Supporting Information (SI) Appendix

Reactive cysteine persulfides and S-polythiolation regulate oxidative stress and redox signaling

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SI Discussion

The Biotin-Switch assay and persulfides: Typically, the biotin switch assay mentioned in the text is used for the specific detection of S-nitrosothiols (RSNO) (for example, 1). Importantly, the lack of specificity of this assay has been discussed (2). As noted above, the biotin-switch assay relies on the reaction of the electrophilic sulfur species MMTS with a free thiol to give a mixed disulfide. Since MMTS will not react with S-nitrosothiols, only free thiols will be modified by MMTS to give a mixed disulfide. However, if MMTS reacts with a protein persulfide, a mixed trisulfide would be generated. The second part of this assay relies on a specific reduction of an S-nitrosothiol to a free thiol by ascorbate, which presumably will not reduce a disulfide (although this has been challenged (3)). If indeed a trisulfide is formed during the assay from the reaction of MMTS with an endogenous persulfide, the next question becomes: is a trisulfide readily reduced by ascorbate? If so, then the presence of persulfides can be misconstrued as indication of the presence of an S-nitrosothiol. Currently, it is not known if trisulfides are readily reduced by ascorbate. However, it is worth noting that increasing the number of sulfurs in polysulfide species would be expected to increase the reduction potential (for example, 4), indicating that some reports of S-nitrosothiol generation may have included a contribution by persulfides.

The use of monobromobimane to detect per- and poly-sulfide species: Monobromobimane (Br-bimane)-coupled bis-S-bimane formation has been employed to measure H_2S/HS^- in biological samples. Our recent study showed, however, that bis-S-bimane was found to be formed indirectly from the reaction of Br-bimane with some types of polysulfides rather than directly with hydrogen sulfide anion (HS^-). For example, Br-bimane can react with Cys or glutathione polysulfides to form bis-S-bimane, although yield is relatively low (about 1%) compared with direct formation of bis-S-bimane adduct from HS^-. Similar but much higher efficacy of bis-S-bimane formation was observed with the high-molecular-weight (HMW) fractions of cell lysates (*SI Appendix*, Fig. S12), which potentially contain protein Cys-bound polysulfides as identified our current polysulfur proteomics (cf. Fig. 5). These protein polysulfides, through rather complicated chemical reactions, may also produce indirectly non-specific bis-S-bimane formation. Therefore, bis-S-bimane formation,

particularly in the presence of high levels of CysS-$(S)_n$-SCys/GS-$(S)_n$-SG or polythiolated proteins, is not a result of HS^- reactivity *per se*. Because this apparent bis-S-bimane formation was possibly due to the putative nucleophilicity of particular sulfur moieties of polysulfides having an HS^--like behavior, such as-yet-unidentified compounds are expressed as 'HS^--like species' herein (cf. Fig. 1*A,B* and legend).

SI Materials and Methods

Materials. GSH, Cys, HCys, NaHS, $Na_2S_2O_3$, Na_2S_4, propylamine NONOate ($CH_3N[N(O)NO]^-(CH_2)_3NH_2^+CH_3$; 1-hydroxy-2-oxo-3-(*N*-methyl-3-aminopropyl)-3-methyl-1-triazene) (P-NONOate), iodine, Br-bimane, *S*-adenosylmethionine (SAM), pyridoxal phosphate (PLP), β-cyanoalanine (BCA), sulfasalazine, Lipofectamine 2000, and Lipofectamine RNAiMAX were obtained from Wako Pure Chemical Industries, Invitrogen, Sigma, and other commercial suppliers. Authentic 8-nitro-cGMP and 8-SH-cGMP were prepared according to the method previously reported (5,6).

Synthesis of Cys polysulfide derivatives

General procedures. Analytical LC-MS data for Cys polysulfide derivatives were acquired by using an Agilent 6510 ESI-TOF mass spectrometer or Agilent 6430 ESI-QQQ mass spectrometer. For the Agilent 6510 system, samples were separated on a reverse phase (RP)-HPLC chip (43 mm × 75 μm inner diameter; Zorbax 300SB-C18, Agilent), with mobile phases A (H_2O + 0.1% formic acid) and B (acetonitrile) with a linear gradient from 5% to 90% B in 7 min and a flow rate of 600 nl/min. For the Agilent 6430 system, samples were separated on a YMC-Triart C18 column (50 × 2.0 mm inner diameter; YMC), with mobile phases A (H_2O + 0.1% formic acid) and B (methanol + 0.1% formic acid) with a linear gradient from 5% to 90% B in 14 min and a flow rate of 0.2 ml/min. In both systems, mass spectra were recorded in the positive ion mode. Preparative HPLC was performed by using the Waters e2695 series with the UV detector set at 210 nm. Samples were injected onto a TSKgel ODS-80Ts column (4.6 × 150 mm; Tosoh), at 40 °C. Mobile phases A (H_2O + 0.1% trifluoroacetic acid) and B (methanol) were used with a linear gradient from 2% to 70% B in 15 min and a flow rate of 1 ml/min. For the separation of oxidized Cys

derivatives, we used Asahipak GS-320 HQ (7.6 × 300 mm; Showa Denko K.K.) with a mobile phase of 5 mM sodium phosphate buffer (pH 2.6) containing 3% acetonitrile at a flow rate of 0.8 ml/min. Small variations in this purification method were made as needed to achieve the ideal separation for each compound.

Bimane derivatives.

CysS-$(S)_n$-bimane adducts. Cys (0.5 mM) was reacted with 0.5 mM NaHS in the presence of P-NONOate (0.5 mM) in 10 mM Tris-HCl buffer (pH 7.4) at room temperature for 30 min. Br-bimane (5 mM) was added to the reaction mixture, which was then incubated at room temperature for 30 min. CysS-$(S)_n$-bimane adducts thus formed were separated on the basis of sulfur number by RP-HPLC. CysSS-bimane, MS (ESI, positive): 344.0 $[M+H]^+$. CysSSS-bimane, MS (ESI, positive): 376.0 $[M+H]^+$. CysSSSS-bimane, MS (ESI, positive): 408.0 $[M+H]^+$. Isotope-labeled CysS-^{34}S-bimane was synthesized by reacting Cys with Na^{34}SH instead of Na^{32}SH. CysS-^{34}S-bimane, MS (ESI, positive): 346.0 $[M+H]^+$.

HCysS-$(S)_n$-bimane adducts. HCys (0.5 mM) was reacted with 0.5 mM NaHS in the presence of P-NONOate (0.5 mM) in 10 mM Tris-HCl buffer (pH 7.4) at room temperature for 15 min. Br-bimane (5 mM) was added to the reaction mixture, which was incubated at room temperature for 30 min. HCysS-$(S)_n$-bimane adducts thus formed were separated by using RP-HPLC. HCysSS-bimane, MS (ESI, positive): 358.1 $[M+H]^+$. HCysSSS-bimane, MS (ESI, positive): 390.0 $[M+H]^+$. HCysSSSS-bimane, MS (ESI, positive): 422.0 $[M+H]^+$. Isotope-labeled HCysS-^{34}S-bimane was synthesized by reacting HCys with Na^{34}SH instead of Na^{32}SH. HCysS-^{34}S-bimane, MS (ESI, positive): 360.1 $[M+H]^+$.

GS-$(S)_n$-bimane adducts. GSH (0.5 mM) was reacted with 0.5 mM NaHS in the presence of P-NONOate (0.5 mM) in 10 mM Tris-HCl buffer (pH 7.4) at room temperature for 15 min. Br-bimane (5 mM) was added to the reaction mixture, which was incubated at room temperature for 30 min. GS-$(S)_n$-bimane adducts thus formed were separated by means of RP-HPLC. GSS-bimane, MS (ESI, positive): 530.1 $[M+H]^+$. GSSS-bimane, MS (ESI, positive): 562.1 $[M+H]^+$. GSSSS-bimane, MS

(ESI, positive): 594.1 $[M+H]^+$. Isotope-labeled GS-^{34}S-bimane was synthesized by reacting GSH with Na^{34}SH instead of Na^{32}SH. GS-^{34}S-bimane, MS (ESI, positive): 532.1 $[M+H]^+$.

Oxidized polysulfides

CysS-$(S)_n$-SCys. Cys (20 mM) was reacted with 20 mM NaHS in the presence of I_2 (20 mM) in 20 mM Tris-HCl buffer (pH 7.4) at room temperature for 15 min. The reaction mixture was subjected to HPLC for purification of CysS-$(S)_n$-SCys derivatives. CysSSSCys, MS (ESI, positive): 273.0 $[M+H]^+$. CysSSSSCys, MS (ESI, positive): 305.0 $[M+H]^+$. CysSSSSSCys, MS (ESI, positive): 337.0 $[M+H]^+$. Isotope-labeled CysS-(^{34}S)n-SCys was synthesized by reacting Cys with Na^{34}SH instead of Na^{32}SH. CysS-^{34}S-SCys, MS (ESI, positive): 275.0 $[M+H]^+$. CysS-$^{34}S_2$-SCys, MS (ESI, positive): 309.0 $[M+H]^+$.

HCysS-$(S)_n$-SHCys. HCys (20 mM) was reacted with 20 mM NaHS in the presence of I_2 (20 mM) in 20 mM Tris-HCl buffer (pH 7.4) at room temperature for 15 min. The reaction mixture was subjected to RP-HPLC for purification of HCysS-$(S)_n$-SHCys derivatives. HCysSSSHCys, MS (ESI, positive): 301.0 $[M+H]^+$. HCysSSSSHCys, MS (ESI, positive): 333.0 $[M+H]^+$. Isotope-labeled HCysS-$(^{34}S)_n$-SHCys was synthesized by reacting HCys with Na^{34}SH instead of Na^{32}SH. HCysS-^{34}S-SHCys, MS (ESI, positive): 303.0 $[M+H]^+$. HCysS-$^{34}S_2$-SHCys, MS (ESI, positive): 337.0 $[M+H]^+$.

GS-$(S)_n$-SG. GSH (20 mM) was reacted with 20 mM NaHS in the presence of I_2 (20 mM) in 20 mM Tris-HCl buffer (pH 7.4) at room temperature for 15 min. The reaction mixture was subjected to RP-HPLC for purification of GS-$(S)_n$-SG derivatives. GSSSG, MS (ESI, positive): 645.1 $[M+H]^+$. GSSSSG, MS (ESI, positive): 677.1 $[M+H]^+$. GSSSSSG, MS (ESI, positive): 709.0 $[M+H]^+$. Isotope-labeled GS-$(^{34}S)_n$-SG was synthesized by reacting GSH with Na^{34}SH instead of Na^{32}SH. GS-^{34}S-SG, MS (ESI, positive): 647.0 $[M+H]^+$. GS-$^{34}S_2$-SG, MS (ESI, positive): 681.0 $[M+H]^+$. GS-$^{34}S_3$-SG, MS (ESI, positive): 715.0 $[M+H]^+$.

LC-MS/MS analysis for polysulfidomics. Samples (10 μl) containing the enzymatic reaction solution, cell lysates, and tissue homogenates were mixed with 50 μl of a methanol solution containing 5 mM Br-bimane and were incubated at 37 °C for 15 min. After centrifugation (10,000 × *g*, 10 min, 4 °C), supernatants were collected. Aliquots of the supernatants were diluted 10-100 times with distilled water containing known amounts of isotope-labeled internal standards. An Agilent 6430 Triple Quadrupole LC/MS was used to perform LC-ESI-MS/MS. Polysulfide derivatives were separated by means of RP-HPLC with a YMC-Triart C18 column (50 × 2.0 mm inner diameter), with a linear 5–90% methanol gradient for 14 min in 0.1% formic acid at 40 °C. A total flow rate of 0.2 ml/min and an injection volume of 20 μl were used. Ionization was achieved by using electrospray in the positive mode with a 3,500 V spray voltage. Nitrogen was the nebulizer gas, with the nebulizer pressure set at 50 psi. The desolvation gas (nitrogen), heated to 350 °C, was delivered at the flow rate of 10 L/min. Collision-induced dissociation (CID) was achieved by using high-purity nitrogen as the collision gas at a pressure of 0.5 MPa. Polysulfide derivatives were identified and quantified by means of MRM. *SI Appendix*, Tables S1 and S2 summarize the MRM parameters for each derivative.

Cell culture. Human adenocarcinoma A549 cells, human neuroblastoma SH-SY5Y cells, monkey kidney cell line COS7 cells, and rat glioma C6 cells were cultured in DMEM that was supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin.

