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Dissecting the role of selenothiol- versus thiol-based catalysis using the model enzyme glutathione peroxidase 4 (GPx4)

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ERKLÄRUNG

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In cherished memory of my loving parents

who always believed in me.

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List of Abbreviations

2-ME	β-mercaptoethanol
AIF	apoptosis inducing factor
Ala	alanine
allCys/Ser	FSH-GPx4 all Cys are changed to Ser
allCys/SerU46C	FSH-GPx4 all Cys are changed to Ser and Sec to Cys
Amp	ampicillin
APAF-1	apoptotic protease activating factor-1
tBOOH	tert-Butyl hydroperoide
BSO	L-buthionine sulfoximine
C_P	peroxidatives Cys, initiates reduction of substrate by formation of an intermolecular bond
C_R	resolving Cys, completes reduction by nucleophilic attack of the intermolecular bond, converting it into an intramolecular disulfidbridge
CMV	cytomegalovirus
COX	cyclooxygenase
Cre	Cre recombinase
Cys	cysteine (reduced)
(Cys)2	cystine (oxidised Cys dimer)
C(x)S	FSH-GPx4 (substitution Cys to Ser on position x)
DCF	dichlorofluorescein
DMSO	dimethylsulfoxide
DTT	dithiothreitol
Ea	alternative exon (of the PHGPx gene)
EDTA	ethylenediamine-N,N,N',N'-tetra-acetic acid
EFsec	Sec-specific elongation factor
eGFP	enhanced green fluorescence protein
FACS	fluorescence associated cell sorting
FAD	flavin adenine dinucleotide
FSH-tag	Flag-Strep-HA-tag
Gln	glutamine
Glu	glutamate
Gly	glycine

GPx	glutathione peroxidase
GSH	glutathione (reduced)
GSH-S	glutathione synthase
GSSG	glutathione dimer (oxidised)
HPLC	high pressure liquid chromatography
HSP90	heat shock protein 90
LOX	lipoxygenase
IRES	internal ribosomal entry site
LMP	agarose low melting point agarose
Lys	lysine
MEFs	murine embryonic fibroblasts
MER	mutated oestrogen receptor
mU46C	Mls-FSH-GPx4 (substitution Sec to Cys on position 46)
mWT	Mls-FSH-GPx4
N137A	FSH-GPx4 (substitution Asn to Ala on position 137)
N137D	FSH-GPx4 (substitution Asn to Asp on position 137)
NAC	N-acetylcysteine
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NF-κB	nuclear factor κB
NOX	NADPH oxidase
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PFA	paraformaldehyde
PHGPx	phospholipid hydroperoxide glutathione peroxidase
PI	propidium iodide
PLA2	phospholipase A2
Q81A	FSH-GPx4 (substitution Gln to Ala on position 81)
Q81E	FSH-GPx4 (substitution Gln to Glu on position 81)
ROS	reactive oxygen species
Se	selenium
SBP2	SECIS binding protein 2
SDS	sodium dodecyl sulfate
Sec	selenocysteine
SECIS	selenocysteine insertion sequence
SelA/B/C/P/R	selenoprotein A/B/C/P/R
Ser	serine
SMCP	sperm mitochondrion-associated cysteine-rich protein

SPS2	selenophosphate synthetase 2
Tam	4-hydroxytamoxifen
TAPe	enhanced tandem affinity purification
TNF	tumour necrosis factor
Toc	α-tocopherol
Trp	tryptophan
Trx	thioredoxin
TrxR	thioredoxin reductase
U46C	FSH-GPx4 (substitution Sec to Cys on position 46)
VitE	vitamin E
W136D	FSH-GPx4 (substitution Trp to Asp on position 136)
WT	FSH-GPx4

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Abstract

Selenocysteine (Sec) is the 21st amino acid. Unlike other amino acids incorporation of Sec into proteins is more complex by far as it is encoded by the opal stop codon UGA. A complex and highly sophisticated incorporation mechanism is required in order to assure the correct co-translational incorporation of Sec into the nascent polypeptide chain. Even after years of intensive research the question as to why selenoproteins are important for mammalian life has not been fully understood. This study aimed to provide answers to this question using gluthathione peroxidase 4 (GPx4) as a model enzyme. It has been shown that GPx4 is essential for early embryogenesis, neuro- and retina-protection and male fertility in mice. But the role of selenothiol- versus thiol-based GPx4 catalysis in cells and mice, as well as the role of the catalytic tetrad in GPx4 catalysis and the subcellular localisation of the different GPx4 isoforms and their impact on cell protection have remained unclear. Therefore, a series of mutant variants of GPx4 were generated by site-directed mutagenesis, stably expressed in tamoxifen-inducible GPx4 knockout cells (Seiler et al., Cell Metab 2008), and functionally analysed. GPx4 mutant variants included those of the peroxidatic Sec, the catalytic tetrad, the mitochondrial leader sequence (Mls) and the non-peroxidatic cysteines. These studies revealed that Cys can functionally replace Sec in the active centre of GPx4 to a large extent in cells, whereas some amino acids of the catalytic site are necessary for GPx4 function. None of the non-peroxidatic Cys were shown to be required for the cell-protective function of GPx4, ruling out a possible resolving Cys in the catalytic cycle of GPx4 unlike homologues in other organisms that use a resolving Cys. The fuction of a resolving Cys is to start a nucleophilic attack upon the intermolecular bond between the peroxidatic Cys and the substrate in order to create an intramolecular disulfidbride. Apparently subcellular localisation of GPx4 in the extramitochondrial/cytosolic compartment is crucial for its cell death preventing qualities as overexpression of mitochondrial GPx4 in the knockout cells failed to rescue cell death induced by endogenous GPx4 disruption.

Zusammenfassung

Bei Selenocystein (Sec) handelt es sich um die 21igste Aminosäure. Im Gegensatz zu anderen Aminosäuren ist der Einbau von Selenocystein in die entstehende Polypeptidkette sehr viel komplexer, da Sec von dem Opal Stopcodon UGA codiert wird. Eine komplexe und hochentwickelte Maschinerie ist erforderlich um den korrekten, ko-translationalen Einbau von Sec in Proteine zu gewährleisten. Auch nach Jahren intensiver Forschung ist es immer noch weitgehend unklar, warum Selenoproteine für das Säugerleben unverzichtbar sind. Diese Studie hatte deshalb zum Ziel, diese Frage etwas genauer zu beleuchten, wobei die Glutathion-Peroxidase 4 (GPx4) als Modellenzym verwendet wurde. Es ist bekannt, dass GPx4 sowohl für die Embryonalentwicklung, den Schutz von Neuronen und der Retina sowie für die männliche Fertilität von Mäusen unverzichtbar ist. Dennoch sind viele Fragen bezüglich der Funktionsweise und möglicher Unterschiede von Selenothiol- zu Thiol-basierter GPx4 Katalyse in Zellen und Mäusen ungeklärt. Ebenso wie die genaue Funktion der katalytischen Tetrade bei der GPx4 Katalyse, und die subzellulare Lokalisation der verschiedenen GPx4 Isoformen und deren Einfluß auf den zellulären Schutz bislang ungeklärt sind. Deswegen wurden in dieser Arbeit eine Reihe von GPx4 Mutanten durch zielgerichtete Mutagenese erzeugt. Diese wurden stabil in Tamoxifen-induzierbaren GPx4 knockout Zellen (Seiler et al., Cell Metab 2008) exprimiert und auf ihre Funktionalität analysiert. Die verschiedenen GPx4 Mutationen beinhalteten Mutanten des Sec im aktiven Zentrum, der katalytischen Tetrade, der mitochondrialen Signalsequenz (Mls) sowie der nicht peroxidativen Cysteine. Die hier vorgelegten Untersuchungen zeigen, dass Cys in der Lage ist das Sec im aktiven Zentrum, unter Aufrechterhaltung eines Großteils der Funktionalität, in der Zelle zu ersetzen, wohingegen einige der Aminosäuren der katalytischen Tetrade unentbehrlich für die Funktionalität von GPx4 sind. Keines der übrigen, in GPx4 vorhandenen, Cys wurde benötigt, um die zelluläre Schutzwirkung von GPx4 aufrecht zu erhalten. Dieses Ergebnis schließt die Existenz eines auflösenden Cys im katalytischen Kreislauf von GPx4 aus. Dies steht im Gegensatz zu den Cys tragenden Homologen in anderen Organismen, welche über ein auflösendes Cys verfügen. Die Funktion des auflösenden Cys besteht darin, einen nukleophilen Angriff auf die intermolekulare Bindung zwischen dem peroxidativen Cys und dem Substrat zu starten. Durch diesen Angriff wird die intermolekulare Bindung in eine intramulekulare Disulfidbrücke umgewandelt. Die Lokalisation von GPx4 im extramitochondrialen/cytosolischen Raum ist zudem entscheidend für den Schutz vor Zelltod, da eine Überexpression der mitochondrialen GPx4 nicht in der Lage war, den durch Ausschalten der endogenen GPx4 verursachten Zelltod zu verhindern.

1 Introduction

1.1 Selenoproteins

The trace element Selenium (Se) is important for some eubacteria, archaea and eukaryotes. In these organisms, Se is mainly found being incorporated into the 21st amino acid, selenocysteine (Sec). Sec differs from cysteine (Cys) only in one atom, selenium replacing sulphur. Sec is co-translationally inserted into Sec-containing proteins, also called selenoproteins. In general Sec is mostly part of the reactive centre and essential for enzyme function (Lu and Holmgren 2009, Steinbrenner and Sies 2009). The widely accepted concept implies that Sec comprises a higher reactivity than its sulphur containing analogue Cys presumably due to a higher nucleophilicity and the ability to be already deprotonated under physiological pH (Gromer, Johansson et al. 2003). Sec is also suggested to allow for a broader range of substrates (Gromer, Johansson et al. 2003).

Selenoproteins are expressed in multiple organisms, including eubacteria, archaea, insects, vertebrates and humans (Lobanov, Hatfield et al. 2009). By contrast, yeast and plants lack selenoproteins, implying that the presence of selenoproteins follows neither a uniform distribution within different organisms nor any correlation between selenoprotein utilisation and increasing complexity of the organism. Likewise, questions have remained unanswered, concerning why mammals require selenoprotein expression for proper embryonic development (Bosl, Takaku et al. 1997, Kumaraswamy, Carlson et al. 2003), whereas other species, which lack selenoproteins, such as *Drosophila Melanogaster (Drosophila)*, only express the respective cysteine containing homologue (Hirosawa-Takamori, Chung et al. 2004). Screening of aquatic organisms' genomes suggested that selenoprotein expression may be linked with the environment, as a higher number of selenoproteins could be found in aquatic organisms when compared to terrestrial organisms (Lobanov, Fomenko et al. 2007, Arner 2010).

Site-directed mutagenesis of Sec to Cys or chemical derivatisation of Sec in various selenoproteins, including glutathione peroxidases (Maiorino, Aumann et al. 1995) and thioredoxin reductases (Lee, Bar-Noy et al. 2000, Zhong, Arner et al. 2000) revealed a remarkable drop of enzyme activity. The reasons for the advantage of Sec- versus Cys-based catalysis are still not fully understood, although some interesting concepts have been developed in case of human thioredoxin reductase (TR1) (see chapter 1.2) (Gromer, Johansson et al. 2003).

In contrast to the other 20 amino acids encoded by the universal genetic code Sec is encoded by the opal stop codon (UGA). Sec incorporation requires unusual rearrangements for correct incorporation during translation (Chambers, Frampton et al. 1986, Zinoni, Birkmann et al. 1986) (see chapter 1.3). Initially, the misinterpretation of UGA codons as simple translational STOP codons precluded the correct identification of proteins as true selenoproteins in many organisms.

Glutathione peroxidase 1 (GPx1) was the first mammalian selenoenzyme to be discovered by two independent groups in 1973 (Flohe, Gunzler et al. 1973, Rotruck, Pope et al. 1973). Eventually it was revealed that mice express 24 and men 25 selenoproteins, respectively (Kryukov, Castellano et al. 2003). GPx6 is the only exception, which is a Cys containing variant in mouse and a selenoprotein in human. Simultaneous disruption of all selenoproteins in the mouse, achieved by targeted deletion of the Sec-specific tRNA gene (*Trsp*), causes early embryonic death at E6.5 (Bosl, Takaku et al. 1997). So far knockout studies for individual selenoproteins revealed, that only cytosolic thioredoxin reductase (Jakupoglu, Przemeck et al. 2005), mitochondrial thioredoxin reductase (Conrad, Jakupoglu et al. 2004) and GPx4 are essential for murine development (Conrad 2009). Since *GPx4*$^{-/-}$ mice die almost as early as *Trsp* null mice (Bosl, Takaku et al. 1997), it is reasonable to conclude that GPx4 is a vitally important selenoprotein in mammals.

Human selenoprotein families relevant for this work are summarized in Table 1 (Kryukov, Castellano et al. 2003).

Table 1: Relevant selenoprotein families.

Name	Chromosomal location (number of exons)	Main catalytic function	Sec location in protein (length aa chain)
GPx1	3p21.31 (2)	H_2O_2 + 2 GSH -> $2H_2O$ + GSSG	47 (201)
GPx2	14q23.3 (2)	H_2O_2 + 2 GSH -> $2H_2O$ + GSSG	40 (190)
GPx3	5q33.1 (5)	H_2O_2 + 2 GSH -> $2H_2O$ + GSSG	73 (226)
GPx4	19p13.3 (7)	ROOH + 2 GSH -> H_2O + GSSG + ROH	73 (197) (long form) 46 (170) (short form)
GPx6	6p22.1 (5)	H_2O_2 + 2 GSH -> $2H_2O$ + GSSG	73 (221)
TrxR1	12q23.3 (15)	Trx-S2 + NADPH + H^+ -> Trx-$(SH)_2$ + $NADP^+$	498 (499)
TrxR2	3q21.2 (16)	Trx-S2 + NADPH + H^+ -> Trx-$(SH)_2$ + $NADP^+$	655 (656)
TrxR3	22q11.21 (18)	Trx-S2 + NADPH + H^+ -> Trx-$(SH)_2$ + $NADP^+$	522 (523)

Abbreviations: Glutathione peroxidases (GPxs), and thioredoxin reductases (TrxRs) are clustered into distinct protein families. Due to its relevance for this work, GPx4 (PHGPx) is highlighted in red (adopted and modified from (Kryukov, Castellano et al. 2003)).

Results obtained by overexpression of individual selenoproteins or by Se depletion/repletion studies in rodents must be interpreted carefully (Weitzel, Ursini et al. 1990). Changes in availability of cellular Se is known to have a significant influence on the expression of specific selenoproteins, which has been described as the “hierarchy” of selenoprotein expression (Schomburg and Schweizer 2009). This hierarchy has been thoroughly investigated for members of the GPx family, which showed that expression of some GPxs, such as GPx1 and GPx3, respond quickly and are lost in response to selenium depletion, whereas selenium repletion causes a fast *de novo* synthesis of these GPxs (Bermano, Nicol et al. 1995, Lei, Evenson et al. 1995, Wingler, Bocher et al. 1999, Fujieda, Naruse et al. 2007). More specifically, even a moderate Se-deficiency leads to a strong decrease in expression of GPx1 and GPx3, whereas GPx2 and GPx4 expression is barely affected under these conditions. Findings mainly obtained from mouse knockout studies indicated that GPx4 is of paramount importance for murine embryogenesis (Conrad 2009), whereas knockout of GPx1, 2 and 3 does not impact on embryonic development nor affects normal life (Conrad and Schweizer 2010).

1.2 The advantage of selenothiol-based catalysis

It has still remained largely unclear why some organisms utilize selenothiol- instead of thiol-based catalysis in a number of proteins. Initial attempts to provide a molecular explanation for this still unclear phenomenon were made by Arnér’s laboratory using human thioredoxin reductase 1 (TrxR1) as a model enzyme (Gromer, Johansson et al. 2003). Mammalian TrxR1 was compared with the non-selenium containing homologue of *Drosophila melanogaster (Drosophila)*. Thioredoxin reductases form homodimers and align in a head to tail fashion. Thereby, the redox centre of the N-terminal region of one monomer interacts with the C-terminal redox centre of the second monomer (Cheng, Sandalova et al. 2009). In mammals the C-terminal active site consists of a peroxidative Sec (initiates the reduction of the substrate by formation of an intermolecular bond) and a resolving Cys (completes reduction of the substarte by a nucleophilic attack on the intermolecular bond, tunring it into a intramolecular bond), the Sec being the penultimate amino acid. Due to the easily oxidisable selenolate anion (lower pK_a), selenoproteins are considered to be by two or three orders of magnitude more active than their Cys-containing counterparts (Lee, Bar-Noy et al. 2000). Yet, there is no notable difference in measurable thioredoxin reductase activity between human and *Drosophila* thioredoxin reductase 1, although the later is a Cys-containing variant (Gromer, Johansson et al. 2003). It appears that the pK_a of the non-selenium catalytic site in

the *Drosophila* enzyme is lowered by the surrounding acidic amino acids (serines), thus enabling an equal activity when compared to the selenium-containing mammalian counterpart. As expected the substitution of Sec by Cys, in the mammalian selenoenzyme was shown to lead to a drastic reduction in its activity (Gromer, Johansson et al. 2003). On the contrary, when the amino acids surrounding the active site of the Sec/Cys variant of mammalian TrxR1 were mutated in a way that resembled the *Drosophila* homologue, the specific activity of mammalian TrxR1 could be increased. In fact, this Sec/Cys variant was equally active as the wild-type TrxR1 enzyme, when tested with the model substrate DTNB (Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid)) or thioredoxin itself (Gromer, Johansson et al. 2003). The only detectable difference was a decreased substrate spectrum of the Sec/Cys variant, in part due to the alterations of the surrounding amino acids, which led to a conformational change in the protein acceptor site (Gromer, Johansson et al. 2003). These studies indicated that Sec may be essential for ensuring full functionality and preserving broad substrate specificity at least for mammalian TrxR1.

1.3 Selenocysteine incorporation mechanisms

The amino acid Sec was discovered as a component of distinctive proteins in bacteria, archaea and eukarya (Low and Berry 1996, Stadtman 1996, Rother, Resch et al. 2001), which is encoded by an in-frame UGA codon that requires various factors in trans and cis for decoding.The mechanism for the insertion of Sec into the nascent polypeptide chain is different between bacteria and mammals. The mammalian incorporation mechanism is more complex than the bacterial one which can be explained by the difference in position and structure of the selenocystein insertion sequence (SECIS) element and the structure of the ribosomes. While in bacteria the SECIS element is positioned immediately downstream of the Sec codon, and therefore also part of the coding region, in mammals it is located in the 3' UTR, and therefore can be quite far away from the actual Sec codon. Due to these intrinsic differences in the SECIS positioning and the different factors involved in selenoprotein decoding, recombinant expression of mammalian selenoproteins in bacteria is strongly hindered. The different mechanisms of bacterial and mammalian selenoprotein translation are illustrated in Figure 1 and Figure 2.

1.3.1 Eubacterial selenoprotein synthesis

In *Escherichia coli (E. coli)* the biosynthesis and co-translational incorporation mechanism of selenocysteine into selenoproteins (Figure 1) requires the products of four genes, *selA*, encoding the selenocysteine synthetase, which catalyses the formation of Sec from Ser bound to $tRNA^{Sec}$, *selB*, the selenocysteine-specific elongation factor, *selC*, the selenocysteine-specific $tRNA^{Sec}$ and *selD*, the selenophosphate synthetase.
SelD catalyses the selenium donor, seleniumphosphate, from ATP and reduced selenium (Sturchler-Pierrat, Hubert et al. 1995). The elongation factor SelB has a N-terminal part that is structurally homologous to the three domains of elongation factor Tu (EF-Tu, which is involved in the incorporation of the other 20 amino acids) (Hilgenfeld, Bock et al. 1996, Tormay, Sawers et al. 1996), and a C-terminal extension involved in the binding of the SECIS element (Kromayer, Wilting et al. 1996). In prokaryotic mRNAs, the SECIS element is located just downstream of the UGA codon as part of the coding region of the selenoprotein, where it may also act as an inhibitor of translational read-through (Engelberg-Kulka, Liu et al. 2001).

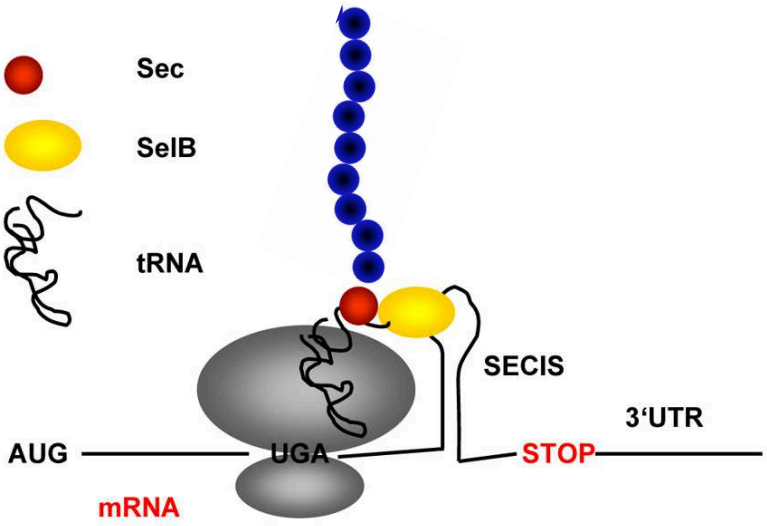

Figure 1: Mechanism of UGA decoding and Sec incorporation in eubacteria. SelB (shown in yellow) is a SECIS binding protein and a specialized translation elongation factor, the nascent polypeptide chain is shown in blue and Sec in red.

Genetic and biochemical studies suggested that SelB catalyses the same functions as EF-Tu. In its GTP-bound form, EF-Tu binds the aminoacylated tRNA forming the ternary complex and transfers the activated tRNA to the ribosome. In the initial selection step, dissimilar ternary complexes are dissociated from the ribosome. When equivalent ternary complexes form proper anticodon-codon interactions, the GTPase activity of EF-Tu is increased, which induces a strong conformational change (Berchtold, Reshetnikova et al. 1993). This leads to (i) a reduction in the affinity for the bound tRNA, (ii) to the release of the

tRNA to the ribosomal A site, and (iii) the dissociation of EF-Tu-GDP from the ribosome. SelB can only recognize the $tRNA^{Sec}$ when it is loaded with selenocysteine (Sec-tRNASec) and not the precursor form loaded with serine (Ser-tRNASec) (Forster, Davies et al. 1990), unlike EF-Tu that is able to recognize 40 different tRNA species loaded with one of the 20 classical amino acids. Most importantly, SelB does not form a ternary, but rather a quaternary complex with selenocysteyl-tRNASec, GTP and the SECIS element, which eventually leads to correct Sec insertion (Heider, Baron et al. 1992, Baron, Westhof et al. 1993, Ringquist, Schneider et al. 1994). The pre-formation of the quaternary complex is essential for the decoding process (Tormay, Sawers et al. 1996). A successful interaction with the ribosome leads to the stimulation of GTP hydrolysis (Huttenhofer and Bock 1998), and it can occur only when the quaternary complex is formed.

1.3.2 Mammalian Sec incorporation mechanism

Selenoprotein translation relies on the incorporation of Sec at the encoding UGA codon through a highly complex mechanism that requires several different cis and trans acting factors (Figure 2) (Allmang, Wurth et al. 2009). Also, the loading of the Sec-specific tRNA with Sec differs significantly from bacteria (Donovan and Copeland 2010). In brief, inorganic selenide is activated to selenophosphate by selenophosphate synthetase. Selenophosphate is processed by selenocysteyl-tRNA-synthetase which catalyses the substitution of Ser to Sec in $Ser\text{-}tRNA^{[Ser]Sec}$, yielding $Sec\text{-}tRNA^{[Ser]Sec}$. In contrast to the bacterial SECIS, the mammalian SECIS element is positioned in the 3´-untranslated region (UTR) of the mRNA. Apart from this cis acting element trans acting elements are also needed, e.g. the SECIS binding protein 2 (SBP2), the ribosomal protein L30 (rpL30), and the EF-Tu-like elongation factor EFSec (Copeland 2003, Caban and Copeland 2006) (Figure 2).

The distance of the UGA codon and the SECIS element can be as far as 4000 bp in eukaryotes in contrast to eubacterial positioning of the SECIS element (Buettner, Harney et al. 1998). Eukaryotic SECIS elements are composed of two helices, an internal and apical loop, and a conserved non-Watson-Crick interacting nucleotide quartet, located at the base of helix 2 (Copeland, Fletcher et al. 2000, Low, Grundner-Culemann et al. 2000). Some large apical loops contain an additional mini-stem that presumably stabilizes the SECIS element. SBP2 is recruited in eukaryotic cells by the SECIS element to form a stable SECIS-SBP2 complex (Copeland, Fletcher et al. 2000, Low, Grundner-Culemann et al. 2000). SBP2 binds the elongation factor EFsec, which in turn recruits Sec-loaded $tRNA^{[Ser]Sec}$ (Fagegaltier, Hubert et al. 2000, Tujebajeva, Copeland et al. 2000). This complex translocates to the

ribosome, where tRNA$^{[Ser]Sec}$ is released to the ribosomal A-site by GTP hydrolysis (Chavatte, Brown et al. 2005). The eukaryotic ribosomal protein L30 has been implicated in Sec incorporation. L30 is considered to replace SBP2 upon binding of the complex to the ribosome, triggering the release of tRNA$^{[Ser]Sec}$. Eventually, the polypeptide chain at the P-site is transferred to the tRNA$^{[Ser]Sec}$, forming a peptide bond with Sec. The elongation factor EFsec is specific for Sec incorporation and differs from EF-Tu, which is involved in the aminoacyl-tRNA delivery of the other 20 amino acids (Chavatte, Brown et al. 2005). Recently, SEC43a has been implicated in selenoprotein biosynthesis. SEC43a forms a complex with tRNA$^{[Ser]Sec}$. Targeted knockdown of this factors reduced overall selenoprotein biosynthesis (Xu, Perez et al. 2005), but detailed characterization of this factor requires further studies. SBP2 along with EFsec constitute the functional equivalent of a single elongation factor in prokaryotic selenoprotein synthesis, designated SelB (Forchhammer, Leinfelder et al. 1989).

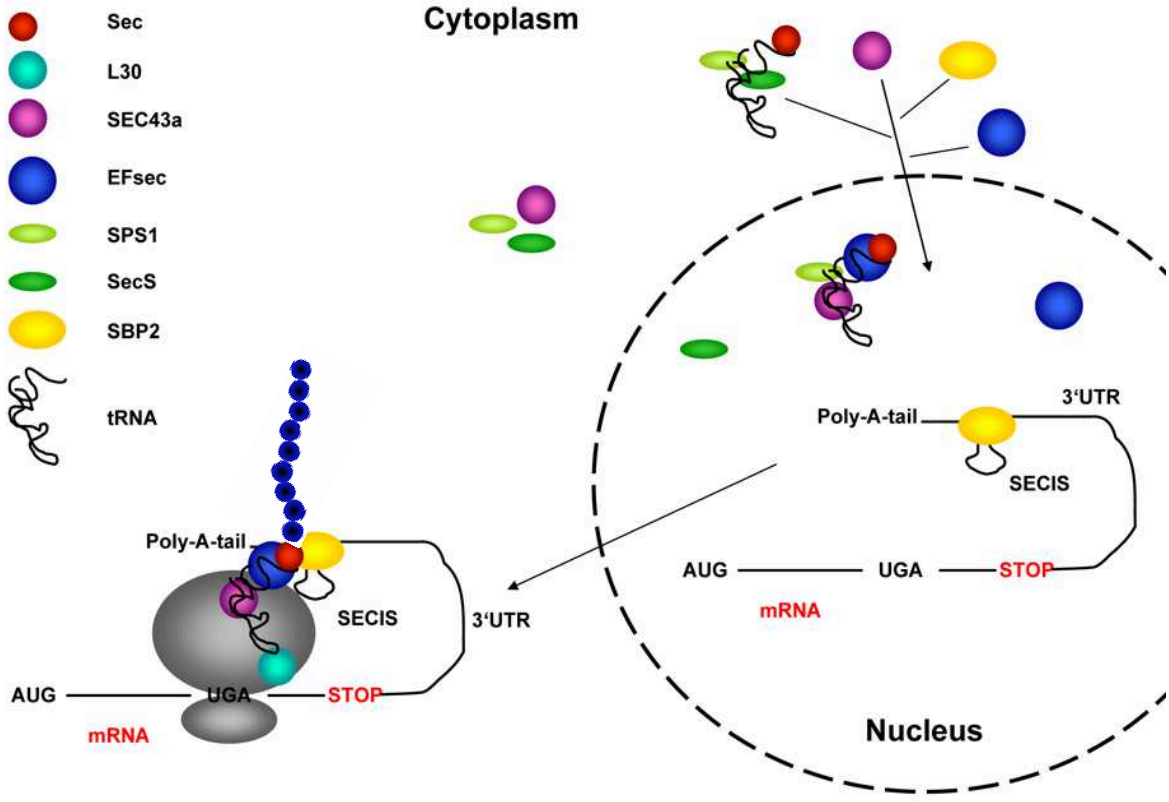

Figure 2: Mechanism of mammalian UGA decoding and Sec incorporation. Depicted is the shuttling of the SPS1/SECp43/EFSec/Sec-tRNA$^{[Ser]Sec}$ complex (light green/ pink/ blue/ black) into the nucleus and association with SBP2 (yellow) and the SECIS element. Cytoplasmic translocation of the SECIS-bound complex is shown on the left, the nascent polypeptide chain in dark blue and Sec in red.

