes 1+2). However, when challenged with copper-supplemented medium (100 μ M CuCl₂) *GRX1*-KO MEFs accumulated approximately 4.5-fold more intracellular copper than wtMEFs (Fig. 1c(i), Lanes 3+4). These results demonstrated that GRX1 is required for homeostatic control of intracellular copper levels, and is consistent with a role for GRX1 in facilitating copper efflux. The absence of GRX1 had no effect on the levels of other metals including iron, zinc (Fig. 1c(ii)+(iii)) and magnesium (not shown).

We next investigated the role of GRX1 in cellular sensitivity to copper (Fig. 1d). Primary wtMEFs gain a proliferative advantage when cultured in copper-supplemented medium (CuCl₂) up to a limit of approximately 200 μ M under our conditions. This, in part, reflects the deficient copper levels in normal medium (<1 μ M). In contrast to wtMEFs, proliferation of *GRX1*-KO MEFs was not augmented in copper-supplemented medium (200 μ M CuCl₂), and they were significantly less tolerant to further rises in supplemented copper (300 & 400 μ M CuCl₂) (Fig. 1d). These results provide further evidence that GRX1 plays a significant role in maintaining cellular copper homeostasis and tolerance.

GRX1 modulates the level of intraneuronal copper.

To investigate the role of GRX1 in regulating neuronal copper homeostasis, we generated a SH-SY5Y cell line that stably overexpressed human GRX1. Immunoblotting revealed a substantial increase in GRX1 expression compared with vector-transfected cells (Fig. 2a) and that GRX overexpression did not significantly alter ATP7A levels (Fig. 2b(i)+(ii)). Using the GRX1-expressing SH-SY5Y cell line, we made comparisons between proliferative and neuronal-like differentiated cells (Fig. 2c-e). Differentiated SH-SY5Y cells develop morphological and biochemical characteristics of mature neurons and are often used as *in vitro* models of neuronal function. To differentiate SH-SY5Y cells, they were cultured in medium with reduced serum (3%) and retinoic acid (RA) (10 mM). Expression levels of both the microtubule-associated protein 2 (MAP2) and

dopamine- β -hydroxylase (DBH) were evaluated as markers of differentiation (Fig. 2c). MAP2 is expressed during the development and maintenance of neuronal morphology, while differentiated sympathetic neurons express the adrenergic marker DBH (Biedler et al., 1978; Matus, 1991). Following differentiation, SH-SY5Y cells expressed significantly more MAP2 and DBH protein (Fig. 2c(i)). This was reproducible across experiments, evident from densitometric analyses, which showed a consistent increase in MAP2 (~2.4-fold) and DBH (~3.5-fold) expression (Fig. 2c(ii)). Consistent with the behaviour of mature neurons, upon differentiation SH-SY5Y cells developed neurites with few branches (Fig. 2d(i)) and their proliferation was inhibited (Fig. 2d(ii)).

Overexpression of GRX1 in both the proliferative and differentiated cells cultured in basal medium, led to a modest but significant increase in intracellular copper levels (Fig. 2e, Lanes 1+2 & 5+6). This result may relate to the high affinity copper-binding capacity of GRX1 (Brose et al., 2014) coupled with reduced need for GRX1 to promote copper efflux at basal copper levels. However, when challenged with copper-supplemented medium (100 μ M CuCl₂), both proliferative and differentiated GRX1-overexpressing SH-SY5Y cells accumulated significantly less copper than vector-transfected cells (Fig. 2e, Lanes 3+4 & 7+8), suggesting that at elevated copper levels, GRX1 is actively involved in promoting copper export. These results are consistent with the above data obtained with GRX1-KO MEFs, which accumulate significantly *more* copper than the wtMEFs when both were challenged with copper (Fig. 1c(i)). In conclusion, these experiments further indicate an important role for GRX1 in maintaining intraneuronal copper levels.

GRX1 protects neurons against copper-mediated ROS production

The above data showed that modulating GRX1 levels in cells affected the amount of copper that accumulated in cells, and the capacity of MEFs to tolerate rises in extracellular copper levels. We then sought to determine the influence of GRX1 overexpression on cell proliferation and copper-induced ROS levels to establish whether GRX1 can confer a growth advantage upon cells and protection from copper-induced oxidative stress. Even in basal medium, the overexpression of GRX1 in SH-SY5Y cells conferred a growth advantage with an approximately 1.4-fold increase in cell proliferation over a 72-hour period, compared with the vector-transfected control cells (Fig. 3a, Lanes 1+2). This likely relates to the GRX1-overexpressing SH-SY5Y cells being better equipped to tolerate the oxidative environment associated with culturing cells *in vitro*.

