Chapter 3

The Important Functions of GSH-Dependent Enzyme Glutaredoxin 2 (Grx2)

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Additional information is available at the end of the chapter

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Abstract

Reactive oxygen species (ROS) are generated at a very high rate throughout our lives as part of normal aerobic life. Glutathione (GSH), normally an antioxidant molecule that scavenges free radicals, oxidizes to form glutathione mixed disulfide (GSSG). As the GSSG/GSH ratio increases, GSSG naturally adds to other proteins, causing protein glutathionylation. Protein glutathionylation, defined as the reversible formation of a mixed disulfide (PSSG) between protein thiols (P-SH) and glutathione (GSH), appears to be the most important mode of thiol oxidation. In my chapter, we will discuss the important roles of GSH and GSH-dependent enzymes in health and disease, with the emphasis on glutaredoxin and thioredoxin systems. Their structures, catalytic reaction mechanisms, major physiological functions, and associations with diseases will be summarized in my chapter. We will also mention how GSH-dependent enzymes play a role in each major organ systems including the nervous, cardiovascular, immune, and visual system.

Keywords: glutathione (GSH), glutaredoxin (Grx), thioredoxin (Trx), the nervous system, cardiovascular system, immune system, visual system

1. Oxidative stress and protein glutathionylation

Reactive oxygen species (ROS) are generated at a very high rate throughout our lives as part of normal aerobic life. In particular, the mitochondria due to its high metabolic capacity are the primary site of endogenous ROS production. With each run of the mitochondrial electron transport chain, 1–2% of free radicals are predicted to escape the mitochondria [1]. With time,

ROS accumulation can be detrimental, as they can cause oxidative damage to proteins, lipids, DNA, and other crucial biological molecules [2]. Among these macromolecules, proteins are very sensitive to oxidative modification. Cysteine residues are particularly reactive with ROS due to the presence of thiol (-SH) group, which can be oxidized to sulfenic (SOH), sulfinic (SO_2H), sulfonic acids (SO_3H), or formed disulfide bonds (S-S). Protein glutathionylation, defined as the reversible formation of a mixed disulfide (PSSG) between protein thiols (P-SH) and glutathione (GSH), appears to be the most important mode of thiol oxidation. GSH, normally an antioxidant molecule that scavenges free radicals, oxidizes to form glutathione mixed disulfide (GSSG). As the GSSG/GSH ratio increases, GSSG naturally adds to other proteins, causing protein glutathionylation. In most cases, the addition of GSSG to proteins renders them inactive, but for specific proteins such as heat shock proteins or peroxiredoxin 6, it may serve as an activation mechanism or a cell signaling event [3, 4]. In small amounts, PSSG may alert the cell that oxidative stress is present and may lead to certain signaling cascades to restore a healthy cellular redox state. Nonetheless, excessive PSSG can be lethal because of severe protein inactivation and damage [5]. Therefore, maintenance of a redox state can translate to increased cell survival and protection.

2. Introduction to the glutaredoxin (Grx) system

To combat oxidative stress, the body is equipped with several antioxidant enzymes in order to restore the redox balance and protect the cell. Several antioxidant enzymes such as catalase, superoxide dismutase (SOD), and thioredoxin (Trx) have been thoroughly researched due to their effectiveness and ability to directly target and scavenge ROS [2, 6–8]. However, as it is becoming more clear that PSSG is an important post-translational modification linked to oxidative stress, recent research has highlighted the glutaredoxin (Grx) system, an antioxidant system capable of reversing PSSG formation. As shown in **Figure 1**, the Grx system

