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Supplementary Materials for

A novel persulfide detection method reveals protein persulfide- and polysulfide-reducing functions of thioredoxin and glutathione systems

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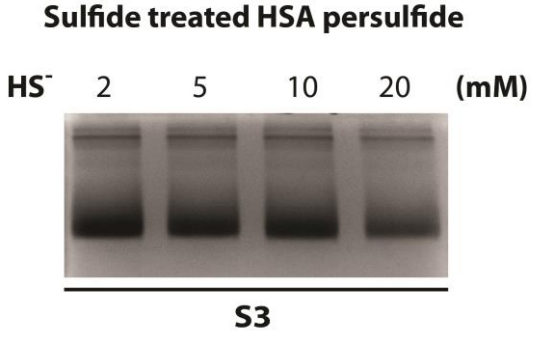

Fig. S1. HSA persulfide is reduced to HSA by sulfide in a dose-dependent manner. Incubation of HSA-SSH with increasing concentrations of sulfide for 30 min before alkylation reduces some of the HSA-SSH by shifting the equilibrium of reaction 1 (see Main text, Fig. 2A'). Note that the indicated sulfide concentrations does not necessarily reflect the equilibrium constant of equation 1 in the main text and further kinetic and thermodynamic experimentation is needed to make quantitative conclusions.

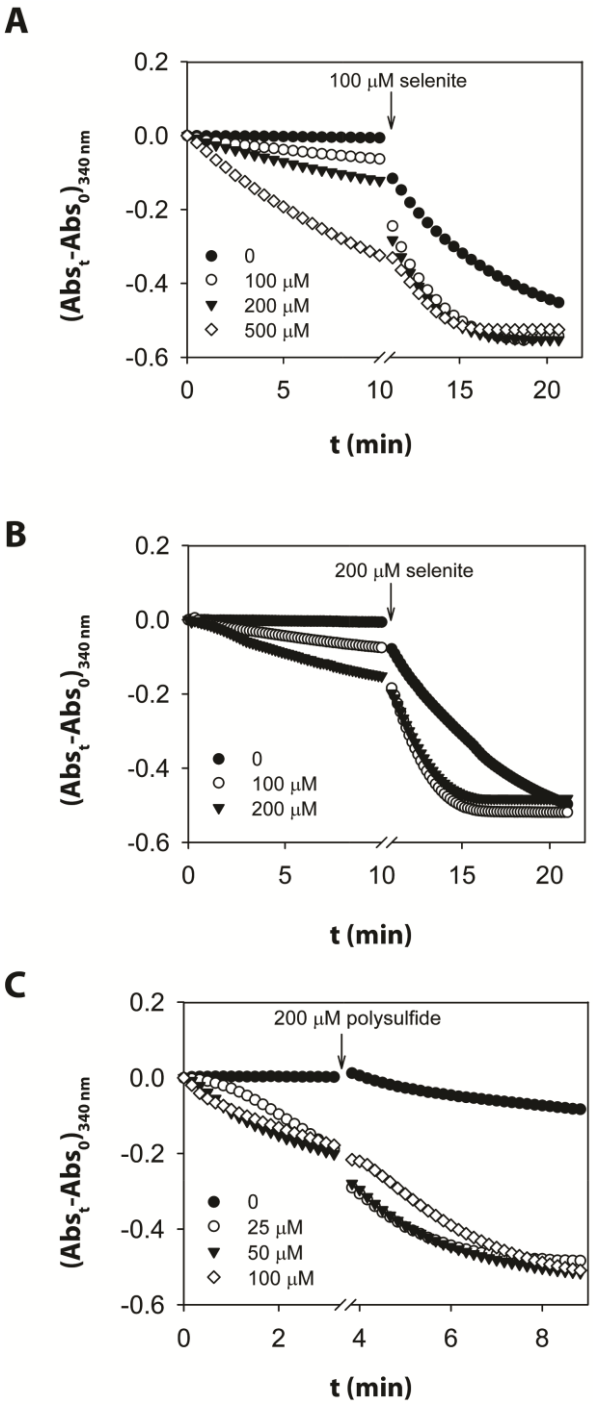

Fig. S2. Selenite-reducing TrxR1 activity is not inhibited by polysulfides. NADPH consumption was followed as the decrease in absorbance at 340 nm over time in a mixture of 250 μM NADPH, 100 nM TrxR1 and (**A**) 0-500 μM polysulfide, (**B**) 0-200 μM polysulfide or (**C**) 0-100 μM selenite. At the indicated time point by the arrow (A) 100 μM selenite, (B) 200 μM selenite or (C) 200 μM polysulfide was added to the corresponding reaction mixtures.

