Electronic Supplementary Material (ESI) for Molecular BioSystems.

Supplementary Information

Clickable Glutathione Approach for Identification of Protein Glutathionylation in Response to Glucose Metabolism

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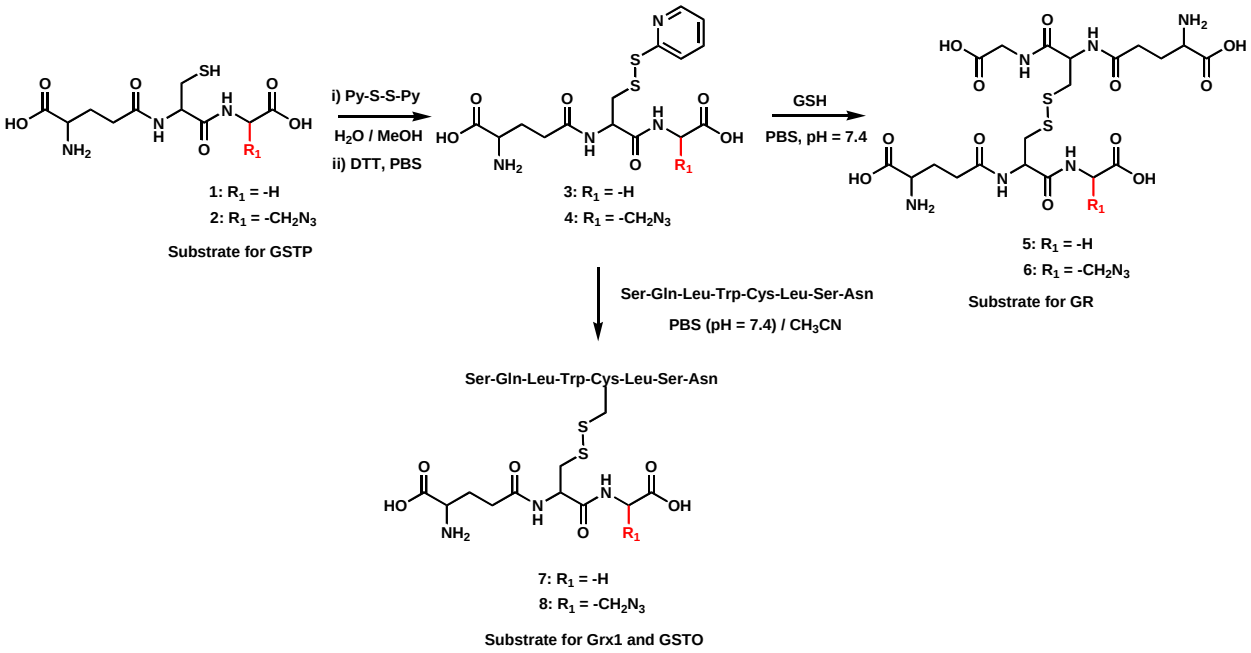
1. Scheme for synthesis of glutathione derivatives for kinetic assays



Scheme S1. Synthesis of glutathione derivatives for kinetic assays with GSTP, GSTO, and GR.

2. Additional Figures (Figure S1-S9) and Table S1

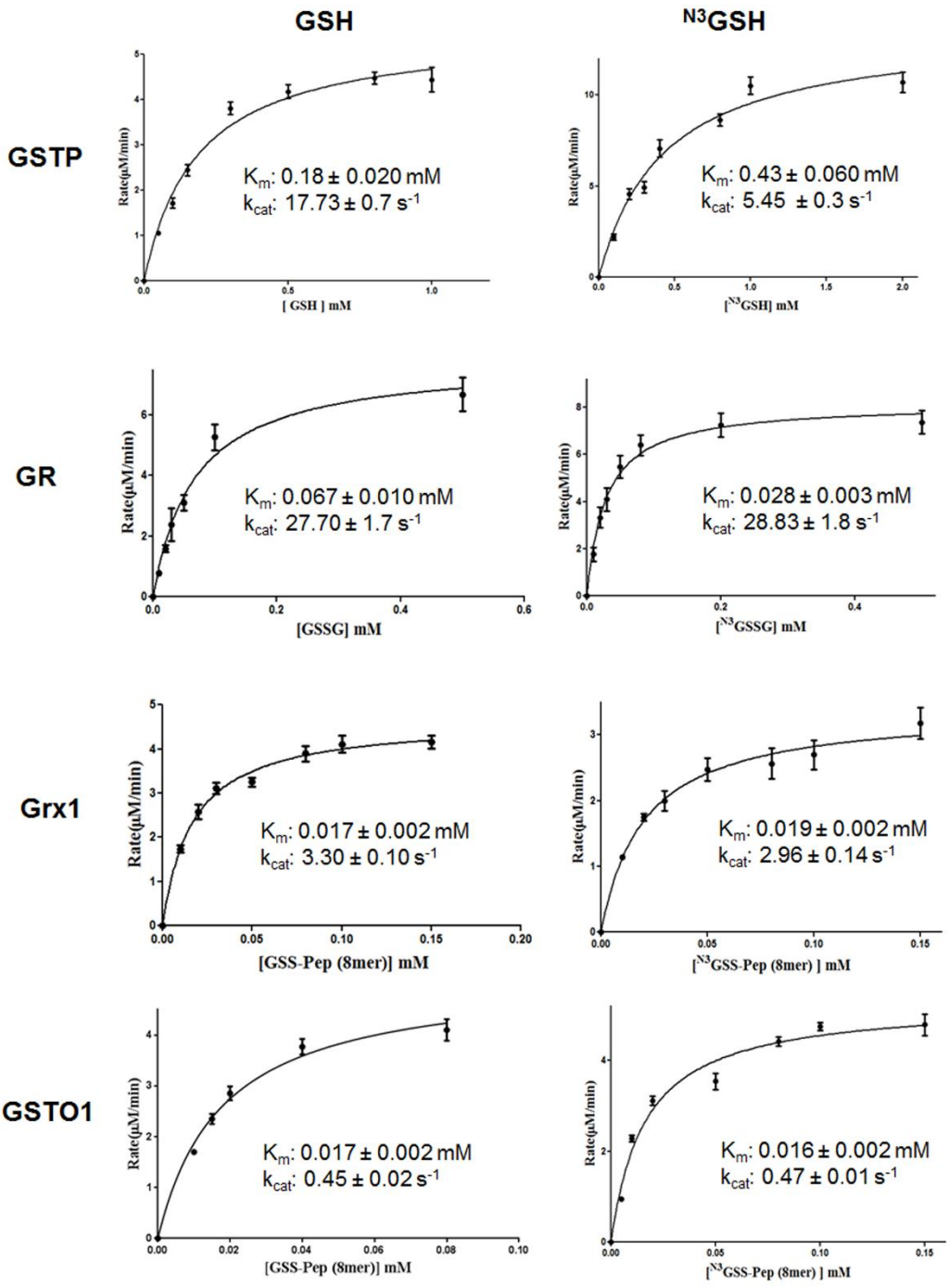


Figure S1. Kinetic analysis of glutathione- and azido-glutathione-containing substrates with GSTP1, Grx1, GR, and GSTO1. To calculate k_{cat} and K_m values, initial reaction rates were plotted with a range of substrate concentrations by using Graphpad Prism.

