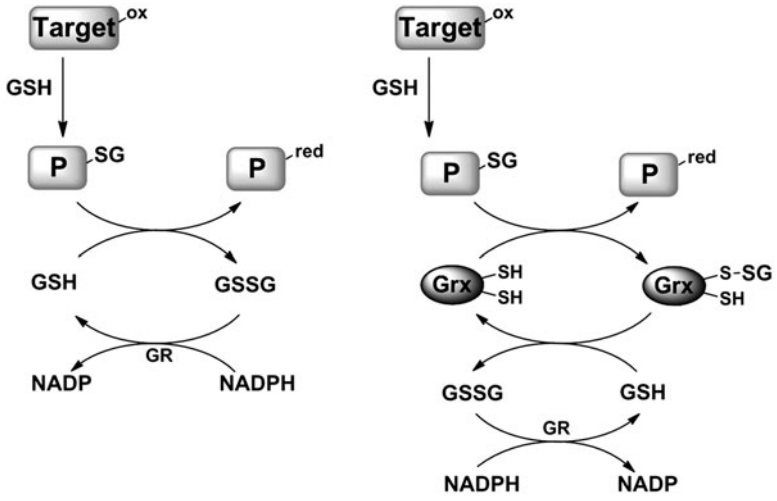
Supplementary Data

Supplementary Materials and Methods

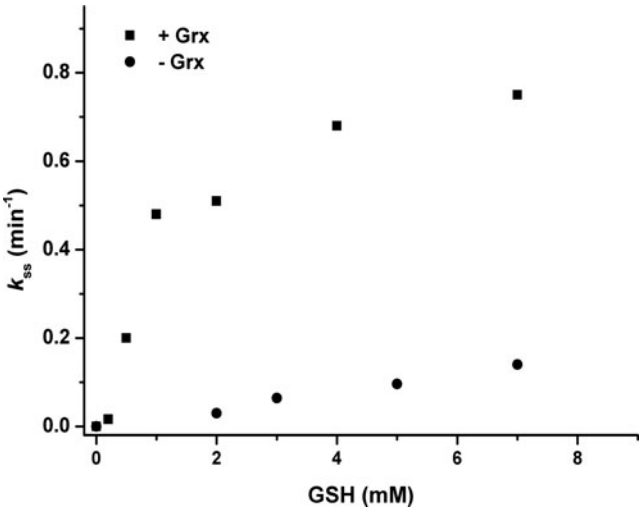
Production and purification of mouse Srx and Prx1

Recombinant *M. musculus* Srx was obtained by cloning a synthetic ORF coding mouse Srx optimized for expression in *Escherichia coli* (GeneArt; Life Technologies, Carlsbad, CA) into a modified pGEX-6P-1 plasmid (GE Healthcare Bio-Sciences, Piscataway, NJ), enabling production of an N-terminal 6xHis/GST fusion protein. Recombinant His-tagged mouse Prx1 was obtained by cloning a synthetic ORF coding mouse Prx1 optimized for expression in *E. coli* (GeneArt; Life Technologies) into the pET28b(+) plasmid (source) between the NdeI and SacI sites. The C41(DE3) [F^- *ompT* *hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3)] *E. coli* strain was used for production of mouse Srx and Prx1.

