Identification of novel cytosolic thioredoxin-1 target proteins in mammalian cells by mechanism-based kinetic trapping

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Summary

Reactive oxygen species (ROS) have been reported to be locally produced after stimulation of various transmembrane receptors, including those for cytokines, growth factors and hormones. Transient ROS generation leads to the reversible oxidation of redox-sensitive proteins, which may either result in their transient activation or inactivation. Even though the role of ROS as a signal transducer is widely accepted, little is known about the identity of redox-sensitive proteins and the reduction mechanism by which they are re-generated. Thioredoxin-1 (Trx1) is an oxidoreductase known to influence a variety of cellular processes including proliferation, apoptosis and gene expression. In higher plants and cyanobacteria different proteomic approaches allowed for the identification of various thioredoxin target proteins. However, no comprehensive study of Trx1 interactions in the cytosol of mammalian cells has been performed so far. In this thesis, mechanism-based kinetic trapping was applied to identify proteins forming mixed disulfide intermediates with Trx1 in the cytosol of a human T cell leukemia line. Most previously established target proteins of Trx1 could be identified, including ribonucleotide reductase, peroxiredoxins and annexin-2, thus confirming the validity of the approach. Interestingly, a substantial number of newly identified proteins currently lacks functional annotation, suggesting that several thioredoxin-regulated pathways still await initial characterization. Of those newly identified proteins with known function, a significant portion is associated with cell cycle control and regulation of apoptosis, while others participate in various signal transduction pathways, cytoskeleton and membrane dynamics, metabolism and transcriptional control. These results strongly support the long-held belief that Trx1 not only plays a role in ROS scavenging and reductive metabolism but is also extensively involved in interactions with key regulatory proteins associated with cellular behavior and fate. While Trx1-interactions are clearly pleiotropic in that they affect a variety of processes simultaneously, we have found strong evidence that they are nevertheless highly target specific. The newly identified Trx1 target proteins cdk6 and caspase-2 were selected for further study. For cdk6 the thioredoxin-interacting cysteine residue was identified and for caspase-2 redox regulation of activity by Trx1 could be demonstrated *in vitro*, lending further support to the biological relevance of Trx-based regulation.

Zusammenfassung

Reaktive Sauerstoffspezies (ROS) werden lokal nach Stimulation verschiedener Transmembranrezeptoren produziert. Die transiente Bildung von ROS bewirkt eine Oxidation redox-sensitiver Proteine, was zu deren Aktivierung oder Inaktivierung führen kann. Trotz der allgemein akzeptierten Rolle von ROS als Botenstoff ist bislang wenig über die Identität und Regeneration oxidations-sensitiver Proteine bekannt. Thioredoxin-1 (Trx1) ist eine reduzierende Thiol-abhängige Oxidoreduktase, die mit zahlreichen zellulären Prozessen, wie z.B. Proliferation, Apoptose und Genexpression in Verbindung steht. In höheren Pflanzen und Cyanobakterien wurden verschiedene Proteom-Analysen zur Identifizierung von Thioredoxin-Zielproteinen erfolgreich angewandt. Entsprechende Studien an Säugerzellen wurden jedoch bislang nicht durchgeführt. Zur Identifizierung von Proteinen die mit Trx1 ein gemischtes Disulfid-Intermediat ausbilden, wurde die sogenannte *mechanism-based kinetic trapping* Technik auf die humane Jurkat T-Zelllinie angewandt. Dabei konnten bereits bekannte Trx1 Zielproteine wie Ribonukleotid-Reduktase, Peroxiredoxine und Annexin-2 identifiziert werden. Ein nicht unerheblicher Anteil der neu identifizierten Proteine ist bislang ohne funktionelle Zuordnung was auf die Existenz noch nicht charakterisierter redox-sensitiver Signalwege hindeutet. Interessanterweise ist ein signifikanter Anteil der mit Trx1 interagierenden Proteine mit der Regulation von Zellzyklus und Apoptose assoziiert, während andere redox-sensitive Proteine an Signaltransduktion, Zytoskelett- und Membrandynamik, Metabolismus und Transkriptionskontrolle beteiligt sind. Diese Daten unterstützen die lange gehegte Auffassung, dass Trx1 nicht nur eine Rolle bei der Neutralisierung von ROS und im Metabolismus spielt, sondern darüber hinaus intensiv an der Regulation von Signaltransduktion, Zellzyklus und Zelltod beteiligt ist. Die in dieser Arbeit beschriebenen Trx1-Interaktionen sind pleiotrop, in dem Sinne, dass sie eine Vielzahl an zellulären Prozessen betreffen, jedoch zugleich sehr spezifisch für bestimmte Zielproteine. Zur Identifizierung der Cysteine die von Trx1 reduziert werden wurden Mutagenese-Studien am neu gefundenen Trx1 Zielprotein cdk6 durchgeführt. Darüber hinaus konnte eine Regulation der Aktivität von Caspase-2 durch Trx1 *in vitro* nachgewiesen werden.

1 Introduction

1.1 Oxygen and its reactive species

Oxygen is the third most abundant element by mass in the universe after hydrogen and helium (Suess & Urey, 1956) constituting 20.9% of the volume of air. Its molecular form (O_2) is essential for the survival of all aerobic organisms which use oxidative phosphorylation to generate ATP, the main energy source of the cell. A flux of electrons through the mitochondrial electron-transport chain (ETC) is used to build a proton gradient across the inner mitochondrial membrane. Four major protein complexes have been identified to form the ETC, NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), cytochrome *bc*$_1$ (complex III) and cytochrome *c* oxidase (complex IV). The proton gradient generated by complex I, III and IV is used by ATP synthase (complex V) to drive synthesis of ATP from ADP. Complex IV catalyzes the complete reduction of O_2 to H_2O without the formation of oxygen radicals. However, some electrons escape the ETC especially from complex I and III, which can react with O_2 to form superoxide anion ($O_2^{\bullet -}$) (Balaban et al, 2005). It has been estimated that 0.1% to 0.5% of the total cellular O_2 reacts to $O_2^{\bullet -}$ (Staniek & Nohl, 2000; St-Pierre et al, 2002) which is spontaneously or enzymatically converted to hydrogen peroxide (H_2O_2). In the presence of transition metal ions H_2O_2 can be converted to the even more reactive hydroxyl radical ($OH^{\bullet}$). These and other O_2 metabolites have been termed reactive oxygen species (ROS) due to their higher reactivity and oxidizing capacity compared to molecular oxygen (Figure 1). Besides the mitochondria, ROS can be generated from other cellular sources including NADPH cytochrome P450 reductase, hypoxanthine/xanthine oxidase, NADPH oxidase, lipoxygenase or cyclooxygenase (COX) (Kamata & Hirata, 1999).

Due to their potential to induce DNA adducts and breaks, lipid peroxidation or protein

			ROS							
	O_2	➡	$O_2^{\bullet -}$	➡	H_2O_2	➡	$OH^{\bullet}$	➡	H_2O
Redox potential (V):		-0.33		+0.89		+0.38		+2.31	
$T_{1/2}$ (sec):			10^{-6}		10^{-5}		10^{-9}		
In vivo concentration (M):			10^{-10}		10^{-7}		10^{-15}		

Figure 1: The O_2 reduction pathway and the family of reactive oxygen species (ROS)
Overview of O_2 reduction to H_2O. Redox potential, half life ($T_{1/2}$) and estimated cellular concentration *in vivo* are listed. Adapted from Giorgio et al (2007) and Forman et al (2002).

carbonylation, ROS have long been considered to be toxic metabolic by-products (Imlay, 2003). The free-radical or oxidative stress hypothesis first formulated by Denham Harman states that oxidizing species are generated during aerobic metabolism which results in a pattern of cumulative damage that eventually leads to tissue dysfunction followed by the onset of aging and development of diseases (Harman, 1956). This hypothesis is in agreement with the fact that the cell normally employs a variety of enzymes and small-molecule scavengers to detoxify ROS. Superoxide dismutase (SOD) accelerates the dismutation of $O_2^{\bullet -}$ to H_2O_2 by 10^4 fold to a nearly diffusion limited rate that effectively prevents most other reactions of $O_2^{\bullet -}$ in cells. Catalase converts H_2O_2 to H_2O and O_2, but in most cells this enzyme is confined to peroxisomes allowing H_2O_2 to react with other proteins before it comes in contact with catalase. The two main enzymatic systems dedicated to H_2O_2 elimination use thiols as reducing agents. Glutathione peroxidases (GPx) are selenocysteine-containing proteins which reduce H_2O_2 to water by using reduced glutathione as an electron donor. Peroxiredoxins (Prx), formerly known as thioredoxin peroxidases, directly reduce H_2O_2 by oxidation of their active site cysteines to a disulfide bond (Forman et al, 2002). In addition to these antioxidant enzymes, ROS can be scavenged non-enzymatically by small molecule antioxidants like vitamin A, C or E.

Oxidative stress has been implicated to play a role in a large number of diseases including atherosclerosis, diabetes mellitus, neurodegenerative diseases, chronic inflammation and cancer (Dröge, 2002). However, ROS are not only produced under pathological conditions. In macrophages, neutrophils and other phagocytes the enzyme NAPDH oxidase (Nox) catalyzes mitochondria-independent production of $O_2^{\bullet -}$ and $NADP^+$ from O_2 and NAPDH during the so-called respiratory burst to efficiently kill bacteria and fungi (Lambeth, 2004). In addition, T cells also express a phagocyte-type NADPH oxidase which is required for regulation of T cell receptor signaling (Jackson et al, 2004). Furthermore, ROS are generated after stimulation by a variety of cytokines and growth factors, including TNF-α, IL-1, IFN-γ, EGF and PDGF (Thannickal & Fanburg, 2000). Therefore, it is now well established that ROS play an important regulatory role also under physiological conditions.

1.2 Redox regulation: Control of protein activity by thiol modifications

The sulphydryl group (–SH) of cysteine residues can react with ROS to different oxidation products. Oxidation of cysteine leads to the formation of sulphenic acid (–SOH) which is highly reactive and likely to react with a second cysteine to form a disulfide bond (–S–S–). If sulphenic acid is further oxidized, sulphinic acid ($–SO_2H$) is generated which can no longer form disulfide bonds. Sulphinic acid in the active site of certain peroxiredoxins can be reduced by the enzyme sulfiredoxin (Srx) (Biteau et al, 2003; Chang et al, 2004). Eventually, oxidation of sulphinic acid leads to the formation of sulphonic acid ($–SO_3H$), and this oxidation step is believed to be irreversible. *S*-Nitrosothiols (–S–NO) are generated by oxidation of cysteine residues by nitric oxide (NO), a reaction called *S*-nitrosylation. Alternatively, cysteines can form of a disulfide bond with glutathione, a modification known as S-glutathionylation (–S–SG) (Figure 2).

Oxidative modification of cysteines in redox-sensitive proteins represents a mechanism through which redox signals can be transmitted within the cell. In this way the redox state of protein thiols serves as a “molecular switch” to reversibly

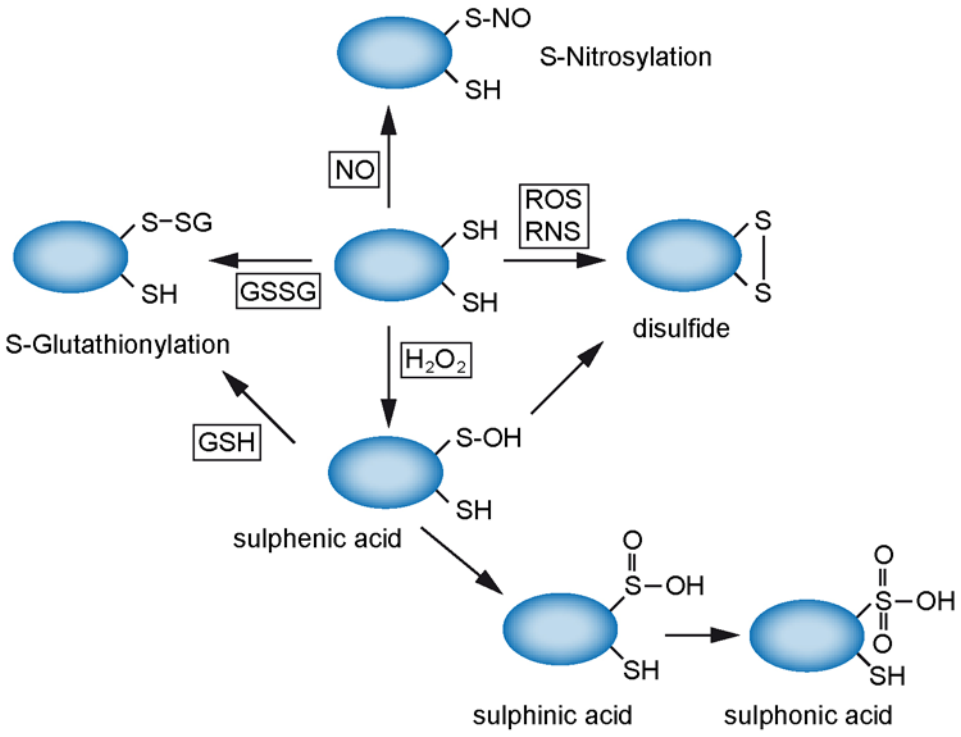

Figure 2: Oxidative modifications of cysteine residues
Oxidation of protein cysteine residues (–SH) to various oxidation products. See text for details. RNS = reactive nitrogen species. Adapted from Paget & Buttner (2003) and Berndt et al (2007).

activate or inactivate protein function. As this process resembles the well-established phosphorylative regulation of protein function by kinases and phosphatases, the term “redox regulation” was coined. Remarkably, kinases and phosphatases as key enzymes in phosphorylative signal transduction are also subject to redox regulation, thereby generating cross-talk between these two chemically distinct regulatory mechanisms.

1.2.1 Redox regulation of protein tyrosine phosphatases

Phosphorylation of protein tyrosine residues is one of the most important regulatory mechanisms of signal transduction controlling a vast number of cellular processes. Cellular phosphotyrosine levels are regulated by the antagonistic activities of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). Due to their central role in the regulation of proliferation, a number of these enzymes function as oncoproteins and tumor suppressors, respectively. Based on the early observation that treatment of cells with H_2O_2 leads to increased phosphorylation of the insulin receptor (Koshio et al, 1988) it was later shown that PTPs are transiently inactivated by oxidation of their active site cysteines thereby inhibiting counter-regulation of PTK activity (Knebel et al, 1996). PTPs hydrolyze phospho-ester bonds via an invariant cysteine residue which has a low p*K*a of 5.0 - 6.7 due to the unique environment of the PTP active site. Therefore, the catalytic cysteine is present as thiolate anion at physiological pH allowing it to act as a nucleophile during catalysis but also rendering it susceptible to oxidation (Salmeen & Barford, 2005; Tonks, 2006). Protein tyrosine phosphatase 1B (PTP1B) has been shown to become inactivated after stimulation of cells with EGF and insulin (Lee et al, 1998; Mahadev et al, 2001; Meng et al, 2004). It could be demonstrated that the SH2 domain-containing protein tyrosine phosphatase-2 (SHP2) is transiently oxidized in response to PDGF stimulation (Meng et al, 2002) which was later also confirmed for T cell receptor stimulation (Kwon et al, 2005a). In addition, phosphatase and tensin homolog (PTEN), a lipid and protein phosphatase (Myers et al, 1997; Maehama & Dixon, 1998) has been shown to be reversibly inactivated in macrophages after stimulation with lipopolysaccharide (LPS) (Leslie et al, 2003). It is essential for the reversible regulation of PTPs that the active site cysteine is not over-oxidized to sulphinic or sulphonic acid, as this would irreversibly inactivate the enzyme (Figure 3A).

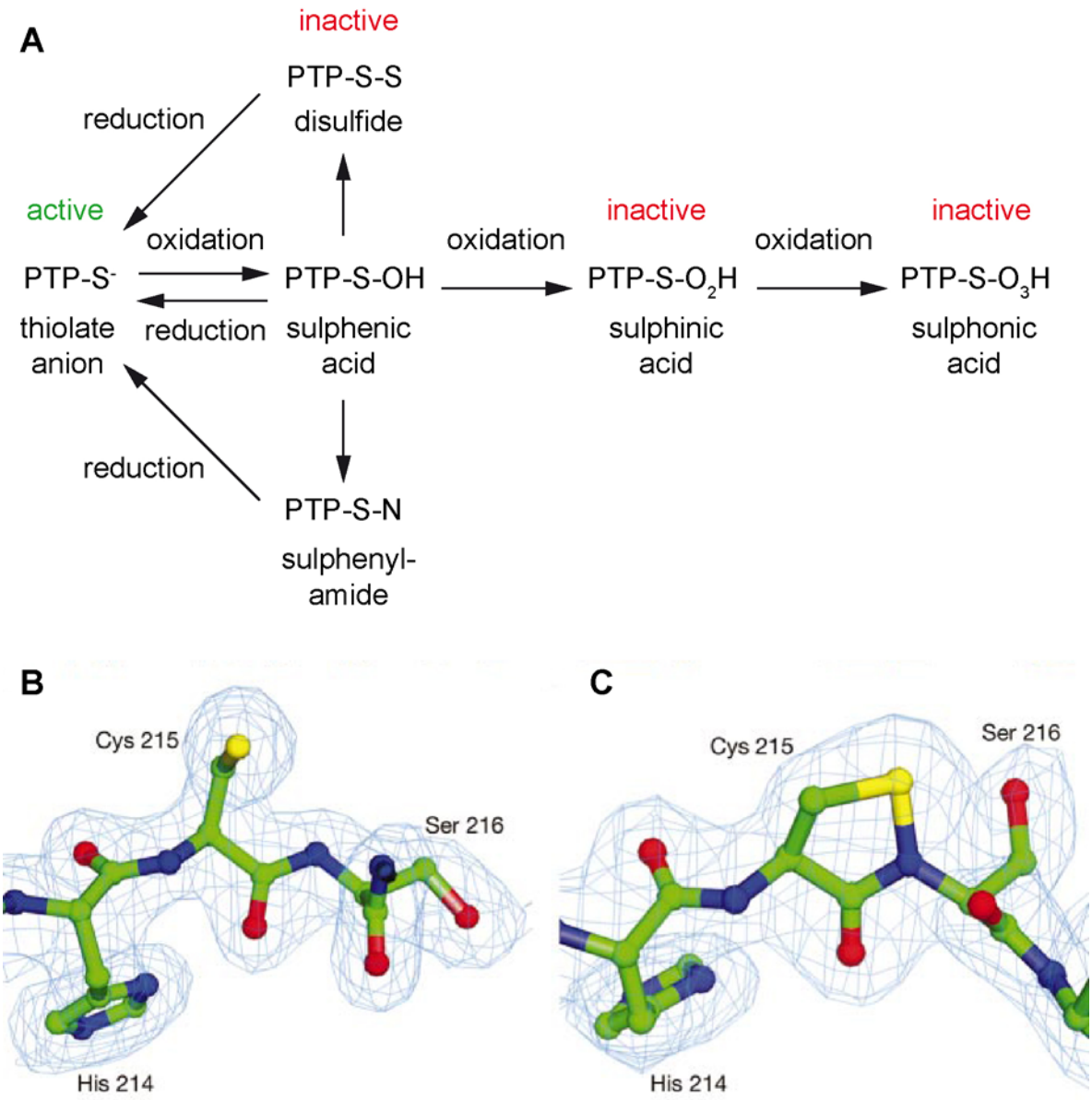

Figure 3: Oxidative inactivation of PTPs
(A) Oxidative inactivation and reactivation of PTPs .Adapted from (den Hertog et al, 2005), (B) reduced PTP1B (C) cyclic sulphenylamide in oxidized PTP1B. (B) and (C) taken from van Montfort et al (2003).

Based on the crystal structure of PTP1B a novel mechanism was identified that allows for reversible redox-regulation of the phosphatase and at the same time prevents over-oxidation. Oxidation of the catalytic cysteine to sulphenic acid is followed by its conversion to a cyclic sulphenylamide species though S-N bonding with the amide nitrogen of the adjacent serine (Figure 3B+C), which induces a conformational change in the active site that disrupts the interaction with its substrate and exposes the oxidized cysteine (Salmeen et al, 2003; van Montfort et al, 2003). Thereby, irreversible inactivation is prevented and re-activation of the phosphatase by reduction is facilitated.

In contrast to classical PTPs like PTP1B, dual specific phosphatases, e.g. PTEN, cdc25 and low molecular weight protein tyrosine phosphatase (LMW-PTP) contain a second cysteine in their active sites which can form a disulfide bond with the adjacent cysteine thereby protecting the protein from over-oxidation. This disulfide bond can be readily reduced allowing tight control of protein activity by redox-regulation (Salmeen & Barford, 2005).

1.2.2 Redox regulation of protein kinases

The best characterized redox-regulated kinase is apoptosis signal-regulating kinase 1 (ASK1) which activates both the c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) cascade by direct phosphorylation of their upstream kinases MKK4/MKK7 and MKK3/MMK6, respectively. ASK1 is activated by oxidative stress (Ichijo et al, 1997), tumor necrosis factor (TNF) receptor stimulation (Nishitoh et al, 1998) or Toll-like receptor 4 (TLR4) activation (Matsuzawa et al, 2005). In addition, it has been demonstrated that ASK1 induces apoptosis by triggering a mitochondria-dependent pathway including cleavage of pro-apoptotic Bcl-2 family members, Bax mitochondrial translocation, cytochrome *c* release and subsequent activation of caspase-9 and caspase-3 (Hatai et al, 2000). In non-stressed cells, reduced thioredoxin-1 (Trx1) tightly binds the N-terminal domain of ASK1, thus inhibiting its multimerization and activation. Upon oxidative stress, Trx1 is oxidized and dissociates from ASK1, thereby promoting kinase activation (Saitoh et al, 1998; Liu et al, 2000). Furthermore, glutaredoxin-1 has been shown to inhibit ASK1 at its C-terminus and dissociate from ASK1 upon induction of oxidative stress by glucose deprivation (Song et al, 2002). In contrast to the described Trx1 oxidation model, a novel mechanism was proposed according to which oxidation not only induces phosphorylation of ASK1 but also formation of disulfide-linked ASK1 multimers required for JNK activation. In this model, Trx1 only transiently interacts with ASK1 to negatively regulate its activity by multimer reduction (Nadeau et al, 2007). Even though disulfide-linked ASK1 multimers may play a role for its activation the classical Trx1-oxidation model has not been disproven and more work needs to be done to elaborate the physiological function of these conjugates.

Other kinases have been suggested to be regulated by direct redox modifications. The proto-oncogenic kinase c-Src forms high molecular weight complexes upon treatment with NO donors, leading to phosphorylation-independent activation

(Akhand et al, 1999). The opposite effect was observed for Janus kinase-2 (JAK2) which is inactivated by NO treatment, thus inhibiting activation of quiescent BaF3 cells (Duhé et al, 1998). Very recently, disulfide-mediated regulation of different kinases has been reported. The Src homology 2 (SH2) domain of C-terminal Src kinase (Csk) contains a unique disulfide bond that is not present in other SH2 domains. Reduction of this disulfide *in vitro* increases Csk kinase activity by an order of magnitude compared to the oxidized protein (Mills et al, 2007). Even though the *in vivo* relevance of this regulatory disulfide still needs to be demonstrated, these data suggest that Csk may be redox-regulated in a way similar to non-classical PTPs like PTEN and LMW-PTP. Similarly, cyclic AMP-dependent protein kinase (PKA), a key regulator of lipolysis, is inhibited by H_2O_2 and re-activated by dithiothreitol (DTT) or the thioredoxin system (see chapter 1.3.2). Phylogenetic analysis suggests that a regulatory disulfide bond is formed between the regulatory and catalytic subunit of PKA (de Piña et al, 2008).

Oxidation does not always lead to inhibition of protein function as has been demonstrated for protein kinase C (PKC) which was found to be activated by oxidants (Gopalakrishna et al, 2008). Moreover, cGMP-dependent protein kinase isoform α (PKGIα) forms an intermolecular disulfide bond between its subunits leading to direct activation of the kinase and increased affinity for substrates (Burgoyne et al, 2007). Interestingly, inter-subunit crosslinking changed kinase activity of PKGIα and PKA in opposite directions (de Piña et al, 2008) showing that disulfide bond formation can have different consequences in closely related proteins.

In comparison to phosphorylation-based regulation of signal transduction where kinases and phosphatases counter-regulate protein activity, redox-regulation by protein oxidation is antagonized by thiol-disulfide oxidoreductases that reduce disulfide-bonds and thereby ensure reversible modulation of protein function.

1.3 Thiol-disulfide oxidoreductases

In addition to those enzymes directly involved in ROS scavenging, like catalase, superoxide dismutase or peroxiredoxins (see chapter 1.1), the cell also utilizes disulfide reductases to maintain cellular thiol redox homeostasis. Thioredoxin and glutaredoxin comprise the family of thioredoxin thiol-disulfide oxidoreductases which is characterized by the thioredoxin fold with a common CxxC active site motif (Martin, 1995). These proteins contribute to a reduced intracellular redox state by reduction of protein thiols and thereby play an important role in the oxidative stress response (Holmgren, 2000). Importantly, thioredoxin and glutaredoxin also contribute to redox-regulation by modulating the activity of kinases, phosphatases, transcription factors and other mediators of signal transduction (Ghezzi, 2005).

1.3.1 The glutathione/glutaredoxin system

Glutathione (γ-glutamyl-cysteinyl-glycine) is the most abundant low-molecular-weight thiol in mammalian cells and exist as either reduced (GSH) or oxidized form (glutathione disulfide; GSSG). Under physiological conditions cytosolic glutathione is

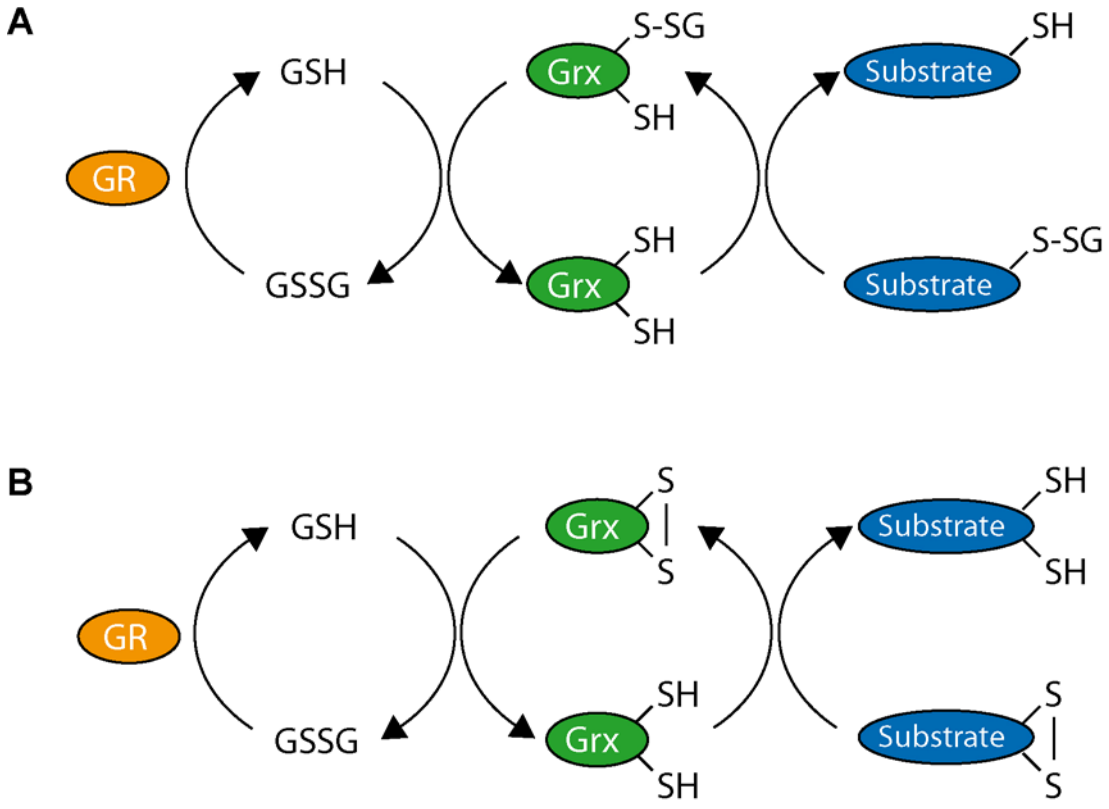

Figure 4: The glutathione/glutaredoxin system
(A) De-glutathionylation of substrate proteins by the mono-thiol mechanism. (B) Disulfide bond reduction by the di-thiol mechanism. GR: glutathione reductase

maintained almost exclusively in the reduced state and serves as intracellular redox buffer (Filomeni et al, 2002).

GSH can function as substrate for glutathione peroxidases which catalyze its reaction with peroxides to GSSG and H_2O. However, the increase in GSSG during oxidative stress is generally transient due to its rapid reduction by glutathione reductase. In addition to its role in the detoxification of electrophilic xenobiotics by glutathione *S*-transferases (GSTs) (Townsend & Tew, 2003), GSH can form a mixed disulfide bond (GS–S–R) with cysteine residues. Protein *S*-Glutathionylation represents another mechanism besides intra- or intermolecular protein-protein disulfide bond formation and generation of cyclic sulphenylamides to prevent over-oxidation of proteins. De-glutathionylation is catalyzed by the oxidoreductase glutaredoxin (Grx), also known as thioltransferase. During the so-called mono-thiol reaction GSH is transferred from a target protein to the active site cysteine of Grx (Figure 4A). Glutathionylated Grx can then be reduced by another GSH molecule, leading to the formation of GSSG (Holmgren, 2000). In addition to the mono-thiol mechanism, Grx has been shown to act as a protein disulfide reductase for bacterial ribonucleotide reductase using a di-thiol mechanism (Holmgren, 1989) comparable to thioredoxin (Figure 4B). However, whether this reaction mechanism has any relevance for other proteins or in other organisms is still a matter of dispute.

Like disulfide-bond formation, glutathionylation often inhibits protein activity as reported for GAPDH (Lind et al, 1998; Mohr et al, 1999), PTB1B (Barrett et al, 1999) or nuclear factor κB (NFκB) (Pineda-Molina et al, 2001), but it may also increase protein activity as shown for HIV-1 protease (Davis et al, 1997) or matrix metalloproteinases (Okamoto et al, 2001).

1.3.2 The thioredoxin system

The thioredoxin system consists of the enzymes thioredoxin (Trx) and thioredoxin reductase (TrxR) as well as nicotinamide adenine dinucleotide phosphate (NADPH). Thioredoxins are ubiquitously expressed proteins with thiol-dependent oxidoreductase activity that reduce protein disulfides using their two active site cysteines. Upon reduction of target proteins Trx is itself oxidized in its active site. Trx is regenerated by TrxR, a homodimeric flavoenzyme that contains a redox active

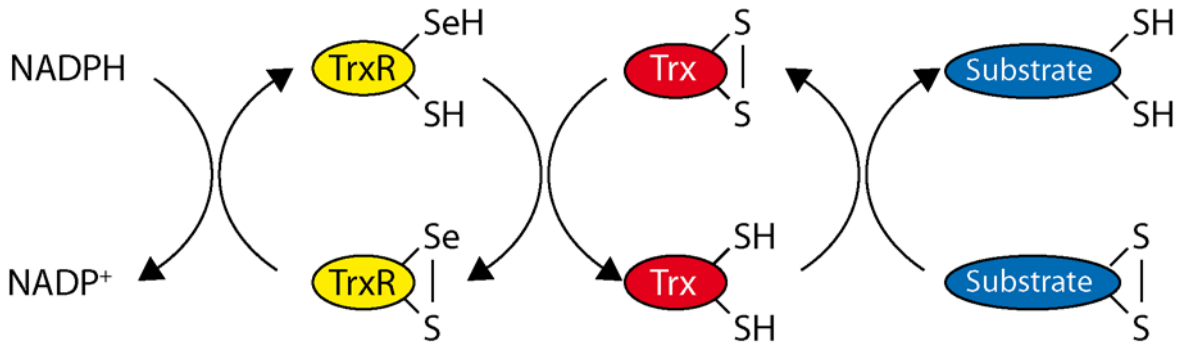

Figure 5: The Trx system
Disulfide bond reduction by Trx. During the catalytic cycle Trx becomes oxidized. It is subsequently reduced by the selenoprotein TrxR under the consumption of NADPH.

disulfide and one tightly bound flavin adenine dinucleotide (FAD) per subunit. The active site of TrxR with the unique sequence Gly-Cys-SeCys-Gly contains a selenocysteine (SeCys) (Gladyshev et al, 1996). The highly reactive selenolate and the accessibility of the active site are the reasons why mammalian TrxR possesses wide substrate specificity and can reduce thioredoxins of other species, a variety of physiological substrates as well as several small compounds (Mustacich & Powis, 2000).

1.3.3 Forms and localization of thioredoxins

Thioredoxin was first identified in *E.coli* as an electron donor for ribonucleotide reductase (Laurent et al, 1964) before it was described in mammals (Moore, 1967) and later purified from rat hepatoma cells (Herrmann & Moore, 1973). In mammalian cells two Trx isoforms encoded by two different genes are expressed.

The *TXN1* gene coding for human Trx1 is organized in five exons separated by four introns and maps to position 9q31 on chromosome 9 (Kaghad et al, 1994; Heppell-Parton et al, 1995). Human Trx1 is a ubiquitous cytosolic 105-amino acid protein with a molecular weight of 12 kDa, which can translocate to the nucleus (Hirota et al, 1999; Kabe et al, 2005) or gets secreted to the extracellular space by various cell types (Rubartelli et al, 1992). Apart from the two active site cysteines (Cys32 and Cys35), human Trx1 contains three additional non-catalytic cysteines (Cys62, Cys69 and Cys73). Trx1 was independently described as IL-1-like autocrine growth factor from a Epstein-Bar virus (EBV) transformed B cell line (Wakasugi et al, 1987), ATL-derived factor (ADF) from HTLV-I transformed T cells (Teshigawara et al, 1985; Tagaya et al, 1989) and early pregnancy factor isolated from ovine placental extracts

(Clarke et al, 1991), which all share the same amino acid sequence with human Trx1 (Deiss & Kimchi, 1991; Gasdaska et al, 1994).

Trx2 encoded by the *TXN2* gene on 22q13.1 contains an N-terminal mitochondrial localization sequence. It is a 116-amino acid protein with a molecular mass of 18 kDa and a conserved thioredoxin active site but no other non-catalytical cysteines (Spyrou et al, 1997). In addition to Trx1 and Trx2 a third variant of human Trx, called Sptrx, is highly expressed in spermatozoa (Miranda-Vizuete et al, 2001).

