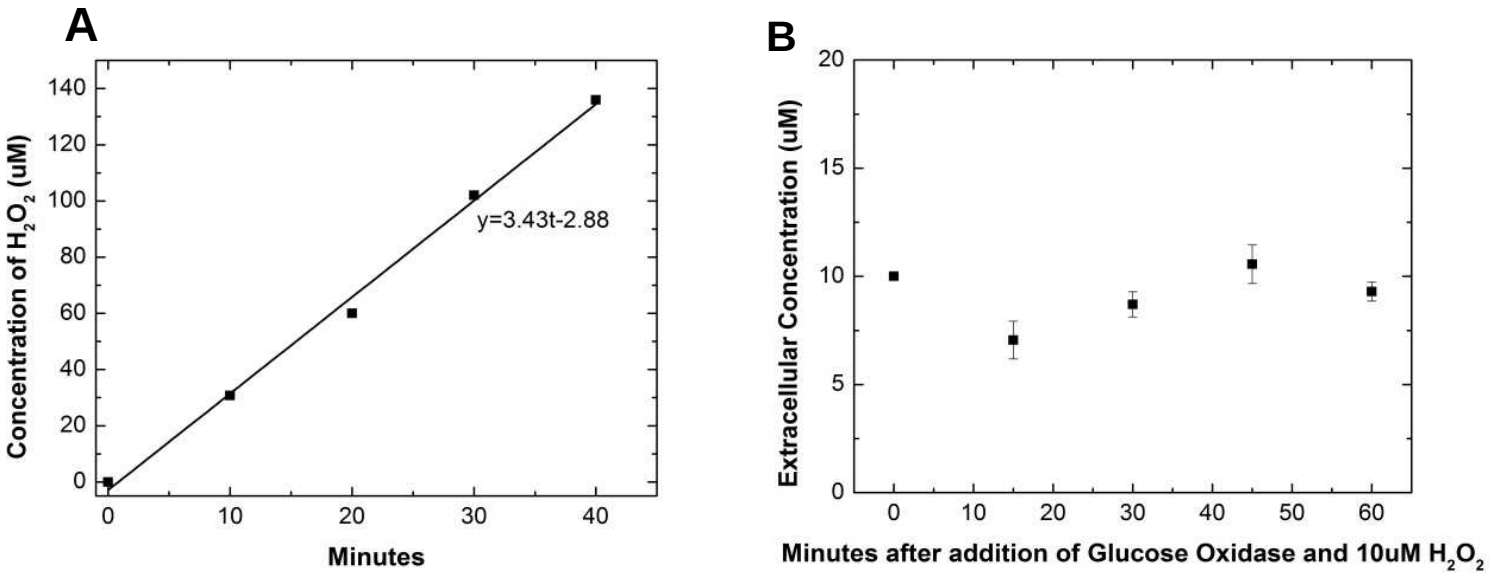
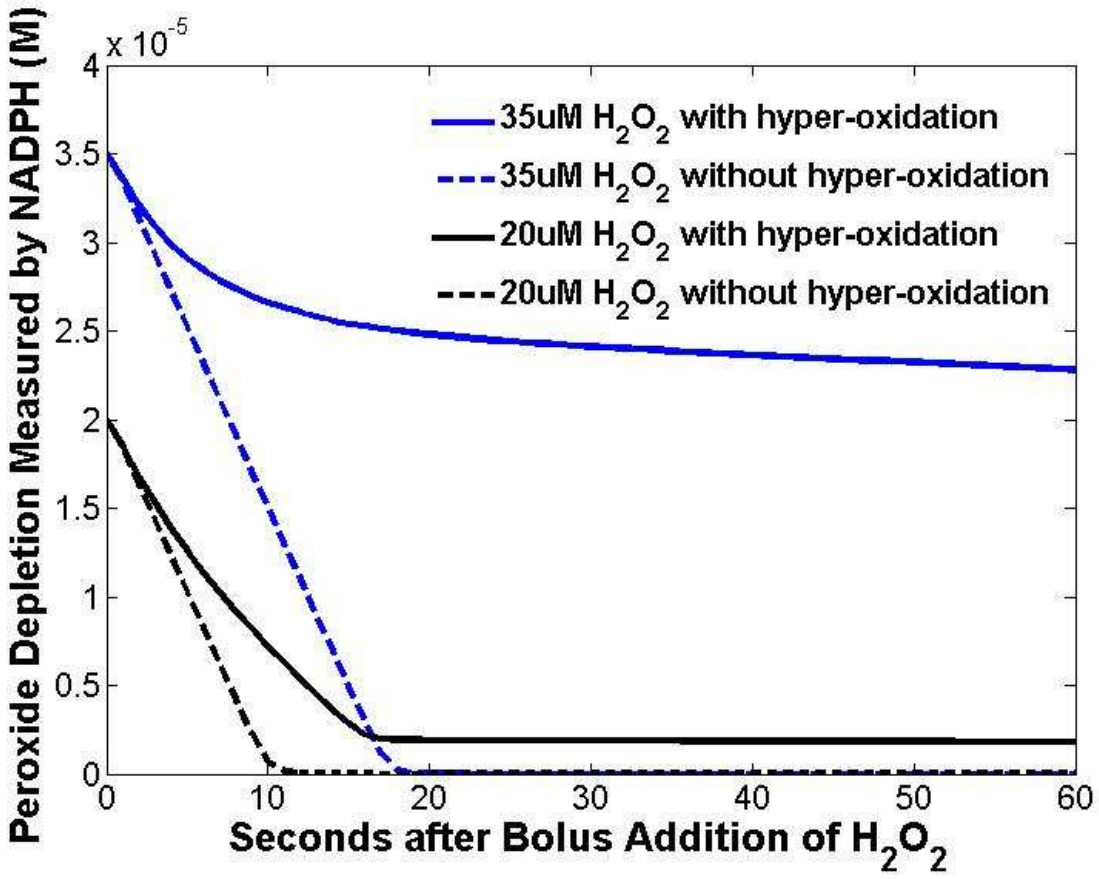
Supplementary Figure 1 (S1)



