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Intricate Aspects of the Thioredoxin System in Redox Signaling

Marcus Cebula

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Front cover: A549 cells expressing v3(Grx)-GFP fusion proteins. Upper left: 2-HMA treated (cytosolic distribution). Upper right: 2-BPA treated (localization to Golgi/ ER). Lower picture: co-localization with actin (red); filopodia are highlighted.

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TO MY FAMILY AND FRIENDS

“Life is nothing but an electron looking for a place to rest”

Albert Szent-Györgyi

(September 16, 1893 – October 22, 1986)

Institutionen för medicinsk biokemi och biofysik

Intricate Aspects of the Thioredoxin System in Redox Signaling

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Abstract

Reversible modifications of redox sensitive protein thiols by reactive oxygen and nitrogen species have emerged as a major posttranslational mechanism that affects the function of the respective proteins and therewith all downstream events. These modifications can be reversed by redox catalysts of which the thioredoxin system forms one of the most prominent. It is ubiquitously expressed and consists of thioredoxin reductase (TrxR) that takes electrons from NADPH to reduce thioredoxin (Trx) as well as a myriad of other substrates. Within this thesis we have studied several aspects of cellular signaling pathways modulated by the Trx system.

Paper I. The Trx system is overexpressed in many types of cancers and considered to contribute to their survival by countering elevated ROS levels that are typical for these cells. Thus, inhibiting TrxR in order to attenuate the antioxidant capacity of cancer cells might tip the balance in favor of ROS induced cell death pathways as a principle of cancer therapy. TrxR1 is a particularly suitable target in this context due to its highly reactive and accessible selenocysteine (Sec) residue within its C-terminal active site. At physiological pH the Sec is mostly de-protonated and thus easily targeted by electrophilic compounds. In characterizing Au, Pt and Pd based salts we found that all inhibited the Sec-depended activity of the enzyme in a specific manner, with Au and Pd being more potent than Pt *in vitro*. In context of cellular TrxR1, however, inhibition and cytotoxicity were mainly dependent on the ligand substituents of the compounds and thus their cellular uptake and metabolism. We furthermore discovered cisplatin triggered covalent complex formation of TrxR1 with either Trx1 or TRP14 (thioredoxin like protein of 14 kDa), which potentially contributes to the mechanism of cisplatin mediated cytotoxicity.

Paper II. TrxR1 has in addition to its main isoform at least five minor splice variants that are distinguished by their N-terminal extensions. These may directly influence the activity of the TrxR1 core module or mediate subcellular localization via potential translocation signals. One of these variants, named "v3" (carrying a unique glutaredoxin domain), was previously shown to associate with the plasma membrane where it provoked dynamic filopodia. Within this study we found that v3 associates with specific membrane raft microdomains upon N-myristoylation and palmitoylation. These membrane structures were shown to serve as signaling platforms, including redox dependent processes, suggesting that v3 is potentially involved in redox signaling.

Paper III. Transcription factors are a specific group of proteins that regulate the rapid transcription of genes. Many are functionally intertwined, activated under redox perturbing conditions and highly controlled by regulatory networks like the thioredoxin and glutathione systems. Signaling pathways leading to their activation are complex and expected to be modulated by numerous factors. In order to simultaneously characterize several transcription factors on single cell level we developed a method that is based on a three-colored fluorescence-based reporter plasmid (pTRAF). We demonstrated the use by quantifying responses of the three medically important transcription factors Nrf2, HIF and NFκB, utilizing HEK293 cells that were subjected to diverse stimulants.

In conclusion, we studied TrxR1 targeting by noble metal based compounds and characterized their ability to transform TrxR1 into its pro-oxidant SecTRAP form as a principle of anti-cancer therapy. We also identified the mechanism behind the intracellular localization of "v3" and show that it is targeted to lipid rafts where it is a potentially important regulator of signaling processes. Finally we developed a tool to study the activation of three redox sensitive, intertwined transcription factors.

Publications

Articles included in this thesis

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

II. **Marcus Cebula**, Naazneen Moolla, Alexio Capovilla and Elias S. J. Arnér.
J *Biol Chem.* 2013; 288:10002-10011.
TXNRD1-encoded v3 is targeted to membrane rafts by N-acylation and induces filopodia independently of its redox active site integrity.

III. Katarina Johansson, **Marcus Cebula**, Olle Rengby, Kristian Dreij, Kristmundur Sigmundsson, Elias S. J. Arnér.
Manuscript.
Simultaneous Determination of Nrf2, HIF and NFκB Activation at Single-Cell Resolution.

Articles not included in this thesis

IV. Irina Pader*, Rajib Sengupta*, **Marcus Cebula**, Jianqiang Xu, Arne Holmgren, Katarina Johansson, Elias S. J. Arnér.
PNAS. 2014; In press.
*Shared first author
Thioredoxin related protein of 14 kDa is an efficient L-cystine reductase and S-denitrosylase.

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.
Manuscript.
The Trp114 residue of thioredoxin reductase 1 is an electron relay sensor for oxidative stress.

Contents

Abbreviations

AP-1	Activator protein 1
ARE	Antioxidant responsive element
ASK-1	Apoptosis signal-regulating kinase 1
ATP	Adenosine-5'-triphosphate
BCL-3	B-cell lymphoma 3
bZIP	Basic Leucine Zipper
CBP	CREB-binding protein
COX-2	Cyclooxygenase-2
Crm1	Chromosome region maintenance 1
CT-B	Cholera toxin subunit B
Cys	Cysteine/ single letter code C
DTT	Dithiothreitol
DUOX	Dual oxidase
EGF	Epidermal growth factor
eIF-4E	Eukaryotic initiation factor-4E
EpRE	Electrophile responsive element
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorting
FAD	Flavin adenine dinucleotide
FIH	Factor inhibiting HIF
Gly	Glycin
GM1	Monosialotetrahexosylganglioside ("prototype" ganglioside)
GPx	Glutathione peroxidase
GR	Glutathione reductase
Grx	Glutaredoxin
GSH/GSSG	Glutathione, reduced/ oxidized form
GST	Glutathione-S-transferase
HIF	Hypoxia-inducible factor
HRE	Hypoxia response element
IκB	Inhibitors of κB
IKK	IκB kinase
IL-1	Interleukin-1
Keap1	Kelch-like ECH-associated protein 1
LPS	Lipopolysaccharides
MAPK	Mitogen-activated protein kinase
MMP-9	Matrix metallopeptidase 9
Msr	Methionine sulfoxide reductase
Mst1/2	Mammalian sterile 20-like kinases 1 and 2
NADPH	Nicotinamide adenine dinucleotide phosphate
NEMO	NFκB essential modulator
NES	Nuclear export signal

NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NFκB-RE	NFκB response element
NLS	Nuclear localization signal
NOS	Nitric oxide synthase
NOX	NADPH oxidase
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
ODDD	Oxygen-dependent degradation domain
p70S6K	p70S6 kinase
PAMP	Pathogen-associated molecular pattern
PDGF	Platelet-derived growth factor
PDI	Protein disulfide isomerase
PHD	Prolyl hydroxylase
PI3K	Phosphatidylinositol 3 kinase
PKC	Protein kinase C
pTRAF	Plasmid for transcription factor reporter activation based upon fluorescence
Prx	Peroxiredoxin
PTEN	Phosphatase and tensin homolog
PTP	Protein tyrosine phosphatase
pVHL	Hippel-Lindau tumor suppressor protein
Ref-1	Redox effector factor-1
RHD	Rel homology domain
RNR	Ribonucleotide reductase
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RSS	Reactive sulfur species
RTK	Receptor tyrosine kinase
Sec	Selenocysteine/ single letter code U
SecTRAP	**S**elenium **c**ompromised **t**hioredoxin **r**eductase-derived **a**poptotic **p**rotein
SHP-1/2	SH2-containing phosphatase 1/2
SOD	Superoxide dismutase
TAD	Transactivation domain
TGR	Thioredoxin glutathione reductase
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
TNFR	Tumor necrosis factor receptor
TRP14	Thioredoxin related protein of 14 kDa
Trx	Thioredoxin
TXNRD	Thioredoxin reductase gene, human
TrxR	Thioredoxin reductase
TXNIP	Thioredoxin interacting protein
VEGF	Vascular endothelial growth factor

1 Introduction

The overall aim of research in redox biology is to understand the coordination, regulation and final consequence of electron flows in living organisms as well as their general organization. As such, it is at the heart of life science and touches almost all areas of biology covering aspects like selenium and sulfur chemistry, oxidative stress, metabolism, free radicals and signaling[1-4].

Its centrally relevant elements are **red**uction-**ox**idation (**redox**) reactions that modify cellular components by either increasing or decreasing their oxidation state, which in turn modulates their respective functions. The realization that especially thiol groups of cysteine residues are redox sensitive and that their modifications are major posttranslational regulators of protein function changed the view to the current understanding that these transient processes are as important and as common as phosphorylation **(Fig. 1)**.

Over the last decades it has been recognized that reactive oxygen and nitrogen species (ROS, RNS) as well as a number of other reactive molecules operate as second messengers in redox pathways. Hydrogen peroxide (H_2O_2) is among all species the most relevant in the context of cell signaling. Several studies suggest that the production, diffusion and life time of ROS in general, and H_2O_2 in particular, is a tightly regulated and highly complex process. But despite its eminent importance, the exact mechanism behind the regulation of specific and efficient oxidation in redox signaling is still not fully understood **(Fig. 1a)**.

The mechanisms to reverse these oxidative modifications are slightly better characterized, but numerous questions still remain before a thorough understanding of this complex processes can be achieved. The two most prominent systems in this context are the glutathione (GSH) and the thioredoxin (Trx) systems with the latter being the focus of this thesis. Both employ coupled redox active enzymes to constantly reduce their respective intracellular substrates. These systems are essential regulators for a myriad of cellular processes as well as for antioxidant defense and redox homeostasis **(Fig. 1b)**.

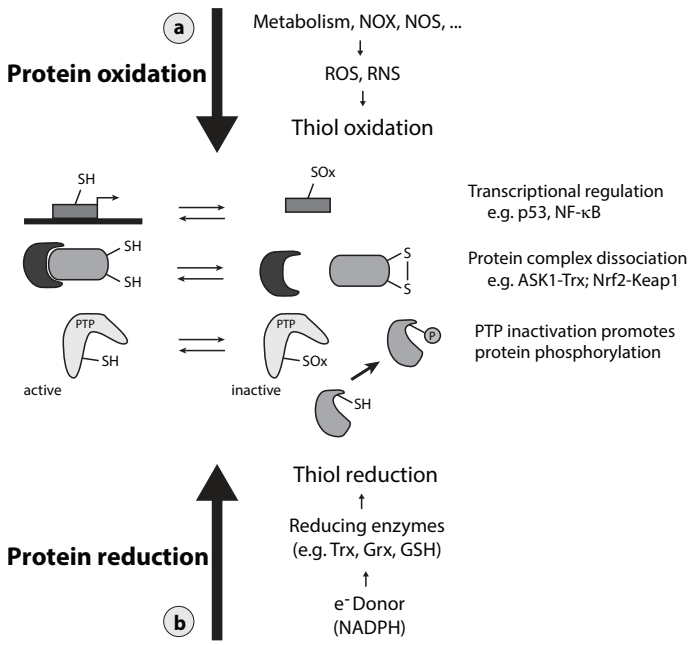


Figure 1. Principle regulation of thiol proteins via the interplay of oxidation and reduction pathways. a) ROS mediated thiol oxidation (SOx) can affect a number of different processes such as transcriptions factor activities, protein interaction or enzyme activities. **b)** These modifications, and thus their effects on the protein, can be reversed by reducing enzyme systems such as the GSH or the Trx system. The cooperation of oxidizing and reducing mechanisms regulates the function of thiol proteins and thus the respective pathways.

The overall aim of this thesis was to further understand how the thioredoxin system is involved redox regulation. For this we concentrated our efforts on the following three distinct aspects:

Paper I	The thioredoxin system as target in anti-cancer therapy.
Paper II	Compartmentalization of the "v3" splice variant of TrxRl and effects on cell motility.
Paper III	Developing a method to analyze the activation of redox sensitive transcription factors on single cell level.

The following chapters aim to briefly introduce some of the key aspects in redox regulation and signaling: I) reactive species in redox signaling, II) reducing enzymes with a focus on the thioredoxin system, III) redox modifications of thiols and IV) redox regulated transcription factors.

2 Reactive species in biological systems

2.1 Introduction

Reactive oxygen species (ROS) and oxidative stress

Reactive oxygen species (ROS) refers to a group of reactive intermediates that are formed during incomplete oxygen reduction **(see chapter 2.2 for more details)**. They are produced under non-stressed conditions and in response to various stimuli. At low concentrations, ROS are essential and have been shown to function as second messengers in the regulation of many physiological processes including the activation/deactivation of transcription factors and enzymes or the modulation of calcium-dependent and phosphorylation pathways[5-8]. At high and unbalanced concentrations however, ROS can inflict damage to cellular constituents causing fatal alterations and eventually cell death. This condition is commonly referred to as oxidative stress and associated with several severe conditions such as diabetes, atherosclerosis, cancer and neurodegenerative disorders[9-13] **(Fig. 2)**.

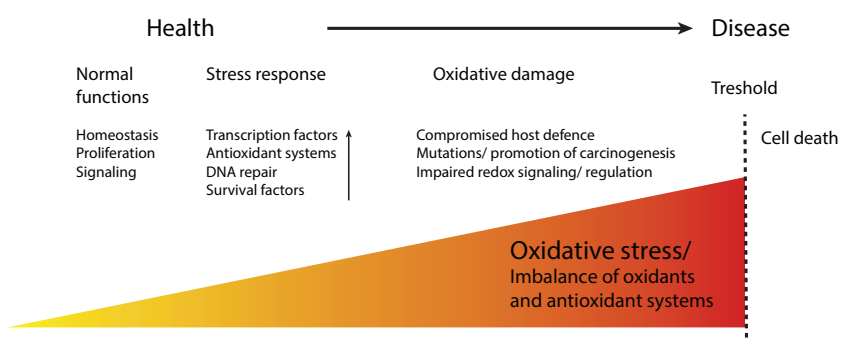


Figure 2. Oxidative stress in the development of diseases. The balance between ROS and antioxidant systems is essential for normal cell function and redox homeostasis. Disruption is referred to oxidative stress and affects the cell in numerous ways depending on the degree of the imbalance. Small to moderate levels of oxidative stress have been shown to stimulate stress responses that lead to cell proliferation and the activation of survival pathways. If prolonged, this condition can alter cellular processes and promote the development of diseases. The continued increase of oxidative stress exceeds at some point the beneficial effects, causing extensive oxidative damage that eventually leads to cell death. Therapeutic approaches in most oxidative stress related conditions, with the exception of cancer, is thus typically based on either boosting the antioxidant capacity or in inhibiting the source of ROS production[14-16].

ROS are thought to exert their physiological function via site-specific, covalent modifications of proteins to modulate the enzymatic activity, folding or intracellular location of those. Particularly the cysteine residues in these proteins, which are often essential for proper protein function, can be easily oxidized and are thus suitable targets for signal transmission and regulation[17, 18].

Reactive nitrogen species (RNS)

Reactive nitrogen species (RNS) are reactive molecules that are equally important second messengers in redox signaling pathways. They have been shown to modify thiol groups by the incorporation of nitric oxide in a process termed S-nitrosylation. The exact chemistry behind S-nitrosylation *in vivo* is still not completely clear, but it was shown that this modification can affect redox sensitive pathways by modulating the activity of transcription factors such as HIF and NFκB[19, 20], of proteases like MMP-9[21] or of caspases[22, 23]. The predominant variant is nitric oxide ($^{\bullet}$NO), which was shown to regulate various processes including vasodilation/ relaxation[24, 25] and neurotransmission[26, 27] as well as proliferation, apoptosis and host defense[28]. $^{\bullet}$NO can be metabolized to other reactive forms that may participate in redox signaling and contribute to RNS associated physiological and pathological conditions **(see chapter 2.2)**.

Reactive sulfur species (RSS)

Occasionally a third group consisting of reactive sulfur species (RSS) is considered in redox signaling processes based on the concept that these reactive sulfur intermediates are capable of propagating their redox modifications analogous to ROS and RNS[29, 30]. This group includes amongst others oxidized thiol groups such as disulfides, sulfenic acid or S-nitrosothiols[31, 32], but also inorganic sulfur-containing species like hydrogensulfide (H_2S) or thiocyanate (SCN^-)[33-35]. Particularly H_2S has been discussed in redox signaling and is considered to function similar to carbon monoxide (CO) or $^{\bullet}$NO as a physiological vasorelaxant[36-38]. Another example is the concept of targeted protein oxidation via oxidized peroxiredoxin intermediates (further discussed in chapter 3.3)[39]. The overall physiological significance of RSS is however, not well established and thus not discussed in detail within this thesis[31, 36, 40].

2.2 Formation

The following chapters aim to provide an overview on the formation and reactivity of ROS and RNS with a focus on biologically relevant molecules in the context of redox signaling. A general overview is provided in **Figure 3**.

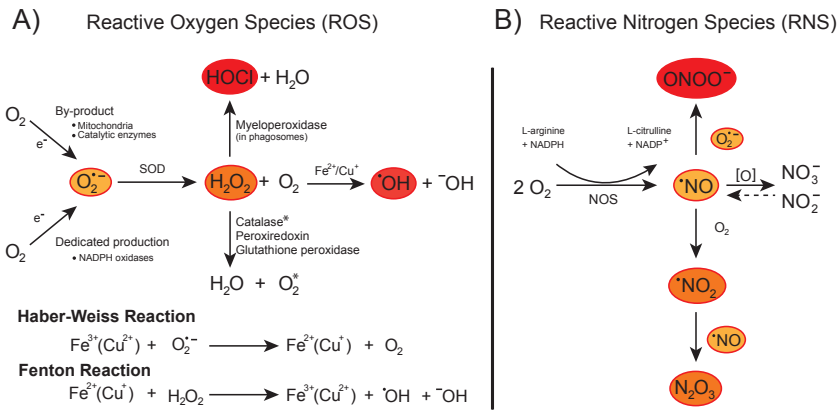


Figure 3. Metabolism of reactive oxygen and nitrogen species. A) Superoxide formation via electron transfer as either by-product or volitional. Subsequent dismutation to hydrogen peroxide (H_2O_2) and oxygen is performed by superoxide dismutases (SODs). H_2O_2 in turn is either i) removed by catalase, peroxiredoxin or glutathione peroxidase, ii) used to produce hypochlorous acid (HOCl) in phagosomes by myeloperoxidases to facilitate efficient killing of engulfed bacteria or iii) involved in hydroxyl radical ($^{\bullet}OH$) formation together with $O_2^{\bullet-}$ via Fenton and Haber-Weis chemistry. **B)** Nitric oxide ($^{\bullet}NO$) formation is mediated by $^{\bullet}NO$ synthases enzymes (NOSs). It is subsequently autoxidized to nitrite (NO_2^-) and nitrate (NO_3^-), but can potentially be reduced to $^{\bullet}NO$ again. Via reaction with molecular oxygen $^{\bullet}NO$ can be transformed to the nitrogen dioxide radical ($^{\bullet}NO_2$) and subsequently to dinitrogen trioxide (N_2O_3) by radical coupling with $^{\bullet}NO$. Additionally, peroxynitrite ($ONOO^-$) can be formed in the presence of superoxide. Color intensity correlates to the relative reactivity. The figure was modified from Paulsen and Carroll, 2013[40].

Reactive oxygen species

Out of all reactive oxygen species, H_2O_2 is the most suitable in terms of redox signaling. It is only mildly reactive, has a long half life (~ 1 ms), shows selective reactivity and can diffuse through membranes either freely or via aquaporins[8, 41-44]. Overall cellular concentrations are in the nanomolar to low micromolar range and constantly regulated by the antioxidant enzymes glutathione peroxidases (GPxs), peroxiredoxins (Prxs) and catalase[41, 42]. Direct H_2O_2 production is only facilitated by a handful of oxidases such as glucose-, xanthine-, lysyl-, monoamine-, and D-amino acid-oxidases, which are yet mostly uncharacterized in terms of redox signaling[45-47]. The majority of H_2O_2 however, is produced in rapid dismutation reactions of

superoxide ($O_2^{\bullet-}$) to H_2O_2 either spontaneous (~10^5 M^{-1} s^{-1}) or facilitated by superoxide dismutases (SODs) (~10^9 M^{-1} s^{-1})[48, 49]. Cellular $O_2^{\bullet-}$ concentrations are thus merely in the low picomolar range despite the constant formation as by-products of respiration at the mitochondria[42, 50] and during the catalytic activities of enzymes[51-55]. Mitochondrial $O_2^{\bullet-}$ formation is estimated to be 0.15 – 2% of all consumed oxygen. Under normal conditions this output is tightly regulated by several antioxidant systems[56-58] and facilitates regulation of various redox sensitive pathways including inflammatory responses[59-61], autophagy[62], HIF activation[63-65] or metabolic feedback regulation[66].

Unlike the aforementioned sources, $O_2^{\bullet-}$ and thus H_2O_2 is deliberately produced by NADPH oxidases (NOXs) in response to various forms of cell stress and stimuli[67, 68] for the propose of activating redox sensitive signaling pathways, including transcription factor activation, PTP inhibition, ion channel activation or regulation of enzyme activities[69, 70]. The NOX family comprises in total seven core members (NOX 1-5, Duox1 and Duox2) that are expressed in a distinct cell type and tissue specific manner[69, 71]. These enzymes are evolutionarily conserved multisubunit complexes that require the translocation and assembly of several cofactors and activators in order to function[69, 72-76]. These factors are regulated and display stimulation and cell type dependent intracellular localization and activation patterns as a means of specific, regulated H_2O_2 formation. NOX activity has been reported to localize to the ER[77-79] and the nucleus[80, 81] as well as to various specific membrane compartments such as lipid rafts[82], focal adhesions[83], activated receptors[72, 84] or invadopodia[85].

Superoxide is indirectly important for redox signaling processes via the formation of H_2O_2, but unlikely to be involved in direct thiol oxidation. Rate constants are typically below 10^3 M^{-1} s^{-1} and thus far outcompeted by dismutase reactions[86, 87]. Only iron-sulfur proteins could react fast enough with superoxide, but this leads to their inhibition and is typically associated with $O_2^{\bullet-}$ toxicity[88]. Likewise, other ROS variants are unsuitable for signaling purposes based on their high reactive and indiscriminate choice of reaction partners[41].

Some of these highly reactive variants include hypochlorous acid (HOCl) and hydroxyl radicals ($^{\bullet}OH$). HOCl formation from H_2O_2 is catalyzed by myeloperoxidases in

neutrophil phagosomes to facilitate efficient killing of engulfed bacteria[89, 90]. HOCl can additionally react with $O_2^{\bullet-}$ to produce the hydroxyl radical ($^{\bullet}OH$), which also contributes to the activity of neutrophils[91]. Hydroxyl radicals can furthermore be formed via Fenton and Haber-Weiss chemistry under conditions were trace metal ions are available[31]. Particularly $^{\bullet}OH$ is very reactive and formation is thus strongly restricted under normal conditions[92].

Reactive nitrogen species

Nitric oxide ($^{\bullet}NO$) is the prototypic reactive nitrogen species (RNS) and was the first reactive molecule to be recognized as second messenger, with Furchgott, Murad and Ignarro getting the Nobel Prize in Physiology or Medicine in 1998 for this discovery[25] . Overall physiological concentrations are estimated to be in the range of 100 pM to 5 nM with a half-life of approx. 0.1 to 2 s[93], which reflects its modest reactivity compared to H_2O_2. It can freely diffuse through membranes[94] and thus function as paracrine, autocrine and endocrine signal within a 200 μM radius[95, 96]. $^{\bullet}NO$ formation is catalyzed by three distinct nitric oxide synthase (NOS) isoforms – endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS)[97-99]. eNOS and nNOS express $^{\bullet}NO$ constitutively in a Ca^{2+} dependent manner in contrast to iNOS, whose activity is Ca^{2+} independent and regulated by cytokines and interleukins[100]. Regulation of these and thus of $^{\bullet}NO$ formation is complex and reviewed elsewhere in detail[40].