Naturally occurring mutations in the human SBP2 were shown to result in a complex phenotype (Schoenmakers, Agostini et al. 2010). Azoospermia, combined with a failure of spermatogenesis in later stages, is probably due to a lack or decreased expression of certain selenoproteins in testis. Also, an axial muscular dystrophy could be found, showing features comparable to myopathies caused by mutations in selenoprotein N (SEPN1) (Arbogast,

Beuvin et al. 2009, Papp, Holmgren et al. 2010). Due to deficiencies in antioxidant selenoenzymes, cellular reactive oxygen species (ROS) were increased in peripheral blood cells in patients carrying SBP2 mutations. Additionally telomere shortening could be detected as part of the phenotype. Strangely enough, increased ROS levels were associated with enhanced systemic and cellular insulin sensitivity (Schoenmakers, Agostini et al. 2010). Cellular depletion of SBP2 leads to oxidative stress and induction of caspase-3 and cytochrome c-dependent apoptosis (Papp, Lu et al. 2010). SBP-2 deletion also caused higher levels of ROS, and an increase in 8-oxo-7,8-dihydroguanine (8-oxo-dG) DNA lesions, stress granule formation, lipid peroxidation and cell death. Interestingly, antioxidants like N-acetylcysteine, glutathione, and alpha-tocopherol only slightly reduced ROS levels and were unable to rescue SBP-2 knockdown cells fully from apoptosis. This indicates that apoptosis might be mediated by various selenoproteins simultaneously, indicating that SBP2 is required for protection against ROS-induced cellular damage and cell survival (Papp, Lu et al. 2010).

It has been reported that read-through of the UGA in mammalian selenoprotein mRNAs is only ~2 % of that of a cysteine codon in the same position (Berry, Maia et al. 1992, Kollmus, Flohe et al. 1996, Suppmann, Persson et al. 1999). Yet it is still unknown whether this is due to chain termination at the UGA competing with Sec insertion or to kinetic impairment due to the highly complex insertion mechanism in eukaryotes.

1.4 Glutathione peroxidase 4 (GPx4)

1.4.1 Genetic and molecular structure

GPx4 (also known as phospholipid hydroperoxide glutathione peroxidase, PHGPx) was first purified from pig liver in 1982 by Ursini and colleagues and characterized as a protein efficiently protecting liposomes and biomembranes from peroxidative degradation in the presence of glutathione (GSH) (Ursini, Maiorino et al. 1982). GPx4 shares the least sequence homology with the other GPxs and significantly differs from its family members in terms of its monomeric structure, low substrate specificity, and its necessity for mouse development (Imai, Hirao et al. 2003, Yant, Ran et al. 2003, Conrad, Schneider et al. 2007, Seiler, Schneider et al. 2008). Three different forms of mammalian GPx4 exist, which show distinctive subcellular localization and tissue-specific expression. Each variant is transcribed from a distinct start codon within the same gene (Figure 3). Alternative transcription initiation leads to the expression of either the short (cytosolic) form (GPx4) (170 aa/20 kDa), the mitochondrial (long) form (mGPx4) (197 aa/23 kDa), with an N-terminal mitochondrial leader sequence, or the 34 kDa nuclear form of GPx4 (253 aa/nGPx4), previously referred to as sperm nuclei-specific GPx (snGPx) (Pfeifer, Conrad et al. 2001).

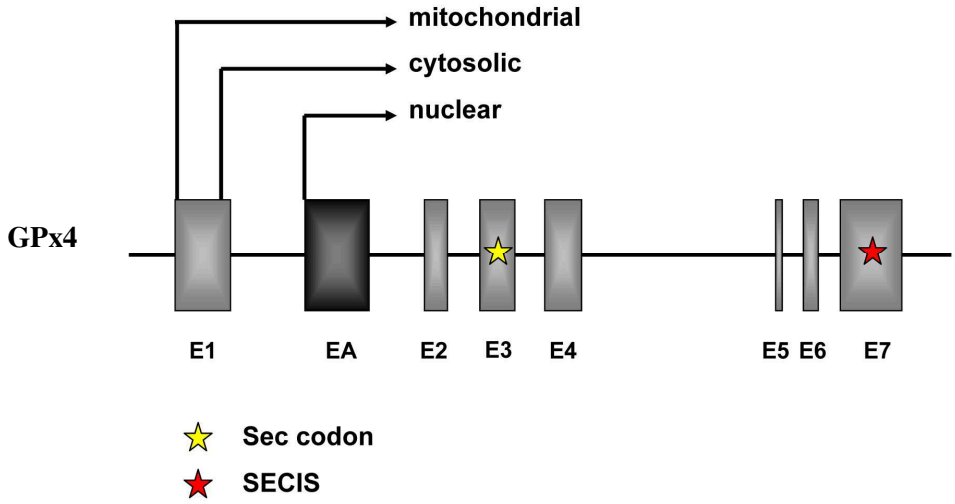

Figure 3: Exon-intron structure of mammalian *GPx4*. *GPx4* consists of 7 "classical" exons (E) and one alternative exon (EA), located between exon 1 and 2. The mitochondrial (197 aa) and cytosolic (170 aa) start codons are encoded by E1. The sperm nuclei-specific form of GPx4 (253 aa) is transcribed from the EA. The EA encodes a nuclear targeting signal and clusters rich in basic amino acids, most probably required for binding of nuclear GPx4 to DNA. The Sec codon (yellow asterisk) is encoded for by exon 3. The SECIS element (red asterisk) is located on E7. For the short and long forms of GPx4 EA is spared by splicing.

The alternative exon (EA), localised between the classical exons 1 and 2, encodes a nuclear targeting sequence as well as clusters rich in arginine and lysine, which are also found in protamines. Protamines are small, arginine-rich, nuclear proteins that replace histones in

spermatids. They are essential for sperm head condensation and DNA stabilization (Balhorn 2007). The cytosolic form of GPx4 is ubiquitously expressed, whereas the mitochondrial and the nuclear form are mainly expressed in testis (Pushpa-Rekha, Burdsall et al. 1995, Pfeifer, Conrad et al. 2001, Schneider, Vogt Weisenhorn et al. 2006). The primary protein structure of the short and long form of GPx4 is identical except for the Mls at the N-terminus (Figure 4). The bulk amount of the long form is exclusively expressed in male testis (Ursini, Heim et al. 1999, Maiorino, Roveri et al. 2005).

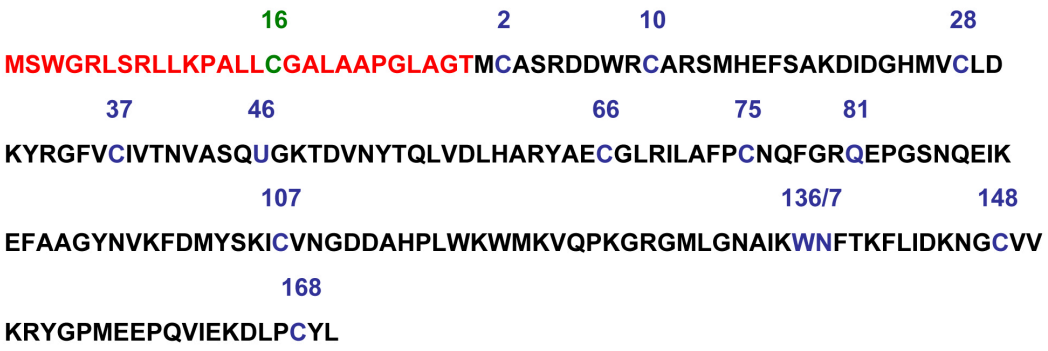

Figure 4: Primary amino acid sequence of mammalian *GPx4.* Shown is the amino acid sequence of the primary strcture of the short form of *GPx4* (black). The long form consists of the same amino acid sequence with an additional 27 amino acid Mls sequence (red). Mutated aa are shown in colour green (Mls) and blue (common sequence) including their position within the respective polypeptide chain.

Although the overall amino acid sequence homology of GPx4 is less than 40 % compared with other Sec-containing GPxs, they do share conserved motifs, particularly the catalytic tetrad, made up of Sec, Gln, Trp and Asn (Figure 5).

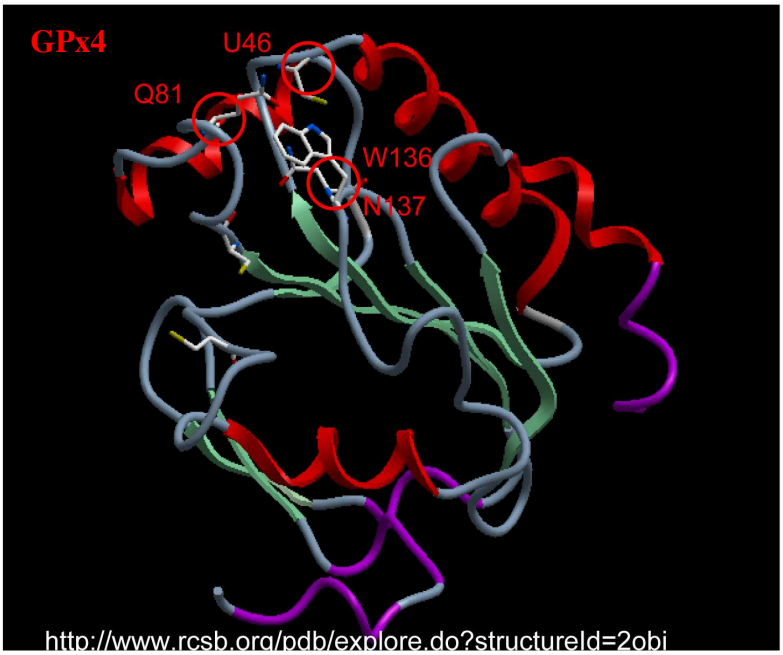

Figure 5: Crystral structure of human *GPx4.* The 3D model of GPx4 was rotated and labelled to show the catalytic tetrad of human *GPx4 (U46, Q81, W136 and N137)*, which is labelled in red. Structural features are encoded as follows; α-helices in red, β-sheets in light green, flexible linker regions in violet and other aa in grey.

This conserved tetrad has been shown to form a catalytic centre in which the selenol group of Sec is stabilized and activated by hydrogen bonds, provided by the Gln and Trp residues (Maiorino, Aumann et al. 1995). An initial mutational and biochemical approach by Maiorino et al. revealed that the conversion of Sec to Cys in pig GPx4 causes a decrease of GPx4 activity by about three orders of magnitude in the recombinant protein (Maiorino, Aumann et al. 1995). Furthermore biochemical *in vitro* experiments suggested that a change in pH, due to the substitution of amino acids in the catalytic tetrad might have a negative effect upon GPx4 function (Tosatto, Bosello et al. 2008).

1.4.2 Catalytic mechanism of GPx4

The catalytic mechanism appears to be similar among all GPxs. Except for GPx4 the intermediate steps have not been formally proven e.g. by the use of mass spectrometry. GPx4 catalysis involves redox shuttling of the Sec residue within the active site (Figure 6). The dissociated selenol (-Se^-) from Sec is oxidized by a hydroperoxide to yield selenenic acid (-SeOH) and water. The selenenic acid reacts with a free thiol (-SH), typically GSH, to form an intermediate selenenyldisulfide, which in turn is resolved by a second GSH molecule (Figure 6 A).

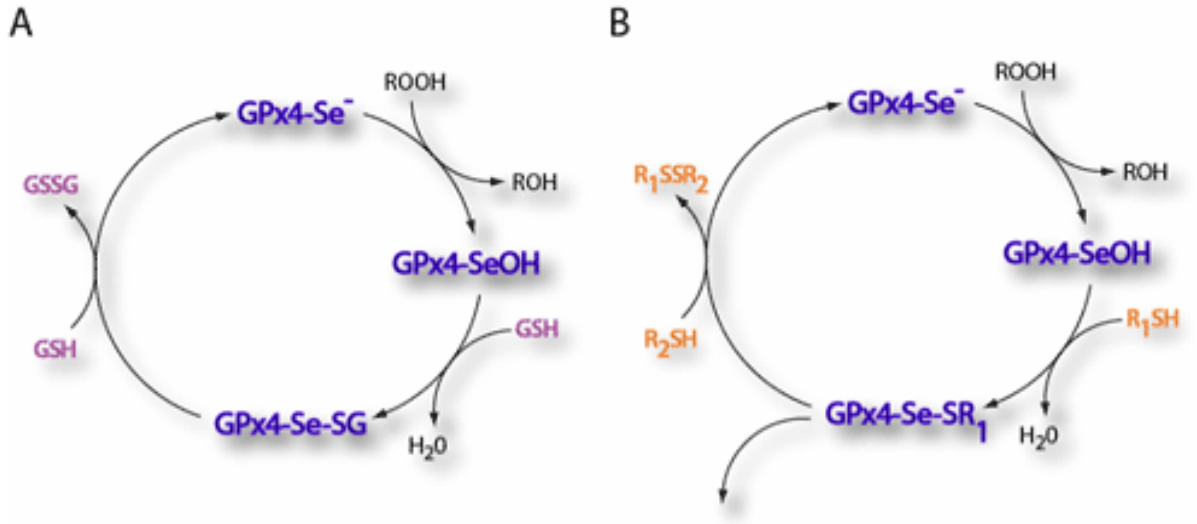

Figure 6: The catalytic cycle of GPx4. The selenol (GPx4-Se^-) is first oxidized by a hydroperoxide to selenenic acid (GPx4-SeOH), following reduction to an intermediate selenodisulfide with GSH (A) or other thiol containing substrate (B). GPx4 is reconstituted into its ground state by a second reducing equivalent, yielding an oxidized GSH dimer (GSSG) (A) or another thiol containing substrate (B) (adopted from (Conrad 2009)) R, R_1 and R_2 mark protein moieties.

In contrast to other GPxs, GPx4 does not only use GSH as a reducing factor. It has been shown for instance in mature spermatozoa, that GPx4 reacts with thiols in proteins, in

particular when GSH is limited. In mature spermatozoa nGPx4 acts as a protein thiol peroxidase by introducing disulfide bridges into protamines, and thus contributes to the structural stability of sperm chromatin (Conrad, Moreno et al. 2005) (Figure 6 B).
In addition to H_2O_2 and lipid hydroperoxides, both common substrates of glutathione peroxidases, GPx4 efficiently reduces phospholipid hydroperoxides within biological membranes and in lipoprotein particles (Ursini, Maiorino et al. 1982). For this reason, GPx4 has been implicated in the protection of cellular membranes against oxidative damage. Two structural features may explain the broad substrate specificity of GPx4. Firstly, GPx4 acts as a monomer and the active site selenol is displayed on the hydrophobic surface of the enzyme. Secondly, arginine residues that direct GSH to the active site are present in GPx1, GPx2 and GPx3, but are missing in GPx4. Instead, two lysine residues (Lys48, Lys125), which are less specific for GSH, guide the sulphur atom of various thiol-containing molecules to the active site of GPx4 (Ursini, Maiorino et al. 1997, Ursini, Heim et al. 1999, Roveri, Ursini et al. 2001, Mauri, Benazzi et al. 2003).

1.4.3 Cellular functions of GPx4

1.4.3.1 The cytosolic form of GPx4

Due to its peroxidative function, GPx4 has been frequently linked with apoptosis regulation. For instance, forced expression of GPx4 protects from many apoptotic triggers like pro-oxidants, DNA damaging agents, glucose depletion and irradiation (reviewed in (Conrad, Schneider et al. 2007)). GPx4 also protects Burkitt's lymphoma cells from oxidative stress-induced cell death imposed by low cell density (Brielmeier, Bechet et al. 2001).
Besides its "antiapoptotic" function, GPx4 regulates the arachidonic acid metabolising enzymes lipoxygenases (LOX) and cyclooxygenases (COX) like other GPxs. COX converts arachidonic acid into prostaglandin (PG)G_2 and later to PGH_2, the precursor of various prostanoids (Funk 2001). LOX oxygenates arachidonic acid at different positions and the resulting hydroperoxyeicosatetraenoic acids (HPETE) are subsequently either reduced to hydroxyeicosatetraenoic acid (HETE) or transformed into leukotrienes, hepoxilins or lipoxins (Funk and Cyrus 2001). As LOX and COX require a certain cellular peroxide level for initial activation and full activity (Ivanov, Saam et al. 2005), GPx4 must be considered as one of the main regulators of both types of enzymes. This hypothesis is supported by the fact that GPx4 knock-down leads to up-regulation of 12-LOX and COX 1 enzym activities in a human

carcinoma cell line (Chen, Huang et al. 2003), while GPx4 overexpression impairs arachidonic acid metabolism and leukotriene secretion. Furthermore, GPx4 controls also 5-, 12-, and 15-LOX activities (reviewed in (Conrad, Schneider et al. 2007)).

Interestingly, it was shown that only the short form of GPx4 is essential for survival of mice (Liang, Yoo et al. 2009). In the same study it was also shown that it can localise to mitochonria, despite the lack of a mitochondrial signalling peptide at its N-terminus. The presence of the short form of GPx4 in mitochondria was identical with the one observed in wild-type mice but male mice expressing only the short form of GPx4 showed sperm malformations and were infertile (Schneider, Forster et al. 2009). It therefore seems that the short form of GPx4 is responsible for proper embryonic development and function of adult tissues, whereas the long form of GPx4 is only important for male fertility (Schneider, Forster et al. 2009).

1.4.3.2 The long (mitochondrial) form of GPx4 (mGPx4)

As described above, a peculiarity of GPx4 is its less restricted specificity for its reducing agent GSH (Ursini, Maiorino et al. 1997). Ursini and colleagues were the first to provide *in vivo* proof that GPx4 is able to inactivate itself during sperm maturation (Ursini, Heim et al. 1999). It was discovered that GPx4 acts as the major structural protein of the mitochondrial capsule of spermatozoa where it is cross-linked with other capsular proteins. Due to this cross-linking it appears to be present in an inactive form as it lacks GPx4 enzyme activity. Later it was also shown, that GPx4 is cross-linked with other capsular proteins via (selenenyl)disulfide bridges (Ursini, Heim et al. 1999, Maiorino, Roveri et al. 2005). As maturing sperm cells are practically devoid of GSH, the mechanism of inactivation is thought to be related to GSH depletion (Shalgi, Seligman et al. 1989).

Knockout studies of mice lacking exclusively the mitochondrial form revealed that mGPx4 null mice are fully viable (Schneider, Forster et al. 2009). GPx4 expression levels were shown to be strongly decreased in testis while other organs did not display any differences, demonstrating for the first time that mGPx4 is the prevailing form in testis. As male *mGPx4*$^{-/-}$ mice are infertile it became clear that mGPx4 plays an essential role in spermatogenesis (Schneider, Forster et al. 2009). Ultrastructural analysis revealed severe sperm abnormalities in the midpiece of mature spermatozoa, which are typical features of sperm derived from selenium-deprived rodents (Wallace E. 1983, Watanabe and Endo 1991). Apparently, mGPx4 is required as an essential structural component of mitochondrial capsules in sperm, but it may also be involved in the cross-linking of capsular proteins. These studies firmly established that mGPx4 confers the vital role of selenium in male fertility (Schneider, Forster

et al. 2009) after first observations that rodents kept on a Se low diet develop male infertility (Gunn, Gould et al. 1967).

1.4.3.3 The nuclear form of GPx4 (nGPx4)

In 2001, a collaborative study between Behne's and Conrad's groups reported on a selenoprotein in rats, which was exclusively expressed in the nuclei of late spermatids. Its appearance coincides with the reorganization of DNA, which leads to a highly condensed chromatin stabilized by cross-linked protamine thiols (Pfeifer, Conrad et al. 2001). This selenoprotein was discovered as a novel form of GPx4, called sperm-nuclei specific GPx4 (previously referred to as snGPx4, now nGPx4). Subsequent studies revealed that nGPx4 is expressed from its own promoter and exclusively expressed in testis (Moreno, Laux et al. 2003). Knockout studies targeting exclusively the nuclear form provided first *in vivo* proof that GPx4 harbors protein thiol peroxidase activity *in vivo* (Moreno, Laux et al. 2003). Yet nGPx4 knockout mice are fully viable and only show a delayed chromatin condensation during spermatogenesis. The nuclear form of GPx4 has a long N-terminal extension (~ 15 kDa) encoding for a nuclear localization signal and clusters rich in arginine and lysine residues (Pfeifer, Conrad et al. 2001). The latter are most likely required for binding of nGPx4 to DNA in order to introduce disulfide bridges into protamines to ensure a tightly packed and protected male genome of sperm cells (Conrad, Moreno et al. 2005). Due to the specific role of nGPx4 in sperm chromatin compaction, it was not included in experiments performed in this work.

1.5 Objectives

While the impact of the overall cellular redox balance on cell decisions has been recognized in recent years, systems that sense and transduce changes in redox balance and thus control distinctive cellular pathways are still poorly understood. The main reasons are (i) the high complexity and partial redundancy of cellular redox systems in particular the glutathione and thioredoxin dependent systems, (ii) the very transient nature of redox-regulated processes and thus inherent technical limitations, and (iii) limitations of suitable knockout models allowing to address the molecular mechanisms of cellular redox control. In addition, interacting partners as well as novel substrates of redox enzymes are largely unknown.
The selenoenzyme GPx4 is emerging as an important redox controlling enzyme in mammals. This was confirmed by targeted disruption of GPx4 in mice, which is associated with early embryonic lethality. While some aspects of GPx4, in particular its antioxidant function and its significance in male spermatogenesis, have been analysed to some extent, experimental systems to study its suspected role as a mammalian redox sensor have been lacking so far. The role of GPx4 in the cellular redox network is only beginning to be recognized. Therefore, the specific aims of this study were:

- To elucidate the functional relevance of the catalytically active Sec in the cell death preventing effect of GPx4 based on previous studies performed in our laboratory
- To investigate the significance of the remaining amino acids of the catalytic tetrad of GPx4 in cells
- To study the functional role of the remaining Cys, which would also allow to identify a new Cys as a possible resolving Cys in the catalytic cycle

In conclusion, these studies would provide novel insights concerning the structure-function relationship of distinct Cys (Sec) residues of one of the most central redox-regulating enzymes in mammals, glutathione peroxidase 4, and address a possible advantage of selenothiol- versus thiol-based catalysis in view of a rescuing effect *in vitro.*

2 Materials

2.1 Antibodies

Antibody (source)	Company	Catalog-No.
α -Actin (rabbit)	Santa Cruz Biotechnology, Heidelberg, Germany	sc-58679
α-FLAG (mouse)	Sigma-Aldrich GmbH, Taufkirchen, Germany	F2555
α-HA High Affinity, clone 3F10 (rat)	Roche Diagnostics, Mannheim, Germany	11 867 423 001
α-Mouse-HRP conjugate (goat)	Promega GmbH, Mannheim, Germany	W4021
α-GPx4 mGPx4 1B4 (rat)	Elisabeth Kremmer, HMGU, Munich, Germany	
α-Rabbit-Cy5 conjugate (goat)	Invitrogen, Karlsruhe, Germany	A21244
α-rabbit-HRP conjugate (goat)	Santa Cruz Biotechnology, Heidelberg, Germany	sc-2768
α-Rat-HRP conjugate (goat)	Dianova GmbH, Hamburg, Germany	112-035-062
α-Rat-Cy3 conjugate (mouse)	Jackson Immunoresearch Europe Ltd., UK	212-165-168
α-alpha-Tubulin (mouse)	Sigma-Aldrich GmbH, Taufkirchen, Germany	T6199
Mitotracker Red CMXRos	Invitrogen, Karlsruhe, Germany	M7512

Table 2: Antibodies and dilutions used in this thesis.

Antibody	Used in	Dilution
α -Actin	Immunoblot	1:1000
α-FLAG (mouse)	Immunoblot Immunofluorescence	1:1000 1:200
α-HA High Affinity, clone 3F10 (rat) supernatent	Immunoblot	1:5
α-Mouse-HRP conjugate (goat)	Immunoblot	1:1000
α-GPx4 mGPx4 1B4 (rat) supernatant	Immunoblot Immunofluorescence	1:5 1:1
α-Rabbit-Cy5 conjugate (goat)	Immunofluorescence	1:100
α-rabbit-HRP conjugate (goat)	Immunoblot	1:1000
α-Rat-HRP conjugate (goat)	Immunoblot	1:1000
α-Rat-Cy3 conjugate (mouse)	Immunofluorescence	1:200
α-alpha-Tubulin (mouse)	Immunoblot	1:1000

2.2 Bacteria

Strain	Company
DH5α cells	Invitrogen, Karlsruhe, Germany

2.3 Cell lines

HEK293 T cells were used as packaging cell line for the production of lentiviral particles, based on the third generation lentiviral vector p442-PL1. (Kafri, van Praag et al. 1999)

Immortalized MEFs were derived from primary MEFs, which were isolated from embryos carrying either one or two loxP-flanked *GPx4* alleles, yielding PFa43$^{(flox/wt)}$, PFa1$^{(flox/flox)}$, PFa24$^{(flox/flox)}$, and PFa37$^{(flox/flox)}$ cells, respectively (Seiler, Schneider et al. 2008). MEFs were transfected with pCAG-3SIP-MCM, yielding cell line PFa1$^{(flox/flox)}$-MCM, PFa24$^{(flox/flox)}$-MCM, PFa37$^{(flox/flox)}$-MCM, and PFa43$^{(flox/wt)}$-MCM, respectively(Seiler, Schneider et al. 2008). The cell-lines PFa1$^{(flox/flox)}$-MCM and PFa43$^{(flox/wt)}$-MCM were used for the studies described in this work.

