Discovery of a redox thiol switch: implications for cellular energy metabolism

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Running Title

Quantitative profiling of protein sulfhydromes by the BTA-TMT

Supplemental Figures

Figure S1
Assessment of protein levels in IHH and EndoC-BH3 cells. Western blot analysis for the indicated proteins, of cell extracts isolated from IHH and EndoC-BH3 cells.

Figure S2
Assessment of CRISPR–Cas9-mediated CTH gene knockout in INS1, rat pancreatic beta cells. (**A**) Schematic representation of the rat CTH gene *loci* and two guide RNA targeting sites. The binding sites by the guide RNAs were overlapped with the translation initiation codon in exon1. (**B**) Evaluation of CTH gene knockout by the mismatch detection assay. INS1 cells were transduced with lentivirus to express the indicated sgCTH RNAs, or empty vector (EV) followed by selection with puromycin to enrich for CTH knockouts. Two different PCR primers (set 1 and set 2) were used to amplify the regions flanking the CRISPR targeting sites. Insertion and deletions in these genomic PCR products were detected by the mismatch assay. (**C, D)** Western blot analysis of INS1 cell clones CRISPR-CTH overexpressing ATF4. Individual INS1 cell clones were transfected to express ATF4 for 48h, then lysed. The levels of CTH protein in each clone were evaluated by Western blot analysis.

Figure S3
Verification of CRISPR-cas9 mediated CTH gene knockout in INS1 cells expressing ATF4. Individual clones derived from two different guide RNAs were transfected to express ATF4. The levels of ATF4 protein were evaluated by Western blot analysis.

Figure S4
Detection of glutathionylated proteins by the Biotin Switch Technique (BST). Mouse liver lysates were pretreated with TCEP (20 mM) to remove any reversible cysteine protein modifications. After desalting, the lysates were exposed to GSSG (1 mM) to introduce S-glutathionylation, followed by the BST assay. After biotinylation of GSSG-modified proteins, the lysates were divided into six equal fractions, were bound on streptavidin columns and eluted by the addition of increasing concentrations of TCEP (1-20 mM). The eluates were analyzed by SDS–PAGE electrophoresis and silver stained.

Figure S5
(**A**) Diamide treatment increases oxidation of intracellular glutathione (GSH). INS1 cells were exposed with diamide at the indicated concentrations for 1h. The intracellular GSH/GSSG ratios were quantified by the GSH/GSSG-Glo™ assay. Results are the mean of triplicate determinations. (**B**) Protein synthesis rate is unaffected by diamide treatment.

Protein synthesis was measured by [^{35}S] Met/Cys incorporation into proteins in INS1 cells treated with diamide at the indicated concentrations for 1h. Results are the mean of triplicate determinations.

Figure S6

Optimization of the BioGEE assay for the detection of glutathionylated proteins *in vivo*. (**A**) Optimization of the BioGEE concentrations used for glutathionylated protein labeling by the BioGEE assay. MIN6 cells were treated with diamide (0.1 mM) for 1h, then incubated with various concentrations of BioGEE for 2h. The BioGEE-treated cells were subsequently subjected to the BioGEE assay. The TCEP eluates were analyzed by SDS–PAGE electrophoresis and silver staining of proteins. (**B**) Optimization of the incorporation time for the BioGEE assay. MIN6 cells were treated with diamide (0.1 mM) for 1h, then incubated with BioGEE (0.03 mM) for the indicated times. The BioGEE-treated cells were subsequently subjected to the BioGEE assay. The TCEP eluates were analyzed by SDS–PAGE electrophoresis and silver staining of proteins. (**C**) MIN6 cells were treated with or without diamide (0.1 mM) for 1h, then followed by the BioGEE assay. The levels of glutathionylated GAPDH and PCK2 were analyzed by Western blotting.

Figure S7

Schematic workflow to identify and quantify RTS^{GS} targets by the TMT-BTA assay. MIN6 cell lysates were pretreated with TCEP, then incubated with GSSG. The GSSG-treated lysates were exposed to the H_2S donor at the indicated concentrations (10, 50 and 100 μM, NaHS), then subjected to the TMT-BTA assay. The RTS^{GS} targets were identified and quantified by LC-MS.

Figure S8

Schematic representation of the predicted RTS^{GS} peptides in the eluate of the TMT-BTA assay as a consequence of increasing concentrations of H_2S. At low concentrations (10 μM), the modified cysteine residues including disulfides and S-glutathionylation are discriminated for S-persulfidation (*top*). The modified cysteine residues with lower reactivity (red) should show a concentration-dependent increases in S-persulfidation (*bottom*). (**B**) Model of a high sensitivity peptide containing two modified cysteine residues, one that can be persulfidated at low H_2S concentrations, and a second cysteine residue that is reduced to a free SH group only at high concentrations (100 μM), leading to the peptide retained to the streptavidin beads. This differential response of close proximity cysteine residues, can explain the decreased S-persulfidation observed for some proteins with the TMT-BTA assay.