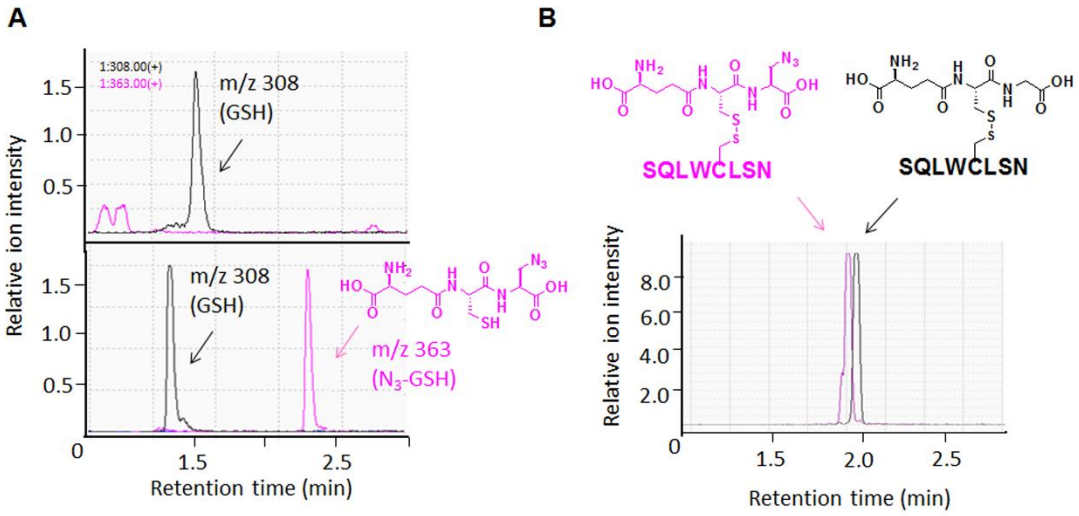


Figure S2. Evaluation of azido-glutathione (^{N3}GSH) biosynthesis and in vitro glutathionylation. (A) HEK293 cells stably expressing GS M4 was incubated with (bottom) or without (top) azido-Ala for 20 h. Cell lysates prepared was injected for LC-MS analysis,[3] detecting the extracted ion mass of endogenous glutathione (black) and azido-glutathione (pink). (B) LC-MS analysis of a model peptide (Pep: SQLWCLSN) incubated with H_2O_2 (1 mM) in vitro in the presence of an 1:1 mixture of endogenous glutathione (GSH, black) and azido-glutathione (^{N3}GSH, pink), showing the similar level of glutathionylated peptide by both glutathione.

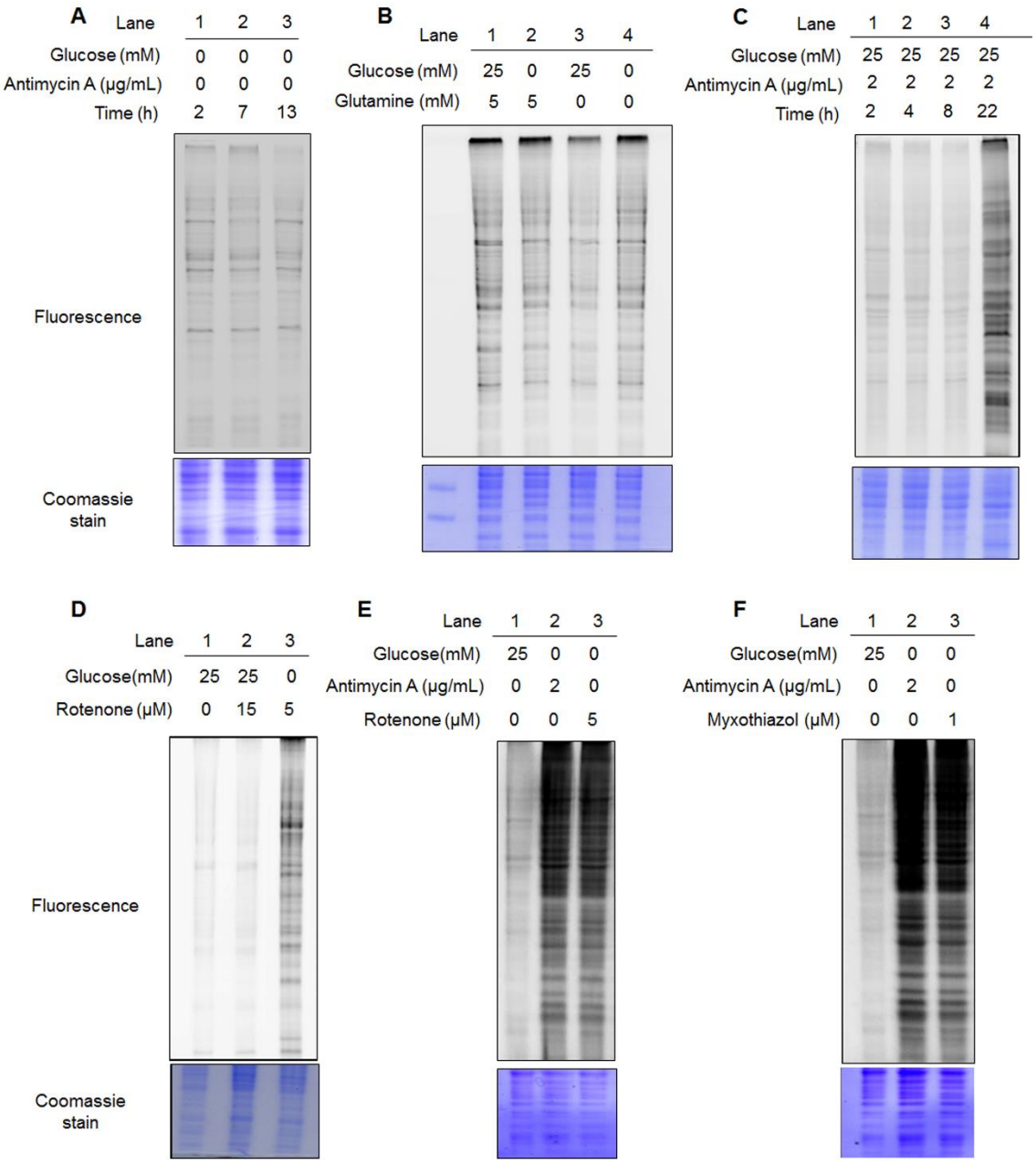


Figure S3. Induction of glutathionylation by glucose starvation together with electron transport chain blockers. After incubation of azido-Ala to HEK293/GS M4 cells for 20 h, cells were subjected to the following stimulus. (A) Cells were incubated in glucose-free medium. (B) Cells were incubated in a combination of glucose starvation and glutamine depletion for 2 h. (C) Cells were incubated with antimycin A alone in indicated conditions. (D) Cells were incubated in glucose starvation with rotenone for 2 h. (E) Glutathionylation in glucose starvation upon treatment of antimycin A or rotenone for 2 h. (F) Glutathionylation in glucose starvation upon treatment of antimycin A or myxothiazol for 2 h. After stimulus, cell lysates were subjected to click reaction with rhodamine-alkyne, and analyzed for fluorescence and Coomassie stain.

