ISOFORMS OF MAMMALIAN GLUTAREDODXIN 2

Christoph Hudemann

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Supervisor
Dr. Christopher Horst Lillig
Department of Medical Biochemistry and Biophysics
Karolinska Institutet Stockholm
&
Institute for Clinical Cytobiology and Cytopathology
Philipps University Marburg

Co-Supervisor
Prof. Arne Holmgren
Department of Medical Biochemistry and Biophysics
Karolinska Institutet Stockholm

Opponent
Prof. Johannes M. Herrmann
Department for Cellbiology
Technical University Kaiserslautern

Coverpage
3D-reconstruction of confocal immunofluorescence detection of Grx2 in round spermatides

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ABSTRACT

Iron-sulfur proteins are characterized by the presence of iron-sulfur clusters containing sulfide linked iron centers in variable oxidation states. Glutaredoxins (Grx) are, among other redoxins, increasingly recognized to be involved at the interface between redox regulation, iron metabolism and iron-cluster biosynthesis. These versatile oxidoreductases are part of a so-called thioredoxin family acting as facilitators against redox dependent dysregulation of numerous cellular pathways (e.g. protein folding and metabolic pathways). They can generally either reduce or oxidize cysteine residues of proteins, the prime target of reactive oxygen species-induced post-translational alteration that affects protein structure and activity. These modifications occur in nearly all subcellular compartments, raising the necessity for practically every oxidoreductase to be present in most compartments. Isoforms are nature’s way to increase protein diversity based on comparably few genes. Previous reports about Grx2 indicated the presence of additional forms, the focus of this thesis lies upon the question how Grx2 activity is regulated as well as how many isoforms of mammalian glutaredoxin 2 there are. Here, we have characterized human Grx2 as the first iron–sulfur center-containing thiol-disulfide oxidoreductase of the thioredoxin fold protein family. It forms a [2Fe-2S] cluster-bridged dimer coordinated by two N-terminal active site thiols of two Grx2 monomers and two molecules of non-covalently bound glutathione. Addition of glutathione disulfide or other oxidants led to the formation of the enzymatic monomer. We therefore proposed that the [2Fe-2S] cluster serves as redox sensor for the activation of Grx2 upon oxidizing conditions. Human and mouse both possess a generally expressed mitochondrial Grx2a, emphasizing the importance of oxidoreductases in mitochondria, the prime site for the formation of ROS. Alternative splicing gives rise to further isoforms. In human we confirmed the presence of Grx2b and identified a third variant named Grx2c, both located in the cytosol and nucleus in testis and cancer cells. Using the glutaredoxin specific HED assay, they essentially displayed the same activity while with thioredoxin reductase as electron donor Grx2b was only half as efficient as hGrx2c. Only Grx2a and Grx2c are able to form an enzymatically inactive iron-sulfur cluster upon normal cellular conditions, their sibling Grx2b is evidently constitutively active. High levels of Grx2 were found in spermatids, spermatogonia and Sertoli cells indicating unknown functions in cellular development and tumor progression. Mouse expression pattern displayed similarities to human with some notable differences. The expression of the cytosolic mGrx2c is, unlike in humans, not restricted to testis. Similar to human, the iron-sulfur cluster was shown for the mitochondrial and the cytosolic Grx2a /2c. Mouse Grx2c displayed specific activity and was also able to donate electrons to ribonucleotide reductase. The putative isoform Grx2d is partially encoded by a single cassette exon IIIb which is unique to mouse. Glutaredoxin specific activity for this protein could not be measured, its functions are to date unknown. To conclude, here we present new forms of glutaredoxin 2 in human and mouse, a new model of regulation and potential functions in tumor progression and cell differentiation.
LIST OF PUBLICATIONS

This thesis is based on the following articles, which will be referred to by their roman numbers:


*these authors contributed equally to the publications

Review articles which are not included in this thesis:


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<td>protein disulfide isomerase</td>
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<td>PICOT</td>
<td>protein kinase C interacting cousin of thioredoxin</td>
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1 INTRODUCTION

Integration of mitochondria into cells sets the beginning of eukaryotic evolution. In mammalian cells aerobic respiration provides the most efficient main source of cellular energy. Mitochondria, virtually present in every living eukaryotic cell, are the primary sites for the production of universally available energy (ATP), via the oxygen consuming electron chain\(^1\). Ironically, due to electron leakage, mitochondria are also the major source of reactive oxygen species (ROS) such as the superoxide anion and following hydrogen peroxide and hydroxy radicals. ROS play a physiological role in the defense against microbes\(^2\), are part of redox-dependent gene expression\(^3\), cell proliferation and growth\(^4\). Environmental factors (pollution, radiation, autooxidation) may also lead to the formation of molecules containing an unpaired electron. They contribute to harmful effects of ROS, threatening the integrity of proteins, DNA and lipids. Globally, redox couples such as NADP+/NADPH, GSSG/GSH or cystine/cysteine provide a basis for discrete redox pathways. Subcellularly, each compartment comprises a specific network of redox regulating players that contributes to the individual function of the organelle, its redox state and the communication between the compartments\(^5\). Once a thermodynamic disequilibrium between thiol-disulfide systems occurs and fails to remove oxidative damage, crucial cellular processes like apoptosis or growth are disturbed and can lead to cancer\(^6\), neuro-degenerative diseases\(^7\), and numerous other diseases\(^8\),\(^9\). Cells have developed several enzymatic systems and low-molecular weight compounds such as glutathione (GSH) to ensure a steady-state redox equilibrium. The thioredoxin (Trx)- and the glutaredoxin (Grx) system are some of the most thoroughly investigated oxidoreductase systems, performing numerous antioxidant- and redox-regulatory roles\(^10\),\(^11\). The main scope of this thesis will be the GSH-dependent oxidoreductase glutaredoxin 2, its functional regulation and subcellular distribution.

1.1 THE THIOREDOXIN FAMILY OF PROTEINS

Discovered in 1964 in *Escherichia coli* (*E. coli*) as a hydrogen donor for ribonucleotide reductase\(^12\), Trx became the founding member of an increasing group called the thioredoxin family of proteins. Soon thereafter, the functions of this relatively small protein (9-16kDa) were expanded to a) general functions in all organisms such as protein disulfide reduction\(^13\) or DNA synthesis\(^14\) as well as b) specific functions in diverse organisms. These range from the general reduction of enzymes, redox dependent gene expression to virus interactions\(^15\),\(^16\) and others\(^17\).

The thioredoxin family of proteins includes redundant oxidoreductases like thioredoxins, Grxs, peroxiredoxins (Prx)\(^18\) and protein disulfide isomerases (PDI)\(^19\), but also functionally different enzymes like glutathione S-transferases (GST)\(^20\), the bacterial DsbA group\(^21\), glutathione peroxidases (GPx)\(^22\), the newly discovered Clic family of putative intracellular anion channel proteins\(^23\),\(^24\) as well as the B8 subunit from human complex I\(^25\). Despite low overall similarity, the commonality in all groups lies in the structural motif called thioredoxin fold which contains a highly conserved Cis-proline in the active site region\(^26\).
1.1.1 The thioredoxin fold

The main feature of all oxidoreductases of the thioredoxin fold superfamily is the so-called thioredoxin fold and the enzymatically indispensable cysteine based active site. This distinct and highly conserved structural motif is found in both prokaryotic and eukaryotic proteins. First described in 1975, it consists of a central core of four to five-stranded mixed beta-sheets which are surrounded by three to four alpha-helices. Despite the fact that E. coli Grx1 resembles the most basic representation of the thioredoxin fold, the name remained (Fig. 01). Other members of the thioredoxin fold superfamily have, to a certain extent, additional structures inserted.

