

Monitoring thioredoxin redox with a genetically encoded red fluorescent biosensor

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Thioredoxin (Trx) is one of the two major thiol antioxidants, playing essential roles in redox homeostasis and signaling. Despite its importance, there is a lack of methods for monitoring Trx redox dynamics in live cells, hindering a better understanding of physiological and pathological roles of the Trx redox system. In this work, we developed the first genetically encoded fluorescent biosensor for Trx redox by engineering a redox relay between the active-site cysteines of human Trx1 and rxRFP1, a redox-sensitive red fluorescent protein. We used the resultant biosensor—TrxRFP1—to selectively monitor perturbations of Trx redox in various mammalian cell lines. We subcellularly localized TrxRFP1 to image compartmentalized Trx redox changes. We further combined TrxRFP1 with a green fluorescent Grx1-roGFP2 biosensor to simultaneously monitor Trx and glutathione redox dynamics in live cells in response to chemical and physiologically relevant stimuli.

Along with glutathione, Trx is one of the two primary thiol-dependent antioxidants in mammals¹. The NADPH-dependent reduction of Trx is catalyzed by the flavoenzyme Trx reductase (TrxR), whereas the oxidation of Trx is catalyzed by Trx-dependent peroxiredoxins (Prx), also known as Trx peroxidases (TPx) (Supplementary Results, Supplementary Fig. 1)¹. Consequently, the Trx system shuttles electrons from NADPH to TPx, leading to rapid removal of reactive oxygen species (ROS). Trx also plays important roles in modulating protein activities through the redox modulation of cysteine and methionine residues¹. Trx can reduce oxidized cysteine residues in proteins such as ribonucleotide reductase involved in DNA synthesis and repair², a number of redox-sensitive transcription factors including NF- κ B and Ref-1/AP-1 (refs. 3,4), apoptosis signal regulating kinase 1 (ASK1)⁵, and methionine sulfoxide reductases for the reduction of oxidized methionine residues^{6,7}. In addition, the Trx system has been linked to immune responses⁸, bacterial and viral infection^{9,10}, cell proliferation¹¹, apoptosis¹², and pancreatic β -cell functions¹³. Moreover, abnormality of the Trx system has been observed in cancer¹⁴. The Trx system, especially TrxR, has been considered as a promising target for cancer therapy¹⁵.

Our current understanding of redox biology remains largely nebulous. Redox signaling, which involves diverse reactive oxygen, nitrogen and sulfur species (ROS, RNS and RSS), small-molecule thiols, proteins, and other types of molecules, is incredibly sophisticated¹⁶. Although thermodynamics drives redox reactions in live cells and organisms, the specificity of the reactions is largely determined by the spatial organization of redox signaling components and the kinetics of competitive reactions¹⁷. This complexity is further compounded by the scarcity of research tools that can monitor the redox of specific redox-signaling components in live cells with appropriate specificity and spatiotemporal resolution. Previous studies have reported a family of genetically encoded fluorescent protein (FP)-based biosensors that can sense live-cell redox dynamics^{18,19}. Reversible disulfide bridges have been introduced to the surfaces of a yellow FP (YFP) and green FPs (GFPs), and recently, to the N and C termini of a circularly permuted red FP (cpRFP) to derive redox-sensitive rxYFP, roGFPs, and rxRFPs, respectively^{20–23}. Genetically encoded biosensors (Supplementary Fig. 1) are also available for monitoring various ROS, RNS and RSS such as hydrogen peroxide

(H₂O₂)^{24–26}, organic hydroperoxides (ROOH)²⁷, peroxynitrite (ONOO[–])²⁸ and hydrogen sulfide (H₂S)^{29,30}. Moreover, rxYFP and roGFPs have been genetically linked to glutaredoxin (Grx), resulting in selective biosensors for the redox dynamics of the oxidized and reduced glutathione (GSSG–GSH) pair^{31,32}. Despite this progress, there is currently no genetically encoded biosensor for probing the redox of the Trx system. Effort has previously been made to fuse rxYFP or roGFPs to Trx, but this has failed to yield any effective biosensor³². The current strategy for probing the redox status of Trx is based on the alkylation of Trx thiols in cell lysates followed by electrophoresis³³. This low-throughput method only allows end-point measurements and cannot avoid additional Trx oxidation during sample preparation. The technical difficulty in direct monitoring of the redox status of Trx in live cells has greatly hindered investigations on the physiological and pathological roles of the Trx redox system and the interplays between Trx and other cell signaling components.

Herein we report the first fluorescent probe that can directly monitor the redox status of the active-site cysteines of Trx in live mammalian cells. Our new probe, TrxRFP1, was developed by creating and optimizing a redox relay between human Trx1 and rxRFP1—a redox-sensitive RFP previously developed in our laboratory²². We have used the resultant optimized biosensor, TrxRFP1, to monitor the dynamics of Trx redox induced by various chemicals, such as H₂O₂, auranofin, and arsenic trioxide. We further localized TrxRFP1 to the cell nucleus and mitochondria, validated TrxRFP1 in a variety of mammalian cell lines, and combined TrxRFP1 with a green fluorescent Grx1-roGFP2 biosensor for simultaneous imaging of Trx and glutathione redox dynamics. We observed Trx redox changes under physiologically relevant conditions, including stimulations by serum or epidermal growth factor (EGF).

RESULTS

Engineering of fluorescent biosensors for Trx redox

We genetically linked rxRFP1 to Trx1, the Trx found in cytoplasmic and nuclear compartments in humans. We reasoned that, with an appropriate topological rearrangement, the redox of the active-site cysteine residues (C32 and C35) of Trx1 might be coupled to the redox of the cysteine pair at the termini of rxRFP1 (Fig. 1a). Because Trx1 and rxRFP1 are forced into proximity in the fusion construct,

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such redox coupling may be kinetically favored. Therefore, the fluorescence of rxRFP1 would become an indicator for the redox of Trx1. We next constructed four chimeric proteins varying in the lengths of the linkers and the relative order of Trx1 and rxRFP1 (constructs 1–4 in **Supplementary Fig. 2**). One of the four variants, which has a 30-amino-acid Gly-Ser-rich linker between the C terminus of Trx1 and the N terminus of rxRFP1, showed a four-fold change in fluorescence in response to reduction by recombinant human TrxR1 and NADPH (**Supplementary Fig. 3**). We named this mutant TrxRFP0.1 (**Supplementary Fig. 4**). Oxidized TrxRFP0.1 was unresponsive to various tested reducing reagents such as L-ascorbic acid and L-cysteine at millimolar concentrations, and reduced glutathione only caused a small, <10% fluorescence decrease (**Supplementary Fig. 5a**). In addition, H₂O₂ effectively oxidized pre-reduced TrxRFP0.1 in the presence of recombinant human TPx1, leading to a prompt fluorescence increase (**Supplementary Fig. 5b**). In comparison, rxRFP1 alone in either an oxidized or a reduced state was unresponsive to TrxR1/NADPH and TPx1/H₂O₂ (**Supplementary Fig. 5a,b**), further confirming that the observed fluorescence changes of TrxRFP0.1 were caused by the redox of Trx1. Moreover, we mutated each one or both active-site cysteine residues of Trx1 to serine and replaced Trx1 in TrxRFP0.1 with one of these three mutants. None of them responded to TrxR1/NADPH or TPx1/H₂O₂ (**Supplementary Fig. 5c,d**), supporting the theory that both active-site cysteine residues of Trx1 are directly involved in the redox coupling between Trx1 and rxRFP1.

Trx1 has a total of five cysteine residues. Its three non-active-site cysteine residues (C62, C69, and C73) have been previously reported to have regulatory roles, and an additional disulfide bond may be formed between C62 and C69 to crosstalk with the Grx-glutathione system³⁴. To eliminate possible interferences caused by these non-active-site cysteines, we mutated all three residues to serine to derive TrxRFP0.2 (**Supplementary Fig. 2**). Next, we randomized the gene fragment encoding rxRFP1 and screened for TrxRFP mutants showing increased dynamic ranges. After three rounds of directed evolution, we arrived at TrxRFP0.9, with three additional mutations (G142S, D228G, and V399Q) and a 5.7-fold fluorescence increase from the fully reduced state to the fully oxidized state (**Supplementary Figs. 3 and 4**). Compared to TrxRFP0.1, oxidized and reduced TrxRFP0.9 were more reactive toward TrxR1/NADPH and TPx1/H₂O₂, respectively (**Supplementary Fig. 5e,f**). Pre-reduced TrxRFP0.9 showed a notable response to amounts as low as 10 nM H₂O₂ in our TPx1 oxidation assay and remained unresponsive to 100 μ M H₂O₂ alone.