2.4 Chemicals

Chemicals	Company	Catalog-No.
Acrylamide	Bio-Rad, Munich, Germany	161-0158
Agarose, electrophoresis grade	Invitrogen, Karlsruhe, Germany	15510-027
Agarose, low melting point	Fermentas GmbH, St. Leon-Rot, Germany	R0801
Ammonium persulfate	Sigma-Aldrich GmbH, Taufkirchen, Germany	A3678
Ampicillin	Sigma-Aldrich GmbH, Taufkirchen, Germany	A9518
α-Tocopherol (Toc)	Sigma-Aldrich GmbH, Taufkirchen, Germany	T3251
β-Mercaptoethanol (2-ME)	Roth Carl GmbH & Co., Karlsruhe, Germany	4227.1
Bovine Albumin	MP Biomedicals, Eschwege, Germany	840043
Bromophenol Blue Sodium Salt	Merck KGaA, Darmstadt, Germany	111746
Buthionine Sulfoximine (BSO)	Sigma-Aldrich GmbH, Taufkirchen, Germany	B2640
4´,6-diamidino-2-phenylindole (DAPI)	Invitrogen, Karlsruhe, Germany	D1306
dNTP Mix	Fermentas GmbH, St. Leon-Rot, Germany	R0241
DMSO (Dimethylsulfoxide)	Sigma-Aldrich GmbH, Taufkirchen, Germany	D2650
DTT (Dithiothreitol)	Sigma-Aldrich GmbH, Taufkirchen, Germany	D8161
ECL	GE Healthcare, Freiburg, Germany	RPN2106
EDTA	Sigma-Aldrich GmbH, Taufkirchen, Germany	E9884
EGTA	Sigma-Aldrich GmbH, Taufkirchen, Germany	O3777
Ethanol p.a.	Merck, Darmstadt, Germany	1.00983.2500
Ethidiumbromide	Merck, Darmstadt, Germany	70257083
Foetal Bovine Serum	PAA, Pasching, Austria	A15-043
Glutathione (GSH)	Sigma-Aldrich GmbH, Taufkirchen, Germany	G6013
Glycine	MP Biomedicals, Eschwege, Germany	808831
Gelatin from porcine skin	Sigma-Aldrich GmbH, Taufkirchen, Germany	G2500
Hydroperoxide	Sigma-Aldrich GmbH, Taufkirchen, Germany	H1009
4-Hydroxytamoxifen (Tam)	Sigma-Aldrich GmbH, Taufkirchen, Germany	H7904
Isopropyl-β-d-thiogalactopyranoside (IPTG)	Sigma-Aldrich GmbH, Taufkirchen, Germany	I6758
Iodoacetamide	Sigma-Aldrich GmbH, Taufkirchen, Germany	I6125
Isopropanol p.a.	Merck, Darmstadt, Germany	1.09634.2511
L-Glutamine	Invitrogen, Karlsruhe, Germany	25030
Mounting medium	Dako Cytomation, Hamburg, Germany	S3023
N-Acetyl-L-Cysteine	Sigma-Aldrich GmbH, Taufkirchen, Germany	A9165
Oligonucleotides	Metabion GmbH, Martinsried, Germany	
Pareformaldehyde (PFA)	Roth Carl GmbH & Co., Karlsruhe, Germany	0335.3
Penicillin-Streptomycin	Invitrogen, Karlsruhe, Germany	15140-122
Phenol	Sigma-Aldrich GmbH, Taufkirchen, Germany	P1037
Phenol:Chloroform:Isoamylalcohol	MP Biomedicals, Eschwege, Germany	802520
Propidium Iodide (PI)	Sigma-Aldrich GmbH, Taufkirchen, Germany	P4170
Protease Inhibitor Cocktail	Tablets Roche Diagnostics, Mannheim, Germany	1 697 498
Puromycin	Sigma-Aldrich GmbH, Taufkirchen, Germany	P7255

Chemicals	Company	Catalog-No.
Select Agar	Invitrogen, Karlsruhe, Germany	30391-023
Skim Milk Powder	Fluka Chemie GmbH, Buchs, Switzerland	70166
Sodium Chloride	MP Biomedicals, Eschwege, Germany	194848
Sodium Deoxocholate	Sigma-Aldrich GmbH, Taufkirchen, Germany	D6750
Sodium Dodecyl Sulfate (SDS)	Fluka Chemie GmbH, Buchs, Switzerland	71729
Sodium Hydroxide	Roth Carl GmbH & Co., Karlsruhe, Germany	UN1823
Sodium Pyrophosphate Tetrabasic Decahydrate	Sigma-Aldrich GmbH, Taufkirchen, Germany	S6422
TEMED	Fluka Chemie GmbH, Buchs, Switzerland	87689
Tris-Base	Merck, Darmstadt, Germany	1.08382
Triton X-100	GE Healthcare, Freiburg, Germany	17-1315-01
Trypan Blue (0.4%)	Sigma-Aldrich GmbH, Taufkirchen, Germany	T8154
Trypsin-EDTA	Invitrogen, Karlsruhe, Germany	25300
Tween 20	Sigma-Aldrich GmbH, Taufkirchen, Germany	P5927

2.5 Cloning

Sequence of *GPx4* cDNA: (MIs is shown in red and the codons mutated in this work are shown in bold)

ATGAGCTGGGGCCGTCTGAGCCGCTTACTTAAGCCAGCACTGCTG**TGC**GGGGCTCTGGCTGCGCCTGGTCT
GGCAGGCACCATG**TGT**GCATCCCGCGATGATTGGCGC**TGT**GCGCGCTCCATGCACGAATTCTCAGCCAAGG
ACATCGACGGGCACATGGTCTGCCTGGATAAGTACAGGGGTTTCGTG**TGC**ATCGTCACCAACGTGGCCTCG
CAA**TGA**GGCAAAACTGACGTAAACTACACTCAGCTAGTCGATCTGCATGCCCGATATGCTGAG**TGT**GGTTTA
CGAATCCTGGCCTTCCCC**TGC**AACCAGTTTGGGAGG**CAG**GAGCCAGGAAGTAATCAAGAAATCAAGGAGTT
TGCAGCCGGCTACAACGTCAAGTTTGACATGTACAGCAAGATC**TGT**GTAAATGGGGACGATGCCCACCCACT
GTGGAAATGGATGAAAGTCCAGCCCAAGGGCAGGGGCATGCTGGGAAATGCCATCAAA**TGGAAC**TTTACCA
AGTTTCTCATTGATAAGAACGGC**TGC**GTGGTGAAGCGCTATGGTCCCATGGAGGAGCCCCAGGTGATAGAG
AAGGACCTGCCG**TGC**TATCTCTAG

Plasmids:

Table 3: Different plasmids used in this thesis.

Name	Source	Type	Modification
pDrive-FSH-GPx4	(Seiler, Schneider et al. 2008)	Intermediate	None
pDrive-FSH-GPx4-NheI-kill	This work	Intermediate	deletion of the *Nhe*I site upstream of GPx4 using the *NheI*-kill primer pair
pDrive-FSH-GPx4-U46C-NheI-kill	This work	Intermediate	Mutation of Sec to Cys using the $GPx4^{U46C}$ primer pair
pDrive-FSH-GPx4-C2S-NheI-kill	This work	Intermediate	Mutation of Cys at position 2 to Ser using the $GPx4^{C2S}$ primer pair
pDrive-FSH-GPx4-C10S-NheI-kill	This work	Intermediate	Mutation of Cys at position 10 to Ser using the $GPx4^{C10S}$ primer pair
pDrive-FSH-GPx4-C37S-NheI-kill	This work	Intermediate	Mutation of Cys at position 37 to Ser using the $GPx4^{C37S}$ primer pair
pDrive-FSH-GPx4-C66S-NheI-kill	This work	Intermediate	Mutation of Cys at position 66 to Ser using the $GPx4^{C66S}$ primer pair
pDrive-FSH-GPx4-C75S-NheI-kill	This work	Intermediate	Mutation of Cys at position 75 to Ser using the $GPx4^{C75S}$ primer pair
pDrive-FSH-GPx4-C107S-NheI-kill	This work	Intermediate	Mutation of Cys at position 107 to Ser using the $GPx4^{C107S}$ primer pair

Name	Source	Type	Modification
pDrive-FSH-GPx4-C148S-NheI-kill	This work	Intermediate	Mutation of Cys at position 148 to Ser using the $GPx4^{C148S}$ primer pair
pDrive-FSH-GPx4-C168S-NheI-kill	This work	Intermediate	Mutation of Cys at position 168 to Ser using the $GPx4^{C168S}$ primer pair
pDrive-FSH-GPx4-Q81A-NheI-kill	This work	Intermediate	Mutation of Gln at position 81 to Ala using the $GPx4^{Q81A}$ primer pair
pDrive-FSH-GPx4-Q81E-NheI-kill	This work	Intermediate	Mutation of Gln at position 81 to Glu using the $GPx4^{Q81E}$ primer pair
pDrive-FSH-GPx4-W136D-NheI-kill	This work	Intermediate	Mutation of Trp at position 136 to Asp using the $GPx4^{C2S}$ primer pair
pDrive-FSH-GPx4-N137A-NheI-kill	This work	Intermediate	Mutation of Asn at position 137 to Ala using the $GPx4^{C2S}$ primer pair
pDrive-FSH-GPx4-N137D-NheI-kill	This work	Intermediate	Mutation of Asn at position 137 to Asp using the $GPx4^{C2S}$ primer pair
pDrive-Mls-FSH-GPx4-NheI-kill	This work	Intermediate	Mls sequence was added to the FSH-GPx4 via overlapping PCR
pDrive-Mls-FSH-GPx4-U46C-NheI-kill	This work	Intermediate	Mls sequence was added to the FSH-GPx4-U46C via overlapping PCR
pDrive-FSH-GPx4-NheI-kill-delta-SECIS	This Work	Intermediate	Removal of the SECIS element by *NheI* digestion of pDrive-FSH-GPx4-NheI-kill
pDrive-FSH-GPx4-NheI-kill-U46C-delta-SECIS	This work	Intermediate	Removal of the SECIS element by *NheI* digestion of pDrive-FSH-GPx4-U46C-NheI-kill
FSH-GPx4-allCys/Ser	Geneart	Intermediate	All Cys in GPx4 mutated to Ser
pDrive-FSH-GPx4-allCys/Ser	This work	Intermediate	Transfer of the GPx4 containing fragment (954 Bp) via *BamHI/XbaI* into the same sites of pDrive
pDrive-FSH-GPx4-allCys/Ser-U46C	This work	Intermediate	Transfer of the GPx4 containing fragment (954 Bp) via *BamHI/XbaI* into the same sites of pDrive
442-PL1	Tim Schröder	Expression	pRRL.PPT.SF.IRES-VENUSnucmem
391	Tim Schröder	Expression	K73 pEcoEnv-IRES-puro
392	Tim Schröder	Expression	pRSV_Rev

Name	Source	Type	Modification
393	Tim Schröder	Expression	pMDLg_pRRE
442-PL1-FSH-GPx4	This work	Expression	Transfer of the GPx4 containing fragment (954 Bp) from pDrive into 442-PL1 via *BamHI/XbaI*
442-PL1-FSH-GPx4-U46C	This work	Expression	Transfer of the GPx4 containing fragment (954 Bp) from pDrive into 442-PL1 via *BamHI/XbaI*
442-PL1-FSH-GPx4-C2S	This work	Expression	Transfer of the GPx4 containing fragment (954 Bp) from pDrive into 442-PL1 via *BamHI/XbaI*
442-PL1-FSH-GPx4-C10S	This work	Expression	Transfer of the GPx4 containing fragment (954 Bp) from pDrive into 442-PL1 via *BamHI/XbaI*
442-PL1-FSH-GPx4-C37S	This work	Expression	Transfer of the GPx4 containing fragment (954 Bp) from pDrive into 442-PL1 via *BamHI/XbaI*
442-PL1-FSH-GPx4-C66S	This work	Expression	Transfer of the GPx4 containing fragment (954 Bp) from pDrive into 442-PL1 via *BamHI/XbaI*
442-PL1-FSH-GPx4-C75S	This work	Expression	Transfer of the GPx4 containing fragment (954 Bp) from pDrive into 442-PL1 via *BamHI/XbaI*
442-PL1-FSH-GPx4-C107S	This work	Expression	Transfer of the GPx4 containing fragment (954 Bp) from pDrive into 442-PL1 via *BamHI/XbaI*
442-PL1-FSH-GPx4-C148S	This work	Expression	Transfer of the GPx4 containing fragment (954 Bp) from pDrive into 442-PL1 via *BamHI/XbaI*
442-PL1-FSH-GPx4-C168S	This work	Expression	Transfer of the GPx4 containing fragment (954 Bp) from pDrive into 442-PL1 via *BamHI/XbaI*
442-PL1-FSH-GPx4-Q81A	This work	Expression	Transfer of the GPx4 containing fragment (954 Bp) from pDrive into 442-PL1 via *BamHI/XbaI*
442-PL1-FSH-GPx4-Q81E	This work	Expression	Transfer of the GPx4 containing fragment (954 Bp) from pDrive into 442-PL1 via *BamHI/XbaI*
442-PL1-FSH-GPx4-W136D	This work	Expression	Transfer of the GPx4 containing fragment (954 Bp) from pDrive into 442-PL1 via *BamHI/XbaI*
442-PL1-FSH-GPx4-N137A	This work	Expression	Transfer of the GPx4 containing fragment (954 Bp) from pDrive into 442-PL1 via *BamHI/XbaI*
442-PL1-FSH-GPx4-N137D	This work	Expression	Transfer of the GPx4 containing fragment (954 Bp) from pDrive into 442-PL1 via *BamHI/XbaI*
442-PL1-FSH-GPx4-allCys/Ser	This work	Expression	Transfer of the GPx4 containing fragment (954 Bp) from pDrive into 442-PL1 via *BamHI/XbaI*
442-PL1-FSH-GPx4-allCys/Ser-U46C	This work	Expression	Transfer of the GPx4 containing fragment (954 Bp) from pDrive into 442-PL1 via *BamHI/XbaI*
442-PL1-Mls-FSH-GPx4	This work	Expression	Transfer of the GPx4 containing fragment (995 Bp) from pDrive into 442-PL1 via *BamHI/XbaI*
442-PL1-Mls-FSH-GPx4-U46C	This work	Expression	Transfer of the GPx4 containing fragment (995 Bp) from pDrive into 442-PL1 via *BamHI/XbaI*
442-PL1-MlsC16S-FSH-GPx4	This work	Expression	Mutation of Cys to Ser at position 16 in the Mls using the MlsC16S-GPx4 primer pair

Primer:

Tabel 4: Primers and primer-pairs used in this thesis.

Name	Sequence	Purpose
GPx4^{U46C}	5'-GTGGCCTCGCAATGCGGCAAAACTGACGTAAAC-3' 5'-TTTACGTCAGTTTTGCCGCATTGCGAGGCCACG-3'	Substitution of Sec with Cys at position 46
GPx4^{U46S}	5'-GTGGCCTCGCAAAGCGGCAAAACTGACGTAAAC-3' 5'-TTTACGTCAGTTTTGCCGCTTTGCGAGGCCACG-3'	Substitution of Sec with Ser at position 46
GPx4^{C2S}	5'-GATTACGCTTCTGCATCCCGCGATGATT-3' 5'-AATCATCGCGGGATGCAGAAGCGTAATC-3'	Substitution of Cys with Ser at position 2
GPx4^{C10S}	5'-GATGATTGGCGCTCTGCGCGCTCCATGC-3' 5'-GCATGGAGCGCGCAGAGCGCCAATCATC-3'	Substitution of Cys with Ser at position 10
GPx4^{C37S}	5'-GGTTTCGTGTCCATCGTCACCAACGTGG-3' 5'-CCACGTTGGTGACGATGGACACGAAACC-3'	Substitution of Cys with Ser at position 37
GPx4^{C66S}	5'-CGATATGCTGAGTCTGGTTTACGAATCC-3' 5'-GGATTCGTAAACCAGACTCAGCATATCG-3'	Substitution of Cys with Ser at position 66
GPx4^{C75S}	5'-CTGGCCTTCCCCTCCAACCAGTTTGGGA-3' 5'-TCCCAAACTGGTTGGAGGGGAAGGCCAG-3'	Substitution of Cys with Ser at position 75
GPx4^{C107S}	5'-TGTACAGCAAGATCTCTGTAAATGGGGACGATGC-3' 5'-GCATCGTCCCCATTTACAGAGATCTTGCTGTACA-3'	Substitution of Cys with Ser at position 107
GPx4^{C148S}	5'-AAGAACGGCTCCGTGGTGAAGCGCTATG-3' 5'-CATAGCGCTTCACCACGGAGCCGTTCTT-3'	Substitution of Cys with Ser at position 148
GPx4^{C168S}	5'-GACCTGCCGTCCTATCTCTAGCTAGCCC-3' 5'-GGGCTAGCTAGAGATAGGACGGCAGGTC-3'	Substitution of Cys with Ser at position 168
GPx4^{Q81A}	5'-CCAGTTTGGGAGGGCGGAGCCAGGAAGTAATC 5'-GATTACTTCCTGGCTCCGCCCTCCCAAACTGG	Substitution of Gln with Ala at position 81
GPx4^{Q81E}	5'-CAGTTTGGGAGGGAGGAGCCAGGAAGTA-3' 5'-TACTTCCTGGCTCCTCCCTCCCAAACTG-3'	Substitution of Gln with Glu at position 81
GPx4^{W136D}	5'-GGAAATGCCATCAAAGACAACTTTACCAAGTTTC-3' 5'-GAAACTTGGTAAAGTTGTCTTTGATGGCATTTCC-3'	Substitution of Trp with Asp at position 136
GPx4^{N137A}	5'-GCCATCAAATGGGCCTTTACCAAGTTTCTC-3' 5'-GAGAAACTTGGTAAAGGCCCATTTGATGGC-3'	Substitution of Asn with Ala at position 137
GPx4^{N137D}	5'-GCCATCAAATGGGACTTTACCAAGTTTCTC-3' 5'-GAGAAACTTGGTAAAGTCCCATTTGATGGC-3'	Substitution of Asn with Asp at position 137
GPx4$^{\Delta SECIS\ U46C}$	5'-GTGGCCTCGCAATGCGGCAAAACTGACGTAAAC-3' 5'-TTTACGTCAGTTTTGCCGCATTGCGAGGCCACG-3'	Substitution of Sec with Cys at position 46
Mls^{C16S}-GPx4	5'-GCCAGCACTGCTGTCCGGGGCTCTGGCTGCG-3' 5'-CGCAGCCAGAGCCCCGGACAGCAGTGCTGGC-3'	Substitution of Cys with Ser at position 16 of the Mls
NheI-kill	5'-CGAGAAAGGAGCCAGCTACCCATACGATGTTCC-3' 5'-GGCGATAGAAGGCGATGCGCTGCGAATCG-3'	Deletion of the *NheI* site upstream of GPx4
pRTS-GPx4	5'-CCAAGGTGATCATTAGGCAGCCAGG-3' 5'-GCGTCGATTTAAATCGCCACCATGG-3'	Addition of a *BclI* restriction site upstream and *SwaI* restriction site downstream of GPx4 for cloning of FSH-GPx4 or variant

Name	Sequence	Purpose
Oligo Mls-GPx4-f1	5'-ATTGGATCCGAGATGAGCTGGGGCCGTC-3'	introduction of a *BamHI* site 5' from the Mls
Oligo Mls-GPx4-f2	5'-CGCCTGGTCTGGCAGGCACCTACCCATACGATGTTCCAGA-3'	introduction of an overlap corresponding to the end of the Mls in front of FSH-GPx4 and elimination of FSH-tag AUG
Oligo Mls-GPx4-f3	5'-CGCCTGGTCTGGCAGGCACCGATTATAAAGATGATGATGA-3'	introduction of an overlap corresponding to the end of the Mls in front of HA-GPx4 and elimination of HA-tag AUG
Oligo Mls-GPx4-r1	5'-TCTGGAACATCGTATGGGTAGGTGCCTGCCAGACCAGGCG-3'	Introduction of an overlap corresponding to the beginning of the FSH-tag behind the Mls
Oligo Mls-GPx4-r2	5'-GGCCACGTTGGTGACGATGC-3'	amplification of FSH-GPx4 and HA-GPx4, respectively
Oligo Mls-GPx4-r3	5'-TCATCATCATCTTTATAATCGGTGCCTGCCAGACCAGGCG-3'	Introduction of an overlap corresponding to the beginning of the HA-tag behind the Mls

All oligonucleotides were obtained from Metabion GmbH, Martinsried, Germany.

2.6 Disposables and Kits

Item	Company	Catalog-No.
DMEM	Invitrogen, Karlsruhe, Germany	41966
Gel Extraction Kit	QIAGEN GmbH, Hilden, Germany	28704
Hybond-C super membrane	GE Healthcare, Freiburg, Germany	RPN203G
Hybond-N_+ membrane	GE Healthcare, Freiburg, Germany	RPN303B
Hyperfilm	GE Healthcare, Freiburg, Germany	RPN3103K
X-ray film	GE Healthcare, Freiburg, Germany	RPN16778K
JETstar Plasmid purification system	Genomed GmbH, Löhne, Germany	220020
LightCycler Capillaries	Roche Diagnostics, Mannheim, Germany	11 909 339 001
Plasmid Maxi Kit	QIAGEN GmbH, Hilden, Germany	12163
PCR Cloning Kit	QIAGEN GmbH, Hilden, Germany	231122
RNeasy Mini Kit	QIAGEN GmbH, Hilden, Germany	74104
Rediprime II random prime labelling system	GE Healthcare, Freiburg, Germany	RPN1633
Sephadex G-50 columns	GE Healthcare, Freiburg, Germany	27-5330-01
Sep-Pak C 18 cartridges	Waters GmbH, Eschborn, Germany	WAT051910

2.7 Enzymes

Enzyme	Company	Catalog-No.
BamHI	Fermentas GmbH, St. Leon-Rot, Germany	ER0051
BclI	Fermentas GmbH, St. Leon-Rot, Germany	ER0721
DNA Polymerase I (Klenow)	New England Biolabs GmbH, Frankfurt, Germany	M0210S
DpnI	Fermentas GmbH, St. Leon-Rot, Germany	ER1701
EcoRI	Fermentas GmbH, St. Leon-Rot, Germany	ER0271
HindIII	Fermentas GmbH, St. Leon-Rot, Germany	ER0501
NheI	Fermentas GmbH, St. Leon-Rot, Germany	ER0971
NotI	Fermentas GmbH, St. Leon-Rot, Germany	ER0591
Pfu DNA Polymerase	Promega GmbH, Mannheim, Germany	M774A
Phosphatase, alkaline	Roche Diagnostics, Mannheim, Germany	713 023
Proteinase K	Roth Carl GmbH & Co., Karlsruhe, Germany	7528.1
Restriction Endonucleases	New England Biolabs GmbH, Frankfurt, Germany	
T4 DNA Ligase	Promega GmbH, Mannheim, Germany	M180A
Taq DNA Polymerase	Invitrogen, Karlsruhe, Germany	18038-026
XbaI	Fermentas GmbH, St. Leon-Rot, Germany	ER0681

2.8 Equipment

Equipment	Company
FACS Calibur	BD GmbH, Heidelberg, Germany
GenAmp PCR system 2700	Applied Biosystems, Darmstadt, Germany
Gene Pulser II System	Bio-Rad, Munich, Germany
Gene Pulser Electroporation Cuvettes, 0.4 cm gap	Bio-Rad, Munich, Germany
Heraeus Incubator, Modell B 5060	Heraeus Holding GmbH, Hanau, Germany
Leica DM IRBE confocal microscope	Leica Microsystems GmbH, Wetzlar, Germany
Leica TCS SP2 scanner	Leica Microsystems GmbH, Wetzlar, Germany
Light-Cycler 1.5	Roche Diagnostics, Mannheim, Germany
LS 5000 TA Scintillation Counter	Beckman Coulter GmbH, Krefeld, Germany
Microscope Axiovert 135	Carl Zeiss Jena GmbH, Göttingen, Germany
Microscope Axiovert 200M	Carl Zeiss Jena GmbH, Göttingen, Germany
Mini-PROTEAN 3 Electrophoresis Cell	Bio-Rad, Munich, Germany
Mini-Sub Cell GT Electrophoresis Cell	Bio-Rad, Munich, Germany
OTD Combi Ultracentrifuge	Sorvall, Langenselbold, Germany
Photometer Bio	Eppendorf, Hamburg, Germany
PowerPac 200 Power Supply	Bio-Rad, Munich, Germany

Spectrophotometer DU-64	Beckman Coulter GmbH, Krefeld, Germany
Speed Vac Concentrator SVC100H	Savant Instruments NC, Farmingdale, N.Y. USA
Trans-Blot Semi-Dry	Bio-Rad, Munich, Germany
RF-10A XL Fluorescence Spectrophotometer	Shimadzu, Duisburg, Germany
UZ-PA-38,5-1 Ultracentrifuge Tubes	Kisker GbR, Steinfurt, Germany

2.9 Expression vectors

442-PL1

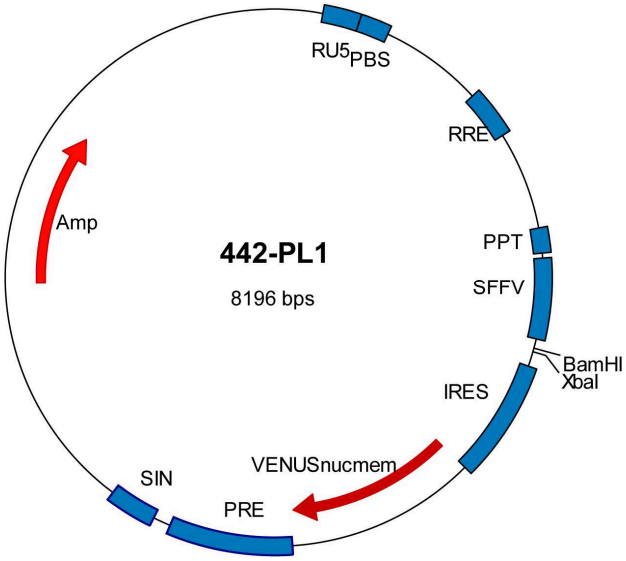

Figure7: Map of the lentiviral expression vector p442-PL1. ampR (*beta lactamase*), RU5 (RU5 LTR), PBS (primer binding site), RRE (Rev responsive element), PPT (poly-purine tract), SFFVp (spleen focus forming virus promoter), *BamHI/XbaI* (restriction sites used for cloning), IRES (internal ribosome entry site), PL1nucmem (fluorescent protein with nuclear membrane anchor), PRE (post regulatory element), SIN (self-inactivating 3' LTR).

pDrive

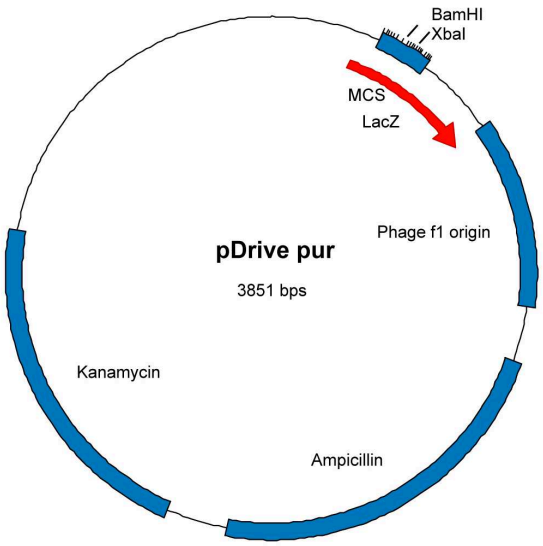

Figure 8: Map of the expression vector pDrive. (Qiagen, Hilden). MCS (multiple cloning site) containing *BamHI* and *XbaI* restriction sites used for cloning, Ampicillin resistance (*beta lactamase*) and Kanamycin resistance (*kanamycin kinase* gene).

3 Methods

3.1 Cell culture and related techniques

3.1.1 Murine embryonic fibroblasts (MEFs)

MEF cells were cultured with standard DMEM medium at 20 % O_2 and 5 % CO_2. Cells were splitted every second to third day, depending upon confluency, at a 1:10 ratio.

Standard DMEM: DMEM, 4.5 g/l glucose, 10 % FCS, 1 % glutamine, 50 U/ml penicillin G, 50 µg/ml streptomycin

3.1.2 Determination of cell number

Cells were harvested from cell culture dishes by trypsinization and suspended by adding 5 volumes of Standard DMEM. 30 µl of the cell suspension was mixed with an equal volume of 0.4 % trypan blue solution and cells were counted using a Fuchs-Rosenthal haemocytometer. Trypan blue is excluded from viable cells, whereas dead cells stain blue due to plasma membrane disruption.

Standard DMEM: DMEM, 4.5 g/l glucose, 10 % FCS, 1 % glutamine, 50 U/ml penicillin G, 50 µg/ml streptomycin

3.1.3 Cryopreservation and thawing of cells

Stocks of all cell lines were stored in liquid nitrogen. Cells were grown in 10 cm cell culture dishes until they reached approximately 80 % confluency. Cells were washed with PBS trypsinized and collected by centrifugation. The cell pellets were resuspended in FCS containing 10 % DMSO, transferred to cryo vials in 1 ml aliquots and frozen in isopropanol containers at -80 °C over night. Subsequently, vials were transferred to a liquid nitrogen tank. Frozen cells were defrosted in a water bath at 37 °C, immediately transferred to 5 ml Standard DMEM and collected by centrifugation. Cell pellets were resuspended in 10 ml Standard DMEM and seeded onto a 10 cm cell culture dish.