A truncated form a Trx1 (Trx80) comprising the 80 N-terminal amino acids is generated by proteolytic cleavage. Trx80 has been found to be secreted by various cell lines (Sahaf et al, 1997) and seems to be identical to eosinophil cytotoxicity enhancing factor (ECEF) secreted by phorbol ester (PMA)-stimulated U937 cells (Silberstein et al, 1993). Trx80 has no catalytic activity but functions as a mitogenic cytokine for resting human peripheral blood mononuclear cells (Pekkari et al, 2000). Furthermore, Trx80 induces activation and IL-12 secretion of monocytes (Pekkari et al, 2001).

Plants possess two thioredoxin systems that can be distinguished by the electron donor and the enzyme that catalyzes thioredoxin reduction. The chloroplastic FRT/Trx system is composed of ferredoxin, ferredoxin-thioredoxin reductase (FRT), thioredoxin *m* and *f*. In the light, electrons are transferred from photosystem I to the iron-sulfur protein FRT via ferredoxin. In turn, FRT reduces thioredoxin *m* and *f* which reduce their targets involved in carbon assimilation, photorespiration and metabolism, thereby conferring a strict, light-sensitive control of both the assimilatory and dissimilatory pathway. In contrast, the NAPDH-thioredoxin reductase (NTR) system uses NAPDH for the reduction of cytosolic thioredoxin *h* which plays a role in seed germination and oxidative protection (Baumann & Juttner, 2002). Unlike in bacteria and animals, a large number of genes encode for thioredoxins in plants. In the genome of *Arabidopsis thaliana* 19 different thioredoxins have been identified and evidence suggest that individual tissues express characteristic members of the thioredoxin family (Buchanan & Balmer, 2004).

1.3.4 Structure of Trx1

Oxidized *E.coli* Trx was the first thioredoxin to be structurally solved (Holmgren et al, 1975), subsequently refined to 1.68 Å resolution (Katti et al, 1990). Later, also reduced *E.coli* Trx (Jeng et al, 1994) as well as human thioredoxin were crystallized (Qin et al, 1994; Weichsel et al, 1996) showing that Trx is a compact globular protein with a five-stranded β-sheet surrounded by four α-helices. Its active site disulfide is located at a C-terminal end of a β-strand (β_2) and the beginning of the α_2-helix (Figure 6). It turned out that thioredoxins from archaebacteria to humans show large variations in the amino acid sequence (27-69% sequence identity to *E.coli* thioredoxin) but still have similar three-dimensional structures (Eklund et al, 1991). This so-called thioredoxin fold is also present in other proteins like protein disulfide-isomerase (PDI) (Eklund et al, 1991). Structures of oxidized and reduced Trx are very similar with only slight differences in the active site and hydrogen bonding pattern (Jeng et al, 1994; Holmgren, 1995).

Recombinant human Trx1 forms covalent disulfide-linked homodimers via Cys73, in the presence of oxidants or when stored at high concentrations (Ren et al, 1993; Weichsel et al, 1996). As the active site is buried in the dimer interface, Trx1 homodimers are catalytically inactive. It remains unknown if Trx dimer formation occurs in intact cells under physiological conditions.

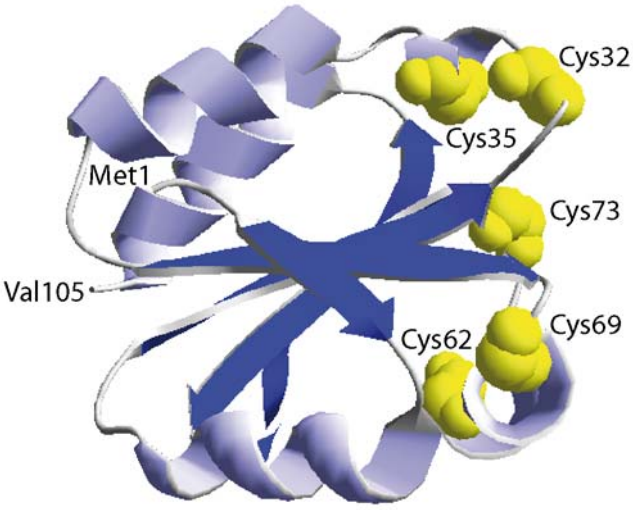

Figure 6: Crystal structure of reduced human Trx1
X-ray structure of human Trx1 generated from the Protein Data Bank (PDB) entry 1ERT (Weichsel et al, 1996) using Swiss-PbdViewer 4.0

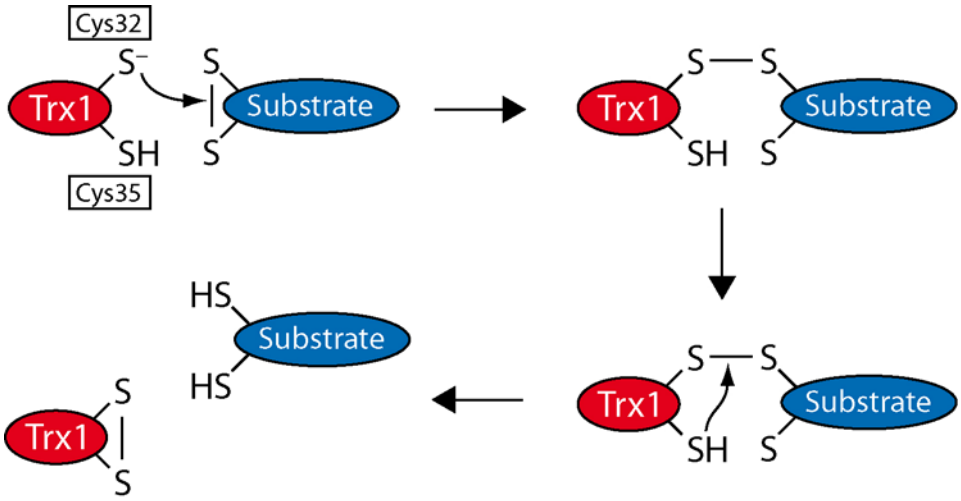

Figure 7: Reaction mechanism of Trx1
Disulfide bond reduction by Trx1 is initiated by a nucleophilic attack of Trx1 Cys32 leading to formation of a mixed disulfide which is resolved by Trx1 Cys35.

1.3.5 Catalytic mechanism of Trx1

All thioredoxins, including human Trx1, Trx2 and Sptrx, contain a conserved Cys-Gly-Pro-Cys motive in the their active site (Holmgren, 1989). Upon binding of oxidized substrates to the hydrophobic surface of Trx1, the thiolate anion of the N-terminal Cys32 acts as a nucleophile and attacks the target disulfide bond, leading to the formation of a covalent mixed-disulfide bond between Trx1 and its target protein (Kallis & Holmgren, 1980). In a second step, the mixed disulfide is resolved by a nucleophilic attack of the second active site cysteine (Cys35), thus generating a reduced substrate and an oxidized Trx1 (Figure 7). The standard redox potential of Trx1 is –230 mV (Watson et al, 2003) and it has been shown that Trx1 is 95% reduced in the cytoplasm (Nkabyo et al, 2002) and in the nucleus (Watson & Jones, 2003) under basal conditions.

In addition to its established function as a reductase, it has been proposed that Trx1 may also catalyze the reverse reaction under oxidative conditions (Watson et al, 2004). However, so far there is no direct evidence that Trx1 acts as a protein oxidase in mammalian cells.

1.3.6 Regulation of Trx1 expression and activity

Thioredoxin-1 activity is regulated at the transcriptional level, by post-translational modifications and by protein-protein interactions. The Trx1 promoter contains binding sites for different transcription factors including AP-1, NFκB and Oct-1 (Kaghad et al,

1994) as well as an oxidative stress response element (Masutani et al, 1996; Taniguchi et al, 1996). Trx1 expression is induced by a variety of stress stimuli including hypoxia (Berggren et al, 1996), O_2 (Das et al, 1999), H_2O_2 (Sachi et al, 1995; Higashikubo et al, 1999), UV irradiation (Hoshi et al, 1997) or LPS (Ejima et al, 1999). On the protein level, Trx1 activity may be regulated by redox-modifications of its cysteines. Glutathionylation of Trx1 at Cys73 after oxidative stress was reported to inhibit its enzymatic activity (Casagrande et al, 2002) and may represent a crosstalk between the glutathione and the thioredoxin system. Besides the disulfide bond between Cys32 and Cys35, formed during the catalytic cycle of Trx1, a second intramolecular disulfide between Cys62 and Cys69 was identified after treatment with diamide (Figure 8A). Interestingly, this second disulfide inhibited reduction of the active site disulfide by TrxR (Watson et al, 2003), suggesting that the interaction with TrxR is influenced by the α3-helix within which these two cysteines are located. This possibility is supported by the finding that homo-dimerisation of recombinant Trx1 via Cys73 made the active site inaccessible for reduction (Gasdaska et al, 1996). However, whether disulfides involving non-catalytic cysteines are also formed *in vivo* under physiological conditions remains to be proven. Recently, *in vitro* disulfide bond formation between Cys62 and Cys69 was investigated by another group of researchers (Hashemy & Holmgren, 2008). However, in contrast to the observations made by Watson and colleagues, the Cys62-Cys69 disulfide bond could only be induced by H_2O_2 treatment, whereas treatment with diamide led to formation of disulfide-linked dimers and multimers. It has been suggested that H_2O_2 generated by NADPH oxidases may transiently inactivate Trx1 (via Cys62-Cys69 disulfide bond formation or glutathionylation) to inhibit Trx1-mediated regeneration of peroxiredoxins. The resulting increase in H_2O_2 concentration could then facilitate the oxidation of redox-regulated proteins. In the same line, transient inactivation of Trx1 could lead to prolonged oxidative inactivation of PTPs, thus enhancing phosphorylation-driven signaling events (Hashemy & Holmgren, 2008).

In addition to disulfide bond formation and glutathionylation, Trx1 is potentially also regulated by *S*-nitrosylation. In endothelial cells, Trx1 has found to be *S*-nitrosylated at Cys69 which appeared to increase its enzymatic activity (Haendeler et al, 2002), whereas another study showed that Trx1 was nitrosylated at Cys73 *in vitro* (Mitchell & Marletta, 2005). In the recent study by Hashemy and Holmgren (2008),

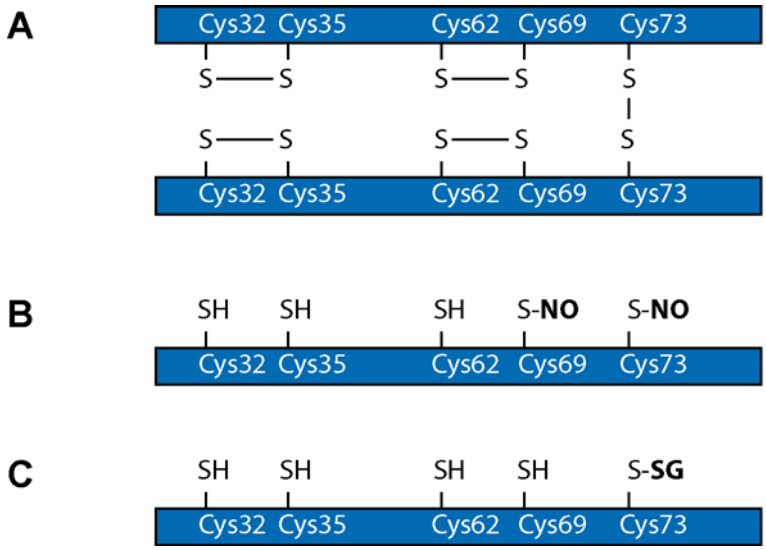

Figure 8: Cysteine modifications of Trx1
(A) Disulfide bond formation of Cys32/Cys35, Cys62/Cys69 and disulfide-linkage of Trx1 monomers via Cys73. (B) *S*-Nitrosylation of Cys69 and Cys73. (C) Glutathionylation of Cys73.

recombinant Trx1 was found to be nitrosylated at both Cys69 and Cys73 resulting in the inhibition of Trx1 activity (Figure 8B). The conflicting results regarding the site(s) of nitrosylation and its influence on Trx1 activity may derive from differences in the thiol-disulfide redox status of the Trx1 samples used.

Additionally, Trx1 is regulated by the interaction with vitamin D_3 up-regulated protein 1 (VDUP1), also known as thioredoxin-interacting protein (Txnip) or thioredoxin binding protein-2 (TBP-2). VDUP1 was initially discovered as a 46 kDa protein up-regulated after vitamin D_3 treatment in HL-60 cells (Chen & DeLuca, 1994) and later found to bind Trx1 in a yeast two-hybrid screen. VDUP1 binds reduced, but not oxidized Trx1, nor to a Trx1 mutant in which the two active site cysteines were replaced by serine. The interaction inhibits Trx1 activity both *in vitro* and *in vivo*. Furthermore, over-expression of VDUP1 in HEK or COS-7 cells reduced expression levels of endogenous Trx1 (Nishiyama et al, 1999a; Yamanaka et al, 2000). Due to its ability to inhibit Trx1 activity and to downregulate Trx1 expression, VDUP1 has been proposed to function as mediator of oxidative stress (Junn et al, 2000).

Besides its role as Trx1 inhibitor, VDUP1 has multiple Trx1-indepedent functions in diverse cellular responses including proliferation, differentiation or apoptosis and has been demonstrated to act as tumor suppressor (Han et al, 2003; Kim et al, 2007).

1.3.7 Biological functions of Trx1

The importance of Trx1 for embryonic development has been demonstrated with mice carrying a targeted disruption of the Trx1 gene. Homozygous animals die shortly after implantation whereas heterozygous animals are viable, fertile and appear normal (Matsui et al, 1996). Consistent with this phenotype, Trx1 is expressed in various different organs during fetal development (Fujii et al, 1991a).

Besides providing electrons for ribonucleotide reductase (Laurent et al, 1964), the function first recognized to depend on Trx1, various other functions have since then been identified. One of the main functions of Trx1 is its role as an antioxidant, namely to maintain the activity of peroxide-scavenging peroxiredoxins (Prx) by reducing their oxidized forms. So far, six mammalian Prx isoforms have been described which can be classified into 2-Cys, atypical 2-Cys and 1-Cys peroxiredoxins. Upon oxidation, the 2-Cys peroxiredoxins, which include Prx1, -2, -3 and -4, form a Trx-reducible inter-subunit disulfide bond, whereas the atypical 2-Cys peroxiredoxin Prx5 forms an intra-subunit disulfide bond. In contrast, the 1-Cys peroxiredoxin Prx6 does not form a disulfide intermediate and is also not a substrate for Trx1. Among the 2-Cys peroxiredoxins, only Prx1 and Prx2 are localized in the cytoplasm, while Prx3 is confined to the mitochondria and Prx4 is found in the ER as well as in the extracellular space (Rhee et al, 2005). During the catalytic cycle of typical 2-Cys peroxiredoxins, the conserved H_2O_2-sensitive peroxidative cysteine is selectively oxidized to the sulfenic acid which then reacts with a conserved C-terminal cysteine of another Prx subunit to form a disulfide-linked homodimer. The dimer is subsequently reduced by Trx1 to its monomeric form (Chae et al, 1994a; Chae et al, 1994b). Of note, plasma Trx1 has been shown to reduce glutathione peroxidase 3 (Björnstedt et al, 1994), a process indicating an extracellular antioxidative function of Trx1.

Trx1 was reported to activate a number of transcription factors, including NFκB which plays an important role during the immune response, in inflammation and cancer (Karin, 2006). Binding of NFκB to DNA can be promoted by Trx1 (Hayashi et al, 1993) and this effect depends on Cys62 in the p50 subunit of NFκB. Oxidation of Cys62 leads to the formation of a disulfide-linked p50 homodimer, which interferes with DNA binding and can be reduced by Trx1 (Matthews et al, 1992). Later it has been shown that Trx1 has opposing roles in NFκB regulation in the cytosol and the

nucleus. In the cytoplasm Trx1 inhibits NFκB activation by blocking degradation of the cytosolic inhibitor IκBα. Upon stimulation with TNF-α, UV irradiation or PMA Trx1 translocates to the nucleus where it reduces and activates NFκB p50 (Hirota et al, 1999). Trx1 interacts with redox-factor 1 (Ref-1) to activate the transcription factors activator protein 1 (AP-1) (Hirota et al, 1997; Wei et al, 2000) and hypoxia-inducible factor-1 alpha (HIF-1α) (Ema et al, 1999). Other transcription factors regulated by Trx1 include the glucocorticoid receptor (Grippo et al, 1985; Makino et al, 1999) and estrogen receptor (Hayashi et al, 1997). Furthermore, Trx1 reduces a disulfide-linked complex formed between the heat-shock protein DnaJb5 and class II histone deacetylases (HDACs) thereby inhibiting its nuclear import independently of its phosphorylation status. This Trx1-mediated modulation of HDAC nucleocytoplasmic shuttling has been linked to the regulation of cardiac hypertrophy (Ago et al, 2008). The activity of the tumor suppressor p53 is regulated by Ref-1 and it has been shown that Trx1 augments DNA binding of p53 in a Ref-1-dependent manner, which leads to an enhanced p53-dependent expression of the cell cycle inhibitor p21 (Ueno et al, 1999). Another tumor suppressor, the lipid and protein phosphatase PTEN, is also regulated by Trx1. Upon exposure to H_2O_2, PTEN is inactivated by the formation of an intramolecular disulfide bond involving its catalytic cysteine, which can be reduced by Trx1, at least in vitro (Lee et al, 2002). In stark contrast, according to other researchers, Trx1 forms a disulfide bond with a cysteine in the C2 domain, thereby inhibiting PTEN activity through steric interference (Meuillet et al, 2004). Most recently, it was reported that mouse embryonic fibroblasts (MEFs) from VDUP1-deficient mice show increased oxidation of PTEN and elevated Akt activation, which has been explained by reduced Trx1 activity in $VDUP^{-/-}$ MEFs (Hui et al, 2008). However, this explanation is in conflict with to the described function of VDUP1 as negative regulator of Trx1. Thus, Trx1 seems to regulate PTEN activity by thiol-disulfide exchange, but the exact mechanism still awaits further clarification. In addition to PTEN other PTPs have been shown to serve as substrate for Trx1 including cdc25 (Sohn & Rudolph, 2003) and PTP1B (Lee et al, 1998). LMW-PTP has been shown to be redox regulated by Grx1 (Kanda et al, 2006). However the influence of Trx1 on this phosphatase has not been analyzed so far and a possible co-regulation of LMW-PTP by both redox systems can not be excluded.

Trx1 is secreted to the extracellular space by a variety of normal and transformed

cells including EBV-positive B cells (Wakasugi et al, 1987; Wakasugi et al, 1990), T lymphocytes (Ericson et al, 1992) and various cancer cells (Rubartelli et al, 1992). Secretory Trx1 can act as growth factor for fibroblasts (Oblong et al, 1994), lymphocytes (Wakasugi et al, 1990) and tumor cells (Nakamura et al, 1992). This mitogenic effect depends on the active site cysteines of Trx1 and can not be mimicked by *E.coli* thioredoxin (Oblong et al, 1994). Trx1 does not act as a classical growth factor via any known growth factor receptor, but instead can sensitize cells to autocrine growth factors like IL-2 or bFGF (Gasdaska et al, 1995; Powis et al, 1997). Expression of IL-2, IL-6, IL-8 and TNF-α was increased by extracellular Trx1 in several cell lines (Schenk et al, 1996). Trx1 was show to mediate reduction of the TNFR-family member CD30 on the cell surface of lymphocytes, which affects both CD30 ligand binding and effector function of these cells (Schwertassek et al, 2007). Apart from its function as growth factor Trx1 is a chemoattractant for neutrophils, monocytes and T cells (Bertini et al, 1999; Miller et al, 2000). Secretion of Trx1 is enhanced after stimulation with PMA, calcium ionophores, LPS or oxidative stress (Sahaf & Rosen, 2000) but the molecular mechanism is still unknown. Neither brefeldin A nor dinitrophenol, two inhibitors of the exocytic pathway can block secretion of Trx1, showing that Trx1 secretion is not mediated via the classical ER/golgi pathway. Furthermore, Trx1 cannot be detected in the membrane-bound compartment of secreting cells (Rubartelli et al, 1992) and its secretion does not depend on the redox state of the active site cysteines (Tanudji et al, 2003). Very recently, caspase-1 has been demonstrated to be required for unconventional secretion of Trx1 from UVB irradiated keratinocytes and LPS/ATP stimulated macrophages (Keller et al, 2008) thereby providing a novel link between inflammation and non-conventional protein secretion.

S-Nitrosylation of Trx1 has been observed repeatedly. However, its influence on Trx1 activity still remains controversial (Haendeler et al, 2002; Sumbayev, 2003; Hashemy et al, 2007). Interestingly, Trx1 regulates caspase-3 activity not by disulfide-reduction but instead by trans-nitrosylation involving the active site thiol of the caspase (Mitchell et al, 2007). Upon stimulation of the death-receptor CD95, caspase-3 is denitrosylated by Trx1 and Trx2, a process required for its activation and initiation of apoptosis (Benhar et al, 2008). This finding is consistent with data showing that Trx1 can promote denitrosylation *in vitro* after treatment with nitrosylating agents

(Stoyanovsky et al, 2005; Sengupta et al, 2007) and reveals a novel role for Trx1 in NO-mediated signal transduction besides its well established function as protein-disulfide reductase.

1.3.8 Trx1 in human diseases

As Trx1 influences a broad range of cellular functions including control of the cellular redox balance, promotion of cell growth, inhibition of apoptosis and modulation of inflammation, it apparently also plays an important role in a broad range of human diseases. As a growth-promoting anti-apoptotic protein, Trx1 is frequently linked to tumor development. Indeed, Trx1 is over-expressed in various cancers including liver (Nakamura et al, 1992; Kawahara et al, 1996), lung (Gasdaska et al, 1994; Kim et al, 2003), colon (Berggren et al, 1996) and cervix cancer (Fujii et al, 1991b). Trx1 was shown to be associated with increased proliferation of tumor cells, inhibition of apoptosis, aggressive tumor growth and decreased patient survival (Lincoln et al, 2003; Raffel et al, 2003). In addition to inhibiting ASK1-mediated apoptosis and tumor suppression by PTEN, Trx1 further augments vascularization of the tumor by increasing the expression level and activity of HIF-1α which leads to enhanced production of vascular endothelial growth factor (VEGF) (Welsh et al, 2002; Kim et al, 2005b).

Based on its growth-promoting activity Trx1 has been proposed to be classified as an oncogene (Grogan et al, 2000) but this proposal has not received wide acceptance. Even though Trx1 is over-expressed in many tumors, elevated expression levels are also observed in non-cancerous cells and tissues (Arner & Holmgren, 2006). The strongest refutation of the oncogene hypothesis stems from mice over-expressing Trx1 under the control of a β-actin promoter. These mice develop normally and show no increase in malignancies compared to wild-type mice, instead they have an extended life span (Takagi et al, 1999; Mitsui et al, 2002). Thus, high expression levels of Trx1 in tumors appears to be a consequence of malignancy, associated with metabolic adaptation and a high proliferation rate, rather than being the cause of malignant transformation (Lillig & Holmgren, 2007). Nevertheless, Trx1 may play an important role for tumor progression due to its anti-apoptotic effects which directly influence the efficiency of chemotherapy. Accordingly, reduction of Trx1 expression levels has been shown to sensitize human bladder cancer cells to doxorubicin, mitomycin C, etopsoside and UV irradiation (Yokomizo et al, 1995). Conversely,

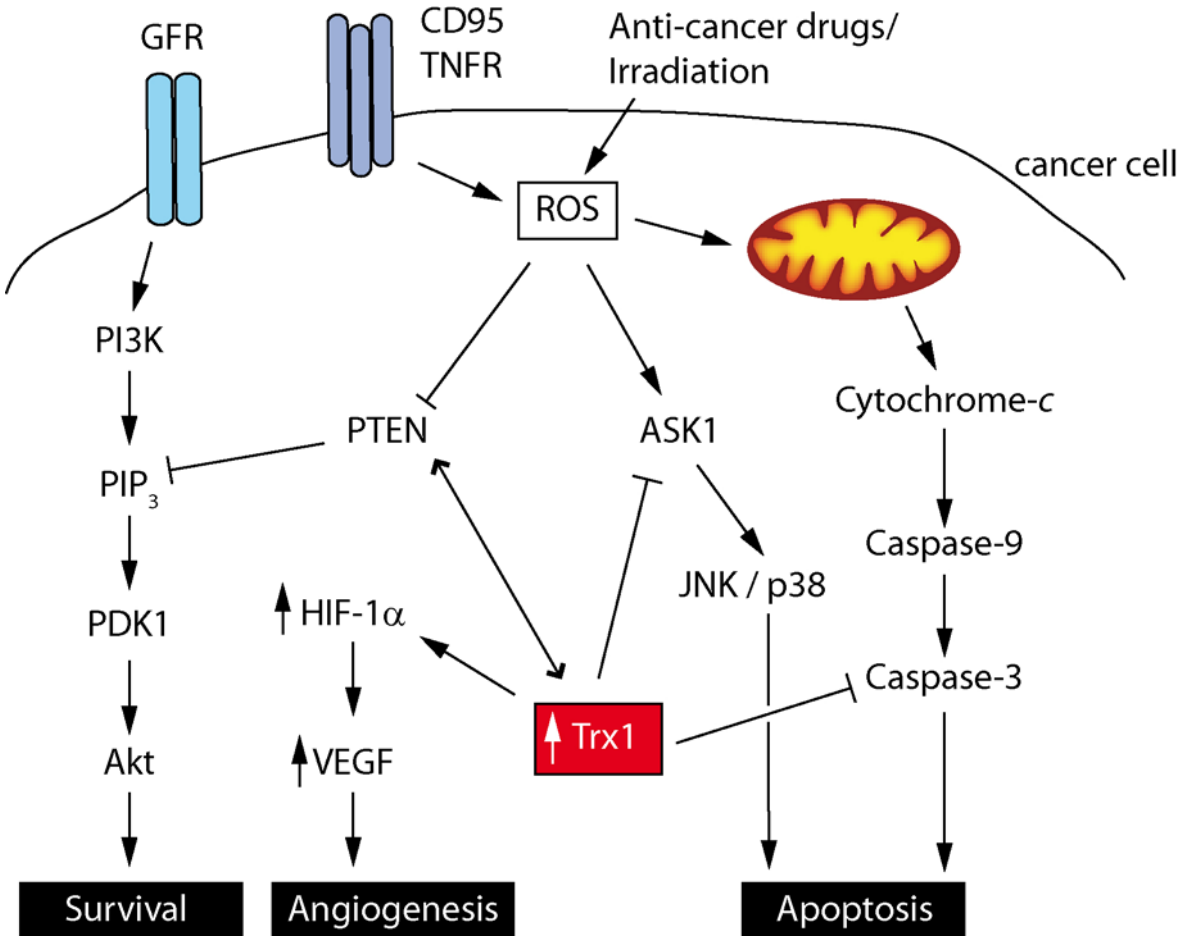

Figure 9: Trx1 in cancer and drug resistance
Trx1 regulated pathways influencing tumor development and cancer drug resistance. See text for details. Adapted from Kaimul et al (2007).

over-expression of Trx1 in fibrosarcoma cells results in drug resistance (Sasada et al, 1996) and high levels of Trx1 are associated with increased resistance to docetaxel in primary breast cancer (Kim et al, 2005a).

In addition to its function in neoplastic diseases, Trx1 plays a role in retroviral infections. Human T cell leukemia virus type 1 (HTLV-1) infection can induce the development of adult T cell leukemia (ATL) which leads to up-regulation of the IL-2 receptor α chain induced by ATL-derived factor (ADF) (Teshigawara et al, 1985), later found to be identical to Trx1 (Tagaya et al, 1989). Moreover, Trx1 is up-regulated in HTLV-1 positive T cells (Makino et al, 1992), potentially contributing to increased proliferation and inhibition of apoptosis in these cells. The increased levels of Trx1 may result from transactivation by the viral oncoprotein tax (Masutani et al, 1996) or down-regulation of VDUP1 (Nishinaka et al, 2004). Epigenetic silencing of VDUP1 results in loss of responsiveness to IL-2 in HTLV-1 infected cells which

further adds to uncontrolled proliferation of these cells (Ahsan et al, 2006). Furthermore, it has been proposed that Trx1 suppresses gene expression from the HTLV-1 long terminal repeat (LTR) thereby negatively regulating viral gene expression (Sasada et al, 2002).

Infection of cells with human immunodeficiency virus 1 (HIV-1) results in down-regulation of Trx1 and loss of cells that highly express Trx1 in lymphoid tissue (Masutani et al, 1992) whereas Trx1 plasma levels are increased in HIV-1 infected patients (Nakamura et al, 1996). The plasma concentration of Trx1 inversely correlates with the number of CD4 T cells and the life expectancy of HIV-1 infected patients, an observation which may result from the interference of Trx1 with virus-induced chemotaxis of immune cells (Nakamura et al, 2001a; Nakamura et al, 2001b). On the host cell, the CD4 co-receptor serves as primary ligand for HIV-1 gp120 to trigger fusion of the virus with the cell membrane and to mediate entry of the virus. The extracellular portion of CD4 consists of four immunoglobin-like domains (D1 to D4). The D2 domain contains a disulfide bond which can be reduced by Trx1 and locking of CD4 and the Trx1 active site in a reduced state blocked HIV-1 entry in susceptible cells (Matthias et al, 2002). Reduction of CD4 is enhanced in the presence of gp120 and it has been reported that Trx1 can also reduce disulfide bonds in gp120 (Ou & Silver, 2006). Even though the exact mechanism of how Trx1 regulates HIV-1 entry still needs to be clarified, these data provide strong evidence for an important role of Trx1 in HIV-1 infection.

Furthermore, Trx1 has been identified as growth factor secreted by an EBV-positive B cell line (Wakasugi et al, 1987) and it was shown to prevent EBV-transformed cells from entering the lytic phase of virus replication (Sono et al, 1999). In addition, Trx1 is thought to be involved in various non-viral diseases including reperfusion injury (Tao et al, 2004); Alzheimer's disease (Akterin et al, 2005) or cardiovascular disorders (Berndt et al, 2007).

1.4 Objective of this work

Although causing oxidative stress and damage at higher concentrations, ROS fulfill positive roles by regulating protein functions through reversible oxidation of regulatory cysteine residues. Cysteine-based redox-regulation includes the formation of disulfide bonds which are known to modulate the structure and activity of proteins. Trx1 catalyzes protein disulfide reduction to counteract oxidation and to influence the redox-dependent activity of its target proteins as, for example, transcription factor NFκB (Matthews et al, 1992), phosphatase cdc25 (Sohn & Rudolph, 2003) and PTP1B (Lee et al, 1998). Proteomic studies led to the identification of candidate H_2O_2-sensitive thiol proteins in mammalian cells (Ghezzi & Bonetto, 2003; Baty et al, 2005) but so far no proteome-wide identification of cytosolic Trx1 target proteins has been performed. The major obstacle for the detection of Trx1 target proteins is the short half-life of the thiol-disulfide exchange intermediate. In order to overcome this limitation, activity-based kinetic trapping was used to stabilize transient conjugates between Trx1 and its target proteins. This method is based on the catalytic reaction mechanism of the oxidoreductase in which the two active cysteines are involved.
Reduction of target disulfide bonds by Trx1 is mediated by the attack of the N-terminal cysteine in the CXXC active site motive, resulting in the formation of a mixed disulfide that covalently links Trx1 and its target protein. In a second reaction step, this intermolecular disulfide bond is attacked by the second active site cysteine, Cys35. Following the so-called escape reaction, the substrate is reduced. In turn, Trx1 itself is oxidized and will be reduced and thereby re-activated by TrxR under the consumption of NAPDH (Kallis & Holmgren, 1980) (Figure 10). Because of the escape reaction, disulfide-linked Trx1 target complexes are very short-lived and difficult to characterize. In order to stabilize these transient complexes, Cys35 was replaced by serine, thus prolonging the half-life of the intermediary enzyme-substrate conjugate (Qin et al, 1995; Wynn et al, 1995). Thus, the so-called trapping mutant Trx1(C35S) remains covalently linked to it substrates and can be isolated by an affinity tag attached to it.

Kinetic trapping has been used previously to study individual enzyme:substrate interactions of the bacterial periplasmatic oxidoreductase DsbA (Kishigami et al, 1995), plant thioredoxin f (Brandes et al, 1996), human Trx1 (Hirota et al, 1997) and the thiol oxidoreductase ERp57 (Dick et al, 2002; Dick & Cresswell, 2002). Later this

approach was used to perform proteomic screens for thioredoxin substrates in yeast (Verdoucq et al, 1999), plants (Hisabori et al, 2005) and recently on the surface of mammalian cells (Schwertassek et al, 2007). In order to identify cytosolic protein targets of Trx1 in mammalian cells, a proteome-wide Trx1 kinetic trapping screen was performed on cytosolic preparations of the human Jurkat T cell line. Conjugate formation between Trx1 and selected target proteins was analyzed by immunoblotting and target cysteine(s) were identified by site-directed mutagenesis.

In addition, novel tools for the analysis of Trx1-medited redox regulation were developed. A doxycycline-dependent expression system of the Trx1 trapping mutant was generated in Jurkat T cells in order to perform kinetic trapping of Trx1 target proteins in intact cells. Moreover, an inducible shRNA-based Trx1 knockdown system was established in HeLa cells in order to overcome the lack of specific Trx1 inhibitors for the analysis of Trx1-dependent processes.

