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CHARACTERIZATION OF THIOREDOXIN RELATED PROTEIN OF 14 KDA AND ITS ROLE IN REDOX SIGNALING

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Characterization of Thioredoxin Related Protein of 14 kDa and its Role in Redox Signaling

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To my family

Redox regulation, though now becoming fashionable, remains confusing.

Leopold Flohé, Redox Pioneer

ABSTRACT

Reversible reduction/oxidation (redox) reactions play key roles in cellular signaling pathways. Particularly cysteine residues in proteins can be modified by reactive oxygen-, nitrogen- or sulfur species (ROS, RNS, RSS), thereby altering the functions of the respective proteins. These modifications can be reversed by two major reductive systems in mammalian cells – the thioredoxin (Trx) and glutathione (GSH) systems. Both contain various representatives of the Trx fold family of proteins, among them the name-giving Trxs being the most prominent. In the cytosolic Trx system, electrons are transferred from NADPH to Trx reductase 1 (TrxR1) and subsequently to Trx1, which reduces a multitude of cellular substrates. Thioredoxin-related protein of 14 kDa (TRP14, *TXNDC17*) is a sparsely characterized, but evolutionarily well-conserved member of the Trx system. The studies comprising this thesis examined TRP14 in several aspects of redox signaling.

In **Paper I** we investigated the inhibition of TrxR1 by noble metal compounds and their effect on cancer cell survival. Inhibition of the Trx system as anti-cancer strategy is thought to attenuate the antioxidant capacity of cancer cells, thereby leading to cell death. We found that gold (Au), platinum (Pt), and palladium (Pd) compounds all inhibited TrxR1 *in vitro*, but in a cellular context, the inhibition and cytotoxicity were mainly dependent on the ligand substituents and cellular uptake. Furthermore, we found a covalent crosslink between TrxR1 and TRP14 upon treatment of cells with the antitumor agent cisplatin. We concluded that noble metals are potent TrxR1 inhibitors but Pt compounds, especially cisplatin, trigger highly specific cellular effects, including the covalent complex formation.

In **Paper II** we studied the role of the Trx system in reactivation of oxidized protein tyrosine phoshatases (PTPs) in platelet derived growth factor (PDGF) signaling. Using fibroblasts that lacked TrxR1 (*Txnrd1*$^{-/-}$), we found both an increased oxidation of PTP1B and phosphorylation of the PDGFβ receptor (PDGFβR). Consequently, we showed that both Trx1 and TRP14, coupled to TrxR1, are able to reduce oxidized PTP1B *in vitro*. This study demonstrated that the Trx system, including both Trx1 and TRP14, impacts the oxidation of specific PTPs and can thereby modulate PDGF signaling.

In **Paper III** we established TRP14 as an efficient TrxR1-dependent reductase and denitrosylase. Using several low molecular weight disulfide compounds, we found that, dependent on the substrate, TRP14 can be at least as efficient as Trx1. We also suggested TRP14 instead of Trx1 to be a major intracellular cystine reductase, because Trx1 does not reduce cystine once a preferred substrate such as insulin is present. Acting in parallel with Trx1, we also provide evidence of TRP14 being an efficient cellular reductase for nitrosylated proteins and concluded that TRP14 should be considered as an integral part of the Trx system.

In **Paper IV** we developed a novel method for the detection of protein persulfides named Protein Persulfide Detection Protocol, ProPerDP. The formation of persulfide (-SSH) moieties at regulatory cysteine residues is emerging as a major pathway of hydrogen sulfide (H_2S) mediated redox signaling. Using ProPerDP we discovered that both the Trx and the GSH system are potent reduction pathways for poly- and persulfides in cells.

These studies reinforce the notion that TrxR1-dependent pathways are not only mediated via its well-known substrate Trx1. We show that TRP14 is yet another cytosolic oxidoreductase with various intracellular functions, including reduction of PTPs, disulfides, nitrosothiols and persulfides. TRP14 is thereby potentially involved in a variety of different redox signaling pathways.

LIST OF SCIENTIFIC PAPERS

I. Stefanie Prast-Nielsen*, Marcus Cebula*, **Irina Pader**, Elias S J. Arnér.
Noble metal targeting of thioredoxin reductase – covalent complexes with thioredoxin and thioredoxin-related protein of 14 kDa triggered by cisplatin.
Free Radic Biol Med 49:1765-1778; 2010.

II. Markus Dagnell, Jeroen Frijhoff, **Irina Pader**, Martin Augsten, Benoit Boivin, Jianqiang Xu, Pankaj Mandal, Nicholas K. Tonks, Carina Hellberg, Marcus Conrad, Elias S. J. Arnér, Arne Östman.
Selective activation of oxidized PTP1B by the thioredoxin system modulates PDGF-beta receptor tyrosine kinase signaling.
Proc Natl Acad Sci USA; 110:(33), 13398-13403; 2013.

III. **Irina Pader***, Rajib Sengupta*, Marcus Cebula, Jianqiang Xu, Jon O. Lundberg, Arne Holmgren, Katarina Johansson, Elias S. J. Arnér.
Thioredoxin-related protein of 14 kDa is an efficient L-cystine reductase and S-denitrosylase.
Proc Natl Acad Sci U S A; 111(19), 6964-6969; 2014.

IV. Éva Dóka, **Irina Pader**, Adrienn Bíró, Katarina Johansson, Qing Cheng, Krisztina Ballagó, Justin R. Prigge, Daniel Pastor-Flores, Tobias P. Dick, Edward E. Schmidt, Elias S. J. Arnér, Péter Nagy.
Novel persulfide detection method reveals protein persulfide and polysulfide reducing functions of thioredoxin- and glutathione systems.
Science Advances 2:e1500968, 2016.

*Equal contribution.

Articles not included in this thesis

V. Jianqiang Xu, Sofi E. Eriksson, Marcus Cebula, Tatyana Sandalova, Elisabeth Hedström, **Irina Pader**, Qing Cheng, Charles R. Myers, William E. Antholine, Péter Nagy, Ulf Hellman, Galina Selivanova, Ylva Lindqvist, Elias S. J. Arnér.
The Trp114 residue of thioredoxin reductase 1 is an electron relay sensor for oxidative stress. *Cell Death & Disease* 6:e1616, 2015.

CONTENTS

LIST OF ABBREVIATIONS

AP-1	Activator protein 1
ARE	Antioxidant response element
ASK1	Apoptosis signaling kinase 1
ATP	Adenine triphosphate
CBS	Cystathionine-β-synthase
CSE	Cystathionine-γ-lyase
cDDP	Cisplatin (cis-diamminedichloroplatinum(II))
Cys	Cysteine
DTT	Dithiothreitol
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
ER	Endoplasmatic reticulum
ERK	Extracellular regulated kinase
FAD	Flavine adenine dinucleotide
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPx	Glutathione peroxidase
GR	Glutathione reductase
Grx	Glutaredoxin
GSH/GSSG	Glutathione (γ-glutamyl-cysteinyl-glycine)/GSH disulfide
GST	Glutathione S-transferase
HED	Hydroxyethyldisulfide (dithiodiethanol)
HIF-1	Hypoxia inducible factor 1
IκB	Inhibitor of κB
IKK	IκB kinase
KEAP1	Kelch-like associated protein 1
MAPK	Mitogen activated protein kinase
MSR	Methionine sulfoxide reductase
MST	3-mercaptopyruvate sulfurtransferase
NADPH	Nicotinamide adenine dinucleotide phosphate
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
NOS	Nitric oxide synthase
NOX	NADPH oxidase
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
PI3K	Phosphatidyl-inositol-3-kinase
PDGF	Platelet-derived growth factor
PDGFβR	Platelet-derived growth factor β receptor
PDI	Protein disulfide isomerase
Prx	Peroxiredoxin
Pt	Platinum
PTP	Protein tyrosine phosphatase
PTEN	Phosphatase and tensin homolog
Ref-1	Redox factor 1
RNR	Ribonucleotide reductase
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RSS	Reactive sulfur species
RTK	Receptor tyrosine kinase
Sec	Selenocysteine
SecTRAP	Selenium compromised TrxR-derived apoptotic protein
Ser	Serine
TNFα	Tumor necrosis factor α
TRP14	Thioredoxin related protein of 14 kDa
Trx	Thioredoxin
TrxR	Thioredoxin reductase
TXNIP	Thioredoxin interacting protein

1 INTRODUCTION

1.1 INTRODUCTION TO THE FIELD AND THE PRESENT THESIS

"Life is nothing but electrons looking for a place to rest"
Albert Szent-Györgyi (Nobel laureate, 1893-1986)

This famous quote illustrates one of the most fundamental processes in life: the flow of electrons. It means that electrons in high-energy states, which sit on top of "energy hills", roll down the hill towards a low-energy state and thereby dissipate energy [1]. It is easily conceivable how this is part of our daily life, e.g. as electricity and the batteries in our smartphones. But it is far more than that: flowing electrons drive a myriad of cellular processes, including the synthesis of ATP, the cellular energy currency.

Unfortunately, we know more about the electron flow in modern electronics than in biologic systems!

Referring to the picture of the energy hill, we transfer electrons from fuel molecules (e.g. the glucose in our food, on top of the hill) to the terminal acceptor (molecular oxygen, the bottom of the hill), which results in water. Chemically speaking, the fuel molecule becomes **oxidized**, because it loses electrons, and the oxygen molecule becomes **reduced**, because it gains electrons. To make a long story short: this is the basis for **redox reactions**, which are at the center of the field of **redox biology**. The term **redox signaling** thereby refers to cellular signaling pathways that involve redox reactions.

This thesis will cover several aspects of redox signaling:

- Oxygen (O_2) has to take up four electrons to be completely reduced. An incomplete reduction leads to reactive intermediates, **reactive oxygen species ("ROS")**, which play a central role in redox biology. In addition, **reactive nitrogen** and **reactive sulfur species** (**"RNS"** and **"RSS"**) also participate in cellular redox reactions.
- ROS, RNS and RSS can act as signaling molecules by modifying **redox-sensitive cysteine (Cys) residues** in proteins, thereby altering the functions of the respective proteins.
- In turn, these modifications can be reduced by the **Glutathione (GSH)** and **Thioredoxin (Trx)** systems. The major focus of this thesis is the Trx system, particularly the Trx related protein of 14 kDa (TRP14) and its role in redox signaling.
- A prominent example of a cellular process that involves redox reactions is growth factor signaling. Notably, the oxidation of **protein tyrosine phosphatases (PTPs)** leads to their inhibition and thereby stimulation of **receptor tyrosine kinase (RTK)** mediated phosphorylation cascades.

1.2 CONCEPTS OF REDOX SIGNALING

Generally speaking, signaling pathways have to fulfill minimal requirements. i) They need an unambiguous signal, which is ii) received by a cellular sensor and processed to evoke a specific cellular response and iii) the signaling process has to be terminated to restore basal conditions [2]. In higher organisms, signaling networks are very complex due to simultaneous competitive reactions and crosstalk between different pathways – and still, the signal has to be fast and specifically transduced to the respective effector molecule [3,4]. The best characterized way to transfer information in biological systems is via protein phosphorylation cascades. But how can signaling pathways be dependent on reduction-oxidation (redox) reactions?

For a long time it was believed that ROS were randomly produced, detrimental metabolic byproducts. Nowadays it has been widely appreciated that reactive species like superoxide ($O_2^{\bullet-}$) or hydrogen peroxide (H_2O_2) regulate signaling pathways by reversibly modifying specific proteins [5-7]. Redox signaling thereby involves both a specific response to certain receptor ligands (e.g. growth factors) that activate cells to produce oxidant species, and a stress response to excessive oxidant production (e.g. in pathological conditions) [8]. This section aims at summarizing some of the key concepts of redox signaling (see Fig. 1 for a simplified scheme).

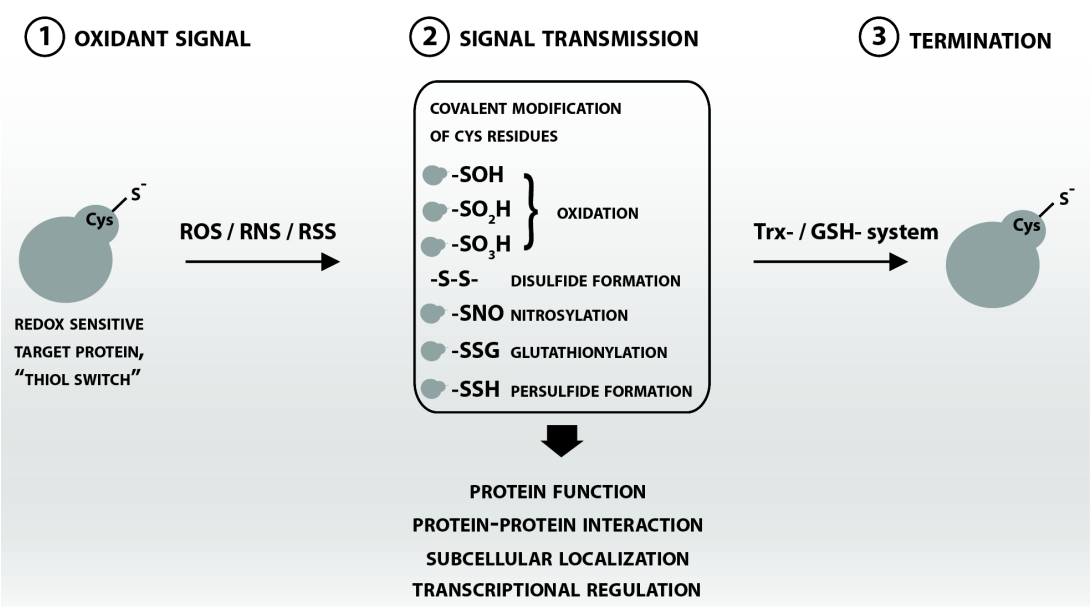

Figure 1. Simplified overview of the general concepts of redox signaling. (1) Redox sensitive target proteins, also called "thiol switches" due to the presence of a reactive Cys thiol (or the deprotonated thiolate), can be reversibly modified by ROS, RNS and RSS. These different protein modifications **(2)** (oxidation to sulfenic- (-SOH), sulfinic- ($-SO_2H$) and sulfonic- ($-SO_3H$) acids, formation of disulfides (-S-S-), nitrosylation (-SNO), glutathionylation (-SSG) and formation of persulfides (-SSH) can affect a number of different processes such as protein function, protein-protein interaction, subcellular localization or transcriptional regulation. **(3)** Reductive enzyme systems like the Trx and the GSH systems can reverse most of the Cys modifications. The cooperative actions of oxidizing and reducing mechanisms can regulate cellular signaling pathways and are a general principle of redox signaling.

1.2.1 Oxidants: What is the ideal Signal?

Out of all physiological reactive species (reviewed in depth in section 1.3), H_2O_2 is suggested to be one of the main oxidants in redox signaling [8,9]. In contrast, free radicals (e.g. $O_2^{\bullet-}$) are less suitable signaling molecules due to their high reactivity and lack of specificity, and evidence for direct thiol oxidation is limited [8,10]. The only radical that is currently accepted to be a genuine signaling molecule is nitric oxide ($NO^{\bullet}$), but for reasons discussed later, it is typically not counted as a redox-signaling molecule [3]. Instead, often the secondary products of free radicals are used for signaling purposes, e.g. the formation of H_2O_2 from $O_2^{\bullet-}$, or the formation of peroxynitrite ($ONOO^-$) from $NO^{\bullet}$ and $O_2^{\bullet-}$ [9].

1.2.2 Sensing and Transmitting the Signal

A central concept for redox signaling is that specific Cys residues in proteins act as so called "thiol switches" [11]. Their reversible modification by oxidants transiently changes the functional properties of the respective protein, which works similarly to phosphorylation/dephosphorylation. Redox-dependent switches do not operate only via ROS induced oxidation of the thiol moiety; other modifications like nitrosylation, glutathionylation and persulfidation exist as well (section 1.4).

Cys modifications have been often considered a result of global changes in the "redox balance" or the "redox homeostasis" – a perception that has recently been questioned [12-15]. In general, redox reactions in a thermodynamic equilibrium can be described using the Nernst equation. The resulting redox potential provides useful information of the ratio of oxidized to reduced form of a certain redox-couple. However, a living organism is not in equilibrium and cellular redox reactions should therefore be regarded by their kinetic and enzymatic parameters, and not by their redox potential [16,17].

The major question in the redox field that remains debated is how specificity can be achieved with "promiscuous" oxidant species. One essential property is a low acid dissociation constant (pKa) for key Cys residues. At physiological pH these residues can be deprotonated to the more nucleophilic thiolate (RS^-), which is a prerequisite for the reaction with oxidants such as H_2O_2. However, a low pKa is unlikely to be the only determinant of thiol reactivity and selectivity in redox signaling. This is illustrated by the fact that Peroxiredoxins (Prxs) and PTPs such as PTP1B have a similar pKa value, but the rate constant of Prxs with H_2O_2 is million times higher than for PTP1B [4]. On the other hand, several studies have demonstrated that stimulation with growth factors results in a burst of H_2O_2 production [18,19] and a concomitant oxidation of PTPs [20-24]. How can PTPs be specifically oxidized when enzymes like Prxs and Glutathione Peroxidases (GPxs) are around, that both can react with H_2O_2 at almost diffusion-controlled rates? PTPs should be easily outcompeted by thiol peroxidases [14]. Several hypotheses have been discussed and debated over the last years.

One of the earliest theories is the "**floodgate theory**". It suggests an over-oxidation and thereby inactivation of Prxs that in turn generates a localized zone in which H_2O_2 is available to react with less reactive cellular targets such as PTPs [25]. Based on thermodynamic

considerations this seems to be unlikely *in vivo,* and specificity for redox signaling cascades has also been doubted [14,16,26]. Additionally, in the context of signaling events, no hyperoxidized Prxs have been detected yet [27]. However, following up on the inactivation of Prxs, another study suggests inhibition of PrxI by phosphorylation in confined membrane associated areas, which would also create a local "hotspot" of H_2O_2 [28].

In line with spatially confined H_2O_2 accumulation, several studies have proposed **localized redox signaling**. Subcellular localization of NOX complexes and PTPs in lipid raft regions and ER membranes, as well as compartmentalization of additional signaling proteins, could result in specific redox signaling platforms [29-32]. Considering the limitations in diffusion distances of oxidant species, it seems feasible that they are predominantly generated in certain compartments [16]. Locally confined redox signaling has also been reported in so-called "redoxosomes", which are signaling endosomes that are triggered by growth factors and cytokines [33].

The latest concept suggests that certain thiol peroxidases that have an exceptionally high affinity for peroxides act as sensors for oxidants and that they transfer this signal to a target protein – thus acting as "**redox relays**" or "**redox mediators**" [8,14]. A recent study shows that PrxII acts as a direct peroxide sensor that oxidizes the transcription factor STAT3 [34]. Similar evidence is reported for PrxI and its action on ASK1 [35].

In summary, the current understanding of redox signaling mechanisms involves thiol oxidation either via direct reactions or facilitated via sensor proteins, thereby transmitting the signal. The floodgate theory seems to be rather outdated and latest research suggests that peroxidases are most likely involved in regulating H_2O_2 mediated signaling pathways, and also that the localization of the signaling partners plays an important role. Many questions still remain but future studies will certainly add more pieces to the puzzle.

1.2.3 Switching off the Signaling Pathway: Reducing Systems

Most oxidative modifications are reversible via the GSH and the Trx systems, whereof the latter is the main focus of this thesis. Both are essential regulators of multiple cellular processes and will be further discussed in section 1.5. In addition, cells utilize a variety of other enzymes and low molecular weight compounds as antioxidants. Important antioxidant enzymes are superoxide dismutases (SODs) that catalyze the dismutation of $O_2^{\bullet-}$ to H_2O_2 and catalases (Cat) that can degrade millions of H_2O_2 molecules every second to O_2 and water (section 1.3.2). Some well known non-enzymatic antioxidants include the vitamins A and E, as well as ascorbate (vitamin C) and lipoic acid [36].

1.3 REACTIVE SPECIES IN BIOLOGY

1.3.1 Overview

The perception of reactive oxygen/nitrogen/sulfur species (ROS, RNS, RSS) in biology has evolved a lot over the last years. The concept of "oxidative stress" has been described for the first time in 1985 as a disturbance in the cellular equilibrium between antioxidants and oxidants; often depicted as a scale, which is tipped in favor of the oxidants [5]. As already addressed in section 1.2, physiological concentrations of reactive species can regulate signaling pathways by reversibly modifying protein targets [5,7,37]. It should be emphasized that ROS, RNS and RSS are *not* single entities and should not be used without a proper definition, as they differ enormously in their reactivity and cellular functionality [38]. This section therefore shall give an overview about the different biologically relevant reactive signaling species.

Before addressing the different ROS, RNS and RSS in detail, it is helpful to start with some definitions (adapted from [8,39]).

A **free radical** is any species capable of independent existence with one or more unpaired electrons, e.g. nitric oxide ($NO^{\bullet}$) [40].

One electron ($1e^-$) oxidants are either free radicals or transition metals that accept electrons. Physiologically relevant species are superoxide ($O_2^{\bullet-}$), the hydroxyl radical ($^{\bullet}OH$), nitrogen dioxide ($NO_2^{\bullet}$) and the carbonate radical ($CO_3^{\bullet-}$).

Two electron ($2e^-$) oxidants are non-radicals that accept electrons to give non-radical products. Physiologically relevant species are hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), and peroxynitrite ($ONOO^-$).

Figure 2 shows an overview of the different reactive species, which throughout this section are classified into ROS, RNS and RSS and described in the main text according to their numbering in the Figure.

In analogy to a quote from Christine Winterbourn, "Feasibility is a long way from reality" [41], this section emphasizes biological reactions of ROS, RNS and RSS.

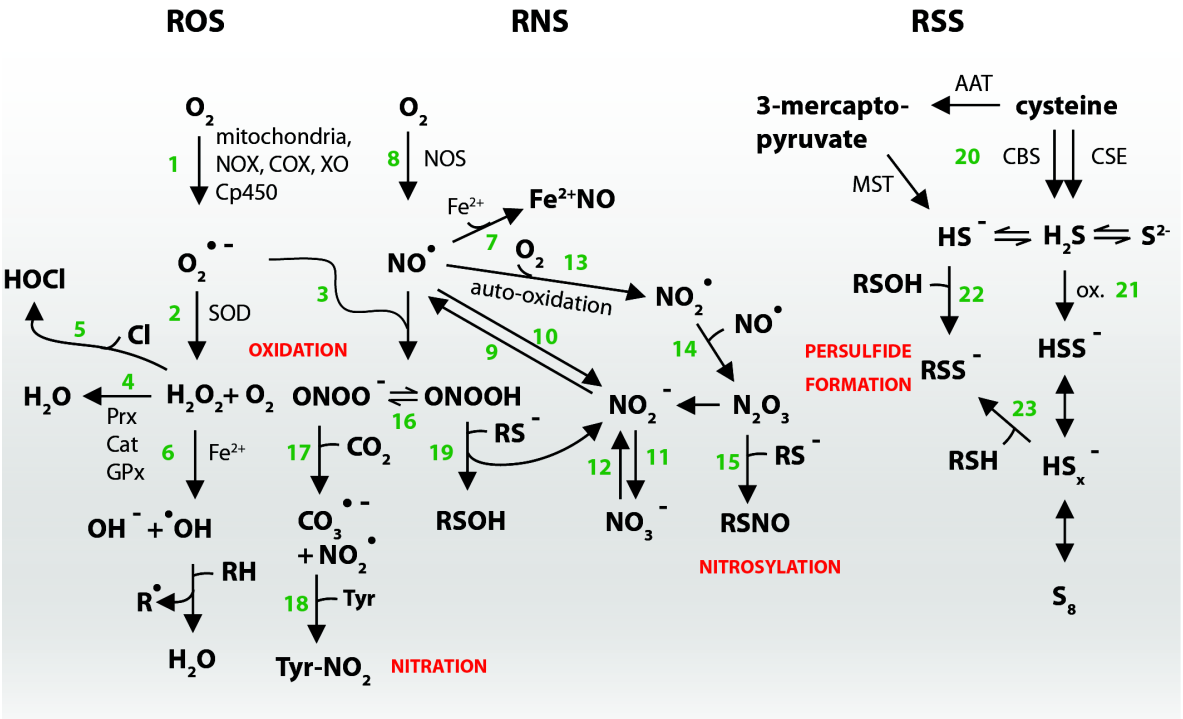

Figure 2. Production and reaction of different reactive oxygen, nitrogen and sulfur species (ROS, RNS, RSS). The reactions are not balanced and the Figure intends to give an overview about the main physiologically relevant ROS, RNS and RSS. All reactions are explained in the main text of section 1.3 according to their numbering. **1)** Production of superoxide ($O_2^{\bullet-}$) via one electron reduction of O_2 in the electron transport chain in the mitochondria, specific NADPH oxidases (NOXs) or as byproduct of cyclooxygenases (COX), xanthine oxidase (XO) or cytochrome p450 enzymes (Cp450). **2)** Dismutation of $O_2^{\bullet-}$ to H_2O_2 and O_2 either spontaneously or catalyzed by superoxide dismutases (SODs). **3)** Spontaneous reaction of $O_2^{\bullet-}$ with nitric oxide ($NO^{\bullet}$) to peroxynitrite ($ONOO^-$). **4)** Reduction of H_2O_2 by peroxiredoxins (Prxs), catalase (Cat) and GSH peroxidases (GPxs). **5)** H_2O_2 may react with chloride anions, yielding hypochlorous acid (HOCl). **6)** The metal-catalyzed Fenton reaction yields hydroxyl anions and hydroxyl radicals. The latter is extremely reactive and can engage in further radical reactions. **7)** Reaction of $NO^{\bullet}$ with metals, e.g. in ferrous heme proteins (Fe^{2+}) like soluble guanylate cyclase to form ferrous nitrosyl-complexes. **8)** Production of $NO^{\bullet}$ by nitric oxide synthases (NOSs). **9)** Nitrate-nitrite-NO pathway to generate $NO^{\bullet}$. **10) + 11)** Oxidation of $NO^{\bullet}$ to nitrite (NO_2^-) and nitrate (NO_3^-). **12)** Reduction of NO_3^-. **13 + 14)** Auto-oxidation of $NO^{\bullet}$ to nitric dioxide ($NO_2^{\bullet}$) and dinitrogen trioxide (N_2O_3). **15)** S-nitrosylation of thiols by N_2O_3. **16)** $ONOO^-$, the product of reaction **3)**, can be reversibly protonated to peroxynitrous acid (ONOOH). **17)** Reaction of $ONOO^-$ with carbon dioxide (CO_2) to carbonate radical ($CO_3^{\bullet-}$) and $NO_2^{\bullet}$. **18)** Tyrosine (Tyr) nitration by $NO_2^{\bullet}$. **19)** ONOOH can react with protein thiolates, yielding protein sulfenic acids. **20)** Hydrogen sulfide (H_2S) may be the product of cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), or the cooperative actions of aspartate/cysteine aminotransferase (AAT) and 3-mercaptopyruvate sulfurtransferase (MST). **21)** Oxidation of H_2S to polysulfides (HS_x^-). **22)** Protein persulfide formation via the reaction of oxidized Cys derivates (e.g. sulfenic acid) with sulfide or **23)** via the reaction of reduced Cys thiols with polysulfides. The Figure has been adapted in parts from [42].

1.3.2 Reactive Oxygen Species (ROS)

"Oxygen has been a trouble-maker since the very beginning"
(Doris Abele, Researcher in Stress Physiology and Ageing) [40]

We all require oxygen (O_2) as the final electron acceptor for efficient energy production in our cells. The complete reduction to H_2O requires four electrons (two per oxygen atom). According to the Pauli principle of electron spins, these electrons have to be transferred separately and not all at once [43]. If O_2 is only partial reduced during that process, reactive oxygen species (ROS) can be formed.

Superoxide ($O_2^{\bullet-}$)

The one-electron reduction of O_2 yields superoxide ($O_2^{\bullet-}$, **reaction 1**). The largest intracellular contributors to $O_2^{\bullet-}$ production are thought to be the mitochondria, mainly as a result of electron leakage in complexes I and III of the electron transport chain [6]. Earlier reports estimated that 0.15-2% of consumed O_2 results in the conversion to $O_2^{\bullet-}$ [44-46]. $O_2^{\bullet-}$ is also the byproduct of several enzymes like cyclooxygenases (COX), xanthine oxidase (XO), cytochrome p450 enzymes and lipoxygenases [47-49]. However, NADPH-dependent oxidases (NOXs) produce $O_2^{\bullet-}$ as their main product [50] (see below). $O_2^{\bullet-}$ can react with thiols, but the reaction is very slow as shown with GSH ($k \sim 200\ M^{-1}s^{-2}$) [4,51]. Even if $O_2^{\bullet-}$ can be produced with a high rate, the steady state concentration is estimated to be in the low picomolar range due to a rapid rate constant for spontaneous ($k \sim 10^5\ M^{-1}s^{-2}$) or superoxide dismutase (SOD) catalyzed ($k \sim 10^9\ M^{-1}s^{-2}$) dismutation to H_2O_2 and O_2 (**reaction 2**) [52]. Recent reports suggest that SOD should not be purely considered as a scavenging enzyme, but that the produced H_2O_2 is important in metabolic regulation [53]. $O_2^{\bullet-}$ can also readily react with other radicals to form e.g. peroxynitrite ($ONOO^-$) with the $NO^{\bullet}$ radical at almost diffusion-controlled rates ($\sim 10^9$-$10^{10}\ M^{-1}s^{-2}$) (**reaction 3**, see section 1.3.3) [39]. Due to these fast secondary reactions, redox signaling is most likely indirectly mediated by $O_2^{\bullet-}$.

Hydrogen peroxide (H_2O_2)

Of all ROS, H_2O_2 is suggested to be the most relevant signal transmitter in redox signaling [9,41,54]. It is the main product from $O_2^{\bullet-}$, and among all ROS, it has the longest cellular half-life (ca. 1 ms) [40]. Direct reactions with most thiols are slow, but H_2O_2 shows exceptional reactivity with e.g. transition metals, selenothiols and selected thiol proteins [14,54,55]. Its intracellular concentration ranges between nM and µM and is constantly regulated by GPxs, Prxs and catalase (**reaction 4**). Even if H_2O_2 is hardly reactive with most biological molecules due to a high activation energy barrier, it is especially associated with receptor-mediated redox signaling [55]. H_2O_2 can also directly oxidize methionine (Met) to Met sulfoxide, but there is large body of evidence in the literature suggesting that Cys is the most sensitive amino acid residue to H_2O_2 mediated oxidation [52].

NADPH-dependent oxidases (NOXs)

NOX enzymes are protein complexes that assemble in response to various stimuli to activate signaling pathways via e.g. transcription factor activation, inhibition of protein tyrosine phosphatases (PTPs), ion channel activation or regulation of enzyme activities [50,56]. NOX complexes produce extracellular $O_2^{\bullet-}$ by transporting electrons from cytoplasmic NADPH through their FAD and heme cofactors to O_2. $O_2^{\bullet-}$ in turn, dismutates to H_2O_2, which can enter the cytoplasm either by diffusion or by aquaporin channels [52,57].

Neutrophils use $O_2^{\bullet-}$ produced by NOX to generate highly reactive oxidants, including hypochlorous acid (HOCl) by myeloperoxidase (MPO) (**reaction 5**), which is used as part of the host defense to inactivate microorganisms in phagosomes [58]. This classic phagocytic response to stimulation is known as the "respiratory burst". An illustrative example is found in patients with a mutation in NOX2, which results in a condition called granulomatous disease. They suffer from recurring infections due to the inability to produce $O_2^{\bullet-}$ as part of their immune system [6]. It should be noted, however, that peroxidases like MPO are largely restricted to cells involved in the host defense and should not be considered as a universal role for H_2O_2 mediated cellular signaling [55].

Transition metals and Fenton Chemistry

Most of the damaging effects of ROS are due to the reaction of H_2O_2 with iron and the formation of hydroxyl radicals ($^{\bullet}OH$) via Fenton Chemistry (**reaction 6**) [39,40]. $^{\bullet}OH$ has a very short cellular half-life (10^{-9} s). It is a strong oxidant and reacts at diffusion-limited rates with proteins, DNA and lipids in the cell [54,59]. There are no enzymes that neutralize $^{\bullet}OH$, but in healthy cells the formation is considered very low due to a tightly regulated H_2O_2 and iron metabolism [52].