Figure S9

KEGG pathway analysis of the RTS^{GS} targets from the **Figure S7**. Numbers on top of the bars indicate the number of proteins/category.

Figure S10

Treatment of INS1 cells with H_2S donors inhibits glucose-stimulated insulin secretion (GSIS). INS1 cells were treated with two different H_2S donors (NaHS, GYY4137) at the indicated concentrations for 1h, then subjected to the GSIS assay. The levels of released insulin were quantified by an ELISA assay. 5 replicates of each condition were used.

Figure S11

MS-annotated S-persulfidated human insulin

Supplemental tables

Table S1

Optimization of the reaction buffer used for persulfidated peptide labeling by the iodoTMT tags. Cell extracts treated NaHS (100 μM) were subjected to the TMT-BTA assay. After biotinylation and trypsin digestion, the sample was divided into four equal fractions. Each fraction was incubated with the same amount of streptavidin beads, the biotinylated peptides were eluted with the different buffers as described below:

A. 50 mM TEAB (triethylamonium bicarbonat) and 10 mM TCEP, pH 8.0
B. 50 mM HEPES-NaOH and 10 mM TCEP pH 8.0
C. 50 mM TEAB and 20 mM TCEP, pH 8.0
D. 50 mM HEPES-NaOH and 20 mM TCEP, pH8.0

The BST eluates were mixed with iodoTMT reagents and incubated for 1h. The reactions were stopped by adding DTT (20 mM). After desalting to remove excess chemicals. All the samples were combined for LC-MS identification and quantification.

Table S2

Assessment of the TMT-BTA assay. MIN6 cell extracts treated NaHS (100 μM) were subjected to the TMT-BTA assay. After biotinylation and trypsin digestion, the peptide sample was divided into six equal fractions. Each fraction was incubated with the same amount of streptavidin beads; the biotinylated proteins were incubated with or without TCEP for 45 min. 3 replicates for each condition. The eluates were labeled with iodoTMT 6plex reagents, and then subjected for LC-MS analysis. The samples treated with TCEP were labeled by iodoTMT129-131, the samples treated without TCEP were labeled by iodoTMT 126-128.

Table S3
Quantitative profiling of protein sulfhydromes in MIN6 cells by the TMT-BTA assay. Mouse pancreatic beta cells were incubated with increasing concentrations of NaHS followed by the TMT-BTA method. Control without NaHS (iodoTMT126), 5uM (iodoTMT127), 20 uM (iodoTMT128), 80 uM (iodoTMT129), 200 uM (iodoTMT131).

Table S4
Quantitative profiling of protein sulfhydromes between human hepatocytes and pancreatic beta cells by the TMT-BTA approach. 3 biological replicates of each cell type. Human hepatocytes (IHH, iodoTMT126-128),human pancreatic beta cells (endoC-BH3, iodoTMT129-131).

Table S5
KEGG pathway of highly enriched persulfided proteins from IHH and EndoC-BH3 cells.

Table S6
Label-free MS quantification (LFQ) to determine the relative protein abundance in human hepatocytes (IHH) and pancreatic beta cells (EndoC).

Table S7
KEGG pathway of most abundant proteins in IHH and EndoC-BH3 cells. The most abundant proteins with $\log_2$ ratios greater than 2 were selected for the pathway analysis.

Table S8
Quantitative profiling of protein sulfhydromes in rat pancreatic beta cells (INS1) expressed ATF4 transcription factor. 3 biological replicates of each condition. The TMT-BTA assay was applied to IINS1 cells expressed ATF4 (iodoTMT129-131) or GFP (iodoTMT126-128).

Table S9
Quantitative profiling of protein sulfhydromes in response to the depletion of CTH in ATF4-expressing INS1 cells. 3 biological replicates. The TMT-BTA assay was applied to the CTH KO (sgCTH, iodoTMT129-131) or control (EV, iodoTMT126-128) INS1 cells expressed ATF4.

Table S10
Identification of S-glutathionylated proteins by the BST assay.

Table S11
Absolute metabolite flux of metabolites from MIN6 cells treated as follows: 0.1 mM diamide, 0.1 mM diamide and 10 μM NaHS. Control as untreated.

