Supplementary Information

Proteomic Identification of Protein Glutathionylation in Cardiomyocytes

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Table of Contents:

Figure S-1. Investigation of levels of redox enzymes and cellular thiols.

Figure S-2. Detection of protein glutathionylation.

Figure S-3. A scheme of enrichment and elution of glutathionylated peptides.

Figure S-4. Optimization of elution and digestion of glutathionylated proteins.

Figure S-5. Detection of fragmentation of glutathione during LC-MS/MS analysis.

Figure S-6. Analysis of identified glutathionylated peptides

Figure S-7. STRING and cluster analysis of two merged networks.

Figure S-8. Analysis of glutathionylated Cys residues in Complex I supernumerary subunits.

Figure S-9. Analysis of glutathionylation in complex II.

Table S-1: A list of glutathionylated proteins and peptides in the positive and negative samples.

Table S-2: A list of 125 identified proteins that belong to both networks of 'glutathionylation' and 'cardiomyopathy'.

Supplementary Methods: cellular thiol concentrations, Western blotting, pull-down of glutathionylated proteins, and the sequence motif for glutathionylation.

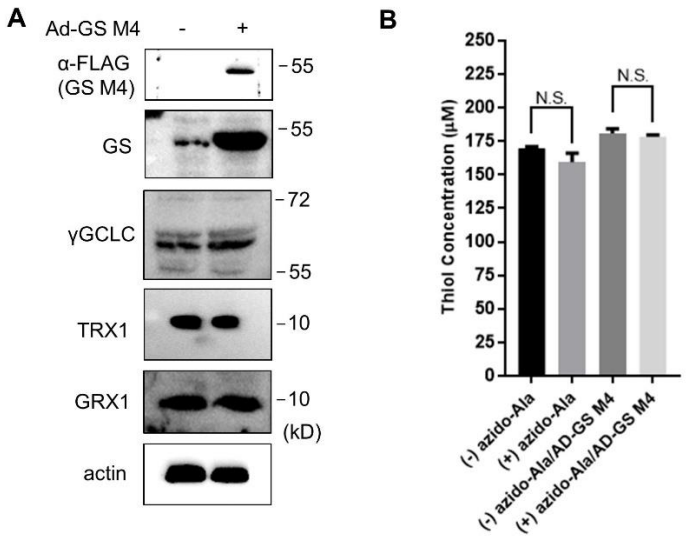


Figure S-1. Investigation of levels of redox enzymes and cellular thiols upon application of a clickable glutathione approach. HL-1 cells were infected by adenovirus expressing a mutant of glutathione synthetase (Ad-FLAG-GS M4) for 48 h. (**A**) Levels of GS M4, glutathione biosynthesis enzymes, such as glutathione synthetase (GS) and γ-glutamylcysteine ligase catalytic subunit (GCLC), and other redox enzymes. Lysates were analyzed by Western blots. Blots are the representation of at least 2 independent experiments. (**B**) A level of thiols in cells after expressing GS M4 and incubation of azido-Ala. Thiol concentrations in protein-free lysates were measured by bromobimane assay. Data represent the mean ± SD, n = 2 independent experiments. Difference is significant by one-way ANOVA followed by Tukey's *post-hoc* test.

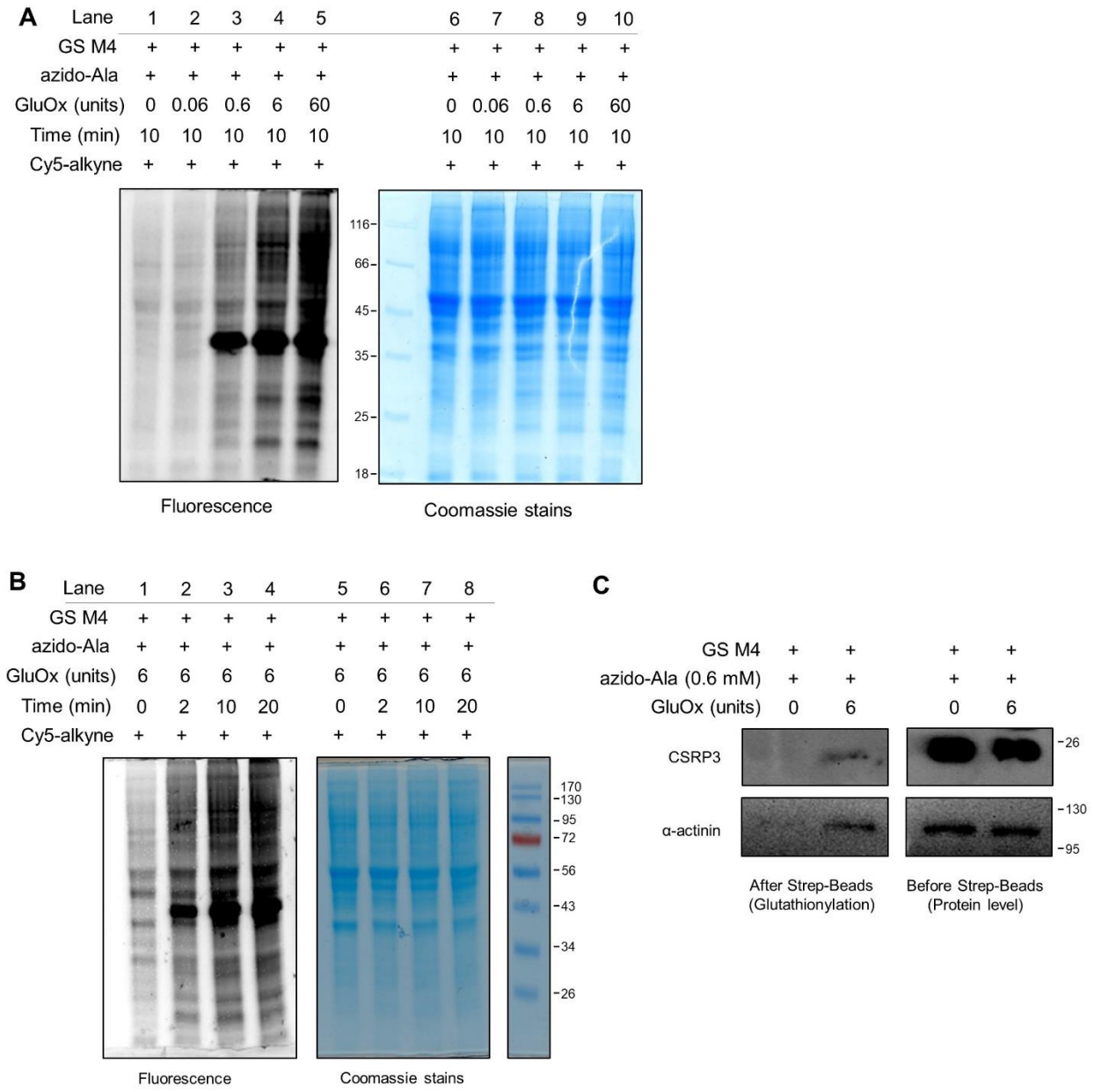


Figure S-2. Detection of protein glutathionylation upon addition of glucose oxidase (GluOx). Glutathione synthetase mutant (GS M4) was expressed in HL-1 cells. After incubation of azido-Ala, cells were incubated with GluOx to induce glutathionylation. (**A**) Increasing concentrations of GluOx. (**B**) Increasing incubation times of GluOx. Collected lysates were subjected to click reaction with Cy5-alkyne, separated by SDS-PAGE, and examined for fluorescence or by Coomassie stains. (**C**) Identification of glutathionylation of specific proteins. After incubation of GluOx for 10 min, collected lysates were subjected to click reaction with biotin-alkyne. Biotinylated glutathionylated proteins were incubated with streptavidin-agarose (strep-beads) for 3 h, eluted by an SDS-loading buffer, and examined by Western blotting with specific antibodies to CSRP3 (Abcam, ab172952) or α-actinin (Abcam, ab137346)