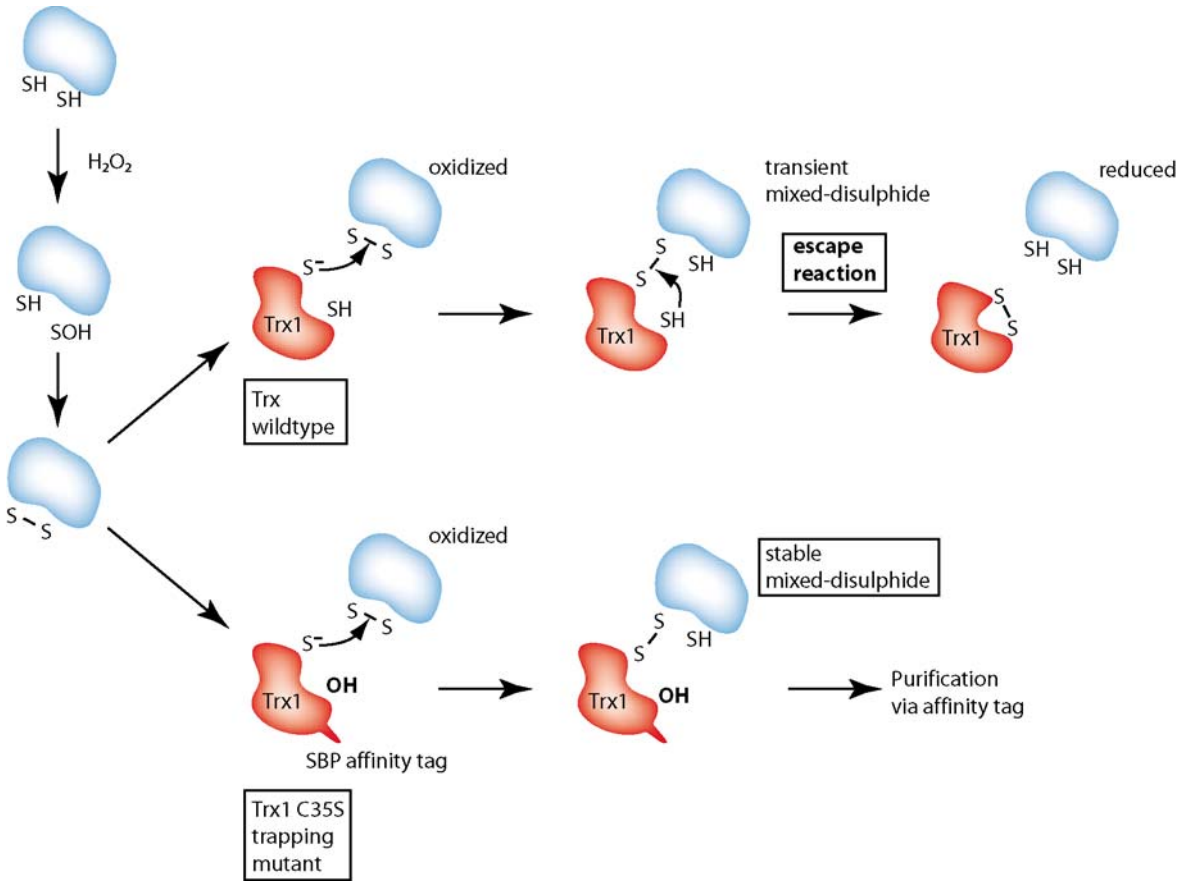

Figure 10: Principle of kinetic trapping
Wildt-ype Trx1 WT forms a transient mixed-disulfide with its target protein, which is rapidly resolved by the second active site cysteine (Cys35). Trx1:substrate complexes can be stabilized by mutation of Cys35 to serine (-OH). Addition of an SBP affinity tag to Trx1(C35S) allows for purification of disulfide-linked complexes with streptavidin sepharose.

2 Materials

2.1 Chemicals

Chemicals were from Sigma (Munich), Roth (Karslruhe), AppliChem (Darmstadt), Merck (Darmstadt), Serva (Heidelberg) or Perbio (Bonn) if not otherwise stated.

2.2 Laboratory equipment

Device	Source
Precision balance (AG287)	Mettler
Analytical balance (PB602-S)	Mettler
freezer -20°C	Liebherr
freezer -80°C	Sanyo
Darkroom film processor (Classic E.O.S)	AGFA
HLPC system (ÄKTA purifier)	GE Healthcare
Centrifuge (5415 R)	Eppendorf
Centrifuge (Heraeus Multifuge 3 S-R)	Kendro
Centrifuge (Heraeus Multifuge 4 KR)	Kendro
Centrifuge (Sorvall Evolution RC)	Kendro
PCR cycler (DNA Engine PTC-200)	MJ Research
Microplate reader (GENios)	TECAN
GelAir Drying System	Bio-Rad
Gel Dryer (Model 583)	Bio-Rad
CO_2 incubator (Heraeus BBD 6220)	Kendro
Flow hood (Heraeus Hera Safe)	Kendro
Water bath (Heraeus Julabo TW20)	Kendro
Incubator Shaker (Innova 4200)	New Brunswick Scientific
NuPAGE® Novex 4-12% Bis-Tris Gels (1.0 mm; 9 well)	Invitrogen
NuPAGE® Novex 4-12% Bis-Tris Gels (1.5 mm; 2D well)	Invitrogen

Electrophoresis Power Supply (EPS 3501XL)	GE Healthcare
Power supply (PowerPac Basic)	Bio-Rad
Power supply (PowerPac 1000)	Bio-Rad
Electrophoresis System (Mini PROTEAN® 3 Cell)	Bio-Rad
Electrophoresis System (SE 400 Sturdier)	GE Healthcare
Thermomixer (compact)	Eppendorf
UV/Vis Photospectrometer (Ultraspec 3100 pro)	GE Healthcare
Electrophoresis System (XCell Sure Lock Mini-Cell)	Invitrogen
Semi-dry blotter (Trans-Blot SD cell)	Bio-Rad
X-ray films (CL-XPosure)	Perbio

2.3 Buffers

Phosphate buffered saline (PBS)	137 mM	NaCl
	2.7 mM	KCl
	10 mM	Na_2HPO_4
	2 mM	KH_2PO_4
PBS-T	0.1 % (v/v)	Tween 20 in PBS
Tris buffered saline (TBS)	10 mM	Tris-HCl (pH 7.4)
	150 mM	NaCl
HEPES buffer saline (HBS)	50 mM	HEPES (pH 7.0)
	12 mM	Dextrose
	10 mM	KCl
	280 mM	NaCl
	1.5 mM	Na_2HPO_4
Lysis buffer	1 % (v/v)	Triton X-100 in TBS (pH 7.5)
		Complete protease inhibitor (Roche)

Streptavidin binding buffer	40 mM	Tris-HCl (pH 7.4)
	300 mM	KCl
	2 mM	EDTA (pH 8.0)
	0.1 % (v/v)	Triton X-100
DNA loading buffer (5x)	0.25 % (w/v)	bromophenol blue
	30 % (v/v)	glycerol
	10 mM	EDTA
	10 mM	Tris-HCl (pH 7.4)
TAE (50x)	2 M	Tris
	50 mM	EDTA (pH 8.0)

His-Tag protein purification:

Washing buffer	50 mM	NaH_2PO_4
	300 mM	NaCl
	20 mM	Imidazole
		pH 8.0
Elution buffer	50 mM	NaH_2PO_4
	300 mM	NaCl
	500 mM	Imidazole
		pH 8.0

SDS-PAGE:

Separation gel buffer	1.5 M	Tris/HCl pH 8.7
	0.1 % (w/v)	SDS
Stacking gel buffer	0.5 M	Tris/HCl pH 6.8
	0.1 % (w/v)	SDS
Running buffer (10x)	250 mM	Tris
	2.5 M	Glycine
	1 % (w/v)	SDS

SDS sample buffer (10x)	10 % (w/v)	SDS
	500 mM	Tris-HCl (pH 7.4)
	20 % (v/v)	Glycerol
	0.02 % (w/v)	bromophenol blue
MES running buffer (20x)	1 M	MES
	1 M	Tris
	0.1 % (w/v)	SDS
	20 mM	EDTA
Transfer buffer	25 mM	Tris
	192 mM	Glycine
	20 % (v/v)	Methanol
Stripping buffer	62.5 mM	Tris pH 6.8
	2% (w/v)	SDS
	0.7% (v/v)	β-mercaptoethanol
Silver staining:		
Fixing	30 % (v/v)	Ethanol
	10 % (v/v)	CH_3COOH
	60 % (v/v)	ddH_2O
Sensitization	0.02 % (w/v)	$Na_2S_2O_3 \cdot 5H_2O$
Impregnation	0.2 % (w/v)	$AgNO_3$
	0.075% (v/v)	formaldehyde
Developer	6 % (w/v)	Na_2CO_3
	2% (v/v)	sensitization solution
	0.05% (v/v)	Formaldehyde
Stop solution	4 % (w/v)	Tris
	2 % (v/v)	CH_3COOH

Colloidal Coomassie solution	10 % (w/v)	$(NH_4)_2SO_4$
	0.1 % (v/v)	H_3PO_4
	0.1 % (w/v)	Coomassie brilliant blue G250
	20 % (v/v)	methanol (add freshly before use)
Coomassie solution	0.25 % (w/v)	Coomassie brilliant blue R250
	10 % (v/v)	CH_3COOH
	45 % (v/v)	methanol

Other buffers and solutions are listed in the corresponding sections.

2.4 Antibodies

Specificity	Clone	Isotype, species	Stock conc.	Working dilution	Source	Cat. No.
beta-actin	AC-15	IgG1 mouse monoclonal	-	1:2.000	Sigma	A5441
Annexin-1	29	IgG1 mouse monoclonal	-	1:5.000	BD Biosciences	610066
Annexin-2	5	IgG1 mouse monoclonal	-	1:5.000	BD Biosciences	610068
Annexin-7	5	IgG1 mouse monoclonal	250 µg/ml	1:1.000	BD Biosciences	610668
Annexin-11	16	IgG1 mouse monoclonal	250 µg/ml	1:15.000	BD Biosciences	611878
caspase-2	35	IgG1 mouse monoclonal	250 µg/ml	1:1.000	BD Biosciences	611022
caspase-3	-	rabbit polyclonal	-	1:2.000	Cell Signaling	9662
caspase-8	C15	IgG2B mouse monoclonal	-	1:100	P. Krammer	-
caspase-9	F-7	IgG2A mouse monoclonal	0.2 mg/ml	1:1.000	Santa Cruz	sc-17784
cdk1	17	IgG2A mouse monoclonal	0.2 mg/ml	1:1.000	Santa Cruz	sc-54
cdk2	-	rabbit polyclonal	0.5 mg/ml	1:1.000	I. Hoffmann	-
cdk4	C-22	rabbit polyclonal	0.2 mg/ml	1:1.000	Santa Cruz	sc-260
cdk6	C-21	rabbit polyclonal	0.2 mg/ml	1:1.000	Santa Cruz	sc-177
Flag-tag	M2	IgG1 monoclonal mouse	1 mg/ml	1:1.000	Sigma	F1804
Peroxiredoxin-1	-	rabbit polyclonal	-	1:5.000	Upstate	07-609
p120RasGAP	B4F8	mouse monoclonal	1 mg/ml	1:1.000	Upstate	05-178

SBP-tag	-	rabbit polyclonal	1 mg/ml	1:10.000	M. Gutscher	-
Thioredoxin-1	-	rabbit polyclonal	1 mg/ml	1:10.000	M. Preuss	-

Antibody dilutions were stored in PBS-T / 5% (w/v) BSA / 0.01% (v/v) NaN_3 at 4°C.

Secondary HRP-conjugated antibodies

Specificity	Clone	Isotype, species	Stock conc.	Working dilution	Source	Cat. No.
mouse	-	goat polyclonal	0.8 mg/ml	1:10.000	Dianova	115-035-146
rabbit	-	goat polyclonal	0.8 mg/ml	1:10.000	Dianova	111-035-144

2.5 Media for eukaryotic cells and bacteria

Media for eukaryotic cells

Dulbecco's modified Eagle medium (DMEM), Gibco
Iscove's modified Dulbecco's medium (IMDM), Gibco
Roswell Park Memorial Institute medium (RPMI), Gibco
Fetal calf serum (FCS) (Gibco), working concentration: 10% (v/v)
Penicillin, streptomycin (Gibco), working conc. 100 U/ml and 100 µg/ml, respectively
Neomycin, G418 (Gibco), stock: 500 mg/ml, working concentration: 1 mg/ml
Puromycin (Gibco), stock: 1 mg/ml, working concentration: 1 µg/ml
Hygromycin (Invitrogen), stock: 50 mg/ml, working concentration: 250 µg/ml
Doxycycline (Sigma), stock: 1 mg/ml, working concentration: 1 µg/ml

Media for bacteria

LB broth (Luria/Miller) dry medium containing 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, pH7.0 $\pm$ 0.2 was obtained from Roth (Karlsruhe). 25 g/l dry medium were dissolved in ddH_2O and and sterilized by autoclavation.

LB agarose plates were prepared by addition of 1.5% (w/v) agarose prior to

sterilization.

For selection medium or plates the following antibiotics were added after autoclavation:

Ampicillin: stock solution 100 mg/ml in ddH_2O, working concentration: 100 µg/ml

Kanamycin: stock solution: 25 mg/ml in ddH_2O, working concentration: 25 µg/ml

2.6 Biological material

E.coli strains

Strain	Characteristics
E.coli M15 [pREP4]	NalS StrS RifS Thi^- Lac^- Ara^+ Gal^+ Mtl^- F^- $RecA^+$ Uvr^+ Lon^+
E.coli XL1-Blue	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F´ proAB $lacI^qZ\Delta M15$ Tn10 (Tet^r)]
E.coli DH5-alpha	F' Phi80dlacZ DeltaM15 Delta(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rK-mK+)phoA supE44 lambda- thi-1

Eukaryotic cell lines

Line	Characteristics	Medium	Selection
HeLa T-REx	HeLa cells expressing the Tet repressor (TetR) (Invitrogen)	DMEM	-
HEK 293T	human embryonic kidney cell line expressing the SV40 large T-antigen	DMEM	-
Phoenix-Ampho	human embryonic kidney cell line stably expressing a amphotrophic viral capsid protein	DMEM	-
J16-145	Subclone of the human Jurkat acute lymphoblastic leukemia T cell line J16 (Gülow et al, 2005)	IMDM	-
HH	T cell line established from the blood of a 61 year old patient with an aggressive cutaneous T-cell leukemia/lymphoma (Starkebaum et al, 1991)	RPMI	-
MyLa	Sezary T-cell leukemia line obtained from a plaque biopsy of a patient with mycosis fungoides (Kaltoft et al, 1992)	RPMI	-
Hut78	T cell lymphoma derived from peripheral blood of a 50 year old male patient with Sezary syndrome (Gootenberg et al, 1981)	RPMI	-

Molt-4	T-cell line derived from the peripheral blood of a 19 year old male with acute lymphoblastic leukemia in relapse (Minowada et al, 1972)	RPMI	-
CEM	T lymphoblastoid line obtained from the peripheral blood of a 4 year old Caucasian female with acute lymphoblastoid leukemia (Foley et al, 1965)	RPMI	-
J16-M2.15	J16-145 transduced with the rtTA-M2, clone 15	IMDM	Puromycin
J16-M2 -Trx1 WT -CSCCC -CCAAA -CSAAA	J16-M2.15 transduced with pRevTight-Trx1 WT, CSCCC, CCAAA or CSAAA See section 4.5.1	IMDM	Puromycin Hygomycin
HeLa T-REx -212 -252 -281 -NCR	HeLa T-REx transduced with pSR-TRE-NG shTrx1 212, 252, 281 or NCR See section 4.5.2	DMEM	Neomycin

2.7 Expression vectors

Plasmid	**Source**
pSUPER-Retro-NeoGFP	Oligoengine
pTER	Hans Clevers (van de Wetering et al, 2003)
pCMV-3Tag-4A	Stratagene
pcDNA3-cdk6-HA	Addgene #1868 (van den Heuvel & Harlow, 1993)
pQE60-hTrx1-SBP-His	described earlier (Schwertassek et al, 2007)
pcDNA3	Invitrogen
Rc/CMV cyclin D3	Addgene #10912 (Hinds et al, 1992)
pUHrT 62-1	Herman Bujard (Urlinger et al, 2000)
pRev-TRE	Clontech
pTRE-Tight	Clontech
pMXs-IN-Puro	Adelheid Cerwenka (Tessarz et al, 2007)

2.8 Enzymes and kits

Pfu Turbo polymerase	Stratagene
shrimp alkaline phosphatase	Promega
Quick Ligation Kit	New England Biolabs
BCA Protein Assay Kit - Reducing Agent Compatible	Perbio
QuickChange Multi Site-Directed Mutagenesis Kit	Stratagene
Gel extraction kit NucleoSpin Extract II	Macherey-Nagel
NucleoSpin Plasmid QuickPure	Macherey-Nagel
NucleoBond AX	Macherey-Nagel
Chemiluminescent Substrate SuperSignal West Femto	Perbio
Chemiluminescent Substrate SuperSignal West Pico	Perbio
1 kb Plus DNA Ladder	Invitrogen
Precision Plus Protein Dual Color Standard	Bio-Rad

2.9 Software

The following computer software was used: Microsoft Office 2003, Adobe Acrobat Professional 7.0, Adobe Illustrator CS3, Adobe Photoshop CS2, Textco Gene Construction Kit 2.5, Thomson EndNote 6, BD CellQuest Pro, Deep View Swiss-PdbViewer 4.0, ClustalW.

3 Methods

3.1 Methods in molecular biology

3.1.1 Small scale isolation of plasmid DNA from *E.coli*

For analytical preparation of plasmid DNA the NucleoSpin Plasmid QuickPure kit (Macherey-Nagel) was used. An *E.coli* culture volume of 3 ml was resuspended in RNase-containing buffer and plasmid DNA was liberated by SDS/alkaline lysis. After neutralization and clarification of the lysate, the plasmid was bound onto a silica membrane and washed with ethanol-containing buffer. Finally, pure plasmid DNA was eluted in 50 µl H_2O.

3.1.2 Large scale isolation of plasmid DNA from *E.coli*

Quantitative preparation of plasmid DNA was performed with the NucleoBond AX kit (Macherey-Nagel). Overnight cultures of 250 to 500 ml were lysed in alkaline buffer supplemented with RNase, neutralized and plasmid DNA-containing supernatant was loaded onto a macroporous silica matrix functionalized with methyl-amino-ethanol (MAE). The positively charged MAE group specifically binds the negatively charged phosphate backbone of plasmid DNA under acidic pH. After washing, plasmid DNA is eluted in a high-salt elution buffer and precipitated with 0.7 volumes of isopropanol. NucleoBond Finalizer silica membranes (Macherey-Nagel) were used to concentrate and desalt the precipitated plasmid DNA. Finally, elution was done with 0.5 to 1 ml ddH_2O, depending on the copy number of the plasmid.

3.1.3 Restriction digest of DNA

Restriction enzymes were from Promega and New England Biolabs. For cloning 3 µg DNA were digested for 1 h at 37°C. 1 U shrimp alkaline phosphatase (Promega) was added to preparative vector digestions to avoid re-ligation of the digested plasmid.

3.1.4 Polymerase chain reaction (PCR)

Selective amplification of DNA sequences was performed by using the polymerase chain reaction (PCR), which is based on cyclic changes in temperature, which provides optimal conditions for the different reaction steps. After denaturation of template DNA, specific oligonucleotides (primers) hybridize with single stranded DNA to form a double stranded starting point for DNA polymerase. In the second

elongation step double stranded DNA is synthesized from the single stranded DNA template. The newly synthesized DNA double strand is separated in the denaturation step and will serve as template for the next amplification cycle.
Reaction mix for one sample:

2.5 µl	10x *Pfu* reaction buffer
1 µl	dNTP mix (25 mM each)
1 µl	forward primer (10 pmol/µl)
1 µl	reverse primer (10 pmol/µl)
1 µl	template DNA (200 ng/µl)
1 µl	*Pfu* Turbo polymerase (2.5 U/µl)
17.5 µl	ddH_2O

PCR program:

Initial denaturation		5 min 95°C
30 cycles	denaturation	5 min 95°C
	annealing	1 min 50°C*
	elongation	5 min** 68°C
Final elongation		10 min 68°C

* annealing temperature depends on the primers used
** elongation time depends on the product size (speed of *Pfu* Turbo: 500 bp/min)

3.1.5 Site-directed mutagenesis

The QuikChange site-directed mutagenesis kit (Stratagene) was used to introduce point mutations into plasmid DNA. Two primers each complementary to opposite strands of the vector and containing the desired mutation were designed and used for mutagenesis PCR with *PfuTurbo* polymerase. This enzyme replicates both plasmid strands with high fidelity without displacing the mutated primers which leads to the generation of mutated nicked plasmids. Thereafter, *Dpn*I was added to specifically digest methylated parental plasmid DNA and select for mutation-containing DNA. Nicks in the mutated plasmid are repaired in *E.coli* after transformation.

3.1.6 Agarose gel electrophoresis

The agarose powder (standard gel 1% (w/v) agarose) was dissolved in TAE buffer by boiling in a microwave. After cooling down to approximately 50°C, 0.5 µg/ml ethidium bromide was added to stain the DNA. The DNA sample was mixed with DNA loading dye and loaded on the gel. The gel was run 1 h at 100 V using a Power Pack Basic power supply (Bio-Rad). Pictures of the gel were taken with a GelDoc 2000 (Bio-Rad).

3.1.7 DNA extraction from agarose gels

The DNA band of interest was visualized with mild UV light and cut out of the gel with a scalpel. The DNA was purified with a NucleoSpin Extract II kit (Macherey&Nagel) according to the manufacturer's protocol and eluted in ddH_2O. The same kit was used to desalt DNA from PCRs.

3.1.8 Ligation of DNA fragments

Purified vector and insert DNA were mixed together in a ratio of 1:9 filled up with ddH_2O to 10 µl. Then 10 µl 2x Quick Ligase buffer and 1 µl T4 Quick Ligase (New England Biolabs #M2200L) was added and the mixture was incubated for 10 min at RT.

3.1.9 Cloning of expression vectors

The expression vector pcDNA3-ckd6-HA was obtained from Addgene (#1868) (van den Heuvel & Harlow, 1993). Cysteine-to-serine mutations were introduced with the QuikChange site-directed mutagenesis kit (Stratagene) using the following oligonucleotides:

Oligo	Sequence
C7S_F	GAAGGACGGCCTGTCCCGCGCTGACCAG
C7S_R	CTGGTCAGCGCGGGACAGGCCGTCCTTC
C15S_F	GACCAGCAGTACGAATCCGTGGCGGAGATCG
C15S_R	CGATCTCCGCCACGGATTCGTACTGCTGGTC
C83S_F	CAGGTTGTTTGATGTGTCCACAGTGTCACGAACAG
C83S_R	CTGTTCGTGACACTGTGGACACATCAAACAACCTG
C207S_F	CTCTGGAGTGTTGGCTCCATATTTGCAGAAATGTTTCG
C207S_R	CGAAACATTTCTGCAAATATGGAGCCAACACTCCAGAG
C280S_F	GGCAAAGACCTACTTCTGAAGTCTTTGACATTTAACCCAG
C280S_R	CTGGGTTAAATGTCAAAGACTTCAGAAGTAGGTCTTTGCC
C306S_F	CAGGACCTGGAAAGGTCCAAAGAAAACCTGGATTCC
C306S_R	GGAATCCAGGTTTTCTTTGGACCTTTCCAGGTCCTG

Cdk6-mutants were cloned into pCMV-3Tag-4A in order to replace the HA tag with a 3xMyc Tag. The cdk6 coding sequence lacking the stop codon was amplified by PCR and inserted into pCMV-3Tag-4A with *Hind*III and *Xho*I using the following primers:

HindIII_cdk6_F	CCCAAGCTTATGGAGAAGGACGGCC
cdk6_XhoI_R	CCCCTCGAGGGCTGTATTCAGCTC

Eventually, using *Xho*I and *Kpn*I the 3xMyc tag of pCMV-3Tag-4A-cdk6 was replaced by a 3xFlag tag which was generated by annealing the following oligonucleotides:

3xFlag sense	TCGAGGATTACAAGGATGACGACGATAAGGACTATAAGGACGATGATGAC AAGGACTACAAAGATGATGACGATAAATAAGGGCCCGGTAC
3xFlag anti-sense	CGGGCCCTTATTTATCGTCATCATCTTTGTAGTCCTTGTCATCATCGTCC TTATAGTCCTTATCGTCGTCATCCTTGTAATCC

3.1.10 Transformation of *E.coli*

Competent *E.coli* DH5α or *E.coli* XL1-blue were thawn on ice. The ligated DNA was added to the bacteria and incubated for 10 min on ice. Heat-shock transformation for 2 min at 42°C was performed in a Thermomixer compact (Eppendorf). After addition of 500 µl fresh LB medium without antibiotics, the bacteria were incubated with shaking for 1 h at 37°C to recover. Finally the bacteria were plated on LB-agar plates containing 100 µg/ml Ampicillin or 25 µg/ml Kanamycin.

3.2 Methods in cell biology

3.2.1 Cultivation of cell lines

All cells were cultivated at 37°C, 5% CO_2. HEK 293T and HeLa T-REx cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS / 100 U/ml penicillin / 100 µg/ml streptomycin. Jurkat J16-145 cells were cultivated in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FCS / 100 U/ml penicillin / 100 µg/ml streptomycin. HeLa T-REx cells transfected with pSR-TRE-NeoGFP shRNA plasmids were cultivated in the presence of 1 mg/ml G418 (Neomycin).

3.2.2 Cryoconservation of cell lines

For long-term storage, 1 x 10^6 cells were resuspended in 1 ml FCS / 10% DMSO and transferred into cryovials, which were cooled down to -80°C with 1°C/min. After 24-48 h cells were stored in liquid nitrogen. For thawing, frozen cells were incubated at 37°C in the water bath and washed twice with 10 ml pre-warmed PBS. After washing cells were resuspended in 5 ml cell culture medium and transferred to a 25 cm^2 cell culture flask.

3.2.3 Transfection of eukaryotic cell lines

Exponentially growing HEK 293T cells were harvested 24 h before transfection by trypsinization and replated at a density of 1 x 10^5 to 4 x 10^5 cells/cm^2 in a 175 cm^2 tissue culture flask in 25 ml DMEM / 10% FCS. For the preparation of the calcium phosphate-DNA coprecipitate, 50 µg DNA were added to 2.2 ml H_2O, combined with 305 µl 2.5 M $CaCl_2$ and incubated 5 min at room temperature. Meanwhile 25 mM chloroquine diphosphate was diluted 1:1,000 directly into the medium. Afterwards 2.5 ml 2x HEPES buffered saline (HBS) (50 mM HEPES, 12 mM dextrose, 10 mM KCl, 280 mM NaCl, 1.5 mM Na_2HPO_4, pH 7.0) were added to the calcium chloride-DNA solution and air was blown into the mixture for 10 sec. After additional 30 sec the calcium phosphate-DNA suspension was transferred into the medium above the cell monolayer. The flask was gently rocked to mix the medium which will become turbid. After 6-7 hours the medium and DNA precipitate was removed by aspiration and fresh pre-warmed DMEM medium was added. The cells were analyzed 48 h after transfection.

3.2.4 Retroviral transduction of eukaryotic cells

Retrovirus-based transduction was used to stably integrate a gene of interest in the genome of eukaryotic target cells. As retrovirus producer cell line the moloney murine leukemia virus (MMULV)-based amphotropic cell line phoenix ampho was used. Phoenix ampho cells were harvested 24 h before transfection by trypsinization and 1.5 x 10^6 cells were replated in a 25 cm^2 tissue culture flask in 4 ml DMEM / 10% FCS. Before transfection, 25 mM chloroquine diphosphate was diluted 1:1,000 directly into the medium. The transfection was performed as described above using 10 µg DNA, 37 µl 2 M $CaCl_2$ and H_2O to a final volume of 300 µl. Formation of calcium phosphate precipitates was induced by addition of 300 µl 2x HBS. After the

calcium phosphate-DNA suspension was added, the cells were transferred to an incubator with biosafety level 2 and medium was changed after 6-7 hours. After 48 h the virus-containing supernatant was harvested and filtered through a 0.45 µM filter. Infection was performed by addition of 1.5 ml of virus-containing supernatant to 5 x 10^5 target cells cultivated in a 24-well plate. To further enhance the infection rate, 1 µg/µl polyprene (8 mg/ml) was added. Spin-infection was performed with suspension cells by centrifugation for 2 h at 1,350 x g. Adherent cells were not subjected to spin-infection, but directly placed into an incubator for 24 h. After washing with medium, cells were seeded into 25 cm^2 cell culture flasks. To select for stably transduced target cells, antibiotics were added after 48 h.

3.2.5 Inducible short-hairpin RNA (shRNA)-mediated knockdown of Trx1

The administration of small-interfering RNA (siRNA) has been described to efficiently suppress gene expression in many eukaryotic cells. Because this reduction in gene expression by siRNA is only transient, mammalian expression vectors have been developed for the synthesis of short-hairpin RNA (shRNA), which are processed intracellularly into short duplex RNAs with siRNA-like properties. Even though cells stably transfected with shRNA vectors can maintain long-term suppression of gene expression, these cells could undergo adaptation by reducing shRNA expression or other compensatory mechanisms. To overcome the problem of adaptation for the knockdown of Trx1, a silencing vector for the doxycycline-dependent expression of Trx1-specific shRNA was generated. For this purpose the H1 promoter of pSUPER-retro-NeoGFP (Oligoengine) was replaced with the TetO binding site containing H1 promoter of pTER (kindly provided by Hans Clevers, Utrecht, Netherlands) using *Eco*RI and *Hind*III. Trx1-specific target sequences were computed using the SiSearch algorithm (Chalk 2004). For each of the four different targeting sequences two complementary 63mers were synthesized (see below). 3 µg of each were resuspended in annealing buffer (10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA) and heated to 90°C for 5 min on a thermocycler. The oligonucleotides were cooled down to 4°C with 0.1°C/sec to generate double-stranded DNA, which was then cloned into pSR-TER-NeoGFP with *Bgl*II and *Xho*I. HeLa T-REx cells (Invitrogen) stably expressing the Tet-repressor were retrovirally transduced with pSR-TRE-NeoGFP and selected with 1 mg/ml G418/neomycine after 48 h post infection. Trx1 knockdown was induced by addition of 1 µg/ml doxycycline (Sigma).

Oligo	Sequence
212 forward	GATCCCCCAACGTGATATTCCTTGATTCAAGAGATCAAGGAATATCACGTTGGTTTTTGGAAC
212 reverse	TCGAGTTCCAAAAACCAACGTGATATTCCTTGATCTCTTGAATCAAGGAATATCACGTTGGGG
252 forward	GATCCCGGATGTTGCTTCAGAGTGTTTCAAGAGAACACTCTGAAGCAACATCCTTTTTTGGAAC
252 reverse	TCGAGTTCCAAAAAGGATGTTGCTTCAGAGTGTTCTCTTGAAACACTCTGAAGCAACATCCGG
281 forward	GATCCCGCATGCCAACATTCCAGTTTTCAAGAGAAACTGGAATGTTGGCATGCTTTTTTGGAAC
281 reverse	TCGAGTTCCAAAAAGCATGCCAACATTCCAGTTTCTCTTGAAAACTGGAATGTTGGCATGCGG
NCR forward	GATCCCCCAGTTGCCATCTGCGTGATTCAAGAGATCACGCAGATGGCAACTGGTTTTTTGGAAC
NCR reverse	TCGAGTTCCAAAAACCAGTTGCCATCTGCGTGATCTCTTGAATCACGCAGATGGCAACTGGGG

3.3 Methods in protein biochemistry

3.3.1 Purification of recombinant proteins from *E.coli*

E.coli M15 [pREP4] (Qiagen) were transformed with Trx1 constructs according to standard protocol (Sambrook et al., 2001). LB medium supplemented with Ampicillin [100 µg/ml] and Kanamycin [25 µg/ml] was inoculated with transformed bacteria and incubated over night at 37°C until the OD [600 nm] reached 0.7-0.8. Protein expression was induced by addition of 1 mM IPTG and cultures were incubated for 3 h at 37°C. Bacteria were harvested in 250 ml-centrifugation buckets at 6000 rpm, 4°C, 15 min (Rotor: SLA 1500; Sorvall Evolution RC). Pellets were solubilized in 40 ml B-PER Bacterial Protein Extraction Reagent (Pierce) supplemented with 10 mM Imidazole and incubated for 10 min at RT with end-over-end rotation. Extracts were centrifuged at 20,500 rpm, 4°C, 15 min (Rotor: SS34; Sorvall Evolution RC). Supernatants were mixed with 2 ml of HIS-Select Nickel Affinity Gel (Sigma-Aldrich) equilibrated with extraction reagent, and incubated for 1 h at 4°C with end-over-end rotation. Beads were loaded onto 5 ml-Polypropylene columns (Qiagen) and flow-through was collected for a second incubation with Nickel beads. Columns were washed four times with 5 ml washing buffer (for protein purification) and proteins were eluted in four steps with 1.5 ml of elution buffer and 5 min incubation after addition of the buffer. Beads from the second incubation (flow-through) were treated the same.

Alternatively, extract supernatants were frozen in liquid nitrogen after centrifugation and stored at -80°C. After thawing, supernatants were centrifuged again at 20,500 rpm, 4°C, 15 min (Rotor: SS34; Sorvall Evolution RC) to remove precipitates. Protein was purified on an ÄKTA purifier using HisTrap FF columns (1 ml). Washing and elution buffer were the same as for manual purification except that 1 M imidazol

was added to the elution buffer. Proteins were eluted in 1 ml-fractions. Purified protein was stored in 1-ml aliquots supplemented with 5 mM DTT and 5 mM EDTA in PBS at 4°C. Protein concentration was determined using the BCA Protein Assay Kit - Reducing Agent Compatible (Pierce) according to the manufacturer's protocol.

3.3.2 Immunoprecipitation of protein complexes

Proteins were immunoprecipitated from lysate of 1 x 10^8 cells by addition of 5 µg of the corresponding antibody and 30 µl protein G sepharose beads (GE Healthcare). After 1 h end-over-end rotation, the beads were washed five times with 1 ml lysis buffer, resuspended in 50 µl SDS-sample buffer and boiled for 5 min at 95°C. Precipitated proteins were analyzed by immunoblotting.

3.3.3 Mechanism-based kinetic trapping

For substrate trapping of intracellular Trx1 target proteins, Jurkat cells (5 x 10^7) were washed twice with PBS and permeabilized with TBS / 0.01% digitonin (pH 7.5) for 5 min on ice. Streptavidin sepharose beads (Amersham Biosciences) were loaded with recombinant oxidoreductase in streptavidin binding buffer (40 mM Tris-HCl (pH 7.4), 300 mM KCl, 2 mM EDTA, 0.1% T-X100) with 20 mM DTT and subsequently washed to remove the reductant. The beads were added to the supernatant of digitonin-permeabilized cells and incubated for 1h at 4°C. The reaction was stopped with 20 mM N-ethylmaleimide (NEM) and beads were washed two times with TBS / 500 mM NaCl /10 mM NEM /1% T-X100 (pH 7.5), once with TBS / 1 mM NEM / 1% T-X100 (pH 7.5), once with TBS / 1 M urea / 1 mM NEM / 1% T-X100 (pH 7.5) and once with TBS / 0.1% T-X100 (pH 7.5) (300 x g, 4°C, 3 min). Protein complexes were eluted with 2 mM biotin in PBS for 30 min at 4°C, treated with 40 mM NEM and separated by SDS-PAGE under non-reducing or reducing conditions. Proteins were visualized by silver staining and analyzed by LC-MS/MS.

For re-capturing of denatured Trx1 target complexes, they were separated from streptavidin sepharose beads by boiling for five minutes in 10 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1% SDS, 1 mM iodoacetamide. Before fresh streptavidin sepharose beads were added, the SDS concentration was reduced to 0.1% by dilution with 10 mM Tris/HCl (pH 7.4), 150 mM NaCl. Washing and elution was performed as described above.