S1. (A) Hydrogen peroxide production by glucose oxidase. Glucose oxidase was diluted to concentrations between 3-5 mU/mL in in PBS (pH 7.4) + 2 g/L glucose. The production of peroxide by the enzyme was measured using the ABTS assay. (B) A steady-state hydrogen peroxide level obtained using external glucose oxidase as a generator and Hyper HeLa cells as scavengers. HyPer HeLa cells were re-suspended at 0.8 million cells/mL in PBS + 2 g/L glucose. The amount of glucose oxidase producing 2.8 μM hydrogen peroxide/min was added simultaneously with 10 μM of hydrogen peroxide. The extracellular peroxide concentration was monitored for a period of 1 hour using the ABTS assay. A constant extracellular concentration verifies the k_{intact} value.

Supplementary Figure 2 (S2).



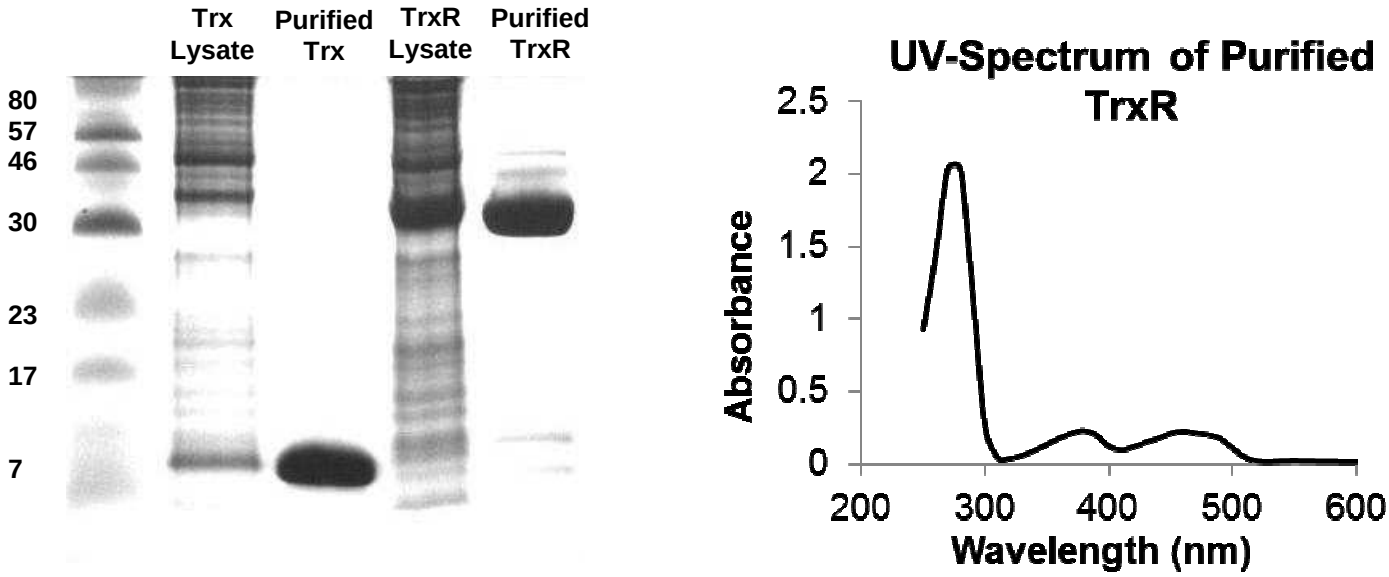
S2. Depletion of hydrogen peroxide due to 2-cys Prx activity in cell lysates with and without hyper-oxidation effects.