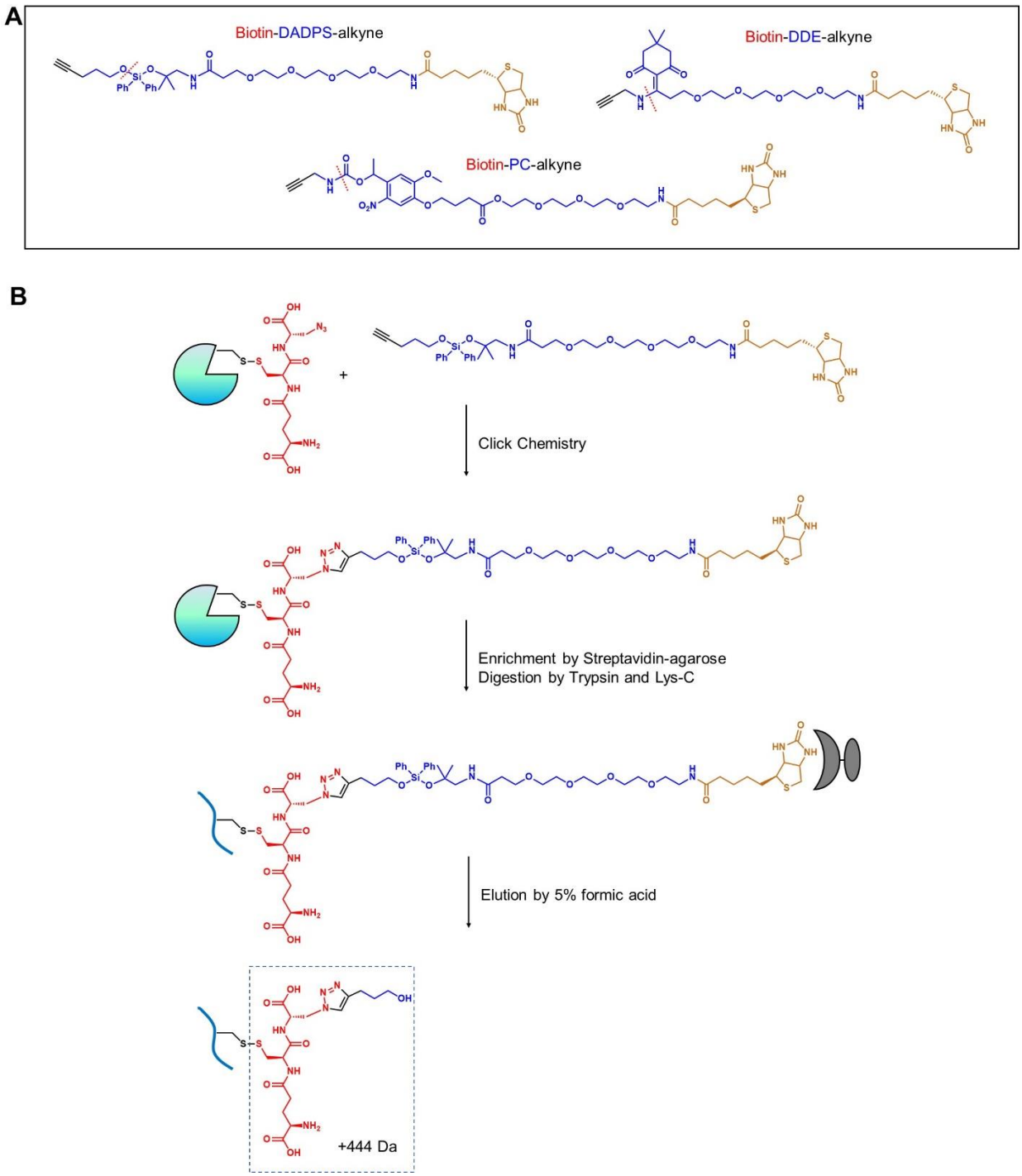


Figure S-3. A scheme of enrichment and elution of glutathionylated peptides after click reaction with biotin-alkyne containing a cleavable linker. (**A**) Biotin-alkyne derivatives with three different cleavable linkers. The cleavage site is shown by a red dotted line. (**B**) A scheme for identification of glutathionylated peptides by LC-MS/MS. After click reaction with biotin-DADPS-alkyne, biotinylated glutathionylated proteins were bound to streptavidin-agarose, digested by trypsin/Lys-C, eluted in an acidic condition, which leaves +444 Da modification on the Cys residue.

