Supplementary Data 1

Oligonucleotides used for subcloning

Oligo name	sequence
XmaI_roGFP2Orp1_F	5′-AA**CCCGGG**ATCCACCGGTCGCCACCATGGCAGTGAGCAAGGGCGAGGAG-3′
NotI_roGFP2Orp1_R	5′-AA**GCGGCCGC**CTAGCTAGAGTCGCGGCCTTCCACCTCTTTCAAAAG-3′
XmaI_Grx1roGFP2_F	5′-AA**CCCGGG**ATCCACCGGTCGCCACCATGGCTCAAGAGTTTGTGAACTGC-3′
NotI_Grx1roGFP2_R	5′-AA**GCGGCCGC**TTACTTGTACAGCTCGTCCATGCCGAGAGTGATCCCGGC-3′

Oligonucleotides used in the study

Supplementary Data 2

Material and methods: Cloning

Plasmids pEIGW/Grx1-roGFP2[1] and pEIGW/roGFP2-Orp1[2] were gifts from Dr. Tobias Dick (Addgene plasmid # 64990 and # 64993, respectively). pPalmitoyl-mTurquoise2[3] was a gift from Dr. Dorus Gadella (Addgene plasmid # 36209). Palmitoylated redox sensors were constructed from original plasmids pEIGW/Grx1-roGFP2 (Grx1-roGFP2^{Cyt}), pEIGW/roGFP2-Orp1 (roGFP2-Orp1^{Cyt}). Oligonucleotides used in the study are listed in Supplementary Data 1. To generate palmitoylated version of Grx1-roGFP2, entire region of Grx1-roGFP2 was amplified by PCR using DNA primers XmaI_Grx1roGFP2_F and NotI_Grx1roGFP2_R and pEIGW/Grx1-roGFP2 as a template. The resulting fragment was digested with XmaI and NotI. pPalmitoyl-mTurquoise2 plasmid was digested using the same set of restriction enzymes and subsequently treated with Antarctic phosphatase (New England BioLabs). Fragments were resolved on 1% agarose gel, isolated using QIAEX II Gel Extraction Kit (QIAGEN), and ligated using T4 DNA ligase (New England BioLabs). Ligated plasmds were introduced into XL10-Gold Ultracompletent cells (Agilent Technologies) by flash heat shock and transformed cells were selected on a culture plate containing 50 ug/ml kanamycine. The resulting plasmid pPalmitoyl-Grx1-roGFP2 (Grx1-roGFP2^{Palm}) was propagated in the same bacterial host and extracted using Plasmid Midi Kit (QIAGEN). Plasmid DNA was further purified by Phenol/Chloroform/Isoamyl alcohol extraction and ethanol precipitation following standard laboratory procedures. Exactly the same strategy was adopted to construct pPalmitoyl-roGFP2-Orp1 (roGFP2-Orp1^{Palm}) except that PCR was performed using primers XmaI_roGFP2Orp1_F and NotI_roGFP2Orp1_R and pEIGW/roGFP2-Orp1 as a template. The entire coding regions of Grx1-roGFP2^{Palm} and roGFP2-Orp1^{Palm} were sequenced for validation. DNA concentration was estimated by absorbance (λ=260 nm) using NanoDrop ND-1000 UV-Vis spectrophotometer.

References

[1] M. Gutscher, A.-L. Pauleau, L. Marty, T. Brach, G.H. Wabnitz, Y. Samstag, A.J. Meyer, T.P. Dick, Real-time imaging of the intracellular glutathione redox potential., Nat. Methods. 5 (2008) 553–9.

[2] M. Gutscher, M.C. Sobotta, G.H. Wabnitz, S. Ballikaya, A.J. Meyer, Y. Samstag, T.P. Dick, Proximity-based Protein Thiol Oxidation by H 2 O 2 -scavenging Peroxidases, J. Biol. Chem. 284 (2009) 31532–31540.