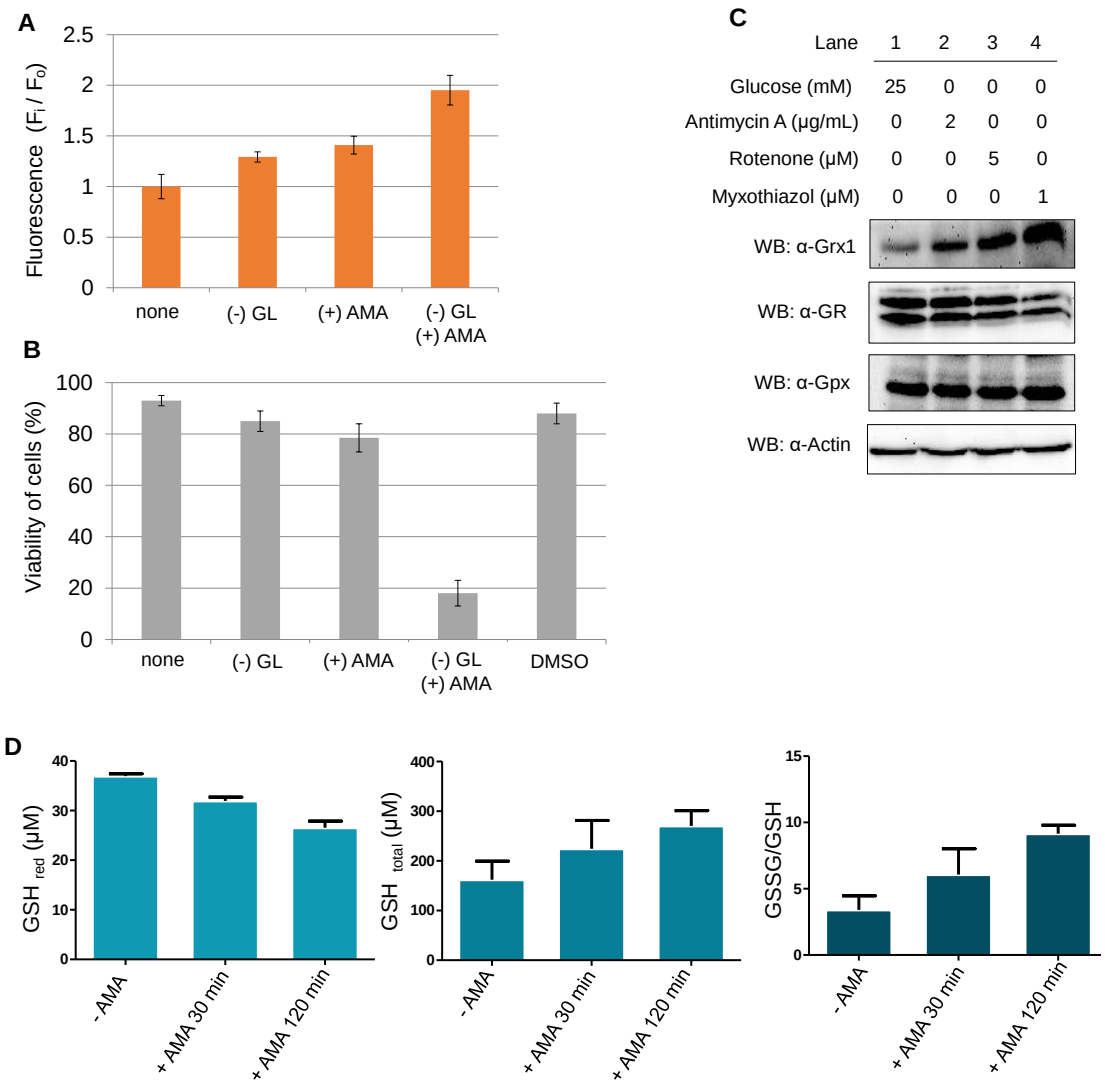


Figure S4. Redox changes and viability of cells under glucose starvation with treatment of antimycin A. (A) ROS induction assays: HEK293 cells were subjected to indicated conditions for 2-h, and cells were tested for ROS increases by 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) assay: none (25 mM glucose), (-) GL (0 mM glucose), (+) AMA (2 μg/mL antimycin A), and (-) GL (+) AMA (0 mM glucose, 2 μg/mL antimycin A). (B) Viability assays: HEK293 cells were subjected to indicated conditions for 24-h: none (25 mM glucose), (-) GL (0 mM glucose), (+) AMA (1 μg/mL antimycin A), and (-) GL (+) AMA (0 mM glucose, 1 μg/mL antimycin A). Cells were then tested for viability by Trypan blue assay. (C) Levels of redox enzymes: after HEK293 cells were subjected to indicated conditions for 2 h, individual antibodies were used for Western blotting. (D) The level of cellular thiols: after HEK293 cells were subjected to indicated conditions for 2-h, thiol concentrations in cell lysates free from proteins was measured by bromobimane assay in the presence and absence of TCEP, which were used to determine the total, the reduced form, and oxidized form of thiols (glutathione), respectively, in cell lysates.

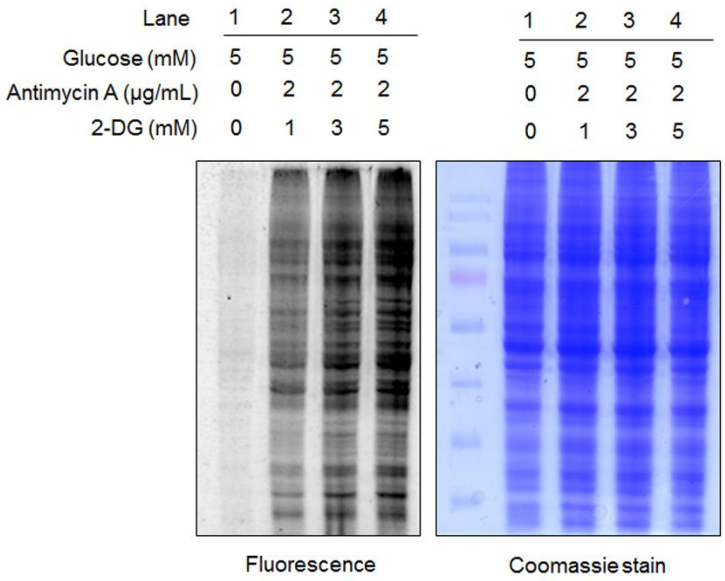


Figure S5. Induction of glutathionylation by treatment of 2-deoxyglucose (2-DG) with electron transport chain blockers. After incubation of azido-Ala to HEK293/GS M4 cells for 20 h, cells were serum-starved for 4-h. Cells were then incubated in glucose (5 mM) and antimycin A (2 μg/mL) with an increasing concentration of 2-DG for 2 h. After stimulus, cell lysates were subjected to click reaction with rhodamine-alkyne, and analyzed for fluorescence and Coomassie stain.

A ratio of spectral count in positive vs. negative			# of protein
10 ≤	ratio		1291
5 ≤	ratio	< 10	28
2 ≤	ratio	< 5	64
0 ≤	ratio	< 2	145

Figure S6. Analysis of proteomic data of glutathionylated proteins. Identified proteins in glucose starvation with antimycin A for 2-h (positive) or in the presence of glucose without antimycin A (negative) were compared for the ratio of spectral counts in the positive versus negative sample. All spectral counts are the average of at least triplicate experiments of identical conditions.