3.3.4 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed using either Mini-PROTEAN 3 Cell (Bio-Rad) for small gels or SE 400 Sturdier Vertical Slab Gel Electrophoresis Units (GE Healthcare) for large gels:

Stacking gels:

	4%	**6%**
4x Upper Buffer [ml]	6.25	6.25
30% Acrylamid/N,N'-Methylen-bis(acrylamid) [29:1] [ml]	5	3.33
10% APS [µl]	100	100
TEMED [µl]	25	25
H_2O [ml]	12.5	14.2
Total [ml]	24	24

Separation gels:

	8%	**10%**	**12%**	**15%**
4x Lower Buffer [ml]	15	15	15	15
30% Acrylamid/N,N'-Methylen-bis(acrylamid) [29:1] [ml]	16	20	24	30
10% APS [µl]	500	500	500	500
TEMED [µl]	50	50	50	50
H_2O [ml]	28	24	20	14
Total [ml]	60	60	60	60

For non-reducing SDS-PAGE, protein samples were mixed with SDS sample buffer and boiled for 5 min at 95°C. For reducing SDS-PAGE, 20 mM DTT was added before boiling. After cooling to RT, samples were mixed with 40 mM NEM and incubated for 5 min at RT before loading on the gel. Precision Plus Protein™ Dual Color Standard (Bio-Rad) was used as a standard protein marker. Large gels were run for 16 h at 80 V, small gels were run for 1-2 h at 100 V.

3.3.5 2D diagonal SDS-PAGE (non-reducing/reducing)

For the identification of Trx1 target proteins, conjugates from 1.5 x 10^9 J16-145 cells were loaded onto a 5-12% gradient SDS-PAGE under non-reducing conditions. The lane was excised and incubated with SDS sample buffer supplemented with 250 mM DTT for 20 min at 56°C. DTT was removed by washing with DTT-free SDS sample buffer and NEM was added to a final concentration of 100 mM. After incubation for 20 min at 21°C, the lane was washed again and transferred to a 5-12% SDS-PAGE gradient gel. Proteins were visualized with colloidal Coomassie and analyzed by mass spectrometry. Pre-cast 4-12% NuPAGE Bis-Tris gradient gels (Invitrogen) were

used for non-preparative N/NR gel using the same protocol as for preparative gels. Protein complexes from these gels were visualized by silver-staining.

3.3.6 Colloidal Coomassie staining

For staining with colloidal Coomassie, gels were washed two times for 5 min in ddH_2O and stained in colloidal Coomassie staining solution (12.5% (w/v) $(NH_4)_2SO_4$, 1.45% (v/v) H_3PO_4, 0.125% (w/v) Coomassie Blue G-250, 20% methanol) over night at RT. The staining solution was exchanged and gels were incubated again for 2 h at RT. Gels were destained in ddH_2O. For staining with Coomassie Brilliant Blue, gels were treated as described above but stained in Coomassie staining solution and destained in the same solution without dye.

3.3.7 Silver staining

Gels were incubated in fixing solution (10% acetic acid, 30% ethanol, 60% H_2O) over night or for 1 h at RT. Gels were washed three times 15 min with 30% ethanol, sensitized for 30 min at RT in sensitization solution (0.02% (w/v) $Na_2S_2O_3$ x 5 H_2O) washed in ddH_2O twice for 1 min and incubated in impregnation solution (0.2% (w/v) $AgNO_3$, 0.075% (v/v) formaldehyde) for 1 h. After briefly rinsing twice with ddH_2O, gels were developed in developer solution (6% (w/v) Na_2CO_3, 2% (v/v) sensitization solution, 0.05% (v/v) formaldehyde) until bands were clearly visible and the reaction was stopped with stop solution (4% (w/v) Tris, 2% acetic acid).

3.3.8 Immunoblotting

Proteins separated by SDS-PAGE were transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon-P, Millipore) by semi-dry blotting. PVDF membranes were activated with methanol for 15 sec, washed twice with ddH_2O and equilibrated with Towbin buffer for 15 min. Semi-dry blotting was performed using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad) according to the manufacturer's protocol for 1 h at 15 V. Membranes were blocked with TBS / 5% non-fat milk for 1 h at RT or ON at 4°C, before the primary antibody was added for 1 h at RT or ON at 4°C. Working dilutions for primary antibodies are listed in section 2.4. After washing three times with PBS-T, horseradish peroxidase (HRP)-coupled secondary antibodies were added for 45 min at RT, followed by additional five washing steps for at least 5 min each. Peroxide-luminol solution (Supersignal West, Pierce) was added and chemo luminescence was detected with x-ray films.

3.3.9 Stripping of immunoblots

Bound antibodies were removed from PVDF membranes by so-called “stripping”. Freshly prepared stripping buffer (62.5 mM Tris/HCl, 2% (v/v) SDS, 0.7% (v/v) β-mercaptoethanol, pH 6.8) was added to membranes and incubated for 20 min at 65°C. Before the membrane was blocked again with PBS-T / 5% non-fat milk, it was briefly washed 10 times with ddH_2O, shaked in ddH_2O for 5 min and again briefly washed 10 times in ddH_2O.

3.3.10 Mass spectrometry

Individual spots were excised, reduced with DTT, alkylated with iodoacetamide and digested with trypsin (Catrein et al, 2005) using a Digest pro MS liquid handling system (Intavis AG). Following digestion, tryptic peptides were extracted from the gel pieces with 50 % acetonitrile/0.1 % TFA and concentrated. For MALDI-TOF mass spectrometry (Ultraflex, Bruker), the sample was desalted using ZipTip and spotted onto a steel target using a-cyano-4-hydroxy cinnamic acid as matrix, and the peptide mass fingerprint (PMF) was acquired after external calibration (peptide calibration standard II, Bruker). For protein identification by PMF, peptide masses were labeled manually without smoothing and background subtraction using a sample from an empty area of the same gel as background control. The PMF was searched against the NCBInr database (download 04.01.2007) using Mascot 2.1 (Matrix Science). The algorithm was set to use *homo sapiens* as taxonomy (189945 entries), trypsin as enzyme, allowing at maximum for one missed cleavage site and assuming carbamidomethyl as a fixed modification of cysteine and oxidized methionine as a variable modification. Mass tolerance was set to 100 ppm. Protein hits were taken as identified if the mascot score exceeded the significance level ($p<0.05$).

3.3.11 Caspase-2 activity assay

For the detection of caspase-2 activity 1 x 10^6 SWK 6.4 cells were left untreated or stimulated in 1 ml RPMI / 10% FCS with 10 µg/ml anti-APO for 1 h. Cells were lysed in 300 µl caspase assay buffer without DTT (50 mM HEPES, 100 mM NaCl, 0.1% (w/v) CHAPS, 10% (w/v) sucrose, pH 7.4) (Lavrik et al, 2006). Trx1 was pre-reduced with 30 mM DTT and eluted 2 mM biotin to a final concentration of 100 µM. 5 µl reduced Trx1 was mixed with 45 µl lysate. The caspase-2 substrate N-Acetyl-Val-Asp-Val-Ala-Asp-7-amido-4-trifluoromethylcoumarin (Ac-VDVAD-AFC) was obtained from Sigma (#A-5345) and a 20 mM stock solution was prepared in DMSO. A

100 µM working dilution was prepared in caspase-2 assay buffer. 50 µl 100 µM Ac-VDVAD-AFC was mixed with 50 µl Trx1-containing lysate in a 96 well microtiter plate. Fluorescence at 505 nm was measured after excitation at 400 nm every 5 min for 2 h.

For the *in vitro* caspase-2 assay, 0.25 units of recombinant caspase-2 (Alexis # ALX-201-057-U025) were incubated with 10 µM pre-reduced Trx1, 2.5 µM TrxR and 1 mM NAPDH in 50 µl caspase-2 assay buffer. After addition of 50 µl 100 µM Ac-VDVAD-AFC fluorescence was recorded as described above.

3.3.12 *In vitro* kinase assay

Activity of cdk6 was measured by its ability to phosphorylate a recombinant retinoblastoma (Rb) peptide *in vitro*. 5 x 10^6 HEK 293T cells were co-transfected with SBP-tagged cdk6 and its regulatory subunit cyclin D3 and lysed on ice in cdk6 lysis buffer (40 mM Tris/HCl, 0.4% (v/v) NP-40, 150 mM NaCl, 2 mM EDTA, 0.5 mM EGTA, 5 mM NaF, 10 mM glycerol-2-phosphate, Complete protease inhibitors (Roche), pH 7.5). Pre-clear of the lysate was done twice for 30 min at 4°C with 100 µl (50% slurry) CL-4B sepharose (GE Healthcare) equilibrated in lysis buffer. Cyclin D3 – cdk6 complexes were isolated by addition of 60 µl streptavidin (SA) sepharose (50 % slurry, GE Healthcare) for 1 h at 4°C. After washing three times with 3 ml lysis buffer, cdk6-loaded SA beads were equilibrated in 1 ml kinase assay buffer (KAB) (50 mM Tris/HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.5), and a 50% slurry was prepared. From this slurry, 10 µl beads were mixed with 7.5 µl KAB, 2.5 µl recombinant Rb peptide (2 µg/µl, amino acids 773-928, Upstate #12-439) and 1 µl ^{32}P γ-ATP (Perkin Elmer #NEG502A250UC, 10 mCi/ml, 3000 Ci (111 TBq)/mmol). The reaction was incubated at 30°C for 10 min while shaking with 650 rpm and stopped by addition of 4 µl 5x reducing SDS sample buffer and heating for 5 min at 90°C. Free ^{32}P γ-ATP was separated from phosphorylated Rb by SDS-PAGE (10%, 45 mA), the gel was washed twice with ddH_2O and dried on a whatman paper with a gel vacuum dryer for 2 h at 80°C. Finally, audioradiography was performed by exposing an x-ray film to the dried gel for 6 to 24 h, depending on signal intensity.

4 Results

4.1 Kinetic trapping of cytosolic Trx1 target proteins

4.1.1 Cloning and purification of Trx1(C35S)

To generate a Trx1 trapping mutant, the second cysteine of the ^{32}CxxC^{35} active site motif was mutated to serine, thereby preventing the escape reaction to occur. A double affinity tag was introduced to meet the different requirements of bacterial purification and target protein isolation. High-yield purification of the recombinant trapping mutant from *E.coli* lysates requires an affinity tag with high capacity. For this purpose, Trx1(C35S) was C-terminally fused to a hexahistidine (His_6) tag that binds to HisTrap nickel columns with a capacity of approximately 40 mg protein/ml matrix. However, due to its moderate affinity the His_6 tag is not suitable for purification of Trx1 target proteins from cytosolic preparations. Therefore, Trx1(C35S) was additionally tagged with the high-affinity streptavidin-binding peptide (SBP) that binds to the biotin-binding pocket of streptavidin with nanomolar affinity (Keefe et al, 2001) and allows for specific, highly efficient and quantitative purification (Call et al, 2002).

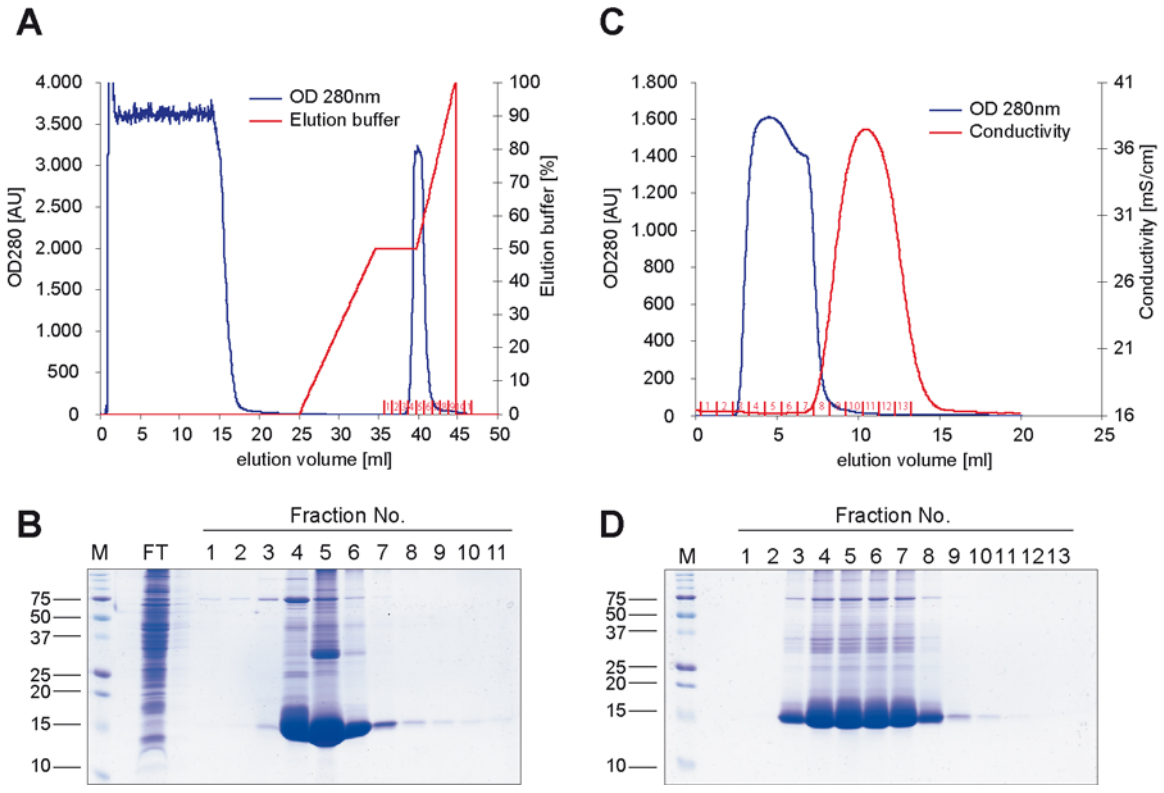

Figure 11: Purification of hTrx1(C35S)-SBP-His
(A) Chromatogram of a typical nickel chelate purification on an ÄKTA purifier. (B) Visualization of collected fractions by Coomassie staining. FT = flow through (C) Chromatogram of Trx1 desalting with HiTrap columns. (D) Coomassie staining of Trx1 fractions after desalting. M = molecular weight marker

The Trx1 trapping mutant C35S was generated by site-directed mutagenesis using the bacterial expression vector pQE60-hTrx1-SBP-His as a template (Schwertassek et al, 2007). Trx1(C35S) was expressed in the *E.coli* strain M15 and purified via the His_6 tag using nickel chelate chromatography on an ÄKTA purifier HPLC system (GE Healthcare). The chromatogram shows that high amounts of His-tagged Trx1 can be eluted (Figure 11A). Coomassie staining of the eluted fractions revealed that even though several other proteins were co-purified as bacterial contaminants, Trx1 was highly enriched in fractions four to six (Figure 11B). Up to 10 mg trapping mutant could be purified from 1 l of *E.coli* culture. For buffer exchange and removal of imidazol, gel filtration chromatography was performed. Protein-containing fractions could be efficiently separated from the imidazol-containing fraction as measured by conductivity and the optical density at 280 nm (Figure 11C). Trx1 was recovered and slightly diluted after desalting as shown by Coomassie staining (Figure 11D).

4.1.2 Kinetic trapping stabilizes Trx1-target protein conjugates

Trx1(C35S) was bound to streptavidin sepharose via its SBP tag before it was incubated with cytosol for kinetic trapping of target proteins. Even though Trx1 preparations are stored with 20 mM DTT it cannot be excluded that the protein is oxidized over time. Catalytically inactive disulfide-linked Trx1 dimers have been described to form *in vitro* after purification (Holmgren, 1977). Trx1 can also form a second intramolecular disulfide bond between Cys62 and Cys69 suggested to decrease Trx1 activity (Watson et al, 2003). Moreover, the active site can be oxidized by oxygen during storage. To fully reduce Trx1 and thereby ensure maximal activity, the protein was bound to streptavidin sepharose in the presence of DTT. After removal of unbound protein and DTT, Trx1(C35S) coupled beads were incubated with cytosol preparations of Jurkat T cells. Conjugates formed by trapping after 60 min were protected by addition of the alkylating agent N-ethylmaleimide (NEM). NEM prevents any further thiol oxidation or reduction during the subsequent washing steps. Due to the high affinity of the SBP tag, harsh washing conditions involving high salt and urea could be used to decrease the amount of non-covalently bound proteins. Thereafter, Trx1(C35S) conjugates were eluted with biotin and visualized by silver staining and immunoblotting.

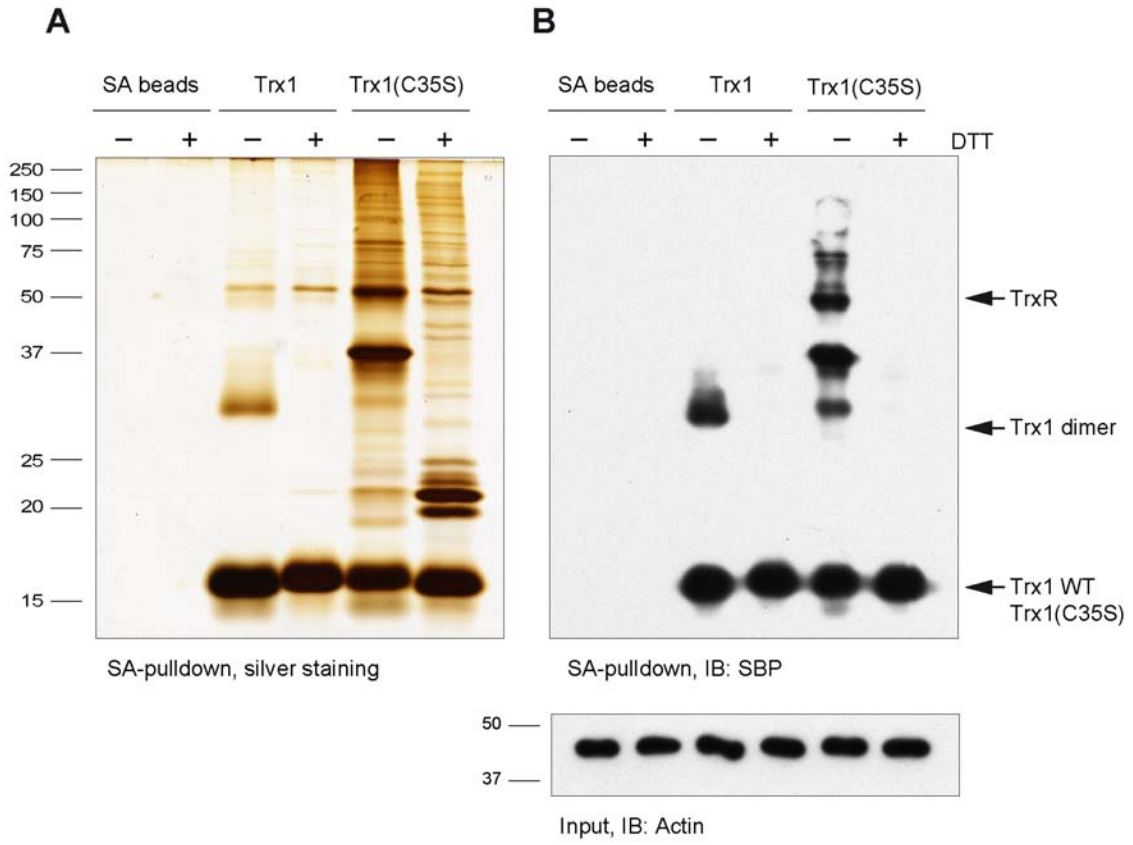

Figure 12: Specific purification of Trx1 target proteins
(A) Trx1(C35S) was added to Jurkat cytosol and after washing, eluted protein complexes were visualized by silver staining. (B) Immunoblotting of Trx1 trapping conjugates with an anti-SBP antibody. Input lysate was blotted against actin as loading control.

Streptavidin beads alone did not show any non-specific binding of proteins as judged by silver staining. Beads coupled to wild type (WT) Trx1 showed binding of two proteins in addition to recombinant Trx1 (17 kDa) used as bait (Figure 12A). Under non-reducing conditions a prominent band of 34 kDa was observed which has been identified as a disulfide-linked Trx1 dimer by mass spectrometry. Consistent with that, this band is no longer detectable under reducing conditions. Interestingly, the Trx1 dimer is hardly detectable for Trx1(C35S) which suggests that in this case formation of mixed-disulfide conjugates with target proteins is favored over homodimer formation. The other protein is bound to WT Trx1 under both reducing and non-reducing conditions and was identified as one of the isoforms of thioredoxin reductase. In contrast to WT Trx1 numerous proteins were bound to Trx1(C35S) demonstrating that Trx1 target proteins can be efficiently trapped in the cytosol of mammalian cells. The most prominent Trx1 target protein has a molecular weight of approximately 21 kDa and forms a 1:1 complex with Trx1 (38 kDa). As expected, most of the trapped proteins undergo a shift of 17 kDa upon reduction, indicating that

they have been disulfide-linked to the Trx1 trapping mutant (Figure 12A).

In order to verify that the protein bands purified with Trx1(C35S) indeed represent complexes containing Trx1 as a covalent binding partner, they were analyzed by immunoblotting against the SBP tag (Figure 12B). Under non-reducing conditions Trx1(C35S)-SBP-His containing complexes were clearly identified. As expected, these complexes were destroyed by reduction which separates Trx1(C35S)-SBP-His from its targets (Figure 12B+13B).

4.1.3 Trx1 target proteins are not covalently captured by a Grx1 trapping mutant

To further confirm that trapping by Trx1 is specific, Trx1(C35S) was directly compared to the corresponding trapping mutant of another member of the Trx superfamily, glutaredoxin-1 (Grx1). Like Trx1, Grx1 is a cytosolic disulfide reductase

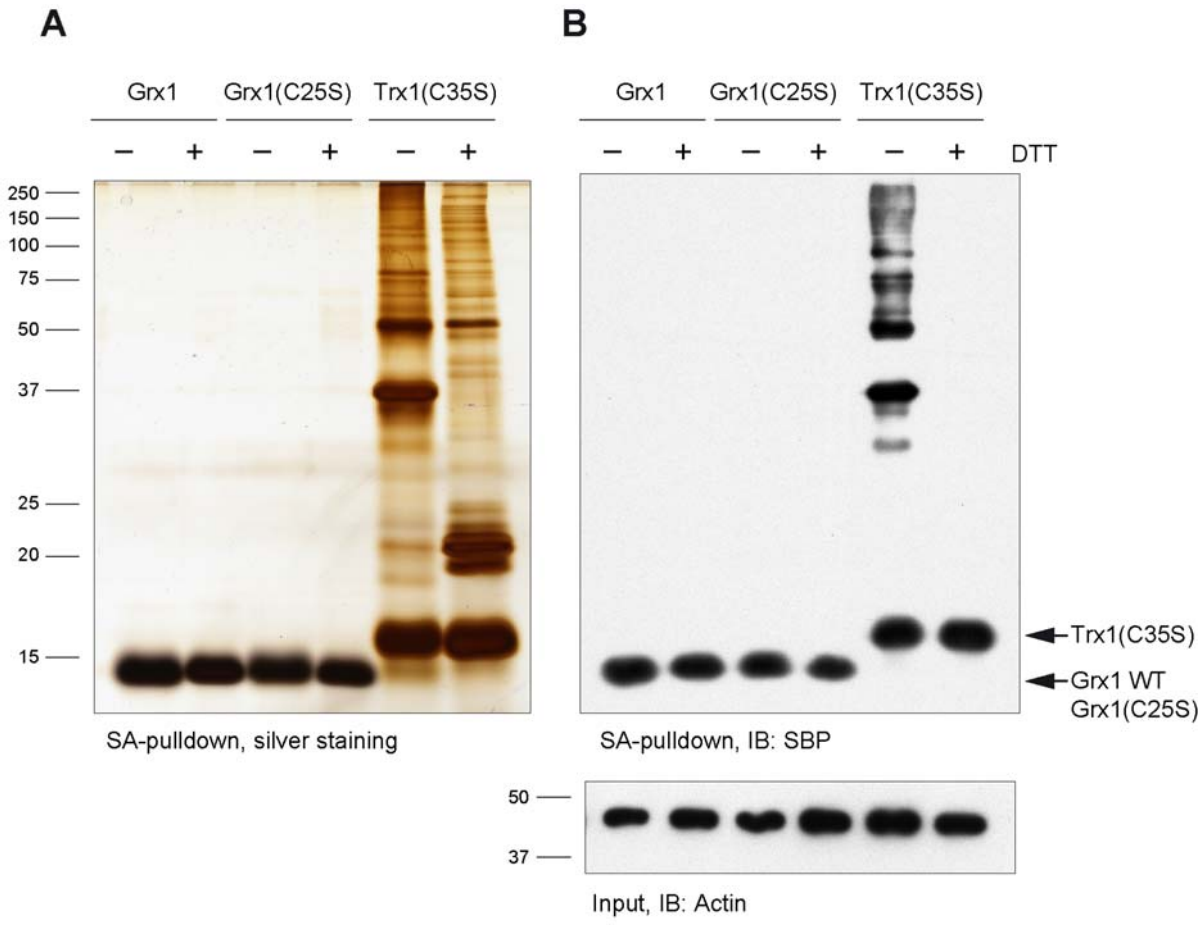

Figure 13: A Grx1 trapping mutant does not form mixed-disulfide intermediates with cytosolic target proteins
(A) Trx1(C35S) or Grx(C25S) were incubated with Jurkat cytosol and after washing, eluted protein complexes were visualized by silver staining. (B) Immunoblotting of Trx1 trapping conjugates with an anti-SBP antibody. Input lysate was blotted against actin as loading control.

also using the CxxC active site motive for substrate reduction. However, in contrast to Trx1, Grx1 reduces protein-glutathione mixed-disulfide bonds and forms mixed-disulfides with glutathione rather than with target proteins (Yang et al, 1998; Peltoniemi et al, 2006). In agreement with this reaction mechanism Grx1 did not form mixed-disulfide conjugates with other proteins and thus no protein trapping could be detected for WT Grx1 or the Grx1 trapping mutant by silver staining or immunoblotting (Figure 13A+B). These results demonstrate that the mere availability of an active site thiol is not sufficient for the generation of the trapping profile observed with Trx1. In fact it suggests that target protein reduction by Trx1 requires specific protein-protein interactions.

4.1.4 The pattern of Trx1 target proteins differs in different T cell lines

The expression level of Trx1 varies between different cell types (Sahaf et al, 1997) and this could influence the oxidation state of Trx1 target proteins. In order to analyze whether differences in target protein expression and/or redox state influence the Trx1 trapping pattern, kinetic trapping was performed on cytosol preparations of six different T cell lines. Three lymphoblastic T cell lines (Jurkat, CEM, Molt-4) were compared with three cutaneous T cell lymphoma lines (Hut78, MyLa, HH), either un-stimulated or stimulated with PMA/ionomycin. Although no differences in the Trx1

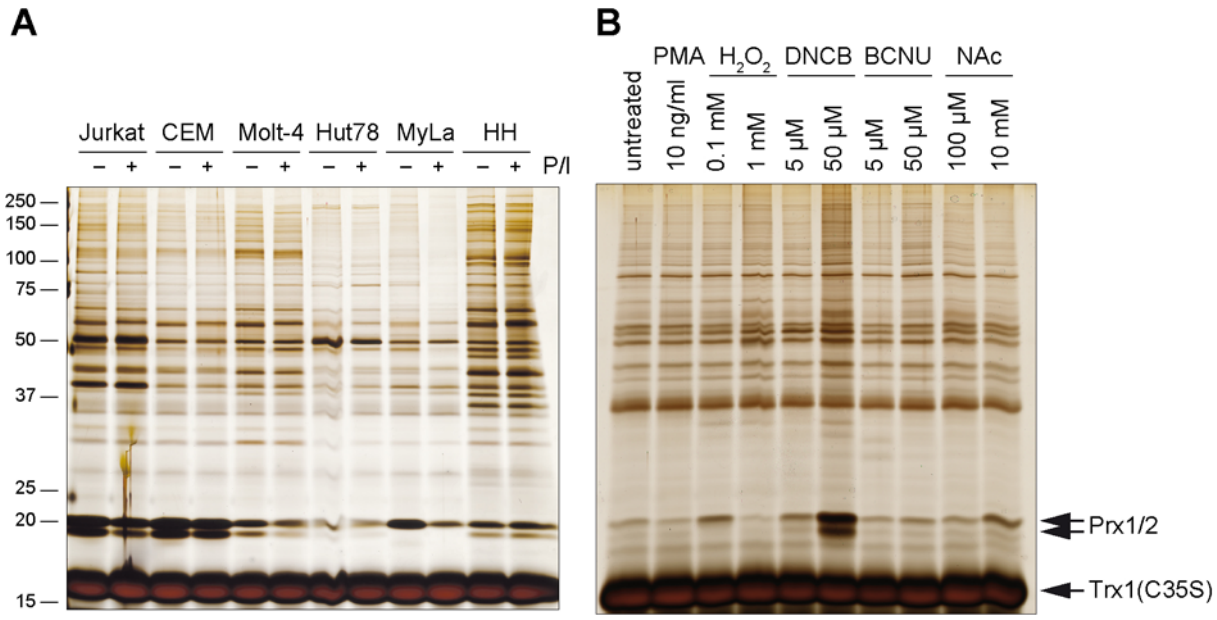

Figure 14: Comparisons and modulation of the Trx1 trapping profile
(A) Trx1(C35S) was added to cytosol prepared from different T cell lines either left untreated or treated with 10 ng/ml PMA and 1 µM ionomycin for 30 min. After washing, eluted protein complexes were reduced before separation and visualized by silver staining. (B) Jurkat T cells were treated with the indicated compounds for 30 min. Trx1 trapping conjugates were reduced before separation and visualized by silver staining. P/I = PMA/Ionomycin

trapping profile were observed 30 minutes after T cell stimulation, both qualitative and quantitative differences were detected among the cell lines tested (Figure 14A). Interestingly, the lymphoblastic T cell lines among each other showed a very similar profile, except for some quantitative differences. Trapping of two proteins with a molecular weight of approximately 40 and 42 kDa as well as a 50 kDa target was enhanced in Jurkat T cells, whereas trapping of a 110 kDa target was increased in Molt-4 cells. In contrast, the trapping profile of cutaneous T cell lines Hut78, MyLa and HH was much less comparable to the pattern of the lymphoblastic lines tested. Target protein trapping was decreased in Hut78 and MyLa, while the overall amount of trapped cytosolic target proteins was the highest in HH T cells. Interestingly, the ratio of trapped Prx to other Trx1 target proteins is very different in the cell lines tested. Whereas Prx represents the predominant Trx1 target in Jurkat and CEM T cells, the amount of trapped Prx in Molt-4 and HH T cells is comparable to the amounts of other Trx1 target proteins. As the protein content of the cytosol preparations and the amount of trapping mutant were matched, these data suggest that differences in the oxidation of Trx1 target proteins exist and can be visualized by kinetic trapping. However, some differences observed might also be due to unequal expression levels of Trx1 target proteins.

4.1.5 Inhibition of TrxR leads to an increase in Trx1 trapping

Because T cell stimulation with PMA/ionomycin did not alter the Trx1 trapping profile after 30 minutes (Figure 14A) redox-active compounds were applied to Jurkat T cells in order to test their influence on the oxidation state of Trx1 target proteins. No effect was seen after treatment of cells for 30 minutes with different concentrations of H_2O_2, the GSH reductase inhibitor BCNU (also known as Carmustine) (Frischer & Ahmad, 1977; Karplus et al, 1988) or the anti-oxidant N-acetyl cysteine (NAc) (Figure 14B). However, treatment of Jurkat T cells with the TrxR inhibitor DNCB (Arner et al, 1995) resulted in an overall increase in the formation of Trx1(C35S) conjugates, in agreement with the assumption that inhibition of TrxR leads to increased oxidation of Trx1 target proteins. In subsequent experiments increased trapping of Trx1 target proteins was observed shortly (5 minutes) after treatment with 100 µM H_2O_2, thus indicating that 30 minutes after oxidant treatment proteins have already returned to their reduced ground state (data not shown). In agreement with this observation, treatment of cells with redox-active compounds did not induce major long-term changes in protein oxidation, most likely due to the efficient action of the intracellular

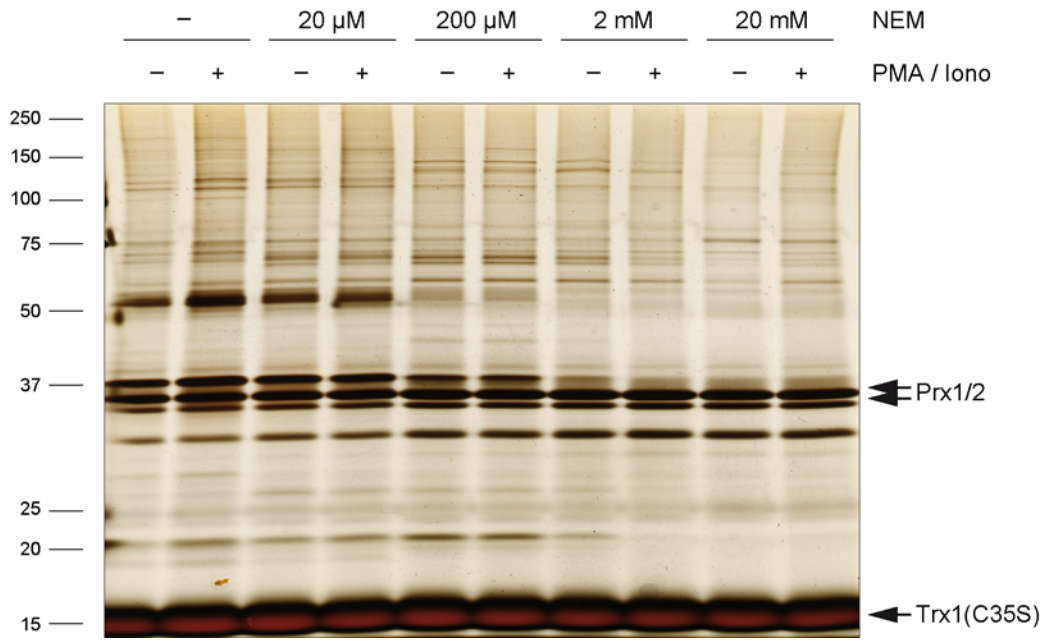

Figure 15: Trx1 target proteins are differently affected by thiol alkylation
Jurkat T cells were either not treated or treated with 10 ng/ml PMA and 1 µM ionomycin for 30 min. After stimulation cells were treated with the indicated concentrations of NEM for 5 min. Trx1(C35S) was added to cytosol preparations of these cells and after washing, eluted protein complexes were visualized by silver staining.

scavenging and reducing systems.