Table S12
Evaluation of the changes in protein S-persulfidation and S-glutathionylation in rat pancreatic beta cells (INS1) upon exposure to diamide followed by H_2S treatment. The BioGEE and BTA assays were employed to identify S-glutathionylated and S-persulfidated proteins. Eluates were labeled by iodoTMT 6plex tags followed a LC-MS quantification. The BTA assay was employed for identification of S-persulfidated proteins. Control of untreated (iodoTMT126), Diamide-treated (iodoTMT127), diamide and NaHS-treated (iodoTMT128). BioGEE assay was employed for identification of S-glutathionylated proteins. Control of untreated (iodoTMT129), diamide-treated (iodoTMT130), diamide and NaHS-treated (iodoTMT131).

Table S13
Identification and quantification of RTS^{GS} protein targets from S-glutathionylation to S-persulfidation. Lysates from MIN6 cells were exposed to GSSG. An equal amount of GSSG-treated MIN6 cell extracts were incubated with NaHS at different concentrations 10 (iodoTMT126-127), 50 (iodoTMT128-129) and 100 μM (iodoTMT130-131) in duplicates, then subjected to the TMT-BTA assay for LC-MS analysis.

Table S14
Most enriched KEGG pathway among all the TMT-labeled proteins from BioGEE and BTA assays.

Table S15
KEGG pathway of the RTS^{GS} target proteins with great redox sensitive cysteine residues to H_2S

Figure S1

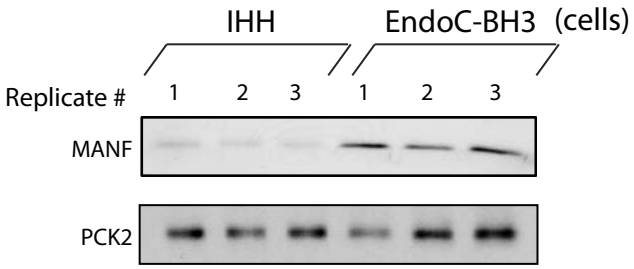
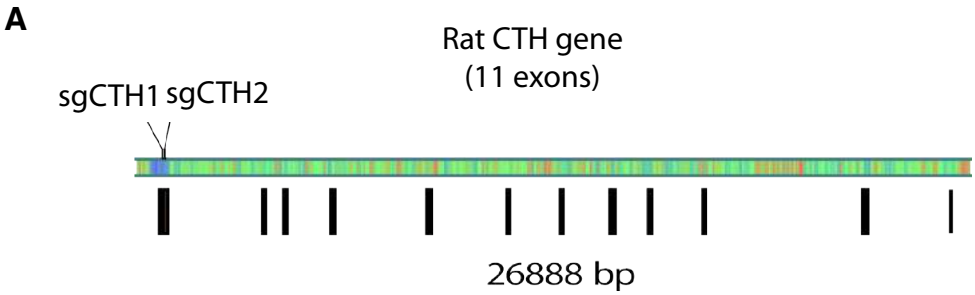
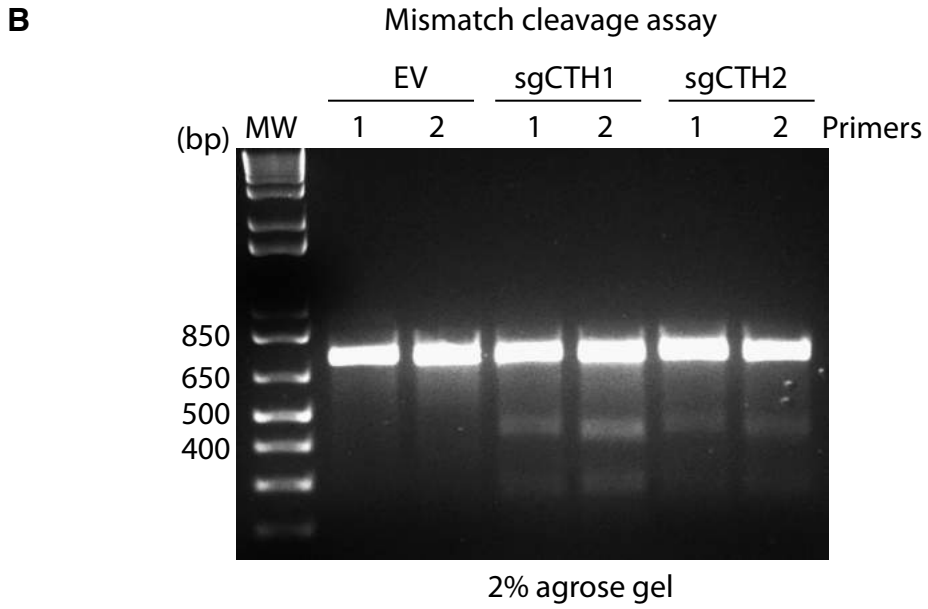
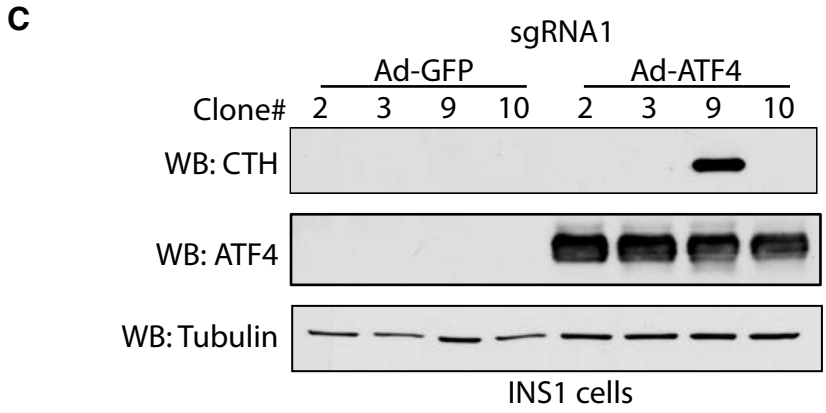