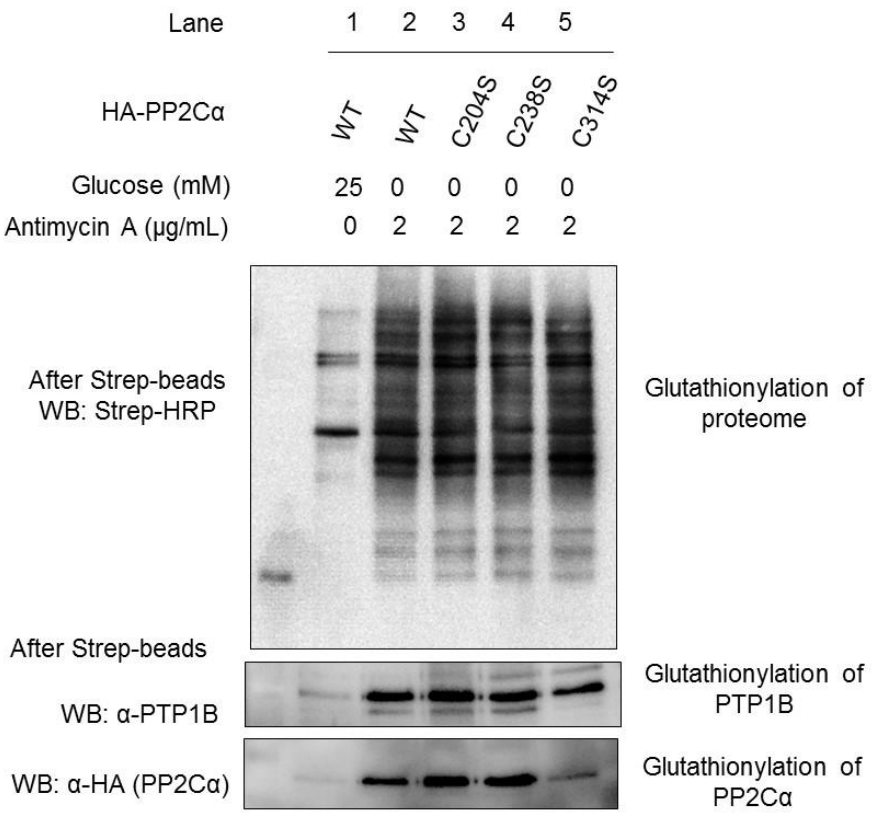


Figure S7. Identification of global glutathionylation and PP2Cα glutathionylation in glucose starvation with antimycin A. After incubation of azido-Ala in HEK293/GS M4 cells transfected with PP2Cα WT and mutants, cells were incubated in glucose starvation with antimycin A for 2-h. Cell lysates were subjected to click reaction with biotin-alkyne, and incubated with streptavidin-agarose. The enriched proteins were probed for global glutathionylation by streptavidin-HRP, or for individual proteins, PP2Cα and PTP1B. In PP2Cα C314S transfected cells, PP2Cα glutathionylation is significantly decreased while PTP1B glutathionylation did not change.

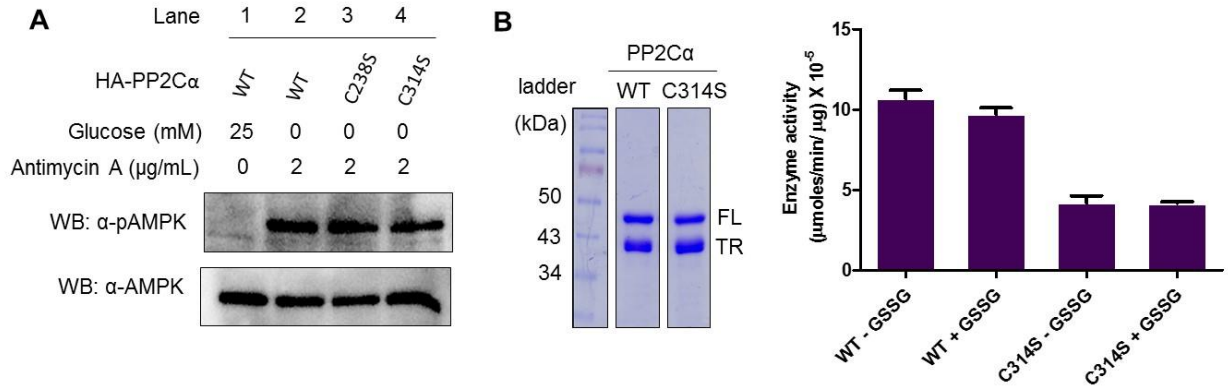


Figure S8. PP2Cα activity upon glutathionylation. (A) PP2Cα glutathionylation does not change the level of AMPK phosphorylation. After inducing glutathionylation, cell lysates were probed for AMPK (bottom) and its phosphorylation (top) (B) (Left panel) A gel after purification of His-PP2Cα, which gave a full-length (FL) and a truncated form (TR) that appears to be lack of about 10 kD size of the C-terminus domain. His-tag is on the N-terminus. (Right panel) PP2Cα dephosphorylation assay with p-nitrophenyl phosphate (pNPP): PP2Cα (50 ng/μL) and pNPP (20 mM) were incubated with and without oxidized glutathione (GSSG) (0.5 mM) for 30 min at 37^{o}C, and absorbance was measured at 405 nm.

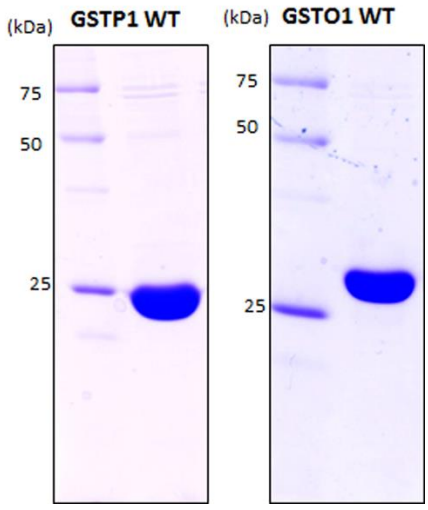


Figure S9. Purity of GSTP1 and GSTO1 after purification.

Table S1. A list of glutathionylated proteins identified in mass analysis that have high spectral counts and high enrichment in the positive over negative sample.

[a]Neg and Pos = spectral count of the negative and positive samples, respectively.
[b]Loc = Localization (C: cytosol, N: nucleus, EC: extracellular, L: lysosome, M: mitochondrion)
[c]Function = molecular function determined by DAVID GO analysis
[d]Process = biological processes determined by DAVID GO analysis
[e]G = glutathionylation proteomic result by exogenous H_2O_2[1]
[f]S = sulfenylation proteomic result by exogenous H_2O_2 [2]

Gene	Protein	Neg[a]	Pos[a]	Loc[b]	Function[c]	Process[d]	G[e]	S[f]
ACE	Angiotensin-converting enzyme	0.0	79.8	EC	Peptidase	Cell proliferation	+	-
TBA1A	Tubulin alpha-1A chain	0.0	59.5	C	GTPase	Cell division	+	-
CTPS1	CTP synthase 1	0.0	43.5	C	ligase	B cell proliferation	-	+
RRM1	Ribonucleoside-diphosphate reductase large subunit	0.0	35.0	C	ATP binding	Cell proliferation	-	+
GMPS	GMP synthase	0.0	30.5	C	Ligase	Metabolic	-	+
PPP2R1A	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	0.0	29.0	C	Phosphatase regulatory	Apoptosis	-	-
NPEPPS	Puromycin-sensitive aminopeptidase	0.0	28.8	C, N	Peptidase	Stress response	-	-
DHX15	Putative pre-mRNA-splicing factor ATP-dependent RNA helicase	0.0	28.3	N	Helicase	mRNA processing	+	-
GSPT1	Eukaryotic peptide chain release factor GTP-binding subunit ERF3A	0.0	24.8	C	GTPase	Cell cycle	-	+
PARP1	Poly [ADP-ribose] polymerase 1	0.0	23.5	N	Glycosyltransferase	DNA repair	+	-
NAA15	N-alpha-acetyltransferase 15, NatA auxiliary subunit	0.0	23.0	C	Acetyltransferase	Angiogenesis	+	+
VARS	Valine-tRNA synthetase	0.0	20.0	C	Ligase	Protein biosynthesis	-	+
CAND1	Cullin-associated NEDD8-dissociated protein 1	0.0	19.5	C, N	Ubiquitin conjugation	Proteolysis	-	+
CSTB	Cystatin-B	0.0	19.0	C, N	Peptidase inhibitor	proteolysis	-	-
USP7	Ubiquitin carboxyl-terminal hydrolase 7	0.0	18.0	C, N	Peptidase	DNA repair	-	-
DIS3	Exosome complex exonuclease RRP44	0.0	18.0	C,N	Endonuclease	rRNA processing	-	-
TUBA3D	Tubulin alpha-3C/D chain	0.0	18.0	C	GTPase	Protein folding	-	
TPP2	Tripeptidyl-peptidase 2	0.0	17.5	C, N	Peptidase	Proteolysis	+	+
RBBP7	Histone-binding protein RBBP7	0.0	16.8	N	RNA binding	DNA replication	+	-
HSPA4L	Heat shock 70 kDa protein 4L	0.0	16.8	C, N	Chaperone	Stress response	-	+