In addition to the two cysteine residues forming the reversible disulfide bond, rxRFP1 has two additional cysteine residues. We next created a mutant of TrxRFP0.9 with all nonessential cysteines removed (C217Y C397S). The C217Y mutation was chosen because mApple³⁵, the parental protein of rxRFP1, has a tyrosine residue at this site, whereas the C397S mutation has previously been shown to have little impact on the spectral and redox properties of rxRFP1 (ref. 23). This final mutant was named TrxRFP1 (**Supplementary Fig. 4**) and was subjected to thorough characterization.

Initial characterization of TrxRFP1

Our freshly purified TrxRFP1, which was fully oxidized by molecular oxygen in air during preparation, showed an excitation peak at 576 nm and an emission peak at 600 nm (**Fig. 1b**), identical to the peaks of rxRFP1 (ref. 22). In the presence of dithiothreitol (DTT) or TrxR1/NADPH, TrxRFP1 was reduced quickly, showing a nearly six-fold fluorescence decrease (**Fig. 1c**; **Supplementary Figs. 3 and 6a**). The speed and extent of the reduction were dependent of the concentrations of TrxR1. By contrast, rxRFP1 could be reduced by DTT, but not by TrxR1/NADPH. TrxRFP1 inherited excellent specificity from TrxRFP0.1, as millimolar L-cysteine and L-ascorbic acid did not decrease the fluorescence of oxidized TrxRFP1.

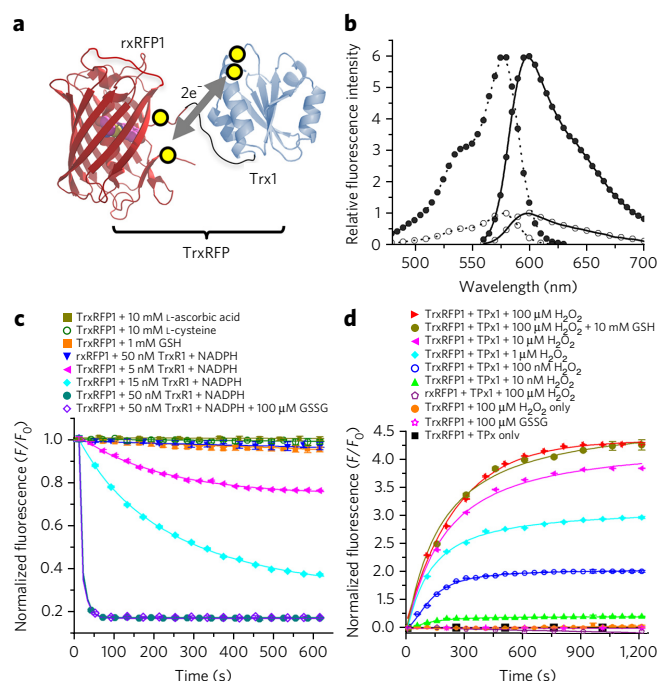


Figure 1 | Design and fluorescence characterization of TrxRFP biosensors.

(a) Schematic representation of the mechanism for TrxRFP sensors. The yellow circles indicate cysteine residues involved in the redox coupling. (b) Excitation (dotted line) and emission (solid line) spectra of reduced (open circle) and oxidized (filled circle) TrxRFP1. Spectra were normalized to the maximal fluorescence of the reduced form. (c) Kinetic traces for the reduction of oxidized proteins. (d) Kinetic traces for the oxidation of reduced proteins. TrxR1 used in these experiments was a commercial rat TrxR1 containing ~50% of the full-length selenocysteine (Sec)-containing enzyme.

Interestingly, through this evolution process, the sensitivity of TrxRFP1 to glutathione was further reduced. Millimolar reduced glutathione alone triggered little fluorescence response, and in the presence of 100 μ M oxidized glutathione, which is considered an upper concentration limit for typical mammalian cells³⁶, the reduction of TrxRFP1 by TrxR1/NADPH was not impacted (**Fig. 1c**). The fully reduced TrxRFP1, freshly prepared by DTT reduction and dialysis in a nitrogen (N₂)-filled anaerobic chamber, responded to TPx1-catalyzed oxidation by H₂O₂ (**Fig. 1d**). This response was dependent of the concentrations of H₂O₂. In parallel, reduced TrxRFP1 was unresponsive to 100 μ M H₂O₂ or oxidized glutathione. In the presence of 10 mM reduced glutathione, oxidation of reduced TrxRFP1 by TPx1/H₂O₂ was also not affected (**Fig. 1d**). We further investigated the response of TrxRFP1 to reducing and oxidizing enzymes in the same reaction vessel. As expected, TrxRFP1 reduced by TrxR1/NADPH could be re-oxidized by addition of TPx1/H₂O₂ (**Supplementary Fig. 6b**). When the concentrations of TrxR1 and NADPH were kept constant, the degree of re-oxidation was dependent of the concentrations of H₂O₂ and TPx1.

We also tested the responses of TrxRFP1 to Trx-like, redox-sensitive proteins such as glutaredoxin 1 (Grx1) and Trx-related protein TRP14. Neither oxidation nor reduction took place (**Supplementary Fig. 7**), suggesting that the redox states of these proteins are kinetically separated and that TrxRFP1 is a specific biosensor. Next, we compared the enzyme kinetics of TrxRFP1 and Trx1 using a commercial rat TrxR1 enzyme (**Supplementary Fig. 8**). We derived similar Michaelis–Menten constants (K_M values, as fit value $\pm$ s.e.m. of fit, are 2.63 ± 0.49 μ M or 2.83 ± 0.69 μ M for TrxRFP1 and Trx1, respectively), supporting the idea that TrxRFP1 and Trx1 respond to TrxR1 similarly.

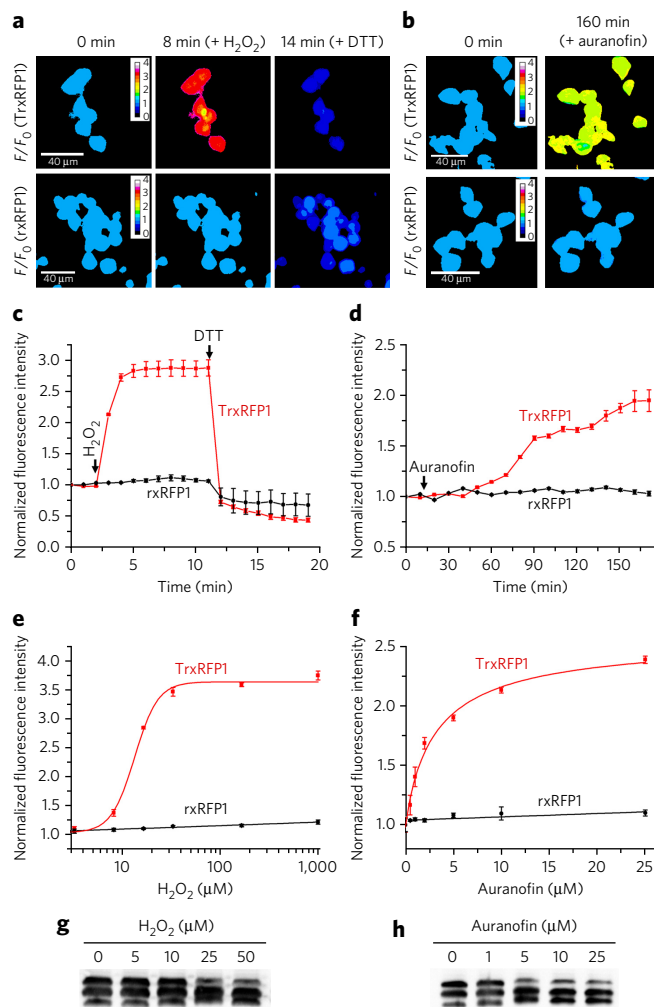


Figure 2 | Characterization of TrxRFP1 in HEK 293T cells. (a,b) Time-lapse pseudocolored fluorescence images (F/F_0) of HEK 293T cells expressing either TrxRFP1 or rxRFP1 sequentially treated with 16.7 μ M H_2O_2 and 10 mM DTT (a) or with 10 μ M auranofin (b), showing H_2O_2 - and auranofin-induced fluorescence changes of TrxRFP1 but not of rxRFP1. H_2O_2 and auranofin were added at $t = 2$ min and $t = 12$ min, respectively (scale bars, 40 μ m). (c,d) Fluorescence intensity traces for TrxRFP1 or rxRFP1 in HEK 293T cells. The intensities were normalized to the value at $t = 0$ min and shown as the mean and s.d. of eight randomly selected cells from three independent replicates. The red and black lines are for TrxRFP1 and rxRFP1, respectively. The arrows indicate the time points for addition of chemicals. (e,f) Fluorescence responses of TrxRFP1 (red) or rxRFP1 (black) in HEK 293T to various concentrations of H_2O_2 (e) or auranofin (f) suggesting that TrxRFP1 can selectively sense the redox changes of Trx in live cells. Data are shown as mean and s.d. of three independent experiments. (g,h) Redox urea-PAGE and immunoblotting analysis of TrxRFP1 in HEK 293T cells treated with H_2O_2 (g) or auranofin (h) at the indicated concentrations, showing the increase of protein oxidation in response to the increase of H_2O_2 or auranofin (full gels for g and h are in **Supplementary Fig. 21d,e**). The three bands, from top to bottom, are interpreted as the TrxRFP1 protein containing no disulfide bond, one disulfide bond, and two disulfide bonds, respectively.