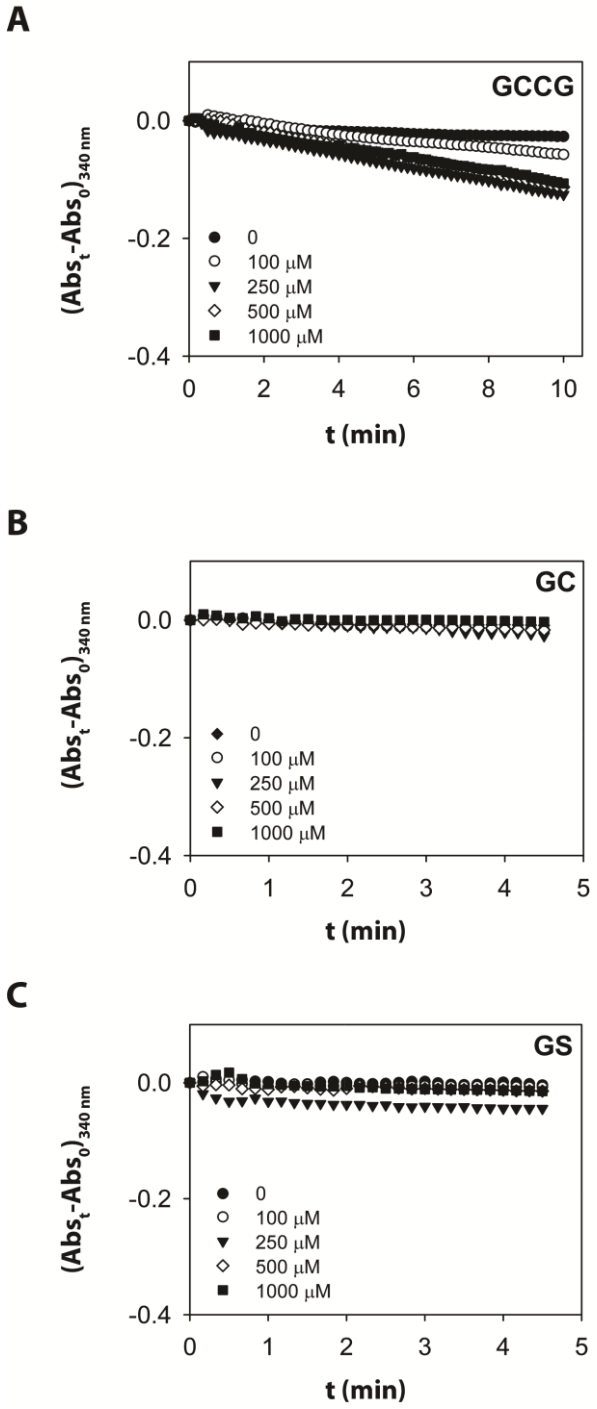

Fig. S3. Compromised polysulfide reduction by TrxR1 mutants lacking the Sec residue. The Sec residue at the C-terminal active site of TrxR1 was mutated to Cys (**A**) or the conventional -GCUG active site of the enzyme was truncated to GC (**B**) or truncated and mutated to GS (**C**). Polysulfide (0-1000 μM) was mixed with 50 nM of the corresponding TrxR1 mutant enzyme in the presence of 250 μM NADPH with kinetic traces recorded spectrophotometrically at λ = 340 nm, following the consumption of NADPH (see also the GCSG mutant in main text Fig. 3D).

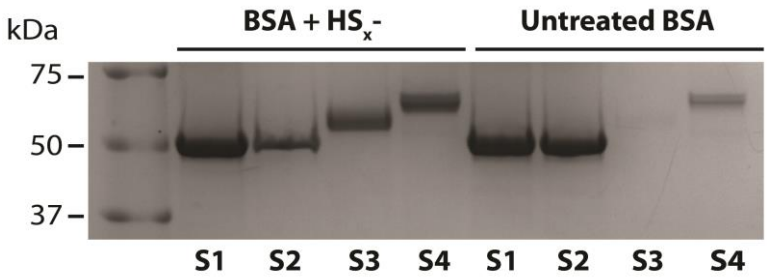

Fig. S4. Detection of persulfide formation on BSA. The ProPerDP method was here used with 1 mg/ml purified BSA. Protein samples were treated with 1 mM polysulfide or only buffer (untreated control) for 30 minutes in TE buffer, followed by alkylation with 1 mM IAB for 30 minutes (*S1*). The alkylated proteins were pulled down from the mixture with streptavidin agarose resin (non alkylated residual proteins are depicted in *S2*) then cleaved off with 5 mM TCEP (*S3*). The remaining portion (representing the thioethers of alkylated Cys residues) was eluted from the beads by boiling in SDS sampling buffer at 100 °C for 3 minutes (*S4*). *S1-S4* refers to sampling as shown in Fig. 1 of the main text.