MAP4	Microtubule-associated protein 4	0.0	16.3	C	Microtubule binding	Cell division	+	+
HSPH1	Heat shock protein 105 kDa	0.0	15.8	C	ATP binding	Stress response	-	+
TIPRL	TIP41-like protein	0.0	14.8	C	PP2A inhibitor	Cell cycle	+	-
HUWE1	E3 ubiquitin-protein ligase	0.0	14.8	C, N	Ligase	DNA repair	-	+
PPP2R2A	Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B alpha isoform	0.0	14.5	C	Phosphatase	Cell cycle	-	+
MCMBP	Mini-chromosome maintenance complex-binding protein	0.0	14.3	N	Chromatin binding	Cell cycle	+	-
UBA6	Ubiquitin-like modifier-activating enzyme 6	0.0	14.3	C	ATP binding	Protein ubiquitination	+	+
USP5	Ubiquitin carboxyl-terminal hydrolase 5	0.0	14.3	L	Hydrolase	Protein catabolism	-	-
SNRNP200	U5 small nuclear ribonucleoprotein 200 kDa helicase	0.0	14.3	N	Helicase	mRNA processing	-	-
GSTM3	Glutathione S-transferase Mu 3	0.0	14.0	C	Glutathione binding	Detoxification	+	-
DNM1L	Dynamin-1-like protein	0.0	14.0	C	Hydrolase	Apoptosis	+	-
KHSRP	Far upstream element-binding protein 2	0.0	14.0	N	DNA binding	mRNA processing	-	-
AIMP2	Aminoacyl tRNA synthase complex-interacting multifunctional protein 2	0.0	14.0	C, N	Development-al protein	Apoptosis	+	+
PABPC4	Polyadenylate-binding protein 4	0.0	14.0	C	mRNA binding	mRNA processing	-	+
NCAPD2	Condensin complex subunit 1	0.0	14.0	N	Chromatin binding	Cell cycle	-	+
MARS	Methionine--tRNA ligase, cytoplasmic	0.0	13.8	C	tRNA ligase	Gene expression	-	-
UNC45A	Protein unc-45 homolog A	0.0	13.8	C	Chaperone	Cell differentiation	-	-
STIP1	Stress-induced-phosphoprotein 1	0.0	13.5	C, N	RNA binding	Stress response	+	+
CORO1C	Coronin-1C	0.0	12.8	C	Actin binding	cytokinesis	-	+
MSH6	DNA mismatch repair protein Msh6	0.0	12.5	N	DNA binding	DNA repair	+	-
IARS	Isoleucine--tRNA ligase, cytoplasmic	0.0	12.5	C	tRNA editing	Gene expression	-	-
PABPC1	Polyadenylate-binding protein 1	0.0	12.0	C, N	RNA binding	mRNA processing	-	+
EIF4G1	Eukaryotic translation initiation factor 4 gamma 1	0.0	12.0	C	Translation factor	Translation	-	+
SPAG9	C-Jun-amino-terminal kinase-interacting protein 4	0.0	11.5	C	Kinase binding	Cell differentiation	-	-
KHDRBS1	KH domain-containing, RNA-binding, signal transduction-associated protein 1	0.0	11.5	N	DNA binding	Cell cycle	-	-
PSMD2	26S proteasome non-ATPase regulatory subunit 2	0.0	11.3	C	MAPKK activator	Cell proliferation	+	-
ABCE1	ATP-binding cassette sub-family E member 1	0.0	11.0	C	ATPase activity	RNA catabolism	-	+

GFPT1	Glucosamine--fructose-6-phosphate aminotransferase 1	0.0	11.0	C	Carbohydrate binding	metabolism	-	+
SND1	Staphylococcal nuclease domain-containing protein 1	0.0	11.0	C, N	Nuclease	Transcription	+	-
RNPEP	Aminopeptidase B	0.0	10.8	EC	peptidase	Proteolysis	-	-
SMC4	Structural maintenance of chromosomes protein 4	0.0	10.8	N	ATP binding	Cell cycle	-	-
IPO11	Importin-11	0.0	10.5	C	Protein transporter	Protein transport	-	-
USP9X	Probable ubiquitin carboxyl-terminal hydrolase FAF-X	0.0	10.5	C	Hydrolase	Cell cycle	-	-
CRKL	Crk-like protein	0.0	10.3	C	MAPKK activator	Cell proliferation	+	+
TBCD	Tubulin-specific chaperone D	0.0	10.3	C	chaperone	Protein folding	+	-
PSMD1	26S proteasome non-ATPase regulatory subunit 1	0.0	10.3	C	MAPKK activator	Cell proliferation	+	-
GTF2I	General transcription factor II-I	0.0	10.3	C, N	DNA binding	Transcription	+	+
LARP1	La-related protein 1	0.0	10.3	C	mRNA binding	Cell proliferation	+	+
TTLL12	Tubulin--tyrosine ligase-like protein 12	0.0	10.0	C	ATP binding	Tubulin PTM	-	-
EPRS	Bifunctional glutamate/proline--tRNA ligase	1.0	68.5	C	Ligase	Translation	-	+
PFAS	Phosphoribosylformylglycinamidine synthase	0.7	34.5	C	Ligase	Purine biosynthesis	-	+
NASP	Nuclear autoantigenic sperm protein	0.7	33.0	C, N	Protein binding	cell proliferation	+	+
UBE2L3	Ubiquitin-conjugating enzyme E2 L3	0.7	32.8	C, N	Transferase	Transcription	-	-
GARS	Glycine--tRNA ligase	0.7	32.0	C	Ligase	Translation	-	+
PRMT1	Protein arginine N-methyltransferase 1	0.7	25.0	N	Methyl transferase	Gene expression	-	+
FSCN1	Fascin	0.7	25.0	C	Actin binding	Cell migration	+	-
HNRNPK	Heterogeneous nuclear ribonucleoprotein K	1.0	36.8	C, N	Pre-mRNA binding	Apoptosis	-	+
XPO1	Exportin-1	1.0	36.5	C, N	Protein transporter	Gene expression	+	+
PPA1	Inorganic pyrophosphatase	1.0	31.0	C	Phosphatase	Gene expression	-	+
PAICS	Multifunctional protein ADE2	0.7	20.3	C	Decarboxy-lase	Purine biosynthesis	-	+
PREP	Prolyl endopeptidase	0.7	20.3	C	Peptidase	Proteolysis	-	+
CSE1L	Exportin-2	0.7	18.0	C, N	Protein transport	Apoptosis	-	+
EEF2	Elongation factor 2	8.3	201.3	C, N	GTPase	Translation	-	+
PCMT1	Protein-L-isoaspartate(D-aspartate) O-methyltransferase	2.3	54.3	C	Methyl transferase	Protein repair	-	-
NPM1	Nucleophosmin	1.0	23.0	N	Chaperone	Cell proliferation	-	-
PLS3	Plastin-3	1.3	27.5	C	Calcium binding	Cell differentiation	-	+