1.3.3 Reactive Nitrogen Species (RNS)

RNS are derivatives of nitric oxide ($NO^{\bullet}$) with distinct properties in terms of reactivity and biological half-life. Analogous to ROS, RNS are suggested to play major roles in cellular signaling processes and pathological conditions. Some terms that are frequently confused in the $NO^{\bullet}$-field are nitrosylation, nitrosation and nitration. The general consensus is that nitrosylation is a direct addition of $NO^{\bullet}$ to a macromolecule, while nitrosation is the attachment of a nitrosonium (NO^{+}) ion (see section 1.4.3). Nitration, however, is the attachment of a NO_2 (nitro) group [40].

Nitric oxide ($NO^{\bullet}$)

$NO^{\bullet}$ is a colorless gas that, in general, has a modest reactivity with biological molecules [40]. Due to its membrane solubility it can diffuse within a 100-200 μm radius of its production site [60]. Concentrations vary between 100 pM to 5 nM and the half-life from 0.1-2 s [61]. One of its main physiological roles and fastest reactions is the reversible binding to heme prosthetic groups, e.g. in guanylate cyclase (**reaction 7**) [62]. The subsequent conformational change in

the enzyme results in the production of the second messenger cyclic guanosine monophosphate (cGMP), which in turn causes smooth muscle relaxation and vasodilation [40,63]. $NO^{\bullet}$ is highly reactive with other radicals; it reacts at almost diffusion controlled rates with $O_2^{\bullet-}$, becoming $ONOO^-$ (**reaction 3**). The most damaging effect of $NO^{\bullet}$ is considered to be its reaction with the tyrosyl radical in ribonucleotide reductase (RNR) [40].

$NO^{\bullet}$ is synthesized by a family of enzymes called nitric oxide synthases (NOSs) that use L-arginine, O_2, and NADPH to produce $NO^{\bullet}$ and L-citrulline (**reaction 8**). There are three types of NOS that differ in their rate of $NO^{\bullet}$ production and the mechanisms that regulate their activity. Inducible NOS (iNOS) exists in a variety of cells, including neuronal cells, hepatocytes and phagocytic cells. It can be expressed in response to bacterial products and certain cytokines to produce large amounts of $NO^{\bullet}$ as part of the host defense or inflammatory response [64]. Endothelial NOS (eNOS) and neuronal NOS (nNOS) are both constitutively expressed, and in contrast to iNOS, furthermore regulated by Ca^{2+}. Functions of nNOS include modulation of neurotransmission in the brain. As the name implies, eNOS is mostly expressed in endothelial cells where it regulates vasodilation and blood pressure. Its functions can be modulated by a variety of modifications including phosphorylation after growth factor stimulation and also nitrosylation [64,65]. Interestingly, conditions of oxidative stress and inflammation have been shown to convert eNOS from an $NO^{\bullet}$ producing enzyme to a $O_2^{\bullet-}$ producing enzyme; a process that has been referred to as eNOS uncoupling [66].

Another recently emerging $NO^{\bullet}$-generating mechanism is the "nitrate-nitrite-NO pathway" [67]. Compared to the NOS mediated production of $NO^{\bullet}$, the reduction of nitrate (NO_3^-) and nitrite (NO_2^-) provides an O_2 independent pathway to generate $NO^{\bullet}$ and has been shown to be active during hypoxic conditions (**reaction 9**) [68]. Nitrate and nitrite are either taken up via the diet or are endogenously produced via the oxidation of $NO^{\bullet}$. The *in vivo* mechanisms of the oxidation remain elusive [69], but formation of NO_2^- by ceruloplasmin (**reaction 10**) [70] and formation of NO_3^- by oxyhemoglobin (**reaction 11**) [71] have been reported. In turn, $NO^{\bullet}$ can be regenerated from these oxidized derivatives by hemoglobin, myoglobin, xanthine oxidase, ascorbate and polyphenols (see [67] and references therein). It should be noted, however, that NO_3^- is considered an inert end product of $NO^{\bullet}$ oxidation that first needs to be reduced to NO_2^- – a process that requires bacteria in the oral cavity or gastrointestinal tract (**reaction 12**) [67].

$NO^{\bullet}$ does not react directly with thiol groups under biological conditions (S-nitrosylation, see section 1.4.3). Instead this type of redox modification is generally mediated by its oxidation products. However, the oxidation of $NO^{\bullet}$ is a very complex process. For example, the one-electron oxidation of $NO^{\bullet}$ to give free NO^+, which would be required for reaction with a thiol, is very unfavorable [72,73]. The first auto-oxidation product of $NO^{\bullet}$ is nitric dioxide ($NO_2^{\bullet}$), a highly reactive free radical (**reaction 13**) [74,75]. In aqueous solution it can react with another $NO^{\bullet}$ molecule (**reaction 14**) to give dinitrogen trioxide (N_2O_3), which can indeed react with thiolates to nitrosothiols (**reaction 15**) [72,75]. Under normal physiological conditions this auto-oxidation process is considered to be too slow to be significant, and controversy about the

biological mechanism of N_2O_3 generation (and thereby also S-nitrosylation) still exists [72]. However, some studies suggest that this reaction is accelerated in hydrophobic compartments [52,74].

Peroxynitrite ($ONOO^-$)

$ONOO^-$ is the product of the combination of $NO^\bullet$ and $O_2^{\bullet-}$ (**reaction 3**). The reaction has a greater rate constant than the reaction of $NO^\bullet$ with heme or the reaction of $O_2^{\bullet-}$ with SOD [40]. $ONOO^-$ can be continuously formed under basal metabolic conditions, but it has a very short half-life and only nanomolar steady state levels [76]. Its formation sites are highly dependent on the formation of its precursor radicals. In contrast to $NO^\bullet$, $O_2^{\bullet-}$ is unstable and does not diffuse as freely, thereby restricting the production of $ONOO^-$ to the production site of $O_2^{\bullet-}$. The chemistry of $ONOO^-$ is very intricate. Its reactivity is pH dependent (pKa~6.8) and both the peroxynitrite anion ($ONOO^-$) and peroxynitrous acid (ONOOH) are present under physiological conditions and can participate in $1e^-$ and $2e^-$ reactions (**reaction 16**) [76]. The homolysis of ONOOH to $OH^\bullet$ and $NO_2^\bullet$ is slow and unlikely to be physiologically relevant [39].

Physiologically relevant reactions of $ONOO^-$ are e.g. with carbon dioxide (CO_2), thiol- and selenol-containing proteins, and transition metal centers in metalloproteins (e.g. Mn-SOD and heme proteins) [76,77]. The biological concentration of CO_2 is relatively high and the reaction yields the carbonate radical ($CO_3^{\bullet-}$) and $NO_2^\bullet$ (**reaction 17**), which both are potent $1e^-$ oxidants that can have various effects. For example, $NO_2^\bullet$ can directly react with $NO^\bullet$ to form the potent nitrosating species N_2O_3 and $CO_3^{\bullet-}$, but can also participate in Tyr nitration reactions (**reactions 14+18**) [78]. The $2e^-$ oxidation of thiols by ONOOH yields sulfenic acids and nitrite (**reaction 19**) [39]. Examples of proteins that have high rate constants with $ONOO^-$ are Prxs ($k\sim10^6$-$10^7\ M^{-1}s^{-1}$) [78] and GPxs ($k\sim10^8\ M^{-1}s^{-1}$) [79]. The reaction of $ONOO^-$ with transition metal centers yields, via several steps, $NO_2^{\bullet-}$ and a strongly oxidizing oxo-metal complex [78]. All of the secondary radical species resulting from $ONOO^-$ mediated reactions (e.g. $CO_3^{\bullet-}$, oxo-metal complexes, $NO_2^\bullet$) are involved in promoting the nitration of Tyr residues in proteins, forming 3-nitrosotyrosine [80].

1.3.4 Reactive Sulfur Species (RSS)

The prototype of low molecular weight RSS is hydrogen sulfide (H_2S). For a long time, it was only considered as a toxic gas and an environmental hazard, but recently it has gained more attention as potential signaling molecule [81,82]. H_2S is a colorless gas with an unpleasant smell of rotten eggs. It is both water and lipid soluble, and at physiological pH it occurs primarily in its thiolate form (HS^-) ($H_2S \Leftrightarrow HS^- + H+ \Leftrightarrow S^{2-} + H^+$) [52,83]. Important to note, the two dissociable protons can be substituted with other functional groups. This property allows H_2S to form bridges, e.g. in per- and polysulfides (R-S-SH and R-S-S_n-S-R) [84]. Another important aspect is that H_2S is a fully reduced sulfur species with a formal oxidation state of -2 and can, as such, only be oxidized. The biological concentration of H_2S has been controversial and it is believed that H_2S is rapidly undergoing diverse reactions (Fig. 3),

which makes a reliable estimation of its physiological concentration difficult. Depending on the method, the concentration of free H_2S was estimated to be in the submicromolar range in plasma and liver homogenates [85]. However, recent reports suggest a labile pool of sulfur containing species in biological systems that can liberate H_2S [86].

H_2S can be produced from Cys by at least three different enzymatic systems, Cystathionine-β-synthase (CBS), Cystathionine-γ-lyase (CSE) and the cooperative actions of aspartate/cysteine aminotransferase (AAT), and 3-mercaptopyruvate sulfurtransferase (MST) (**reaction 20**) [87,88]. Although they all use Cys as precursor, their kinetic and mechanistic properties are very different. In addition, the expression of these enzymes is tissue specific, e.g. CBS is predominantly expressed in the brain and CSE in peripheral tissues [88]. Both CBS and CSE can catalyze many reactions with different substrates. CBS, for example, is the first enzyme in the transsulfuration pathway and converts homocysteine to cystathionine, but can also efficiently generate H_2S from Cys and homocysteine [89]. Which of these reactions is the predominant mechanism, and under which conditions, is currently unclear.

The biological functions of H_2S are very complex and many uncertainties still exist about the exact signaling mechanisms (Fig. 3). Predominantly, effects on the cardiovascular system and in inflammation have been described, with the major targets believed to be membrane ion channels, e.g. the ATP-sensitive potassium channel, which mediates vasorelaxation [90]. The mechanisms how H_2S activates ion channels are not fully elucidated yet, but evidence suggests that the respective channels are persulfidated [91,92]. H_2S can also react with hemoglobin to form sulfhemoglobin. This process is irreversible and thereby probably rather a metabolic sink for H_2S and not relevant for signaling [85,93,94]. H_2S can theoretically react with other endogenous oxidant species, but due to its low concentrations, this has been suggested to be unlikely *in vivo* [95]. In the mitochondria, H_2S can be rapidly oxidized in several steps to sulfate (SO_4^{2-}), which has both been considered as a pathway to eliminate H_2S but also a site for generation of RSS such as GSH persulfide (GSSH) [93].

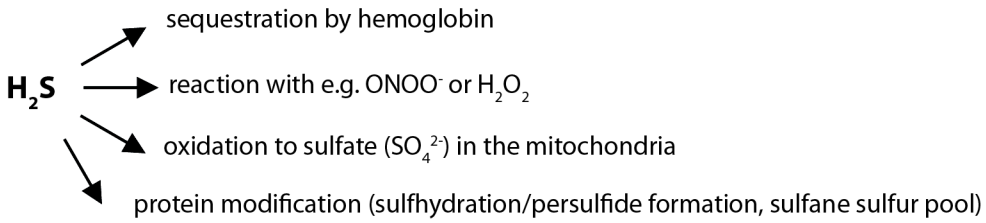

Figure 3. Scheme of potential fates of H_2S is the cell. H_2S can readily react with hemeproteins, e.g. hemoglobin to form sulfhemoglobin or with other cellular oxidant species like for instance $ONOO^-$ or H_2O_2. Sulfide catabolism mainly occurs in the mitochondria, where it is rapidly oxidized to sulfate. The formation of persulfides on protein Cys residues (-SSH), called sulfhydration or persulfidation, is an emerging mechanism of persulfide mediated cellular signaling.[85,96]

Persulfidation at reactive Cys residues is increasingly recognized as the likely signaling mechanism of H_2S. However, H_2S does not react with thiol groups directly, and parts of the physiological roles of H_2S are potentially mediated via partially oxidized sulfane-sulfur containing species (**reaction 21**) [87,97-99].

Sulfane-sulfur is a form of sulfur with six valence electrons (represented by S^0), which does not exist in free form, but is always attached to another sulfur atom (e.g. in elemental sulfur S_8, persulfides R-S-SH and polysulfides R-S-S_n-S-R) [100]. Polysulfides are unstable sulfide oxidation products, mainly generated with oxidants such as HOCl, H_2O_2 and other peroxides [87]. The general formula of polysulfides can be depicted as H_2S_x, but since they are expected to be in their anionic form at physiological pH, the formula HS_x^- is recommended. Polysulfides can vary in chain length, containing one sulfide and 1-8 sulfane-sulfurs. At the point the number reaches eight, the molecule cyclizes and separates from polysulfides as colloidal elemental sulfur [100].

Protein persulfides can either be formed via the reaction of oxidized Cys derivatives with sulfide (**reaction 22**) or via the reaction of polysulfides with Cys thiols (**reaction 23**) [87]. However, how this occurs *in vivo* is still not completely elucidated yet and despite the growing literature on protein persulfidation, the exact sources and identities of RSS are still controversial (see also section 1.4.4).

1.4 MODIFICATION OF CYS RESIDUES

1.4.1 Overview

In proteins, Sec, Cys, Met, and aromatic amino acids can be reversibly oxidized and thus take part in redox signaling processes [101-103]. The oxidation of Met to Met sulfoxide (MetO) and the reduction by MetO reductases (MSRs) is a sparsely characterized, but emerging signaling mechanism [104,105]. Sec is only present in 25 mammalian proteins, mostly in the active site, e.g. in the major redox enzymes TrxR and GPx (section 1.5) [106,107]. This section will focus on the modification of Cys residues because they are considered the major targets for redox signaling processes.

The mammalian genome encodes 214 000 Cys residues of which 10-20% are redox active under physiological conditions [101]. At least 18 biologically occurring non-radical modifications of Cys thiols have been described, with varying stability and biological impacts [108]. The thiol (-SH) group is the sulfur analogue of the hydroxyl group (-OH), but compared to oxygen, sulfur is less electronegative and has a valence shell radius twice as large. Consequently, the SH bond is less polarized and becomes more easily deprotonated to the more nucleophilic thiolate form ($-S^-$) at physiological pH [103]. The acid dissociation constant (pKa) for free Cys is around 8.3 and for glutathione around 8.8 [52,109]. However, the local protein environment can dramatically influence the pKa value and thus the Cys reactivity.

Cys is a non-essential amino acid, which is either synthesized from Met via the transsulfuration pathway or imported as Cys or cystine (Fig. 4) [110]. The availability of Cys is the rate-limiting step for the synthesis of GSH [111]. This makes the transsulfuration pathway an important metabolic pathway as it can maintain the pool of GSH in conditions of oxidative or

xenobiotic challenges [112,113]. A recent study showed that mice genetically engineered to lack both TrxR1 and GSH reductase (GR) in mouse liver remained viable because they used the transsulfuration pathway to produce GSH for redox homeostasis [114]. The enzymes of the transsulfuration pathway, CBS and CSE, are furthermore important in the generation of H_2S (see section 1.3.4).

Both Cys and its oxidized form cystine (the predominant form of the amino acid in circulation) are imported via several transport systems [115]. Cys is imported via the alanine-serine-cysteine (ASC) and X_{AG-} systems (also called excitatory amino acid transporters (EAATs)) in a Na^+-dependent manner [116-118]. Cystine, on the other hand, is imported in a Na^+ independent manner by system b^{0+}, which seems to be involved in the renal absorption of cystine, and by the cystine/glutamate antiporter X_c^- [119,120]. X_c^- is highly inducible by O_2, electrophilic agents, TNFα, and the transcription factor Nrf2 [120-123]. Also, recent reports suggest that X_c^- is induced by standard cell culture conditions and essential for the maintenance of intracellular GSH in cultured cells [120,124]. After being imported, cystine is rapidly reduced to Cys. The exact mechanism is still not completely understood, but thought to involve the GSH and Trx systems [125].

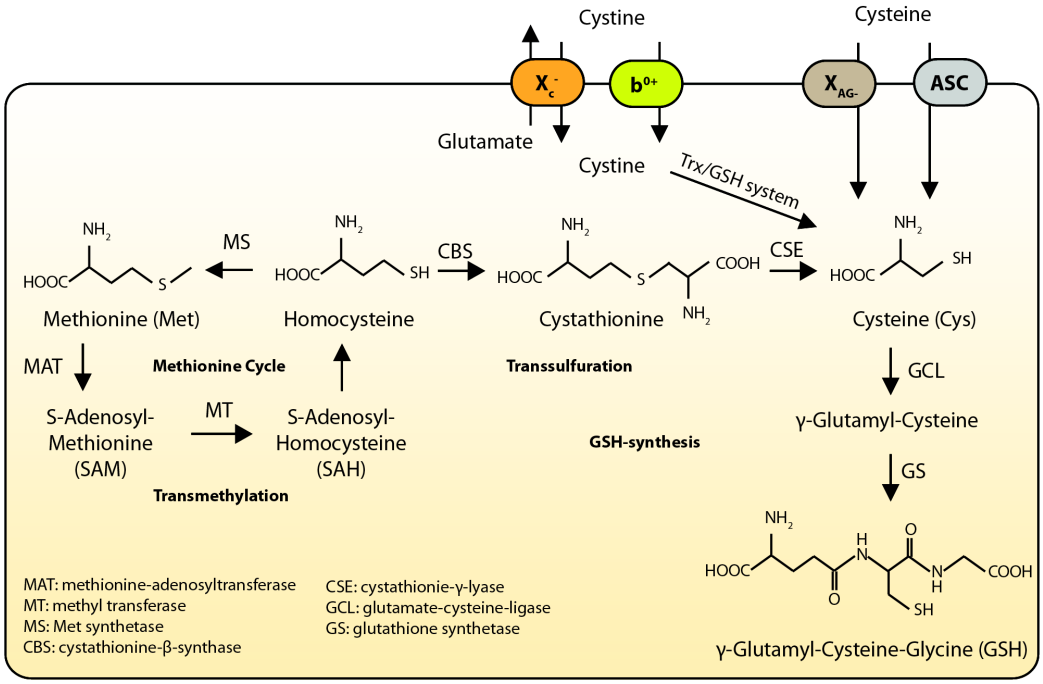

Figure 4. Overview of the transsulfuration pathway and the synthesis of GSH. Cys is the rate-limiting precursor for the synthesis of GSH, a two-step process catalyzed by glutamate-cysteine-ligase (GCL) and glutathione synthetase (GS). Cys, or its oxidized form cystine, can be imported via different transporters. Cystine is reduced intracellularly, but the mechanisms are not fully elucidated and suggested to be mediated via the GSH and Trx systems. Alternatively, Cys can be produced from the essential amino acid methionine (Met) and via transsulfuration, a pathway that is particularly active in the liver. In this pathway, Met is converted via the enzyme methionine-adenosyltransferase (MAT) to S-adenosylmethionine (SAM), the most important methyl donor in the body. In a transmethylation reaction catalyzed by methyltransferase (MT), S-adenoysl-homocysteine (SAH) is generated. SAH can subsequently be hydrolyzed to homocysteine. Homoysteine in turn can either be remethylated via Met synthetase (MS) to form Met, or converted to Cys in the transsulfuration pathway via cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE). For simplicity, the reactions are not balanced and only the main reaction products are shown. This Figure has been adapted from [110,113].

1.4.2 Thiol Oxidation

Most oxidative thiol modifications are reversible and a central regulating mechanism in redox signaling [41]. Oxidation can occur via the one-electron (radical) or the two-electron (non-radical) pathway, whereof the latter is preferred in signaling pathways (Fig. 5).

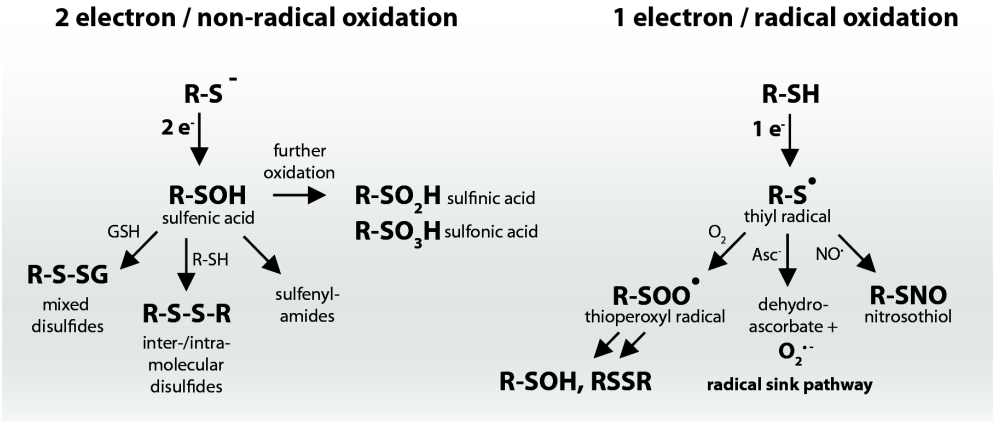

Figure 5. Non-radical ($2e^-$) and radical ($1e^-$) pathways of thiol oxidation. Non-radical oxidation of a thiolate gives sulfenic acid, which is a transient intermediate and in most cases undergoes further reactions. Its reversible condensation with an adjacent amide gives a sulfenylamide (e.g. in PTP1B). Further oxidation can result in sulfinic and sulfonic acids. Radical oxidation of a thiol results in the formation of a thiyl radical, which can have various fates. Asc^-: ascorbate. The reactions are not balanced and just provide an overview of the main pathways. The Figure has been adapted from [41].

The **$2e^-$ oxidation** of thiolates with oxidants (H_2O_2, ROOH, HOCl, ONOOH) yields sulfenic acid (R-SOH), which is generally unstable and short-lived [39,41]. It can subsequently react with protein thiols or GSH to form intra- or intermolecular disulfides. In absence of other thiols, the reaction with a backbone amide can result in a cyclic sulfenyl amide. All these forms can be reduced by the Trx and GSH systems. Sulfenic acids can be further oxidized to sulfinic ($R\text{-}SO_2H$) and sulfonic ($R\text{-}SO_3H$) acids, a process that is generally considered to be irreversible and slower than the initial oxidation [52]. The only protein known to reduce sulfinic acids, e.g. in Prxs, are Sulfiredoxins [126]. The reactivity of thiols with $2e^-$ oxidants like H_2O_2 is determined by the pKa of the Cys residue. Thiol peroxidases like Prxs and GPxs show an exceptionally high reactivity with H_2O_2, which is a result of a highly conserved active site architecture and a specific transition-state activation of H_2O_2 in Prxs [41,127].

The oxidation of Cys can also occur via **$1e^-$ oxidation** of the thiol group to a thiyl radical. Under physiological conditions, the main reactions of a thiyl radical are with oxygen, thiol groups, and ascorbate, which are believed to be mainly protective mechanisms to prevent deleterious cellular reactions [39,128]. The reaction with O_2 results in thioperoxyl radicals ($RSOO^\bullet$) that can undergo a variety of different reactions to e.g. sulfenic acids and disulfides. The reaction with ascorbic acid leads to the formation of $O_2^{\bullet-}$, which is removed in the radical sink pathway involving SODs and peroxidases [129]. It is important to note that thiyl radicals can also react with $NO^\bullet$ and thereby provide a mechanism for protein S-nitrosylation [130].

1.4.3 S-Nitrosylation

In order to discuss the formation of S-nitrosothiols, it is worthwhile to address some of its basic chemistry. The term "S-nitrosylation" generally describes the direct addition of $NO^{\bullet}$ to macromolecules, which is the underlying mechanism for the reaction of $NO^{\bullet}$ with metal centers, for example in guanylate cyclase. However, as described in section 1.3.3, $NO^{\bullet}$ does not react directly with thiol groups. Instead, the formation of a S-nitrosothiol involves the addition of a nitrosonium ion (NO^{+}), which from a chemical perspective, is therefore a "nitrosation reaction". The nitrosonium ion is the unstable $1e^{-}$ oxidation product of $NO^{\bullet}$. Accordingly, biological S-nitrosation reactions involve so-called NO^{+}-donors [72]. Since the literature to date has used the term "S-nitrosylation" also for reactions that are formally nitrosation reactions, this nomenclature will be used throughout this thesis.

Even if the exact molecular and biological mechanisms are not fully elucidated yet, several mechanisms have been proposed for the formation of S-nitrosothiols [52,131]:

- Conversion of $NO^{\bullet}$ to the nitrosating compound N_2O_3
- Radical reaction between $NO^{\bullet}$ and a thiyl radical
- Metal-center catalyzed transfer of NO^{+} from a heme group to a Cys, demonstrated with e.g. hemoglobin [132]
- Transnitrosylation from one S-nitrosothiol to a thiolate

Transnitrosylation is an emerging concept for the formation of S-nitrosothiols *in vivo* [133-135] and was demonstrated for several proteins including GAPDH [136], Trx1 [137], and caspase 3 [138]. In terms of redox signaling, this concept of forming S-nitrosothiols provides specificity, as it involves protein-protein interaction [139]. Other mechanisms that have been suggested to determine the selectivity of Cys residues for S-nitrosylation are i) proximal localization of the target Cys to the source of NO production, ii) a so-called "signature motif" consisting of basic and acidic amino acids and iii) hydrophobic compartments close to the Cys residue [135,140].

A recent literature review identified 233 endogenously S-nitrosylated proteins under physiological conditions as determined with the Biotin-Switch method [131,141], while another recent database identified >2000 mammalian proteins as targets for S-nitrosylation [142]. A comprehensive analysis of all these proteins would be beyond the scope of this thesis, but examples include inhibitory effects of nitrosylation on caspases [143,144], the p65 unit of NFκB [145] and also PTPs [146,147]. Aberrant S-nitrosylation has been correlated to neurodegenerative diseases such as Parkinsons and Alzheimers [140]. Although significant efforts have been made in the field of S-nitrosylation, there is still debate about which proteins become S-nitrosylated, the context of the S-nitrosylation and the overall physiological significance.

Denitrosylation

Compared to the formation of S-nitrosothiols, the mechanism of the abstraction of the nitroso-moiety (denitrosylation), is less characterized [148]. Nevertheless, denitrosylation reactions seem to be tightly regulated, as some proteins including caspases, NOSs and IκB kinase β are S-nitrosylated and only become denitrosylated in response to a specific stimulus [65,143,149]. Two enzyme systems have emerged as physiological relevant denitrosylating pathways: the GSNO reductase (GSNOR) and the Trx system. GSNOR is a major enzyme for the denitrosylation of GSNO. It uses NADH as an electron donor and is ubiquitously expressed in mammalian cells [150-152]. It does not denitrosylate proteins directly, but a knockout of GSNOR has been correlated to increased levels of intracellular nitrosoproteins [150]. The Trx system, coupled to NADPH, can denitrosylate a multitude of cellular proteins and low molecular weight nitrosothiols [153-155]. Several other studies have demonstrated denitrosylating activity of enzymes including PDI [156], xanthine oxidase [157], SOD [158], GPx1 [159] and carbonyl reductase [160].

The Trx system and its regulation of $NO^{\bullet}$-dependent processes is very complex and far from being fully understood. Compiled evidence suggests denitrosylation and transnitrosylation reactions mediated by Trx in cellular processes including apoptosis and inflammation [161]. Caspase 3 is a prominent example of a protein that becomes denitrosylated by Trx1 [153,162,163]. However, at the same time, Cys46 in Trx1 has emerged as a target for nitrosylation that can perform transnitrosylation reactions to caspase 3 and Prx [137,164,165]. These opposing reactions of Trx1 in either promoting or suppressing apoptosis via de- or transnitrosylation are still a conundrum in the field. Also the interplay with TXNIP, which seemingly inhibits the denitrosylating activity of Trx1 adds complexity to the picture [166].

Trx1 mediated denitrosylation could follow two different mechanisms. After the attack of the nucleophilic Cys on the nitrosothiol, either an intramolecular disulfide between Trx1 and the protein could be formed [153] or this Cys could become nitrosylated and subsequently denitrosylated by the resolving Cys (Fig. 6) [167,168]. A recent study applied the reaction mechanism via the intramolecular disulfide as "trapping" strategy with a mutant Trx1 lacking the resolving Cys to identify a large number S-nitrosylated protein targets, including the mitogen-activated protein kinase (MAPK) MEK1 and the transcription factor STAT3. [169].

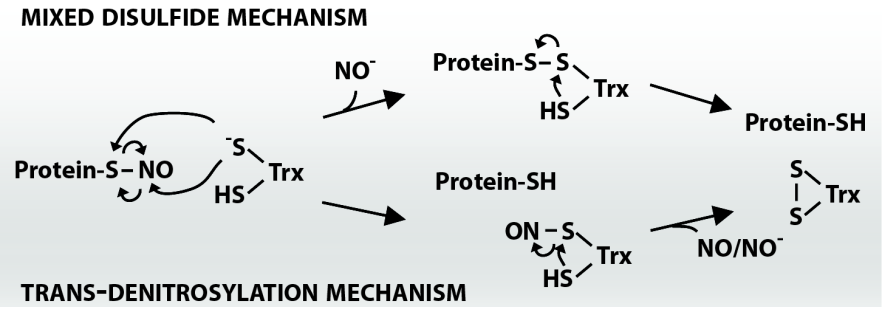

Figure 6. Potential mechanisms of denitrosylation by Trx1. Either, an intermolecular disulfide intermediate between Trx1 and the substrate protein could be formed (upper part, mixed disulfide mechanism) or Trx1 becomes transiently nitrosylated (lower part, trans denitrosylation mechanism). [161]

1.4.4 Persulfide Formation

S-sulfhydration, e.g. the formation of persulfides (–SSH), is an emerging mechanism for H_2S mediated signaling processes. From a chemical perspective, the term persulfide formation/persulfidation is preferred and will therefore be used throughout this thesis [87].

As briefly discussed in section 1.3.4, H_2S cannot directly interact with a thiol group. Thus, persulfide formation can only occur via i) reactions of oxidized Cys derivatives with sulfide or ii) reactions of Cys thiols with oxidized sulfide derivatives. The exact mechanisms are still under debate, but the major hypotheses can be summarized as follows:

- **Reaction of oxidized Cys derivatives (disulfides or sulfenic acids) with sulfide** [86,170-172]

  $$R-S-S-R' + HS^- \rightarrow RSS^- + R'SH \qquad (1)$$

  $$R-SOH + HS^- \rightarrow RSS^- + H_2O \qquad (2)$$

  Both reactions have been intensively discussed, but currently the reaction of the sulfenic acid with sulfide (reaction 2) seems to be more feasible. Based on kinetic and thermodynamic considerations, reaction 1 has been suggested to be unlikely under physiological conditions [84]. However, it should not be completely disregarded as it could be a possible pathway in more oxidizing environments including mitochondria and ER [173]. Reaction 2 is influenced by the microenvironment of the sulfenic acid and local concentrations of sulfide vs. GSH, as protein S-glutathionylation is a competing reaction.
- **Reaction of thiols with polysulfides** [86,99]

  The formation of polysulfides has already been discussed in section 1.3.4 and efficient polysulfide mediated persulfidation of PTEN and GFP was recently reported [99]. This mechanism is dependent on the initial oxidizing agent to generate polysulfides, the respective stability of the polysulfides, and the pKa of the thiol [87].

Interestingly, a recent study suggested that enzymes of the transsulfuration pathway can generate persulfides, e.g. the formation of Cys persulfide (Cys-SSH) from cystine, which in a persulfide exchange reaction with GSH forms GSH persulfide (GSSH) [98]. This has, however, been contradicted by another report which suggested that the reducing cellular environment favors the synthesis of H_2S over persulfides [174]. The question if low molecular weight persulfides act as sulfur donors in protein persulfidation reactions is still under active debate. In addition, the abundance of protein persulfides *in vivo* is also still a matter of controversy, mainly due to the methodological challenges in detection. Current methods like the Modified Biotin Switch Assay (based on the "traditional" Biotin Switch Assay to determine protein S-nitrosylation) [91,141] or the recently developed Tag Switch Assay [175] give different results and illustrate that the very similar reactivity of Cys persulfides and thiols poses a major problem in studying persulfidation.

It has been increasingly recognized that while the S-nitrosylation of a Cys residue typically results in inhibition of the respective protein, persulfide formation instead results in activation of enzyme activity [176]. The most prominent example is GAPDH, where S-nitrosylation inhibits the glycolytic function and results in the translocation to the nucleus [177]. The

persulfide formation on the same Cys residue instead enhances the glycolytic activity of GAPDH [91]. Another example is NFκB, where S-nitrosylation of the p65 subunit inhibits NFκB signaling, while persulfidation promotes the transcription of antiapoptotic genes [178]. In contrast, persulfidation of the active site Cys in PTP1B has been reported to inhibit its phosphatase activity similarly to oxidation or nitrosylation, and thereby to contribute to the ER stress response [179].

