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Identification and Functional Characterization of Novel Thioredoxin Systems

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To my family

ABSTRACT

Thioredoxins (Trx) are a class of small multifunctional 12-kDa proteins that are characterized by the redox active site Trp-Cys-Gly-Pro-Cys (WCGPC). In the oxidized (inactive) form of Trx (Trx-S₂), the two cysteines at the active site form a disulfide bond. This can then be reduced by thioredoxin reductase (TrxR) and NADPH, the so-called thioredoxin system, to a dithiol (Trx-(SH)₂), which can then act as a general protein disulfide reductase. Thioredoxins are present in all living organisms and have been isolated and characterized from a wide variety of prokaryotic and eukaryotic cells. In this thesis we describe the identification and functional characterization of novel members of the thioredoxin superfamily.

We present evidence for a novel Trx (Trx2) in *Escherichia coli*. The *E. coli* Trx2 contains two domains: an N-terminal domain of 32 amino acids containing two CXXC motifs and a C-terminal domain with high homology to the prokaryotic thioredoxins, containing the conserved active site. Trx2 together with TrxR and NADPH can reduce ribonucleotide reductase as well as the interchain disulfide bridges of insulin.

Thioredoxins are ubiquitously expressed in all tissues within the same organism. We have identified the first tissue specific Trx (Sptrx1) exclusively expressed in human spermatozoa. Sptrx1 is an active thioredoxin which under native conditions appears to have a multimeric structure.

We also identify and characterize a complete thioredoxin system (Trx2, TrxR2) located in mitochondria. We show that Trx2 overexpressing cells have a higher mitochondrial membrane potential that is dependent on the function of the ATP synthase complex. Furthermore, overexpression of Trx2 was found to protect cells against the cytotoxic effects of etoposide, a drug commonly used in anticancer treatment.

In addition, we showed that the second compound of the mitochondrial thioredoxin system, TrxR2, is capable of reducing cytochrome c and could protect cells against the cytotoxic effects of antimycin and myxothiazol, chemicals that inhibit the function of complex III in the mitochondrial electron transport chain.

Furthermore, we identified an alternative splicing variant of cytosolic thioredoxin reductase (TrxR1b) that could bind to the Estrogen Receptors (ER) α and β . As a result of this binding, a distinct subnuclear localization of TrxR1b was observed, co localizing with both ER α and β . TrxR1b can act as a coactivator and enhance the transcriptional activity of ER in the classical activation pathway, which relies on the binding of the ER to an ER response element on the DNA. By contrast, TrxR1b is a co-repressor in the alternative pathway where ER activates AP-1 transcription independently of its DNA binding activity.

In summary, the results presented in this thesis give a better understanding of Thioredoxin systems in both prokaryotes and eukaryotes, with the introduction of new members in this redox superfamily of proteins. This study, which shows a wide spectrum of functions for these Thioredoxin systems in influencing various redox mechanisms and processes in biological systems, indicates that there is still a great deal of work yet to be done in this expanding field of research.

LIST OF PUBLICATIONS

- I. Miranda-Vizueté A, **Damdimopoulos AE**, Gustafsson JÅ and Spyrou G, Cloning, expression, and characterization of a novel *Escherichia coli* thioredoxin. *J Biol Chem* 272(49): 30841-7, 1997.
- II. Miranda-Vizueté A, Ljung J, **Damdimopoulos AE**, Gustafsson JÅ, Oko R, Pelto-Huikko M and Spyrou G, Characterization of Sptrx, a novel member of the thioredoxin family specifically expressed in human spermatozoa. *J Biol Chem* 276(34): 31567-74, 2001.
- III. **Damdimopoulos AE**, Miranda-Vizueté A, Pelto-Huikko M, Gustafsson JÅ and Spyrou G, Human mitochondrial thioredoxin. Involvement in mitochondrial membrane potential and cell death. *J Biol Chem* 277(36): 33249-57, 2002.
- IV. Miranda-Vizueté A, **Damdimopoulos AE**, Pedrajas JR, Gustafsson JÅ and Spyrou G, Human mitochondrial thioredoxin reductase cDNA cloning, expression and genomic organization. *Eur J Biochem* 261(2): 405-12, 1999.
- V. Nalvarte I, **Damdimopoulos AE**, Spyrou G, Human Mitochondrial Thioredoxin Reductase Reduces Cytochrome c and Confers Resistance to Complex III Inhibition. (Manuscript)
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TABLE OF CONTENTS

1. INTRODUCTION	10
1.1 Oxygen and Energy Production	10
1.2 Oxygen Paradox – Reactive Oxygen Species	12
1.3 Aging	13
1.4 ROS, Oxidative Stress and Cellular Signaling	14
1.4.1 Mitogen Activated Protein Kinases (MAPK)	16
1.4.1.1 <i>ERK</i>	16
1.4.1.2 <i>JNK and p38 (SAPK)</i>	17
1.4.1.3 <i>Targets of MAPK</i>	18
1.4.2 PI3-Kinase/Akt	18
1.4.3 p53	18
1.4.4 NF-κB	19
1.4.5 JAK/STAT	20
1.4.6 Heat Shock Proteins (Hsp)	20
1.5 ROS and infection	21
1.6 Antioxidant Defense Mechanisms	21
1.6.1 Non enzymatic antioxidants	22
1.6.1.1 <i>Glutathione</i>	22
1.6.1.2 <i>Vitamins</i>	22
1.6.1.3 <i>Ubiquinone (Q10)</i>	23
1.6.1.4 <i>Polyphenols</i>	23
1.6.2 Enzymatic antioxidant systems	24
1.6.2.1 <i>Superoxide Dismutase (SOD)</i>	24
1.6.2.2 <i>Catalase</i>	25
1.6.2.3 <i>Glutathione Peroxidase (GPx)</i>	25
1.6.2.4 <i>Peroxiredoxins (Prx)</i>	26
1.6.2.5 <i>Glutaredoxins (Grx)</i>	26
1.7 Thioredoxin superfamily	28
1.7.1 Escherichia coli Trx1 and Trx2	29
1.7.2 Mammalian Trx1	32
1.7.2.1 <i>Extracellular Trx1</i>	32
1.7.2.2 <i>Gene regulation</i>	33

1.7.2.3 Cellular localization of Trx1	33
1.7.2.4 Regulation of signaling pathways and transcription factors by Trx1	34
1.7.3 Thioredoxin like protein (Txl).....	36
1.7.4 Organelle specific thioredoxins	36
1.7.4.1 Mitochondrial Thioredoxin (Trx2)	36
1.7.4.2 ERdj5	37
1.7.4.3 Transmembrane Thioredoxin (TMX)	38
1.7.4.4 Nucleoredoxin (NRX)	38
1.7.5 Tissue specific thioredoxins	39
1.7.5.1 Sperm specific Trx1 (Sptrx1)	39
1.7.5.2 Sperm specific Trx2 (Sptrx2)	40
1.7.5.3 Thioredoxin- like protein 2 (Txl2)	40
1.7.6 Thioredoxin Reductases.....	41
1.7.6.1 Cytosolic Thioredoxin Reductase (TrxR1)	41
1.7.6.2 Mitochondrial Thioredoxin Reductase (TrxR2)	42
1.7.6.3 Thioredoxin and Glutathione Reductase (TGR)	43
2. RESULTS AND DISCUSSION.....	44
2.1 Paper I	44
2.2 Paper II.....	44
2.3 Paper III	45
2.4 Paper IV	45
2.5 Paper V	46
2.6 Paper VI	46
2.7 General Discussion	47
3. CONCLUSIONS.....	50
3.1 Paper I	50
3.2 Paper II.....	50
3.3 Paper III	50
3.4 Paper IV	51
3.5 Paper V	51
3.6 Paper VI	51
4. ACKNOWLEDGMENTS.....	52
5. REFERENCES	55

ABBREVIATIONS

ASK	Apoptosis Signal-Regulating Kinase
ATP	Adenosine Triphosphate
DTT	Dithiothreitol
ER	Estrogen Receptor
ERK	Extracellular Signal-Regulated Kinases
FAD	Flavin Adenine Dinucleotide
GR	Glutathione Reductase
Grx	Glutaredoxin
JNK	c-Jun N-terminal kinases
MAPK	Mitogen Activated Protein Kinases
MRC	Mitochondrial Respiratory Chain
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NF- κ B	Nuclear Factor Kappa B
Prx	Peroxiredoxin
RNR	Ribonucleotide Reductase
ROS	Reactive Oxygen Species
SAPK	Stress Activated Protein Kinases
SOD	Superoxide Dismutase
TNF	Tumour Necrosis Factor
Trx	Thioredoxin
TrxR	Thioredoxin Reductase
UQ	Ubiquinone
UV	Ultraviolet

1. INTRODUCTION

1.1 Oxygen and Energy Production

Oxygen is essential for aerobic life due to its function in mitochondria and energy production. In the inner membrane of mitochondria a series of oxidoreductase multienzyme complexes reside, the so called mitochondrial respiratory chain (MRC) or the electron transport chain (ETC) (Fig. 1). The MRC receives electrons from NADPH through complex I or from succinate through the complex II. The electrons are then passed over to ubiquinone and this enables complex I to pump protons from the mitochondrial matrix into the intermembrane space. By contrast, complex II is not capable of proton pumping. From ubiquinone the electrons are then transferred to complex III which uses them to reduce cytochrome c and this is once again followed by proton pumping into the intermembrane space. Cytochrome c then becomes oxidized while reducing complex IV. Finally, oxygen is used as the terminal acceptor of the electrons and become reduced to water by complex VI, while protons are transferred from the mitochondrial matrix to the intermembrane space (Figure 1).

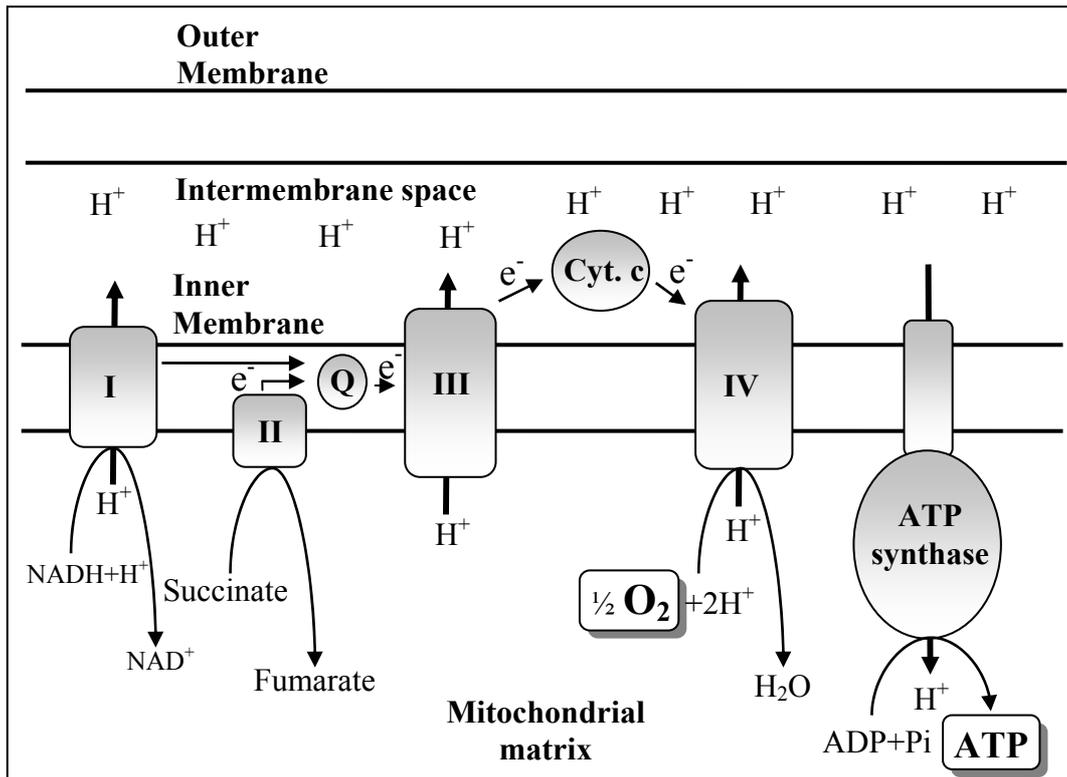


Figure 1. The mitochondrial respiratory chain. A scheme illustrating the mitochondrial respiratory chain (MRC) and its function in the production of ATP. The flow of electrons through the MRC to oxygen creates a proton gradient that drives the production of ATP by ATP synthase. Ubiquinone is illustrated as Q and cytochrome c as Cyt. c.

