Supporting Information

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SI Materials and Methods

Chemicals and Reagents. Chemicals and reagents were purchased from Sigma-Aldrich Chemicals, unless stated otherwise. Rat thioredoxin reductase 1 (TrxR1) (~32 U/mg), human wild-type thioredoxin 1 (Trx1), and human glutaredoxin 1 (Grx1) were recombinantly produced and purified as described previously (1–3). Yeast glutathione reductase (GR) and cathepsin B (human liver) were purchased from Calbiochem (Merck Millipore). S-nitrosocysteine (L-CysSNO) was prepared as described elsewhere (4). Concentrations of nitrosothiols were calculated from the absorbance at 334 nm using the extinction coefficient 767 $M^{-1}\cdot cm^{-1}$ for S-nitrosogutathione (5) and 900 $M^{-1}\cdot cm^{-1}$ for L-CysSNO (6). Solutions used in the denitrosylation experiments were prepared in deionized and Chelex-100–treated water or potassium phosphate buffer containing 0.1 mM EDTA.

Cloning and Mutagenesis of Human Thioredoxin-Related Protein of 14 kDa and Trx1. The ORF of a cDNA clone (imaGenes) encoding human thioredoxin-related protein of 14 kDa (TRP14) was amplified by PCR using the forward primer TRP14_f (5′-GCCA-TATGGCCCGCTATGAGGAGGTGAGC-3′) containing a NdeI site (underlined) and the start codon (boldface type) in combination with the reverse primer TRP14_r (5′CGCGGATCC**TTA**-ATCTTCAGAGAACAACATTTCC-3′) containing a BamHI site (underlined) and stop codon (boldface type). The PCR product was ligated into a pET20b expression vector (Invitrogen), thus introducing an N-terminal His-tag. The three TRP14 mutant proteins, with active-site Cys residues replaced by Ser (C43S, C46S and C43/46S double mutant), were generated by PCR-mediated site-directed mutagenesis. For mutagenesis of Trx1, a plasmid for wild-type human Trx1 (1) was used as template for introduction of an N-terminal His-tag and the active-site mutations. All final plasmids were transformed into *Escherichia coli* BL21 (DE3) competent cells (Invitrogen), and ORFs were verified through commercial DNA-sequencing services (GATC Biotech).

Expression and Purification of TRP14 and Trx1 Variants. *E. coli* BL21 (DE3) cells transformed with the respective expression plasmids were cultured at 37 °C in LB medium supplemented with 0.1 mg/mL ampicillin and propagated until the OD_{600} reached ~0.6, whereupon protein expression was induced using 0.4 mM isopropyl-β-D-thiogalactopyranosid with subsequent culturing for ~12 h at 18 °C. Cells were harvested and lysed in purification buffer (50 mM Tris, 500 mM NaCl, 10% (vol/vol) glycerol, pH 7.5) containing 25 mM imidazole. Nucleic acids were removed with DNase (Roche), and cell debris were removed by centrifugation at 34,500 × *g* for 30 min. Cleared cell lysates were filtered (0.2 μM) whereupon His-tagged proteins were purified using Nickel-Sepharose Fast Flow beads or HisTrap columns, according to the manufacturer's instructions (GE Healthcare). Protein absorbance was followed at 280 nm, and fractions were analyzed using SDS/PAGE (Invitrogen). In some cases, the target proteins were further purified with size-exclusion chromatography (Superdex G200, GE Healthcare). As the activities of TRP14 or Trx1 were not compromised by the presence of the His-tag, it was not removed. The Bradford method (Bio-Rad) was used to estimate protein concentrations, with BSA as standard. Total yields of purified proteins were about 15 mg per liter of culture, and all TRP14 or Trx1 preparations were pure near homogeneity, as judged by Coomassie-stained SDS/PAGE analyses.

Immunoblot Analyses. For immunoblot analyses, proteins were separated on SDS/PAGE and subsequently transblotted to nitrocellulose membranes (Invitrogen). Equal loading of cell lysate proteins (10 μg per lane) was confirmed using Ponceau staining (Sigma). Primary antibodies were used according to the manufacturer's instructions (anti-TRP14 (Fig. 1) (Atlas Antibodies), anti-TRP14 (Fig. 6) (R&D Systems), anti-Trx1 (sc-58439, Santa Cruz), anti-TrxR1 (sc-58444, Santa Cruz), and anti-GAPDH (sc-25778, Santa Cruz). Signals were detected using a Bio-Rad ChemiDoc XRS scanner, and densitometry analyses were performed using the Quantity One 4.6.7 software.