The main cellular targets for $^{\bullet}NO$ are other reactive species such as superoxide or metals. A prominent example is reversible binding to the heme in soluble guanylyl cyclase, which activates the enzyme to stimulate vasodilation[101]. The reaction with $O_2^{\bullet-}$ on the other hand promotes the formation of peroxynitrate ($ONOO^-$) with rate constants of 10^{10} M^{-1} s^{-1} that even exceed SOD catalyzed dismutation reactions. Peroxynitrate is highly reactive and damages cellular components. Its production is often associated with a number oxidative stress related conditions such as cardiovascular, neurodegenerative and inflammatory diseases[102].

$^{\bullet}NO$ can furthermore autooxidize to either nitrite (NO_2^-) or nitrate (NO_3^-). These variants have mainly been considered as by-products, but are also discussed in $^{\bullet}NO$ signaling based on the existence of enzymes such as xanthine oxidase[103] or

deoxyhemoglobin[104] that are capable of reducing NO_2^- to $^{\bullet}NO$ again. $^{\bullet}NO$ can additionally react with molecular oxygen to form nitrogen dioxide ($^{\bullet}NO_2$) that is further converted to dinitrogen trioxide (N_2O_3) by radical-radical interaction with a second $^{\bullet}NO$ molecule[105]. N_2O_3 in turn can directly S-nitrosylate thiolate groups in proteins or low molecular weight compounds. This might however, be only relevant at higher concentrations of $^{\bullet}NO$ as this process is limited by dinitrogen trioxide formation. Alternatively, $^{\bullet}NO$ might S-nitrosylate a thiyl radical that was initially formed during one-electron oxidation of a thiolate group by radicals such as $^{\bullet}NO$ or $O_2^{\bullet-}$[106, 107].

An interesting property of $^{\bullet}NO$ mediated modifications is the ability to transfer this modification to another thiolate in a process called transnitrosylation. The reaction of GSH with an S-nitrosothiol for example can result in a free thiol and GSNO. GSNO in turn can be reduced by GSNO reductase to give GSH and HNO[108]. S-nitrosylated low molecular weight thiols such as GSNO or S-nitroso-L-cysteine (L-CysSNO) are furthermore able to S-nitrosylate other protein thiols and thus frequently used in S-nitrosylation studies.

2.3 Antioxidants and redox regulatory enzymes

Introduction

Cells utilize a variety of low molecular weight antioxidants, antioxidant enzymes and repair systems to protect against oxidative damage, but also to reverse oxidative modifications in order to regulate signaling pathways[109]. The composition varies between tissues and cells and is affected by nutrition and cellular redox states. Some of the well known non-enzymatic antioxidants include Vitamin A and E, ascorbate, lipoic acid, ubiquinone and GSH[41]. Their functionality and intracellular localization is variable, but involves essentially the scavenging of highly reactive radicals or the chelation of transition metal ions. They may also be either directly or indirectly regenerated by various antioxidant enzymes with the GSH and Trx systems being considered the most important. The latter is central to this thesis and thus discussed in more detail in **chapter 3**.

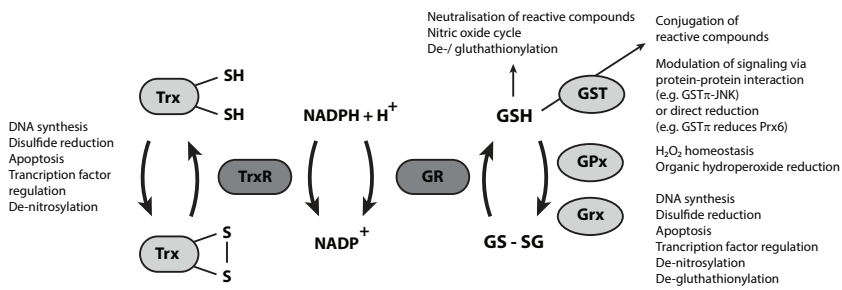


Figure 4. Illustration of the GSH and Trx systems. The Trx system has a diverse set of functions as illustrated further in chapter 3. Its key enzyme is TrxR that takes electrons from NADPH to reduce Trx as well as a number of other substrates. The GSH system fulfills similar functions. At its core is GSH that is used as cofactor for GST, GPx and Grx, but also acts directly in a number of processes.

Glutathione system

The tripeptide glutathione (GSH; Glu-Cys-Gly) is present in low millimolar concentrations and thus the most abundant low molecular weight antioxidant[109, 110]. It can scavenge electrophilic and oxidizing compounds either directly or catalyzed by glutathione-S-transferases (GSTs), which has also been shown to be important in the modulation of signaling pathways[111, 112]. Additionally, GSH is used as a cofactor by glutathione peroxidases (GPxs) to reduce hydroperoxides and by glutaredoxins (Grxs) that operate as disulfide reductases and de-glutathionylation enzymes[113] **(Fig. 4)**.

After donating the electrons, two GSH molecules form a glutathione disulfide (GSSG) via an intermolecular disulfide bridge. This oxidized form is in turn reduced by glutathione reductase (GR), which uses NADPH as electron donor[114]. Interestingly, GSH also influences redox signaling events via glutathionylation of reactive thiol-groups in key cysteine residues. This protects them from oxidative modifications and electrophilic compounds[115].

The glutathione peroxidase (GPx) family contains eight isoforms that are expressed in various tissues and with different subcellular localizations. GPx1-4 and GPx6 in humans have a peroxidatic Sec residue whereas the other isoforms contain a Cys instead. GPx4 is unique as it can also reduce hydroperoxides in complex lipids such as phospholipids or cholesterol. In addition to being important antioxidant enzymes they are also discussed in redox signaling and the regulation of physiological processes. An interesting example is given by the currently discussed GPx7 and GPx8 isoforms. Both reside in the endoplasmatic reticulum and have shown to be able to transfer their oxidation state to PDI *in vitro*. This was previously demonstrated for Prx4 and is considered an important process for targeted oxidation[116]. The GPx family was recently reviewed in detail by Brigelius-Flohé and Mairino[117].

Peroxiredoxins

Peroxiredoxins (Prxs) form an important antioxidant and redox regulatory enzyme family that contain several protein isoforms (Prx1-6) that differ in their cellular localization, substrate specificity and reaction mechanism **(Table 1)**. For a long time they have only been discussed in the prevention of oxidative stress by reducing hydrogen peroxide, organic hydroperoxides, lipid hydroperoxides and peroxinitrite. However, the realization that hydroperoxides are important mediators of physiological processes brought about a change of view. Now their roles are increasingly discussed in the context of signal transduction by either regulating the concentration of hydroperoxidic mediators and thus the respective signaling pathways or by acting as sensors that may transfer an oxidative modification to a specific target protein via protein-protein interaction. The peroxiredoxin enzyme family has been extensively discussed in these recent reviews[118, 119].

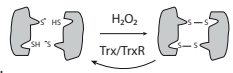
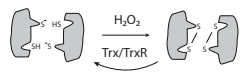
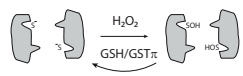
Peroxiredoxins			
Prx1 Prx2 Prx3 Prx4	Cytosol Cytosol Mitochondria Endoplasmatic reticulum	2-Cys subfamily. The conserved peroxidatic Cys is present as thiolate and easily oxidized to sulfenic acid, which next forms an intermolecular disulfide with the resolving cysteine of the other subunit.	
Prx5	Cytosol Mitochondria Peroxisomes	Atypical 2-Cys variant. Forms an intermolecular dithiol with the resolving cysteine of the same subunit during oxidation.	
Prx6	Cytosol	1-Cys subfamily. Does not contain a resolving cysteine. Sulfenic acid form is reduced by GSH in the presents of GSTπ, but not Grx or Trx.	

Table 1. Localization and general catalytic mechanism of peroxiredoxins (Prxs). All peroxiredoxins form anti-parallel homodimers. Localization and expression is dependent on cell type and the environment. Prx5 and 6 are less sensitive to overoxidation, but the overoxidized forms are not reduced by sulfiredoxin. The main function is the reduction of hydrogen peroxide, organic peroxides, lipid peroxides and peroxynitrite, but they have also been discussed in oxidizing other thiols.

Additional antioxidant enzymes

Many of the antioxidant enzymes have very distinct functions and exhibit high reaction rates. The aforementioned superoxide dismutases for example, specialize in the rapid dismutation of superoxide to the less reactive hydrogen peroxide. The regulation of superoxide levels is essential since it can either react with nitric oxide to form the highly reactive peroxynitrite or hydroxyl radicals via the Fenton reaction **(Fig. 3)**. Mammalian cells contain two variants: MnSOD (SOD2) that is located in the matrix of the mitochondria and the predominantly cytosolic CuZnSOD (SOD1), which can also be found in the nucleus, lysosomes and the intermembrane space of the mitochondria[120]. Catalase is another antioxidant enzyme that however, specializes on the decomposition of hydrogen peroxide to water in peroxisomes[121]. Other than controlling the levels of reactive molecules, some enzymes are specialized on the reversal of damaging oxidative modifications. The enzyme family of methionine sulfoxide reductases (Msrs) is for example able to reduce oxidized methionine residues[122]. Another repair enzyme is sulfiredoxin that is able to reduce sulfinic acid back to sulfenic acid in a subset of peroxiredoxins[123].

3 The thioredoxin system

The thioredoxin system is one of the key redox regulatory systems in the cell and as such involved in defense against oxidative stress[124, 125], cell proliferation and viability[16, 126] as well as protein folding and signal transduction[127, 128]. It consists of thioredoxin reductase (TrxR) that uses NADPH as electron donor to reduce its main substrate thioredoxin (Trx)[129, 130], which in turn sustains a number of pathways by either providing enzymes with electrons or via protein-protein interactions[16, 125] **(Fig. 4)**. TrxR furthermore catalyzes the reduction of various additional thiol-proteins as well as several low-molecular weight compounds and thus displays a functional spectrum that exceeds the mere reduction of Trx[131]. In mammals these functions are essentially carried out by cytosolic TrxR1/Trx1 and mitochondrial TrxR2/Trx2 isoenzymes.

Its central functions in redox regulation and antioxidant defense link the thioredoxin system to numerous pathophysiological conditions that are related to an oxidative imbalance and thus identify it as a promising therapeutic target. Conditions such as cardiovascular and neurodegenerative diseases, inflammation and those related to aging are often associated with elevated ROS levels while having a low antioxidant capacity and might therewith be countered by boosting the thioredoxin system[16, 132]. However, a high antioxidant activity might on the other hand also be unfavorable in some cases such as certain types of cancer where it promotes proliferation and associates with resistance to chemotherapy and a poor patient prognosis. Particularly TrxR is discussed as a potential anti-cancer target due to its central role in the Trx system and as a prime target for electrophilic drugs[133, 134].

A more comprehensive overview with regard to the physiologic importance is provided by the recent reviews of Mahmood et al.[16] and Lu et al.[125].

3.1 Thioredoxin and the thioredoxin fold family of proteins

Trxs are 12-kDa large oxidoreductases that typically catalyze thiol-disulfide exchange reactions via their conserved -CGPC- active site motif[127]. They are ubiquitously expressed and characterized by a specific structure that consists of a four-stranded beta sheet surrounded by three alpha helices[135-137]. Variations of this so called Trx fold are also part of Grxs[138] and Prxs[139] as well as GPxs[140], GSTs[141] and protein disulfide isomerases (PDIs)[142] – all of which were shown to exhibit oxidoreductase activity. Trx fold proteins are further characterized by their active site motif – containing either one or two cysteine residues together with different adjacent amino acid residues, resulting in different catalytic mechanisms.

The main mammalian isoforms of Trx are the cytosolic Trx1 and the mitochondrial Trx2. Both are major disulfide reductases with various specific functions in redox regulation and antioxidant defense[127] **(Fig. 5)**. Trx1 has in contrast to Trx2 three additional cysteines that are suggested to be the subject of post-translational modifications such as glutathionylation, S-nitrosylation and disulfide formation that may contribute to its redox regulatory functions in different physiologic contexts[143-146]. The formation of a second disulfide bond between the non active site Cys62 and Cys69 leads for example to an inactivated form that can not directly be reduced by TrxR1, but requires the Grx system instead[147, 148].

Trx1 is predominantly located in the cytosol where it provides RNR with electrons and catalyzes the reduction of Prxs[149] and Msrs[150]. It can however, also translocate into the nucleus under oxidative conditions where it regulates gene expression by modulating the binding of various transcription factors to DNA including NFκB, HIF, p53, Nrf2, AP-1 and the glucocorticoid receptor[151-158]. Furthermore, reduced Trx1 directly binds PTEN, which is a major tumor suppressor that prevents survival signaling by deactivating the PI3K/Akt pathway. Trx1 binding inhibits the phosphatase activity of PTEN and promotes thus cell proliferation and tumor growth while also inhibiting apoptosis[159]. Trx is also an important regulator of apoptosis signal regulating kinase 1 (ASK1). In its reduced form Trx is binding and thus inhibiting ASK1. However, high levels of ROS promote oxidation of Trx and thus ASK1 release leading to subsequent

apoptosis[160]. ASK1 release may also be promoted by the Trx interacting protein (TXNIP), an endogenous inhibitor of Trx that binds to reduced Trx and thus competes with ASK1[161]. Interestingly TXNIP binding mediates also Trx1 translocation to the plasma membrane, which is proposed to enable inflammation in endothelial cells by promoting cell survival and vascular endothelial growth factor signaling during oxidative stress[162]. Additionally, Trx together with a truncated variant (Trx80) can be found in the extracellular environment where it exhibits an oxidoreductase independent chemokine-like activity[109, 163].

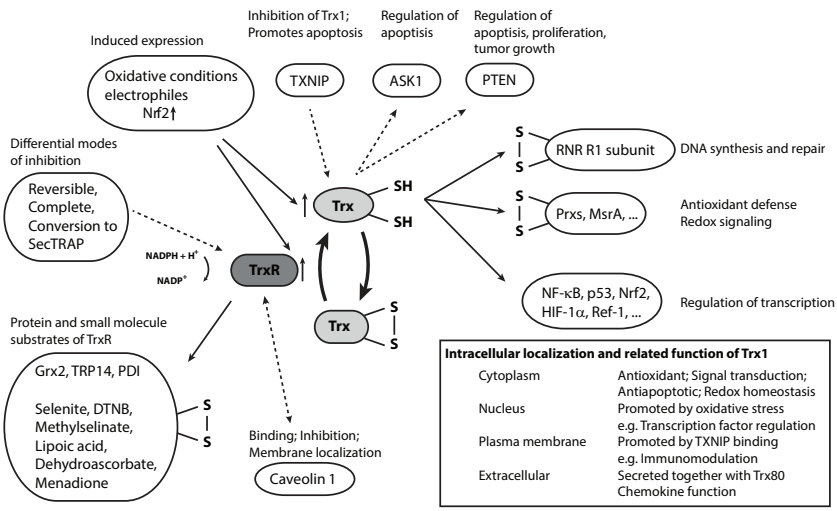


Figure 5. Substrates and principle functions of the thioredoxin system. Doted lines indicate direct binding whereas solid lines show thiol-disulfide exchange reactions. Oxidative stress promotes Nrf2 activation, which in turn induces Trx and TrxR expression. For further details see text. Figure was modified from Lu et al.[164].

3.2 Thioredoxin reductases

Classification and catalytic activity

TrxRs are dimeric flavoenzymes that belong to the pyridine nucleotide disulfide oxidoreductase family, which also includes glutathione reductase[131, 165]. They exist in two forms: low-M_r TrxRs (ca. 35 kDa/ subunit) that can be found in lower eukaryotes and prokaryotes and high-M_r TrxRs (ca. 55 kDa/ subunit) that are present in higher eukaryotes[166, 167]. Low-M_r TrxRs contain one FAD and one redox active disulfide motif, but lack the additional C-terminal -XCU/CX active site that distinguishes the larger TrxR variants[166, 168]. The selenocysteine containing -XCUX motif is characteristic for most high-M_r TrxRs such as mammalian TrxRs, whereas the -XCCX motif containing TrxRs can be found in certain parasites and insects such as *D. melanogaster*[169].

The principle catalytic mechanism for mammalian TrxR has been extensively studied and involves the transfer of electrons from NADPH to the oxidized N-terminal disulfide motif via the enzyme bound FAD. The thereby reduced dithiol exchanges these electrons with the selenenylsulfide in the C-terminal active site of the other subunit to form a reduced selenolthiol motif that in turn facilitates reduction of most substrates such as Trx and DNTB[170, 171] **(Fig. 6A)**. Several substrates, including certain quinones do not require an intact Sec-residue and may be directly reduced via the N-terminal C59/ C64 dithiol motif[172].

Nonetheless, the detailed analysis of the TrxR mediated catalysis is an ongoing process and essential to fully understand the underlying mechanisms. Important features that are currently discussed include the need for selenocysteine in the active site[131, 173-175], the structure of the flexible C-terminal tail[175, 176], the function of key amino acids in the TrxR/substrate interface or in the vicinity of the active site as well as the exact electron flow during catalysis[175, 177]. Recent studies showed for instance that the C-terminal amino acids 407-422 in TrxR1 form a “guiding bar” like structure that restricts the movement of the C-terminal and supports the electron transfer from the N-terminal dithiol to the C-terminal selenenylsulfide motif. This arrangement promotes Sec-dependent catalysis while simultaneously restricting the direct reduction of substrates

via the N-terminus[176]. TrxR2 in contrast, is lacking this supporting "guiding bar" and allows for a greater access to the N-terminal active site, which in turn promotes alternative catalytic mechanisms, makes the enzyme less dependent on the Sec residue and influences its substrate specificity[175].

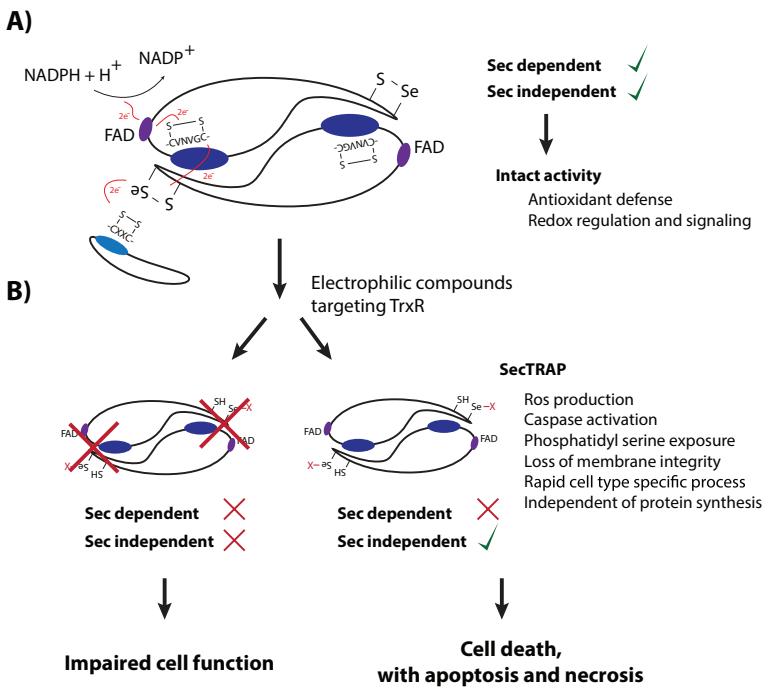


Figure 6. Principle electron flow in normal catalysis and differential modes of inhibition. A) Scheme of the head-to-tail homodimer confirmation of mammalian TrxR. The principle electron flow during normal catalysis is indicated. **B)** Targeting via electrophilic compounds can either leave the enzyme complete inactive (left) or transform it into its pro-oxidant SecTRAP form, which might lead to cell death via apoptosis and necrosis. Figure was modified from Anestål et al.[178]. See text for further details.

TrxR inhibition and SecTRAP formation

The Sec residue in the flexible C-terminal active site is easily accessible and highly reactive due to its strong inherent nucleophilicity. These properties are important for its catalytic efficiency; however, they also make its reduced selenolate form prone to attacks by electrophilic compounds[131]. The list of identified inhibitors is extensive and includes naturally occurring substances such as flavonoids[179, 180], the lipid peroxidation product 4-hydroxy-2-nonenal (HNE)[181] or curcumin[182] as well as many constructed electrophilic compounds of which some are already in clinical use. Prominent examples include gold compounds (auranofin[183], aurothioglucose[184]), platinum compounds

(cisplatin[185], oxaliplatin[186]), arsenic oxide[187], nitrosoureas[188] or dinitrohalobenzenes[189]. Despite their overall similarity, TrxR1 and TrxR2 are differentially targeted by electrophiles. This might in part be due to their different intracellular localization, but it has also been shown that their inherent catalytic properties play a role as well[175, 190].

The effect of various inhibitors on the activity of TrxR1 is diverse – some diminish the enzymatic activity completely while others transform the enzyme into its pro-oxidant SecTRAP form (**s**elenium **c**ompromised **t**hioredoxin **r**eductase-derived **a**poptotic **p**rotein)[178, 191] **(Fig. 6B)**. SecTRAPs are presumably formed by compounds that specifically derivatize the Sec residue. These enzymes have lost the ability to catalyze Sec dependent reactions, but gained a potent NADPH oxidase activity by being able to redox cycle with certain substrates via the intact FAD and N-terminus active site. Recent studies propose that the increased access to the N-terminus is promoted by conformational changes that are caused by the modification of the Sec residue[175, 177]. In the unmodified enzyme this access and thus electron leakage at the N-terminus is thought to be prevented by an efficient electron transfer to the Sec residue[177].

SecTRAPs were previously shown to induce cell death via apoptosis and necrosis and might thus contribute to the cytotoxic profile of many TrxR inhibitors[178, 192-194]. An interesting study showed for instance that A549 cells with high TrxR1 levels are more susceptible towards the SecTRAP forming compound cisplatin[195] than A549 cells with low levels[196]. The same trend was illustrated in thiophosphate treated HCT116 and selenite treated NIH 3T3 cells. Selenite stimulated the expression of TrxR1 and rendered thus these cells more sensitive towards cisplatin, whereas thiophosphate on the other hand promoted a more resistant phenotype based on the stimulated expression of a less reactive, but also less sensitive Sec-to-Cys variant[197].

Transcriptional regulation and isoforms of human TrxRs

Mammals possess three separate genes that guide the transcription of several isoforms. In human, the *TXNRD1* gene encodes the predominantly cytosolic TrxR1[129], whereas the mainly mitochondrial TrxR2 is expressed from the *TXNRD2* gene[198]. A third isoenzyme, named thioredoxin glutathione reductase (TGR), can essentially be found in testis and is encoded by the *TXNRD3* gene. TGR is unique as it contains an additional

monothiol glutaredoxin domain as N-terminal fusion to the TrxR core module[199]. It is mainly expressed in male germ cells and has been implicated in disulfide bond formation during sperm maturation[200].

The transcriptional regulation of *TXNRD1* and *TXNRD2* is complex and involves extensive alternative splicing yielding several mRNAs that in turn encode protein variants that differ in their N-terminal extensions[131, 201]. Most of these are minor variants and not extensively studied. Nonetheless, they might carry potential translocation signals and protein binding sequences and thus exhibit specific, localized functions or operate as back-up systems[202, 203]. Several splice variants of *TXNRD2* were, for example, shown to exhibit cytosolic expression patterns due to the lack of the mitochondrial targeting sequence in the 5'-end. They also showed a similar catalytic activity in the reduction of cytosolic Trx1 compared to mitochondrial Trx2, which indicates a potential back-up mechanism[203, 204]. Another example is given by the TXNRD1_v2 isoform of TrxR1, which contains an LQKLL nuclear receptor binding motif[205]. As a result, TXNRD1_v2 is translocated to the nucleus where it binds and regulates the estrogen receptors alpha and beta[206].

The TXNRD1_v3 isoform ("v3" in short) is among all splice variants of TrxR1 particularly unusual as it contains an atypical glutaredoxin domain as N-terminal extension that is encoded by three 5' exons located upstream of the TrxR1 core promoter. This unique constellation requires the activation of a yet uncharacterized alternative upstream promoter as well as a simultaneous shut-down of the TrxR1 core promoter during transcription[205, 207, 208]. The protein is predominantly expressed in the Leydig cells of the testis, but transcripts could also be detected in other tissues and in several cancer cell lines, where its expression could be induced by estradiol or testosterone treatment[209]. The glutaredoxin domain contains an atypical -CTRC- active site motif that was shown to be inactive in typical Grx assays unless mutated to the more common -CPYC- active site found in Grxs[208]. Previous overexpression experiments of GFP-TXNRD1_v3 fusion proteins (either only the Grx domain or a Sec-deficient full length variant) in HEK293, Hela and MCF7 cells demonstrated an intracellular localization pattern that was characterized by a strong perinuclear localization as well as a dotted cytoplasmic and plasma membrane appearance. At the

membrane, GFP-TXNRD1_v3 co-localized with actin and induced dynamic cell protrusions that were later identified as filopodia[209, 210]. Also notably, the protein is only found in higher vertebrates like human, bovine, dog or chimpanzee, but not in rat or mouse[208].