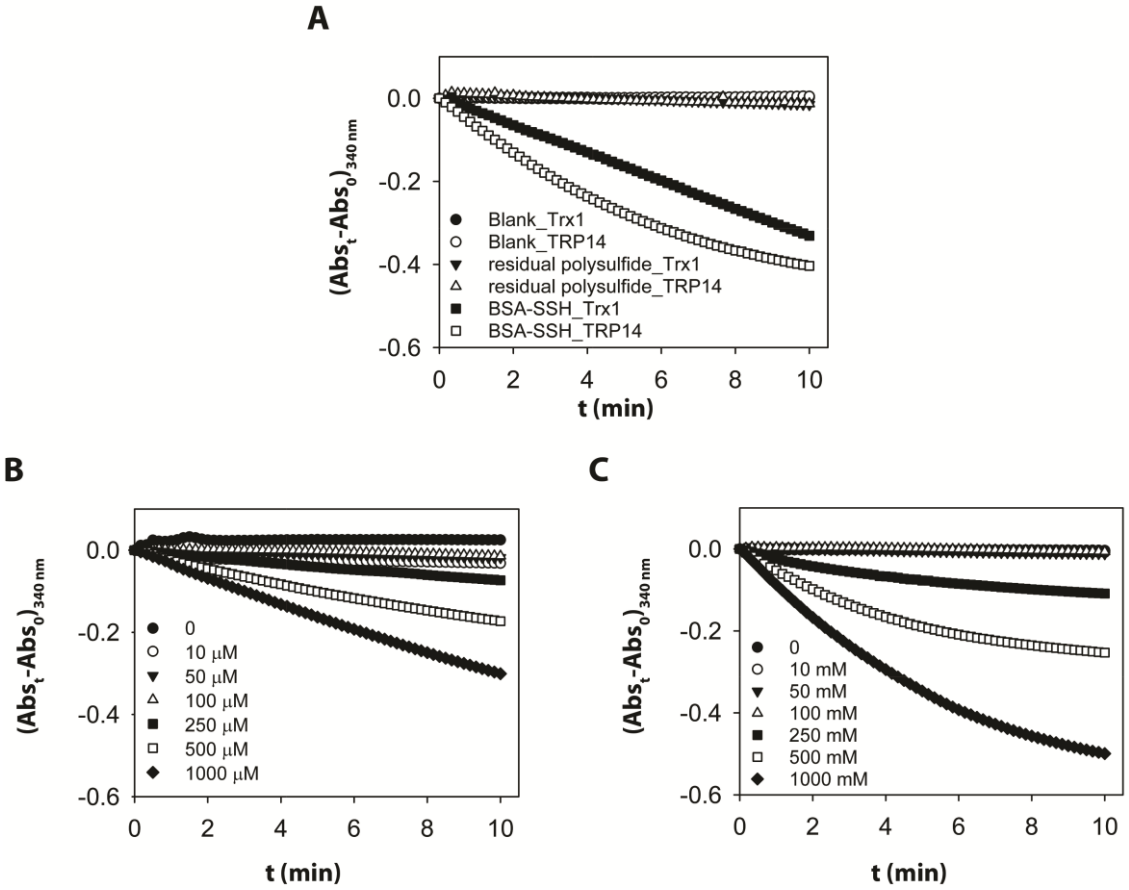

Fig. S5. BSA-SSH reduction by NADPH/TrxR1-coupled Trx1 and TRP14. (**A**) 18 mg/ml (270 μM) BSA was treated with 1 mM polysulfide and desalted with a 7K MWCO desalting spin column. In a control sample, an equal volume of 1 mM polysulfide solution in the absence of protein was passed through a spin column. The flow-throughs were mixed with 50 nM TrxR1 and 5 μM Trx1 or 2 μM TRP14. No detectable activities were observed for Trx1 (▼) or TRP14 (Δ) in the protein free polysulfide control samples indicating that the desalting steps were effective and the measured activities for BSA-SSH (■,□ and on Fig. 3H and Fig. S5B&C) were not due to residual HS_x^- contaminations. Control kinetic runs were also included in the absences of polysulfide and BSA both for Trx1 (●) and TRP14 (○).