The knowledge of the reduction of protein persulfides and polysulfides is also very limited. Recent studies have reported that Trx1 can reduce active site persulfides in MST and PTP1B [179,180]. Both studies noted faster reactions with the Trx system compared to DTT and GSH, and also higher rates of Trx1 with the persulfidated PTP1B compared to the nitrosylated and oxidized versions.

1.5 BIOLOGICAL SYSTEMS RELEVANT FOR REDOX SIGNALING

1.5.1 Overview

Most oxidative modifications of Cys residues can be reversed by the Trx and the GSH systems, which are the main topic of this section. Both systems mediate electron flux from NADPH through TrxR and GR to members of the Trx fold family of proteins and GSH respectively (Fig. 7) [42,108]. First, the Trx family of proteins will be discussed and the regulation of redox signaling pathways by the Trx and GSH systems highlighted. TRP14, the major topic of this thesis, will be discussed separately in section 1.6. At the end of this section, protein tyrosine phosphatases (PTPs), which regulate receptor-tyrosine kinase signaling through oxidation/reduction of their Cys residue, will also be addressed [21].

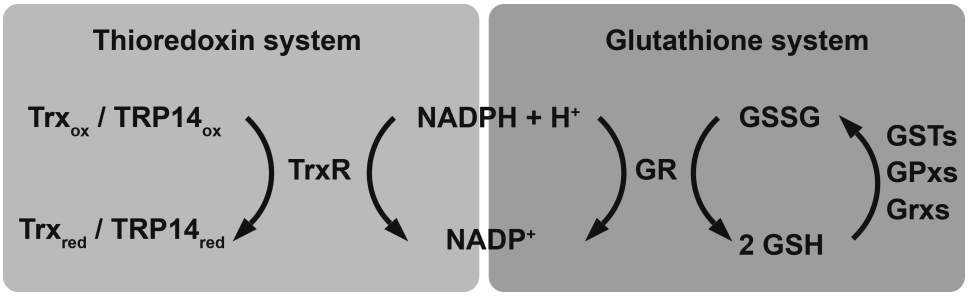

Figure 7. Schematic overview of the complementary Trx and GSH systems in mammals. The Trx system (left) depends on TrxRs that reduce isoforms of Trx. Trx1 and TRP14 can be reduced by cytosolic TrxR1. The GSH system (right) uses GSH to support the functions of GSTs, GPxs and Grxs. Oxidized GSH (GSSG) is reduced by GR. Both systems depend on NADPH (provided by the pentose phosphate pathway) and exhibit significant crosstalk and overlapping functions, such as protein disulfide reduction and sustaining DNA synthesis via ribonucleotide reductase.

The Keap1/Nrf2 system

The major regulator to coordinate cellular responses to oxidative and electrophilic stress is the transcription factor **Nrf2** [3,181]. A full discussion of the Nrf2 signaling pathway would go beyond the scope of this thesis, but for the understanding of redox signaling the main concept is briefly summarized. Endogenous signaling molecules as presented in section 1.3 (e.g.

H_2O_2, ROOH, $ONOO^-$), but also exogenous electrophilic compounds can activate Nrf2. Under basal conditions, Nrf2 is bound to its inhibitor Keap1 in the cytosol, which targets Nrf2 for proteasomal degradation. The oxidation and concomitant inhibition of Keap1 leads to a nuclear import of Nrf2, where it binds to the antioxidant response element (ARE) and induces the expression of a wide range of enzymes that promote cell survival and defense against oxidative and electrophilic stress. Examples of typical Nrf2 targets are members of the Trx and GSH systems, e.g. Trx1 and TrxR1, GR, GSTs, the cystine transporter X_c^-, and enzymes for the synthesis of GSH [3,182-185].

1.5.2 The Thioredoxin Family of Proteins

Many key regulators of redox signaling belong to the Trx fold family of proteins. Its members are ubiquitously expressed in all organisms, tissues, cell types, and organelles [42]. They typically share a common structural motif, which is called the "Trx fold": a central core of a four-stranded beta sheet that is surrounded by three alpha helices [186,187]. A recent study estimated that 723 proteins contain at least one Trx fold domain [188]. In addition, Trx family proteins are characterized by having one or two Cys residues in their active site, which are essential for their catalytic activity [42]. The reduction of protein disulfides is catalyzed by Trxs and Grxs via the so-called dithiol-mechanism and depends on a CXXC active site motif. This mechanism involves the nucleophilic attack of the N-terminal active site thiol which results in a transient mixed disulfide intermediate, followed by reduction through the C-terminal thiol. Deglutathionylation works via the monothiol mechanism and is only catalyzed by Grxs. After reduction of the target protein, the active site thiol forms a mixed disulfide intermediate with GSH, which is reduced by a second molecule of GSH. Some members of the Trx family, e.g. certain protein disulfide isomerases (PDIs), just have one Cys in the active site motif. Due to the absence of a second Cys longer stable disulfides can be formed which can be supportive in the oxidative protein folding in the endoplasmatic reticulum (ER).

Many proteins of the Trx superfamily are not characterized yet, which results in a diffuse nomenclature and an incomplete understanding of their physiological functions. Besides the already mentioned, classical representatives of the family (Trxs, Grxs, Prxs), many so-called Trx-related proteins have recently emerged. Table 1 highlights members of the Trx family that are consecutively listed as *thioredoxin-domain-containing*-(*TXNDC*) proteins in NCBI and also includes the isoforms of the Trx and Grx proteins. Prxs and PDIs, as well as GPxs and GSTs are Trx fold proteins that are not included in this Table. Prxs are separately discussed, and for a thorough discussion of PDIs the interested reader is referred to an excellent review of the topic [189].

As illustrated in Table 1, Trx-like proteins have diverse active sites with not only CXXC, but also C/S/A-XX-S/R variations. Also, the composition of the XX dipeptide in the active site can be very different. In terms of localization, these proteins seem to be distributed over almost all cellular compartments and in some cases they are even secreted. The proteins that are relevant for this thesis are mostly located in the cytosol, but a major part of the Trx-like proteins are also classified as PDIs and located in the ER. Some representatives are also

transmembrane proteins (TMX1-TMX4). They are all targeted to the ER membrane, but only TMX1 has oxidoreductase activity, while the others are thought to be more similar to PDIs [190-193]. In addition, TMXs have recently been implicated in inflammatory liver injury [194]. Humans also possess spermatozoa-specific Trx (*TXNDC2* and *TXNDC3* and *TXNDC8*) with various activities [195,196].

Nucleus-located representatives of the Trx-like proteins are the **Nucleoredoxins** (Nxn [197], Nxnl1 [198], Nxnl2 [199]). Nxn is a 55 kDa protein located both in the cytosol and nucleus [200,201] that functions as a redox-sensitive regulator of the Wnt/b-catenin pathway and toll-like receptor signaling [202,203].

Another emerging protein is Thioredoxin-like protein 1 (Txl), also known as **Thioredoxin-related protein of 32 kDa** [204,205]. It is a substrate for TrxR1 and due to its association with the proteasome, suggested to be the link between reduction and proteolysis [206,207]. It can be induced by prostaglandins [208] and in contrast to Trx1 and TRP14, it reduces the phosphatase of regenerating liver (PRL) [209]. A recent study also suggested a role in cisplatin resistance [210].

Peroxiredoxins (Prxs) are substrates for Trxs and ubiquitous enzymes that scavenge H_2O_2, lipid peroxides and $ONOO^-$ [211]. The view of Prxs has evolved significantly over the last years, from being "just" oxidative stress defenders to regulators of cell signaling processes. Several studies showed that overexpression of Prxs results in dampened growth factor signaling, while a deficiency causes elevated H_2O_2 levels and thereby oxidative inactivation of phosphatases and PDGF receptor activation [212,213]. Also, regulatory functions in apoptosis and Wnt signaling have been documented [214,215]. Six different Prxs exist in humans, PrxI-VI, which are categorized into three groups based on their structure and their catalytic mechanism: 2-Cys-Prxs (PrxI-PrxIV), atypical 2-Cys-Prx (PrxV) and 1-Cys-Prx (PrxVI). The first step in the catalytic mechanism is the oxidation of the N-terminal thiolate to a sulfenic acid intermediate. In a second step the resolving Cys residue, either within the same protein (PrxV) or from another subunit within the homodimeric complex (2-Cys-Prxs, PrxI-IV), forms a disulfide bridge, which is subsequently reduced by Trx1. 1-Cys Prxs do not have a resolving Cys residue and can be reduced by GSH catalyzed by GSTpi [14,42,211]. Instead of being reduced, the sulfenic acid in Prxs can also be further oxidized to sulfinic acid (and even sulfonic acid), which blocks the enzyme for further catalysis [216]. The finding that already very low concentrations of H_2O_2 can result in Prx hyperoxidation lead to the formulation of the floodgate hypothesis [25] (see section 1.2). The hyperoxidation of Prxs to sulfinic acid can be reversed by Sulfiredoxins [126].

Prxs are very efficient catalysts with rate constants for some enzymes as high as 10^7-10^8 M^{-1} s^{-1} [14,39]. These rates are comparable to selenocysteine (Sec) containing GPxs and Prxs are therefore considered to be prime H_2O_2 sensors in the cell [8]. The activity of Prxs is most likely only limited by the reducing capacity of the Trx system [14]. Prxs can also be regulated by posttranslational modifications such as phosphorylation [28], glutathionylation [217], nitrosylation [218], and nitration [219].

Table 1. Members of the Trx fold family of proteins in the human genome. The genes are listed according to their official designation (in bold) by the Human Gene Nomenclature Committee (HGNC) in NCBI. Trx domain containing (*TXNDC*) proteins, Trxs, Nrxs and Grxs are shown. Prxs, PDIs, GPxs and GSTs have been omitted. a.a.: amino acids. Adapted from [42].

gene	official name	also known as	cellular compartment	a.a.	active site
TMX1 (*TXNDC1*)	Trx-related transmembrane protein 1	TMX, TXNDC/TXNDC1 PDIA11	ER	280	CPAC
TXNDC2	Trx domain containing 2 (spermatozoa)	SpTrx, SpTrx1	cytosol	553	CGPC
NME8 (*TXNDC3*)	Trx domain containing 3 (spermatozoa) NME/NM23 family member 8	SpTrx2, TXNDC3	cytosol	558	CGPC
ERP44 (*TXNDC4*)	endoplasmatic reticulum protein 44	ER protein 44 (ERp44) PDI family A member 10 (PDIA10), TXNDC4	ER	406	CRFS
TXNDC5	Trx domain containing 5	ER protein 46 (ERp46) PDI family A member 15 (PDIA15), Trx like protein p46	ER	432	CGHC
TXNDC6	NME family member 9	TXNDC6 NME gene family member 9 (NM23-H9) Trx-like protein 2 (TXL2)		174	CGPC
PDIA6 (*TXNDC7*)	PDI family A member 6	P5, ERP5, TXNDC7	ER	440	CGHC
TXNDC8	Trx domain containing 8 (spermatozoa)	SpTrx3, Trx6		108	CGPC
TXNDC9	Trx domain containing 9	APACD, PHLP3 phosphoducin-like protein 3		226	
TMX3 (*TXNDC10*)	Trx related transmembrane protein 3	TXNDC10, PDI TMX3 PDI family A, member 13 (PDIA13)	ER	454	CGHC
TXNDC11	Trx domain containing 11	EF-hand binding protein 1 (EFP1)	ER	985	CELC, CGFR
TXNDC12	Trx domain containing 12	Anterior gradient homolog 1 (AG1) ER protein 18 (ERP18) PDI family A member 16 (PDIA16)	ER	172	CGAC
TMX4 (*TXNDC13*)	Trx related transmembrane protein 4	TXNDC13, PDI family A, member 14 (PDIA14)	ER	349	CPSC
TMX2 (*TXNDC14*)	Trx related transmembrane protein 2	TXNDC14, PDI family A member 12 (PDIA12) cell prolif. induc. gene 26 protein (PIG26)	ER	372	SNDC
TXNDC15	Trx domain containing 15	C5orf14	membrane	360	CRFS
TXNDC16	Trx domain containing 16	TXNDC16, ERp90, KIAA1344	secreted (serum)	825	
TXNDC17	Trx domain containing 17	Trx-related protein of 14 kDa (TRP14) Trx-like protein 5 (TXNL5) Protein 42-9-9	cytosol	123	CPDC
TXNL1	Trx-like 1, 32 kDa Trx related protein	Txl1, txnl, TRP32	cytosol	289	CGPC
NXN	Nucleoredoxin	Nrx, Nrx1, TRG-4	nucleus, cytosol	435	CPPC
NXNL1	Nucleoredoxin-like 1	rod-derived cone viability factor (RDCVF), TXNL6	nucleus	212	CPQC
NXNL2	Nucleoredoxin-like 2	rod-derived cone viability factor 2 (RDCVF2), C9orf121	nucleus	156	CAPS
TXN	Thioredoxin-1	Trx, Trdx, Trx1	cytosol, nucleus, secreted	105 (80)	CGPC
TXN2	Thioredoxin-2	Trx2	mitochondria	166	CGPC
GLRX	Glutaredoxin 1	Grx, Grx1, thioltransferase 1	cytosol, nucleus, secreted	106	CPYC
GLRX2	Glutaredoxin 2	Grx2, thioltransferase 2	mitochondria	164	CSYC
GLRX3	Glutaredoxin 3	Grx3, Grx4, PKC interacting cousin of Trx (PICOT), Trx	cytosol, nucleus	335	APQC, CGFS CGFS
GLRX5	Glutaredoxin 5	Grx5, Grx related protein 5, monothiol Grx 5	mitochondria	157	CGFS

1.5.3 The Thioredoxin System

The Trx system, consisting of NADPH and isoforms of TrxR and Trx, is a major disulfide reductase system that transfers electrons to a multitude of enzymes [220]. As such, it is critical for DNA synthesis and cell proliferation as well as redox signaling and defense against oxidative stress [221,222]. A knockout of Trx or the TrxR genes in mice is embryonically lethal, showing the importance of the system for normal cell function and development [223-227].

Trx (discovered by Peter Reichard in 1964) is an electron donor for ribonucleotide reductase (RNR) and thereby essential for the *de novo* synthesis of 2'-deoxyribonucleotides from ribonucleotides in DNA replication [228,229]. Trx is ubiquitously expressed and conserved through all species [230]. The main mammalian isoforms are cytosolic Trx1 and mitochondrial Trx2 and both contain the highly conserved CGPC active site motif. Oxidized Trx is reduced by electrons from NADPH via TrxR [231]. Despite the lack of a nuclear localization signal, Trx1 is additionally found in the nucleus where it interacts with several transcription factors, e.g. Ref-1, AP-1, NFκB and p53 [232-236]. A truncated form of Trx1, Trx-80, can be found extracellularly and is involved in T-cell activation and possibly in Alzheimers disease [222,237].

Beside the active site, Trx1 contains three additional Cys residues: C62, C69 and C73. C73 protrudes from the surface and can thereby form disulfide-linked homodimers that cannot be reduced by TrxR1 [238,239]. The formation of an intramolecular disulfide bond between C62 and C69 is believed to have a regulatory function, as this modification also cannot be reduced by TrxR1, but instead requires the GSH system [240,241]. The additional Cys are furthermore subject to post-translational modifications, e.g. nitrosylation and glutathionylation [242].

Cytosolic Trx1 acts as electron donor for Prxs [243], MSR [244] and also binds PTEN [245], thereby inhibiting PTEN and thus promoting tumorigenesis. Trx1 is furthermore involved in apoptosis signaling via apoptosis signal regulating kinase 1 (ASK1). ASK1 is an upstream MAPKKK that regulates c-Jun-N-terminal kinases (JNK) and p38 MAPK leading to stress-induced apoptosis and inflammation. Reduced Trx1 binds ASK1 thereby inhibiting the kinase activity [246]. Oxidation or nitrosylation of Trx1 releases ASK1, thereby initiating apoptosis signaling [247,248].

The function of Trx1 can be regulated by Trx-interacting protein (TXNIP, also known as thioredoxin binding protein 2/vitamin D3 upregulated protein). TXNIP does not belong to the Trx fold family of proteins and forms a disulfide bond with C32 in the active site of Trx1, thereby inactivating the enzyme [249,250]. TXNIP has both redox dependent and independent functions, its expression is for example widely regulated by nutrition status [251]. The release of ASK1 and subsequent apoptosis induction is likely also promoted by TXNIP [252].

Trx reductase (TrxR)

TrxRs are dimeric flavoenzymes of the pyridine nucleotide disulfide oxidoreductase family [253]. Three different isoforms of TrxR1 are expressed in mammalian cells, of which the cytosolic TrxR1 will be the focus of this thesis. Further isoforms are localized in the mitochondria (TrxR2) and one isoform named Trx GSH reductase (TGR) is predominantly expressed in testis. TrxR1 is a homodimeric enzyme. Each subunit contains one FAD and two redox-active sites: one N-terminal, close to the FAD; the other at the C-terminus. The C-terminal active site contains a highly reactive and accessible selenocysteine (Sec) residue, which is cotranslationally incorporated and essential for the catalytic function [254,255]. Sec, often referred to as the 21st amino acid, is the selenium-containing analogue of Cys. Compared to Cys, Sec has a lower pKa and is therefore more reactive [256].

TrxR1 has a multitude of cellular functions, mainly via its substrate Trx1, which thereby makes the whole Trx system dependent on the trace element selenium [253]. Other protein substrates of TrxR1 include TRP14 [163,257], TRP32 [206], PDIs [258] and mitochondrial Grx2 [259]. TrxR1 is also known to reduce various small molecules, including quinones like 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB, Ellmans reagent) [260], selenite [261], selenoglutathione [262], ebselen [263], H_2O_2 [255], lipid peroxides [264], lipoic acid and lipoamide [265], dehydroascorbate [266] and menadione [260]. The principal catalytic mechanism in the head-to-tail arrangement of two TrxR1 subunits involves the transfer of electrons from NADPH to the N-terminal active site motif (-CVNVGC-) via the enzyme bound FAD. The resulting dithiol exchanges its electrons with the selenyldisulfide motif in the other subunit, which in turn facilitates reduction of the different TrxR1 substrates (Fig. 8) [253]. Some substrates, like 5-hydroxy-1,4-naphtalenedione (juglone), do not require an intact Sec residue and may be directly reduced via the N-terminal motif [267].

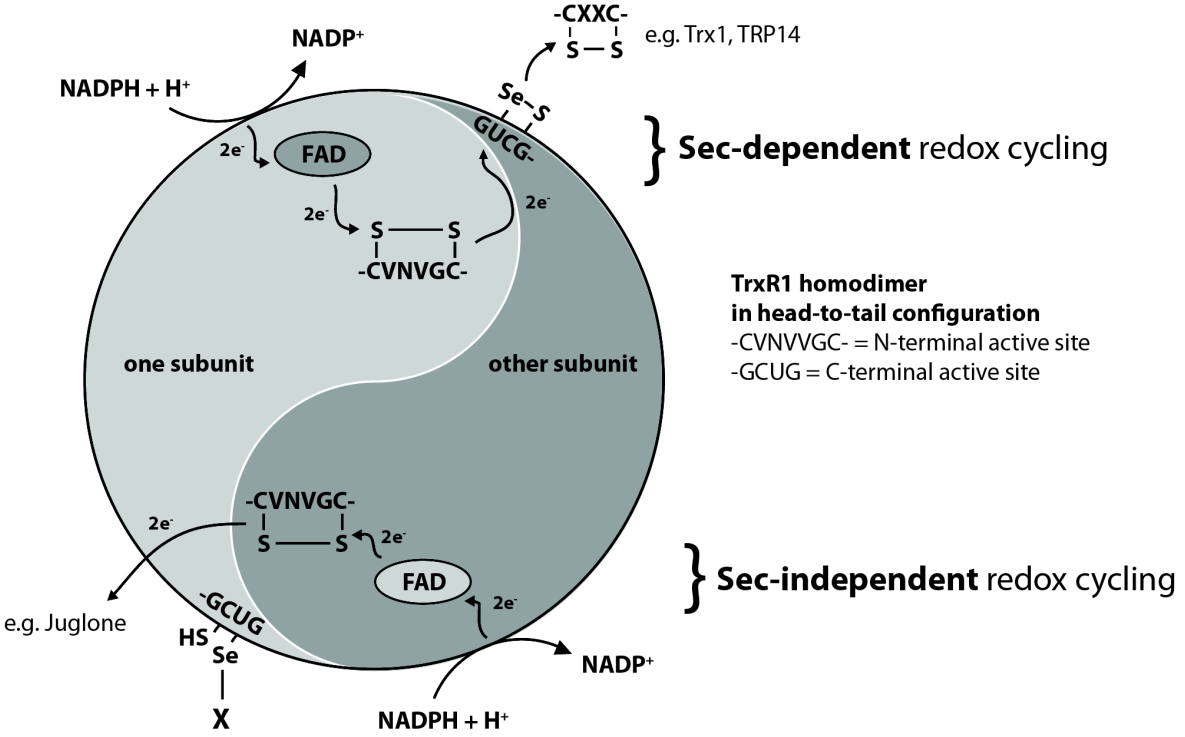

Figure 8. Catalytic mechanism of TrxR1. The enzyme is depicted as "Yin-Yang", illustrating the homodimer in head-to-tail configuration. For convenience, two possible catalytic mechanisms are depicted on the same enzyme. Sec-dependent redox cycling (upper part): The electrons flow from NADPH via the FAD moiety to the N-terminal disulfide motif of one subunit and subsequently to the C-terminal selenylsulfide motif of the other subunit. The thereby reduced selenothiol motif allows reduction of substrates such as Trx and TRP14. Sec-independent redox cycling (lower part): The selenolate of the C-terminus can be derivatized with electrophilic agents (indicated with X), which results in an enzyme species that is irreversibly inhibited for the normal substrate reduction via the C-terminal active site. However, NADPH can still donate electrons to FAD and the functional -CVNVGC- motif, which promotes Sec-independent reduction of certain substrates such as juglone and other quinones. For further details, see text. This Figure was adapted from [253].

TrxR1 inhibition and the SecTRAP concept

The accessible and highly nucleophilic Sec residue is essential for the catalytic activity of TrxR1, but makes the enzyme at the same time susceptible to inhibition by electrophilic compounds. A complete list of inhibitors of TrxR1 would be beyond the scope of this thesis and the interested reader is referred to a recent compilation of TrxR1 inhibitors in relation to the Nrf2 system [184]. However, prominent examples of TrxR1 inhibitors include naturally occurring compounds such as flavonoids [268], curcumin [269], sulforaphane [270] and interestingly, also various components in red wine [271]. Also 4-hydroxynonenal, the end product of lipid peroxidation, is a potent, irreversible inhibitor of TrxR1 [272].

Some of the most potent inhibitors of TrxR1 are transition-metal containing compounds (Fig. 9). Gold (Au) compounds, like the FDA approved drug auranofin, are well known inhibitors and have long been used in the treatment of rheumatoid arthritis, a condition that is associated with elevated levels of TrxR1 [273-275]. Auranofin also seems to be effective as chemotherapeutic agent and is currently in clinical trials for the treatment of cancer [276]. Platinum (Pt) compounds, like the prototype cis-diamminedichloroplatinum(II) (cDDP,

cisplatin), are also effective inhibitors of TrxR1 [277,278]. Cisplatin was "accidentally" discovered in the 1960s, when Barnett Rosenberg found that electrolysis products of platinum electrodes inhibited cell division in *E. coli* [279]. Today, Pt containing drugs are used to treat a variety of cancers. Cisplatin was one of the first anticancer compounds found to interact with the Trx system. Additionally, the therapeutic effect may involve other mechanisms including DNA adducts [278,280], induction of ROS [281] and ER stress [282]. Severe side-effects of cisplatin and acquired drug-resistance have limited its application and promoted the development of next-generation Pt-based agents [283]. Besides Pt compounds, also silver, ruthenium and gadolinium-based compounds have been shown to inhibit TrxR1 [284].

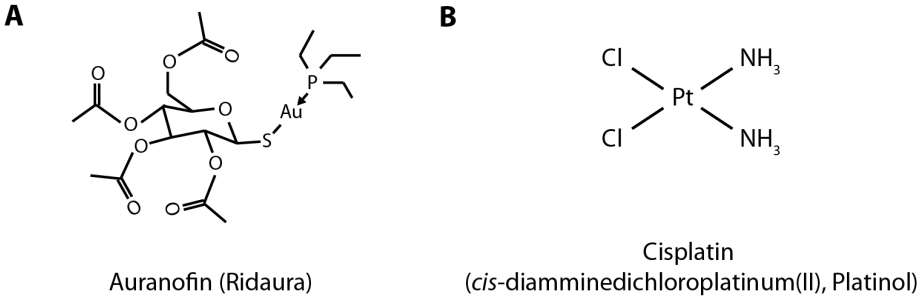

Figure 9. Structures of auranofin (Ridaura®) and cisplatin.

A proposed effect of certain TrxR1 inhibitors that target the Sec residue of the protein is the ability to convert TrxR1 into a **se**lenium **c**ompromised **t**hioredoxin **r**eductase-derived **a**poptotic **p**rotein (**SecTRAP**). These SecTRAPs have lost their ability to catalyze Sec dependent reactions, but are still capable of redox cycling via the intact FAD and N-terminal active site [285,286]. One explanation is that compromising the Sec residue causes a conformational change, so that the N-terminus is more accessible, because otherwise the electron transfer within the homodimer is very efficient and should not result in electron leakage [287,288]. Several studies have shown that SecTRAPs can induce cell death and that the formation of reactive oxygen species is part of their cytotoxic mechanism [286,289,290]. Recent reports correlate the inhibition of TrxR1 by the compounds N-acetyl-p-benzoquinone imine (NAPQI), the active metabolite of acetaminophen [291], Shikonin [292] and MJ25 [293] to the formation of SecTRAPs. Interestingly, the overexpression of TrxR1 in cells involves expression of a truncated version of the enzyme, which also has SecTRAP-like effects and causes rapid cell death. Supportive of the SecTRAP concept is also the finding that targeting the Sec residue in TrxR1 results in more severe effects than caused by TrxR1 knockdown (see [253] and references therein).

1.5.4 The Glutathione System

The GSH system is another NADPH-dependent redox-regulatory system. One of its major functions is the detoxification of xenobiotics and their metabolites; either directly by GSH or catalyzed by Glutathione-S-transferases (GSTs) [111]. In addition, GSH is used as a cofactor for GPxs and glutaredoxins (Grxs). The oxidized form of GSH, glutathione disulfide (GSSG), is reduced by the flavoprotein Glutathione reductase (GR).

GSH

Reduced GSH is a 307 Da tripeptide (γ-glutamyl-cysteine-glycine) present in millimolar concentrations in most cells [111]. 90% of the cellular GSH is in the cytosol, 10% in the mitochondria and a small percentage in the ER [294,295]. The turnover in rat liver is estimated to be about 2-3 h, and it is important to note that due to the γ-carboxylgroup, GSH cannot be degraded intracellularly. The only enzyme capable of that reaction is gamma-glutamyltranspeptidase (GGT), which is present on external surfaces of certain cell types [111]. GSH is synthesized from Cys, which is either imported into the cell or provided by the transsulfuration pathway (see section 1.4.1). Under conditions involving severe oxidative stress, GSSG will accumulate and subsequently be exported. Since cells cannot take up GSSG, they require *de novo* synthesis of GSH [296].

Protein glutathionylation can be a regulatory function or a protective mechanism against irreversible overoxidation [297]. Possible mechanisms include thiol-disulfide exchange reactions with GSH or the condensation of GSH with Cys sulfenic acids or nitrosothiols [52]. More than 200 mammalian proteins are reported to be glutathionylated, e.g. PrxII as a mechanism to protect against overoxidation [217,298,299].

GSTs

GSTs are important detoxifying enzymes that catalyze the conjugation of GSH to various endogenous and exogenous electrophilic compounds [300,301]. A key feature of GSTs is to aid in the deprotonation of GSH. The resulting thiolate anion (GS^-) is a strong nucleophile and required for the conjugation to the electrophilic substrates [302]. Many GSTs are expressed in response to Nrf2 activation as part of the cellular defense against electrophiles and oxidative stress. In most cases, the GSH-conjugated compound is less reactive, more water soluble and subsequently excreted from cells via cellular efflux systems [303]. Sometimes the conjugation of GSH can also result in metabolites that are more reactive than the parental compound, a feature that could be used in cancer therapy to produce reactive metabolites that selectively target tumor cells with upregulated expression of GSTs [304,305]. In addition to their detoxifying activities, GSTs have metabolic functions and catalyze the synthesis of e.g. steroids, leukotriens, prostaglandins, testosterone, and progesterone [300,302]. GSTs also exhibit peroxidases activities in reducing lipid hydroperoxides and are furthermore suggested to perform the glutathionylation of proteins [185,306].

GPxs

GPxs catalyze the GSH-dependent reaction with various types of hydroperoxides [307]. Eight GPxs are expressed in humans; most of them are selenoproteins (GPx1, GPx2, GPx3, GPx4, GPx6) while the remaining enzymes have a Cys instead of a Sec in their active site (GPx5, GPx7, GPx8) [308]. The Sec containing GPxs reduce H_2O_2 with rate constants higher than 10^7 $M^{-1}s^{-1}$ while the regeneration of the enzyme by GSH is estimated to be near 10^5 $M^{-1}s^{-1}$, which seems reasonable to keep the enzyme reduced under most physiological conditions. This makes them, together with the Prxs, prime targets for the reaction with H_2O_2 in redox signaling pathways [14]. The most studied GPx is GPx1. It is ubiquitously expressed in the cytosol and the mitochondria and was the first selenoprotein ever described [309]. A knockout of GPx1 in mice has only mild effects, but the overexpression of GPx1 (the so-called "supermouse") resulted in a phenotype resembling type II diabetes and showed that GPx1 is involved in regulating insulin signaling [310]. According to the current hypothesis, GPx1 "steals" the H_2O_2 that is required for efficient inactivation of PTP1B, which is a prerequisite for efficient insulin signaling [14]. Interestingly, the only knockout of a GPx that is embryonically lethal is Gpx4. It seems to be unique in reducing specific lipid peroxides generated by 12,15-lipoxygenase and has thereby additional regulatory functions in the cell [307,311].

Grxs

Grxs were first identified as electron donor for RNR and as GSH-dependent reductases [312-314]. Grxs catalyze both the formation and reduction of mixed disulfides between protein thiols and GSH and can be divided into dithiol and monothiol Grxs. Dithiol Grxs have a characteristic CXXC active site motif, whereas monothiol Grxs lack the C-terminal Cys and instead possess a CXXS active site. Oxidized Grxs are reduced by GSH [315]. Human Grx1 exists in the cytosol, nucleus, mitochondria, as well as in the plasma and therefore has a variety of functions. Examples include the regulation of transcription factors like NF-1 [316], NFκB and AP-1 [201] as well as the reactivation of PTP1B after glutathionylation [317]. Furthermore it is involved in apoptosis signaling by binding ASK1 [318] and regulating caspase 3 cleavage [319]. The activity of Grx1 is possibly also regulated via S-nitrosylation [320].

1.5.5 Targeting Redox Signaling Pathways as Anti-Cancer Strategy

Cancer is still a leading cause of death worldwide, with a tendency to double until 2030 (source: who.org). Cancer is, however, a collective name for multiple genetic disorders that result in individual tumors, consisting of heterogenic populations of aberrant proliferating cells. Douglas Hanahan and Robert Weinberg originally described common hallmarks of cancer [321], which now have been expanded with additional stress phenotypes, called non-oncogenic addictions [322]. One of these additional cancer-supporting characteristics is an excessive ROS production in cancer cells, accompanied by elevated antioxidant mechanisms [323,324]. What first sounds like a paradox, is the result of a redox adaptation. The activation of oncogenic pathways leads to increased production of ROS via multiple mechanisms, which in turn promotes diverse signaling events and contributes to tumorigenesis [325]. At the same time, oncogenesis is further promoted by antioxidant mechanisms that are induced to counteract the ROS overproduction and its effects [326,327]. As a result, both ROS and enzymatic systems such as the Trx and GSH systems are elevated at the same time. A promising, recently emerging anti-cancer strategy exploits this feature by either increasing the generation of ROS or decreasing the capacity of the antioxidant systems to specifically eliminate cancer cells, leading to an "overload" of ROS (Fig. 10) [323,328]. According to this concept, cancer cells would be more sensitive to further ROS accumulation than normal cells, as they are already at the limit of their antioxidant capacity. Several drugs that are in clinical use, including anthracyclines such as doxorubicin, but also Pt complexes like cisplatin, are known to increase ROS, which might exceed the tolerated threshold and thus be part of their therapeutic efficacy [278,281,329,330].