Animal studies. All protocols using mice were approved by the Animal Care and Use Committees of Kumamoto University, and Osaka Prefecture University.
Ten-week-old male C57BL/6J mice purchased from Kiwa Laboratory Animals were maintained one mouse per cage in a controlled 12-h light/dark cycle with free access to water and a standard diet for 1 week. The mice were then randomly assigned to one of four dietary groups consuming chemically defined AIN-93-based diets with protein replaced by different amino acid mixtures (see below table) for 32 days: *1*) Control-fed (0.39% L-methionine, 0.24% L-cystine); *2*) cystine deficient (0.39% L-methionine, 0%

L-cystine); *3*) methionine-50% restricted (0.19% L-methionine, 0% L-cystine); and *4*) methionine-25% restricted (0.10% L-methionine, 0% L-cystine). To compensate for the reduced amino acid content in the methionine-restricted diet, cellulose content was raised on an equal weight basis. Food and water were provided ad libitum. Body weights were monitored twice a week for the duration of the study. At the end of the study, the animals were sacrificed. Blood was collected and plasma was prepared, flash frozen and stored at −80 °C until analyzed. Liver, heart, kidney and brain were harvested, flash frozen and stored at −80 °C until processed.

	Control-fed	Cystine deficient	Cystine deficient & methionine-50% restricted	Cystine deficient & methionine-25% restricted
	Amino acid content (%) [wt/wt]			
L-Methionine	0.39	0.39	0.19	0.10
L-Cystine	0.24	0	0	0
L-Isoleucine	0.70			
L-Leucine	1.22			
L-Lysine	1.30			
L-Phenylalanine	0.66			
L-Tyrosine	0.73			
L-Threonine	0.54			
L-Tryptophan	0.16			
L-Valine	0.88			
L-Histidine	0.39			
L-Arginine	0.48			
L-Alanine	0.39			
L-Aspartic acid	0.92			
L-Glutamic acid	2.76			
L-Glycine	0.24			
L-Proline	1.51			
L-Serine	0.68			

Preparation of recombinant enzymes. Recombinant rat CSE and CBS were prepared according to the literature (7-9). cDNAs of CSE and CBS were kind gifts from Dr. N. Nishi (Kagawa Medical School, Japan) and J. P. Kraus (University of Colorado School of Medicine), respectively. Recombinant human GAPDH was prepared as reported

previously (10).

Analyses for Cys persulfide formation by enzymes, cell lysates, cells, and tissues. Recombinant rat CSE (20 or 50 μg/ml) or CBS (5 μg/ml) was incubated in the presence of substrates such as CysSSCys in 30 mM HEPES buffer (pH 7.5) containing 50 μM PLP at 37 °C. For the CBS reaction, SAM (100 μM) was added. The enzymatic reaction was terminated by adding a 5 vol excess of a methanol solution containing 5 mM Br-bimane. Mixtures were then incubated at 37 °C for 20 min, followed by centrifugation (10,000 × *g*, 10 min) to collect supernatants. These supernatants were diluted with distilled water containing isotope-labeled polysulfide standards for polysulfidomics as mentioned above.

Polysulfide derivatives formed in cultured cells were extracted from cell lysates after derivatization with Br-bimane as just mentioned. Knockdown of CBS, CSE, xCT, and SNAT2 was performed as reported recently (6) by using the following small interfering RNAs (siRNAs): CBS, HSS101428 (Invitrogen); CSE, HSS102447 (Invitrogen); xCT, D-007612-04-005 (Thermo Scientific); and SNAT2, D-007559-01-005 (Thermo Scientific). siRNA transfection was performed by using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. In brief, A549 cells seeded in 24-well plates (5×10^4 cells per well) were incubated for 24 h. For transfection, we mixed 30 pmol per well of siRNA duplex or 1 μl per well of Lipofectamine RNAiMAX with 50 μl of Opti-MEM (Invitrogen) in a tube. Before siRNA and transfection reagent solutions were added to the cells, solutions were mixed together and incubated for 10 min at room temperature. To the cells were added the solutions, and incubation proceeded for 48 h. In some experiments, cell lysates were then incubated *in vitro* with CysSSCys at 37 °C for indicated periods.

For overexpression of hemagglutinin (HA)-tagged human CBS or CSE, cells were transfected with the expression plasmid pME18S-CBS-HA or pME18S-CSE-HA by using Lipofectamine 2000 (Invitrogen). At 24 h after transfection, cell lysates were prepared for polysulfidomics.

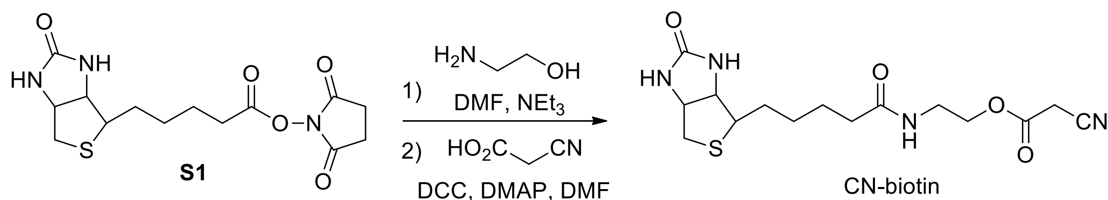
Mouse tissues (100 mg each) were homogenized by using a Polytron homogenizer with 0.5 ml of a methanol solution containing 5 mM Br-bimane. The resultant

solution was incubated at 37 °C for 30 min, followed by centrifugation (14,000 × *g*, 10 min, 4 °C), and the supernatants were collected and subjected to polysulfidomics as mentioned above. Mouse and human plasma samples (0.1 ml) were sonicated in 0.5 ml of a methanol solution containing 5 mM Br-bimane, incubated at 37 °C for 30 min, and centrifuged (14,000 × *g*, 10 min, 4 °C). The supernatants obtained were subjected to polysulfidomics.

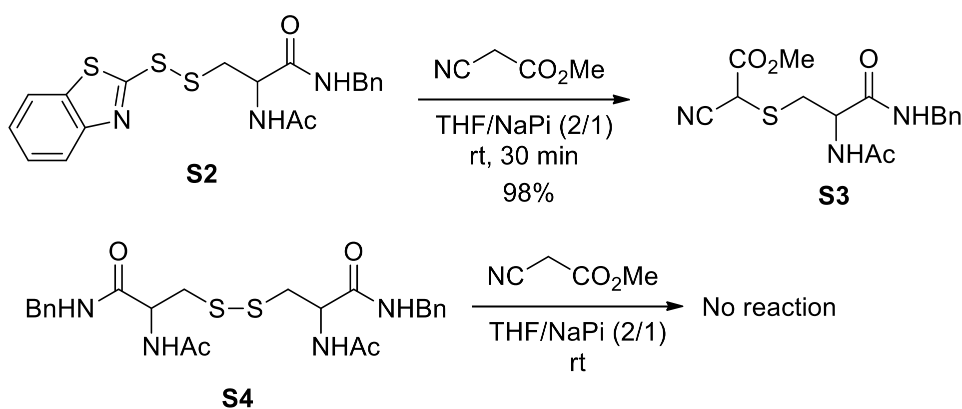
Polysulfide imaging. Cellular polysulfide imaging was performed by using the recently developed SSP2 (11). A549 cells were seeded in four-well glass chamber slides at a density of 4×10^5 cells per well. In some experiments, cells were pre-treated with CSE vector, CBS and CSE siRNAs, sodium cyanide (NaCN), or BCA (CSE inhibitor). Cells were washed once with serum-free DMEM, followed by incubation with 50 μM SSP2 in serum-free DMEM containing 500 μM cetyltrimethylammonium bromide (CTAB) at 37 °C for 20 min. Cells not treated with SSP2 were used as negative controls. After removing the excess probes from the cells and washing them with PBS, they were incubated in PBS for 30 min at 37 °C. Cells were then washed twice with PBS, followed by measurement of fluorescence in images, at an excitation wavelength of 488 nm, with a Nikon EZ-C1 confocal laser microscope. Images were edited with ImageJ software (National Institutes of Health) and Adobe Photoshop CS4 version 11 (Adobe Systems).

Polysulfide determination with SSP2. In addition to fluorescence microscopic imaging, polysulfides levels were determined with the use of SSP2 in a quantitative manner. Samples of interest were reacted with SSP2 (10 or 50 μM final) in 20 mM Tris-HCl (pH 7.4) in the presence of 1 mM CTAB in the dark for 10 min at room temperature. Fluorescence intensities of the resultant solutions were determined with excitation wavelength (482 nm) and emission wavelength (518 nm) by using microplate reader (SH-9000, Corona Electric Co., Ltd.). In separate experiments, polysulfides were pre-treated with NaCN in 200 mM Tris-HCl (p 7.4) at 37 °C for 60 min, followed by reacting with SSP2.

Preparation of CN-biotin. CN-biotin was prepared from compound **S1** in two steps: to a solution of **S1** (1.1 g, 3.24 mmol) in dry dimethylformamide (DMF) (66 mL) was added 1.5 equivalents of 2-aminoethanol (4.86 mmol, 0.29 mL). Then, 2 equivalents of triethylamine (6.48 mmol, 0.9 mL) was added into the mixture and the reaction was stirred overnight at room temperature. The solvent was removed under reduced pressure and the resulting residue was purified by flash chromatography (CH_2Cl_2 : MeOH = 50 :1 to CH_2Cl_2 : MeOH = 7 :1) to provide the intermediate (biotin-OH). This intermediate was dissolved in DMF (~3.24 mmol in 50 mL DMF). 1.2 equivalents of cyanoacetic acid (3.89 mmol, 331 mg) was added into the solution, followed by the addition of *N,N'*-dicyclohexylcarbodiimide (DCC) (4.2 mmol, 867 mg) and *N,N*-dimethyl-4-aminopyridine (DMAP) (0.32 mmol, 40 mg). The mixture was stirred overnight at room temperature and the solvent was removed under reduced pressure to give the crude product, which was purified by flash chromatography (CH_2Cl_2 : MeOH = 50 :1 to CH_2Cl_2 : MeOH = 7 :1) to afford the final product CN-biotin as a white solid (804 mg). Yield: 70% for two steps. mp 132-134 ^oC; ^1H NMR (300 MHz, DMSO) δ 7.96 (t, J = 5.3 Hz, 1H), 6.43 (s, 1H), 6.37 (s, 1H), 4.30 (dd, J = 7.7, 4.8 Hz, 1H), 4.11 (m, 3H), 3.97 (s, 2H), 3.36 – 3.23 (m, 2H), 3.16 – 3.03 (m, 1H), 2.82 (dd, J = 12.4, 5.0 Hz, 1H), 2.57 (m, 1H), 2.06 (t, J = 7.3 Hz, 2H), 1.68 – 1.37 (m, 4H), 1.29 (m, 2H); ^{13}C NMR (75 MHz, DMSO) δ 172.3, 164.3, 162.6, 114.9, 64.4, 60.9, 59.1, 55.3, 39.7, 37.1, 34.9, 28.1, 27.9, 25.0, 24.5. FT-IR (thin film, cm^{-1}) 3270.6, 3079.7, 2931.3, 2264.3, 2200.7, 1742.4, 1696.7, 1549.2, 1264.7, 1032.1, 726.8. MS (ESI) m/z, calcd for $C_{15}H_{22}N_4NaO_4S$ $[M+Na]^+$ 377.1, found 377.1.



Model reactions to prove the selectivity of the 'Tag-Switch' technique



Methyl cyanoacetate (MCA) was used as a model compound to demonstrate the selectivity of the Tag-Switch method. As shown above, **S2** is a MSBT-blocked product from persulfides. **S2** reacted with MCA rapidly in tetrahydrofuran (THF)/phosphate buffers to give the tag-switched product **S3** in good yield. Compound **S3** (as 1:1 diastereomers): ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.47 – 7.14 (m, 6H), 6.95 (m, 1H), 4.90 – 4.72 (m, 1H), 4.59 (d, J = 8.8 Hz, 1H), 4.39 (m, 2H), 3.82 (s, 3H), 3.29 – 3.01 (m, 2H), 1.96 (s, 3H).

However, MCA did not react with normal disulfides such as **S4**.