The series of oxidoreductase reactions followed by proton pumping leads to the creation of a proton gradient in the mitochondria, where the intermembrane space has a higher concentration of protons compared to the mitochondrial matrix, giving rise to a high membrane potential over the inner membrane. This gradient drives the import of protons back to the matrix, which is possible only through the ion channels of the ATP synthase complex and is accompanied by the formation of ATP, the major energy currency in the cell. Hence, the function of oxygen as the final acceptor of electrons drives the MRC to create a proton gradient that is coupled to ATP synthesis. This renders oxygen essential for aerobic life, as its depletion would lead to a severely impaired energy production unable to sustain life in higher organisms.

An example of the importance of the proper function of the MRC is well illustrated by the extremely toxic chemical cyanide. Cyanide is a potent inhibitor of complex IV in the MRC and blocks the reduction of oxygen to water, so although oxygen is present, the cells are unable to use it. The cells are then incapable of proton

pumping and ATP production leading to the well known severe, many times lethal, consequences of cyanide poisoning.

1.2 Oxygen Paradox – Reactive Oxygen Species

The use of oxygen for energy production is a double edged sword because, although essential for life, oxygen can be the source of reactive oxygen species (ROS), which are highly reactive molecules produced due to the leakiness of the MRC during aerobic respiration. Incomplete reduction of oxygen by other components of the MRC, mainly complex III [1, 2], produces superoxide radical ($O_2^{\bullet-}$) which can be dismutated to hydrogen peroxide (H_2O_2) that in the presence of transition metal ions (Fe^{2+} or Cu^+) can further react to produce the highly reactive hydroxylradical ($\bullet OH$) in a reaction known as the Fenton reaction. $O_2^{\bullet-}$ and $\bullet OH$ are also so called free radicals, a term used to describe molecules that have one unpaired electron in their outer orbit [3]. It has been calculated that between 1% and 3% of the oxygen in mitochondria give rise to $O_2^{\bullet-}$ instead of being reduced to water [4, 5].

ROS can be very harmful, especially under conditions where their production is enhanced which then exceeding cellular antioxidant defenses, a condition known as oxidative stress. ROS can target cellular biomolecules and cause severe damage such as lipid peroxidation [6, 7], protein oxidation/inactivation [8], DNA damage/mutations [9, 10]. They also regulate cell proliferation and cell death (Figure 2). Recent findings suggest an important role of oxidized low-density lipoproteins (Ox-LDL) in atherogenesis. Lipids are not the only targets for oxidation in LDL. Apoprotein B (apoB) is also a target for modification. Ox-LDL has modified properties compared to minimally oxidized LDL (MM-LDL). Unlike MM-LDL, it is metabolized through macrophage scavenger receptors and can cause foam cell formations [6]. In addition, evidence has been provided that demonstrates the presence of Ox-LDL in atherosclerotic lesions, but not in normal arteries, of humans and rabbits [11] and several antioxidants have been proven to be effective protectors against atherosclerosis [12-15].

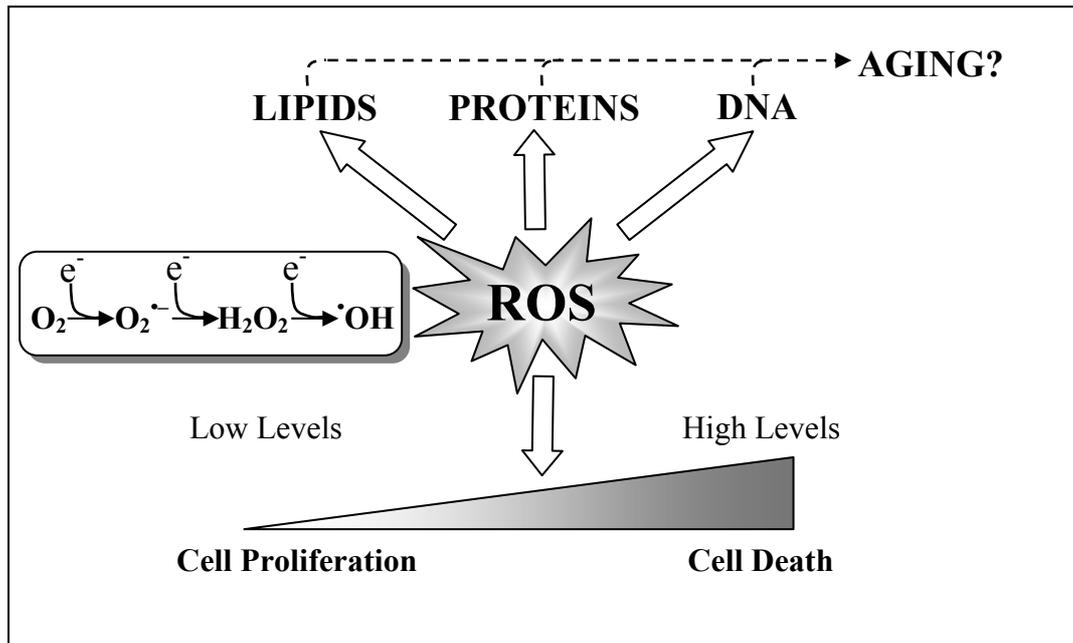


Figure 2. The reactive oxygen species. Potential targets of oxidative damage and the cellular functions of reactive oxygen species (ROS). Boxed are ROS commonly produced in the cell.

1.3 Aging

ROS have been implicated in the process of aging, largely due to the damage they inflict upon the cellular macromolecules [16-18]. The free radical theory of aging states that ROS are the cause of aging due to their cumulative damage of macromolecules, as discussed above [19-22]. Indeed, the level of oxidative damage and the rate of aging show a strong correlation [19]. It has been shown that human diploid fibroblasts grown at low oxygen tension have a prolonged life span [23] but at hyperoxic conditions they are characterized by shortened telomeres and compromised cellular life span [24]. *Drosophila* strains that display enhanced longevity have been shown to have an upregulation of genes associated with antioxidant defenses [25, 26]. Furthermore, caloric restriction has been shown to extend the life span in several species [27], which has been proposed to be due to decreased oxidative stress [5]. A restricted food intake would decrease the metabolism meaning lower use of the MRC and ATP production, which in turn would minimize the production of ROS as well. It has been shown in mice grown under conditions of caloric restriction, that the rate of oxidant generation is significantly lower compared to ad libitum-fed mice and that the age-

associated accumulative oxidative damage on lipids, proteins and DNA is minimized as well [27].

The aging process has been extensively studied in the nematode worm, *Caenorhabditis elegans*. An extremely important gene for life span is *daf-2*, a gene that codes for an insulin receptor-like transmembrane tyrosine kinase [28]. *C. elegans daf-2* mutants revealed a doubling of life span compared to the wild type worms [29]. Furthermore, those mutants were also more resistant to several stress inducers, such as ultraviolet irradiation (UV) [30], heavy metals [31] and oxidative stress [32], all known to be mediated at least to some degree by the production of ROS. It has been shown that these mutants have an upregulation of enzymes that scavenge ROS [32, 33] which could explain the increased resistance to oxidative stress and the longevity of the mutants. Another equally important gene is *isp-1* that codes for the “Rieske” iron sulfur protein of complex III in the MRC. *C. elegans isp-1* mutants have a decreased oxygen consumption but an increased life span [34], similar to that of *daf-2* mutants. It is not known how, but the *isp-1* mutants are thought to have a modified complex III function that leads to a reduced ROS production leading to the long lived phenotype.

Another way to modify the ROS levels is through the gene *clk-1* which encodes for an enzyme required for the synthesis of ubiquinone (UQ), an antioxidant and integral part of the MRC. In the absence of *clk-1*, UQ cannot be synthesized and its precursor demethoxyubiquinone (DMQ) accumulates in mutant mice, yeast and worms and replaces to some extent the function of UQ [35-38]. Interestingly, DMQ might actually be a better antioxidant than UQ with a redox cycle that does not tend to produce as much ROS as UQ [39]. *C. elegans clk-1* mutants show a slowing of a variety of developmental and physiological processes. While, they appear to have a normal respiration [40-42], they accumulate oxidative damaged biomolecules more slowly [42].

1.4 ROS, Oxidative Stress and Cellular Signaling

ROS might be very dangerous to cells and their content, but it is becoming more and more evident that they take part in processes necessary for normal cellular function such as acting as second messengers in several transduction pathways [43]. The concept of second messengers was introduced with the discovery of cAMP, which is produced from ATP by an adenylyl cyclase activated through a stimulatory GTPase

[21, 22]. Second messenger is the definition of a molecule that acts to amplify the information from the membranes to the cytoplasm or nucleus. Several second messengers have been found apart from cAMP, such as phosphatidic acid, ceramide, sphingosine 1-P and polyphosphoinositides. It is now known that ROS are able to act as second messengers and serve critical functions in response to several stresses. They can consequently regulate cellular growth and death through various transduction pathways (Figure 3).

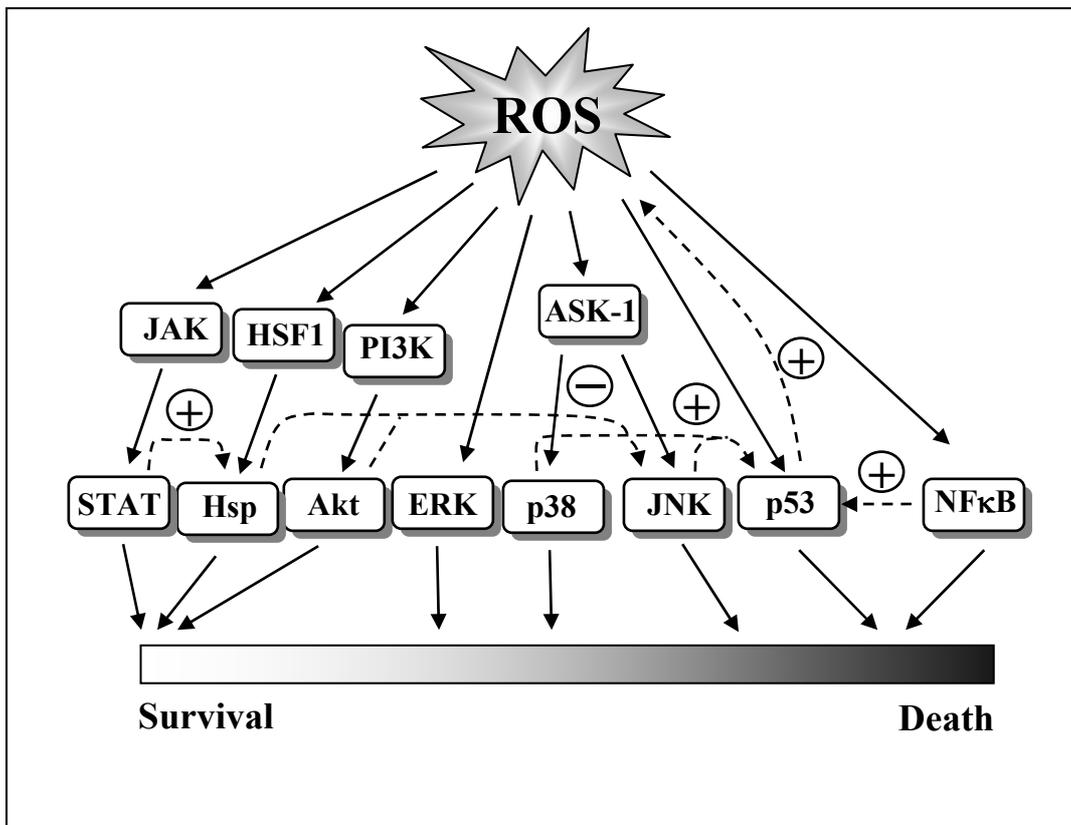


Figure 3. ROS and signaling pathways. Several signaling pathways are targeted by various ROS which can then work to either prevent or promote cell death. Many of the pathways have been found to be able to do both, and what they will finally promote can depend on factors such as the cell type, the oxidant insult and the duration of the stress. Furthermore, several of those pathways can either enhance (+) or suppress (-) other signaling pathways.

1.4.1 Mitogen Activated Protein Kinases (MAPK)

Mitogen Activated Protein Kinases (MAPKs) are a large family of serine/threonine kinases regulating a wide range of cellular processes, such as proliferation, differentiation and apoptosis. They are divided into three subfamilies: a) the extracellular signal-regulated kinases (ERK) b) the c-Jun N-terminal kinases (JNK) and c) the p38 kinases. The ERK pathway is mostly linked to cellular proliferation while JNK and p38 pathways are commonly associated to stress responses. Therefore the JNK and p38 subfamilies are many times tied together and referred to as stress activated protein kinases (SAPK) [44-46].