4.1.6 Trx1 target proteins have different susceptibilities to oxidation

Some redox-sensitive proteins undergo rapid oxidation when exposed to increased levels of oxygen, as is observed for members of the peroxiredoxins family (C. Winterbourn, personal communication). In order to test whether pre-lysis thiol alkylation influences the quantity or quality of proteins targeted by Trx1(C35S), Jurkat T cells were pre-treated with increasing concentrations of NEM prior to lysis. Less trapping by Trx1(C35S) was observed for some proteins when cells were pre-treated with 200 µM or higher concentrations of NEM, indicating that some proteins may undergo post-lysis oxidation during lysis or incubation with the Trx trapping mutant (Figure 15). Interestingly, Trx1 target proteins are affected by alkylation in different ways, indicating individual susceptibilities for oxidation. It should be considered that unreacted NEM could inactive the active site of the Trx1 trapping mutant which may account for some of the observed effects. Removal of NEM by gel filtration before application of the trapping mutant could circumvent this problem in the future.

4.1.7 Separation of covalent from non-covalent Trx1 interaction partners

Even though immunoblotting against the SBP tag confirmed that Trx1(C35S) forms

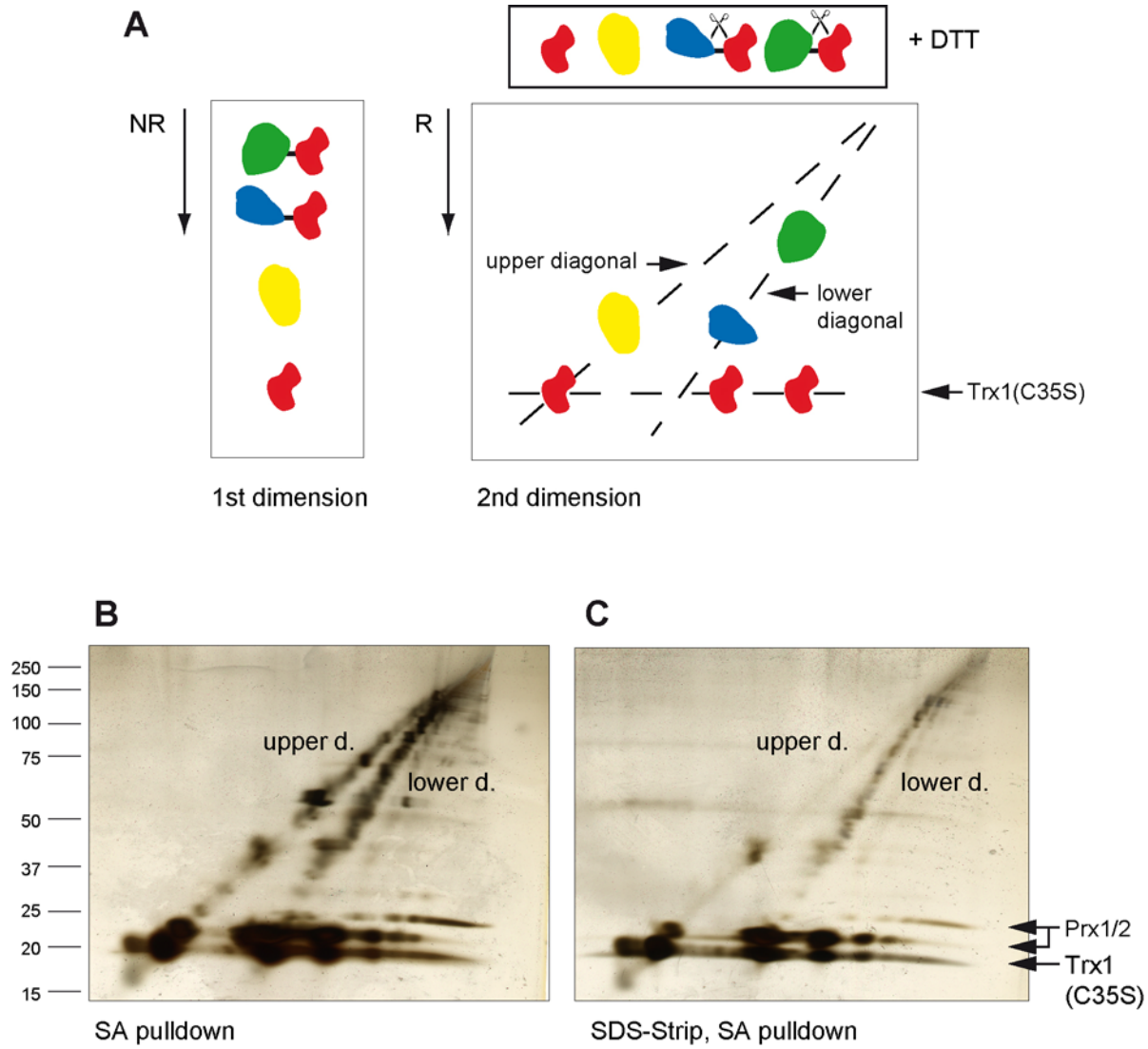

Figure 16: Visualization of Trx1 trapping complexes by NR/R 2D SDS-PAGE
(A) Schematic overview of NR/R 2D SDS-PAGE. See text for details. (B) Trx1(C35S) was applied to Jurkat cytosol. After washing, eluted trapping conjugates were separated by NR/R 2D electrophoresis and visualized by silver staining. (C) Trapping was performed as in (B), conjugates bound to SA beads were denatured by boiling for 5 min in TBS / 1% SDS / 1 mM iodoacetamide. The supernatant was diluted to a final concentration of 0.02% SDS and trapping conjugates were re-captured on fresh SA beads. After washing, eluted trapping conjugates were separated by NR/R 2D electrophoresis and visualized by silver staining.

disulfide-linked conjugates with target proteins (Figure 12) it is likely that, despite intensive washing, some proteins remain non-covalently attached to the trapping mutant. On one-dimensional gels these proteins cannot be easily differentiated from covalently linked Trx1 target proteins due to the complexity of the trapping profile.

In order to separate disulfide-linked Trx1 targets from non-covalently interacting proteins, eluted Trx1 conjugates were analyzed by non-reducing/reducing (NR/R) 2D-SDS-PAGE. In contrast to conventional 2D electrophoresis separating proteins by

isoelectric point and molecular mass, both dimensions of NR/R 2D electrophoresis separate proteins by molecular weight. In the first dimension disulfide-linked conjugates are preserved in the absence of reducing agents. A gel slice containing the separated non-reduced proteins is then treated with DTT and the reduced proteins are separated again on a second gel. Proteins that were not disulfide-linked to other proteins show very similar mobility in both dimensions and will therefore come to lie on a diagonal, whereas Trx1-linked proteins will appear on a diagonal below the main diagonal as a result of separation under reducing conditions (Figure 16A). Most of the proteins captured by the Trx1 trapping mutant appeared below the main diagonal on a NR/R 2D gel, showing that they have in fact formed a disulfide-linked conjugate with Trx1(C35S). As expected, the mobility difference between the main diagonal and the lower diagonal corresponds to 17 kDa, the size of recombinant Trx1(C35S)-SBP-His (Figure 16B). It should be noted that proteins running on the main diagonal may include some disulfide-linked Trx1 complexes in case of inefficient reduction. Interestingly, in addition to the lower diagonal shifted by 17 kDa additional minor diagonals could be detected, likely explained by disulfide-linked oligomers (Figure 16+17).

To confirm that proteins appearing on the main diagonal are non-covalent interaction partners, Trx1 complexes were depleted of non-covalent interactions by boiling in 1% SDS. Disulfide-linked Trx1 conjugates were then re-captured via the SBP tag on streptavidin sepharose and analyzed by NR/R 2D electrophoresis. Following the stripping and Re-IP procedure hardly any proteins were detectable on the main diagonal, whereas covalently bound Trx1 target proteins still appeared on the lower diagonal (Figure 16C). These results clearly demonstrate that Trx1(C35S) forms mixed-disulfide conjugates with its target proteins, which can be separated by NR/R 2D electrophoresis.

4.1.8 Depletion of endogenous Trx1 influences the Trx1 trapping profile

Treatment with pro- or antioxidants did not permanently alter the Trx1 trapping profile in Jurkat T cells, most likely because of efficient counter-regulation by the endogenous thioredoxin system. However, the TrxR inhibitor DNCB changed the Trx1 trapping pattern when tested after 30 minutes (Figure 14B). In order to exclude non-specific effects of this inhibitor and to test whether the expression level of endogenous Trx1 also influences the Trx1(C35S) trapping profile, endogenous Trx1

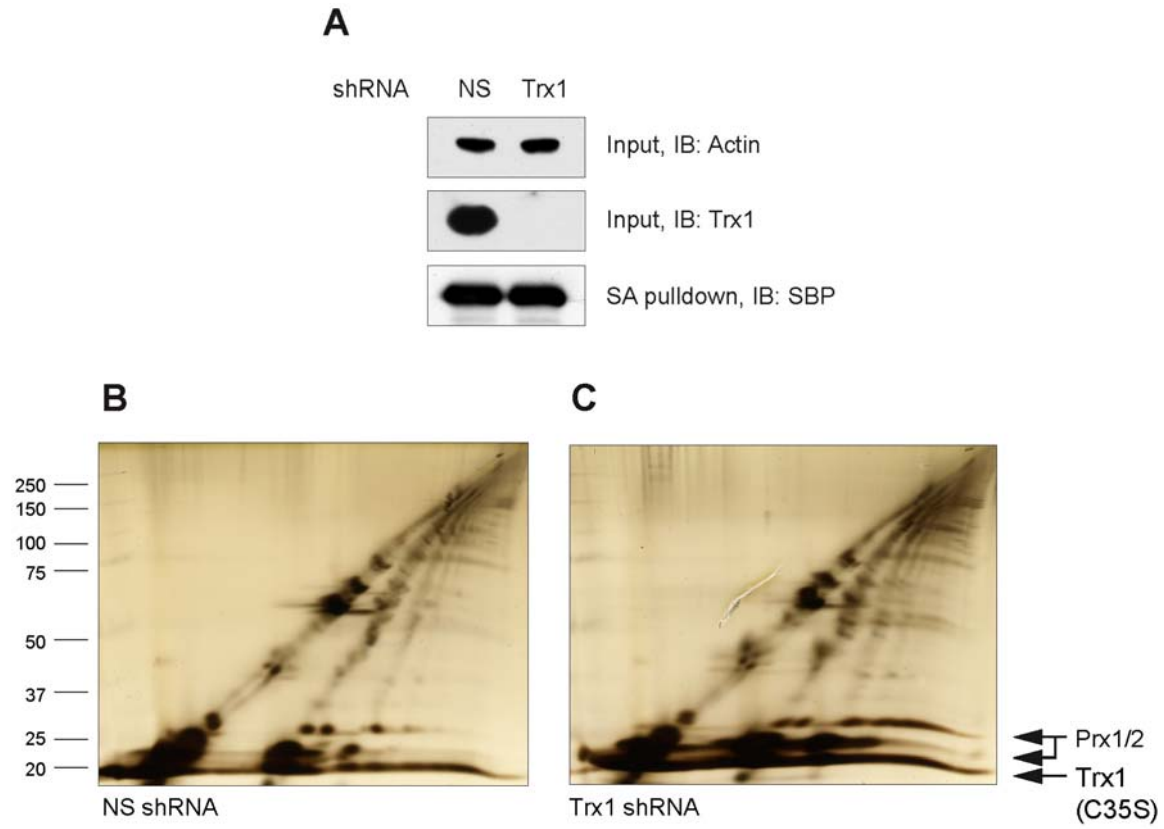

Figure 17: Depletion of endogenous Trx1 influences the Trx1 trapping profile
(A) Endogenous Trx1 was down-regulated by shRNA in HeLa T-REx cells after addition of 1 µg/ml doxycycline. NS = non-silencing shRNA construct. Total cell lysate was analyzed by immunoblotting against Trx1 and actin. After trapping, Trx1(C35S) conjugates were reduced with DTT and the amounts of trapping mutant were visualized by immunoblotting against the SBP tag. (B) NR/R 2D electrophoresis and silver staining of trapping conjugates from HeLa T-REx cells expressing a non-silencing shRNA construct. (C) NR/R 2D electrophoresis and silver staining of trapping conjugates from HeLa T-REx cells with reduced Trx1 expression.

was down-regulated in HeLa cells by shRNA. To avoid cellular adaptation to reduced levels of Trx1, shRNA-mediated Trx1 knockdown was induced by the addition of doxycycline (see section 4.5.2 for details). A comparison of trapping profiles of HeLa cells transfected with non-silencing and silencing shRNA constructs (Figure 17A) showed that overall trapping of target proteins was only weakly affected by the Trx1 knockdown (Figure 17B+C). Possibly, differences in protein oxidation may be lost during or after cell lysis which could account for the weak effect of the Trx1 knockdown on the profile of trapped target proteins. Alkylation could protect proteins from artificial oxidation or reduction, thereby preserving the altered redox status of Trx1 target proteins. Nevertheless, peroxiredoxins were clearly trapped more extensively in knockdown cells, most likely due to impaired reduction by endogenous Trx1.

4.2 Preparative purification and identification of cytosolic Trx1 target proteins

After having confirmed the specificity of kinetic trapping, large-scale preparation of Trx1 conjugates was performed. WT Trx1 was used as control to ensure that complex formation was based on the catalytic mechanism of thioredoxin (Figure 18).

4.2.1 Identification of cytosolic Trx1(C35S) interaction partners

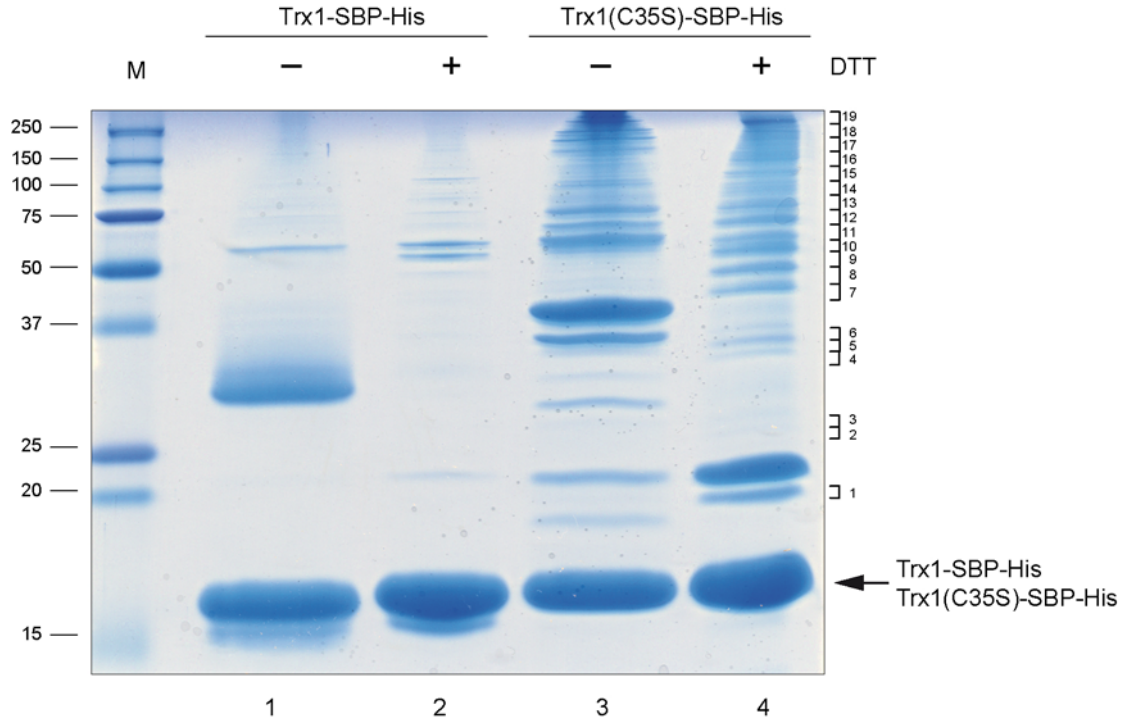

Figure 18: Purification of Trx1 targets for mass spectrometry
Cytosol from 3 x 10^9 Jurkat T cells was incubated with 0.75 ml streptavidin sepharose coupled with 500 µg pre-reduced Trx1(C35S)-SBP-His. NEM was added to preserve the disulfide-linked Trx1 complexes and prevent their reduction during washing. Eluted Trx1 complexes were separated by SDS-PAGE under non-reducing and reducing conditions and visualized by colloidal Coomassie staining. Band labels for lane 4 are shown on the right. M = molecular weight marker

For the identification of proteins interacting with Trx1 in the cytosol of mammalian cells, proteins captured by the Trx1 trapping mutant were separated under reducing conditions (Figure 18, lane 4), excised, digested with trypsin and subjected to liquid chromatography coupled electrospray ionization mass spectrometry (LC-MS/MS). In total 73 proteins were identified by mass spectrometry. These were classified into different functional categories based on Swiss-Prot entries and published data (Table 1). As many proteins act in several functional contexts and others are poorly characterized this functional categorization can only be considered a simplification. However, it does provide a broad overview.

Category	UniProt	Protein name	Band(s)	Score	Mass	Pep	Cover
Cytoskeleton	Q549M8	CLE7	2	131	28	3	15
	CNN2	Calponin 2	4, 5	122	34	3	12
	ACTB	Actin beta	7	534	42	13	44
	TBB5	Tubulin beta-5 chain	9, 10	781	50	18	43
	TBA3C	Tubulin alpha-2 chain	9	412	50	11	31
	TBB2C	Tubulin beta-2 chain	10	574	50	14	40
	PLSL	L-plastin	11, 12	640	70	12	27
	KIF2C	Mitotic centromere-associated kinesin	14	109	81	3	15
	FLII	Flightless-1 homolog	16	219	145	6	5
	DREB	Drebrin	16, 17	131	71	2	5
	DIAP1	Diaphanous homolog 1	16	118	141	3	3
	CKAP5	Cytoskeleton-associated protein 5	18	328	218	9	6
	MAP4	Microtubule-associated protein 4	18	310	121	8	10
Metabolism	RPIA	Ribose-5-phosphate isomerase	2, 3	112	26	3	18
	G3P	Glyceraldehyde-3-phosphate dehydrogenase	5	68	36	2	8
	RIR1	Ribonucleoside-diphosphate reductase M1 chain	14	595	90	16	18
	K6PP	Phosphofructokinase 1	14	520	86	12	19
	GFPT1	Glutamine:fructose 6 phosphate amidotransferase 1	14	382	77	9	16
	PYR1	CAD protein	18	82	240	2	2
	FAS	Fatty acid synthase	18	80	272	1	2
Redox	PRDX2	Peroxiredoxin-2	1	430	22	10	41
	PRDX4	Peroxiredoxin-4	2, 3	367	31	9	40
	TRXR1	Thioredoxin Reductase	10, 11	1005	54	19	41
Signaling	RAC2	Ras-related C3 botulinum toxin substrate 2	1	59	21	2	13
	MTMRE	Myotubularin-related protein 14	12	80	72	1	4
	DPYL2	Dihydropyrimidinase-related protein 2	11, 12, 13	737	62	17	43
	KHDR1	P21 Ras GTPase-activating protein-associated p62	13	122	68	3	10
	TBK1	TANK-binding kinase 1	14	175	83	5	11
	PSD4	PH and SEC7 domain-containing protein 4	17	58	116	1	1
	FNBP2	SLIT-ROBO Rho GTPase-activating protein 2	17	137	124	3	3
	SLK	STE20-like serine/threonine-protein kinase	18	381	142	9	11
	PPP6	Serine/threonine-protein phosphatase 6	4	127	36	3	11
	TTK	Dual specificity protein kinase TTK	15	430	97	11	20
	MCM5	DNA replication licensing factor MCM5	15	116	82	2	3
	AKP8L	Helicase A-binding protein 95	15	90	73	2	4
	RB	Retinoblastoma-associated protein	16, 17	491	106	12	15
	PDS5A	Sister chromatid cohesion protein 112	17	141	146	3	3
Transcription	SFRS7	Splicing factor, arginine/serine-rich 7	1	47	19	1	8
	HNRPF	Heterogeneous nuclear ribonucleoprotein F	8	184	46	5	18
	HNRPK	Heterogeneous nuclear ribonucleoprotein K	11, 12	506	51	13	38
	IKZF1	Ikaros family zinc finger protein 1	11	78	58	1	3
	DDX59	DEAD box protein 59	13	147	68	5	11
	LRRF1	Leucine-rich repeat flightless-interacting protein 1	16, 17	257	86	6	10
	NCOR1	Nuclear receptor corepressor 1	19	117	270	4	10
	DCPS	Scavenger mRNA-decapping enzyme DcpS	5	55	39	1	5
Translation	EF1D	Elongation factor 1 delta	6	115	71	2	8
	EF1A1	Elongation factor 1 alpha 1	4, 5, 8	510	50	13	32
	SYFA	Phenylalanyl-tRNA synthetase alpha chain	10	200	57	4	9
	SYCC	Cysteinyl-tRNA synthetase	13	772	85	19	30
	HBS1L	HBS1-like protein	14	152	75	5	12
	EF2	Elongation factor 2	15	1151	95	32	38

	JHD2A	JmjC domain-containing histone demethylation protein 2A	17	136	147	3	3
Folding	HSP7C	Heat shock 70kDa protein 8	12	856	71	16	36
	HSP76	Heat shock 70kDa protein 6	12	142	71	3	5
	HS105	Heat shock protein 105 kDa	16	967	106	22	30
Transport	RANB3	Ran-binding protein 3	12	100	60	2	5
	SC23B	Sec23B	13	102	86	3	5
	SC16A	Sec16A	19	638	234	16	11
	NADAP	Solute carrier family 4 (anion exchanger), member 1	17	135	89	3	4
Degradation	CUL5	Cullin-5	14	160	91	5	7
	TRI33	E3 ubiquitin-protein ligase TRIM33	16	160	122	3	3
	UBAP2	Ubiquitin-associated protein 2	17	133	117	3	4
	ATG7	Autophagy-related protein 7	12	100	78	2	3
Others	IAN-1	Immune associated nucleotide	4	110	35	2	10
	CHRD1	Cysteine and histidine-rich domain-containing protein 1	5	456	37	11	42
	CCD22	Coiled-coil domain-containing protein 22	12	64	71	1	2
	CB044	WD repeat-containing protein C2orf44	13	194	79	6	10
	CG027	HEAT repeat-containing protein C7orf27	13	133	88	3	7
	K1967	Deleted in breast cancer gene 1 protein	17	329	103	10	16
	DOCK7	Dedicator of cytokinesis protein 7	18	487	174	13	7
	FNBP4	Formin-binding protein 4	18	443	114	10	10
	ANXA2	Annexin A2	4, 5	871	39	18	53
	ZFY16	Zinc finger FYVE domain-containing protein 16	18	294	168	7	7

Table 1: Candidate Trx1 target proteins in Jurkat T cells identified from 1D SDS-PAGE
Proteins identified from lane 4 (Figure 18) by ESI LC MS/MS. *UniProt*: protein entry name in the UniProtKB/Swiss-Prot database, *Band(s)*: band on gel from which protein was identified, *Score*: Protana significance score, *Mass*: molecular weight of the identified protein [kDa], *Pep*: number of unique peptides sequenced, *Cover*: coverage of total protein sequence by identified peptides [%].

A majority of proteins was found to be associated with the cytoskeleton, including structural components actin and tubulin as well as regulatory proteins like L-plastin (Janji et al, 2006), flightless-1 homolog (Claudianos & Campbell, 1995), dihydropyrimidinase-related protein 2 (Fukata et al, 2002) or Map4 (MacRae, 1992). Even though actin and tubulin are highly abundant and frequently found in proteomic screens as false positives, there are reports describing redox-regulation of both actin (Tang et al, 1999; Fiaschi et al, 2006) and tubulin (Landino et al, 2004; Britto et al, 2005). Thus, both proteins can be considered candidate Trx1 target proteins despite their abundance. Most previously known Trx1 target proteins could be identified in this screen, including peroxiredoxins (Björnstedt et al, 1994), annexin-2 (Kwon et al, 2005b), GAPDH (Baalmann et al, 1995; Kumar et al, 2004) and ribonucleoside-diphosphate reductase (RIR1) (Laurent et al, 1964), thus demonstrating the capability of kinetic trapping to identify well established Trx1 target proteins. Besides RIR1 and GAPDH five other metabolic enzymes were trapped by Trx1. These are

involved in glycolysis (phosphofructokinase 1), nucleotide sugar biosynthesis (glutamine:fructose 6 phosphate amidotransferase 1) and fatty acid synthesis (fatty acid synthase). In addition, the trifunctional enzyme CAD which is required for pyrimidine synthesis as well as ribose-5-phosphate isomerase, a key enzyme of the pentose phosphate cycle, were found to be targeted by Trx1. Interestingly, besides these metabolic enzymes, several target proteins are involved in signal transduction. G protein-mediated signal transduction is regulated by candidate Trx1 target proteins Rac2 (Didsbury et al, 1989), p62 (Wong et al, 1992), PSD4 (Derrien et al, 2002) and SrGAP2 (Wong et al, 2001). Surprisingly, most of the identified regulatory proteins are regulators of the cell cycle. Retinoblastoma-associated protein (Rb) is a tumor suppressor and key regulator of cell cycle entry by acting as transcriptional repressor of E2F1 target genes (Chau & Wang, 2003). Protein phosphatase PP6 restricts G1 to S phase progression in human cancer cells (Stefansson & Brautigan, 2007). Msp1 is a regulator of the spindle-assembly checkpoint (Musacchio & Salmon, 2007) and mediates correct attachment of the chromosomes to the mitotic spindle (Jelluma et al, 2008). During early G1 phase of the cell cycle, the MCM5 is recruited as a subunit of the prereplication complex that assembles at replication origins (Tye, 1999) and also facilitates STAT1-mediated transcription (Snyder et al, 2005). Thus, Trx1 may not only have a regulatory function in various signaling pathways but might furthermore regulate cell cycle progression at different stages. Two members of the heterogeneous nuclear ribonucleoprotein (hnRNP) family which belong to a multiprotein complex involved in pre-mRNA processing were identified (Krecic & Swanson, 1999). Among the proteins involved in translation, EF1A1 and EF2 have already been described to be targeted by Trx1 in *Dictyostelium* (Brodegger et al, 2004) and *Arabidopsis* (Yamazaki et al, 2004), respectively. Finally, a number of proteins with unknown functions were trapped, including C2orf44, C7orf27 and CCD22.

4.2.2 Separation of Trx1 trapping conjugates by NR/R 2D SDS-PAGE

The first trapping screen identified proteins which remained bound to Trx1(C35S) after intensive washing with high salt und urea-containing buffers, suggesting that most of them were disulfide-linked to the trapping mutant. As already discussed, it could not be excluded that some of the potential Trx1 targets listed in table 1 were purified due to non-covalent association. Therefore, Trx1 trapping conjugates were again purified and re-analyzed on a NR/R 2D gel, which allows for discrimination

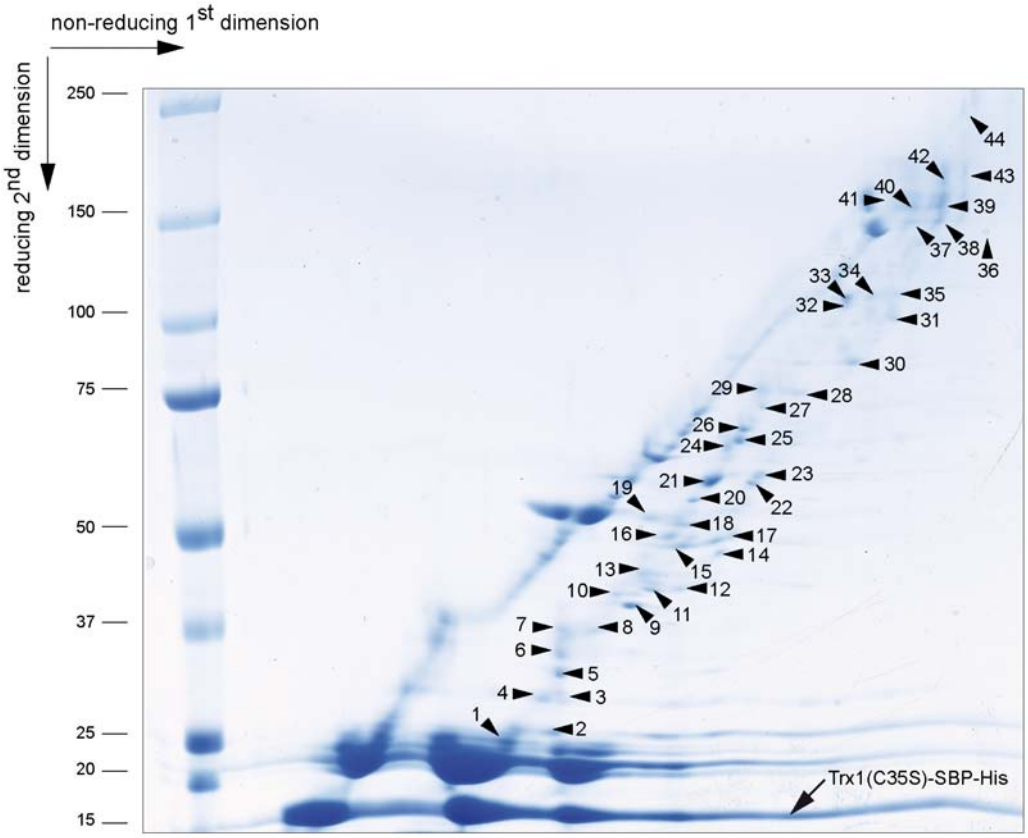

Figure 19: Preparative separation of proteins covalently trapped by Trx1(C35S) by diagonal NR/R 2D SDS-PAGE
Cytosol from 3 x 10^9 Jurkat T cells was incubated with 500 µg Trx1(C35S)-SBP-His bound to streptavidin sepharose beads. NEM was added to preserve the disulfide-linked Trx1 complexes and prevent further thiol interactions during washing. Trapping conjugates were separated by NR/R 2D electrophoresis and visualized by colloidal Coomassie staining.

between disulfide-linked and non-covalently bound proteins (see also Figure 16). For improved resolution, a 4–12% gradient gel was used to separate target proteins. Only proteins from below the main diagonal were selected for further analysis (Figure 19).

4.2.3 Identification of disulfide-linked Trx1 target proteins

Because many spots contained more than one protein, 62 candidate Trx1 targets were identified from 44 spots by tandem mass spectrometry (Table 2). Of those, 23 proteins were also found in the first trapping screen. Compared to the first screen, the number of cytoskeleton-associated proteins was reduced, suggesting that some of these proteins may have interacted non-covalently. The number of metabolic enzymes was increased whereas the number of candidate target proteins involved in signal transduction was equally high (Figure 20).

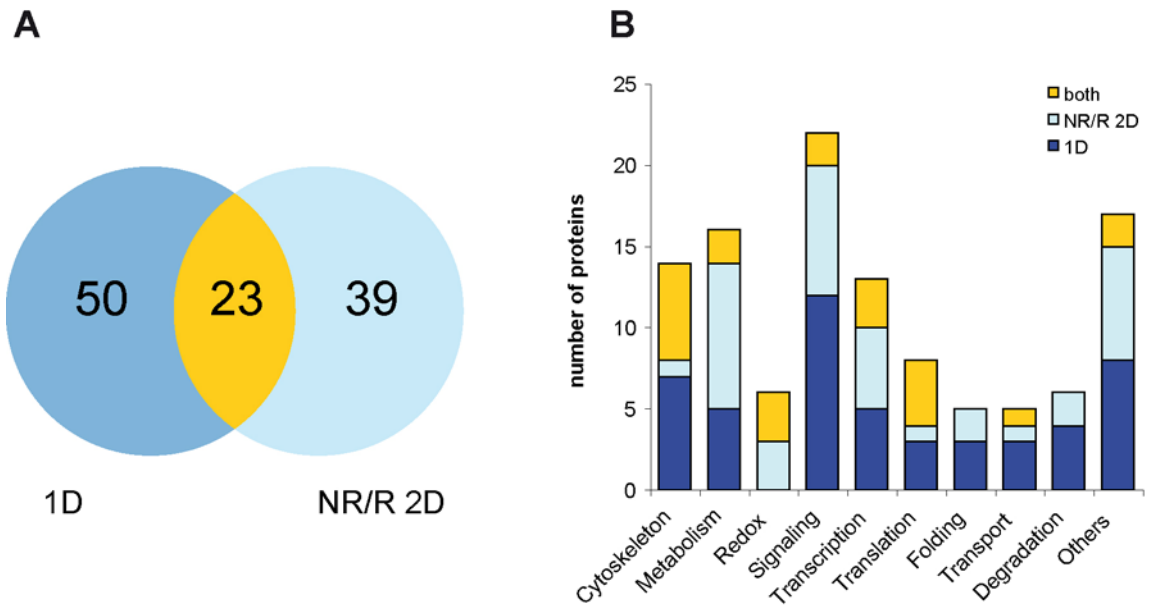

Figure 20: Comparison of candidate Trx1 target proteins identified from 1D and NR/R 2D gels
(A) Venn diagram showing the total number of Trx1 target proteins identified in each trapping experiment and the number of target proteins indentified in both screens. (B) Classification of identified Trx1 target proteins in functional groups according to published data and database entries.

The metabolic enzymes identified from the 2D gel have diverse functions ranging from acyl-CoA hydrolysis (cytosolic acyl coenzyme A thioester hydrolase) and nucleotide synthesis (guanine monophosphate synthetase) to epoxide detoxification (cytosolic epoxide hydrolase). In addition to ribose-5-phosphate isomerase, found in the first screen, glucose-6-phosphate 1-dehydrogenase (G6PD) was identified as a second enzyme involved in the pentose phosphate pathway. Besides elongation factors-1 alpha and -2, hnRNPs could be confirmed as targets for Trx1.

Additionally, p120 RasGAP was identified as a candidate Trx1 target protein. This enzyme stimulates the weak intrinsic GTP-hydrolysis activity of the Ras oncogene, thereby inactivating it (Bernards & Settleman, 2004). PKN1, also known as protein kinase C-related kinase 1 (PRK1), is a serine/threonine protein kinase, which has a catalytic domain highly homologous to that of protein kinase C (PKC). NimA-related kinase 9 (NEK9) plays a central role for cdc2-mediated initiation of mitosis in the G2 phase of the cell cycle. Interestingly, the third kinase found is also involved in cell cycle regulation. Cyclin-dependent kinase 6 (cdk6) cooperates with cdk4 in G1/S phase transition. As already suggested from the first Trx1 interaction screen, the identification of further cell cycle regulators as candidate Trx1 target proteins strengthens the possibility that Trx1 plays an important role in redox-regulation of cell cycle progression.