Figure S2


A
Rat CTH gene
(11 exons)
sgCTH1 sgCTH2
26888 bp


B
Mismatch cleavage assay
EV
sgCTH1
sgCTH2
(bp) MW 1 2 1 2 1 2 Primers
850
650
500
400
2% agrose gel


C
sgRNA1
Ad-GFP
Ad-ATF4
Clone# 2 3 9 10 2 3 9 10
WB: CTH
WB: ATF4
WB: Tubulin
INS1 cells

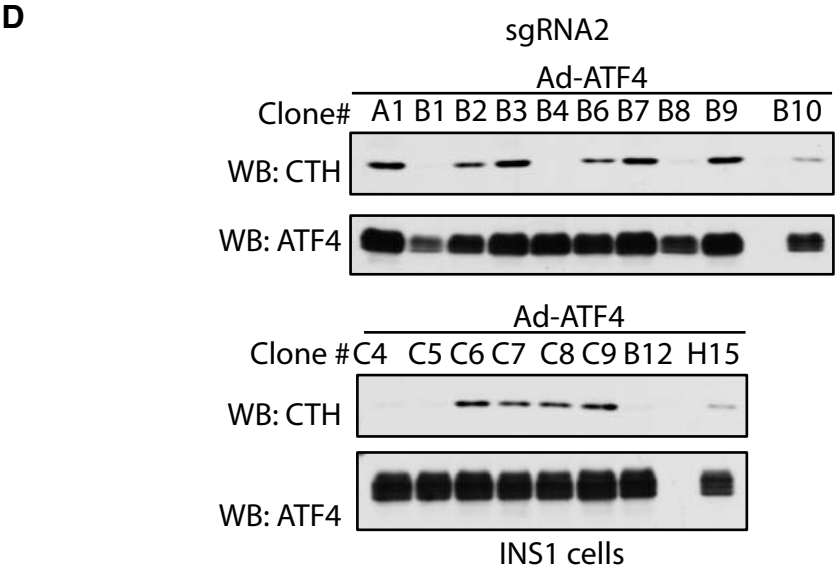

D
sgRNA2
Ad-ATF4
Clone# A1 B1 B2 B3 B4 B6 B7 B8 B9 B10
WB: CTH
WB: ATF4
Ad-ATF4
Clone # C4 C5 C6 C7 C8 C9 B12 H15
WB: CTH
WB: ATF4
INS1 cells

Figure S3

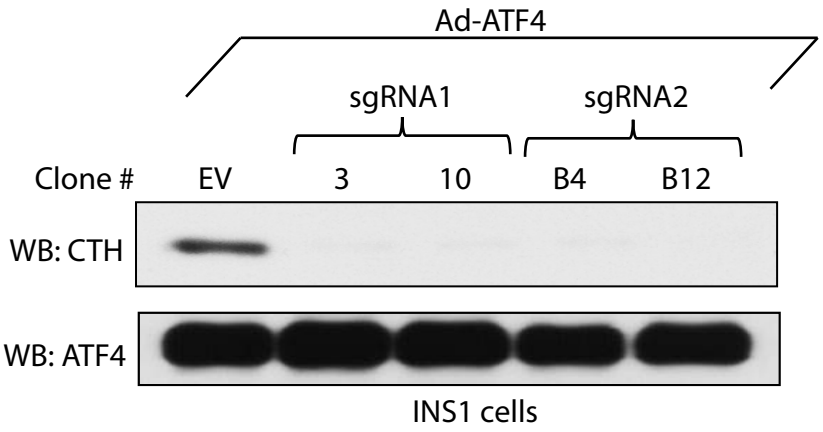
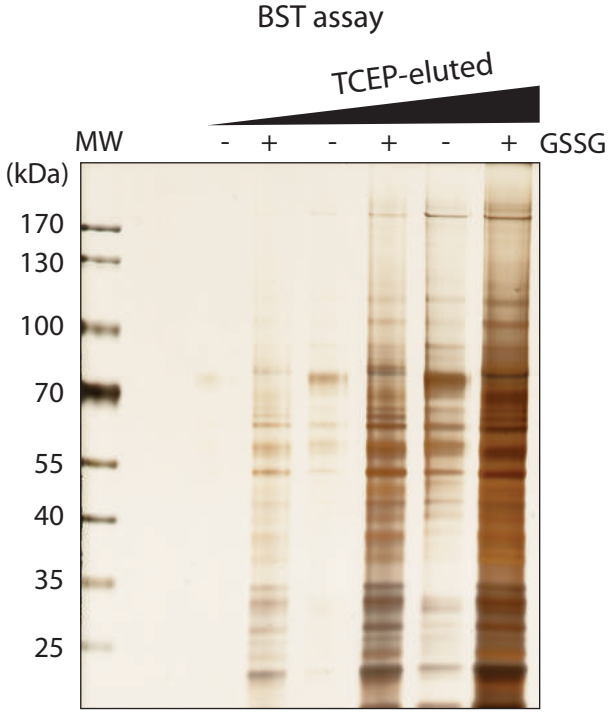