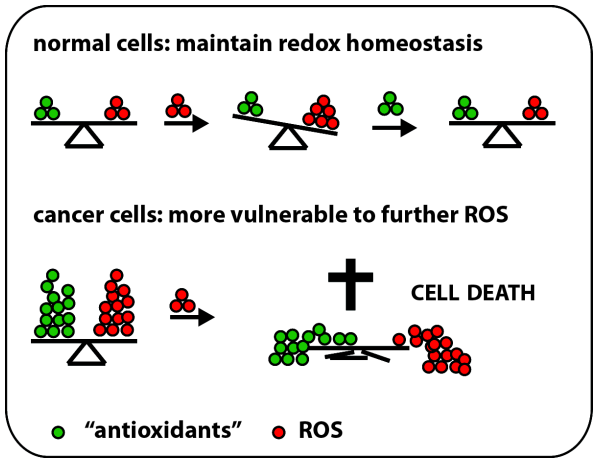

Figure 10. Simplified scheme of the redox homeostasis in normal and cancer cells. Normal cells have basal levels of ROS and antioxidant systems. In contrast, cancer cells have elevated basal ROS levels that are balanced by an increased antioxidant capacity. This redox adaptation helps cancer cells to survive, but makes them at the same time more vulnerable for further oxidative stress modulation. A further increase of ROS (either by using ROS generating agents or suppression of the increased antioxidant activities of cancer cells) may be sufficient to cause cell death in cancer cells but not in normal cells. Normal cells can tolerate a certain level of oxidative stress by upregulating their antioxidant capacity and restoring their basal "redox balance". Cancer cells already exhibit elevated ROS levels and antioxidant systems, so that the already fragile "redox balance" cannot tolerate any further oxidative stress, thereby leading to cancer cell death.

The Trx system, in particular TrxR1, has been extensively discussed as an anti-cancer target [222,331-333]. TrxR1 is often overexpressed in tumors and the therapeutic effect of several

anti-cancer drugs has been connected to the inhibition of TrxR1 and oxidative stress [278,334-338]. These observations therefore support the notion of TrxR1 being an attractive anti-cancer drug target. Whether the inhibition of TrxR1 alone would be sufficient for anti-cancer efficacy or if the inhibition of the enzyme is just part of the cytotoxic effect is still a complex and active research field.

Recent studies have also suggested a simultaneous targeting of the Trx and GSH systems as an effective anti-cancer strategy [339-341]. As either one of the two systems is required for cell survival [342], this approach still poses many unanswered questions and requires further insights into the regulation of the Trx and GSH systems both in normal and malignant cells.

1.5.6 Redox Signaling and Protein Tyrosine Phosphatases (PTPs)

A number of critical cellular processes like proliferation and differentiation are regulated by receptor tyrosine kinases (RTKs) [343]. All RTKs have a similar architecture consisting of an extracellular ligand binding domain, a single transmembrane helix, a cytoplasmic region that contains the protein tyrosine kinase domain and additional c-terminal regulatory regions. The binding of a growth factor induces receptor dimerization and auto-phosphorylation of Tyr residues, which then mediate the cellular response through circuits of protein interactions and signaling cascades (Fig. 11) [344].

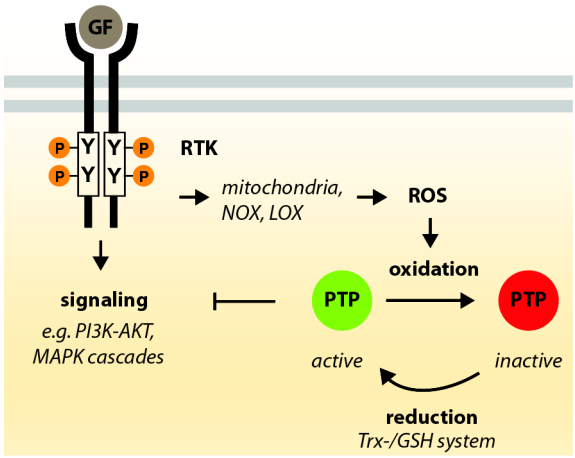

Figure 11. Simplified overview of the regulation of receptor tyrosine kinase (RTK) signaling by reversible oxidation of protein tyrosine phosphatases (PTPs). RTK activation through growth factor (GF) binding can cause the production of ROS by mitochondria, NOX and lipoxygenase (LOX) enzymes, thereby inactivating PTPs and allowing for RTK mediated signaling processes. Note: NOX enzymes produce ROS extracellularly (see more detailed discussion in section 3.2).

Growth factors like insulin-like growth factor 1 (IGF1), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and also insulin act through RTKs [345]. They can activate key signal transduction pathways, including the phosphatidylinositol 3-kinase (PI3K)-AKT signaling pathway and mitogen-activated protein kinase (MAPK) cascades, thereby promoting cell proliferation, nutrient uptake and cell survival [346]. RTK signaling is negatively regulated by protein tyrosine phosphatases (PTPs), which will be the focus of this section. Since RTK signaling is important for critical cellular processes, a dysregulation of both RTKs and PTPs is associated with a variety of diseases [347].

PTPs are not just simple "housekeeping" proteins that oppose RTK signaling; their activity is tightly regulated and involves the reversible, inhibitory oxidation of their active site Cys residue upon receptor stimulation [348]. The specificity towards phosphotyrosine is determined by a deep active site pocket, for which phosphoserine and phosphothreonine are too small [32,349]. The structural environment of the active site is organized in a way, that it lowers the pKa of the catalytic Cys, making it more nucleophilic, but at the same time also susceptible to oxidation [348]. It has been well established that the stimulation of growth factor receptors results in a production of ROS and a concomitant oxidation of PTPs [21,350]. Possible sources of ROS include i) NOX enzymes [351], ii) mitochondria [352-354] and iii) lipid peroxides [355,356]. Collectively, these events cause an increase in receptor phosphorylation and activation of downstream signaling pathways (more detailed discussion in context of paper II, section 3.2). The PTP family consists of 107 members [357], of which three that are important for this thesis will be briefly introduced:

PTP1B was the first PTP that was purified and characterized [358,359]. It is encoded by the *PTPN1* gene and has a hydrophobic C-terminal sequence that targets PTP1B to the membranes of the ER [360]. Despite this anchoring, PTP1B is able to access its substrates by different mechanisms, e.g. by endocytosis, the movement of the ER network or by generation of a truncated, soluble form [360,361]. PTP1B dephosphorylates and thus inactivates the EGF receptor (EGFR), PDGF receptor (PDGFR) and the insulin receptor (IR) [362]. PTP1B knockout mice (*Ptpn1*$^{-/-}$) are healthy, but remain insulin-sensitive when put on a high-fat diet [363]. Overexpression of PTP1B abrogates insulin receptor signaling [364]. In 1998, the first study reported that the EGF mediated H_2O_2 production resulted in oxidation and thereby inactivation of PTP1B [24]. The same study provided the first evidence that the Trx system could reactive PTP1B, which has been further confirmed by other laboratories [179,365,366].

SHP2 belongs to non-transmembrane, Src homology 2 (SH2) domain-containing PTPs [367,368]. The SH2 domains regulate the intracellular location and catalytic activity. They are specifically targeted to Tyr phosphorylation sites in receptors and scaffolding adaptor proteins [369]. SHP2 is required for the full activation of the RTK induced MAPK-ERK signaling pathway. It dephosphorylates PDGFR, but also binds scaffolding molecules like members of the Gab/Dos and insulin receptor substrate (IRS) families [369]. In the basal state, SHP2 exhibits low activity because the active site is occluded by intramolecular interaction with the N-terminal SH2 domain opposite of the pTyr binding site. Upon stimulus with appropriate pTyr ligands, a conformational change results in release of the autoinhibitory loop and activation of the enzyme. Thus, SHP2 only becomes activated when it is recruited to the correct signaling complex [32].

The phosphatase and tensin homologue (PTEN) is another PTP that becomes reversibly oxidized in response to EGFR activation [23]. PTEN is a dual protein and lipid phosphatase as well as a negative regulator of the PI3K/AKT signaling pathway [370]. It is worthwhile to note, that PTEN is one of the most frequently lost or mutated tumor suppressors in human cancer, which further emphasizes the importance of tight regulation in RTK/PTP signaling [371].

1.6 THIOREDOXIN RELATED PROTEIN OF 14 KDA (TRP14)

TRP14 was first identified and characterized by the laboratory of Sue Goo Rhee in 2004 [257]. It is a ubiquitous protein with a highly conserved WCPDC active site and most likely a predominantly cytosolic protein due to the lack of a signal peptide. As judged by immunoblot analysis on rat tissues, it seems to be most abundant in liver, kidney, pancreas and uterus [257]. In humans, high levels have been indicated in gall- and urinary bladder, parts of the digestive system, kidney, ovaries, cerebellum and thyroid gland (according to the human protein atlas, www.proteinatlas.org and not verified in follow up studies [372]). Recent studies have investigated TRP14 in lancelets [373], saltwater clam [374] and a type of cod, showing tissue specific expression, as well as antioxidant and metal stress induced functions [375].

Human TRP14 shares 20% sequence identity with human Trx1 and analyses with different cell lines suggest that it is usually present in less amounts than Trx1 [163,257]. It should be noted that TRP14 is a substrate of TrxR1 but not TrxR2, that its pKa and redox potential are lower compared to Trx1 and that it does not reduce many typical substrates of Trx1 (RNR, MSR, PrxI and insulin) (see Tab. 2). Similar to Trx1, TRP14 possesses three additional Cys. While these seem to have regulatory function in Trx1 (see section 1.5.3), they seem to be inaccessible in TRP14 [257]. Suggested substrates for TRP14 are dynein light chain (LC8 or DLC8), cofilin, and ribosomal protein L30 [376]. Whether these proteins are substrates for Trx1 is currently unknown. TRP14 and Trx1 also have a number of overlapping substrates including oxytocin, vasopressin and H_2O_2. PTP1B is also a common substrate for both enzymes, whereas PTEN can only be reduced by Trx1 and not by TRP14 [377]. The phosphatase of regenerating liver (PRL) can neither be reduced by Trx, nor by TRP14 [209,378].

Table 2. Biochemical parameters of Trx1 and TRP14 in comparison. Adapted from [379].

	Trx1	TRP14
MW (kDa / no. of aa)	12 / 105	14 / 123
active site motif	CGPC	CPDC
active site cys	C32, C35	C43, C46
additional cys	C62, C69, C73	C64, C69, C110
pKa of N-terminal Cys	7.3	6.1
redox potential (mV)	-270	-257
electron donors	TrxR1, TrxR2	TrxR1
substrates		
RNR	●	●
MSR	●	●
PrxI	●	●
insulin	●	●
Oxytocin	●	●
Vasopressin	●	●
H_2O_2	●	●
LC8	●	●
Cofilin	●	●
L30	●	●
PTEN	●	●
PTP1B	●	●
ASK1	●	●
Cystine	●	●
HED	●	●

● substrate ● unknown ● not a substrate

The structure of TRP14 shows a similar overall topology compared to Trx1 (Fig.12) [380]. However, there are several important structural differences that might explain the different substrate specificity of both proteins. In TRP14, the helix that corresponds to helix α3 in Trx1, is divided into two separate helices (α3a and α3b) with four residues in between. Helix α3 is clearly shifted towards the active site and seems to compromise its accessibility. The access is furthermore blocked by a protruding loop structure (loop β2-α2), which is seven residues longer compared to the equivalent loop in Trx. Both helices and the loop seem to significantly influence the substrate accessibility in TRP14. Another important parameter is the different hydrogen bonding network in the active site. The residues between the vicinal Cys (Gly-Pro in Trx1 and Pro-Asp in TRP14) clearly influence the pKa of the N-terminal Cys. The thiolate of Cys43 in TRP14 is stabilized by the partial positive charge of the helix α2 dipole and by additional hydrogen bonding between the amide nitrogen of Asp45 and the N-terminal Cys sulfur. This bonding network is not possible in Trx1 because of the proline residue. Important to note, the surface surrounding the active site is highly charged in TRP14, but mainly hydrophobic in Trx1. Comparing the structures, it appears that TRP14 and Trx1 have evolved to recognize different substrates. The significantly different architecture and accessibility of the active site strongly suggests that TRP14 is designed to have a more selected set of substrates [380]. Interestingly, yeast Grx8 shares the same active site motif as human TRP14 and a recent study concluded that both proteins should have evolved in a way to constrain the active site to increase substrate specificity [381].

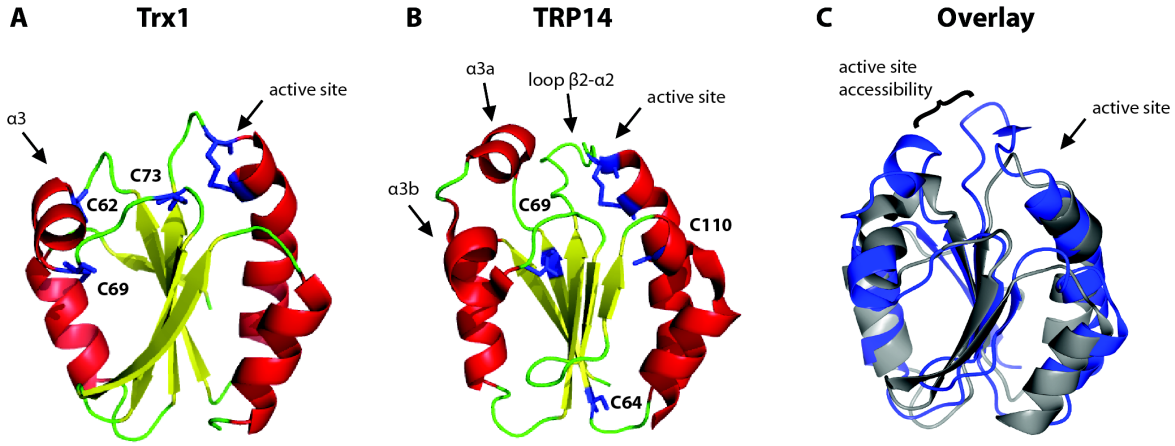

Figure 12. Structural comparison of human Trx1 and TRP14. A) Structure of human Trx1 (1ERU). The locations of the active site (C32, C35) and additional Cys residues (C62, C69, C73) are indicated in blue. B) Structure of human TRP14 (1WOU). The active site (C43, C46) and additional Cys residues (C64, C69, C110) are also indicated in blue. C) Overlay of human Trx1 (1ERU, grey) with human TRP14 (1WOU, blue). The approximate location of the active site in both structures is indicated.

TRP14 has been implicated to be involved in the NFκB (nuclear factor kappa light chain enhancer of activated B cells) pathway. NFκB regulates the expression of more than hundred genes of the immune and inflammatory response and was the first transcription factor suggested to be redox regulated and activated by ROS [382,383]. Different pathways activate NFκB, but for the scope of this thesis focus will be given on the typical, or canonical pathway (Fig. 13). This pathway is triggered by e.g. TNFα, IL-1 and LPS, that activate the IκB-kinase complex (IKK complex) through phosphorylation, which in turn phosphorylates IκB. This leads to a release of IκB from the NFκB complex (typically the p65+p50 complex). IκBs are targeted for degradation, while NFκB is phosphorylated by proteinkinase A and translocates to the nucleus. There it binds together with its coactivators CREB binding protein (CBP) and p300 to the NFκB responsive element [3]. The binding requires the reduction of a specific Cys residue in the p50 subunit.

According to recent studies by Rhee and coworkers, Trx1 and TRP14 regulate the NFκB pathway differently. The role of Trx1 in NFκB regulation seems to be confined to the nucleus, where it reduces the Cys residue in the p50 subunit that in turn enables DNA binding (Fig. 13) [234,384,385]. The overexpression of Trx1 results in an inhibition of NFκB activation, which is most likely an effect on the reduction of the Prxs that compete for hydroperoxides that are essential to maintain the NFκB pathway active [232]. TRP14, however, appears to have a predominant role in the cytosolic regulation of NFκB and was reported to inhibit the TNFα induced NFκB activation via its substrate LC8 [376]. In that study, RNA interference experiments showed that depletion of TRP14 resulted in an increase of TNFα induced phosphorylation and degradation of IκBα to a greater extent than Trx depletion [376]. In a follow up study the authors suggested that LC8 acts as a negative regulator of NFκB [386], which is consistent with an earlier study, linking TRP14 to prevention of cytosolic NFκB activation. How the initial oxidation of LC8 occurs is not clear yet. A direct oxidation by H_2O_2 seems unlikely, but no intermediate proteins have been described [3].

Several studies have correlated the NFκB activation with an induction of NOX enzymes and thereby a simultaneous production of $O_2^{\bullet-}$, H_2O_2 and lipid hydroperoxides [3,33,387,388], which is consistent with the suggested concept of localized redox signaling (section 1.2). The role of H_2O_2 in NFκB signaling has been very controversial in the literature, but present consensus seems to be that H_2O_2 is a fine-tuning regulator of NFκB dependent processes [389,390].

A first hint on the biological significance of NFκB regulation by TRP14 was reported recently [391]. In this study, TRP14 depletion enhanced the receptor activator of NFκB ligand (RANKL) induced osteoclast differentiation, thereby providing evidence for a regulatory role of TRP14 in bone resorption.

Besides its role in NFκB signaling, TRP14 has been associated with chemo-resistance. Based on a quantitative proteomics approach, a recent study identified elevated expression of TRP14 in chemo-resistant ovarian cancer cells [392]. The authors also correlated TRP14 expression to Beclin 1, a key regulator for autophagy and concluded that TRP14 promotes autophagy and chemo-resistance in ovarian cancer.

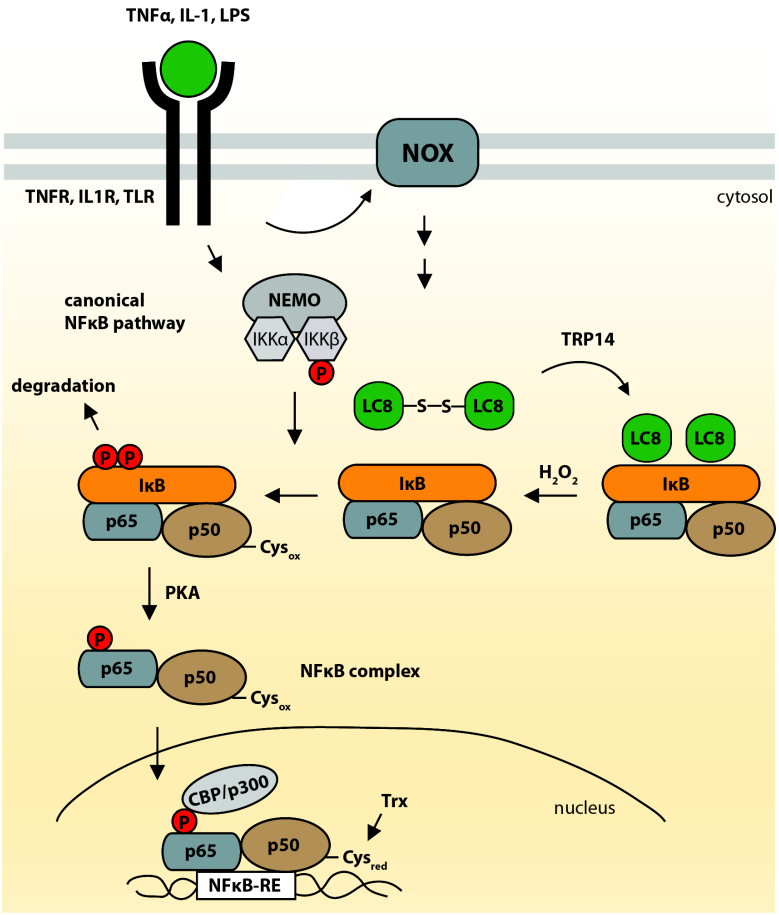

Figure 13. Simplified overview of the putative role of TRP14 in the canonical NFκB pathway. This model has been adapted in parts from [379]. In unstimulated cells, LC8 binds to IκB and inhibits its phosphorylation by the IKK complex. A stimulation of the NFκB pathway activates the IKK complex and leads to a production of H_2O_2 by NOXs. This can result in oxidation and dissociation of LC8 from IκB. IκB is then be phosphorylated by IKK and degraded by the proteasome. The NFκB complex (consisting of p65 and p50) can then translocate to the nucleus and initiate gene transcription. LC8 is a substrate for TRP14, which coupled to TrxR1, maintains LC8 in a reduced form and thereby counteracts NFκB stimulation. Trx1 reduces a critical Cys residue of the NFκB complex in the nucleus, which is required for DNA binding. NFκB-RE: NFκB response element, CBP: CREB binding protein. NEMO: NFκB essential modulator.

2 PRESENT INVESTIGATION

2.1 AIMS OF THIS THESIS

The overall aim of this thesis was to investigate the oxidoreductase TRP14 in different contexts.

Paper I

- Investigating the inhibition of TrxR1 by noble metal compounds and their effect on cancer cell survival.
- Analyzing the mechanism of inhibition and the potential of forming SecTRAPs in cells.

Paper II

- Studying the role of the Trx system in reactivation of oxidized protein tyrosine phoshatases (PTPs) in the context of platelet derived growth factor (PDGF) signaling.

Paper III

- Characterizing enzymatic properties of TRP14 in reducing disulfide substrates and nitrosylated proteins.

Paper IV

- Developing a method to detect protein persulfides in a cellular context and studying the role of the Trx and GSH systems in the reduction of polysulfides and protein persulfides.

2.2 METHODOLOGY

This section provides a short introduction into some of the methods used in paper I-IV. For more detailed information see the Materials and Methods part of each study.

Cell culture (paper I-IV)

Most cell culture experiments described in paper I-IV are based on commercially available cell lines (summarized in Tab. 3). For many experiments A549 cells were used, because of their exceptionally high levels of TrxR1 [336,393]. HEK293 cells are commonly known to be a good transfection host (source: ATCC), which is why they were used for transient (paper III) and stable (paper IV) knockdown of TRP14, Trx1 and TrxR1. Also mouse embryonic fibroblasts (MEFs) were used, which have been generated by the laboratories of Marcus Conrad (*Txnrd1*$^{-/-}$) [226], Edward Schmidt (*Txnrd1*$^{-/-}$) [225] and Benjamin Neel (*Ptpn1*$^{-/-}$) [394] from the respective mice (paper II and IV).

Table 3. Commercial cell lines used within this thesis. Reference: American Type Culture Collection (ATCC).

	cell line	tissue	diagnosis	paper
human	A549	lung	carcinoma	I, III, IV
	H23	lung	adenocarcinoma	I
	HCT116	colon	colorectal carcinoma	I
	HeLa	cervix	adenocarcinoma	I
	A431	skin/epidermis	epidermoid carcinoma	III
	HEK293	embryonic kidney	n.a.	III, IV
	HT29	colon	colorectal carcinoma	III
	THP1	monocyte	acute monocytic leukemia	III
mouse	NIH3T3	embryo	fibroblasts	II

Activity Assays for TrxR1, Trx1 and TRP14 (paper I-IV)

We measured the activity of members of the Trx and GSH system in recombinant enzyme preparations, as well as in biological samples. Some of the basic principles of these assays are summarized in Fig. 14.

The activity of recombinant TrxR1 can be measured using Ellman's reagent (5,5-dithio-bis(2-nitrobenzoic acid), DTNB) [395]. TrxR1 directly reduces DTNB to two TNB^- molecules, which absorb at 412 nm. This method is used to determine the specific activity of TrxR1, because one unit (U) of TrxR1 is defined as the amount of enzyme catalyzing the reduction of 1 mol DTNB (formation of 2 mol TNB^-) per minute.

Another available assay is the insulin-coupled Trx assay, which can be used to determine the activity of Trx1 and TrxR1 in recombinant enzyme preparations as well as in biological samples. The assay is typically performed in an endpoint format and involves saturating levels of NADPH and insulin as well as a relative excess of either Trx1 or TrxR1, depending on which of the proteins is measured [396]. The reaction mixture is incubated at 37°C and

stopped with guanidine hydrochloride (GuHCl) and DTNB. The number of TNB^- ions reflects the number of TrxR1- or Trx1-dependent reduced insulin thiols in the sample. In contrast to biological samples, recombinant proteins can also be measured by following the consumption of NADPH at 340 nm. An option to increase the sensitivity of measuring cellular Trx1 is to use fluorescent-labeled insulin [397].

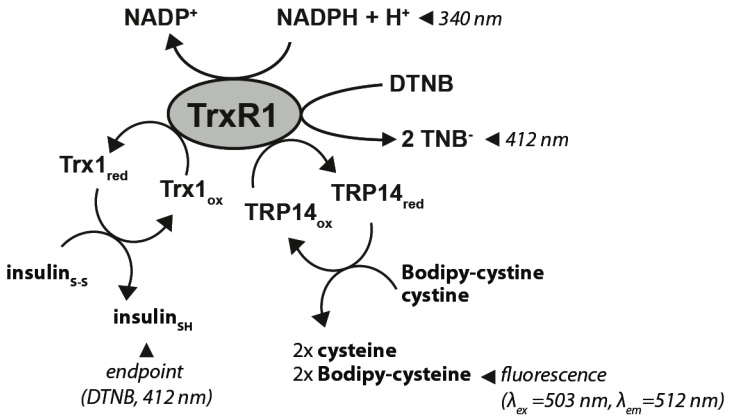

Figure 14. Principles of the activity assays used in this thesis. All reactions depend on the activity of TrxR1.

In paper III, we established activity assays for TRP14. They base on the same principle as Trx1 activity assays, with the difference that TRP14 does not reduce insulin. In contrast, cystine and also the fluorescent-labeled Bodipy-cystine are exceptionally good substrates for recombinant TRP14 (paper III). The activity of TRP14 in cell extracts has been assessed in paper III using cystine as substrate and measuring NADPH consumption at 340 nm. The use of Bodipy-cystine in biological samples is still being optimized.

Grxs are commonly measured using β-hydroxyethylene disulfide (HED) as substrate. The reaction is coupled to GSH and GR, whereof the latter uses NADPH that can be measured at 340 nm [398].

Measurement of protein nitrosylation and denitrosylation (paper III)

Analysis of protein nitrosylation is often difficult due to technical limitations, the complex chemistry of the reactants and the relative instability of the -SNO moiety. Therefore the use of at least two different methods is generally recommended [399].

One of the most sensitive methods to measure $NO^\bullet$ is by chemiluminescence detection. In paper III a commercially available $NO^\bullet$ analyzer, that monitors the chemiluminescence of the reaction of $NO^\bullet$ with ozone (O_3), was used (Fig. 15) [400]. To measure nitrosoproteins, ascorbic acid was used in the reaction vessel to maintain a steady-state level of Cu^+ over Cu^{2+}, which is known to release $NO^\bullet$ from RSNO moieties [154,401]. The reaction of $NO^\bullet$ with O_3 generates nitric dioxide in an excited state (NO_2^*), which decays back to its ground state, thereby releasing light. Denitrosylation reactions were thereby monitored by measuring the remaining $NO^\bullet$ content of each sample after certain time intervals using the $NO^\bullet$ analyzer.

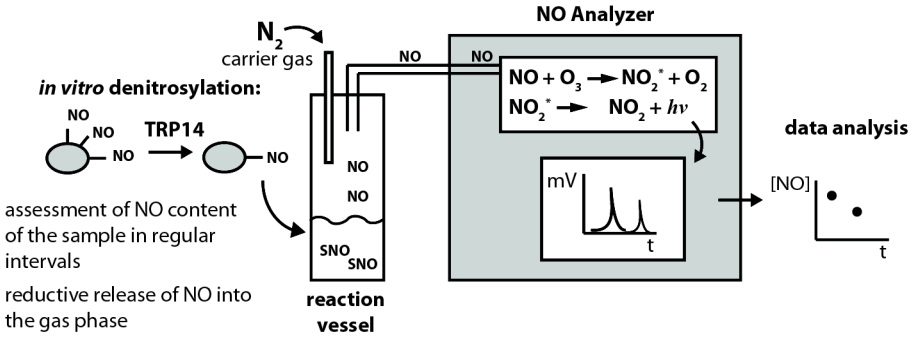

Figure 15. Assessment of TRP14 mediated denitrosylation activities using a NO• Analyzer. The denitrosylating activity of TRP14 or Trx1 is monitored by adding an aliquot of the reaction mixture to the NO• analyzer after certain time intervals. In the reaction vessel, NO• is released by Cu-mediated reduction into the gas phase. There it reacts with ozone (O_3) to form nitric dioxide in an excited state (NO_2^*). The latter emits light when decaying to its ground state, which in turn is recorded by a detector and plotted against the time (t) course over which the denitrosylation was followed. The area under each peak is proportional to the NO• released from each injected sample and can be used to determine the rate of denitrosylation. Adapted from [400].

Nitrosoproteins can also be measured with the 2,3-diaminonapthalene (DAN) assay. Here, NO• is released from SNO bonds by $HgCl_2$, which subsequently reacts with DAN to form a fluorescent product [399,402].

Gene silencing by RNA interference (Paper III and IV)

In paper III and IV we used RNA interference (RNAi) to induce gene silencing of Trx1, TrxR1 and TRP14 – a technique that was awarded the Noble Prize in 2006 [403]. RNAi is based on a cellular post-transcriptional gene regulatory mechanism that uses endogenously produced, ~22 nucleotide containing single stranded RNAs to induce gene silencing via the RNA induced silencing complex (RISC) that targets specific mRNAs [404]. In contrast to small interfering (si) RNAs, which only work under transient transfection conditions, plasmid-based short hairpin RNAs (shRNAs) allow for a direct synthesis of siRNAs in the cell [405] and were thus used in papers III and IV. The short RNA sequence folds into a short hairpin and is processed by the RNAi machinery to be used by the RISC complex. In addition, a selection marker in the plasmid allows for an enrichment of transfected cells and thereby a persistent suppression of gene expression in a cell population. Using that approach, we generated a stable knockdown of TrxR1 and TRP14 in HEK293 cells in paper IV.

3 SUMMARY AND DISCUSSION

3.1 PAPER I

Noble metal targeting of thioredoxin reductase – covalent complexes with thioredoxin and thioredoxin-related protein of 14 kDa triggered by cisplatin.

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Free Radicals in Biology and Medicine 49:1765-1778; 2010
*Equal contribution.

Background

In this paper, we investigated the effects of noble metal compounds on the activity of TrxR1. The platinum (Pt) containing drug cisplatin and the gold (Au) containing drug auranofin are noble metal compounds that are in clinical use today; cisplatin against several types of cancer and auranofin against rheumatoid arthritis. Earlier studies suggest that the therapeutic effects of these compounds are partly due to inhibition of TrxR1 [280,406]. Here we used chloride salts of palladium (Pd), Pt and Au (Fig. 16) together with auranofin and cisplatin to investigate differences in the inherent properties of these metals on the inhibition of TrxR1.

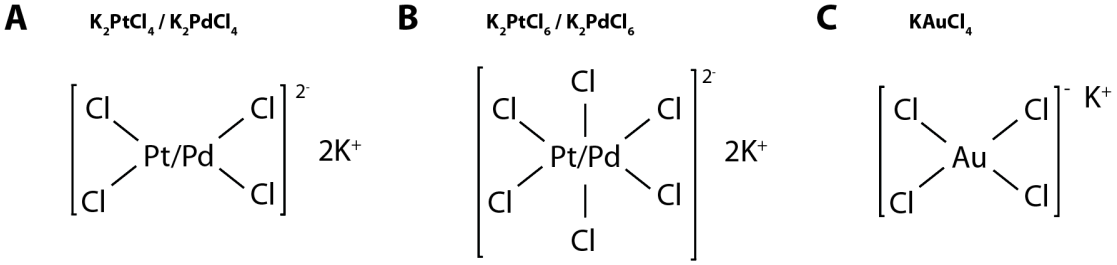

Figure 16. Coordination complexes of the noble metal salts used in paper I. A) K_2PtCl_4, potassium tetrachloroplatinate (II) and K_2PdCl_4, potassium tetrachloropalladate (II). B) K_2PtCl_6, potassium hexachloroplatinate (IV) and K_2PdCl_6, potassium hexachloropalladate (VI). C) $KAuCl_4$, potassium gold (III) chloride.