Tag-Switch assay and proteomics of protein S-polythiolation. Biotinylation of polysulfides was performed via Tag-Switch-Tag labeling of polysulfides using MSBT as the Tag reagent and CN-biotin as the Switch-Tag reagent (*SI Appendix* Fig. S9*A*). GS-$(S)_n$-H compounds generated via GSR-catalyzed reduction of GSSSG were used as model polysulfides. GSSSG (0.5 mM) was incubated with GSR (1 U/ml) and NADPH (1 mM) in 40 mM sodium phosphate buffer (pH 7.4) at room temperature for 10 min. GS-$(S)_n$-H compounds formed were reacted with 5 mM MSBT at room temperature for 15 min to tag sulfhydryls. Resultant solutions were then reacted with 10 mM CN-biotin at room temperature for 30 min to switch-tag sulfhydryls. Reactions were terminated by adding 0.2 M citric acid buffer (pH 2.5) at one-fourth the volume of the reaction buffer, followed by LC-MS analysis to identify formation of biotinylated GS-$(S)_n$-H compounds.

S-Thiolated proteins were also biotinylated via this labeling method. Lysates of

A549 cells (0.4 mg of protein in RIPA buffer [10 mM Tris-HCl, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, pH 7.4]) were reacted with 10 mM MSBT in 20 mM Tris-HCl (pH 7.4) at 37 °C for 30 min. These solutions were then reacted with 20 mM CN-biotin at 37 °C for 30 min, after which protein samples were subjected to Western blotting with neutrAvidin-horseradish peroxidase (HRP). For quantification of biotinylated bands, densitometric analyses were performed with the signal intensity of Western blotting images measured by using Image J software (National Institute of Health) as reported previously (6). Total band intensities ranging from 30 to 150 kDa were measured. In separate experiments, protein samples were purified with the 2-D Clean-Up kit (GE Healthcare UK Ltd.), according to the manufacturer's instructions. After this clean-up, protein samples were dissolved in a rehydration buffer and subjected to isoelectric focusing with immobilized pH gradient strips (7 cm, pH 3-10 NL; GE Healthcare), as reported recently (12). Proteins were then separated by SDS-PAGE (10% acrylamide), followed by silver staining or Western blotting with neutrAvidin-HRP. Spots were detected by means of a chemiluminescence reagent (ECL Plus Western Blotting Reagent; GE Healthcare) and a luminescent image analyzer (LAS-2000; Fujifilm).

S-Thiolated proteins were identified by means of LC-MS/MS-based proteomics. The silver-stained gel image was covered with the biotin Western blotting image to reveal protein spots containing S-thiolated proteins. These spots were subjected to in-gel digestion and LC-MS/MS analysis to identify proteins, as previously reported (12).

LC-MS/MS identification of protein-bound Cys-CN-biotin. Cys modification with CN-biotin occurring in proteins was further identify by mass spectrometry as illustrated in Fig. 5*B*. Protein samples labeled with CN-biotin were digested with Pronase (Calbiochem) to liberate free cysteine-CN-biotin adduct (Cys-CN-biotin). After enrichment with monomeric avidin agarose, Cys-CN-biotin was analyzed by means of LC-MS/MS.

Authentic Cys-CN-biotin was prepared by reacting Cys (1 mM) with P-NONOate (0.1 mM) and NaHS (0.1 mM) in 20 mM sodium phosphate buffer (pH 7.4) at 37 °C for 30 min, followed by reacting with 2 mM MSBT for 15 min and 4 mM CN-biotin for 15

min. Cys-CN-biotin was purified by RP-HPLC monitoring with *m/z* of 474.1. MRM parameters for Cys-CN-biotin was determined as follows: precursor ion (*m/z*), 474.1; product ion (*m/z*), 288.1; fragmentor voltage, 130 V; and CID, 13, at positive mode.

Lysates of A549 cells overexpressing CSE (3 mg/ml, 0.2 mL) were reacted with 2 mM MSBT at 37 °C for 20 min, followed by reacting with 6 mM CN-biotin at 37 °C for 20 min. Proteins were then precipitated by adding 0.6 mL of ice-cold acetone to remove unreacted MSBT and CN-biotin. The protein pellets were washed once with 1 mL ice-cold acetone, and were then dissolved with 0.05 mL of 20 mM sodium phosphate buffer (pH 6.0) containing 6 M urea. To the resultant protein solution was then added 0.3 mL of 20 mM sodium phosphate buffer (pH 6.0) to dilute the urea concentration below 1 M, followed by incubating with Pronase (0.5 mg/ml) at 37 ° C for 2 h. In separate experiments, the protein pellets were dissolved with 0.05 mL of PBS containing 1% SDS, followed by subjecting for SDS-PAGE. After electrophoresis, gels corresponding to certain molecular size were cut and dehydrated with acetone. To the dried gel pieces were added 0.1 mL of Pronase (0.5 mg/ml in 20 mM sodium phosphate buffer, pH 6.0) and incubated at 37 °C for 2 h.

Pronase-treated protein samples were applied to ultracentrifugation (Amicon Ultra, 10 kD cut-off, Millipore) to remove undigested proteins and Pronase. Cys-CN-biotin was enriched by using monomeric avidin agarose (Thermo Scientific) according to the manufacture's protocol. In brief, the filtrates as obtained above were applied to the monomeric avidin agarose-filled column (0.05 mL), washed 3 times with 0.15 mL of 20 mM sodium phosphate buffer (pH 6.0) and eluted with 0.1 mL of 20 mM sodium phosphate buffer (pH 6.0) containing 2 mM biotin. Cys-CN-biotin was determined by LC-MS/MS with MRM parameters as mentioned above.

Determination of antioxidant activity of Cys-related persulfides. Antioxidant activity of Cys-related persulfides was determined by measuring the peroxide-scavenging potential of persulfides. GS-$(S)_n$-H compounds were generated via GSR-catalyzed reduction of GSSSG. GSSSG (0.2 mM) was incubated with 1 U/ml GSR and 0.4 mM NADPH in 20 mM Tris-HCl (pH 7.4) at room temperature for 5 min, followed by reacting with H_2O_2 at a final concentration of 0.1 mM at 37 °C for 10 min. GSSG was used as the control substrate. Peroxides remaining in the reaction

mixtures were quantified by using an H_2O_2 colorimetric detection kit (catalogue number ADI-907-015; Enzo Life Sciences) according to the manufacturer's protocol. Similar experiments were performed for GSH and NaHS instead of GSSSG with GSR/NADPH.

The effects of CSE overexpression on oxidant-mediated cytotoxicity were also examined. A549 cells, seeded in a 96-well plate at a density of 10^4 cells per well, were cultured overnight. Cells were then treated with CSE vector for 36 h or were untreated. Cells were washed with PBS and incubated with 10% FBS-DMEM containing H_2O_2 (0, 1,000, and 2,000 μM) for 24 h. Cell viability after treatment with H_2O_2 was determined by using Cell Counting Kit-8 (Dojindo Laboratories) according to the manufacturer's protocol.

Regulation of electrophilic signaling by Cys persulfides. Polysulfide-dependent conversion of 8-nitro-cGMP to 8-SH-cGMP was studied *in vitro*, in cell culture and *in vivo*. *In vitro*, 8-nitro-cGMP (1 mM) was reacted with 0.1 mM NaHS in the presence or absence of 0.1 mM GSH and 0.1 mM P-NONOate in 20 mM Tris-HCl (pH 7.4) at 37 °C. In some experiments, DTT (10 mM) was added to the reaction mixtures to promote reduction of disulfide bond in 8-GS-$(S)_n$-cGMPs. Formation of 8-GS-$(S)_n$-cGMPs as reaction intermediates was determined by means of LC-MS/MS with the use of an Agilent 6430 Triple Quadrupole LC/MS.

In cell culture, C6 cells were stimulated with 10 μg/ml lipopolysaccharide (*E. coli*; L8274) (Sigma), 100 U/ml interferon-γ, 100 U/ml tumor necrosis factor α, and 10 ng/ml interleukin-1β (all cytokines from R&D Systems, Inc.) for 12 to 42 h. In some experiments, CBS was knocked down with siRNA. 8-SH-cGMP was extracted from cells by methanol precipitation as reported previously (5,6).

In vivo, mouse tissues (100 mg each) were homogenized by using Polytron homogenizer with 1 mL of methanol solution containing 2% acetic acid. After centrifugation (14,000 × *g* 10 min 4 °C), the supernatants were collected and subjected to anion-exchange purification with Oasis WAX cartridge (Waters). After the cartridge was washed with methanol, cGMP derivatives were collected into the elution with 1 mL of methanol containing 15% aqueous ammonia. Eluted samples were dried in vacuo and then redissolved in distilled water (0.2 mL).

8-SH-cGMP extracted from cell lysates and mouse tissues were quantitated by means of LC-MS/MS. A TSQ Vantage (Thermo Scientific) for LC-ESI-MS/MS was used, after RP-HPLC with a YMC-Triart C18 column (50 × 2.0 mm inner diameter), and a linear 0-100% methanol gradient in 0.1% formic acid and 0.0005% aqueous ammonia for 14 min at 40 °C. A total flow rate of 0.2 ml/min and an injection volume of 50 μl were used. Ionization was achieved by using electrospray in the positive mode with a 2,500 V spray voltage. Nitrogen was the nebulizer gas; nebulizer pressure was 50 psi. The desolvation gas (nitrogen), heated to 400 °C, was delivered at the flow rate of 10 L/min. CID was then achieved by using high-purity nitrogen as the collision gas. Nucleotide derivatives were quantitated using the MRM mode as reported recently (6) with slight modification. MRM parameters for cGMP and 8-SH-cGMP were given below. Isotope-labeled 8-SH-c[$^{13}C_{10}$]GMP and c[$^{15}N_5$]GMP (final concentration for each: 200 nM) were used as internal standards.

Analyte	Precursor ion (*m/z*)	Product ion (*m/z*)	S_lens (a.u.)	CID (V)
cGMP	346	152	106	23
c[$^{15}N_5$]GMP	351	157	106	23
8-SH-cGMP	378	184	93	19
8-SH-c[$^{13}C_{10}$]GMP	388	189	93	19

Western blotting. Cells were washed twice with PBS and were then solubilized with RIPA buffer that contained protease inhibitors. Cell lysate proteins were heat-denatured and separated via SDS-PAGE and were then transferred to polyvinylidene fluoride membranes (Immobilon-P; Millipore). Membranes were blocked with TTBS (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.6) containing 5% skim milk (Difco Laboratories), after which they were incubated with antibodies in TTBS containing 5% skim milk at 4 °C overnight. Antibodies used in Western blotting were as follows: anti-CBS (clone 3E1; Abnova), anti-GAPDH (sc-25778; Santa Cruz Biotechnology), anti-CSE (clone 4E1-1B7; Abnova), anti-β-actin (C-11; Santa Cruz Biotechnology). Membranes were washed three times in TTBS and were then incubated with an HRP-conjugated secondary antibody for 1 h at room temperature. After three washes in TTBS, a chemiluminescence reagent (ECL Plus

Western Blotting Reagent; GE Healthcare) with a luminescent image analyzer (LAS-2000; Fujifilm) was used to detect immunoreactive bands. Densitometric analyses were performed to quantify these bands, with the signal intensity of the Western blotting images measured via ImageJ software. Protein S-thiolation was visualized via biotin labeling, as mentioned above, and detection via neutrAvidin-HRP (Invitrogen) blotting.

Molecular weight-based fractionation of cell lysates. A549 cell lysate (1 mg/ml, 0.1 ml) was loaded to ultracentrifugation tube with cut-off size of 3,000 Da (Amicon Ultra 3K, Millipore). Tubes were then subjected for centrifugation (14,000 × *g*, 10 min, 4 °C). Filtrates were collected as low-molecular-weight (LMW) fractions. HMW fractions retained on the ultracentrifugation membrane was recovered with lysis buffer of equal volume of LMW fractions. HMW and LMW fractions thus obtained and untreated lysates (total fractions) were incubated with Br-bimane (5 mM) at 37 °C for 30 min. Polysulfide-bimane adducts as well as bis-S-bimane were determined for each fraction.

Statistical analysis. All data are expressed as means ± SD. Data for each experiment were acquired from at least three independent experiments. Statistical analyses were performed by using Student's *t*-test, with significance set at $P < 0.05$.

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Table S1. MRM parameters for bimane adducts (positive mode)

Analyte	Precursor ion (*m/z*)	Product ion (*m/z*)	Fragmentor voltage (V)	CID
GSSSS-bimane	594	192	130	33
GSSS-bimane	562	192	130	33
GS^{34}S-bimane*	532	192	130	33
GSS-bimane	530	192	130	33
GS-bimane*	501	225	140	29
GS-bimane	498	225	140	29
Bis-^{34}S-bimane*	417	193	130	13
Bis-S-bimane	415	193	130	13
HCysS^{34}S-bimane*	360	192	90	17
HCysSS-bimane	358	192	90	17
HCys-bimane*	330	193	130	13
HCys-bimane	326	193	130	13
CysSSSSS-bimane	440	192	90	13
CysSSSS-bimane	408	192	90	13
CysSSS-bimane	376	192	110	17
CysS^{34}S-bimane*	346	192	90	13
CysSS-bimane	344	192	90	13
Cys-bimane*	313	192	140	21
Cys-bimane	312	192	140	21
$H^{34}S_2O_3$-bimane*	309	227	110	9
HS_2O_3-bimane	305	225	110	9

*Stable isotope-labeled derivatives.