Ad-ATF4
sgRNA1
sgRNA2
Clone #
EV
3
10
B4
B12
WB: CTH
WB: ATF4
INS1 cells

Figure S4



Silver stained gel

Figure S5

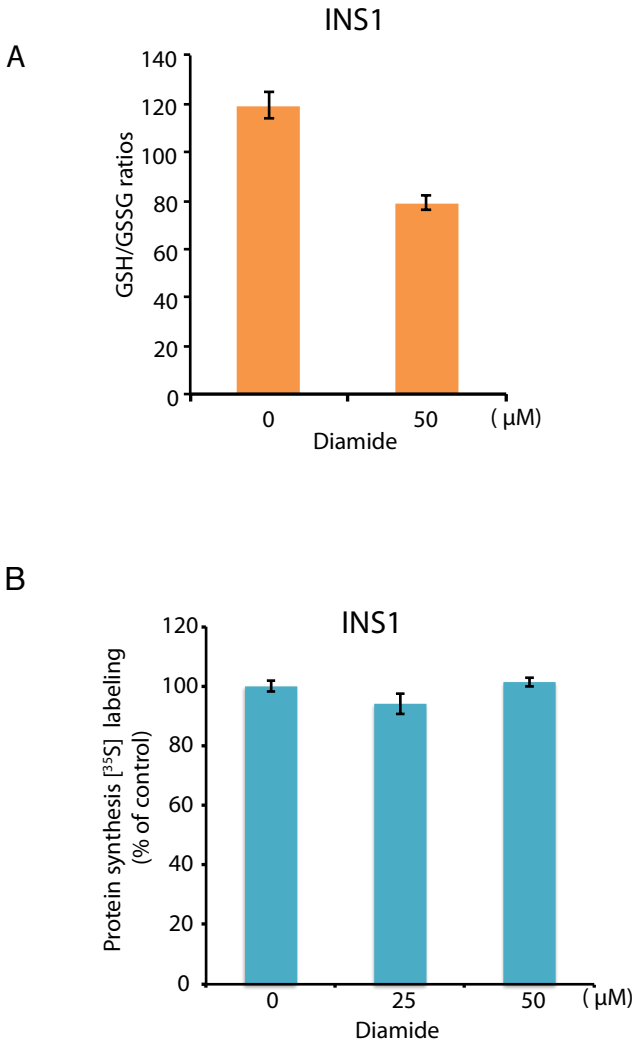
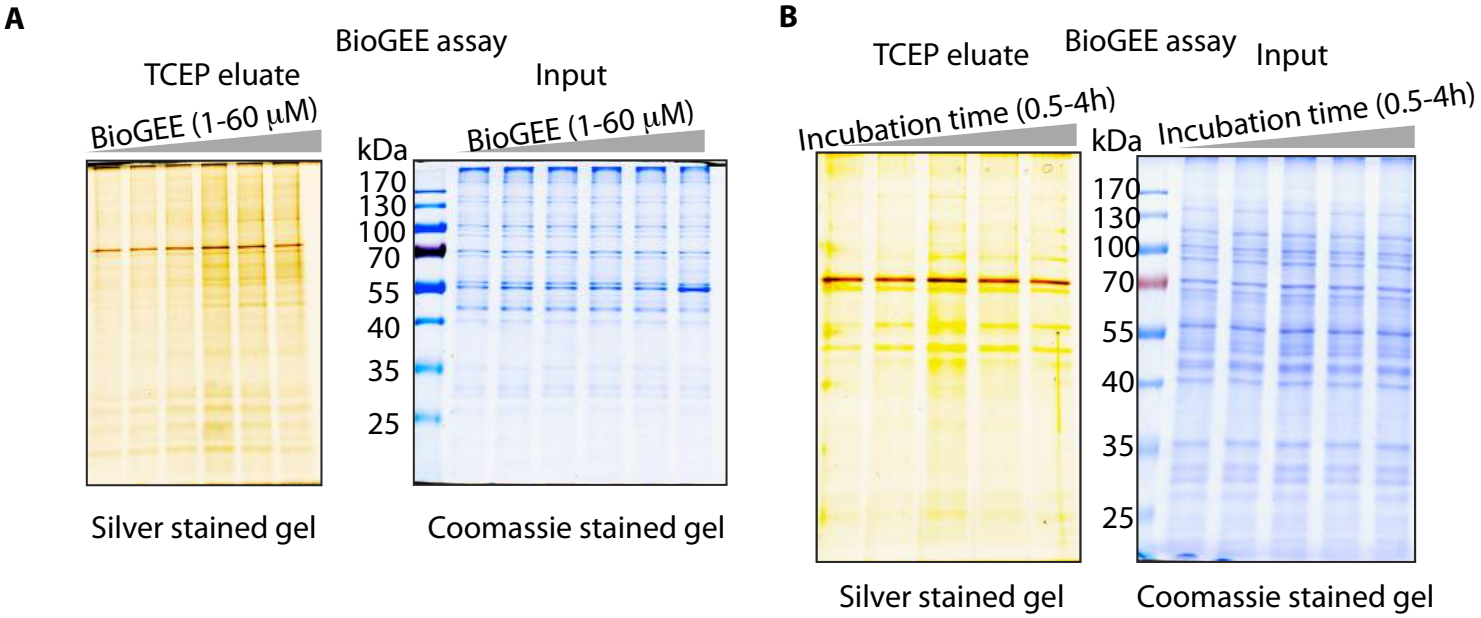