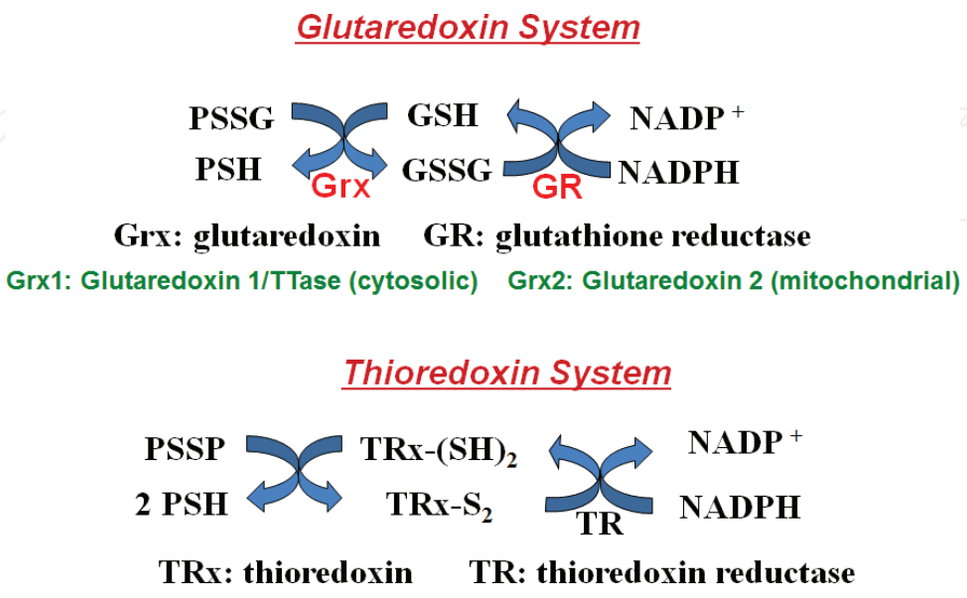

Figure 1. Glutaredoxin and thioredoxin system.

has two main subsets in humans: the cytosolic glutaredoxin 1 (Grx1) and the mitochondrial glutaredoxin 2 (Grx2) [7]. Grx1 has garnered much interest due to its similarity with Trx1 in promoting cytosolic protection, yet Grx2 with its primary mitochondrial localization may hold a crucial role in preventing cell death. This is especially important considering that mitochondria are the primary sites of ATP and ROS production and are critically involved in balancing pro- and anti-apoptotic signals [1, 4]. However, to date, only a few studies have been published to highlight Grx2's potential roles in humans. This chapter hopes to emphasize Grx2 as an imperative and crucial antioxidant enzyme, encourage more research studying Grx2's possible roles and protein targets, and provide support for Grx2 activating drugs to treat oxidative stress-induced diseases.

3. Glutaredoxin 2 (Grx2)

3.1. Grx2 localization and expression

Grx2 was first discovered simultaneously by two research groups leading by Holmgren and Gladeshev in the same year 2001. Both teams characterized Grx2 as a 18 kDa oxidoreductase enzymes capable of reversing PSSG and working as electron donors for ribonucleotide reductase [9]. There are several alternative spliced forms in different types of organisms, but in humans specifically, there are two main subsets: the cytosolic Grx1 and the mitochondrial Grx2. Grx1 has also been implicated in the mitochondrial inner membrane space but in miniscule amounts. Grx2 is rather unique, in that depending on cell type and organism, may be present in different cellular components. Grx2 has three alternatively spliced forms: Grx2a, Grx2b, and Grx2c. Grx2a is the mitochondrial isoform, whereas Grx2b and Grx2c mainly reside in the cytoplasm and nucleus. Grx2a is ubiquitous in every cell, except in the testes [6, 10, 11]. In spermatids, Grx2c is prominently present in the cytoplasm, yet Grx2a is absent (found to be less than 1% in the mice testes) [12, 13]. In embryos, Grx2 expression tends to surpass other dominant antioxidant enzymes such as Trx1, Trx2, and Grx1. During the gestation of E11 embryos, more than 50% of Grx2 mRNA transcripts compose of Grx2a [13]. This is not remarkable when considering that aerobic respiration and mitochondria formation takes precedent in this stage of gestation. Surprisingly, Grx2b and Grx2c are rarely found in normal human cells, but its presence has been discovered in HeLa cells and certain cancer cell lines. In patients with hepatocellular carcinoma and underlying metabolic syndrome that increases a person's risk for diabetes, stroke, and heart disease, Grx2 expression was particularly high [14]. In non-small cell lung cancer and adenocarcinoma, proliferative effects are correlated with higher cytoplasmic and nuclear Grx2 levels [15]. Along with several studies supporting Grx2 is highly expressed in cancer cells, Grx2 may have a distinct and special function in cancer cells, possibly in enhancing cancer cell survival and proliferation [15–17].

Patterns of Grx2 expression vary from species to species. Unsurprisingly, Grx2's localization in mice showed that Grx2a was present in all tissue types. However, it was also identified that Grx2b and Grx2c mRNA transcripts were found at various expression levels in several tissues with higher concentrations in the heart, liver, kidney, and eyes [12, 13]. Another study looking at porcine ocular tissues showed that Grx2 is abundantly present in the ciliary body and

retina but is lacking in the vitreous humor [18]. Both the ciliary body and retina have direct access to blood vessels and are also known to be more sensitive to oxidative stress, increasing the risk in these areas for a variety of oxidative stress-induced eye diseases. Therefore, Grx2's concentration in these areas indicate Grx2's potential role in preventing oxidative-induced damage in these exposed tissues.