Category	UniProt	Protein name	Spot(s)	1D	Score	Mass	Pep	Cover
Cytoskeleton	ACTB	Actin beta	37	✓	338	42	7	24
	TBB2A	Tubulin beta-2A chain	15, 17	✓	778	50	15	28
	CNN2	Calponin 2	5	✓	60	35	1	4
	FLII	Flightless-I homolog	41	✓	1789	144	48	31
	PLSL	L-plastin	21, 23	✓	1884	71	35	49
	DIAP1	Diaphanous homolog 1	39	✓	88	139	5	3
	PLST	T-plastin	21		526	65	9	15
Metabolism	BACH	Cytosolic acyl coenzyme A thioester hydrolase	10		199	42	3	11
	HYES	Cytosolic epoxide hydrolase	18		578	64	15	26
	DLDH	Dihydrolipoyl dehydrogenase	18		49	57	3	5
	G6PD	Glucose-6-phosphate 1-dehydrogenase	16		80	60	3	5
	G3P	Glyceraldehyde-3-phosphate dehydrogenase	37	✓	59	36	2	5
	GUAA	Guanine monophosphate synthetase	24		300	78	11	13
	HCD2	Hydroxysteroid (17-beta) dehydrogenase 10	1		113	27	3	12
	KPRB	Phosphoribosyl pyrophosphate synthetase-associated protein 2	8		98	42	2	7
	RIR1	Ribonucleoside-diphosphate reductase M1 chain	28, 29	✓	712	90	20	20
	SPTC2	Serine palmitoyltransferase, subunit 2	19		275	64	7	13
	GLU2B	Glucosidase II subunit beta	29		329	59	7	13
Redox	PRDX1	Peroxiredoxin 1	1-3, 6-8		560	22	12	55
	PRDX2	Peroxiredoxin 2	1, 6-8	✓	361	16	7	27
	PRDX3	Peroxiredoxin 3	1, 2, 6-8		616	28	12	36
	PRDX4	Peroxiredoxin 4	1, 2, 7	✓	570	31	14	45
	SQRD	Sulfide:quinone oxidoreductase	13		148	51	3	6
	TRXR1	Thioredoxin reductase	19	✓	214	56	6	10
Signaling	CDK6	Cyclin-dependent kinase 6	6		272	37	7	24
	DPYL2	Dihydropyrimidinase-related protein 2	21, 24-26	✓	1420	68	27	44
	MTMRE	Myotubularin related protein 14	23	✓	142	74	3	5
	NEK9	Never in mitosis A-related kinase 9	33, 34		327	107	9	9
	PKN 1	Protein kinase N1	34		468	105	13	11
	RASA1	Ras GTPase-activating protein 1 (p120)	32		116	102	4	4
	S10A9	S100 calcium-binding protein A9	37		51	13	1	13
	ARL15	ADP-ribosylation factor-like 15	1		67	23	2	8
	APIP	APAF1 interacting protein	3, 4		335	28	13	44
	CASP2	Caspase 2	12		87	52	4	11
Transcription	HNRPF	Heterogeneous nuclear ribonucleoprotein F	12	✓	269	46	8	23
	HNRPH1	Heterogeneous nuclear ribonucleoprotein H1	14		480	50	12	27
	HNRPH2	Heterogeneous nuclear ribonucleoprotein H2	14		370	50	10	20
	PTBP1	Heterogeneous nuclear ribonucleoprotein I	16		162	58	5	9
	HNRPK	Heterogeneous nuclear ribonucleoprotein K	22	✓	152	41	2	7
	*	Histone 2B	6		68	14	3	24
	LRRF1	Leucine-rich repeat flightless-interacting protein 1	36-43	✓	1563	89	36	47
	THOC3	THO complex 3	8		575	39	12	27
Translation	BZW1	Basic leucine zipper and W2 domains 1	9		700	48	15	27
	SYCC	Cysteinyl-tRNA synthetase	26	✓	597	84	16	22
	EF1A1	Elongation factor 1 alpha 1	13, 37	✓	268	44	4	13
	EF2	Elongation factor 2	30	✓	1153	95	30	30
	HBS1L	HBS1-like	27, 29	✓	699	77	17	28
Folding	HS90B	Heat shock 90kDa protein 1, beta	28		51	40	3	9
	HSPB1	Heat shock protein 27	37		130	23	2	13
Transport	SC24A	Sec24A	34		261	120	10	8
	NADAP	Solute carrier family 4 (anion exchanger), member 1	32-35	✓	1993	90	41	41

Degradation	EDEM3	ER degradation enhancer, mannosidase alpha-like 3	31		508	100	15	17
	UBR1	Ubiquitin protein ligase E3 component n-recognin 1	43		155	200	5	2
Others	ANXA2	Annexin A2	5, 37	✓	700	39	16	41
	ATD3A	ATPase family, AAA domain containing 3A	20		609	66	11	24
	CCDC88	Coiled-coil domain containing 88	42		48	165	2	1
	DOCK7	Dedicator of cytokinesis 7	44	✓	65	239	5	1
	EFHD2	EF hand domain family, member D2 (swiprosin 1)	4		60	27	1	3
	LAP2B	Lamina-associated polypeptide 2, isoforms beta/gamma	8, 16		344	42	7	23
	LMBRL	Lipocalin-1-interacting membrane receptor	21		51	46	1	2
	TBC15	TBC1 domain family, member 15	24, 25		973	79	23	26
	TB22B	TBC1 domain family, member 22B	17		210	59	6	13

Table 2: Cytosolic Trx1 target proteins from Jurkat T cells identified from below the diagonal in NR/R 2D gel electrophoresis
UniProt: protein entry name in the UniProtKB/Swiss-Prot database, *Spot(s)*: on gel from which protein was identified, *1D*: checked if protein was also found in first screen (see Table 1), *Score*: MASCOT score, *Mass*: molecular weight of the identified protein [kDa], *Pep*: number of unique peptides sequenced, *Cover*: coverage of total protein sequence by identified peptides [%]. * Histone 2B sutype could not be identified.

Finally, also regulators of cell death were trapped by Trx1. Apaf-1 interacting protein (APIP) competes with caspase-9 for binding to Apaf-1 thereby inhibiting apoptosis whereas caspase-2 is can initiate programmed cell death after stimulation with TNF-α, CD95 or etoposide (Troy & Shelanski, 2003).

Overall, the collective data from both trapping screens suggest the Trx1 does not only function as antioxidant but may play important additional roles in regulating metabolic processes, cell cycle progression or induction of apoptosis.

4.3 Characterization of Trx1-linked conjugates by immunoblotting

4.3.1 Identification of multimeric Trx1 trapping conjugates

In order to validate the data obtained from mass spectrometry, selected proteins trapped by Trx1(C35S) were analyzed by immunoblotting. WT Trx1 was included as a negative control to ensure that proteins were in fact captured by the trapping mechanism. Interestingly, among the candidate targets tested only annexin-2 and RasGAP formed a single 1:1 disulfide-linked complex with Trx1, whereas other proteins showed a more complex trapping pattern consisting of multiple forms (Figure 21). Prx1 represents the major cytosolic target protein of Trx1. It forms a 1:1 complex of 39 kDa and, in addition, a second complex of around 55 kDa. The subunit composition of the larger complex remained unclear. Judged by its size it may be a Trx1-$(Prx1)_2$ or $(Trx1)_2$-Prx1 complex, expected to give rise to 61 kDa and 56 kDa bands, respectively. Furthermore, it cannot be excluded that the 55 kDa complex

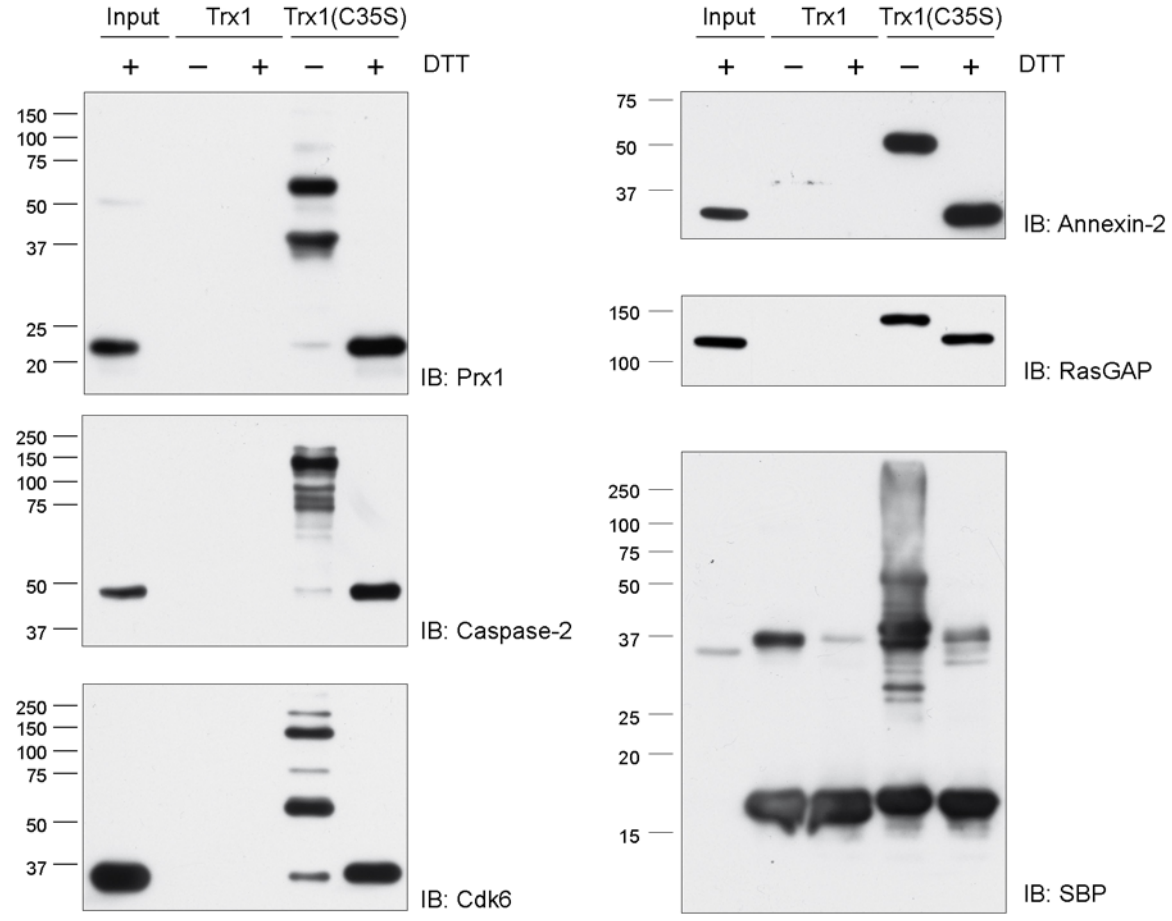

Figure 21: Analysis of Trx1-containing disulfide conjugates by specific immunoblotting
Trx1 trapping conjugates were separated by SDS-PAGE under reducing and non-reducing conditions. Conjugate formation was analyzed by immunoblotting against indicated proteins.

represents a hetero-trimer of Trx1, Prx1 and a third (unknown) component with a molecular weight of approximately 15–20 kDa. Besides the two main Prx1-Trx1 complexes several additional conjugates of higher molecular weight could be detected after prolonged exposure of the immunoblot, all of which resolved upon addition of DTT.

Similar to Prx1, cdk6 forms two major complexes with Trx1, a putative heterodimer of 54 kDa and a second complex of higher molecular weight. The cdk6 complex of around 140 kDa is too large to represent a hetero-trimer of Trx1 and cdk6 alone. Caspase-2 and Trx1 form an expected 1:1 complex of 69 kDa. However, caspase-2 forms various additional complexes, comparable to the trapping patterns of cdk6 and Prx1. Thus, it seems that Trx1 target proteins show a divergent redox behavior ranging from only one oxidized redox form (e.g. annexin-2) to various different oxidized forms (e.g. caspase-2) targeted by Trx1.

These differences in redox behavior are also reflected by the distribution of cysteines in annexin-2 and caspase-2. Annexin-2 contains only four cysteines, of which Cys133 and Cys262 are in close enough proximity to allow for formation of an intramolecular disulfide bond which would then be targeted by Trx1 to form the observed 1:1 disulfide-linked complex (Figure 22A). In contrast, the unprocessed form of caspase-2 contains a total of 18 cysteines of which 11 belong to the active

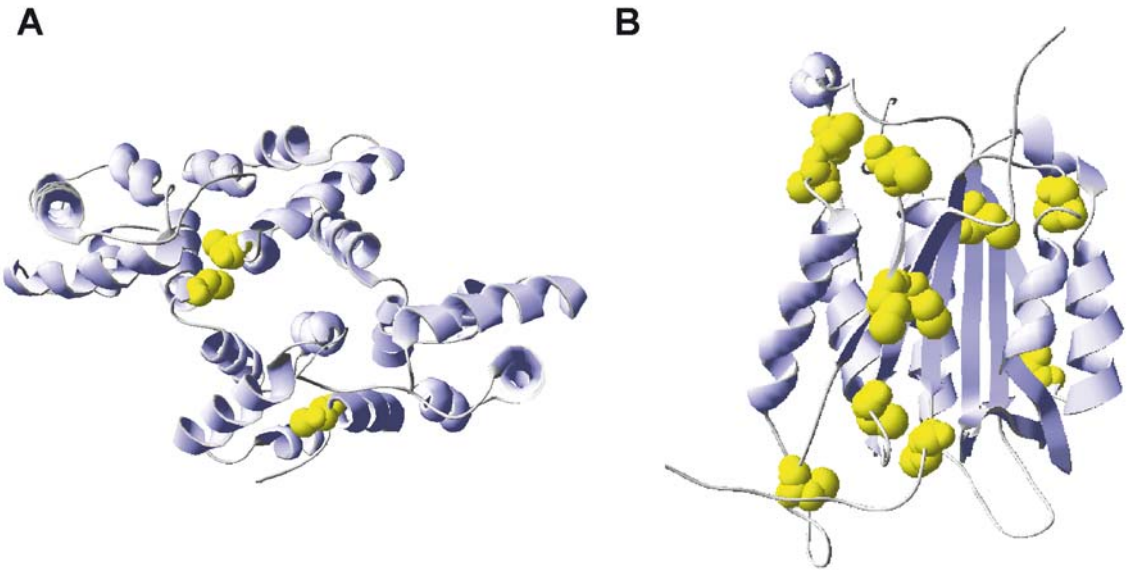

Figure 22: Cysteines in annexin-2 and caspase-2
(A) Crystal structure of annexin-2 (aa 29-339) generated from PDB file 1W7B, Cys9 is not depicted. (B) Crystal structure of the active p12/p18 caspase-2 heterodimer generated from PDB file 1PYO. Cysteine residues are highlighted in yellow. Images generated with Swiss-PdbViewer 4.0

p12/p18 heterodimer (Figure 22B). The high number of cysteines could lead to the formation of several (alternative) intra- and intermolecular disulfide bonds, thus generating a complex trapping pattern.

4.3.2 Redox interactions between Trx1 and its target proteins are highly selective

Specific formation of disulfide-linked Trx1 target conjugates was demonstrated using a Grx1 trapping mutant as negative control (Figure 13) and NR/R 2D gel electrophoresis (Figure 16). To test in addition the selectivity of Trx1 reactivity with regard to closely related proteins, members of the annexin, caspase and cdk families were tested by immunoblotting for their propensity to form Trx1 target complexes. Out of five annexins tested only annexin-2 was targeted by Trx1(C35S) (Figure 23A). Concerning caspases, in addition to caspase-2 also caspase-8 was found to be trapped by Trx1. Caspase-8 had not been identified by mass spectrometry, most likely due to insufficient sensitivity or incomplete sampling. In contrast, caspase-3 and -9 were not trapped by Trx1(C35S) (Figure 23B). Interestingly, although cdk6 is a target for Trx1, neither cdk1 (cdc2), cdk2 nor cdk4 (Figure 23C) showed any reactivity, despite the fact that cdk6 and cdk4 are closely related and share two conserved cysteines (Cys83 and Cys207). These results clearly demonstrate that although Trx1 interactions are pleiotropic with regard to target protein function, they are nevertheless highly selective.

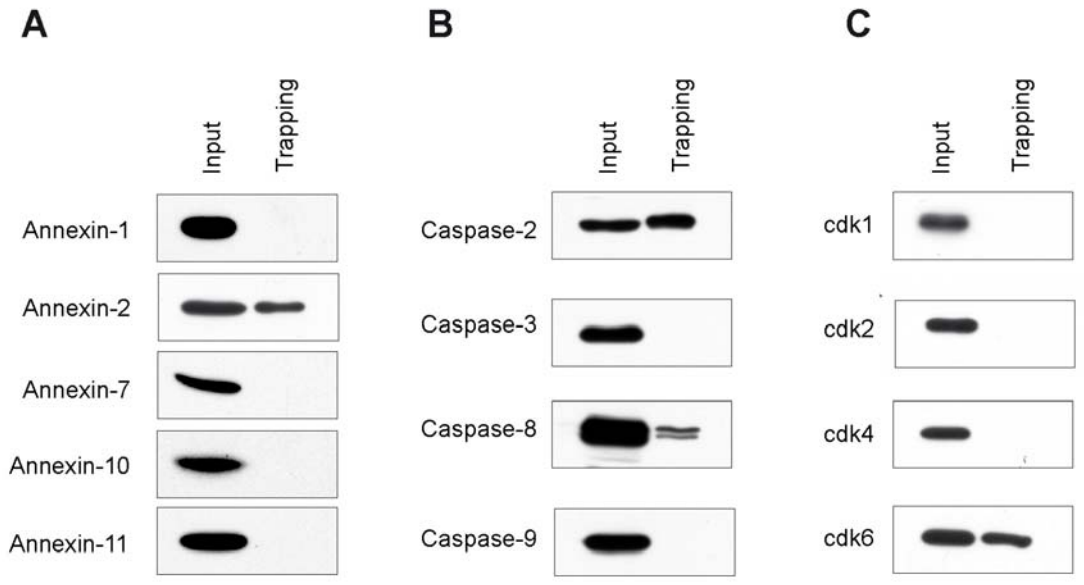

Figure 23: Trx1 selectively targets only certain members of protein families
Input and Trx1 trapping conjugates from Jurkat T cell cytosol were separated by SDS-PAGE under reducing conditions. Trapping of proteins by Trx1(C35S) was analyzed by immunoblotting against indicated proteins.

4.4 Functional analysis of Trx1 target proteins

4.4.1 Caspase-2 activity is regulated by the Trx1 system

Following the identification of numerous novel Trx1 target proteins, the influence of Trx1 on the activity of a selected target protein, Caspase-2, was analyzed. Caspase-2 activity was measured in lysates of SKW 6.4 T cells stimulated with an anti-CD95 antibody which was shown to efficiently activate caspase-2 (Lavrik et al, 2006) as measured with the fluorogenic substrate Ac-VDVAD-AFC. Addition of 10 mM DTT to the lysate increased caspase-2 activity over time (Figure 24A) which was strictly dependent on CD95 stimulation since untreated control cells did not shown any caspase-2 activity (data not shown). Interestingly, the Trx1 system can efficiently replace DTT in promoting caspase-2 activation. By comparison, caspase-2 activity was not significantly enhanced by the inactive Trx1 mutants C35S and C32S/C35S or TrxR alone (Figure 24A), showing that the enzymatic activity of Trx1 was responsible for the activation of caspase-2. In conclusion, caspase-2 appears to be inactivated by oxidation which is counteracted by Trx1. Even though the peptide substrate VDVAD is thought to be specific for caspase-2, Douglas Green and colleagues could recently show that this substrate is cleaved by caspase-3 even more efficiently (McStay et al, 2008). In order to exclude side-effects of caspase-3 or any other caspase, the influence of Trx1 on caspase-2 activity was also measured in a cell-free system using recombinant proteins. Because of auto-catalytic processing, recombinant caspase-2 is already pre-activated, but further reduction is required to

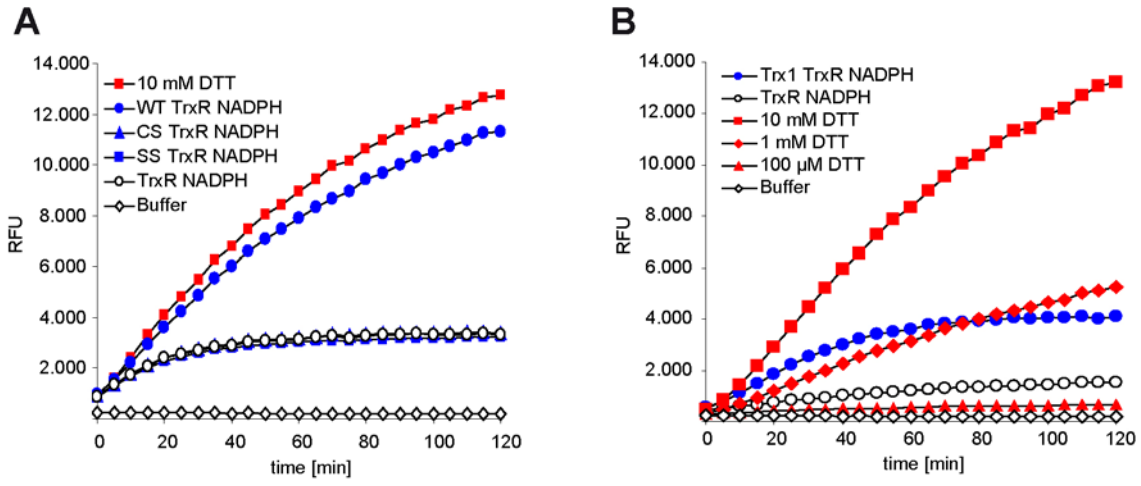

Figure 24: Regulation of caspase-2 activity by Trx1
(A) SKW 6.4 cells were stimulated for 1h with 10 µg/ml anti-CD95 antibody (anti-APO, kindly provided by Peter Krammer), lysed and caspase-2 activity was measured in the presence or absence of DTT or pre-reduced Trx1. CS: Trx1(C35S), SS: Trx1(C32S/C35S). (B) Activity of recombinant caspase-2 was measured in the presence of DTT or the Trx1 system. RFU = relative fluorescence units

completely activate the enzyme. Again, Trx1 could substitute for DTT in reducing caspase-2, although the recovered activity was lower compared to lysate preparations (Figure 24B). This could either result from cleavage of VDVAD by caspase-3 in the lysate or lower concentrations of caspase-2 in the cell-free system. An involvement of caspase-3 seems unlikely as it is not a target of Trx1 redox activity (Figure 23). From these experiments it was concluded that Trx1 is likely to play a role in the reduction of caspase-2 after oxidative inactivation.