Some members (GPx, DsbA and Trx) are also individually characterized by additional residues located N-terminally of the thioredoxin fold. Hallmarks of the thioredoxin fold are the cis-Pro upstream of beta sheet three and the Cys-X-Y-Cys active site. X and Y are usually hydrophobic amino acids and are, among other factors, believed to play a crucial role for the physico-chemical properties of proteins as oxidants or reductants. Evolutionary exchange led to a significant change of midpoint potential, e.g. E. coli Trx1 (Cys-Gly-Pro-Cys) $\Delta E'_0=-270mV$ compared to the bacterial DsbA (Cys-Pro-His-Cys), with an $\Delta E'_0=-89mV^{29,30}$. Mutational studies have shown that by exchanging the active site of E. coli Trx1 to mimic PDI altered the redox potential similar to the natural occurring PDI. The redox active cysteines in glutaredoxins are located at the loop that connects beta-sheet one and alpha sheet one. The enzymatic reaction performed by all the oxidoreductases of the thioredoxin family are based on the switch between the reduced (dithiol) and the oxidized (disulfide) active site which induces only minor structural alterations. This so-called dithiol mechanism is, due to a low pKa value, initiated by an attack of the solvent exposed N-terminal active site cysteine on the targets disulfides. Subsequently, the newly formed mixed disulfide intermediate is

Figure 01 - The thioredoxin fold (A) consists of a four stranded beta sheet and three surrounding alpha helices (location of active site indicated by asterix) (B) is represented in its basic form as E. coli Grx1 (PDB code: 1EGO).
attacked by the C-terminal active site cysteine, yielding a reduced substrate and an oxidized enzyme (E-S\(_2\)). The resulting disulfide in the enzyme's active site is, in case of glutaredoxins, reduced by GSH. The first molecule of GSH leads to the formation of a mixed disulfide between the N-terminal active site cysteine and GSH, a second molecule GSH initiate the separation between the now fully reduced enzyme and the oxidized GSSG. NADPH is constantly synthesized in the pentose phosphate cycle and functions in both the thioredoxin and the glutaredoxin system as primary electron donor (Fig. 02).

Characteristic for the glutaredoxin family described below is the ability to reduce GSH-mixed disulfides requiring only the N-terminal active site cysteine. In this "monothiol mechanism", this cysteine forms a glutathione mixed disulfide intermediate (Grx-SG) which is subsequently reduced by a second molecule of GSH. Oxidized GSSG and the reduced substrate protein are released\(^{31,33}\).

1.2 THE GLUTAREDOXIN FAMILY

The term glutaredoxin (glutathione dependent reductase and oxidase) was first introduced for a novel protein functioning as an alternative electron donor for ribonucleotide reductase. Grx was able to restore growth in an *E. coli* ΔTrx mutant\(^{34,35}\). Glutaredoxins can be found in viruses\(^{36}\) and most living organisms including prokaryotes\(^{34}\), as well as eukaryotes (plants\(^{37}\) and algae\(^{38}\), *Plasmodium falciparum*\(^{39}\), yeast\(^{40}\) and human\(^{41}\)). They represent a group of glutathione dependent thiol-disulfide oxidoreductases of small molecular weight. Grxs play an important role in many cellular pathways, their functions can be essentially divided into three categories. First, they can act as electron donor in a broad range of metabolic and signaling pathways. Ribonucleotide reductase (RNR), the key enzyme for deoxyribonucleotide biosynthesis, is efficiently reduced by Grxs in *E. coli*\(^{35}\) and mammalian cells\(^{42}\). In *S. cerevisiae*, deletion of the cytosolic Trx resulted in a viable
strain, implying Grx as an alternative electron donor system for RNR in that manner. Furthermore, PAPS reductase, arsenate reductase, OxyR and dehydroascorbate were shown to act as substrates for Grxs.

Second, glutaredoxins are able to catalyze the formation or reduction of protein-GSH mixed disulfides via the monothiol mechanism. When the GSH/GSSG ratio is comparably high (100:1), protein GSH dithiols are reduced. Under more oxidizing conditions Grxs are believed to rather form glutathionylated protein disulfides, even though the reduction of disulfide bonds are the favored reaction mechanism. Glutathionylation has been shown to be a common posttranslational redox modification that alters protein function. Lind et al. identified a broad range of S-glutathionylated cellular proteins during oxidative stress and constitutive metabolism. Glutaredoxin essentially requires the N-terminal active site cysteine for deglutathionylation activity, mutating the C-terminal active site cysteine to a serine in pig Grx increased this activity even further. In contrast, a C14S active site Grx1 mutant in E. coli displayed 38% of the GSH disulfide oxidoreductase activity compared to the wildtype Grx1.

Third, an increasing number of reports connect Grxs with iron which is the most abundant transition metal in the living organism. This redox active metal is crucial for, among others, the formation of iron-sulfur proteins functioning during electron transfer, enzyme catalysis and regulation. The monothiol Grx5 in S. cerevisiae is localized in mitochondria and facilitates Fe-S cluster assembly. Gene disruption studies showed impaired mitochondrial Fe-S cluster biogenesis and respiratory growth. An increase of iron loaded scaffold proteins was observed, indicating a role of Grx5 during the transfer of the iron-sulfur cluster to the target proteins. Mutants of the Fe/S machinery led to the upregulation of the Aft1/Aft2 (activator of ferrous transport)-dependent iron regulon. In addition, the monothiol Grx3 and Grx4 are also critical for the iron inhibition of Aft1 in yeast cells. The N-terminal conserved cysteine of the CGFS motif is essential for binding to Aft1 likely in the nucleus. Deficiency of Grx5 in zebrafish D. rerio mutants caused loss of Fe-S cluster biogenesis and subsequently hypochromic anemia. The human counterpart led to iron regulatory protein (IRP) upregulation, as a result haem production was impaired. Ultimately, sideroblastic-like anemia and iron overload due to defective heme synthesis was the result. D. rerio Grx5 was able to substitute for the loss of Grx5 in S. cerevisiae.

Donating electrons and catalyzing reversible glutathionylation is achieved by the systemic interplay of all members of the glutaredoxin system. Electrons flow from NADPH, the ultimate electron donor, to the dimeric glutathione reductase (GR), which in turn reduces GSSG to GSH. Reduced Grx reduces an oxidized target protein and becomes oxidized. GSH in turn reduces the oxidized Grx with the formation of GSSG (Fig. 03).
1.2.1 Classification of glutaredoxins