It was reported that the reduction of oxidized Trx1 by glutathione is enzymatically coupled with Grx1, glutathione reductase, and NADPH³⁷, and we therefore further examined our oxidized TrxRFP1 under this condition. In both of our *in vitro* fluorescence and NADPH absorbance assays, no reaction was detectable for TrxRFP1 (**Supplementary Fig. 9**). We also created a TrxRFP1

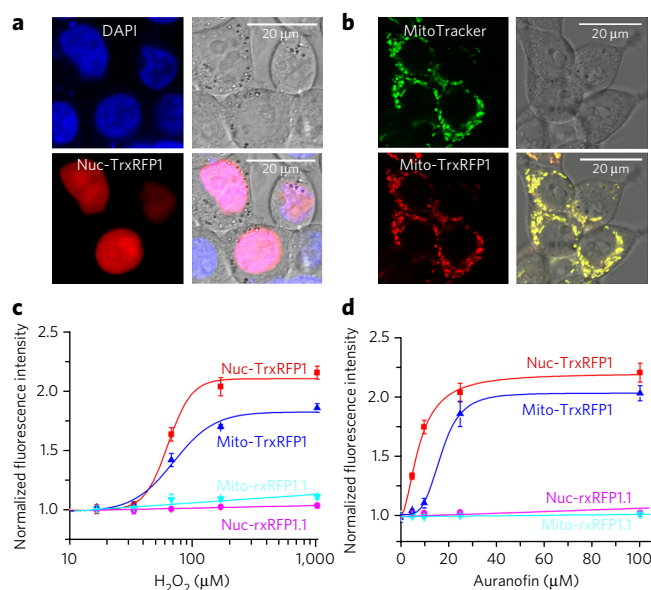


Figure 3 | Subcellularly localized TrxRFP1. (a,b) Colocalization of nuclear (a) and mitochondrial (b) TrxRFP1 with a nuclear stain DAPI and a mitochondrial stain MitoTracker Green, respectively (scale bars, 20 μ m). (c,d) Fluorescence responses of nuclear (red) and mitochondrial (blue) TrxRFP1 in HEK 293T to various concentrations of H_2O_2 (c) or auranofin (d), suggesting that TrxRFP1 can selectively sense the subcellular redox changes of Trx in live cells. Fluorescence responses of nuclear (cyan) and mitochondrial (magenta) rxRFP1.1 are also shown as the controls. Data are represented as mean and s.d. of three independent experiments.

mutant (C32S C35S S62C S69C) in which only the non-active-site disulfide bond can form. This new mutant was reduced under this condition, as observed from the NADPH absorbance change at 340 nm. Next, we prepared Trx1 and several Trx1 variants, including Trx1 with an active-site disulfide and three non-active-site free thiols, and tested their responses to the Grx and glutathione reduction (**Supplementary Fig. 10**). Our results collectively suggest that the Grx–glutathione system is involved in the reduction of the non-active-site disulfide bond, but not the active-site disulfide bond, of Trx1.

To examine the performance of TrxRFP1 in live cells, we transiently expressed it in human embryonic kidney (HEK) 293T cells. Live mammalian cells express TPx to transform ROS and, at the same time, oxidize Trx (**Supplementary Fig. 1**). Not surprisingly, addition of a single bolus of 16.7 μ M H_2O_2 to the imaging medium caused an immediate and dramatic increase in fluorescence for TrxRFP1-expressing cells (**Fig. 2a**; **Supplementary Fig. 11**; **Supplementary Video 1**), suggesting the change of Trx to a more oxidized state. The TrxRFP1 sensor remained oxidized during our imaging period until the addition of 10 mM DTT 9 min later. We also imaged H_2O_2 -treated TrxRFP1-expressing cells for a longer period or with less H_2O_2 and found that the oxidation of TrxRFP1 could be spontaneously reverted (**Supplementary Fig. 12**), suggesting the presence of an efficient intracellular system for maintaining the redox homeostasis of Trx. Furthermore, we treated TrxRFP1-expressing cells with 10 μ M auranofin, a previously reported gold(i)-containing TrxR reductase inhibitor³⁸. Like H_2O_2 , auranofin also shifted Trx to a more oxidized state, indicated by the gradual fluorescence increase over the monitored period (**Fig. 2b**; **Supplementary Fig. 11**; **Supplementary Video 2**). In comparison, we did not observe substantial fluorescence increase for H_2O_2 - or auranofin-treated control cells expressing either rxRFP1 or a genetically encoded pH indicator, pHRFPP²² (**Fig. 2c,d**; **Supplementary Fig. 13**).

To further explore the sensitivity of TrxRFP1 in live cells, we characterized the responses of TrxRFP1 to various concentrations of H_2O_2 and auranofin (Fig. 2e,f). The EC_{50} s (the concentration to give a half-maximal response) for 10 min H_2O_2 treatment and 3 h auranofin treatment were $13.5 \pm 0.7 \mu M$ (fit value $\pm$ s.e.m. of fit) and $3.2 \pm 1.1 \mu M$, respectively. In the tested concentration ranges, H_2O_2 and auranofin did not appreciably increase the fluorescence of rxRFP1-expressing HEK 293T cells. We further performed redox urea-PAGE and immunoblotting analysis³⁴ of TrxRFP1 in HEK 293T cells treated with different concentrations of H_2O_2 or auranofin. We treated cells with the neutral alkylating agent iodoacetamide, subsequently reduced cell lysates, and further treated the lysates with acidic alkylating agent iodoacetic acid. We were able to separate the TrxRFP1 protein based on its redox states. As the concentration of H_2O_2 or auranofin increases, the oxidation of TrxRFP1 increases (Fig. 2g,h), corroborating our measurements based on the fluorescence of TrxRFP1. These results further confirm that TrxRFP1 is a selective indicator for Trx redox and that the engineered redox relay in TrxRFP1 is necessary for sensing Trx redox changes.

It is a general concern that the use of redox sensors in live cells could inevitably perturb intrinsic physiology. Expression of TrxRFP1 may result in the additional Trx1 domain and rxRFP1, which also contains cysteines. Fortunately, Trx1 is a highly abundant protein in mammalian cells³⁹, whereas cysteines in rxRFP1 are not very reactive, as rxRFP1 alone does not respond to micromolar or even low millimolar H_2O_2 *in vitro* and in live cells (Figs. 1d and 2e). We assessed the effect of TrxRFP1 expression in mammalian cells, in terms of cell viabilities, in response to treatment with H_2O_2 and auranofin, which are both modulators of the thioredoxin redox system. After 24-h incubation with H_2O_2 or auranofin, we did not observe any substantial difference in the viabilities of mammalian cell lines (e.g., HEK 293T, HeLa, MCF-7, and SW620 cells) with or without TrxRFP1 overexpression (Supplementary Fig. 14). We also compared the proliferation of HEK 293T cells expressing or not expressing TrxRFP1 and observed no difference (Supplementary Fig. 15).