A
INS1
GSH/GSSG ratios
140
120
100
80
60
40
20
0
0
50
(μM)
Diamide
B
INS1
Protein synthesis [35S] labeling
(% of control)
120
100
80
60
40
20
0
0
25
50
(μM)
Diamide

Figure S6


A
BioGEE assay
TCEP eluate
Input
BioGEE (1-60 μM)
BioGEE (1-60 μM)
kDa
170
130
100
70
55
40
35
25
Silver stained gel
Coomassie stained gel
B
TCEP eluate
BioGEE assay
Input
Incubation time (0.5-4h)
Incubation time (0.5-4h)
kDa
170
130
100
70
55
40
35
25
Silver stained gel
Coomassie stained gel

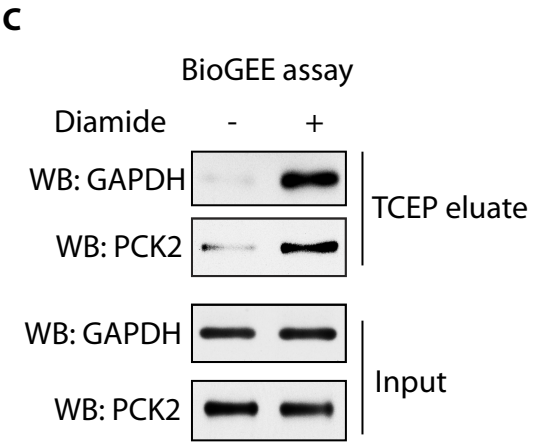

C
BioGEE assay
Diamide
-
+
WB: GAPDH
WB: PCK2
TCEP eluate
WB: GAPDH
WB: PCK2
Input

Figure S7

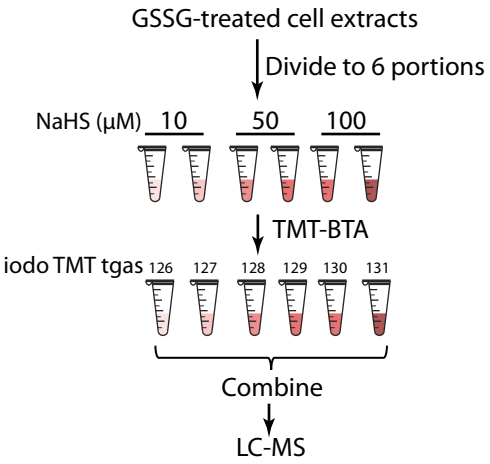

GSSG-treated cell extracts
Divide to 6 portions
NaHS (μM)
10
50
100
TMT-BTA
iodo TMT tgas
126
127
128
129
130
131
Combine
LC-MS