By using artificial cloning of recombinant human Grx2 (hGrx2) in *E. coli* and HeLa cells, Grx2 was able to be fully expressed in the mitochondria and nucleus. Closer analysis suggests that Grx2a has a distinctive mitochondrial N-terminal signal sequence, yet Grx2a and Grx2b both have an arginine and lysine rich C-terminal sequence that highly resembles a nuclear translocation sequence [11]. Staining also suggested that Grx2a was found exclusively in the mitochondria, whereas Grx2b had a strong presence in the cytoplasm and a weaker presence in the nucleus. This contrasted to data previously shown that in Jurkat cells, Grx2 preferentially is found in the nucleus compared to the mitochondria [11]. Nonetheless, the reasons for these differences are unclear, other than the obvious alternative splicing of Grx2 transcripts and the absence of a translocation signal in Grx1 transcripts. Moreover, what prompts the predilection of Grx2a in the mitochondria despite having a nuclear translocation signal and the role of Grx2b and Grx2c in human cells still remain unsolved mysteries.

3.2. Grx2 structure

Compared to the exclusively monomeric and constitutively expressed Grx1, Grx2 is normally 18 kDa and found in an inactive dimeric form with an iron-sulfur cluster [19, 20]. As shown in **Figure 2**, the iron-sulfur cluster is crucial for Grx2's typical functioning, as it is able to detect oxidative stress in the environment. In terms of voltage differences, one study claimed that Grx2's dimeric form only disassociated with pulses greater than 0.5 V [21]. When oxidative stress is present, Grx2 cleaves to its active 16 kDa monomeric form and directly scavenges free radicals and reverses PSSG. Of all the isoforms, Grx2b does not have the specific iron–sulfur cluster and remains a monomer in non-stressed and stressed conditions [10, 21].

3.3. Grx2 mechanism

Grx1 and Grx2 have a particular active site template: Cys-X-Tyr-Cys. Grx1 has a proline residue after the first cysteine residue, whereas Grx2 has a serine instead. Although this gives Grx2 a slightly slower reaction rate, the serine essentially gives Grx2 a higher affinity for glutathionylated substrates [22]. Both monothiol and dithiol mechanisms can be performed, yet the monothiol mechanism using the N-terminal Cys is more accepted. Both Grx1 and

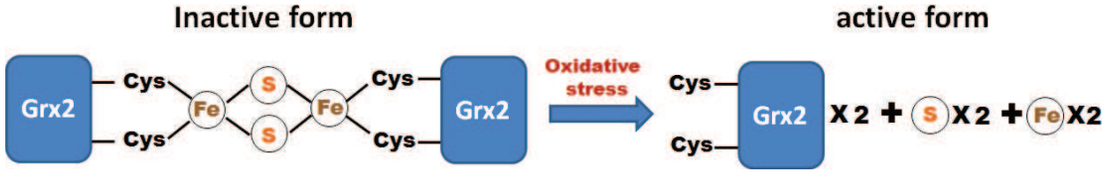

Figure 2. Glutaredoxin 2 as an iron-sulfur (Fe-S) protein.

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Grx2 employ the same mechanism to remove GSH from its protein targets: it accepts the GSH from the protein adduct and becomes Grx-SSG only to be returned back to its reduced form (Grx-SH) by glutaredoxin reductase (GR) and the reducing power of NADPH. GSSG is further processed and recycled to GSH by the enzyme glutathione reductase [23, 24].

The cysteines in the active site of Grx2 play a major role for Grx2 to function. Along with active site Cys residues, there are several disulfide bridges between non-active site Cys residues that promote further stability in Grx2 structure [25]. This attribute has been remarkably the only conserved feature found in all vertebrate-specific Grx2 forms, indicating the necessity of this disulfide bridge in promoting Grx2's potency. However, other modifications of Cys residues may inhibit Grx activity. A study performed by Hashemy et al. indicated that compared to its counterpart Grx1, Grx2 enzyme activity was relatively initiated by S-nitrosylation, leading to the conversion of Grx2 into its active monomeric form [17]. Grx1's cysteine oxidation either by S-nitrosylation or intradisulfide bonds only lead to significant structural changes and inactivation. Therefore, compared to Grx1, Grx2 is more robust in highly oxidative stressed environments and is still able to function despite Cys modifications [17]. Moreover, this evidence highlights the ability of Grx2 to be exclusively activated under oxidative stress conditions.