Table S2. MRM parameters for oxidized polysulfides (positive mode)

Analyte	Precursor ion (*m/z*)	Product ion (*m/z*)	Fragmentor voltage (V)	CID
GS^{34}S^{34}S^{34}SSG*	715	375	170	21
GSSSSSG	709	371	170	21
GS^{34}S^{34}SSG*	681	341	170	21
GSSSSG	677	339	170	21
GS^{34}SSG*	647	389	170	21
GSSSG	645	387	170	21
GSSG*	619	361	90	9
GSSG	613	355	90	9
HCysS^{34}S^{34}SSHCys*	337	169	90	9
HCysSSSSHCys	333	167	90	9
HCysS^{34}SSHCys*	303	136	90	9
HCysSSSHCys	301	136	90	9
HCysSSHCys*	277	140	90	5
HCysSSHCys	269	136	90	5
CysS^{34}S^{34}S^{34}SSCys*	343	120	90	13
CysSSSSSCys	337	120	90	13
CysS^{34}S^{34}SSCys*	309	155	90	9
CysSSSSCys	305	153	90	9
CysS^{34}SSCys*	275	122	90	9
CysSSSCys	273	122	90	9
CysSSCys*	243	121	90	13
CysSSCys	241	120	90	13

*Stable isotope-labeled derivatives.

Table S3. Intracellular levels of polysulfides in A549 cells

Analyte	Concentration (μM)		
	Control	CBS overexpression	CSE overexpression
GSH	23,086 ± 4,418	18,636 ± 1,505	30,647 ± 1,372
GSSH	4.2 ± 0.1	5.7 ± 1.1	10.7 ± 0.8**
GSSSH	0.18 ± 0.01	0.34 ± 0.08*	0.33 ± 0.08*
Cys	1,239 ± 112	1,121 ± 103	1,585 ± 124
CysSSH	2.7 ± 0.1	6.9 ± 0.7**	20.5 ± 4.1**
HCys	10.0 ± 0.6	7.16 ± 1.0	13.2 ± 1.2
HCysSSH	N.D.	N.D.	N.D.
GSSG	3,669 ± 283	1,529 ± 184*	4,905 ± 305
GSSSG	0.60 ± 0.03	0.76 ± 0.03	1.51 ± 0.01*
GSSSSG	N.D.	0.31 ± 0.16	N.D.
CysSSCys	11.9 ± 0.5	11.9 ± 0.1	6.6 ± 0.2*
CysSSSCys	N.D.	N.D.	N.D.
CysSSSSCys	2.4 ± 0.1	5.2 ± 0.1*	2.4 ± 0.1
CysSSSSSCys	0.67 ± 0.35	N.D.	N.D.

N.D., not detected.

Data are means ± SD (n = 3). *$P < 0.05$, **$P < 0.01$ versus control.

Table S4. Identification of polysulfides formed in various organs in mice

Analyte	Concentration (μM)			
	Heart	Liver	Brain	Lung
GSH	1,378 ± 92	4,418 ± 123	3,036 ± 45	2,089 ± 232
GSSH	54 ± 2	60 ± 19	154 ± 4	4 ± 1
GSSSH	0.67 ± 0.02	0.20 ± 0.05	0.32 ± 0.03	0.08 ± 0.01
Cys	13.3 ± 0.4	68 ± 17	90 ± 13	48 ± 3
CysSSH	3.9 ± 0.2	1.0 ± 0.4	2.2 ± 0.2	0.2 ± 0.0
HCys	0.15 ± 0.05	1.13 ± 0.27	0.07 ± 0.02	0.29 ± 0.03
HCysSSH	0.18 ± 0.07	0.56 ± 0.30	0.19 ± 0.03	0.02 ± 0.00
GSSG	203 ± 24	507 ± 33	157 ± 29	394 ± 37
GSSSG	0.10 ± 0.01	0.75 ± 0.15	0.07 ± 0.02	0.247 ± 0.03
GSSSSG	0.15 ± 0.06	0.06 ± 0.01	0.59 ± 0.19	0.03 ± 0.01
CysSSCys	N.D.	0.20 ± 0.01	N.D.	0.05 ± 0.01
CysSSSCys	N.D.	N.D.	N.D.	N.D.
CysSSSSCys	0.43 ± 0.02	0.60 ± 0.11	N.D.	0.45 ± 0.14
CysSSSSSCys	0.35 ± 0.14	N.D.	N.D.	0.46 ± 0.17

N.D., not detected.

Data are means ± SD (n = 3).

Table S5. Proteomic identification of S-sulfhydrated proteins in A549 cells overexpressing CSE

Spot #		Protein	Molecular mass	Score	Matches	Sequence	pI	Sequence coverage
1	GRP78	78 kDa glucose-regulated protein	72,288	1,316	73 (53)	30 (26)	5.07	47%
2	-	Not determined						
3	HSP7C	Heat shock cognate 71 kDa protein	70,854	1,482	82 (62)	32 (30)	5.37	51%
	GRP75	Stress-70 protein, mitochondrial	73,635	510	29 (20)	15 (12)	5.87	27%
4	GRP75	Stress-70 protein, mitochondrial	73,635	1,608	66 (51)	28 (24)	5.87	46%
5	CH60	60 kDa heat shock protein, mitochondrial	61,016	235	19 (10)	10 (8)	5.70	19%
6	CH60	60 kDa heat shock protein, mitochondrial	61,016	1,230	71 (51)	30 (25)	5.70	70%
7	PDIA3	Protein disulfide-isomerase A3	56,747	495	27 (23)	15 (12)	5.98	36%
8	PDIA3	Protein disulfide-isomerase A3	56,747	249	19 (11)	12 (8)	5.98	27%
9	PDIA6	Protein disulfide-isomerase A6	48,091	330	20 (15)	10 (8)	4.95	24%
10	PRS6A	26S protease regulatory subunit 6A	49,172	492	32 (26)	13 (13)	5.13	31%
	F10A1	Hsc70-interacting protein	41,305	225	13 (7)	7 (5)	5.18	18%
	DCTN2	Dynactin subunit 2	44,204	185	12 (8)	8 (5)	5.10	22%
11	-	Not determined						
12	PLIN3	Perilipin-3	47,046	286	13 (9)	9 (7)	5.30	27%
13	-	Not determined						
14	STML2	Stomatin-like protein 2, mitochondrial	38,510	141	16 (6)	8 (4)	6.88	28%
15	-	Not determined						
16	-	Not determined						
17	-	Not determined						
18	-	Not determined						
19	-	Not determined						
20	-	Not determined						
21	-	Not determined						
22	-	Not determined						
23	-	Not determined						
24	-	Not determined						
25	-	Not determined						
26	FUBP1	Far upstream element-binding protein 1	67,518	594	25 (9)	16 (7)	7.18	28%
27	ENOA	Alpha-enolase	47,139	118	4 (1)	4 (1)	7.01	12%
28	ENOA	Alpha-enolase	47,139	459	52 (19)	22 (13)	7.01	56%
29	ENOA	Alpha-enolase	47,139	595	32 (6)	16 (4)	7.01	43%
30	ENOA	Alpha-enolase	47,139	1108	70 (38)	25 (16)	7.01	68%
	ENOB	Beta-enolase	46,957	244	14 (5)	5 (2)	7.59	13%
	ENOG	Gamma-enolase	47,239	172	10 (5)	4 (2)	4.91	11%
31	-	Not determined						
32	ALDOA	Fructose-bisphosphate aldolase A	39,395	65	9 (2)	4 (1)	8.30	12%
33	PGK1	Phosphoglycerate kinase 1	44,586	331	35 (13)	16 (8)	8.30	44%
	PGK2	Phosphoglycerate kinase 2	44,767	143	10 (7)	5 (4)	8.74	12%
	ALDOA	Fructose-bisphosphate aldolase A	39,395	70	15 (4)	7 (3)	8.30	25%
34	PGK1	Phosphoglycerate kinase 1	44,586	338	29 (19)	16 (13)	8.30	44%
35	PGK1	Phosphoglycerate kinase 1	44,586	519	39 (18)	19 (11)	8.30	51%
36	AK1C2	Aldo-keto reductase family 1 member C2	36,712	60	8 (3)	4 (2)	7.13	9%
	AK1C3	Aldo-keto reductase family 1 member C3	36,830	53	6 (2)	3 (1)	8.06	6%
37	AK1C2	Aldo-keto reductase family 1 member C2	36,712	76	13 (4)	6 (3)	7.13	12%
38	AK1C1	Aldo-keto reductase family 1 member C1	36,765	37	4 (1)	2 (1)	8.02	4%
39	G3P	Glyceraldehyde-3-phosphate dehydrogenase	36,030	33	8 (1)	6 (1)	8.57	17%
40	G3P	Glyceraldehyde-3-phosphate dehydrogenase	36,030	317	30 (15)	13 (9)	8.57	45%
	ROA2	Heterogeneous nuclear ribonucleoproteins A2/B1	37,407	253	13 (9)	7 (5)	8.97	23%
	MDHM	Malate dehydrogenase, mitochondrial	35,481	196	15 (10)	8 (6)	8.92	30%

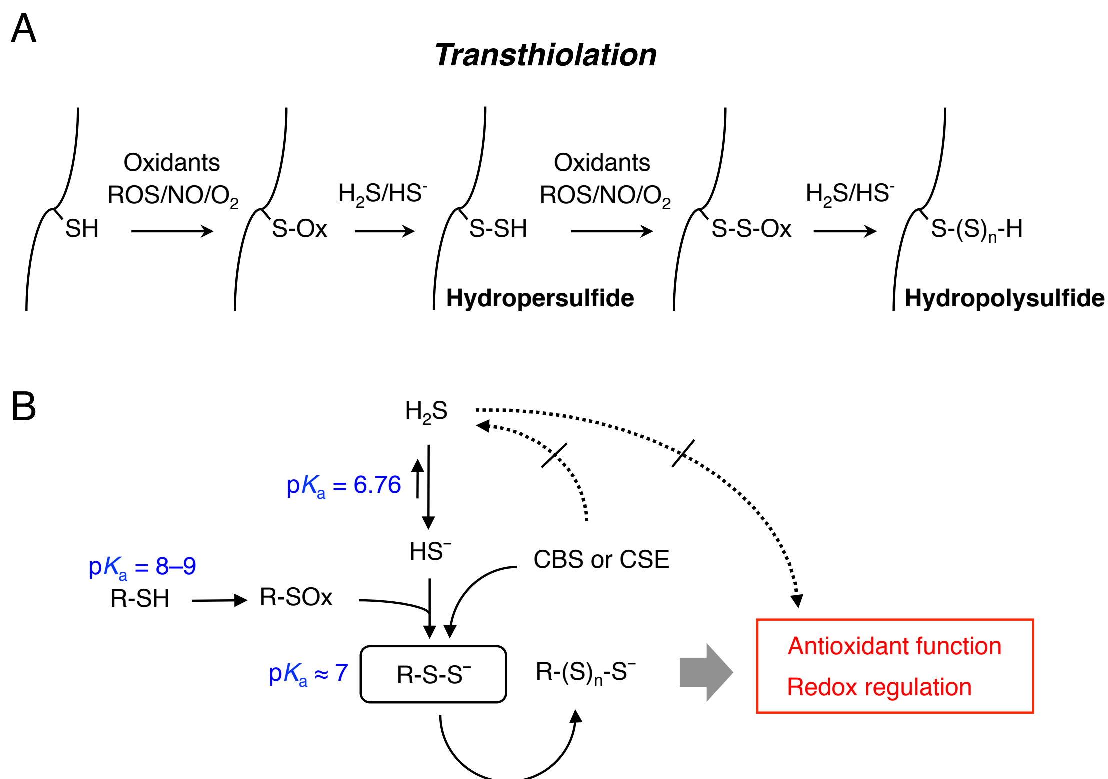
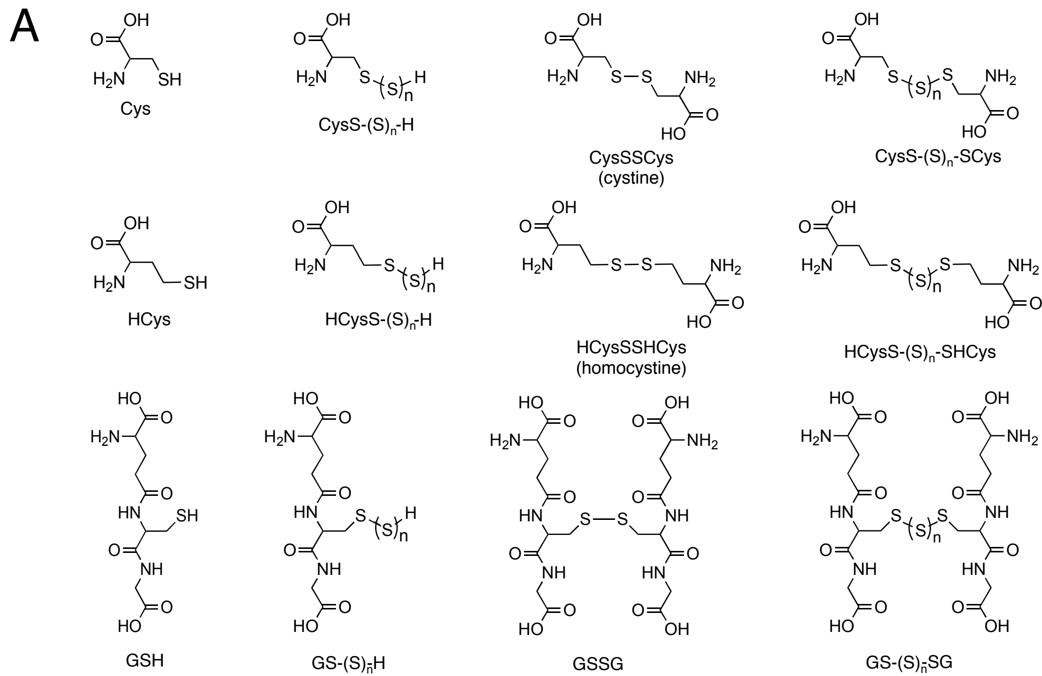