Main findings

- *TrxR1 inhibition by Pt, Pd and Au depends on several parameters*
  Using recombinant protein or cell lysates, we found that Pd and Au salts were more efficient inhibitors of TrxR1 than Pt salts. In contrast, cellular TrxR1 activity was clearly inhibited by Pt and Au compounds (predominantly cisplatin and auranofin), whereas the Pd compounds were less potent (Tab. 4). In addition, the ligand substituents of the metal compounds influenced the extent of inhibition.
- *Mechanism and SecTRAP formation*
  The inhibition of TrxR1 by the noble metal compounds was irreversible. The compounds most likely modified the Sec-residue of the enzyme (based on the findings that i) prior reduction of TrxR1 by NADPH was necessary and ii) that the

inhibited enzyme had a sustained ability to redox cycle with juglone, which is an activity of the enzyme known to be independent of an intact Sec).

- *Cisplatin induced complex formation*
  With platinum compounds (cisplatin and to some extent K_2PtCl_4) intracellular covalent complexes between TrxR1 and either Trx1 or TRP14 were formed. This complex formation was cell-line, time- and cisplatin-concentration dependent.

Table 4. Inhibition profile of TrxR1 using noble metal compounds.

recombinant protein/ cell lysate		noble metal compound	cellular activity (A549 cells)	
k_{inact}	+++	auranofin	++++	influenced by cellular uptake and metabolism
	+++	$KAuCl_4$	++	
	++	K_2PdCl_4	+	
	+	cisplatin	+++	
	+	K_2PtCl_4	++	

+ = relative degree of inhibition of TrxR1
k_{intact} = second order rate inactivation constant

Discussion

Targeting the Trx system as anti-cancer strategy is still challenging and the underlying mechanisms are far from being fully understood. This paper further supports TrxR1 as a potential anti-cancer target and strengthens the concept of SecTRAP formation as part of the cytotoxic mechanism of targeting TrxR1.

Severe side effects of cisplatin and the observed high rates of cancers developing resistance to the drug propels the need for further development of transition metal based compounds [407-409]. Based on our findings, new Pd-based anti-cancer drugs may be designed, but other parameters that contribute to the pharmacological profile of noble metal compounds clearly need to be considered. The inhibitory profile of Pd on TrxR1 is very encouraging, but our results suggest that a more efficient uptake of the Pd compound is required. Pd-based complexes are closely related to their Pt analogues due to a similar structure and coordination chemistry of the two metals. However, the high hydrolysis rates of Pd complexes compared to the Pt variants are still problematic and current research focuses on finding ligands that slow down the dissociation rates to make them therapeutically more applicable [410]. Nonetheless, Pd-based complexes are emerging as anti-tumor agents, and the engineering of suitable ligands is an active field of research. In line with our results, a recent study demonstrated *in vitro* inhibition of TrxR1 with Pd and Pt complexes, but could similarly not correlate it to the cytotoxicity profile, which again emphasizes the importance of suitable ligands [411]. It will be very interesting to assess emerging Pd-based anti-cancer complexes for their inhibitory profile on TrxR1 in cells [412]. Intriguing new approaches also try to improve

drug delivery and selectivity by encapsulating metal-based drugs in macrocycles or by attaching them to nanoparticles [413].

In the context of cellular TrxR1 inhibition, it is essential to elucidate the exact mechanism, e.g. if the complete TrxR1 activity is lost or if the N-terminal active site is still intact, thereby potentially forming a cytotoxic SecTRAP. Our results are compatible with the SecTRAP hypothesis, as our data suggest a preferential targeting of the Sec residue in TrxR1. We did not prove a direct derivatization of the Sec residue via mass spectrometry (MS), but our results indirectly show that a targeting of the Sec is likely to be the case. Our data also agree with earlier studies that showed the susceptibility of the reduced Sec residue towards electrophilic compounds [278] and a direct binding of a Pt(II) complex to the selenolate of TrxR1 [414].

As we have already discussed in section 1.5.3, the SecTRAP formation could account for parts of the cytotoxic effect and cellular ROS production seen with several electrophilic compounds that target the Sec residue in TrxR1. However, the cellular formation and mechanisms of SecTRAPs are not very well understood and it is unclear if all TrxR1 inhibitors promote that effect. Despite the loss of the C-terminal activity of TrxR1, the enzyme can still redox cycle via the N-terminal active site with certain compounds, e.g. DNCB [415], curcumin [269] and juglone [267], which in turn can lead to $O_2^{\bullet-}$ production. Also, a direct NADPH oxidase activity of TrxR1 in absence of any other substrate is possible [416]. Currently, the scenario of cellular superoxide production by derivatized TrxR1 is conceivable, but not exclusively proven. Nonetheless, SecTRAPs may explain the attenuation of cisplatin toxicity in cells expressing a Sec-to-Cys variant of TrxR1 [417] and why high levels of TrxR1 in A549 cells are critical to the effectiveness of cisplatin [336]. For all compounds, however, any cellular effects beside TrxR1 inhibition should not be excluded.

The concept of targeting TrxR1 as anti-cancer strategy is further strengthened by this study, because for most of the compounds the inhibition of TrxR1 correlated well with a reduction in cell viability. Based on this, our laboratory recently developed a high-throughput screening assay to discover novel small molecule inhibitors for TrxR1 [418]. Based on that assay, a large quantitative screen was performed with >300 000 compounds. The lead compound irreversibly inhibits TrxR1 in cellular systems in agreement with the SecTRAP concept, and also shows anti-cancer efficacy in different mouse models (Stafford et al., *manuscript in preparation*).

We here also discovered the intracellular formation of covalently linked TrxR1-Trx1 and TrxR1-TRP14 complexes upon treatment with cisplatin and K_2PtCl_4. How the proteins are cross-linked is currently unknown and until today, we were unable to resolve the exact nature of the complexes via MS. As argued in this paper, all active sites, with the exception of the C-terminal active site of TrxR1, could be detected via MS, which made it unlikely that the complex formation occurred via the active sites. However, the molecular mechanism of cisplatin in the cell involves hydrolysis to a potent electrophile that can react with nucleophiles including thiol/selenol-groups on proteins and nitrogen atoms on nucleic acids

[407,419]. It therefore seems likely that the complex formation of TrxR1 with Trx1 and TRP14 respectively occurs via the same mechanism as in DNA crosslinking and therefore presumably via the active sites of the enzymes. This is strengthened by a recent study showing a crosslink of TrxR1 and Trx1 via their active sites by nitrogen mustard in A549 cells [420]. However, all mechanisms are purely speculative at the moment and further research is needed to understand the exact nature of the complex.

If and how the complexes contribute to the cytotoxic mechanism of cisplatin is currently unknown. But the following differences between the Trx1 versus the TRP14 complex formation should be noted: i) only a minor fraction of Trx1 compared to basal levels of the enzyme seemed to be trapped in the complex as shown with Western Blot, ii) cisplatin treatment gave no reduction in overall Trx1 activity and iii) much higher amounts of TRP14 seemed to be in the complex compared with basal levels of TRP14. The biological relevance of the complex formation is unknown, but it could be possible that disruption of TRP14 mediated pathways could be part of the cytotoxic mechanism of cisplatin. If and how this correlates with the concept of SecTRAP formation and if the complex itself works as a SecTRAP should clearly be a focus of further studies. Despite the finding that TrxR1 and TRP14 associate and seem to interact closely within the cellular context, not much is known about this protein. A further characterization thus formed the basis for the other papers presented within this thesis.

This paper does not exclusively provide any evidence that the complex forms under physiological conditions. It should certainly be noted, however, that we have recently detected the TrxR1-TRP14 complex in liver and kidney of mice that had been treated with cisplatin (*unpublished*). This implies a potential therapeutic effect of the cisplatin mediated complex *in vivo* and should also be further investigated.

3.2 PAPER II

Selective activation of oxidized PTP1B by the thioredoxin system modulates PDGF-beta receptor tyrosine kinase signaling.

Markus Dagnell, Jeroen Frijhoff, **Irina Pader**, Martin Augsten, Benoit Boivin, Jianqiang Xu, Pankaj Mandal, Nicholas K. Tonks, Carina Hellberg, Marcus Conrad, Elias S. J. Arnér, Arne Östman.
Proceedings of the National Academy of Sciences USA; 110:(33), 13398-13403; 2013

Background

In this paper we investigated the reactivation of two different oxidized PTPs, namely PTP1B and SHP2, by the Trx system. The stimulation of the platelet-derived growth factor β receptor (PDGFβR) typically mediates cell proliferation and migration and results in an oxidation of these two PTPs. How they are reactivated, and thereby terminate growth factor signaling is still largely unknown.

Main findings

- *The Trx system can reduce PTP1B but not SHP2*
  Using MEFs that lack TrxR1 (*Txnrd1*$^{-/-}$) we found that PTP1B oxidation was increased and its activity decreased, whereas SHP2 was unaffected. We did not observe any changes in overall ROS levels (as determined by CellROX fluorescence), suggesting that the increase in PTP1B oxidation was not due to oxidative stress, but rather that TrxR1 is required to reduce PTP1B. Using recombinant PTP1B as well as immunoprecipitated PTP1B and SHP2 from H_2O_2-treated NIH3T3 cells, we could confirm that oxidized PTP1B but not SHP2, is indeed efficiently reduced by the Trx system. Additionally, using a substrate trapping mutant of Trx1 (C35S) we found that the active site Cys32 of Trx1 was forming an intermolecular disulfide intermediate with PTP1B.
- *The Trx system regulates PDGFβR signaling via PTP1B*
  Compared to wildtype MEFs, *Txnrd1*$^{-/-}$ MEFs showed enhanced PDGFβR phosphorylation at Tyr579/581 upon PDGF stimulation. This phosphorylation site is known to be regulated by PTP1B. In contrast, we found no changes in the SHP2-regulated PDGFβR site pY771, which is consistent with SHP2 not being a substrate of the Trx system. Phosphorylation at Tyr579/581 is known to promote a growth-stimulatory response and *Txnrd1*$^{-/-}$ MEFs consequently showed an increase in proliferation. We furthermore used MEFs deficient in PTP1B (*Ptpn1*$^{-/-}$) that are also characterized by an enhanced phosphorylation of the PDGFβR upon stimulation to validate that TrxR1 affects PDGFβR phosphorylation via PTP1B. As expected, treatment with the TrxR1 inhibitor auranofin did not increase the phosphorylation of PDGFβR in *Ptpn1*$^{-/-}$ cells, only in MEFs that had been reconstituted with PTP1B (*hPtpn1*).

- *The Trx system can reactivate PTP1B also via TRP14*
  Using recombinant PTP1B, we found that TRP14 can reduce oxidized PTP1B, but not SHP2, in a dose dependent manner. This suggests that also TRP14 can contribute to the observed differences in PTP1B oxidation and PDGFβR phosphorylation in *Txnrd1*$^{-/-}$ MEFs.

Discussion

Cellular signaling via the PDGF receptor has been extensively studied, but detailed knowledge of the redox regulation of this pathway still remains elusive [421]. Some potential mechanisms of redox regulation within PDGF signaling pathways start to unravel and are, for a better understanding, summarized in Figure 17. Several reports have established that stimulation of the PDGF receptor results in increased H_2O_2 levels and a concomitant oxidation of PTPs [18,24,422]. This is most likely mediated via the activation of NOX enzymes by the phosphatidylinositol-3 kinase (PI3K) pathway and the GTPase Rac [351,423-425]. The NOX enzymes produce $O_2^{\bullet-}$ extracellularly, which rapidly dismutates to H_2O_2 and is, in turn, imported via aquaporins [57]. Receptor stimulation has furthermore been linked to PrxI inactivation through phosphorylation via the Src kinase, which presumably leads to a spatially restricted increase of H_2O_2 [28]. The current idea is that phosphatases such as PTP1B and SHP2 are inactivated by this local "hotspot" of H_2O_2, or possibly also by lipid peroxides [355,426] and thereby allow for sustained receptor activation and downstream signaling. How oxidized PTPs are reduced and thereby PDGF signaling terminated is not fully understood. This study shows that oxidized PTP1B can be reactivated by the Trx system and thereby provides evidence of a crosstalk between the Trx system and PDGF signaling, which is in line with earlier reports [24,365]. Similar to the study by *Schwertassek et al.*, we also detected a Trx1-PTP1B conjugate using a Trx1-C35S trapping approach. Furthermore, our *Txnrd*$^{-/-}$ MEFs clearly illustrate that a disruption of the Trx system increases the cellular PTP1B oxidation state and thereby PDGF signaling.

This paper further illustrates that the TrxR1-dependent regulation of PTP1B is possibly not only mediated via Trx1, but also via TRP14. Using *in vitro* PTP activity assays, we found TRP14 to be less efficient in reactivating PTP1B compared to Trx1, but this nonetheless is the first phosphatase to be reported as a substrate for TRP14. Earlier reports could not measure any activity of TRP14 with the phosphatases PTEN [377] and PRL [209,378]. Additionally, both Trx1 and TRP14 were unable to reactivate SHP2, which is interesting regarding the fact that the GSH system is reported to be more efficient in reducing SHP1 and SHP2 compared to PTP1B [24,427]. This suggests selectivity of the Trx and GSH systems in the reactivation of certain PTPs.

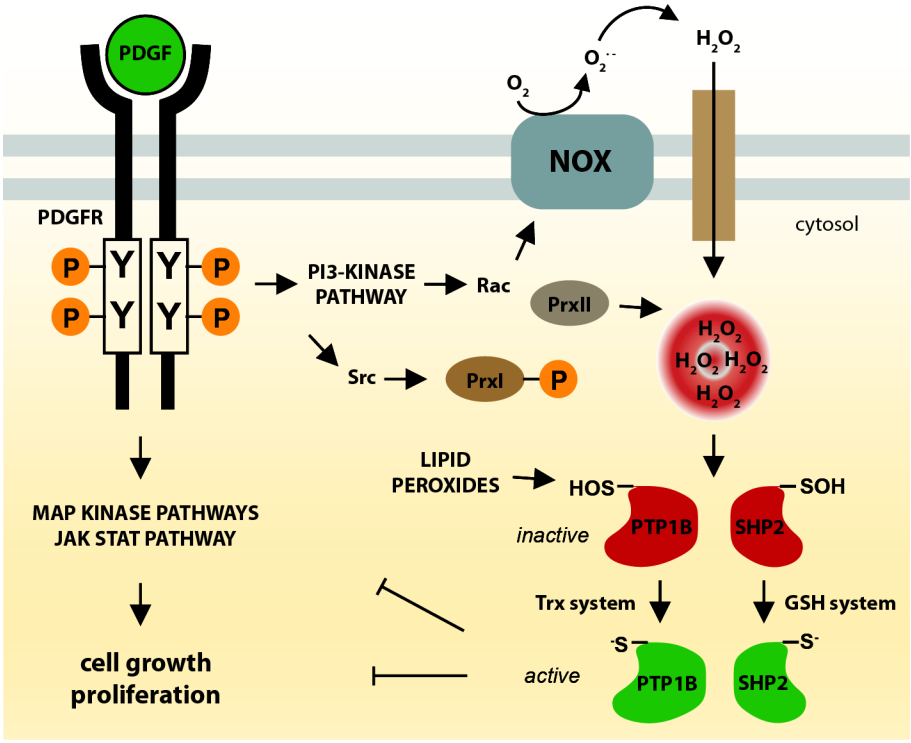

Figure 17. Simplified model of PDGF signaling and its redox regulation. Activated platelets and many other cell types including endothelial, epithelial and inflammatory cells secrete PDGFs, which upon binding to PDGFRs results in a stimulation of cell growth and proliferation. PDGFRs activate MAP kinase cascades and further non-receptor tyrosine kinases, including JAK kinases (JAK-STAT pathway) and Src-family kinases. The activation of phosphatidylinositol-3 kinase (PI3K) leads to the production of the second messenger phosphatidylinositol-3,4,5-triphosphate (PIP_3), which can activate further pathways, including AKT (also known as protein kinase B, PKB). The PI3K pathway leads to an activation of NOX enzymes via the small GTPase Rac, resulting in the production of $O_2^{\bullet-}$, which undergoes dismutation to H_2O_2. Activated NOX complexes seem to be confined to subcellular compartments, e.g. lipid rafts, thereby generating H_2O_2 within restricted regions of the cell. The activated receptor also induces phosphorylation of cell membrane associated PrxI via Src-family kinases, resulting in a localized inactivation of PrxI that otherwise would reduce H_2O_2. The role of PrxII is still unclear, it has not been shown to be phosphorylated or hyperoxidized. The localized H_2O_2 "hotspot" results in an inactivation of the PTPs PTP1B and SHP2. The reactivation of oxidized PTP1B and SHP2 can be mediated via the Trx and GSH systems.

An interesting question to ask is what determines the specificity of the Trx system for PTP1B over SHP2? Several factors should be considered, e.g. the molecular and structural basis for interaction, which has not been determined yet. Interesting to note, the type of oxidative modification might be crucial, especially since PTP1B has been reported to be oxidized (to sulfenic acid as well as to sulfenylamide) but also nitrosylated, glutathionylated and sulfhydrated [179,317,428,429]. The biological context where these modifications occur and how they are reversed is still incompletely understood. An aspect that we have not looked into yet is the specific cellular localization of redox signaling events. Especially PTP1B is known for being spatially confined within the cell [361], and how this relates to its access by members of the Trx system should also be part of future studies.

Future research into redox regulation of PTPs should certainly also include global analyses of the oxidation state of PTPs (the "PTPome") [430]. As we are continuously expanding our genetic "toolbox" for mouse models of members of the Trx system, future studies have the potential to investigate which subsets of PTPs are regulated by e.g. Trx1 and TRP14.

3.3 PAPER III

Thioredoxin-related protein of 14 kDa is an efficient L-cystine reductase and S-denitrosylase.

Irina Pader*, Rajib Sengupta*, Marcus Cebula, Jianqiang Xu, Jon O. Lundberg, Arne Holmgren, Katarina Johansson, Elias S. J. Arnér.
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*Equal contribution.

Background

The discovery of the cisplatin induced covalent TrxR1-TRP14 complex in Paper I and the lack of information about TRP14 at that time encouraged us to further characterize this enzyme. We expressed it as a recombinant protein and analyzed its activities in reducing disulfides and nitrosothiols in comparison to Trx1.

Main findings

- *TRP14 is expressed in different human cell lines*
  We have determined the expression levels of TRP14 and Trx1 in five different human cell lines, which revealed that different cells express different amounts of the two proteins. TRP14 is expressed in all cell types, but generally in lower amounts compared to Trx1.
- *TRP14 is an efficient TrxR1-dependent reductase*
  We established activity assays for TRP14 and found that both TRP14 and Trx1 can reduce cystine, HED and H_2O_2. Importantly, TRP14 seems to be a qualitatively more dedicated cystine reductase in cells because i) it is approx. fivefold more efficient than Trx1 and ii) in contrast to Trx1, its activity with cystine was not affected by the presence of another protein disulfide substrate of Trx1, such as insulin. In contrast to Trx1, TRP14 is not inhibited with higher concentrations of H_2O_2. Furthermore, both TRP14 and Trx1 required TrxR1 as an electron donor and could not be reduced by GSH coupled to GR.
- *TRP14 is a TrxR1-dependent denitrosylase*
  We found that both TRP14 and Trx1 can reduce nitrosothiols including GSNO, nitroso-caspase 3 (caspase 3-SNO) and nitroso-cathepsin B (cathepsin-SNO). In addition, both proteins could reduce a mixture of nitrosylated proteins from HEK293 cell lysates.

Discussion

It was previously thought that Trx1 alone is the most significant substrate of TrxR1. However, we have found that TrxR1 can reduce TRP14 as efficiently as Trx1, making it an integral component of the Trx system.

Our data showing that TRP14 is expressed in a variety of cell lines, agrees well with the first study by *Jeong et al*. identifying TRP14 as a ubiquitous cellular oxidoreductase with a tissue specific expression pattern [257]. The subcellular localization of TRP14 has not been exclusively determined yet. It seems to be a predominantly cytosolic protein (which agrees with our hypothesis that it is acting in the cytosol in parallel with Trx1), but it also might exert its function in other cellular compartments. An interesting possibility is for example nuclear translocation to regulate transcription factor binding as shown for Trx1.

Earlier reports have suggested a crosstalk between the Trx and the GSH systems, e.g. that TrxR1 can reduce Grx2 [259] and that Grx1 coupled to GR and GSH acts as a backup reducing system for Trx1 [241,431]. Based on this we tested if GSH may be able to donate electrons to TRP14 or Trx1, but found this not to be the case. However, if Grxs can instead catalyze the electron transfer to TRP14 is currently unknown and should be followed up in future studies.

To this day the exact mechanisms of the intracellular reduction of cystine are still debated. Earlier studies have suggested that once cystine is imported via system X_C^-, it is rapidly reduced by either the GSH or the Trx system [124,125]. Our hypothesis is that due to the exceptional high *in vitro* activity, TRP14 instead of Trx1 likely contributes to the cytosolic cystine reduction. This was further strengthened by our observation that Trx1 does not reduce cystine once a preferred substrate like insulin is present. We concluded that Trx1 has a variety of intracellular substrates; whereas TRP14 is much more specific and can reduce cystine while Trx1 is "occupied" with its basal cellular functions (e.g. RNR). One should, however, not rule out the possibility that cystine reduction could also be mediated via GSH-dependent enzymes and further studies are needed to establish the primary cystine reduction mechanism in cells. Another aspect of cystine reduction that we have not looked into yet is the rather new field of cysteinylation as a posttranslational modification. A few studies have demonstrated cysteinylation as a protective mechanism to shield thiols from irreversible oxidation [432-435] and it will be very interesting to investigate if TRP14 could function as a regulator of protein cysteinylation.

Interestingly, the reduction of H_2O_2 by TRP14 is, in contrast to Trx1, maintained at high concentrations. As we have already discussed in the paper, the used concentrations of H_2O_2 most likely do not represent physiological concentrations, but suggest that TRP14 could still be functional under conditions of oxidative stress. Also the results we obtained with SDS-PAGE, should evidently be followed up in further studies. As determined earlier with DTNB [257] and judged from the structure (section 1.6), the additional Cys of TRP14 do not seem to be surface exposed. However, TRP14 clearly forms oligomers via amino acid residues outside the active site, which suggests different structural dynamics and regulation

compared to Trx1. We were not able to express a TRP14 variant where the additional Cys residues were mutated to alanines (*unpublished*), which suggested that they are at least partially required for the structural integrity of the protein. Future experiments are clearly needed to assess the accessibility of the additional Cys residues and to elucidate if potentially other surface exposed residues could be involved in the regulation of TRP14.

In this paper we furthermore provide evidence that TRP14 is another TrxR1-dependent denitrosylase. Other members of the Trx family, including Trx1 and PDI, have earlier been reported to act as denitrosylases [153,155,156]. The study by *Benhar et al*., assessed TRP14-depleted lysates of HeLa cells for denitrosylating activity on caspase 3-SNO, which compared to Trx1-depletion, was unaffected [153]. Considering our results from protease activity assays and the NO• analyzer we still hypothesize that TrxR1 can mediate denitosylase activity via both Trx1 and TRP14. In addition, the knockdown and overexpression of TRP14 in our experiments clearly had an effect on the levels of cellular nitrosoproteins in HEK293 cells. We therefore suggest that the contributions of Trx1- vs. TRP14- mediated denitrosylation *in vivo* certainly depends on the cell type and the specific nitrosylated protein substrate, which should evidently be the focus of further studies. Specific nitrosylated protein substrates of TRP14 are currently unknown, but we have started a mass spectrometry based substrate trapping approach using a TRP14 active site "trapping mutant" as previously described for Trx1 [169]. This approach will certainly contribute to a better understanding of the role of TRP14-mediated denitrosylation in a cellular context.

This study should emphasize that TRP14 is another, yet sparsely characterized, member of the Trx system with redox activities that are complementing those of Trx1.

3.4 PAPER IV

Novel persulfide detection method reveals protein persulfide and polysulfide reducing functions of thioredoxin- and glutathione systems.

Éva Dóka, **Irina Pader**, Adrienn Bíró, Katarina Johansson, Qing Cheng, Krisztina Ballagó, Justin R. Prigge, Daniel Pastor-Flores, Tobias P. Dick, Edward E. Schmidt, Elias S. J. Arnér, Péter Nagy.
Science Advances 2:e1500968, 2016

Background

To overcome challenges and uncertainties of previous detection methods, we here developed and validated a novel method to detect persulfides of recombinant proteins and in biological samples (Protein Persulfide Detection Protocol, ProPerDP). In addition, reduction pathways for poly- and persulfides are still largely unknown. We therefore used the ProPerDP method to investigate the role of the Trx and GSH systems on these sulfur modifications.

Main findings

- *Development and validation of ProPerDP*
  ProPerDP is based on three steps: i) the alkylation of thiol and persulfide groups with Iodoacetyl-PEG_2-Biotin, ii) subsequent pull down by Streptavidin and iii) reduction with DTT. The last step will only cleave, and thereby detach from the Streptavidin, the alkylation products originating from protein persulfides in the sample (R-SS-alkylating agent) and not from thiols (R-S-alkylation agent) (for a detailed scheme of the method see Fig. 1 in paper IV). Using human albumin as a model system to validate this method we could detect persulfidation on recombinant protein as well as in polysulfide treated human plasma. We furthermore detected protein persulfides in HS_x^- treated A549 cells and identified several proteins known toe be susceptible to persulfidation using mass spectrometry. In addition, CBS and CSE deleted yeast strains showed lower levels of protein persulfides, thereby further validating ProPerDP.
- *Roles of the Trx and GSH systems in the reduction of poly- and persulfides*
  We first tested the reduction of polysulfides and albumin persulfide *in vitro*. Both could directly be reduced by TrxR1 and required an intact Sec residue in the active site. The activity was further enhanced by adding Trx1 or TRP14 to the reaction mixture. Additionally, polysulfides and albumin persulfide could be reduced by GSH coupled to GR, which was further enhanced by adding Grx1. While assessing the reduction of persulfides in a cellular context we found elevated levels of protein persulfides upon stable knockdown of TrxR1 and TRP14 in HEK293 cells. To further establish *in vivo* relevance and suitability of the assay for tissue samples, we assessed protein persulfides in mouse liver and found higher levels of protein persulfides in livers that lack GR (“GR null”) as well as both TrxR1 and GR (“TR/GR null”).

Discussion

Until today, the development of detection methods for persulfides remains challenging due to the similarity of the persulfide (-S-SH) and thiol (-SH) moiety. We therefore developed a method that is more sensitive and can moreover be used to study persulfidation in intact cells, which is not possible with previously described methods (Fig. 18):

- **Modified Biotin Switch Assay (Fig. 18A)**
  The Modified Biotin Switch Assay was the first method available to detect protein persulfides and is used in its original form to detect protein S-nitrosylation [141,436]. The essential steps in the original Biotin Switch Assay are i) blocking of thiols with methylmethanthiosulfonate (MMTS), ii) conversion of nitrosothiols to thiols via transnitrosylation with ascorbate and iii) S-biotinylation of the previously nitrosylated thiols with Biotin-HPDP. In contrast, the Modified Biotin Switch Assay only requires steps i) and iii) [91]. Persulfides are believed to not react with MMTS, thus they are available for conjugation with biotin-HPDP in step iii). However, the essential assumption that MMTS does not react with persulfides has been questioned due to the higher nucleophilicity of -SSH moieties compared to -SH moieties [437].
- **Cysteinyl-labeling assay (Fig. 18B)**
  Another recently developed method bases on the previously established PTP cysteinyl-labeling assay [179,438]. Here, in step i) both -SSH and -SH moieties are blocked using alkylating agents such as iodoacetic acid (IAA) or iodoacetamide (IAM). Subsequently, in step ii) the persulfide adducts are reduced by DTT to form free SH groups, which in step iii) are labeled with IAM-linked Biotin. The disadvantage with this protocol is that the DTT reducing step is not selective for the persulfide adducts. Protein disulfides will also be reduced by DTT and labeled in step iii).
- **Tag-Switch Assay (Fig. 18C)**
  In the first step i), -SH and -SSH moieties are labeled with methylsulfonyl benzothiazole (MSBT). Subsequenltly ii) biotinylated cyanoacetate (CN-Biotin) is added and thought to only react with -SS-MSBT thereby "switching" the persulfide tag to -SS-CN-Biotin [175]. However, a background reaction with protein disulfides is possible and has not been experimentally excluded yet.
- **Maleimide Assay (Fig. 18D)**
  The maleimide assay is based on the modification of free thiols and persulfides with a fluorescently labeled maleimide compound [178]. A subsequent treatment with DTT cleaves disulfide bonds and thereby detaches the fluorescent label from persulfides, but not unpersulfidated proteins, which can be visualized by in-gel fluorescence. The advantage is that it is relatively specific, but does not allow for a direct persulfide labeling for e.g. proteomic analysis.

ProPerDP (Fig. 18E) has the advantage that it can be used on intact cells to study sulfide signaling in a more physiological relevant setting. Our validation experiments have furthermore demonstrated its specificity and reliability. However, a drawback with this

method is that proteins with extra thiol groups that are IAB labeled may not be detected. While other methods can easily give false-positive information, ProPerDP thus runs the risk of losing persulfidated proteins in the DTT reduction step. The suggested mass-spectrometry based method improvement is currently under development.

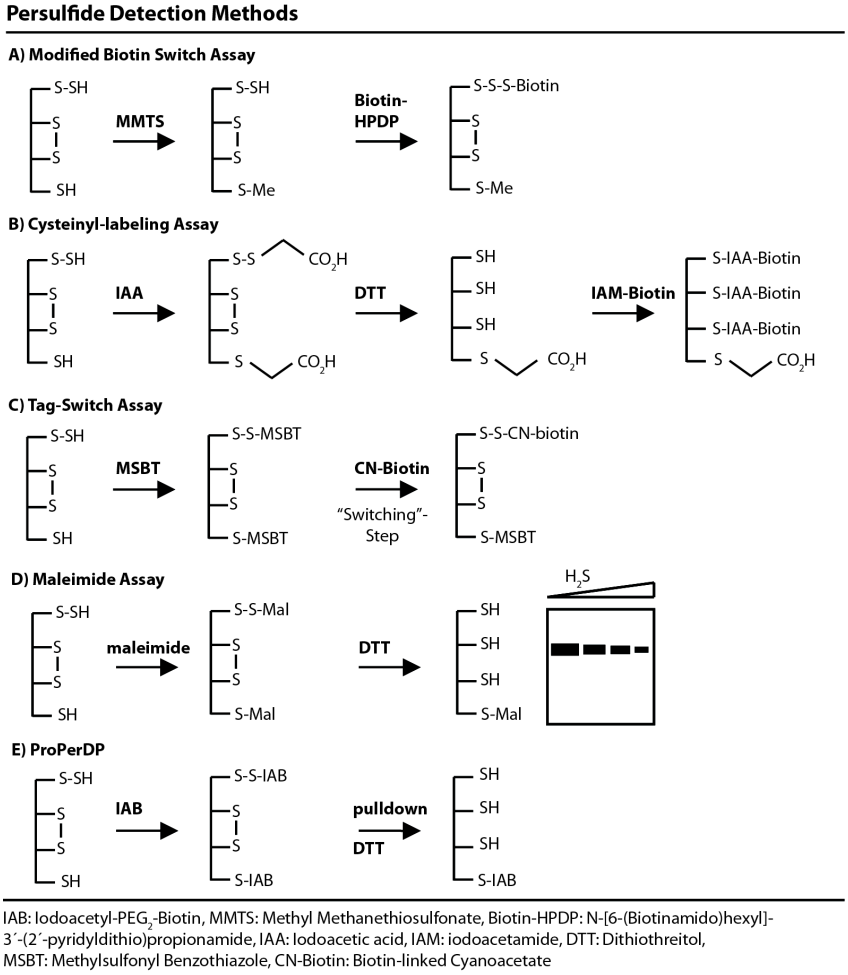

Figure 18. Principles of the currently available methods to detect protein persulfides. For details, see main text.

Using this method, several questions can be addressed in the future. Which proteins are regulated *in vivo* via persulfide modifications? Which of them are reduced by the GSH and the Trx system, respectively? And within the Trx system, what are the relative contributions of Trx1 versus TRP14? Both NADPH-dependent reductase systems are able to reduce poly- and persulfides *in vitro*, which is further supported by our experiments with the stable knockdown HEK293 cells and the TR/GR null mouse livers. However, a direct comparison of the relative efficiencies of both systems *in vitro* is difficult and future genetic models are needed to elucidate specific physiological persulfide signaling mechanisms. In analogy to S-nitrosylation, a persulfide based trapping approach using Trx1 and TRP14 proteins as "baits" is conceivable and could shed light on the regulation of specific signaling pathways.