1.4.1.1 ERK

ERK is the major pathway used to transfer proliferation signals from the growth factor receptors in the membrane to the nucleus. [44, 47]. Several oxidative stress inducers such as H₂O₂, asbestos, UV irradiation and arsenite have been shown to activate/phosphorylate a wide range of growth factor receptors, such as epidermal growth factor, platelet-derived growth factor, and the T-cell receptor complex [48-53]. The expression of mutant receptors has been shown to lessen the ERK response by oxidants [48]. In agreement with the activation of the growth factor receptors and their signaling pathways by oxidants is the observation that H₂O₂ in low amounts can act as a mitogenic factor [54].

Several studies have shown that the activation of ERK in many cell types by a broad spectrum of oxidant stress inducers is associated with cell survival [55-60] although conflicting results have been produced in other model systems, suggesting that ERK activation leads to an apoptotic response instead of cell survival [61-65]. What determines whether ERK activation will lead to cell survival or death is not known, but it has been speculated [43] that when the activation is rapid and transient cell survival is favored [55-57], whereas when the stimulus is prolonged with a delayed activation, there is a pro-apoptotic response instead [65, 66].

1.4.1.2 JNK and p38 (SAPK)

JNK and p38 are known for being activated by several stresses such as cytokines, radiation, osmotic shock, heat stress and oxidative damage. The pathways leading to their activation are extremely complex and involve several MAPKK and MAPKKKs. One of these MAPKKKs is the apoptosis signal-regulating kinase (ASK-1). ASK-1 is regulated by thioredoxin1 (Trx1) and is an upstream regulator of the JNK and p38 [67]. Under normal conditions Trx is bound to ASK-1 suppressing the apoptotic signal, but under oxidative conditions, Trx becomes oxidized and disassociates, allowing the activation of the downstream targets of ASK-1, JNK and p38 and the subsequent apoptotic response of the cell. Similar regulation has been found on the level of JNK by glutathione S-transferase (GST), which seems to be able to bind JNK to hinder its activation, but during oxidative stress JNK will be released [68].

The functional consequences of JNK activation during oxidative stress are controversial, with studies presenting both pro-survival and pro-death effects of the pathway. Many studies have shown the apoptotic effects of JNK activation by oxidative inducers [69]. Mouse embryo fibroblasts (MEF) from JNK1 and JNK2 knockout mice showed that the disruption of either gene had any significant effect on UV induced apoptosis. However, MEF from JNK1 and JNK2 double knockouts lost the ability to undergo apoptosis through the release of cytochrome c from mitochondria when irradiated by UV [70]. Similarly, MEF from ASK1 knockouts were more resistant to H₂O₂ treatment [71].

However, reports have also implicated the JNK pathway in cell survival during oxidative insult. Disruption of MEKK1, rendered embryonic stem cell-derived cardiac myocytes, extremely sensitive towards oxidant-induced apoptosis when the cells were oxidatively challenged by H₂O₂. The MEKK1 disrupted cells failed to activate JNK which is thought to have pro-survival abilities due to its ability to inhibit the production of tumor necrosis factor α (TNF α) [72].

p38 activation has also given rise to conflicting results about the consequences of its activation. Experiments using pharmacological inhibitors for the α and β isoforms of p38 have shown pro-apoptotic functions [73-76] as well as anti-apoptotic [77, 78]. Furthermore, whether p38 will act as pro or anti-apoptotic might depend on the oxidative insult, as it has been shown that apoptosis induced by singlet

oxygen requires p38 which is not required when H₂O₂ is used to induce apoptosis [76]. In addition, p38 activation, under conditions of minor oxidative stress, is involved in the mitotic arrest of the cell [79].

1.4.1.3 Targets of MAPK

The most studied target of the MAPK pathway in response to oxidative stress is c-Jun. Similarly to its upstream regulator, JNK, both pro and anti-apoptotic properties have been assigned to c-Jun [80-82]. Another target is the tumor suppressor p53, which can be phosphorylated by both JNK and p38, both of which have also been implicated in the regulation the p53 protein stability during stress [74, 83]. It has been reported that inhibition of p38 activity also reduces UV-dependent apoptosis in a p53 dependent manner. The relationship between JNK and p53 is not as clear though, as accumulation of p53 during UV exposure is not JNK-dependent [70] and likewise p53 is not necessary in JNK-induced apoptosis [69].

1.4.2 PI3-Kinase/Akt

The PI3-Kinase/Akt pathway is inhibited as a result of a wide variety of stresses that can lead to cellular death such as UV, ionizing radiation and hyperosmotic stress, suggesting an important role for PI3-Kinase/Akt in the survival of the cell [84-86]. However, it has been shown that Akt can be activated by oxidative damage and insults that lead to oxidative stress [87-91]. Using the PI3 inhibitor wortmannin, cells treated with H₂O₂ were not able to activate Akt which lead to increased cell death, further confirming the importance of this pathway for the survival of the cell during oxidative insult [90]. In addition, overexpression of Akt led to enhanced survival of HeLa and NIH3T3 cells that were treated with H₂O₂ [91]. Interestingly, Akt also regulates ASK1 [92] through phosphorylation of the protein, which renders ASK1 unable to activate JNK and can therefore protect cells from H₂O₂-induced apoptosis.

1.4.3 p53

p53 is a transcription factor that regulates genes essential for the regulation of cellular growth arrest and cell death. p53 has been found to be activated by

many chemotherapeutic agents and agents such as cytokines, that are not thought to damage the DNA, through the production of ROS. However, ROS are also acting downstream of the p53 activation as mediators in the apoptotic process [72, 93].

The activation of p53 involves a series of complex phosphorylations and adenylation steps, leading to the stability of the protein and enhanced DNA binding. How ROS can activate p53 is not completely understood. One alternative would be by the direct DNA damage caused by ROS. In addition, as previously mentioned p53 is a downstream phosphorylation target of the MAPK family members p38 and JNK which are in turn also activated by ROS. Another way would be through NF κ B which has been shown to upregulate p53 in H₂O₂ treated T cells [94].

Although ROS are potent activators of p53, p53 in turn enhances the production of ROS, creating a positive feedback loop which increases the level of oxidative stress thought to play an important role in the execution of p53-dependent apoptosis [93, 95]. The production of ROS by p53 is not completely understood, however, as a transcription factor, p53 could regulate genes that are involved in the antioxidant defenses of the cell. Indeed, it has been reported that one of those transcriptional targets is manganese superoxide dismutase (MnSOD) as it is repressed by p53 activation [96]. In addition, when overexpressed, MnSOD provides enhanced survival against p53 dependent apoptosis and the absence of p53 confers resistance to oxidant/pro-oxidant treatments [96, 97].

1.4.4 NF- κ B

It has been shown that in several cell types ROS are potent activators of NF- κ B signaling. NF- κ B is bound to the inhibitory protein I κ B that upon phosphorylation disassociates and undergoes degradation, freeing NF- κ B which then translocates into the nucleus and functions as a transcription factor. Several kinases are capable of the I κ B phosphorylation such as MEKK1 [98, 99] and Akt [100] both of which have been previously discussed as targets of redox control.

Although ROS can activate NF- κ B they can also inhibit its action. For NF- κ B to be able to bind to DNA it must be in a reduced form and oxidants like diamide can decrease the DNA binding activity while reducing agents like dithiothreitol (DTT) allow for an enhanced activity. It has been shown that thioredoxin (Trx) can act

on two levels to regulate NF- κ B activity. In the cytosol Trx can be an inhibitor that prevents the phosphorylation of I κ B while in the nucleus it activates NF- κ B via reduction, enhancing its DNA-binding activity [101].

1.4.5 JAK/STAT

Janus protein tyrosine kinases (JAK) activate, through tyrosine phosphorylation, members of the signal transducers and activators of transcription (STAT) family, which will then translocate into the nucleus to regulate gene transcription. Oxidative stress, in the form of H₂O₂ is a potent activator of the STAT factors STAT1 and STAT3, and the STAT kinases JAK2 and TYK2[102]. Furthermore, antioxidants are capable of inhibiting the STAT activation by ROS as neither superoxide nor nitric oxide could activate STATs. One of the target genes identified that is in part regulated by the JAK/STAT pathway is heat shock protein 70 (Hsp70). It has been shown that STAT can bind to the Hsp70 promoter after H₂O₂ treatment and that inhibition of JAK2 decreased the activation of Hsp70 by H₂O₂ [103].

1.4.6 Heat Shock Proteins (Hsp)

Hsp are proteins that function as molecular chaperones in aiding the folding of proteins. Hsp are transcriptionally regulated by heat shock transcription factor 1 (HSF1). Hsps are induced during oxidative stress, with several oxidants being capable of activating them. The signal thought to cause the activation is oxidative damage to proteins. On the other hand, antioxidants before heat stress seem to attenuate the Hsp response leading to the apoptotic death of the cell [104]. Many studies have shown the antiapoptotic effects of Hsp under a broad range of stresses. These proteins are also implicated in the chemotherapeutic resistance of tumors and carcinogenesis [105, 106]. Furthermore, apart from protecting the cell from commitment to apoptotic death Hsps will also reduce oxidative damage to DNA, lipids and proteins [107-109]. Finally, a crosstalk between the Hsp pathway and the JNK pathway exists, as the Hsp family members, Hsp70 and Hsp72, can inhibit the apoptotic function of JNK [110-112].

1.5 ROS and infection

Although mitochondria are the major source of ROS other sites for the production of ROS have been shown to exist, such as the NADPH oxidase. In contrast to the MRC where the production of ROS is thought to be an unfortunate by-product of aerobic respiration, the NADPH oxidase deliberately produces ROS. NADPH oxidase is a complex multicomponent enzyme that resides in the plasma membrane and was first described to exist in phagocytes. When inactive however, several components of the complex are located in the cytoplasm. Upon activation by phagocytosis the various components assemble into a complex in the cell membrane [113]. When activated, NADPH oxidase is responsible for the addition of one electron to oxygen to produce superoxide ($O_2^{\bullet-}$) which is a precursor to other ROS such as hypochlorous acid (HOCl) and the hydroxyl radical ($\bullet OH$) that are used for the neutralization of invading bacteria in the phagosomes [114].

In humans, several mutations of the subunits of the NADPH oxidase complex have been described which lead to the loss of function of the enzyme. This disorder is termed chronic granulomatous disease (CGD) and is inherited. Patients with this disease are characterized by the inability of their phagocytes to produce $O_2^{\bullet-}$, leading to a higher susceptibility to bacterial and fungal infections and suggesting a major role of the NADPH oxidase in our defense against microbes.

1.6 Antioxidant Defense Mechanisms

To overcome the harmful effects of ROS there are several antioxidant defense mechanisms being utilized by the cells. Antioxidants serve to protect the cells against oxidative damage by either repairing damage inflicted by oxidants on cellular biomolecules, or by scavenging the ROS. The antioxidant defense mechanisms can be roughly divided into two categories, the enzymatic systems and the non enzymatic, consisting of low molecular weight compounds with antioxidant properties.

1.6.1 Non enzymatic antioxidants

1.6.1.1 Glutathione

One of the major antioxidants that belong to this category is glutathione (GSH), a tri-peptide synthesized of the amino acids glutamate, cysteine and glycine. It is the most abundant antioxidant present in the cell with concentrations in the millimolar range. Glutathione can scavenge ROS and reduce cysteine thiols on proteins but by doing so, it becomes oxidized, forming a glutathione radical (GS[•]) which is a pro-oxidant. However, this radical can react with another GS[•] to form GS-SG which can then be reduced back to two GSH by glutathione reductase [115]. As previously mentioned, ROS can act as secondary messenger molecules and glutathione is the main redox buffer of the cell and as such has been shown to regulate numerous transduction pathways and transcription factors. It has been shown that the protein kinase C (PKC) family (except for nPKCdelta) are inactivated by S-glutathionylation induced by diamide [116]. Protein-tyrosine phosphatases (PTPs) are also targets for negative regulation by glutathione as it has been shown for PTP-1B where binding of glutathione to redox-active cysteines inactivates the protein [117]. Also transcription factors such as p53, NFκB and AP-1 may be regulated by glutathione suggesting an important role in cellular proliferation and differentiation [118].

1.6.1.2 Vitamins

Vitamins also serve important antioxidant functions. Vitamin E (α -tocopherol) is recognized as one of the most important antioxidants and as a lipid soluble molecule it is especially important in preventing the generation of lipid peroxyl radicals and thereby protecting membranes from lipid peroxidation [119]. Hence, the absence of vitamin E has been shown to cause liver lipid peroxidation as well as neurologic and cardiovascular disorders [120, 121]. Furthermore, it has been reported that mice with a disrupted gene for the α -tocopherol transfer protein, a protein essential for the transportation of vitamin E from the liver to the blood, have increased brain lipid peroxidation and neurodegeneration thought to be caused by chronic oxidative stress [122].