PBS: 80.0 g NaCl, 2.0 g KCL, 14.4 g Na_2HPO_4, 2.4 g KH_2PO_4 in 1 l H_2O, pH 7.4
Standard DMEM: DMEM, 4.5 g/l glucose, 10 % FCS, 1 % glutamine, 50 U/ml penicillin G, 50 µg/ml streptomycin

3.2 Gene transfer methods

3.2.1 Lentiviral infection

Lentivirus-based gene transfer methods mediate an efficient gene transfer into primary cells. The lentiviral infection method proved to be the most efficient technique to transfer DNA into immortalised MEFs due to integration and stable expression of transgenes in dividing as well as resting cells. Lentiviral particles were produced in HEK 293 T packaging cells with a third generation lentiviral packaging system, which provides maximal biosafety. Yet this method is more laborious, because it involves the simultaneous transfection of four different plasmids into the producer cell line. Three plasmids encode all necessary structural proteins for lentivirus production, including pEcoEnv-IRES-puro (contains glycoprotein Env), plasmid pMDLg_pRRE (contains the main structural protein Gag and the enzyme cluster Pol) and pRSV_Rev (contains the post-transcriptional regulator Rev). Third generation packaging systems are Tat independent, because the transfer vector pRRL.PPT.SF.IRES-VENUSnucmem (p442) contains an additional SFFV promoter. 5 x 10^6 HEK 293 T packaging cells were seeded in Standard DMEM on 10 cm cell culture dishes and incubated for 24 h. Plasmids (2 µg pEcoEnv-IRES-Venus, 5 µg pMDLg_pRRE, 10 µg pRSV_Rev, 5 µg p442-PL1-based vectors) were diluted in 500 µl 12.5 mM $CaCl_2$ solution and mixed with 500 ml HBS by air bubbling. Cell culture medium was replaced with TF medium, and chloroquine was added to a final concentration of 25 µM. For calcium phosphate transfection, the plasmid mix was vortexed, applied to the cell culture dish, and the cells were incubated for 8-12 h. The transfection medium was replaced with fresh TF medium and the transfected packaging cells were incubated for further 36 h. Virus-enriched TF medium was recovered from the cell culture dish, filtrated through a 0.22 mm sterile filter and centrifuged in a swing rotor ultracentrifuge for at least 16 hours at 16,000 x g. The virus containing pellet was resuspended in 200 µl Standard DMEM and stored at -80 °C. 3 x 10^5 MEFs were infected in a 3.5 cm cell culture dish by adding 50 µl of the virus concentrate and a subsequent incubation for 48 h. Infection efficiency was monitored by fluorescence microscopy, screening for VENUSnucmem expression and quantification was performed by FACS analysis.

Chloroquine (1000x): 25 mM chloroquine in PBS

HBS (2x): 50 mM HEPES, 280 mM NaCl, 1.5 mM Na_2HPO_4, pH 7.05 adjusted with NaOH

PBS: 80.0 g NaCl, 2.0 g KCL, 14.4 g Na_2HPO_4, 2.4 g KH_2PO_4 in 1 l H_2O, pH 7.4

Standard DMEM: DMEM, 4.5 g/l glucose, 10 % FCS, 1 % glutamine, 50 U/ml penicillin G, 50 µg/ml streptomycin

TF medium: Standard DMEM, 20 mM HEPES

3.2.2 Electroporation

3.2.2.1 Transfection of mammalian cells by electroporation

Cells were harvested by trypsinization, washed twice in PBS and resuspended in 250 µl FCS-free DMEM. Primary MEFs (1 x 10^6 cells) were electroporated with a Gene Pulser II (Bio Rad, Munich, Germany) in cuvettes with 0.4 cm gap width. For each transfection, 20 µg of linearized plasmid DNA were transfected into cells with an electric pulse of 230 V and a capacitance of 940 µF. FCS (500 µl) was applied to the cells immediately after the pulse. The cell suspension was gently mixed by pipetting, and cells were seeded on a 10 cm cell culture dish in Standard DMEM. Selection was initiated 24 hours after electroporation by the addition of 150 µg/ml G418.

PBS: 80.0 g NaCl, 2.0 g KCL, 14.4 g Na_2HPO_4, 2.4 g KH_2PO_4 in 1 l H_2O, pH 7.4
Standard DMEM: DMEM, 4.5 g/l glucose, 10 % FCS, 1 % glutamine, 50 U/ml penicillin G, 50 µg/ml streptomycin

3.2.2.2 Transformation of bacterial cells (XL1-Blue) by electroporation

Twenty µl of electro-competent cells were transferred into a pre-cooled 1.5 ml Eppendorf tube. 1-2 µl of diluted DNA was added to the bacteria, mixed briefly and transferred to a 1 mm cuvette. Cells were pulsed with 1.5 (KV), 25 µF and 100 Ohm. Immediately after pulsing, 1 ml of SOC medium was added to the cuvette. The cells were shaken shortly and then incubated at 37 °C for 30 min. After incubation, the cells were centrifuged for 2 min at 700 x g. Most of the supernatant was discarded and the bacteria were resuspended in the remaining SOC medium. Cells were platted on two LB agar plates at either 10 % or 90 %, of the resuspended bacteria, respectively, and plates were incubated over night at 37 °C.

SOC medium: 20 g Bacto Tryptone, 5 g Bacto Yeast Extract, 2 ml of 5 M NaCl, 2.5 ml of 1 M KCl, 10 ml of 1 M $MgCl_2$, 10 ml of 1 M $MgSO_4$, 20 ml of 1 M glucose adjust to 1 l with dH_2O
Ampicillin: 100 µg/ml

3.3 Cloning techniques

3.3.1 Preparation of competent cells

Competent cells were prepared by the rubidium chloride procedure using DH5α *E. coli* cells. 250 ml LB medium containing 20 mM $MgSO_4$ were inoculated with a single colony from an LB plate and incubated over night at 37 °C with shaking until the OD_{600} reached 0.4-0.6. Cells were collected at 4,500 x g for 5 min at 4 °C. All subsequent steps were performed in a 4 °C cold room with pre-chilled pipettes, tubes and flasks. Cells were resuspended in 100 ml of ice cold TFB1, incubated on ice for 5 min and pelleted at 4,500 x g for 5 min at 4 °C. Cells were resuspended gently in 10 ml TFB2, incubated on ice for 30 min and 200 µl aliquots were snap-frozen in liquid nitrogen. Aliquots of competent cells were stored at -80 °C. Cells were used no longer than 4 months after preparation.

LB: yeast extract (5 g/l), tryptone (10 g/l), NaCl (0.5–10 g/l), pH 7
TFB1: 30 mM potassium acetate, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 100 mM RbCl, 15 % glycerol, pH 5.8
TFB2: 10 mM 3-(N-morpholino) propansulfonic acid (MOPS), 75 mM $CaCl_2$, 10 mM RbCl, 15 % glycerol, pH 6.5

3.3.2 Transformation of bacteria by heat shock

For transformation, 200 µl aliquots were defrosted on ice and 10 µl of the ligation mix or 10 ng purified plasmid DNA were added to the cell suspension. Cells were incubated for 15 min on ice, heated to 42 °C for 90 sec and replaced on ice for 2 min. 800 µl LB medium were added to the cell suspension and incubated for 45 min at 37 °C with vigorous shaking. All plasmids used in this work contained a *beta lactamase* cassette for selection. 100 µl of the transformation mixture was plated on a LB plate containing 50 µg/ml ampicillin. Remaining cells were harvested by brief centrifugation at 10,000 x g for 20 sec to remove excess LB. The pellet was resuspended in 100 µl LB and plated onto a second LB plate with ampicillin. Agar plates were incubated for 12-20 hours at 37 °C until single cell colonies became visible.

LB: yeast extract (5 g/l), tryptone (10 g/l), NaCl (0.5–10 g/l), pH 7

3.3.3 Preparation of plasmid DNA

After transformation, single colonies were picked from LB plates and used to inoculate 2 ml LB medium, containing 50 µg/ml ampicillin. The inoculum was incubated at 37 °C for 12-20 hours with shaking. Cells were harvested by centrifugation at 10.000 x g for 20 sec and resuspended in 200 µl buffer E1 by vortexing. 200 µl of denaturing buffer E2 was added to the cell suspension, mixed by inverting the tube and incubated for 5 min at room temperature. To stop the denaturation reaction, 200 µl of buffer E3 was added, mixed by inverting and centrifuged for 6 min at 10,000 x g. To extract proteins and lipids, 400 µl of phenol-chloroform was added, vortexed and centrifuged for 3 min at 10,000 x g. Plasmid DNA dissolves in the aqueous upper phase, which was recovered and added to 500 ml isopropanol for DNA precipitation. The precipitated DNA was harvested by centrifugation for 12 min at 4 °C at 10,000 x g. The DNA pellet was washed with 70 % ethanol, dried for 30 min at room temperature and resuspended in 50 µl TE. The purified plasmids were subjected to analytic restriction digestions to identify the correct construct and orientation of the insert. After identification of the correct clones, selected single cell clones were used to augment plasmid DNA according to Qiagen Plasmid Maxi Kit instructions. Plasmid concentrations were determined by measuring the absorbance at 260 nm in a spectrophotometer.

E1: 50 mM Tris, 10 mM EDTA, pH 8,0
E2: 200 mM NaOH, 1 % (w/v) SDS
E3: 3.1 M potassium acetate, adjust to pH 5.5 with acetic acid
TE: 10 mM Tris pH 7.5, 1 mM EDTA

3.3.4 Isolation of DNA fragments

DNA restriction digestion was performed with respective endonucleases according to manufacturers' instructions (New England Biolabs GmbH or MBI Fermentas GmbH). DNA fragments were separated by electrophoresis in a 0.8 % low melting point (LMP) agarose gel at 60 V in 1 x TAE buffer. The desired fragment was isolated from the gel with a scalpel and processed by phenol-butanol-DNA extraction as follows. Isolated LMP-gel-fragments were melted at 68 °C for 10 min. After melting, 300 µl of pure phenol were added and briefly mixed and centrifuged for 6 min at 10,000 x g. The upper aqueous phase was recovered and the volume was reduced to 50 µl by butanol extraction. The butanol phase was removed and DNA was precipitated by adding 2.5 x volumes of ethanol and 50 mM NaCl, followed by centrifugation at 4 °C with 10,000 x g. The DNA precipitate was washed with 70 % ethanol, centrifuged as above and air-dried for 30 min. The dried DNA pellet was dissolved in 30 µl TE and stored at -20 °C.

TAE (50x): 2 M Tris-acetate (2 M Tris-base and 5.71 % v/v acetic acid); 50 mM EDTA/NaOH pH 8.0
TE: 10 mM Tris pH 7.5; 1 mM EDTA

3.3.5 Phenol-chloroform extraction and ethanol precipitation of DNA

Linearized fragments, obtained from restriction enzyme digestions, were purified by phenol-chloroform treatment and subsequent ethanol precipitation. Therefore, an equal volume of phenol-chloroform-isoamylalcohol was added to the digestion reaction, briefly mixed and centrifuged for 6 min at 10,000 x g. The upper aqueous phase was recovered and DNA was precipitated by adding 2.5 x volumes of ethanol and 50 mM NaCl, followed by centrifugation at 4 °C with 10,000 x g. The DNA precipitate was washed with 70 % ethanol, centrifuged as above and air dried for 30 min. The dried DNA pellet was dissolved in 30 µl TE and stored at -20 °C.

TE: 10 mM Tris pH 7.5, 1 mM EDTA

3.3.6 DNA ligation

Ligation reactions were prepared with T4 DNA ligase according to standard procedures, described in the manufacturers' instructions (Promega GmbH). The solutions contained 3-5 ng vector and 5-10 ng insert DNA and were incubated at a 16 °C – 0 °C gradient for 12-24 hours. To quantify the specificity of the ligation reaction, respective backbone and insert DNA were subjected to separate control ligations. The ligation reactions were used for subsequent heat shock transformation of chemically competent bacteria.

TE: 10 mM Tris pH 7.5, 1 mM EDTA

3.3.7 Dephosphorylation of linearized plasmid DNA

Vector DNA was dephosphorylated with alkaline phosphatase according to manufacturers' instructions (Roche Diagnostics). Dephosphorylation of backbone DNA favours the integration of DNA inserts since religation of the backbone is prevented. After dephosphorylation, DNA fragments were purified by phenol-chloroform extraction as described above. The standard procedure was incubation for 1 hour at 37 °C.

3.4 Cloning of expression vectors

3.4.1 Site-directed PCR Mutagenesis

Mutations of single amino acids within GPx4 were performed by site-directed PCR mutagenesis with pDrive-Flag-Strep-HA-GPx4 as a template (Seiler, Schneider et al. 2008) in a volume of 50 µl. Primers of 30-33 nucleotides were designed, such that the primer sequence consists of a mutated codon, flanked by wild type sequences of at least 10 nucleotides. Mutated codons were chosen according to the highest murine codon usage of the respective amino acid. The codon substitution used is shown in Table 5. The PCR-reactions were performed with reduced primer annealing temperature depending on the substitution used, due to the mismatch of primer and wild type GPx4 sequence. The list of primers used can be found in the Materials section. Prior to the transformation of competent cells, PCR reactions were digested with *DpnI* for 60 min to remove methylated template DNA. Mutations were verified by sequencing in pDrive with primers ”T7 promoter” and “SP6 promoter”. The mutated sequences were cloned in the lentiviral expression vector p442-PL1 as described above, yielding the according p442-FSH-GPx4-variants. Products were sequenced again to ascertain the correct coding sequence.

Tabel 5: The different codon substitutions used to replace the corresponding amino acid.

Nomenclature	Mutation	Nomenclature	Mutation
GPx4	sequence see Materials	**GPx4$^{All\ Cys}$**	From GeneArt
GPx4^{U46C}	TGA → TGC	**GPx4^{Q81A}**	CAG → GCG
GPx4^{U46S}	TGA → AGC	**GPx4^{Q81E}**	CAG → GAG
GPx4^{C2S}	TGT → TCT	**GPx4^{W136D}**	TGG → GAC
GPx4^{C10S}	TGT → TCT	**GPx4^{N137A}**	AAC → GCC
GPx4^{C37S}	TGT → TCC	**GPx4^{N137D}**	AAC → GAC
GPx4^{C66S}	TGT → TCT	**GPx4$^{\Delta SECIS}$**	none
GPx4^{C75S}	TGC → TCC	**GPx4$^{\Delta SECIS\ U46C}$**	TGA → TGC
GPx4^{C107S}	TGT → TCT	**Mls-GPx4**	none
GPx4^{C148S}	TGC → TCC	**Mls-GPx4^{U46C}**	TGA → TGC
GPx4^{C168S}	TGC → TCC	**Mls^{C16S}-GPx4**	TGC → TCC

The positions of the respective codons are marked in Materials 2.5

3.4.2 Generation of the Mls-FSH-GPx4

The mitochondrial localization signal (Mls) was introduced upstream of the FSH-GPx4 by overlap and extension PCR using the following primers: Oligo Mls-GPx4-f1, Oligo Mls-GPx4-f2, Oligo Mls-GPx4-f3, Oligo Mls-GPx4-r1, Oligo Mls-GPx4-r2 and Oligo Mls-GPx4-r3. The isolation of the Mls sequence from genomic DNA was the first step. For the second step the appropriate restriction site *(BamHI)* was added at the 5'-end and a 20 bp sequence representing the start of the FSH-GPx4 was added at the 3'-end. These base-pairs included a substitution of the start codon (AUG) with an ACG sequence. The FSH-GPx4 was isolated from the pDrive plasmid. For this cloning step the start codon was replaced as described above and a 20 bp overhang was added to the 5'-end representing the last 20 bp of the Mls-sequence. Both fragments were purified and isolated by LMP gel electrophoresis and subsequent phenol-butanol extraction. Both purified fragments were used as templates for the overlapping PCR. The resulting overlap PCR product was sequenced after cloning into pDrive. Thereafter, the construct was transferred in the lentiviral expression vector as described below.

3.4.3 Generation of SECIS-less GPx4

The GPx4 SECIS element was predicted by using Seleno DB (www.selenodb.org, SPS00000047). The pDrive vector carrying either WT or U46C, which contains two *NheI* restriction sites flanking the SECIS element, was digested with *NheI*. After digestion, the linearized vectors were isolated, religated and cloned. This led to the generation of SECIS-less wild type (WT) and U46C cDNA´s.

3.4.4 p442-PL1-based GPx4 expression vectors

The 3rd generation lentiviral vector p442-PL1 (a kind gift from Dr. Timm Schröder, Helmholtz Zentrum München, Munich, Germany) was used for efficient gene transfer and expression in mosue embryonic fibroblasts (MEFs) by viral infection. Gene expression in p442-PL1 is regulated by an SFFV promoter, synthesizing a bicistronic mRNA that is linked by an internal ribosome entry site (IRES). The fluorescence marker VENUSnucmem including a nuclear membrane anchor (nucmem) is encoded at the 3' position of the bicistronic expression cassette. As described below, various genes were cloned into the 5' position of the expression cassette, upstream of the IRES.

3.5 Immunoblotting and Immunocytochemistry

3.5.1 Western blot

For whole cell lysate preparation, cells were lysed in LCW Lysis Buffer, containing Protease Inhibitor Cocktail (Roche Diagnostics), sonicated (Duty cycle set at 80, Output set at 10) for 40 sec and incubated for 30 min on ice. Cell debris was removed by centrifugation for 30 min at 4 °C at 15,000 x g. Protein concentration of the supernatant was determined by the DC Protein Assay (Bio-Rad), according to manufacturers' instructions. Equal amounts of protein lysates were mixed with sample loading buffer, boiled for 5 min at 95 °C, and proteins were separated on a 12 % SDS-PAGE at 160 V in a Mini-PROTEAN 3 Electrophoresis Cell (Bio-Rad, Munich, Germany). Proteins were transferred onto a Hybond-C super nitrocellulose membrane in blotting buffer with a Trans-Blot Semi-Dry Blotter at 20 V for 30 min (Bio-Rad, Munich, Germany). Membranes were blocked with 5 % skim milk powder in TBST for 30 min and probed with a primary antibody at 4 °C for at least 12 hours. Membranes were washed three times in TBST for 5 min, following HRP-conjugated secondary antibody incubation for 60 min. The nitrocellulose membranes were repeatedly washed in TBST (3 x 5 min) and proteins were visualized by ECL detection on Hyperfilm. Efficient stripping of Hybond-C super membranes was performed with 0.4 M NaOH for 10 min. Prior to second incubation (usually with a primary antibody against β-Tubulin), membranes were blocked with 5 % skim milk powder in TBST for 60 min. All following steps were performed as described above.

Blotting buffer: 10 % running buffer (10x), 20 % methanol
Sample loading buffer (2x): 100 mM Tris, 4 % SDS, 20 % glycerol, 0.1 % bromophenol blue, 100 mM DTT, pH 6.8
LCW lysis buffer: 0.5 % TritonX-100, 0.5 % sodium deoxycholate salt, 150 mM NaCl, 20 mM TRIS, 10 mM EDTA, 30 mM Na-pyrophosphate, pH 7.5
Running buffer (10x): 250 mM TRIS, 1 % SDS, 2.5 M glycine
TBST: 25 mM TRIS, 125 mM NaCl, 0.1 % Tween-20, pH 8.0

3.5.2 Determination of relative protein amount

In order to be able to compare the different protein expression levels of every variant of GPx4, a semi-quantitative approach using immunoblotting data was used. Protein was extracted from cells and used in a dilution series (1:4). Resulting western blot results were analyzed by Image J software and normalized to internal β-tubulin controls.

3.5.3 Immunocytochemistry and confocal microscopy

MEFs were seeded on UV-sterilized and ethanol-cleaned cover slips in 6-well cell culture dishes. Cells were cultivated to semi-confluency in standard DMEM. Cells were incubated for 30 min with a 100 nM solution of Mitotracker Red CMXRos in serum free DMEM and washed 3 x 5 min with medium. For fixation of adherent cells, medium was aspirated from the cell culture dish, and cover slips were rinsed with PBS. Cells were fixed for 10 min in a 4 % PFA solution. Cover slips were washed 3 x with PBS and permeabilized for 3 x 5 min with 0.1 % Triton X-100 in PBS. Subsequently, cells were blocked for 3 x 15 min with 1 % BSA in PBS. For detection of FSH-tagged GPx4, cover slips were incubated over night with the primary antibody α-Flag High Affinity (Sigma M2) at 4 °C in a humidified chamber. Glasses were washed 3 x 10 min with PBS/Tween and subsequently incubated with the secondary antibody α-rabbit-Cy5 conjugate protected from light (45 min). Cover slips were washed 3 x 10 min with PBS/Tween and then incubated for 10 min in DAPI solution. Glasses were briefly rinsed in PBS and mounted onto microscope slides, using VECTASHIELD® Mounting Medium. Excess liquid was removed and slides were sealed with nail polish and stored at 4 °C. Pictures were taken by confocal-laser microscopy with Leica DM IRBE microscope, Leica TCS SP2 scanner and Leica Confocal software.

α-FSH (Sigma M2) antibody solution: 1:200 dilution in 1 % (w/v) BSA in PBS
α-rabbit-Cy5 solution: 1:200 dilution in 1 % (w/v) BSA in PBS
Mitotracker Red CMXRos: 100 nM in serum free DMEM
DAPI-solution: 1 µg/ml DAPI diluted in PBS (5 mg/ml stock solution in H_2O)
PBS: 80.0 g NaCl, 2.0 g KCL, 14.4 g Na_2HPO_4, 2.4 g KH_2PO_4 in 1 l H_2O, pH 7.4
PBS/Tween: 0.1 % (v/v) Tween 20 in PBS
PFA solution: 4 % (w/v) PFA in PBS, pH 7.4 adjusted with HCl
Standard DMEM: DMEM, 10 % FCS, 1 % glutamine, 50 U/ml penicillin G, 50 µg/ml streptomycin
Triton X-100 solution: 0.1 % (v/v) Triton X-100 in PBS

3.6 Flow cytometry (FACS analysis)

3.6.1 Determination of infection efficiency by flow cytometry

Cells were harvested from an 80 % - 90 % confluent 6 well. Cell were trypsinised and transferred into medium, collected by centrifugation for 5 min at 1200 rpm. Supernatant was aspirated, and cells were washed once in PBS. After centrifugation cells were resuspended in PBS containing 1 % FCS. Resuspended cells were applied to a flow cytometry, whereby cell suspensions were excited with 488 nm UV line argon ion laser and Venus emission was recorded on channel FL1 at 524 nm.

PBS: 80.0 g NaCl, 2.0 g KCL, 14.4 g Na_2HPO_4, 2.4 g KH_2PO_4 in 1 l H_2O, pH 7.4
Standard DMEM: DMEM, 10 % FCS, 1 % glutamine, 50 U/ml penicillin G, 50 µg/ml streptomycin

3.6.2 Cell viability detection by flow cytometry

Cell viability was determined by propidium iodide (PI) staining. The fluorogenic dye PI enters only dead cells and intercalates into double stranded DNA. PI does not cross the intact membrane of viable cells, thus its fluorescence of above 630 nm can be used as a measure for cell viability. Adherent cells were washed with PBS and trypsinized. Cells were collected by centrifugation (180 x g, 5 min), washed in PBS three times and resuspended in 200 µl PBS. PI was added to a final concentration of 1 µg/ml. Cell suspensions were excited with 488 nm UV line argon ion laser and PI emission was recorded on channel FL3 at 661 nm.

PI solution: 1 mg/ml PI in PBS
PBS: 80.0 g NaCl, 2.0 g KCL, 14.4 g Na_2HPO_4, 2.4 g KH_2PO_4 in 1 l H_2O, pH 7.4
Standard DMEM: DMEM, 10 % FCS, 1 % glutamine, 50 U/ml penicillin G, 50 µg/ml streptomycin

3.7 GPx4-specific activity

3.7.1 Substrate Preparation

Phosphatidylcholine hydroperoxide (PCOOH) was generated by oxygenation of phosphatidylcholine catalysed by lipoxygenase. This enzyme has the capability to act on phospholipids provided that deoxycholate is present. After a given incubation of phosphatidylcholine with lipoxygenase in the presence of deoxycholate, deoxycholate was removed and PCOOH concentrated by solid phase extraction.

Oxygenation of PCOOH was started by adding 200 µl of soybean lipoxygenase (corresponding to 660 U) to the incubation mixture. After 20 min continuous stirring, the whole mixture was loaded onto reverse phase Sep-Pak C 18 cartridges (Waters GmbH), and purified via reverse phase HPLC. Prior to loading, the cartridges were equilibrated with 5 ml methanol followed by 10 ml H_2O. After loading, the cartridge was washed with 20 ml H_2O and then air-dried. PCOOH was eluted with 1.5 ml of methanol. The first drop of the eluate was discarded as it contained most of the deoxycholate still bound to the matrix. PCOOH methanolic solution was stored at -20 °C, where it is stable for several months. During storage, a white precipitate may appear. This is mostly deoxycholate, which was not eliminated during the procedure described above, but can be removed by centrifugation.

PCOOH was quantified by measuring the total NADPH consumption after adding 15-30 µl methanolic solution as substrate. Calculations were made by using $E_{(NADPH)}$= 6.22 $mM^{-1}cm^{-1}$.

Buffer: 0.2 M Tris-HCl, pH 8.6, 3 % Na-deoxycholate (DOC), 39 mg soy bean phosphatidylcholine, (PC), SIGMA cat. P-6263) dried and mixed with 1 ml DOC 3 %

HPLC grade methanol, PCOOH

Incubation mixture: 24.37 ml Tris buffer, 170 ml PC, 2.26 ml DOC

Soybean Lipoxygenase type IV: SIGMA cat. L-3004

SEP Pack C18: Reverse phase cartridges for solid phase extraction (Waters-Millipore c51910)

3.7.2 Measurement of GPx4-specific activity

GPx4 activity was measured through recording the specific NADPH oxidation at 340 nm in the presence of GSH, GSSG reductase, and phosphatidylcholine hydroperoxides, according to published protocols (Ursini, Maiorino et al. 1985; Zhang, Maiorino et al. 1989; Maiorino, Gregolin et al. 1990). A stirrer was used to improve the quality of recording. 200 µl of whole cell lysate containing GPx4 was added to 0.5 ml of the prepared mixture as described below. The volume was adjusted to 2.5 ml by addition of dH_2O in order to achieve the following final concentrations: 0.1 M Tris-HCl, 5 mM EDTA, 0.16 mM NADPH, 1 mM NaN_3, 3 mM GSH, 0.1 % Triton X-100, 1 U/ml Glutathione reductase. After recording of the baseline, reaction was started with 15-30 µl of methanolic solution including PCOOH, prepared as described (3.7.1). A volume of 8 µl PCOOH was chosen in order to have a minimal concentration of approx. 0.016 mM PCOOH.

Tris-HCl: 1 M, pH 7.4
EDTA: 0.5 M, pH 8.0 neutralization (with NaOH) is required for solution
NADPH: 10 mM
NAN_3: 0.1 M
GSH: 0.1 M
Glutathione reductase: SIGMA cat. G-4759
Triton X-100: 20 %
Mixture for 20 assays: 5 ml Tris-HCl, 0.5 ml EDTA, 0.8 ml NADPH, 0.5 ml NaN_3, 1.5 ml GSH, 0.25 ml Triton X-100, 2.5 U/ml Glutathione reductase, 1.4 ml dH_2O

3.8 MTT Assay

3.8.1 Determination of cell viability by MTT assay

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma M-5655) is a yellow tetrazole and in living cells it is reduced by the mitochondrial reductase, thereby forming the insoluble purple formazan. For the MTT assay, cells were plated on a 96 well dish containing 90 µl of cell culture medium in a given concentration and incubated for 4 hours at 37 °C after the addition of 10 µl of MTT reagent. The reaction was terminated by adding 200 µl of acidified isopropanol, a solubilisation solution that is needed to dissolve the otherwise insoluble purple formazan product into a colored solution. The solubilisation is greatly enhanced by repeatedly pipetting up and down the solution followed by gently shaking at RT for 1-2 hours. The absorbance (OD) was measured in an ELISA reader at 570 nm (650 nm reference). Raw data was imported in Excel and statistical analysis was done by Sigmastat 3.0.