Fig. S1. Formation and physicochemical/physiological properties of polysulfides. (*A*) Mechanisms of S-polythiolation via chemical pathways. Schematic drawing of chemical S-polythiolation of oxidized Cys thiols induced by HS^-. Cys oxidation can be induced by using various oxidants such as ROS, NO, and I_2. (*B*) Physicochemical properties and physiology of polysulfides. This scheme also applies to metabolism of electrophiles derived from endogenous and environmental origins.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CysS-(S)n-SCys
HCys
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(homocystine)
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GSH
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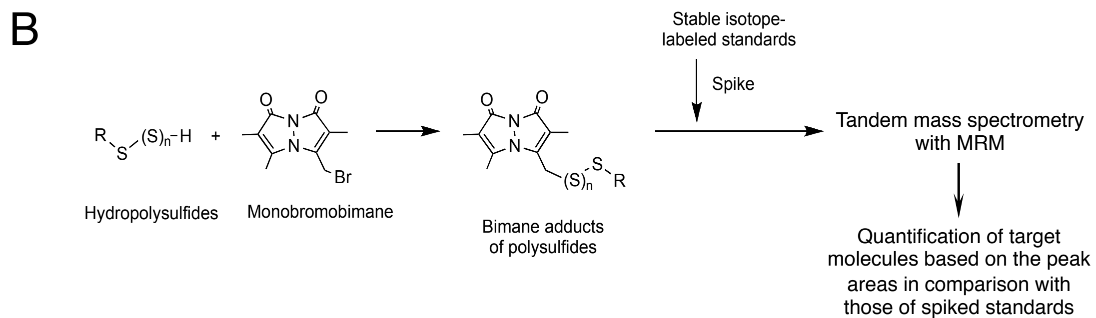
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Monobromobimane
Bimane adducts
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Quantification of target
molecules based on the peak
areas in comparison with
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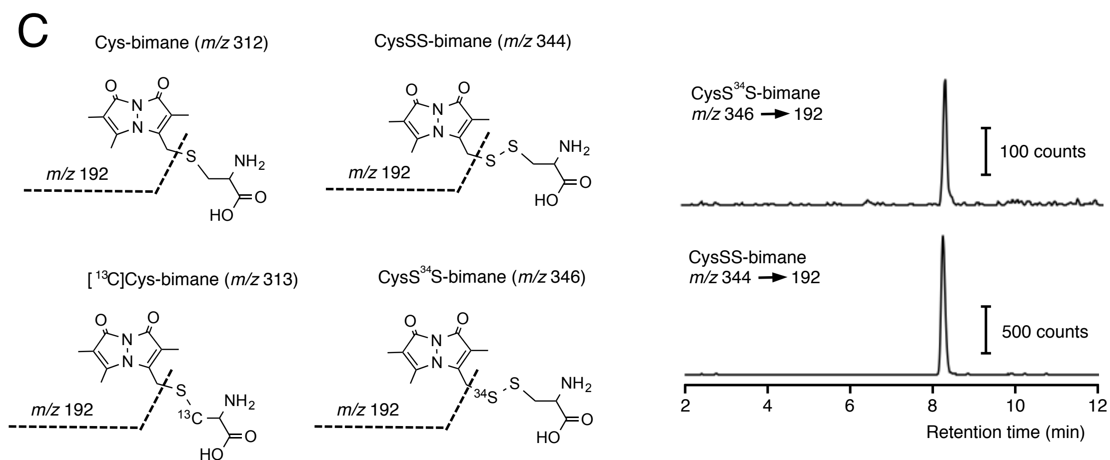
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CysSS-bimane (m/z 344)
[13C]Cys-bimane (m/z 313)
CysS34S-bimane (m/z 346)
m/z 192
CysS34S-bimane
m/z 346 → 192
100 counts
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m/z 344 → 192
500 counts
Retention time (min)

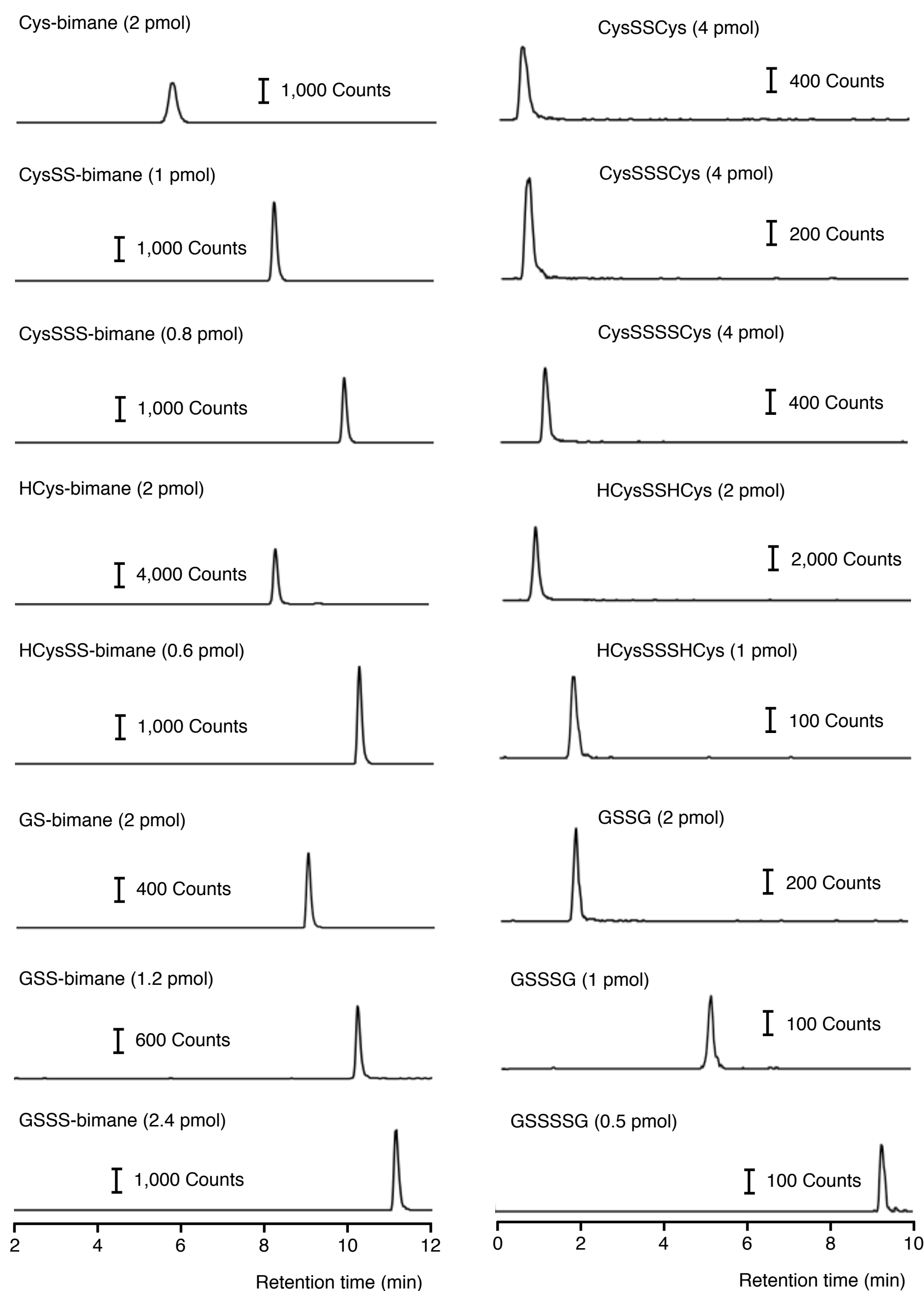

Fig. S2. Chemical structures of polysulfides (*A*), bimane assay principle (*B*), representative MS/MS fragmentation patterns for Cys-bimane adducts (*C*), and LC-MS/MS profiles of purified polysulfide standards (*D*). Hydropolysulfides were derivatized with monobromobimane. MRM parameters shown in *SI Appendix*, Tables S1 and S2 were used.