Future challenges also include the crosstalk of sulfide and $NO^{\bullet}$ signaling. This relatively new area of research is not without controversies, but in consideration of the reducing activities of Trx1 and TRP14 for both nitrosothiols and protein persulfides, future experiments are clearly required to understand mechanistic principles and the underlying physiological relevance.

4 CONCLUSIONS AND FUTURE PERSPECTIVES

Within this thesis we have investigated the redox protein TRP14 in different contexts. We have focused on a biochemical characterization and obtained indications for its functions in cancer chemotherapy, growth factor signaling as well as nitric oxide and sulfide signaling respectively. All herein presented results cumulatively suggest that TRP14 is a substantial, and likely underestimated, member of the Trx system. It is important to emphasize that TRP14 is a substrate for TrxR1, which until its initial characterization in 2004 was unknown. Our results here clearly show that TrxR1 powered reactions do not necessarily have to be mediated through Trx1, but depending on the final substrate can also occur via TRP14. In theory, it is possible that many functions that had so far been attributed only to Trx1, could also be mediated via TRP14.

A major challenge for future experiments will be to identify specific substrates for TRP14 and to elucidate its role in relation to other members of the Trx system. During the progress of this thesis we have not followed up on earlier published substrates, e.g. cofilin, LC8 and L30 [257]. Among them, cofilin seems to be a promising candidate to investigate with regards to TRP14. Cofilin is an essential component of actin dynamics in cell migration and its oxidation has been related to the activity of NOXs and the induction of apoptosis [439-441]. Further work will certainly shed more light on the potential redox regulation of the cell cytoskeleton by TRP14.

Redox processes can regulate cell survival at several different levels, including signal transduction cascades, transcription factors and cell death pathways [442]. How is TRP14 involved in these redox-signaling processes? At this stage we can only speculate because i) we lack definite *in vivo* evidence and ii) we have not identified any specific substrates for TRP14 (compared to Trx1) yet. It seems that TRP14 has structurally evolved to reduce a more restricted set of substrates compared to Trx1, but as of today, there are also many overlapping functions and further experiments are needed. Nevertheless, the results presented here indicate that TRP14 could be involved in all levels of redox regulation for cell survival. Growth factor induced signaling cascades, e.g. by EGF, PDGF and insulin, involve an inactivation of PTP1B, which we have here found to be reactivated by TRP14 (paper II). Earlier reports have established a modulatory role of TRP14 on the redox regulated transcription factor NFκB [379]. In addition, our results in paper III point towards a regulatory role in apoptosis signaling involving caspases and cathepsins. Already ongoing experiments will help for a more comprehensive understanding of the cellular functions of TRP14:

- In collaboration with Moran Benhar in Israel we have initiated the already mentioned "trapping" approach to screen for specific nitrosylated protein substrates of TRP14, which his laboratory has successfully applied with Trx1 [169].
- In collaboration with Ed Schmidt in the USA, we are currently developing novel mouse models for whole-animal and conditional tissue-specific deletion of *Txndc17*, the gene encoding TRP14. His laboratory has already developed several genetically

engineered mouse models with distinct aberrations of key redox enzymes (TrxR1, Trx1 and GR). Given that TRP14 has earlier been suggested to be involved in inflammatory signaling cascades and bone remodeling, the new mouse models will certainly provide some more insights into the physiological functions of TRP14.

- Besides NFκB, a variety of transcription factors have been recognized to be redox-regulated [3]. How the Trx system can modulate these transcription factors is largely unknown. We have recently developed a new tool, pTRAF (plasmid for transcription factor reporter activation based upon fluorescence), which can be used to determine the activities of three different transcription factors simultaneously within single cells [443]. We are currently using the pTRAF technology together with the stable TRP14 and TrxR1 knockdown HEK293 cells (paper IV) in order to investigate if and how the Trx system and especially TRP14, is involved in the regulation of any of the transcription factors NFκB, Nrf2 and HIF-1.

To summarize, the aim of this PhD project was to examine and characterize TRP14 in different aspects. Within this thesis we thus expanded the knowledge of TRP14 by providing evidence of its potential functions in redox regulated signaling pathways (Fig. 19).

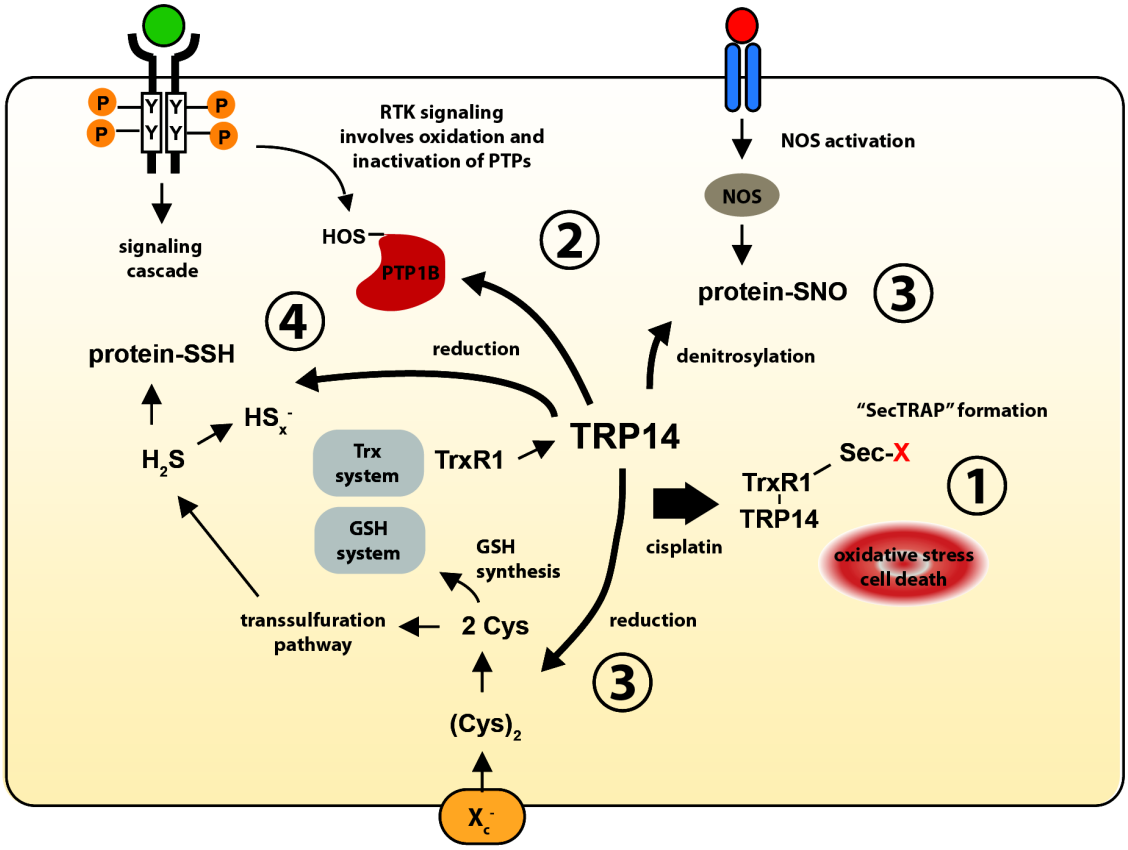

Figure 19. Schematic overview of the findings of paper I-IV in relation to TRP14. For simplicity, the functions of the Trx and the GSH system are omitted. Paper 1: Cisplatin induced complex between TrxR1 and TRP14 and potential SecTRAP formation. Paper 2: Reduction and thereby activation of oxidized PTP1B by TRP14. Paper 3: Reduction of cystine and nitrosothiols by TRP14. Paper IV: TRP14 mediated reduction of polysulfides (HS_x^-) and protein persulfides.

5 ACKNOWLEDGEMENTS

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Working with this project has been a great experience. The research presented within this thesis was generated in several team efforts and I am proud to have had the opportunity to work with so many brilliant scientists. I have learned a lot during the past five years and this book would not have been possible without the support, help and guidance of many people. In particular I would like to thank:

My supervisor **Elias Arnér**, for accepting me as a project student six years ago, agreeing to supervise my german "Diplom" and eventually registering me as a PhD student. You have been the best supervisor a PhD student could wish for! I admire your passion for science; you have taught me a lot about redox biology and science as a whole. I cannot thank you enough for always encouraging me to think beyond the lab bench and allowing me travel to so many places to "mingle"; both for my professional but also personal development.

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6 REFERENCES

1. Herrmann, J. M. & Dick, T. P. Redox Biology on the rise. *Biological chemistry* **393**, 999-1004 (2012).
2. Berg, J. M., Tymoczko, J.L., Stryer L. Biochemistry. *W H Freeman* **5th edition** (2002).
3. Brigelius-Flohe, R. & Flohe, L. Basic principles and emerging concepts in the redox control of transcription factors. *Antioxidants & redox signaling* **15**, 2335-2381 (2011).
4. Winterbourn, C. C. & Hampton, M. B. Thiol chemistry and specificity in redox signaling. *Free radical biology & medicine* **45**, 549-561 (2008).
5. Sies, H. Oxidative stress: a concept in redox biology and medicine. *Redox biology* **4C**, 180-183 (2015).
6. Holmstrom, K. M. & Finkel, T. Cellular mechanisms and physiological consequences of redox-dependent signalling. *Nature reviews. Molecular cell biology* **15**, 411-421 (2014).
7. Finkel, T. Signal transduction by reactive oxygen species. *The Journal of cell biology* **194**, 7-15 (2011).
8. Winterbourn, C. C. & Hampton, M. B. Redox biology: signaling via a peroxiredoxin sensor. *Nature chemical biology* **11**, 5-6 (2015).
9. Forman, H. J., Maiorino, M. & Ursini, F. Signaling functions of reactive oxygen species. *Biochemistry* **49**, 835-842 (2010).
10. Mitchell, L., Hobbs, G. A., Aghajanian, A. & Campbell, S. L. Redox regulation of Ras and Rho GTPases: mechanism and function. *Antioxidants & redox signaling* **18**, 250-258 (2013).
11. Leichert, L. I. & Dick, T. P. Incidence and physiological relevance of protein thiol switches. *Biological chemistry* **396**, 389-399 (2015).
12. Schafer, F. Q. & Buettner, G. R. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free radical biology & medicine* **30**, 1191-1212 (2001).
13. Kemp, M., Go, Y. M. & Jones, D. P. Nonequilibrium thermodynamics of thiol/disulfide redox systems: a perspective on redox systems biology. *Free radical biology & medicine* **44**, 921-937 (2008).
14. Flohe, L. The impact of thiol peroxidases on redox regulation. *Free radical research*, 1-17 (2015).
15. Berndt, C., Lillig, C. H. & Flohe, L. Redox regulation by glutathione needs enzymes. *Frontiers in pharmacology* **5**, 168 (2014).
16. Winterbourn, C. C. Reconciling the chemistry and biology of reactive oxygen species. *Nature chemical biology* **4**, 278-286 (2008).
17. Flohe, L. The fairytale of the GSSG/GSH redox potential. *Biochimica et biophysica acta* **1830**, 3139-3142 (2013).
18. Sundaresan, M., Yu, Z. X., Ferrans, V. J., Irani, K. & Finkel, T. Requirement for generation of H2O2 for platelet-derived growth factor signal transduction. *Science* **270**, 296-299 (1995).
19. Bae, Y. S. *et al.* Epidermal growth factor (EGF)-induced generation of hydrogen peroxide. Role in EGF receptor-mediated tyrosine phosphorylation. *The Journal of biological chemistry* **272**, 217-221 (1997).
20. Nakashima, I. *et al.* Redox control of catalytic activities of membrane-associated protein tyrosine kinases. *Archives of biochemistry and biophysics* **434**, 3-10 (2005).
21. Truong, T. H. & Carroll, K. S. Redox regulation of epidermal growth factor receptor signaling through cysteine oxidation. *Biochemistry* **51**, 9954-9965 (2012).
22. Meng, T. C., Buckley, D. A., Galic, S., Tiganis, T. & Tonks, N. K. Regulation of insulin signaling through reversible oxidation of the protein-tyrosine phosphatases TC45 and PTP1B. *The Journal of biological chemistry* **279**, 37716-37725 (2004).
23. Kwon, J. *et al.* Reversible oxidation and inactivation of the tumor suppressor PTEN in cells stimulated with peptide growth factors. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 16419-16424 (2004).

24. Lee, S. R., Kwon, K. S., Kim, S. R. & Rhee, S. G. Reversible inactivation of protein-tyrosine phosphatase 1B in A431 cells stimulated with epidermal growth factor. *The Journal of biological chemistry* **273**, 15366-15372 (1998).

25. Wood, Z. A., Poole, L. B. & Karplus, P. A. Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. *Science* **300**, 650-653 (2003).

26. Thamsen, M., Kumsta, C., Li, F. & Jakob, U. Is overoxidation of peroxiredoxin physiologically significant? *Antioxidants & redox signaling* **14**, 725-730 (2011).

27. Choi, M. H. *et al.* Regulation of PDGF signalling and vascular remodelling by peroxiredoxin II. *Nature* **435**, 347-353 (2005).

28. Woo, H. A. *et al.* Inactivation of peroxiredoxin I by phosphorylation allows localized H(2)O(2) accumulation for cell signaling. *Cell* **140**, 517-528 (2010).

29. Ushio-Fukai, M. Compartmentalization of redox signaling through NADPH oxidase-derived ROS. *Antioxidants & redox signaling* **11**, 1289-1299 (2009).

30. Chen, K., Kirber, M. T., Xiao, H., Yang, Y. & Keaney, J. F., Jr. Regulation of ROS signal transduction by NADPH oxidase 4 localization. *The Journal of cell biology* **181**, 1129-1139 (2008).

31. Dwivedi, G. & Kemp, M. L. Systemic redox regulation of cellular information processing. *Antioxidants & redox signaling* **16**, 374-380 (2012).

32. Tonks, N. K. Protein tyrosine phosphatases--from housekeeping enzymes to master regulators of signal transduction. *The FEBS journal* **280**, 346-378 (2013).

33. Oakley, F. D., Abbott, D., Li, Q. & Engelhardt, J. F. Signaling components of redox active endosomes: the redoxosomes. *Antioxidants & redox signaling* **11**, 1313-1333 (2009).

34. Sobotta, M. C. *et al.* Peroxiredoxin-2 and STAT3 form a redox relay for H2O2 signaling. *Nature chemical biology* **11**, 64-70 (2015).

35. Jarvis, R. M., Hughes, S. M. & Ledgerwood, E. C. Peroxiredoxin 1 functions as a signal peroxidase to receive, transduce, and transmit peroxide signals in mammalian cells. *Free radical biology & medicine* **53**, 1522-1530 (2012).

36. Young, I. S. & Woodside, J. V. Antioxidants in health and disease. *Journal of clinical pathology* **54**, 176-186 (2001).

37. Jones, D. P. Redefining oxidative stress. *Antioxidants & redox signaling* **8**, 1865-1879 (2006).

38. Forman, H. J. *et al.* Even free radicals should follow some rules: a guide to free radical research terminology and methodology. *Free radical biology & medicine* **78**, 233-235 (2015).

39. Trujillo, M., Alvarez, B. & Radi, R. One- and two-electron oxidation of thiols: mechanisms, kinetics and biological fates. *Free radical research*, 1-59 (2015).

40. Halliwell, B., Gutteridge, J. . Free Radicals in Biology and Medicine. *Oxford University Press: New York* (2007).

41. Winterbourn, C. C. Are Free Radicals Involved in Thiol-Based Redox Signaling? *Free radical biology & medicine* (2014).

42. Hanschmann, E. M., Godoy, J. R., Berndt, C., Hudemann, C. & Lillig, C. H. Thioredoxins, glutaredoxins, and peroxiredoxins--molecular mechanisms and health significance: from cofactors to antioxidants to redox signaling. *Antioxidants & redox signaling* **19**, 1539-1605 (2013).

43. Fridovich, I. Oxygen toxicity: a radical explanation. *The Journal of experimental biology* **201**, 1203-1209 (1998).

44. Chance, B., Sies, H. & Boveris, A. Hydroperoxide metabolism in mammalian organs. *Physiological reviews* **59**, 527-605 (1979).

45. St-Pierre, J., Buckingham, J. A., Roebuck, S. J. & Brand, M. D. Topology of superoxide production from different sites in the mitochondrial electron transport chain. *The Journal of biological chemistry* **277**, 44784-44790 (2002).

46. Brand, M. D. The sites and topology of mitochondrial superoxide production. *Experimental gerontology* **45**, 466-472 (2010).

47. Nishino, T., Okamoto, K., Eger, B. T., Pai, E. F. & Nishino, T. Mammalian xanthine oxidoreductase - mechanism of transition from xanthine dehydrogenase to xanthine oxidase. *The FEBS journal* **275**, 3278-3289 (2008).

48. Zangar, R. C., Davydov, D. R. & Verma, S. Mechanisms that regulate production of reactive oxygen species by cytochrome P450. *Toxicology and applied pharmacology* **199**, 316-331 (2004).

49. Kukreja, R. C., Kontos, H. A., Hess, M. L. & Ellis, E. F. PGH synthase and lipoxygenase generate superoxide in the presence of NADH or NADPH. *Circulation research* **59**, 612-619 (1986).

50. Jiang, F., Zhang, Y. & Dusting, G. J. NADPH oxidase-mediated redox signaling: roles in cellular stress response, stress tolerance, and tissue repair. *Pharmacological reviews* **63**, 218-242 (2011).

51. Jones, C. M., Lawrence, A., Wardman, P. & Burkitt, M. J. Electron paramagnetic resonance spin trapping investigation into the kinetics of glutathione oxidation by the superoxide radical: re-evaluation of the rate constant. *Free radical biology & medicine* **32**, 982-990 (2002).

52. Paulsen, C. E. & Carroll, K. S. Cysteine-mediated redox signaling: chemistry, biology, and tools for discovery. *Chemical reviews* **113**, 4633-4679 (2013).

53. Reddi, A. R. & Culotta, V. C. SOD1 integrates signals from oxygen and glucose to repress respiration. *Cell* **152**, 224-235 (2013).

54. Giorgio, M., Trinei, M., Migliaccio, E. & Pelicci, P. G. Hydrogen peroxide: a metabolic by-product or a common mediator of ageing signals? *Nature reviews. Molecular cell biology* **8**, 722-728 (2007).

55. Winterbourn, C. C. The biological chemistry of hydrogen peroxide. *Methods in enzymology* **528**, 3-25 (2013).

56. Bedard, K. & Krause, K. H. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiological reviews* **87**, 245-313 (2007).

57. Miller, E. W., Dickinson, B. C. & Chang, C. J. Aquaporin-3 mediates hydrogen peroxide uptake to regulate downstream intracellular signaling. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 15681-15686 (2010).

58. Winterbourn, C. C. & Kettle, A. J. Redox reactions and microbial killing in the neutrophil phagosome. *Antioxidants & redox signaling* **18**, 642-660 (2013).

59. Wood, P. M. The potential diagram for oxygen at pH 7. *The Biochemical journal* **253**, 287-289 (1988).

60. Malinski, T. *et al.* Diffusion of nitric oxide in the aorta wall monitored in situ by porphyrinic microsensors. *Biochemical and biophysical research communications* **193**, 1076-1082 (1993).

61. Hall, C. N. & Garthwaite, J. What is the real physiological NO concentration in vivo? *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society* **21**, 92-103 (2009).

62. Broniowska, K. A. & Hogg, N. The chemical biology of S-nitrosothiols. *Antioxidants & redox signaling* **17**, 969-980 (2012).

63. Murad, F. Shattuck Lecture. Nitric oxide and cyclic GMP in cell signaling and drug development. *The New England journal of medicine* **355**, 2003-2011 (2006).

64. Forstermann, U. & Sessa, W. C. Nitric oxide synthases: regulation and function. *European heart journal* **33**, 829-837, 837a-837d (2012).

65. Erwin, P. A., Lin, A. J., Golan, D. E. & Michel, T. Receptor-regulated dynamic S-nitrosylation of endothelial nitric-oxide synthase in vascular endothelial cells. *The Journal of biological chemistry* **280**, 19888-19894 (2005).

66. Landmesser, U. *et al.* Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *The Journal of clinical investigation* **111**, 1201-1209 (2003).

67. Lundberg, J. O., Weitzberg, E. & Gladwin, M. T. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nature reviews. Drug discovery* **7**, 156-167 (2008).

68. Lundberg, J. O., Gladwin, M. T. & Weitzberg, E. Strategies to increase nitric oxide signalling in cardiovascular disease. *Nature reviews. Drug discovery* **14**, 623-641 (2015).

69. Hill, B. G., Dranka, B. P., Bailey, S. M., Lancaster, J. R., Jr. & Darley-Usmar, V. M. What part of NO don't you understand? Some answers to the cardinal questions in nitric oxide biology. *The Journal of biological chemistry* **285**, 19699-19704 (2010).

70. Shiva, S. *et al.* Ceruloplasmin is a NO oxidase and nitrite synthase that determines endocrine NO homeostasis. *Nature chemical biology* **2**, 486-493 (2006).

71. Moncada, S. & Higgs, A. The L-arginine-nitric oxide pathway. *The New England journal of medicine* **329**, 2002-2012 (1993).

72. Heinrich, T. A. *et al.* Biological nitric oxide signalling: chemistry and terminology. *British journal of pharmacology* **169**, 1417-1429 (2013).

73. Czapski, G. & Goldstein, S. The role of the reactions of .NO with superoxide and oxygen in biological systems: a kinetic approach. *Free radical biology & medicine* **19**, 785-794 (1995).

74. Moller, M. N. *et al.* Membrane "lens" effect: focusing the formation of reactive nitrogen oxides from the *NO/O2 reaction. *Chemical research in toxicology* **20**, 709-714 (2007).

75. Hughes, M. N. Chemistry of nitric oxide and related species. *Methods in enzymology* **436**, 3-19 (2008).

76. Ferrer-Sueta, G. & Radi, R. Chemical biology of peroxynitrite: kinetics, diffusion, and radicals. *ACS chemical biology* **4**, 161-177 (2009).

77. Trujillo, M., Ferrer-Sueta, G. & Radi, R. Peroxynitrite detoxification and its biologic implications. *Antioxidants & redox signaling* **10**, 1607-1620 (2008).

78. Radi, R. Peroxynitrite, a stealthy biological oxidant. *The Journal of biological chemistry* **288**, 26464-26472 (2013).

79. Briviba, K., Kissner, R., Koppenol, W. H. & Sies, H. Kinetic study of the reaction of glutathione peroxidase with peroxynitrite. *Chemical research in toxicology* **11**, 1398-1401 (1998).

80. Radi, R. Protein tyrosine nitration: biochemical mechanisms and structural basis of functional effects. *Accounts of chemical research* **46**, 550-559 (2013).

81. Abe, K. & Kimura, H. The possible role of hydrogen sulfide as an endogenous neuromodulator. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **16**, 1066-1071 (1996).

82. Szabo, C. Hydrogen sulphide and its therapeutic potential. *Nature reviews. Drug discovery* **6**, 917-935 (2007).

83. Olson, K. R. A practical look at the chemistry and biology of hydrogen sulfide. *Antioxidants & redox signaling* **17**, 32-44 (2012).

84. Ono, K. *et al.* Redox chemistry and chemical biology of H2S, hydropersulfides, and derived species: implications of their possible biological activity and utility. *Free radical biology & medicine* **77**, 82-94 (2014).

85. Li, L., Rose, P. & Moore, P. K. Hydrogen sulfide and cell signaling. *Annual review of pharmacology and toxicology* **51**, 169-187 (2011).

86. Nagy, P. *et al.* Chemical aspects of hydrogen sulfide measurements in physiological samples. *Biochimica et biophysica acta* **1840**, 876-891 (2014).

87. Nagy, P. Mechanistic chemical perspective of hydrogen sulfide signaling. *Methods in enzymology* **554**, 3-29 (2015).

88. Paul, B. D. & Snyder, S. H. H(2)S signalling through protein sulfhydration and beyond. *Nature reviews. Molecular cell biology* **13**, 499-507 (2012).

89. Chen, X., Jhee, K. H. & Kruger, W. D. Production of the neuromodulator H2S by cystathionine beta-synthase via the condensation of cysteine and homocysteine. *The Journal of biological chemistry* **279**, 52082-52086 (2004).

90. Zhao, W., Zhang, J., Lu, Y. & Wang, R. The vasorelaxant effect of H(2)S as a novel endogenous gaseous K(ATP) channel opener. *The EMBO journal* **20**, 6008-6016 (2001).

91. Mustafa, A. K. *et al.* H2S signals through protein S-sulfhydration. *Science signaling* **2**, ra72 (2009).

92. Jiang, B., Tang, G., Cao, K., Wu, L. & Wang, R. Molecular mechanism for H(2)S-induced activation of K(ATP) channels. *Antioxidants & redox signaling* **12**, 1167-1178 (2010).

93. Mishanina, T. V., Libiad, M. & Banerjee, R. Biogenesis of reactive sulfur species for signaling by hydrogen sulfide oxidation pathways. *Nature chemical biology* **11**, 457-464 (2015).

94. Whiteman, M. *et al.* The novel neuromodulator hydrogen sulfide: an endogenous peroxynitrite 'scavenger'? *Journal of neurochemistry* **90**, 765-768 (2004).

95. Carballal, S. *et al.* Reactivity of hydrogen sulfide with peroxynitrite and other oxidants of biological interest. *Free radical biology & medicine* **50**, 196-205 (2011).

96. Li, L. & Moore, P. K. Putative biological roles of hydrogen sulfide in health and disease: a breath of not so fresh air? *Trends in pharmacological sciences* **29**, 84-90 (2008).

97. Cuevasanta, E. *et al.* Reaction of Hydrogen Sulfide with Disulfide and Sulfenic Acid to Form the Strongly Nucleophilic Persulfide. *The Journal of biological chemistry* **290**, 26866-26880 (2015).

98. Ida, T. *et al.* Reactive cysteine persulfides and S-polythiolation regulate oxidative stress and redox signaling. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 7606-7611 (2014).

99. Greiner, R. *et al.* Polysulfides link H2S to protein thiol oxidation. *Antioxidants & redox signaling* **19**, 1749-1765 (2013).

100. Toohey, J. I. Sulfur signaling: is the agent sulfide or sulfane? *Analytical biochemistry* **413**, 1-7 (2011).

101. Go, Y. M. & Jones, D. P. Thiol/disulfide redox states in signaling and sensing. *Critical reviews in biochemistry and molecular biology* **48**, 173-181 (2013).

102. Stadtman, E. R. & Levine, R. L. Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. *Amino Acids* **25**, 207-218 (2003).

103. Marino, S. M. & Gladyshev, V. N. Analysis and functional prediction of reactive cysteine residues. *The Journal of biological chemistry* **287**, 4419-4425 (2012).

104. Drazic, A. & Winter, J. The physiological role of reversible methionine oxidation. *Biochimica et biophysica acta* **1844**, 1367-1382 (2014).

105. Kaya, A., Lee, B. C. & Gladyshev, V. N. Regulation of Protein Function by Reversible Methionine Oxidation and the Role of Selenoprotein MsrB1. *Antioxidants & redox signaling* **23**, 814-822 (2015).

106. Gladyshev, V. N. & Hatfield, D. L. Selenocysteine-containing proteins in mammals. *Journal of biomedical science* **6**, 151-160 (1999).

107. Kryukov, G. V. *et al.* Characterization of mammalian selenoproteomes. *Science* **300**, 1439-1443 (2003).

108. Go, Y. M., Chandler, J. D. & Jones, D. P. The cysteine proteome. *Free radical biology & medicine* (2015).

109. Tajc, S. G., Tolbert, B. S., Basavappa, R. & Miller, B. L. Direct determination of thiol pKa by isothermal titration microcalorimetry. *Journal of the American Chemical Society* **126**, 10508-10509 (2004).

110. Banerjee, R. Redox outside the box: linking extracellular redox remodeling with intracellular redox metabolism. *The Journal of biological chemistry* **287**, 4397-4402 (2012).

111. Lu, S. C. Regulation of glutathione synthesis. *Molecular aspects of medicine* **30**, 42-59 (2009).

112. Mosharov, E., Cranford, M. R. & Banerjee, R. The quantitatively important relationship between homocysteine metabolism and glutathione synthesis by the transsulfuration pathway and its regulation by redox changes. *Biochemistry* **39**, 13005-13011 (2000).

113. Belalcazar, A. D., Ball, J. G., Frost, L. M., Valentovic, M. A. & Wilkinson, J. t. Transsulfuration Is a Significant Source of Sulfur for Glutathione Production in Human Mammary Epithelial Cells. *ISRN biochemistry* **2013**, 637897 (2014).

114. Eriksson, S., Prigge, J. R., Talago, E. A., Arner, E. S. & Schmidt, E. E. Dietary methionine can sustain cytosolic redox homeostasis in the mouse liver. *Nature communications* **6**, 6479 (2015).

115. Bannai, S. Transport of cystine and cysteine in mammalian cells. *Biochimica et biophysica acta* **779**, 289-306 (1984).

116. Christensen, H. N. Role of amino acid transport and countertransport in nutrition and metabolism. *Physiological reviews* **70**, 43-77 (1990).

117. Knickelbein, R. G., Seres, T., Lam, G., Johnston, R. B., Jr. & Warshaw, J. B. Characterization of multiple cysteine and cystine transporters in rat alveolar type II cells. *The American journal of physiology* **273**, L1147-1155 (1997).

118. Chen, Y. & Swanson, R. A. The glutamate transporters EAAT2 and EAAT3 mediate cysteine uptake in cortical neuron cultures. *Journal of neurochemistry* **84**, 1332-1339 (2003).

119. Peters, T. *et al.* A mouse model for cystinuria type I. *Human molecular genetics* **12**, 2109-2120 (2003).

120. Lewerenz, J. *et al.* The cystine/glutamate antiporter system x(c)(-) in health and disease: from molecular mechanisms to novel therapeutic opportunities. *Antioxidants & redox signaling* **18**, 522-555 (2013).

121. Bannai, S., Sato, H., Ishii, T. & Sugita, Y. Induction of cystine transport activity in human fibroblasts by oxygen. *The Journal of biological chemistry* **264**, 18480-18484 (1989).

122. Bannai, S. Induction of cystine and glutamate transport activity in human fibroblasts by diethyl maleate and other electrophilic agents. *The Journal of biological chemistry* **259**, 2435-2440 (1984).

123. Sato, H., Fujiwara, K., Sagara, J. & Bannai, S. Induction of cystine transport activity in mouse peritoneal macrophages by bacterial lipopolysaccharide. *The Biochemical journal* **310 (Pt 2)**, 547-551 (1995).

124. Conrad, M. & Sato, H. The oxidative stress-inducible cystine/glutamate antiporter, system x (c) (-) : cystine supplier and beyond. *Amino Acids* (2011).

125. Mandal, P. K. *et al.* System x(c)- and thioredoxin reductase 1 cooperatively rescue glutathione deficiency. *The Journal of biological chemistry* **285**, 22244-22253 (2010).

126. Woo, H. A. *et al.* Reversing the inactivation of peroxiredoxins caused by cysteine sulfinic acid formation. *Science* **300**, 653-656 (2003).

127. Hall, A., Parsonage, D., Poole, L. B. & Karplus, P. A. Structural evidence that peroxiredoxin catalytic power is based on transition-state stabilization. *Journal of molecular biology* **402**, 194-209 (2010).

128. Nauser, T., Koppenol, W. H. & Schoneich, C. Protein thiyl radical reactions and product formation: a kinetic simulation. *Free radical biology & medicine* **80**, 158-163 (2015).

129. Winterbourn, C. C. Superoxide as an intracellular radical sink. *Free radical biology & medicine* **14**, 85-90 (1993).

130. Jourd'heuil, D., Jourd'heuil, F. L. & Feelisch, M. Oxidation and nitrosation of thiols at low micromolar exposure to nitric oxide. Evidence for a free radical mechanism. *The Journal of biological chemistry* **278**, 15720-15726 (2003).

131. Gould, N., Doulias, P. T., Tenopoulou, M., Raju, K. & Ischiropoulos, H. Regulation of protein function and signaling by reversible cysteine S-nitrosylation. *The Journal of biological chemistry* **288**, 26473-26479 (2013).

132. Gow, A. J. & Stamler, J. S. Reactions between nitric oxide and haemoglobin under physiological conditions. *Nature* **391**, 169-173 (1998).