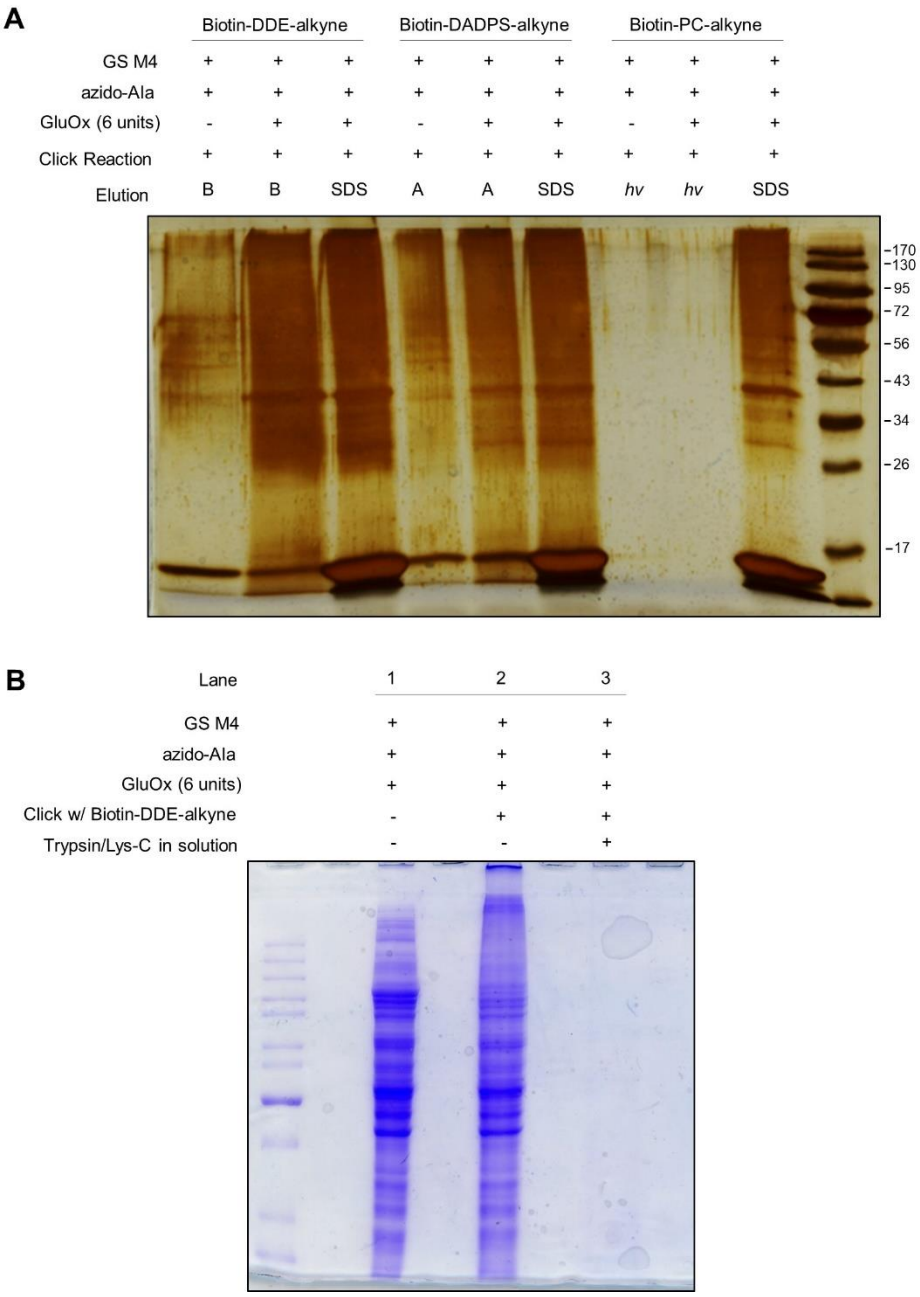
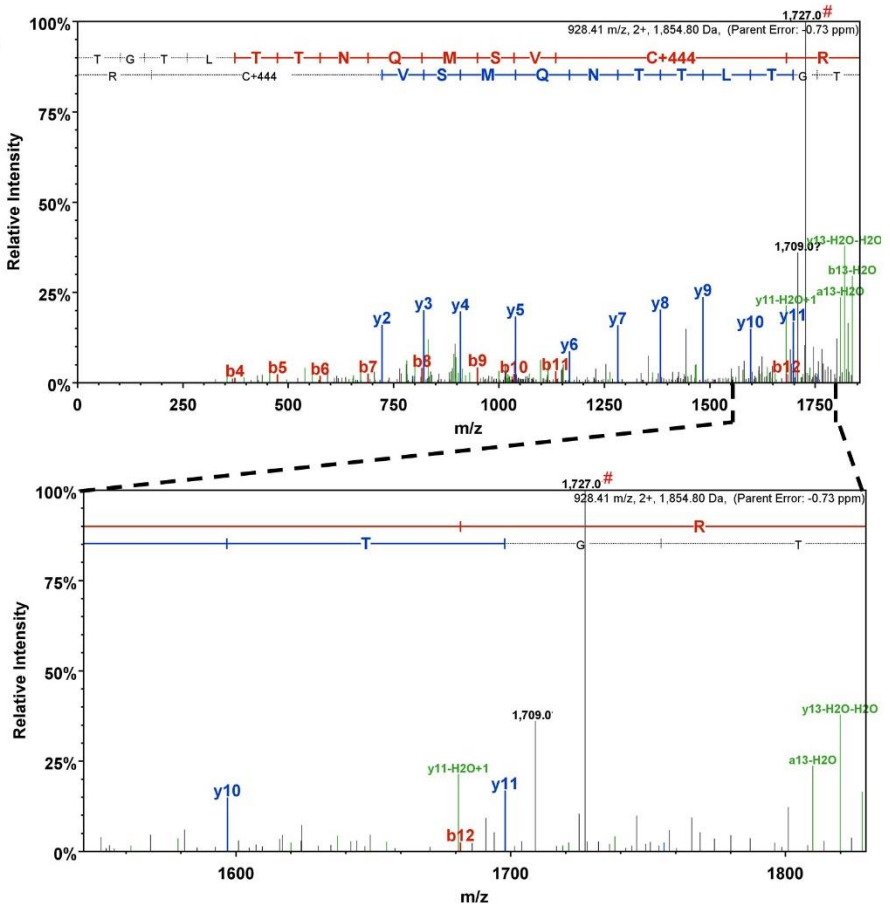


Figure S-4. Optimization of elution and digestion of glutathionylated proteins. (**A**) Elution of glutathionylated proteins. After click reaction with individual cleavable biotin-alkyne derivatives, glutathionylated proteins were bound to streptavidin-agarose and eluted in the specified conditions for 30 min twice: [A] acidic 10% aq. formic acid, [B] basic 2% hydrazine in PBS with 0.1% SDS, [*hv*] UV irradiation at 365 nm, [SDS] SDS-loading dye. Eluted proteins were analyzed by silver stains. (**B**) Digestion of glutathionylated proteins. Lysates containing glutathionylated proteins were precipitated by cold acetone and resuspended (lane 1), subjected to click reaction, followed by precipitation and resuspension (lane 2), and digested by Trypsin/Lys-C in resuspended solution (lane 3). The sample volume of resuspended solution was loaded and analyzed by SDS-PAGE and Coomassie stains.

A



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1	102.1			84.0	T	1,855.8	928.4	1,838.8	1,837.8	13
2	159.1			141.1	G	1,754.8	877.9	1,737.7	1,736.8	12
3	260.1			242.1	T	1,697.7	849.4	1,680.7	1,679.7	11
4	373.2			355.2	L	1,596.7	798.9	1,579.7	1,578.7	10
5	474.3			456.2	T	1,483.6	742.3	1,466.6	1,465.6	9
6	575.3	288.2		557.3	T	1,382.6	691.8	1,365.5	1,364.6	8
7	689.3	345.2	672.3	671.3	N	1,281.5	641.3	1,264.5	1,263.5	7
8	817.4	409.2	800.4	799.4	Q	1,167.5	584.2	1,150.4	1,149.5	6
9	948.4	474.7	931.4	930.4	M	1,039.4		1,022.4	1,021.4	5
10	1,035.5	518.2	1,018.5	1,017.5	S	908.4		891.3	890.4	4
11	1,134.5	567.8	1,117.5	1,116.5	V	821.3		804.3		3
12	1,681.7	841.4	1,664.7	1,663.7	C+444	722.3		705.2		2
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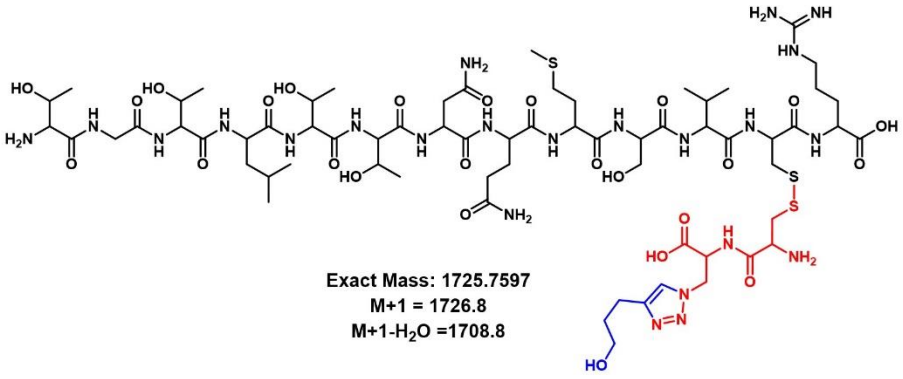