(**B&C**) 20 mg/ml (300 μM) BSA was treated with 0-1000 μM polysulfide for 30 minutes. After desalting the protein persulfide samples were mixed with 50 nM TrxR1 and (B) 5 μM Trx1 or (C) 5 μM TRP14.

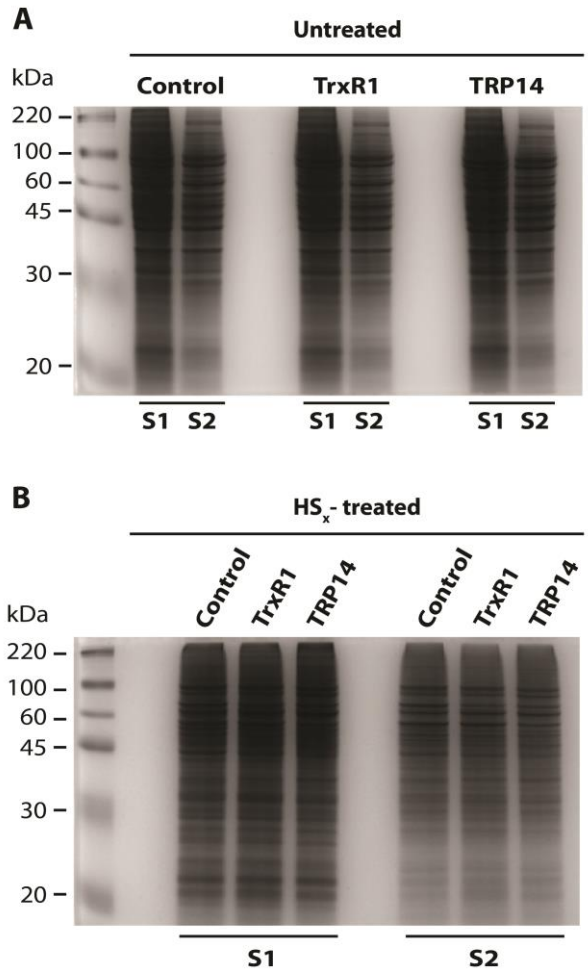

Fig. S6. Relative efficiency of alkylation and streptavidin pulldown in the ProPerDP method applied to control and TrxR1 or TRP14 knockdown HEK293 cells. (**A**) Intact cells were alkylated by IAB. After IAB was washed away, cells were lysed and 1 mg/ml cell lysates were incubated with equal volumes of streptavidin coated magnetic microparticles. *S1* and *S2* refer to total protein content and supernatant fractions after bead enrichment, respectively (i.e. Sample 1 and Sample 2 according to main text Fig. 1). (**B**) Intact cells were treated with 200 µM inorganic polysulfides for 1 h. Polysulfide solution was removed and the cells were then processed as in (A). The corresponding *S3* samples are shown in Fig. 5B & D, respectively.

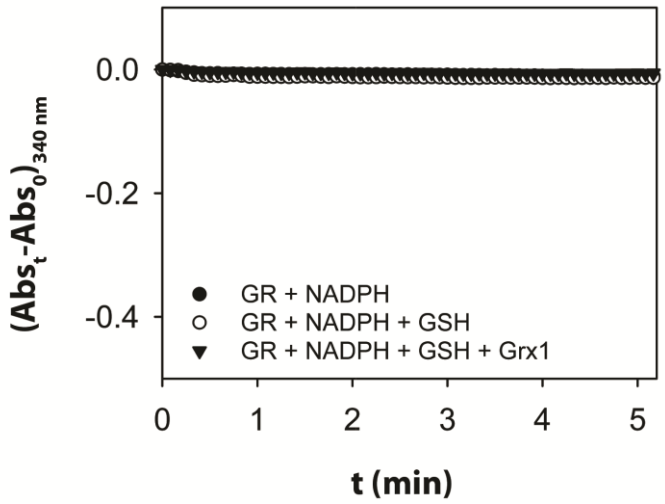

Fig. S7. Control experiment to the BSA-SSH reduction by the NADPH/GR/GSH-coupled Grx1 experiment (see Fig. 6 in the main text). 60 mg/ml BSA was treated with 1 mM polysulfide and desalted with a 7K MWCO desalting spin column. As a control sample, an equal volume of 1 mM polysulfide solution in the absence of protein was passed through a spin column. The flow-throughs were mixed with 6 μg/ml GR and 250 μM NADPH (●), in the presence of 1 mM GSH (o) or 1 mM GSH and 1 μM Grx1 (▼). No detectable activities were observed in the protein free polysulfide control samples indicating that the desalting steps were effective and the measured activities for BSA-SSH on Fig. 6D were not due to residual HS_x^- contamination.