Standard DMEM: DMEM, 10 % FCS, 1 % glutamine, 50 U/ml penicillin G, 50 µg/ml streptomycin
MTT solution: 5 mg/ml MTT in PBS
Acidified isopropanol: 0.04 M HCl in isopropanol

3.8.2 Toxicity assays

Four different toxins, hydrogen peroxide (H_2O_2, an oxidative stress inducing ROS), tertiary-butyl hydroperoxide (tBOOH, an oxidative stress inducing ROS), L-buthionine sulfoximine (a potent and specific inhibitor of γ-glutamylcysteine synthetase) and doxorubicin (an anthracycline antibiotic that works by intercalating into DNA/RNA thereby inhibiting DNA and RNA synthesis; it also inhibits the topoiosomerase II enzyme, thereby preventing the relaxation of supercoiled DNA and thus blocking DNA transcription and replication) were used in five different concentrations to determine the resistance of the different GPx4 variants: H_2O_2 (0; 50; 100; 200; 250; 500 µM), tBOOH (0; 1; 5; 10; 20; 50 µM), BSO (0; 0.75; 1.5; 2; 3.5; 5.5 µM) and doxorubicin (0; 0.5; 1; 2; 5; 10 µM). Cells expressing the different GPx4 variants were plated on a 96 well dish in a concentration of 4000 cells/well and were left for 4 hours to adhere to the plate. After 4 hours, cell stress was applied by replacing the medium by medium containing the different compounds. This was considered as time point 0 hours. Viability of the cells was measured 0 hour, 24 hours and 48 hours after toxin treatment by the MTT assay.

Standard DMEM: DMEM, 10 % FCS, 1 % glutamine, 50 U/ml penicillin G, 50 µg/ml streptomycin
MTT solution: 5 mg/ml MTT in PBS
H_2O_2: stock solutions of 50, 100, 200, 250 and 500 mM
tBOOH: stock solutions of 1, 5, 10, 20 and 50 mM
BSO: stock solutions of 0.75, 1.5, 2, 3.5 and 5.5 mM
Doxorubicin: stock solutions of 0.5, 1, 2, 5 and 10 mM

3.9 Tet-inducible expression system

The WT and the U46C constructs were cloned in the pRTS1 vector (Bornkamm, Berens et al. 2005) using the *SwaI* and *BclI* restriction sites. Pfa1 cells were transfected with the construct by electroporation and selected by hygromycin (100 µg/ml) for 3 weeks following transfection. After selection, the cells were treated with tamoxifen to induce the GPx4 knockout. At the same time, doxycycline (dox) was added at 10 nM to the medium to prevent cell death induced by disruption of the endogenous *GPx4* gene. Dox was washed out by several washing steps, and cells were then cultivated with different amounts of dox from up to 1 µM to induce different levels of expression of WT and U46C, respectively.

3.10 Determination of mRNA levels

3.10.1 RNA isolation and cDNA synthesis

Total RNA from cells was isolated by RNeasy Mini Kit (Quiagen) according to manufacturers' instructions. Cells were harvested from 10 cm cell culture plates with a cell scraper. Disruption of cells occurred by addition of 600 µl Buffer RLT which contains 10 µl/ml 2-ME. In order to avoid DNA contamination the accessory RNase-Free DNase Set (Qiagen) was used. RNA was eluted with 30 µl of elution buffer from the column. Final RNA concentration was determined by measureing the absorbance at 260 nm in a spectrophotometer. The Reverse Transcription System (Promega) was used according to manufacturers' instructions on 1 µg of isolated mRNA in order to yield single-stranded cDNA. To increase the yield of cDNA the incubation time was prolonged to 45 min. The cDNA amplification was performed with random primers at 42 °C. The initial 20 µl first strand cDNA synthesis reaction mix was finally diluted to 100 µl with nuclease-free waterand subject to qRT-PCR

3.10.2 Quantitative RT-PCR

The synthesized cDNA was amplified and detected with LightCycler FastStart DNA MasterPLUS SYBR Green I Kit together with LightCycler 1.5 System (Roche). Each qRT-PCR reaction was composed as follows, 1 µl Master Mix, 1 µl primer mix, 6 µl H_2O and 2 µl cDNA which were replaced by H_2O in the H_2O control. Liquids were mixed in a LightCycler Capilary and were pre-cooled in a centrifuge adapter. Primers for the qRT-PCR were designed to yield 200-300 nucleotide long products of GPx4 RNA (Forward 5'-GCAACCAGTTTGGGAGGCA GGAG-3'; Reverse 5'-CCTCCATGGGACCATAGCGCTTC-3'). H_2O control was used to detect potential primer dimerization.

Primer Mix: 5 µl of 100 µM forward primer, 5 µl of 100µM reverse primer, 90 µl H_2O
TE: 10 mM Tris pH 7.5; 1 mM EDTA

3.11 Statistics

Statistics were performed using SigmaPlot software and Man-Whitney U-tests and ANOVA on ranks respectively. Significant results were marked with an asterisk (<0.05); highly significant results with two asterisks (<0.001).

4 Results

4.1 The role of the catalytically active Sec and the remaining Cys residues in mammalian glutathione peroxidase 4 (GPx4)

4.1.1 Generation and expression of various mutant forms of the short form of GPx4 in inducible GPx4 null cells

In order to address the supposedly essential role of the catalytically important Sec in one of the most important mammalian selenoproteins, a recently established inducible *GPx4* knockout cell system was used (Figure 9 A) (designated as PFa1 cells in the following (Seiler, Schneider et al. 2008)). PFa1 cells harbour two loxP-flanked *GPx4* alleles and stably express the MerCreMer (Mer = mutated estrogen receptor) fusion protein. Only upon 4-hydroxy-tamoxifen (Tam) administration, MerCreMer is liberated from the cytosolic hsp90 complex and translocates to the nucleus, where Cre-mediated deletion of both *GPx4* alleles is induced.

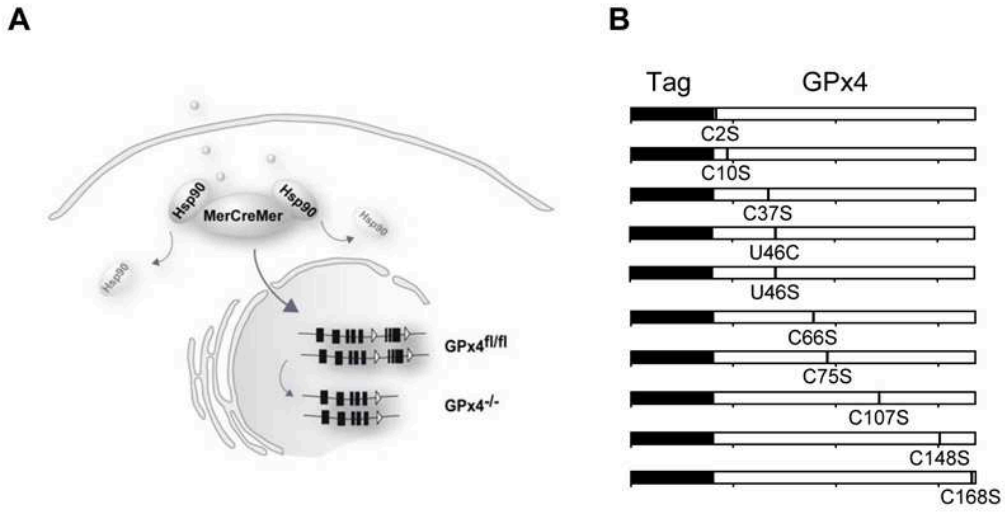

Figure 9: Schematic representation of the cellular system used for inducible disruption of *GPx4*$^{-/-}$ (PFa1 cells) for the *in vivo* analysis of the GPx4 mutant forms. (A) Shown is the Tamoxifen (Tam)-inducible *GPx4* knockout system in MEFs. Addition of Tam (grey balls) to the cell culture medium, liberates MerCreMer (Mer = mutated estrogen receptor) from cytosolic hsp90 (Verrou, Zhang et al. 1999), and thus allows MerCreMer to translocate to the nucleus, where Cre-mediated deletion of the last three loxP-flanked (fl; white triangular) exons (black bars) occurs. This leads to disruption of the *GPx4* allele (Seiler, Schneider et al. 2008). (B) Depicted is the relative position of the mutated codons within the *GPx4* mRNA.

To study the functional relevance of distinct GPx4 residues *in vitro*, a highly efficient lentivirus-based add-back system was used in combination with the inducible *GPx4*$^{-/-}$ cells. Various mutant forms of GPx4 were generated and expressed in these cells (Figure 9 B). For detection and purification of potential GPx4 binding partners, all mutant forms were N-terminally tagged with an FSH tag (Gloeckner, Boldt et al. 2007). The FSH tag consists of Flag-Strep-linker-Strep-HA domains. To be able to detect the expression of the different mutant forms of GPx4 in these cells, WT GPx4 and all mutants were expressed in a bi-cistronic manner using VENUS, a yellow fluorescent protein, as a detectable marker.

The first set of substitutions was introduced in order to address the catalytically active site Sec in GPx4. Sec was mutated either to a Cys (U46C) or a Ser (U46S) residue at this position. Cells reconstituted with FSH-tagged wild-type GPx4 (in the following referred to as wildtype, WT) or empty vector-tranduced cells (mock), were used as positive and negative controls, respectively. In addition, mutations of the remaining non-peroxidatic Cys residues were created whereby each Cys residue was replaced by a Ser individually (C2S, C10S, C37S, C66S, C75S, C107S, C148S, and C168S) or simultaneously as shown in Figure 10.

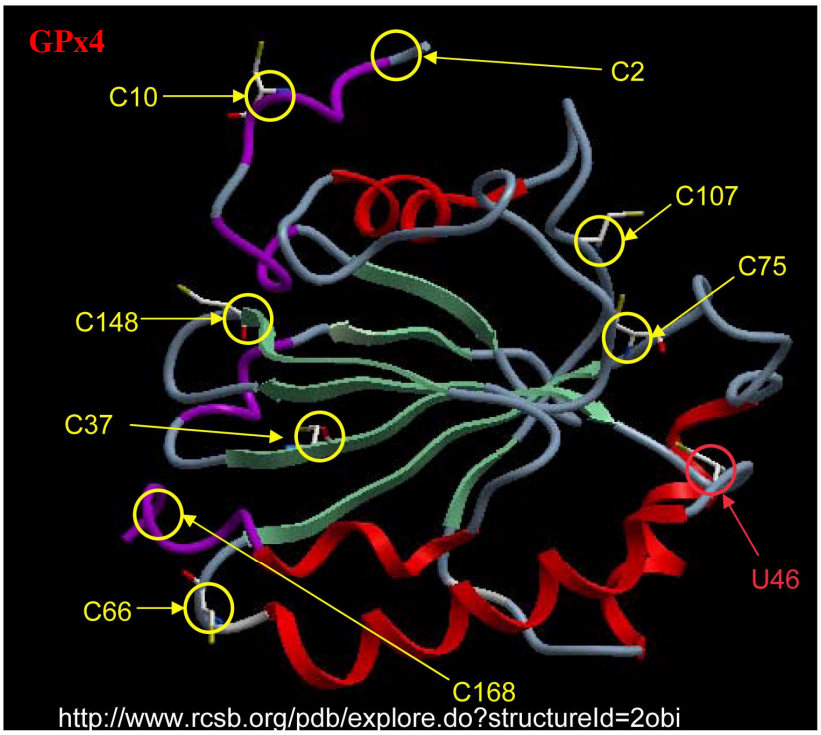

Figure 10: Map showing the position of the Cys residues in mammalian *GPx4*. The 3D model of GPx4 was rotated and labelled to show the the positions of Cys (C2, C10, C37, C66, C75, C107, C148 and C168) using the human *GPx4*, which are labelled in yellow and the Sec (U46) which is labelled in red. Structural features are encoded as follows; α-helices in red, β-sheets in light green and flexible linker regions in violet other aa in grey.

The two sets of substitutions were deemed necessary for two reasons: first, it was shown for the yeast homologue of GPx4, glutathione peroxidase 3 (yGPx3), that Cys 36 of yGPx3 is able to form a transient disulfide bridge with the peroxidatic Cys 598 of Yap1 during the

catalytic cycle of yGPx3 (Tolando, Jovanovic et al. 2000); second, invertebrates and terrestrial plants are practically devoid of Sec containing glutathione peroxidases, instead they express glutathione peroxidases that besides the redox active Cys (= peroxidatic Cys, C_P) have an additional resolving Cys (C_R). Upon oxidation of C_P, the C_R forms a transient disulfide-bridge with C_P thereby releasing the reduced substrate. The intramolecular disulfide-bridge between the catalytically active Cys and the resolving Cys can be reduced by thioredoxin (Maiorino, Ursini et al. 2007).

Finally, amino acids forming the proposed catalytic tetrad of GPx4 were included in these mutational studies (Figure 11) (Maiorino, Ursini et al. 2007).

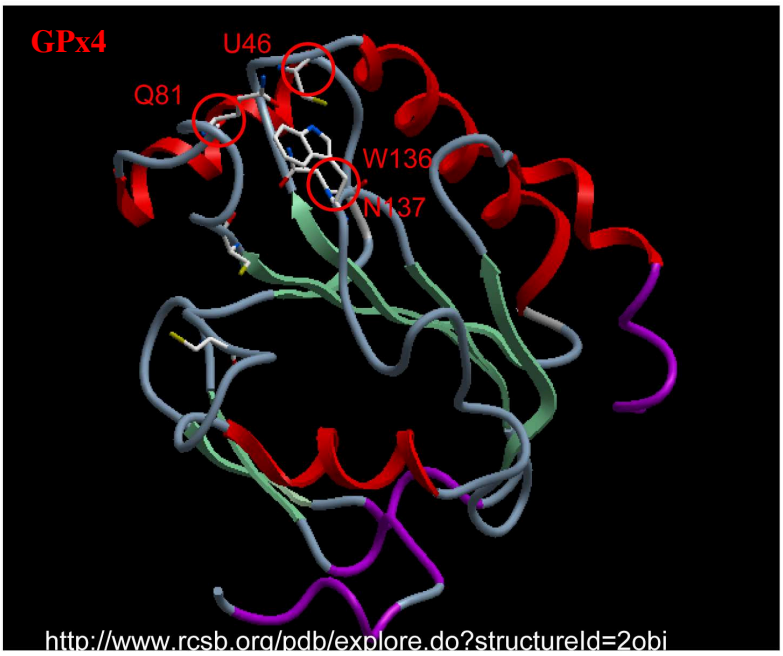

Figure 11: Map showing the position of the catalytic tetrade in mammalian *GPx4.* The 3D model of GPx4 was rotated and labelled to show the catalytic tetrad of human *GPx4 (U46, Q81, W136 and N137)*, which is labelled in red. Structural features are encoded as follows; α-helices in red, β-sheets in light green and flexible linker regions in violet other aa in grey.

After the cloning of all constructs, lentiviruses were generated and used for transduction of the PFa1 cell line. 3-6 passages after lentiviral transduction, expression of the different constructs was studied by immunoblotting using an HA-specific antibody (Figure 12). This revealed that all variants, with the exception of the U46C and U46S, were expressed to almost even levels in the cells. An increase in protein level could be detected for U46C and U46S expressing cells that was about three times higher for the Cys variant and about six times higher for the Ser variant when compared with WT GPx4.

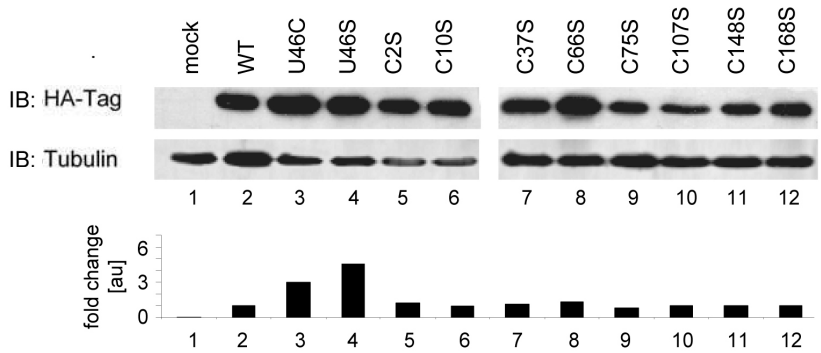

Figure 12: Expression of the mutants was determined by immunoblotting using an HA-specific antibody. β-Tubulin was used to assess loading of protein lysates. Below, expression efficiency was quantified as described in Materials and Methods (3.5.2). Expression of the individual mutants was normalized to β-Tubulin levels presented as fold increase/decrease compared with wild type GPx4 expression arbitrarily set as 1.

Expression of the different GPx4 variants was monitored over a period of 50 passages in the absence of Tam using flow cytometry. As no additional selection pressure was imposed on lentivirus-infected cells, transfected cells were repeatedly analysed by measuring a fluorescent marker protein (VENUS) produced by the bi-cistronic *GPx4* mRNA. Figure 13 illustrates that exogenous GPx4 expression was stable for many passages in cell culture for WT GPx4 and U46C GPx4 expressing cells (Figure 13 A), as well as for all cysteine variants (Figure 13 B). Only expression of mock transduced cells and U46S transduced mutant form was gradually lost during subsequent passaging of cells (Figure 13 B), indicating that the expression of these forms does not provide growth advantage to the cells.

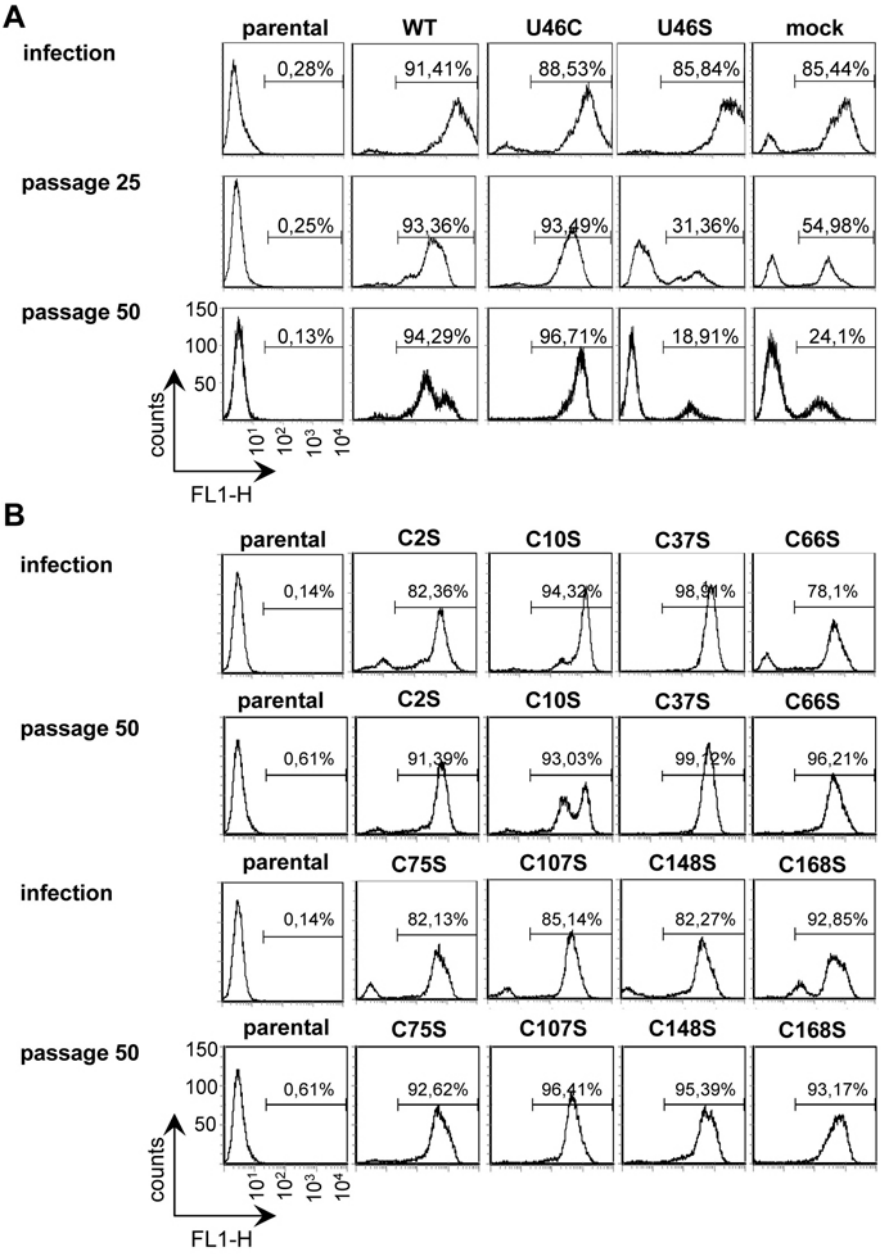

Figure 13: Stability of expression of the different GPx4 constructs in lentivirus-infected PFa1 cells. Cells were infected by lentiviruses with the indicated constructs. To be able to monitor infection efficiencies and stability of the constructs, expression was performed in a bi-cistronic manner using VENUS as a detectable marker. Expression was monitored by FACS analysis of VENUS-positive cells (FL1-H) after gating for living cells. (A) Cells carrying the WT, U46C, U46S GPx4 or mock construct. (B) Cells carrying the construct containing individual mutations of the non-peroxidatic Cys (C2S, C10S, C37S, C66S, C75S, C107S, C148S, C168S, respectively). Note that the WT and U46C expressing cells and all the individual non-peroxidatic Cys mutants, but not U46S and mock expressing cells, showed stable expression even after 25 and 50 passages without selective pressure.

4.1.2 The U46C mutant of GPx4 rescues GPx4 depleted cells from cell death

To investigate whether the catalytically active Sec is essential for the supposedly vital role of GPx4 in cell survival and proliferation, cells expressing the different constructs (WT, U46C, U46S, mock and CxS') were either treated with Tam to disrupt endogenous GPx4 expression or left untreated (control). For these analyses, freshly transduced cells (passage 3-6) were used to ensure that expression was still high in cells. Cell proliferation was monitored over a period of 60 hours (Figure 14 A and B, left panel). No significant differences in cell proliferation were detected between the different cell lines in the absence of the inducer. When cells were treated with Tam, parental, mock and U46S expressing cells died between 36 and 48 hours after Tam administration (Figure 14 A, right panel). In stark contrast, U46C expressing cells survived like WT GPx4 expressing cells. No significant difference could be seen in the Cys to Ser variants (Figure 14 B right panel).

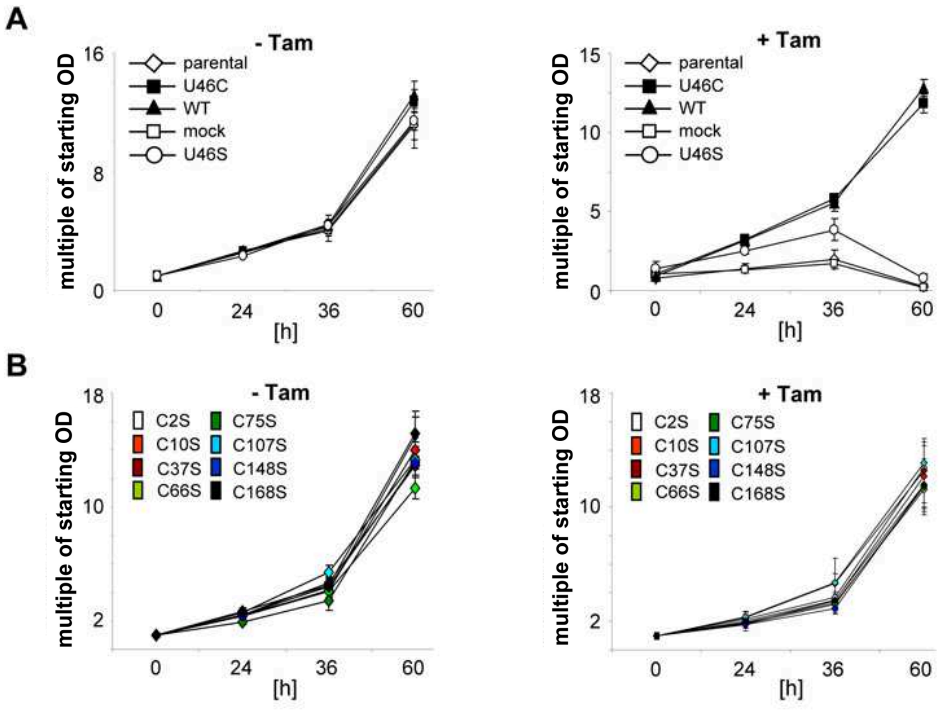

Figure 14: The U46C mutant form of GPx4 rescues from cell death induced by endogenous GPx4 disruption. (A, B, left panel) In the absence of Tam, cell proliferation was comparable between the different cell lines. (A, right panel) Unexpectedly, the U46C mutant was able to rescue cells from cell death induced by endogenous GPx4 disruption in a manner similar to WT GPx4 expressing cells. (B, right panel) None of the remaining Cys residues of GPx4 were required for the essential cell death inhibiting function of GPx4. PFa1 cells expressing the different constructs were treated with vehicle (0.07% EtOH; -Tam) or with Tam (+Tam) to disrupt endogenous *GPx4.* First measurement was set as 1. Shown is one representative experiment out of 2 independent biological experiments (mean ± SD out of 6 technical replicas).

This was unexpected and surprising as it was previously shown that the U46C variant of pig GPx4 has less than 1 ‰ activity compared to WT when analyzed in *in vitro* biochemical

assays (Maiorino, Aumann et al. 1995). When treated with Tam in order to induce deletion of the endogenous GPx4, all the eight Cys to Ser variants of GPx4 survived similar to WT GPx4 expressing cells (Figure 14 B, right panel), suggesting that none of the remaining cysteines is essential for the rescuing effect of GPx4.

4.1.3 Cells expressing the Sec/Cys mutant are equally resistant to stress conditions as WT expressing cells

Since the U46C variant of GPx4 (and all other serine mutants of the non-peroxidatic cysteines) prevented cell death of GPx4 knockout cells, it was hypothesized that a difference in the rescuing activity between WT GPx4 and U46C (and the other Cys/Ser variants) may be unmasked when these cell lines are subjected to several stress inducing agents. Hydrogen peroxide (H_2O_2) and tert-Butyl hydroperoxide (tBOOH) were chosen since they are known to cause oxidative stress. Buthionine sulfoximine (BSO), a highly specific and irreversible inhibitor of the GSH synthesizing enzyme γ-glutamylcysteine synthetase (γ-GCS), was used because it efficiently deprives cells from intracellular GSH. Doxorubicin intercalates into DNA, induces double strand breaks, and stabilises the topoisomerase II complex, thereby preventing DNA repair.

As shown in Figure 15 A - D, the U46C variant was almost as effective as WT GPx4 in preventing cell death induced by treatment with BSO, H_2O_2, tBOOH or doxorubicin. Only when cells were treated with increasing concentrations of tBOOH, the U46C expressing cells appeared to be slightly less resistant (Figure 15 C).

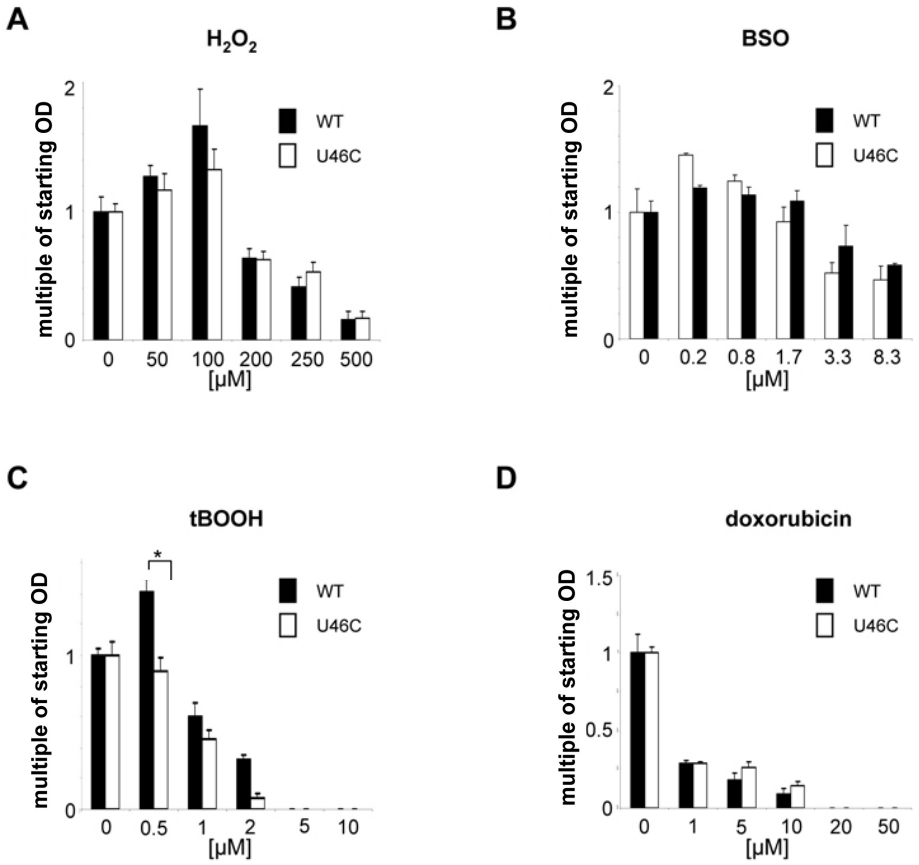

Figure 15: U46C expressing cells are almost as stress-resistant as WT expressing cells. Upon Tam treatment, both cell lines were treated with increasing concentrations of (A) hydrogen peroxide (H_2O_2), (B) the γ-glutamylcysteine-synthetase inhibitor L-buthionine sulfoximine (BSO), (C) tert-Butyl hydroperoxide (tBOOH) and (D) doxorubicin as indicated. 48 hours after treatment. First measurement was set as 1. Shown is one representative experiment out of 3 independent biological experiments (mean ± SD out of 6 technical replicas). Statistically significant results are marked with a star ($p<0.05$) and highly significant results with two stars ($p<0.001$).