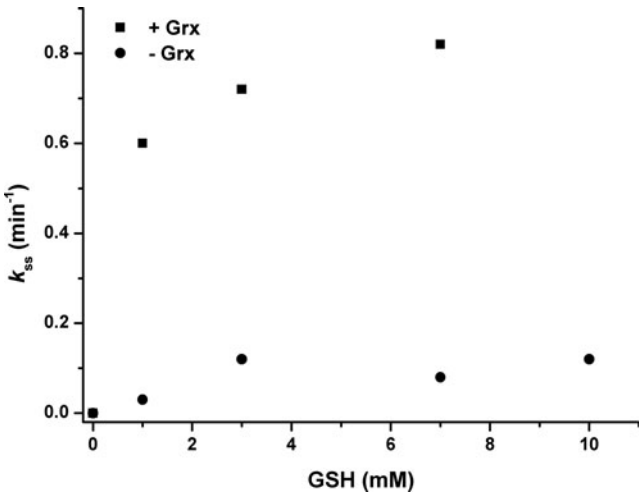
For protein purification, cells were harvested by centrifugation, resuspended in a minimal volume of buffer (50 m*M* potassium phosphate buffer, 50 m*M* imidazole, 0.5 *M* KCl, pH7) and sonicated. The fusion His tagged proteins contained in the soluble fraction were purified on an Ni-Sepharose column that was connected to an AKTA FPLC system (GE Healthcare Bio-Sciences), and eluted by a 0.5 *M* imidazole step. For mouse Srx, the HT-GST tag was cleaved by the PreScission protease (GE Healthcare Bio-Sciences) overnight at 4°C, before final purification by gel filtration on a superdex 75 column. Both purified proteins were stored at −20°C, in the presence of 15% glycerol and 10 m*M* DTT. Molecular concentrations were determined spectrophotometrically, using theoretical extinction coefficients at 280 nm of 13,400 $M^{-1}.cm^{-1}$ for mouse Srx and 20,065 $M^{-1} \cdot cm^{-1}$ for mouse Prx1.



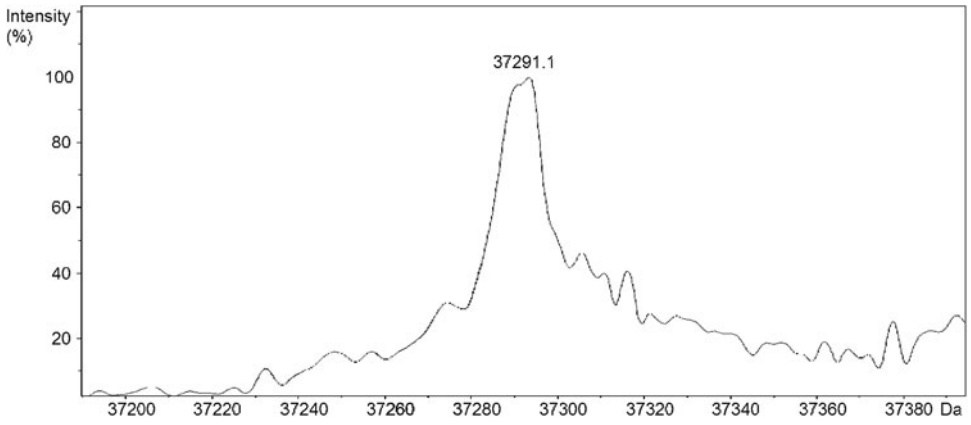
SUPPLEMENTARY FIG. S1. Schematic principle of the GSH/GR (*left*) and GSH/Grx/GR (*right*) coupled assays. The oxidized target is reduced by a molecule of GSH, which results in formation of a glutathionylated protein (P). The glutathionyl moiety is then transferred to a second GSH molecule either directly (*left*) or *via* Grx (*right*). The oxidized glutathione GSSG produced is reduced to GSH by GR coupled to NADPH oxidation. GR: glutathione reductase.