Localization of TrxRFP1 to subcellular compartments

In addition to the cytosol, the Trx redox system exists in the nucleus and mitochondria of mammalian cells, and we therefore subcellularly localized TrxRFP1 to monitor compartmentalized Trx redox dynamics. We appended a nuclear localization sequence (NLS) or a mitochondrial targeting sequence (MTS) to TrxRFP1. This led to the proper localization of TrxRFP1 in either the mammalian cell nucleus or mitochondria, as confirmed by the colocalization of TrxRFP1 with a nuclear stain DAPI (Fig. 3a) or a mitochondrial stain MitoTracker Green (Fig. 3b), respectively. Treating the cells expressing nuclear or mitochondrial TrxRFP1 with H_2O_2 or auranofin resulted in robust fluorescence increases (Supplementary Fig. 16). However, both nuclear and mitochondrial TrxRFP1 are less sensitive to exogenous H_2O_2 and auranofin than to unlocalized TrxRFP1, as higher concentrations are needed to trigger comparable fluorescence responses (Fig. 3c,d). In particular, the EC_{50} s for those with 10 min H_2O_2 treatment were $62.6 \pm 3.6 \mu M$ (fit value $\pm$ s.e.m. of fit) and $69.6 \pm 9.7 \mu M$ for nuclear and mitochondrial TrxRFP1, respectively. The EC_{50} s for 3 h auranofin treatment were 7.9 ± 0.8 and $17.1 \pm 1.2 \mu M$ for nuclear and mitochondrial TrxRFP1, respectively. These observations are reasonable, as exogenous H_2O_2 and auranofin have to cross cellular space and layers of lipid membranes to reach the nuclear or mitochondrial Trx redox system. Moreover, compared to the cytosol, the cell nucleus and mitochondria may possess sophisticated systems to quickly degrade ROS. We used a HyPer3 biosensor⁴⁰ to directly monitor cytosolic and nuclear H_2O_2 , and observed more robust fluorescence changes for cytosolic HyPer3 than for nuclear HyPer3 (Supplementary Fig. 17), suggesting that the effective concentrations of H_2O_2 in the cytosol are

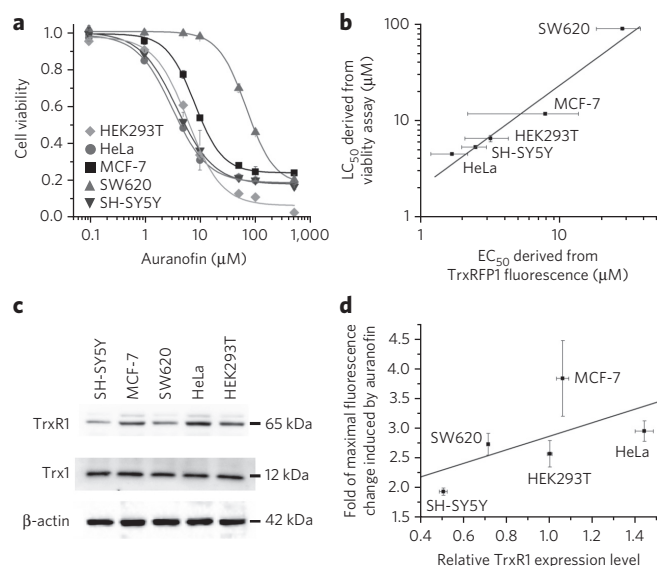


Figure 4 | Use of TrxRFP1 in various mammalian cell lines. (a) The viabilities of indicated cell lines in response to 24-h auranofin treatment. (b) The correlation between EC_{50} values derived from TrxRFP1 fluorescence and LC_{50} values derived from viability assays of various cell lines ($R^2 = 0.95$). (c) Western blots of endogenous TrxR1, Trx1, and β -actin in various cell lines (full gels are in Supplementary Fig. 21a–c). (d) A plot for relative TrxR1 expression levels and the fold of fluorescence changes induced by auranofin across various cell lines ($R^2 = 0.35$). Data in a are shown as mean and s.d. of three independent experiments. Error bars in b and d are s.e.m. from curve fitting or s.d. from quantification of western blot bands of three independent replicates.

indeed higher than those in the nucleus upon extracellular addition of H_2O_2 . Furthermore, the Trx redox system in mammalian mitochondria is based on Trx2 and TrxR2 (ref. 41), which is homologous to, but distinct from, the cytosolic and nuclear Trx redox system based on Trx1 and TrxR1. Therefore, although our Trx1-based TrxRFP1 biosensor displayed cross-reactivity as expected⁴², it may not have the optimal kinetics to sense the redox of the mitochondrial Trx system.

Chemical-induced Trx redox dynamics in mammalian cells

To demonstrate the general applicability of TrxRFP1, we expressed TrxRFP1 in several additional mammalian cell lines, including cervical cancer HeLa cells, breast cancer MCF-7 cells, colon carcinoma SW620 cells, and neuroblastoma SH-SY5Y cells. Fluorescence responses to H_2O_2 - and auranofin-induced Trx oxidation were observed in all conditions (Supplementary Figs. 18 and 19; Supplementary Videos 3 and 4). We further compared the responses of TrxRFP1 in different cell lines to various concentrations of auranofin after a 3-h incubation period. Each showed different sensitivity to auranofin, with an EC_{50} of $1.7 \pm 0.5 \mu M$ (fit value $\pm$ s.e.m. of fit) for HeLa, $7.9 \pm 5.7 \mu M$ for MCF-7, $28.1 \pm 9.8 \mu M$ for SW620, and $2.5 \pm 0.5 \mu M$ for SH-SY5Y (Supplementary Fig. 18). Moreover, the magnitudes of their fluorescence responses were different, with approximately 2.95-fold, 3.83-fold, 2.73-fold, 1.94-fold maximal changes for HeLa, MCF-7, SW620, and SH-SY5Y, respectively. To understand the biological meanings of these differences across cell lines, we further determined the viability responses of various cell lines to auranofin after 24-h incubation. The LC_{50} s (the concentration needed to kill half of the cells) were determined to be ~ 6.5 , 4.5, 11.7, 88.4, and $5.3 \mu M$ for HEK 293T, HeLa, MCF-7, SW620, and SH-SY5Y cells, respectively (Fig. 4a). These LC_{50} values roughly correlate with the EC_{50} values derived from the fluorescence measurements of TrxRFP1 (Fig. 4b), suggesting that TrxR is