Like already observed for the U46C expressing cells, the different CxS variants of GPx4 were almost as efficient as WT GPx4 expressing cells in protecting the cells from the different stress-inducing agents (Figure 16 A - D). An increase at low to medium concentrations of H_2O_2 is not surprising as it is known that such circumstances have a positive effect upon cell proliferation.

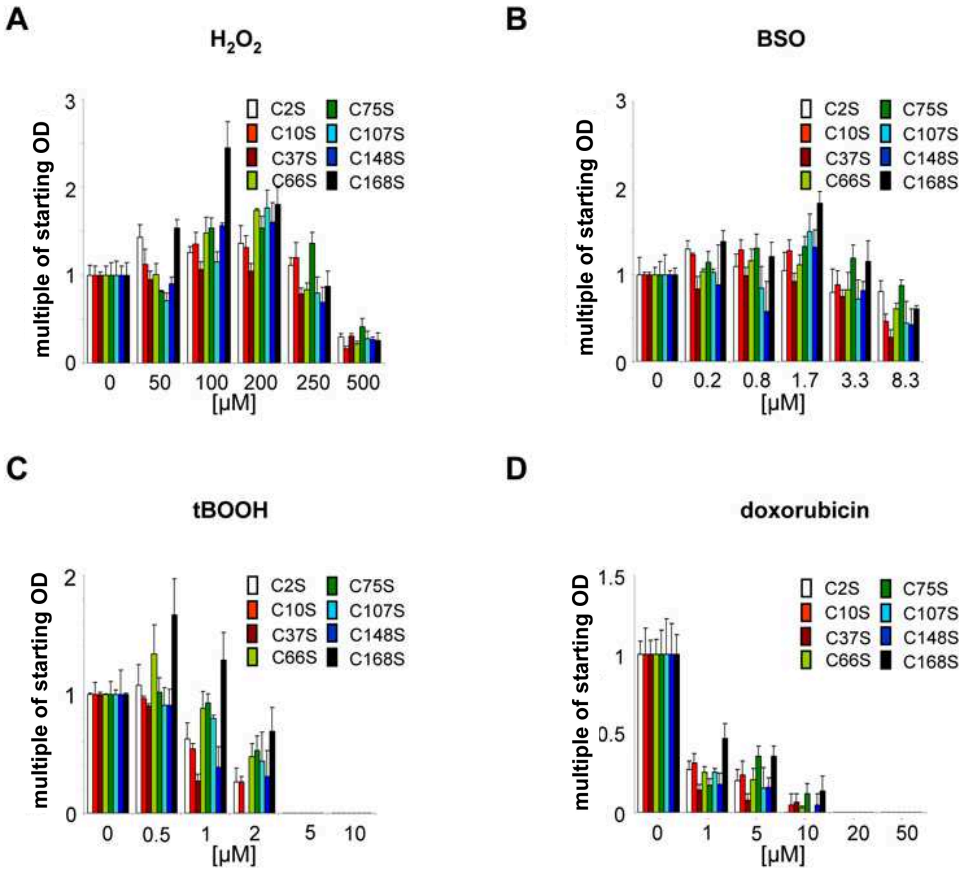

Figure 16: CxS expressing cells are as stress-resistant as WT expressing cells. Upon Tam treatment, all CxS expressing cell lines were treated with increasing concentrations of (A) hydrogen peroxide (H_2O_2), (B) the γ-glutamylcysteine-synthetase inhibitor L-buthionine sulfoximine (BSO), (C) tert-butyl hydroperoxide (tBOOH) and (D) doxorubicin as indicated. 48 hours after treatment. First measurement was set as 1. Shown is one representative experiment out of 3 independent biological experiments (mean ± SD out of 6 technical replicas). No statistical differnces could be found.

As a more "physiological" stress inducing condition, cells were exposed for 6 or 12 hours to either hypoxia (0.5 - 2 % O_2) (Figure 17 A) or to hyperoxia (95 % O_2) (Figure 17 B), respectively. Additionally, a combination of both conditions was used to mimic ischemia/reperfusion conditions. The first condition was a relatively short period for hypoxia (1 hour followed by 1 hour reperfusion with various oxygen levels (5 %, 20 %, and 95 %)) (Figure 17 C), whereas the second model used longer times for both, hypoxia (12 hours) and reperfusion (6 hours) (Figure 17 D); all experiments were conducted with 5 % CO_2 and in the presence of Tam (Figure 17 A - D).

The WT and U46C expressing cells showed an increased resistance to hyperoxia, probably due to the higher expression of the respective GPx4 variants compared to parental cell lines (Figure 17 B). Notably, cells overexpressing GPx4 also showed a higher tolerance for hypoxic conditions. Although it is still unclear whether hypoxia causes increased or decreased oxidative stress in cells, these results may indicate a yet unrecognized function of GPx4 during hypoxia.

Reoxygenation following hypoxic conditions is known to cause massive endogenous oxidative stress and cell death, for instance as observed in tissues and organs of stroke patients or patients suffering from cardiac infarction. Again, the U46C variant was as protective as WT GPx4 indicating that the U46C variant is as efficient as WT GPx4 at least with respect to these conditions (Figure 17 C - D).

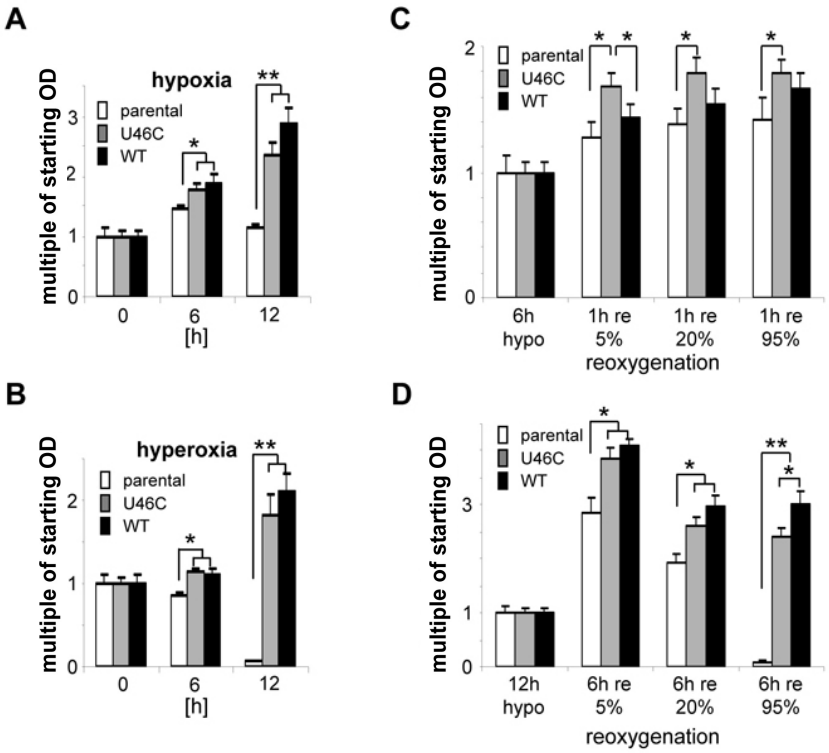

Figure 17: Resistance to hypoxia, hyperoxia and hypoxia/reoxygenation of *GPx4*$^{-/-}$ cells expressing WT or U46C GPx4. (A - D) PFa1 cells (parental) or PFa1 cells depleted of endogenous GPx4 by Tam but stably expressing WT GPx4 or U46C GPx4 were subjected to hypoxia or hyperoxia or hypoxia/reoxygenation for the time indicated. Effects of 6 or 12 hours of (A) hypoxia or (B) hyperoxia. (C) Effect of 6 hours of hypoxia followed by 1 hour re-oxygenation (re) with the indicated concentrations of O_2. (D) Effect of 12 hours of hypoxia followed by 6 hours re-oxygenation with the indicated concentrations of O_2. First measurement was set as 1. Shown is one representative experiment out of 3 independent biological experiments (mean ± SD out of 6 technical replicas). Statistically significant results are marked with a star ($p<0.05$) and highly significant results with two stars ($p<0.001$).

In order to test whether a prolonged exposure time to hypoxia and hyperoxia may be able to unveil differences between WT and U46C expressing cells, the different cell lines were subjected to either 24 hours of hypoxia, hyperoxia or 24 hours of hypoxia followed by 24 hours of reperfusion under different oxygen levels (5 %, 20 % or 95 %). Normal culturing conditions (20 % oxygen) were used as control (Figure 18 A). No significant difference could be seen between the different cell lines under hypoxia or normal cell culture conditions. Only upon exposure to hyperoxic conditions a significant difference could be detected between the parental and WT or U46C overexpressing cells, respectively (Figure 18 A). When exposed to the prolonged hypoxia/reperfusion conditions, the WT overexpressing cells showed a

siginifcantly better survival under 5 % and 20 % oxygen when compared to the parental and U46C expressing cells. Yet no significant difference could be detected between WT and U46C overexpressing cells when subjected to 95 % oxygen conditions during reperfusion (Figure 18 B).

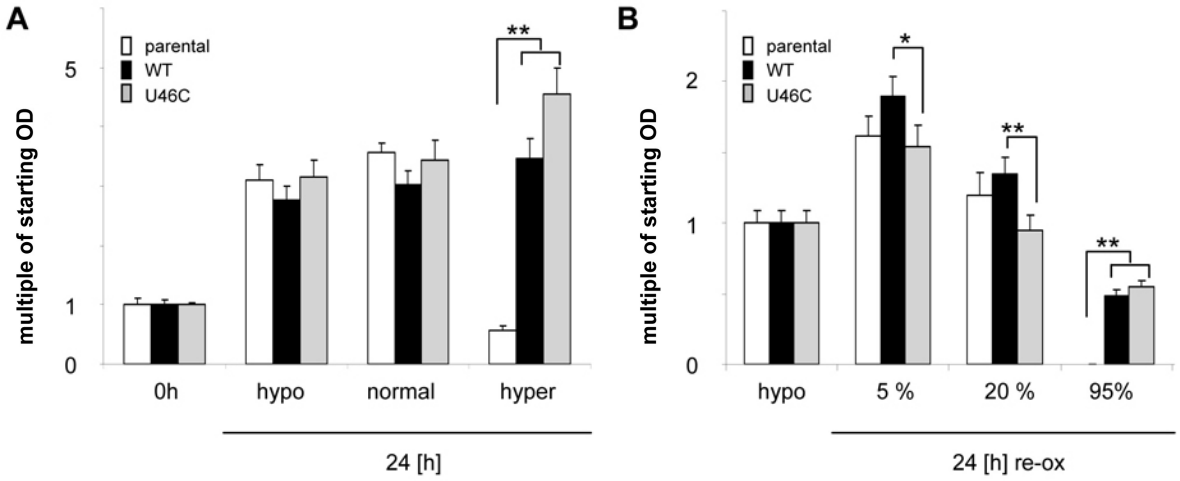

Figure 18: Survival under hypoxia, hyperoxia and hypoxia/reoxygenation of *GPx4*$^{-/-}$ cells expressing WT or U46C GPx4 compared to parental cells. (A) PFa1 cells (parental) or PFa1 cells depleted of endogenous GPx4 by Tam but stably expressing WT GPx4 or U46C GPx4 were subjected to 24 hours hypoxia or hyperoxia or normal growth (20 % oxygen). (B) PFa1 cells (parental) or PFa1 cells depleted of endogenous GPx4 by Tam but stably expressing WT GPx4 or U46C GPx4 were subjected to 24 hours of hypoxia followed by 24 hours of reoxygenation with the indicated oxygen concentrations. First measurement was set as 1. Shown is one representative experiment out of 3 independent biological experiments (mean ± SD out of 6 technical replicas). Statistically significant results are marked with a star ($p<0.05$) and highly significant results with two stars ($p<0.001$).

4.1.4 Sec is incorporated at the UGA codon in WT GPx4

Recent work by Lu et al. showed that under selenium-deficiency Cys instead of Sec is incorporated at the active site of the selenoprotein thioredoxin reductase 1 (Lu, Zhong et al. 2009). To investigate the possibility that Cys instead of Sec may be incorporated at the UGA codon, which would also allow proliferation of GPx4-depleted cells, the SECIS element was deleted in the cDNAs coding for either WT GPx4 or U46C GPx4 (Figure 19 A). As expected no effect was observed in the absence of Tam (Figure 19 B). However, Tam treatment caused cell death of parental cells, mock-transfected cells and WT GPx4 expressing cells lacking the SECIS element (Figure 19 C), whereas the U46C expressing cells survived without the SECIS element. Thus, one can conclude that Cys is not incorporated at the UGA codon of WT GPx4 construct. The lack of protein expression for SECIS-less WT GPx4 was confirmed by immunoblotting using an HA-tag-specific antibody (Figure 19 D).

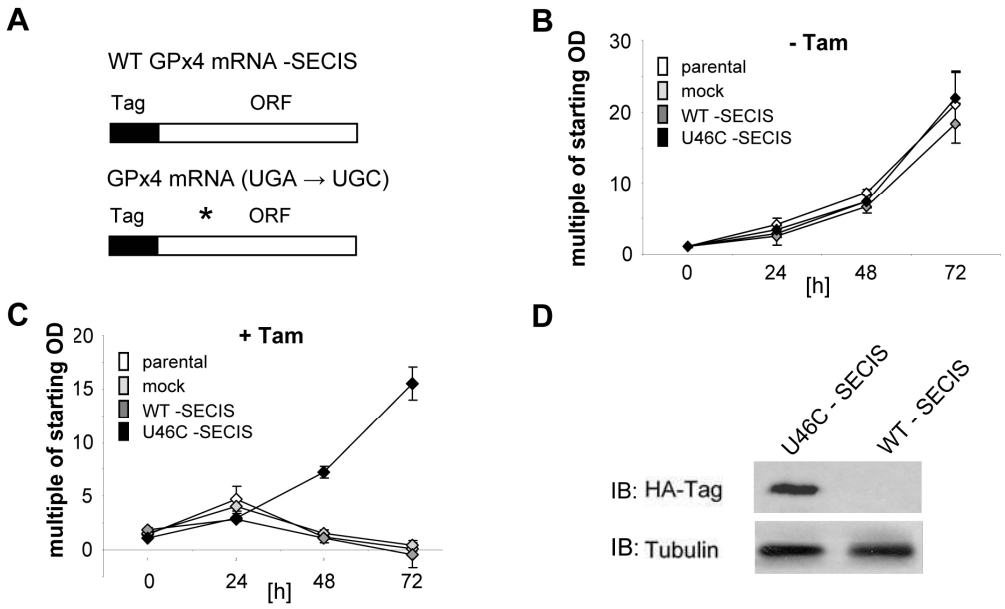

Figure 19: The SECIS element is essential for the correct expression of WT GPx4, but not for the U46C variant. Recent studies indicated that under selenium deficiency, Cys instead of Sec is incorporated at the UGA codon of thioredoxin reductase 1. (A) To rule out that the rescuing effect of WT relies in part on Cys incorporation, the SECIS element was removed from both, the WT and the U46C variant. (B) In the absence of Tam, proliferation rates were comparable between all cell lines, ruling out any dominant negative effect of SECIS-less GPx4 variants. (C) Only the U46C mutant was able to rescue endogenous GPx4 deficiency while the SECIS-less WT GPx4 failed to do so. (D) Immunoblotting confirmed that no WT protein was expressed in the absence of the SECIS element. First measurement was set as 1. Shown is one representative experiment out of 2 independent biological experiments (mean ± SD out of 6 technical replicas).

4.1.5 Doxycycline inducible expression of WT and U46C variant in the GPx4 null background reveals decreased efficiency of U46C in rescuing cell death

Intrigued by the finding that the U46C variant is almost as effective as WT GPx4 in rescuing cell death induced by the loss of endogenous GPx4, it was investigated whether it may be possible to detect differences in the rescuing effect when both variants are expressed at very low levels in a controllable manner. Therefore, both forms were cloned into the doxycycline (dox)-inducible pRTS1 expression vector (Bornkamm, Berens et al. 2005). Cells that showed stable expression were obtained after selection with hygromycin over 3 weeks. Following selection, cells were cultured in the presence of Tam and 1 ng/ml dox in order to guarantee cell survival.

qRT-PCR analysis of dox-inducible WT and U46C expressing cells revealed that expression of both constructs could be increased depending on the amount of inducer (Figure 20 A). The low expression obtained in the absence of dox suggested that there was minimal leakiness, which, however, did not preclude the use of the system for the experimental question. Although mRNA levels could be detected by qRT-PCR (Figure 20 A), verification of protein expression proved to be elusive at 0.001 ng/ml dox, and even at 0.1 ng/ml only faint expression could be detected (Figure 20 B).

As shown in Figure 20 C, as little as 0.001 ng/ml of dox was sufficient to allow proliferation of WT GPx4 expressing *GPx4*$^{-/-}$ cells. No further increase in proliferation was observed at higher dox concentrations (Figure 20 C, left panel). In U46C expressing cells, 0.001 ng/ml dox was also sufficient to allow proliferation of GPx4-deficient cells, although proliferation of U46C expressing cells was clearly reduced when compared to WT GPx4 expressing cells at the same concentration (Figure 20 C, right panel). In contrast to WT expressing cells, higher dox concentrations allowed an increase in the proliferation rate of U46C expressing cells. Only concentrations of 100 µg/ml dox and higher were toxic to the cells (data not shown). Therefore, it is tempting to speculate that minute amounts of WT GPx4 protein is sufficient to allow survival and proliferation of cells, whereas higher expression levels of WT GPx4 have an adverse impact upon proliferation. This may be due to reduced peroxide levels, which would otherwise stimulate proliferation. In contrast, higher amounts of U46C expression are required to reach a similar proliferation rate as WT GPx4 expressing cells, while a higher expression level of U46C in the respective cells does not impair cell proliferation in contrast to WT GPx4 expressing cells.

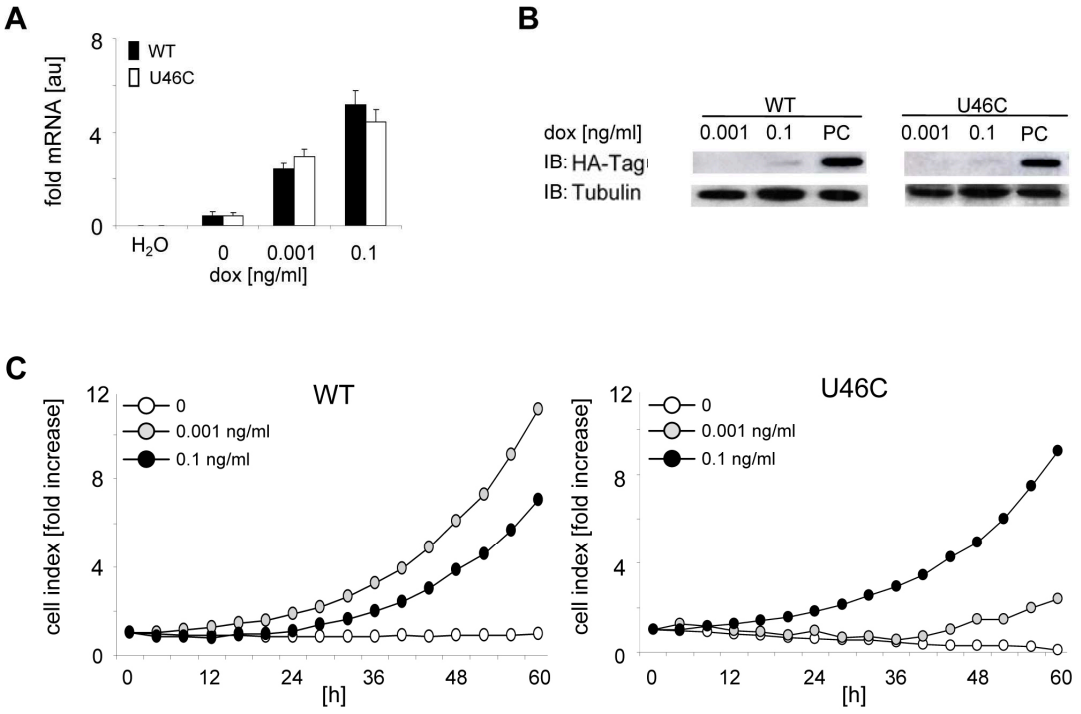

Figure 20: Expression and cell survival/proliferation of *GPx4*$^{-/-}$ cells expressing controllable amounts of WT or U46C. Tam-treated PFa1cells carrying the doxycycline (dox)-inducible pRTS1 expression vector (Bornkamm, Berens et al. 2005) containing WT or U46C were treated with the indicated amounts of dox. Expression was monitored by (A) quantitative RT-PCR (water was used as to check for primerdimerisation) and by (B) immunoblotting using the HA-tag specific antibody (PC = positive control, PFa1 cells stably expressing WT). (C) Changes in cell coverage of the well was measured in real time by the xCELLigence system ('cell index' is proportional to cell proliferation). Shown is one out of 2 independent experiments with similar results.

4.1.6 Only the (seleno)thiol in the catalytically active site is essential for the rescuing effect

In addition to the catalytically important Sec, the short form of GPx4 contains eight other cysteine residues of yet unknown function. In 2002, Toledano and colleagues proposed a dual role for yGPx3, the homologue of GPx4: one corresponds to the “classical” peroxidising function, whereas the second is a redox-sensing function involving the formation of a disulfide bridge between the catalytically active Cys37, which corresponds to GPx4 Sec46, and the Cys82, which, has no corresponding Cys in mouse GPx4 (Delaunay, Pflieger et al. 2002).

To investigate whether one or several cysteine(s) besides the catalytic site Sec are essential for the rescuing effects, variants of WT GPx4 and U46C were generated, which lacked all cysteines but the catalytically important Sec/Cys of the active site (Figure 21 A). Two stably outgrowing clones were established, one for each cell line (allCys/Ser and allCys/SerU46C) (Figure 21 B).

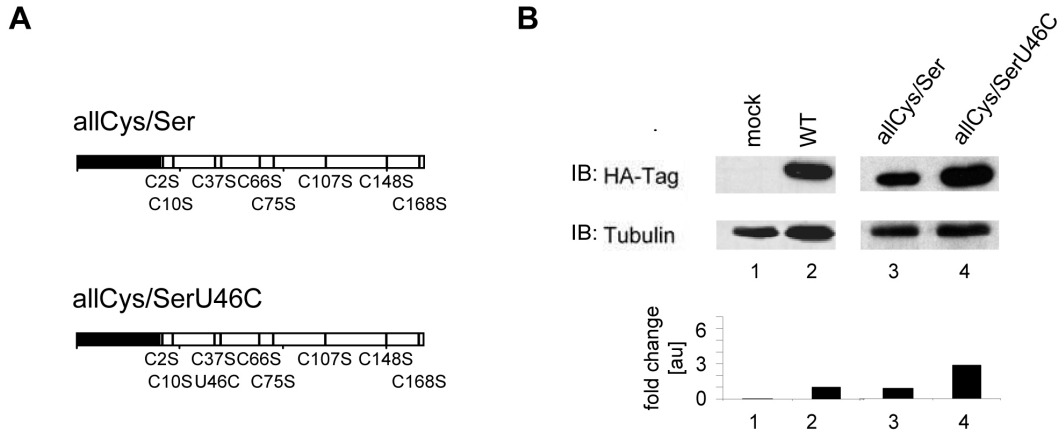

Figure 21: Expression of the allCys/Ser mutants was determined by immunoblotting using an HA-specific antibody. (A) Positions of the different Cys mutated to Ser in the allCys/Ser variants, allCys/Ser and allCys/SerU46C. (B) Protein expression was monitored using an HA-tag-specific antibody. β-Tubulin was used to assess similar loading of protein lysates. Below, expression efficiency was quantified as described in Materials and Methods (3.8.2). Expression of the individual mutants was normalized to β-Tubulin levels presented as fold increase/decrease compared with wild type GPx4 expression arbitrarily set as 1.

As illustrated in Figure 22 no significant difference in cell proliferation rates could be detected between the different GPx4 variants in the absence of Tam (Figure 22 A, left panel). Interestingly, both cell lines rescued cell death induced by disruption of endogenous GPx4 like that already observed for U46C GPx4 or of all mutants carrying single substitutions of the individual non-peroxidatic cysteine residues (Figure 22 A, right panel). Additional experiments with tBOOH as a stress inducing agent revealed a similar resistance of allCys/Ser and allCys/SerU46C to oxidative stress like WT GPx4 expressing cells (Figure

22 B). tBOOH was chosen as it was the only oxidative stress inducing agent, where a slight difference between WT and U46C could be detected (see also Figure 15).

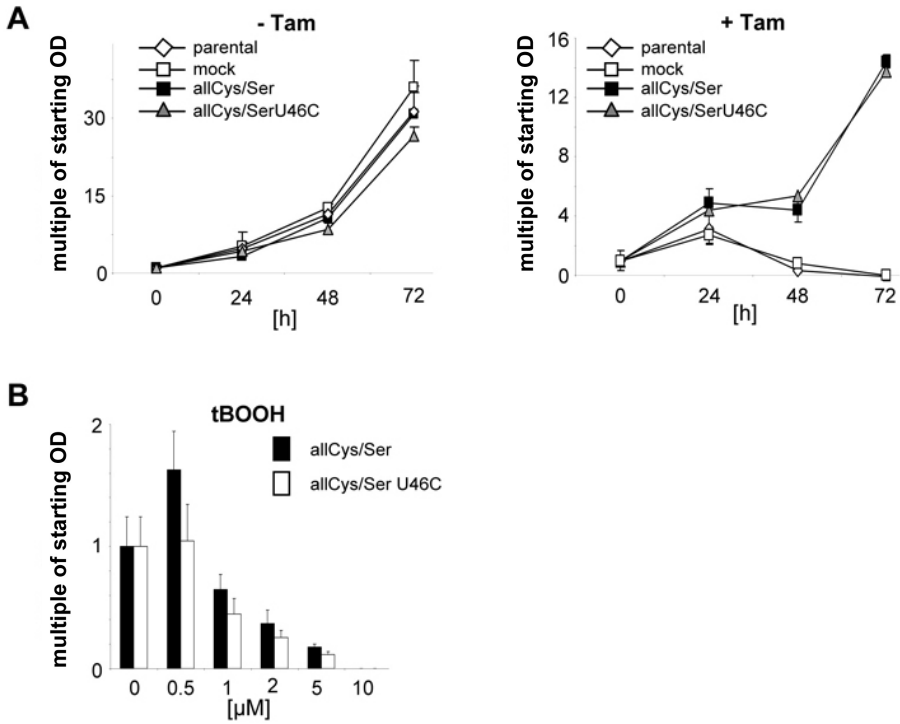

Figure 22: Viability of *GPx4*$^{+/+}$ and *GPx4*$^{-/-}$ cells stably expressing WT GPx4 or the allCys/Ser or allCys/SerU46C variants. (A) PFa1 cells, expressing mock, allCys/Ser and allCys/SerU46C in the absence (left panel) or presence (right panel) of Tam. In the absence of Tam no differences in proliferation could be observed (left). The allCys/Ser and allCys/SerU46C variants rescued cell death induced by GPx4 disruption (right). (B) allCys/Ser and allCys/SerU46C were subjected to oxidative stress triggered by tBOOH. First measurement was set as 1. Shown is one representative experiment out of 3 independent biological experiments (mean ± SD out of 6 technical replicas). Statistically significant results are marked with a star ($p<0.05$) and highly significant results with two stars ($p<0.001$).