Another vitamin with important antioxidant properties is vitamin C (ascorbic acid) but unlike vitamin E, C is water soluble and is capable of scavenging various ROS [123]. A well known disease that is associated with vitamin C deprivation is scurvy. Vitamin C is crucial for the activity of hydroxylase and oxygenases as it reduces the active central ions of these enzymes. These enzymes are involved in the synthesis of procollagen, carnitine and neurotransmitters [124]. Even though vitamin C is water soluble, it is nonetheless important in the protection of membranes from oxidative damage. The lipid protective function of vitamin C is thought to be indirect because it relies on its ability to reduce vitamin E radicals that arises from reactions with lipid hydroperoxides. It has been shown that the combination of both vitamins is important in the prevention of atherosclerotic progression in hypercholesterolemic persons [125]. Furthermore, it has been reported that the combined vitamin C and E supplementation can inhibit the early progression of coronary arteriosclerosis in patients after heart transplantation [126].

1.6.1.3 Ubiquinone (Q10)

Another major lipid soluble antioxidant is ubiquinone. It is composed of a redox active quinone ring, for which the precursor is thought to be the aromatic amino acids tyrosine and phenylalanine [127], and an all-*trans*-isoprenoid side chain, that has various lengths in different species. In humans the predominant ubiquinone homologue contains the longest known isoprene side chain with ten isoprene units and is therefore also termed Q10 [127]. As previously mentioned, ubiquinone is an essential part of the MRC, however, its redox capabilities render it an effective antioxidant. It has been shown to prevent lipid peroxidation in liposomes, lipid emulsions, phospholipids and LDL [128-134]. It has also been demonstrated that ubiquinone is as effective as vitamin E in preventing lipid peroxidation and even more effective in the protection of LDL from oxidative damage [129, 134, 135].

1.6.1.4 Polyphenols

A group of compounds that have been shown to have strong antioxidant capabilities are the polyphenols, including an important subgroup, the flavonoids. Polyphenols can be found in a wide range of plants and agricultural products with

particularly high concentrations in tea, grapes, wine, nuts and whole grain cereals. Several studies on tea polyphenols have shown their beneficial effects against oxidative stress. They are able to enhance the resistance towards oxidative stress in red blood cells [136], and are capable scavengers of superoxide and hydroxyl radicals [137]. In addition, they have been shown to inhibit the growth and induce apoptosis of several human cancer cell lines [138] as well as increase the average life span of male and female houseflies and *Drosophila melanogaster* [139].

1.6.2 Enzymatic antioxidant systems

Apart from the low molecular weight antioxidants, cells have developed a series of enzymes to scavenge ROS and repair oxidative stress related damage on cellular biomolecules (Fig. 4).

1.6.2.1 Superoxide Dismutase (SOD)

To neutralize formed $O_2^{\bullet-}$, cells utilize a class of enzymes termed superoxide dismutases (SOD), which convert $O_2^{\bullet-}$ into H_2O_2 and O_2 . Three types of SOD have been identified in mammals, defined not only by their sequence but also their prosthetic group and cellular localization. SOD1 is localized in the cytoplasm and uses copper and zinc to maintain its catalytic activity and is therefore also known as Cu/Zn-SOD. SOD2 is also called Mn-SOD due to its manganese prosthetic group. It resides in mitochondria and in contrast to the constitutive expression of Cu/Zn-SOD, Mn-SOD can be induced by oxidative stress. A Cu/Zn-SOD is also secreted from the cells to the extracellular matrix (EC-SOD) [140] and is characterized by a heparin binding domain that can be cleaved in the cells before the release of the protein and can affect its extracellular distribution [141]. Mn-SOD appears to be more important for cellular function than Cu/Zn-SOD; knock-out mice lacking the protein suffer dilated cardiomyopathy, hepatic lipid accumulation and early neonatal death and using Mn-SOD mimetic drugs can prolong their life span [142]. Cu/Zn-SOD defective mice, however, appear to be developing normally but show enhanced susceptibility to central nervous system injury [143]. Furthermore, it has been shown that mutations in the SOD1 gene account for 20% of the neurodegenerative disease familial amyotrophic

lateral sclerosis [144]. Other neurodegenerative disorders where SOD is thought to play a role are Parkinson's and Alzheimer's disease [145].

1.6.2.2 Catalase

Catalase is an enzyme that is predominantly localized in the peroxisomes. It exists as a tetramer of four identical subunits all of them containing a prosthetic heme group and catalyses the decomposition of H_2O_2 into H_2O and O_2 . Thereby, the risk of H_2O_2 forming the highly reactive $\cdot OH$ through the Fenton reaction is minimized. In addition, each subunit also binds an NADPH which serves to protect the enzyme from oxidative damage [146].

1.6.2.3 Glutathione Peroxidase (GPx)

Another weapon in the antioxidant arsenal of the cell, employed to counteract the threat of H_2O_2 are the selenoenzymes glutathione peroxidases (GPx). They are able to reduce H_2O_2 and other peroxides using glutathione as the source of electrons needed for the reaction. There are at least four different GPxs described (GPx1-4). GPx1 and 4 are both cytosolic and mitochondrial proteins and are found in most tissues. GPx2 is mainly expressed in the gastrointestinal tract and GPx3 in the kidney [147-150]. Knockout mice lacking the gene for GPx1 are more sensitive to oxidative stress when challenged with paraquat or diquat and show a higher degree of lethality. This suggests that GPx1 is essential in the protection against acute oxidative stress [151]. GPx4 is also called phospholipid hydroperoxide GPx (PHGPx) and can reduce H_2O_2 , similarly to GPx1. However, unlike GPx1, it is also capable of reducing more lipophilic substrates such as phospholipid hydroperoxides [152]. In addition, GPx4 has been shown to be important in the prevention of hydroperoxide formation [150]. Cells overexpressing the mitochondrial but not the cytosolic GPx4 variant are resistant to apoptosis induced by 2-deoxyglucose [153]. Furthermore, GPx4 has been found to have multiple functions during sperm maturation. In the early spermatids GPx4 exists as monomeric and active enzyme, but in mature spermatozoa it changes its physical properties and exists as an inactive and insoluble protein that is oxidatively cross linked and composes more than 50% of the capsule that embeds the mitochondria. [154]. A third function that has been assigned to a GPx4 variant is specific to the

nucleus, where GPx4 is necessary for the protamine thiol cross-linking during sperm maturation [155]

1.6.2.4 Peroxiredoxins (Prx)

Another group of enzymes that also exert their antioxidant properties through their peroxidase activity are the peroxiredoxins (Prx). They have been found to reduce and detoxify H₂O₂, peroxynitrate and a wide range of organic hydroperoxides [156-161]. Although Prxs are predominantly found in the cytosol, they are also localized in mitochondria, peroxisomes and nucleus and are associated with membranes. They are abundantly expressed, constituting 0.1-0.8% of the soluble protein in mammalian cells [156, 162, 163]. Prxs can be divided into two categories, the 1-Cys and 2-Cys Prxs, depending on the number of cysteines involved in their catalytic active site [164]. After oxidation of their active site, 2-Cys Prxs rely on thioredoxin to provide electrons for the reduction and reactivation of their active site cysteines [165]. The regeneration of 1-Cys Prxs is still unclear but glutathione, lipoic acid and cyclophilin have all been proposed to reduce them [160, 166, 167]. Prxs can save thyroid cells from oxidative stress-induced apoptosis by H₂O₂ [161] and can protect Molt-4 leukemia cells from various insults that can initiate apoptosis by a mechanism that is thought to work upstream of Bcl-2 [168]. Furthermore, they can prevent the p53 induction of ROS and thereby inhibit the subsequent apoptotic response [169] and several Prxs have been found to be overexpressed in malignant mesothelioma [170].

1.6.2.5 Glutaredoxins (Grx)

Glutaredoxins are a group of low molecular weight (9-12kDa) redox active enzymes and are characterized by their dithiol active site, Cys-Pro-Tyr-Cys. Glutaredoxins rely on the reducing capacity of glutathione to reactivate the dithiol catalytic site after oxidation. There are two glutaredoxins identified in mammalian cells, Grx1 and Grx2. Grx1 is predominantly cytosolic while two isoforms of Grx2 exist with separate cellular localizations. Alternative splicing at the 5' end of the Grx2 mRNA directs whether a mitochondrial targeting signal will be inserted or not. In the absence of a mitochondrial signal a nuclear localization signal targets the protein to the nucleus

instead of the mitochondria [171]. Furthermore, the active site in Grx2 differs from Grx1 in that the proline is replaced by a serine.

Grx has been found to reduce oxidized vitamin C, one of the major antioxidant vitamins [172]. Furthermore, Grx has been shown to regenerate proteins inactivated by S-glutathiolation such as PTP-1B and PKC-alpha by reducing the mixed disulfides formed between glutathione and cys on PTP and PKC. In this way it is able to regulate cellular signal transduction pathways [117] [173]. The ability of Grx to deglutathionylate proteins is essential since treatment of cells with cadmium leads to the inactivation of Grx, resulting in an increased content of S-glutathiolated proteins in the presence of H₂O₂ and the initiation of the apoptotic response [174]. Grx has also been implicated in the incidence of cancer. Grx was induced in pancreatic ductal carcinoma compared to normal tissue [175] and was associated with resistance to the anti cancer drugs such as cis-diamminedichloroplatinum and Adriamycin as cells resistant to those drugs also show elevated Grx levels [175, 176].

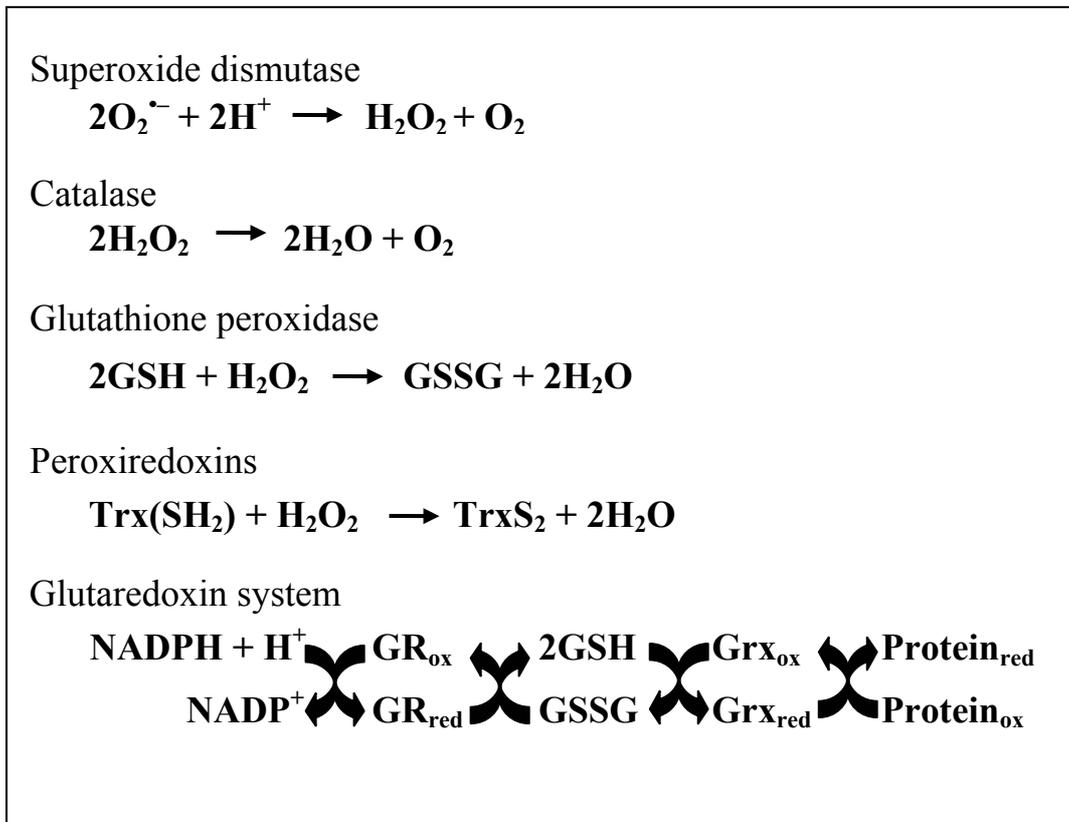


Figure 4. Enzymatic antioxidants. A list of enzyme based antioxidant defence mechanisms that can be found in cells. Shown are the reactions they catalyze. GR; Glutathione reductases, GSH; Glutathione, Grx; Glutaredoxin, Trx; Thioredoxin

1.7 Thioredoxin superfamily

Thioredoxins (Trx) are a class of small multifunctional 12-kDa proteins that are characterized by the redox active site Trp-Cys-Gly-Pro-Cys (WCGPC). In the oxidized (inactive) form of Trx (Trx-S₂), the two cysteines at the active site form a disulfide bond. This can then be reduced by thioredoxin reductase (TrxR) and NADPH, the so-called thioredoxin system, to a dithiol (Trx-(SH)₂), which can then act as a general protein disulfide reductase [177, 178] (Figure 5).