Glutaredoxins can be divided into three classes according to their structure and mechanism of action. The first class is characterized by their dihedral consensus sequence Cys-Pro-Tyr-Cys. Most of them can catalyze both dihedral- and monothiol reactions. Dihedral-Grxs are ubiquitously expressed in all kingdoms of life. They can be found in *E. coli* (Grx1,2,3), *S. cerevisiae* (Grx1,2), mammals (Grx1,2), *Arabidopsis thaliana* (Grx1,2,3,4) and many more (reviewed in 10). The second group, monothiol Grxs, is characterized by the consensus Cys-Gly-Phe-Ser active site sequence. One can further divide them into two subclasses, those with a single glutaredoxin domain (*S. cerevisiae* Grx5, human Grx5, *E. coli* Grx4) and those with a thioredoxin-like region followed by one or more glutaredoxin domains (e.g. *S. cerevisiae* Grx3,4, human Grx3). While all bacterial monothiol Grxs are of the single Grx domain type, in eukaryotes both the Grx-type and the hybrid Trx-Grx-type monothiol Grxs co-exist. The third category are proteins displaying glutaredoxin like oxidoreductase activity but are structurally more related to the GSTs. This class of Grxs is defined by *E. coli* Grx2. High resolution structure analysis revealed a N-terminal Grx domain containing active site residues followed by a 11 residue linker and a highly helical C-terminal domain. Despite low sequence homology, *E. coli* Grx2 belongs to the group of GST-like proteins displaying oxidoreductase activity59. Other structurally related members of this group are the human δ class GSTs60, the human GSTO161, the mouse GST δ-like stress response protein62 and the Clic 1 family of putative intracellular chloride channel63. Most of them act as detoxifiers of xenobiotics, they conjugate GSH to electrophilic target molecules (reviewed in64).
1.2.2 Redox dependent thiol modifications

Most proteins contain cysteine residues which add a sulfhydryl group side chain. This is a rather labile residue which makes it a chemical hot spot for a wide variety of biochemical interactions. Lately, increasing focus in the redox field was drawn on the question which cysteines are oxidized or reduced as part of their catalytic mechanism and which ones are vulnerable to oxidative damage. Surface oriented cysteine residues are usually kept reduced and may therefore serve as ‘redox sensors’ of the cells, fulfilling specific roles in catalysis, regulation, or electron transfer. Caused by dysregulation of oxidative or nitrosative oxygen species, subsequent thiol modifications with different effects can occur. They can be irreversible (e.g. formation of sulfonic or sulfonic acid) or reversible (e.g. thiolation, formation of sulfenic acid). Glutathionylation can also be induced by nitric oxide. Given the reversible nature of most forms of thiol oxidation and the presence of additional cysteines in members of the thioredoxin superfamily, it has been suggested that thiol modifications play important roles in redox signaling. Human Trx1 comprises, apart from the active site cysteines, three structural Cys residues in positions 62, 69, and 73. Upon oxidation or S-nitrosoglutathione-mediated S-nitrosylation, the reduced Trx1 became reversibly inactivated. Human Grx1 and Grx2 contain three and two additional structural cysteine protein residues, respectively. Here, only Grx1 was partly inactivated by both S-nitrosylation and oxidation. On the other hand, Grx2 did not loose activity upon oxidation, did not form disulfide-bonded dimers or oligomers, and could not be S-nitrosylated. Furthermore, E. coli Trx2 has been shown to contain a H2O2-labile zinc site influencing the redox and thermodynamic properties representing a novel regulatory mechanism.

1.2.3 The mammalian Glutaredoxin system

In the Grx system, glutaredoxin is reduced by GSH which is kept reduced by GR. This dimeric flavoprotein contains a conserved FAD$^+$ binding region at the center of the subunit, an interface domain and a NADP$^+$ domain. The binding sites for NADPH and GSSG are on opposite sides of each subunit of the dimer. FAD and an adjacent cysteine moiety mediate transfer of reduction equivalents to GSSG leading to the formation of GSH. Human and mouse GR (GeneName: GSR) are highly similar, consisting of 13 exons and spanning over 50kb. Exon 1 contains a mitochondrial targeting sequence and two in-frame start codons that function as alternative translation initiation sites giving rise to a mitochondrial and a cytosolic form of GR. Nuclear presence has been reported, however, the mechanism of translocation is not known yet.

All dithiol glutaredoxins are characterized by three evolutionary conserved regions. A GSH-binding site allows them to utilize GSH as a substrate. A solvent accessible surface area allows protein-substrate interaction and generally seems to increase upon reduction. A characteristic Cys-Pro-Tyr-Cys consensus active site sequence enables Grxs to perform mono- or dithiol reactions. Mammalian cells contain four distinct glutaredoxins, Grx1 and Grx2 with a dithiol active site, and Grx3 and Grx5 with monothiol active sites.
1.2.3.1 Glutaredoxin 1

Grx1 is a relatively small dithiol oxidoreductase (~12kDa) (GeneName: GLRX1), exclusively reduced by GSH and inhibited by oxidation75,76. Compared to thioredoxin, it specifically reduces GSH-mixed disulfides with greater efficiency (ca. 5000 fold $k_{cat}/K_m$), playing a major role for regulatory mechanism in response to redox dysregulated conditions. The rate of reduction falls significantly when the GSH/GSSG ratio decreases or when the intracellular pH drops49,50. Its location is mainly cytosolic, recent studies have shown the presence of Grx1 in nucleus77 and the intermembrane space of mitochondria78. Furthermore, secretion of Grx1 into extracellular space implicates a role in formation of extracellular disulfides, thus it might be responsible for, for instance, HIV-virion – cell interaction79,80. The levels of intracellular Grx1 in human vary significantly, they seem to be generally 20 fold higher than Grx279, Grx1+/- knockout mice were viable in contrast to Trx+/- mice81. No isoforms were reported for Grx1.

1.2.3.2 Glutaredoxin 2

Grx2 (GeneName: GLRX2) is comparably larger (18kDa unprocessed, 14kDa processed) and exhibits a 34% sequence identity compared to Grx184,85. In contrast to the Grx-consensus Cys-Pro-Tyr-Cys active site sequence present in Grx1, Grx2 contains a dithiol Cys-Ser-Tyr-Cys motif. Grx2 is an efficient catalyst of protein-deglutathionylation with an increased catalytic efficiency ($k_{cat}/K_m$) compared to Grx1. Mutational studies showed that the active site Pro to Ser exchange decreases the activity of Grx2 but increases its affinity for glutathionylated substrates. Furthermore, the oxidized active site of Grx2 is a substrate for thioredoxin reductase (TR) whose activity is not sensitive to either changes in the glutathione redox buffer or decrease in the intracellular pH86. This feature makes Grx2 less dependent on cellular pH levels or the GSH redox buffer. Knockdown of Grx2 in HeLa cells using short interfering RNA (siRNA) led to a dramatical sensitivity towards cell death inducing doxorubicin and phenylarsine oxide. No difference in oxidative damage with respect to carbonylation and glutathionylation was found, indicating a crucial role for human Grx2 in the regulation of the mitochondrial redox status87. Cytochrome c release and following apoptosis is efficiently reduced upon overexpression of Grx2 in HeLa cells. Remarkably, only the mitochondrial Grx2a but not the cytosolic Grx2c prevented cell death induced by redox active 2-Deoxy-D-glucose or doxorubicin88. Transgenic mice overexpressing Grx2a also showed a reduced ischemia/reperfusion-mediated loss of mitochondrial cardiolipin, a decrease of reactive oxygen species (ROS) and a preserved GSH/GSSG ratio89. A greater tolerance towards doxorubicin toxicity is evidently based on the increase in protein S-glutathionylation in heart mitochondria90.
1.2.3.3 Monothiol Glutaredoxins

In addition to the before described dithiol Grxs, monothiol Grxs are characterized by a monothiol Cys-Gly-Phe-Ser active site. Grx3, also known as protein kinase C interacting cousin of thioredoxin, PICOT (GeneName: GLRX3) is composed of a N-terminal Trx domain and two C-terminal monothiol Grx domains both containing a Cys-Gly-Phe-Ser active site. This 37kDa and cytosolic protein is a homologue to S. cerevisiae Grx3/Grx4 and was shown to translocate into the nucleus upon oxidative stress in human leukemic Jurkat T-cells. Several studies reported a role of human Grx3 as a regulator in cardiac hypertrophy. Grx3 binds to a muscle LIM protein (MLP) which prevents calcium-dependent phosphatase CnA activity and ultimately disrupts downstream signaling and dephosphorylation of NFAT (calcineurin-CnA–nuclear factor of activated T cells). Grx3 overexpressing cardiomyocytes displayed enhanced contractility based on an increased rate of sarcoplasmic reticulum Ca$^{2+}$ re-uptake, transgenic mice had increased ventricular function. Homozygot knockout ∆Grx3 mice were embryonic lethal indicating that PICOT plays an essential role during embryogenesis.