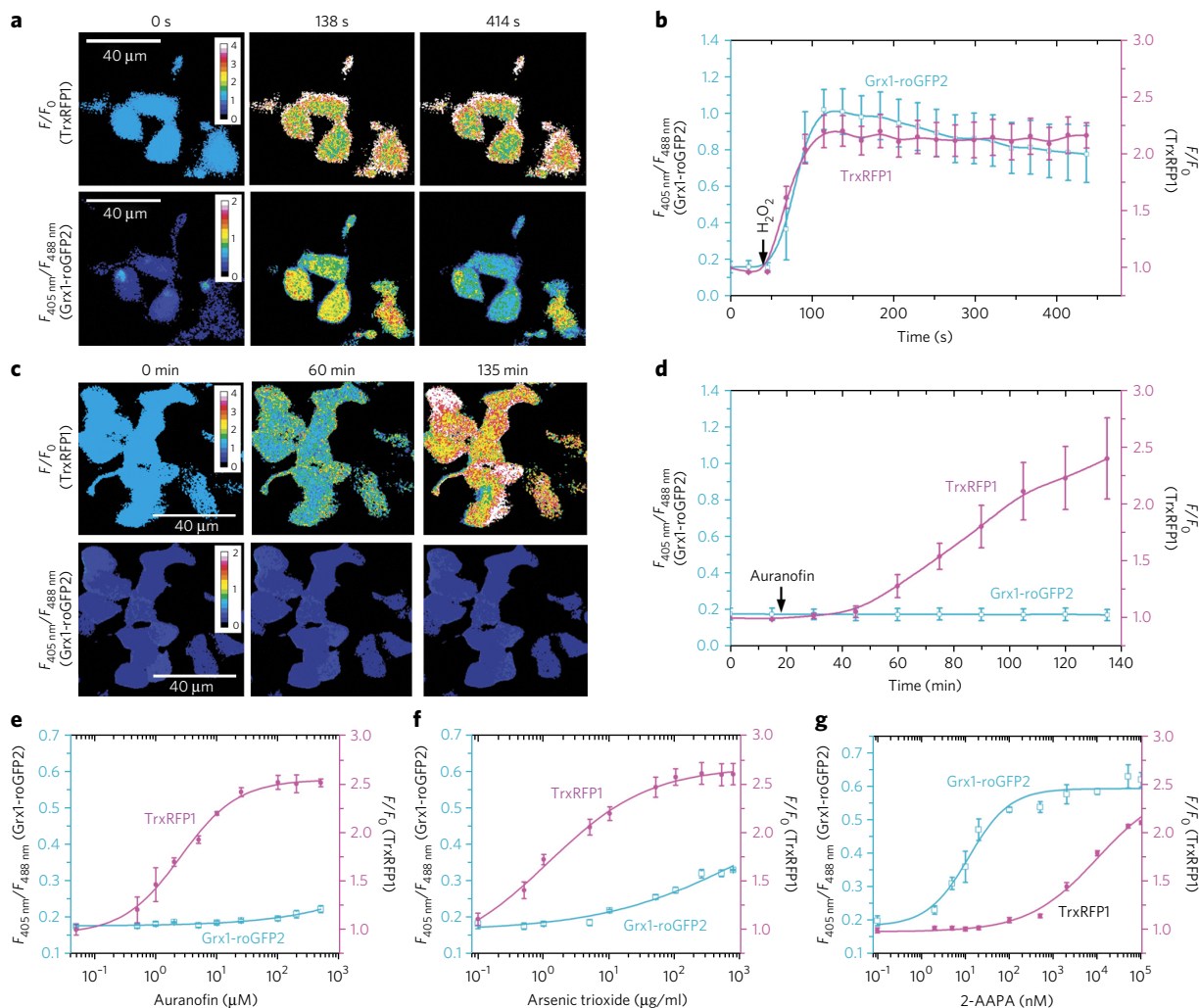


Figure 5 | Simultaneous monitoring of thioredoxin and glutathione redox dynamics using TrxRFP1 and Grx1-roGFP2. (a,c) Time-lapse pseudocolored fluorescence images of HEK 293T cells expressing both TrxRFP1 and Grx1-roGFP2 treated with 13.3 μM H₂O₂ (a) or 15 μM auranofin (c), indicating that H₂O₂ induces changes in both thioredoxin and glutathione redox systems, whereas auranofin induces the oxidation of thioredoxin but not glutathione (Scale bars, 40 μm). In the top row are pseudocolored ratiometric images (F/F₀) for TrxRFP1, and in the bottom row are pseudocolored ratiometric images (405 nm excitation/488 nm excitation) for Grx1-roGFP2. H₂O₂ and auranofin were added at *t* = 46 s and *t* = 16 min, respectively. (b,d) Ratio traces for TrxRFP1 or Grx1-roGFP2 in HEK 293T cells in a and c shown as mean and s.d. of six individual cells from three independent replicates. The magenta and cyan lines are for TrxRFP1 and Grx1-roGFP2, respectively. The arrows indicate the time points for addition of H₂O₂ or auranofin. (e-g) Fluorescence responses of TrxRFP1 (red) and Grx1-roGFP2 in HEK 293T to various concentrations of auranofin (e), arsenic trioxide (f), or 2-AAPA (g), suggesting that the Trx redox system and the glutathione redox system can be individually perturbed. Data are shown as mean and s.d. of three independent experiments.

indeed a cellular target of auranofin, causing cell growth inhibition and death. The differences in EC₅₀ and LC₅₀ values across various cell lines were likely due to differences in bioavailability and drug permeability⁴³. On the other hand, it is difficult to rationalize the differences in the magnitudes of auranofin-induced fluorescence changes across various cell lines. We probed the expression levels of Trx1 and TrxR1 in various cell types (Fig. 4c), and did not observe much difference for the expression of Trx1, but observed dramatic differences for TrxR1 across various cell lines. SH-SY5Y, which has a low level of TrxR1, indeed showed a relatively small fluorescence change in response to high concentrations of auranofin, suggesting that Trx1 in untreated SH-SY5Y is likely more oxidized than Trx1 in other tested cell lines. However, when all tested cell lines are considered together, the correlation between the maximal fluorescence changes and the TrxR1 levels is weak (Fig. 4d). We reason that the redox of Trx1 is determined by the interplay between Trx1 oxidation and reduction and that the TrxR1 expression level is not the only factor. Moreover, intracellular TrxRFP1 was not fully oxidized

by high concentrations of auranofin (Fig. 2h), suggesting that cells have complicated systems for regulating Trx redox in response to auranofin and that such systems may differ in various types of cells. This theory may also contribute to the observed differences in TrxRFP1 responses across various cell lines.

Simultaneous monitoring of Trx and glutathione redox

TrxRFP1 is a red fluorescent biosensor spectrally orthogonal to common green fluorescent biosensors. A green fluorescent excitation-ratiometric Grx1-roGFP2 biosensor was previously reported to monitor glutathione redox dynamics in mammalian cells³². As glutathione and Trx are the two major thiol-dependent antioxidants in mammals, we next examined the use of TrxRFP1 and Grx1-roGFP2 to simultaneously monitor the two redox systems. We expressed both probes in HEK 293T cells and treated the cells with H₂O₂. Because H₂O₂ is coupled to the redox of both glutathione and Trx (Supplementary Fig. 1), we observed concurrent fluorescence changes for both biosensors (Fig. 5a,b and Supplementary Video 5).

We next treated cells co-expressing the two biosensors with auranofin, a small molecule preferably targeting TrxR. A prominent fluorescence change was observed for TrxRFP1 but not for Grx1-roGFP2 (Fig. 5c,d and Supplementary Video 6). We further examined the responses of the cells co-expressing the two biosensors to various concentrations of auranofin, arsenic trioxide, and 2-AAPA (Fig. 5e–g). No change in Grx1-roGFP2 fluorescence was observed for a wide range of auranofin concentrations (Fig. 5e), further supporting the theory that auranofin is a selective inhibitor of TrxR over the glutathione redox system. Arsenic trioxide also preferably triggered the response of TrxRFP1, but, at high concentrations (>10 µg/ml), the fluorescence of Grx1-roGFP2, represented as fluorescence excitation ratios ($F_{405\text{nm}}/F_{488\text{nm}}$), also started to change (Fig. 5f). This result corroborates the previous finding that TrxR is a target for arsenic trioxide⁴⁴. 2-AAPA is a glutathione-reductase inhibitor⁴⁵. At low-nanomolar concentrations, it induced the oxidation of Grx1-roGFP2, but not TrxRFP1 (Fig. 5g). When its concentration increased above 100 nM, the fluorescence of TrxRFP1 started to rise, indicating the oxidation of both Trx and glutathione is induced by high concentrations of 2-AAPA. All of these results collectively suggest that TrxRFP1 and Grx1-roGFP2 can be used simultaneously to monitor the redox dynamics of Trx and glutathione in mammalian cells, that the Trx and glutathione redox systems can be individually perturbed, and that a thermodynamic equilibrium for Trx1 and glutathione is not reached in live mammalian cells.

Responses of TrxRFP1 to physiological stimuli

The sensitivity of TrxRFP1 to nanomolar H_2O_2 *in vitro* suggests that the Trx system in live cells could be oxidized by low concentrations of H_2O_2 generated under physiologically relevant conditions. To test this, we treated HEK 293T cells co-expressing TrxRFP1 and Grx1-roGFP2 with 10% FBS post a 6-h serum starvation. FBS contains a mixture of growth factors and hormones that are expected to stimulate various signaling pathways. A robust oxidation of Trx1 was observed, as the fluorescence of TrxRFP1 increased rapidly in the first 10 min post-stimulation (Fig. 6a,b). By contrast, we observed only a very small fluorescence change for Grx1-roGFP2. We further tested the response of HEK 293T cells to epidermal growth factor (EGF), which is known to induce the biological production of H_2O_2 (ref. 46). We observed fluorescence changes for both TrxRFP1 and Grx1-roGFP2, suggesting the oxidation of both Trx1 and glutathione under this condition (Fig. 6c,d and Supplementary Video 7). In comparison to FBS stimulation after serum starvation, the EGF-induced TrxRFP1 response was relatively slow. It is not yet possible for us to fully rationalize these observations. Potentially, these different processes can activate different compartmentalized sources of H_2O_2 or induce different antioxidant systems. These results, however, at least support the idea that TrxRFP1 could detect Trx redox changes in live cells under physiologically relevant conditions. We further repeated our serum and EGF stimulation experiments with control HEK 293T cells expressing the pH indicator pHRF22. None of these conditions increased the fluorescence of pHRF22 (Supplementary Fig. 20), suggesting that the observed fluorescence increase of TrxRFP1 was not caused by pH changes.