[3] J. Goedhart, D. von Stetten, M. Noirclerc-Savoye, M. Lelimousin, L. Joosen, M.A. Hink, L. van Weeren, T.W.J. Gadella, A. Royant, A. Royant, Structure-guided evolution of cyan fluorescent proteins towards a quantum yield of 93%., Nat. Commun. 3 (2012) 751.

Supplementary Data 3

Primary antibodies

antibody name	host animal	Manufacturer	catalogue number
Primary antibodies			
Anti-EGFR	Mouse, monoclonal	Thermo Fisher	MA5-13070
Anti-TUFM	Mouse, monoclonal	ATLAS antibodies	AMAb90964
Anti-ABCD3	Mouse, monoclonal	ATLAS antibodies	AMAb90995
Anti-LAMP1	Mouse, monoclonal	ATLAS antibodies	AMAB91170
Secondary antibodies			
Alexa Fluor 568 Anti-mouse IgG(H+L)	Goat, polyclonal	Life Technologies	A11004

Antibodies used in the study

Supplementary Data 4

Material and methods: Calculations for ratiometric analyses of the redox sensors

Degree of roGFP oxidation and redox potentials were calculated according to previously described procedures[1,2]. The percentage of sensor oxidation was calculated by the following Hanson's equation;

$$OxD = \frac{R - R_{red}}{I488_{min}/I488_{max}(R_{ox} - R) + (R - R_{red})}$$

where R stands for the ratio of I405 and I488 (I405/I488), R_{red} for the R value in completely reducted condition, R_{red} for the R value in completely reducted condition, $I488_{max}$ and $I488_{min}$ for I488 values in reduced and oxidized conditions. For glutathione sensor, redox potential of glutathione pair ($E_{GSH/GSSG}$) was further calculated using the obtained OxD value. Redox potential of $roGFP_{ox}$/$roGFP_{red}$ pair (E_{roGFP}) was calculated using the following Nernst equation;

$$E_{roGFP} = E^0_{roGFP} - \frac{RT}{zF}\frac{(1 - OxD)}{OxD}$$

where R stands for gas constant (8.315 $J \cdot K^{-1} \cdot mol^{-1}$), T for the absolute temperature (298 K), z for the number of electrons transferred in the redox reaction (z=2), F for the Faraday constant (96485 $C \cdot mol^{-1}$), and E^0_{roGFP} for the standard redox potential of roGFP2 (-280 mV[3]). Assuming that the redox exchange between Grx1-roGFP2 and surrounding glutathione is equilibrated, redox potential of glutathione ($E_{GSH/GSSG}$) equals E_{roGFP}.

References

[1] B. Morgan, M.C. Sobotta, T.P. Dick, Measuring E(GSH) and H2O2 with roGFP2-based redox probes., Free Radic. Biol. Med. 51 (2011) 1943–51.

[2] M. Gutscher, A.-L. Pauleau, L. Marty, T. Brach, G.H. Wabnitz, Y. Samstag, A.J. Meyer, T.P. Dick, Real-time imaging of the intracellular glutathione redox potential., Nat. Methods. 5 (2008) 553–9.

[3] C.T. Dooley, T.M. Dore, G.T. Hanson, W.C. Jackson, S.J. Remington, R.Y. Tsien, Imaging Dynamic Redox Changes in Mammalian Cells with Green Fluorescent Protein Indicators, J. Biol. Chem. 279 (2004) 22284–22293.

Supplementary Data 5

Supplementary Data 5.avi

Time-lapse movie showing constitutively oxidized glutathione pair near the cytosolic vesicles.

Grx1-roGFP2Palm was transfected into HeLa cells and I405/I488 time-lapse imaging was performed by live-cell confocal microscopy. *R* value (I405/I488) represents the degree of sensor oxidation and shown in a false-color scale. Representative images are shown in the main figure 4A. Time is indicated in each image (h:m:s).