IMPDH2	Inosine-5'-monophosphate dehydrogenase 2	0.7	13.8	C, N	Oxidoredu-ctase	Purine biosynthesis	-	-
CLTC	Clathrin heavy chain 1	1.7	33.5	C	Protein or RNA binding	Protein transport	-	+
PRKDC	DNA-dependent protein kinase catalytic subunit	2.3	46.8	N	kinase	DNA repair	+	+
YWHAQ	14-3-3 protein theta	0.7	13.3	C	Protein binding	Apoptosis	-	+
ACLY	ATP-citrate synthase	2.0	39.5	M	Transferase	Lipid metabolism	+	+
EEF1G	Elongation factor 1-gamma	3.0	58.3	C	Transferase	Gene expression	-	+
RCC2	Protein RCC2	1.3	25.3	N	GTPase activator	Cell cycle	+	+
GART	Trifunctional purine biosynthetic protein adenosine-3	5.0	78.8	C	Ligase	Purine biosynthesis	-	+
CPNE1	Copine-1	0.7	10.5	N	Repressor	Differentiation	-	+
CCT2	T-complex protein 1 subunit beta	4.3	66.5	C	Chaperone	Protein folding	-	+
FASN	Fatty acid synthase	11.0	165.3	C	Hydrolase, Transferase	Fatty acid biosynthesis	-	+
AHCY	Adenosylhomocysteinase	1.3	20.0	C	Hydrolase	One-carbon metabolism	-	-
DYNC1H 1	Cytoplasmic dynein 1 heavy chain 1	1.3	19.5	C	ATPase	Transport	-	+
PTBP1	Polypyrimidine tract-binding protein 1	1.7	24.0	N	Pre-mRNA binding	mRNA processing	-	+
TARS	Threonine--tRNA ligase, cytoplasmic	1.0	13.3	C	Ligase	Translation	-	-
HSP90AB 1	Heat shock protein HSP 90-beta	10.7	137.3	C	Chaperone	Stress response	-	+
DDX3X	ATP-dependent RNA helicase DDX3X	1.0	12.8	N	Helicase/ ATPase	Transcription	+	+
TCP1	T-complex protein 1 subunit alpha	2.7	33.8	C	Chaperone	Protein folding		+
IPO5	Importin-5	1.7	21.0	C, N	GTPase inhibitor	Protein transport	+	+
GSPT2	Eukaryotic peptide chain release factor GTP-binding subunit ERF3B	1.0	12.5	C	GTPase	Cell cycle	-	-
CFL1	Cofilin-1	4.7	56.8	N	Actin binding	Cytokinesis	-	+
CCT6A	T-complex protein 1 subunit zeta	1.0	11.0	C	Chaperone	Protein folding	-	+
PRDX2	Peroxiredoxin-2	2.3	24.3	C, M	Peroxidase	Oxidative stress response	-	-
PHGDH	D-3-phosphoglycerate dehydrogenase	1.3	13.8	C	Oxidoredu-ctase	Amino-acid biosynthesis	-	+
GCN1L1	Translational activator GCN1	2.3	21.5	C	RNA binding	Translation	-	+
PRDX1	Peroxiredoxin-1	2.7	24.5	C, M	Peroxidase	Oxidative stress response	-	-
UBA1	Ubiquitin-like modifier-activating enzyme 1	6.7	57.3	C,M	Ligase	proteolysis	+	+
PKM	Pyruvate kinase isozymes M1/M2	6.3	53.5	C, N	Kinase	Glycolysis	-	+
RPL18A	60S ribosomal protein L18a	2.0	16.3	C	RNA binding	Translation	-	-

DDX1	ATP-dependent RNA helicase	1.3	10.8	N	Helicase	Transcription	+	-
HNRNPF	Heterogeneous nuclear ribonucleoprotein F	1.7	11.5	N	RNA binding	mRNA processing	-	+
HSP90AA1	Heat shock protein HSP 90-alpha	20.7	140.5	C	Chaperone	Stress response	-	+
PRDX6	Peroxiredoxin-6	6.0	38.0	C	peroxidase	Oxidative stress response	+	+
QARS	Glutamine--tRNA ligase	1.7	10.5	C, M	Ligase	Translation	-	+
HNRNPL	Heterogeneous nuclear ribonucleoprotein L	5.0	25.5	C, N	RNA binding	mRNA processing	-	+
EEF1A2	Elongation factor 1-alpha 2	12.3	60.5	N	GTPase	Translation	-	-
XRCC6	X-ray repair cross-complementing protein 6	2.7	13.0	N	Helicase	DNA repair	-	-
XRCC5	X-ray repair cross-complementing protein 5	6.3	29.5	N	Helicase	DNA repair	-	+
EIF5AL1	Eukaryotic translation initiation factor 5A-1-like	5.7	25.8	C, N	mRNA binding	mRNA transport	-	-
EEF1A1	Elongation factor 1-alpha 1	18.3	79.8	C, N	GTPase	Translation	-	+
CAD	CAD protein	2.7	11.5	C, N	Hydrolase, ligase	Pyrimidine biosynthesis	-	-
YWHAE	14-3-3 protein epsilon	4.0	14.8	C	HDAC binding	Apoptosis	-	+
HSPA8	Heat shock cognate 71 kDa protein	3.0	11.0	C	ATPase, Chaperone	Transcription	-	+
ALDOA	Fructose-bisphosphate aldolase A	3.0	10.0	C	Lyase	Glycolysis	+	+
RPL10	60S ribosomal protein L10	4.7	14.0	C	RNA binding	Translation	-	+
CCT5	T-complex protein 1 subunit epsilon	6.0	16.0	C	ATP binding	Protein folding	-	+
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	30.7	77.3	C	Oxidoreductase/transferase	Glycolysis	-	+
MCM3	DNA replication licensing factor MCM3	4.0	10.0	N	Helicase	DNA replication	+	+
NME1	Nucleoside diphosphate kinase A	7.3	18.3	C, N	Kinase	Cell differentiation	-	-
PGAM1	Phosphoglycerate mutase 1	8.0	17.5	C	Isomerase	Glycolysis	+	-
PPIA	Peptidyl-prolyl cis-trans isomerase A	7.7	15.5	C, EC	Isomerase	Protein folding	+	-

3. Experimental procedure

3.1 Material

All chemicals were purchased from Sigma unless otherwise stated. 3-Azido-L-alanine HCl (Azido-Ala) was purchased from Jena Biosciences (Germany). EDTA-free protease inhibitor cocktail tablets were purchased from Roche. All cell culture reagents and medium were purchased from Life Technologies. Streptavidin-horseradish peroxidase (HRP) and high capacity streptavidin agarose beads were purchased from Thermo Scientific. HRP-conjugated anti-mouse and anti-rabbit secondary antibodies were purchased from GE healthcare. Light chain specific HRP-conjugated mouse and rabbit secondary antibodies were purchased from Jackson ImmunoResearch and ECM Biosciences, respectively. Polyethyleneimine-MAX was purchased from Polysciences, Inc (USA). Reduced glutathione (Sigma), oxidized glutathione (Acros), 2,2-dithiodipyridine (Acros), γ-glutamyl-cysteine (Sigma), 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma), human glutathione reductase (Sigma), and 2,7-dichlorodihydrofluorescein diacetate (DCF-DA) (Sigma) were purchased. Glutaredoxin 1 (Grx1) was purified as reported previously.[3]

3.2. Synthesis of glutathione derivatives

3.2.1. Synthesis of azido-glutathione (2)

Clickable glutathione containing L-azido-Ala (azido-glutathione, ^{N3}GSH) was synthesized using GS M4 enzyme, which catalyze L-azido-Ala in place of Gly. 3.5 mM azido-alanine (azido-Ala, 5.9 mg), 3.0 mM γ-glutamyl-cysteine, 10 mM ATP, 50 mM $MgCl_2$, 300 mM NaCl, 200 mM Tris buffer (pH 7.4) and GS M4 (80 ng) were mixed in 10 ml final reaction mixture and incubated overnight at 37°C. After acidification (pH 2-3) by adding 1 M HCl, the mixture was centrifuged at 10,000 rpm for 5 min. The supernatant aqueous solution was subjected to reverse-phase C18 column HPLC purification (gradient mobile phase: H_2O and CH_3CN), and the disulfide of azido-glutathione was obtained as a white solid after lyophilization. The purity and mass were confirmed by ESI-MS and LC-MS. Disulfide of azido-glutathione was reduced using dithiothreitol (DTT) (2 equivalent) in 100 mM PBS (pH 7.4) at room temperature for 1 h. The mixture was acidified by addition of 1 M HCl (pH 2-3), and centrifuged at 10,000 rpm for 4 min. The supernatant acidic aqueous layer was subjected to reverse-phase C18 column HPLC purification, and the reduced azido-glutathione was obtained as a white solid after lyophilization, giving azido-glutathione (**2**) (8 mg, 56%). The purity and mass were confirmed by ESI-MS and LC-MS. Calculated mass: 362.10 Found mass: ESI m/z 363.07 (M+H) 725.18 (2M+H).