DISCUSSION

Trx is a cellular protein containing highly reactive cysteines that is important for redox homeostasis and signaling. Prior to our work, there were no genetically encoded biosensors for monitoring Trx redox in live cells. To develop a selective biosensor for Trx redox, we linked a redox-sensitive rxRFP1 protein with Trx1. A similar fusion strategy based on roGFPs and rxYFP has been previously used to create biosensors for glutathione and H_2O_2 (refs. 25,32), but it failed to create them for Trx^{17,32,47}. Unlike roGFPs and rxYFP, rxRFP1 adopts a circularly permuted topology to allow a new fusion

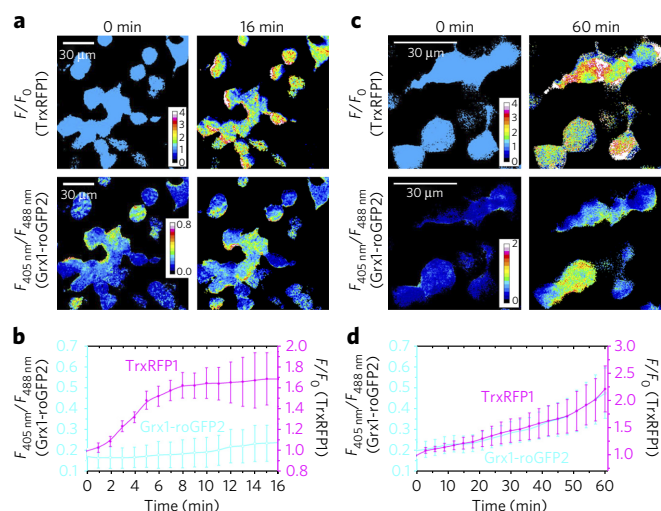


Figure 6 | Responses of TrxRFP1 to physiological stimuli in HEK 293T cells.

(a,b) Time-lapse responses of TrxRFP1 and Grx1-roGFP2 to serum stimulation at $t = 0$ min, indicating a robust oxidation of Trx (Scale bars, 30 µm). Prior to the 10% FBS treatment, cells were subjected to 6-h serum starvation. (c,d) Time-lapse responses of TrxRFP1 and Grx1-roGFP2 to epidermal growth factor (EGF, 500 ng/ml) treatment at $t = 0$ min, indicating the oxidation of both Trx and glutathione (Scale bars, 30 µm). Pseudocolored ratiometric images (F/F_0) for TrxRFP1 and pseudocolored ratiometric images (405 nm excitation/488 nm excitation) for Grx1-roGFP2 are shown in a and c. Ratio traces for TrxRFP1 and Grx1-roGFP2 are presented in b and d as mean and s.d. of eight individual cells from three independent replicates. The magenta and cyan lines are for TrxRFP1 and Grx1-roGFP2, respectively.

geometry. This indeed resulted in novel biosensors, in which the disulfide exchange between rxRFP1 and Trx1 is possible. We localized TrxRFP1 to the cell nucleus and mitochondria and successfully monitored compartmentalized Trx redox dynamics in live mammalian cells. We also used TrxRFP1 to monitor Trx redox dynamics induced by H_2O_2 and auranofin in various cell lines. In particular, the fluorescence responses of TrxRFP1 to 3-h auranofin treatment were indicative of cell viabilities determined after 24-h auranofin treatment. This correlation is due to the fact that TrxR is a major cellular target of auranofin. On this basis, our TrxRFP1 biosensor may be used for mechanistic studies on the interactions between molecules of interest and the Trx redox system. It may also be used in high-throughput screening assays to identify selective molecular modulators of the Trx redox system. Considering the importance of the Trx system and its implications in various diseases, TrxRFP1 may open the door for a large array of exciting studies.

Trx1 is typically considered as a reductant for oxidized cysteines in proteins¹. It was not surprising that the fluorescence of fully oxidized TrxRFP1 decreased quickly in response to TrxR/NADPH, because the disulfide in Trx1 reduced by TrxR/NADPH can subsequently exchange with the disulfide in rxRFP1. Furthermore, fully reduced TrxRFP1 responded to TPx/ H_2O_2 , whereas rxRFP1 alone was unresponsive, suggesting that Trx1 oxidized by TPx/ H_2O_2 can further oxidize the two cysteine residues in reduced rxRFP1. Chemically, such reversibility is not surprising, because oxidation is an exact reverse of the reduction reaction. Biologically, the result indicates that the reversibility may also be important *in vivo* and that Trx may be able to modulate the redox of its interacting proteins in both directions.

The red fluorescent color of TrxRFP1 makes it highly attractive, not only because it enhances tissue penetration and minimizes phototoxicity, but also because it can be paired with many existing GFP-based biosensors to monitor more than one parameter.

We have demonstrated the use of TrxRFP1 and Grx1-roGFP2 to simultaneously monitor glutathione and Trx redox dynamics in live cells. Furthermore, we show that the redox of glutathione and the Trx1 active site can be perturbed individually without shifting the other. These data align with the results of our *in vitro* enzymes assays, which indicate that the Grx–glutathione system reduces the non-active-site disulfide bond, but not the active-site disulfide bond, of Trx1. This finding also corroborates the emerging perspective that various cellular redox couples are quasi-independent from each other and a thermodynamic equilibrium is not reached in live cells⁴⁸.

The specificity of TrxRFP1 is partially due to the low reactivity of rxRFP1 toward H₂O₂ and its slow reaction with the glutathione couple. There is essentially no response for rxRFP1 to micromolar and low-millimolar H₂O₂ (ref. 22). By contrast, roGFP2 is more reactive toward H₂O₂ (refs. 25,32). Therefore, rxRFP1 is a preferable scaffold to roGFP2 for construction of redox-relay-based biosensors. This unique circularly permuted topology of rxRFP1 may also be advantageous, as shown in this study. Moreover, despite the high specificity of TrxRFP1, rxRFP1 serves as an excellent control to further confirm the connection between the observed fluorescence changes of TrxRFP1 and the redox dynamics of Trx.

Humans express several Trx isozymes. Although we demonstrated that the Trx1-based TrxRFP1 biosensor could be used to monitor the dynamic of the mitochondrial Trx redox system, a better mitochondrial biosensor may be engineered from Trx2 and rxRFP1. Furthermore, although the data presented here were mainly based on experiments in live human cell lines, TrxRFP1 or similar biosensor may be applicable in other mammalian organisms, other eukaryotic systems, or even prokaryotic cells because of the strong homology between the Trx redox systems across species. There is an interest to monitor Trx redox dynamics in pathogenic bacteria or parasites, as inhibition of bacterial or parasitic thioredoxin reductase has been considered as new therapeutic mechanisms^{10,49}. This work may be extended to these studies.

In conclusion, by engineering a redox relay between Trx1 and rxRFP1, we developed the first genetically encoded fluorescent biosensor that allows the observation of real-time dynamics of the Trx redox system in live mammalian cells. We further combined the resultant TrxRFP1 sensor with a green fluorescent Grx1-roGFP2 biosensor to simultaneously monitor Trx and glutathione redox dynamics in live cells in response to chemical and physiologically relevant stimuli. With these new capabilities, a large array of new studies will be enabled to further our understanding of redox biology.

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METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the [online version of the paper](#).

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Author contributions

H.A. conceived and supervised the project. Y.F. performed all experiments, except for that M.M. assisted Y.F. with cell culture and transfection and M.X.W. assisted Y.F. with plasmid preparation and SDS–PAGE. H.A. and Y.F. analyzed the data and wrote the manuscript.

Competing financial interests

The authors declare no competing financial interests.

Additional information

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ONLINE METHODS

Reagents and general methods. The human Trx1 gene fragment and all other synthetic DNA oligonucleotides were purchased from Integrated DNA Technologies. The genes for human TrxR1 and TPx1 were purchased from DNASU Plasmid Repository. Restriction endonucleases were purchased from Thermo Scientific Fermentas. Accura high-fidelity DNA polymerase and EconoTaq DNA polymerase were purchased from Lucigen. Products of PCR and restriction digestion were purified using gel electrophoresis and Syd Laboratories Gel Extraction columns. Plasmid DNA was purified using Syd Laboratories Miniprep columns. DNA sequences were analyzed by Retrogen. All other chemicals were purchased from Sigma-Aldrich, Fisher Scientific, and VWR unless otherwise noted. Anti-human Trx1 antibody (Cat. # 11538) and recombinant rat TrxR1 (Cat. # 14638) were purchased from Cayman Chemical. Anti-human/mouse/rat TrxR1 antibody (Cat. # MAB7428-SP) was purchased from R&D Systems. Secondary antibodies and the anti- β -actin antibody (Cat. # sc-130656) were purchased from Santa Cruz Biotechnology. Human recombinant EGF (Cat. # 354052) was purchased from Corning. Glutathione reductase (Cat. # G3664) was purchased from Sigma-Aldrich.