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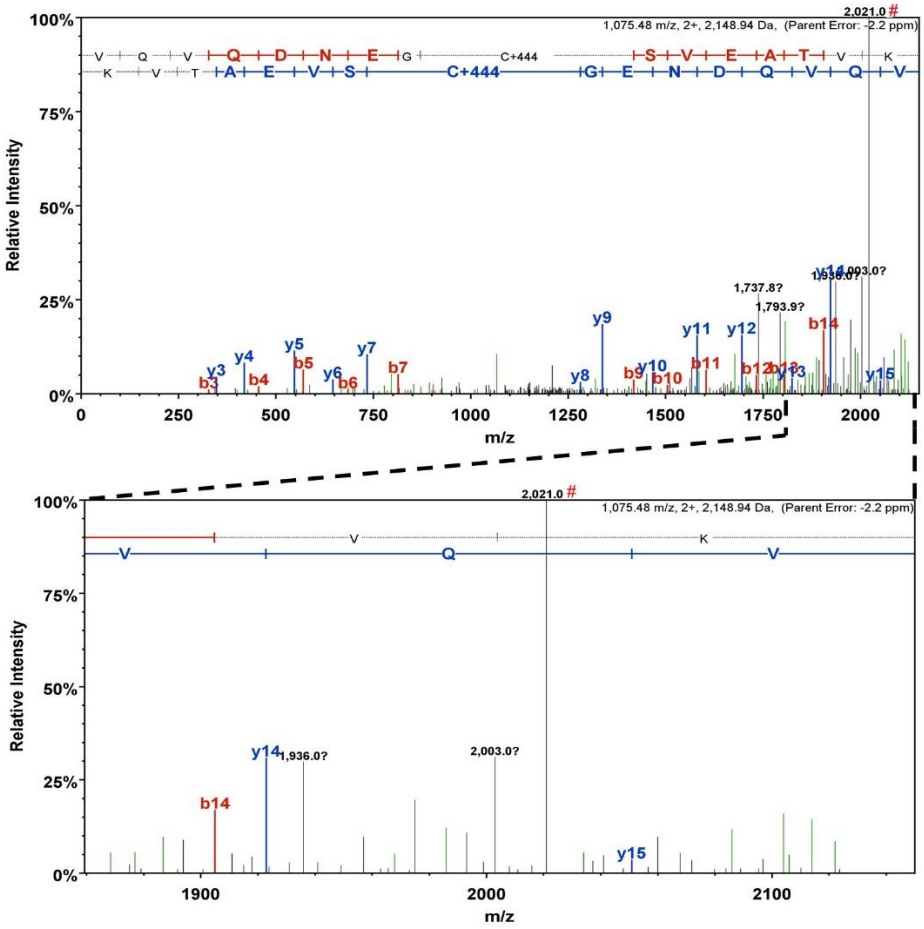
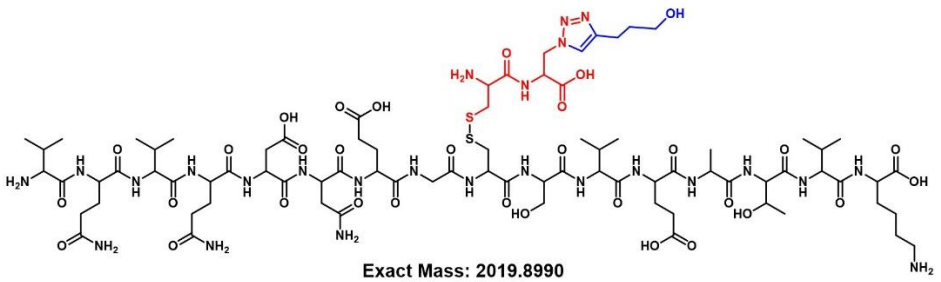
B



B	B Ions	B+2H	B-NH3	B-H2O	AA	Y Ions	Y+2H	Y-NH3	Y-H2O	Y
1	100.1				V	2,149.9	1,075.5	2,132.9	2,131.9	16
2	228.1		211.1		Q	2,050.9	1,025.9	2,033.9	2,032.9	15
3	327.2		310.2		V	1,922.8	961.9	1,905.8	1,904.8	14
4	455.3		438.2		Q	1,823.8	912.4	1,806.7	1,805.7	13
5	570.3		553.3	552.3	D	1,695.7	848.4	1,678.7	1,677.7	12
6	684.3	342.7	667.3	666.3	N	1,580.7	790.8	1,563.6	1,562.7	11
7	813.4	407.2	796.3	795.4	E	1,466.6	733.8	1,449.6	1,448.6	10
8	870.4	435.7	853.4	852.4	G	1,337.6	669.3	1,320.6	1,319.6	9
9	1,417.5	709.3	1,400.5	1,399.5	C+444	1,280.6	640.8	1,263.5	1,262.6	8
10	1,504.6	752.8	1,487.6	1,486.6	S	733.4	367.2	716.4	715.4	7
11	1,603.6	802.3	1,586.6	1,585.6	V	646.4	323.7	629.4	628.4	6
12	1,732.7	866.8	1,715.7	1,714.7	E	547.3		530.3	529.3	5
13	1,803.7	902.4	1,786.7	1,785.7	A	418.3		401.2	400.3	4
14	1,904.8	952.9	1,887.7	1,886.8	T	347.2		330.2	329.2	3
15	2,003.8	1,002.4	1,986.8	1,985.8	V	246.2		229.2		2
16	2,149.9	1,075.5	2,132.9	2,131.9	K	147.1		130.1		1