4. Grx2 is a potent antioxidant and anti-apoptotic enzyme in different systems

4.1. The nervous system

Numerous research studies have defined Grx2 as a potent antioxidant and anti-apoptotic enzyme in the brain. Compared to other tissues, the brain is more vulnerable and sensitive to oxidative stress. Moreover, mitochondrial dysfunction is a similar theme found in several neurodegenerative diseases, including Parkinson's, amyotrophic lateral sclerosis (ALS), and Alzheimer's disease [26]. Because mitochondria are heavily involved in the apoptotic pathway, the ability to prevent mitochondrial induction of cell death and repair oxidatively damaged neurons are methods believed to be the future treatments for neurodegenerative disorders.

In motor neurons, aberrant SOD1, misfolded protein aggregation, oxidative stress, and mitochondrial dysfunction are all key players in ALS pathogenesis [27]. Ferri et al. was able to determine that Grx1 overexpression may increase mutant SOD1 solubility in the cytosol but has no effect in its solubility in the mitochondria or motor neuron apoptosis [27]. The accumulation of mutant SOD1 is attributed to higher rates of apoptosis due to ATP and GSH depletion and unusual mitophagy. A previous study in yeast has shown that GSH is a main contributor for SOD1 activation and glutathionylation especially when using oxidative stress agents like menadione are used and is crucial for lifespan development [28]. Rather, Grx2 overexpression cleared mutant SOD1 in the mitochondria, resulting in decreased mitochondrial fragmentation and abolishing neuron apoptosis [27]. Optic atrophy 1 (OPA1), a pro-fusion protein, and dynamin-related protein 1 (DRP1), a mitochondrial fragmentation protein, are often imbalanced due

to mutant SOD1. However, Grx2 overexpression leads to the phosphorylation and activation of DRP1, restoring mitochondrial morphology and the mitochondrial GSH pool [27]. Overall, Grx2 was able to better reduce mutant SOD1 toxicity and increase neuronal cell survival, perhaps by working to correct oxidized disulfide bonds found in aggregated mutant SOD1.

The role of Grx2 in preventing Parkinson's pathology is also a recent research interest. The reduction of mitochondrial GSH pools may indirectly contribute to Grx2 impairment and the development of Parkinson's disease. Several studies have suggested that in dopaminergic neurons of the substantia nigra, the high GSSG/GSH ratio leads to Grx2 inhibition, causing a mitochondrial iron overload that deters the functions of complex I and aconitase, a tricarboxylic acid cycle (TCA) enzyme involved in converting citrate to isocitrate [29]. As a result, mitochondrial dysfunction due to reduced mitochondrial biogenesis and iron–sulfur cluster regeneration is rampant. When 1-methyl-4-phenyl-1,2,3,6,tetrahydropyridine (MPTP) is used to induce neurodegeneration in the extrapyramidal system, Grx2 responded with an increase in mRNA and protein levels after 4 h of treatment and helped alleviate MPTP toxicity and apoptosis [30]. Correspondingly, inhibition of Grx2 lead to compromised complex I activity, indicating Grx2's potential role in restoring complex I activity in times of oxidative stress [30, 31]. Another oxidative stress agent, 6-hydroxydopamine (6-OHDA), used to stimulate a Parkinson's disease model in neuroblastoma cell line, and *C. elegans* was found to increase the expression of Trx1, Trx2, Grx1, and Grx2. Grx2 was found to reduce 6-OHDA and thus prevent its cytotoxicity in dopaminergic neurons [29].

In an ischemia/reperfusion (I/R) model of the rat brain, Grx2 was the most affected, having rebounded from low levels during hypoxia to normal levels by reoxygenation [32]. When Grx2 was successively silenced, neuronal integrity was severely impacted, as there were more distinct areas of neuron damage and death. Furthermore, considering the use of perinatal rat brains, Grx2 was vital in the development of a normal neuronal phenotype [32].

Previous studies have also supported that Grx2 is fundamental for neuronal development. In a zebrafish model, Grx2c was found to be important for the development of the axonal scaffold, as silencing of Grx2c ultimately prevented the neuron differentiation and promoted neuron cell death in nearly 97% of embryos [33]. In particular, Grx2 mediated the redox regulation of collapsin response mediator protein 2 (CRMP2/*DPYSL2*), a protein that mediates axonal outgrowth, cytoskeleton remodeling, and neuronal cell migration [34]. However, their study also found that knockdown of Grx2 did not particularly induce carbonyl oxidative stress in the embryos. A follow-up study characterized the Cys-504-Cys-504 dithiol-disulfide switch as the regulatory mechanism for CRMP2 to control axonal development and neuronal differentiation [32]. Grx2 can reduce the two cysteines, causing CRMP2 conformational changes and activation for proper neuronal development [33, 34].