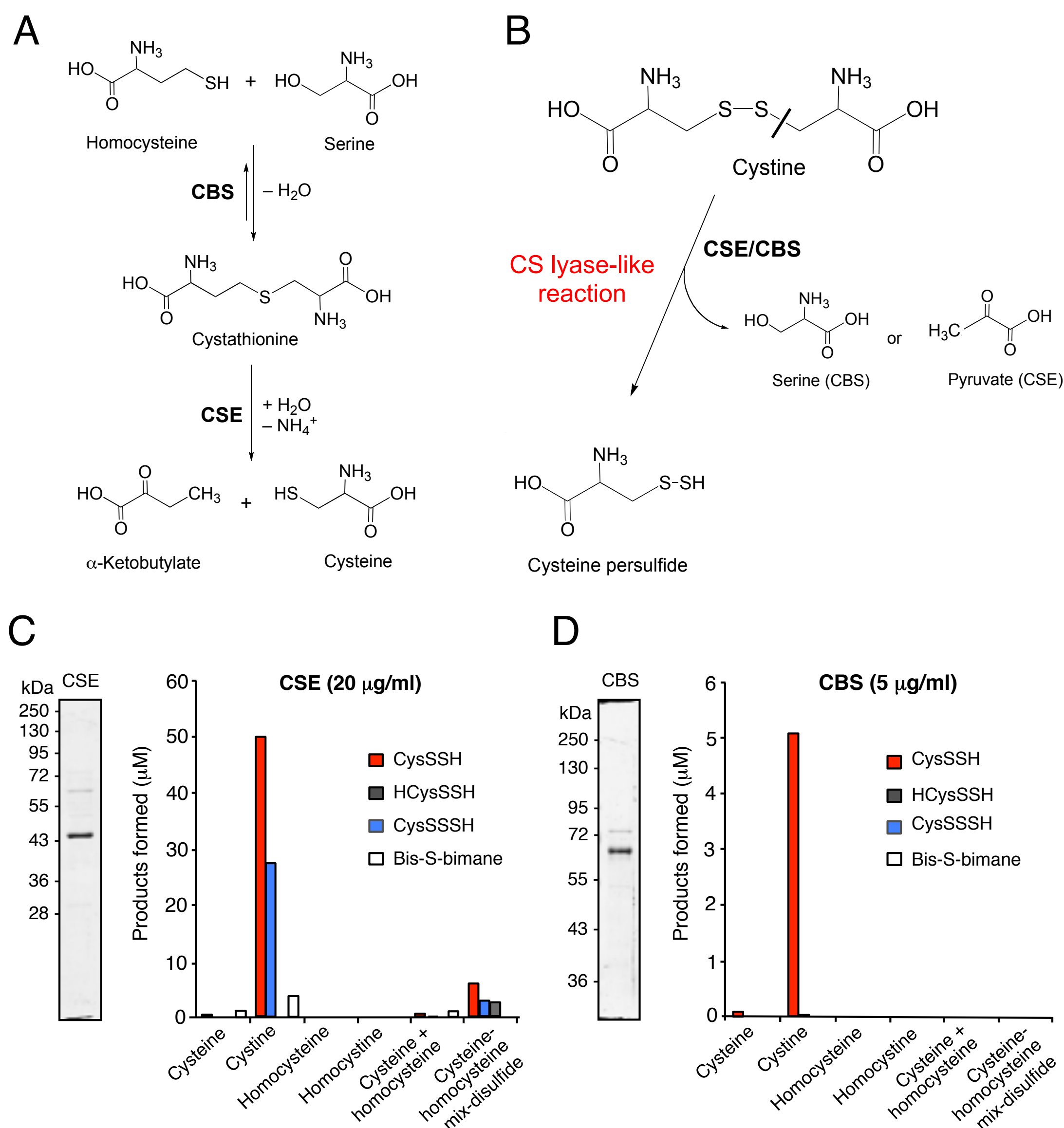

Fig. S3. Enzymatic reaction scheme and substrate specificities. (*A*) Transsulfuration from homocysteine to cysteine catalyzed by CBS and CSE. (*B*) Cysteine persulfide formation via CS lyase-like reaction on CysSSCys catalyzed by CBS and CSE. Formation of serine in the reaction of cystine with CBS was identified by means of LC-MS. Substrate specificities for enzyme reactions of CSE (*C*) and CBS (*D*). Gel electrophoretic images of recombinant proteins were shown in each left panel. Reaction products of CSE and CBS with various substrates were determined by LC-MS/MS-based polysulfidomics. Substrates (0.5 mM) used were: Cys, CysSSCys (cystine), HCys, HCysSSHCys (homocystine), Cys plus HCys, and CysSSHCys mixed disulfide. Enzyme reactions were carried out with CSE (20 μg/ml) or with CBS (5 μg/ml) in 30 mM HEPES buffer (pH 7.5) in the presence of 50 μM PLP for 30 min. For CBS reactions, 100 μM SAM was further added.

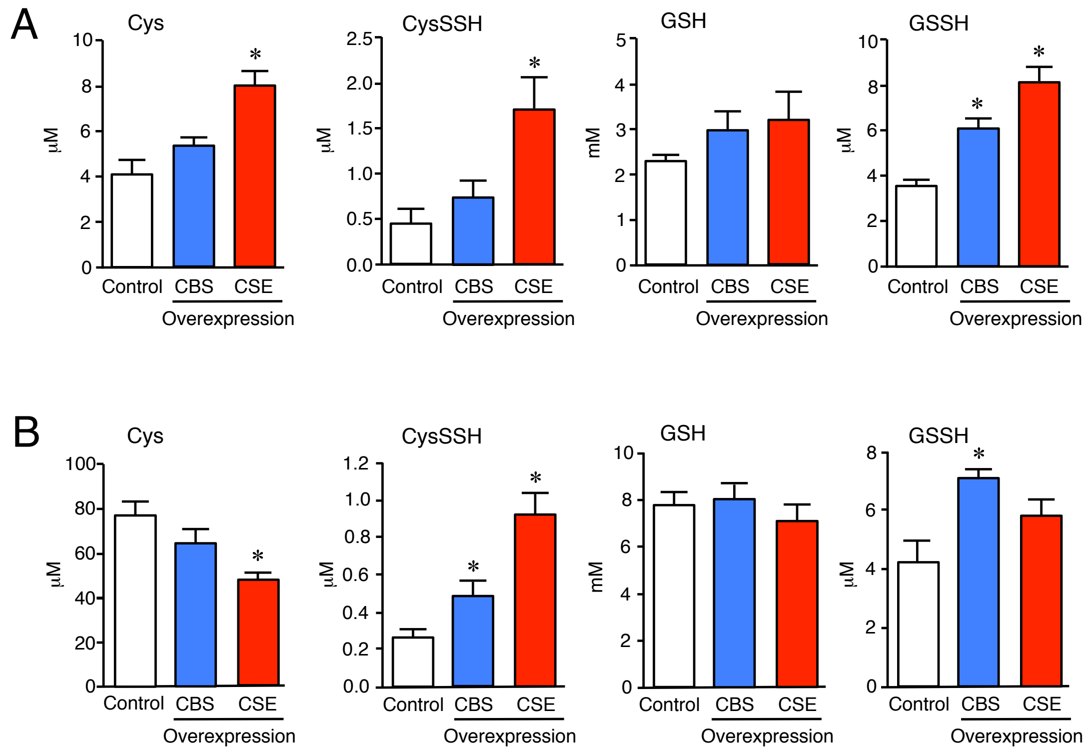

Fig. S4. CBS- and CSE-dependent formation of Cys and GSH polysulfides in SH-SY5Y cells (*A*) and C6 cells (*B*). Cys and GSH polysulfides in SH-SY5Y cells and C6 cells with or without overexpression of CBS and CSE were determined by means of LC-MS/MS-based polysulfidomics. Data are means ± SD (n = 3). $^*P < 0.05$ versus control.

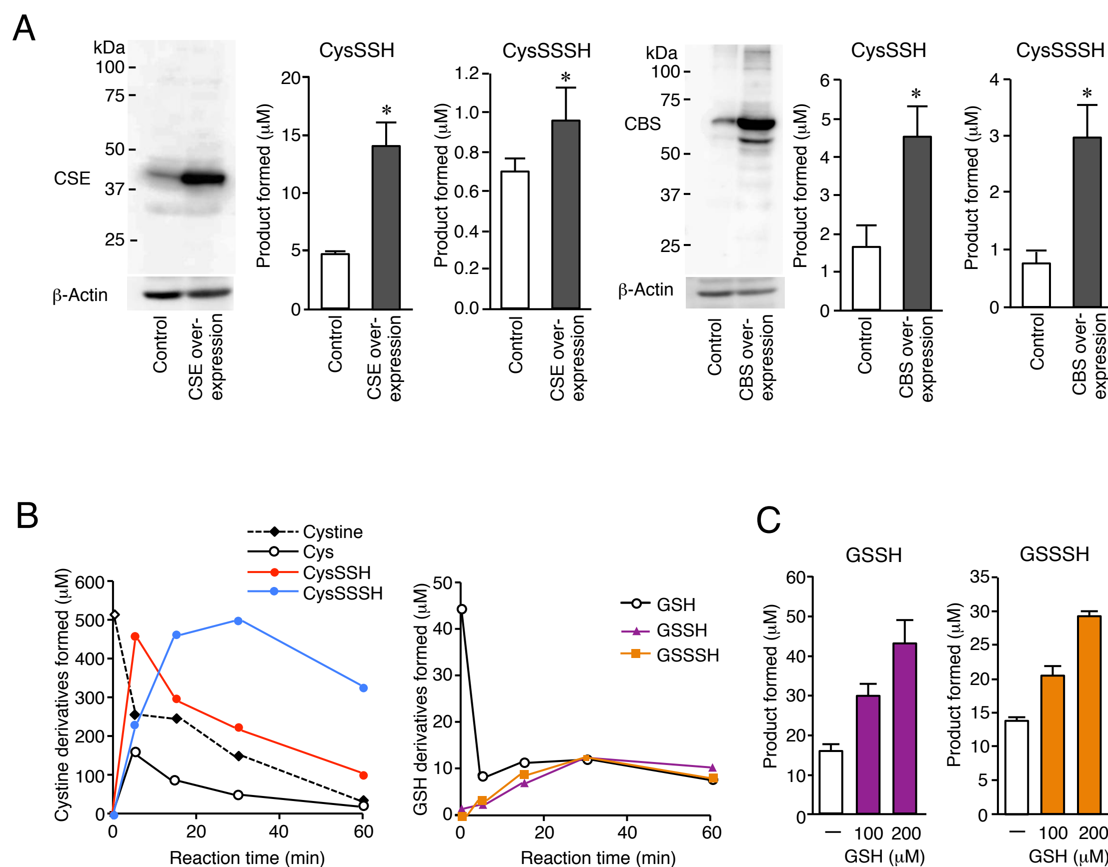

Fig. S5. Analyses of Cys and GSH polysulfide formation using cell lysates. (*A*) Polysulfide production dependent on CSE and CBS in A549 cell lysates with or without overexpression of CSE (left panels) and CBS (right panels), together with Western blotting for CSE and CBS expression. CysSSCys (0.5 mM) was incubated with cell lysates (1 mg protein/ml) at 37 °C for 15 min. Data represent means ± SD (n = 3). *P < 0.05 versus control. (*B, C*) Formation of Cys and GSH polysulfides in the reaction of CysSSCys with lysates of COS7 cells overexpressing CSE. CysSSCys (0.5 mM) was incubated with lysates (1 mg protein/ml) of CSE-overexpressed COS7 cells in 30 mM HEPES buffer (pH 7.5) in the presence of 50 μM PLP at 37 °C. In Fig. S6*C*, effects of exogenous addition of GSH was examined. GSH polysulfide formation was markedly increased by addition of GSH (yields after a 60-min incubation).

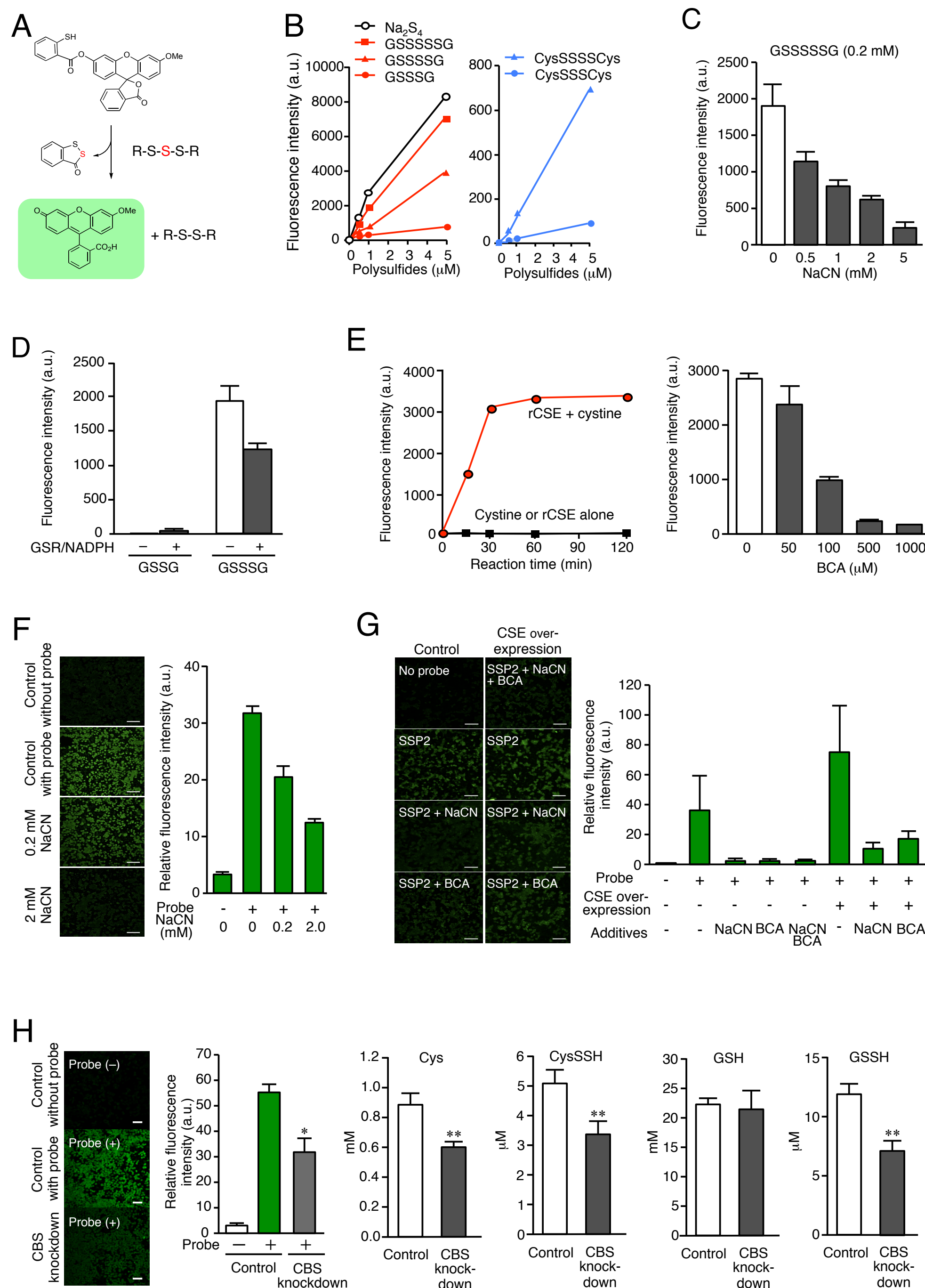

Fig. S6. SSP2-based determination and imaging of polysulfides. (*A*) Schematic illustration for the principle of fluorescence generation from the reaction of polysulfides and SSP2. (*B*) Various polysulfides were reacted with SSP2 (50 μM), followed by measuring fluorescence intensities. (*C*) Cyanolysis of polysulfides. GSSSSSG (2 μM) was reacted with NaCN (0 – 5 mM) in 200 mM Tris-HCl (pH 7.4) at 37 °C for 60 min. Data are means ± SD (n = 3). (*D*) SSP2 responses of reduced and oxidized GSH polysulfides. GSSG (10 μM) or GSSSG (10 μM) was treated with GSR (1 U/ml) and