All in all, the complex transcriptional regulation and the additional features of the variable N-terminal extensions are parameters that potentially contribute to the extensive spatial and temporal regulation of redox sensitive pathways.

3.3 Thioredoxin reductases as targets for anticancer therapy

Cancer collectively refers to a whole group of complex genomic disorders. In simple terms it can be viewed as the abnormal and uncontrolled proliferation of cells with an altered physiology. Although cancer types vary in several aspects, they often exhibit increased glycolysis, based on the need for energy to fuel their abnormal proliferation, as well as elevated ROS levels[211, 212]. ROS is not only a consequence of an increased cell metabolism, but has been implicated to be an essential factor in the development and progression of tumors as well[213, 214]. Moderately increased levels promote for instance the inactivation of the tumor suppressor PTEN[215] and of several PTPs[216] by oxidizing critical cysteine residues. Inhibition of PTPs in turn promotes the phosphorylation and activation of mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) pathways. These are only some examples by which cell survival and proliferation is stimulated by ROS[217].

Cancer cells typically also boost their redox regulatory and antioxidant capacities in parallel in order to balance the consistently high production of ROS and to avoid oxidative damage[218] **(Fig. 7)**. Inhibition of these cellular antioxidant systems might thus provide a therapeutic approach to promote cancer cell death via the rapid accumulation of ROS[219, 220].

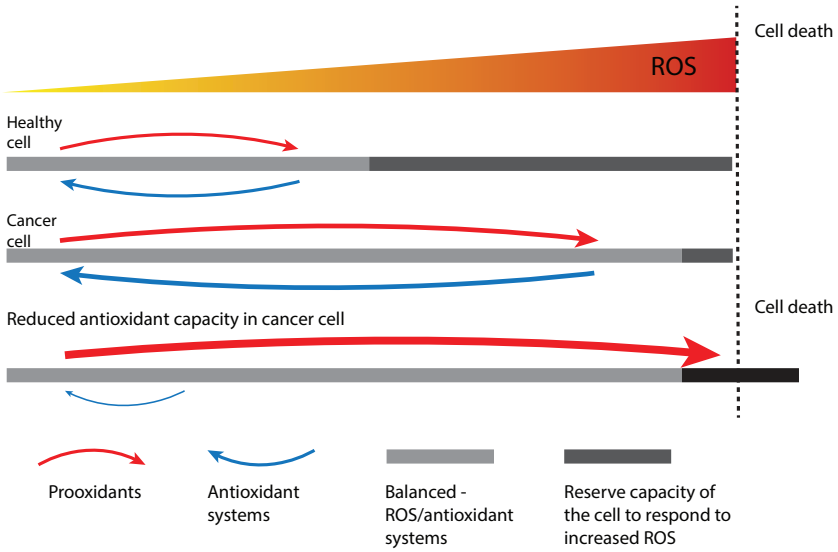


Figure 7. Redox homeostasis in normal and cancer cells. Normal cells have low levels of ROS, which are balanced by antioxidant systems. They also have a certain reserve capacity in order to buffer elevated ROS levels under stressed conditions and can thus prevent cell death. Cancer cells on the other hand, exhibit already elevated basal ROS levels that are balanced adequately by induced antioxidant systems. This balance is fragile however, as only a small reserve capacity is available before the threshold is reached. A decrease of the antioxidant capacity, for instance via chemotherapy that targets the antioxidant systems, might thus cause a massive accumulation of ROS and tip the balance in favor of cell death[220]. This approach of killing cancer might also be further amplified by stimulating an increase in ROS, which can be buffered by normal, but potentially not by cancer cells.

The Trx system, particularly TrxR1, has been identified as a promising target for anti-cancer therapy[221]. TrxR and Trx are both upregulated in numerous cancers with high Trx levels having been associated with aggressive cancers, poor patient prognosis and resistance to chemotherapy[222, 223]. TrxR1 is not only easily inhibited by electrophilic compounds, but may also be converted into its pro-oxidant SecTRAP form, whereby it further promotes accumulation of ROS as a means of inducing cancer cell death. Over the years evidence is increasingly accumulating that links TrxR1 targeting to cancer cell death and thus strengthens this approach[187, 189, 196, 224-226].

4 Thiols in redox regulation

ROS may modify histidine, tryptophan, tyrosine, and methionine in addition to cysteine residues. Tryptophan, histidine and tyrosine oxidation is, however, less favored and considered to be irrelevant for normal physiological processes[227, 228]. Methionine oxidation is physiologically observed and can be reversed via methionine sulfoxide reductases[122]. This process is usually considered a repair mechanism and its contribution to redox signaling is thus not fully established yet. So far all evidence suggest that cysteine residues are the prime residues involved in redox signaling. It is important to mention though that most of the evidence is obtained from individual studies of known thiol proteins. The sulfenic acid oxidation for example is unstable in most proteins and thus difficult to observe. Current research puts therefore a lot of effort into the development of methods to monitor, identify and analyze redox sensitive cysteine residues[229].

4.1 Cysteine & selenocysteine as targets for oxidative modifications

Cysteine

Cysteine is a polar, sulfur containing amino acid that is characterized by its mildly acidic thiol side chain (RSH). It is among the least abundant amino acids, but often directly involved in catalysis, protein binding, stabilization and other functions. Its general biochemical properties are difficult to characterize as they are highly influenced by the local environment. Exposed cysteine residues in low molecular weight thiols, like GSH, have typically pK_a values around 8-9, whereas the pK_a in thiol-proteins can be as low as 3.5 due to a suitable microenvironment[18, 230].

A low pK_a is considered a key property for oxidative reactivity as it promotes the formation of the more reactive deprotonated thiolate (RS^-) at physiologic pH[229]. This dependence is illustrated by H_2O_2 mediated oxidation of low molecular weight thiols that requires the thiolate anion[87]. However, several observations clearly show that this is not the sole determinant of oxidative susceptibility. A common example is the

comparison of the protein tyrosine phosphatase PTP1B and peroxiredoxin 2. Both have a similar pK_a, whereas the reaction rate constant of Prx2 with H_2O_2 is a million times higher than for PTP1B[231, 232]. This feature was recently attributed to the additional activation of the peroxide substrate via a unique and conserved hydrogen bonding network that is formed by the amino acids (Pro, Thr, Arg and either His, Glu or Gln)[232, 233]. Noteworthy, the ability of sulfur to adopt a wide range of oxidation states (-2 to +6) is based on its electron configuration and available d-orbitals, thus enabling a number of different modifications[234] **(Fig. 9)**.

Selenocysteine

Selenocysteine (Sec; U in one letter code) is a reactive analogue of cysteine. It is considered the 21st naturally occurring amino acid[235] and distinguished by a selenium containing selenol group in place of the sulfur containing thiol group **(Fig. 8)**. It has unique biochemical properties such as a high nucleophilicity and a low pK_a of 5.3. The selenol group is thus several magnitudes more acidic and reactive in redox reactions compared to its thiol counterpart[236, 237]. This characteristic was shown for the selenoproteins GPx and TrxR1. The reactivity of both was drastically reduced as the Sec residue was replaced by Cys[238, 239].

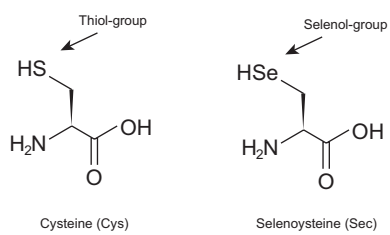


Figure 8. Comparison of cysteine and selenocysteine.

However, this does not imply that selenoproteins have a higher capacity to regulate redox processes in general. The thiol protein Prx2, for example, has a similar reaction rate as the selenoprotein GPx when catalyzing the decomposition of H_2O_2[232]. Another example is the non-selenium TrxR variant of *Drosophila melanogaster*. The wild type variant, having an -SCCS- active site, showed a similar catalytic activity as when mutated to the mammalian -GCUG- motif[173].

All in all, the presence of a Sec residue does not reflect a higher activity *per se*. Other parameters, as for example the microenvironment of the active site and the substrate, are likely contributing to the kinetics and need thus to be considered as well[173, 229]. Some kind of advantage can nevertheless be expected by Sec over Cys, particularly since the cells have developed a specific machinery for selenoprotein production that is considered "costly and inefficient"[173]. Potential advantages might include a greater flexibility towards substrates, efficient support for one-electron-transfer reactions, its high nucleophilicity that leads to fast reaction rates with electrophiles or its resistance to inhibition via overoxidation[173, 240-243]. Interestingly it was also shown that Sec can be substituted by Cys in TrxR1 either *in vivo* under selenium deficient conditions[244, 245] or when cells were treated with thiophosphate thus showing that the less reactive variant can potentially serve as backup[197, 245].

4.2 Relevant thiol modifications

As mentioned above, cysteine residues whose side chains are deprotonated at physiological pH, are prime targets for reversible oxidative modifications that regulate their function and thus important pathways. A number of physiologically relevant modifications include disulfide bonds (RS-SR'), sulfenic acid (SOH), sulfinic acid (SO_2H), sulfonic acid (SO_3H), S-glutathionylation (RS-SG), S-nitrosylation (RSNO) and sulfhydration (RSSH) as summarized in **Figure 9**. They each have distinct chemical properties, functions and formation pathways.

Sulfenic acid is the first oxidation state that is induced by H_2O_2 exposure. It is unstable, reactive, short lived and thus difficult to monitor[246]. Due to its unique chemical properties it is recognized as a critical intermediate in redox signaling – it can be either oxidized further or directly reduced back to a thiol[234]. The most common subsequent modification is the formation of inter- and intramolecular disulfide bonds, which have been shown to affect enzyme activity, subcellular localization and protein-protein interactions[234, 247]. Disulfide bonds are relatively stable and typically cleaved via thiol-disulfide exchange reactions with enzymes such as Trx or Grx. A high and prolonged exposure to H_2O_2 on the other hand, promotes oxidation to the point of forming the essentially irreversible sulfinic and sulfonic acids[248, 249]. This cysteine overoxidation is typically correlated to oxidative stress and can be prevented by other reversible cysteine

modifications such as GSH/GSSG mediated S-glutathionylation[250] **(Fig. 9)**. An example is PTP1B were S-glutathionylation was shown to display a regulatory role by preventing complete inhibition[251]. In addition to its protective role it is also involved in the modulation of enzyme activity, protein-protein interaction and transcription factor regulation[250, 252, 253].

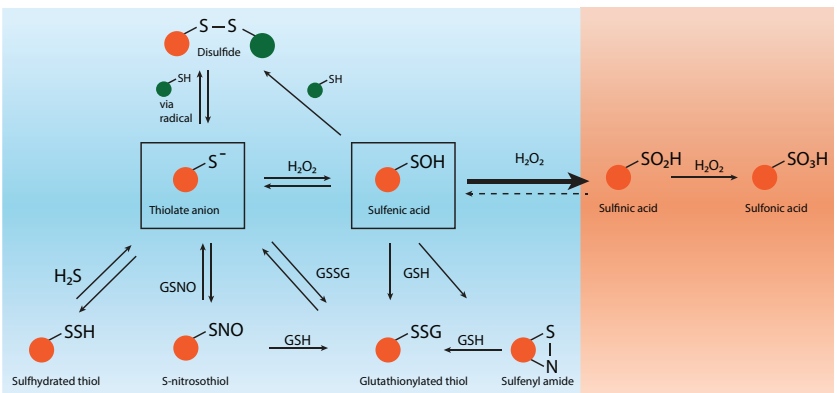


Figure 9. Overview of relevant oxidative thiol modifications. The blue highlighted variants refer to a number of reversible modifications that are important in redox signaling. Oxidizing molecules are highlighted. Removal of the modifications is facilitated by the Trx and GSH systems respectively. Sulfinic and sulfonic acid, highlighted in red, occur in the presence of elevated H_2O_2 levels and are often seen as markers of oxidative stress. However, it has been shown that sulfiredoxin is able to reduce sulfinic acid back to sulfenic acid in a subset of peroxiredoxins[123]. This figure was modified from Chung et al. 2013[254].

Two more reversible cysteine modifications include S-nitrosylation[146, 255-257] and sulfhydration[40]. The later was for example shown to modulate PTP1B activity as a key aspect of the ER stress response[258]. In general, the study of physiological relevant sulfhydration is at the very beginning and thus the understanding still very limited. S-nitrosylation on the other hand as already been implied to be an important regulator of numerous thiol-proteins and their signaling pathways[259]. For more information on S-nitrosylation see chapter 2.2 “reactive nitrogen species”.

4.3 Principle mechanisms in redox signaling

There are three principle concepts described by which redox signaling may be mediated[8]:

The thermodynamic concept

The thermodynamic principle implies that all couples of oxidized and reduced thiols are in an equilibrium, which is determined by the cellular redox potential[260]. The thiol/disulfide ratio of the main redox buffer glutathione[261] ($[GSH]^2$:GSSG), is thus used to correlate redox signaling processes to the cellular redox potential as a whole. A change in the $[GSH]^2$:GSSG ratio as response to oxidizing or reducing conditions is thought to affect the thiol/disulfide ratios of other proteins depending on their redox potential and therewith their function[260, 262]. However, the concept of the $[GSH]^2$:GSSG ratio being the actual cause of a response and therewith a central mediator of redox signaling as well as the usefulness of thermodynamic and electrochemical parameters in redox signaling in general is increasingly questioned[263]. Catalyzed reactions by enzymes like Trx or Grx are far superior in terms of kinetics than the slow direct thiol-disulfide exchange reactions and thus better suitable to regulate dynamic redox signaling processes[127, 264]. Nonetheless might the $[GSH]^2$:GSSG ratio have an indirect functional influence on redox signaling processes. A change in the ratio may affect protein S-glutathionylation as a whole and thus H_2O_2 mediated signaling.

Direct oxidation of sensitive thiol proteins

This concept favors, in contrast to the thermodynamic model, a more specific cellular response. As discussed before, thiols are differently susceptible towards oxidative modifications with only a few being sensitive enough to participate in redox events. An oxidative signal, as for example a burst of H_2O_2, could thus transiently oxidize some of these thiol proteins in order to activate or deactivate them as a principle of signal transmission. The activation of various pathways and the strength of the response could be regulated via the levels of the oxidants, compartmentalization of the oxidative signal or the deactivation of the reducing and oxidant scavenging enzymes.

However, according to kinetic modeling are Prxs the only known cellular thiol proteins that are sufficiently abundant and reactive towards H_2O_2[265]. Direct oxidation of other thiols should thus not be relevant in their presence. A potential solution is the "floodgate" hypothesis that suggests local H_2O_2 signaling as a consequence of Prx inhibition via overoxidation, which could be observed in H_2O_2 exposed cells[266-268]. However, this is argued by recent studies showing that relevant H_2O_2 concentrations for Prx overoxidation are not reached during signaling[269, 270]. Instead it was demonstrated that Prx1 is locally inhibited via transient phosphorylation by activated Src family kinases in response to growth factor stimulation[39, 269]. Two other kinases that also regulate Prx1 activity via phosphorylation in response to H_2O_2 are the tumor suppressors Mst1 and Mst2[271]. Nonetheless, this may not entirely explain the frequently observed oxidation of phosphatases such as PTPs and PTEN[215, 231, 272-277]. They are only mildly reactive and would require high H_2O_2 concentrations to be sufficiently oxidized during fast signaling events.

Most data emanate from *in vitro* models and might thus not always reflect the *in vivo* situation were numerous additional parameters potentially contribute to the process. For example, the reactivity may be affected by posttranslational modifications as in case of the just mentioned phosphorylation of Prx1. Another example is given by caspase-3, which was shown to react 40 times faster with H_2O_2 in the presence of its substrate[278]. Yet another example is related to conformational changes of the Src kinase. Normally it exhibits a closed inactivated confirmation that is insensitive to H_2O_2. In response to growth factor cytokine stimulation it is, dephosphorylated whereby it acquires an open confirmation. In this state it exhibits redox sensitive cysteine residues that further stimulate the activity if oxidized and also prevent the kinase from getting inactivated[279].

Sensor protein mediated oxidation

A solution to the aforementioned direct oxidation of thiol proteins is a third model that proposes regulated oxidation of target proteins via thiol exchange reactions with specific sensor proteins of which peroxidases are suggested to be the best candidates[39]. An example is given by the yeast transcription factor Yap1 that is oxidized via the glutathione peroxidase-like protein GPx3[280] and the thioredoxin peroxidase Tsa1[281]. In principle, the sulfenic acid of the oxidized peroxidase forms an interdisulfide bond with

Yap1. The subsequent exchange with a second cysteine in Yap1 generates in turn an intramolecular disulfide and recycles GPx3 or Tsa1. An example in mammals involves the transfer of the oxidation state of Prx4 to PDI, which in turn promotes the disulfide formation in its target protein[116]. Nonetheless, further studies are clearly needed to show the physiological relevance of these processes. A particularly interesting possibility in this context is the Prx mediated oxidation of PTPs, which is often discussed, but has not been shown yet.

5 Redox sensitive transcription factors

Gene transcription is regulated by the effects of the chromatin structure and the interactions of transcription factors, which are regulatory proteins that bind to specific DNA sequences in order to mediate rapid transcriptional responses. The specific actions of these factors are regulated by an exceedingly complex network of protein-protein interactions and post translational modifications such de-/phosphorylation and redox modifications. All of these processes occur on several levels: i) regulation of transcription, mRNA stability and translation; ii) stability and prevention of degradation; iii) cytoplasm-nuclear transport; iv) DNA binding and nuclear transactivation of the transcription factor as well as v) regulation of co-activators and repressors[282, 283].

The precise mechanisms of redox regulated gene transcription are still rather poorly characterized. It was only in coherence with the growing acceptance that oxidants act as mediators and modulators of signaling pathways and protein function that these aspects received attention. A notable finding in the earlier days was the direct correlation between NFκB activity and oxidative molecules, making it the first mammalian transcription factor shown to be redox regulated[282, 284, 285].

Several additional transcription factors such as AP-1, Nrf2, HIF and p53 have since then been shown to be either directly or indirectly regulated by redox processes that are primarily mediated by the Trx and GSH systems[283]. Interestingly, many of these transcription factors are closely linked to cell survival and cell development due to their importance in stress response, proliferation, differentiation as well as apoptosis. Their deregulation is thus often the basis of various pathophysiological conditions, which further highlights the importance of studying the underlying regulatory mechanisms[282, 283].

Moreover, various pathways that regulate the activation of transcription factors may be functionally intertwined. For instance, Nrf2 activation that is typically mediated by oxidative stress or via reactive electrophiles, promotes the transactivation of antioxidant and detoxifying enzymes, including components of the Trx and GSH systems.

Upregulation of these systems affects in turn oxidant levels and redox sensitive processes such phosphorylation mediated signaling cascades, which is for instance highlighted in the H_2O_2 supported activation of NFκB[286-288]. The interested reader is referred to the recent reviews by Brigelius-Flohé, R. and Flohé, L.[282] as well as Marinho, H. et al.[283] for further details on the overall concepts of redox regulation in gene transcription.

The following chapters will briefly introduce the basic concepts of NFκB, HIF and Nrf2 regulation in reference to **Paper III**.

NFκB

NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) refers to a family of dimeric transcription factors that consist of the p65 (RelA), RelB, c-Rel, p50 and p52 subunits, with the last two being produced from their precursors p105 and p100[289]. All of these proteins have a N-terminal Rel homology domain (RHD) that contains a nuclear localization signal (NLS) and that is also responsible for DNA and protein binding. However, only the three Rel subfamily proteins contain additional C-terminal transactivation domains (TADs)[289]. Homodimers of the p50 and p52 proteins are thus often discussed as repressors as they can bind to the DNA, but are unable to activate transcription[290].

The NFκB system can be found in almost all cells and is primarily involved in the immune and inflammatory response as well as in cellular growth and apoptosis. It is typical activated by cytokines (e.g. IL-1, TNFα), bacterial and viral antigens (e.g. LPS, PAMPs) or growth factors (e.g. EGF) that initiate a response by stimulating different receptor families (e.g. RTK, TLR, TNFR) that in turn activate NFκB through distinct phosphorylation cascades[282]. The list of NFκB targets encompasses several hundred genes including cytokines/chemokines, growth factors, adhesion molecules, receptors, stress response genes, regulators of apoptosis, enzymes and many more. The incorrect regulation of NFκB is furthermore associated with many diseases such as muscular dystrophy, cancer, diabetes and atherosclerosis[291]. An extensive summary of most aspects regarding NFκB, including extensive lists of activators and related diseases, can be found on the web-page of the "The Gilmore Lab" (http://www.bu.edu/nf-kb/) **(Fig. 10)**.

NFκB belongs to the type of transcription factor that is present in the cytosol in an inactivated state. Upon stimulation NFκB is released from its inhibitor IκB and translocates into the nucleus where it binds to specific promoter regions of its target genes together with the transcriptional coactivators CBP and p300. This setup enables a rapid initial responds to harmful stimuli as is does not require new protein synthesis **(Fig. 10)**.

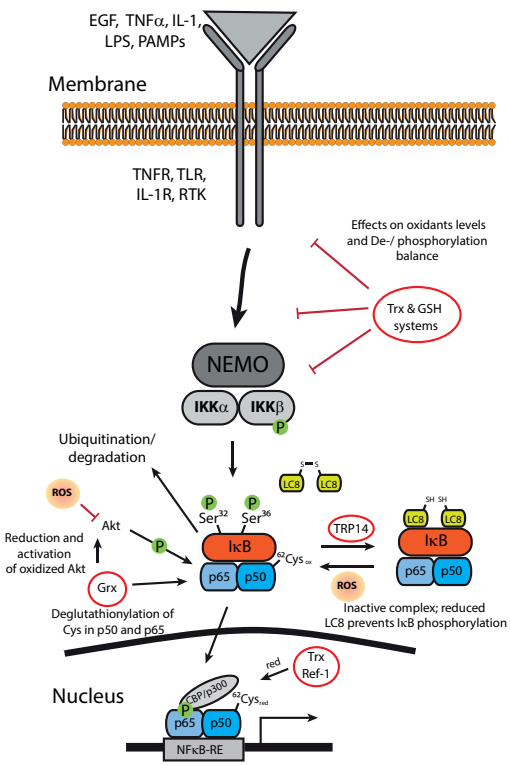


Figure 10. Typical activation pathway for NFκB. See text for further details. The predominant cytosolic NFκB complex is considered to be p50/p65/IκBα. Activation is initiated by receptor mediated phosphorylation cascades in response to stimulation. The central event is IκB phosphorylation via IKK, whereupon IκB is ubiquitinated and degraded. The p65/p50 dimer translocates to the nucleus and binds to specific sequences within the promoter regions. The coactivators CBP and p300 are also recruited and gene transcription is initiated. NFκB activity is modulated by the Trx and GSH systems in several ways as indicated. The here depicted scheme is only one of several pathways. Especially the initiating phosphorylation cascades and the specific response to certain stimulations is complex[292]. This figure was modified from[282].

In the absence of stimulation, NFκB dimers are sequestered in the cytosol by members of the IκB family (Inhibitors of κB) such as IκBα (major variant), IκBβ, IκBε, IκBγ and BCL-3[293]. These proteins contain several ankyrin repeats that mediate binding to the RHD, which in turn prevents NFκB from binding to the DNA and from translocating into the nucleus by interfering with the NLS. The key event in NFκB activation is thus the release of the NFκB dimer from the cytosolic complex[288]. This is achieved by signal-induced phosphorylation of IκB, which leads to ubiquitination and subsequent proteasomal degradation of the inhibitor[294]. IκB phosphorylation is

primarily catalyzed by the IκB kinase (IKK) – a complex that is composed of IKKα, IKKβ and two IKKγ subunits that form the NFκB essential modulator (NEMO)[295] **(Fig. 10)**. Notably, the upstream events leading to NFκB activation as depicted in **Figure 10** are complex and depend amongst other things on the receptor involved.