133. Doulias, P. T. *et al.* Structural profiling of endogenous S-nitrosocysteine residues reveals unique features that accommodate diverse mechanisms for protein S-nitrosylation. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 16958-16963 (2010).

134. Nakamura, T. & Lipton, S. A. Emerging Role of Protein-Protein Transnitrosylation in Cell Signaling Pathways. *Antioxidants & redox signaling* (2012).

135. Marino, S. M. & Gladyshev, V. N. Structural analysis of cysteine S-nitrosylation: a modified acid-based motif and the emerging role of trans-nitrosylation. *Journal of molecular biology* **395**, 844-859 (2010).

136. Kornberg, M. D. *et al.* GAPDH mediates nitrosylation of nuclear proteins. *Nature cell biology* **12**, 1094-1100 (2010).

137. Mitchell, D. A., Morton, S. U., Fernhoff, N. B. & Marletta, M. A. Thioredoxin is required for S-nitrosation of procaspase-3 and the inhibition of apoptosis in Jurkat cells. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 11609-11614 (2007).

138. Nakamura, T. *et al.* Transnitrosylation of XIAP regulates caspase-dependent neuronal cell death. *Molecular cell* **39**, 184-195 (2010).

139. Jia, J. *et al.* Target-selective protein S-nitrosylation by sequence motif recognition. *Cell* **159**, 623-634 (2014).

140. Nakamura, T. & Lipton, S. A. Protein S-Nitrosylation as a Therapeutic Target for Neurodegenerative Diseases. *Trends in pharmacological sciences* **37**, 73-84 (2016).

141. Jaffrey, S. R. & Snyder, S. H. The biotin switch method for the detection of S-nitrosylated proteins. *Science's STKE : signal transduction knowledge environment* **2001**, pl1 (2001).

142. Chen, Y. J. *et al.* dbSNO 2.0: a resource for exploring structural environment, functional and disease association and regulatory network of protein S-nitrosylation. *Nucleic acids research* **43**, D503-511 (2015).

143. Mannick, J. B. *et al.* Fas-induced caspase denitrosylation. *Science* **284**, 651-654 (1999).

144. Rossig, L. *et al.* Nitric oxide inhibits caspase-3 by S-nitrosation in vivo. *The Journal of biological chemistry* **274**, 6823-6826 (1999).

145. Kelleher, Z. T. *et al.* Thioredoxin-mediated denitrosylation regulates cytokine-induced nuclear factor kappaB (NF-kappaB) activation. *The Journal of biological chemistry* **289**, 3066-3072 (2014).

146. Li, S. & Whorton, A. R. Regulation of protein tyrosine phosphatase 1B in intact cells by S-nitrosothiols. *Archives of biochemistry and biophysics* **410**, 269-279 (2003).

147. Mikkelsen, R. B. & Wardman, P. Biological chemistry of reactive oxygen and nitrogen and radiation-induced signal transduction mechanisms. *Oncogene* **22**, 5734-5754 (2003).

148. Benhar, M., Forrester, M. T. & Stamler, J. S. Protein denitrosylation: enzymatic mechanisms and cellular functions. *Nature reviews. Molecular cell biology* **10**, 721-732 (2009).

149. Reynaert, N. L. *et al.* Nitric oxide represses inhibitory kappaB kinase through S-nitrosylation. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 8945-8950 (2004).

150. Liu, L. *et al.* A metabolic enzyme for S-nitrosothiol conserved from bacteria to humans. *Nature* **410**, 490-494 (2001).

151. Staab, C. A., Hellgren, M. & Hoog, J. O. Medium- and short-chain dehydrogenase/reductase gene and protein families : Dual functions of alcohol dehydrogenase 3: implications with focus on formaldehyde dehydrogenase and S-nitrosoglutathione reductase activities. *Cellular and molecular life sciences : CMLS* **65**, 3950-3960 (2008).

152. Jensen, D. E., Belka, G. K. & Du Bois, G. C. S-Nitrosoglutathione is a substrate for rat alcohol dehydrogenase class III isoenzyme. *The Biochemical journal* **331 (Pt 2)**, 659-668 (1998).

153. Benhar, M., Forrester, M. T., Hess, D. T. & Stamler, J. S. Regulated protein denitrosylation by cytosolic and mitochondrial thioredoxins. *Science* **320**, 1050-1054 (2008).

154. Sengupta, R. *et al.* Thioredoxin catalyzes the denitrosation of low-molecular mass and protein S-nitrosothiols. *Biochemistry* **46**, 8472-8483 (2007).

155. Nikitovic, D. & Holmgren, A. S-Nitrosoglutathione is cleaved by the thioredoxin system with liberation of glutathione and redox regulating nitric oxide. *J. Biol. Chem.* **271**, 19180-19185 (1996).

156. Sliskovic, I., Raturi, A. & Mutus, B. Characterization of the S-denitrosation activity of protein disulfide isomerase. *The Journal of biological chemistry* **280**, 8733-8741 (2005).

157. Trujillo, M., Alvarez, M. N., Peluffo, G., Freeman, B. A. & Radi, R. Xanthine oxidase-mediated decomposition of S-nitrosothiols. *The Journal of biological chemistry* **273**, 7828-7834 (1998).

158. Jourd'heuil, D., Laroux, F. S., Miles, A. M., Wink, D. A. & Grisham, M. B. Effect of superoxide dismutase on the stability of S-nitrosothiols. *Archives of biochemistry and biophysics* **361**, 323-330 (1999).

159. Hou, Y., Guo, Z., Li, J. & Wang, P. G. Seleno compounds and glutathione peroxidase catalyzed decomposition of S-nitrosothiols. *Biochemical and biophysical research communications* **228**, 88-93 (1996).

160. Bateman, R. L., Rauh, D., Tavshanjian, B. & Shokat, K. M. Human carbonyl reductase 1 is an S-nitrosoglutathione reductase. *The Journal of biological chemistry* **283**, 35756-35762 (2008).

161. Benhar, M. Nitric oxide and the thioredoxin system: a complex interplay in redox regulation. *Biochimica et biophysica acta* **1850**, 2476-2484 (2015).

162. Sun, N. *et al.* GluR6-FasL-Trx2 mediates denitrosylation and activation of procaspase-3 in cerebral ischemia/reperfusion in rats. *Cell death & disease* **4**, e771 (2013).

163. Pader, I. *et al.* Thioredoxin-related protein of 14 kDa is an efficient L-cystine reductase and S-denitrosylase. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 6964-6969 (2014).

164. Mitchell, D. A. & Marletta, M. A. Thioredoxin catalyzes the S-nitrosation of the caspase-3 active site cysteine. *Nature chemical biology* **1**, 154-158 (2005).

165. Wu, C. *et al.* Redox regulatory mechanism of transnitrosylation by thioredoxin. *Mol Cell Proteomics* **9**, 2262-2275 (2010).

166. Forrester, M. T. *et al.* Thioredoxin-interacting protein (Txnip) is a feedback regulator of S-nitrosylation. *The Journal of biological chemistry* **284**, 36160-36166 (2009).

167. Kneeshaw, S., Gelineau, S., Tada, Y., Loake, G. J. & Spoel, S. H. Selective protein denitrosylation activity of Thioredoxin-h5 modulates plant Immunity. *Molecular cell* **56**, 153-162 (2014).

168. Sengupta, R. & Holmgren, A. The role of thioredoxin in the regulation of cellular processes by S-nitrosylation. *Biochimica et biophysica acta* **1820**, 689-700 (2012).

169. Ben-Lulu, S., Ziv, T., Admon, A., Weisman-Shomer, P. & Benhar, M. A substrate trapping approach identifies proteins regulated by reversible S-nitrosylation. *Mol Cell Proteomics* **13**, 2573-2583 (2014).

170. Nagy, P. & Winterbourn, C. C. Rapid reaction of hydrogen sulfide with the neutrophil oxidant hypochlorous acid to generate polysulfides. *Chemical research in toxicology* **23**, 1541-1543 (2010).

171. Kabil, O., Motl, N. & Banerjee, R. H2S and its role in redox signaling. *Biochimica et biophysica acta* **1844**, 1355-1366 (2014).

172. Francoleon, N. E., Carrington, S. J. & Fukuto, J. M. The reaction of H(2)S with oxidized thiols: generation of persulfides and implications to H(2)S biology. *Archives of biochemistry and biophysics* **516**, 146-153 (2011).

173. Vasas, A., Doka, E., Fabian, I. & Nagy, P. Kinetic and thermodynamic studies on the disulfide-bond reducing potential of hydrogen sulfide. *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society* **46**, 93-101 (2015).

174. Yadav, P. K. *et al.* Biosynthesis and Reactivity of Cysteine Persulfides in Signaling. *Journal of the American Chemical Society* **138**, 289-299 (2016).

175. Zhang, D. *et al.* Detection of protein S-sulfhydration by a tag-switch technique. *Angewandte Chemie* **53**, 575-581 (2014).

176. Altaany, Z., Ju, Y., Yang, G. & Wang, R. The coordination of S-sulfhydration, S-nitrosylation, and phosphorylation of endothelial nitric oxide synthase by hydrogen sulfide. *Science signaling* **7**, ra87 (2014).

177. Hara, M. R., Cascio, M. B. & Sawa, A. GAPDH as a sensor of NO stress. *Biochimica et biophysica acta* **1762**, 502-509 (2006).

178. Sen, N. *et al.* Hydrogen sulfide-linked sulfhydration of NF-kappaB mediates its antiapoptotic actions. *Molecular cell* **45**, 13-24 (2012).

179. Krishnan, N., Fu, C., Pappin, D. J. & Tonks, N. K. H2S-Induced sulfhydration of the phosphatase PTP1B and its role in the endoplasmic reticulum stress response. *Science signaling* **4**, ra86 (2011).

180. Mikami, Y. *et al.* Thioredoxin and dihydrolipoic acid are required for 3-mercaptopyruvate sulfurtransferase to produce hydrogen sulfide. *Biochemical Journal* **439**, 479-485 (2011).

181. Taguchi, K., Motohashi, H. & Yamamoto, M. Molecular mechanisms of the Keap1-Nrf2 pathway in stress response and cancer evolution. *Genes to cells : devoted to molecular & cellular mechanisms* **16**, 123-140 (2011).

182. Ishii, T. *et al.* Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. *The Journal of biological chemistry* **275**, 16023-16029 (2000).

183. Harvey, C. J. *et al.* Nrf2-regulated glutathione recycling independent of biosynthesis is critical for cell survival during oxidative stress. *Free radical biology & medicine* **46**, 443-453 (2009).

184. Cebula, M., Schmidt, E. E. & Arner, E. S. TrxR1 as a Potent Regulator of the Nrf2-Keap1 Response System. *Antioxidants & redox signaling* **23**, 823-853 (2015).

185. Higgins, L. G. & Hayes, J. D. Mechanisms of induction of cytosolic and microsomal glutathione transferase (GST) genes by xenobiotics and pro-inflammatory agents. *Drug metabolism reviews* **43**, 92-137 (2011).

186. Martin, J. L. Thioredoxin--a fold for all reasons. *Structure* **3**, 245-250 (1995).

187. Pedone, E., Limauro, D., D'Ambrosio, K., De Simone, G. & Bartolucci, S. Multiple catalytically active thioredoxin folds: a winning strategy for many functions. *Cellular and molecular life sciences : CMLS* **67**, 3797-3814 (2010).

188. Qi, Y. & Grishin, N. V. Structural classification of thioredoxin-like fold proteins. *Proteins* **58**, 376-388 (2005).

189. Kozlov, G., Maattanen, P., Thomas, D. Y. & Gehring, K. A structural overview of the PDI family of proteins. *The FEBS journal* **277**, 3924-3936 (2010).

190. Matsuo, Y. *et al.* Identification of a novel thioredoxin-related transmembrane protein. *The Journal of biological chemistry* **276**, 10032-10038 (2001).

191. Meng, X. *et al.* Cloning and identification of a novel cDNA coding thioredoxin-related transmembrane protein 2. *Biochemical genetics* **41**, 99-106 (2003).

192. Haugstetter, J., Blicher, T. & Ellgaard, L. Identification and characterization of a novel thioredoxin-related transmembrane protein of the endoplasmic reticulum. *The Journal of biological chemistry* **280**, 8371-8380 (2005).

193. Sugiura, Y. *et al.* Novel thioredoxin-related transmembrane protein TMX4 has reductase activity. *The Journal of biological chemistry* **285**, 7135-7142 (2010).

194. Matsuo, Y. *et al.* The protective role of the transmembrane thioredoxin-related protein TMX in inflammatory liver injury. *Antioxidants & redox signaling* **18**, 1263-1272 (2013).

195. Miranda-Vizuete, A. *et al.* Characterization of Sptrx, a novel member of the thioredoxin family specifically expressed in human spermatozoa. *The Journal of biological chemistry* **276**, 31567-31574 (2001).

196. Sadek, C. M. *et al.* Sptrx-2, a fusion protein composed of one thioredoxin and three tandemly repeated NDP-kinase domains is expressed in human testis germ cells. *Genes to cells : devoted to molecular & cellular mechanisms* **6**, 1077-1090 (2001).

197. Kurooka, H. *et al.* Cloning and characterization of the nucleoredoxin gene that encodes a novel nuclear protein related to thioredoxin. *Genomics* **39**, 331-339 (1997).

198. Leveillard, T. *et al.* Identification and characterization of rod-derived cone viability factor. *Nature genetics* **36**, 755-759 (2004).

199. Chalmel, F. *et al.* Rod-derived Cone Viability Factor-2 is a novel bifunctional-thioredoxin-like protein with therapeutic potential. *BMC molecular biology* **8**, 74 (2007).

200. Funato, Y. & Miki, H. Nucleoredoxin, a novel thioredoxin family member involved in cell growth and differentiation. *Antioxidants & redox signaling* **9**, 1035-1057 (2007).

201. Hirota, K. *et al.* Nucleoredoxin, glutaredoxin, and thioredoxin differentially regulate NF-kappaB, AP-1, and CREB activation in HEK293 cells. *Biochemical and biophysical research communications* **274**, 177-182 (2000).

202. Funato, Y., Michiue, T., Asashima, M. & Miki, H. The thioredoxin-related redox-regulating protein nucleoredoxin inhibits Wnt-beta-catenin signalling through dishevelled. *Nature cell biology* **8**, 501-508 (2006).

203. Hayashi, T. *et al.* Nucleoredoxin negatively regulates Toll-like receptor 4 signaling via recruitment of flightless-I to myeloid differentiation primary response gene (88). *The Journal of biological chemistry* **285**, 18586-18593 (2010).

204. Miranda-Vizuete, A., Gustafsson, J. A. & Spyrou, G. Molecular cloning and expression of a cDNA encoding a human thioredoxin-like protein. *Biochemical and biophysical research communications* **243**, 284-288 (1998).

205. Lee, K. K. *et al.* Purification, molecular cloning, and characterization of TRP32, a novel thioredoxin-related mammalian protein of 32 kDa. *The Journal of biological chemistry* **273**, 19160-19166 (1998).

206. Jimenez, A., Pelto-Huikko, M., Gustafsson, J. A. & Miranda-Vizuete, A. Characterization of human thioredoxin-like-1: potential involvement in the cellular response against glucose deprivation. *FEBS letters* **580**, 960-967 (2006).

207. Andersen, K. M. *et al.* Thioredoxin Txnl1/TRP32 is a redox-active cofactor of the 26 S proteasome. *The Journal of biological chemistry* **284**, 15246-15254 (2009).

208. Kim, K. Y. *et al.* Expression of a thioredoxin-related protein-1 is induced by prostaglandin E(2). *International journal of cancer. Journal international du cancer* **118**, 1670-1679 (2006).

209. Ishii, T., Funato, Y. & Miki, H. Thioredoxin-related protein 32 (TRP32) specifically reduces oxidized phosphatase of regenerating liver (PRL). *The Journal of biological chemistry* **288**, 7263-7270 (2013).

210. Xu, W. *et al.* TXNL1-XRCC1 pathway regulates cisplatin-induced cell death and contributes to resistance in human gastric cancer. *Cell death & disease* **5**, e1055 (2014).

211. Perkins, A., Nelson, K. J., Parsonage, D., Poole, L. B. & Karplus, P. A. Peroxiredoxins: guardians against oxidative stress and modulators of peroxide signaling. *Trends in biochemical sciences* **40**, 435-445 (2015).

212. Kang, S. W. *et al.* Mammalian peroxiredoxin isoforms can reduce hydrogen peroxide generated in response to growth factors and tumor necrosis factor-alpha. *The Journal of biological chemistry* **273**, 6297-6302 (1998).

213. Kang, S. W. *et al.* Cytosolic peroxiredoxin attenuates the activation of Jnk and p38 but potentiates that of Erk in Hela cells stimulated with tumor necrosis factor-alpha. *The Journal of biological chemistry* **279**, 2535-2543 (2004).

214. Kim, S. Y., Kim, T. J. & Lee, K. Y. A novel function of peroxiredoxin 1 (Prx-1) in apoptosis signal-regulating kinase 1 (ASK1)-mediated signaling pathway. *FEBS letters* **582**, 1913-1918 (2008).

215. Lu, W. *et al.* Peroxiredoxin 2 knockdown by RNA interference inhibits the growth of colorectal cancer cells by downregulating Wnt/beta-catenin signaling. *Cancer letters* **343**, 190-199 (2014).

216. Karplus, P. A. A primer on peroxiredoxin biochemistry. *Free radical biology & medicine* **80**, 183-190 (2015).

217. Peskin, A. V. *et al.* Glutathionylation of the Active Site Cysteines of Peroxiredoxin 2 and Recycling by Glutaredoxin. *The Journal of biological chemistry* (2015).

218. Engelman, R. *et al.* Multilevel regulation of 2-Cys peroxiredoxin reaction cycle by S-nitrosylation. *The Journal of biological chemistry* **288**, 11312-11324 (2013).

219. Randall, L. M. *et al.* Nitration transforms a sensitive peroxiredoxin 2 into a more active and robust peroxidase. *The Journal of biological chemistry* **289**, 15536-15543 (2014).

220. Lu, J. & Holmgren, A. The thioredoxin antioxidant system. *Free radical biology & medicine* **66**, 75-87 (2014).

221. Lillig, C. H. & Holmgren, A. Thioredoxin and related molecules-from biology to health and disease. *Antioxidants & redox signaling* **9**, 25-47 (2007).

222. Mahmood, D. F., Abderrazak, A., El Hadri, K., Simmet, T. & Rouis, M. The thioredoxin system as a therapeutic target in human health and disease. *Antioxidants & redox signaling* **19**, 1266-1303 (2013).

223. Matsui, M. *et al.* Early embryonic lethality caused by targeted disruption of the mouse thioredoxin gene. *Developmental biology* **178**, 179-185 (1996).

224. Nonn, L., Williams, R. R., Erickson, R. P. & Powis, G. The absence of mitochondrial thioredoxin 2 causes massive apoptosis, exencephaly, and early embryonic lethality in homozygous mice. *Molecular and cellular biology* **23**, 916-922 (2003).

225. Bondareva, A. A. *et al.* Effects of thioredoxin reductase-1 deletion on embryogenesis and transcriptome. *Free radical biology & medicine* **43**, 911-923 (2007).

226. Jakupoglu, C. *et al.* Cytoplasmic thioredoxin reductase is essential for embryogenesis but dispensable for cardiac development. *Molecular and cellular biology* **25**, 1980-1988 (2005).

227. Conrad, M. *et al.* Essential role for mitochondrial thioredoxin reductase in hematopoiesis, heart development, and heart function. *Molecular and cellular biology* **24**, 9414-9423 (2004).

228. Laurent, T. C., Moore, E. C. & Reichard, P. Enzymatic Synthesis of Deoxyribonucleotides. Iv. Isolation and Characterization of Thioredoxin, the Hydrogen Donor from Escherichia Coli B. *The Journal of biological chemistry* **239**, 3436-3444 (1964).

229. Zahedi Avval, F. & Holmgren, A. Molecular mechanisms of thioredoxin and glutaredoxin as hydrogen donors for Mammalian s phase ribonucleotide reductase. *The Journal of biological chemistry* **284**, 8233-8240 (2009).

230. Holmgren, A. Thioredoxin. *Annu. Rev. Biochem.* **54**, 237-271 (1985).

231. Arner, E. S. & Holmgren, A. Physiological functions of thioredoxin and thioredoxin reductase. *Eur J Biochem* **267**, 6102-6109 (2000).

232. Schenk, H., Klein, M., Erdbrugger, W., Droge, W. & Schulze-Osthoff, K. Distinct effects of thioredoxin and antioxidants on the activation of transcription factors NF-kappa B and AP-1. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 1672-1676 (1994).

233. Hirota, K. *et al.* AP-1 transcriptional activity is regulated by a direct association between thioredoxin and Ref-1. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 3633-3638 (1997).

234. Hirota, K. *et al.* Distinct roles of thioredoxin in the cytoplasm and in the nucleus. A two-step mechanism of redox regulation of transcription factor NF-kappaB. *The Journal of biological chemistry* **274**, 27891-27897 (1999).

235. Ueno, M. *et al.* Thioredoxin-dependent redox regulation of p53-mediated p21 activation. *The Journal of biological chemistry* **274**, 35809-35815 (1999).

236. Schroeder, P., Popp, R., Wiegand, B., Altschmied, J. & Haendeler, J. Nuclear redox-signaling is essential for apoptosis inhibition in endothelial cells--important role for nuclear thioredoxin-1. *Arteriosclerosis, thrombosis, and vascular biology* **27**, 2325-2331 (2007).

237. Pekkari, K. & Holmgren, A. Truncated thioredoxin: physiological functions and mechanism. *Antioxidants & redox signaling* **6**, 53-61 (2004).

238. Ren, X., Bjornstedt, M., Shen, B., Ericson, M. L. & Holmgren, A. Mutagenesis of structural half-cystine residues in human thioredoxin and effects on the regulation of activity by selenodiglutathione. *Biochemistry* **32**, 9701-9708 (1993).

239. Weichsel, A., Gasdaska, J. R., Powis, G. & Montfort, W. R. Crystal structures of reduced, oxidized, and mutated human thioredoxins: evidence for a regulatory homodimer. *Structure* **4**, 735-751 (1996).

240. Watson, W. H. *et al.* Redox potential of human thioredoxin 1 and identification of a second dithiol/disulfide motif. *The Journal of biological chemistry* **278**, 33408-33415 (2003).

241. Du, Y., Zhang, H., Zhang, X., Lu, J. & Holmgren, A. Thioredoxin 1 is inactivated due to oxidation induced by peroxiredoxin under oxidative stress and reactivated by the glutaredoxin system. *The Journal of biological chemistry* **288**, 32241-32247 (2013).

242. Wu, C. *et al.* Thioredoxin 1-mediated post-translational modifications: reduction, transnitrosylation, denitrosylation, and related proteomics methodologies. *Antioxidants & redox signaling* **15**, 2565-2604 (2011).

243. Chae, H. Z., Chung, S. J. & Rhee, S. G. Thioredoxin-dependent peroxide reductase from yeast. *The Journal of biological chemistry* **269**, 27670-27678 (1994).

244. Lee, B. C., Dikiy, A., Kim, H. Y. & Gladyshev, V. N. Functions and evolution of selenoprotein methionine sulfoxide reductases. *Biochimica et biophysica acta* **1790**, 1471-1477 (2009).

245. Meuillet, E. J., Mahadevan, D., Berggren, M., Coon, A. & Powis, G. Thioredoxin-1 binds to the C2 domain of PTEN inhibiting PTEN's lipid phosphatase activity and membrane binding: a mechanism for the functional loss of PTEN's tumor suppressor activity. *Archives of biochemistry and biophysics* **429**, 123-133 (2004).

246. Lu, J. & Holmgren, A. Thioredoxin system in cell death progression. *Antioxidants & redox signaling* **17**, 1738-1747 (2012).

247. Saitoh, M. *et al.* Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *The EMBO journal* **17**, 2596-2606 (1998).

248. Sumbayev, V. V. S-nitrosylation of thioredoxin mediates activation of apoptosis signal-regulating kinase 1. *Archives of biochemistry and biophysics* **415**, 133-136 (2003).

249. Hwang, J. *et al.* The structural basis for the negative regulation of thioredoxin by thioredoxin-interacting protein. *Nature communications* **5**, 2958 (2014).

250. Spindel, O. N., World, C. & Berk, B. C. Thioredoxin interacting protein: redox dependent and independent regulatory mechanisms. *Antioxidants & redox signaling* **16**, 587-596 (2012).

251. Yoshihara, E. *et al.* Thioredoxin/Txnip: redoxisome, as a redox switch for the pathogenesis of diseases. *Frontiers in immunology* **4**, 514 (2014).

252. Yoshihara, E., Chen, Z., Matsuo, Y., Masutani, H. & Yodoi, J. Thiol redox transitions by thioredoxin and thioredoxin-binding protein-2 in cell signaling. *Methods in enzymology* **474**, 67-82 (2010).

253. Arner, E. S. Focus on mammalian thioredoxin reductases--important selenoproteins with versatile functions. *Biochimica et biophysica acta* **1790**, 495-526 (2009).

254. Tamura, T. & Stadtman, T. C. A new selenoprotein from human lung adenocarcinoma cells: purification, properties, and thioredoxin reductase activity. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 1006-1011 (1996).

255. Zhong, L. & Holmgren, A. Essential role of selenium in the catalytic activities of mammalian thioredoxin reductase revealed by characterization of recombinant enzymes with selenocysteine mutations. *The Journal of biological chemistry* **275**, 18121-18128 (2000).

256. Johansson, L., Gafvelin, G. & Arner, E. S. Selenocysteine in proteins-properties and biotechnological use. *Biochimica et biophysica acta* **1726**, 1-13 (2005).

257. Jeong, W., Yoon, H. W., Lee, S. R. & Rhee, S. G. Identification and characterization of TRP14, a thioredoxin-related protein of 14 kDa. New insights into the specificity of thioredoxin function. *The Journal of biological chemistry* **279**, 3142-3150 (2004).

258. Lundstrom-Ljung, J., Birnbach, U., Rupp, K., Soling, H. D. & Holmgren, A. Two resident ER-proteins, CaBP1 and CaBP2, with thioredoxin domains, are substrates for thioredoxin reductase: comparison with protein disulfide isomerase. *FEBS letters* **357**, 305-308 (1995).

259. Johansson, C., Lillig, C. H. & Holmgren, A. Human mitochondrial glutaredoxin reduces S-glutathionylated proteins with high affinity accepting electrons from either glutathione or thioredoxin reductase. *The Journal of biological chemistry* **279**, 7537-7543 (2004).

260. Luthman, M. & Holmgren, A. Rat liver thioredoxin and thioredoxin reductase: purification and characterization. *Biochemistry* **21**, 6628-6633 (1982).

261. Kumar, S., Bjornstedt, M. & Holmgren, A. Selenite is a substrate for calf thymus thioredoxin reductase and thioredoxin and elicits a large non-stoichiometric oxidation of NADPH in the presence of oxygen. *Eur J Biochem* **207**, 435-439 (1992).

262. Bjornstedt, M., Kumar, S. & Holmgren, A. Selenodiglutathione is a highly efficient oxidant of reduced thioredoxin and a substrate for mammalian thioredoxin reductase. *The Journal of biological chemistry* **267**, 8030-8034 (1992).

263. Zhao, R., Masayasu, H. & Holmgren, A. Ebselen: a substrate for human thioredoxin reductase strongly stimulating its hydroperoxide reductase activity and a superfast thioredoxin oxidant. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 8579-8584 (2002).

264. Bjornstedt, M., Hamberg, M., Kumar, S., Xue, J. & Holmgren, A. Human thioredoxin reductase directly reduces lipid hydroperoxides by NADPH and selenocystine strongly stimulates the reaction via catalytically generated selenols. *The Journal of biological chemistry* **270**, 11761-11764 (1995).

265. Arner, E. S., Nordberg, J. & Holmgren, A. Efficient reduction of lipoamide and lipoic acid by mammalian thioredoxin reductase. *Biochemical and biophysical research communications* **225**, 268-274 (1996).

266. May, J. M., Mendiratta, S., Hill, K. E. & Burk, R. F. Reduction of dehydroascorbate to ascorbate by the selenoenzyme thioredoxin reductase. *The Journal of biological chemistry* **272**, 22607-22610 (1997).

267. Cenas, N. *et al.* Interactions of quinones with thioredoxin reductase — A challenge to the antioxidant role of the mammalian selenoprotein. *J. Biol. Chem.* **279**, 2583-2592 (2004).

268. Lu, J. *et al.* Inhibition of Mammalian thioredoxin reductase by some flavonoids: implications for myricetin and quercetin anticancer activity. *Cancer research* **66**, 4410-4418 (2006).

269. Fang, J., Lu, J. & Holmgren, A. Thioredoxin reductase is irreversibly modified by curcumin: a novel molecular mechanism for its anticancer activity. *The Journal of biological chemistry* **280**, 25284-25290 (2005).

270. Hu, Y. *et al.* Glutathione- and thioredoxin-related enzymes are modulated by sulfur-containing chemopreventive agents. *Biological chemistry* **388**, 1069-1081 (2007).

271. Wallenborg, K. *et al.* Red wine triggers cell death and thioredoxin reductase inhibition: effects beyond resveratrol and SIRT1. *Exp Cell Res* **315**, 1360-1371 (2009).

272. Fang, J. & Holmgren, A. Inhibition of thioredoxin and thioredoxin reductase by 4-hydroxy-2-nonenal in vitro and in vivo. *Journal of the American Chemical Society* **128**, 1879-1885 (2006).

273. Gromer, S., Arscott, L. D., Williams, C. H., Jr., Schirmer, R. H. & Becker, K. Human placenta thioredoxin reductase. Isolation of the selenoenzyme, steady state kinetics, and inhibition by therapeutic gold compounds. *The Journal of biological chemistry* **273**, 20096-20101 (1998).

274. Kean, W. F. & Kean, I. R. Clinical pharmacology of gold. *Inflammopharmacology* **16**, 112-125 (2008).

275. Kabuyama, Y. *et al.* Involvement of thioredoxin reductase 1 in the regulation of redox balance and viability of rheumatoid synovial cells. *Biochemical and biophysical research communications* **367**, 491-496 (2008).

276. Roder, C. & Thomson, M. J. Auranofin: repurposing an old drug for a golden new age. *Drugs in R&D* **15**, 13-20 (2015).

277. Arner, E. S. *et al.* Analysis of the inhibition of mammalian thioredoxin, thioredoxin reductase, and glutaredoxin by cis-diamminedichloroplatinum (II) and its major metabolite, the glutathione-platinum complex. *Free radical biology & medicine* **31**, 1170-1178 (2001).

278. Witte, A. B., Anestal, K., Jerremalm, E., Ehrsson, H. & Arner, E. S. Inhibition of thioredoxin reductase but not of glutathione reductase by the major classes of alkylating and platinum-containing anticancer compounds. *Free radical biology & medicine* **39**, 696-703 (2005).

279. Rosenberg, B., Vancamp, L. & Krigas, T. Inhibition of Cell Division in Escherichia Coli by Electrolysis Products from a Platinum Electrode. *Nature* **205**, 698-699 (1965).

280. Sasada, T. *et al.* Possible involvement of thioredoxin reductase as well as thioredoxin in cellular sensitivity to cis-diamminedichloroplatinum (II). *Free radical biology & medicine* **27**, 504-514 (1999).

281. Berndtsson, M. *et al.* Acute apoptosis by cisplatin requires induction of reactive oxygen species but is not associated with damage to nuclear DNA. *International journal of cancer. Journal international du cancer* **120**, 175-180 (2007).

282. Mandic, A. *et al.* Calpain-mediated Bid cleavage and calpain-independent Bak modulation: two separate pathways in cisplatin-induced apoptosis. *Molecular and cellular biology* **22**, 3003-3013 (2002).

283. Han, X., Sun, J., Wang, Y. & He, Z. Recent Advances in Platinum (IV) Complex-Based Delivery Systems to Improve Platinum (II) Anticancer Therapy. *Medicinal research reviews* **35**, 1268-1299 (2015).

284. Gandin, V. & Fernandes, A. P. Metal- and Semimetal-Containing Inhibitors of Thioredoxin Reductase as Anticancer Agents. *Molecules* **20**, 12732-12756 (2015).

285. Anestal, K. & Arner, E. S. Rapid induction of cell death by selenium-compromised thioredoxin reductase 1 but not by the fully active enzyme containing selenocysteine. *The Journal of biological chemistry* **278**, 15966-15972 (2003).