Since GRX1 overexpression reduced cellular copper accumulation in copper-challenged cells (Fig. 2e), we postulated that this would correspond with the cells being markedly more tolerant to rises in extracellular copper. In support of this idea, when the SH-SY5Y cells were challenged with copper-supplemented medium (100 μ M or 200 μ M CuCl₂) over a 72-hour period, GRX1-overexpressing cells maintained a relatively consistent level of proliferation. In contrast, the proliferation of vector-transfected cells was significantly decreased with increasing medium copper levels (Fig. 3a, Lanes 3+4 & 5+6). With both copper concentrations (100 μ M and 200 μ M CuCl₂), there was almost a two-fold (1.8 & 1.9-fold, respectively) increase in the number of GRX1-overexpressing SH-SY5Y cells compared to the vector control cells (Fig. 3a, Lanes 3+4 & 5+6). Addition of 400 μ M CuCl₂ to the growth medium inhibited the proliferation of both cell lines (Fig. 3a, Lanes 7+8), indicating a threshold of copper tolerance conferred through overexpression of GRX1. These results demonstrated that GRX1 levels can influence cellular tolerance to elevated copper.

We next investigated whether this GRX1-mediated increase in copper tolerance corresponds with an enhanced capacity of cells to handle copper-mediated ROS production (Fig. 3b). ROS generation was investigated using FACS-based detection of fluorescence emitted by the peroxide-sensitive fluorophore, 2',7'-Dichlorofluorescein Diacetate (DCF-DA), to detect and quantify H₂O₂ produced intracellularly (Wolyniec et al., 2013). Overexpression of GRX1 in both proliferative and differentiated SH-SY5Y cells cultured in basal medium had no significant effect on intracellular ROS levels (Fig. 3b, Lanes 1+2 & 5+6). However, when challenged with copper-supplemented medium (200 μ M CuCl₂), both proliferative and differentiated GRX1-overexpressing SH-SY5Y cells had significantly reduced levels of ROS compared with vector-transfected cells (Fig. 3b, Lanes 3+4 & 7+8). These results demonstrated that GRX1 could regulate the level of ROS that is directly generated by increased copper, and consequently confers upon cells a greater tolerance to rises in extracellular copper concentrations.

Discussion

In the present study, modulation of GRX1 expression led to altered cellular copper levels: *GRX1* knockout led to increased copper and GRX1 overexpression led to reduced copper levels in copper-challenged cells, but not in cells grown in basal medium (see Fig. 1c, 2e). These observations are consistent with compromised or enhanced ATP7A-mediated copper export when GRX1 was absent or overexpressed, respectively.

Accordingly, in the presence of excess copper, ROS levels were decreased in GRX1-expressing SH-SY5Y cells compared with the vector-transfected cells (Fig. 3b). Consequently, consistent with reduced oxidative burden, wtMEFs or GRX1-expressing SH-SY5Y cells had a growth advantage, with increased cell proliferation compared with GRX1-KO MEFs and vector-transfected SH-SY5Y cells, respectively (see Fig. 1d, 3a). GRX1-KO MEFs were previously shown to be sensitive to diquat or paraquat, but not to H₂O₂ or diamide (Ho et al., 2007). The authors suggested that damage induced by different oxidants occurs via different mechanisms, some of which may not be attenuated by GRX1. In this study, we showed that GRX1-KO MEFs are sensitive to copper-induced toxicity and that GRX1 can protect against copper-induced cell damage. In addition, the ability to replicate the effects of GRX1 expression in differentiated SH-SY5Y cells suggested that the data accurately reflect a role for GRX1 in maintaining copper levels in terminally differentiated neurons of the adult central nervous system.

We previously revealed for the first time new roles for GSH and GRX1 in copper homeostasis through copper-responsive redox regulation of ATP7A/7B activity (Lim et al., 2006; Singleton et al., 2010). We proposed a model based on our data and what was known about GRX1 function, which suggested that (i) this regulation occurs via ATP7A/7B glutathionylation/deglutathionylation in response to cellular copper levels, (ii) this process may act as a redox switch to turn ATP7A/7B “on” or “off” depending on copper levels, and (iii) GRX1/GSH modulates copper-binding by these copper-ATPases. Our recent data demonstrated that GRX1 can bind copper(I) with high (femtomolar) affinity at the active site (Cys²³-xx-Cys²⁶); that both active site Cys residues are essential for high affinity copper(I) binding but only Cys²³ is needed for GRX1 catalytic activity; and that copper(I) binding at the active site inhibits GRX1 catalytic activity. Consequently, a single GRX1 molecule cannot simultaneously bind copper and retain catalytic activity (Brose et al., 2014). Further, it became apparent that the dominant oxidised form of ATOX1 or GRX1 (and probably other Cys-xx-Cys containing proteins such as the metal binding domains (MBDs) of the copper-ATPases) is the disulfide form (P-SS), rather than the glutathionylated form (P-(SSG)₂); and that the oxidised form ATOX1(disulfide) can be reduced by GSH in a reaction catalysed by GRX1, in presence of copper(I) that is available to bind to the reduced ATOX1 (Brose et al., 2014). The reduction process appears to involve initial nucleophilic attack of GRX1 on the protein disulphide bond, followed by sequential glutathionylation and deglutathionylation of the catalyst GRX1 (Brose et al., 2014). Consistent with this, a recent study by Hatori et al (2012) also