4.2. Cardiovascular and immune systems

Cardiac cells depend on mitochondrial metabolism for the constant ATP supply necessary to pump blood throughout the body. Compared to the other body organs, the heart has the most number of mitochondria per cell with nearly 5000 mitochondria per cardiac muscle cell [38]. Considering the importance of mitochondria in cardiac function, it is hypothesized that Grx2 plays a major role in protecting the heart's mitochondria.

Inhibition of Grx2 in zebrafish embryos' hearts prevent cardiac neural cells from entering the primary heart region [39]. This results in obstructed blood flow to the aorta and common cardinal vein. Proper heart looping and cardiac neural crest cell migration were also hindered by Grx2 knockdown. Because migration is highly dependent on actin polymerization, Grx2 knockdown would result in more globular actin (G-actin) production and prevent appropriate cell migration [37, 40]. Grx2 has also been attributed to vascular development in embryos. Sirtuin 1 (SIRT1) is prone to redox regulation with approximately 17 cysteines and 5 cysteines specifically known to be glutathionylated. Conserved Cys204 in the catalytic site is around the NAD+ binding site. NAD+ is a crucial cofactor that allows for SIRT1 functioning. Glutathionylation of the key Cys residue would block the cofactor binding site, leading to decreased SIRT1 activity [41]. Therefore, inhibition of Grx2 would promote glutathionylation of the active site, causing SIRT1 inactivation and prevent angiogenesis [41].

Grx2 gene Knockout (KO) mice have decreased aerobic respiration and ATP production due to complex I glutathionylation and inhibition [39]. As a side effect, hypertension ensued with increased cardiac glucose uptake. KO hearts also especially developed left ventricular hypertrophy and fibrosis, yet cardiac contraction and relaxation remained the same between wildtype and KO mice [39, 42].

Usually, with aging, there is a decline in antioxidant expression and activity. Interestingly, Gao et al. first suggested that Grx2 is found to be increased nearly twofold in the mitochondrial matrix of cardiac muscle cells [43]. However, most of the Grx2 is prominently in the inactive dimer formation. Only by exogenous superoxide production by xanthine oxidase was the dimer able to cleave to monomeric form [43]. H_2O_2 had no effect on Grx2 activation. When complex I inhibitor was used to induce endogenous ROS, there was not enough of an ROS threshold to activate Grx2 [41, 43].

Although there is no direct evidence hinting on the connection between Grx2 and immune cells, macrophages depend heavily on actin polymerization to migrate to areas of injury and inflammation. Actin has two prominent forms: filamentous F-actin and globular G-actin. G-actin is often glutathionylated, which prevents its conversion to F-actin [44]. Therefore, Grx2 activation can lead to increased F-actin production, which can cause increased cell migration, differentiation, and proliferation [24, 37, 44].

4.3. Skeletal muscle and adipose tissue

Research is still relatively scarce on how Grx2 may affect the cell bioenergetics, despite the consensus being that Grx2's regulation of complex I glutathionylation is a huge determinant on total ATP production.

In skeletal muscle, the GSH/GSSG mitochondrial pool is relatively high to account for high respiratory rates, which may be a consequence of having somewhat high levels of Grx2 and Trx2 in the mitochondria. Uncoupling protein 3 (UCP3) reversible glutathionylation is carefully mediated by Grx2 and plays a crucial role in promoting glucose metabolism and controlling oxidative stress in skeletal muscle [45, 46]. On the other hand, brown adipose tissue (BAT) is specifically saturated with mitochondria rich in iron, giving BAT its unique color

and is highly concentrated in GSSG rather than GSH. Grx2 is lacking in BAT mitochondria, whereas Trx2 has slightly greater expression to most likely compensate for the lack of Grx2 [45]. Uncoupling protein 1 (UCP1) is similarly regulated by glutathionylation for thermogenesis, but the smaller need for immediate regulation prompts Grx1 and Trx2 to perform UCP1 reduction, albeit at a much lower efficiency [45]. Differences in the choice of substrates, demand, and rate for metabolism in stages of fasting and exercise could explain the lesser role glutathionylation has on BAT mitochondria bioenergetics. Moreover, to maintain a steadfast high iron environment in mitochondria, Grx2 may have to be suppressed, considering Grx2's distinctive iron-sulfur core that differs from the rest of the thioredoxin superfamily. Also, an oxidizing GSSG pool is preferred in BAT mitochondria, and Grx2 expression could alter bioenergetics more to a reducing GSH pool and hinder BAT's ROS production to generate heat. These are all possibilities that could explain why Grx1 and Trx2 are favorably expressed in BAT mitochondria unlike Grx2's key localization in skeletal muscle mitochondria. The selectivity of cysteine glutathionylation in UCP1 and UCP3 provides a great insight into skeletal muscle and BAT functioning [45–47]. The mechanism or the relative importance of Grx2 in regulating these functions remains unseen but does highlight Grx2's potential role in curbing obesity and insulin resistance in diabetes.