As mentioned above, NFκB is the first transcription factor that has been shown to be redox regulated. In contrast to early ideas, which implied that H_2O_2 could directly initiate NFκB activation, are current concepts promoting the view that H_2O_2 rather supports the activation by other stimuli[287, 288]. The earlier notion that H_2O_2 directly activates NFκB is thought to have come from the overlapping effects of NOX activators and NFκB enhancers[282, 286].

The beneficial effect of oxidants on NFκB activation has been mainly attributed to the modulation of redox sensitive de-/phosphorylation events[296]. Oxidative inactivation of phosphatases for example, leads to increased phosphorylation states within all levels of the NFκB activation cascades and thus enhanced signaling[297]. The diminishing effects on NFκB activation by Trx overexpression are therewith suggested to involve the reactivation of phosphatases and the antioxidant properties of the system[286, 298]. Interestingly, nuclear Trx1 is, in contrast to its preventive role in the cytosol, essential for DNA binding of the p65/p50 complex by catalyzing the reduction of the critical Cys62 in the p50 subunit[151, 299]. Another regulatory mechanism of the Trx system is given by TRP14, which has been implied in the prevention of NFκB activation by keeping the dynein light chain LC8 reduced. Reduced LC8 binds IκB and prevents IKK mediated phosphorylation and subsequent degradation[300]. Furthermore, the Grx system has been implied to modulate NFκB activation via deglutathionylation of kinases and phosphatases. It was also shown that Grx mediated deglutathionylation of critical cysteines in the p65 and p50 subunits possess a regulatory mechanism[301, 302].

HIF

Hypoxia inducible factors (HIFs) are transcription factors that mediate the response to changes in oxygen tension during physiological and pathological conditions such as cancer or cardiovascular disease at systemic and cellular levels. Their activation has thus been implicated in angiogenesis, erythropoiesis and glycolysis[303].

HIFs are dimeric proteins composed of the constitutive HIF-1β subunit and one of the three inducible HIFα proteins (HIF-1α, HIF-2α, HIF-3α), with HIF-1α being the most extensively studied form. HIF-1β is present in excess so that the HIF-1α levels determine the overall transcriptional activity[304].

All HIF-1α proteins contain an oxygen-dependent degradation domain that has two conserved proline residues. At normal oxygen levels, at least one is hydroxylated by prolyl hydroxylases (PHDs). The hydroxylated protein is recognized by the Hippel-Lindau tumor suppressor protein (pVHL), which recruits an ubiquitin ligase that targets HIF-1α for subsequent proteasomal degradation[305]. The catalytic activity of the PHDs is oxygen dependent and thus inhibited at low oxygen tension, enabling HIF-1α stabilization and transcriptional activation[306]. Notably, HIF-1α can also be hydroxylated at an asparaginyl residue in an oxygen dependent manner by the factor inhibiting HIF (FIH). This modification blocks association with the p300 coactivator and represses thus HIF dependent transactivation[307, 308].

The finally stabilized HIF-1α dimerizes with HIF-1β and binds to the specific hypoxia response elements (HRE) that are characterized by the (G/ACGTG) motif in the promoter region of its target genes to initiate transactivation[309]. FIH mediated hydroxylation is also inactivated under hypoxic conditions allowing thus recruitment of the CBP/p300 coactivators[310] **(Fig. 11)**.

Although HIF activation is predominantly mediated by low oxygen levels it has been shown that additional, hypoxia-independent, mechanisms activate this transcription factor as well. For instance, HIF activation in normoxia was observed upon stimulation with growth factors, cytokines or hormones[311]. The underlying mechanism is suggested to be a combination of NOX mediated H_2O_2 production and NFκB activation[312], which

promotes HIF-1α transcription based upon a NFκB-binding site in the HIF-1α promoter region[313, 314]. Other studies also showed that lipopolysaccharide stimulates HIF-2α signaling via NFκB activation, thus further linking these transcription factors. The connection between these two pathways is also often discusses in cross-tolerance as for example an inflammatory stimulus may protect an organ from an subsequent ischemic insult[315].

The remaining question is how HIF-1α stabilization is achieved under normoxic conditions. HIF-1α has been reported to be redox sensitive, but as to how this affects its stabilization is not known yet. Instead it is suggested that H_2O_2 oxidizes the catalytic Fe^{2+} in PHD to Fe^{3+} thus inactivating the enzyme[316]. For instance, H_2O_2 production due to glucose oxidase in the cell culture medium was shown to promote HIF-1α stabilization by inhibiting hydroxylation[317]. Other HIF-1α stabilizing examples include NOX1 overexpression, mitochondria-derived ROS or the aforementioned H_2O_2 production during NFκB activation[64, 65, 318]. HIF, like many other transcription factors, is furthermore regulated by H_2O_2 via effects on redox sensitive proteins within the translation and proteasomal degradation machineries[283] **(Fig. 11)**.

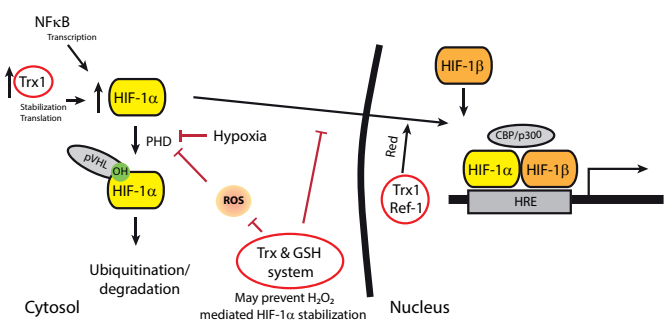


Figure 11. Principle regulation of HIF. See text for further details. HIF-1α and HIF-1β are the predominant and most studied variants. HIF-1α is hydroxylated by PHD, which marks the protein for subsequent ubiquitination and degradation under normal conditions. The catalytic function of PHD is oxygen dependent and thus inhibited in hypoxia. Furthermore, PHD is inhibited by ROS, thus enabling HIF activation also in normoxia. PHD inhibition results in the translocation of HIF-1α into the nucleus where it dimerizes with HIF-1β. The complex binds to specific HRE sequences within the promoter region of target genes and thus activates transcription. The role of the Trx system in the regulation of HIF is yet largely unclear. However, a number have been proposed as indicated.

Despite the case that reactive oxygen species are able to promote HIF-1α stabilization did neither TrxR1 overexpression nor depletion display a notable effect on HIF stabilization or function[319, 320]. This lack of effect can potentially be attributed to a number of reasons: i) the presence of the GSH system, which effectively controls

reactive oxygen levels in the cytosol; ii) residual TrxR1 levels in knockdown cells may be sufficient for Trx1 reduction; iii) back-up systems for Trx1 reduction[321, 322] or that iv) catalytic mechanisms are not that essential.

Interestingly however, numerous studies proposed that redox active Trx1 may modulate stabilization, translation, translocation into the nucleus as well as DNA binding and transactivation of HIF-1α[320, 323-329]. For instance, it is assumed that the Trx1 (or via Ref-1) catalyzed reduction of cysteine 800 in HIF-1α is required for CBP/p300 recruitment and thus transactivation[323, 325] **(Fig. 11)**. Furthermore did Kim et al. propose that Trx1 promotes nuclear localization and prevents degradation of HIF-1α by promoting the dissociation of pVHL and HIF-1α, as shown via Trx1 knockdown and overexpression experiments[326]. These results are well in line with several other studies showing that Trx1 overexpression markedly enhanced HIF-1α protein levels and target gene expression (e.g. VEGF, COX-2) under hypoxic and partly also normoxic conditions[324, 326, 327, 329]. Welsh et al. furthermore pointed out that this effect did not account for changes in mRNA levels[324], which was later confirmed by Zhou et al., who additionally demonstrated that Trx1 overexpression promotes cap-dependent translation of HIF-1α by activating p70S6K and eIF-4E[329]. Activation of these components is essential for translation initiation and was furthermore shown to be mediated by the PI3K/Akt and MAPK signaling pathways. The exact mechanism is not known, but it was proposed that Trx1 mediates activation of these pathways by binding to and thus inhibiting PTEN, which is a negative regulator of these signaling pathways[159, 329, 330] **(Fig. 11)**. In addition there are several studies that further illustrate the connection between "Trx1 – PI3K/Akt/MAPK – HIF activation" or more general "PI3K/Akt/MAPK – HIF activation" in a number of conditions[331, 332]. These results are furthermore in agreement with the aforementioned studies that reported HIF-1α stabilization and activation to be largely independent of TrxR1 as PTEN inhibition is facilitated via protein-protein binding.

One aspect that is still debated is whether Trx1 is essential for HIF-1 activation or whether the reported mechanisms can only be attributed to elevated Trx1 levels. Naranjo-Suarez et al. showed recently that Trx1 is neither essential for HIF-1α stabilization nor its activity[320]. These results are however, in stark contrast to other

studies showing that Trx1 knockdown, expression of a redox inactive variant or inhibitors could markedly decrease HIF activation[324, 326, 328, 333].

Nrf2

Nrf2 (Nuclear factor (erythroid-derived 2)-like 2) is one of the most important transcription factors in regulating detoxification and oxidative stress response. Activation is typically mediated by a variety of exogenous and endogenous stressors such as electrophilic agents and reactive oxygen species[334]. It binds to a common DNA regulatory element called the antioxidant responsive element (ARE; alternatively called EpRE for electrophile responsive element) in the promoter region of genes that collectively promote cell survival such as detoxifying enzymes, antioxidant proteins (Trx, TrxR, Prx1, GPx2), enzymes for glutathione synthesis (γ-glutamyl-cysteine synthetase), receptors, transcription factors, proteases and many more[111, 131, 282, 335, 336].

Nrf2 is bound to its inhibitor Keap1 under normal conditions, which targets Nrf2 constantly for proteasomal degradation. Keap1 is also acting as sensor for Nrf2 activating compounds causing it to undergo a conformational change by either binding to or oxidizing critical cysteine residues[337, 338]. As a consequence, the Nrf2-Keap1 binding is partly disrupted so that Nrf2 ubiquitination and degradation is blocked. Nrf2 is however not released, but instead occupies the now inactive Keap1 so that newly synthesised Nrf2 can freely translocate into the nucleus where it forms heterodimers with bZIP transcription factors such as Mafs (predominantly), c-Jun or ATF4 prior binding to ARE[339] **(Fig. 12)**. It has been, and still is, debated whether Nrf2 also dissociates from Keap1[282]. However, a recent study showed clearly that Nrf2 is indeed not released from Keap1[340]. In agreement with this it was also demonstrated that Nrf2 activation is strongly affected by H_2O_2 on the translational level. Steady state concentrations of 12.5 μM promote Nrf2 synthesis with a rate that exceeds nuclear translocation, which furthermore suggests that there has to be a very potent, but yet unknown H_2O_2 sensor[283, 341].

Keap1 is a homodimer that functions as adaptor of the Cullin-3-based E3 ligase. Each Keap1 subunit contains furthermore 27 cysteine residues of which 9 have been predicted to be reactive due to a basic microenvironment[342]. Based on the broad

chemical heterogeneity of Nrf2 activators it is also suggested that these are targeted differently by different electrophiles, which may furthermore translate into specific cellular responses[343]. Cys151, Cys273 and Cys288 are crucial examples among these residues. Cys151 was for instance shown to be important for H_2O_2 mediated Nrf2 activation by forming an intermolecular disulfide with Cys151 of the second Keap1 molecule, which releases Cullin-3 that is required for degradation and Keap1 dimerization[344, 345]. The Cys273 and Cys288 residues are furthermore Zn-correlated and essential for the response to many Nrf2 activators. A modification of those disrupts the Zn-stabilised conformation of Keap1 and thus inhibits degradation[346] **(Fig. 12)**.

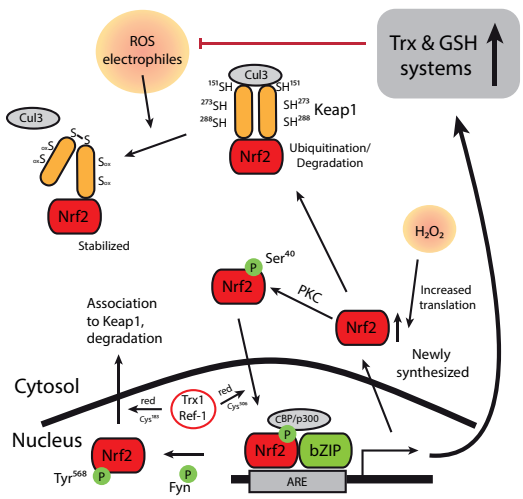


Figure 12. Principle regulation of Nrf2. See text for further details. Nrf2 is bound to its inhibitor Keap1, which targets it for proteasomal degradation. Keap1 also serves as redox sensor and changes conformation in response to oxidation or alkylation of its crucial cysteines. The complex is thus stabilized and degradation prevented. Newly synthesized Nrf2 bypasses the loaded Keap1 and is translocated into the nucleus where it binds to specific ARE sequences. Nuclear Trx1 is important for the reduction of critical cysteines in Nrf2. One is important for DNA binding, whereas the other is involved in nuclear export. Nrf2 is additionally subject to phosphorylation, which further modulates its activation. Among the Nrf2 target genes are enzymes of the Trx & GSH system. The upregulation of these counters the initial Nrf2 activating conditions and facilitates detoxification and antioxidant defense. This figure was modified from[282].

Not only Keap1, but also Nrf2 itself is subject to redox regulation. It contains at least two redox sensitive cysteine residues within its nuclear localization signal (NLS) and nuclear export signal (NES) sequences. Oxidation of Cys183 in the NES site interferes with Crm1 (chromosome region maintenance 1; exportin) binding and thus retains Nrf2 in the nucleus[347, 348]. A similar effect was also reported for nuclear Keap1, which thus further prevents the nuclear export of Nrf2[349]. These oxidations may be reversed by the GSH or Trx systems. Trx1 was for example shown to promote nuclear export of Nrf2[158]. This particular function is highlighted in *txnrd1*$^{-/-}$ cells that showed a prolonged Nrf2 transactivation that was attributed to Nrf2 persistence in the nucleus[350]. Reduction of Cys506 in the NLS region that is catalyzed by Trx1/Ref-1[152, 158] is furthermore also

important for the interaction with the coactivators CBP/p300 as well as for DNA binding[351] **(Fig. 12)**.

Nrf2 is additionally regulated by phosphorylation – certain events promote Nrf2 activation, whereas it is diminished by others. The Ser40 residue is for example phosphorylated by the redox sensitive protein kinase C (PKC), which prevents binding to Keap1 and promotes nuclear translocation[352]. On the other hand, Nrf2 can be phosphorylated by Fyn at Tyr568 in the nucleus, promoting Crm1 interaction and thus nuclear export. Activation and nuclear translocation of Fyn is seen several hours after Nrf2 activation and regulated by a H_2O_2 activated phosphorylation cascade[353] **(Fig. 12)**. Processes that involve phosphatases and kinases are furthermore susceptible to cross-talk between different signaling pathways – an aspect that has been compiled by Brigelius-Flohé R. and Flohé L. in the context of Nrf2[282, 354].

6 Projects

6.1 Aims

The thioredoxin system has been discussed in a variety of different contexts and many of its important roles in health and disease have been discovered. However, its involvement in cellular pathways is versatile and countless aspects that contribute to the final outcome of the cell are still unknown. With this in mind we studied three separate aspects of the Trx system that had the following specific aims:

Paper I

- TrxR1 targeting and inhibition by Pt, Au and Pd based compounds in relation to cancer cell survival.
- Characterizing the mechanism of inhibition and the potential formation of SecTRAPs *in vitro* and in cell culture.

Paper II

- Analyzing the underlying mechanism of the intracellular localization pattern of the v3 splice variant of TrxR1.
- Clarifying whether the effects on actin polymerization can be attributed to a potential redox activity of the Grx-domain of v3 or whether other properties are essential.

Paper III

- Developing a method to simultaneously study the behavior of several functionally intertwined transcription factors with single cell resolution.
- Applying this method to study the regulation of Nrf2, HIF and NFκB, which are all in part regulated by the Trx system.

6.2 Paper I

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

Stefanie Prast-Nielsen*, **Marcus Cebula***, Irina Pader, Elias S. J. Arnér. *Free Radic Biol Med*. 2010; 49: 1765-1778. *Equal contribution

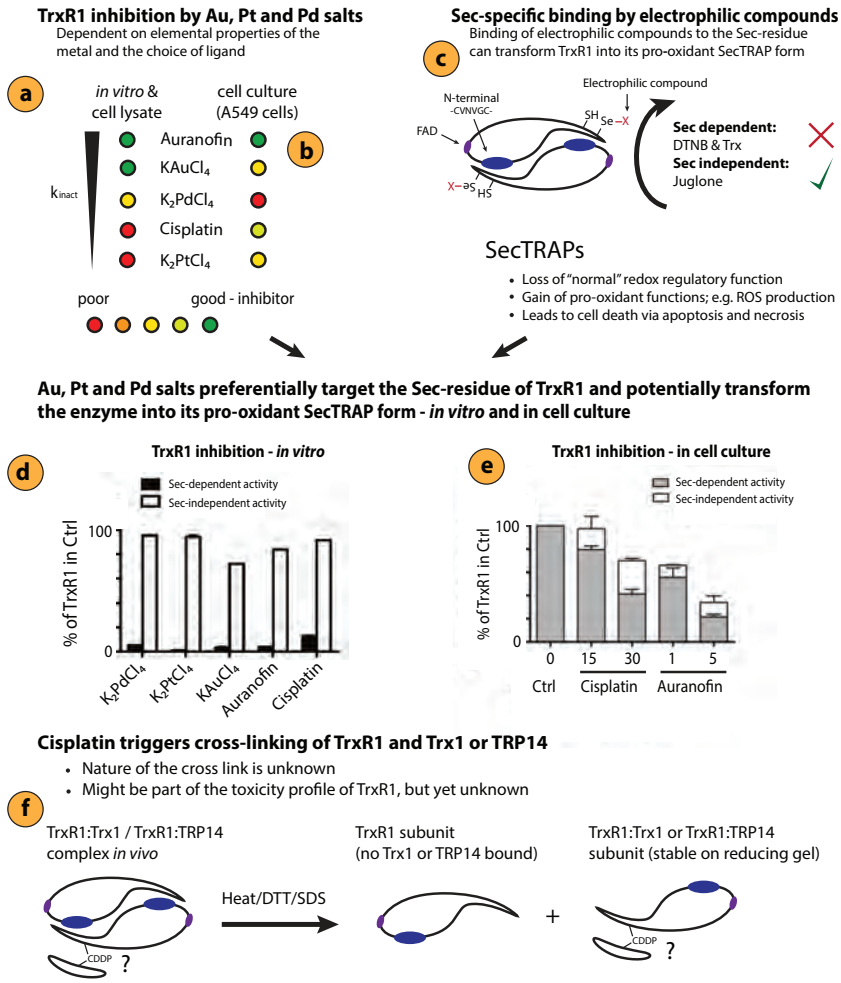


Figure 13. Summary of Paper I. See text for a detailed description. a, b) The inhibition profile is dependent on the elemental properties of the metal and the choice of ligand. Cellular uptake and inhibition is particular affected. **c, d)** The various salts are preferentially targeting the Sec residue of TrxR1 and potentially converting the enzyme to its pro-oxidant SecTRAP form. **e)** Cisplatin and auranofin could also both modify the enzyme into its potentially Sec-TRAP form within cells. **f)** Cisplatin has additional features with regard to TrxR1 as it cross-links Trx1 and TRP14 to its subunits.

Results

Elemental properties of the metal and choice of ligands are the major parameters effecting uptake and TrxR1 inhibition

Pd based compounds have already been synthesized and validated in terms of anticancer effectiveness and cytotoxicity in comparison to Pt based drugs on account of their chemical similarity[355-358]. In line with these studies we demonstrated that Pd as well as Au based salts were exceedingly more potent than their Pt counterparts in terms of inhibiting TrxR1 *in vitro* as well as in cell extracts **(Fig. 13a)**. The ligand substituents on the other hand affected the inhibition rates only marginally - $KAuCl_4$ for example, performed similar to auranofin as the Pt salts did compared to cisplatin. This pattern was in contrast to the cellular inhibition profile where cisplatin and auranofin both inhibited TrxR1 several fold more efficiently than their $KAuCl_4$ and K_2PtCl_4 counterparts. Particularly, the comparable Pd salt K_2PdCl_4 had surprisingly mild effects given that it was an efficient inhibitor in crude cell extract **(Fig. 13b)**. This rather indicates a limited cellular uptake than extensive off target binding.

Furthermore, the inhibition of TrxR1 correlated well with a reduction in cell viability, suggesting that the disruption of the thioredoxin system might to some extent contribute to the cytotoxic profile of the respective compounds. Off target effects can however, not entirely be excluded due to the electrophilic nature of the compounds as indicated for instance by $KAuCl_4$, that not only inhibited TrxR1, but also Trx1 in cell culture.

In vitro characterization of SecTRAP formation

In context of intracellular TrxR1 targeting it is furthermore important to study the specific mechanism of inhibition, i.e. whether the complete enzyme activity is inhibited or whether "only" the Sec-dependent functions are impaired by which the enzyme may transform into its pro-oxidant SecTRAP form. The gain of function in this case would likely support the anti-cancer efficiency compared to a mere decrease in cellular TrxR1 activity, which was shown to exhibit rather mild effects in a number of cases[196, 359] **(Fig. 13c)**.

Using recombinant TrxR1 we noted that the inhibition profile was strongly suggestive of preferential binding to the highly nucleophilic selenolate anion in the C-terminal active site. The evidence for this mode of action includes: I) NADPH dependent reduction of the selenosulfide prior incubation with the compounds was needed for inhibition; II) approximate reaction order of 1 (stoichiometric ratio between metal compound and enzyme); III) diminished DNTB (Sec-dependent), but sustained juglone (Sec-independent) reduction and thus NADPH oxidase activity; IV) no inhibitory effect on the structurally related glutathione reductase as well as V) fast inhibition kinetics that are in agreement with preferential targeting of the highly reactive and accessible selenolate. Although the direct binding to the selenolate was not explicitly proven in this study it shall be noted that the susceptibility of a Sec-residue towards electrophilic compounds was reported by others[131, 186, 360-362], including a study showing direct derivatization of the Sec-residue in TrxR1 by platinum compounds via X-ray crystallography[363] **(Fig. 13d)**.

Identification of intracellular SecTRAP formation as proof of principle

In order to test if such TrxR1 derivatives can be formed intracellularly we performed immunoprecipitation studies on cell extracts of cisplatin and auranofin treated cancer cells followed by activity measurements as a proof of principle. In agreement with the SecTRAP concept we found that the ability to redox cycle with juglone (Sec-independent) was maintained to a larger extend than the capacity to reduce Trx1 (Sec-dependent). When extrapolated to the cell extract we estimated that cisplatin could derivatize up to 40% of the enzyme, whereas auranofin treatment led foremost to a strong decrease in overall TrxR1 activity with only up to 20% derivatized enzyme **(Fig. 13e)**. However, the yield and the recovery of the enzyme was probably not qualitatively and the whole handling procedure may have further altered the activity of the enzyme as well. A solid conclusion regarding the intracellularly formed SecTRAPs is thus difficult, but we believe that this data further support the pro-oxidant concept of derivatized TrxR1 enzymes.

TrxR1:Trx1 and TrxR1:TRP14 complex formation and potential functions

Surprisingly, we also discovered the intracellular formation of covalently linked complexes of TrxR1 with either Trx1 or TRP14 upon treatment of cells with cisplatin, and to some extent with the Pt salt, but not with any of the Au and Pd compounds **(Fig. 13f)**. A potential function or contribution of these complexes to cisplatin mediated cytotoxicity and anticancer efficacy is not established yet. Considering the two observations that Trx1 activity was not inhibited by cisplatin and that only a minor fraction of the protein was actually trapped in the complex suggests that inactivation of Trx1 is not part of a potential mechanism. On the other hand, the trapping involved rather high amounts of TRP14 compared to their basal levels, indicating that disruption of TRP14 dependent functions could be an important consequence.

While analyzing tryptic digests of the complex via mass spectrometry we could not identify the nature of the cross-link. With the exception of the C-terminal active site in TrxR1 we detected however, all other respective active sites, which were also essentially unmodified. Cisplatin might thus not link both enzymes via their active sites despite its known ability to cross-link nucleotides and proteins. Potential alternative mechanisms might involve cisplatin mediated cross-linking at the interface between the enzymes or conformational changes due to intraprotein cross-linking. Another possibility is cross-linking via surface exposed residues that have undergone oxidative modifications. These modifications may be mediated by potentially high local ROS concentrations due to the NADPH oxidase activity of the cisplatin transformed enzyme.