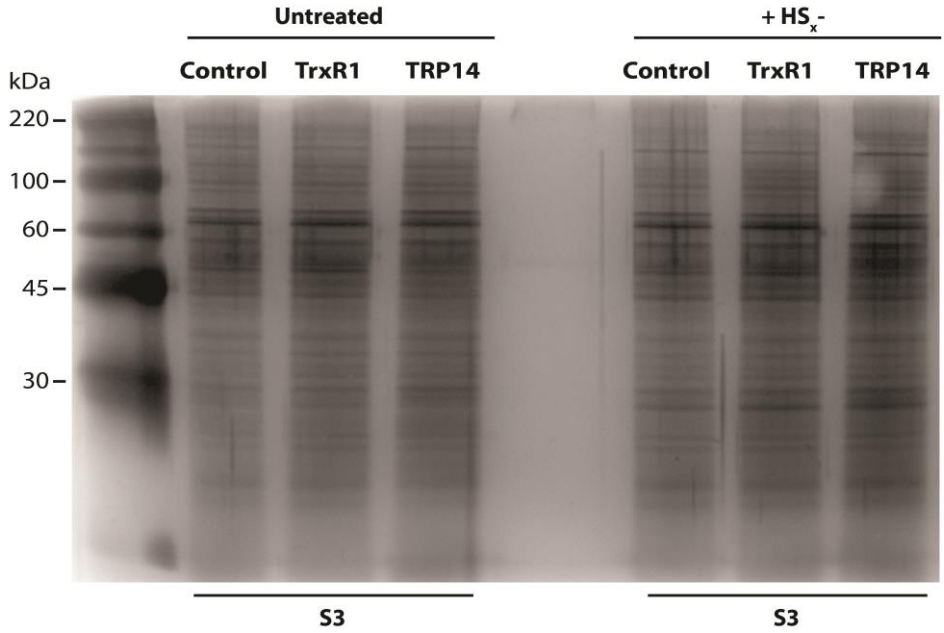

Fig. S8. Endogenously detected protein persulfides exhibit elevated levels in HS_x^--treated TrxR1, TRP14 knockdown, or control HEK293 cells. 200 μM polysulfide treated and untreated (at 30 min incubation) intact cells were alkylated by IAB. After IAB was washed away, cells were lysed and 1 mg/ml cell lysates were incubated with equal volumes of streptavidin coated magnetic microparticles. *S3* refer to persulfidated protein content according to Fig. 1 Sample 3.