Human Grx5 (GeneName: GLRX5) was named after its closely related sibling in yeast. This monothiol glutaredoxin consists of only one Grx domain with the same active site sequence (Cys-Gly-Phe-Ser) like Grx3 and a mitochondrial localization sequence (MLS). It is localized in mitochondria and does not display any disulfide reductase activity. The loss of Grx5 in S. cerevisiae led to accumulation of iron-sulfur cluster on the scaffold protein ISU. As a consequence, dramatic inactivation of iron–sulfur (Fe–S) cluster containing enzymes can be observed and suggests a crucial role for Grx5 during transfer of the iron-sulfur cluster from the scaffold to the target protein. For further implications regarding glutaredoxins and their functions in iron homeostasis read section 1.2.

1.3 Subcellular Compartmentalization and Isoforms of Redoxins

Phosphorylation and thiol redox state changes constitute the two major cellular redox signaling mechanisms. Oxidative phosphorylation is the essential step for the production of energy in eukaryotes. The major factor generally responsible for low redox potential and free SH level inside cells is, apart from NADH, NADPH, cysteine and thioredoxin (Trx), the tripeptide GSH. GSH is present in millimolar concentrations and kept reduced by NADPH and glutathione reductase. Mitochondria are evidently the organelle contributing to about 90% of the electron transfer to oxygen as the terminal electron acceptor which is essential for all aerobic organisms. Anaerobic bacteria have terminal redoxes individualized to their terminal acceptor. As a result, the major redox couple GSH/GSSG in mitochondria has the most reduced state of about -280mV at approximately 5-11mM. Since GSH is synthesized in the cytosol, GSH transport plays a major role in compartmentalization of local redox conditions. The cytosolic GSH/GSSG redox state is usually more oxidized (ca. -200mV). Also, overall
cellular redox conditions affect the respective cellular state. The GSH/GSSG ratio in the cytosol can range from -260mV (proliferation) over -220mV (differentiation) to -170mV (apoptosis). The cytosolic and redox-sensitive transcription factor Nrf-2 is activated by an oxidative signal in the cytoplasm and differently affected by the Trx and the GSH system, supporting the theory that cellular redox systems are distinct both within and between compartments. The nucleus appears to have a more reducing environment, part of the GSH pool seem to be independently regulated from the cytosol. Many nuclear systems depend on Cys-containing proteins. Nuclear GSH maintains the redox state of critical protein sulfhydrys, which are necessary for nuclear integrity, DNA and protein protection from redox changes and others. Not only cellular compartments need to be taken into account separately during redox signaling and cycling, but also membrane related activities such as nuclear translocation and membrane lipid peroxidation promoting oxidative stress mediated cell death were shown to be under redox control. Based on these subcellular differences and their specific functions, cells have developed distinct sets of redoxins for each compartment and specific cell types. Translocation upon certain stimuli occurs as well as complementation between the systems.

1.3.1 Isoforms of mammalian glutaredoxins

The human Grx2 gene (GLRX2) is located on chromosome 1 and consists of five exons. The functional core part of Grx2 is encoded by exon II-IV, while exon Ia and Ib are alternative first exons. Exon Ia encodes a MLS which is conserved among mammals. Tagging this protein with a C-terminal green-fluorescent protein (GFP) tag resulted as expected in a clear co-staining with a mitochondrial marker in fixed HeLa cells. In contrast, transcription initiation at exon Ib – IV would give rise to Grx2b and tagging it to a C-terminal GFP tag displayed a dotted fluorescence pattern located predominantly in the perinuclear area. Further analysis using Western blotting on subcellular fractions of Jurkat cells indicated the presence of Grx2 in the microsomal fraction, Grx2b was suggested to be the nuclear isoform of Grx2. Analysis of mouse Grx2 pattern is to some extend different. A putative Expressed Sequence Tag (EST) search by Gladyshev et al. revealed a more complex pattern for exons upstream of exon II in rodent Grx2. Despite the lack of a coding counterpart of human exon Ib, four different alternative sequences upstream of exon II were found in mouse. In rat, only a truncated exon Ia with an in-frame Stop signal was identified. Quantifications of mRNA levels concluded that Grx2a mRNA isoform accounted for 40% of all available Grx2 mRNAs in each of the mouse organs. In contrast, Grx2a mRNA levels in testis accounted for only 1% of the total Grx2 level, raising the question for the presence of further unknown isoforms.

1.3.2 Isoforms of the thioredoxin systems

Mammalian cells contain essentially two thioredoxin systems. Trx1 together with thioredoxin reductase 1 (TR1) represents the predominantly cytosolic system. In general, Trx1 regulates cytosolic proteins through the reduction of disulfides. The
Trx system has been shown to affect a broad range of protein systems (overview including clinical applications in 111). In contrast to Trx2, Trx1 has three structural cysteine residues which were implied in Trx-dimer formation and other regulatory processes 112, 65. Even though Trx1 does not have a clear nuclear localization sequence (NLS) 113, translocation into the nucleus of transformed or treated cells has been observed 114, 115, 116. A basic region including Lys-81, 82, and 85 was proposed to contribute to nuclear translocation 117. Also, despite the lack of a typical signal sequence usually present in proteins secreted via the endoplasmic reticulum (ER), numerous conditions have been described where secretion of Trx1 to either the outer membrane 118 or to the extracellular space occurs. Here, a leaderless pathway has been proposed which does not involve the Golgi apparatus and is independent of Trx1’s redox activity 119, 120. Extracellular Trx performs a variety of physiological and pathophysiological functions that range from potentiating the effect of cytokines 121, acting as a chemotactic factor 122 and many more 123. Several splice variants of Trx1 have been described 124, 125, 126. One cleavage product of Trx1, comprising only the first 80 amino acids, was named Trx80. It is mainly present on the cell surface, but studies with blood donors revealed plasma concentrations ranging from 1-170ng/ml. Its functions are significantly different from Trx1, e.g., strong cytokine functions for monocytes 127. Transgenic Trx1-mice are more resistant towards oxidative stress 128, 129, while knockout of Trx1 or TR1 caused early embryonic lethality 130, 131. Noteworthy, only Trx1 appears to be essential for cardiovascular development, TR1 is dispensable. A total of five different isoforms of TR1 have been identified, all carrying alternative N-terminal domains and one with an N-terminal Grx-domain 132.