4.4.2 Identification of the Trx1 target cysteine in cdk6

The interaction between the Trx1 trapping mutant and its target proteins depends on thiol-disulfide exchange, it should therefore be disrupted by mutation of the cysteines involved. Mutation of the N-terminal active site cysteine in the Trx1 trapping mutant to serine (C32S/C35S) completely abolishes target protein trapping (Schwertassek et al, 2007) and data not shown). Accordingly, the replacement of cysteines forming the target disulfide bond in the target protein should also abolish trapping. This notion was further tested for candidate Trx1 target protein cdk6. As amino acids with important regulatory functions are conserved during evolution, a multiple sequence alignment of cdk6 orthologs was performed (Figure 25). Six cysteines are present in human cdk6 of which Cys7 is unique among the species analyzed, Cys306 is weakly conserved, whereas Cys280 is conserved between four out of the seven orthologs. Cys83 and Cys207 appear to be absolutely conserved amongst the animal kingdom, suggesting the possibility that these cysteine residues form the disulfide bond which is targeted by Trx1.

```
                                    |7      |15           |83        |207
Homo sapiens                    MEKDGLCRADQQYECVAEI … VRLFDVCTV … LWSVGCIFAEMF …
Mus musculus                    MEKDSLSRADQQYECVAEI … VRLFDVCTV … LWSVGCIFAEMF …
Rattus norvegicus               MEKDSLSRADQQYECVAEI … VRLFDVCTV … LWSVGCIFAELF …
Gallus gallus                   MDKDGTNLADQQYECVAEI … VRLFDVCTV … LWSVGCIFAEMF …
Danio rerio                     MDKE---SSTQQYEPVAEI … VRLFDVCTV … LWSVGCIFAEMF …
Xenopus laevis                  MSKE----MKGQYEPVAEI … VKLMDVCAS … LWSVGCIFAEMF …
Drosophila melanogaster      … QAKKFGDGDPFNYQELNII … VKLYEVCQF … IWSAACIIFEMF …

                                      |280           |306
Homo sapiens                 … GKDLLLKCLTFNPA … YFQDLERCKENLDSHLPP …
Mus musculus                 … GKDLLLKCLTFNPA … YFQDLERYKDNLNSHLPS …
Rattus norvegicus            … GKDLLLKCLTFNPA … YFQDLERYKDNLHSHLSS …
Gallus gallus                … GKDLLLKCLAFNPA … YFHDLEKCKENLDSHMSS …
Danio rerio                  … GKSLLLSLDSTS-- … ----------------FPS …
Xenopus laevis               … GADLLLAMLTFSPQ … FFADDPQACSKQEHFTHI
Drosophila melanogaster      … ADDLLNKMLSYDLH … YFQQEPL----------- …
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Figure 25: Conservation of cdk6 cysteine residues of during evolution
Multiple sequence alignment of cdk6 protein sequences. Orthologs of human cdk6 from the indicated species were aligned using the ClustalW algorithm. Numbers indicate cysteine positions in human cdk6.

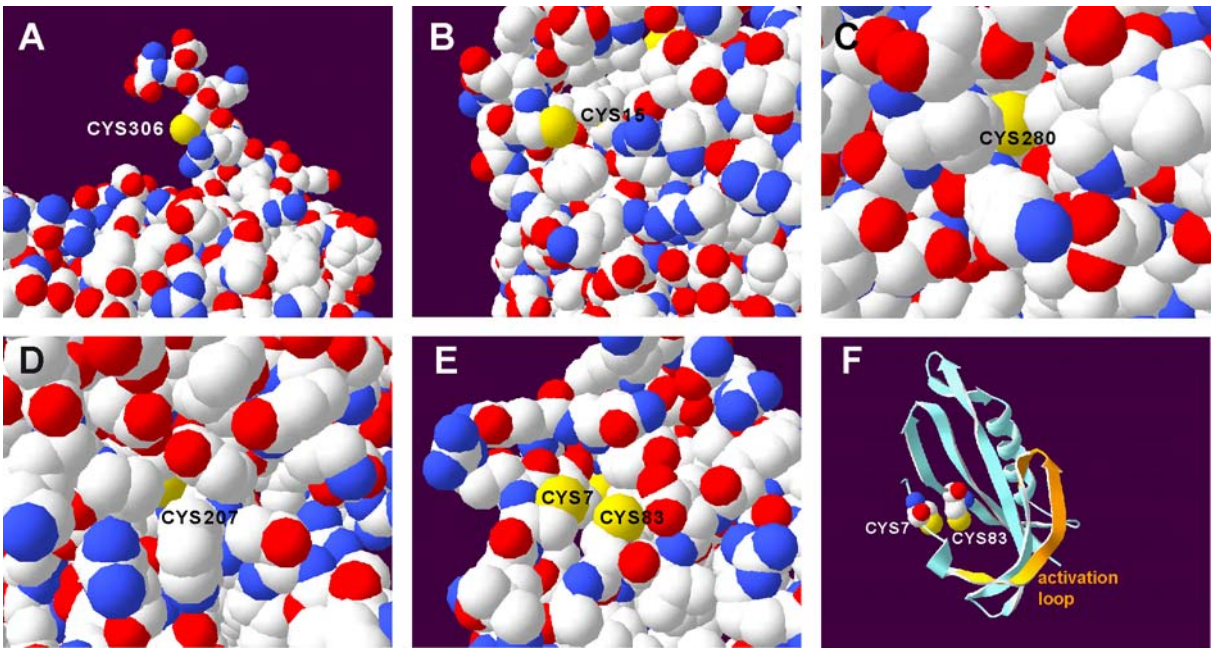

Figure 26: Cysteine localization in the crystal structure of human cdk6
(A)-(E) Space-filling model of human cdk6 with highlighted cysteines. The sulphur atom of cysteine residues is depicted in yellow. (F) Ribbon model of the human cdk6 activation loop. Structure generated from PDB entry 1JOW using Swiss-Pdb Viewer 4.0

According to the crystal structure of cdk6 four of six cysteines are exposed at the protein surface (Figure 26A-E). Interestingly, the highly conserved Cys207 residue is buried inside the protein whereas Cys7 and Cys83 are in close proximity on the surface. As Cys7 is located on a flexible N-terminal loop, it is conceivable that upon oxidation the protein undergoes a conformational change which would allow these two cysteines to form a disulfide bond (Figure 26F). Moreover, Cys7 is located N-terminally of the so-called activation loop which buries the phosphate binding pocket in the inactive state of cdk6. Thus, the redox state of Cys7 and Cys83 may have a direct effect on cdk6 activity.

For the identification of the Trx1 target cysteines in cdk6, site directed mutagenesis was performed to generate six single-cysteine cdk6 mutants which were tagged with a 3xFlag tag to discriminate them from endogenous cdk6. After co-expression of these mutants with SBP-tagged Trx1(C35S) in HEK 293T cells, Trx1 complexes were purified with streptavidin sepharose and the ability of the cdk6 cysteine mutants to serve as a target protein for Trx1 was determined by immunoblotting against the Flag tag. Surprisingly, all cdk6 mutants except C7S were trapped by Trx1(C35S) (Figure 27A).

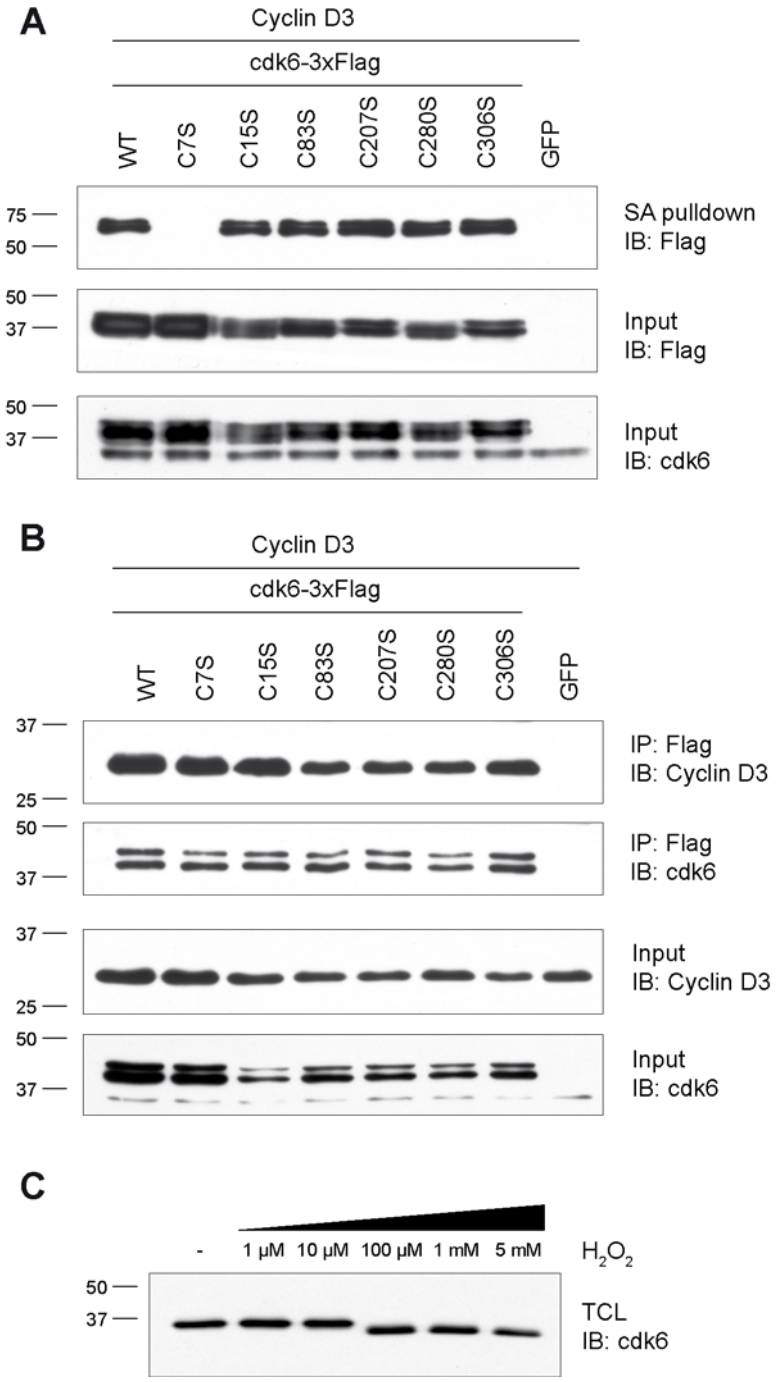

Figure 27: Identification of the Trx1 target cysteine in cdk6
(A) HEK 293T cells were transiently transfected with cyclin D3 and the indicated cdk6 mutants, C-terminally tagged with a 3xFlag tag. GFP was used as control. Lysate from these cells was incubated with Trx1(C35S) and trapping of the cdk6 mutants was determined by immunoblotting against Flag. Immunoblotting of input material against cdk6 detects both endogenous and Flag-tagged cdk6. (B) Cells from (A) were lysed and cdk6 mutants were immunoprecipitated with anti-3xFlag antibodies. Immunoprecipitates were analyzed for co-precipitation of cyclin D3. (C) Jurkat T cells were treated with increasing concentrations of H_2O_2 and lysed in the presence of NEM. Total cell lysate (TCL) was loaded under non-reducing conditions and the redox state of cdk6 was determined by immunoblotting.

If an intramolecular disulfide bond was targeted by Trx1, two of the cdk6 cysteine mutants should resist trapping by Trx1(C35S). Instead, it seems that Cys7 is forming an intermolecular disulfide bond potentially with another cdk6 molecule (Cys7-Cys7 homodimer), an unknown protein or small thiol compound. To exclude the possibility, that mutagenesis of Cys7 interfered with correct folding of cdk6, the interaction with cyclin D3 was tested. Cyclin D3 could be co-immunoprecipitated with all mutants to the same extent as with WT cdk6 (Figure 27B). Furthermore, all mutants showed that same activity in phosphorylating recombinant Rb *in vitro* (data not shown) which demonstrates that cysteine mutagenesis of cdk6 does not interfere with protein folding or activity.

Independently of target cysteine mapping, the redox status of endogenous cdk6 was determined after treatment with H_2O_2. At a concentration of 100 µM H_2O_2 cdk6 showed increased mobility on a non-reducing SDS-PAGE (Figure 27C), a strong indication for the formation of an intramolecular disulfide bond. This finding is in contrast to the observation that only Cys7 is required for trapping by Trx1(C35S), suggesting that cdk6 forms two alternative disulfide bonds upon oxidation. Most likely an intermolecular disulfide bond is formed by Cys7 (Figure 27A) in addition to an alternative intramolecular disulfide bond that could be formed between Cys7 and Cys83 (Figure 23E). In order to identify the cysteines involved in the formation of the intramolecular disulfide bond, cdk6 cysteine mutants need to be tested for their redox status after H_2O_2 treatment.
Formation of the intramolecular disulfide could be favored because no disulfide-linked complex of cdk6 could be detected by immunoblotting. Alternatively, the intermolecular disulfide could be rapidly reduced by Trx1 thereby impeding the identification of the interaction partner. In case that a small thiol compound is covalently linked to cdk6 Cys7, the detection could be limited by its low molecular weight.

4.5 Development of novel tools for the analysis of Trx1-mediated redox-regulation

4.5.1 Inducible expression of tagged Trx1mutants in Jurkat cells

Oxidation by O_2 after or during cell lysis may lead to artificial alterations of protein redox states, a common problem in the redox field. Reagents like N-ethylmaleimide (NEM), iodoacetamide (IAM) or methylmethanethiosulfonate (MMTS) can be used to alkylate cysteine residues and thereby conserve their redox state, but if these agents are applied to cells they impede further analysis by trapping as recombinant Trx1(C35S) is easily inactivated in the continued presence of alkylating agents. Transient transfection of a Trx1 trapping mutant would circumvent this problem because Trx1 trapping conjugates can form before the addition of alkylation agents, yet this approach has quantitative limitations which make it less applicable for large scale proteomic screens. Alternatively, cells could be stably transduced with retroviral expression vectors coding for the Trx1 trapping mutant, which would allow for their expansion on a scale suitable for redox proteomics. However, constitutive over-expression of Trx1(C35S) likely disturbs intracellular redox homeostasis and leads to cellular crisis and subsequent adaptive changes. To attenuate the problems of constitutive expression, an inducible expression system for Trx1 was established.

The Tet-system is an inducible expression system based on the essential regulatory components of the *E coli* Tn*10* tetracycline-resistance operon. The repressor (TetR) of this operon binds specifically to its operator (*tetO*). This is prevented in the presence of tetracycline, particularly by doxycycline (Dox). By fusing TetR with transcription activation domains, tetracycline-controlled transactivators (tTAs) were generated which have been shown to activate minimal promoters fused downstream of *tetO* sequences (P_{tet}) in the absence of Dox (Gossen & Bujard, 1992; Baron et al, 1997). In this Tet-Off system addition of Dox inhibits p_{tet} activation. A tTA containing mutations in the tetracycline-binding site requires Dox for activation of P_{tet}. This so-called reverse tTA (rtTA) has been shown to activate transcription in mammalian cells in a tetracycline-dependent way and established the basis for the Tet-On system (Gossen et al, 1995).

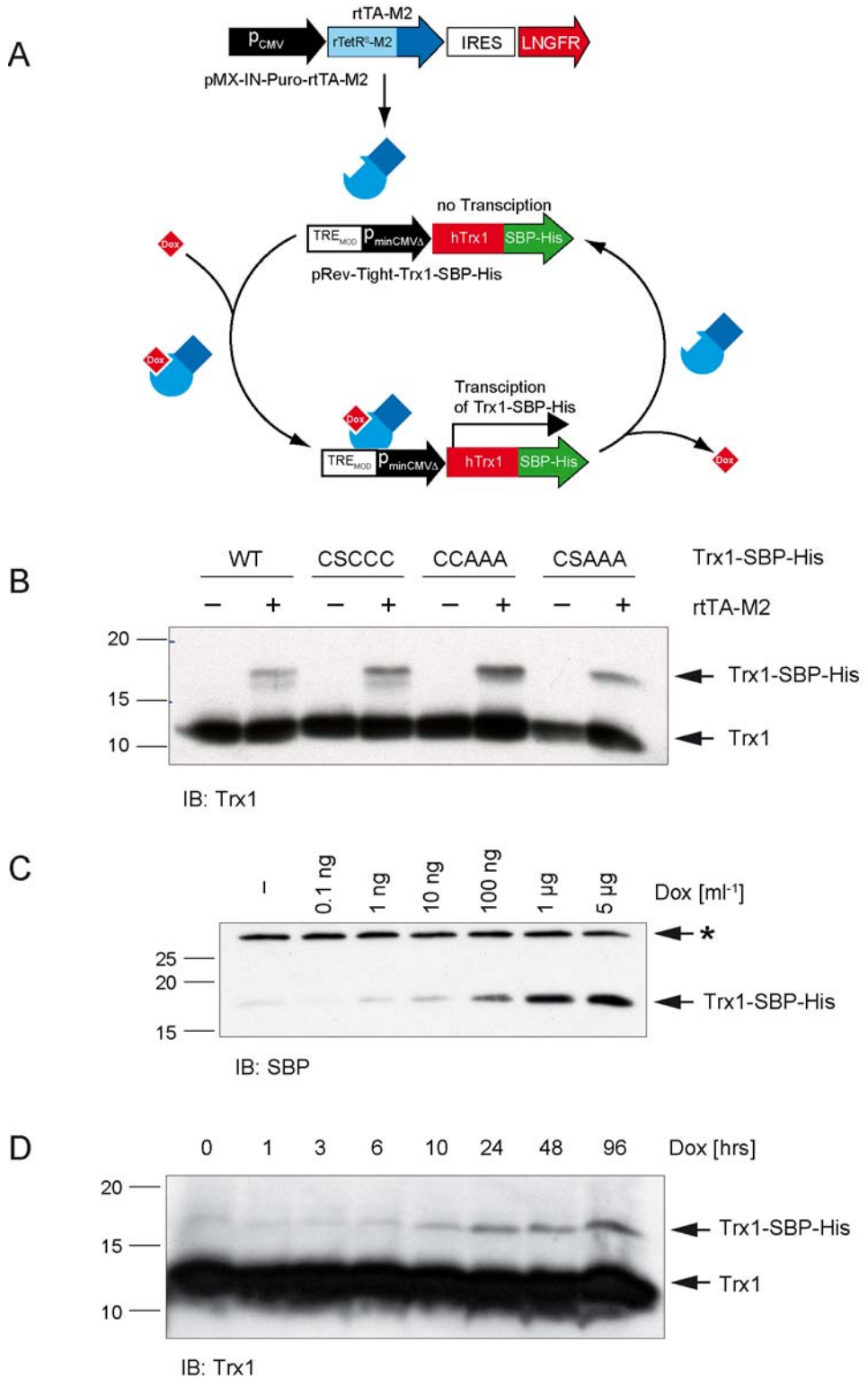

Figure 28: Doxycycline-dependent expression of Trx1-SBP-His
(A) Overview of the Tet-On system for Dox-regulated Trx1 expression. (B) HeLa cells were transiently transfected with pMXs-IN-Puro rtTA-M2 and pRev-Tight Trx1-SBP-His. CS: C35S, CCAAA: C62A/C69A/C73A, CSAAA: C35S/C62A/C69A/C73A. (C) Jurkat cells stably transfected rtTA-M2 and pRev-Tight Trx1-SBP-His CSAAA were treated with increasing concentrations of Dox for 48h. * unspecific band served as loading control (D) The same cells as in (C) were treated with 1 µg/ml Dox for various time points. Expression of endogenous and SBP-tagged Trx1 was analyzed by immunoblotting against Trx1.

For the regulated expression of SBP-tagged Trx1, a Tet-On system was developed in Jurkat T cells using the improved transactivator rtTA-M2 (kindly provided by Hermann Bujard, ZMBH) which functions at lower Dox concentrations and causes no background expression in the absence of Dox (Urlinger et al, 2000). Jurkat T cells were transduced with a vector coding for rtTA-M2 and the truncated human low-affinity nerve growth factor receptor (LNGFR) separated by an internal ribosome entry site (IRES). LNGFR-positive cells were transduced with a response-plasmid for Trx1-SBP-His under the control of the Dox-responsive promoter consisting of seven *tetO* sites just upstream of the minimal CMV promoter (pRev-Tight) and expression of SBP-tagged Trx1 was induced by the addition of Dox (Figure 28A).

In addition to WT Trx1, the Trx1 trapping mutant C35S was introduced into pRev-Tight to analyze trapping conjugate formation in intact cells. To test the influence of non-catalytic cysteines on Trx1 function and/or trapping behaviour, the two Trx1 mutants C62A/C69A/C73A (CCAAA) and C35S/C62A/C69A/C73A (CSAAA) were also cloned into pRev-Tight.

The functional capability of the newly generated expression vectors was tested by expression in HeLa cells in the presence of Dox for 48 h. Only if the transactivator rtTA-M2 was present, Trx1-SBP-His was expressed from the response-plasmid in the presence of Dox, even though to a lesser extent as endogenous Trx1 (Figure 28B). Trx1-SBP-His expression was dependent on Dox, as tested in an independent experiment (data not shown), demonstrating that both transactivator and response-plasmid were working properly.

After the functional validation of the system Jurkat T cells stably expressing the Tet-On Trx1 system were generated. First, Jurkat cells were transduced with rtTA-M2 and selected with puromycin followed by magnetic-activated cell sorting (MACS) of LNGFR-positive cells. Single Jurkat clones were tested for a functional rtTA-M2 with a pRev-Tight-EGFP reporter by flow cytometry. The clone with the highest EGFP expression after Dox treatment was chosen for transduction with pRev-Tight-Trx1-SBP-His followed by double selection with puromycin and hygromycin. Trx1-SBP-His expression could be tightly regulated and correlated with the applied concentration of Dox (Figure 28C). Maximal expression was reached after 48 to 96 h with 1 µg/ml Dox, however the expression level was very low compared to endogenous Trx1 (Figure 28D).

Because of the low expression of SBP-tagged Trx1 this system is not suitable for

large-scale proteomic screens but it can be used for the analysis of Trx1 trapping conjugates in intact cells. Low inducibility is a common problem of Tet-On systems (Gopalkrishnan et al, 1999; Munoz et al, 2004) which may result form epigenetic silencing of the CMV promoter that drives expression of rtTA-M2 (Pikaart et al, 1998; Berens & Hillen, 2003). The Trx1 Tet-On system could be further improved by replacement of the CMV promoter with the chicken β-actin promoter, which has been described to be less prone to epigenetic silencing and thus facilitates stable expression of rtTA-M2 (Welman et al, 2005).

4.5.2 Inducible shRNA-mediated Trx1 knockdown

For the analysis of Trx1-regulated pathways, inhibition of endogenous Trx1 or TrxR is often required. Unfortunately, substances which have been reported to specifically inhibit the Trx system have been shown to induce unspecific off-target effects. PX-12 is claimed to be a specific inhibitor of Trx1 and TrxR (Kirkpatrick et al, 1998) and has already completed phase I clinical trails in cancer patients (Ramanathan et al, 2007). Very recently it was reported, that PX-12 not only inhibits the Trx system but also other thiol-containing proteins like tubulin and cysteine-dependent proteases (Huber et al, 2008). Auronofin is also referred to as specific TrxR inhibitor, even though it has been shown to inhibit glutathione reductase, glutathione peroxidase (Gromer et al, 1998) as well as PKC (Wong et al, 1990). DCNB is also used in the clinic and has been shown to inhibit TrxR, it strongly increases the NADPH-oxidase-like function of this enzyme leading to the formation of superoxide (Arner et al, 1995). To avoid the problems associated with pharmacologic inhibitors, Trx1 expression can be specifically depleted using shRNA. Similar to over-expression, constitutive down-regulation of Trx1 will lead to adaptation of the cell. Therefore, an inducible shRNA-based knockdown system was established in HeLa cells which allows for the analysis of Trx1-dependent signaling pathways.

To develop an inducible Trx1 knockdown system, again the Tet system was utilized. In contrast to transactivator-based Tet-On or Tet-Off systems the original TetR from *E.coli* was used to suppress expression of Trx1 shRNA. HeLa cells stably expressing the TetR were obtained from Invitrogen (HeLa T-REx). For the expression of Trx1-specific shRNA pSUPER-Retro-NeoGFP (OligoEngine) was used, a retroviral vector based on pSUPER (Brummelkamp et al, 2002) in which a neomycin resistance gene and a GFP marker were introduced. With this vector shRNA is constitutively

transcribed from an unmodified H1 promoter by RNA polymerase I. In order to create an inducible expression vector for shRNA, the H1 promoter of pSUPER-Retro-NeoGFP was replaced with the modified H1 promoter of pTER (van de Wetering et al, 2003) kindly provided by Hans Clevers, Utrecht. The modified H1 promoter contains a *tetO* sequence directly upstream of the transcription start site which can bind TetR and thereby inhibit shRNA transcription. Trx1-specific shRNA were cloned into this newly generated vector (pSUPER-Retro-TRE-NG) downstream of the H1 promoter. Targeting sequences specific for human Trx1 were generated using the SiSearch algorithm (Chalk et al, 2004) which is primarily based on thermodynamic properties but also incorporates previously published criteria for shRNA design (Reynolds et al, 2004; Jagla et al, 2005). Using this algorithm, four different Trx1 targeting sequences were selected (Table 3).

HeLa T-REx cells were transduced with a retrovirus carrying the pSUPER-Retro-TRE-NG expression vector, selected with neomycin and sorted for GFP expression by flow cytometry. In these cells expression of Trx1 specific shRNA is inhibited by the TetR which blocks access to the H1 promoter for RNA polymerase I. In the presence of Dox the TetR dissociates from the H1 promoter and Trx1 specific shRNA is transcribed (Figure 29A) which targets Trx1 mRNA for degradation by the RISC complex. HeLa T-REx cells stably transduced with Trx1 shRNA constructs were cultivated in the presence or absence of Dox for one week and the expression level of Trx1 was analyzed by immunoblotting. Construct 281 had no effect on Trx1 expression, whereas construct 212 reduced Trx1 expression by 60%. Two of the four shRNA constructs (252 and NCR) showed a strong down-regulation above 90% in the presence of Dox (Figure 29B-D). Possible off-target effects of RNAi-mediated gene knockdowns (Boutros & Ahringer, 2008) can be reduced by using these two independent shRNA constructs which both induce down-regulation of Trx1

Region	Start	End	GC	human Trx1 mRNA target sequence	Score	unique	dG Diff
CDS	212	230	42.11%	CCAACGUGAUAUUCCUUGA	6	True	-1.0
CDS	252	270	47.37%	GGAUGUUGCUUCAGAGUGU	6	True	-1.5
CDS	281	299	47.37%	GCAUGCCAACAUUCCAGUU	6	True	-2.6
3'NCR	91	109	57.89%	CCAGUUGCCAUCUGCGUGA	3	True	-0.6

Table 3: shRNA targeting sequences generated with SiSearch
Region: CDS (coding sequence), NCR (non-coding region), Score: Stockholm rules score (0-7) (Chalk et al, 2004), dG Diff: energy difference between sense and antisense ends (Schwarz et al, 2003)

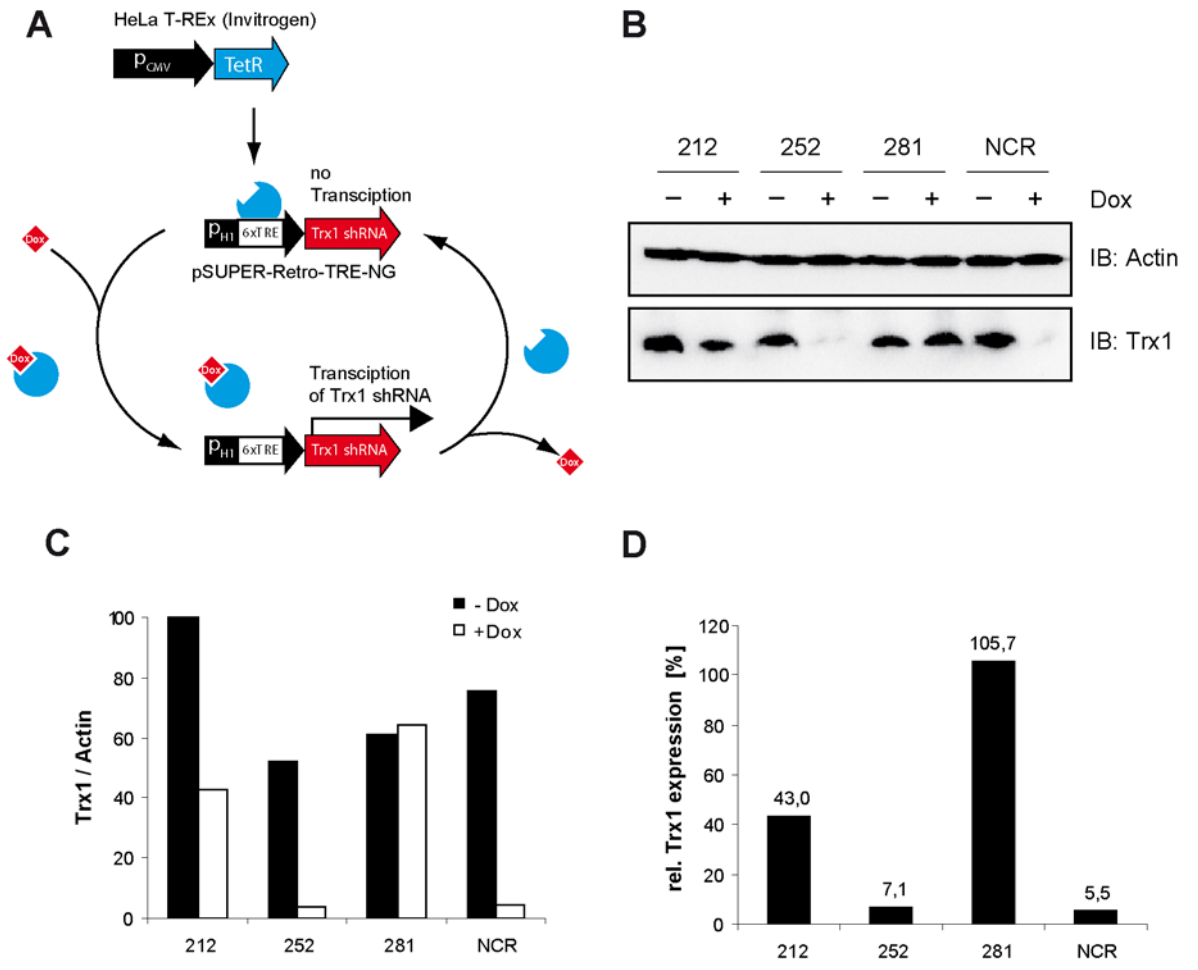

Figure 29: Inducible shRNA-mediated knockdown of Trx1
(A) Overview of the Dox-dependent shRNA-mediated down-regulation of Trx1 expression. For details see text. (B) HeLa T-REx cells expressing the Trx1 shRNA constructs listed in table 3 were cultivated for one week in the absence or presence of 1 mg/ml Dox. Cells were lysed and Trx1 expression was analyzed by immunoblotting. Actin was used as loading control. (C) Actin and Trx1 expression was quantified with a Lumi-Imager F1 (Roche Diagnostics) and relative expression levels were calculated. (D) Relative Trx1 expression after Dox treatment normalized to actin expression.

expression. Moreover, as one silencing construct targets Trx1 mRNA in the non-coding region, Trx1 expression can be restored by transfection of Trx1 cDNA lacking this sequence. Thus, specificity of this targeting sequence can be easily demonstrated by reconstitution of Trx1.

This inducible Trx1 knockdown system provides a powerful tool for the analysis of Trx1-mediated redox-regulation because it is very specific compared to the use of thioredoxin inhibitors. Furthermore it circumvents the problem of cellular long-term adaption to low Trx1 expression levels.

5 Discussion

5.1 Trapping of cytosolic Trx1 target proteins in mammalian cells

A number of redox-sensitive proteins is known to be regulated by Trx1, including NFκB (Hayashi et al, 1993), Ref-1 (Hirota et al, 1997) and PTP1B (Lee et al, 1998). However, no comprehensive analysis of Trx1 target proteins in the cytosol of mammalian cells has been performed so far. The characterization of proteins interacting with Trx1 was limited to yeast two-hybrid screens leading to the identification of p40phox (Nishiyama et al, 1999b), VDUP1 (Yamanaka et al, 2000) and pro-α 1 type 1 collagen (Matsumoto et al, 2002) as Trx1 interaction partners. Notably, these screens were done with WT Trx1 and therefore the proteins identified do not necessarily interact with Trx1 by thiol-disulfide exchange. Characterization of Trx1 substrate proteins is difficult due to the short half-life of the mixed-disulfide intermediates which are generated during disulfide bond reduction by Trx1 (Figure 7). Mutation of the second active site cysteine of thioredoxin has been shown to stabilize this otherwise transient reaction intermediate (Wynn et al, 1995). A yeast two-hybrid screen using a human brain cDNA library and the Trx(C35S) trapping mutant as a bait led to the identification of Jun activation domain-binding protein 1 (Jab1). However, Jab1 also co-immunoprecipitated with WT Trx1, thus indicating that the interaction does not depend on Trx1 catalytic activity (Hwang et al, 2004). On a cautionary note, the redox status of gene products ectopically expressed in yeast may not be representative of the mammalian cellular environment. Moreover, the high number of false positives and the inability to differentiate between non-covalent interactions and true enzyme-substrate (i.e. disulfide exchange) relationships are major drawbacks of the two-hybrid approach.

For the specific large-scale identification of novel mammalian cytosolic Trx1 target proteins recombinant Trx1 trapping mutant was incubated with cytosolic preparations of human Jurkat T cells. Several controls were implemented in order to minimize the number of false positives and to assure specificity of the kinetic trapping technique. Firstly, recombinant WT Trx1 was used as a negative control in all experiments. After stringent washing the only protein found to interact with WT Trx1 was TrxR, whereas a substantial number of proteins were reproducibly trapped by Trx1(C35S) (Figure 12), thus demonstrating the specificity of kinetic trapping for the identification

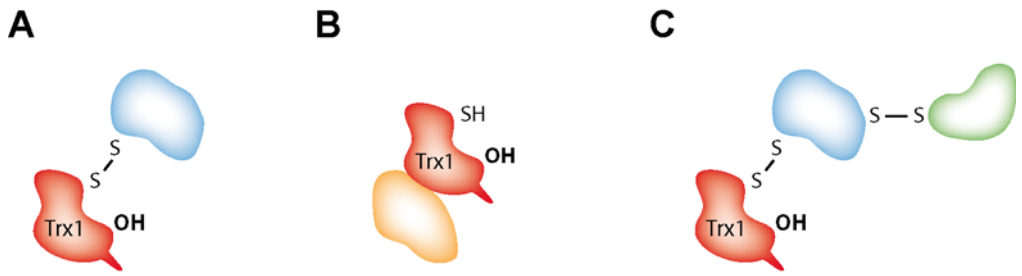

Figure 30: Possible protein-protein interactions of Trx1(C35S)
Schematic overview of possible covalent and non-covalent protein-protein interactions of Trx1(C35S). (A) direct trapping of a protein target. (B) non-covalent interaction. (C) indirect trapping of a protein which is disulfide-linked to a Trx1 target protein.

of disulfide exchange interactions. These and other experiments confirmed that mixed disulfide conjugates which form between Trx1 and target proteins are stabilized in the absence of the resolving cysteine in the active site. Furthermore, disulfide-linked Trx1 targets (Figure 30A) could be efficiently separated from non-covalent interaction partners (Figure 30B) by non-reducing/reducing 2D gel electrophoresis (Figure 16A). Denaturating re-precipitation (so-called 'Strip-Re-IP') of conjugates almost completely removed non-covalently attached proteins (Figure 16B). However, even though these measures can dramatically decrease the number of non-covalent Trx-binding proteins, it could not be excluded that a few proteins were captured indirectly, because they form a disulfide bond with a Trx1 target protein, rather than with Trx1 itself (Figure 30C).

No proteins were captured by the corresponding Grx1 trapping mutant, showing that substrate trapping is based on protein-protein-interactions and not simply due to the exposure of a reactive thiol, which is a common feature of both Grx and Trx (Figure 13). Moreover, these data not only confirm target specificity of Trx1-based trapping but also provide important insights in the reaction mechanism of Grx1. It is widely accepted that Grx1 catalyzes de-glutathionylation by a mono-thiol mechanism (Figure 4A). In addition, Holmgren and colleagues introduced the concept that Grx1, precisely like Trx1, can act by a di-thiol mechanism to reduce protein disulfide bonds (Figure 4B). However, this notion is based on a single experiment, namely the observation that *E.coli* glutaredoxin lacking the second active site cysteine does not reduce ribonucleotide reductase (Bushweller et al, 1992). However, protein disulfide bond reduction by Grx has not been described in eukaryotes and experiments demonstrate that mammalian Grx1 is a reductase specific for protein-glutathionyl mixed disulfide bonds (Gravina & Mieyal, 1993). Nevertheless, the possibility of a

dithiol reaction mechanism has not been generally disproven. If Grx1 could catalyze protein disulfide reduction via the proposed dithiol mechanism, mutation of the second active site cysteine should stabilize the transient reaction intermediate analogous to Trx1 substrate trapping. As no protein substrates could be trapped with the human Grx1(C25S) mutant (Figure 13), Grx1 does not seem to exhibit any protein disulfide reductase activity under the conditions of our experiments. In agreement with this finding, other studies concluded that Grx1 only reduces protein-glutathione mixed-disulfide bonds (Yang et al, 1998; Peltoniemi et al, 2006) and that Grx1(C25S) only forms mixed-disulfide intermediates with glutathione. Moreover, the lack of protein trapping clearly demonstrates that Grx1 specifically attacks the sulfur atom of the glutathionyl residue but not that of the protein cysteine residue, thus reflecting the structurally precise recognition of protein-SSG conjugates by Grx1. This conclusion is also supported by a structural comparison of the thioredoxin and glutaredoxin active sites. The thioredoxin surface exhibits a hydrophobic and elongated groove to interact with a continuous peptide chain of a target protein. In contrast, Grx exhibits a conformation that seems inappropriate for the recognition of substrate proteins (Maeda et al, 2006). Instead, reduction of glutathionylated proteins proceeds via binding of the glutathionyl moiety, but not of the glutathionylated protein itself (Fernandes & Holmgren, 2004).

Even though our experiments strongly suggest that Grx1 does not reduce any protein disulfides, the opposite situation should be considered as a possibility: Does Trx reduce glutathione-protein mixed disulfide bonds? If so, the Trx1 trapping mutant may not only trap substrates with an intra- or intermolecular protein-protein disulfide bond but also glutathionylated proteins. Structural restrictions argue against a direct reduction of glutathionylated proteins by Trx. It has been suggested that the hydrophobic surface of the Trx active site lacks groups that complement the charged and polar groups of glutathione (Nordstrand et al, 1999).
To clarify a possible role for Trx1 in de-glutathionylation radiolabeled ^{35}S-GSSG could be used in future experiments. Treatment of a cytosolic preparation with ^{35}S-GSSG and recombinant Grx1 should lead to radiolabeled glutathionylated proteins. Following removal of unbound GSSG, recombinant Trx1 can be tested for its ability to de-glutathionylate proteins which would show as a decrease in protein-associated radioactivity.

Recently it has been shown that Trx1 can catalyze denitrosylation of caspase-3 (Benhar et al, 2008). However, it remains to be confirmed that this is a general phenomenon affecting many proteins. To address this issue on a proteomics scale, cells could be treated with nitrosylating compounds like S-nitrosoglutathione (GSNO), blocked with NEM, incubated with recombinant Trx1 and analyzed for a reduction in nitrosylation using the biotin-switch method (Jaffrey et al, 2001).

To perform Trx1 target trapping on cytosolic preparations, proteins were released from their physiological environment. Upon release, cytosolic proteins become exposed to increased oxygen levels and, potentially, to redox enzymes released from non-cytosolic compartments. Accordingly, the redox status of the trapped proteins may be altered during lysis. In order to prevent leakage of oxidoreductases and GSSG from the endoplasmic reticulum, cytosolic proteins can be released by plasma membrane permeabilization rather than general membrane solubilization. However, even though some disulfide bonds targeted by Trx1(C35S) in lysates may have formed by artificial oxidation during or after lysis, trapped proteins nevertheless are likely to represent proteins specifically recognized by Trx1. In order to verify that the same proteins which were identified by lysate trapping are also targeted by Trx1 in intact cells under more physiological conditions, expression of the trapping mutant was induced in intact cells expressing the reverse Tet-transactivator rtTA-M2 and Trx1(C35S) under control of a doxycycline-dependent promoter (Figure 28D). Before lysis and pulldown of Trx1-linked conjugates the cells were treated with NEM to preserve the redox states of intracellular thiol groups. The resulting trapping pattern was comparable to the one obtained using recombinant trapping mutant albeit the total amount of Trx1 conjugates was less due to the lower expression level of the trapping mutant (data not shown). These data indicate that most Trx1 target proteins identifiable in cytosolic preparations also act as Trx1 substrates in intact cells. Accordingly, similar results were obtained by transient expression of Trx1(C35S) in HEK 293T cells which have been treated with NEM prior to lysis and isolation of Trx1-linked conjugates.

Notably, it appears that only a minor fraction of each Trx1 target protein's total amount was trapped in our experiments. No absolute quantification was performed, which would require the use of recombinant calibrators and normalizers (Schilling et

al, 2005). This observation is in accordance with the concept that proteins in the cytosol are normally reduced and that local generation of ROS leads to transient and spatially limited oxidation of redox-sensitive proteins.

5.2 The influence of T cell stimulation on the Trx1 trapping profile

Treatment of Jurkat T cells with PMA/ionomycin for 30 minutes was reported to induce ROS production as measured by H_2DCFDA-staining (Gülow et al, 2005). However, 30 minutes after stimulation we could not detect any significant difference in the Trx trapping pattern. The lack of a Trx1 trapping response upon T cell stimulation might have several reasons. Firstly, the kinetics of ROS generation and decomposition may limit detection of protein oxidation in the Trx1 trapping assay. Inside the cell H_2DCFDA is hydrolyzed to H_2DCF which is in turn oxidized by peroxides to fluorescent DCF. As DCF is stable, its fluorescence will accumulate and persist over time even if the initial ROS signal has already vanished. Thus, the oxidative state induced by T cell stimulation could be gone after 30 minutes, even though the oxidative signal is detectable by DCF fluorescence. In fact, subsequent experiments have shown that treatment of cells with a single bolus of H_2O_2 substantially increased Trx1 trapping, but only for up to 5 minutes. The notion that pro-oxidative states can be extremely short-lived is also supported by independent measurements with biosensors in live imaging experiments (Gutscher et al, 2008). In conclusion, the issue of Trx1 trapping after T cell stimulation needs to be re-addressed in future experiments at earlier time points.

Secondly, differences in protein oxidation may be lost during or after cell lysis. Following disruption of the cell oxidant-sensitive proteins may become oxidized independently of their initial redox state inside the intact cell. However, this explanation seems unlikely as inhibition of TrxR by DCNB (Figure 14B) and knockdown of endogenous Trx1 (Figure 17B) changed the Trx1 substrate trapping pattern. In order to diminish the danger of post-lysis oxidation free thiol groups need to be rendered inert to oxidation prior to cellular disruption. However, treatment of cells with the membrane-permeable alkylating agent NEM just before lysis would interfere with subsequent Trx1 substrate trapping as unreacted NEM in the lysate would also alkylate the active site cysteine of the Trx1 trapping mutant. A possibility to circumvent this problem is to remove unreacted NEM by gel filtration before application of the trapping mutant. The conservation of protein redox states by trichloroacetic acid (TCA) precipitation (Leichert & Jakob, 2004) is not compatible with the trapping procedure, which requires the target proteins to maintain their native structure.

Thirdly, even though it has been shown that ROS are generated by T cell stimulation, it not clear if and to which extent protein disulfide bonds are formed and subsequently reduced by the thioredoxin system. It is possible that the glutathione/ glutaredoxin system plays a more important role under the specific conditions of T cell activation. Stimulation of primary T cells leads to oxidation of the cytosolic glutathione pool (Gutscher et al, 2008) and it is therefore possible that redox-sensitive proteins are mainly subject to glutathionylation rather than intramolecular disulfide bond formation (Figure 2). In this scenario, changes in the Trx1 trapping pattern are not expected to follow as a consequence of T cell activation. A detailed characterization of the protein subsets targeted by Trx1 and Grx1 would provide a better understanding of the oxidative events induced by T cell stimulation.

