Quantitative H$_2$S-mediated protein sulfhydration reveals metabolic reprogramming during the Integrated Stress Response

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Abstract

The sulfhydration of cysteine residues in proteins is an important mechanism involved in diverse biological processes. We have developed a proteomics approach to quantitatively profile the changes of sulfhydrated cysteines in biological systems. Bioinformatics analysis revealed that sulfhydrated cysteines are part of a wide range of biological functions. In pancreatic β cells exposed to endoplasmic reticulum (ER) stress, elevated H_2S promotes the sulfhydration of enzymes in energy metabolism and stimulates glycolytic flux. We propose that transcriptional and translational reprogramming by the Integrated Stress Response (ISR) in pancreatic β cells is coupled to metabolic alternations triggered by sulfhydration of key enzymes in intermediary metabolism.

3

48 **Introduction**

49 Posttranslational modification is a fundamental mechanism in the regulation of

50 structure and function of proteins. The covalent modification of specific amino

51 acid residues influences diverse biological processes and cell physiology across

52 species. Reactive cysteine residues in proteins have high nucleophilicity and low

53 pKa values and serve as a major target for oxidative modifications, which can

54 vary depending on the subcellular environment, including the type and intensity

55 of intracellular or environmental cues. Oxidative environments cause different

56 post-translational cysteine modifications, including disulfide bond formation (-S-

57 S-), sulfenylation (-S-OH), nitrosylation (-S-NO), glutathionylation (-S-SG), and

58 sulfhydration (-S-SH) (also called persulfidation) (Finkel, 2012; Mishanina et al.,

59 2015). In the latter, an oxidized cysteine residue included glutathionylated,

60 sulfenylated and nitrosylated on a protein reacts with the sulfide anion to form a

61 cysteine persulfide. The reversible nature of this modification provides a

62 mechanism to fine tune biological processes in different cellular redox states.

63 Sulfhydration coordinates with other post-translational protein modifications such

64 as phosphorylation and nitrosylation to regulate cellular functions (Altaany et al.,

65 2014; Sen et al., 2012). Despite great progress in bioinformatics and advanced

66 mass spectroscopic techniques (MS), identification of different cysteine-based

67 protein modifications has been slow compared to other post-translational

68 modifications. In the case of sulfhydration, a small number of proteins have been

69 identified, among them the glycolytic enzyme glyceraldehyde phosphate

70 dehydrogenase, GAPDH (Mustafa et al., 2009). Sulfhydrated GAPDH at Cys^{150}

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71 exhibits an increase in its catalytic activity, in contrast to the inhibitory effects of

72 nitrosylation or glutathionylation of the same cysteine residue (Mustafa et al.,

73 2009; Paul and Snyder, 2012). The biological significance of the Cys[150]

74 modification by H_2S is not well-studied, but H_2S could serve as a biological switch

75 for protein function acting via oxidative modification of specific cysteine residues

76 in response to redox homeostasis (Paul and Snyder, 2012). Understanding the

77 physiological significance of protein sulfhydration requires the development of

78 genome-wide innovative experimental approaches. Current methodologies based

79 on the modified biotin switch technique do not allow detection of a broad

80 spectrum of sulfhydrated proteins (Finkel, 2012). Guided by a previously reported

81 strategy (Sen et al., 2012), we developed an experimental approach that allowed

82 us to quantitatively evaluate the sulfhydrated proteome and the physiological

83 consequences of H_2S synthesis during chronic ER stress. The new methodology

84 allows a quantitative, close-up view of the integrated cellular response to

85 environmental and intracellular cues, and is pertinent to our understanding of

86 human disease development.

87 The ER is an organelle involved in synthesis of proteins followed by various

88 modifications. Disruption of this process results in the accumulation of misfolded

89 proteins, causing ER stress (Tabas and Ron, 2011; Walter and Ron, 2011),

90 which is associated with development of many diseases ranging from metabolic

91 dysfunction to neurodegeneration (Hetz, 2012). ER stress induces

92 transcriptional, translational, and metabolic reprogramming, all of which are

93 interconnected through the transcription factor Atf4. Atf4 increases expression of

94   genes promoting adaptation to stress via their protein products. One such gene

95   is the H$_2$S-producing enzyme, γ-cystathionase (CTH), previously shown to be

96   involved in the signaling pathway that negatively regulates the activity of the

97   protein tyrosine phosphatase 1B (PTP1B) via sulfhydration (Krishnan et al.,

98   2011). We therefore hypothesized that low or even modest levels of reactive

99   oxygen species (ROS) during ER stress may reprogram cellular metabolism via

100   H$_2$S-mediated protein sulfhydration (Figure 1A).

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102   **Results**

103   We have previously shown that the insulin-producing mouse pancreatic β cells

104   MIN6, known for their high metabolic activity, are very susceptible to ER stress

105   (Guan et al., 2014; Krokowski et al., 2013a). We tested whether or not MIN6 cells