While hyper-oxidation of 2-cys Prx in intact cells has only been detected for extracellular additions of >100 μM, it is important to consider hyper-oxidation effects for lower peroxide concentrations when the sample consists of cell lysates rather than intact cells.

The kinetics of Prx activity in the experimental system was modeled using a system of ODEs implemented using Matlab (SI). The situation where Prx can undergo hyper-oxidation (solid) versus without hyper-oxidation (dotted) was compared for bolus additions of 20 μM (black) and

35 μM (blue) hydrogen peroxide to a solution of HeLa cell lysate. The possibility of hyper-oxidation changes the kinetics of hydrogen peroxide depletion significantly, and this effect is greater for the 35 μM addition in comparison with the 20 μM addition. From this simulation, we conclude that for both concentrations, hyper-oxidation needs to be taken into account when fitting the Prx kinetic data to a rate equation. Other parameters used in the simulation were Trx=30 μM, k_1*[Prx_{active}]= $3.8s^{-1}x10^{-9}$ $s^{-1}cell^{-1}L$ and a cell density of 5.8 million cells/mL.

Supplementary Figure 3 (S3).



S3. Expression and purification of yTrx and TrxR. After induction with IPTG and expression for 4 hours, Trx and TrxR overexpression was detected in the lysate fractions. After chromatographic separations with anionic (Q) and gel filtration (Superdex75) columns using an AKTA FPLC, a single 11kD fragment for Trx was observed using SDS PAGE. Similarly, TrxR was also purified using Q and Phenyl hydrophobic columns, and a strong band of 35 kD was detectable. The UV-spectra for the purified TrxR showed the correct peaks at 280 nm, 359 nm and 452 nm.

Supplementary Information (SI)

GPx kinetics

The reaction mechanism of GPx consists of an oxidation-reduction cycle where GPx is oxidized by H_2O_2 and reduced by GSH:

$$H_2O_2 + GPx_{red} + H^+ \xrightarrow{k_1'} GPx_{ox} + H_2O$$

$$GPx_{ox} + GSH \xrightarrow{k_2'} GS - GPx + H_2O$$

$$GS - GPx + GSH \xrightarrow{k_3'} GPx_{red} + GSSG + H^+$$

The GSSG is then reduced using electrons supplied by NADPH inside the cell. By adding excess NADPH to cell lysate, we can indirectly track the consumption of H_2O_2 by GPx. These depletion kinetics are fitted to the integrated rate law (equation 16) reported in *Materials and Methods.*

2-Cys Prx kinetics

Similar to GPx, 2-cys Prx requires a partner, Trx, to return it to the reduced form after oxidation by hydrogen peroxide. The difference between GPx and Prx is that Prx can react with a second hydrogen peroxide molecule to form sulfinic or sulfonic acid. This over-oxidized form reverts very slowly back to Prx-SOH through reaction with sulfiredoxin. The competing reaction to hyper-oxidation is the formation of a disulfide bond between the –SOH and –SH groups of adjacent 2-cys Prx molecules to form Prx-SS. This disulfide bond is reduced by thioredoxin/thioredoxin reductase back to the Prx-$(SH)_2$ form.