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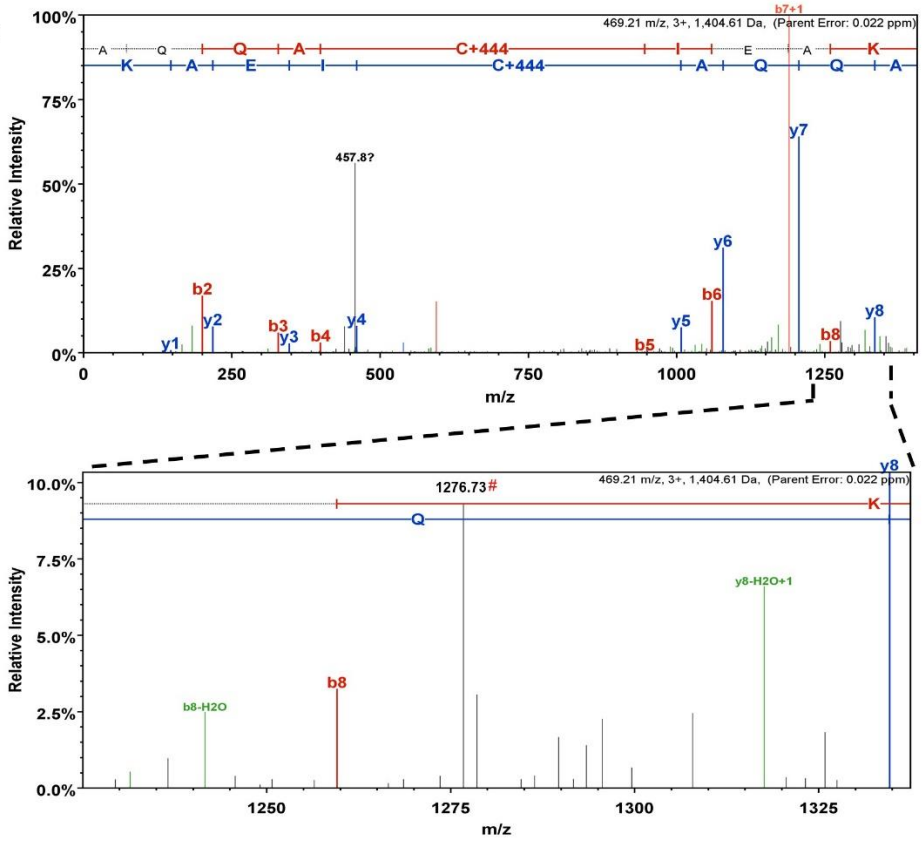
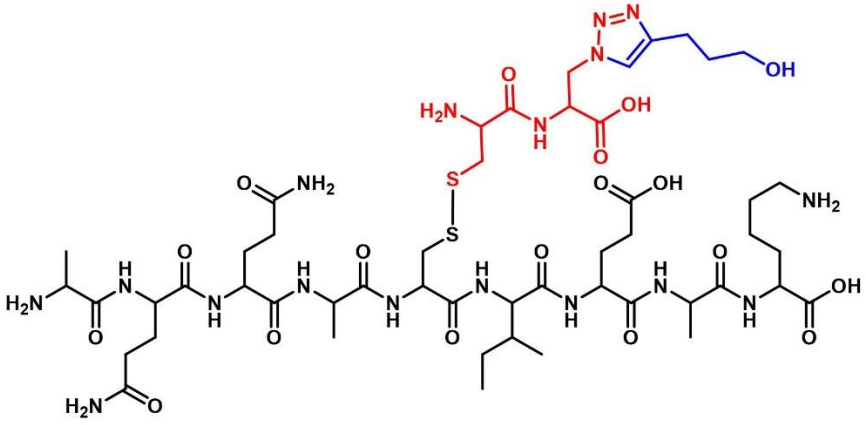
C



B	B Ions	B+2H	B-NH3	B-H2O	AA	Y Ions	Y+2H	Y-NH3	Y-H2O	Y
1	72.0	36.5			A	1,405.6	703.3	1,388.6	1,387.6	9
2	200.1	100.6	183.1		Q	1,334.6	667.8	1,317.6	1,316.6	8
3	328.2	164.6	311.1		Q	1,206.5	603.8	1,189.5	1,188.5	7
4	399.2	200.1	382.2		A	1,078.5	539.7	1,061.4	1,060.5	6
5	946.4	473.7	929.3		C+444	1,007.4	504.2	990.4	989.4	5
6	1,059.4	530.2	1,042.4		I	460.3	230.6	443.3	442.3	4
7	1,188.5	594.7	1,171.5	1,170.5	E	347.2	174.1	330.2	329.2	3
8	1,259.5	630.3	1,242.5	1,241.5	A	218.1	109.6	201.1		2
9	1,405.6	703.3	1,388.6	1,387.6	K	147.1	74.1	130.1		1



Exact Mass: 1275.5700
M+1=1276.57

Figure S-5. Detection of fragmentation of glutathione during LC-MS/MS analysis. MS2 spectrums of three peptides, TGTLTTNQMSVC*R from SERCA (**A**), VQVQDNEGC*SV EATVK from Filamin A (**B**), and AQQAC*IEAK from CHIP (**C**). In each protein (**A-C**), MS2 spectrums (top) of individual peptides are shown with an expanded area (the second from top) that show the peak (marked with #) corresponding to the identical peptides except with cleavage of γ-glutamyl-cysteine linkage on glutathione. Table shows the identified b or y ion peaks in each spectrum (the third from top). The structure and mass of identified peptides with the cleavage of γ-glutamyl-cysteine linkage are shown (bottom).

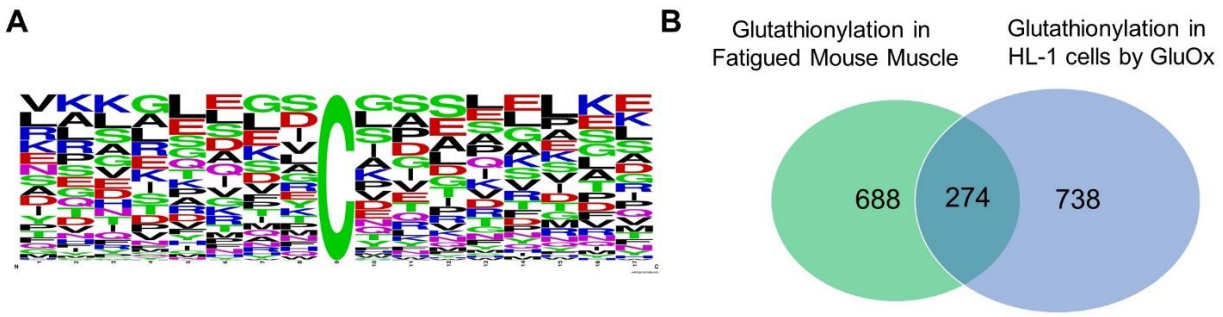


Figure S-6. Analysis of identified glutathionylated peptides. (**A**) Sequence motif analysis of glutathionylated peptides. 200 out of 1,767 identified glutathionylated peptides were randomly selected and aligned to show 17 amino acids containing Cys residue in the middle. These 200 peptide sequences were analyzed by WebLogo online tool (https://weblogo.berkeley.edu/logo.cgi) to generate the sequence motif. (**B**) Comparison of our list of glutathionylated proteins (1,012 proteins) with a previous report of glutathionylated proteins (962 proteins) from fatigue mouse skeletal muscle.[1] Accession numbers of our identified proteins were compared to the data available in reference 1.