Determination of Kinetic Parameters. Activities of the TRP14 or Trx1 variants were determined from TrxR1-dependent NADPH consumption as assessed following decrease of absorbance at 340 nm (7) using a VersaMax microplate reader (Molecular Devices). To measure activities in hydroxyethyldisulfide (HED) reduction, typical Grx assay conditions were used (8). The protocols were modified to use in 96-well microtiter plates with total reaction volumes of 200 μL, and all experiments were carried out in TE buffer (50 mM Tris·HCl, 2 mM EDTA, pH 7.5) at ~20 °C with protein concentrations (TRP14, Trx1, TrxR1, Grx1, and GR) as indicated. For TrxR1-coupled assays, reactions were initiated by simultaneous addition of NADPH and TrxR1 to a reaction mixture containing substrates and either TRP14 or Trx1. For the Grx assays, a mixture of GR, GSH, and NADPH was prepared in TE buffer, and reactions were initiated by addition of HED and Grx1, Trx1, or TRP14. If not stated otherwise, background samples containing all compounds except Grx1, Trx1, or TRP14 were analyzed in parallel, with any background absorbance subtracted afterward. Specific activities and, where applicable, kinetic parameters were calculated using absorbance values of NADPH standard curves. Fits to Michaelis–Menten equations were made using the Prism 5.0 software.

Fluorescence Kinetic Assays. The NADPH consumption assay described above was modified to a fluorescence-based assay, in which Bodipy-labeled L-cystine (Invitrogen) was used as substrate. The assay was performed in a total reaction volume of 200 μL, and fluorescence changes over time were determined using a Victor3 plate reader (Perkin-Elmer) with 405 nm of excitation and 520-nm emission filters. The experiments were carried out in TE buffer (50 mM Tris·HCl, 2 mM EDTA, pH 7.5) at ~20 °C with concentrations as indicated. Unless stated otherwise, background samples were included for every sample and subtracted afterward.

Cell Cultures and Preparation of Cell Lysates. A549 (lung carcinoma) and A431 (epidermoid carcinoma) cells were cultured in Dulbecco's Modified Eagle's Medium containing 4.5 g/L glucose (Invitrogen) further supplemented with 2 mM glutamine. To A431 cell medium, 1 mM sodium pyruvate was added. HT-29 (colorectal adenocarcinoma) cells were cultured in McCoy's 5a medium (Lonza) supplemented with 2 mM glutamine. THP1 (acute monocytic leukemia) cells were cultured in RPMI medium 1640 (ATCC) supplemented with 0.05 mM 2-mercaptoethanol. HEK293 cells were cultured in Eagle's Minimum Essential Medium (ATCC). All cell media were complemented with 10% (vol/vol) FBS (PAA laboratories), 100 U/mL penicillin, and 100 μg/mL streptomycin (Biochrom AG). Cells were kept in logarithmic growth phase at 37 °C in humidified air containing 5% CO_2. For preparation of extracts, cells were harvested at

a confluence of ~80% using trypsination (Gibco) and resuspended in lysis buffer (50 mM Tris·HCl, 5 mM EDTA, 150 mM NaCl, 1% Triton-X-100) including protease inhibitor mixture Complete (Roche), followed by three rapid freeze/thaw cycles. Lysates were cleared by centrifugation at 16,000 × *g* for 30 min at 4 °C, and protein concentrations were determined using the Bradford method with BSA as a standard (Bio-Rad). Cell lysates were stored at −20 °C until further analysis.

Measurement of Nitrosothiol Content with a NO Analyzer. Nitrosothiol quantities were determined following their Cu^{+}-catalyzed decomposition to the corresponding thiols and NO, with chemiluminescence measurements of the latter in gas phase using a NO analyzer (CLD 77:00 AM; ECO Physics, Inc.) as described previously (9). The chemiluminescence signals were integrated and plotted as arbitrary units on the *y* axis against time of incubation before injection into the NO analyzer on the *x* axis. All NO analyzer experiments were repeated at least two times, and similar results were obtained.