Thioredoxin 2 (Trx2) contains a MLS and is, together with thioredoxin reductase 2 (TR2), the mitochondrial part of the thioredoxin system 133. Due to the lack of extra cysteines, Trx2 is more resistant to oxidative stress and plays an important role in mitochondrial antioxidant and in apoptosis systems 133, 134. Trx2-/- homozygous mouse embryos are not viable, time of death is consistent with the formation of active mitochondria. Knockdown of TR2 led to fatal dilated cardiomyopathy suggesting a specific role of TR2 in detoxification in mitochondria and cardiac development 135. In general, both the cytosolic Trx1 and the mitochondrial Trx2 have similar catalytic activities, as do the two TR isoenzymes (TR1 and TR2). The third TR, named thioredoxin/glutathione reductase (TGR), contains in addition to the dimerization and oxidoreductase domain specific for TRs a glutaredoxin domain similar to the isoform TR1_v3. It exhibits specific activity for both the thioredoxin and the glutathione systems 136, broadening even more the substrate specificity shown by thioredoxin reductases. In mammals this mainly testis specific form has a broad subcellular distribution including cytoplasm and nucleus 137, resembling a striking similarity to GPx IV 138. In Schistosoma mansoni TGR is ubiquitously expressed and seems to have replaced TR and GR entirely 139. All three reductases (TR1, TR2 and TGR) are selenoproteins with their disulfide reductase activities involving a carboxyterminally located selenocysteine-containing active site (Gly-Cys-Sec-Gly). Some data indicate even further crosstalk between the Grx and the Trx system. E. coli Trx can, compared with GSH, efficiently reduce the yeast mitochondrial monocysteinic Grx5 140, poplar Trx can be reduced by
poplar Grx or E. coli Grxs. Drosophila melanogaster which completely lacks GR was shown to use the Trx system as a substitute to support GSSG reduction. Based on these studies, it is clear that the two systems have without doubt specific functions, but they also display a number of overlapping and complementary activities.

Sertoli cells, spermatocytes, and spermatids express little or no cytosolic Trx. The finding of a new testis/spermatid Trx system (Sptrx-1, Sptrx-2, Sptrx-3) in combination with TGR suggested new functions of the Trx system in mammalian spermatogenesis. Sptrx-1 lacks any translocation signal, a GFP-fusion protein resulted in cytosolic localization. It consists of two distinct domains, a N-terminal domain containing 23 repeats of a 15-residue motif and a C-terminal Trx domain. It is believed to participate in the regulation of fibrous sheath assembly by supporting the formation of disulfide bonds during sperm tail morphogenesis. In contrast to Sptrx-1 and 2, Sptrx-2 did not display any thioredoxin enzymatic activity using NADPH and thioredoxin reductase, or dithiothreitol as electron donors. It is a fusion protein consisting of a N-terminal Trx domain linked to three nucleoside diphosphate (NDP) kinase domains. A C-terminally tagged GFP-fusion protein used in HEK293-cells displayed a subcellular distribution within the cytosol, nucleus and presumably mitochondria. Sptrx-3 was found to translocate into the Golgi-apparatus of pachytene spermatocytes and, to a lesser extent, spermatids. Unlike Sptrx-1 and 2, Sptrx-3 is composed of only one thioredoxin domain. Alternative usage of two forms of exon VI defines two subgroups named the α- and the β-variants.

TXNIP (thioredoxin interacting protein), also know as TBP2 or VDUP1, is primarily thought to be an endogenous inhibitor of Trx. It is involved in the maintenance of Trx mediated redox regulation and is dysregulated in various diseases. Subcellular localization of TXNIP is believed to be cytoplasmic, functioning as an inhibitor of nuclear translocation of Trx. However, Nishinaka et al. postulate a rather nuclear presence.

Nucleoredoxin (Nrx) was found during the mapping of the nude locus and named after its sequence similarity to Trx and its predominant nuclear localization in COS-7 cells. A typical NLS could not be found, a recent study claims a rather cytosolic distribution. Here, Funato and co-workers report an interaction between Nrx and the cytosolic Dishevelled, a member of the Wnt–β-catenin pathway. A redox dependent association between Nrx and Dishevelled indicate a possible nucleocytosolic shuttling in form of a complex between those two proteins. Nrxs and orthologues can be found in mammalian and non mammalian species. They usually contain two N-terminal Trx-like domains with the catalytic motif (WCPPC) which differs from that of Trx (WCGPC). The C-terminal region of mammalian Nrx contains an acidic region that resembles the b domain of PDI proteins where it is required for substrate recognition.

1.3.3 Peroxiredoxins

Mammalian Prxs comprise a family of peroxidases which essentially can be divided into three subfamilies. 2-Cys Prxs contain both the N- and C-terminal-conserved
Cys residues and require both of them for catalytic function (PrxI, II, III, IV); atypical 2-Cys Prxs contain only the N-terminal Cys but require one additional, non-conserved Cys residue for their catalytic activity (PrxV); and 1-Cys Prx proteins contain only the N-terminal Cys and require only the conserved one for catalytic function (PrxVI). Mammalian Prxs occupy a central position between ROS and nitric oxide signaling pathways, they can reduce both peroxinitrite and hydroperoxides. Even though located primarily in the cytosol, Prxs are also found within mitochondria and peroxisomes, associated with nuclei and membranes, and are also being exported. PrxI and PrxV appear to have the widest subcellular distribution, as they are found in the cytoplasm, mitochondria, peroxisomes and, to a smaller extent, in the nucleus. Together, they typically constitute 0.1 to 0.8% of total soluble protein. PrxII and IV are mainly located in the cytosol, expression of PrxIII is restricted to mitochondria. PrxIV is also present in the endoplasmic reticulum. Secretion was shown for PrxIV and PrxI despite the lack of any signaling peptide. Together with the mitochondria specific Trx and TR, Prx III is suggested to provide a primary line of defense against \( \text{H}_2\text{O}_2 \) produced by the mitochondrial respiratory chain.

### 1.4 IRON-SULFUR CLUSTER PROTEINS

Iron-sulfur clusters [Fe/S] are ubiquitous co-factors of proteins found in all kingdoms of life. They essentially consist of iron cations (Fe\(^{2+/3+}\)) and anionic sulfide (S\(^{-}\)). In proteins, the iron ions are usually bound to cysteine-sulfur groups, but coordination by nitrogen atoms of arginine, histidine or small molecules (CN\(^{-}\), homocitrate) can also be observed. Fe/S clusters have versatile electrochemical properties with a reduction potential ranging from +300 mV to –500 mV. Given their remarkable structural versatility, they are involved in structural aspects, electron transport, regulation of gene expression and enzyme catalysis. They can be most frequently found as rhombic [Fe\(_2\)S\(_2\)], cubic [Fe\(_4\)S\(_4\)] clusters or its oxidation product [Fe\(_3\)S\(_4\)]. After the discovery of the first [Fe/S]-protein, a ferrodoxin in *Clostridium pasteurianum*, the general belief was that they act as single electron transfer complex between redox couples. This dogma was broken with the finding of cytosolic aconitase, which, in its native state, contains a \([4\text{Fe}-4\text{S}]^{2+}\) cluster. When iron is scarce, the apo-form (also called the iron regulatory protein, IRP) prevails, blocking ferritin synthesis and protecting transferrin receptor mRNAs. When iron becomes available again, holo-aconitase is reconstituted from IRP so that the protein loses its function as IRP, but can now be recognized by its aconitase activity with the non-ligated iron serving as a Lewis acid. Eukaryotic Fe/S proteins can be found in mitochondria, cytosol, and nucleus. Nuclear Fe/S proteins are involved in DNA interaction and ribosome assembly, cytosolic Fe/S proteins function in nucleotide metabolism, iron uptake regulation, receptor signaling and Fe/S biosynthesis. The mitochondrion contains Fe/S proteins in the respiratory chain and is also the place of Fe/S biosynthesis (for review, see).
1.4.1 Biosynthesis of iron-sulfur cluster proteins