Then, these cell lines (allCys/Ser and allCys/SerU46C overexpressing cells) were subjected to hypoxia, hyperoxia and hypoxia/reperfusion as performed already for WT and U46C overexpresssing cells (Figure 23 A). Similar to WT and U46C overexpressing cells, no significant difference could be observed between the different cell lines under hypoxia or normal cell culture condition. Only upon exposure to hyperoxic conditions a significant difference could be detected between the parental and allCys/Ser or allCys/SerU46C overexpressing cells (Figure 23 A). When exposed to the prolonged hypoxia/reperfusion model, the allCys/Ser overexpressing cells showed a better survival rate at 5 % oxygen tension compared to the parental and U46C expressing cells. Yet no significant difference could be detected between allCys/Ser and allCys/SerU46C expressing cells when subjected to 20 % or 95 % oxygen tension during the reperfusion phase (Figure 23 B).

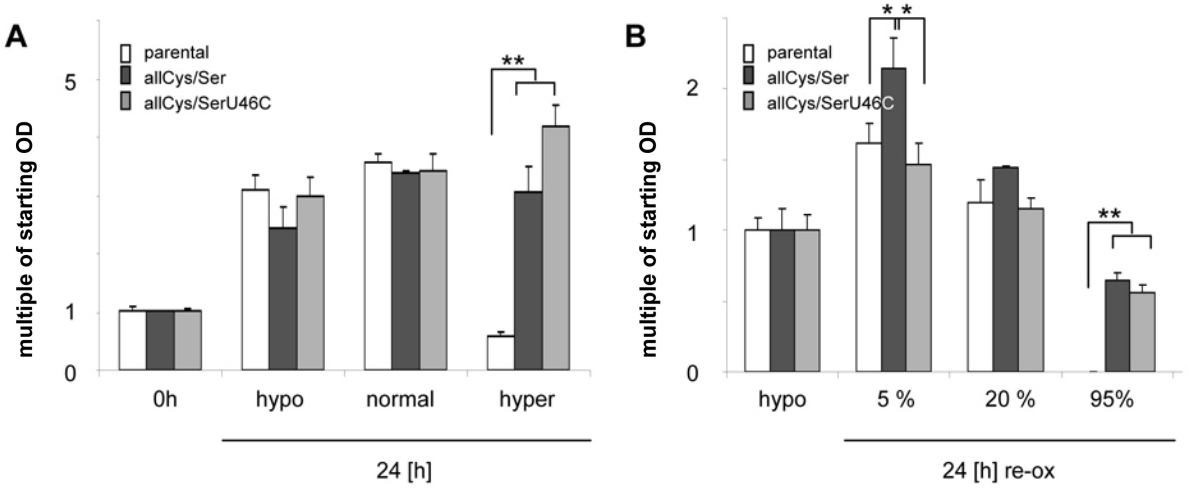

Figure 23: Resistance to hypoxia, hyperoxia and hypoxia/reoxygenation of *GPx4*$^{-/-}$ cells expressing allCys/Ser or allCys/SerU46C GPx4 compared to parental cells. (A) PFa1 cells (parental) or PFa1 cells depleted of endogenous GPx4 by Tam but stably expressing allCys/Ser or allCys/SerU46C were subjected to 24 hours hypoxia or hyperoxia or normal growth (20 % oxygen). (B) PFa1 cells (parental) or PFa1 cells depleted of endogenous GPx4 by Tam but stably expressing allCys/Ser or allCys/SerU46C were subjected to 24 hours of hypoxia followed by 24 hours of reoxygenation with the indicated oxygen concentrations. First measurement was set as 1. Shown is one representative experiment out of 2 independent biological experiments (mean ± SD out of 6 technical replicas). Statistically significant results are marked with a star ($p<0.05$) and highly significant results with two stars ($p<0.001$).

4.1.7 The Sec/Cys mutant shows decreased GPx4-specific activity compared to WT GPx4

Previously, it was demonstrated that the U46C mutant of pig GPx4 has approximately 1000-fold reduced GPx4-specific activity compared to wild type GPx4, when tested with the model substrate phosphatidylcholine hydroperoxide (PCOOH) in an *in vitro* glutathione peroxidase activity measurement (Maiorino, Aumann et al. 1995). Therefore, the specific GPx4 activity was investigated in crude lysates of cells expressing WT GPx4, U46C or U46S; mock-transfected cells were used as negative control. Prior to analysis, the knockout of GPx4 was induced by Tam in all cell lines and 1 µm α-Toc was included in the medium to prevent cell death of U46S and mock-transfected cells due to depletion of endogenous GPx4. In fact, the U46C expressing cells showed a very small percentage of specific GPx4 activity when compared to WT expressing cells (Figure 24 A, left panel). The measured activity could not be unambiguously discriminated from the activity of the background controls (mock) and of U46S expressing cells, which is the enzymatically inactive form.

The substitution of all non-catalytic Cys to Ser did not affect GPx4-specific activity (Figure 24 B, right panel). Only when the catalytically active Sec was substituted by Cys the activity dropped again, as it did with the U46C variant (see Figure 24 A, left panel). Also, the relative expression of the allCys/SerU46C was about three times higher than that of WT or

allCys/Ser (Figure 24 B, right panel), as already observed for U46C (Figure 24 A, right panel).

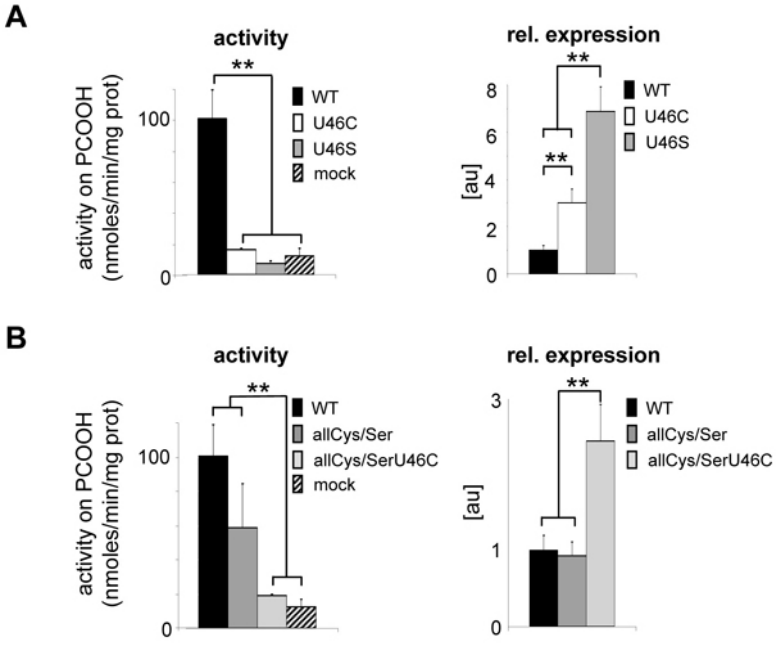

Figure 24: The Cys containing mutant variants of GPx4 do not show any detectable GPx4-specific activity when analysed *in vitro*. (A) Cell lysates from the different cell lines were subjected to the "classical" GPx4-specific activity assay using phosphatidylcholine hydroperoxide as model substrate. Similar to what has been published for the Sec/Cys variant of pig GPx4 (Maiorino, Aumann et al. 1995), mouse U46C GPx4 showed a sharp drop in GPx4-specific activity compared to WT expressing cells, despite higher expression of the U46C variants ([au] stands for arbitrary units). (B) Although the allCys/Ser variant did not show a significant decrease in activity, the allCys/SerU46C variant again showed a significant drop in activity (left) and an increase in protein expression level (right). Shown is one out of 2 independent biological experiments (mean ± SD of 3 technical replicas). Statistically significant results are marked with a star ($p<0.05$) and highly significant results with two stars ($p<0.001$).

From this it can be concluded, that despite a clear drop of GPx4 activity of the U46C variant, the U46C variant is able to sustain basic cell functions of GPx4-deficient cells, even when challenged with different stress conditions.

4.2 Investigating the function of the long form of GPx4

4.2.1 Generation and stable expression of the long form of GPx4 and mutant forms thereof in inducible *GPx4*$^{-/-}$ cells

All of the above described experiments were performed with the cytosolic variant of GPx4 (cGPx4), also called the short form of GPx4. To analyse whether the mitochondrial form of GPx4 (mGPx4), also called the long form, may rescue cell death induced by disruption of the endogenous GPx4 gene, a construct was generated, which included the N-terminal mitochondrial leader sequence (Mls) as an extension of the cytosolic GPx4. To this end, the Mls was cloned in front of the FSH-tagged GPx4 and the U46C variant, yielding mWT and mU46C, respectively. Furthermore, the Cys at position 16 in the Mls was replaced with a Ser, resulting in MlsC16S. The respective protein expression was verified by western blot analyses (Figure 25). Like previously observed, conversion of Sec to Cys caused a strong increase in expression while MlsC16S was similarly expressed as mGPx4 (Figure 25, mWT).

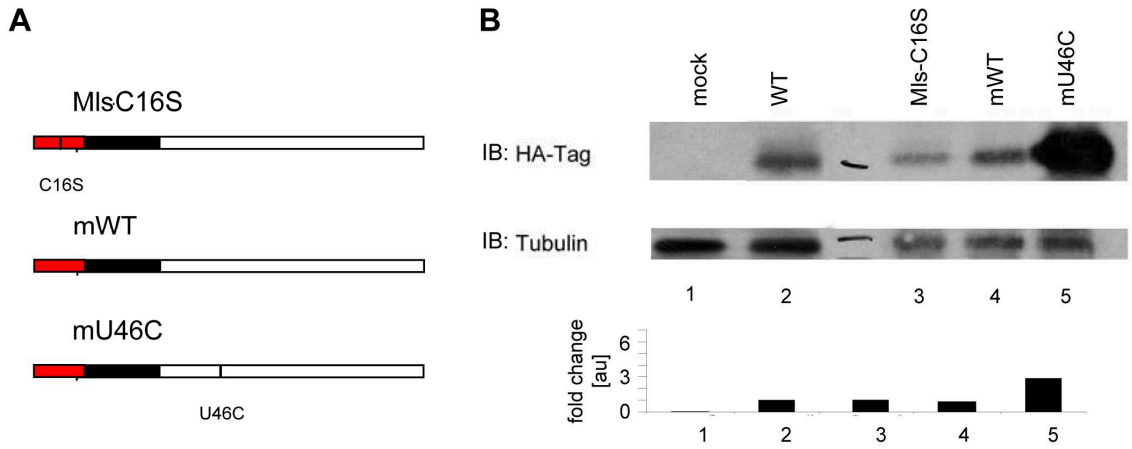

Figure 25: Expression of MlsC16S, mWT and mU46C. (A) Shown is the corresponding position of the inserted mutation. The Mls is shown in red, the FSH-tag in black and the ORF of GPx4 in white. (B) Expression of the different mutants was determined by immunoblotting using an HA-specific antibody. α-Tubulin was used to assess protein loading. Below, expression efficiency was quantified as described in Materials and Methods (3.5.2). Expression of the individual mutants was normalized to β-Tubulin levels presented as fold increase/decrease compared with wild type GPx4 expression arbitrarily set as 1.

To determine whether the Mls tag indeed conferred mitochondrial localisation, the localisation pattern of GPx4 was investigated by immuno-fluorescence and confocal laser microscopy (Figure 26). A co-localization study was conducted using Mitotracker red, and a rat GPx4-specific primary antibody visualized by means of a Cy5-conjugated α-rat, secondary antibody (fluorescing deep red). To ascertain that only reconstituted mGPx4 protein will be detected, Pfa1 cells, expressing the different forms, were treated with Tam

prior to immuno-fluorescence analysis in order to deprive cells from endogenous GPx4. Following this, all cell lines were cultured in the presence of 1 µM α-Toc. The cells expressing the transduced GPx4 variants were recorded using the FITC-channel (Figure 26). As expected, no GPx4 signals could be detected in the parental cells after knockout induction, whereas GPx4 protein was detectable in all reconstituted cell lines.

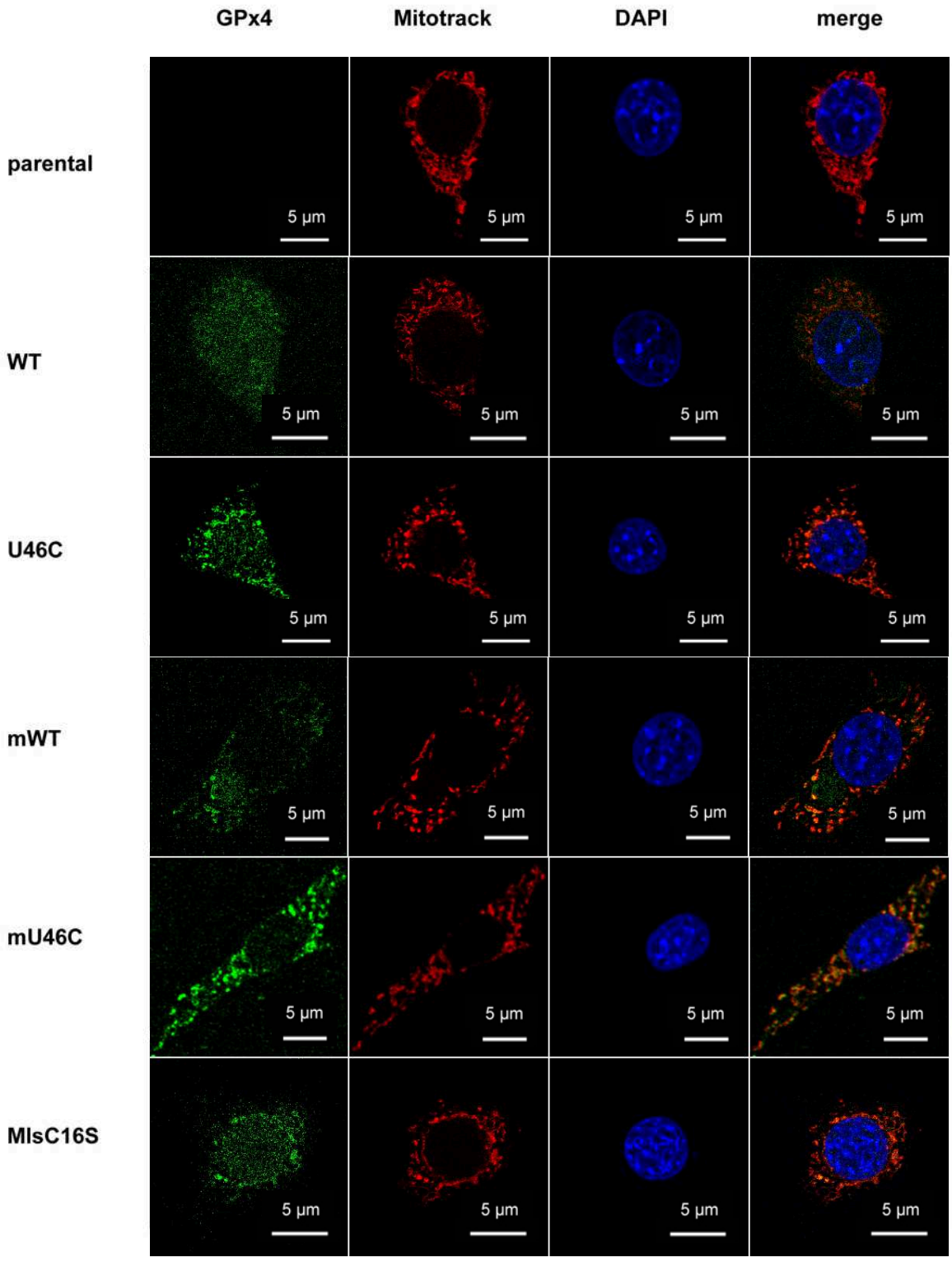

Figure 26: Subcellular localisation of GPx4 knockout cells expressing different GPx4 isoforms. Images were acquired using confocal laser microscopy. Mitochondria were stained with MitoTracker Red, GPx4 with a GPx4-specific antibody (1B4) that was detected by a secondary, Cy5-conjugated antibody (green). Nuclei were

counterstained with DAPI. Prior to analysis cells were treated with Tam to deplete cells from endogenous GPx4 and cultured in the presence of 1 µM α-Toc to prevent cell death. As expected, no GPx4 immuno-reactivity was observed in the parental cells. Protein expression in cells expressing the U46C variants was clearly higher than that in WT expressing cells. Colocalization is seen as yellow in the merge. Background was substracted by Image J programand fake colours were added to greyscale phtoos for better visibility..

Intriguingly, the cGPx4 could be detected in the cytosol as well as inside mitochondria. By contrast, localisation of mWT and mU46C was predominantly in mitochondria. However, when the Cys in the Mls was mutated to Ser, the localisation of MlsC16S changed and it was also detectable in the cytosol (Figure 26).

4.2.2 Mitochondrial expression of GPx4 does not rescue GPx4 knockout cells from cell death

Next, it was asked whether mGPx4 is capable of rescuing cell death induced by endogenous GPx4 deficiency. Previously it was shown by several groups that overexpression of mGPx4 has an anti-apoptotic effect in cells and in transgenic mice (reviewed in (Conrad, Schneider et al. 2007; Conrad 2009)).

In the absence of Tam, cell proliferation rates of the different variants were comparable between overexpressing cells and mock-transfected cell lines as well as parental cell lines (Figure 27 A). However, when endogenous GPx4 was disrupted by Tam treatment, only the MlsC16S variant was able to prevent cell death, whereas the mGPx4 and mU46C failed to rescue (Figure 27 B). This demonstrates that mitochondrial localisation of GPx4 is not sufficient to rescue cells from cell death and supports the idea that cytosolic localisation of GPx4 is prerequisite to prevent cell death induced by endogenous GPx4 disruption.

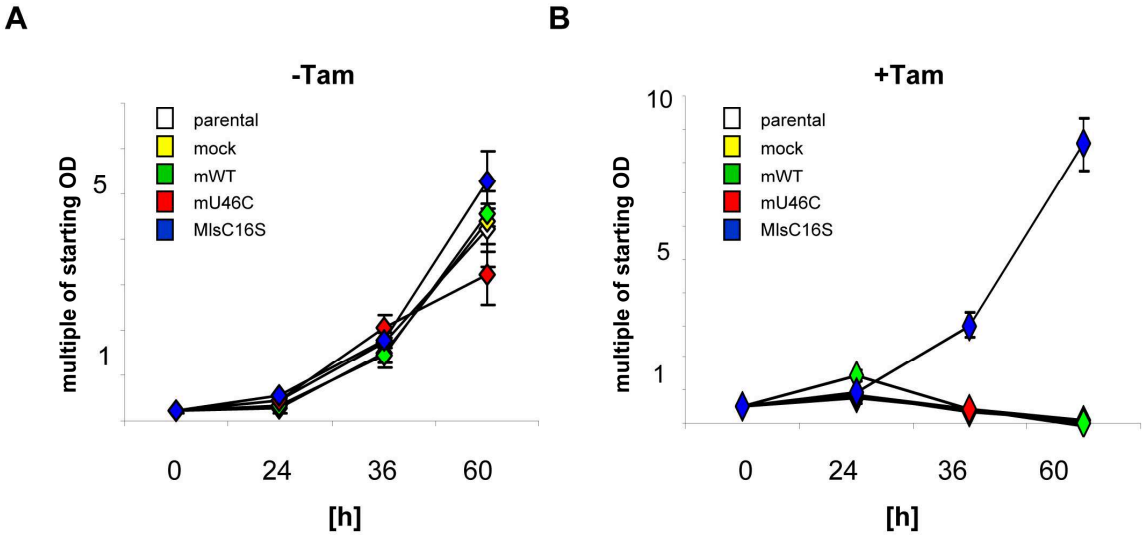

Figure 27: Overexpression of the long form (= mitochondrial form) of GPx4 fails to prevent cell death induced by the disruption of endogenous GPx4. (A) In the absence of Tam, no significant difference in the proliferation rate could be detected. (B) Tam treatment revealed that neither mWT nor mU46C and only MlsC16S

was able to rescue the cells from cell death. First measurement was set as 1. Shown is one representative experiment out of 2 independent biological experiments (mean ± SD out of 6 technical replicas).

Intrigued by these findings, the question arose whether the inability of mWT and mU46C to rescue the cells from cell death was due to its localisation inside mitochondria or to its insertion into sub-mitochondrial structures such as the outer membrane like a transmembrane protein. Due to a considerable amount of MlsC16S being localized in the cytosol, *in silico* prediction of a possible transmembrane domain (TMB) was performed using TMdas. This analysis revealed that the N-terminus of Mls-GPx4 seems to carry a transmembrane domain (Figure 28).

Apparently, there are two possible explanations for this: i) substitution of the Cys to a Ser at this point is weakening the signal and seems to prevent an insertion of the MlsC16S into the mitochondrial membrane, thereby enabling it to rescue the otherwise lethal phenotype of GPx4 deletion, or ii) the substitution disrupts or at least impairs the ability of mGPx4 to be imported into mitochondria.

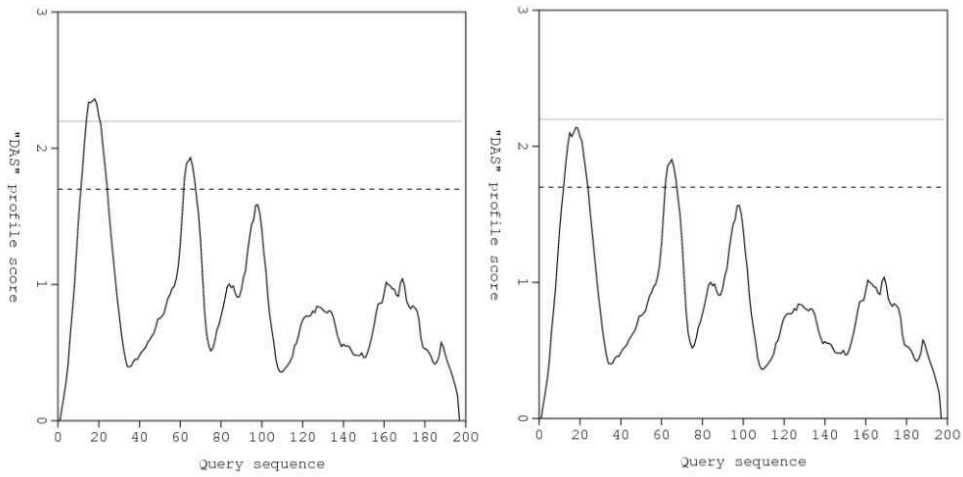

Figure 28: Transmembrane domain (TMD) prediction using TMdas (http://www.sbc.su.se/~miklos/DAS/). A loose cut off for a possible TMD is set at 1.7 (dashed line), and a strict cut off is set at 2.2 (solid line). Results are shown for mWT on the left panel and MlsC16S on the right, indicating that the prediction value drops below the needed value to be a cognate TMD.

4.2.3 Overexpression of MlsC16S protects cells from oxygen induced cell death

The stress model encompassing hypoxia, hyperoxia and hypoxia/reperfusion was applied to MlsC16S overexpressing cells (Figure 29 A). Interestingly, the MlsC16S variant showed a significantly decreased protection of cells under hypoxic and hyperoxic conditions, when compared to WT expressing cells, whereas no siginificant difference in survival could be detected during normal cell culture conditions (Figure 29 A). When exposed to the prolonged hypoxia/reperfusion model the MlsC16S overexpressing cells showed a siginifcantly better survival when compared to parental cells only under 95 % oxygen, while no significant difference could be detected between WT and MlsC16S overexpressing cells under any of the investigated conditions (Figure 29 B).

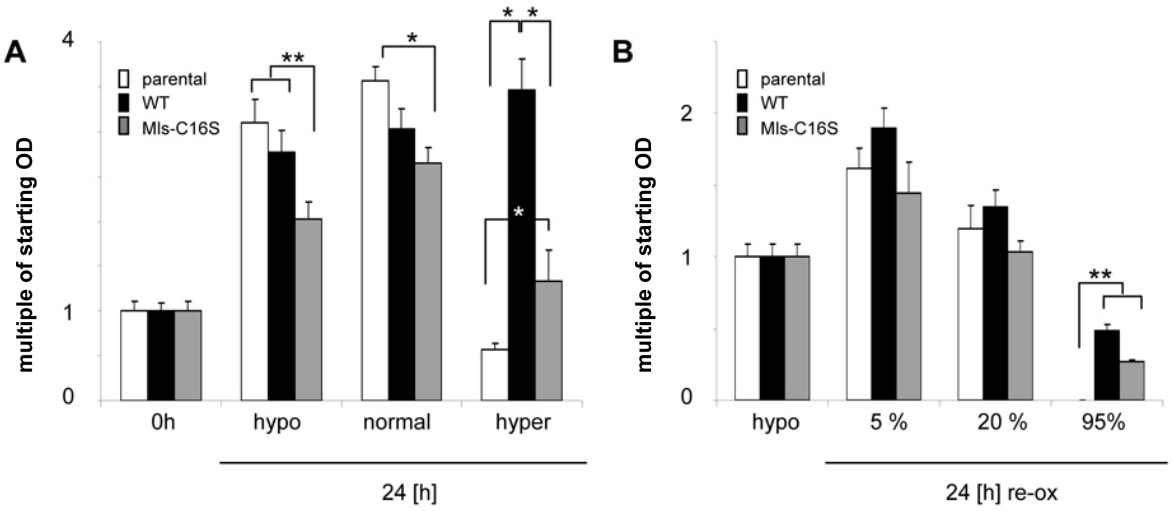

Figure 29: **Resistance to hypoxia, hyperoxia and hypoxia/reoxygenation of *GPx4*$^{-/-}$ cells expressing MlsC16S compared to WT expressing cells.** (A) PFa1 cells (parental) or PFa1 cells depleted of endogenous GPx4 by Tam but stably expressing WT or MlsC16S, respectively, were subjected to 24 hours hypoxia or hyperoxia or normal growth (20 % oxygen). (B) PFa1 cells (parental) or PFa1 cells depleted of endogenous GPx4 by Tam but stably expressing WT or MlsC16S, respectively, were subjected to 24 hours of hypoxia followed by 24 hours of reoxygenation for with the indicated oxygen concentrations. First measurement was set as 1. Shown is one representative experiment out of 2 independent biological experiments (mean ± SD out of 6 technical replicas). Statistically significant results are marked with a star ($p<0.05$) and highly significant results with two stars ($p<0.001$).

4.3 Addressing the cellular relevance of the remaining amino acids of the catalytic tetrad of GPx4

Since Sec is part of a catalytic tetrad made up of U46, Q81, W136 and N137, it was interesting to see how the other three amino acids contribute to GPx4 catalysis, and thus to its rescuing function. In order to address this, five different variants were chosen in accordance with experiments previously performed by Maiorino's laboratory utilizing DmGPx4 variants (Tosatto, Bosello et al. 2008). The following substitutions were generated by site-directed PCR mutagenesis as outlined above: the uncharged polar glutamine (Q81) was replaced either by a charged polar, acidic amino acid (glutamic acid, Q81E), which represents an exchange with a similar amino acid or by a nonpolar Alanine (Q81A). The nonpolar tryptophan (W136) was substituted by the acidic, uncharged polar aspartic acid (W136D). Finally, the uncharged polar, amino acid of Asp (N137) was replaced either by a nonpolar alanine (N137A) or by an acidic, uncharged polar aspartic acid (N137D), which again represents an exchange with a similar amino acid (Figure 30 A). Protein levels were detected by immunoblot and estimated as described above. All the different variants showed expression levels similar to WT GPx4 (Figure 30 B).

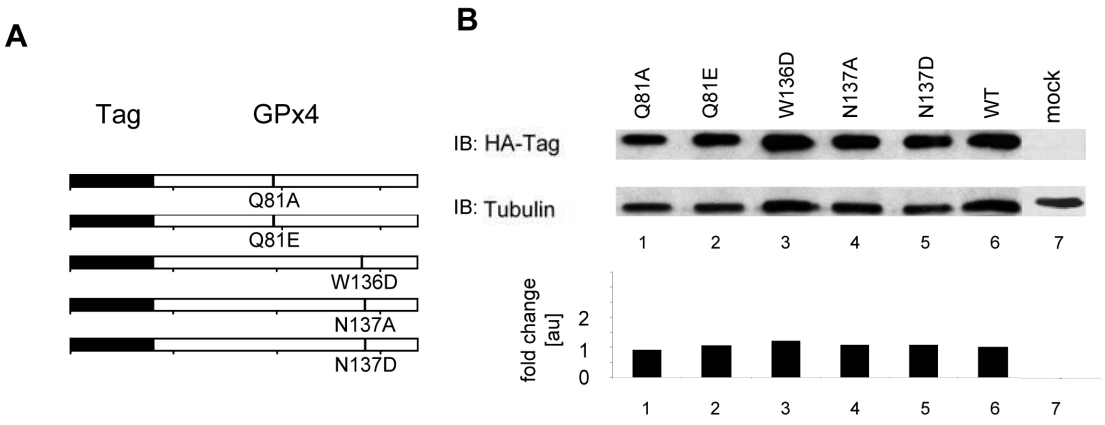

Figure 30: Expression of the mutants of the catalytic tetrad was determined by immunoblotting using an HA-specific antibody. (A) Positions of the different site-directed alterations in GPx4. (B) β-Tubulin was used to assess loading of protein lysates. Below, expression efficiency was quantified as described in Materials and Methods (3.5.2). Expression of the individual mutants was normalized to β-Tubulin levels presented as fold increase/decrease compared with wild type GPx4 expression arbitrarily set as 1.