Figure S8

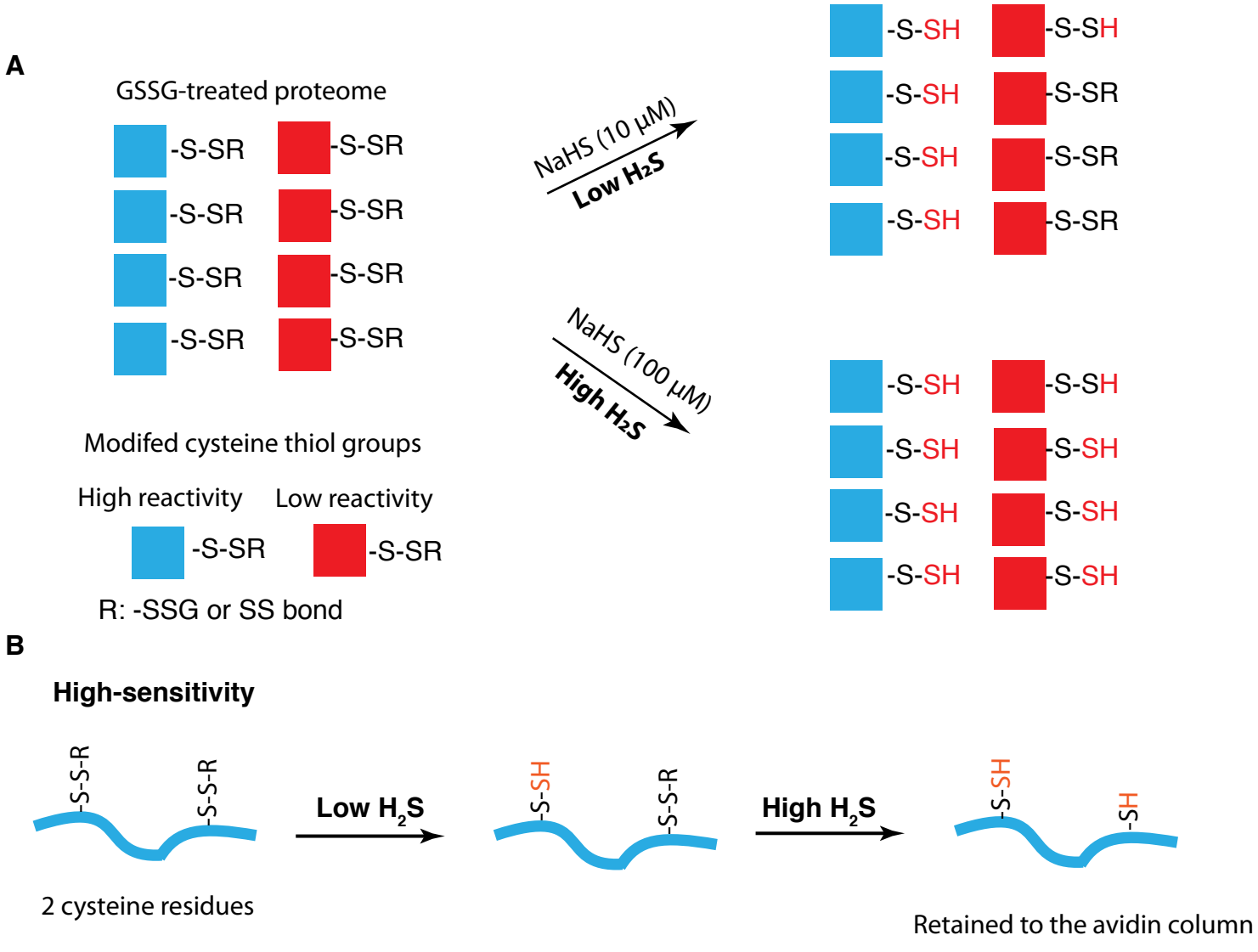
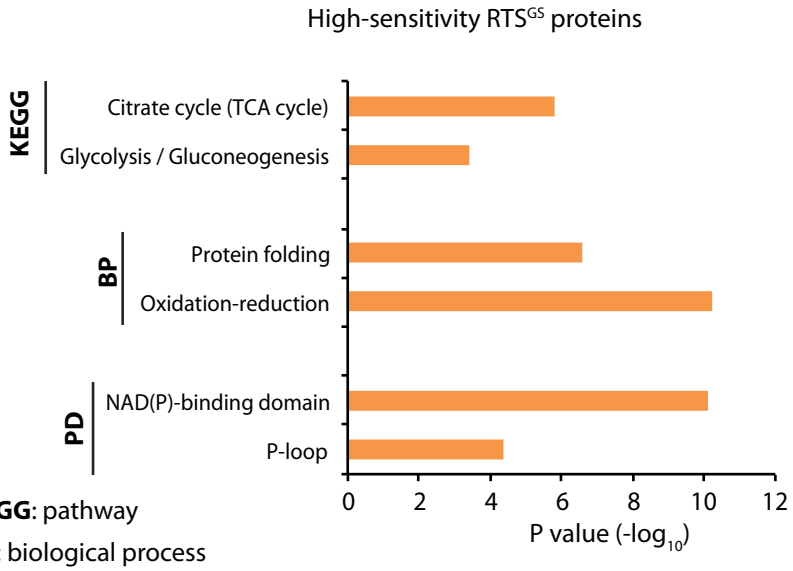