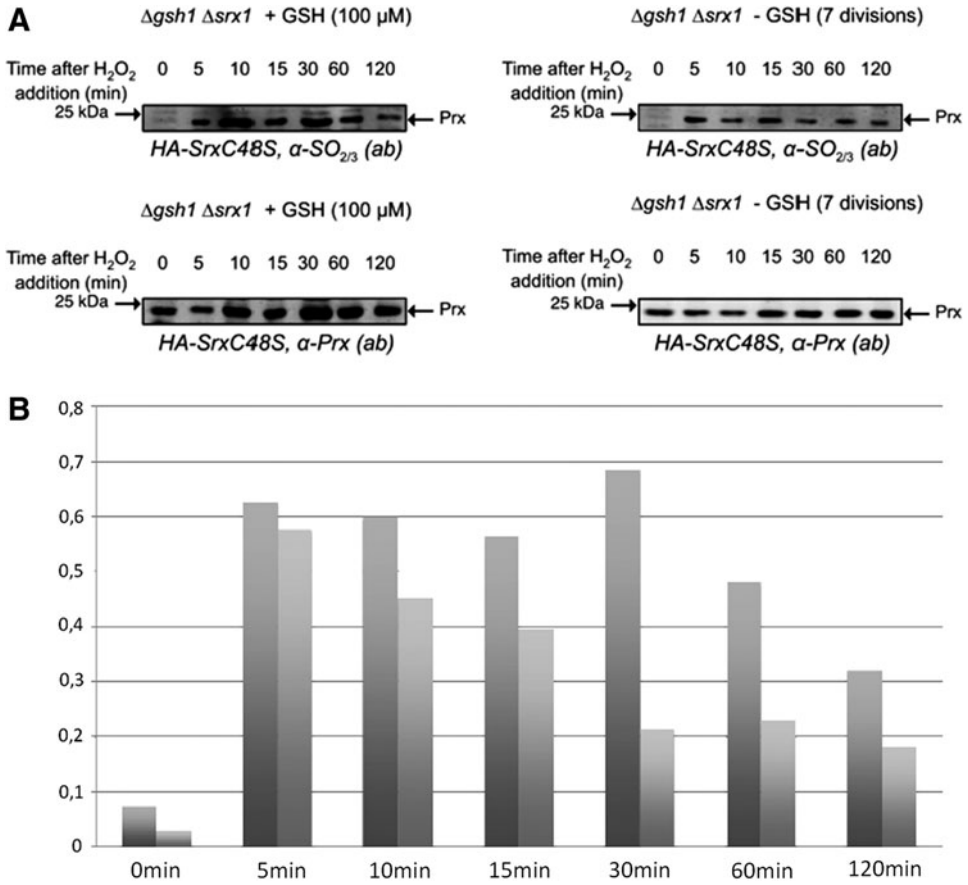
SUPPLEMENTARY FIG. S2. Steady-state kinetics of Srx^{C84} using wild-type Prx–SO_2 substrate, recycled by the GSH/Grx/GR system. The reaction of 25 μM Prx–SO_2 with 10 μM Srx^{C84} in the presence of 1 mM $MgCl_2$ was followed using the GSH/GR (0.5 μM GR and 200 μM NADPH, *circles*), GSH/Grx/GR (50 μM Grx, 0.5 μM GR, and 200 μM NADPH, *squares*) coupled assay. The assay was initiated by the addition of 1 mM ATP. Initial rate measurements were carried out at 30°C in buffer TK by following the decrease in absorbance at 340 nm due to the oxidation of NADPH. The lower maximum rate constant of 0.8 *versus* 1.2 min^{-1} obtained for C171A Prx–SO_2 is due to the use of a Prx–SO_2 concentration of 25 μM, which is slightly sub-saturating relative to Srx.



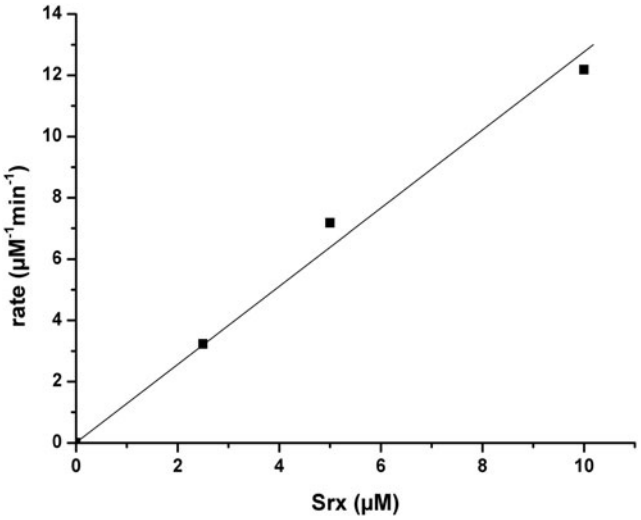
SUPPLEMENTARY FIG. S3. Mouse Srx is recycled by the GSH/Grx/GR system in the steady state. The reaction of 50 μM mouse hyperoxidized Prx1 with 10 μM mouse Srx in the presence of 1 mM $MgCl_2$ was followed using the GSH/GR (0.5 μM GR and 200 μM NADPH, *circles*), or GSH/Grx/GR (50 μM *E. coli* Grx, 0.5 μM GR, and 200 μM NADPH, *squares*) coupled assays. The assay was started by addition of 1 mM ATP. Initial rate measurements were carried out at 30°C in buffer TK by following the decrease in absorbance at 340 nm due to the oxidation of NADPH.