4.4. Cancer

Cancer cells are in a constant oxidative stress environment. In order to combat excessive oxidative stress-induced cell death and increase survival, cancer cells often overexpress antioxidant enzymes [2, 14, 15]. With higher expression of antioxidant enzymes, there is increased drug resistance to anti-cancer drugs such as doxorubicin whose primary purpose is to increase oxidative stress and create DNA damage to cause cancer cell death. Several previous studies have highlighted that hepatocellular carcinoma and adenocarcinoma tend to have higher amounts of Grx2 compared to healthy tissue [14, 15]. The role of Grx2 in cytoprotection and promoting drug resistance is still uncertain, but some studies have attempted to decipher how the knockdown or inhibition of Grx2 would affect cancer cell survival.

Enoksson et al. overexpressed Grx2 in HeLa cells, which caused less sensitivity to doxorubicin and 2-deoxy-D-glucose (2-DG). Grx2 was found to inhibit cytochrome c release [48]. Cardiolipin binds to cytochrome c to the inner mitochondrial membrane as an anti-apoptotic measure. When cardiolipin is reduced or lost, cytochrome c is free to move to the cytoplasmic membrane and initiate apoptosis. Without cytochrome c release, caspase is not activated, and apoptosis is thwarted [49]. It is believed that Grx2 decreases cytochrome c release by enhancing cardiolipin expression through prevention of cardiolipin glutathionylation [48, 49].

Similarly, Lillig et al. discovered that siRNA-mediated silencing of Grx2 sensitized HeLa cells to doxorubicin and phenylarsine oxide (PAO) severely. For doxorubicin, the ED50 dropped from 40 to 0.7 μM, whereas PAO's ED50 dropped from 200 to 5 nM [50]. When treated with a Grx1 inhibitor, HeLa sensitivity remained the same. Carbonyl stress was also slightly increased with Grx2 inhibition and doxorubicin treatment. This indicates that Grx2 potentially enhances drug resistance by altering the redox state of the HeLa cell. Conversely, it was also shown that Grx2 overexpression attenuated apoptosis by enhancing mitochondrial protection [48, 50].

In a renal I/R model, Grx2's increased expression in proximal tubular cells corresponded with less oxidative damage and injury after hypoxic conditions. Moreover, there was increased cellular survival and proliferation in HEK293 and HeLa cells after reoxygenation [16]. Considering Grx2's optimal localization in the kidney tubules, this may explain regeneration and anti-apoptotic effects even after an ischemic attack.

4.5. Cataract

Compared with other tissues, the ocular tissues are more susceptible to oxidative stress due to daily exposure of sunlight and relatively high oxygen consumption. Many major blind-inducing diseases, like cataract, age-related macular degeneration (AMD), glaucoma, and diabetic retinopathy are all proved to be closely related with oxidative stress. Therefore, anti-oxidant enzymes play a fundamental role in regulating redox homeostasis and maintaining overall ocular health. Lou's group is the first research team to investigate Grx2 distribution and compare its enzyme activity in ocular tissues. Their data have shown that Grx2 is present in all ocular tissues except vitreous humor. Grx2 level was highest in the mitochondrial-rich and vascular-rich tissues such as the retina while lowest in the tissues that are non-vascular or low mitochondria presence, such as the lens [18]. Fernando et al. first explored the function of Grx2 in lens epithelial cells and determined that human Grx2 has direct peroxidase activity and is capable of using GSH and thioredoxin reductase (TR) as electron donors [35]. Mouse Grx2 did not have as much of a potent peroxidase activity. Moreover, Grx2 could directly detoxify H_2O_2, tert-butyl hydroperoxide (tBHP), and cumene hydroperoxide with greater affinity for tBHP and cumene hydroperoxide [23, 35]. *Grx2* KO in lens epithelial cells also has been shown to less cell viability, decreased ATP levels, and increased complex I inactivation and glutathionylation when treated with H_2O_2 [36].