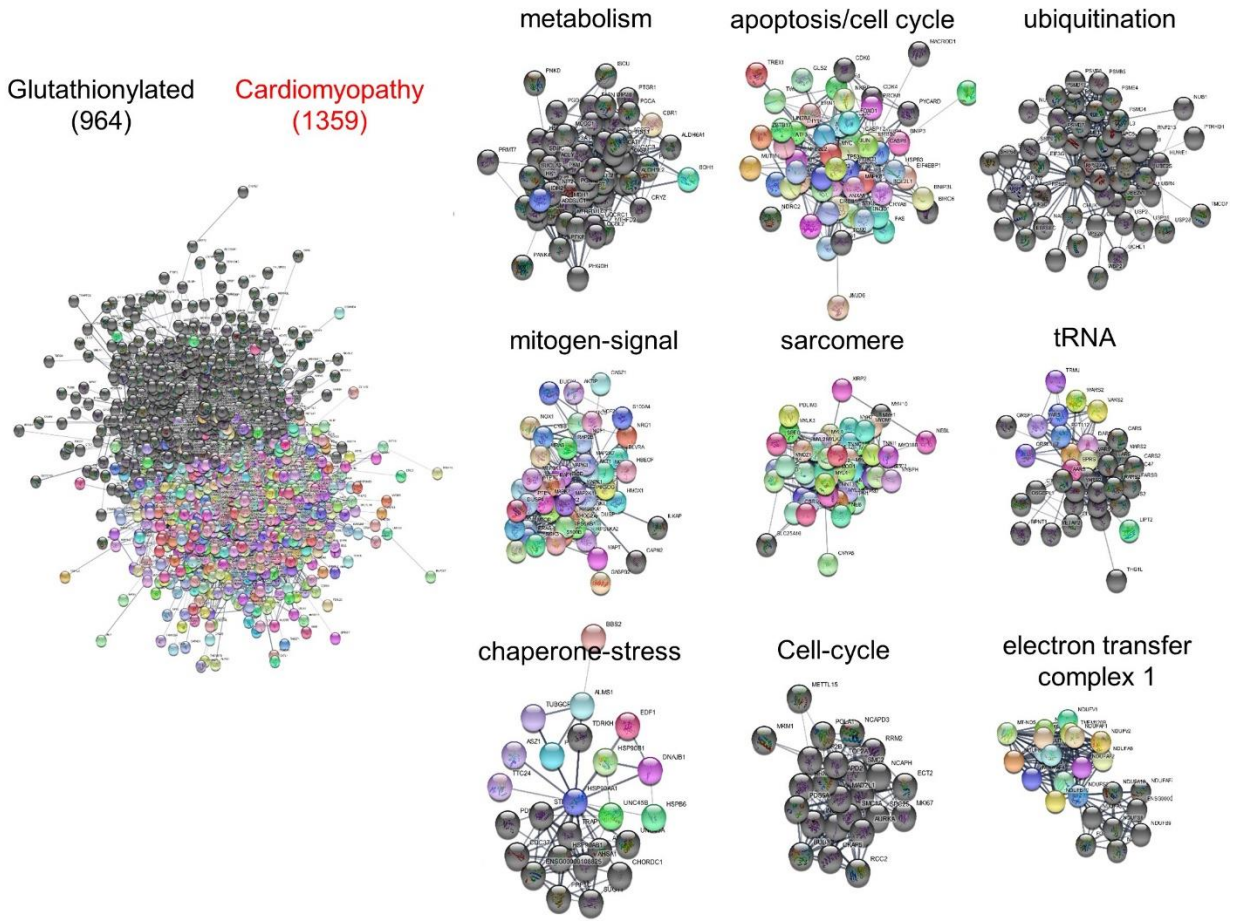


Figure S-7. STRING and cluster analysis of two merged networks, 'glutathionylated proteins' and 'cardiomyopathy-relevant gene'. A list of glutathionylated proteins from mouse HL-1 cells were converted to human orthologs (964 proteins, black circle), which were merged with a list of genes (1,359 proteins, color circle) relevant to cardiomyopathy from STRING disease query. Two merged networks were clustered, and each cluster was assigned by the process associated with genes that belong to individual clusters.

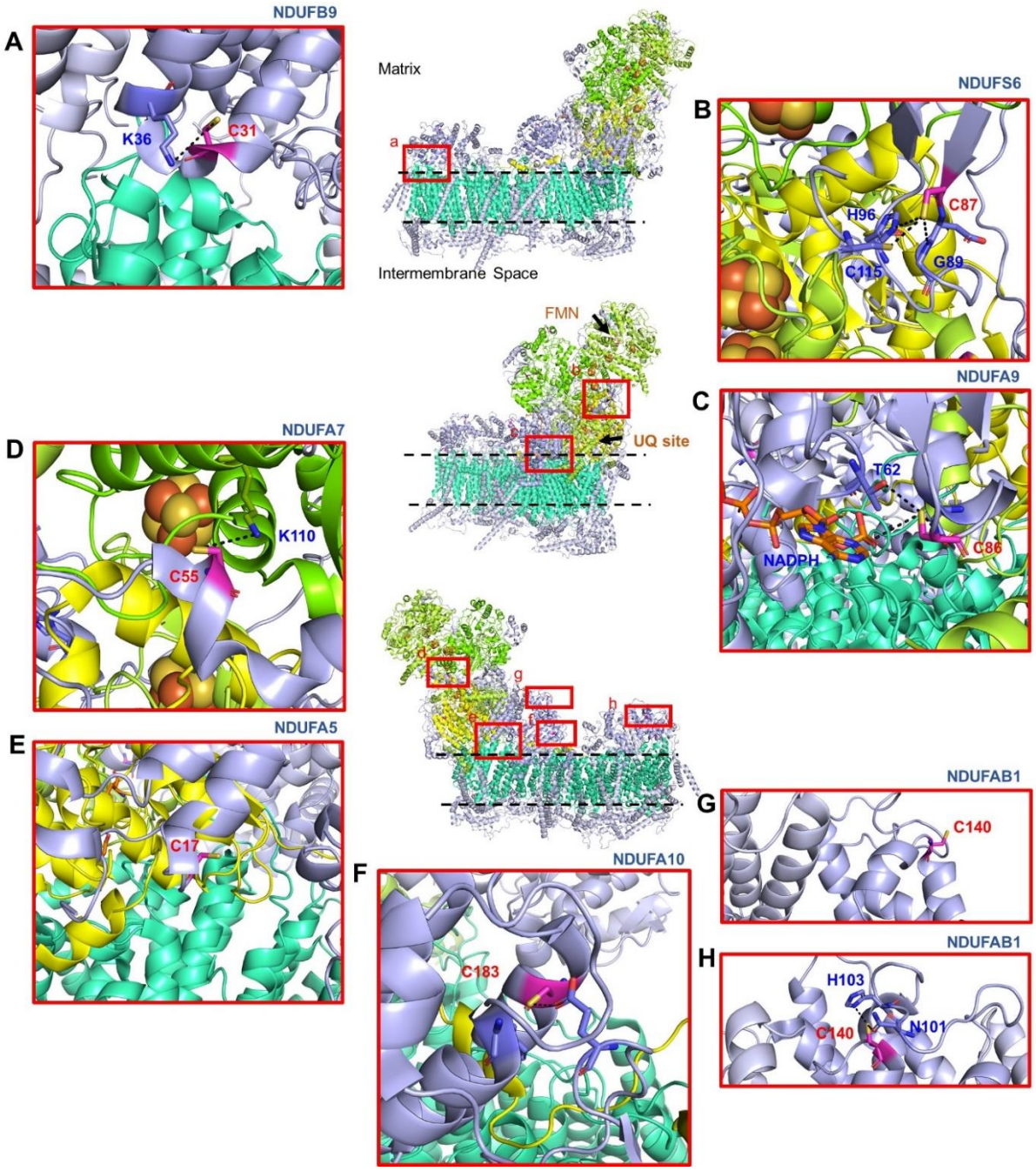


Figure S-8. Analysis of glutathionylated Cys residues in Complex I supernumerary subunits. A structure of bovine complex I (PDB: 5XTD) that contains 14 core subunits (7 cores in matrix, shown in lime, green, yellow, and 7 cores in membrane, shown in greencyan) and 31 supernumerary subunits (light blue). Glutathionylated Cys residues in supernumerary subunits are in rectangular boxes (a-h, red). (**A-H**) The enlarged structures of boxed areas (a-h), showing the position of glutathionylated Cys residues in supernumerary subunits. All numberings are based on the residue number in bovine complex I. Note that C87 in bovine NDUFS6 in (b) corresponds to C79 in mouse NDFUS6.