106   expressed the essential components of H$_2$S synthesis and protein sulfhydration

107   in response to ER stress induced by thapsigargin (Tg, Figure 1). Upon Tg

108   treatment, MIN6 cells exhibited higher levels of intracellular ROS, decreased

109   GSH/GSSG ratios, and increased CTH protein levels via transcriptional activation

110   (Figure 1B-D, Figure 1-figure supplement 1 and 2A). This transcriptional

111   reprogramming also was seen in mouse and human islets subjected to

112   physiological or pharmacologically-induced ER stress (Figure 1D, Figure 1-figure

113   supplement 2B-C and 3). In agreement with increased CTH expression, H$_2$S

114   levels increased during the chronic phase of the stress response (Figure 1E).

115   Coordinated induction of expression of the gene encoding the glutamate/cystine

116   exchanger, Slc7a11, also was increased (Figure 1D), and this induction was

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117   associated with increased glutamate/cystine flux (Figure 1- figure supplement 4).

118   Slc7a11 mediates the exchange of oxidized extracellular cystine with intracellular

119   glutamate. Once cystine is imported into the cells it gets reduced to cysteine,

120   then serves as a substrate for GSH and H_2S synthesis. These data support the

121   idea that both increased uptake of the CTH substrate (cysteine) and increased

122   levels of CTH contribute to increased H_2S levels in cells under ER stress.

123   The functional significance of increased H_2S synthesis was shown by measuring

124   the catalytic activity of GAPDH (Mustafa et al., 2009), which gradually increased

125   in response to elevated H_2S production in MIN6 cells during ER stress (Figure

126   1F). This increase in activity was independent of GAPDH protein levels (Figure

127   1F). As noted above, GAPDH has the unusual feature of being catalytically

128   inactive when Cys^{150} is oxidatively modified, except when it undergoes

129   sulfhydration which restores/increases its activity (Mustafa et al., 2009). We

130   therefore tested the protective effects of Cys^{150} sulfhydration by H_2S on its

131   catalytic activity in the presence of H_2O_2-induced oxidation. Recombinant

132   GAPDH was incubated with H_2O_2 in the presence or absence of the H_2S donor,

133   NaHS. The inhibition of GAPDH activity by H_2O_2 was significantly reversed by

134   H_2S treatment (Figure 1G). Furthermore, incubation of purified GAPDH with

135   oxidized glutathione (GSSG) resulted in formation of inactive glutathionylated

136   GAPDH (Gao et al., 2010), which was significantly rescued by treatment with H_2S

137   as well as DTT reduction (Figure 1H). HPLC-MS confirmed that recombinant

138   GAPDH exposed to NaHS or GSSG was modified predominantly at Cys^{150}

139   (Figure 1-figure supplement 5, Figure 2-figure supplement 7B-C). These data

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140　confirm that H_2S is a positive regulator of GAPDH activity. Increased GAPDH

141　activity is directly linked to H_2S production, as shown by the loss of induction

142　(Figure 1I) upon treatment with the CTH inhibitor, propargylglycine (PAG). PAG

143　inhibits H_2S synthesis and therefore is expected to decrease Cys^{150} modification.

144　Taken together, these results indicate that regulation of H_2S synthesis during ER

145　stress might regulate the catalytic activity of other metabolic pathway proteins.

146　The latter is raising the possibility that the ATF4-mediated sulfhydration of

147　proteins is part of the Integrated Stress Response (ISR), and has regulatory

148　effects on cellular metabolism.

149　Atf4 increases gene expression of CTH (Dickhout et al., 2012), the cystine

150　transporter Slc7a11, as well as the ROS-producing enzyme Ero1α (Han et al.,

151　2013; Tabas and Ron, 2011). We hypothesized that during ER stress this

152　network of ATF4 target genes promotes protein sulfhydration (Figure 2A).

153　Knockdown of ATF4 in MIN6 cells during Tg-induced ER stress caused inhibition

154　of H_2S synthesis with a parallel loss of induction of CTH protein levels (Figure

155　2B). In contrast, in the absence of stress, ATF4 overexpression increased CTH

156　and Ero1α levels and H_2S synthesis, in agreement with increased GAPDH

157　activity (Figure 2C-E, Figure 2-figure supplement 1). The levels of GSH and the

158　activity of the glutamate/cystine exchanger also increased with ATF4

159　overexpression (Figure 2F-G). These data support the hypothesis that ATF4 is a

160　master regulator of protein sulfhydration in pancreatic β cells during ER stress.