In summary, we show that *in vitro* inhibition kinetics are strongly affected by the elemental properties of Au, Pd and Pt. All compounds targeted preferentially the Sec-residue and potentially converted the enzyme into a pro-oxidant SecTRAP. Cellular TrxR1 inhibition was however, strongly dependent on the ligand substituents. Additionally, cisplatin mediated the formation of covalently linked complex of TrxR1 and either Trx1 or TRP14 with yet unknown consequences for the cell.

Discussion and future perspectives

These results collectively demonstrated strong elemental differences in TrxR1 targeting and inhibition as well as a substantial dependence on the ligand substituent in context of cellular uptake and metabolism. The inhibitory effect by Pd together with the positive correlation between TrxR1 inhibition and reduction in cell viability identify Pd as a promising candidate for the development of a new class of TrxR targeting drugs if a suitable ligand substitution can be established. In any case, it would be interesting to study the effects of TrxR1 targeting in a more complex system. Many clinically used drugs such as auranofin and cisplatin are thought to exhibit part of their function via inducing oxidative stress by targeting antioxidant systems. However, a direct correlation between specific TrxR1 inhibition and anti-tumor efficacy in a mouse model for example was not yet established.

Previous studies that characterized the rapid cell killing effects of SecTRAPs relied on protein delivery systems to directly transfer the derivatized enzyme into the cell[178, 195]. This method likely caused a rapid intracellular accumulation of the pro-oxidant enzyme form, which in turn influenced the cellular response by promoting rapid and severe oxidative stress[195]. Intracellular SecTRAP formation via drug treatment on the other hand might lead to less rapid effects given that the pro-oxidant form accumulates probably slower and steadier. Given the slower dynamics, the cells might upregulate compensatory antioxidant enzymes that might diminish a cell killing effect. Such a situation was previously characterized where a truncated form of TrxR1, which was also classified as a SecTRAP[195], was overexpressed in cancer cells via DNA transfection[364]. It is thus important for future studies to characterize the dynamics of intracellular SecTRAP formation – whether it accumulates fast enough to promote efficient cell killing and how the cell reacts to a steady increase in the pro-oxidant form. Novel Pd based TrxR1 inhibitors might thus transform the enzyme efficient enough to make the difference.

One aspect that we have not looked into, but that might be interesting for future mechanistic studies, is the potential influence of the Trx1 or TRP14 attachment on the pro-oxidant functions of the cisplatin derivatized enzyme. To know if the NADPH

oxidase activity is diminished or aggravated might have a huge impact on the SecTRAP concept and the design of future compounds and studies. This might not only concern stable and DTT resistant complexes such as the cisplatin induced variant, but also potential complexes that are sensitive towards DTT reduction while being stable in solution. For instance, a truncated TrxR2 variant (missing the Sec residue) was found to interact stronger with Trx1 *in vitro*[203]. Another study established a crystal structure of the TrxR1-Trx1 complex using double mutants of TrxR1 [C497S,U498C] and Trx1 [C35S,C73S][177], thus showing that non-covalently linked TrxR1:Trx1 complexes may be formed independently of cisplatin. Further evidence is presented in paper V, which is however, not included in this thesis. We show there that a compromised Sec-residue is sufficient for DTT sensitive complex formation. All in all, this shows that further research is needed to solidify the concept of a pro-oxidant function of Sec-compromised TrxR1 *in vivo* as well as their influences on the cell.

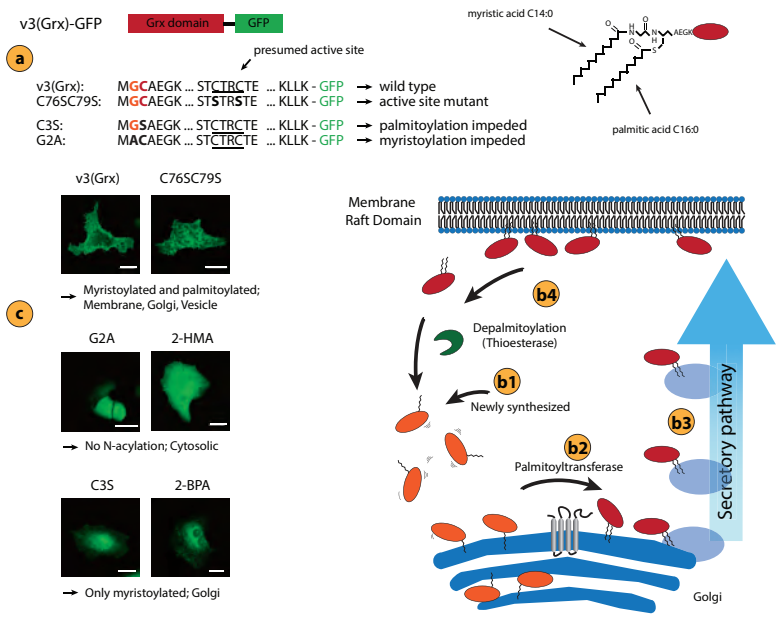
Upon the discovery of TRP14 as component of the cisplatin mediated complexes we also aimed to further characterize this yet sparsely studied enzyme. Within paper 4, which is not included in this thesis, we characterized its kinetic parameters as substrate of TrxR1 and demonstrate that it is an efficient L-cystine reductase and S-denitrosylase.

6.3 Paper II

TXNRD1-encoded v3 is targeted to membrane rafts by N-acylation and induces filopodia independently of its redox active site integrity.

Marcus Cebula, Naazneen Moolla, Alexio Capovilla and Elias S. J. Arnér. J *Biol Chem.* 2013; 288:10002-10011.

v3(Grx)-GFP fusion constructs and mutants thereof were used to show that v3 is myristoylated and palmitoylated at the N-terminus.



v3(Grx) is targeted to CT-B positive membrane raft microdomains with expression being correlated to the induction of filopdia independently of an intact active site motif.

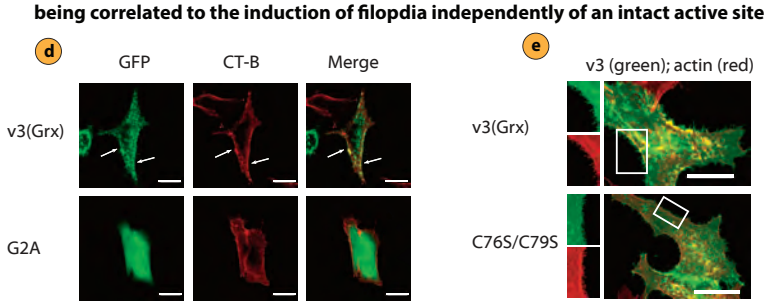


Figure 14. Summary of Paper II. See text for a detailed description. a) The glutaredoxin domain of v3 and variants thereof were expressed as GFP-fusion proteins to study the mechanism of intracellular localization. **b-d)** We show that myristoylation at the Gly2 residue targets the enzyme to the golgi where it is subsequently palmitoylated at Cys3 and transported to membrane raft microdomains as shown via colocalization with the known raft marker CT-B. After performing its function, v3 is depalmitoylated and diffuses back to the Golgi where the cycle starts anew. **e)** At the membrane v3 induced the formation of filopodia independently of an intact active site.

Results

Intracellular localization of v3 is dependent on myristoylation and palmitoylation at the N-terminal MGC-motif

We applied, in accord with previous studies, constructs that express only the glutaredoxin domain of v3 in fusion to GFP and variants thereof throughout the whole study **(Fig. 14a)**. To our surprise, the intracellular localization pattern and filopodia formation were completely independent of an intact -CTRC- active site motif. Instead, all features were dependent on the integrity of the N-terminal MGCAEG-sequence, which we found to meet the requirements of a myristoylation and palmitoylation site. In combination, these two processes form a rather well characterized two-step N-acylation that effectively dictates the intracellular sorting and localization of the protein. While being newly synthesized, v3 is first co-translationally modified by linking myristic acid (C14:0) irreversibly to the N-terminal Gly2 via an amide bond **(Fig. 14b1)**. The resulting increase in hydrophobicity serves to promote transient membrane interaction that in turn is essential for stable membrane association, subcellular localization and protein function[365]. Myristoylated v3 associates with golgi membranes were it gains access to membrane-bound DHHC domain palmitoyltransferases that catalyze the addition of palmitic acid (C16:0) to the adjacent cysteine residue at position 3, thus further solidifying v3 into the membrane **(Fig. 14b2)**. Palmitoylation is in contrast to other lipid modifications reversible and regulated and facilitates subcellular trafficking between different membrane compartments[366]. v3 is as such transported via the secretory pathway to its functional site at the plasma membrane **(Fig. 14b3)**. After facilitating its task, v3 is depalmitoylated and detaches from this membrane domains **(Fig. 14b4)**. The hydrophobicity of myristoyl group guides the protein subsequently back to the golgi where the cycle starts anew. These dynamics have been extensively characterized by several N-acylated proteins, including Ras, eNos, GAP43, and $G_i\alpha_1$[367-369]. A particularly neat study characterizes the palmitoylation dependent trafficking between Golgi and plasma membrane of H- and N-Ras, which yields a localization patterns highly similar to those found here for v3[369].

In expressing fusion proteins of GFP with either wild type v3 or its G2A and C3S mutants, known to impede myristoylation and palmitoylation, we could confirm this modifications as only the natural variant was targeted to the plasma membrane. The myristoylated, but not palmitoylated C3S variant displayed a strong perinuclear staining that is typical for this type of modification whereas the G2A mutant showed a cytosolic distribution similar to GFP. These patterns were additionally verified using the myristoylation and palmitoylation inhibitors 2-hydroxymyristic acid (2-HMA) and 2-bromopalmitic acid (2-BPA) **(Fig. 14c)**.

Palmitoylation targets v3 to CT-B positive membrane raft domains

Palmitoylation not only solidifies proteins into membranes, but also facilitates subcellular trafficking into membrane subdomains such as lipid rafts[366]. These microdomains are highly dynamic and able to form larger platforms that are thought to be stabilized by actin and essential for the assembly of functional complexes and signaling events[370-372]. To examine if v3 localizes into these lipid raft structures as consequence of its palmitoylation we performed co-localization studies using cholera toxin subunit B (CT-B) as a marker for ganglioside GM1 rich domains. In doing so we could verify that the v3 was raft associated and that palmitoylation was a requirement **(Fig. 14d)**. Interestingly we also found v3 dimers in purified CT-B positive lipid raft fractions indicating that membrane raft patching could have occurred. The induction of filopodia has indeed already been linked to membrane raft stimulation and patching in previous studies[373-375]. Thus a potential explanation is that actin polymerization and filopodia formation is induced as a means to stabilize raft platforms with are formed due to the extensive v3 accumulation **(Fig. 14e)**.

In summary, we found that v3 is myristoylated and palmitoylated at its N-terminal motive. As a consequence it is targeted to CT-B positive membrane rafts where it correlates with the induced formation of filopodia. All observed features were independent of an intact -CTRC- motif and the presence of the TrxR1 core module.
Its functions are yet unknown, but the here identified membrane raft association of the v3 splice variant of TrxR1 clearly expands the spectrum of the Trx system.

Discussion and future perspectives

Potential impact of N-acylation on the catalytic activity of v3 in relation to previous studies

The catalytic activity of recombinant v3 was previously characterized by Gladyshev et al., showing that the enzyme was unable to use Trx1 as substrate, but could efficiently reduce DTNB[208]. A truncated variant, missing the last two amino acids (Sec-Gly) displayed a similar activity as the Sec-containing enzyme, which is in strong contrast to TrxR1 where DTNB reduction is considered highly Sec-dependent. Interestingly, a similar behavior was reported for TGR that has a monothiol Grx domain as N-terminal to a thioredoxin reductase core module, thus indicating that the N-terminal domains have a strong influence on the catalytic mechanism and that both enzymes potentially share similarities in their catalytic mechanisms[199]. In contrast to v3, TGR was able to reduce Trx1 and also showed typical Grx activity – a property that v3 only gained after its unusual -CTRC- active site was mutated to the more common -CPYC- motif found in glutaredoxins[208].

However, these properties need to be potentially re-evaluated. The acylated, lipophilic N-terminal renders a homodimer with a symmetric head-to-tail confirmation of two identical v3 subunits unlikely as both opposite ends are unable to attach to the membrane simultaneously. It is thus reasonable to assume that v3 is either present as monomer or that it might form a heterodimer with only one subunit being v3 and the other the main variant of TrxR1. A monomeric form would be uncommon, but not implausible. It was for instance demonstrated that the monothiol active site motif of the Grx domain in TGR was able to receive electrons from either the thioredoxin reductase domain of TGR or TrxR1[199]. A dimer, consisting of the main TrxR1 variant and v3, might be also able to efficiently reduce Trx1 and exhibit a different catalytic profile than reported so far.

Potential functions at membrane rafts

In general, it would be of major interest to further probe potential functions and substrates of v3. The membrane raft association locates v3 in close proximity to several redox systems and proteins involved in signaling such as NOX, eNOS, Src family kinases, phosphatases, cytoskeleton-binding proteins and many more[371, 372]. The activity and function of several of those is regulated via reversible oxidative modifications. For instance, NOX mediated superoxide production is stimulated by EGF, PDGF and insulin-receptor signaling. The resulting ROS promotes in turn oxidation of redox sensitive proteins such as Prxs, kinases and PTPs as well as their downstream signaling events, thus affecting numerous cellular processes such as differentiation, proliferation and migration[269, 273, 376, 377] **(see Fig. 16 in summary section 6.5)**. v3 might in this setup potentially act as a local regulator of certain growth factor or hormone stimulated events via reactivation of certain PTPs or peroxiredoxins or via reduction of kinases such as Src. Previous studies have already shown that the Trx system catalyzes the efficient reduction of several peroxiredoxins as well as of PTPs such as PTP1B, PTEN as well as Cdc25A and B[258, 277, 377-380]. Interestingly, Grx was also shown to reduce PTP1B[377] as well as LMW-PTP, suggesting that the Grx domain of v3 might be able to mediate similar functions[381].

It shall be emphasized again that v3 was in previous studies not able to reduce Trx1 nor was its Grx domain active in typical assays whereas it should not be involved in the just mentioned processes. However, as illustrated above, v3 might exhibit a different catalytic profile if expressed in mammalian cells compared to the studies using recombinantly expressed enzyme. Alternatively, v3 might also reduce certain substrates either directly or via Trx-like proteins such as TRP14, which was also not studied yet.

Intriguing possibilities for v3 function reminiscent to the close correlation with actin are podosomes and invadopodia. These are actin rich invasive microdomains that are specialized for matrix degradation and potentially important for metastasis and cell movement through the matrix. They are formed by cancer and normal cells, respectively. Initiation, maturation and regulation of this structures is complex and involves numerous factors including growth factor and adhesion signaling, NOX

activation, PTP oxidation, kinases activation, actin regulatory elements, membrane rafts and many more[382]. Extensive ROS production is considered to be essential for these structures, but the regulation of redox processes is yet only sparsely analyzed[85, 383]. v3 function within specialized structures such as podosomes and invadopodia might also explain its general low abundance.

Potential future experiments

All evidence so far describes v3 as a rare enzyme. The regulation of its expression is only sparsely characterized on mRNA as well as protein level. Its presumably low expression levels are a major handicap for functional studies, whereas most studies need to rely on DNA transfection and overexpression. This is a general problem of low abundant membrane proteins and much effort is required to optimize detection methods[384].

Potential future experiments might involve overexpression of the Sec-containing full length v3 and mutants thereof in mammalian cells with subsequent growth factor or hormone stimulation while studying the cellular response. This approach might also be combined with the pTRAF methodology as described in **Paper III** to characterize the potential impact on the activation of transcription factors. Single active site mutants may be used to trap and identify potential binding partner. To distinguish v3 from other TrxR1 variants one can either use antibodies raised against the unique Grx domain or implement a FLAG-tag ca. 10-20 amino acids away from the N-acylation site. The characterization of the alternative v3 promoter as well as the expression of the endogenous variant on mRNA and protein level in relation to different cell types may also be further pursued.

6.4 Paper III

Simultaneous Determination of Nrf2, HIF and NFκB Activation at Single-Cell Resolution.

Katarina Johansson, **Marcus Cebula**, Olle Rengby, Kristian Dreij, Kristmundur Sigmundsson, Elias S. J. Arnér. Manuscript.

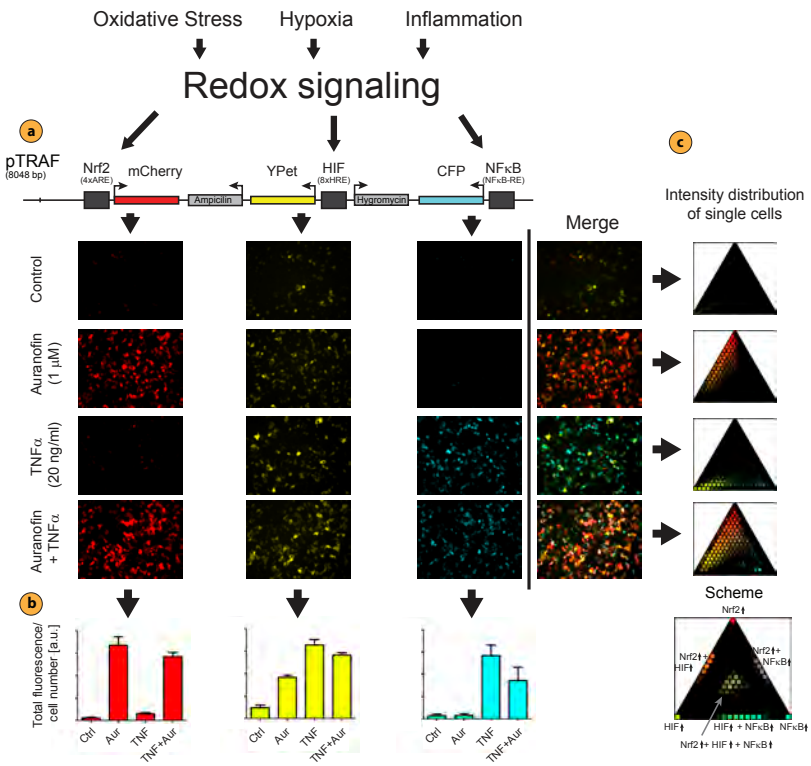


Figure 15. Summary of Paper III. See text for a detailed description. a) We constructed, characterized and utilized the pTRAF plasmid to analyze Nrf2, HIF and NFκB activation in response to various stimuli by measuring corresponding fluorescent signals on single cell level. The fluorescence intensities are analyzed as either **b)** an accumulated signal to display the total response of all cells or in **c)** single cell distribution plots to show the stochastic degree of the events. The data in the scheme were acquired at 1% oxygen tension.

Results

Construction and validation of the pTRAF method

We developed a method that utilizes fluorescent proteins to monitor the activation of three separate transcription factors. For this we constructed a single reporter plasmid (**pTRAF**: **p**lasmid for **t**ranscription factor **r**eporter **a**ctivation based upon **f**luorescence) that carries three separately transcribed "response element - fluorescent protein" combinations. This arrangement enables the simultaneous analysis of their intertwined regulation within a single cell without the need of a separate transfection control.

As a proof of principle we tested this system on the three major transcription factors Nrf2, HIF and NFκB that are all in part regulated by the Trx and GSH systems and involved in different pathological conditions such as cancer, chronic inflammation as well as cardiovascular and degenerative diseases. This particular pTRAF variant thus carried the following cassettes: "Nrf2 - mCherry (red)", "HIF - YPet (yellow)" and "NFκB - CFP (cyan)" **(Fig. 15a)**. For validation we stimulated pTRAF transfected HEK293 cells with the known Nrf2 and NFκB inducing drugs auranofin and TNFα at 1% and 21% oxygen tension and compared the results with traditional luciferase assays. The results agreed well at an accumulated cell-culture level with pTRAF having the advantage of a simultaneous analysis whereas the luciferase approach required three separate experiments **(Fig. 15b)**. We also verified that pTRAF does not alter the regular cellular response by quantifying transcripts of known downstream target genes for Nrf2, HIF or NFκB using quantitative PCR and comparing the result to non-transfected cells.

Validation of the pTRAF method for high throughput applications

To further characterize the performance of pTRAF, specifically to validate its usability for high throughput applications, we included several additional treatments and analyzed the corresponding transcriptional responses of Nrf2, HIF and NFκB using the Operetta® high-content imaging system. The results were highly reproducible and led to interesting observations. The combinatory treatment of doxorubicin and TNFα for instance stimulated a strong synergistic NFκB activation that was much higher than for

TNFα alone, whereas doxorubicin gave no NFκB activation by itself. Another interesting observation was the effect on HIF activation with a number of non-classified HIF inducers including those that activated NFκB **(Fig. 15b)**.

The main advantage of using pTRAF however, is the ability to validate the activation of all three transcription factors within each cell separately based on the arrangement of all cassettes being located on the same plasmid. Thus by comparing the intensities of mCherry, YPet and CFP to each other it is possible to study the relative activation of all three transcription factors in single cells without the need to control for transfection. We visualized these data by plotting the cells of a whole culture in a triangular plot. The resulting pattern shows the treatment dependent distribution of single cell transcription factor activities and thus the level of stochastic cellular events **(Fig. 15c)**. A potential improvement that would be beneficial if implemented might be to quantify the stochastic degree to be able to better compare various treatments.

In summary, we developed and validated the pTRAF method for the simultaneous analysis of three different transcription factors on single cell level. We initially focused on Nrf2, HIF and NFκB responses, but the methodology can also be adapted to other combinations of three response elements.

Discussion and future perspectives

Based on these preliminary, but very encouraging results, we aim to further improve the pTRAF method and to expand the scope of its applications. The main aspect that we wish to follow up is to apply this tool to study how Nrf2, HIF and NFκB are intertwined and regulated on single cell level. A potential approach could involve the combination with additional methods. For instance may cells with a specific response pattern be separated via FACS and subsequently analyzed regarding their unique mRNA or protein composition in order to understand why they respond the way they did.

Improvements on the pTRAF methodology

The fluorescent proteins mCherry, YPet and CFP were chosen as they are stable, monomeric proteins with a high quantum yield and a short maturation time while enabling an optimal separation between the channels. However, the high demand on suitable fluorescent proteins for imaging pushes the development of more suitable variants constantly forward. For example, a highly improved cyan fluorescent protein, termed mTurquoise2, was recently developed that might strongly improve the signal-to-noise ration when analyzing NFκB activation with pTRAF[385]. Other examples are given by far red and near-infrared fluorescent proteins[386]. They might be more suitable than mCherry depending on the technical specifications of the setup as the suitable margin for optimal excitation and emission is increased.

So far experiments relied on transient transfection to transfer the pTRAF plasmid with an approximate efficiency of 50% into the cells. To achieve either higher transfection rates or to be completely independent on transient transfection procedures altogether we wish to develop a viral transfection routine and to establish a cell line that has the pTRAF plasmid incorporated into its genome.

An alternative approach in using pTRAF is to exchange one response element with a constitutively active promoter. This specific variant would give the possibility to set the activation of the two remaining transcription factors in relation to a well established and characterized promoter, thus enabling to further quantify the absolute response of a single cell. In addition we also wish to further implement different response elements. For instance would it be also of interest to monitor p53 or c-Myc in cancer cells for instance.

6.5 Summary and conclusion

Within this thesis we investigated different aspects of the Trx system in the context of redox signaling with **Figure 16** giving a graphical summary that combines all three papers in the cellular context.

In **Paper I** we focused as such on the inhibition of TrxR1 by noble metal based drugs and showed that all used compounds efficiently targeted the Sec-residue and thus potentially transformed the enzyme into its pro-oxidant SecTRAP form. This transformation not only diminishes all antioxidant and redox regulatory downstream functions such as those mediated by Trx1 or TRP14, but also promotes additional ROS formation by its attained NADPH oxidase activity. The increased ROS levels in turn may cause random oxidative damage, trigger apoptosis via ASK1 release from oxidized Trx1 or promote various response pathways such as stress adaptation by activating Nrf2, which leads to a boost of the Trx- and GSH-systems *inter alia* **(Fig. 16 I)**. This differential response to TrxR1 inhibition is indeed favorably for anti-cancer therapy – healthy cells may be protected by the concomitant Nrf2 response while cancer cells that are already at their anti-oxidant limit will rather suffer from a further increase in ROS by oxidative damage and cell death pathway induction. Further studies, particularly in more complex systems such as mouse models, are clearly needed. Nonetheless, may the specific, drug based inhibition of TrxR be a clear asset for the future treatment of certain conditions such as cancer.