So far, three different systems for the biosynthesis of iron-sulfur cluster proteins are known, all of them involve a cysteine desulfurase providing sulfur ligands and a scaffold protein for the de novo synthesis of the [Fe/S] cluster. The nitrogen-fixation system (NIF) was first found in *Azotobacter vinelandii* and is generally required for generation of the metalloclusters in nitrogenase. The mitochondrial “iron-sulfur cluster” assembly system (ISC) is required for the formation of most cellular Fe/S proteins. In yeast and higher nonphotosynthetic organisms the ISC-like system exclusively exists in the mitochondria. ISC is ubiquitously found in prokaryotes and in the mitochondria of both nonphotosynthetic and photosynthetic eukaryotes, whilst the sulfur mobilization system (SUF) is present in bacteria and plant plastids. SUF plays a more general role during harmful conditions and is present in bacteria and plant plastids. Even though in most bacterial species only one of these machineries is encoded, in some like *E. coli* the ISC and SUF systems are present in parallel. Photosynthetic eukaryotes like plants and algae display, due to the presence of plastids and mitochondria, a more complex pattern.

The CIA (cytosolic iron-sulfur protein assembly) system is regarded as a cytosolic iron-sulfur protein assembly system. Present in eukaryotic organisms, most components of CIA have also counterparts in *A. thaliana* based on amino acid sequence similarity. Current beliefs state that the interplay between the mitochondrial ISC system, the ISC export machinery and the cytosolic CIA machinery give rise to cytosolic, mitochondrial and nuclear Fe/S proteins in eukaryotes. The implications of glutaredoxins in iron-sulfur cluster assembly and iron homeostasis are discussed in 1.2.
2 PRESENT INVESTIGATION
2.1 AIMS OF THIS STUDY
Current estimations range from 20,000-25,000 human genes\textsuperscript{166} giving rise to a proteome of about 200,000 proteins. Several mechanisms like alternative splicing and different transcription initiation sites lead to the formation of isoforms of proteins generated from a single gene and contribute to the vast variety of proteins. Glutaredoxins are versatile GSH dependent oxidoreductases that are able to counteract destructive effects of cellular redox dysregulation. They are increasingly found to be involved in iron homeostasis, iron sulfur cluster assembly and to contain iron-sulfur cluster themselves. In 2001, human Grx2 was first introduced and characterized\textsuperscript{167,84}. Both studies revealed a gene giving rise to a mitochondrial (hGrx2a) and suggested a nuclear (hGrx2b) variant. Even though the mouse Grx2 gene lacks the alternative exon that gives rise to Grx2b, gene expression pattern analysis revealed intriguing differences between mRNA levels of the mitochondrial and the absolute gene expression of Grx2, especially in testis\textsuperscript{110}. Based on those results, we systematically started to investigate the presence, localization and function of Grx2 isoforms in human and mouse and to characterize the proteins. The initial questions we asked were the following:

- Which isoforms are capable to complex an Fe/S cluster?
- What is the structural mechanism behind the formation of an iron-sulfur cluster in Grxs?
- How many isoforms arise from the respective GLRX2 gene in human and mouse?
- What is the expression profile of Grx2 isoforms in normal compared to transformed tissues and cells, what are their sub-cellular localizations?
- How are these isoforms regulated in terms of activity and interaction partners, what are their characteristics?
- Could there be any clinical relevance for the presented findings, can Grx2 be used as representative marker genes for oxidative stress-inducing drugs?
2.2 RESULTS

2.2.1 Paper I


In this study we investigated the nature of brown freshly purified human Grx2. Using gel filtration chromatography, we separated a larger (34kDa) fraction containing the chromophore from a colourless smaller one of approximately 16.9kDa. Both fractions yielded one similar band of 16kDa in SDS-page identified as Grx2 by Western blotting. The smaller, enzymatically active fraction had a characteristic peak at 280nm in the UV-visible spectrum. The larger enzymatically inactive fraction displayed additional bands at 320 and 428nm indicating the presence of an iron-sulfur cluster. To further characterize the nature of the cluster, we purified Grx2 from E. coli grown in 57Fe-enriched mineral medium. Subsequent Mößbauer spectroscopy demonstrated the presence of a [2Fe-2S]2+ cluster bound dimer of Grx2. To analyse the coordination of the cluster, we performed mutational analysis on both C-terminal active site-cysteines (C40S) as well as on extra cysteinyl mutants (C28S, C118S). Human Grx2 Cys-28-Ser and Grx2 Cys-118-Ser lost their additional characteristic peaks. We therefore proposed a [2Fe-2S] bridged holo-dimer of Grx2 coordinated by cysteine 113 and cysteine 28. The holo-complex was easily destabilized, the addition of GSSG or one electron carriers like dithionite led to monomerization of Grx2 whereas GSH stabilized it. Co-immunoprecipitation from two different cell lines revealed in vivo that approx. 0.8 molecules Fe are bound to one molecule Grx2, cytosolic Grx1 on the other hand did not bind iron at all. In summary, we characterized Grx2 as the first iron-sulfur protein of the thioredoxin family. We proposed the formation of an [2Fe-2S] cluster coordinated holo-dimer of Grx2 in vivo as a novel and redox dependent sensor which leads to activation of Grx2 upon oxidative stress conditions at the crossroad between cell death and survival.

2.2.2 Paper II


As shown in paper I, iron bound in Grx2 is present in tetrahedral sulfur coordination. Why does GSH stabilize this holo-dimer? For the present investigation we developed a reconstitution assay for holo-Grx2, based on the iron-cluster synthesis machinery in E. coli involving IscA and IscS. Interestingly, besides iron and sulfide, GSH was needed for the reconstitution of the dimer and is also in constant equilibrium with a pool of free GSH. Following, we used site directed mutagenesis to further characterize cluster coordination. We focused not only on the mutation of four cysteiny1 residues in Grx2, but also on Thr95Arg, which potentially impairs GSH binding due to a missing hydrogen bond and electrostatic disturbance. Also, we produced the Ser38Pro mutant, mimicking the active site of
Grx1 in Grx2 rendering Grx2 unable to form a [Fe/S] cluster. Among the cysteine mutants, only the N-terminal active site Cys37Ser mutant lost its ability to form a holo-dimer in vitro. The extra cysteine mutants Cys28Ser and Cys113Ser displayed an overall decreased stability as shown by heat induced denaturation, underlining their importance for the structural integrity of holo- and apo-protein, but not for cluster binding itself. Mutating Thr95 was predicted to affect the interaction between Grx2 and non covalently bound GSH. Indeed, we registered a less stable iron-sulfur cluster and an about two-fold increased specific activity of the apo-protein.

Grx1 contains the consensus active site sequence Cys-Pro-Tyr-Cys and has no homologues to either the extra cysteines or the Thr95 from Grx2. By exchanging the Pro23Ser and thereby mimicking Grx2, we were able to reconstitute a brownish Grx1 displaying similar spectral peaks like the reconstituted holo-dimer of Grx2. Evidently, the characteristic active site Cys-Ser-Tyr-Cys enables Grx2 to form an inactive holo-dimer upon normal intracellular conditions. At present, more Grxs were found to form the same holo-complex.