In the absence of Tam, cells expressing the different variants showed no significant difference in proliferation when compared to each other (Figure 31 A). However, when endogenous GPx4 expression was abolished by Tam, only the Q81E and N137A variants were able to prevent the cells from cell death, whereas Q81A, W136D and N137D failed to rescue (Figure 31 B). This indicates that the composition of the aminoacids of the catalytic tetrad (Figure 11) plays an important role in enzymatic activity.

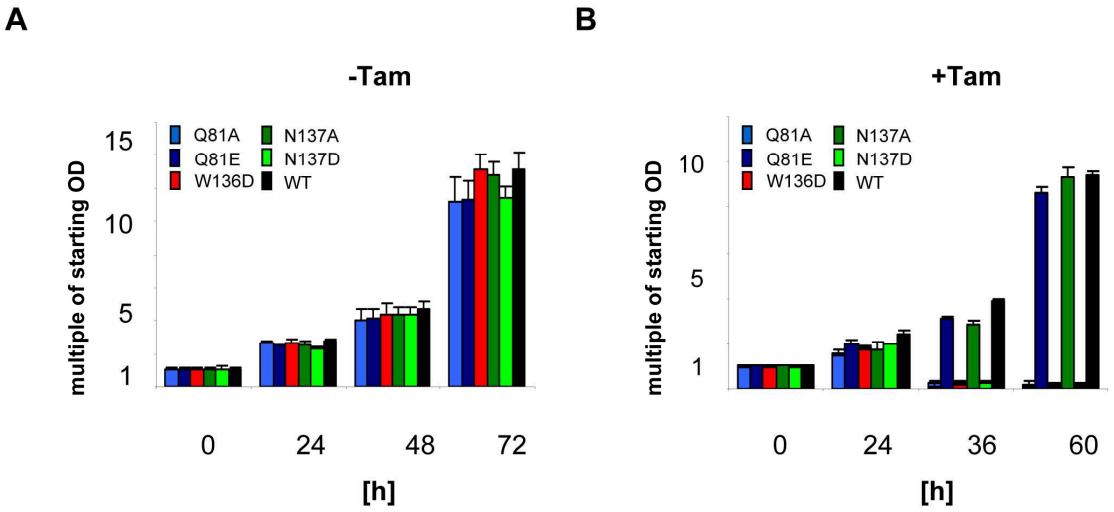

Figure 31: Addressing the in vivo relevance of individual amino acids of the catalytic tetrad involved in GPx4 catalysis in cell survival and proliferation. (A) In the absence of Tam proliferation rates were comparable between all cell lines, ruling out any dominant negative effects. (B) Only the Q81E and N137A mutants were able to rescue endogenous GPx4 loss. First measurement was set as 1. Shown is one representative experiment out of 2 independent biological experiments (mean ± SD out of 6 technical replicas).

5 Discussion

5.1 *Elucidating the catalytic mechanisms of GPx4 in cells and mice*

5.1.1 A Cys variant of the wild type GPx4 selenoenzyme rescues cell death induced by GPx4 knockout despite non-detectable *in vitro* catalytic activity

The conceptual basis for this work is built on three previous key findings concerning GPx4 activity and function. Firstly, GPx4 is unique as its short (cytosolic) form is, so far, the only glutathione peroxidase, which has been shown to be essential for cell survival/proliferation and early mouse embryogenesis as well as neuronal development in newborn mice (Imai, Hirao et al. 2003, Yant, Ran et al. 2003, Seiler, Schneider et al. 2008, Wirth, Conrad et al. 2010) By stark contrast, the nuclear and mitochondrial forms of GPx4 confer important functions only in sperm maturation (Pfeifer, Conrad et al. 2001, Conrad, Moreno et al. 2005, Schneider, Forster et al. 2009). Using tamoxifen-inducible GPx4 knockout cells (designated as PFa1 cells), it has been shown previously in our laboratory that the inducible depletion of endogenous GPx4 expression leads to an increase in 12/15-LOX activity and lipid hydroperoxide generation (Seiler, Schneider et al. 2008). In the same study it was also proposed that this ultimately causes apoptosis inducing factor (AIF)-mediated and caspase independent cell death. Cell death progression downstream of GPx4 inactivation could be interrupted either by inhibitors directed against 12/15-LOX, α-TOC or siRNA-mediated knockdown of AIF (Loscalzo 2008, Seiler, Schneider et al. 2008). Most recent data indicate that relocation of AIF from mitochondria to the nucleus requires truncation, and thus activation of the pro-apoptotic molecule Bid (Tobaben, Grohm et al. 2011). Secondly, it was shown, that the substitution of the Sec by Cys within the catalytically active centre of GPx4 leads to a decrease of the protein activity by approximately three orders of magnitude (Maiorino, Aumann et al. 1995). Thirdly, it has been known that Cys-containing homologues of GPx4 do exist in other phyla like insects, plants or yeast.

The findings and facts described above raised the fundamental question whether the complex incorporation of Sec into GPx4 is necessary for its function or whether specific alterations of the catalytic site are tolerated with respect to its rescuing activity. In order to shed light in this unsolved mystery, the Sec codon in GPx4 was substituted by either a Cys (U46C) or a Ser (U46S) codon. The Ser mutation served as a negative control as this mutant

does not have any catalytic activity due to its hydroxyl group instead of the seleno- or thiol-group (Seiler, Schneider et al. 2008). All three mutated forms of GPx4 carried a FSH (Flag-Strep-HA) tag at their N-termini (Gloeckner, Boldt et al. 2007), which would not only allow purification of yet-to-be identified binding partners/novel substrates of GPx4 but also the unambiguous detection of GPx4 in cells and tissues by immuno(histo)chemical means. A lenti-viral system was chosen to ensure high transduction efficiencies of the PFa1 cells as well as robust and stable expression of the different cGPx4 variants.

To address a potentially rescuing capability of the respective cGPx4 variants, cells expressing the different forms were experimentally deprived of endogenous GPx4 expression by conditional knockout of the floxed *GPx4* gene by tamoxifen application. As expected, lentivirus-based reconstitution of WT expression was able to rescue the cells from cell death in contrast to the U46S variant, which failed to rescue the cells. Most surprisingly, the U46C variant was able to prevent cell death induced by endogenous GPx4 knockout like WT expressing cells. This finding was unexpected in light of a previous report by Maiorino and colleagues who showed by kinetic analysis of the purified pig GPx4 homologue, that a Sec/Cys substitution leads to an enzyme with an at least three orders of magnitude decreased catalytic efficiency, when measured in the classical GPx4-specific activity assay (Maiorino, Aumann et al. 1995). As observed in the study with purified pig GPx4 homologue (Maiorino, Aumann et al. 1995), the *in vitro* measured activity of PFa1 cells expressing the U46C mutant was also indistinguishable from background levels (Figure 24). Therefore, it remains obscure what the actual activity of the Sec/Cys mutant in intact cells is. It seems that despite its significantly decreased *in vitro* activity the U46C mutant enzyme still has sufficient activity in cells to prevent cell death and confers resistance to various stress inducing agents as discussed below. This indicates that the Cys mutant of GPx4 is particularly instable during experimental manipulations and/or during the activity assay, when it is susceptible to a possibly irreversible over-oxidation due to the oxidizing conditions in a cell-free system. This over-oxidation would lead to an inactivation of the enzyme and therefore calls for an optimised experimental procedure in order to prevent possible artefactual over-oxidation for instance during cell lysis or purification of heterologously expressed recombinant proteins. One solution to this technical limitation might be to perform these experiments under anoxic conditions.

Another question that arises from these somehow unexpected findings on the Cys mutant of GPx4 is whether the biochemical measurements are indeed representative of the cellular mechanism required to protect the cells from cell death. At least two possibilities may be envisaged: firstly, the protection from cell death induced by lipid hydroperoxides results in an over-oxidised U46C variant which is subsequently degraded. Secondly, reduction of the oxidised U46C protein to its ground state is no longer achieved by two molecules of GSH

and therefore would be difficult to be determined by the classical GPx4-specific assay (Maiorino, Gregolin et al. 1990). A mixed disulfide (E-S-SG) will be barely resolved by a second GSH, however, it could be reduced by glutaredoxin (deglutathionylation) like any other glutathionylated protein (Holmgren 1979). Recently S-glutathionylation has been shown to be important in a number of critical oxidative signaling events and has been proposed to act as a biological switch (Xiong, Uys et al. 2011). It also influences protein structure/function and cellular regulatory events. Recently, increasing numbers of Cys-based redox switches are being found in many different regulatory and signalling systems (Spadaro, Yun et al. 2010). It has also been shown, that S-glutathionylation is also involved in cell death signalling, as Fas becomes *S*-glutathionylated on Cys 294 in the death domain (Janssen-Heininger, Aesif et al. 2010). *S*-glutathionylation of Fas does occur following degradation of Grx1 by caspases eight- and/or three. This promotes the recruitment of Fas into lipid rafts and assembly of the death inducing signalling complex (DISC). Importantly, overexpression of Grx1 attenuates Fas-induced apoptosis, while ablation of Grx1 increases the sensitivity of cells to undergo Fas-induced apoptosis.

The results obtained from the cell culture experiments imply that, at least under normal *in vitro* cell culture conditions, the complex incorporation of Sec into GPx4 is dispensable and that at least a thiol group is essential for the rescuing effect. This is in line with the fact that other less complex organisms do not require a Sec residue in the GPx4 homologues in order to develop and survive (Diechtierow and Krauth-Siegel 2011). Moreover, it still remains unclear whether these findings are just representative for cellular studies or whether these findings can be translated into in an *in vivo* setting.

5.1.2 Slight differences in the rescuing activity between WT and U46C GPx4 upon stress conditions

To unravel a possible difference in oxidative stress susceptibility of cells expressing WT and the U46C variant, a series of cellular studies were conducted. To induce oxidative stress, WT and U46C expressing cells were exposed to BSO, H_2O_2, Doxorubicin or tBOOH. Additionally, cells were exposed to hypoxia, hyperoxia or conditions mimicking hypoxia/reperfusion for different time intervals as a more pathophysiological stress condition (Bunn and Poyton 1996, Lee and Choi 2003, Freiberg, Krieg et al. 2006, Kulkarni, Kuppusamy et al. 2007). Thereby, it could be shown in this work that no difference could be detected between the cells expressing either the WT or U46C variant when exposed to different concentrations of BSO, H_2O_2 or Doxorubicin. Only the treatment of cells with low (0.5 µM) tBOOH

concentrations unraveled a difference in the rescuing activity. As an independent means to impose oxidative stress, cells were either kept under hypoxia, hyperoxia or hypoxia/reperfusion for different time periods. While no difference could be detected between the WT and U46C expressing cells during short (6 hours), medium (12 hours) or long (24 hours) exposure to hypoxic or hyperoxic conditions, respectively, a difference could be unmasked by the hypoxia/reperfusion experiment. While the U46C form seemed to have a slight survival advantage during the shorter intervals of exposure, it became apparent that the WT form was able to protect the cells more efficiently during longer exposure times. This is in line with the biochemical findings, concerning the lower (hardly detectable) activity of U46C despite a higher expression level. While the cells expressing the U46C variant express approximately three-fold higher protein levels, which may confer protection for a short period of time, the higher activity of WT GPx4 is able to provide a survival advantage over a longer period towards chronic oxidative stress. This assumption seems to be reasonable, as the cells expressing the U46S variant fail to survive the knockout of endogenous GPx4, whereas the expression of the U46C variant allows for survival of cells under these conditions.

5.1.3 The catalytically important tetrad is required for the rescuing effect of GPx4 and its variants

In order to investigate the necessity of a catalytically active site in greater detail, a closer look was taken at the three remaining amino acids, Q81, W136 and N137, known to form the catalytic tetrad along with U46 (Flohe, Toppo et al. 2011). Therefore, the three amino acids were replaced by selected amino acids according to a report by Maiorino's laboratory (Tosatto, Bosello et al. 2008) using site-directed mutagenesis of WT GPx4. This yielded Q81A, Q81E, W136D, N137A, and N137D, respectively. The substitutions were chosen in order to shift the pK_a value of the Sec moiety towards more alkaline values. Additionally, the substitution of W136D removes the aromatic ring provided by Trp and is thereby able to perturb a possible electron shift between Sec and Trp. In line with the findings of Tosatto and colleagues (Tosatto, Bosello et al. 2008), the rescuing activity of GPx4 was fully abolished by the Q81A and N137D substitutions, which were shown to be inactive variants. The inability to rescue cells from cell death by these forms probably results from a change in the environment around the catalytically active Sec, which is an unfavourable condition for deprotonation by shifting the pK_a value to a basic value (Maiorino et al., 1998). Thus, these alterations are considered to have an adverse impact on Sec deprotonation and thereby impair proper GPx4 function. It is also conceivable that the changes result in the inability to

reduce Sec back into its ground state by interfering with the ability of GSH to form a selenenyldisulfide. It is noteworthy to mention, that the N137A substitution, which rescued the cells, leads to a pK_a of around 7.8 (Tosatto, Bosello et al. 2008), which is closer to the WT scenario (pK_a of 7.2) than that of the N137D variant, which results in a pK_a of around 10.2. Only very low activity could be detected for the N137A variant, whereas no activity could be detected for the N137D form (Tosatto, Bosello et al. 2008). As the W136D mutation also proved to be lethal, further investigations including substitutions of Trp by phenylalanine (Phe) could be used to shed more light in a possible electron shift between Sec and Trp. This would also strengthen the idea that a lowered activity of GPx4 is sufficient to rescue cells form cell death caused by endogenous GPx4 depletion and prove that a catalytically active centre is crucial for the rescuing effect of the GPx4 variants.

5.1.4 Inducible re-expression of WT and U46C GPx4 variants in GPx4 knockout cells reveals a difference in rescuing function

Intruiged by the higher protein expression of U46C variant in combination with the non-detectable enzymatic activity it was decided to investigate whether the amount of GPx4 expression could have an impact on survival/proliferation following disruption of endogenous GPx4. To achieve this, an expression system was established where the amount of WT or U46C expression could be tightly controlled by the inducer dox at sub-nanomolar levels (Bornkamm, Berens et al. 2005). A comparable expression of the mRNAs of the two different proteins, controlled by the amount of inducer, allowed unravelling that cells expressing the U46C construct require a higher expression level of mRNA and enzyme than WT expressing cells to obtain a survival effect and maximal proliferation (Figure 20). Interestingly, the level of mRNA expression required to produce the maximal effect on cell proliferation in WT expressing cells was detectable by qRT-PCR, yet at this condition GPx4 could not be detected at the protein level by immunoblotting. This probably mimics the condition of many somatic cells, where GPx4 is expressed at very low levels (Roveri, Maiorino et al. 1994). To obtain a comparable rescuing function, a larger amount of the Cys mutant is required and this corresponds reasonably well with the lower catalytic activity of the Cys- as compared to the Sec-containing enzyme in reducing hydroperoxides.

Interestingly, higher expression levels of GPx4 proved to attenuate the proliferation rate in WT but not in U46C expressing cells. This may suggest that, besides its well established antiapoptotic function, wild-type GPx4 is involved in the control of cell cycle progression by the modulation of receptor tyrosine kinase signalling. It is a well established paradigm that

protein tyrosine phosphatases (PTPs) are regulated through reversible oxidation of the active-site cysteine by H_2O_2 (Boivin, Zhang et al. 2008; Boivin and Tonks 2010). The mechanism relies on the transient oxidation of the thiolate by H_2O_2 or other hydroperoxides (Gracanin and Davies 2007). In a recent report published by our group (Conrad, Sandin et al. 2010), it was shown for the first time that the 12/15-LOX metabolic intermediate, 15-OOH-eicosatetraenoic acid, a lipid hydroperoxide, is able to efficiently induce PTP oxidation at much lower concentration than H_2O_2, the classical inducer of PTP oxidation. Consequently, PDGF β-receptor specific phosphorylation was increased in GPx4 knockout cells due to increased oxidation of PTPs by lipid hydroperoxides. Hence, a higher expression of wild-type GPx4 may lead to lower peroxide levels in cells, and consequently to an increased steady-state active stage of phosphatases with an adverse impact on proliferation.

Unlike prevention of apoptosis, which among peroxidases is specific for GPx4, the function of attenuating the proliferation rate is shared with GPx1, a selenoperoxidase localized together with GPx4 in the cytosol and mitochondria of somatic cells (Handy, Lubos et al. 2009, Liang, Yoo et al. 2009). In view of the pivotal role of hydroperoxides in both survival and proliferation (D'Autreaux and Toledano, 2007; Schumacker, 2006), and the different substrate specificity of the two peroxidases, this observation might support the notion that survival and proliferation are controlled by different hydroperoxides of distinct chemical identity. For instance, membrane hydroperoxides, which are specific substrates of GPx4, are relevant for apoptosis (Seiler, Schneider et al. 2008), whereas small hydroperoxides including H_2O_2 and free fatty acid hydroperoxides, both common substrates of GPx1 and GPx4, are relevant for proliferation possibly through redox-regulated cell signalling (Chen, Cai et al. 2006, Conrad, Sandin et al. 2010). Elucidation of the nature of the distinct oxidants involved in the plethora of the proliferative pathways is emerging a major challenge for the near future. This suggests that efficiency, in conjunction with expression level of WT GPx4, are key factors to properly regulate the balance between survival and proliferation of cells.

5.1.5 The catalytic cycle of U46C does not involve a resolving Cys (C_R)

It has been known that the CysGPxs of invertebrates and plants which swiftly react with hydroperoxide carry a resolving Cys (C_R) (Trujillo, Mauri et al. 2006, Hall, Karplus et al. 2009). Therefore, it was an interesting hypothesis to see whether the U46C variant of GPx4 may exploit a similar catalytic mechanism in order to remain in a functional state.

To address this concept, a variant form of U46C was generated which lacks all but the catalytically active Cys (allCys/SerU46C). The corresponding form of WT (allCys/Ser) served

as control. Like WT GPx4, the allCys/Ser variant was able to rescue the cells from cell death induced by disruption of endogenous GPx4. The allCys/Ser form showed no significant difference in GPx4-specific activity or stress resistance when compared to WT. Likewise, the allCys/SerU46C variant behaved similarly to the U46C variant. This data provides conclusive evidence that none of the non-catalytical Cys residues is necessary for proper GPx4 activity in WT GPx4, nor for its rescuing activity. Hence, neither of the Cys residues is used as a default C_R in the U46C variant.

In conclusion, this set of data points towards a higher resistance of GPx4 against over-oxidation when carrying a Sec in the active site. Yet it does not exclude the possibility that a C_R would be favourable for full enzymatic activity in case of a Cys replacement in the active site (as it is found in the majority in non-vertebrate glutathione peroxidases), but it appears not to be essential for the ability of U46C to protect cells from cell death. Whether these non-catalytic Cys residues might only play a role in forming the mitochondrial capsule by becoming cross-linked with other capsular proteins (Mauri 2005, Ursini Science 1999) or in chromatin condensation by forming disulfide bridges with protamines (Pfeifer FASEB 2001, Conrad MCB 2005) can only be addressed by generating mice expressing exclusively the WT (allCys/Ser) form.

5.2 *Cellular investigations concerning the long form of GPx4*

5.2.1 Expression of the long form of GPx4 fails to protect cells from cell death

Previous work in our laboratory demonstrated that neither the mitochondrial (long) form (mGPx4) (Schneider, Forster et al. 2009), nor the nuclear form of GPx4 are required for embryogenesis and survival of mice (Conrad, Moreno et al. 2005). As this work has shown that the short form of GPx4 is able to protect the cells from cell death induced by disruption of endogenous GPx4, it was obvious to ask whether the long form (mGPX4) could provide a similar protective mechanism. In order to address this question, the WT and U46C variants of mGPx4 were cloned and expressed in PFa1 cells. Respective variants of the nuclear form were not investigated as it is only expressed in testis (Pfeifer, Conrad et al. 2001, Conrad, Moreno et al. 2005, Schneider, Vogt Weisenhorn et al. 2006).

As expected the expression of the mU46C variant was higher than that of mWT, which is consistent with the phenotype already observed for the short variants of WT and U46C. Interestingly, neither the WT nor the U46C variant of mGPX4 was able to protect the cells from cell death induced by inducible disruption of endogenous GPx4 expression. This is consistent with recent findings of Liang and collaborators, who showed that expression of the short form of GPx4 in transgenic mice promotes survival and normal development, whereas expression of the long form of GPx4 failed to rescue the lethal phenotype (Liang, Yoo et al. 2009). The only obvious phenotype they showed in mice expressing the short form was male infertility (due to the lack of the mitochondrial form), which confirms findings by our groups (Schneider, Forster et al. 2009). These findings are compatible with two possibilities: (i) GPx4 exerts its cell death protecting function exclusively in the cytoplasm and not in mitochondria, or (ii) the short form is required for survival in the cytosol as well as in mitochondria. The later case implies that the short form lacking a mitochondrial localization sequence is imported into mitochondria through a yet unknown mechanism.

5.2.2 The importance of subcellular localisation

To furher corroborate the importance of the intracellular localization of GPx4 for protection against cell death, another form of GPx4 was designed which carries a substitution of Cys by Ser on position 16 of the Mls (MlsC16S). According to *in silico* analysis (http://www.sbc.su.se/~miklos/DAS/), this mutation would weaken a potential transmembrane domain positioned in the Mls. Co-localisation studies were conducted using confocal laser microscopy to determine the localisation of the different GPx4 variants. As expected the mGPx4 was found almost exclusively in mitochondria. By contrast, the MlsC16S variant was detected in mitochondria as well as in the cytosol. It is noteworthy in this context that the short form of GPx4 has also been found in the cytosol as well as in mitochondria. When deprived of endogenous GPx4, cells expressing the MlsC16S variant survived, as did the GPx4 expressing cells in contrast to mGPx4 expressing cells. This clearly indicates that the subcellular localisation (i.e. in the cytosol) of GPx4 determines its rescuing activity. However, it became obvious during hypoxia and hyperoxia experiments, that the cells expressing the MlsC16S form were less resistant than WT or U46C expressing cells. These results imply that the localisation the GPx4 is important for its full function. Liang et al. hypothesized that the rescuing effect of GPx4 is primarily due to its inner membrane localisation in mitochondria and its ability to repair cardiolipin (CL) peroxidation (Liang, Ran et al. 2009). CL is an important component of the inner mitochondrial membrane, where it constitutes about

20% of the total lipid composition. The only other place where cardiolipin can be found is in the membranes of most bacteria (Pangborn 1946). CL regulates aggregate structures and is believed to play a role in membrane fusion (Ortiz, Killian et al. 1999). Complex V of the oxidative phosphorylation machinery displays a high binding affinity for CL, binding four molecules of CL per molecule of complex V (Eble, Coleman et al. 1990). Recently it has been shown, that CL and horse cytochrome c can interact (Ascenzi, Polticelli et al. 2011) with either proapoptotic (i.e. catalysing lipid peroxidation), or antiapoptotic outcome (i.e. scavenging of peroxynitrite) by protecting mitochondria from reactive nitrogen and oxygen species (Ascenzi, Polticelli et al. 2011). An apoptotic chain of events is suggested, involving changes in cytochrome c phosphorylation, dephosphorylation being proapoptotic (Huttemann, Pecina et al. 2011), increased ROS levels via increased mitochondrial membrane potential, and oxidation of cardiolipin by cytochrome c followed by its release from the mitochondria (Kagan, Wipf et al. 2009, Huttemann, Pecina et al. 2011).

Another finding points to a possible role of GPx4 in mitochondria. Upon deletion of the endogenous GPx4 gene cell death is mediated by translocation of AIF from mitochondria into the nucleus (Lorenzo, Susin et al. 1999, Susin, Lorenzo et al. 1999, Galluzzi, Joza et al. 2008, Seiler, Schneider et al. 2008, Joza, Pospisilik et al. 2009, Norberg, Orrenius et al. 2010, Sevrioukova 2011). Whether and how mitochondrially localised cGPx4 is involved in this cell death inducing mechanism remains to be studied.

A putative role of the short form in mitochondria raised the question as to how the short form GPx4 is imported into mitochondria in the absence of a “classical” Mls-sequence. Several different mechanisms are known for the import and sorting of proteins in mitochondria. The classical one is guided by an N-terminal pre-sequence (Bolender, Sickmann et al. 2008), but there are also different forms of Mls-sequences for proteins anchored in the inner or outer mitochondrial membrane (Rapaport 2003). Interestingly, more than 50 % of the mitochondrial proteins do not use the classical pathway for their import into mitochondria (Bolender, Sickmann et al. 2008). One of the well described alternative pathways is the Mia40 – Erv1 system, which is responsible for the import of proteins into the intermembrane space (IMS) (Stojanovski, Muller et al. 2008). This system relies on certain characteristic motives in the target proteins such as CXC or CX_9C (Hofmann, Rothbauer et al. 2005), which, however, are absent in GPx4. This import system functions basically by trapping imported proteins inside the IMS due to introduction of an intramolecular disulfide bond after their transit trough the outer mitochondrial membrane mediated by the transport outer membrane (TOM)-system (Stojanovski, Muller et al. 2008). As the Mia40 – Erv1 system relies on a redox cycle linked to the respiratory chain via cytochrome c, it was suggested that it could be used to sense oxygen levels in the cell. Thereby, it might act as a molecular switch that adapts the mitochondrial activities to cellular oxygen levels by affecting cytochrome c (Bihlmaier,

Mesecke et al. 2008). In this case the presence of GPx4 inside mitochondria as a freely moving molecule could have a regulatory effect upon this suggested molecular switch function by regulating mitochondrial ROS levels (Huttemann, Pecina et al. 2011).

Moreover, recent studies conducted in *Arabidopsis thaliana* showed that mitochondrial carriers (MC) as part of mitochondrial carrier families (MCF) of inner membrane transporters can display a variety of different substrate specificities (Palmieri, Pierri et al. 2011). MCs are proteins from the solute carrier family which transfer molecules across the membranes of mitochondria (Nury, Dahout-Gonzalez et al. 2006). All MCs are encoded by nuclear genes. All carriers contain a common sequence, referred to as the MCF motif in each repeated region, with some variation in one or two signature sequences (Nury, Dahout-Gonzalez et al. 2006). Further experiments are certainly necessary to investigate a possible involvement of GPx4 in the suggested Mia40-Erv1 pathway with special emphasis on how the Mls-less, short form of GPx4 actually translocates to mitochondria.

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8 Publications

Mannes AM, Seiler A, Bosello V, Maiorino M, Conrad M. Cysteine mutant of mammalian GPx4 rescues cell death induced by disruption of the wild-type selenoenzyme. FASEB J. 2011 Jul;**25**(7):2135-44. Epub 2011 Mar 14.

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Hepperger C, **Mannes A**, Merz J, Peters J, Dietzel S. Three-dimensional positioning of genes in mouse cell nuclei. Chromosoma. 2008 Dec;**117**(6):535-51. Epub 2008 Jul 3.

9 Erklärung

Hiermit erkläre ich, dass die vorliegende Arbeit mit dem Titel

„Dissecting the role of selenolthiol- versus thiol-based catalysis using the model enzyme glutathione peroxidase 4 (GPx4) “

von mir selbständig und ohne unerlaubte Hilfsmittel angefertigt wurde, und ich mich dabei nur der ausdrücklich bezeichneten Quellen und Hilfsmittel bedient habe. Diese Arbeit wurde weder in der jetzigen noch in einer abgewandelten Form einer anderen Prüfungskommission vorgelegt.

München, im September 2013

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10 Curriculum Vitae

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