The main function of the lens is to maintain transparency so that the light can be transmitted and properly focused on the retina for vision. The eye lens contains high concentration GSH as the first line of defense against free radicals to protect critical enzymes and structural proteins from oxidation. However, with aging, the GSH pool decreases and oxidized GSH (GSSG) may attack protein thiols and formed PSSG. Many of lens structural proteins contain high level of thiol residues which are highly susceptible to oxidative damage. For example, alpha A-crystallin is one of the important and abundant structural proteins in the lens. It functions as a chaperone to facilitate protein folding and stabilize other lens proteins in a soluble form. However, under oxidative stress, many of its -SH groups could be glutathionylated, resulting in its chaperonelike function to be drastically lost. By using shotgun proteomic analysis, Giblin's group studied the glutathionylation sites of crystallins in the lens of the guinea pig under oxidative stress. Their data showed that almost all major crystallins, except $\alpha\beta$, were glutathionylated after the oxidative insult. More than 70% of the –SH groups were conjugated with GSH, cysteine, or both molecules. Interestingly, our previous data have shown that mice lacking Grx2 showed faster progression of cataract during aging. In the Grx2 null mice, the lens nuclear opacity began at 5 months, 3 months sooner than that of the control mice and the progression of cataract were also much faster than the age-matched controls. Importantly, this early cataract formation is closely associated with alpha A-crystallin glutathionylation accumulation and compromised mitochondrial functions in

Grx2 gene KO mice [37]. These data suggested that oxidative stress-induced thiol/disulfide imbalance at the stage of PSSG accumulation is the first critical checkpoint to prevent the cascading events leading to cataract formation. The findings also suggest that Grx2 protein may be a potential anticataract agent for delaying or slowing down the age-related cataracts in humans.

5. Grx2 interactions and protein targets

Ribonucleotide reductase has been classified as a primary target for electron donation from several members of the thioredoxin superfamily including Trx1, Trx2, Grx1, and Grx2. In DNA nucleotide synthesis, ribonucleotide reductase performs the rate-limiting step by converting ribonucleotides to deoxyribonucleotides. dNTPs are crucial for DNA reproduction and repair. Therefore, by mediating ribonucleotide reductase, Grx2 essentially allows for sustained DNA production even in times of oxidative damage. Evidence shows that Grx2 uses a monothiol mechanism to donate electrons with activity highly dependent on its cofactor, GSH, concentration.

Several studies have demonstrated Grx2's ability to protect classical mitochondrial targets, such as complex I and IV. Particularly, Wu et al. have shown that complex I and IV glutathionylation is reversed by Grx2 in retinal pigment epithelial (RPE) cells and furthermore, prevents hydrogen peroxide (H_2O_2)-induced damage [31]. UCP1 in BAT and UCP3 in skeletal muscle are involved in thermogenesis and ROS production and signaling [45, 46]. Allosteric control of these proteins relies on Grx1 and Grx2's reversible glutathionylation. *Grx2* KO mice displayed slightly significant lower body mass, accompanied by denser gonadal white adipose tissue and less reactivity to H_2O_2-induced activation of UCP3 [46]. Without Grx2, UCP3 activation ceased. TCA cycle metabolites such as succinate and pyruvate were decreased in the liver, compared to a decrease in 2-oxoglutarate in skeletal muscle [51]. 2-Oxoglutarate dehydrogenase (Ogdh) is another enzyme that has been shown to be directly regulated by ROS-induced glutathionylation. Grx2 works to deglutathionylate Ogdh, and Ogdh in return also controls mitochondrial redox balance by preserving normal ROS levels. With H_2O_2 treatment, GSH initially feeds the amplifying cascade of ROS production by Ogdh, but ROS levels are strictly controlled by Grx2's activation and direct peroxidase activities [51]. The inhibition of Grx2 leads to the limitation of Ogdh activity and could explain lower amounts of 2-oxoglutarate in *Grx2* KO mice.

Grx2 has also been implicated to interact with TR to reduce certain substrates. With the help of TR, Grx2's primary protein targets are coenzyme A (CoA) and other small molecule disulfides [7, 22, 23]. Because of Grx2's high affinity to oxidized targets, CoA mixed disulfide and glutathione disulfide were easily reduced due to TR's ability to reduce both disulfide bridges and mixed glutathione disulfide at Grx2's catalytic Cys sites [22, 52, 53]. Interestingly, there is also evidence that supports that Grx2 may also use TR as an electron donor to reduce the GSSG mitochondrial pool especially in times of GSH deficiency or mitochondrial dysfunction [26, 35].