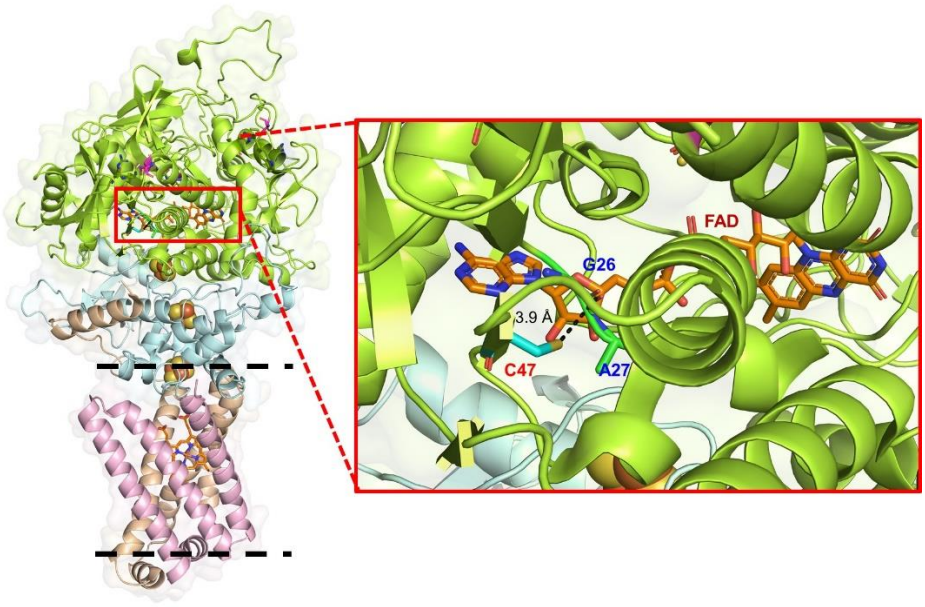


Figure S-9. Analysis of glutathionylation of a previously reported Cys residue in complex II. A structure of porcine complex II (PDB: 1ZOY) with SDHA subunit (green) that contains a previously reported glutathionylation at Cys 90,[2] which corresponds to Cys 47 in porcine structure.

Table S2. A list of 125 identified proteins that belong to both networks of 'glutathionylated proteins' and 'cardiomyopathy'

	Gene	Name	Disease score
O95817	BAG3	BCL2-associated athanogene 3	5.00
P50461	CSRP3	cysteine and glycine-rich protein 3 (cardiac LIM protein)	5.00
Q8NEU6	DES	desmin	5.00
P15924	DSP	desmoplakin	5.00
Q5TCJ2	LMNA	lamin A/C	5.00
A0A0C4DGG7	LDB3	LIM domain binding 3	5.00
Q14896	MYBPC3	myosin binding protein C, cardiac	5.00
Q96DL0	NEXN	nexilin (F actin binding protein)	5.00
P48544	KCNJ5	potassium inwardly-rectifying channel, subfamily J, member 5	5.00
Q92736	RYR2	ryanodine receptor 2 (cardiac)	5.00
Q13424	SNTA1	syntrophin, alpha 1	5.00
P42166	TMPO	thymopoietin	5.00
P12235	SLC25A4	solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 4	4.37
Q14315	FLNC	filamin C, gamma	4.33
F8WCK2	TSFM	Ts translation elongation factor, mitochondrial	4.20
P31040	SDHA	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	4.19
O60313	OPA1	optic atrophy 1 (autosomal dominant)	4.16
P16615	ATP2A2	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	2.15
Q06124	PTPN11	protein tyrosine phosphatase, non-receptor type 11	2.03
H3BQA7	OBSCN	obscurin, cytoskeletal calmodulin and titin-interacting RhoGEF	1.85
P40939	HADHA	hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), alpha subunit	1.82
O43772	SLC25A20	solute carrier family 25 (carnitine/acylcarnitine translocase), member 20	1.81
P02144	MB	myoglobin	1.75
P55084	HADHB	hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), beta subunit	1.60
Q8N904	XIRP1	xin actin-binding repeat containing 1	1.59
O95822	MLYCD	malonyl-CoA decarboxylase	1.54
O43815	STRN	striatin, calmodulin binding protein	1.46
P23381	WARS	tryptophanyl-tRNA synthetase	1.44
Q09EX5	DYSF	dysferlin, limb girdle muscular dystrophy 2B (autosomal recessive)	1.41
P04406	GAPDH	glyceraldehyde-3-phosphate dehydrogenase	1.39
P40763	STAT3	signal transducer and activator of transcription 3 (acute-phase response factor)	1.36
Q92667	AKAP1	A kinase (PRKA) anchor protein 1	1.34
Q15796	SMAD2	SMAD family member 2	1.34
P34932	HSPA4	heat shock 70kDa protein 4	1.34

P10599	TXN	thioredoxin	1.31
P05091	ALDH2	aldehyde dehydrogenase 2 family (mitochondrial)	1.31
Q13557	CAMK2D	calcium/calmodulin-dependent protein kinase II delta	1.27
P08133	ANXA6	annexin A6	1.25
Q8IWX7	UNC45B	unc-45 homolog B (C. elegans)	1.24
Q9NZD0	TP53	tumor protein p53	1.24
Q04446	GBE1	glucan (1,4-alpha-), branching enzyme 1	1.23
Q9UBG5	MYLK	myosin light chain kinase	1.23
P09874	PARP1	poly (ADP-ribose) polymerase 1	1.22
A6NFY7	SDHAF1	succinate dehydrogenase complex assembly factor 1	1.20
Q6PCB7	SLC27A1	solute carrier family 27 (fatty acid transporter), member 1	1.18
O75127	PTCD1	pentatricopeptide repeat domain 1	1.18
Q13418	ILK	integrin-linked kinase	1.18
P11216	PYGB	phosphorylase, glycogen; brain	1.17
Q86YB5	KIAA0391	KIAA0391	1.17
P27797	CALR	calreticulin	1.15
P06732	CKM	creatine kinase, muscle	1.14
Q96HG5	ACTB	actin, beta	1.12
Q53S33	BOLA3	bolA homolog 3 (E. coli)	1.12
Q7L6B3	DNM1L	dynamin 1-like	1.12
P11142	HSPA8	heat shock 70kDa protein 8	1.11
P30825	SLC7A1	solute carrier family 7 (cationic amino acid transporter, y+ system), member 1	1.10
Q9NQC3	RTN4	reticulon 4	1.09
Q6S381	PLEC	plectin	1.08
Q96T22	CASP8	caspase 8, apoptosis-related cysteine peptidase	1.07
Q04917	YWHAH	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide	1.06
Q5HYG0	EIF4G1	eukaryotic translation initiation factor 4 gamma, 1	1.05
Q96HK4	PRDX3	peroxiredoxin 3	1.03
Q02338	BDH1	3-hydroxybutyrate dehydrogenase, type 1	1.02
P53701	HCCS	holocytochrome c synthase	1.00
Q00325	SLC25A3	solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3	0.98
P12829	MYL4	myosin, light chain 4, alkali; atrial, embryonic	0.96
O14558	HSPB6	heat shock protein, alpha-crystallin-related, B6	0.95
P13535	MYH8	myosin, heavy chain 8, skeletal muscle, perinatal	0.89
P07900	HSP90AA1	heat shock protein 90kDa alpha (cytosolic), class A member 1	0.88
Q15942	ZYX	zyxin	0.86
Q01449	MYL7	myosin, light chain 7, regulatory	0.86
P16152	CBR1	carbonyl reductase 1	0.86
P50552	VASP	vasodilator-stimulated phosphoprotein	0.85
Q7M4R4	YWHAE	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide	0.85
Q13496	MTM1	myotubularin 1	0.85