161　To profile genome-wide changes in protein sulfhydration by ER stress and the

162　integrated stress response, we exploited the different reactivity of cysteine

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163 persulfides (Cys-S-SH) and thiols (Cys-SH) to alkylating agents (Nishida et al.,

164 2012; Pan and Carroll, 2013; Paul and Snyder, 2012), to develop a new thiol

165 reactivity-based approach called BTA (Biotin Thiol Assay, (Figure 2H and Figure

166 2-figure supplement 2). We employed the following steps (Figure 2H): (1)

167 labeling of reactive Cys-SH or Cys-S-SH groups by a biotin-conjugated

168 maleimide (maleimide-PEG2-biotin, NM-biotin) (Weerapana et al., 2010), (2)

169 binding of the biotin-labeled proteins on an avidin column, (3) elution of the

170 retained proteins that contain a persulfide bridge using DTT and (4) analysis of

171 the eluted proteins.

172 The BTA approach was validated by testing the hypothesis that a lower

173 concentration of the thiol-alkylating reagent (NM-biotin) will result in a selective

174 labeling of highly reactive cysteine SH groups, and that this selectivity will be lost

175 as the concentration of NM-biotin increases (Weerapana et al., 2010). We tested

176 this hypothesis in extracts isolated from Tg-treated MIN6 cells in the presence of

177 increasing concentrations of NM-biotin (0.05 to 1 mM). The eluate containing the

178 sulfhydrated proteins was analyzed with SDS-PAGE and the proteins were

179 visualized by Coomassie blue staining. Indeed, increasing the concentration of

180 NM-biotin beyond 0.5 mM decreased the levels of eluted proteins (Figure 2-figure

181 supplement 3), consistent with cysteine residues having free -SH groups

182 becoming alkylated at high concentrations of NM-biotin. No protein was eluted

183 from the column with the addition of DTT at high concentrations of NM-biotin

184 (Figure 2-figure supplement 3), confirming that the biotin was attached to

185 proteins via a disulfide bond (Figure 2-figure supplement 4A). This selectively

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186   labeling behavior not only relies on the probe concentration, but also is

187   dependent on the protein structure conformations. We found that no protein was

188   detected in the eluates if the BTA was performed on the denatured cell extracts

189   (data not shown).

190   When Tg-treated MIN6 lysates were pretreated with DTT (to decrease the level

191   of sulfhydrated proteins), the signal was significantly reduced compared to

192   untreated lysates (Figure 2-figure supplement 4B), confirming that DTT reduces

193   an intermolecular disulfide bond of cysteine persulfides labeled with biotin. These

194   data show that sulfhydrated cysteine residues are the primary targets of NM-

195   biotin at low concentrations, thus making the BTA a unique tool to identify the

196   sulfhydrated proteome (Figure 2-figure supplement 2).

197   We next assessed the BTA assay using recombinant GAPDH, which contains six

198   cysteine residues including the redox-regulated Cys[150]. GAPDH was incubated

199   with either H_2O_2 or NaHS. The presence of sulfhydrated GAPDH was evaluated

200   by the BTA method following Western blot analysis. We found that only the

201   NaHS-treated GAPDH was eluted as an H_2S-modified target, indicating that the

202   assay distinguishes sulfhydration or free SH groups from other forms of cysteine

203   modifications (Figure 2-figure supplement 5B). This was independently confirmed

204   with the red malemide assay (Figure 2-figure supplement 6), which discriminates

205   between free -SH groups and sulfhydrated-SH (Sen et al., 2012). We next tested

206   if the BTA can identify modification of a specific cysteine on GAPDH by using

207   recombinant wild-type and Cys[150]Ser GAPDH mutant, which were incubated with

208   H_2O_2 or NaHS. We found that NaHS treatment induced sulfhydration in the wild-

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209 type GAPDH (Figure 2-figure supplement 7A), a result that was also confirmed

210 by high-resolution quadruple MS analysis (Figure 2-figure supplement 7B-C), and

211 that this modification was absent in the Cys^{150}Ser mutant. Finally, a proteome-

212 wide view of sulfhydration was obtained from Tg-treated MIN6 cell extracts

213 subjected to BTA and further analyzed by LC-MS/MS (Figure 2-figure

214 supplement 8). We identified 150 proteins, including several known targets for

215 sulfhydration (Figure 2-figure supplement 8D) (Mustafa et al., 2009). Similar

216 results were obtained from analysis of mouse liver (data not shown), a tissue

217 known to exhibit high levels of H_2S synthesis (Kabil et al., 2011). Taken together,

218 this shows that BTA discriminates between protein sulfhydration and other

219 oxidative modifications.