showed that the redox state of ATOX1 is maintained by appropriate GSH/GSSG balance and that the ATOX1 intramolecular disulfide can be reduced and made available for copper binding by high GSH concentration. However, GRX1 was required for ATOX1 reduction under low GSH (Hatori et al., 2012).

These new data not only indicate a role for GRX1 in regulating the redox sulphur chemistry and copper(I)-binding by ATOX1 and likely other similar proteins or protein domains, but also suggest an additional model whereby the observed copper-ATPase/GRX1 interaction may involve GRX1/GSH-catalysed reduction of the P-SS sites within the MBDs to allow copper(I) binding, and potentially also the formation of Cu(I)-bridged complexes between GRX1 and the MBDs during the interaction process. Hence, GRX1 may function in copper(I) buffering and in regulating ATOX1-mediated copper(I) delivery to the copper-ATPases, and could also itself feasibly act in a manner comparable to ATOX1 in copper(I)-delivery to ATP7A/7B (Brose et al., 2014).

When copper is in excess, the reaction of copper with H_2O_2 produces the highly reactive hydroxyl radical via Fenton chemistry, which is particularly damaging to biomacromolecules (Barnham et al., 2004). The interaction of GRX1 with ATP7A may serve a dual purpose – (i) as a regulatory mechanism to activate the transporter (by reduction of MBD disulfides) for copper delivery to cuproenzymes and to efflux the excess copper before it leads to the generation of ROS and oxidative stress; and (ii) to activate ATP7A during recovery from oxidative stress mediated by copper (and other oxidants). In the GRX1-KO MEFs challenged with elevated copper, the accumulation of copper relative to wtMEFs, points to reduced activity of ATP7A. Potentially, the oxidative stress resulting from increased copper/ROS levels likely leads to reduced GSH levels, which will be partly due to increased protein glutathionylation. This change in the GSSG/GSH ratio would favour conversion of the ATP7A MBDs to the oxidised (disulfide or glutathionylated) form. With low GSH and in the absence of GRX1 for ATOX1/ATP7A (MBD) reduction, copper-binding by ATOX1 and ATP7A, and consequently copper transport is compromised and copper accumulates. In contrast, SH-SY5Y cells overexpressing GRX1 (both proliferative and differentiated), when challenged with excess copper, accumulated less copper than vector-transfected cells, had reduced levels of ROS, and the proliferative cells had a growth advantage. These data point to enhanced activity of ATOX1 and ATP7A due to increased GRX1 for disulfide reduction and activation of ATOX1/ATP7A, and potentially direct GRX1-mediated copper delivery to ATP7A for export of the excess copper.

A recent study also demonstrated altered copper metabolism in SH-SY5Y cells that constitutively overexpressed GRX1 (De Benedetto et al., 2014). However, in contrast to the observations we report here, this study reported increased copper accumulation when these cells were copper-challenged, but did not conduct any experiments to examine the effects of GRX1 depletion on copper levels (De Benedetto et al., 2014). We also observed a modest but significant increase in copper levels in GRX1-overexpressing cells, but only under basal conditions, and not under elevated copper conditions. These observations may in part be explained by the copper-buffering/sequestration capacity of GRX1 (Brose et al., 2014). Further studies are needed to clarify the reason for the observed discrepancy.

Oxygen consumption in the brain is high (20% of total body oxygen) and this may lead to increased levels of ROS compared with other tissues. When coupled with low levels of antioxidants and antioxidant enzymes, and high levels of heavy metal ions, this may result in an increased susceptibility of neurons to elevated ROS and oxidative stress (Kozlowski et al., 2009). Correct redox control is therefore essential for all cells, particularly for post-mitotic cells such as neurons, and heavily relies on the activity of antioxidant systems. GRX1-mediated protection is well documented in a variety of oxidative stress-related conditions (3-7), and in the context of neurodegenerative disease, GRX1 was shown to protect mitochondrial integrity, membrane potential and complex I (Diwakar et al., 2007; Diwakar et al., 2006; Kenchappa et al., 2004; Kenchappa et al., 2002; Kenchappa and Ravindranath, 2003; Saeed et al., 2008). Hence, GRX1 is also likely to play a key role in neuroprotection. This study supports a neuroprotective role for GRX1, in particular in regulating neuronal copper homeostasis and in protecting cells and the integrity of essential copper transport proteins during metal-induced oxidative stress. This information is important for understanding the protective mechanisms available to neuronal cells during pathological processes where copper and oxygen chemistry is perturbed, such as in neurodegenerative disease, and may have therapeutic implications if GRX1 levels can be physiologically manipulated.