In order to obtain an analytic solution, we make a quasi-steady state assumption of the intermediate species $\mathrm{Prx-SOH}$, $\mathrm{Prx-(SO_2H)}$ and $\mathrm{Prx-SS}$, and by adding excess yeast $Trx_{reduced}$ and TrxR to the system, we can assume that concentration to be constant. Since the conversion of $\mathrm{Prx-(SO_2H)}$ back to $\mathrm{Prx-SOH}$ is very slow [35], we can also assume that the oxidation reaction k_5 is irreversible for the kinetic time frame we are considering. Furthermore,

at hydrogen peroxide of less than < 62 μM, only 1% of the mammalian 2-cys Prx enzymes are over-oxidized per cycle [28]. This allowed us to simply the expression $\frac{k_1[H_2O_2]}{k_3[H_2O_2]+k_2}$ to $\frac{k_1[H_2O_2]}{k_2}$. Solving the system of ODEs (equations 9-15) give us an implicit equation of hydrogen peroxide related to time:

$$(k_1 Prx_{active}\left(\frac{1}{k_2}+\frac{1}{k_5[Trx]}\right)-\frac{k_3}{k_2})\ln\left(\frac{Prx_{active}+\frac{k_3}{k_2}[H_2O_2]_0}{Prx_{active}+\frac{k_3}{k_2}[H_2O_2]_1}\right)+\frac{k_3}{k_2}\ln\left(\frac{[H_2O_2]_0}{[H_2O_2]_1}\right)=\Delta t$$

What is notable about the equation is that the rate constants k_1, k_2, k_3 and k_4 are needed in order to fit the $[H_2O_2]$ against Δt, since the implicit form is not as elegant as GPx that allow us to determine k_1'*[GPx_{total}] from the slope of expression involving only hydrogen peroxide terms and time. Thus, the most accurate way of determining k_1*[Prx_{active}] will be to numerically simulate a model with the system of ordinary differential equations as shown above, with rate constants and initial values as shown in the table below, and fit our experimental data to the model.

Reaction	**Rate Constant**
k_1	$1.3*10^{7}$ M^{-1} s^{-1} [17]
k_2	2 s^{-1} [34]
k_3	$1.2*10^{4}$ M^{-1} s^{-1} [34]
k_4	$3*10^{-3}$ s^{-1} [35]
k_5	$1*10^{5}$ M^{-1} s^{-1} [32]
k_6	$2*10^{7}$ M^{-1} s^{-1} [33]
Species	**Initial Concentration**
H_2O_2	20-25 μM

Prx_{active}	Parameter to fit
Prx-SOH	1e-14 M
Prx-SO_2H	1e-14 M
Prx-SS	1e-14 M
$Trx_{reduced}$	20-50 μM
$Trx_{oxidized}$	1e-14 M
NADPH	300 μM

The system of ODEs was simulated using MatLab2013, time step size of 1s. k_1=1.3*10^{7} M^{-1} s^{-1}, k_5= 2 s^{-1} and k_3=1.2*10^{4} M^{-1} s^{-1} were obtained from *in vitro* data with 2-cys Prx II [17,34], a close homolog of Prx I that also exists in the cytoplasm. k_4 =3*10^{-3} s^{-1} was obtained from experimental data with purified human Prx I protein interaction with human Srx [35]. k_5=1*10^{5} M^{-1} s^{-1} comes from experimental data with human Prx I and a yeast Trx/TrxR system [32]. k_6=2*10^{7} M^{-1} s^{-1} is derived from experimental data measuring mammalian Trx reduction with the mammalian Trx [33]. The initial conditions for hydrogen peroxide and $Trx_{reduced}$ are given as experimental inputs. NADPH initial condition is 300 μM. The variable to fit is the initial concentration of active reduced 2-cys peroxiredoxin in the system. k_{prx} is equal to k_1*[Prx_{active}].