NADPH (50 μM) at room temperature for 5 min or untreated, followed by subjecting for SSP2 reaction. Data are means ± SD (n = 3). (*E*) The enzyme reaction mixture containing CysSSCys (1.25 mM), CSE (50 μg/ml), PLP (50 μM) in 30 mM HEPES buffer (pH 7.5) at indicated reaction time were reacted with SSP2 (10 μM) (upper panel). CysSSCys alone and CSE alone were used as negative controls. Effects of the CSE inhibitor BCA on CSE-mediated Cys polysulfide formation as determined by SSP2 fluorescence generation (lower panel). CysSSCys (1 mM) was incubated with CSE (50 μg/ml) in the presence of indicated concentrations of BCA at 37 °C for 30 min. Data are means ± SD (n = 3). (*F*) A549 cells were treated with NaCN (0.2, 2 mM) or untreated for 30 min, followed by subjecting for SSP staining. (*G*) Effects of NaCN (2 mM, 60 min) and BCA (1 mM, 30 min) on SSP responses of A549 cells with or without CSE overexpression. Data are means ± SD (n = 3). (*H*) SSP2-induced fluorescence imaging (left two panels) and levels of Cys and GSH polysulfides in A549 cells suppressed by CBS knockdown (right four panels). Data are means ± SD (n = 3). *$P < 0.05$, **$P < 0.01$ versus control. Scale bars, 50 μm. a.u., arbitrary unit.

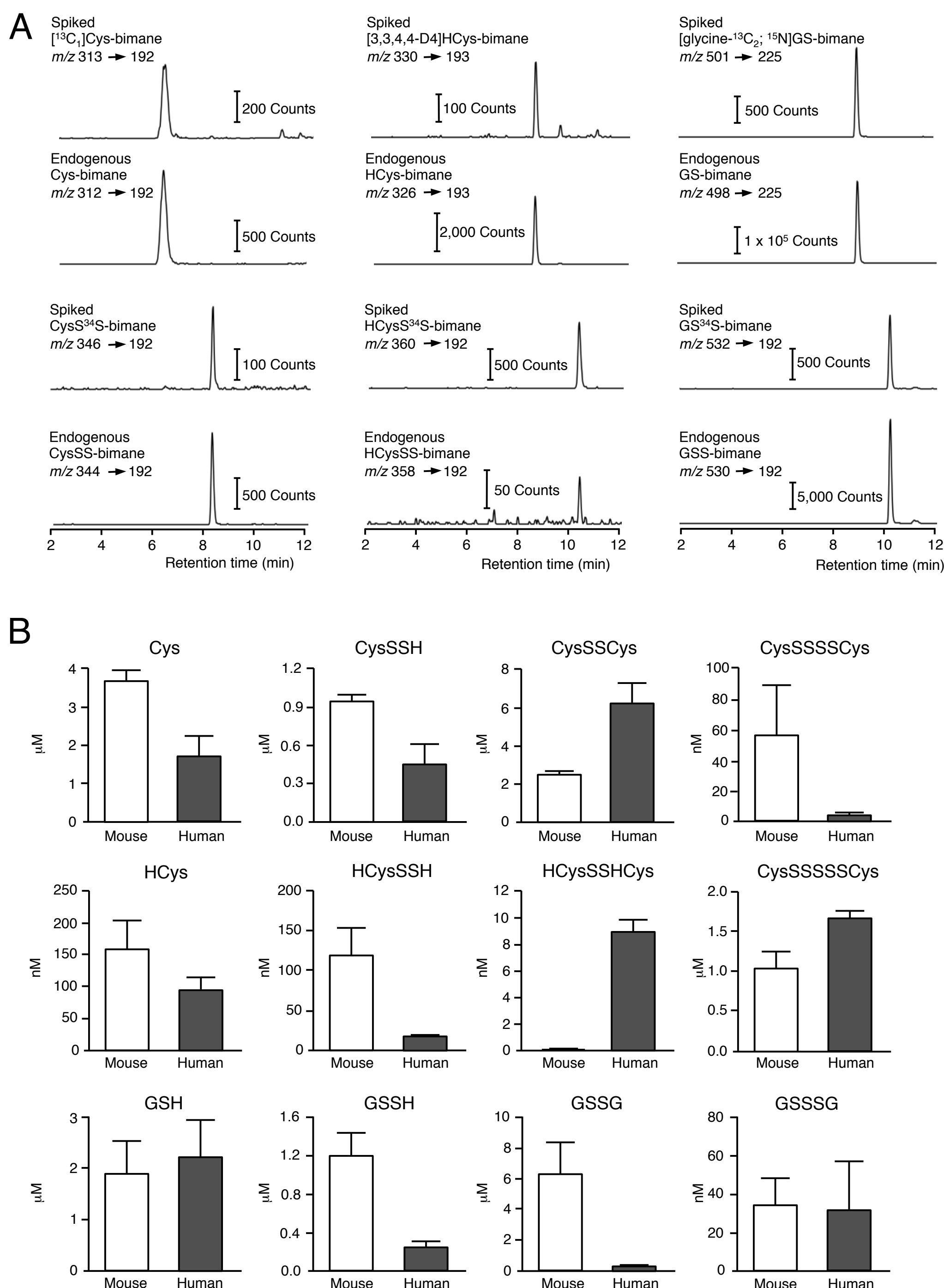

Fig. S7. Polysulfur metabolomics *in vivo*. (*A*) Representative LC-MS/MS chromatograms of polysulfides identified in mouse heart. MRM parameters shown in *SI Appendix* Tables S1 and S2 were used. (*B*) Identification of various Cys-related polysulfides formed in plasma of mice and humans. Hydrosulfide, after stabilization via bimane adduct formation, as well as oxidized polysulfides were quantified by LC-MS/MS. Data are means ± SD (n = 3).

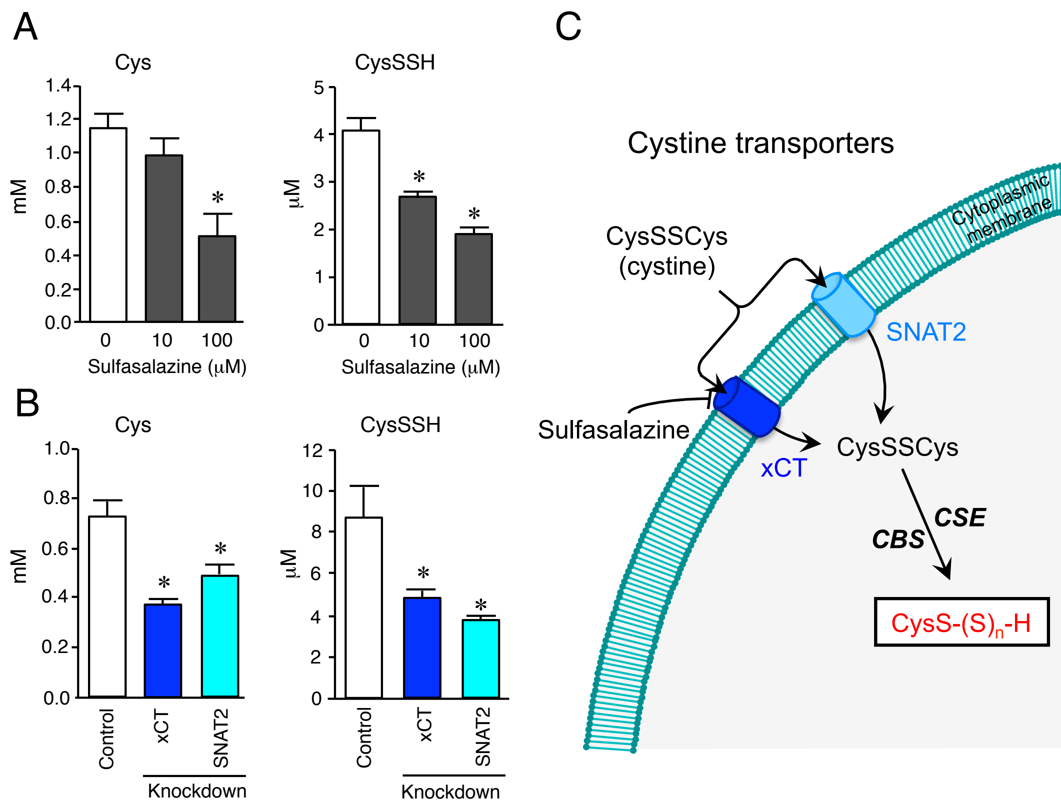

Fig. S8. Roles of cystine uptake on cellular formation of Cys polysulfides. (*A*) Effects of xCT inhibitor sulfasalazine on Cys persulfide formation. A549 cells were treated with indicated concentrations of sulfasalazine for 3 h, followed by determining Cys persulfides. Data are means ± SD (n = 3). *P < 0.05 versus control (no sulfasalazine). (*B*) Effects of knockdown of cystine transporters (xCT and SNAT2) on Cys persulfide formation. A549 cells were treated with siRNA of xCT and SNAT2 for 48 h, followed by subjecting to polysulfide determination. Data are means ± SD (n = 3). *P < 0.05 versus control. (*C*) Schematic representation of roles of cystine transporters on cellular Cys persulfide formation.