In **Paper II** we further expanded the functional spectrum of the Trx system in demonstrating that the v3 splice variant of TrxR1 associates with lipid raft microdomains, which have been shown to serve as redox signaling platforms. Despite its yet unknown functions, v3 is potentially involved in the regulation of specific redox dependent processes. It might for instance play a role in cell surface receptor mediated signaling, which includes kinase activation and local H_2O_2 formation via NOXs. The H_2O_2 production in the vicinity of the receptor modulates the activities of certain PTPs and kinases to enable proper downstream signaling as for example in case of MAPK pathway mediated gene expression, NFκB activation as well as a myriad of other processes **(Fig. 16 II)**. v3 might regulate the redox status of a number of enzymes in this setup either directly via its unique Grx domain or through redoxins such as Trx1 or TRP14.

The Trx- and GSH-systems are furthermore directly or indirectly involved in the redox regulation of transcription factors such as Nrf2, HIF or NFκB. For example, Trx1 promotes the transactivation in the nucleus by reducing crucial cysteine residues in these transcription factors. In the cytosol, Trx1 may diminish the activation of Nrf2, NFκB by regulating H_2O_2, whereas it promotes HIF activation by stimulating its translation. An example is given by TRP14, a Trx-like substrate of TrxR1 that was previously reported to reduce the LC8 chain, which binds and protects IκB from phosphorylation and prevents thus NFκB from being activated **(Fig. 16 III)**. The overall mechanisms by which redox regulation of transcription factors is mediated in general and by the Trx-system in particular, is however, still largely unknown and thus one of the key intention behind the study in **Paper III**. Furthermore, several transcription factors are functionally intertwined. NFκB activation that is stimulated via receptor signaling also activates HIF. This transcription factor is typically induced by low oxygen levels – a condition which may affect ROS production and thus the redox state of key elements within other signaling pathways such as Nrf2 or NFκB activation. Nrf2 activation that is typically mediated by oxidative stress or via reactive electrophiles, promotes furthermore the transactivation of antioxidant and detoxifying enzymes, including components of the Trx-and GSH-systems. The upregulation of these systems affects in turn the redox regulation within the cell and is for instance considered to mediate the downregulation of NFκB. In constructing pTRAF within **Paper III** we developed a tool that enables us to monitor the activation of three transcription factors simultaneously within a single cell in order to study their intertwined activation pathways.

In conclusion, we expanded the functional spectrum of the Trx-system to lipid rafts, showed that TrxR1 can be efficiently targeted by electrophilic compounds that potentially convert the enzyme into its pro-oxidant SecTRAP form and developed a tool that enables simultaneous determination of three transcription factor activities within single cell level to study their functional interplay.

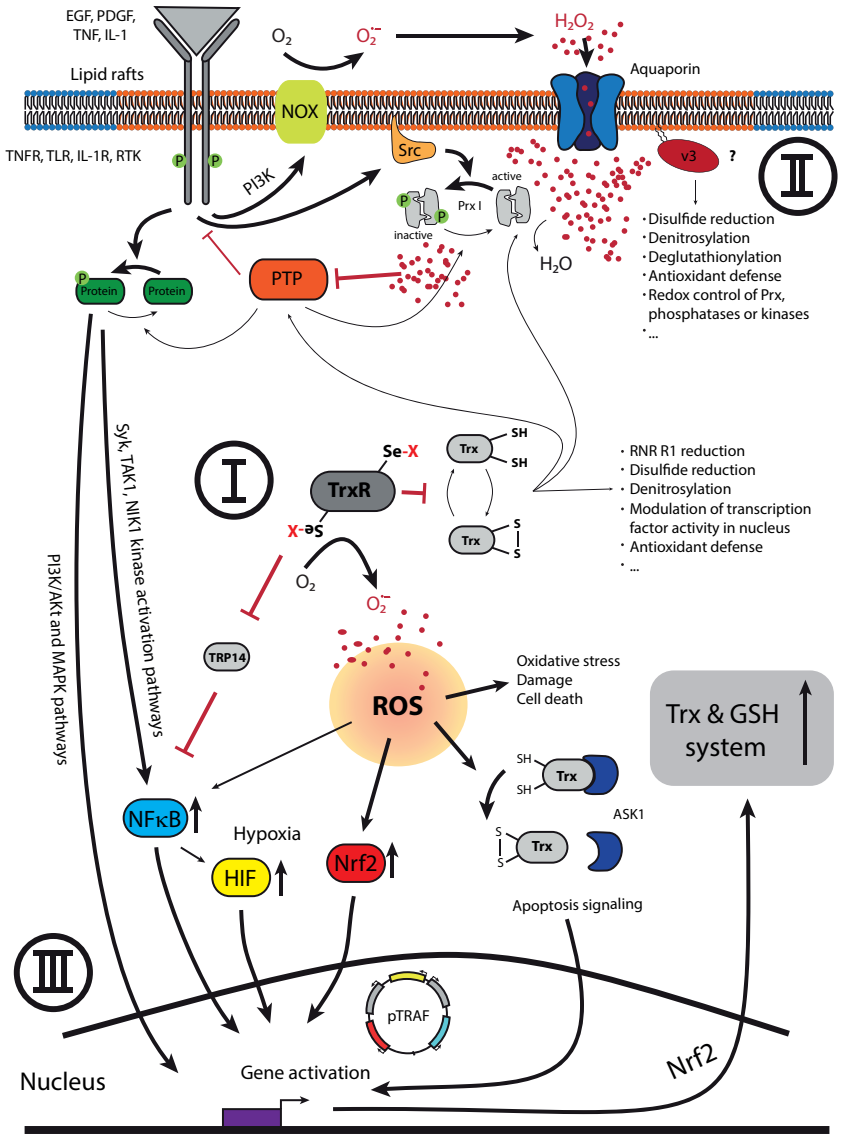


Figure 16. Schematic overview of Paper I to III in relation to the Trx-system. Roman numerals indicate the various aspects of the three papers. **See text for further details.**

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8 References

1. Banerjee, R. in Redox Biochemistry. (eds. R. Banerjee, D.F. Becker, M.B. Dickman, V.N. Gladyshev & S.W. Ragsdale) (John Wiley & Sons, Inc., 2008).
2. Flohé, L. in Methods in Enzymology; Thiol Redox Transitions in Cell Signaling, Part A: Chemistry and Biochemistry of Low Molecular Weight and Protein Thiols, Vol. 473. (eds. J.N. Abelson & M.I. Simon) (Elsevier, Inc., 2010).
3. Herrmann, J.M. & Dick, T.P. Redox Biology on the rise. *Biological chemistry* **393**, 999-1004 (2012).
4. Rhee, S.G., Chae, H.Z. & Kim, K. Peroxiredoxins: a historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling. *Free radical biology & medicine* **38**, 1543-1552 (2005).
5. D'Autreaux, B. & Toledano, M.B. ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. *Nature reviews. Molecular cell biology* **8**, 813-824 (2007).
6. Finkel, T. Reactive oxygen species and signal transduction. *IUBMB life* **52**, 3-6 (2001).
7. Rhee, S.G. Cell signaling. H2O2, a necessary evil for cell signaling. *Science* **312**, 1882-1883 (2006).
8. Winterbourn, C.C. & Hampton, M.B. Thiol chemistry and specificity in redox signaling. *Free radical biology & medicine* **45**, 549-561 (2008).
9. Grek, C.L. & Tew, K.D. Redox metabolism and malignancy. *Current opinion in pharmacology* **10**, 362-368 (2010).
10. Henriksen, E.J., Diamond-Stanic, M.K. & Marchionne, E.M. Oxidative stress and the etiology of insulin resistance and type 2 diabetes. *Free radical biology & medicine* **51**, 993-999 (2011).
11. Hirooka, Y., Sagara, Y., Kishi, T. & Sunagawa, K. Oxidative stress and central cardiovascular regulation. - Pathogenesis of hypertension and therapeutic aspects. *Circulation journal : official journal of the Japanese Circulation Society* **74**, 827-835 (2010).
12. Koh, C.H. et al. Chronic exposure to U18666A is associated with oxidative stress in cultured murine cortical neurons. *Journal of neurochemistry* **98**, 1278-1289 (2006).
13. Suh, Y.A. et al. Cell transformation by the superoxide-generating oxidase Mox1. *Nature* **401**, 79-82 (1999).
14. Stanner, S.A., Hughes, J., Kelly, C.N. & Buttriss, J. A review of the epidemiological evidence for the 'antioxidant hypothesis'. *Public health nutrition* **7**, 407-422 (2004).
15. Lassegue, B. & Griendling, K.K. NADPH oxidases: functions and pathologies in the vasculature. *Arteriosclerosis, thrombosis, and vascular biology* **30**, 653-661 (2010).
16. 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).
17. Reddie, K.G. & Carroll, K.S. Expanding the functional diversity of proteins through cysteine oxidation. *Current opinion in chemical biology* **12**, 746-754 (2008).
18. Banerjee, R. (John Wiley & Sons, Inc., 2008).
19. Li, F. et al. Regulation of HIF-1alpha stability through S-nitrosylation. *Molecular cell* **26**, 63-74 (2007).
20. Marshall, H.E. & Stamler, J.S. Inhibition of NF-kappa B by S-nitrosylation. *Biochemistry* **40**, 1688-1693 (2001).
21. Gu, Z. et al. S-nitrosylation of matrix metalloproteinases: signaling pathway to neuronal cell death. *Science* **297**, 1186-1190 (2002).
22. Li, J., Billiar, T.R., Talanian, R.V. & Kim, Y.M. Nitric oxide reversibly inhibits seven members of the caspase family via S-nitrosylation. *Biochemical and biophysical research communications* **240**, 419-424 (1997).
23. Sengupta, R. et al. Thioredoxin catalyzes the denitrosation of low-molecular mass and protein S-nitrosothiols. *Biochemistry* **46**, 8472-8483 (2007).
24. Ignarro, L.J., Buga, G.M., Wood, K.S., Byrns, R.E. & Chaudhuri, G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proceedings of the National Academy of Sciences of the United States of America* **84**, 9265-9269 (1987).

25. Palmer, R.M., Ferrige, A.G. & Moncada, S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* **327**, 524-526 (1987).
26. Roman, L.J., Martasek, P. & Masters, B.S. Intrinsic and extrinsic modulation of nitric oxide synthase activity. *Chemical reviews* **102**, 1179-1190 (2002).
27. Alderton, W.K., Cooper, C.E. & Knowles, R.G. Nitric oxide synthases: structure, function and inhibition. *The Biochemical journal* **357**, 593-615 (2001).
28. Bogdan, C. Nitric oxide and the immune response. *Nature immunology* **2**, 907-916 (2001).
29. Gruhlke, M.C. & Slusarenko, A.J. The biology of reactive sulfur species (RSS). *Plant physiology and biochemistry : PPB / Societe francaise de physiologie vegetale* **59**, 98-107 (2012).
30. Giles, G.I., Tasker, K.M. & Jacob, C. Hypothesis: the role of reactive sulfur species in oxidative stress. *Free radical biology & medicine* **31**, 1279-1283 (2001).
31. Hurd, T.R. et al. Redox Signaling and Regulation in Biology and Medicine. (Wiley-VCH, Weinheim, Germany; 2009).
32. Jacob, C. A scent of therapy: pharmacological implications of natural products containing redox-active sulfur atoms. *Natural product reports* **23**, 851-863 (2006).
33. Nagy, P. & Ashby, M.T. Reactive sulfur species: kinetics and mechanisms of the oxidation of cysteine by hypohalous acid to give cysteine sulfenic acid. *Journal of the American Chemical Society* **129**, 14082-14091 (2007).
34. Nagy, P., Wang, X., Lemma, K. & Ashby, M.T. Reactive sulfur species: hydrolysis of hypothiocyanite to give thiocarbamate-S-oxide. *Journal of the American Chemical Society* **129**, 15756-15757 (2007).
35. Ashby, M.T. & Aneetha, H. Reactive sulfur species: aqueous chemistry of sulfenyl thiocyanates. *Journal of the American Chemical Society* **126**, 10216-10217 (2004).
36. Li, L., Rose, P. & Moore, P.K. Hydrogen sulfide and cell signaling. *Annual review of pharmacology and toxicology* **51**, 169-187 (2011).
37. Hosoki, R., Matsuki, N. & Kimura, H. The possible role of hydrogen sulfide as an endogenous smooth muscle relaxant in synergy with nitric oxide. *Biochemical and biophysical research communications* **237**, 527-531 (1997).
38. 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).
39. Rhee, S.G., Woo, H.A., Kil, I.S. & Bae, S.H. Peroxiredoxin functions as a peroxidase and a regulator and sensor of local peroxides. *The Journal of biological chemistry* **287**, 4403-4410 (2012).
40. Paulsen, C.E. & Carroll, K.S. Cysteine-mediated redox signaling: chemistry, biology, and tools for discovery. *Chemical reviews* **113**, 4633-4679 (2013).
41. Halliwell, B. & Gutteridge, J. Free Radicals in Biology and Medicine. (Oxford University Press: New York; 2007).
42. 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).
43. Reth, M. Hydrogen peroxide as second messenger in lymphocyte activation. *Nature immunology* **3**, 1129-1134 (2002).
44. Bienert, G.P., Schjoerring, J.K. & Jahn, T.P. Membrane transport of hydrogen peroxide. *Biochimica et biophysica acta* **1758**, 994-1003 (2006).
45. Finkel, T. Oxygen radicals and signaling. *Current opinion in cell biology* **10**, 248-253 (1998).
46. Kamata, H. & Hirata, H. Redox regulation of cellular signalling. *Cellular signalling* **11**, 1-14 (1999).
47. Niimura, Y., Poole, L.B. & Massey, V. Amphibacillus xylanus NADH oxidase and Salmonella typhimurium alkyl-hydroperoxide reductase flavoprotein components show extremely high scavenging activity for both alkyl hydroperoxide and hydrogen peroxide in the presence of S. typhimurium alkyl-hydroperoxide reductase 22-kDa protein component. *The Journal of biological chemistry* **270**, 25645-25650 (1995).
48. Hsu, J.L. et al. Catalytic properties of human manganese superoxide dismutase. *The Journal of biological chemistry* **271**, 17687-17691 (1996).
49. McCord, J.M. & Fridovich, I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *The Journal of biological chemistry* **244**, 6049-6055 (1969).

50. Ott, M., Gogvadze, V., Orrenius, S. & Zhivotovsky, B. Mitochondria, oxidative stress and cell death. *Apoptosis : an international journal on programmed cell death* **12**, 913-922 (2007).
51. de Groot, H. & Littauer, A. Hypoxia, reactive oxygen, and cell injury. *Free radical biology & medicine* **6**, 541-551 (1989).
52. Lacy, F., Gough, D.A. & Schmid-Schonbein, G.W. Role of xanthine oxidase in hydrogen peroxide production. *Free radical biology & medicine* **25**, 720-727 (1998).
53. Cross, A.R. & Jones, O.T. Enzymic mechanisms of superoxide production. *Biochimica et biophysica acta* **1057**, 281-298 (1991).
54. Tyler, D.D. Polarographic assay and intracellular distribution of superoxide dismutase in rat liver. *The Biochemical journal* **147**, 493-504 (1975).
55. Finkel, T. Signal transduction by reactive oxygen species. *The Journal of cell biology* **194**, 7-15 (2011).
56. Lambert, A.J. & Brand, M.D. Reactive oxygen species production by mitochondria. *Methods Mol Biol* **554**, 165-181 (2009).
57. 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).
58. Chance, B., Sies, H. & Boveris, A. Hydroperoxide metabolism in mammalian organs. *Physiological reviews* **59**, 527-605 (1979).
59. Bulua, A.C. et al. Mitochondrial reactive oxygen species promote production of proinflammatory cytokines and are elevated in TNFR1-associated periodic syndrome (TRAPS). *The Journal of experimental medicine* **208**, 519-533 (2011).
60. Nakahira, K. et al. Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. *Nature immunology* **12**, 222-230 (2011).
61. Zhou, R., Yazdi, A.S., Menu, P. & Tschopp, J. A role for mitochondria in NLRP3 inflammasome activation. *Nature* **469**, 221-225 (2011).
62. Scherz-Shouval, R. et al. Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4. *The EMBO journal* **26**, 1749-1760 (2007).
63. Brunelle, J.K. et al. Oxygen sensing requires mitochondrial ROS but not oxidative phosphorylation. *Cell metabolism* **1**, 409-414 (2005).
64. Guzy, R.D. et al. Mitochondrial complex III is required for hypoxia-induced ROS production and cellular oxygen sensing. *Cell metabolism* **1**, 401-408 (2005).
65. Mansfield, K.D. et al. Mitochondrial dysfunction resulting from loss of cytochrome c impairs cellular oxygen sensing and hypoxic HIF-alpha activation. *Cell metabolism* **1**, 393-399 (2005).
66. Nemoto, S., Takeda, K., Yu, Z.X., Ferrans, V.J. & Finkel, T. Role for mitochondrial oxidants as regulators of cellular metabolism. *Molecular and cellular biology* **20**, 7311-7318 (2000).
67. 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).
68. Lambeth, J.D. NOX enzymes and the biology of reactive oxygen. *Nature reviews. Immunology* **4**, 181-189 (2004).
69. Bedard, K. & Krause, K.H. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiological reviews* **87**, 245-313 (2007).
70. Dickinson, B.C., Peltier, J., Stone, D., Schaffer, D.V. & Chang, C.J. Nox2 redox signaling maintains essential cell populations in the brain. *Nature chemical biology* **7**, 106-112 (2011).
71. Aguirre, J. & Lambeth, J.D. Nox enzymes from fungus to fly to fish and what they tell us about Nox function in mammals. *Free radical biology & medicine* **49**, 1342-1353 (2010).
72. Yazdanpanah, B. et al. Riboflavin kinase couples TNF receptor 1 to NADPH oxidase. *Nature* **460**, 1159-1163 (2009).
73. Choi, H. et al. Mechanism of angiotensin II-induced superoxide production in cells reconstituted with angiotensin type 1 receptor and the components of NADPH oxidase. *The Journal of biological chemistry* **283**, 255-267 (2008).
74. Kim, Y.S., Morgan, M.J., Choksi, S. & Liu, Z.G. TNF-induced activation of the Nox1 NADPH oxidase and its role in the induction of necrotic cell death. *Molecular cell* **26**, 675-687 (2007).
75. Sharma, P. et al. Redox regulation of interleukin-4 signaling. *Immunity* **29**, 551-564 (2008).

76. Honda, F. et al. The kinase Btk negatively regulates the production of reactive oxygen species and stimulation-induced apoptosis in human neutrophils. *Nature immunology* **13**, 369-378 (2012).
77. Basuroy, S., Bhattacharya, S., Leffler, C.W. & Parfenova, H. Nox4 NADPH oxidase mediates oxidative stress and apoptosis caused by TNF-alpha in cerebral vascular endothelial cells. *American journal of physiology. Cell physiology* **296**, C422-432 (2009).
78. Serrander, L. et al. NOX4 activity is determined by mRNA levels and reveals a unique pattern of ROS generation. *The Biochemical journal* **406**, 105-114 (2007).
79. Weyemi, U. et al. ROS-generating NADPH oxidase NOX4 is a critical mediator in oncogenic H-Ras-induced DNA damage and subsequent senescence. *Oncogene* **31**, 1117-1129 (2012).
80. Hahn, N.E. et al. NOX2, p22phox and p47phox are targeted to the nuclear pore complex in ischemic cardiomyocytes colocalizing with local reactive oxygen species. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* **27**, 471-478 (2011).
81. Meischl, C. et al. Ischemia induces nuclear NOX2 expression in cardiomyocytes and subsequently activates apoptosis. *Apoptosis : an international journal on programmed cell death* **11**, 913-921 (2006).
82. Chen, K., Craige, S.E. & Keaney, J.F., Jr. Downstream targets and intracellular compartmentalization in Nox signaling. *Antioxidants & redox signaling* **11**, 2467-2480 (2009).
83. Wu, R.F. et al. Subcellular targeting of oxidants during endothelial cell migration. *The Journal of cell biology* **171**, 893-904 (2005).
84. Paulsen, C.E. et al. Peroxide-dependent sulfenylation of the EGFR catalytic site enhances kinase activity. *Nature chemical biology* **8**, 57-64 (2012).
85. Diaz, B. et al. Tks5-dependent, nox-mediated generation of reactive oxygen species is necessary for invadopodia formation. *Science signaling* **2**, ra53 (2009).
86. Benrahmoune, M., Therond, P. & Abedinzadeh, Z. The reaction of superoxide radical with N-acetylcysteine. *Free radical biology & medicine* **29**, 775-782 (2000).
87. Winterbourn, C.C. & Metodiewa, D. Reactivity of biologically important thiol compounds with superoxide and hydrogen peroxide. *Free radical biology & medicine* **27**, 322-328 (1999).
88. Liochev, S.I. & Fridovich, I. Superoxide and iron: partners in crime. *IUBMB life* **48**, 157-161 (1999).
89. Babior, B.M., Kipnes, R.S. & Curnutte, J.T. Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. *The Journal of clinical investigation* **52**, 741-744 (1973).
90. Klebanoff, S.J. Myeloperoxidase: friend and foe. *Journal of leukocyte biology* **77**, 598-625 (2005).
91. Miller, R.A. & Britigan, B.E. Role of oxidants in microbial pathophysiology. *Clinical microbiology reviews* **10**, 1-18 (1997).
92. Halliwell, B., Gutteridge, J.M. & Aruoma, O.I. The deoxyribose method: a simple "test-tube" assay for determination of rate constants for reactions of hydroxyl radicals. *Analytical biochemistry* **165**, 215-219 (1987).
93. 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).
94. Moller, M. et al. Direct measurement of nitric oxide and oxygen partitioning into liposomes and low density lipoprotein. *The Journal of biological chemistry* **280**, 8850-8854 (2005).
95. 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).
96. Lukacs-Kornek, V. et al. Regulated release of nitric oxide by nonhematopoietic stroma controls expansion of the activated T cell pool in lymph nodes. *Nature immunology* **12**, 1096-1104 (2011).
97. Knowles, R.G. & Moncada, S. Nitric oxide synthases in mammals. *The Biochemical journal* **298 (Pt 2)**, 249-258 (1994).
98. Marletta, M.A. Nitric oxide synthase: aspects concerning structure and catalysis. *Cell* **78**, 927-930 (1994).
99. Nathan, C. & Xie, Q.W. Nitric oxide synthases: roles, tolls, and controls. *Cell* **78**, 915-918 (1994).