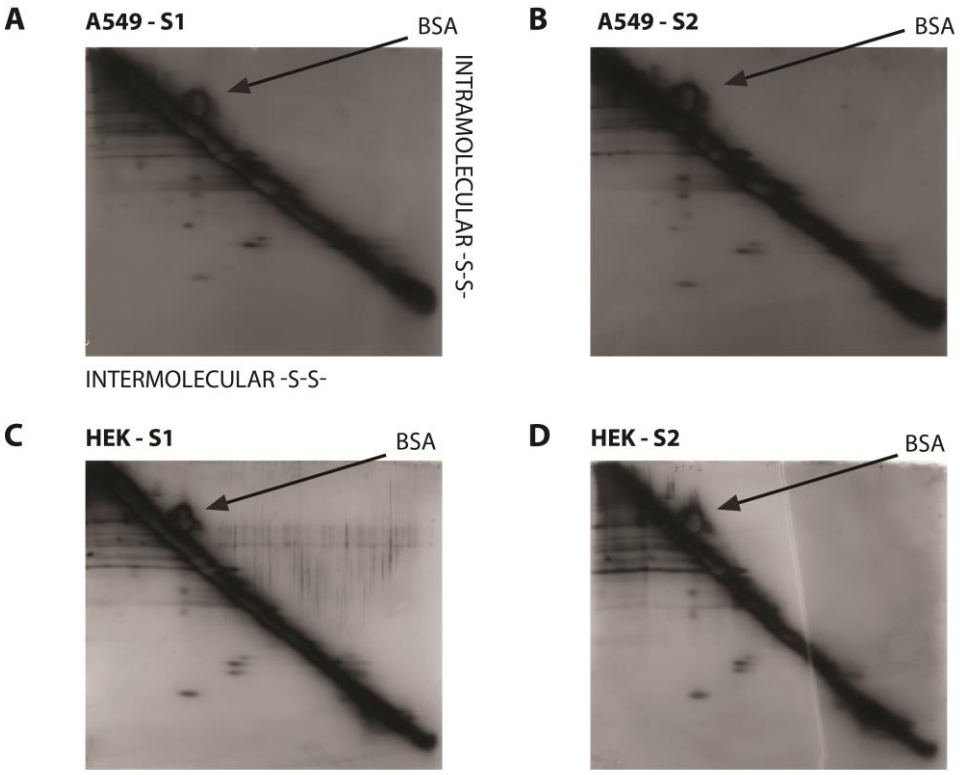

Fig. S9. Diagonal gel electrophoresis experiment corroborates that false-positive hits outlined on Fig. 8B by ProPerDP are of minor importance. 2D diagonal gel electrophoresis was carried out (with a nonreducing and a reducing dimension) on the *S1* and *S2* samples (see Fig. 1 for explanation of fractions) from A549 (**A** and **B**, respectively) and HEK293 cells (**C** and **D**, respectively). We could not detect significant differences in the patterns of protein spots between the *S1* and *S2* samples below the diagonal, representing the intermolecular disulfide pools. This corroborates that false positive hits by ProPerDP via the mechanism shown in main text Fig. 8B is likely to be of minor importance. BSA was applied as a loading control; note that it contains intramolecular/structural disulfide bridges and therefore appears above the diagonal.

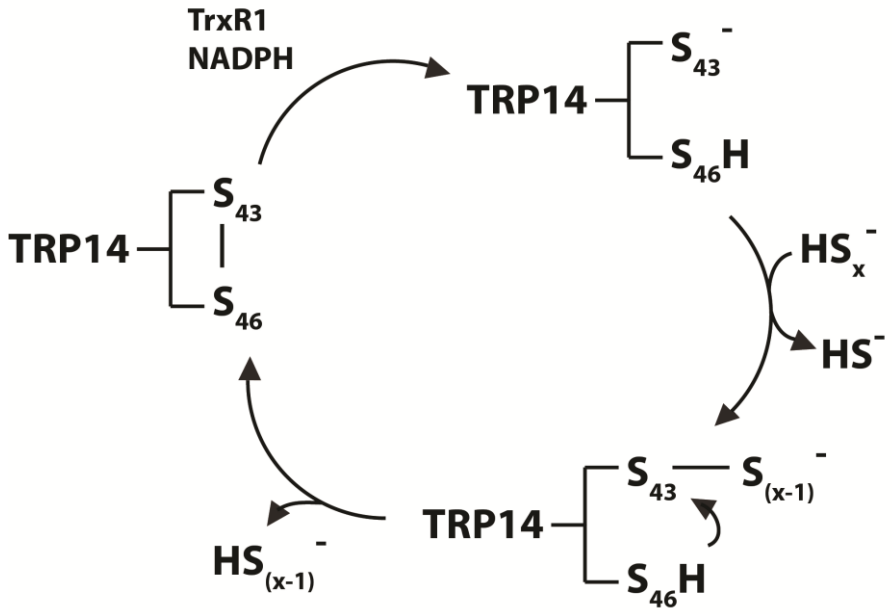

Fig. S10. Suggested scheme for polysulfide reduction by the NADPH/TrxR1-coupled TRP14 system. The fact that polysulfides did not inhibit TRP14 activities but were catalytically reduced by TrxR1 coupled with TRP14 led us to suggest that TRP14-persulfide intermediate species at the proposed nucleophilic Cys43 residue of its active site are likely to be relatively unstable and rapidly regenerated by the neighboring Cys46 residue, resulting in release of $HS_{(x-1)}^-$ and HS^-. An initial persulfidation reaction at Cys43 may either produce Cys43-SSH with direct release of $HS_{(x-1)}^-$ and subsequent release of HS^- upon the formation of an active site disulfide or, as depicted here, the analogous reactions occurring in the reverse order. The additional Cys residues of TRP14 (Cys64, Cys69 or Cys110) may also be involved in the catalysis, which needs to be addressed by future studies.