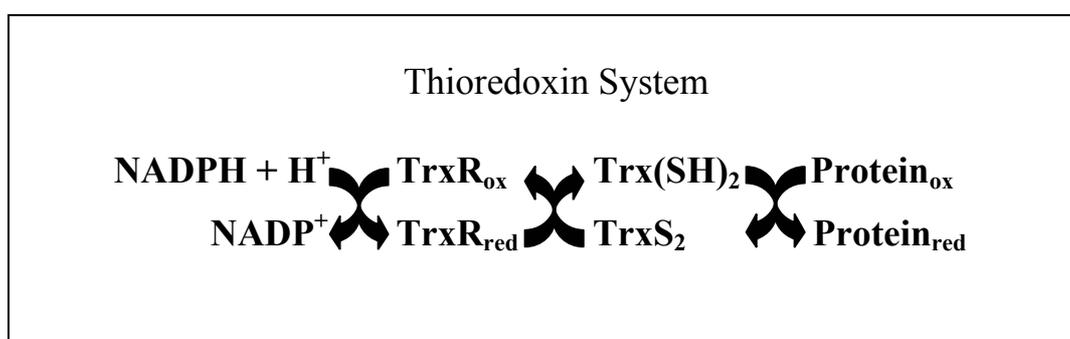


Figure 5. The thioredoxin system. Trx uses the reducing equivalents provided by NADPH and TrxR to reduce oxidized proteins.

Thioredoxin is present in all living organisms and has been isolated and characterized from a wide variety of prokaryotic and eukaryotic cells [179]. The thioredoxin family is an expanding group of proteins with several novel members being cloned and characterized in recent years. Those new members range from proteins being true thioredoxins to thioredoxin-like proteins, namely proteins which have a non-conserved active site or where the thioredoxin part is just one of many domains. Here follows a short introduction to some of the members of the Trx superfamily most relevant for this thesis (Figure 6).

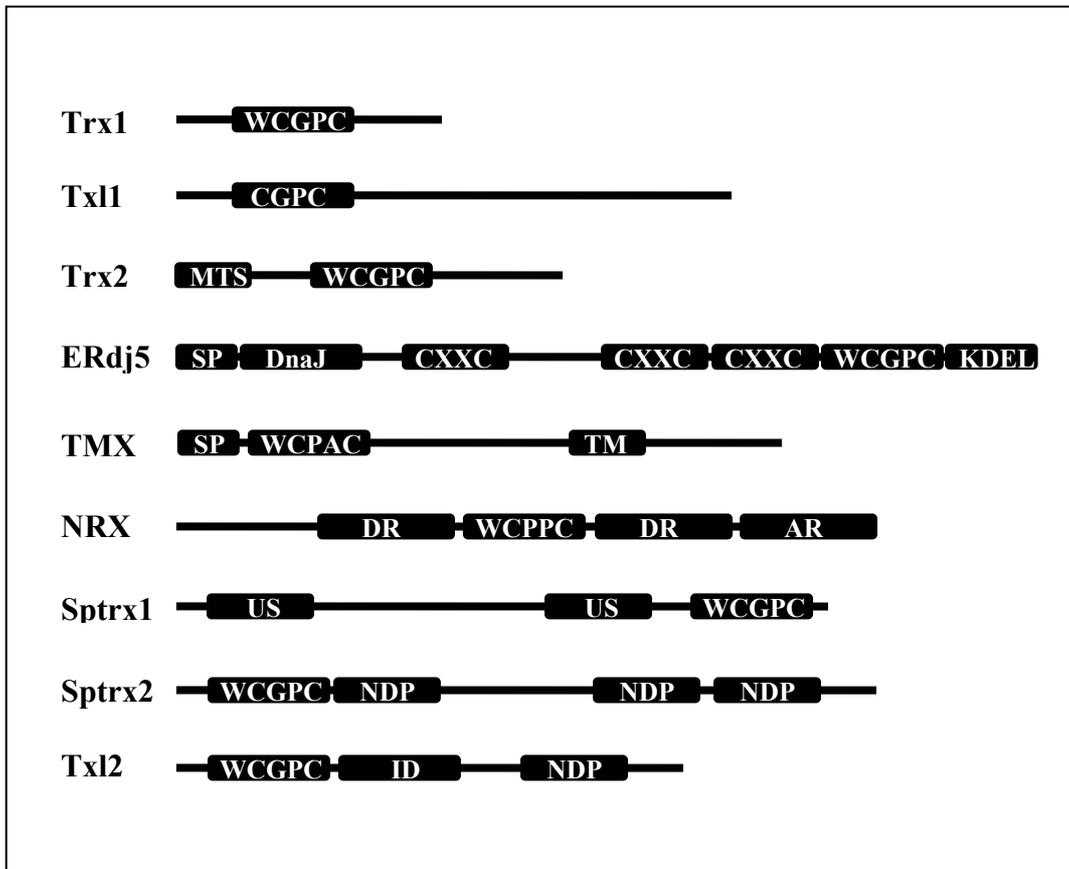


Figure 6. Thioredoxin Superfamily. MTS; mitochondrial translocation signal, SP; signal peptide, DnaJ; DnaJ domain, KDEL; endoplasmatic reticulum retention signal, TM; transmembrane domain, DR; direct repeat, AR; acidic region, US; ubiquitination site, NDP; nucleoside-diphosphate kinase domain; ID, interface domain.

1.7.1 *Escherichia coli* Trx1 and Trx2

The *E. coli* Trx1 was first purified in 1964 and characterized as the electron donor for ribonucleotide reductase (RNR), the enzyme responsible for the reduction of ribonucleotides to deoxyribonucleotides, the building blocks for DNA synthesis [180]. Its amino acid sequence including its active site (WCGPC) was reported in 1968 [181]. Another target of the reducing capacity of Trx1 is 3'-phosphoadenosine 5'-phosphosulfate reductase (PAPS), an enzyme important for the reduction of sulfate to sulfite [182]. Furthermore, Trx1 can reduce methionine sulfoxide reductase which is responsible for the reduction of methionine sulfoxide to methionine [183]. Thioredoxin can act as a general protein reductase [184]. Other functions of Trx1

include its participation in the life cycle of several bacteriophages. Trx1 constitutes a part of the T7 DNA polymerase complex, where in a reduced state it enhances the activity of the polymerase 100-fold. Furthermore, Trx1 is essential for the assembly of the filamentous phages [185-187]

Although Trx1 is a reducing protein, it has been suggested that in its oxidized state it can act to oxidize other proteins instead. When expressed with the alkaline phosphatase target signal, Trx1 translocates to the periplasm of *E. coli*. There, due to the absence of TrxR, Trx1 will exist in an oxidized form and has been found to promote the formation of disulfide bonds. This function of Trx1 can partially circumvent the phenotype of a *dsbA*⁻ strain defective for disulfide bond formation. Therefore, the function of Trx1 can depend on the redox conditions of its environment [188].

Thioredoxin-negative mutants (*trxA*⁻) of *E. coli* are viable [186], and analysis of these mutants led to the identification of a novel cofactor, glutaredoxin-1 (Grx1), as an efficient substitute of thioredoxin for RNR and PAPS reductase enzymatic activity [189, 190]. However, Grx1 could neither substitute for thioredoxin in methionine sulfoxide reduction nor in bacteriophage growth or assembly, which thus remained typical phenotypes of *E. coli* thioredoxin mutants. The isolation of an *E. coli* double mutant in thioredoxin/glutaredoxin-1 allowed the identification of two novel glutaredoxins, Grx2 and Grx3, but only Grx3 is able to serve as a hydrogen donor for RNR [191, 192]. However, a triple Trx, Grx1, and Grx3 mutant was viable [184], indicating the presence of an alternative protein capable of reducing RNR *in vivo*. Furthermore, *trxB* null strains lacking TrxR showed high levels of disulfide formation in the *E. coli* cytoplasm as measured by the alkaline phosphatase (AP) method. However, this excessive disulfide bond formation was not simply due to the absence of the reduced form of Trx1, since mutants lacking thioredoxin alone had little effect on AP activity. In addition, since the double mutant *trxB trxA* exhibited the same activity as the *trxB* mutant alone, the properties of the *trxB* mutant could not be explained by its allowing the accumulation of oxidized thioredoxin [184, 193]. Taking into account that the *E. coli* TrxR is highly specific for the Trx, these results raised the possibility of the presence of a second Trx.

Indeed, as presented in this thesis a second Trx (Trx2) is present in *E. coli*. Trx2 is homologous to Trx1 and contains the conserved active site WCGPC found in all

thioredoxins. However, Trx2 also contains a distinct N-terminal domain of 32 amino acids that includes two additional CXXC motifs. Trx2 was found to be an active redox protein, capable of reducing disulfides using the insulin reduction assay and being an efficient electron donor of RNR. Although, its biochemical properties were similar to those of Trx1, Trx2 was more sensitive to oxidation as it needed preincubation with DTT to be as active as Trx1. This suggested that cysteines, other than those in the active site, were involved in the regulation of Trx2 activity. Indeed, Trx2 protein lacking the N-terminal domain with the extra CXXC motifs was as active as Trx1. The N-terminal domain of Trx2 also conferred heat sensitivity to Trx2 since the truncated protein was as thermostable as Trx1 [194] (Paper I).

As previously mentioned, Trx1 in the presence of an oxidizing environment can take on the role of an oxidant instead of its normal role as a reducer. Similarly, it has been shown that Trx2 can also act as an oxidant as long as there is no source of electrons that can reduce Trx2. The presence of either Trx is required in TrxR defective *E. coli* for disulfides to form in the cytoplasm. In triple mutant *E. coli* lacking TrxR as well as both Trxs, no disulfide formation occurs in the cytoplasm, but the addition of a plasmid expressing Trx2 results in the formation of cytoplasmic disulfides. However, this is not the normal role of Trx and is unlikely to occur in wild type *E. coli*. Furthermore, Trx2 is capable of performing many of the *in vivo* tasks of Trx1. Triple mutant *E. coli* lacking Trx1, Trx2 and glutaredoxin 1 are non-viable and can be rescued only in the presence of a plasmid expressing any of the above proteins e.g. Trx2, suggesting that Trx2 is indeed capable of substituting for Trx1 [195].

Although, Trx2 can replace Trx1 and reduce proteins such as PAPS, RNR and DsbC via DsbD, there are two instances where there is no overlap in the functions of Trx1 and Trx2: a) Trx1 is more efficient in reducing methionine sulfoxide reductase and b) Trx2 is incapable of restoring the T7 phage infection cycle [196, 197]. Despite all the similarities in their enzyme activity, the two thioredoxins seem to be differentially regulated on the transcriptional level. Trx2 is regulated by the transcriptional activator OxyR during oxidative stress. Oxidized OxyR can directly bind to the promoter of *trxC* (the gene coding for Trx2) in response to H₂O₂ exposure or disruption of one of the other cytoplasmic redox pathways present in *E. coli* [196]. Interestingly, mutants lacking both thioredoxins were found to be more resistant to H₂O₂ exposure. This is thought to occur due to the lack of thioredoxins leading to a more oxidized environment and hence more oxidized OxyR (active) which will then be

capable of inducing the expression of several proteins participating in the defense against oxidative stress [196].

1.7.2 Mammalian Trx1

The mammalian Trx1 was first purified in 1972 from rat liver [198]. Since then it has been purified and cloned from several species including rat, mouse, chicken, bovine and human. Trx1 has a molecular mass of 12 kDa and, similarly to the *E. coli* Trx1, contains the conserved redox active (WCGPC) typical for thioredoxins and is able to reduce insulin disulfides as well as RNR, PAPS and methionine sulfoxide reductase. However, a feature that sets the mammalian Trx1 apart from its *E. coli* homologue is the presence of cysteines in its C-terminal domain, apart from the two located in the active site. Those are not involved in the Trx1 catalytic redox reactions but they seem to regulate the activity of the protein in an indirect way. They can form disulfide bonds leading to the aggregation of the protein rendering it a poor substrate for TrxR, which greatly diminishes the reductive activity of Trx1 [199-201]. Another way through which Trx1 has been reported to be regulated is by nitrosylation. Trx1 is a regulator of the intracellular content of S-nitrosylated molecules (S-NO content), but in turn Trx1 becomes itself S-nitrosylated on Cys 69 which is a cysteine essential for the redox activities and antiapoptotic functions of Trx1 [202].