A
GSSG-treated proteome
-S-SR
-S-SR
-S-SR
-S-SR
-S-SR
-S-SR
-S-SR
-S-SR
NaHS (10 μM)
Low H2S
NaHS (100 μM)
High H2S
-S-SH
-S-SR
-S-SH
-S-SR
-S-SH
-S-SR
-S-SH
-S-SR
-S-SH
-S-SH
-S-SH
-S-SH
-S-SH
-S-SH
-S-SH
-S-SH
Modifed cysteine thiol groups
High reactivity
Low reactivity
-S-SR
-S-SR
R: -SSG or SS bond
B
High-sensitivity
-S-S-R
-S-S-R
Low H2S
-S-SH
-S-S-R
High H2S
-S-SH
-SH
2 cysteine residues
Retained to the avidin column

Figure S9



KEGG: pathway

BP: biological process

PD: protein domain

Figure S10

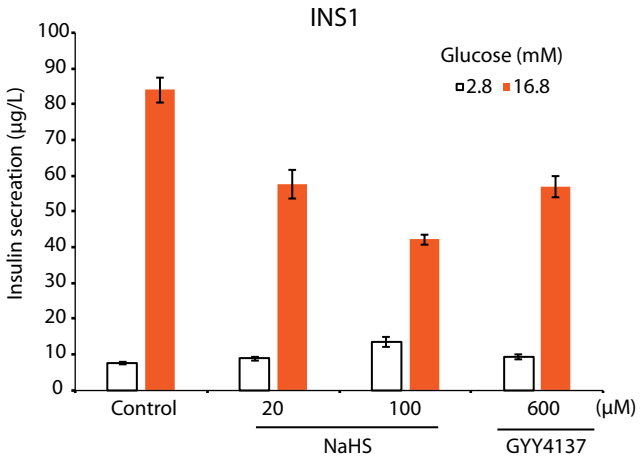
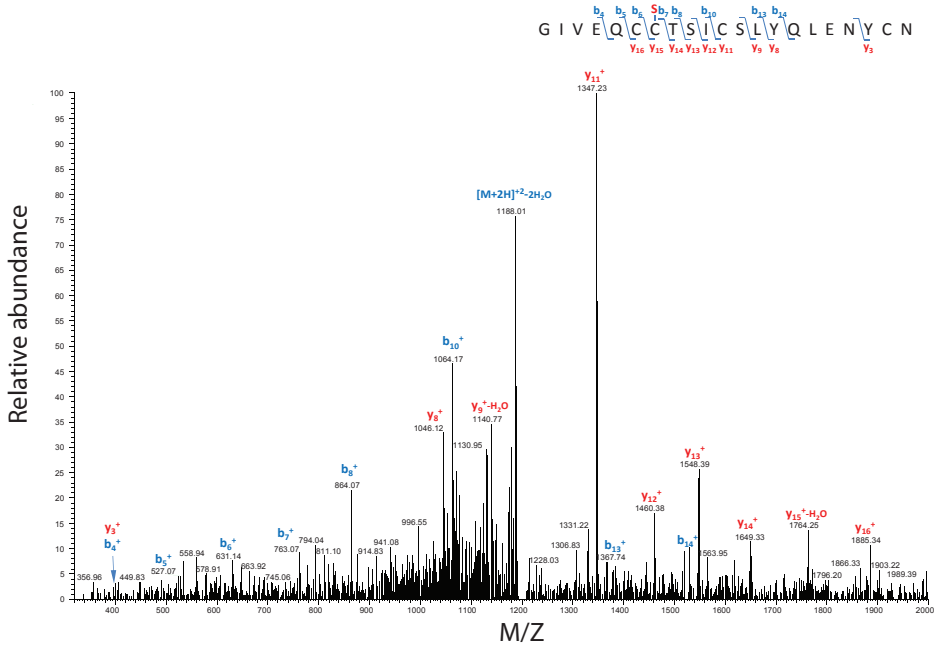

INS1
Glucose (mM)
2.8
16.8
Insulin secreation (μg/L)
100
90
80
70
60
50
40
30
20
10
0
Control
20
100
600
(μM)
NaHS
GYY4137

Figure S11

Human recombinant insulin treated with NaHS



Human recombinant insulin treated with CTH and L-cysteine