3.2.2. Synthesis of GS-S-Pyr (3) and ^{N3}GS-SPyr (4)

Glutathione (GSH, **1**) (10.0 mg, 1 equivalent) or azido-glutathione (^{N3}GSH, **2**) (8.0 mg, 1 equivalent) and 2, 2-dithiodipyridine (15.0 mg, 2 equivalent) were mixed in the mixture of water and methanol (1:1 by volume) and stirred for 15-20 h at room temperature. The mixture was concentrated to remove methanol, and the aqueous layer was extracted several times with dichloromethane. The aqueous layer was acidified by addition of 1 M HCl (pH 2-3), and the acidified aqueous portion was then subjected to reverse phase C-18 column HPLC purification, yielding S-(2-thiopyridyl)glutathione (GS-S-Pyr, **3**, 11.1 mg, 82%) or S-(2-thiopyridyl)azido-glutathione (^{N3}GS-S-Pyr, **4**, 8.1 mg, 78%) as a white solid after lyophilization. The purity and mass were confirmed by ESI-MS and LC-MS. Calculated mass for S-(2-thiopyridyl)glutathione (**3**): 416.08. Found mass: ESI m/z 417.04 (M+H) and 833.11 (2M+H). Calculated mass for ^{N3}GS-S-Pyr (**4**): 471.10. Found mass: ESI m/z 472.10 (M+H), 943.21 (2M+H).

3.2.3. Synthesis of ^{N3}GSSG (6)

S-(2-thiopyridyl)azido-glutathione (^{N3}GS-S-Pyr, **4**, 7.0 mg, 1 equivalent) and glutathione (GSH, **1**, 4.6 mg, 1 equivalent) were dissolved in 100 mM PBS buffer (pH 7.4), and the reaction mixture was stirred for 2 h at room temperature. The mixture was acidified by addition of 1 M HCl (pH 2-3), and centrifuged at 10,000 rpm for 4 min. The acidic aqueous supernatant was separated by the reverse phase C-18 column HPLC, yielding the mixture disulfide of azido-glutathione with glutathione (^{N3}GSSG, **6**, 7.3 mg, 74%). Calculated mass for ^{N3}GSSG (**6**): 667.17. Found mass: ESI m/z 668.14 (M+H), 334.59 [(M+2H)/2].

3.2.4. Synthesis of GSS-Pep (8mer) (7) and ^{N3}GSS-Pep (8mer) (8)

S-(2-thiopyridyl)glutathione (GS-S-Pyr, **3**, 3.0 mg, 1 equivalent) or S-(2-thiopyridyl)azido-glutathione (^{N3}GS-S-Pyr, **4**, 3.0 mg, 1 equivalent) was mixed with 8-mer peptide (Pep: SQLWCLSN), which was synthesized by standard Fmoc solid phase peptide synthesis, in a mixture of PBS buffer (100 mM, pH 7.4) and acetonitrile (4:1 volume). The mixture was stirred for 2 h at room temperature. The aqueous layer was then washed with dichloromethane for five times to remove the thione by-product. The aqueous layer was then acidified by addition of 1 M HCl (pH 2-3). The acidic aqueous portion was subjected to the reverse phase C-18 column HPLC purification, and GSS-Pep (**7**, 6.3 mg, 71%) or ^{N3}GSS-Pep (**8**, 5.7 mg, 69%) was obtained as white

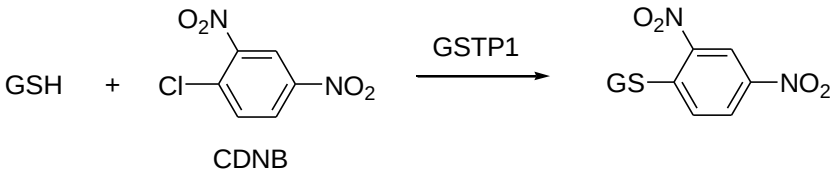
solids after lyophilization. Calculated mass for GSS-Pep (**7**): 12553.81. Found mass: ESI m/z 1254.80 (M+1). Calculated mass for ^{N3}GSS-Pep (**8**): 1308.59. Found mass: ESI m/z 1309.86 (M+H), 1331.65 (M+Na).

3.3. Purification of GSTO and GSTP

Human GSTP1 cDNA clone (OriGene) and Human GSTO1 cDNA clone (gift from Dr. Young-Tae Chang, National University of Singapore) were subcloned in to pET-28a(+) bacterial expression vector using NheI/XhoI (GSTP1) and NdeI/XhoI (GSTO1) restriction sites. PCR reaction was performed using forward primer (5'- GCG CGC GCT AGC ATG CCG CCC TAC ACC GTG GTC TAT TTC -3') with NheI and reverse primer (5'- GCG CGC CTCGAG TCA CTG TTT CCC GTT GCC ATT GAT GGG -3') with XhoI for human GSTP1 cDNA clone. Forward primer (5'- GCG CGC CAT ATG ATG TCC GGG GAG TCA GCC AGG-3') with NdeI and reverse primer (5'-GCG CGC CTC GAG TCA GAG CCC ATA GTC ACA GGC C-3') with XhoI were used for the PCR reaction of human GSTO1 cDNA clone. Both PCR product and the empty pET-28a(+) vector were double digested with above restriction enzymes accordingly and ligated using T4 DNA ligase. All cloned plasmids were confirmed by DNA sequencing of entire open reading frames. All the bacterial expression constructs (pET28-His-GSTP1 and pET28-His-GSTO1) were transformed in to BL21 (DE3) cells. Cells were inoculated in 1 L LB medium, and grown at 37^{o}C until OD_{600} reached about 0.6. The protein expression (GSTP1 or GSTO1) was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) and was incubated 4-5 h at 37^{o}C. Cells were harvested by centrifugation at 5000 rpm for 20 min and lysed by passing through French Press (1,000 psi) three times. Cell lysate was then centrifuged at 15,000 rpm for 20 min, and soluble fraction was purified by Ni-NTA affinity column. The eluted proteins were dialyzed by a buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1 mM DTT and 5% glycerol) and concentrated. Protein concentrations were determined by Bradford assay.

3.4. Enzyme assays of GSTP1, GSTO, and GR

3.4.1. Spectrophotometric assay of GSTP1 activity

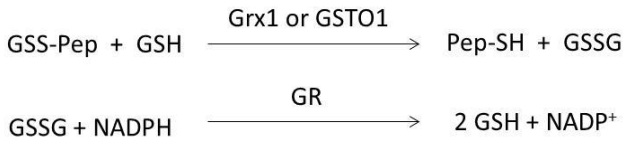


Enzyme assay was carried out in 0.1 M PBS (pH 6.5) with 1 mM EDTA and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) at 25ºC. In this assay, alkylation production (GS-DNB or ^{N3}GS-DNB) between CDNB and glutathione (**1**) or azido-glutathione (**2**) was monitored by measuring absorbance at 340 nm using DU730-Beckman coulter UV-VIS Spectrophotometer. The rate of spontaneous conjugation (non-enzymatic reaction) of GSH or ^{N3}GSH with CDNB was measured and subtracted from rates of GSTP1-catalyzed reactions. The extinction coefficient (9.6 $mM^{-1}cm^{-1}$)[4] for the GS-DNB at 340 nm was used to calculate the enzyme kinetics. Initial velocity versus substrate concentration plots were fitted to the Michaelis-Menten equation using Graphpad prism 5.01 and the kinetic constants (K_m, k_{cat} and V_{max}) for glutathione and azido-glutathione were calculated accordingly.