1.7.2.1 Extracellular Trx1

Trx1 was also identified and characterized as adult T cell leukemia-derived factor (ADF). ADF is a factor produced and secreted by the adult T-cell leukemia – 2 (ATL-2) cell line which is infected by human T cell lymphotropic virus-I (HTLV-I). Trx1 was found to cause the abnormal expression of the interleukin 2 receptor (IL-2R α) and exert a growth-promoting activity [203]. Subsequent N-terminal amino acid sequencing of the protein and cloning of the ADF cDNA concluded that ADF is in fact Trx1 [204]. Furthermore, extracellular Trx1 has a function as a chemotactic agent for monocytes, neutrophils, and T lymphocytes. For chemotaxis, Trx1 uses a pathway different from other known chemokines as it is G protein-independent, but requires the presence of its redox active site [205]. A shorter form of Trx1, composed of the 80-84 N-terminal amino acids (Trx80) has been found to be

secreted as well from certain cell types and can promote eosinophilic cytotoxicity and function as a mitogenic cytokine for human peripheral blood mononuclear cells [206-208]. The pathway underlying the excretion of Trx1 is not fully understood. Using brefeldin A and dinitrophenol to block the exocytotic pathway did not prevent the release of Trx1 from the cell, suggesting that the export does not involve the classic Endoplasmatic Reticulum-Golgi pathway [209, 210].

1.7.2.2 Gene regulation

Trx1 is a stress inducible protein. Several known oxidative stress inducing agents and conditions in various model systems have been shown to increase the expression of Trx1, such as H₂O₂, phorbol esters, O₂, hypoxia, viral infection, UV irradiation and X-ray radiation [211-217]. In the promoter region of the Trx1 gene there are two AP-1 consensus sequences and one for NF-κB, however, those appear not to play a major role in the induction of Trx1 during oxidative stress. A novel promoter sequence, that showed no homology with any known consensus sequences for DNA binding factors, was found to be involved in the oxidative stress induced expression of Trx1 [218]. However, even non stress inducing compounds, such as vitamin A, estradiol and prostaglandin E1, can induce the expression of Trx1 [219-223].

1.7.2.3 Cellular localization of Trx1

The exposure to oxidative stress, affects not only the expression of Trx1 but also the intracellular localization of the protein. Trx1 is primarily a cytoplasmic protein in resting cells and does not contain any specific localization signals. Exposure of HeLa cells to UVB irradiation caused the quick translocation of Trx1 from the cytoplasm to the nucleus [224]. Other treatments that promote the nuclear translocation of Trx1 are PMA, H₂O₂ and hypoxia [225-227]. However, the mechanism behind the event of translocation is not clear. Trx1 could bind to proteins that contain a nuclear translocation signal and are transported into the nucleus during oxidative stress thereby “carrying” Trx1 into the nucleus as well.

1.7.2.4 Regulation of signaling pathways and transcription factors by Trx1

As previously discussed in the introduction, ROS are highly involved in the regulation of intracellular signaling pathways. Trx1 as a redox active protein has been shown to be a capable regulator of several transduction pathways and transcription factors. The effects exerted by Trx1 in intracellular signaling can be assigned to its functions as a ROS scavenger or as a protein disulfide reductase. Trx1 has been shown to directly quench singlet oxygen ($^1\text{O}_2$) and scavenge hydroxyl ($\cdot\text{OH}$) radicals. The mammalian Trx1 was far more efficient than the *E. coli* Trx1, suggesting that the non catalytic structural cysteines present in the mammalian protein are of importance. Indeed, mutated Trx1 lacking the three non catalytic cysteines was a poor $^1\text{O}_2$ quencher and $\cdot\text{OH}$ scavenger. Furthermore, although the redox state of Trx1 was not important, the catalytic cysteines played a significant role in ROS scavenging by Trx1 [228]. In addition, Trx1 is also indirectly involved in the elimination of H_2O_2 since it is an electron donor for the peroxiredoxins.

Apart from scavenging ROS, Trx1 can also interact with proteins involved in the signal transduction pathways, repair oxidatively damaged transcription factors and restore their functions. One protein that Trx1 regulates is ASK1, which is a MAPKKK for JNK and p38. Trx1 can bind to ASK1 in a redox dependent manner. In its reduced form Trx1 binds to ASK1 and inhibits its kinase activity and subsequent ASK1-dependent apoptosis. Oxidized Trx1, however, cannot bind to ASK1 and is therefore not able to prevent ASK1-dependent apoptosis [67]. This provides for an elegant model of how Trx1 and the redox environment of the cell can regulate the apoptotic cascade.

NF- κB is also a target for redox regulation. NF- κB is sensitive to thiol-modulating agents and Trx1 has been shown to fully restore the DNA binding activity of oxidized NF- κB . Transfection of Trx1 and a reporter gene for NF- κB , showed that Trx1 could enhance the transactivation function of NF- κB . Cysteine 62 in the p50 subunit of NF- κB has been identified as the target for thiol modification. A mutant p50 where cysteine 62 was substituted for a serine was no longer sensitive for oxidation, but on the other hand the DNA binding activity was reduced and could not be stimulated by Trx1 [229, 230]. However, the activation of NF- κB is a two step process. An oxidized environment in the cytoplasm is advantageous for its activation and the presence of

antioxidants can inhibit the phosphorylation and dissociation of I- κ B from NF- κ B. In the nucleus however, the NF- κ B DNA binding activity is dependent of the reducing power of Trx1 [101, 231-235].

The transcription factor activator protein 1 (AP-1) is also a redox sensitive factor, where the presence of antioxidants and thioredoxin enhance its activity [232, 233]. However, Trx1 is not capable of directly reducing AP-1, but instead uses the redox factor 1 (Ref-1) as an intermediate step to activate AP-1 [226, 236]. Trx1 also uses Ref-1 to enhance the transcriptional activity of the hypoxia-inducible factor 1 α (HIF-1 α). By reducing a cysteine residue of HIF-1 α through Ref-1, Trx1 allows the interaction of HIF-1 α with the p300/CBP and is essential for the hypoxia induced transcription [225]. Another redox sensitive transcription factor is the tumor suppressor p53. Ref-1 has been reported to stimulate oxidized p53 and, in the presence of antioxidants, Ref-1 becomes an extremely powerful activator of p53. Trx1 as a reducer of Ref-1 becomes an important regulator of p53 activation. Trx1 augments the DNA binding activity of p53 and further potentiates Ref-1-enhanced p53 activity. Furthermore, Trx1 enhanced p53-dependent expression of p21 and further intensified Ref-1-mediated p53 activation. In addition, overexpression of a transdominant negative mutant Trx1 suppressed the effects of Trx1 or Ref-1.[237, 238]

Trx1 also regulates the glucocorticoid receptor and the estrogen receptor (ER) which belong to the nuclear receptor superfamily. The glucocorticoid receptor is sensitive to oxidation, which decreases its ability to bind hormone as well as DNA, thereby inhibiting glucocorticoid receptor-dependent gene expression. Trx1 has been shown to restore the hormone binding activity of glucocorticoid receptor. Furthermore, Trx1 can directly interact with the DNA-binding domain (DBD) of glucocorticoid receptor, restoring its DNA-binding activity glucocorticoid receptor -dependent gene expression under oxidative conditions such as exposure to H₂O₂ [227, 239, 240]. The ER transcription activity is downregulated in the presence of H₂O₂. Transfection of a Trx1 expression construct, however, restored ER activity. The importance of Trx1 in the regulation of ER was further confirmed by transfection with an antisense Trx1 plasmid which diminished ER activity. Furthermore, the DNA-binding activity of recombinant ER was compromised by sulfhydryl-modifying reagents but restored by the addition of recombinant Trx1 protein. [241].

1.7.3 Thioredoxin like protein (Txl)

A human cDNA that encodes a new Thioredoxin-like protein (Txl) that belongs to the expanding family of thioredoxins based on sequence comparison of the deduced amino acid sequence, has been reported [242]. The coding sequence predicts a protein of 289 amino acids with two distinct domains: an N-terminal domain of 105 residues homologous to the rest of mammalian thioredoxins containing the conserved active site (CGPC) and a C-terminal domain of 184 residues with no homology with any other protein in the database. Northern blot analysis reveals a 1.3 Kb mRNA that is ubiquitously expressed in human tissues with the highest expression in stomach, testis and bone marrow. Although there is a high similarity of the active site sequence with the conserved Trx active site, the protein was found to be inactive in the insulin reduction assay. To exclude the possibility of the C-terminal domain interfering with the N-terminal Trx domain of the protein, a truncated form of the protein was expressed, including the first 105 amino acids. However, even this form of the protein was redox inactive. A possible explanation for this fact could be the substitution of the tryptophan residue at the active site for a glycine. Indeed, the tryptophan has been shown to stabilize the three dimensional structure of the active site and its substitution in the *E. coli* Trx1 to alanine, leads to the decrease of the activity of the protein to only 4% of the wild type [243, 244].

1.7.4 Organelle specific thioredoxins

1.7.4.1 Mitochondrial Thioredoxin (Trx2)

Trx2 possesses the conserved thioredoxin-active site, WCGPC, and has a dithiol-reducing enzymatic activity which, in conjunction with mammalian TrxR and NADPH, reduces the interchain disulfide bridges of insulin. However, Trx2 differs from Trx1 by the presence of a 60-amino acid extension at the N terminus which has properties characteristic of a mitochondrial translocation signal. Cleavage at a putative mitochondrial peptidase cleavage site would give a mature protein of 12.2 kDa. The mitochondrial localization of Trx2 was confirmed by western blot analysis on cytosolic, peroxisomal, and mitochondrial rat liver cell fractions. A novel feature of Trx2 was the

absence of structural cysteines present in all mammalian thioredoxins. The lack of non-catalytic cysteines rendered Trx2 more resistant to oxidation compared to Trx1 [245].

Trx2 is involved in the death processes of the cell. Stably transfected HEK-293 cells overexpressing Trx2 are more resistant toward the anti-cancer drug etoposide and have a higher mitochondrial membrane potential, $\Delta\Psi_m$ [246]. Furthermore, Trx2 is an essential protein for cell viability as Trx2-deficient cells undergo apoptosis and show an accumulation of intracellular ROS. Upon silencing of the Trx2 gene, cytochrome c is released from mitochondria, and caspases, the executioners of the apoptotic cascade, are activated [247]. Corroborating those results are the studies performed on knock-out mice. Homozygous mutant embryos do not survive to birth and die after implantation at Theiler stage 15/16. These mice display an open anterior neural tube and show massively increased apoptosis at 10.5 days postcoitus and die by 12.5 days postcoitus. The timing of the embryonic lethality coincides with the maturation of the mitochondria, since they begin oxidative phosphorylation during this stage of embryogenesis. In addition, embryonic fibroblasts cultured from homozygous Trx-2-null embryos were not viable [248].

Trx2 mRNA and protein are highly expressed in neurons in several brain regions, including the olfactory bulb, frontal cortex, hippocampus, some hypothalamic and thalamic nuclei, cerebellum and numerous brainstem nuclei. In addition, the Trx2 mRNA expression was elevated in paraventricular hypothalamic nucleus and reticular thalamic nucleus by dexamethasone treatment, but other brain areas were unaffected by the presence of dexamethasone [249].

1.7.4.2 ERdj5

ERdj5 is an endoplasmatic reticulum resident protein. It is comprised of a number of distinct domains; a DnaJ domain, normally acts as a cochaperone for Hsp70; three protein disulfide isomerase like domains, responsible for disulfide bonding within the endoplasmatic reticulum, and a Trx domain. *In vitro* experiments demonstrated that ERdj5 interacts via its DnaJ domain with BiP in an ATP-dependent manner. ERdj5 is a ubiquitous protein and is particularly abundant in secretory cells. Upstream of the ERdj5 ATG an endoplasmatic reticulum stress element (ERSE) is present, suggesting the possibility of the ERdj5 expression being regulated by endoplasmatic reticulum stress. Indeed, exposure of cells to known inducers of the unfolded protein response

(UPR) such as tunicamycin and DTT caused the transcriptional induction of ERdj5, suggesting potential roles for ERdj5 in protein folding and translocation across the endoplasmatic reticulum membrane [250].

1.7.4.3 Transmembrane Thioredoxin (TMX)

Another ER localized Trx protein is TMX. The TMX protein possesses an N-terminal signal peptide followed by a thioredoxin (Trx)-like domain and a potential transmembrane domain. The catalytic active site in the Trx domain of TMX has the unique sequence, WCPAC, which is not the typical, conserved Trx active site sequence. Nevertheless, a recombinant protein containing the Trx domain of TMX was able to reduce the interchain disulfide bridges in insulin *in vitro*. TMX is widely expressed in normal human tissues, and western blot analysis on subcellular fractionations and immunofluorescence staining revealed that TMX is predominantly localized in the ER. TMX has antiapoptotic activity as HEK293 cells with induced expression of the protein were resistant towards brefeldin A (BFA), an inhibitor of endoplasmatic reticulum-Golgi transport and inducer of apoptosis. The antiapoptotic function is thought to be dependent on the catalytic activity of TMX, since mutant TMX where the Cys residues in the active site were replaced by Ser was incapable of protecting the cells against BFA-induced apoptosis. This suggests that the Trx-like activity of TMX may help relieve endoplasmatic reticulum stress caused by BFA [251].