100. Roman, L.J. et al. The C termini of constitutive nitric-oxide synthases control electron flow through the flavin and heme domains and affect modulation by calmodulin. *The Journal of biological chemistry* **275**, 29225-29232 (2000).
101. Pacher, P., Beckman, J.S. & Liaudet, L. Nitric oxide and peroxynitrite in health and disease. *Physiological reviews* **87**, 315-424 (2007).
102. Szabo, C., Ischiropoulos, H. & Radi, R. Peroxynitrite: biochemistry, pathophysiology and development of therapeutics. *Nature reviews. Drug discovery* **6**, 662-680 (2007).
103. Li, H., Samouilov, A., Liu, X. & Zweier, J.L. Characterization of the magnitude and kinetics of xanthine oxidase-catalyzed nitrite reduction. Evaluation of its role in nitric oxide generation in anoxic tissues. *The Journal of biological chemistry* **276**, 24482-24489 (2001).
104. Cosby, K. et al. Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. *Nature medicine* **9**, 1498-1505 (2003).
105. 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).
106. Keszler, A., Zhang, Y. & Hogg, N. Reaction between nitric oxide, glutathione, and oxygen in the presence and absence of protein: How are S-nitrosothiols formed? *Free radical biology & medicine* **48**, 55-64 (2010).
107. Chen, C.A. et al. Superoxide induces endothelial nitric-oxide synthase protein thiyl radical formation, a novel mechanism regulating eNOS function and coupling. *The Journal of biological chemistry* **286**, 29098-29107 (2011).
108. Liu, L. et al. A metabolic enzyme for S-nitrosothiol conserved from bacteria to humans. *Nature* **410**, 490-494 (2001).
109. Nordberg, J. & Arner, E.S. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free radical biology & medicine* **31**, 1287-1312 (2001).
110. 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).
111. Hayes, J.D., Flanagan, J.U. & Jowsey, I.R. Glutathione transferases. *Annual review of pharmacology and toxicology* **45**, 51-88 (2005).
112. Laborde, E. Glutathione transferases as mediators of signaling pathways involved in cell proliferation and cell death. *Cell death and differentiation* **17**, 1373-1380 (2010).
113. 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).
114. Thieme, R., Pai, E.F., Schirmer, R.H. & Schulz, G.E. Three-dimensional structure of glutathione reductase at 2 A resolution. *Journal of molecular biology* **152**, 763-782 (1981).
115. 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).
116. Tavender, T.J., Springate, J.J. & Bulleid, N.J. Recycling of peroxiredoxin IV provides a novel pathway for disulphide formation in the endoplasmic reticulum. *The EMBO journal* **29**, 4185-4197 (2010).
117. Brigelius-Flohe, R. & Maiorino, M. Glutathione peroxidases. *Biochimica et biophysica acta* **1830**, 3289-3303 (2013).
118. Rhee, S.G. & Woo, H.A. Multiple functions of peroxiredoxins: peroxidases, sensors and regulators of the intracellular messenger H(2)O(2), and protein chaperones. *Antioxidants & redox signaling* **15**, 781-794 (2011).
119. Poole, L.B., Hall, A. & Nelson, K.J. Overview of peroxiredoxins in oxidant defense and redox regulation. *Curr Protoc Toxicol* **Chapter 7**, Unit7 9 (2011).
120. Fridovich, I. Superoxide radical and superoxide dismutases. *Annual review of biochemistry* **64**, 97-112 (1995).
121. Fritz, R. et al. Compartment-dependent management of H(2)O(2) by peroxisomes. *Free radical biology & medicine* **42**, 1119-1129 (2007).
122. Schoneich, C. Methionine oxidation by reactive oxygen species: reaction mechanisms and relevance to Alzheimer's disease. *Biochimica et biophysica acta* **1703**, 111-119 (2005).
123. Lowther, W.T. & Haynes, A.C. Reduction of cysteine sulfinic acid in eukaryotic, typical 2-Cys peroxiredoxins by sulfiredoxin. *Antioxidants & redox signaling* **15**, 99-109 (2011).

124. Gromer, S., Urig, S. & Becker, K. The thioredoxin system--from science to clinic. *Medicinal research reviews* **24**, 40-89 (2004).
125. Lu, J. & Holmgren, A. The thioredoxin antioxidant system. *Free radical biology & medicine* **66**, 75-87 (2014).
126. Arner, E.S. & Holmgren, A. The thioredoxin system in cancer. *Seminars in cancer biology* **16**, 420-426 (2006).
127. Lillig, C.H. & Holmgren, A. Thioredoxin and related molecules--from biology to health and disease. *Antioxidants & redox signaling* **9**, 25-47 (2007).
128. Matsuzawa, A. & Ichijo, H. Redox control of cell fate by MAP kinase: physiological roles of ASK1-MAP kinase pathway in stress signaling. *Biochimica et biophysica acta* **1780**, 1325-1336 (2008).
129. Luthman, M. & Holmgren, A. Rat liver thioredoxin and thioredoxin reductase: purification and characterization. *Biochemistry* **21**, 6628-6633 (1982).
130. Holmgren, A. Thioredoxin. *Annual review of biochemistry* **54**, 237-271 (1985).
131. Arner, E.S. Focus on mammalian thioredoxin reductases--important selenoproteins with versatile functions. *Biochimica et biophysica acta* **1790**, 495-526 (2009).
132. Fu, C. et al. Elucidation of thioredoxin target protein networks in mouse. *Molecular & cellular proteomics : MCP* **8**, 1674-1687 (2009).
133. Tonissen, K.F. & Di Trapani, G. Thioredoxin system inhibitors as mediators of apoptosis for cancer therapy. *Molecular nutrition & food research* **53**, 87-103 (2009).
134. Dal Piaz, F., Braca, A., Belisario, M.A. & De Tommasi, N. Thioredoxin system modulation by plant and fungal secondary metabolites. *Current medicinal chemistry* **17**, 479-494 (2010).
135. Eklund, H., Gleason, F.K. & Holmgren, A. Structural and functional relations among thioredoxins of different species. *Proteins* **11**, 13-28 (1991).
136. Qi, Y. & Grishin, N.V. Structural classification of thioredoxin-like fold proteins. *Proteins* **58**, 376-388 (2005).
137. Martin, J.L. Thioredoxin--a fold for all reasons. *Structure* **3**, 245-250 (1995).
138. Fernandes, A.P. & Holmgren, A. Glutaredoxins: glutathione-dependent redox enzymes with functions far beyond a simple thioredoxin backup system. *Antioxidants & redox signaling* **6**, 63-74 (2004).
139. Wood, Z.A., Schroder, E., Robin Harris, J. & Poole, L.B. Structure, mechanism and regulation of peroxiredoxins. *Trends in biochemical sciences* **28**, 32-40 (2003).
140. Toppo, S., Vanin, S., Bosello, V. & Tosatto, S.C. Evolutionary and structural insights into the multifaceted glutathione peroxidase (Gpx) superfamily. *Antioxidants & redox signaling* **10**, 1501-1514 (2008).
141. Armstrong, R.N. Structure, catalytic mechanism, and evolution of the glutathione transferases. *Chemical research in toxicology* **10**, 2-18 (1997).
142. Hatahet, F. & Ruddock, L.W. Protein disulfide isomerase: a critical evaluation of its function in disulfide bond formation. *Antioxidants & redox signaling* **11**, 2807-2850 (2009).
143. Holmgren, A. Antioxidant function of thioredoxin and glutaredoxin systems. *Antioxidants & redox signaling* **2**, 811-820 (2000).
144. Wu, C. et al. Thioredoxin 1-mediated post-translational modifications: reduction, transnitrosylation, denitrosylation, and related proteomics methodologies. *Antioxidants & redox signaling* **15**, 2565-2604 (2011).
145. Hashemy, S.I. & Holmgren, A. Regulation of the catalytic activity and structure of human thioredoxin 1 via oxidation and S-nitrosylation of cysteine residues. *The Journal of biological chemistry* **283**, 21890-21898 (2008).
146. Sengupta, R. & Holmgren, A. Thioredoxin and thioredoxin reductase in relation to reversible S-nitrosylation. *Antioxidants & redox signaling* **18**, 259-269 (2013).
147. 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).
148. 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).
149. Rhee, S.G., Kang, S.W., Chang, T.S., Jeong, W. & Kim, K. Peroxiredoxin, a novel family of peroxidases. *IUBMB life* **52**, 35-41 (2001).

150. 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).
151. 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).
152. 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).
153. Freemerman, A.J., Gallegos, A. & Powis, G. Nuclear factor kappaB transactivation is increased but is not involved in the proliferative effects of thioredoxin overexpression in MCF-7 breast cancer cells. *Cancer research* **59**, 4090-4094 (1999).
154. Grippo, J.F., Holmgren, A. & Pratt, W.B. Proof that the endogenous, heat-stable glucocorticoid receptor-activating factor is thioredoxin. *The Journal of biological chemistry* **260**, 93-97 (1985).
155. Ueno, M. et al. Thioredoxin-dependent redox regulation of p53-mediated p21 activation. *The Journal of biological chemistry* **274**, 35809-35815 (1999).
156. Go, Y.M. & Jones, D.P. Redox control systems in the nucleus: mechanisms and functions. *Antioxidants & redox signaling* **13**, 489-509 (2010).
157. Arai, R.J. et al. Nitric oxide induces thioredoxin-1 nuclear translocation: possible association with the p21Ras survival pathway. *Biochemical and biophysical research communications* **348**, 1254-1260 (2006).
158. Hansen, J.M., Watson, W.H. & Jones, D.P. Compartmentation of Nrf-2 redox control: regulation of cytoplasmic activation by glutathione and DNA binding by thioredoxin-1. *Toxicological sciences : an official journal of the Society of Toxicology* **82**, 308-317 (2004).
159. 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).
160. Saitoh, M. et al. Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *The EMBO journal* **17**, 2596-2606 (1998).
161. 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).
162. World, C., Spindel, O.N. & Berk, B.C. Thioredoxin-interacting protein mediates TRX1 translocation to the plasma membrane in response to tumor necrosis factor-alpha: a key mechanism for vascular endothelial growth factor receptor-2 transactivation by reactive oxygen species. *Arteriosclerosis, thrombosis, and vascular biology* **31**, 1890-1897 (2011).
163. Pekkari, K. & Holmgren, A. Truncated thioredoxin: physiological functions and mechanism. *Antioxidants & redox signaling* **6**, 53-61 (2004).
164. Lu, J. & Holmgren, A. Thioredoxin system in cell death progression. *Antioxidants & redox signaling* **17**, 1738-1747 (2012).
165. Mustacich, D. & Powis, G. Thioredoxin reductase. *The Biochemical journal* **346 Pt 1**, 1-8 (2000).
166. Arner, E.S. & Holmgren, A. Physiological functions of thioredoxin and thioredoxin reductase. *European journal of biochemistry / FEBS* **267**, 6102-6109 (2000).
167. Arscott, L.D., Gromer, S., Schirmer, R.H., Becker, K. & Williams, C.H., Jr. The mechanism of thioredoxin reductase from human placenta is similar to the mechanisms of lipoamide dehydrogenase and glutathione reductase and is distinct from the mechanism of thioredoxin reductase from Escherichia coli. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 3621-3626 (1997).
168. Williams, C.H. et al. Thioredoxin reductase two modes of catalysis have evolved. *European journal of biochemistry / FEBS* **267**, 6110-6117 (2000).
169. Bauer, H. et al. The mechanism of high Mr thioredoxin reductase from Drosophila melanogaster. *The Journal of biological chemistry* **278**, 33020-33028 (2003).
170. Cheng, Q., Sandalova, T., Lindqvist, Y. & Arner, E.S. Crystal structure and catalysis of the selenoprotein thioredoxin reductase 1. *The Journal of biological chemistry* **284**, 3998-4008 (2009).

171. Zhong, L., Arner, E.S. & Holmgren, A. Structure and mechanism of mammalian thioredoxin reductase: the active site is a redox-active selenolthiol/selenenylsulfide formed from the conserved cysteine-selenocysteine sequence. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 5854-5859 (2000).
172. Cenas, N., Prast, S., Nivinskas, H., Sarlauskas, J. & Arner, E.S. Interactions of nitroaromatic compounds with the mammalian selenoprotein thioredoxin reductase and the relation to induction of apoptosis in human cancer cells. *The Journal of biological chemistry* **281**, 5593-5603 (2006).
173. Gromer, S. et al. Active sites of thioredoxin reductases: why selenoproteins? *Proceedings of the National Academy of Sciences of the United States of America* **100**, 12618-12623 (2003).
174. Lothrop, A.P., Ruggles, E.L. & Hondal, R.J. No selenium required: reactions catalyzed by mammalian thioredoxin reductase that are independent of a selenocysteine residue. *Biochemistry* **48**, 6213-6223 (2009).
175. 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).
176. Fritz-Wolf, K., Urig, S. & Becker, K. The structure of human thioredoxin reductase 1 provides insights into C-terminal rearrangements during catalysis. *Journal of molecular biology* **370**, 116-127 (2007).
177. 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).
178. Anestal, K., Prast-Nielsen, S., Cenas, N. & Arner, E.S. Cell death by SecTRAPs: thioredoxin reductase as a prooxidant killer of cells. *PloS one* **3**, e1846 (2008).
179. Brown, K.K., Eriksson, S.E., Arner, E.S. & Hampton, M.B. Mitochondrial peroxiredoxin 3 is rapidly oxidized in cells treated with isothiocyanates. *Free radical biology & medicine* **45**, 494-502 (2008).
180. 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).
181. 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).
182. 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).
183. 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).
184. Smith, A.D., Guidry, C.A., Morris, V.C. & Levander, O.A. Aurothioglucose inhibits murine thioredoxin reductase activity in vivo. *The Journal of nutrition* **129**, 194-198 (1999).
185. 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).
186. 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).
187. 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).
188. Gromer, S., Schirmer, R.H. & Becker, K. The 58 kDa mouse selenoprotein is a BCNU-sensitive thioredoxin reductase. *FEBS letters* **412**, 318-320 (1997).
189. 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).
190. Rackham, O. et al. Substrate and inhibitor specificities differ between human cytosolic and mitochondrial thioredoxin reductases: Implications for development of specific inhibitors. *Free radical biology & medicine* **50**, 689-699 (2011).

191. Cenas, N. et al. Interactions of quinones with thioredoxin reductase: a challenge to the antioxidant role of the mammalian selenoprotein. *The Journal of biological chemistry* **279**, 2583-2592 (2004).
192. Hu, J. et al. Modulation of p53 dependent gene expression and cell death through thioredoxin-thioredoxin reductase by the Interferon-Retinoid combination. *Oncogene* **20**, 4235-4248 (2001).
193. Ma, X. et al. Thioredoxin participates in a cell death pathway induced by interferon and retinoid combination. *Oncogene* **20**, 3703-3715 (2001).
194. Ma, X. et al. Regulation of interferon and retinoic acid-induced cell death activation through thioredoxin reductase. *The Journal of biological chemistry* **276**, 24843-24854 (2001).
195. 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).
196. 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).
197. 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).
198. Rigobello, M.P., Callegaro, M.T., Barzon, E., Benetti, M. & Bindoli, A. Purification of mitochondrial thioredoxin reductase and its involvement in the redox regulation of membrane permeability. *Free radical biology & medicine* **24**, 370-376 (1998).
199. Sun, Q.A. et al. Reaction mechanism and regulation of mammalian thioredoxin/glutathione reductase. *Biochemistry* **44**, 14528-14537 (2005).
200. Su, D. et al. Mammalian selenoprotein thioredoxin-glutathione reductase. Roles in disulfide bond formation and sperm maturation. *The Journal of biological chemistry* **280**, 26491-26498 (2005).
201. Osborne, S.A. & Tonissen, K.F. Genomic organisation and alternative splicing of mouse and human thioredoxin reductase 1 genes. *BMC genomics* **2**, 10 (2001).
202. Sun, Q.A. et al. Heterogeneity within animal thioredoxin reductases. Evidence for alternative first exon splicing. *The Journal of biological chemistry* **276**, 3106-3114 (2001).
203. Turanov, A.A., Su, D. & Gladyshev, V.N. Characterization of alternative cytosolic forms and cellular targets of mouse mitochondrial thioredoxin reductase. *The Journal of biological chemistry* **281**, 22953-22963 (2006).
204. Lescure, A., Gautheret, D., Carbon, P. & Krol, A. Novel selenoproteins identified in silico and in vivo by using a conserved RNA structural motif. *The Journal of biological chemistry* **274**, 38147-38154 (1999).
205. Rundlof, A.K., Janard, M., Miranda-Vizuete, A. & Arner, E.S. Evidence for intriguingly complex transcription of human thioredoxin reductase 1. *Free radical biology & medicine* **36**, 641-656 (2004).
206. Damdimopoulos, A.E., Miranda-Vizuete, A., Treuter, E., Gustafsson, J.A. & Spyrou, G. An alternative splicing variant of the selenoprotein thioredoxin reductase is a modulator of estrogen signaling. *The Journal of biological chemistry* **279**, 38721-38729 (2004).
207. Rundlof, A.K., Carlsten, M. & Arner, E.S. The core promoter of human thioredoxin reductase 1: cloning, transcriptional activity, and Oct-1, Sp1, and Sp3 binding reveal a housekeeping-type promoter for the AU-rich element-regulated gene. *The Journal of biological chemistry* **276**, 30542-30551 (2001).
208. Su, D. & Gladyshev, V.N. Alternative splicing involving the thioredoxin reductase module in mammals: a glutaredoxin-containing thioredoxin reductase 1. *Biochemistry* **43**, 12177-12188 (2004).
209. Dammeyer, P. et al. Induction of cell membrane protrusions by the N-terminal glutaredoxin domain of a rare splice variant of human thioredoxin reductase 1. *The Journal of biological chemistry* **283**, 2814-2821 (2008).
210. Damdimopoulou, P.E., Miranda-Vizuete, A., Arner, E.S., Gustafsson, J.A. & Damdimopoulos, A.E. The human thioredoxin reductase-1 splice variant TXNRD1_v3 is an atypical inducer of cytoplasmic filaments and cell membrane filopodia. *Biochimica et biophysica acta* **1793**, 1588-1596 (2009).

211. Szatrowski, T.P. & Nathan, C.F. Production of large amounts of hydrogen peroxide by human tumor cells. *Cancer research* **51**, 794-798 (1991).
212. Toyokuni, S., Okamoto, K., Yodoi, J. & Hiai, H. Persistent oxidative stress in cancer. *FEBS letters* **358**, 1-3 (1995).
213. Wu, W.S. The signaling mechanism of ROS in tumor progression. *Cancer metastasis reviews* **25**, 695-705 (2006).
214. Behrend, L., Henderson, G. & Zwacka, R.M. Reactive oxygen species in oncogenic transformation. *Biochemical Society transactions* **31**, 1441-1444 (2003).
215. Lee, S.R. et al. Reversible inactivation of the tumor suppressor PTEN by H2O2. *The Journal of biological chemistry* **277**, 20336-20342 (2002).
216. Harris, I.S. et al. PTPN12 promotes resistance to oxidative stress and supports tumorigenesis by regulating FOXO signaling. *Oncogene* **33**, 1047-1054 (2014).
217. Martindale, J.L. & Holbrook, N.J. Cellular response to oxidative stress: signaling for suicide and survival. *Journal of cellular physiology* **192**, 1-15 (2002).
218. Diehn, M. et al. Association of reactive oxygen species levels and radioresistance in cancer stem cells. *Nature* **458**, 780-783 (2009).
219. 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).
220. Gorrini, C., Harris, I.S. & Mak, T.W. Modulation of oxidative stress as an anticancer strategy. *Nature reviews. Drug discovery* **12**, 931-947 (2013).
221. Urig, S. & Becker, K. On the potential of thioredoxin reductase inhibitors for cancer therapy. *Seminars in cancer biology* **16**, 452-465 (2006).
222. Berggren, M. et al. Thioredoxin and thioredoxin reductase gene expression in human tumors and cell lines, and the effects of serum stimulation and hypoxia. *Anticancer research* **16**, 3459-3466 (1996).
223. Tonissen, K.F. Targeting the human thioredoxin system by diverse strategies to treat cancer and other pathologies. *Recent patents on DNA & gene sequences* **1**, 164-175 (2007).
224. 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).
225. 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**, 3576-3583 (2009).
226. Paz, M.M., Zhang, X., Lu, J. & Holmgren, A. A new mechanism of action for the anticancer drug mitomycin C: mechanism-based inhibition of thioredoxin reductase. *Chemical research in toxicology* **25**, 1502-1511 (2012).
227. Ali, F.E., Barnham, K.J., Barrow, C.J. & Separovic, F. Metal catalyzed oxidation of tyrosine residues by different oxidation systems of copper/hydrogen peroxide. *Journal of inorganic biochemistry* **98**, 173-184 (2004).
228. Ji, J.A., Zhang, B., Cheng, W. & Wang, Y.J. Methionine, tryptophan, and histidine oxidation in a model protein, PTH: mechanisms and stabilization. *Journal of pharmaceutical sciences* **98**, 4485-4500 (2009).
229. Marino, S.M. & Gladyshev, V.N. Analysis and functional prediction of reactive cysteine residues. *The Journal of biological chemistry* **287**, 4419-4425 (2012).
230. Nelson, J.W. & Creighton, T.E. Reactivity and ionization of the active site cysteine residues of DsbA, a protein required for disulfide bond formation in vivo. *Biochemistry* **33**, 5974-5983 (1994).
231. Denu, J.M. & Tanner, K.G. Specific and reversible inactivation of protein tyrosine phosphatases by hydrogen peroxide: evidence for a sulfenic acid intermediate and implications for redox regulation. *Biochemistry* **37**, 5633-5642 (1998).
232. Peskin, A.V. et al. The high reactivity of peroxiredoxin 2 with H(2)O(2) is not reflected in its reaction with other oxidants and thiol reagents. *The Journal of biological chemistry* **282**, 11885-11892 (2007).
233. Hall, A., Nelson, K., Poole, L.B. & Karplus, P.A. Structure-based insights into the catalytic power and conformational dexterity of peroxiredoxins. *Antioxidants & redox signaling* **15**, 795-815 (2011).
234. Roos, G. & Messens, J. Protein sulfenic acid formation: from cellular damage to redox regulation. *Free radical biology & medicine* **51**, 314-326 (2011).

235. Bock, A. et al. Selenocysteine: the 21st amino acid. *Molecular microbiology* **5**, 515-520 (1991).
236. Huber, R.E. & Criddle, R.S. Comparison of the chemical properties of selenocysteine and selenocystine with their sulfur analogs. *Archives of biochemistry and biophysics* **122**, 164-173 (1967).
237. Wessjohann, L.A., Schneider, A., Abbas, M. & Brandt, W. Selenium in chemistry and biochemistry in comparison to sulfur. *Biological chemistry* **388**, 997-1006 (2007).
238. Rocher, C., Lalanne, J.L. & Chaudiere, J. Purification and properties of a recombinant sulfur analog of murine selenium-glutathione peroxidase. *European journal of biochemistry / FEBS* **205**, 955-960 (1992).
239. Lee, S.R. et al. Mammalian thioredoxin reductase: oxidation of the C-terminal cysteine/selenocysteine active site forms a thioselenide, and replacement of selenium with sulfur markedly reduces catalytic activity. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 2521-2526 (2000).
240. Nauser, T., Steinmann, D. & Koppenol, W.H. Why do proteins use selenocysteine instead of cysteine? *Amino acids* **42**, 39-44 (2012).
241. Arner, E.S. Selenoproteins-What unique properties can arise with selenocysteine in place of cysteine? *Experimental cell research* **316**, 1296-1303 (2010).
242. Snider, G.W., Ruggles, E., Khan, N. & Hondal, R.J. Selenocysteine confers resistance to inactivation by oxidation in thioredoxin reductase: comparison of selenium and sulfur enzymes. *Biochemistry* **52**, 5472-5481 (2013).
243. Hondal, R.J. & Ruggles, E.L. Differing views of the role of selenium in thioredoxin reductase. *Amino acids* **41**, 73-89 (2011).
244. Lu, J. et al. Penultimate selenocysteine residue replaced by cysteine in thioredoxin reductase from selenium-deficient rat liver. *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **23**, 2394-2402 (2009).
245. Xu, X.M. et al. Targeted insertion of cysteine by decoding UGA codons with mammalian selenocysteine machinery. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 21430-21434 (2010).
246. Poole, L.B., Karplus, P.A. & Claiborne, A. Protein sulfenic acids in redox signaling. *Annual review of pharmacology and toxicology* **44**, 325-347 (2004).
247. Paulsen, C.E. & Carroll, K.S. Orchestrating redox signaling networks through regulatory cysteine switches. *ACS chemical biology* **5**, 47-62 (2010).
248. Andersen, J.K. Oxidative stress in neurodegeneration: cause or consequence? *Nature medicine* **10 Suppl**, S18-25 (2004).
249. Klaunig, J.E. & Kamendulis, L.M. The role of oxidative stress in carcinogenesis. *Annual review of pharmacology and toxicology* **44**, 239-267 (2004).
250. 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).
251. Barrett, W.C. et al. Regulation of PTP1B via glutathionylation of the active site cysteine 215. *Biochemistry* **38**, 6699-6705 (1999).
252. Prinarakis, E., Chantzoura, E., Thanos, D. & Spyrou, G. S-glutathionylation of IRF3 regulates IRF3-CBP interaction and activation of the IFN beta pathway. *The EMBO journal* **27**, 865-875 (2008).
253. Velu, C.S., Niture, S.K., Doneanu, C.E., Pattabiraman, N. & Srivenugopal, K.S. Human p53 is inhibited by glutathionylation of cysteines present in the proximal DNA-binding domain during oxidative stress. *Biochemistry* **46**, 7765-7780 (2007).
254. Chung, H.S., Wang, S.B., Venkatraman, V., Murray, C.I. & Van Eyk, J.E. Cysteine oxidative posttranslational modifications: emerging regulation in the cardiovascular system. *Circulation research* **112**, 382-392 (2013).
255. Liaudet, L., Vassalli, G. & Pacher, P. Role of peroxynitrite in the redox regulation of cell signal transduction pathways. *Front Biosci (Landmark Ed)* **14**, 4809-4814 (2009).
256. Foster, M.W., Hess, D.T. & Stamler, J.S. Protein S-nitrosylation in health and disease: a current perspective. *Trends in molecular medicine* **15**, 391-404 (2009).
257. Benhar, M., Forrester, M.T. & Stamler, J.S. Protein denitrosylation: enzymatic mechanisms and cellular functions. *Nature reviews. Molecular cell biology* **10**, 721-732 (2009).