2.2.3 Paper III


Alternative splicing, pre-mRNA editing, alternative transcription initiation sites and post-translational modifications give rise to protein isoforms originating from the same gene. Isoforms differ in their subcellular localization, interaction partners, function and / or stability and contribute immensely to the diversity of proteins. The human Grx2 gene is located at chromosome 1 (1q31.2-31.3) and stretches over approximately 10 kb. The existence of 5 exons and 4 introns had been suggested before, the lack of experimental evidence on mRNA and protein isoforms prompted us to investigate different normal and cancerous tissues.

Using RT-PCR, we screened for (a) the core part of Grx2 encoded by exon II-IV, (b) the mitochondrial Grx2a encoded by exon Ia-IV, and (c) the previously suggested nuclear isoform by exon Ib-IV. The existence of three essentially different isoforms were verified:

1) transcript variant 1 (encoding hGrx2a) – This mitochondrial variant can be found in both non-transformed and cancerous cell lines underlining its general importance in the defense against oxidative stress in mitochondria. Its localization was confirmed by both GFP-tagged fusion proteins as well as by indirect immunofluorescence.

2) transcript variant 2 (encoding hGrx2b) – This previously as nuclear introduced variant was found exclusively in testis and to some extend in transformed cancer cell lines, as shown by RT-PCR. The refolded hGrx2b displayed about the same activity in both the hydroxyethyl disulfide assay (HED) as well as using TR as electron donor. The protein localized in the cytosol as well as in the nucleus. Surprisingly, hGrx2b was not able to form
an iron-sulfur cluster and may represent a constitutively active cytosolic variant of Grx2.

3) transcript variant 3 (encoding hGrx2c) – Screening for the presence of hGrx2b via RT-PCR, 2 bands were observed. While the bigger one contained the full length transcript variant 2, the 101bp smaller fragment contained a truncated exon Ib. Here, the shortened possible longest open reading frame starts from an ATG codon in exon II. It gives rise to a new third isoform of human Grx2, named Grx2c. It is equally active as hGrx2b in the HED assay, but reduces GSSG with TR as electron donor about twice as fast. Unlike hGrx2b, Grx2c was found to be present in both cytosol and the nucleus. Unlike hGrx2b, Grx2c was able to form a holo-dimer and therefore represents a cytosolic iron-sulfur glutaredoxin.

To clarify in vivo functions of these isoforms, we studied the presence of Grx2 in testis using immunohistochemistry with antibodies raised against the core part of Grx2. Strong staining was found in round and elongated spermatides as well as in spermatogonia and Sertoli cells. The signal was concentrated in the cytosol, suggesting the presence of hGrx2c and hGrx2b in cells. To summarize, in this study we confirmed and identified three isoforms of human Grx2 (hGrx2a,b,c). The individual expression pattern and properties indicate further unknown functions not just in redox regulation, but also in differentiation and carcinogenesis.

2.2.4 Paper IV


Suggested by Jurado et al., only 40% of the mRNA transcripts in normal tissue and 1% in testis referred to the mitochondrial Grx2a, raising the question for further isoforms. Based on an extensive Expressed Sequence Taq (EST) - cluster search and a set of specific RT-PCR, we were able to identify 5 different transcript variants giving rise to three different isoforms of mouse Grx2. In comparison to human isoforms:

1) mouse Grx2a (v1) is present in virtually all tissues but testis (<1%). It is encoded by exon Ia (containing a mitochondrial translocation signal) and the three constitutive exons II, III and IV. This variant is conserved in human and has been described above.

2) the cytosolic mouse Grx2c originates from three different transcript variants resulting in ubiquitous or testis specific expression (v2 and v4/5, respectively). As shown by (UV-Vis), mouse Grx2c also forms an Fe/S-bridged holo-dimer. Ribonucleotide reductase is effectively reduced by Grx2c and is to date the only confirmed co-localizing electron donor in spermatogonia.

3) the third isoform identified, named mGrx2d, originates from v3 and is encoded by exon Ia<sup> trunc </sup>, II, III, the newly discovered single cassette exon IIIb and IV. Its expression was found to be restricted to testis. The open
reading frame only ranges from exon II to IIIb, it lacks residues essential for glutaredoxin function. Enzymatically inactive, its function is to date not clear.

To shed light on in vivo functions of the isoforms identified, we first developed a highly sensitive ELISA and determined total Grx2 content in tissues. Liver contained the lowest amount of Grx2, highest levels were detected in kidney and heart. Following, immuno-histochemical staining and immunofluorescence was used to identify protein localization in mouse testis. Here, prominent staining occurred in cytoplasm of spermatogonia as well as in round and elongated spermatides. Cytosolic Grx2 was also found in enteroendocrine cells of the stomach and the white pulpa of the spleen, not in the red pulpa though. We therefore propose new and unknown functions in multiple mouse tissues and spermatogenesis for mouse glutaredoxin 2 isoforms.
3 CONCLUSIONS

The present investigation provides a new understanding of how activity of mammalian glutaredoxin 2 is regulated. The ability to complex a Fe/S cluster for the dithiol Glutaredoxin 2 and the identification, confirmation and characterization of isoforms of mammalian Glutaredoxin 2 provides new insights on iron-sulfur based redox regulation. In specific, in

Paper I
- introduced Grx2 as the first protein of the thioredoxin fold superfamily to contain an iron-sulfur cluster which may function as regulatory switch

Paper II
- further investigation into the nature of the iron-sulfur cluster revealed that the N-terminal active site cysteine as well as the loss of the active site proline are essential for the ability to form a iron-sulfur cluster bound also to 2 molecules of GSH
- GSH is bound non-covalently to the enzymatic inactive dimer, facing oxidizing intracellular conditions turns the holo-dimer into a enzymatically active monomer

Paper III
- we systematically screened for the presence of isoforms of Grx2 in human and unraveled the presence of three functionally different isoforms with different subcellular localization
- high concentration of Grx2 in sperm cells with different developmental status indicates new functions during cellular differentiation

Paper IV
- we identified three isoforms of Grx2 in mouse which are, compared to human, based on a different set of transcript variants
- ribonucleotide reductase was found to be reduced by Grx2c, together with specific cytosolic IHC staining in testis, spleen ect. we concluded that Grx2 must have specific functions in the cytosol, e.g. in spermatogenesis and during development
4 DISCUSSION AND FUTURE PERSPECTIVE