Measurements of Caspase 3 and Cathepsin B Activities. Protease activities of both proteins were measured fluorometrically using a Perkin-Elmer 2300 EnSpire Multilabel Reader spectrofluorimeter. For caspase 3 Ac-Asp-Met-Gln-Asp-AMC (AMC: 7-amino-4-methylcoumarin; Enzo Life Sciences), and for cathepsin B the Z-Arg-Arg-AMC peptide (Sigma) was used as substrate (10). The nitrosylated forms of caspase 3 and cathepsin B were incubated for 15 min at 37 °C with 5 nM TrxR1, 100 μM NADPH, 5 μM Trx1, and 5 μM TRP14 as indicated. The fluorescent substrates were added to a final concentration of 20 μM, and the change in fluorescence was measured for 10 min.

Plasmids for Cell Transfections. Transient knockdown of TRP14, Trx1, and TrxR1 was performed using shRNA-bearing plasmids for the respective proteins (Sure Silencing, Qiagen). To overexpress TRP14, Trx1, and TrxR1, the ORFs for all three proteins were inserted into the pEGFP-N3 vector (Clontech). A plasmid containing the complete sequence for human of TrxR1 (including the selenocysteine insertion sequence element containing 3′ UTR) was kindly provided by Anastasios E. Damdimopoulos (Karolinska Institutet, Stockholm) (11, 12). The ORF for TrxR1 was amplified using TrxR1-f (5′-GTGGTCTCGATGACGACGATAAGATGAACGGCCCTGAAGATCTTC) and TrxR1-r (5′-GTGGTCTCGGATCCCCATTTCTTGAATTCGCCAAATG) containing Eco31I restriction sites (underlined). The pEGFP-N3 vector backbone was amplified using EGFP-f (5′-GTGGTCTCGGATCCATCGCCACCATGGTGAG) and EGFP-r (5′-GTGGTCTCGTCATCCTTGTAATCCATGGTGGCGAATTCGAAGC). Both PCR products were purified, digested with Eco31I, and ligated. With this procedure we concomitantly added a N-terminal FLAG tag (DYKDDDK) and removed the GFP. Using this plasmid as a template, we next replaced the ORF of TrxR1 by Trx1 and TRP14 using the protein expression plasmids as templates. The following primers containing EcoRI and BamHI restriction sites (underlined) were used: TRP14-f (5′-GTGAATTCGCCACCATGGCCCGCTATGAGGAGGTGAGCG), TRP14-r (5′-GTGGATCCATCTTCAGAGAACAACATTTCCACCAG), Trx1-f (5′-GTGAATTCGCCACCATGGTGAAACAGATCGAG), and Trx1-r (5′-GTGGATCCGACTAATTCATTAATGGTG). All ORFs were verified through commercial DNA-sequencing services (GATC Biotech).

Cell Transfection. HEK cells were seeded in 100-mm dishes at a density of 2.5×10^6 cells/dish 24 h before transfection. For knockdown, 4 μg of plasmid were transfected into cells using 15 μL Attractene (Qiagen) as described by the manufacturer. For overexpression, 3 μg of plasmid was transfected into cells using 6 μL Turbofect (Thermo Scientific). The cells were harvested after 72 h, and cell lysates were prepared for immunoblot analyses and activity assays as described above. Knockdown and overexpression samples were compared with their respective transfection control (a Sure Silencing negative control plasmid and a pEGFP vector expressing solely GFP).

Measurement of Nitrosothiol Content of Cell Lysates with the 2,3-Diaminonaphthalene Assay. HEK cells were seeded in six-well plates at a density of 400,000 cells/well 24 h before transfection. All plasmids were transfected using Turbofect (Thermo Scientific) as transfection reagent with 0.6 μg DNA and 1.2 μL Turbofect per well. After 72 h, cells were treated with 500 μM of L-CysSNO for 15 min at 37 °C, rinsed with phosphate buffered salilne, and then cell pellets were stored at −80 °C until further analysis. Cell lysates were prepared and protein content determined using the Bradford method as described above. For the 2,3-diaminonaphthalene (DAN) assay, 100 μg of total protein was incubated with 200 μM $HgCl_2$ and 200 μM DAN for 30 min (in the dark at ~20 °C), after which 1 M NaOH was added. The quantity of fluorescent 2,3-naphthyltriazole was measured with a Perkin-Elmer 2300 EnSpire Multilabel Reader spectrofluorimeter (λ_{ex} = 375 nm, λ_{ex} = 450 nm) (13). Cell extract from HEK cells that had not been treated with L-CysSNO was included as a negative control, and background fluorescence was subtracted from each experiment.

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