1.7.4.4 Nucleoredoxin (NRX)

Although the putative active site sequence of nucleoredoxin, WCPPC, is not the typical Trx site, a recombinant protein showed oxidoreductase activity towards insulin disulfide bonds with kinetics similar to those of thioredoxin. NRX mRNA is present in all adult tissues examined with particularly high expression in skin and testis; expression seems to be developmentally regulated since it was restricted to the nervous system and the limb buds in Day 10.5-11.5 embryos. The NRX protein is predominantly localized to the nucleus of cells transfected with the NRX expression construct. Its nuclear localization opens up the possibility of the protein to be a regulator for redox sensitive transcription factors [252]. Indeed, NRX was demonstrated to be a potent inducer of NF- κ B activity during TNF α treatment. PMA-induced AP-1 activation was

also enhanced by the presence of NRX and similarly activation of the transcription factor CREB (cAMP response element binding protein) by forskolin was also induced by NRX. In the case of CREB, NRX was more a efficient inducer than both Trx and Grx. NRX could also enhance the AP-1 and CREB activity in the absence of their activators PMA and forskolin, similarly to Trx and Grx [231].

1.7.5 Tissue specific thioredoxins

1.7.5.1 Sperm specific Trx1 (Sptrx1)

Thioredoxins are proteins with ubiquitous expression found in most tissues. Sptrx1 was the first Trx family member to show specific tissue distribution. Northern analysis and *in situ* hybridization showed that Sptrx1 mRNA is only expressed in human testis. Immunostaining of human testis sections identified Sptrx protein in spermatids, while in ejaculated sperm Sptrx1 localized in the cytoplasmic droplet. Sptrx1 is composed of two clear domains: an N-terminal domain consisting of 23 highly conserved repetitions of a 15-residue motif, and a C-terminal domain typical of a thioredoxin conserved active site. Sptrx appears to have a multimeric structure in native conditions and is able to reduce insulin disulfide bonds in the presence of NADPH and thioredoxin reductase but in contrast to Trx1, Sptrx1 was more effective when oxidized [253]. The two domain organization was supported by analysis of circular dichroism spectra of fragments 1-360 and 361-469 and comparison to spectra of full-length Sptrx-1 which showed a largely unstructured N-terminal domain and a folded thioredoxin-like C-terminal domain. *In vitro*, Sptrx-1 behaves as an oxidant when using selenite, but not oxidized glutathione, as electron acceptor. This oxidizing enzymatic activity suggests that Sptrx-1 might be important in the development of the different structures in the developing tail of spermatids and spermatozoa by stabilizing structures through disulfide cross-linking. [254]. In support of that theory, it has been reported that Sptrx1 is transiently expressed during step 9-18 of spermatogenesis. It specifically associates to the fibrous sheath during development but does not become a permanent structural component [255].

1.7.5.2 Sperm specific Trx2 (*Sptrx2*)

Another Trx-like protein specifically expressed in sperm is *Sptrx2*. Similarly to *Sptrx1*, *Sptrx2* appears to consist of two domains as well. However, in *Sptrx2* the Trx domain is located at the N-terminus while at the C-terminus there are three putative nucleoside diphosphate (NDP) kinase domains. Although, the active site present in the Trx-domain is the conserved sequence found in thioredoxins, *Sptrx2* could not function as a reducer of insulin disulfides using calf thymus TrxR1 and NADPH. However, when using DTT as a reducer, a truncated form of *Sptrx2*, expressing only the Trx-domain, was found positive for disulfide reduction. *Sptrx2* was also negative for NDP kinase activity. *Sptrx2* mRNA is exclusively expressed in human testis, mainly in primary spermatocytes, while *Sptrx2* protein expression is detected from the pachytene spermatocytes stage onwards, peaking at round spermatids stage [256].

1.7.5.3 Thioredoxin-like protein 2 (*Txl2*)

Txl2 is expressed in several tissues as determined with real time PCR, with testis and lung showing by far the highest expression. The *Txl-2* open reading frame codes for a protein of 330 amino acids consisting of two distinct domains: an N-terminal domain typical of thioredoxins containing the conserved active site and a C-terminal domain belonging to the NDP kinase family, separated by a small interface domain. However, the protein has neither disulfide reducing activity nor NDP kinase activity. Light and electron microscopy analyses show that the protein is associated with microtubular structures such as lung airway epithelium cilia and the manchette and axoneme of spermatids. Using *in vitro* translated proteins, we demonstrate that full-length *Txl-2* weakly associates with microtubules. In contrast, a splicing variant, lacking exon 5 ($\Delta 5$ *Txl2*) which is also normally expressed in tissues, specifically binds with very high affinity brain microtubule preparations containing microtubule-binding proteins. Importantly, $\Delta 5$ *Txl2* also binds to pure microtubules, proving that it possesses intrinsic microtubule binding capability [257].

1.7.6 Thioredoxin Reductases

Thioredoxin reductase (TrxR) is a selenocysteine (SeCys) containing enzyme which catalyses the reduction of thioredoxin (Trx) in the presence of NADPH [179, 258]. The presence of SeCys is essential for the activity of the protein as mutants where the selenocysteine was replaced for Cys had only 1% of the catalytic activity of the wild type for the reduction of Trx [259]. Except for the presence of SeCys, TrxRs have other typical features such as the presence of NADPH and FAD-binding domains and an active site (CVNVGC) also conserved in glutathione reductase [260, 261]. TrxR1 exists as a dimer of two identical subunits, with one FAD bound per subunit. Using NADPH, the enzyme-bound FAD becomes reduced and subsequently the electrons are transferred to the CVNVGC active site forming a dithiol. Then one subunit of the homodimer reduces the SeCys-Cys pair located at the C-terminus of the other subunit [199, 262, 263]. In mammalian cells three TrxRs have been characterized, a cytoplasmic one (TrxR1), a mitochondria specific TrxR (TrxR2) and, more recently, a testis specific one that can function both as a thioredoxin reductase and a glutathione reductase (TGR) [261, 264].

1.7.6.1 Cytosolic Thioredoxin Reductase (TrxR1)

Mammalian TrxR1 is a very promiscuous protein, as opposed to the *E. coli* enzyme which can only reduce Trx. Mammalian TrxR can, in addition to Trx, reduce proteins such as protein disulfide-isomerase and plasma glutathione peroxidase [262]. Furthermore, it has a wide range of low molecular weight targets such as dehydroascorbic acid, lipoic acid, selenite and selenocysteine. It can cleave S-nitrosoglutathione (GSNO) to glutathione and nitric oxide and can scavenge peroxynitrite [262, 265]. Recently, the reduction of ubiquinone by TrxR1 has been reported, suggesting a novel role of TrxR1 in the antioxidant defenses against oxidative damage towards cell membranes [266].

Similar to Trx, TrxR1 has also been implicated in the regulation of transcription factors such as p53, hypoxia inducible factor (HIF) and AP-1. Prostaglandins have been shown to covalently modify and inhibit the function of TrxR1 leading to the repression of p53 and HIF [267]. Furthermore, TrxR1 is involved in

promoting the cell death effect of combined interferon and retinoic acid, caused by the TrxR1 dependent activation of p53-mediated gene expression [268-271]. In addition, p53 dependent gene expression was inhibited in yeast lacking TrxR due to the oxidation and inactivation of Trx, suggesting that the effect of TrxR1 on p53 activity is mediated by the reduction of Trx[272, 273]. Recently, TrxR1 has also been shown to regulate the activation of AP-1 although this is also thought to occur through its function of reducing Trx1 [274]. These examples indicate that TrxR1, alone and/or through Trx, may impact various transcriptional pathways.

Several mRNA splicing variants exist for human TrxR1 [275]. However, their translation results in only two different proteins. Furthermore, a band of approximately 67 kDa copurifies with TrxR1a from human JPX9 cells, although at significantly lower amounts, suggest that a heterogeneity within animal thioredoxin reductases exist [276]. In this thesis we present evidence for a form of TrxR1 that is a product of alternative splicing (TrxR1b), and can directly interact with estrogen receptors, a process which causes the intranuclear localization of TrxR1b. Furthermore, TrxR1b can regulate the transactivation function of ER at AP-1 sites (Paper VI).

1.7.6.2 Mitochondrial Thioredoxin Reductase (TrxR2)

The presence of a mitochondrial Trx suggested the presence of a TrxR in mitochondria as well. Indeed, a TrxR from rat liver mitochondria was purified, and found to have similar biochemical properties with TrxR1 being able to reduce Trx as well as chemically unrelated compounds such as 5,5'-dithiobis (2-nitrobenzoic acid), selenite, and alloxan. Furthermore, TrxR2 activity was suppressed by classical inhibitors of TrxR1 such as 1-chloro-2,4-dinitrobenzene and 13-cis-retinoic acid.[277]. cDNA cloning and sequencing revealed that TrxR2 is highly homologous to TrxR1, including the NADPH and FAD binding domains as well as the conserved active site CVNVGC. A mitochondrial targeting sequence is located at the N-terminus (Paper IV) [261].

Cells overexpressing the protein were more resistant towards the cytotoxic effects of the chemical inhibition of the mitochondrial complex III compared to control cells. Furthermore, TrxR2 can reduce cytochrome *c in vitro* using both NADPH and NADH as the source of electrons (Paper V). TrxR2 has also been proposed to be involved in the regulation of cell proliferation. The overexpression of a dominant

negative mutant of TrxR2 caused increased H₂O₂ production as well as increased tyrosine phosphorylation in response to epidermal growth factor treatment. In addition, induction of mutant TrxR2 also resulted in the increased rate of progression of G1 to S phase in cell cycle and cell proliferation and affected the expression of many proteins involved in the cell cycle. [278]. It has also been reported that TrxR2 is overexpressed in hepatocellular carcinomas. In 67.2% cases of the samples studied, the levels of TrxR2 expression were higher in tumor tissues than in corresponding adjacent normal tissues [279].

1.7.6.3 Thioredoxin and Glutathione Reductase (TGR)

A pyridine nucleotide disulfide oxidoreductase specifically expressed in testis was cloned and characterized. This novel enzyme contains a selenocysteine residue as well as NADPH and FAD binding domains and the conserved active site common for TrxRs and glutathione reductase. However, it features an unusual N-terminal domain not present in other pyridine nucleotide disulfide oxidoreductases. This domain exhibits strong homology to various glutaredoxins and has a characteristic GSH-binding motif. This unusual fusion of TrxR and glutaredoxin domains renders the enzyme capable of acting both as a thioredoxin and glutaredoxin reductase (TGR) in *in vitro* assays. A mechanism has been suggested whereby the C-terminal selenotetrapeptide plays the role of a protein-linked GSSG and shuttles electrons from the disulfide centre within the TrxR domain to either the glutaredoxin domain or Trx [264, 280].

2. RESULTS AND DISCUSSION

The results upon which this thesis is based are fully presented and discussed in papers I-VI. The following section contains a short presentation of the results from each paper and a general discussion.

2.1 Paper I

In the first paper we describe the cloning of a novel Trx (Trx2) in *Escherichia coli* and characterize the enzymatic activity of the protein. The *E. coli* Trx2 contains two domains: an N-terminal domain of 32 amino acids containing two CXXC motifs and a C-terminal domain with high homology to the prokaryotic thioredoxins, which also contains the conserved active site, WCGPC. The protein was found to be expressed in normally growing *E. coli*. Trx2 together with TrxR and NADPH can reduce ribonucleotide reductase as well as the interchain disulfide bridges of insulin with K_m values similar to those of the previously described *E. coli* thioredoxin (Trx1). The N-terminal domain of Trx2 is important in the regulation of the enzymatic activity of the protein. Preincubation with the reducing agent DTT or cleavage of the 32 amino acid N-terminal leads to a higher activity of the protein, suggesting that the oxidation of the cysteines at the N-terminal impairs the activity of the protein. Furthermore, the N-terminal domain confers heat sensitivity to the protein, in contrast to Trx1.

2.2 Paper II

In the second paper we describe the cloning of a sperm specific Trx (Sptrx). Thioredoxins are ubiquitously expressed in all of the tissues within the same organism. This thioredoxin unlike other thioredoxins was shown to be exclusively expressed in the testis and more specifically in round and elongating spermatids. The protein consists of 486 amino acids and is divided in two distinct domains. An N-terminal domain composed of 23 highly conserved repetitions of a 15-residue motif, and a C-terminal domain homologous to the cytosolic and mitochondrial thioredoxins and

containing the conserved Trx active site. In the presence of TrxR and NADPH, Sptrx was found to have thioredoxin activity, reducing insulin disulfides. Furthermore, under native conditions, Sptrx appears to have a multimeric structure.