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Figure Legends

Fig. 1 GRX1 knockout increases copper accumulation and copper sensitivity in mouse embryonic fibroblasts (MEFs). (a) Verification of *GRX1* knockout in MEFs. Cell lysates from the wt and GRX1-KO MEF cell lines were prepared and analysed by immunoblotting as described in the Methods section. (b) GRX1 knockout does not effect mATP7A expression in MEFs. Cell lysates from the wt and GRX1-KO MEF cell lines were prepared and analysed by immunoblotting as described in the Methods section. (c) *GRX1* knockout increases copper accumulation in MEFs. The wt and GRX1-KO MEFs were cultured for 24 hours in either fresh basal medium, or medium supplemented with 100 μ M CuCl₂. Total intracellular copper (i), iron (ii) and zinc (iii) were measured by ICP-MS. (d) GRX1 knockout increases copper sensitivity of MEFs. Wt and GRX1-KO MEFs were seeded at 3X10⁴ and first cultured for 16 h (o/n) before being treated with either basal medium, or medium supplemented with 200 μ M, 300 μ M, or 400 μ M CuCl₂. After 72 hours of treatment, viable cells were counted. Results shown represent the mean $\pm$ S.D. of quadruplicate measurements

Fig. 2 GRX1 modulates intraneuronal copper levels. (a) GRX1 overexpression in SH-SY5Y cells. Cell lysates from SH-SY5Y cells transfected with either a mammalian expression construct encoding GRX1 (pSLB369), or with the vector (pcDNA3.1) alone, were prepared and analysed by immunoblotting as described in the Methods section. (b) GRX1 overexpression does not effect mATP7A expression in SH-SY5Y cells. Cell lysates from the wt and GRX1-KO MEF cell lines were prepared and analysed by immunoblotting as described in the Methods section. (c) SH-SY5Y differentiation to neuronal-like cells. SH-SY5Y cells were differentiated as described. (i) Lysates from proliferative and differentiated SH-SY5Y cells were prepared and analysed by immunoblotting for endogenous markers of differentiation, MAP2 and DBH. Calnexin was immunolabeled as a protein loading control. (ii) Differentiation of SH-SY5Y cells is reproducible. Densitometric analyses were used to evaluate the consistency of inducing SH-SY5Y differentiation across multiple experiments by evaluating the expression levels of both MAP2 and DBH. Immunoblots from three independent experiments were used for comparisons and results were normalized against calnexin loading controls. (d) Differentiated SH-SY5Y cells develop morphological characteristics of mature neurons and stop proliferating. (i) SH-SY5Y cells were differentiated as described in the Methods.

The development of neurites with few branches was imaged using an Olympus IX51 microscope and 20X magnification. (ii) SH-SY5Y cells seeded at 3×10^4 were cultured in either basal medium, or medium containing reduced serum (3%) and retinoic acid (RA) (10 mM) to induce differentiation. Cells were counted after 72 hours of treatment. (e) Neuronal copper homeostasis requires GRX1. Proliferative and differentiated SH-SY5Y cells stably overexpressing GRX1 or transfected with the vector (pcDNA3.1) alone, were cultured for 24 hours in either fresh basal medium, or medium supplemented with 100 μ M CuCl_2 . Total intracellular copper was measured by ICP-MS. Results shown represent the mean $\pm$ S.D. of quadruplicate measurements

Fig. 3 GRX1 reduces copper-mediated ROS production. (a) GRX1 overexpression confers a growth advantage upon proliferative SH-SY5Y cells. SH-SY5Y cells overexpressing GRX1 or transfected with the vector (pcDNA3.1) alone, were seeded at 3×10^4 and first cultured for 16 h (o/n) before being treated with either basal medium, or medium supplemented with 100 μ M, 200 μ M, or 400 μ M CuCl_2 . After 72 hours of treatment, viable cells were counted. (b) GRX1 regulates copper-mediated ROS production in neurons. Proliferative and differentiated SH-SY5Y cells overexpressing GRX1 or transfected with the vector (pcDNA3.1) alone, were cultured for 16 hours in either fresh basal medium, or medium supplemented with 200 μ M CuCl_2 . The generation of intracellular ROS was measured using the cell permeable fluorogenic probe DCF-DA and flow cytometry. Results shown represent the mean $\pm$ S.D. of quadruplicate measurements

Fig.1

Fig.2

Fig.3