258. 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).
259. Anand, P. & Stamler, J.S. Enzymatic mechanisms regulating protein S-nitrosylation: implications in health and disease. *J Mol Med (Berl)* **90**, 233-244 (2012).
260. 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).
261. Gilbert, H.F. Molecular and cellular aspects of thiol-disulfide exchange. *Advances in enzymology and related areas of molecular biology* **63**, 69-172 (1990).
262. 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).
263. Flohe, L. The fairytale of the GSSG/GSH redox potential. *Biochimica et biophysica acta* **1830**, 3139-3142 (2013).
264. Gallogly, M.M. & Mieyal, J.J. Mechanisms of reversible protein glutathionylation in redox signaling and oxidative stress. *Current opinion in pharmacology* **7**, 381-391 (2007).
265. Winterbourn, C.C. Reconciling the chemistry and biology of reactive oxygen species. *Nature chemical biology* **4**, 278-286 (2008).
266. Yang, K.S. et al. Inactivation of human peroxiredoxin I during catalysis as the result of the oxidation of the catalytic site cysteine to cysteine-sulfinic acid. *The Journal of biological chemistry* **277**, 38029-38036 (2002).
267. Rabilloud, T. et al. Proteomics analysis of cellular response to oxidative stress. Evidence for in vivo overoxidation of peroxiredoxins at their active site. *The Journal of biological chemistry* **277**, 19396-19401 (2002).
268. Wagner, E. et al. A method for detection of overoxidation of cysteines: peroxiredoxins are oxidized in vivo at the active-site cysteine during oxidative stress. *The Biochemical journal* **366**, 777-785 (2002).
269. 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).
270. 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).
271. Rawat, S.J., Creasy, C.L., Peterson, J.R. & Chernoff, J. The tumor suppressor Mst1 promotes changes in the cellular redox state by phosphorylation and inactivation of peroxiredoxin-1 protein. *The Journal of biological chemistry* **288**, 8762-8771 (2013).
272. Monteiro, H.P. & Stern, A. Redox modulation of tyrosine phosphorylation-dependent signal transduction pathways. *Free radical biology & medicine* **21**, 323-333 (1996).
273. Meng, T.C., Fukada, T. & Tonks, N.K. Reversible oxidation and inactivation of protein tyrosine phosphatases in vivo. *Molecular cell* **9**, 387-399 (2002).
274. Mahadev, K., Zilbering, A., Zhu, L. & Goldstein, B.J. Insulin-stimulated hydrogen peroxide reversibly inhibits protein-tyrosine phosphatase 1b in vivo and enhances the early insulin action cascade. *The Journal of biological chemistry* **276**, 21938-21942 (2001).
275. 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).
276. Groen, A. et al. Differential oxidation of protein-tyrosine phosphatases. *The Journal of biological chemistry* **280**, 10298-10304 (2005).
277. Ross, S.H. et al. Differential redox regulation within the PTP superfamily. *Cellular signalling* **19**, 1521-1530 (2007).
278. Hampton, M.B., Stamenkovic, I. & Winterbourn, C.C. Interaction with substrate sensitises caspase-3 to inactivation by hydrogen peroxide. *FEBS letters* **517**, 229-232 (2002).
279. Giannoni, E. & Chiarugi, P. Redox Circuitries Driving Src Regulation. *Antioxidants & redox signaling* (2013).
280. Delaunay, A., Pflieger, D., Barrault, M.B., Vinh, J. & Toledano, M.B. A thiol peroxidase is an H2O2 receptor and redox-transducer in gene activation. *Cell* **111**, 471-481 (2002).

281. Okazaki, S., Naganuma, A. & Kuge, S. Peroxiredoxin-mediated redox regulation of the nuclear localization of Yap1, a transcription factor in budding yeast. *Antioxidants & redox signaling* **7**, 327-334 (2005).
282. Brigelius-Flohe, R. & Flohe, L. Basic principles and emerging concepts in the redox control of transcription factors. *Antioxidants & redox signaling* **15**, 2335-2381 (2011).
283. Marinho, H.S., Real, C., Cyrne, L., Soares, H. & Antunes, F. Hydrogen peroxide sensing, signaling and regulation of transcription factors. *Redox biology* **2**, 535-562 (2014).
284. Schulze-Osthoff, K., Beyaert, R., Vandevoorde, V., Haegeman, G. & Fiers, W. Depletion of the mitochondrial electron transport abrogates the cytotoxic and gene-inductive effects of TNF. *The EMBO journal* **12**, 3095-3104 (1993).
285. Staal, F.J., Roederer, M. & 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).
286. Flohe, L., Brigelius-Flohe, R., Saliou, C., Traber, M.G. & Packer, L. Redox regulation of NF-kappa B activation. *Free radical biology & medicine* **22**, 1115-1126 (1997).
287. Gloire, G., Legrand-Poels, S. & Piette, J. NF-kappaB activation by reactive oxygen species: fifteen years later. *Biochemical pharmacology* **72**, 1493-1505 (2006).
288. 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).
289. Ghosh, S., May, M.J. & Kopp, E.B. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annual review of immunology* **16**, 225-260 (1998).
290. Guan, H., Hou, S. & Ricciardi, R.P. DNA binding of repressor nuclear factor-kappaB p50/p50 depends on phosphorylation of Ser337 by the protein kinase A catalytic subunit. *The Journal of biological chemistry* **280**, 9957-9962 (2005).
291. Kumar, A., Takada, Y., Boriek, A.M. & Aggarwal, B.B. Nuclear factor-kappaB: its role in health and disease. *J Mol Med (Berl)* **82**, 434-448 (2004).
292. Gloire, G. & Piette, J. Redox regulation of nuclear post-translational modifications during NF-kappaB activation. *Antioxidants & redox signaling* **11**, 2209-2222 (2009).
293. Li, N. & Karin, M. Is NF-kappaB the sensor of oxidative stress? *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **13**, 1137-1143 (1999).
294. Roff, M. et al. Role of IkappaBalpha ubiquitination in signal-induced activation of NFkappaB in vivo. *The Journal of biological chemistry* **271**, 7844-7850 (1996).
295. Yamaoka, S. et al. Complementation cloning of NEMO, a component of the IkappaB kinase complex essential for NF-kappaB activation. *Cell* **93**, 1231-1240 (1998).
296. Pantano, C., Reynaert, N.L., van der Vliet, A. & Janssen-Heininger, Y.M. Redox-sensitive kinases of the nuclear factor-kappaB signaling pathway. *Antioxidants & redox signaling* **8**, 1791-1806 (2006).
297. Chen, L.F. & Greene, W.C. Shaping the nuclear action of NF-kappaB. *Nature reviews. Molecular cell biology* **5**, 392-401 (2004).
298. 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).
299. Ando, K. et al. A new APE1/Ref-1-dependent pathway leading to reduction of NF-kappaB and AP-1, and activation of their DNA-binding activity. *Nucleic acids research* **36**, 4327-4336 (2008).
300. 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).
301. Qanungo, S., Starke, D.W., Pai, H.V., Mieyal, J.J. & Nieminen, A.L. Glutathione supplementation potentiates hypoxic apoptosis by S-glutathionylation of p65-NFkappaB. *The Journal of biological chemistry* **282**, 18427-18436 (2007).
302. Pineda-Molina, E. et al. Glutathionylation of the p50 subunit of NF-kappaB: a mechanism for redox-induced inhibition of DNA binding. *Biochemistry* **40**, 14134-14142 (2001).

303. Semenza, G.L. Hypoxia-inducible factors in physiology and medicine. *Cell* **148**, 399-408 (2012).
304. Semenza, G.L. et al. Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. *The Journal of biological chemistry* **271**, 32529-32537 (1996).
305. Ivan, M. et al. HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. *Science* **292**, 464-468 (2001).
306. Epstein, A.C. et al. C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* **107**, 43-54 (2001).
307. Mahon, P.C., Hirota, K. & Semenza, G.L. FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. *Genes & development* **15**, 2675-2686 (2001).
308. Lando, D. et al. FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. *Genes & development* **16**, 1466-1471 (2002).
309. Wenger, R.H., Stiehl, D.P. & Camenisch, G. Integration of oxygen signaling at the consensus HRE. *Science's STKE : signal transduction knowledge environment* **2005**, re12 (2005).
310. Arany, Z. et al. An essential role for p300/CBP in the cellular response to hypoxia. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 12969-12973 (1996).
311. Zhou, J. & Brune, B. Cytokines and hormones in the regulation of hypoxia inducible factor-1alpha (HIF-1alpha). *Cardiovascular & hematological agents in medicinal chemistry* **4**, 189-197 (2006).
312. Haddad, J.J. & Land, S.C. A non-hypoxic, ROS-sensitive pathway mediates TNF-alpha-dependent regulation of HIF-1alpha. *FEBS letters* **505**, 269-274 (2001).
313. Bonello, S. et al. Reactive oxygen species activate the HIF-1alpha promoter via a functional NFkappaB site. *Arteriosclerosis, thrombosis, and vascular biology* **27**, 755-761 (2007).
314. Page, E.L., Robitaille, G.A., Pouyssegur, J. & Richard, D.E. Induction of hypoxia-inducible factor-1alpha by transcriptional and translational mechanisms. *The Journal of biological chemistry* **277**, 48403-48409 (2002).
315. Willam, C. HIF meets NF-kappaB signaling. *Kidney international* **85**, 232-234 (2014).
316. Gerald, D. et al. JunD reduces tumor angiogenesis by protecting cells from oxidative stress. *Cell* **118**, 781-794 (2004).
317. Pan, Y. et al. Multiple factors affecting cellular redox status and energy metabolism modulate hypoxia-inducible factor prolyl hydroxylase activity in vivo and in vitro. *Molecular and cellular biology* **27**, 912-925 (2007).
318. Goyal, P. et al. Upregulation of NAD(P)H oxidase 1 in hypoxia activates hypoxia-inducible factor 1 via increase in reactive oxygen species. *Free radical biology & medicine* **36**, 1279-1288 (2004).
319. Naranjo-Suarez, S. et al. HIF-independent regulation of thioredoxin reductase 1 contributes to the high levels of reactive oxygen species induced by hypoxia. *PloS one* **7**, e30470 (2012).
320. Naranjo-Suarez, S. et al. Regulation of HIF-1alpha activity by overexpression of thioredoxin is independent of thioredoxin reductase status. *Molecules and cells* **36**, 151-157 (2013).
321. Zhang, H., Du, Y., Zhang, X., Lu, J. & Holmgren, A. Glutaredoxin 2 Reduces Both Thioredoxin 2 and Thioredoxin 1 and Protects Cells from Apoptosis Induced by Auranofin and 4-Hydroxynonenal. *Antioxidants & redox signaling* (2014).
322. 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).
323. Huang, L.E., Arany, Z., Livingston, D.M. & Bunn, H.F. Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its alpha subunit. *The Journal of biological chemistry* **271**, 32253-32259 (1996).
324. Welsh, S.J., Bellamy, W.T., Briehl, M.M. & Powis, G. The redox protein thioredoxin-1 (Trx-1) increases hypoxia-inducible factor 1alpha protein expression: Trx-1 overexpression results in increased vascular endothelial growth factor production and enhanced tumor angiogenesis. *Cancer research* **62**, 5089-5095 (2002).
325. Liu, Q. et al. A Fenton reaction at the endoplasmic reticulum is involved in the redox control of hypoxia-inducible gene expression. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 4302-4307 (2004).

326. Kim, W.J., Cho, H., Lee, S.W., Kim, Y.J. & Kim, K.W. Antisense-thioredoxin inhibits angiogenesis via pVHL-mediated hypoxia-inducible factor-1alpha degradation. *International journal of oncology* **26**, 1049-1052 (2005).
327. Csiki, I. et al. Thioredoxin-1 modulates transcription of cyclooxygenase-2 via hypoxia-inducible factor-1alpha in non-small cell lung cancer. *Cancer research* **66**, 143-150 (2006).
328. Jones, D.T., Pugh, C.W., Wigfield, S., Stevens, M.F. & Harris, A.L. Novel thioredoxin inhibitors paradoxically increase hypoxia-inducible factor-alpha expression but decrease functional transcriptional activity, DNA binding, and degradation. *Clinical cancer research : an official journal of the American Association for Cancer Research* **12**, 5384-5394 (2006).
329. Zhou, J., Damdimopoulos, A.E., Spyrou, G. & Brune, B. Thioredoxin 1 and thioredoxin 2 have opposed regulatory functions on hypoxia-inducible factor-1alpha. *The Journal of biological chemistry* **282**, 7482-7490 (2007).
330. Semenza, G.L. Targeting HIF-1 for cancer therapy. *Nature reviews. Cancer* **3**, 721-732 (2003).
331. Chen, B., Nelin, V.E., Locy, M.L., Jin, Y. & Tipple, T.E. Thioredoxin-1 mediates hypoxia-induced pulmonary artery smooth muscle cell proliferation. *American journal of physiology. Lung cellular and molecular physiology* **305**, L389-395 (2013).
332. Kilic-Eren, M., Boylu, T. & Tabor, V. Targeting PI3K/Akt represses Hypoxia inducible factor-1alpha activation and sensitizes Rhabdomyosarcoma and Ewing's sarcoma cells for apoptosis. *Cancer cell international* **13**, 36 (2013).
333. Welsh, S.J. et al. The thioredoxin redox inhibitors 1-methylpropyl 2-imidazolyl disulfide and pleurotin inhibit hypoxia-induced factor 1alpha and vascular endothelial growth factor formation. *Molecular cancer therapeutics* **2**, 235-243 (2003).
334. Schmidt-Kastner, R. et al. Nuclear localization of the hypoxia-regulated pro-apoptotic protein BNIP3 after global brain ischemia in the rat hippocampus. *Brain research* **1001**, 133-142 (2004).
335. Ishii, T. & Yanagawa, T. Stress-induced peroxiredoxins. *Sub-cellular biochemistry* **44**, 375-384 (2007).
336. Banning, A., Deubel, S., Kluth, D., Zhou, Z. & Brigelius-Flohe, R. The GI-GPx gene is a target for Nrf2. *Molecular and cellular biology* **25**, 4914-4923 (2005).
337. Nguyen, T., Sherratt, P.J., Huang, H.C., Yang, C.S. & Pickett, C.B. Increased protein stability as a mechanism that enhances Nrf2-mediated transcriptional activation of the antioxidant response element. Degradation of Nrf2 by the 26 S proteasome. *The Journal of biological chemistry* **278**, 4536-4541 (2003).
338. Kobayashi, A. et al. Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. *Molecular and cellular biology* **24**, 7130-7139 (2004).
339. Itoh, K., Igarashi, K., Hayashi, N., Nishizawa, M. & Yamamoto, M. Cloning and characterization of a novel erythroid cell-derived CNC family transcription factor heterodimerizing with the small Maf family proteins. *Molecular and cellular biology* **15**, 4184-4193 (1995).
340. Baird, L. & Dinkova-Kostova, A.T. Diffusion dynamics of the Keap1-Cullin3 interaction in single live cells. *Biochemical and biophysical research communications* **433**, 58-65 (2013).
341. Covas, G., Marinho, H.S., Cyrne, L. & Antunes, F. Activation of Nrf2 by H2O2: de novo synthesis versus nuclear translocation. *Methods in enzymology* **528**, 157-171 (2013).
342. Dinkova-Kostova, A.T. et al. Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 11908-11913 (2002).
343. Kobayashi, M. et al. The antioxidant defense system Keap1-Nrf2 comprises a multiple sensing mechanism for responding to a wide range of chemical compounds. *Molecular and cellular biology* **29**, 493-502 (2009).
344. Fourquet, S., Guerois, R., Biard, D. & Toledano, M.B. Activation of NRF2 by nitrosative agents and H2O2 involves KEAP1 disulfide formation. *The Journal of biological chemistry* **285**, 8463-8471 (2010).
345. Rachakonda, G. et al. Covalent modification at Cys151 dissociates the electrophile sensor Keap1 from the ubiquitin ligase CUL3. *Chemical research in toxicology* **21**, 705-710 (2008).

346. Zhang, D.D. & Hannink, M. Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. *Molecular and cellular biology* **23**, 8137-8151 (2003).
347. Li, W. & Kong, A.N. Molecular mechanisms of Nrf2-mediated antioxidant response. *Molecular carcinogenesis* **48**, 91-104 (2009).
348. Li, W., Yu, S.W. & Kong, A.N. Nrf2 possesses a redox-sensitive nuclear exporting signal in the Neh5 transactivation domain. *The Journal of biological chemistry* **281**, 27251-27263 (2006).
349. Velichkova, M. & Hasson, T. Keap1 regulates the oxidation-sensitive shuttling of Nrf2 into and out of the nucleus via a Crm1-dependent nuclear export mechanism. *Molecular and cellular biology* **25**, 4501-4513 (2005).
350. Suvorova, E.S. et al. Cytoprotective Nrf2 pathway is induced in chronically txnrd 1-deficient hepatocytes. *PloS one* **4**, e6158 (2009).
351. Bloom, D., Dhakshinamoorthy, S. & Jaiswal, A.K. Site-directed mutagenesis of cysteine to serine in the DNA binding region of Nrf2 decreases its capacity to upregulate antioxidant response element-mediated expression and antioxidant induction of NAD(P)H:quinone oxidoreductase1 gene. *Oncogene* **21**, 2191-2200 (2002).
352. Niture, S.K., Jain, A.K. & Jaiswal, A.K. Antioxidant-induced modification of INrf2 cysteine 151 and PKC-delta-mediated phosphorylation of Nrf2 serine 40 are both required for stabilization and nuclear translocation of Nrf2 and increased drug resistance. *Journal of cell science* **122**, 4452-4464 (2009).
353. Jain, A.K. & Jaiswal, A.K. GSK-3beta acts upstream of Fyn kinase in regulation of nuclear export and degradation of NF-E2 related factor 2. *The Journal of biological chemistry* **282**, 16502-16510 (2007).
354. Lopez-Otin, C. & Hunter, T. The regulatory crosstalk between kinases and proteases in cancer. *Nature reviews. Cancer* **10**, 278-292 (2010).
355. Butour, J.L., Wimmer, S., Wimmer, F. & Castan, P. Palladium(II) compounds with potential antitumour properties and their platinum analogues: a comparative study of the reaction of some orotic acid derivatives with DNA in vitro. *Chemico-biological interactions* **104**, 165-178 (1997).
356. Budzisz, E. et al. Biological evaluation of novel Pt(II) and Pd(II) complexes with pyrazole-containing ligands. *European journal of pharmacology* **502**, 59-65 (2004).
357. Castan, P., Colacio-Rodriguez, E., Beauchamp, A.L., Cros, S. & Wimmer, S. Platinum and palladium complexes of 3-methyl orotic acid: a route toward palladium complexes with good antitumor activity. *Journal of inorganic biochemistry* **38**, 225-239 (1990).
358. Kuduk-Jaworska, J., Puszko, A., Kubiak, M. & Pelczynska, M. Synthesis, structural, physico-chemical and biological properties of new palladium(II) complexes with 2,6-dimethyl-4-nitropyridine. *Journal of inorganic biochemistry* **98**, 1447-1456 (2004).
359. Yoo, M.H. et al. Targeting thioredoxin reductase 1 reduction in cancer cells inhibits self-sufficient growth and DNA replication. *PloS one* **2**, e1112 (2007).
360. Carvalho, C.M., Chew, E.H., Hashemy, S.I., Lu, J. & Holmgren, A. Inhibition of the human thioredoxin system. A molecular mechanism of mercury toxicity. *The Journal of biological chemistry* **283**, 11913-11923 (2008).
361. Millet, R. et al. Synthesis of 5-nitro-2-furancarbohydrazides and their cis-diamminedichloroplatinum complexes as bitopic and irreversible human thioredoxin reductase inhibitors. *Journal of medicinal chemistry* **48**, 7024-7039 (2005).
362. 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).
363. 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).
364. Lindner, D.J., Hofmann, E.R., Karra, S. & Kalvakolanu, D.V. The interferon-beta and tamoxifen combination induces apoptosis using thioredoxin reductase. *Biochimica et biophysica acta* **1496**, 196-206 (2000).
365. Maurer-Stroh, S., Eisenhaber, B. & Eisenhaber, F. N-terminal N-myristoylation of proteins: refinement of the sequence motif and its taxon-specific differences. *Journal of molecular biology* **317**, 523-540 (2002).

366. Aicart-Ramos, C., Valero, R.A. & Rodriguez-Crespo, I. Protein palmitoylation and subcellular trafficking. *Biochimica et biophysica acta* **1808**, 2981-2994 (2011).
367. Salaun, C., Greaves, J. & Chamberlain, L.H. The intracellular dynamic of protein palmitoylation. *The Journal of cell biology* **191**, 1229-1238 (2010).
368. Goodwin, J.S. et al. Depalmitoylated Ras traffics to and from the Golgi complex via a nonvesicular pathway. *The Journal of cell biology* **170**, 261-272 (2005).
369. Rocks, O. et al. An acylation cycle regulates localization and activity of palmitoylated Ras isoforms. *Science* **307**, 1746-1752 (2005).
370. Sonnino, S. & Prinetti, A. Membrane domains and the "lipid raft" concept. *Current medicinal chemistry* **20**, 4-21 (2013).
371. Jin, S., Zhou, F., Katirai, F. & Li, P.L. Lipid raft redox signaling: molecular mechanisms in health and disease. *Antioxidants & redox signaling* **15**, 1043-1083 (2011).
372. Patel, H.H. & Insel, P.A. Lipid rafts and caveolae and their role in compartmentation of redox signaling. *Antioxidants & redox signaling* **11**, 1357-1372 (2009).
373. Harder, T. & Simons, K. Clusters of glycolipid and glycosylphosphatidylinositol-anchored proteins in lymphoid cells: accumulation of actin regulated by local tyrosine phosphorylation. *European journal of immunology* **29**, 556-562 (1999).
374. Gauthier-Campbell, C., Bredt, D.S., Murphy, T.H. & El-Husseini Ael, D. Regulation of dendritic branching and filopodia formation in hippocampal neurons by specific acylated protein motifs. *Molecular biology of the cell* **15**, 2205-2217 (2004).
375. Scorticati, C., Formoso, K. & Frasch, A.C. Neuronal glycoprotein M6a induces filopodia formation via association with cholesterol-rich lipid rafts. *Journal of neurochemistry* **119**, 521-531 (2011).
376. 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).
377. 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).
378. 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).
379. Beillerot, A., Battaglia, E., Bennasroune, A. & Bagrel, D. Protection of CDC25 phosphatases against oxidative stress in breast cancer cells: evaluation of the implication of the thioredoxin system. *Free radical research* **46**, 674-689 (2012).
380. Sohn, J. & Rudolph, J. Catalytic and chemical competence of regulation of cdc25 phosphatase by oxidation/reduction. *Biochemistry* **42**, 10060-10070 (2003).
381. Kanda, M. et al. Glutaredoxin modulates platelet-derived growth factor-dependent cell signaling by regulating the redox status of low molecular weight protein-tyrosine phosphatase. *The Journal of biological chemistry* **281**, 28518-28528 (2006).
382. Murphy, D.A. & Courtneidge, S.A. The 'ins' and 'outs' of podosomes and invadopodia: characteristics, formation and function. *Nature reviews. Molecular cell biology* **12**, 413-426 (2011).
383. Diaz, B. & Courtneidge, S.A. Redox signaling at invasive microdomains in cancer cells. *Free radical biology & medicine* **52**, 247-256 (2012).
384. Kan, A. et al. An improved method for the detection and enrichment of low-abundant membrane and lipid raft-residing proteins. *Journal of proteomics* **79**, 299-304 (2013).
385. Goedhart, J. et al. Structure-guided evolution of cyan fluorescent proteins towards a quantum yield of 93%. *Nature communications* **3**, 751 (2012).
386. Shcherbakova, D.M. & Verkhusha, V.V. Near-infrared fluorescent proteins for multicolor in vivo imaging. *Nature methods* **10**, 751-754 (2013).