Finally, sensitivity could limit detection of stimulation-dependent changes in Trx1 substrate trapping. As only a small subpopulation of Trx1 targets may become oxidized and thus trapped by Trx1(C35S), minor changes in protein oxidation could be too marginal to be detected by silver staining or immunoblotting. Since oxidation of at least some Trx1 target proteins is nevertheless likely to occur after stimulation of ROS-generating signaling pathways, optimized experimental conditions should eventually facilitate identification of stimulation-dependent changes in Trx1 target trapping. A promising approach is rapid analysis at early time points in combination with NEM alkylation and gel filtration. Titrations of stimuli need to be tested at various time points in order to find to optimal parameters for the analysis of these oxidative changes.

5.3 Identification of novel Trx1 substrates

Two independent large-scale trapping experiments were performed to identify cytosolic Trx1 target proteins. First, conjugates were separated under reducing and non-reducing conditions on conventional 1D SDS-PAGE (Figure 18). From this experiment 73 candidate Trx1 target proteins were identified by mass spectrometry (Table 1). In order to differentiate between disulfide-linked Trx1 targets and proteins non-covalently interacting with Trx1, complexes from a second experiment were separated by NR/R 2D SDS-PAGE (Figure 19). Only proteins from below the main diagonal - representing disulfide-linked Trx1 target proteins - were sequenced by tandem mass spectrometry (Table 2). Among the 62 Trx1 targets isolated from the 2D gel, 23 were also found in the first experiment (Figure 20A), thus confirming approximately one third of the target proteins in an independent experiment. The seemingly low proportion of overlap is, however, not unexpected as different mass spectrometry setups and machines were used in the two experiments. Even slight differences in instrument settings are known to influence the collection of tandem mass spectra (Berg et al, 2006). It is furthermore likely, that additional Trx1 targets could be identified by increasing the number of cells used for the experiment or by oxidative treatment of cells to increase target protein oxidation and subsequent Trx1 substrate trapping.

The limitation of current approaches to global redox proteomics is sensitivity. Often the identified proteins are those with relatively high copy numbers, and important target proteins may well be in too low abundance to be detected. This problem should be solvable with techniques actively enriching specific subsets of redox-regulated proteins (Winterbourn & Hampton, 2008). Kinetic trapping provides such a selective method as only Trx1 target proteins are captured, enriched and efficiently separated from highly abundant contaminants. Indeed, high abundance proteins typically contaminating results from proteomics (e.g. ribosomal or proteasomal proteins) were not observed in our experiments. Instead, it appears that mostly proteins of low abundance were identified.

The family of peroxiredoxins represents the major cytosolic target for Trx1 in the Jurkat T cell line as large quantities of these proteins were trapped by Trx1(C35S). Most interestingly, we obtained indications that Prx1 may form disulfide-linked

conjugates not only with Trx1, but also with numerous other proteins (Figure 21). This possibility is also supported by the finding that peroxiredoxins are broadly distributed on the NR/R 2D gel (Figure 10) thus implicating that they are disulfide-linked to a wide variety of Trx1 target proteins. The reasons for this surprising finding are not yet understood, but it seems possible that Prx1 is critically involved in the oxidation of Trx1 target proteins. Peroxiredoxins, representing the primary target of H_2O_2, have been suggested to act as peroxide sensors and protein oxidases for redox-sensitive proteins (Winterbourn, 2008). Thus, the presence of peroxiredoxins as components in a vast number of Trx1 target protein conjugates could result from their role as oxidases for those proteins which are subsequently reduced by Trx1.

Because of the high number of newly identified Trx1 targets, this discussion will focus on selected examples and their physiological function. In addition to peroxiredoxins, flightless-1 homolog (FLII) is a very prominent Trx1 target of which over 40 peptide fragments were detected by mass spectrometry corresponding to a protein coverage over 30% (Table 2). FLII was first identified in *Drosophila melanogaster* (Campbell et al, 1993) and is required for actin distribution during cellularization of the fly embryo (Straub et al, 1996). It contains a C-terminal gelsolin-like domain (GLD) and belongs to the gelsolin superfamily of actin-remodeling proteins (Silacci et al, 2004). Because the GLD of FLII is more divergent from that in other gelsolin family members, it has been suggested that it may have other unique regulatory functions. In addition to the GLD, FLII contains an N-terminal leucine-rich repeat (LRR) domain which has been shown to interact with leucine-rich repeat flightless-interacting protein 1 (LRRFIP1), a protein also found in the Trx1 kinetic trapping screen. Whether LRRFIP1 was directly trapped or bound to FLII by disulfide-linkage still remains to be tested. FLII serves as coactivator for nuclear receptor-mediated transcription (Lee et al, 2004) but suppresses β-catenin dependent gene expression (Lee & Stallcup, 2006). Recently, new functions of FLII have been characterized. FLII interacts with the pro-inflammatory protein caspase-1 and inhibits its activity (Li et al, 2008), activates Ca^{2+}/calmodulin dependent protein kinase type II (CaMK-II) (Seward et al, 2008) and negatively regulates the TLR4-MyD88 pathway (Wang et al, 2006). Thus, this prominent target of Trx1 is involved in actin-remodeling and signal transduction, and provides a novel link to redox-regulation of these processes.

The hematopoietic-specific GTPase Rac2 belongs to the family of Rho GTPases that facilitates cell adhesion and chemotaxis response in macrophages and neutrophils (Van Hennik & Hordijk, 2005). In addition, formation and activation of the phagocyte NADPH oxidase (phox) can be promoted by Rac2 *in vitro* (Knaus et al, 1991). Accordingly, $O_2^{\bullet -}$ production in phagocytes from Rac2 knockout mice is markedly diminished (Roberts et al, 1999; Glogauer et al, 2003) demonstrating a major *in vivo* relevance of Rac2 in phox activation. Upon phagocytosis $p67^{phox}$ and active GTP-bound Rac2 translocate to the cell membrane to activate the cytochrome-b_{558} subunit of phox to produce $O_2^{\bullet -}$ which contributes to the killing of microbes internalized into the phagosome. Furthermore, Rac2 does not only regulate phox activation in phagocytes but also has pivotal roles in lymphocytes. T cell activation is compromised in Rac2 deficient mice due to decreased activation of ERK1/2 and p38 MAPK as well as defective cytoskeletal changes (Yu et al, 2001). As also T cells express a phagocyte-type NADPH oxidase (Jackson et al, 2004), Rac2 may also contribute to ROS formation after T cell receptor stimulation. In B cells Rac2 has recently been shown to be required for immunological synapse formation (Arana et al, 2008). Trx1-mediated regulation of Rac2 may provide a novel mechanism of how NADPH oxidase activity is feedback-regulated by redox state. Interestingly, the Rac2 active site contains a conversed cysteine which may facilitate redox-regulation by Trx1.

Finally, it should be highlighted that several of the newly identified Trx1 target proteins have important functions in sugar metabolism. These include Ribose-5-phosphate isomerase, glutamine:fructose 6 phosphate amidotransferase 1 (GFPT1), phospho-fructokinase-1 (PFK-1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Most interestingly, glucose-6-phosphate dehydrogenase (G6PD) was also identified as a candidate Trx1 target protein. G6PD is a key enzyme of the pentose phosphate pathway, the main source of NADPH in the cytosol. As NADPH serves as the electron donor for the reduction of GSSG by glutathione reductase, and for the regeneration of Trx1 by TrxR, it is tempting to speculate that the interaction between Trx1 and G6PD provides feedback regulation of NADPH production. For example, under oxidative conditions G6PD may be activated through thiol oxidation to promote generation of NADPH. Thioredoxin in the reduced state may act as a negative feedback regulator by reducing a putative disulfide bond in G6PD to downregulate its

activity and NADPH output. However, it is also conceivable that the interaction constitutes a positive feedback loop to enhance reducing conditions.

5.4 Redox regulation of the cell cycle

Proliferation and cell division is regulated by an ordered sequence of events. In the S phase of the cell cycle the cellular DNA is duplicated, in the following G2 phase the cell prepares for mitosis and in M phase the DNA gets segregated and the cell divides into two. In the following G1 phase the cell decides whether to stop proliferation or continue with another round of the cell cycle. This decision is made at the so-called restriction point after which the cell enters S phase and becomes independent on mitogenic signaling. Already in 1969 the finding that the cellular protein disulfide content fluctuates during cell cycle and reaches a minimum at the G1/S transition suggested a redox-dependent control of the cell cycle (Mauro et al, 1969). A requirement for oxidants in cell cycle progression was indicated by the observation that overexpression of catalase inhibits proliferation (Brown et al, 1999) and that treatment with the antioxidant NAc induces cell cycle arrest in the G1 phase (Sekharam et al, 1998; Kim et al, 2001). This arrest has been shown to be accompanied with decreased cyclin D1 levels (Menon et al, 2003). The three D cyclins (cyclin D1, D2 and D3) are major regulators of the G1 phase and represent the regulatory subunit of the G1 cyclin-dependent kinases cdk4 and cdk6. These cyclin-cdk complexes phosphorylate Rb which induces its dissociation from E2F, allowing E2F-dependent transcription and entry into S phase (Ekholm & Reed, 2000; Massague, 2004). Cyclin D expression levels are regulated by *de novo* synthesis after growth factor stimulation and degradation by the ubiquitin-proteasome pathway. Both mechanisms have been described to be regulated by ROS. Incubation of fibroblasts with H_2O_2 leads to accumulation of cyclin D1 even in the presence of protein synthesis inhibitors and MAPK inhibitors, suggesting inhibition of proteasome-dependent cyclin D1 degradation (Martinez Munoz et al, 2001). In addition, stimulation of growth factor and cytokine receptors induce ROS which lead to activation of the MAPK pathway and induction of cyclin D expression (Cakir & Ballinger, 2005).

In yeast, thioredoxin deficiency prolongs S phase and shortens the G1 interval of the cell cycle (Muller, 1991) which was later attributed to its function as electron donor for ribonucleotide reductase during DNA precursor synthesis (Koc et al, 2006). Considering the newly identified as well as established cytosolic Trx1 substrates, Trx1 seems to regulate cell cycle progression at various additional stages besides

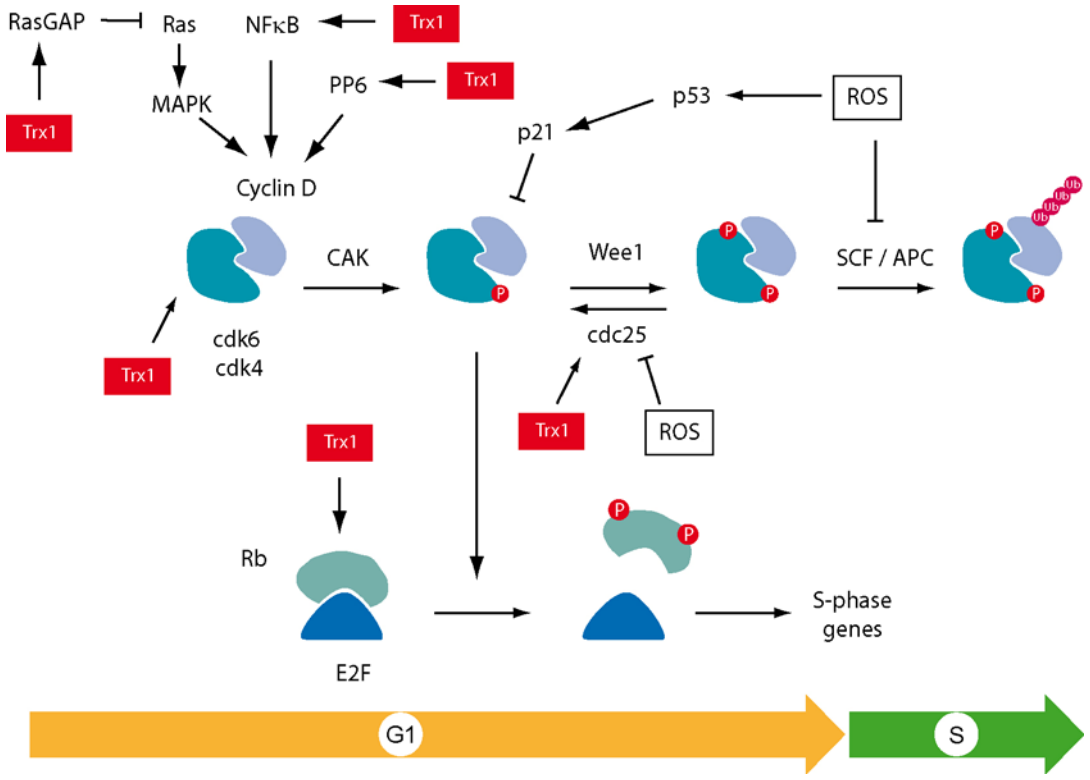

Figure 31: Redox regulation of the G1/S transition by Trx1
Overview of Trx1 target proteins involved in the G1/S transition of the cell cycle. See text for details.

DNA synthesis. Interestingly, most Trx1 substrate proteins identified by kinetic trapping and involved in cell cycle regulation play an important role in G1/S transition (Figure 31). Cyclin D1 expression is up-regulated after growth factor receptor stimulation via the Ras-MAPK pathway. Redox-regulation of the Ras inhibitor RasGAP by Trx1 could influence cell cycle progression by altering cyclin D1 levels. At the transcriptional level cyclin D is regulated by different transcription factors, including AP-1 and NFκB (Burch & Heintz, 2005), both of which have been shown to be substrates of Trx1 (Hirota et al, 1997; Hirota et al, 1999).

Overexpression of protein phosphatase 6 (PP6) induces cell cycle arrest in G1 by suppression of cyclin D1 expression and Rb phosphorylation (Stefansson & Brautigan, 2007). Thus, PP6 reduction by Trx1 could directly influence cyclin D1 levels and transition from G1 to S phase of the cell cycle. This function of Trx1 has already been described for cdc25, a phosphatase that counteracts the kinase wee1 which phosphorylates and thereby inhibits cdks. It has been proposed that the growth-promoting effect of Trx1 could be partially exerted by keeping cdc25 in a reduced and active state (Sohn & Rudolph, 2003). In addition to phosphorylation-based cdk regulation, Trx1 could directly regulate cdk6 by thiol disulfide exchange.

Among the family of cdks, only cdk6 but not cdk1, cdk2 or cdk4 were targeted by the trapping mutant Trx1(C35S) (Figure 23C). Cysteine mutagenesis revealed that Cys7 is required for trapping of cdk6 (Figure 27A) suggesting the formation of a mixed disulfide with another protein. As no such disulfide-linked homo- or heterodimer was detected by immunoblotting the half-life of this conjugate, if it does exist, may be very short, possibly as a consequence of rapid reduction by the endogenous thioredoxin system. When the disulfide bond between cdk6 and the unknown disulfide-linked partner protein is attacked by Trx1(C35S) only one of the two proteins becomes covalently linked to the Trx1 trapping mutant. The alkylating agent MMTS was used to block thiol redox states after H_2O_2 treatment but even under these conditions no disulfide-linked cdk6 conjugates could be detected (data not shown). Depletion of Trx1 by shRNA may serve as a strategy in future experiments to inhibit rapid reduction of cdk6 disulfide-linked conjugates. No difference in kinase activity was measured for the different cdk6 mutants, speaking against a regulatory role of Cys7. For the *in vitro* kinase assay Flag-tagged cdk6 mutants were purified from HEK 293T cells over-expressing cyclin D. However, the status of other cdk6 regulators like p21, CAK, wee1 and cdc25 is poorly defined. A more appropriate strategy to prove the functional relevance of particular cdk6 cysteines would be the depletion of endogenous cdk6 and reconstitution with cdk6 cysteine mutants at a physiological expression level. However, this approach requires that depletion of cdk6 results in a distinct phenotype different from WT cells which can be rescued by re-transfection of cdk6. Unfortunately, cdk6 knockout mice have a very mild phenotype. These mice are viable, have thymic and splenic hypoplasia, and mild defects in hematopoiesis. Only T lymphocytes show a delayed S-phase entry (Malumbres et al, 2004). Apparently, the loss of cdk6 can be largely compensated by cdk4. Cdk4-null mice are viable but sterile and develop diabetes due to abnormal β-islet cell development (Rane et al, 1999). In contrast, cdk4/cdk6 double knockout mice show progressive embryonic lethality from E14.5 onwards and the few surviving animals die soon after birth (Malumbres et al, 2004), clearly demonstrating the redundancy between the two cdks. MEFs deficient in both cdks were only partially able to respond to mitogenic stimulation and to exit quiescence. Thus, the activity of cdk6 cysteine mutants may only be properly analyzed in the absence of cdk4. Recently, mice lacking all interphase cdks (cdk2, cdk3, cdk4 and cdk6) have been shown to undergo organogenesis and MEFs from these cells proliferate *in vitro* (Santamaria et al,

2007). These results indicate that cdk1 is the only essential cell cycle cdk which can execute all events that are required for cell division in the absence of interphase cdks. Thus, the effect of cdk6 on cell cycle progression is difficult to analyze, which becomes even more complicated by the finding that active cdk6 complexes are predominantly nuclear and represent only a minority of the total cdk6 pool (Mahony et al, 1998).

Alternatively, recently identified functions specific to cdk6 may be assayed to assess the relevance of certain cysteine residues for its activity. Ectopic expression of cdk6 circumvents TGF-β mediated growth inhibition in mink epithelial cells (Zhang et al, 2001) and recent findings demonstrate an unexpected role for cdk6 in differentiation of a variety of cell types, a function that is not shared with cdk4. Expression of cdk6 but not cdk4 in mouse astrocytes was associated with de-differentiation to glial progenitor-like cells (Ericson et al, 2003). Accordingly, over-expression of cdk6 blocked osteoblast differentiation without altering cell cycle progression (Ogasawara et al, 2004). Even though the exact mechanism of how cdk6 blocks differentiation is not understood, it may be asked if this cdk4-independent function depends on particular cdk6 cysteine residues and redox-regulation.

As Rb was identified as a Trx1 target protein, cell cycle progression may be influenced by redox-regulation of this tumor suppressor. Moreover, inhibition of Rb by Trx1 could provide an additional explanation for the observation that tumor growth benefits from increased levels of Trx1, adding to the picture that Trx1 can play an important role in tumor development (Figure 9).

5.5 Redox regulation of apoptosis

Programmed cell death or apoptosis was first described in 1972 (Kerr et al, 1972). It is characterized by a tightly regulated cascade of proteolytic events eventually leading to controlled disposal of the cell without induction of inflammation or tissue damage. Apoptosis can be executed by two distinct pathways. The extrinsic pathway is initiated by activation of death receptors including CD95 (APO-1/Fas), TNF-R or TRAIL-R (Bhardwaj & Aggarwal, 2003; Krammer et al, 2007) which induces formation of the so-called death-inducing signaling complex (DISC) and activation of caspase-8. Caspase-3 is a key enzyme at the end of a caspase activation cascade which eventually leads to degradation of cellular caspase substrates and execution of apoptosis.

The intrinsic pathway is activated by cellular stress including cytokine deprivation, mutagenic irradiation or oxidative stress and induces aggregation of the pro-apoptotic Bcl-2 family members Bak and Bax. These BH3-only proteins induce mitochondrial outer membrane permeabilization (MOMP) which leads to release of cytochrome c. Cytoplasmic cytochrome c interacts with caspase-9, apoptotic peptidase activating factor 1 (APAF1) and ATP to form an oligomeric complex, called the apoptosome (Green & Kroemer, 2004). Apoptosome formation eventually leads to activation of caspase-3 and execution of apoptosis. Death receptor mediated apoptosis can be amplified by cleavage of Bid and subsequent cytochrome c release which integrates the extrinsic and intrinsic pathway.

Oxidative stress activates JNK and p38 MAPK via ASK1 (Ichijo et al, 1997). Constitutively active mutants of ASK1 induce cytochrome c release and activation of caspase-3 and -9, but not of caspase-8, indicating that ASK1 activates mainly the intrinsic apoptotic pathway (Hatai et al, 2000). The mechanism of ASK1 regulation by Trx1 has been already been described in chapter 1.2.2. Additionally, Trx1 regulates p53 function which is a direct transcriptional activator of the Bax gene (Miyashita & Reed, 1995).

ROS do not only promote oxidative stress and apoptosis via ASK1 but are also produced after death receptor stimulation (Gulbins et al, 1996; Sato et al, 2004; Medan et al, 2005). Antioxidants like NAc can block CD95-mediated cell death indicating that both the extrinsic and the intrinsic pathway are redox regulated. Kinetic trapping identified so far unknown Trx1 substrates involved in both pathways (Figure 32). Among the four heat-shock proteins found in the trapping screen, Hsp27,

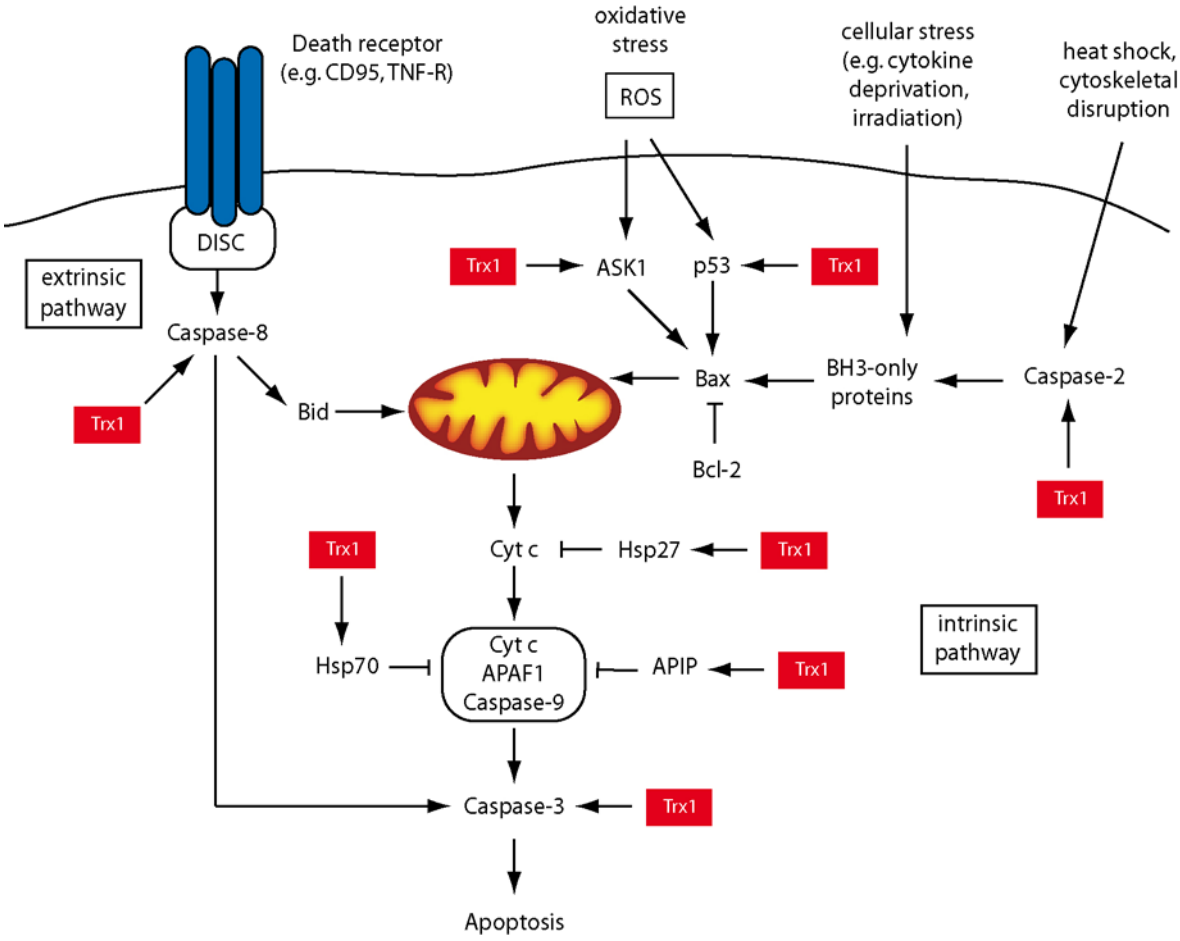

Figure 32: Redox regulation of apoptosis by Trx1
Trx1 target proteins involved in apoptosis. See text for details.

Hsp70 and Hsp90 have been shown to inhibit apoptosis (Garrido et al, 2003; Beere, 2005). Hsp70 inhibits TNF-induced caspase-8 activation, Bid cleavage and cytochrome c release independently of its chaperone activity (Gabai et al, 2002). Moreover, Hsp70 and Hsp90 prevent recruitment of caspase-9 to the apoptosome (Beere et al, 2000; Pandey et al, 2000) and Hsp27 negatively regulates cell death by interacting with cytochrome c (Bruey et al, 2000). Interestingly, Hsp70 also inhibits Trx1-mediated activation of ASK1 (Park et al, 2002). Thus, the heat-shock proteins targeted by Trx1 not only facilitate protein folding but furthermore play a regulatory role in apoptosis.

APAF1-interacting protein (APIP) inhibits cytochrome-c induced activation of caspase-3 and caspase-9 which would otherwise lead to induction of apoptosis (Cho et al, 2004). Recently, it has been shown that APIP also leads to sustained activation of Akt (PKB) and extracellular signal-regulated kinase (Erk) which can suppress hypoxia-induced cell death (Cho et al, 2007).

Regulation of caspase-3 by Trx1 has been described earlier. Recombinant caspase-3 was suggested to be activated by Trx1 (Baker et al, 2000) and inactivated by H_2O_2 (Hampton et al, 2002). It was demonstrated that Trx1 can catalyze the S-nitrosylation of the caspase-3 active site, leading to inhibition of caspase-3 activity (Mitchell & Marletta, 2005). Recently, caspase-3 and GST-tagged Trx1(C35S) were co-expressed in HEK 293T cells and caspase-3 could be co-precipitated with the trapping mutant after treatment with *S*-nitrosocysteine (CysNO). No interaction was observed in untreated cells or cells transfected with GST-tagged WT Trx1, suggesting that Trx1 specifically reduces nitrosylated caspase-3 which can be trapped with Trx1(C35S). Moreover, it was proposed that Trx2 denitrosylates mitochondria-associated caspase-3 to promote CD95-induced apoptosis (Benhar et al, 2008). The observation that the interaction beween Trx1 and caspase-3 strictly depends on nitrosylation is in line with our finding that Trx1 does not form a disulfide-linked conjugate with caspase-3 which is not likely to be nitrosylated in our experimental setup (Figure 23B).

In this study, caspase-2 was found to be targeted by Trx1(C35S). Caspase-2 is classified as initiator caspase due to its long pro-domain and the requirement of dimerization rather than cleavage for activation (Baliga et al, 2004). The mode of caspase-2 activation is very controversial ranging from cytoskeletal disruption (Ho et al, 2008), genotoxic stress (Tinel & Tschopp, 2004), DNA damage (Sidi et al, 2008), ER stress (Upton et al, 2008) and death receptor stimulation (Shin et al, 2005; Lavrik et al, 2006). The role of caspase-2 in heat-shock induced apoptosis was confirmed by the demonstration that caspase-2 deficient cells remain viable after heat-shock (Tu et al, 2006). Heat-shock induces caspase-2 mediated Bid cleavage which results in MOMP and apoptosome formation (Bonzon et al, 2006). It has been proposed that oxidative stress activates caspase-2 (Prasad et al, 2006; Tamm et al, 2008) but this conclusion was drawn from data obtained with the peptide inhibitor z-VDVAD-fmk or the fluorogenic substrate VDVAD-AMC. Recently it has been demonstrated the peptide sequence VDVAD is also targeted by caspase-3 (McStay et al, 2008) which undermines the results from experiments in which this peptide was used in the presence of caspase-3. To address this complication, the effect of Trx1 on caspase-2 activity was reproduced with recombinant caspase-2 (Figure 23B). WT Trx1, but not the active site mutant, could activate recombinant as well as endogenous caspase-2

after stimulation of CD95.

The analysis of Trx1-mediated caspase regulation in intact cells is complicated by the lack of specific caspase substrates, but also by our finding that caspase-8 is another substrate of Trx1. TRAIL-induced caspase-8 activation can be blocked by catalase and vitamin C (Perez-Cruz et al, 2007), indicating that caspase-8 itself could be redox-regulated. Moreover, overexpression of Trx1 enhances interferon and retinoid-induced caspase-8 activation (Ma et al, 2001). However, no mixed-disulfide between Trx1 and caspase-8 has been described prior this study.

5.6 Conclusion and future directions

In this study, activity-based kinetic trapping of Trx1 target proteins was applied for the first time to the cytoplasmic compartment of a mammalian cell line. This work demonstrates that this technique is particularly suitable to selectively capture and identify mammalian cytosolic Trx1 target proteins. While the current work is focused on the identification of Trx1-interactions under steady-state conditions, in the future it will be most interesting to apply this method to comparative studies, i.e. to detect changes in protein redox states specifically associated with changes in cellular behavior, e.g. upon activation, entry into the cell cycle or differentiation. For this purpose the preservation of intracellular protein redox states prior to trapping is a major concern. However, the protection of thiols and disulfides with cell-permeable alkylating agents and the endogenous expression of the Trx1 trapping mutant should allow for comparative studies of Trx1 target protein oxidation in different physiological as well as pathophysiological conditions.

The next step, and another major challenge for the future is to address in detail the physiological relevance of Trx1-mediated redox regulation. Having identified individual Trx1-interacting redox-sensitive proteins, specific questions about the functional changes associated with oxidation and reduction can be asked. In this study, the regulation of caspase-2 activity by oxidation and Trx1-mediated reduction could be demonstrated *in vitro*. However, the functional consequences of caspase-2 redox regulation in intact cells and *in vivo* still need to be investigated. In many cases, the analysis of Trx1-dependent regulation in intact cells is complicated by redundancy. For example, the activity and function of cdk6 is difficult to study in cells which also express cdk4. Moreover, the relevance of Trx1 for certain cellular processes is not easy to analyze due to the lack of specific Trx1 inhibitors. However, the inducible Trx1-knockdown system established in this thesis is likely to provide a valuable tool for future analysis of Trx1-regulated pathways.

Cysteine mutagenesis of cdk6 led to the somewhat unexpected finding that only one cysteine is required for the interaction with Trx1. Even though formation of disulfide-linked homodimers is a likely explanation for this observation, other possibilities may be considered. This raises the more general question which cysteine modifications can actually be targeted by Trx1. In this regard, the long-held assumption that Trx1

only reduces protein disulfide bonds should be reviewed. Recent observations from other groups suggest that Trx1 can target nitrosylated thiols (-SNO). The possibility that Trx1 directly attacks sulfenic acid residues (-SOH) should also be considered. As outlined before, the kinetic trapping procedure can be used to address and answer these important questions.

From two independent proteomic trapping experiments over 100 novel Trx1 candidate target proteins were identified. These are involved in a wide variety of cellular processes including regulation of the cytoskeleton, sugar metabolism, cell cycle and apoptosis. Despite this functional variety, Trx1 target interactions were found to be highly specific. Among the large number of newly identified proteins, flightless-1 homolog stood out as a predominant Trx1 target protein. This makes the gelsolin-like actin-binding protein a prime candidate for further study, as it suggests a major role for the cytoskeleton in the response to oxidants. In addition, it will be especially interesting to address the Trx1-Rac2 redox interaction which potentially regulates the activity of NADPH oxidases, thus providing a mechanism of how ROS production can be feedback-regulated by oxidants.

Another conclusion of this work is that kinetic trapping should be applicable to other members of the thioredoxin family using the same CXXC based reaction mechanism. Other Trx isoforms or Trx-like proteins from different cellular compartments, especially mitochondrial Trx2 may be studied the same way. Such experiments will provide important information on how the different thioredoxin family members differ in their substrate specificity and associated cellular functions.

6 Abbreviations

AP-1	activator protein-1
APIP	Apaf-1 interacting protein
APS	ammonium peroxodisulfate
ASK1	apoptosis signal-regulating kinase 1
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
BSA	bovine serum albumin
CaMK-II	Ca^{2+}/calmodulin dependent protein kinase type II
Cdk	cyclin-dependent kinase
CDS	Coding sequence
Csk	C-terminal Src kinase
ddH_2O	Double distilled water
DISC	death-inducing signaling complex
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNCB	1-chloro-2,4-dinitrobenzene
dNTP	deoxynucleoside
Dox	Doxycycline
DTT	dithiothreitol
E.coli	*Escherichia coli*
EBV	Epstein-Bar virus
EDTA	ethylenediaminetetraacetic acid
EF	Elongation factor
EGF	Epidermal growth factor
Erk	extracellular signal-regulated kinase
ESI	electrospray ionization mass
ETC	electron-transport chain
FAD	flavin adenine dinucleotide
FCS	fetal calf serum
FLII	flightless-1 homolog
FRT	ferredoxin-thioredoxin reductase
G6PD	glucose-6-phosphate 1-dehydrogenase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
GFPT1	glutamine:fructose 6 phosphate amidotransferase 1
GLD	gelsolin-like domain
GPx	Glutathione peroxidase
Grx1	glutaredoxin-1
GSH	Reduced glutathione

GSSG	Oxidized glutathione
GST	glutathione S-transferase
H_2DCFDA	2',7'-dichlorohydrofluorescein diacetate
HBS	HEPES buffered saline
HDAC	histone deacetylase
HEPES	N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid
HIF-1α	hypoxia-inducible factor-1 alpha
HIV-1	human immunodeficiency virus 1
hnRNP	heterogeneous nuclear ribonucleoprotein
HRP	horseradish peroxidase
Hsp	Heat-shock protein
HTLV-I	human T-cell leukemia virus type-I
IAM	Iodoacetamide
IB	Immunoblot
IL	Interleukin
IMDM	Iscove's modified Dulbecco's medium
INF-γ	Interferon-γ
IP	Immunoprecipitation
IPTG	isopropyl-β-D-thiogalactopyranosid
IRES	Internal ribosome entry site
JAK2	Janus kinase-2
JNK	c-Jun N-terminal kinase
kDa	kilodalton
LB	lysogeny broth
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LMW-PTP	low molecular weight protein tyrosine phosphatase
LNGFR	low affinity nerve growth factor receptor
LPS	Lipopolysaccharide
LRR	leucine-rich repeat
LRRFIP1	leucine-rich repeat flightless-interacting protein 1
MACS	magnetic-activated cell sorting
MALDI-TOF	matrix assisted laser desorption/ionisation-time of flight
MAPK	Mitogen-acitvated protein kinase
MEF	mouse embryonic fibroblast
MES	Morpholinoethanesulfonic acid
MMTS	Methylmethanethiosulfonate
MMULV	moloney murine leukemia virus
MOMP	mitochondrial outer membrane permeabilization
NAc	N-acetyl cysteine
NADPH	nicotinamide adenine dinucleotide phosphate
NCR	Non-coding sequence

NEK9	NimA-related kinase 9
NEM	N-ethylmaleimide
NFκB	nuclear factor-κB
NO	nitric oxide
Nox	NAPDH oxidase
NR	Non-reducing
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDB	Protein data base
PDGF	Platelet-derived growth factor
PDI	protein disulfide-isomerase
PKA	cyclic AMP-dependent protein kinase
PKC	protein kinase C
PKN1	Protein kinase N1
PMA	phorbol 12-myristate 13-acetate
PMF	peptide mass fingerprint
PP6	Protein phosphatase 6
Prx	Peroxiredoxin
PTEN	phosphatase and tensin homolog
PTK	protein tyrosine kinase
PTP	protein tyrosine phosphatase
PVDF	polyvinylidene fluoride
R	Reducing
Rb	retinoblastoma
Ref-1	redox-factor 1
RIR1	ribonucleoside-diphosphate reductase
RNA	ribonucleic acid
ROS	Reactive oxygen species
Rpm	rounds per minute
RT	room temperature
rtTA	Reverse tetracycline controlled transactivator
SA	streptavidin
SBP	streptavidin binding peptide
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
SeCys	Selenocysteine
SH2	Src homology 2
SHP2	SH2 domain-containing protein tyrosine phosphatase-2
shRNA	short-hairpin RNA
siRNA	small-interfering RNA
SOD	Superoxide dismutase
Srx	Sulfiredoxin

STAT-1	signal transducer and activator of transcription-1
TBS	Tris buffered saline
TCA	trichloroacetic acid
TEMED	N,N,N',N'-Tetramethylethylenediamine
tetO	Tet operator
TetR	Tet repressor
TFA	trifluoroacetic acid
TGF-β	transforming growth factor-beta
TLR4	Toll-like receptor 4
TNF-α	tumor necrosis factor-α
Tris	tris (hydroxymethyl) aminomethane
Trx1	thioredoxin-1
TrxR	thioredoxin reductase
tTA	tetracycline controlled transactivator
T-X100	Triton X-100
U	Units
VDUP1	vitamin D3 up-regulated protein 1
VEGF	vascular endothelial growth factor
WT	wild type

7 References

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