This thesis provides further insights into the activity, regulation and subcellular distribution of the glutathione-dependent oxidoreductase glutaredoxin 2. Glutaredoxins are increasingly recognized as far more than just a backup system for thioredoxins. Iron-sulfur cluster now connect glutaredoxins not only to redox reactions, but also to iron homeostasis\textsuperscript{97}, related diseases\textsuperscript{168} and entirely new functions\textsuperscript{169}. The papers discussed provide evidence for the formation of an Fe/S cluster as a novel binding mechanism in the dithiol Grx2 and the presence of three isoforms in the sub cellular compartments mitochondria, cytosol and nucleus in human and mouse. Even though, many questions arose which will have to be answered in order to fully understand the whole impact of Grx2 on cells and its possible application in a clinical context. In mammalian cells, two distinct sets of glutaredoxins can be found. The classical dithiol Grxs are characterized by their CPYC active site motif allowing them to reduce β-MESG via a monothiol mechanism in the commonly used hydroxyethyl disulfide (HED) assay. Monothiol Grxs have a conserved CGFS active site and a much more complex structural pattern. Structural analysis of \textit{E. coli} Grx4 revealed a conserved three-dimensional structure common to most other glutaredoxins\textsuperscript{170}. Missing charged residues, responsible for GSH interaction in dithiol Grxs, are a suggested cause for Grx's inactivity in the HED assay. Lately, a global role for monothiol Grxs in iron homeostasis and Fe/S cluster assembly has been suggested. A yeast monothiol Grx5 knockout strain displayed iron accumulation and cellular inactivation of iron sulfur containing enzymes\textsuperscript{171}, in specific the amount of Fe/S clusters bound to the scaffold Isu1p protein increased\textsuperscript{172}. Hence, a crucial role for Grx5 during the transfer of the cluster from the scaffold to the target protein has been suggested. These effects could be reversed by supplementation of Grx5 homologues from various species, confirming the functional conservation of Grx5 homologues throughout evolution\textsuperscript{173}. In mammals, similar effects like can be observed. While in zebrafish a hypochromic anemia mutant caused by lacking expression of Grx5 was identified, decreased expression of Grx5 in human caused sideroblastic-like anemia\textsuperscript{174, 168}. In paper \textbf{I} and \textbf{II} we introduced the concept of the first dithiol Fe/S glutaredoxin as a new way of regulation in the Trx family of thiol-disulfide oxidoreductases (Fig. 04). Unlike in other metal-binding proteins, the Fe/S cluster in Grx2 was remarkably stabilized by reduced GSH. Together with paper \textbf{III} and \textbf{IV} we concluded that it only accounts for the mitochondrial Grx2a and the cytosolic/nuclear Grx2c, but not for Grx2b. Grx2a is translocated into mitochondria and subsequently loaded with the iron-sulfur cluster by the ISC assembly machinery. It remains inactive as a holo-dimer until facing alterations in the mitochondrial redox environment. The maturation of Fe/S proteins in the cytosol and nucleus is less well understood. The cytosolic Fe/S assembly machinery (CIA) is a multicomponent system converting apo- to holo-Fe/S proteins. How and where (cytosol, nucleus) Grx2c interacts with CIA and receives the Fe/S cluster is to date not known. Unlike the cytosolic human Grx1, Grx2c is not inactivated by oxidizing cytosolic conditions, thus potentially representing an alternative redox system in that specific subcellular compartment. Human Grx2b is
a cytosolic / nuclear and constitutively active isoform. In contrast to Grx2c it is similar active in the HED assay but displayed half the specific activity for the reduction of GSSG with TR as electron donor. One might therefore speculate of hGrx2b as a baseline active enzyme supported by hGrx2c whenever changed redox conditions threaten the cellular integrity. Supporting this theory, Elliot S. and Mitra S. proved that the [2Fe-2S] cluster bound Grx2 is stable up to 0.5V poise potential (equivalent to treatment with $\text{H}_2\text{O}_2$). However, under oxidative stress conditions imitated by poise potentials greater than 0.5 V by the direct action of a stronger oxidizing reagent such as the ROS superoxide (+0.89),

Figure 04 - Molecular model for Grx2 regulation. Newly translated apo-Grx2 is loaded by the ISC machinery with an iron-sulfur cluster which involves the N-terminal active site cysteine rendering the protein inactive. 2 molecules of GSH stabilize the cluster and are in constant exchange with the surrounding, upon local changes of the redox state the cluster disintegrates and enzymatically active apo-Grx2 is formed. Disintegration of the cluster occurred174. Lately, other dithiol Grxs like poplar Grx-C1 or monothiol Grxs like 1-C-Grx1 in *Trypanosoma* or the EcGrx4p from the cyanobacterium *Synechocystis* were shown to incorporate the [2Fe-2S] cluster into the homodimeric apoprotein175,176,177, thus raising the question of the function for this new evolutionary mechanisms first described in Paper I. A novel and functionally intriguing subgroup is represented by *S. cerevisiae* Grx6 and Grx7 which are monothiol Grxs (CSYS and CPYS, respectively). They are, due to a N-terminal transmembrane domain, both located in the cis-Golgi network. Unlike other monothiol Grxs, *S. cerevisiae* Grx6 and Grx7 display Grx specific activity and might play individual roles during directed apoptosis induced by intermediates arising from the Golgi apparatus178,179. A recent study by Lee et al. showed that mitochondrial Grx2, like Grx5, may indeed also be involved in iron-sulfur biogenesis. Disrupting a functional Grx2a results in mitochondrial dysfunction and increased iron levels, hallmarks of Parkinson’s disease180. Iron accumulation in mitochondria is further associated with the formation of Alzheimer’s disease and Huntington’s disease. Compared to the mitochondrial Grx2a, the cytosolic Grx2c was shown to be less protective towards increased ROS production88, supporting the general importance of the ubiquitously expressed Grx2a in maintaining the mitochondrial redox status. Investigating iron homeostasis and the specific
interplay with Grx2 might shed more light on why some isoforms form Fe/S cluster. While Grx2a was ubiquitously expressed, its nuclear/cytosolic siblings Grx2c and 2b were shown to be expressed in testis and cancer cells (see Paper III). Testis present a highly specialized tissue with high proliferation rate for sperm cells combined with lower core temperature, demanding a different set of redox proteins, among others. Evidently, numerous proteins were shown to have testis specific isoforms. TR1 is, among other isoforms, expressed as a testis specific isoform TR1_3 containing an additional N-terminal Grx domain. It is believed to act as a guide for actin polymerization\(^{181}\). Testis also lack the cytosolic Trx, but express a new set of thioredoxins, called SpTrx1-3. In addition, TGR is mainly expressed in elongated spermatids, together with the newly identified testis specific human Grx2c and 2b this could represent a novel disulfide bond formation system adapted to the special redox requirement during sperm maturation. Malignant cells are also confronted with an altered redox state caused by local hypoxic conditions. Is the upregulation of hGrx2b/c in cancer cells a defense mechanism? A strong signal for the upregulation of Grx2 in patient neuroblastomas was observed (not published). A role of protecting cells towards oxidative stress induced cell death by doxorubicin and phenylarsine oxide has been shown\(^87\). How can that function be of use to further implement the use of Grx2 in humans to develop new and effective disease treatments? A major issue was to specifically distinguish between the isoforms identified. Since the mitochondrial Grx2a gets processed and essentially looks like Grx2c, and also hGrx2b only differs in the first 40 hypophobic amino acids, specific antibodies were impossible to obtain. Fluorescence in situ hybridization (FISH) based on the detection of specific RNA fragments might be the method of choice to detect specific isoforms in e.g. paraffin embedded human material. Also, DNA methylation as a control mechanisms for gene expression could be of use here. GLRX2 contains two methylation sites in its 5' region, potentially affecting cell cycle or tissue specific expression. Promoter luciferase assays might be able to shed some light on cell cycle specific expression.

In mouse, only mGrx2a and c are able to form a cluster, but not the newly discovered mGrx2d. The remarkable difference here is the ubiquitous expression of the cytosolic Grx2c. Knockout of the cytosolic Grx1 in mouse resulted in mild phenotype with viable pups\(^83\). Could Grx2c function as a backup system for the cytosolic Grx1 here? Further investigations are needed to evaluate the interplay between the different mammalian glutaredoxin systems and their specific functions during spermatogenesis and formation of tumors.
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6 REFERENCES


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