2.3 Paper III

Here we report the cloning and the involvement of the human mitochondrial Trx (Trx2) in cell death and the mitochondrial membrane potential. Trx2 contains a tentative N-terminal mitochondrial signal and using green fluorescent protein (GFP) fusions of the Trx2 we have confirmed its mitochondrial distribution. Trx1 contains, apart from the active site cysteines, two more cysteines, which upon oxidation result in an impaired enzymatic activity of Trx1. Trx2 lacks those two extra cysteines which renders the protein more resistant towards oxidative inactivation which occurs to Trx1. Stably transfected cell lines overexpressing Trx2 were constructed to elucidate the biological functions of the protein. Trx2 was found to protect cells against the cytotoxic effects of etoposide, a drug commonly used in anticancer treatment. Furthermore, Trx2 overexpressing cells have a higher mitochondrial membrane potential which seems to be dependent on the function of the ATP synthase complex, since inhibition with oligomycin lead to a decrease of the potential. This suggests that Trx2 could be involved in the function and regulation of the ATP synthase.

2.4 Paper IV

In this paper we report the cloning of the mitochondrial thioredoxin reductase (TrxR2). Similarly to the previously described cytosolic thioredoxin reductases (TrxR1), TrxR2 contains the conserved active site CVNVGC, as well as the FAD and NADPH-binding domains. However, at the N-terminus 33 extra amino acids are present, with the characteristics of a mitochondrial translocation signal and indeed, when fused to GFP TrxR2 clearly showed the distinct mitochondrial localization pattern. At the 3'UTR a putative selenocysteine insertion sequence (SECIS) is present. The SECIS element forms a stem-loop which dictates the insertion of the amino acid selenocysteine at the UGA codon instead of the termination of the protein. This suggests that similarly to TrxR1, TrxR2 is a selenocysteine-containing protein as well.

2.5 Paper V

To study the biological significance of TrxR2 we established cell lines overexpressing the protein by stable transfection. Mitochondrial extracts from overexpressing cells were assayed for TrxR activity and showed a 7-fold increase in activity compared to control cells. In addition, the TrxR activity was increased when sodium selenite was added to the media suggesting that the TrxR2 is indeed a seleno-containing protein. This was further confirmed by the incorporation of the radiolabeled selenium homologue, ^{75}Se , which also was clearly much higher in the TrxR2-overexpressing cells. TrxR2 was shown to protect cells against the cytotoxic effects of antimycin and myxothiazol, chemicals that inhibit the function of complex III in the MRC. The presence of TrxR2 was proved crucial for the protection against complex III inhibition by the specific inhibition of TrxRs using 1-chloro-2,4-dinitrobenzene (CDNB). In the presence of CDNB, the TrxR2 stable cells no longer showed any significant resistance towards antimycin and myxothiazol compared to the control cells. In addition, TrxR2 is capable of reducing cytochrome c which could explain the cytoprotective effects shown by TrxR2.

2.6 Paper VI

TrxR1 consists of 499 amino acids. In this paper we characterize a novel isoform of TrxR1 that consists of 551 amino acids which we refer to as TrxR1b and is the result of an alternative splicing of the TrxR1 mRNA. TrxR1b differs from the previously described TrxR (TrxR1a) by the presence of an extra 52 amino acid extension at the N-terminus. Antibodies raised against a peptide present within the 52 amino acid extension were used in western blots and confirmed the expression of the protein in normal human tissues as well as in several cell lines. The most distinct feature of the extra amino sequence in TrxR1b is the presence of a nuclear receptor box at amino acids 47-51 with the consensus sequence LXXLL, which is responsible for the binding of various cofactors to nuclear receptors. Subsequent pull down experiments revealed that TrxR1b could bind to the estrogen receptors (ER) α and β . As a result of this binding, a distinct subnuclear localization of TrxR1b was observed, co localizing

with both ER α and β . Furthermore, TrxR1b could act as a coactivator and enhance the transcriptional activity of ER in the classical activation pathway, which relies on the binding of the ER to an ER response element on the DNA. By contrast, TrxR1b was a co-repressor in the alternative pathway where ER activates AP1 transcription independently of its DNA binding activity.

2.7 General Discussion

Redox reactions have evolved to control essential functions and processes in living organisms. The production of energy in mitochondria relies on a series of redox reactions. Folding as well as the biological activity of many proteins is also under the control of redox regulation. While some proteins are activated by oxidation, either directly such as the OxyR in *E. coli* or indirectly, like the ASK1 protein that is activated by the oxidation of Trx1, other proteins like the transcription factors ER, glucocorticoid receptor and p53 are impaired by oxidation.

In biological systems, ROS are the main source of oxidative damage to cellular biomolecules. Although essential for the metabolic processes in aerobic organisms, oxygen also constitutes a constant threat. The incomplete reduction of oxygen leads to the formation of highly reactive oxygen intermediates that can target proteins, lipids and DNA, leading to wide range of cellular responses and damages. To overcome the threat imposed by oxygen and to repair oxidative damages, several antioxidant defense mechanisms have evolved. One of the enzymatic systems employed for such tasks is the thioredoxin system.

In this thesis we present the identification and biochemical characterization of a second Trx in *E. coli*, Trx2. The presence of a second Trx in *E. coli* has been suggested previously based on work on mutant strains lacking Trx and TrxR. The *E. coli* Trx2 could reduce insulin disulfides and was also a capable electron donor for RNR. However, *E. coli* Trx2 contains an extra N-terminal amino acid stretch, with two CXXC domains. This domain is important in the regulation of the redox activity of the protein. It confers sensitivity to oxidation making the reducing power of this protein less effective when oxidized and furthermore it renders the protein sensitive to heat, abolishing the extreme heat stability demonstrated by *E. coli* Trx1. An interesting observation is that the position of the four cysteine residues at the N-terminus of Trx2

resembles the structure of a zinc finger. Zinc finger motifs have been described in various DNA-binding proteins, and the importance of the cysteine residues is supported by the observation that modification of these residues inhibited enzymatic activity [281]. We are currently exploring the possibility that *E. coli* Trx2 might contain a zinc finger.

Another interesting member of the family that we identified is Sptrx1, the first Trx like protein to be shown to have a specific tissue distribution. While other thioredoxins are ubiquitously expressed Sptrx1 is exclusively found in human spermatozoa. The cloning of a Trx in human spermatozoa opens up a new dimension within the thioredoxin field. Its expression during sperm tail formation strongly indicates an important role in this process and therefore a potential target for male factor infertility studies. There are numerous reports describing sperm flagellum pathologies for which the molecular basis is unknown where Sptrx1 could have a crucial role.

Furthermore, a complete Trx system located in mitochondria is described (Trx2, TrxR2). Mitochondria are the energy plant of the cell and the main source of ROS. They also contain proteins that are released and are highly active during the apoptotic response of the cell casting the mitochondria with a protagonist role in the cell death processes [282, 283]. Having a Trx system exclusively located in mitochondria, indicates a possible major role for this system in the death cascade of the cell. Indeed, Trx2 was found to be involved in the regulation of the $\Delta\Psi_m$ and could protect cells against the cytotoxic effects of the anticancer drug etoposide. The cytoprotective function of Trx2 against etoposide coupled with preliminary results that Trx2 can be overexpressed in tumors (*e.g.* astrocytoma, glioblastoma, oligodendroglioma, ependymoma, and meningioma cells) compared to almost no expression in glial cells in normal brain, poses an interesting function of Trx2 in cancer treatment. Trx2 could be used as a marker when characterizing tumors and help in the determination of an appropriate treatment.

TrxR2 was found to have a cytoprotective role against chemical inhibitors that impair the function complex III. Furthermore, TrxR2 could reduce cytochrome c proposing a mechanism where TrxR2 could be used to bypass complex III. Although, this TrxR2 based mechanism is not as effective as complex III, it could be sufficient to minimize the stress to the cell by maintaining the electron transport during inhibition of complex III. The positive effect of TrxR2 upon complex III impairment might be of value in understanding respiratory disorders in diseases such as myopathies and

encephalomyopathies [284, 285], in which the clinical manifestation is neuromuscular Q10 deficiency. However, it is unclear to what extent the complex-bypassing mechanism of TrxR2 or its antioxidant function eliminating ROS, either by itself or with Trx2 and peroxiredoxin, should be credited for the protective roles of TrxR2. What is clear is that the redox balance in the matrix is of great importance for proper function of the respiratory chain. In this context TrxR2 may not only act as an antioxidant regenerator, but probably also can directly reduce members of the respiratory mechanism.

However, are the redox capabilities of these thioredoxin systems the only contribution of biological significance that they have? We describe a novel TrxR1 alternative splicing variant (TrxR1b) that contains the consensus sequence of a nuclear receptor (NR) box, a sequence that is responsible for the binding of coregulatory proteins to nuclear receptors. The NR box in TrxR1b leads the binding of the protein with ER α and ER β and its translocation and colocalization with the ERs in the nucleus. ERs can regulate the expressions of genes that are under the control of ERE or AP1 response elements which leads to the following questions. How can ER distinguish between the two elements, and what regulates whether ER activation will lead to an ERE or AP1 response? TrxR1b was found to selectively repress the ER activity at AP-1 sites while it had no effect or a slightly positive effect at ERE sites. TrxR1b could therefore function as a valve that shifts ER activation towards ERE containing genes, thereby selectively enhancing the classical estrogen response pathway instead of the alternative AP-1 pathway. However, this function does not seem to be dependent on the redox capabilities of TrxR1b. Our results suggest that it is rather the direct protein-protein interaction, where TrxR1b could sterically hinder the binding of ERs to the AP-1 coactivator complex and thereby prevent the ER triggering of AP-1.

Extensive work published concerning thioredoxins mainly focuses Trx1. In this thesis we present new members of the Trx superfamily and even more members have been described lately that are not included in this work. This suggests that the work on the thioredoxin superfamily and the understanding of it, despite the number of publications on the subject, is still in its infancy. The aim of this thesis was to identify novel members, help in their understanding and show the functional diversity of this evolving family of proteins, but hopefully also stimulate further investigation in this exciting field of research.

3. CONCLUSIONS

3.1 Paper I

- The *E. coli* Trx2 contains two domains: an N-terminal domain of 32 amino acids containing two CXXC motifs and a C-terminal domain homologous to the prokaryotic thioredoxins
- The protein was found to be expressed in normally growing *E. coli*.
- Trx2 together with TrxR and NADPH can reduce ribonucleotide reductase as well as the interchain disulfide bridges of insulin
- The N-terminal domain of Trx2 is important in the regulation of the enzymatic activity of the protein.

3.2 Paper II

- Sptrx1 unlike other thioredoxins was shown to be exclusively expressed in round and elongating spermatids.
- The protein is divided in two distinct domains; an N-terminal domain composed of 23 highly conserved repetitions of a 15-residue motif, and a C-terminal domain homologous to thioredoxins.
- In the presence of TrxR1 and NADPH, Sptrx1 was found to have thioredoxin activity.
- Under native conditions, Sptrx1 appears to have a multimeric structure.

3.3 Paper III

- Trx2 is localized in mitochondria.
- Trx2 lacks structural cysteines which renders the protein more resistant towards oxidative inactivation.
- Trx2 protects cells against the cytotoxic effects of etoposide, a drug commonly used in anticancer treatment.
- Trx2 overexpressing cells have a higher mitochondrial membrane potential compared to control cells.

3.4 Paper IV

- TrxR2 is a mitochondrial protein.
- TrxR2 contains the conserved active site CVNVGC, as well as FAD and NADPH-binding domains.
- At the 3'UTR a putative selenocysteine insertion sequence (SECIS) is present suggesting that TrxR2 is a selenocysteine-containing protein.

3.5 Paper V

- Mitochondrial extracts from overexpressing cells were assayed for TrxR activity and showed a 7-fold increase in activity compared to control cells. The activity was further increased when sodium selenite was added to the media
- TrxR2 is a selenocysteine containing protein, confirmed by the incorporation of the radiolabeled selenium homologue, ⁷⁵Se.
- TrxR2 protects cells against the cytotoxic effects of antimycin and myxothiazol, chemicals that inhibit the function of complex III in the MRC.
- TrxR2 is capable of reducing cytochrome c.

3.6 Paper VI

- TrxR1b is an alternative splicing variant of TrxR1.
- TrxR1b differs from the previously described TrxR1 (TrxR1a) by the presence of an extra 52 amino acid extension at the N-terminus which contains a nuclear receptor box, with the consensus sequence LXXLL.
- TrxR1b can bind to Estrogen Receptors α and β .
- As a result of this binding, a distinct subnuclear localization of TrxR1b was observed, co localizing with both ER α and β .
- TrxR1b was a co-repressor in the alternative pathway where ER activates AP1 transcription independently of its DNA binding activity.

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