286. Anestål, K., Prast-Nielsen, S., Cenas, N. & Arnér, E. S. J. Cell Death by SecTRAPs – Thioredoxin Reductase as a Prooxidant Killer of Cells. *PloS one* **3**, e1846 (2008).

287. Lothrop, A. P., Snider, G. W., Ruggles, E. L. & Hondal, R. J. Why is mammalian thioredoxin reductase 1 so dependent upon the use of selenium? *Biochemistry* **53**, 554-565 (2014).

288. Fritz-Wolf, K., Kehr, S., Stumpf, M., Rahlfs, S. & Becker, K. Crystal structure of the human thioredoxin reductase-thioredoxin complex. *Nature communications* **2**, 383 (2011).

289. Cassidy, P. B. *et al.* Thioredoxin reductase is required for the inactivation of tumor suppressor p53 and for apoptosis induced by endogenous electrophiles. *Carcinogenesis* **27**, 2538-2549 (2006).

290. Liu, Z. *et al.* Inhibition of thioredoxin reductase by mansonone F analogues: Implications for anticancer activity. *Chemico-biological interactions* **177**, 48-57 (2009).

291. Iverson, S. V. *et al.* A Txnrd1-dependent metabolic switch alters hepatic lipogenesis, glycogen storage, and detoxification. *Free radical biology & medicine* **63**, 369-380 (2013).

292. Duan, D., Zhang, B., Yao, J., Liu, Y. & Fang, J. Shikonin targets cytosolic thioredoxin reductase to induce ROS-mediated apoptosis in human promyelocytic leukemia HL-60 cells. *Free radical biology & medicine* **70**, 182-193 (2014).

293. Sachweh, M. C. *et al.* Redox effects and cytotoxic profiles of MJ25 and auranofin towards malignant melanoma cells. *Oncotarget* **6**, 16488-16506 (2015).

294. Hwang, C., Sinskey, A. J. & Lodish, H. F. Oxidized redox state of glutathione in the endoplasmic reticulum. *Science* **257**, 1496-1502 (1992).

295. Meredith, M. J. & Reed, D. J. Status of the mitochondrial pool of glutathione in the isolated hepatocyte. *The Journal of biological chemistry* **257**, 3747-3753 (1982).

296. Griffith, O. W. Biologic and pharmacologic regulation of mammalian glutathione synthesis. *Free radical biology & medicine* **27**, 922-935 (1999).

297. Grek, C. L., Zhang, J., Manevich, Y., Townsend, D. M. & Tew, K. D. Causes and consequences of cysteine S-glutathionylation. *The Journal of biological chemistry* **288**, 26497-26504 (2013).

298. Fratelli, M., Gianazza, E. & Ghezzi, P. Redox proteomics: identification and functional role of glutathionylated proteins. *Expert review of proteomics* **1**, 365-376 (2004).

299. Mieyal, J. J. & Chock, P. B. Posttranslational modification of cysteine in redox signaling and oxidative stress: Focus on s-glutathionylation. *Antioxidants & redox signaling* **16**, 471-475 (2012).

300. Hayes, J. D., Flanagan, J. U. & Jowsey, I. R. Glutathione transferases. *Annual review of pharmacology and toxicology* **45**, 51-88 (2005).

301. Mannervik, B., Board, P. G., Hayes, J. D., Listowsky, I. & Pearson, W. R. Nomenclature for mammalian soluble glutathione transferases. *Methods in enzymology* **401**, 1-8 (2005).

302. Wu, B. & Dong, D. Human cytosolic glutathione transferases: structure, function, and drug discovery. *Trends in pharmacological sciences* **33**, 656-668 (2012).

303. Pompella, A., Visvikis, A., Paolicchi, A., De Tata, V. & Casini, A. F. The changing faces of glutathione, a cellular protagonist. *Biochemical pharmacology* **66**, 1499-1503 (2003).

304. van Bladeren, P. J. Formation of toxic metabolites from drugs and other xenobiotics by glutathione conjugation. *Trends in pharmacological sciences* **9**, 295-299 (1988).

305. Tew, K. D. TLK-286: a novel glutathione S-transferase-activated prodrug. *Expert opinion on investigational drugs* **14**, 1047-1054 (2005).

306. Tew, K. D. & Townsend, D. M. Glutathione-s-transferases as determinants of cell survival and death. *Antioxidants & redox signaling* **17**, 1728-1737 (2012).

307. Brigelius-Flohe, R. & Maiorino, M. Glutathione peroxidases. *Biochimica et biophysica acta* **1830**, 3289-3303 (2013).

308. Toppo, S., Flohe, L., Ursini, F., Vanin, S. & Maiorino, M. Catalytic mechanisms and specificities of glutathione peroxidases: variations of a basic scheme. *Biochimica et biophysica acta* **1790**, 1486-1500 (2009).

309. Flohe, L., Gunzler, W. A. & Schock, H. H. Glutathione peroxidase: a selenoenzyme. *FEBS letters* **32**, 132-134 (1973).

310. McClung, J. P. *et al.* Development of insulin resistance and obesity in mice overexpressing cellular glutathione peroxidase. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 8852-8857 (2004).

311. Seiler, A. *et al.* Glutathione peroxidase 4 senses and translates oxidative stress into 12/15-lipoxygenase dependent- and AIF-mediated cell death. *Cell metabolism* **8**, 237-248 (2008).

312. Holmgren, A. Hydrogen donor system for Escherichia coli ribonucleoside-diphosphate reductase dependent upon glutathione. *Proceedings of the National Academy of Sciences of the United States of America* **73**, 2275-2279 (1976).

313. Holmgren, A. Glutathione-dependent synthesis of deoxyribonucleotides. Purification and characterization of glutaredoxin from Escherichia coli. *The Journal of biological chemistry* **254**, 3664-3671 (1979).

314. Holmgren, A. Glutathione-dependent synthesis of deoxyribonucleotides. Characterization of the enzymatic mechanism of Escherichia coli glutaredoxin. *The Journal of biological chemistry* **254**, 3672-3678 (1979).

315. Lillig, C. H. & Berndt, C. Glutaredoxins in thiol/disulfide exchange. *Antioxidants & redox signaling* **18**, 1654-1665 (2013).

316. Bandyopadhyay, S., Starke, D. W., Mieyal, J. J. & Gronostajski, R. M. Thioltransferase (glutaredoxin) reactivates the DNA-binding activity of oxidation-inactivated nuclear factor I. *The Journal of biological chemistry* **273**, 392-397 (1998).

317. Barrett, W. C. *et al.* Regulation of PTP1B via glutathionylation of the active site cysteine 215. *Biochemistry* **38**, 6699-6705 (1999).

318. Song, J. J. *et al.* Role of glutaredoxin in metabolic oxidative stress. Glutaredoxin as a sensor of oxidative stress mediated by H2O2. *The Journal of biological chemistry* **277**, 46566-46575 (2002).

319. Pan, S. & Berk, B. C. Glutathiolation regulates tumor necrosis factor-alpha-induced caspase-3 cleavage and apoptosis: key role for glutaredoxin in the death pathway. *Circulation research* **100**, 213-219 (2007).

320. Hashemy, S. I., Johansson, C., Berndt, C., Lillig, C. H. & Holmgren, A. Oxidation and S-nitrosylation of cysteines in human cytosolic and mitochondrial glutaredoxins: effects on structure and activity. *The Journal of biological chemistry* **282**, 14428-14436 (2007).

321. Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144**, 646-674 (2011).

322. Luo, J., Solimini, N. L. & Elledge, S. J. Principles of cancer therapy: oncogene and non-oncogene addiction. *Cell* **136**, 823-837 (2009).

323. Trachootham, D., Alexandre, J. & Huang, P. Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? *Nature reviews. Drug discovery* **8**, 579-591 (2009).

324. Schumacker, P. T. Reactive oxygen species in cancer cells: live by the sword, die by the sword. *Cancer cell* **10**, 175-176 (2006).

325. Cairns, R. A., Harris, I. S. & Mak, T. W. Regulation of cancer cell metabolism. *Nature reviews. Cancer* **11**, 85-95 (2011).

326. DeNicola, G. M. *et al.* Oncogene-induced Nrf2 transcription promotes ROS detoxification and tumorigenesis. *Nature* **475**, 106-109 (2011).

327. Diehn, M. *et al.* Association of reactive oxygen species levels and radioresistance in cancer stem cells. *Nature* **458**, 780-783 (2009).

328. Gorrini, C., Harris, I. S. & Mak, T. W. Modulation of oxidative stress as an anticancer strategy. *Nature reviews. Drug discovery* **12**, 931-947 (2013).

329. Wondrak, G. T. Redox-directed cancer therapeutics: molecular mechanisms and opportunities. *Antioxidants & redox signaling* **11**, 3013-3069 (2009).

330. Conklin, K. A. Chemotherapy-associated oxidative stress: impact on chemotherapeutic effectiveness. *Integrative cancer therapies* **3**, 294-300 (2004).

331. Arner, E. S. & Holmgren, A. The thioredoxin system in cancer. *Semin Cancer Biol* **16**, 420-426 (2006).

332. Biaglow, J. E. & Miller, R. A. The thioredoxin reductase/thioredoxin system: novel redox targets for cancer therapy. *Cancer biology & therapy* **4**, 6-13 (2005).

333. Urig, S. & Becker, K. On the potential of thioredoxin reductase inhibitors for cancer therapy. *Semin Cancer Biol* **16**, 452-465 (2006).

334. Lu, J., Chew, E. H. & Holmgren, A. Targeting thioredoxin reductase is a basis for cancer therapy by arsenic trioxide. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 12288-12293 (2007).

335. Arner, E. S., Bjornstedt, M. & Holmgren, A. 1-Chloro-2,4-dinitrobenzene is an irreversible inhibitor of human thioredoxin reductase. Loss of thioredoxin disulfide reductase activity is accompanied by a large increase in NADPH oxidase activity. *The Journal of biological chemistry* **270**, 3479-3482 (1995).

336. Eriksson, S. E., Prast-Nielsen, S., Flaberg, E., Szekely, L. & Arner, E. S. High levels of thioredoxin reductase 1 modulate drug-specific cytotoxic efficacy. *Free radical biology & medicine* **47**, 1661-1671 (2009).

337. Peng, X. *et al.* APR-246/PRIMA-1MET inhibits thioredoxin reductase 1 and converts the enzyme to a dedicated NADPH oxidase. *Cell death & disease* **4**, e881 (2013).

338. Hedstrom, E., Eriksson, S., Zawacka-Pankau, J., Arner, E. S. & Selivanova, G. p53-dependent inhibition of TrxR1 contributes to the tumor-specific induction of apoptosis by RITA. *Cell cycle* **8**, 3584-3591 (2009).

339. Mandal, P. K. *et al.* Loss of thioredoxin reductase 1 renders tumors highly susceptible to pharmacologic glutathione deprivation. *Cancer research* **70**, 9505-9514 (2010).

340. Fath, M. A., Ahmad, I. M., Smith, C. J., Spence, J. & Spitz, D. R. Enhancement of carboplatin-mediated lung cancer cell killing by simultaneous disruption of glutathione and thioredoxin metabolism. *Clinical cancer research : an official journal of the American Association for Cancer Research* **17**, 6206-6217 (2011).

341. Harris, I. S. *et al.* Glutathione and thioredoxin antioxidant pathways synergize to drive cancer initiation and progression. *Cancer cell* **27**, 211-222 (2015).

342. Prigge, J. R. *et al.* Hepatocyte DNA replication in growing liver requires either glutathione or a single allele of txnrd1. *Free radical biology & medicine* **52**, 803-810 (2012).

343. Lemmon, M. A. & Schlessinger, J. Cell signaling by receptor tyrosine kinases. *Cell* **141**, 1117-1134 (2010).

344. Ullrich, A. & Schlessinger, J. Signal transduction by receptors with tyrosine kinase activity. *Cell* **61**, 203-212 (1990).

345. Schieber, M. & Chandel, N. S. ROS function in redox signaling and oxidative stress. *Current biology : CB* **24**, R453-462 (2014).

346. Cantley, L. C. The phosphoinositide 3-kinase pathway. *Science* **296**, 1655-1657 (2002).

347. Ostman, A., Hellberg, C. & Bohmer, F. D. Protein-tyrosine phosphatases and cancer. *Nature reviews. Cancer* **6**, 307-320 (2006).

348. Ostman, A., Frijhoff, J., Sandin, A. & Bohmer, F. D. Regulation of protein tyrosine phosphatases by reversible oxidation. *Journal of biochemistry* **150**, 345-356 (2011).

349. Jia, Z., Barford, D., Flint, A. J. & Tonks, N. K. Structural basis for phosphotyrosine peptide recognition by protein tyrosine phosphatase 1B. *Science* **268**, 1754-1758 (1995).

350. Paulsen, C. E. *et al.* Peroxide-dependent sulfenylation of the EGFR catalytic site enhances kinase activity. *Nature chemical biology* **8**, 57-64 (2012).

351. Bae, Y. S. *et al.* Platelet-derived growth factor-induced H(2)O(2) production requires the activation of phosphatidylinositol 3-kinase. *The Journal of biological chemistry* **275**, 10527-10531 (2000).

352. Frijhoff, J. *et al.* The mitochondrial reactive oxygen species regulator p66Shc controls PDGF-induced signaling and migration through protein tyrosine phosphatase oxidation. *Free radical biology & medicine* **68**, 268-277 (2014).

353. Sandin, A. *et al.* Hypoxia followed by re-oxygenation induces oxidation of tyrosine phosphatases. *Cell Signal* **23**, 820-826 (2011).

354. Kuznetsov, A. V. *et al.* Mitochondrial ROS production under cellular stress: comparison of different detection methods. *Analytical and bioanalytical chemistry* **400**, 2383-2390 (2011).

355. Conrad, M. *et al.* 12/15-lipoxygenase-derived lipid peroxides control receptor tyrosine kinase signaling through oxidation of protein tyrosine phosphatases. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 15774-15779 (2010).

356. Covey, T. M., Edes, K. & Fitzpatrick, F. A. Akt activation by arachidonic acid metabolism occurs via oxidation and inactivation of PTEN tumor suppressor. *Oncogene* **26**, 5784-5792 (2007).

357. Alonso, A. *et al.* Protein tyrosine phosphatases in the human genome. *Cell* **117**, 699-711 (2004).

358. Tonks, N. K., Diltz, C. D. & Fischer, E. H. Purification of the major protein-tyrosine-phosphatases of human placenta. *The Journal of biological chemistry* **263**, 6722-6730 (1988).

359. Tonks, N. K., Diltz, C. D. & Fischer, E. H. Characterization of the major protein-tyrosine-phosphatases of human placenta. *The Journal of biological chemistry* **263**, 6731-6737 (1988).

360. Frangioni, J. V., Beahm, P. H., Shifrin, V., Jost, C. A. & Neel, B. G. The nontransmembrane tyrosine phosphatase PTP-1B localizes to the endoplasmic reticulum via its 35 amino acid C-terminal sequence. *Cell* **68**, 545-560 (1992).

361. Bakke, J. & Haj, F. G. Protein-tyrosine phosphatase 1B substrates and metabolic regulation. *Seminars in cell & developmental biology* **37**, 58-65 (2015).

362. Feldhammer, M., Uetani, N., Miranda-Saavedra, D. & Tremblay, M. L. PTP1B: a simple enzyme for a complex world. *Critical reviews in biochemistry and molecular biology* **48**, 430-445 (2013).

363. Elchebly, M. *et al.* Increased insulin sensitivity and obesity resistance in mice lacking the protein tyrosine phosphatase-1B gene. *Science* **283**, 1544-1548 (1999).

364. Kenner, K. A., Anyanwu, E., Olefsky, J. M. & Kusari, J. Protein-tyrosine phosphatase 1B is a negative regulator of insulin- and insulin-like growth factor-I-stimulated signaling. *The Journal of biological chemistry* **271**, 19810-19816 (1996).

365. Schwertassek, U. *et al.* Reactivation of oxidized PTP1B and PTEN by thioredoxin 1. *The FEBS journal* **281**, 3545-3558 (2014).

366. Dagnell, M. *et al.* Selective activation of oxidized PTP1B by the thioredoxin system modulates PDGF-beta receptor tyrosine kinase signaling. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 13398-13403 (2013).

367. Wagner, M. J., Stacey, M. M., Liu, B. A. & Pawson, T. Molecular mechanisms of SH2- and PTB-domain-containing proteins in receptor tyrosine kinase signaling. *Cold Spring Harbor perspectives in biology* **5**, a008987 (2013).

368. Pao, L. I., Badour, K., Siminovitch, K. A. & Neel, B. G. Nonreceptor protein-tyrosine phosphatases in immune cell signaling. *Annual review of immunology* **25**, 473-523 (2007).

369. Neel, B. G., Gu, H. & Pao, L. The 'Shp'ing news: SH2 domain-containing tyrosine phosphatases in cell signaling. *Trends in biochemical sciences* **28**, 284-293 (2003).

370. Leslie, N. R. *et al.* Redox regulation of PI 3-kinase signalling via inactivation of PTEN. *The EMBO journal* **22**, 5501-5510 (2003).

371. Salmena, L., Carracedo, A. & Pandolfi, P. P. Tenets of PTEN tumor suppression. *Cell* **133**, 403-414 (2008).

372. Dammeyer, P. & Arner, E. S. Human Protein Atlas of redox systems - what can be learnt? *Biochimica et biophysica acta* **1810**, 111-138 (2011).

373. Jiang, S., Zhang, S., Vuthiphandchai, V. & Nimrat, S. Human TRP14 gene homologue from amphioxus Branchiostoma belcheri: identification, evolution, expression and functional characterization. *J Anat* **210**, 555-564 (2007).

374. Wang, Q. *et al.* Responses of thioredoxin 1 and thioredoxin-related protein 14 mRNAs to cadmium and copper stresses in Venerupis philippinarum. *Comparative biochemistry and physiology. Toxicology & pharmacology : CBP* **154**, 154-160 (2011).

375. Wei, J., Ji, H., Guo, M., Yan, Y. & Qin, Q. Identification and characterization of TRP14, a thioredoxin-related protein of 14 kDa from orange-spotted grouper, Epinephelus coioides. *Fish & shellfish immunology* **35**, 1670-1676 (2013).

376. Jeong, W., Chang, T. S., Boja, E. S., Fales, H. M. & Rhee, S. G. Roles of TRP14, a thioredoxin-related protein in tumor necrosis factor-alpha signaling pathways. *The Journal of biological chemistry* **279**, 3151-3159 (2004).

377. Lee, S. R. *et al.* Reversible inactivation of the tumor suppressor PTEN by H2O2. *The Journal of biological chemistry* **277**, 20336-20342 (2002).

378. Funato, Y. & Miki, H. Reversible oxidation of PRL family protein-tyrosine phosphatases. *Methods* **65**, 184-189 (2014).

379. Jeong, W., Jung, Y., Kim, H., Park, S. J. & Rhee, S. G. Thioredoxin-related protein 14, a new member of the thioredoxin family with disulfide reductase activity: implication in the redox regulation of TNF-alpha signaling. *Free radical biology & medicine* **47**, 1294-1303 (2009).

380. Woo, J. R. *et al.* Structural basis of cellular redox regulation by human TRP14. *The Journal of biological chemistry* **279**, 48120-48125 (2004).

381. Eckers, E., Bien, M., Stroobant, V., Herrmann, J. M. & Deponte, M. Biochemical characterization of dithiol glutaredoxin 8 from Saccharomyces cerevisiae: the catalytic redox mechanism redux. *Biochemistry* **48**, 1410-1423 (2009).

382. Staal, F. J., Roederer, M., Herzenberg, L. A. & Herzenberg, L. A. Intracellular thiols regulate activation of nuclear factor kappa B and transcription of human immunodeficiency virus. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 9943-9947 (1990).

383. Schreck, R., Rieber, P. & Baeuerle, P. A. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. *The EMBO journal* **10**, 2247-2258 (1991).

384. Hayashi, T., Ueno, Y. & Okamoto, T. Oxidoreductive regulation of nuclear factor kappa B. Involvement of a cellular reducing catalyst thioredoxin. *The Journal of biological chemistry* **268**, 11380-11388 (1993).

385. Matthews, J. R., Wakasugi, N., Virelizier, J. L., Yodoi, J. & Hay, R. T. Thioredoxin regulates the DNA binding activity of NF-kappa B by reduction of a disulphide bond involving cysteine 62. *Nucleic acids research* **20**, 3821-3830 (1992).

386. Jung, Y., Kim, H., Min, S. H., Rhee, S. G. & Jeong, W. Dynein light chain LC8 negatively regulates NF-kappaB through the redox-dependent interaction with IkappaBalpha. *The Journal of biological chemistry* **283**, 23863-23871 (2008).

387. Park, H. S. *et al.* Cutting edge: direct interaction of TLR4 with NAD(P)H oxidase 4 isozyme is essential for lipopolysaccharide-induced production of reactive oxygen species and activation of NF-kappa B. *Journal of immunology* **173**, 3589-3593 (2004).

388. Yazdanpanah, B. *et al.* Riboflavin kinase couples TNF receptor 1 to NADPH oxidase. *Nature* **460**, 1159-1163 (2009).

389. Gloire, G., Legrand-Poels, S. & Piette, J. NF-kappaB activation by reactive oxygen species: fifteen years later. *Biochemical pharmacology* **72**, 1493-1505 (2006).

390. Oliveira-Marques, V., Marinho, H. S., Cyrne, L. & Antunes, F. Role of hydrogen peroxide in NF-kappaB activation: from inducer to modulator. *Antioxidants & redox signaling* **11**, 2223-2243 (2009).

391. Hong, S. *et al.* TRP14 inhibits osteoclast differentiation via its catalytic activity. *Molecular and cellular biology* **34**, 3515-3524 (2014).

392. Zhang, S. F. *et al.* TXNDC17 promotes paclitaxel resistance via inducing autophagy in ovarian cancer. *Autophagy*, 0 (2015).

393. Singh, A. *et al.* Dysfunctional KEAP1-NRF2 interaction in non-small-cell lung cancer. *PLoS medicine* **3**, e420 (2006).

394. Klaman, L. D. *et al.* Increased energy expenditure, decreased adiposity, and tissue-specific insulin sensitivity in protein-tyrosine phosphatase 1B-deficient mice. *Molecular and cellular biology* **20**, 5479-5489 (2000).

395. Arner, E. S., Zhong, L. & Holmgren, A. Preparation and assay of mammalian thioredoxin and thioredoxin reductase. *Methods in enzymology* **300**, 226-239 (1999).

396. Arnér, E. S. J. & Holmgren, A. in *Current Protocols in Toxicology* (eds M. Maines, L. Costa, D. Reed, & S. Sassa) 7.4.1-7.4.14 (John Wiley & Sons, Inc., 2000).

397. Montano, S. J., Lu, J., Gustafsson, T. N. & Holmgren, A. Activity assays of mammalian thioredoxin and thioredoxin reductase: fluorescent disulfide substrates, mechanisms, and use with tissue samples. *Analytical biochemistry* **449**, 139-146 (2014).

398. Holmgren, A. & Åslund, F. Glutaredoxin. *Meth. Enzymol.* **252**, 283-292 (1995).

399. Schonhoff, C. M. & Benhar, M. Analysis of protein S-nitrosylation. *Current protocols in protein science / editorial board, John E. Coligan ... [et al.]* **Chapter 14**, Unit14 16 (2011).

400. Bates, J. N. Nitric oxide measurement by chemiluminescence detection. *Neuroprotocols* **1**, 141-149 (1992).

401. Singh, R. J., Hogg, N., Joseph, J. & Kalyanaraman, B. Mechanism of nitric oxide release from S-nitrosothiols. *The Journal of biological chemistry* **271**, 18596-18603 (1996).

402. Cook, J. A. *et al.* Convenient colorimetric and fluorometric assays for S-nitrosothiols. *Analytical biochemistry* **238**, 150-158 (1996).

403. Fire, A. *et al.* Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* **391**, 806-811 (1998).

404. Cullen, B. R. RNAi the natural way. *Nature genetics* **37**, 1163-1165 (2005).

405. Paddison, P. J., Caudy, A. A., Bernstein, E., Hannon, G. J. & Conklin, D. S. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes & development* **16**, 948-958 (2002).

406. Omata, Y. *et al.* Sublethal concentrations of diverse gold compounds inhibit mammalian cytosolic thioredoxin reductase (TrxR1). *Toxicology in vitro : an international journal published in association with BIBRA* **20**, 882-890 (2006).

407. Miller, R. P., Tadagavadi, R. K., Ramesh, G. & Reeves, W. B. Mechanisms of Cisplatin nephrotoxicity. *Toxins* **2**, 2490-2518 (2010).

408. Siddik, Z. H. Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene* **22**, 7265-7279 (2003).

409. Rabik, C. A. & Dolan, M. E. Molecular mechanisms of resistance and toxicity associated with platinating agents. *Cancer treatment reviews* **33**, 9-23 (2007).

410. Kapdi, A. R. & Fairlamb, I. J. Anti-cancer palladium complexes: a focus on PdX2L2, palladacycles and related complexes. *Chemical Society reviews* **43**, 4751-4777 (2014).

411. Parrilha, G. L. *et al.* Metal complexes with 2-acetylpyridine-N(4)-orthochlorophenylthiosemicarbazone: cytotoxicity and effect on the enzymatic activity of thioredoxin reductase and glutathione reductase. *European journal of medicinal chemistry* **84**, 537-544 (2014).

412. Caires, A. C. Recent advances involving palladium (II) complexes for the cancer therapy. *Anti-cancer agents in medicinal chemistry* **7**, 484-491 (2007).

413. Apps, M. G., Choi, E. H. & Wheate, N. J. The state-of-play and future of platinum drugs. *Endocrine-related cancer* **22**, R219-233 (2015).

414. Lo, Y. C., Ko, T. P., Su, W. C., Su, T. L. & Wang, A. H. Terpyridine-platinum(II) complexes are effective inhibitors of mammalian topoisomerases and human thioredoxin reductase 1. *Journal of inorganic biochemistry* **103**, 1082-1092 (2009).

415. Nordberg, J., Zhong, L., Holmgren, A. & Arner, E. S. Mammalian thioredoxin reductase is irreversibly inhibited by dinitrohalobenzenes by alkylation of both the redox active selenocysteine and its neighboring cysteine residue. *The Journal of biological chemistry* **273**, 10835-10842 (1998).

416. Cheng, Q. *et al.* The selenium-independent inherent pro-oxidant NADPH oxidase activity of mammalian thioredoxin reductase and its selenium-dependent direct peroxidase activities. *The Journal of biological chemistry* **285**, 21708-21723 (2010).

417. Peng, X., Xu, J. & Arner, E. S. Thiophosphate and selenite conversely modulate cell death induced by glutathione depletion or cisplatin: effects related to activity and Sec contents of thioredoxin reductase. *The Biochemical journal* **447**, 167-174 (2012).

418. Prast-Nielsen, S. *et al.* Inhibition of thioredoxin reductase 1 by porphyrins and other small molecules identified by a high-throughput screening assay. *Free radical biology & medicine* **50**, 1114-1123 (2011).

419. Dasari, S. & Tchounwou, P. B. Cisplatin in cancer therapy: molecular mechanisms of action. *European journal of pharmacology* **740**, 364-378 (2014).

420. Jan, Y. H., Heck, D. E., Casillas, R. P., Laskin, D. L. & Laskin, J. D. Thioredoxin Cross-Linking by Nitrogen Mustard in Lung Epithelial Cells: Formation of Multimeric Thioredoxin/Thioredoxin Reductase Complexes and Inhibition of Disulfide Reduction. *Chemical research in toxicology* **28**, 2091-2103 (2015).

421. Demoulin, J. B. & Essaghir, A. PDGF receptor signaling networks in normal and cancer cells. *Cytokine & growth factor reviews* **25**, 273-283 (2014).

422. Meng, T. C., Fukada, T. & Tonks, N. K. Reversible oxidation and inactivation of protein tyrosine phosphatases in vivo. *Molecular cell* **9**, 387-399 (2002).

423. Baumer, A. T. *et al.* Phosphatidylinositol 3-kinase-dependent membrane recruitment of Rac-1 and p47phox is critical for alpha-platelet-derived growth factor receptor-induced production of reactive oxygen species. *The Journal of biological chemistry* **283**, 7864-7876 (2008).

424. Boureux, A., Furstoss, O., Simon, V. & Roche, S. Abl tyrosine kinase regulates a Rac/JNK and a Rac/Nox pathway for DNA synthesis and Myc expression induced by growth factors. *Journal of cell science* **118**, 3717-3726 (2005).

425. Leslie, N. R. The redox regulation of PI 3-kinase-dependent signaling. *Antioxidants & redox signaling* **8**, 1765-1774 (2006).

426. Cho, K. J., Seo, J. M. & Kim, J. H. Bioactive lipoxygenase metabolites stimulation of NADPH oxidases and reactive oxygen species. *Molecules and cells* **32**, 1-5 (2011).

427. Chen, C. Y., Willard, D. & Rudolph, J. Redox regulation of SH2-domain-containing protein tyrosine phosphatases by two backdoor cysteines. *Biochemistry* **48**, 1399-1409 (2009).

428. Chen, Y. Y. *et al.* Cysteine S-nitrosylation protects protein-tyrosine phosphatase 1B against oxidation-induced permanent inactivation. *The Journal of biological chemistry* **283**, 35265-35272 (2008).

429. Salmeen, A. *et al.* Redox regulation of protein tyrosine phosphatase 1B involves a sulphenyl-amide intermediate. *Nature* **423**, 769-773 (2003).

430. Karisch, R. *et al.* Global proteomic assessment of the classical protein-tyrosine phosphatome and "Redoxome". *Cell* **146**, 826-840 (2011).

431. Du, Y., Zhang, H., Lu, J. & Holmgren, A. Glutathione and glutaredoxin act as a backup of human thioredoxin reductase 1 to reduce thioredoxin 1 preventing cell death by aurothioglucose. *The Journal of biological chemistry* **287**, 38210-38219 (2012).

432. Auclair, J. R. *et al.* Post-translational modification by cysteine protects Cu/Zn-superoxide dismutase from oxidative damage. *Biochemistry* **52**, 6137-6144 (2013).

433. Chu, F., Ward, N. E. & O'Brian, C. A. PKC isozyme S-cysteinylation by cystine stimulates the pro-apoptotic isozyme PKC delta and inactivates the oncogenic isozyme PKC epsilon. *Carcinogenesis* **24**, 317-325 (2003).

434. Hochgrafe, F. *et al.* S-cysteinylation is a general mechanism for thiol protection of Bacillus subtilis proteins after oxidative stress. *The Journal of biological chemistry* **282**, 25981-25985 (2007).

435. Regazzoni, L. *et al.* Human serum albumin cysteinylation is increased in end stage renal disease patients and reduced by hemodialysis: mass spectrometry studies. *Free radical research* **47**, 172-180 (2013).

436. Forrester, M. T., Foster, M. W., Benhar, M. & Stamler, J. S. Detection of protein S-nitrosylation with the biotin-switch technique. *Free radical biology & medicine* **46**, 119-126 (2009).

437. Pan, J. & Carroll, K. S. Persulfide reactivity in the detection of protein s-sulfhydration. *ACS chemical biology* **8**, 1110-1116 (2013).

438. Boivin, B., Zhang, S., Arbiser, J. L., Zhang, Z. Y. & Tonks, N. K. A modified cysteinyl-labeling assay reveals reversible oxidation of protein tyrosine phosphatases in angiomyolipoma cells. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 9959-9964 (2008).

439. Kim, J. S., Huang, T. Y. & Bokoch, G. M. Reactive oxygen species regulate a slingshot-cofilin activation pathway. *Molecular biology of the cell* **20**, 2650-2660 (2009).

440. Klamt, F. *et al.* Oxidant-induced apoptosis is mediated by oxidation of the actin-regulatory protein cofilin. *Nature cell biology* **11**, 1241-1246 (2009).

441. Valdivia, A., Duran, C. & San Martin, A. The role of Nox-mediated oxidation in the regulation of cytoskeletal dynamics. *Current pharmaceutical design* **21**, 6009-6022 (2015).

442. Trachootham, D., Lu, W., Ogasawara, M. A., Nilsa, R. D. & Huang, P. Redox regulation of cell survival. *Antioxidants & redox signaling* **10**, 1343-1374 (2008).

443. Johansson, K. *et al.* Cross Talk in HEK293 Cells Between Nrf2, HIF, and NF-kappaB Activities upon Challenges with Redox Therapeutics Characterized with Single-Cell Resolution. *Antioxidants & redox signaling* (2015).