Oxidative stress may lead to inactivation of other fundamental antioxidant enzymes. Compared to other antioxidant enzymes, Grx2 is less prone to inactivation and has higher affinity to glutathionylated substrates, making it ideal antioxidant enzyme in terms of scavenging free radicals in highly oxidizing environments [22]. Other antioxidant enzymes like TR has a sensitive selenocysteine at the active site that makes it susceptible to electrophilic oxidation and inactivation with abundant oxidative stress [54]. A study by Zhang et al. used TR inhibitors such as auranofin and 4-hydroxynonenal (HNE) to determine the effect on Grx2 activity. Seemingly, Grx2 and cytosolic Trx1 and mitochondrial Trx2 act as back-up systems to each other [23]. When Trx1 and Trx2 are compromised, Grx2 expression is increased, and vice-versa. GSH was also shown to directly reduce Trx1 and Trx2, and the addition of Grx2 increased Trx's repair and enzyme activity [7, 55]. When Grx2 overexpression was induced in HeLa cells, HeLa cells were resistant to auranofin and HNE's cytotoxic effects by promoting anti-apoptotic effects and dually reducing and activating oxidized Trx [55]. Interestingly, considering Trx2 and Grx2's principal mitochondrial localization, the two enzymes may also share common targets. Peroxiredoxin 3 (Prx3) is especially located in the mitochondria and has direct peroxidase and signaling activities. Prx3 has two catalytic cysteines, as do most of the typical members of the peroxiredoxin family, but can also be regulated by oxidation of certain structural Cys residues [56]. Hanschmann et al. were able to determine that both Grx2 and Trx2 work as electron donors for Prx3 [57]. Inhibition of either Grx2 or Trx2 does not affect the expression or activation of Prx3, reinforcing the idea that Grx2 and Trx2 may work as back-up systems for each other [7, 23, 56]. However, among dual inhibition, oxidized Prx3 accumulates in the mitochondria. Grx2 also may have functions in preventing the oxidation of ascorbate or vitamin C. Grx2 acts as an electron donor to dehydroascorbate (DHA) reductase, an enzyme responsible for the conversion of DHA into ascorbate [11]. Vitamin C can also then potentially work as an antioxidant and scavenge free radicals in the cell.

With Grx2's mitochondrial and nuclear localization, Grx2 has the potential to regulate the activity and expression of several different proteins. Unfortunately, there is limited research on Grx2 protein targets, yet screening-based assays help identify certain possibilities that may explain why Grx2 is anti-apoptotic or capable of enhancing cytoprotection in oxidative stress environments. Schutte et al. identified that cytoskeleton, chaperone, proteolysis, metabolic, and transcription factor proteins were the most likely candidates to be regulated by Grx2 [24]. Considering Grx2's effects on proliferation and migration, proteins like actin, tubulin, and dihydropyrimidinase-related protein 2 (DPYL2) with key cysteine residues are regulated by Grx2c. Chaperone proteins have multiple functions including proper folding of proteins and response to ER and oxidative stress, so heat shock proteins may be critically controlled by Grx2 enzymatic activity. Grx2's mitochondrial localization makes it a prime candidate for ensuring metabolic activity. Previous studies have highlighted Grx2's management of TCA cycle metabolites and ATP production in the cell [51, 58]. This may explain why such as glyceraldehyde-3-phosphate (G3PD), enoyl CoA hydratase (ECHM), and glycine N-methyltransferase (GNMT) are specific Grx2 targets [24]. Moreover, ubiquitination is closely linked with oxidative stress and autophagy, which may explain why 26S proteasome subunits and certain E3 ubiquitin ligases may be possible Grx2 targets [24]. Regarding Grx2's nuclear localization especially in HeLa cells, this definitely suggests that Grx2 may be

vitally important in activating crucial transcription factors or signaling pathways [2, 11, 50]. The screening-based assay in Schutte's study highlighted multiple key transcription factors including ruvB-like 1 (RUVB1), elongation factor tu (EFTU), and heterogeneous nuclear ribonucleoprotein F (HNRPF) [24]. Although some of these targets have not been confirmed, it still provides insightful information about how Grx2 may facilitate cellular survival and protection.

6. Conclusion: Grx2's anti-apoptotic and cytoprotective roles may make it a potential drug target to treat oxidative stress-induced diseases

Despite being overlooked, research throughout the years has portrayed Grx2 as a capable and robust antioxidant enzyme through direct detoxification of free radicals, cytoprotection, and anti-apoptotic abilities [5, 23]. Grx2's primary mitochondrial and nuclear localization highlights its important role in balancing pro- and anti-apoptotic signals, maintaining metabolic activity, and preventing cellular dysfunction. Furthermore, Grx2 remains inactive as a dimer until oxidative stress is present, indicating that Grx2 expression can be finely manipulated [17, 21]. Several degenerative and oxidative stress-induced diseases involve exacerbated cell death, and Grx2's potent anti-apoptotic effects make it a marketable drug target to combat oxidative stress and injury [2, 5, 26]. More research needs to be focused on novel drug discovery research to find inhibitors for the treatment and drug sensitization of cancer and activators for the treatment of oxidative stress and degenerative diseases.

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