Plasmid construction. To build TrxRFP0.1, oligonucleotide Trx-F was paired with 0.1-R1, 0.1-R2, 0.1-R3, or 0.1-R4 (**Supplementary Table 1**) stepwise in four individual polymerase chain reactions (PCR) using a purchased human Trx1 gene as the template in the first reaction and the products of previous reactions as the templates in the subsequent reactions. Oligonucleotides 0.1-F and pBAD-R were used to amplify an rxRFP1 gene fragment from a previously reported pBAD-rxRFP1 plasmid²². Next, an overlap PCR reaction was performed to assemble the two fragments using Trx-F and pBAD-R to afford a full-length TrxRFP0.1 gene. TrxRFP0.2, TrxRFP0.3, TrxRFP0.4 were constructed similarly using Trx-F/2-R and 2-F/pBAD-R, Trx-F/3-R and 3-F/TrxRFP3-R, and Trx-F/4-R and 4-F/pBAD-R oligonucleotide pairs, respectively. To introduce the C32S mutation to TrxRFP0.1, pBAD-F/C32S-R and C32S-F/pBAD-R were used to amplify two fragments from TrxRFP 0.1, and, subsequently, an overlap PCR reaction was performed using pBAD-F and pBAD-R. Similarly, the C35S mutant and the C32S C35S double mutant of TrxRFP0.1 were made using C35S-F, C35S-R, C32C35S-F, and C32C35S-R. To construct TrxRFP0.2, we used Trx-F/C62S-R, or C69/73S-F/pBAD-R to amplify two fragments from TrxRFP 0.1, which were next linked together by using an overlap PCR reaction with Trx-F and pBAD-R. To introduce the C217Y mutation to TrxRFP0.9, we used a similar overlap PCR strategy based on Trx-F, C217S-R, C217S-F and pBAD-R. Because C397 is close to the N terminus, C397-R was used to replace pBAD-R to introduce the C397S mutation. To create the TrxRFP1_{SSCC} (C32S/C35S/S62C/S69C) mutant, oligonucleotide pairs pBAD-F and 32/35S-R, 32/35S-F and 62/69C-R, and 62/69C-F and pBAD-R were used to amplify three individual fragments from TrxRFP1, which were next assembled in an overlap PCR with pBAD-F and pBAD-R. To construct the plasmid for bacterial expression of human TrxR1, a purchased gene was amplified with oligonucleotides TRND-F and TRND-R. Similarly, PRX-F and PRX-R were used to amplify the gene of human TPx1. To construct the plasmids for human Trx1, Trx1_{CCSS}, and Trx1_{SSCC}, we used Trx-F and Trx-R to amplify the corresponding gene fragments from TrxRFP0.1, TrxRFP1 and TrxRFP_{SSCC}, respectively. To construct the plasmid for bacterial expression of human Grx1, Grx-F and Grx-R were used to amplify the gene from a HEK 293 cDNA library (Biosettia). All aforementioned assembled or amplified gene fragments were digested with Xho I and HindIII, and ligated into a pretreated compatible pBAD/His B plasmid (Life Technologies Cat. # V43001). Oligonucleotides CMV-F1 and CMV-TrxRFP1-R were used to amplify the gene of TrxRFP1 from its corresponding pBAD plasmid. To construct a plasmid for nuclear expression of TrxRFP1, we used CMV-F1, Nuc-R1, Nuc-R2, and Nuc-R3 to amplify the gene of TrxRFP1 and further append three repeats of a nuclear localization sequence (DPKKKKRKV). To construct the plasmid for mitochondrial expression of TrxRFP1, oligonucleotides Mito-F and Mito-TrxRFP-R were used to amplify a mitochondrial targeting sequence (MLSLRQSIRFFKPATRTLCSSRYLL) from pMito-rxRFP1.1 (ref. 23), and Mito-TrxRFP-F and CMV-TrxRFP1-R were used to amplify the TrxRFP1 fragment. The products were assembled in an overlap PCR with Mito-F and CMV-TrxRFP1-R. These assembled or amplified gene fragments were next digested with HindIII and Xba I, and ligated into a pre-digested pcDNA3

plasmid (Life Technologies). To add a nuclear exclusion sequence (NES) to TrxRFP1, NES-F1 and NES-F2 were paired with NES-R for PCR in two steps and the resultant gene was digested with BamH I and Xba I, and ligated into a pre-digested pNES-rxRFP1 plasmid²³. Using similar procedures, we created plasmids for the cytosolic or nuclear expression of HyPer3 from a synthetic HyPer3 gene fragment and oligonucleotides HyPer-NES-F, HyPer-NES-R, HyPer-F, HyPer-Nuc-R1, Nuc-R2 and Nuc-R3. All ligation products were used to transform DH10B *Escherichia coli* cells, which were next plated on LB agar plates supplemented with ampicillin (100 μ g/ml). Individual colonies were used to seed liquid culture for preparation of DNA. All constructs were confirmed by DNA sequencing.

Random mutagenesis and library screening. Randomization was limited to the gene fragment encoding the GS-rich linker and rxRFP1. First, rxRFP0.2 was amplified with oligonucleotides Trx-F/0.2-R and 0.2_F/pBAD_R. The fragments were digested with Xho I and BamH I, and BamH I/HindIII, respectively, and ligated into a pretreated compatible pBAD/His B plasmid. This procedure inserted a new BamH I restriction site downstream to Trx1 without change the protein sequence. Error-prone PCR reactions were next carried out with oligonucleotides 0.2_F and pBAD_R according to a reported procedure⁵⁰. The DNA was digested with BamH I and HindIII and ligated into the pre-digested, aforementioned plasmid. The resultant gene library was introduced into DH10B cells and plated onto LB agar plate supplemented with 100 μ g/ml ampicillin and 0.1% (w/v) L-arabinose. Colonies were imaged using a previously described setup²². Fluorescence before and after spraying of DTT (50 mM) was quantified and the fluorescence ratios of individual colonies were evaluated using the Fiji image analysis software⁵¹. Colonies showing large fluorescence changes were selected and cultured in 96-well plates containing 2YT medium supplemented with 100 μ g/ml ampicillin and 0.2% (w/v) L-arabinose. Bacterial cells were first shaken at 37 °C and 250 r.p.m. for 24 h, and then at room temperature for another 12 h. Cells were pelleted and lysed using B-PER Bacterial Protein Extraction Reagents (Pierce). Cell debris was removed by centrifugation. Fluorescence emission of clear cell lysates was quantified on a BioTek Synergy Mx Microplate Reader before and after treatments of TrxR1 (10 μ M) and NADPH (200 μ M). Three clones with largest dynamic ranges were selected and corresponding plasmids were minipreped. Their mixture was used as the template for the next round of random mutagenesis.

Protein expression and purification. All proteins were expressed and purified as previously described²². The purities of the prepared proteins were analyzed by SDS-PAGE (**Supplementary Fig. 21**). To prepare reduced sensor proteins or the reduced forms of Grx1 and TRP-14, we incubated the proteins with 100 molar equivalent of DTT at room temperature overnight, which were next dialyzed into 1 $\times$ PBS using Thermo Scientific SnakeSkin dialysis tubing (7 k molecular-weight cutoff) in an N₂-filled anaerobic chamber. Purified recombinant TrxR1 and TPx1 proteins were also buffer-exchanged into 1 $\times$ PBS, aliquoted to 10 μ l each at 50 μ M, and stored at -80 °C for long-term use.