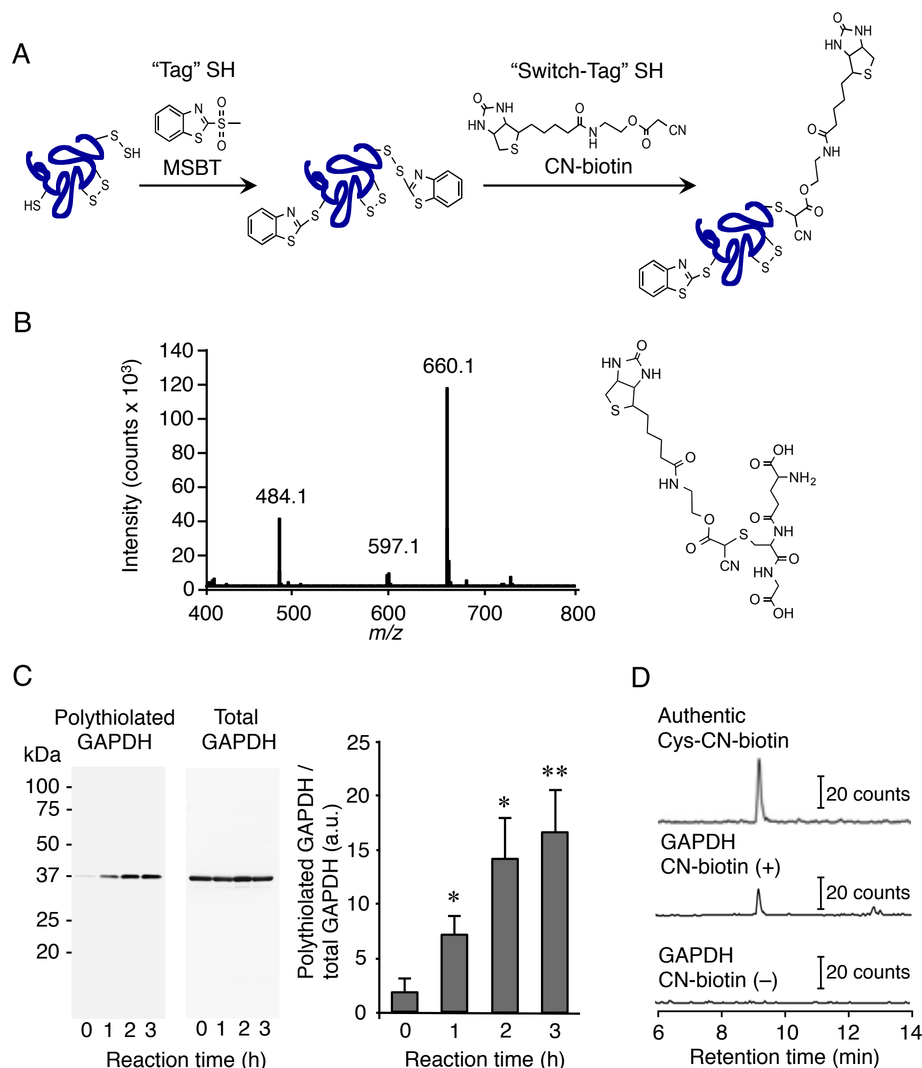

Fig. S9. Polysulfur labeling via Tag-Switch-Tag method using MSBT and CN-biotin. (*A*) The MSBT-CN-biotin labeling reaction of Cys persulfide after sulfhydryl blocking. (*B*) MS identification of biotin-labeled GSSH (*m/z* 660.1). GSSH derived from GSR-catalyzed reduction of GSSSG was biotinylated by using the Tag-Switch-Tag assay. (*C*) Sulfur transfer from CysS-$(S)_n$-H to GAPDH. Human recombinant GAPDH (80 μg/ml), which was pre-treated with mercaptoethanol to reduce Cys residues, was incubated with CSE enzyme reaction mixture (50 μg/ml CSE, 1 mM cystine, 50 μM PLP, 30 mM HEPES pH7.5) at 37 °C for indicated periods. GAPDH polythiolation was then determined by Tag-Switch-Tag method. Increased polythiolation was standardized by total GAPDH as detected by Western blotting (right panel). Data are means ± SD (n = 3). *$P < 0.05$, **$P < 0.01$ versus control (time 0). (*D*) LC-MS/MS identification of Cys-CN-biotin in polythiolated GAPDH. The same GAPDH treated as just above (*C*) was subjected for Pronase digestion, followed by LC-MS/MS as illustrated in Fig. 5*B*. LC-MS/MS chromatograms of GAPDH reacted with CN-biotin [CN-biotin (+)] or without CN-biotin [CN-biotin (-)] were shown with that of authentic Cys-CN-biotin.

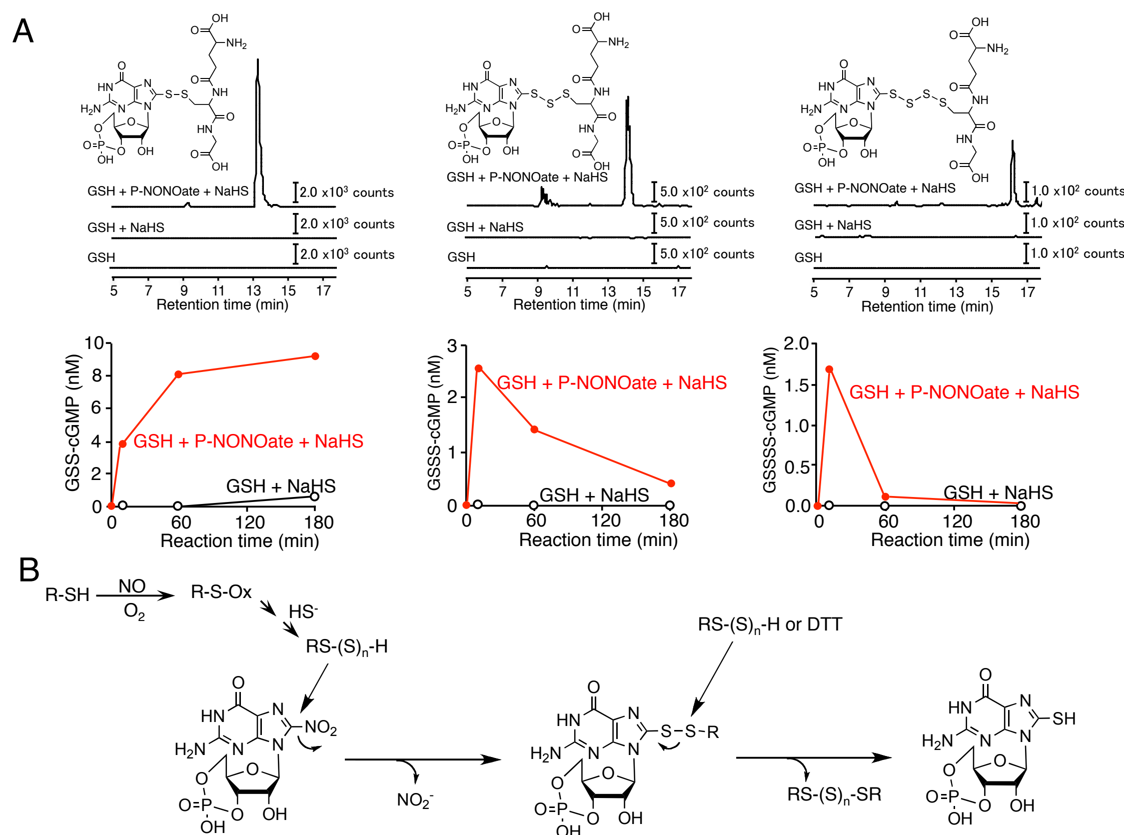

Fig. S10. Thiolation of 8-nitro-cGMP by polysulfides. (*A*) 8-Nitro-cGMP (1 mM) was reacted with 0.1 mM NaHS in the presence or absence of 0.1 mM GSH and 0.1 mM P-NONOate in 20 mM Tris-HCl (pH 7.4) at 37 °C for 30 min. Formation of 8-GS-$(S)_n$-cGMP derivatives was determined by means of LC-ESI-MS/MS. MS/MS chromatograms corresponding to GSS-cGMP (*m/z* 683 → 378), GSSS-cGMP (*m/z* 715 → 378), and GSSSS-cGMP (*m/z* 747 → 378) are given in the upper panels. Time courses for the formation of 8-GS-$(S)_n$-cGMP derivatives were shown in the lower panels. (*B*) The possible reaction mechanism for formation of 8-SH-cGMP from 8-nitro-cGMP via polysulfide-mediated sulfhydration.

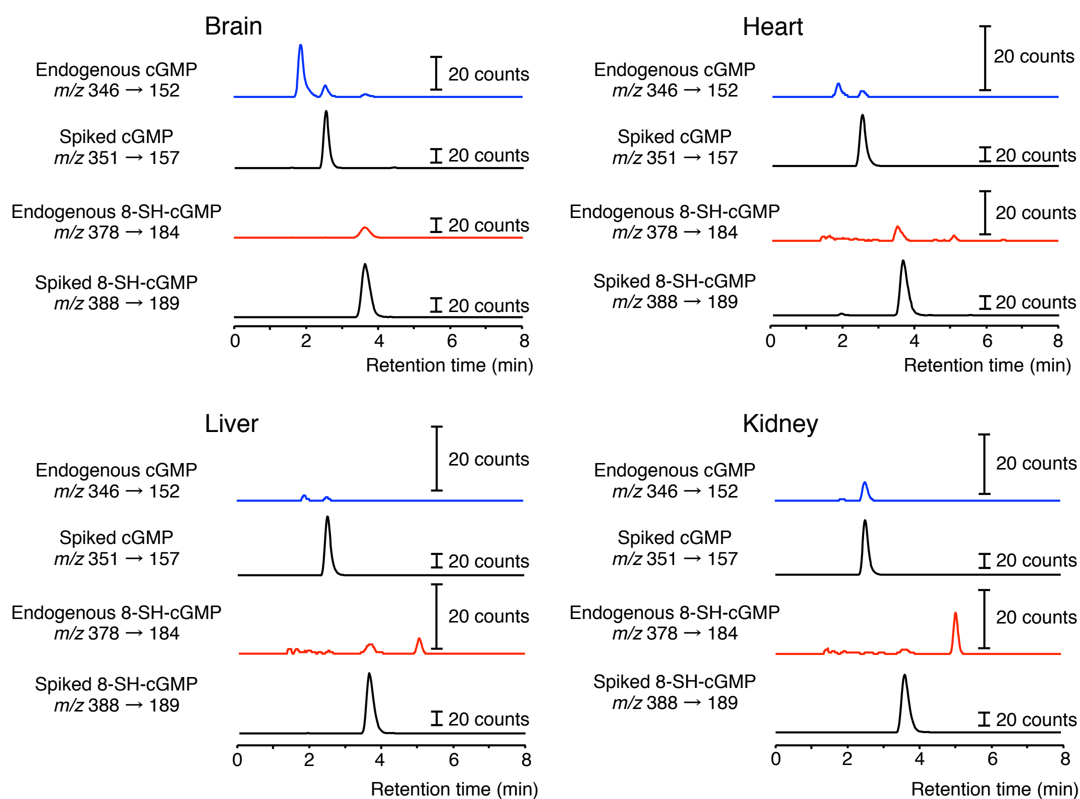

Fig. S11. *In vivo* endogenous formation of 8-SH-cGMP in various organs in mice. LC-ESI-MS/MS chromatograms for cGMP and 8-SH-cGMP were shown. Fig. 7*B* represents quantitative data.

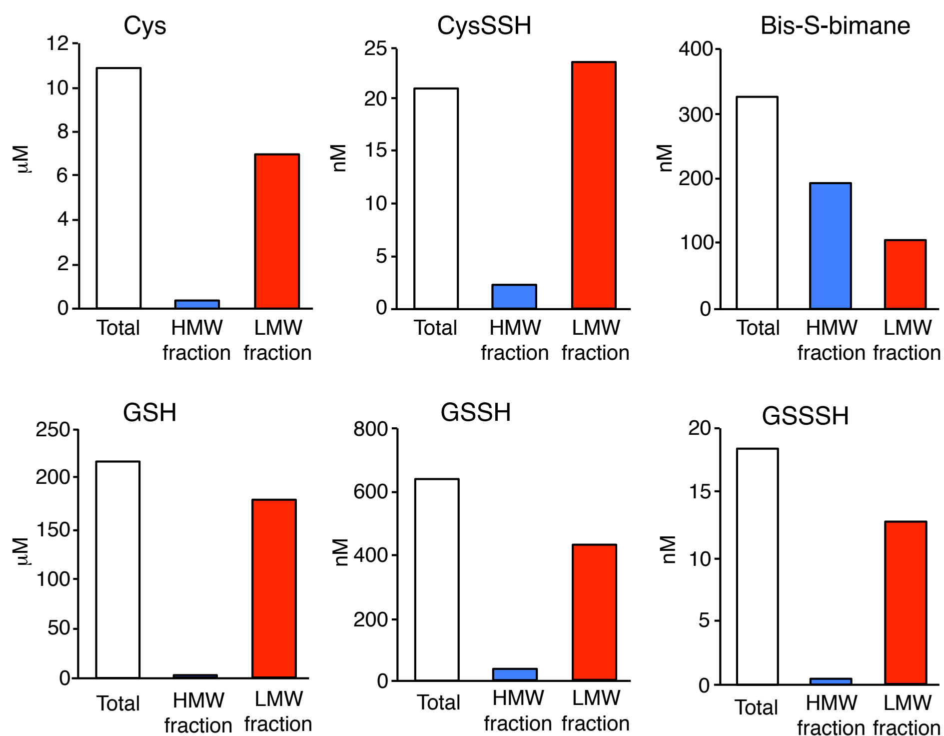

Fig. S12. Bis-S-bimane formation in the reaction of Br-bimane with HMW protein fraction. Bis-S-bimane formation in the reaction of Br-bimane (5 mM) with cell lysates before and after molecular weight-based fractionation. A549 cell lysate (1 mg protein/ml) were subjected for 3,000 Da cut-off ultrafiltration membrane to obtain HMW fraction and LMW fraction. The cell lysate without ultrafiltration was used as the total fraction (Total). Br-bimane was reacted with each fraction, and bimane adducts formed were quantitated by means of polysulfidomics. Remarkable formation of bis-S-bimane in the reaction of Br-bimane with HMW fraction was noted.