P68104	EEF1A1	eukaryotic translation elongation factor 1 alpha 1	0.84
P12814	ACTN1	actinin, alpha 1	0.83
Q9UDE8	LDHA	lactate dehydrogenase A	0.83
P11474	ESRRA	estrogen-related receptor alpha	0.83
Q7Z3X1	IMMT	inner membrane protein, mitochondrial	0.82
P30084	ECHS1	enoyl CoA hydratase, short chain, 1, mitochondrial	0.81
P42704	LRPPRC	leucine-rich pentatricopeptide repeat containing	0.81
Q9UL46	PSME2	proteasome (prosome, macropain) activator subunit 2 (PA28 beta)	0.81
O94901	SUN1	Sad1 and UNC84 domain containing 1	0.79
P50570	DNM2	dynamin 2	0.78
P05141	SLC25A5	solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5	0.77
P26640	VARS	valyl-tRNA synthetase	0.77
P22033	MUT	methylmalonyl CoA mutase	0.76
Q9BT17	GTP	Mitochondrial GTPase 1	0.75
O75306	NDUFS2	NADH dehydrogenase (ubiquinone) Fe-S protein 2, 49kDa (NADH-coenzyme Q reductase)	0.74
Q9Y4R1	SYNE2	spectrin repeat containing, nuclear envelope 2	0.74
P45974	USP5	ubiquitin specific peptidase 5 (isopeptidase T)	0.73
P55884	EIF3B	eukaryotic translation initiation factor 3, subunit B	0.72
P63261	ACTG1	actin, gamma 1	0.71
Q9H9J2	MRPL44	mitochondrial ribosomal protein L44	0.70
P42226	STAT6	signal transducer and activator of transcription 6, interleukin-4 induced	0.70
Q9UNE7	STUB1	STIP1 homology and U-box containing protein 1, E3 ubiquitin protein ligase	0.68
P17540	CKMT2	creatine kinase, mitochondrial 2 (sarcomeric)	0.68
Q9Y613	FHOD1	formin homology 2 domain containing 1	0.67
P20073	ANXA7	annexin A7	0.65
P62633	CNBP	CCHC-type zinc finger, nucleic acid binding protein	0.63
P28482	MAPK1	mitogen-activated protein kinase 1	0.63
Q86X73	EPRS	glutamyl-prolyl-tRNA synthetase	0.63
P35221	CTNNA1	catenin (cadherin-associated protein), alpha 1, 102kDa	0.62
P60604	UBE2G2	ubiquitin-conjugating enzyme E2G 2	0.62
P48735	IDH2	isocitrate dehydrogenase 2 (NADP+), mitochondrial	0.61
O43583	DENR	density-regulated protein	0.60
Q9BQ52	ELAC2	elaC homolog 2 (E. coli)	0.60
O60664	PLIN3	perilipin 3	0.59
O60841	EIF5B	eukaryotic translation initiation factor 5B	0.59
O75116	ROCK2	Rho-associated, coiled-coil containing protein kinase 2	0.59
Q13541	EIF4EBP1	eukaryotic translation initiation factor 4E binding protein 1	0.59
Q9UEQ5	RBM25	RNA binding motif protein 25	0.58
Q32MK0	MYLK3	myosin light chain kinase 3	0.56
Q9Y490	TLN1	talin 1	0.55

Q6PI48	DARS2	aspartyl-tRNA synthetase 2, mitochondrial	0.55
P21399	ACO1	aconitase 1, soluble	0.55
O14980	XPO1	exportin 1 (CRM1 homolog, yeast)	0.55
P51532	SMARCA4	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4	0.54
Q14318	FKBP8	FK506 binding protein 8, 38kDa	0.54
Q9BT27	MYH14	myosin, heavy chain 14, non-muscle	0.54
Q9NW42	IARS2	isoleucyl-tRNA synthetase 2, mitochondrial	0.53
Q99615	DNAJC7	DnaJ (Hsp40) homolog, subfamily C, member 7	0.51
P13639	EEF2	eukaryotic translation elongation factor 2	0.51
P60660	MYL6	myosin, light chain 6, alkali, smooth muscle and non-muscle	0.50

Supplementary Methods

Cellular thiol concentration. Experiment was done as reported previously.[3,4] Briefly, HL-1 cells with and without infection with Ad-GS M4 were treated with 0.6 mM azido-Ala for 24 h and washed with PBS. Cells were then collected and lysed in PBS by freeze-thaw cycles (x3). Lysates were collected after centrifugation. Lysates were centrifuged with 3K centrifugal filter device to remove proteins, and the filtrate (protein-free lysates) was collected. The total thiol contents were analyzed by fluorescence assay using bromobimane (excitation - 390 nm, emission- 478 nm). Bromobimane assay was performed in the following condition: 0.1 M phosphate buffer, 0.5 mM bromobimane, and 10 μL of filtrate. The fluorescence signal was measured by using a plate reader. All the data was duplicated, and the background signal was corrected.

Western blotting. Cells were lysed in RIPA buffer. Lysates were separated by SDS-PAGE and transferred to PVDF membrane. The membrane was blocked with 5% BSA in TBST, and incubated with primary antibody overnight at 4°C. Primary antibodies include TRX1 antibody (Cell Signaling, 2429S) (1:1000 dilution), FLAG antibody (Sigma, F1804) (1:1000 dilution), GRX1 antibody (Santa Cruz, SC-293250) (1:1000 dilution), Actin antibody (Abcam, EPR16769) (1:1000 dilution), GCLC antibody (Boster Biological Technology, PA1492) (1:500 dilution), CSRP3 antibody (Abcam, ab172952), and α-actinin antibody (Abcam, ab137346). Membrane was incubated with secondary HRP-antibody for 1 h at room temperature. Proteins were visualized by chemiluminescence.

Pull-down of glutathionylated proteins. Glutathionylation of individual proteins were detected by pull-down of glutathionylated proteins, followed by Western blotting with specific antibodies, as described previously.[4] Briefly, lysates containing glutathionylated proteins were precipitated by cold-acetone. Proteins were then re-dissolved in PBS and subjected to click reaction with biotin-alkyne (0.4 mM), CuBr (2 mM), and THPTA (2 mM) for 2 h at room temperature. Proteins in the mixture was then precipitated by cold acetone and re-dissolved in PBS containing 1.2% SDS. The resuspended proteins were then incubated with streptavidin-agarose for 3 h at room temperature. After washing with PBS (5 mL) three times, proteins on beads were eluted by an SDS-loading buffer, resolved on SDS-PAGE and analyzed by Western blotting.

Sequence motif for glutathionylation. Out of 1,767 glutathionylated peptides, 200 peptide sequences were randomly selected (every fifth one in alphabetically listed proteins). The total 17 amino acids containing 8 amino acids before and after glutathionylated Cys residue in individual peptides were aligned. The position of Cys was annotated to be 0. These sequences were then used to generate a frequency plot in the WebLogo online tools.[5]

Supplementary References

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