In vitro characterization. Protein concentrations were determined using the Bradford assay. The fluorescence of proteins was measured using a BioTek Synergy Mx Microplate Reader with the excitation wavelength at 540 nm and the emission wavelength at 600 nm. Sensor proteins were diluted to desired concentrations with 1 $\times$ PBS (pH 7.4). Reduction and oxidation kinetics was monitored at room temperature. The wild-type human TrxR1 has a selenocysteine (Sec) residue encoded by a TGA codon upstream to a Sec insertion sequence (SECIS). In our plasmid for the expression of TrxR1, the Sec residue has been replaced with Cys to afford a mutant retaining partial activity⁵². We used this TrxR1 mutant or a commercial rat TrxR1 (Cayman Chemical) that contains ~50% of the full-length Sec-containing protein in our *in vitro* assays. Unless otherwise stated, oxidized sensor proteins (1 μ M) were reduced with our prepared recombinant TrxR1 (10 μ M) or commercial rat TrxR1 (50 nM) and NADPH (200 μ M) or GSH; reduced sensor proteins (0.5 μ M) were mixed with our purified recombinant TPx1 (0.5 μ M) and H₂O₂ at desired concentrations. For reduction and re-oxidation in the same reaction vessel, oxidized TrxRFP1 (1 μ M) was first reduced with our prepared TrxR1 (10 μ M) and NADPH (200 μ M), and then TPx1 and H₂O₂ at the indicated concentrations

were added. For absorbance based kinetic assays, TrxRFP1 (60 μ M) or TrxRFP1_{ssc} (60 μ M) were treated with either NADPH (200 μ M) and TrxR (10 μ M), or a mixture containing NADPH (200 μ M), glutathione reductase (45 nM), Grx1 (5 μ M), and GSH (10 mM). In our effort to re-examine the previously reported result³⁷, different Trx1 mutants (20 μ M) were treated with a mixture containing NADPH (200 μ M), glutathione reductase (60 nM), Grx1 (1 μ M), and GSH (10 mM). The consumption of NADPH was determined by monitoring the absorbance at 340 nm. To determine the K_M values of TrxRFP1 and Trx1 for rat TrxR1, we conducted assays at room temperature with reaction mixtures consisting of 10 nM rat TrxR, 2 mM EDTA, 200 μ M NADPH, and 100 mM sodium phosphate (pH 7.4). The concentrations of Trx1 or TrxRFP1 varied from 1 μ M to 50 μ M. NADPH oxidation was spectrophotometrically monitored at 340 nm to determine reaction rates. K_M values were obtained by fitting the data with the Michaelis–Menten equation.

Cell culture and live-cell imaging. HEK 293T, SW620, MCF-7, and HeLa cells (all purchased from ATCC and tested for mycoplasma by PCR) were cultured in DMEM supplemented with 10% (v/v) FBS, and SH-SY5Y cells were cultured in DMEM/F12 (1:1) supplemented with 10% (v/v) FBS. All cells were kept at 37 °C in humidified air containing 5% CO₂. Cells were seeded and transfected as previously described²², and analyzed 72 h post transfection. Cells were rinsed twice with Dulbecco's Phosphate Buffered Saline (DPBS), and left in the imaging medium containing DPBS supplemented with 1 mM Ca²⁺, 1 mM Mg²⁺ and 1% (w/v) BSA. For the serum stimulation experiment, cells were first rinsed three times with DMEM containing no FBS, maintained in DMEM containing no FBS for 6 h at 37 °C, and then stimulated by adding 10% (v/v) FBS. All single-color images were taken with a Motic AE31 inverted epifluorescence microscope as previously described²². A Leica SP5 confocal microscope was used to simultaneously image TrxRFP1 and Grx1-roGFP2. TrxRFP1 was excited with a 543 nm HeNe laser, and the red fluorescence emission was collected between 580 and 630 nm with a hybrid detector (HyD). Grx1-roGFP2 was excited with a 405 nm UV laser and a 488-nm argon-ion laser sequentially, and the green fluorescence emission was collected between 500 and 530 nm with a PMT (photomultiplier tube) detector. Time-lapse series was acquired at the indicated interval and fluorescence emissions from all channels were scanned sequentially with the 'between frames' mode. All images were analyzed using the Fiji image analysis software⁵¹ and the ratios of the images were processed according to a published procedure⁵³.

Cell responses to chemical treatments, and cell viability and proliferation assays. To study the responses of cells expressing fluorescent biosensors to H₂O₂, transfected cells were rinsed twice with DPBS and left in the abovementioned imaging medium. Fluorescence was quantified after 10 min incubation at 37 °C. For auranofin, arsenic trioxide, and 2-AAPA, chemicals were added into the fresh culture medium and cells were incubated in the medium for 3 h at 37 °C. Cells were collected and cell suspensions were used for quantification. All samples were prepared and measured in triplicates to derive averages and s.d. To determine cell viabilities, cells were treated with chemicals for 24 h at 37 °C, and we followed the manufacturer's protocol and used RealTime-Glo MT cell viability assays (Promega) to determine cell viability. All fluorescence response and viability data were fitted with a modified Hill equation:

$$y = A + \frac{B \cdot x^n}{x^n + k^n}$$

in which k is the EC₅₀ or LC₅₀ reported in the manuscript, n is the Hill coefficient, A is the initial value, and B is the magnitude of change (positive for growth and negative for decay). To compare the growth of cells expressing or not expressing TrxRFP1, we seeded HEK 293T cells in 35-mm culture dishes. They were divided into two groups and one group of cells were transfected with pcDNA3-TrxRFP1 at 70% confluency. Cells were next collected at different time points to determine cell numbers. Briefly, cells were washed with DPBS, trypsinized, and resuspended in DPBS. Cells were further diluted with DPBS and stained with 0.4% trypan blue. Viable cell numbers were determined using a hemocytometer.

Western blotting. To examine the expression levels of Trx1 and TrxR1, we prepared cell lysates from the same number (6×10^6) of HEK 293T, SW620, MCF-7, HeLa, and SH-SY5Y cells using a CellLytic M cell lysis buffer (Sigma-Aldrich) supplemented with cOmplete Protease Inhibitor Cocktail (Roche). The Bradford assay was used to determine total protein concentrations in the cell lysates. 5 μ g of total proteins were loaded to each lane and resolved on a 12% SDS-PAGE. Proteins were next transferred to a nitrocellulose membrane. The membrane was first blocked with 5% skim milk in TBST, and next, incubated with one of the anti-Trx1 (1:5,000), anti-TrxR (1:10,000) and anti- β -actin (1:10,000) antibodies. After washing with TBST, the corresponding horseradish peroxidase (HRP) conjugated secondary antibodies were added. The membrane was visualized with the SuperSignal West Pico Chemiluminescent substrate (Thermo Fisher) on a Bio-Rad Gel Doc XR System.

Redox immunoblotting. We modified a reported procedure³⁴ to performed redox immunoblotting on TrxRFP1 in HEK 293T cells. Untreated, H₂O₂-treated, or auranofin-treated cells (6×10^6) were washed twice quickly with ice-cold 1 $\times$ PBS, pelleted by a brief centrifugation, and lysed with 200 μ l urea lysis buffer (1 mM EDTA, 8 M urea, and 50 mM Tris-HCl, pH 8.3) freshly supplemented with 10 mM iodoacetamide. Free thiols in cells were alkylated with iodoacetamide at 37 °C for 30 min. Cell debris was next removed by centrifugation and proteins in clear cell lysates were precipitated with ice-cold acetone containing 20 mM HCl. The resultant precipitates were washed twice with ice-cold acetone containing 20 mM HCl and resuspended in 50 μ l urea lysis buffer containing 3.5 mM DTT. After incubation at 37 °C for 30 min, 2.5 μ l of freshly prepared 600 mM iodoacetic acid was added, followed by another 30-min incubation at 37 °C to alkylate additional free thiols resulting from DTT reduction. Total protein concentrations were determined using a bicinchoninic acid (BCA) assay and 10 μ g each of total proteins were separated on a 12% urea gel at constant 100 V and 50 °C. Next, protein bands were transferred to a nitrocellulose membrane, which was blocked with 5% skim milk in TBST, incubated with the anti-Trx antibody (1:5,000) overnight. After washing with TBST, an HRP-conjugated anti-goat secondary antibody (Santa Cruz Biotechnology cat. # sc-2354) was added. The SuperSignal West Pico Chemiluminescent substrate was added to visualize the membrane on a Bio-Rad Gel Doc XR System.

Statistical analysis. An unpaired Student's t -test was used to determine all P values. No statistical method was used to predetermine the sample size. This study used *in vitro* enzyme assays and cell lines, so biological variation was not a consideration. Experiments were independently repeated at least three times to minimize technical variation. No sample was excluded from data analysis, and no randomization or blinding was employed. For time-lapse imaging data, we randomly selected cells with sufficient signals to quantify the intensities or ratios of individual cells from three independent duplicates. Unless otherwise indicated, data are shown as mean $\pm$ s.d., and error bars in figures represent s.d.

Data availability. The DNA sequence of TrxRFP1 has been deposited into GenBank under the accession number [KX981912](#). Materials, associated protocols, and other supporting data are available from the corresponding author upon reasonable request. Source data files for **Figures 1–6** are available online.

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