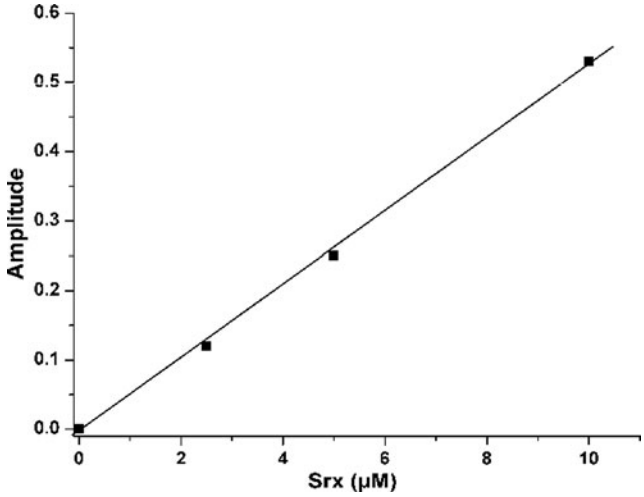
SUPPLEMENTARY FIG. S4. Mass spectrometry analysis of the Prx-Srx complex used in Figure 5A. Deconvoluted mass spectrum of the species eluting in peak **c** (Fig. 4A) after 1 min of incubation of equimolar concentrations (30 μM) of C171A Prx–SO_2 and Srx C84A-C106V (equivalent to Srx^{C84}) in the presence of 1 mM ATP and $MgCl_2$, in buffer TK at 30°C. Expected mass for the Prx–SO–S–Srx species: 37290.5 Da.



SUPPLEMENTARY FIG. S5. Impact of GSH on the kinetics of Prx–SO_2 reduction *in vivo*. **(A)** Cultures of *Δgsh1Δsrx1* carrying pRS316-HA2-SrxC48S, grown in selective minimal medium (SD URA^-) supplemented with 100 μM GSH (*left panels*), or grown for seven divisions in selective minimal medium (SD URA^-) lacking GSH (*right panels*), were exposed during 30 min of 100 μM H_2O_2 to induce *SRX1* expression. After 30 min, cells were exposed again to 500 μM H_2O_2 and lysed after 5, 10, 15, 30, 60, and 120 min by the TCA protocol. Normalized total protein extracts were immunoblotted with anti-Prx–$SO_{2/3}$ or anti-Prx (anti Tsa1) antibodies, after separation by reducing 15% SDS-PAGE. **(B)** The band intensities corresponding to Prx–SO_2 were normalized relative to total Prx, for cells grown in the presence (*light gray*) or absence (*dark gray*) of GSH.



SUPPLEMENTARY FIG. S6. The rate of the Srx-catalyzed reaction in steady state is proportional to Srx. The reaction of 50 μM Prx–SO_2 with 0, 2.5, 5, and 10 μM Srx^{C84} in the presence of 1 mM $MgCl_2$ was followed using the GSH/Grx/GR (50 μM Grx, 0.5 μM GR, and 200 μM NADPH, *squares*) coupled assay in the presence of 1 mM GSH. The assay was started by the addition of 1 mM ATP. Initial rate measurements were carried out at 30°C in buffer TK by following the decrease in absorbance at 340 nm due to the oxidation of NADPH. The slope of the line corresponds to a steady-state rate constant k_{ss} of 1.2 min^{-1}.



SUPPLEMENTARY FIG. S7. Impact of Srx concentration on the kinetics of Srx reduction by GSH under single-turnover conditions. Final concentrations of 40 μM Prx–SO_2, 1 mM ATP, 1 mM $MgCl_2$, and 1 mM GSH were rapidly mixed with 0, 2.5, 5, and 10 μM Srx^{C84} in buffer TK at 30°C in a rapid kinetics spectrofluorometer. Quenching of fluorescence emission intensity of wild-type Prx on going from the oxidized Prx–SO_2 to the disulfide form was recorded on an SX18MV-R stopped-flow apparatus fitted for fluorescence measurements, with excitation wavelength set at 295 nm, and emitted light collected above 320 nm using a cutoff filter. The blank-corrected progress curves were analyzed using a first-order kinetic model to deduce the amplitudes (*squares*) of the process.