3.4.2. Spectrophotometric assay of GR activity

Kinetic parameters for glutathione disulfide reductase (GR) were determined by measuring consumption of NADPH in different substrate concentrations (0.01-0.5 mM). NADPH consumption was monitored at 25ºC by UV absorbance at 340 nm. The reaction mixture (0.2 mL) contains 100 mM PBS (pH 7.4), 1 mM EDTA, 0.2 mM NADPH and 50 ng GR. The extinction coefficient (6.2 $mM^{-1}cm^{-1}$)[5] for the NADPH was used to calculate the enzyme kinetics. The plot of the initial velocity versus substrate concentrations was fitted to the Michaelis-Menten equation using Graphpad prism 5.01 and kinetic constants (K_m, k_{cat} and V_{max}) for GSSG and ^{N3}GSSG were calculated accordingly.

3.4.3. Spectrophotometric assay of Grx1 and GSTO1 activity



The coupled spectrophotometric assay involving above two reactions was used as reported previously.[3] Excess GR was used so that the second step is not a limiting step. Note that GR catalyzes ^{N3}GSH with the similar k_{cat} and lower K_m than GSH (Table 1), indicating that ^{N3}GSSG reduction by GR will not be a limiting step. The assay was performed in 0.1 M PBS (pH 7.4) with 1 mM EDTA, 0.2 mM NADPH, GR (4 units/mL), GSH 0.25 mM, and Grx1 (23 nM) or GSTO1 (0.19 µM). The mixture was incubated for 7 min at 25ºC. The reactions were initiated by addition of the substrate, and the oxidation of NADPH was monitored at 340 nm using DU730-Beckman coulter UV-VIS spectrophotometer. Non-enzymatic reaction at individual substrate concentration was measured and subtracted from the enzyme-catalyzed reaction. The extinction coefficient (6.2 $mM^{-1}cm^{-1}$) for NADPH was used to calculate the enzyme kinetics. The initial velocity versus substrate concentration was plotted, and fitted to the Michaelis-Menten equation using Graphpad prism 5.01. The kinetic constants (K_m, k_{cat} and V_{max}) for ^{N3}GSS-Pep and GSS-Pep were calculated accordingly.

3.5. ROS and viability assays

3.5.1. Cell viability assay

HEK 293 cells were grown on a six well plate by adding same number of cells into each well. After 24 h, cells were incubated in DMEM only for 3 h. Cells were then subjected to different conditions for 24 hrs: 1) no treatment (25 mM glucose, 0 µg/mL antimycin A), 2) glucose starvation (0 mM glucose), 3) antimycin A (1 µg/mL) 4) glucose starvation (0 mM glucose) with antimycin A (1 µg/mL antimycin A), and 5) 0.01% DMSO (amount used to dissolve antimycin A). Antimycin A was dissolved in DMSO. Cells were subjected to trypan blue cell viability assay. Briefly, cells were detached by treating 0.05% trypsin, and diluted with the medium. Then 100 µl of trypan blue reagent was added to the 100 µl of medium with the cells and 20 µl of sample was loaded on to the slide and analyzed the percentage of viable cells by TC 20 automated cell counter (Biorad). The data was shown after duplication of two independent experiments.

3.5.2. ROS assays

HEK293 cells (10,000 cells per well) were seeded and incubated at 37ºC, 5% CO_2 for 24 h for attachment on 96-well black plates with transparent flat bottom. Cells were then washed with warm PBS, and incubated in serum-free medium for 3 h. 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA, 25 μM) was then added, and cells were incubated in the same conditions for another 1 h. Cells were then washed with glucose-free medium without phenol red, followed by treatment at the indicated conditions and incubated for 2 h. Untreated cells acted as a negative control. Fluorescence emission scan was measured at 528 nm after excitation at 485 nm. ROS levels were normalized by a negative control (25 mM glucose without antimycin A).

3.5.3. The level of thiol concentration

HEK293/GS M4 cells were treated with 0.5 mM azido-Ala and cells were washed with PBS after 20 h. Cells were then induced for glutathionylation by glucose starvation with antimycin A (2 μg/mL) for 0.5 h or 2 h. After induction, cells were harvested and lysed in PBS by 3 times of freeze-thaw cycles. The cell lysates were collected after centrifugation. Proteins were removed by 3K centrifugal filter device, and the filtrate (FIL) that contains small molecules (including glutathione) was collected. The total and reduced form of thiol contents (glutathione) were analyzed by fluorescence assay using bromobimane (excitation - 390 nm, emission- 478 nm).[6] Bromobimane assay was performed in 0.1 M phosphate buffer, 0.5 mM bromobimane, and 10 μL of protein free lysates (FIL). The fluorescence signal was measured by using a plate reader. To calculate total GSH levels, protein free lysates (FIL) were incubated with 1 mM TCEP at 37ºC for 30 minutes before the bromobimane assay. All the data was duplicated and the background signal was corrected.

3.6 Cloning, expression, and enzyme assays of PP2Cα

3.6.1 Cloning and mutagenesis

Human PP2Cα cDNA clone was purchased from OriGene and the gene was subcloned into pcDNA3.1/hygro (+) mammalian vector using BamHI and XhoI restriction sites. PCR reaction was performed using forward primer (5'-GGT GGT GGA TCC GCC ACC ATG GGA GCA TTT TTA GAC AAG C-3') containing Kozak sequence and BamHI restriction site and (5'-GGT GGT CTC GAG CTA AGC GTA ATC TGG AAC ATC GTA TGG GTA CCA CAT ATC ATC TGT TGA TGT AGA G-3') as the reverse primer containing HA tag and XhoI restriction site. Both

PCR product and the empty pcDNA3.1 vector were double digested with above two restriction enzymes and ligated using T4 DNA ligase. All the mutants were generated using pcDNA3.1-PP2Cα clone as the template. Quick change mutagenesis was performed using following mutagenic primers. Mutant C204S (forward: 5'-GCC CTT GGG GAT TTT GAT TAC AAA AGT GTC CAT GGA AAA GGT CC-3' and reverse: 5'-GGA CCT TTT CCA TGG ACA CTT TTG TAA TCA AAA TCC CCA AGG GC-3'), C238S (forward: 5'-CAG TTC ATT ATC CTT GCA AGT GAT GGT ATC TGG GAT GTT ATG G-3' and reverse: 5- CCA TAA CAT CCC AGA TAC CAT CAC TTG CAA GGA TAA TGA ACT G-3') and C314S (forward: 5'-GGA CAA GTA CCT GGA AAG CAG AGT AGA AGA AAT CAT AAA GAA GC 3' and reverse: 5'-GCT TCT TTA TGA TTT CTT CTA CTC TGC TTT CCA GGT ACT TGT CC-3'). pcDNA3.1-PP2Cα and empty pET28a vector were double digested using BamHI and XhoI. The digested PP2Cα and pET28a were gel-purified and ligation was performed using T4 DNA ligase at 22^{o}C for 1 h. All plasmids were confirmed by DNA sequencing of entire open reading frames.

3.6.2. Purification of PP2Cα

pET28a-PP2Cα was transformed in to BL21(DE3) cells and single colony was inoculated overnight in LB containing 50 µg/mL kanamycin. 5 mL overnight culture was diluted in to 1 L LB and grew at 37^{o}C until OD_{600} reaches 0.4. Protein overexpression was induced for 4 h at 37^{o}C with 0.5 mM IPTG. Cells were harvested, lysed and purified as described in section 3.3.

3.6.3. Enzyme assays of PP2Cα

PP2Cα enzyme (WT and C314S) activities were calculated by end point assay using *para*-nitrophenyl phosphate (pNPP) as the substrate. Proteins were incubated with or without 0.5 mM GSSG in 20 mM Tris, pH 7.4 for 15 min at 37^{o}C. Phosphatase assay was performed in the colorimetric assay buffer containing 20 mM Tris, pH 7.4, 5 mM $MgCl_2$, 1 mM ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA) and 0.1 mg/mL BSA. Different amounts of each protein were mixed with 20 mM pNPP in 100 µL final volume and incubated for 30 min at 37^{o}C before quenching the reaction with 50 µL of 5 N NaOH. Enzyme activities (µmoles/min/µg) were calculated using absorbance measured at 405 nm.

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