220 Next, the BTA methodology identified the sulfhydrated proteome downstream of

221 the transcription factor Atf4 during ER stress (Figure 2A). MIN6 cells treated with

222 Tg increased GAPDH levels in the DTT eluate, that was abolished by knocking

223 down *Atf4* (Figure 2I). In the absence of stress, ATF4 knockdown resulted in an

224 increase in sulfhydrated GAPDH in MIN6 cells (Figure 2I). Because ATF4-

225 deficient cells have decreased levels of sulfur-containing amino acids (Harding et

226 al., 2003), and sulfur amino acid restriction is linked to an increase in the

227 transulfuration pathway (Hine et al., 2015), it is possible that the increase in

228 GAPDH sulfhydration in ATF4-depleted cells is due to activation of CBS (Niu et

229 al., 2015), the second cytosolic H_2S-producing enzyme. Moreover, we found that

230 in the absence of stress ATF4 overexpression induced GAPDH sulfhydration that

231 is dependent on the CTH activity (Figure 2J-K). The use of PAG decreased

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232   GAPDH sulfhydration (Figure 2K). We conclude that sulfhydration of proteins

233   during ER stress is part of the ISR and is controlled by the transcription factor,

234   Atf4.

235   The BTA requires determining the concentration of NM-Biotin for selectively

236   labeling proteins with reactive, sulfhydrated cysteines rather than the relatively

237   high abundant and less reactive, unmodified (with free SH groups) cysteine

238   residues. However, this labeling step has some limitations under certain

239   circumstances. For example, if free Cys-SH groups are biotinylated on the same

240   protein containing one or more Cys-S-SH groups (Figure 2H and Figure 2-figure

241   supplement 2), then the protein will not be eluted with DTT and will not be

242   identified as a target for sulfhydration. In addition, if a protein contains

243   sulfhydrated cysteines with low reactivity for the probe, this protein will not be

244   captured and identified as an H_2S-modified target. Due to those limitations and

245   in order to extend the capability of the BTA approach, we introduced a proteolytic

246   digestion step before applying the avidin column step. This added step provided

247   not only the isolation of cysteine-containing peptides with persulfide bonds, but

248   also increased the identification of sulfhydrated proteins. The eluted peptides

249   were then sequenced and identified by LC-MS/MS analysis, thereby identifying

250   the modified cysteines on proteins. By using the modified BTA technique, we

251   have identified over ~ 1,000 novel sulfhydrated cysteines in MIN6 cells treated

252   with Tg, corresponding to about 820 proteins (Figure 2- figure supplement 9 and

253   source data 1), including GAPDH, wherein two cysteine-containing peptides were

254   captured: Cys^{150} and Cys^{245}. Remarkably, the Cys^{150} active-site peptide was

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255   highly enriched as compared to the C-terminal of Cys^{245}, supporting prior

256   mutagenesis studies that have shown Cys^{150} as the primary H_2S-modified site on

257   GAPDH, both in vitro and in vivo. One of novel targets including

258   phosphoglycerate dehydrogenase (PHGDH) was also confirmed by Western blot

259   analysis (Figure 2- figure supplement 10). CTH Knockdown mediated by shRNA

260   decreased GAPDH and PHGDH sulfhydration levels, confirming that those

261   proteins identified by the BTA method are bona-fide targets for sulfhydration in

262   vivo (Figure 3-figure supplement 11). Finally, there is no correlation between

263   protein abundance and sulfhydrome (Figure 3-figure supplement 6-7 and source

264   data 2), supporting that lower concentrations of the NM-biotin labeling reveal

265   reactivity of cysteine residues rather than protein abundance.

266   In order to obtain genome-wide stress-induced changes in the sulfhydrome and

267   individual cysteine residues within these proteins, we devised a modified BTA

268   protocol (Figure 3A) by introducing a stable isotope-labeling step after the DTT

269   elution step. This protocol uses (1) digestion of biotinylated cell extracts with

270   trypsin before avidin capture, (2) labeling of free Cys-SH groups on peptides in

271   the column eluent by mass-difference cysteine-alkylating reagents with either

272   NEM-H$_5$ (<u>L</u>ight) or NEM-D$_5$ (<u>H</u>eavy) and (3) quantification by LC-MS/MS analysis

273   of H/L ratios of the individual pair-labeled cysteines in the identified peptides

274   based on a mass-difference labeling. In addition to quantifying changes in protein

275   sulfhydration, this modified BTA approach allows the detection of additional

276   proteins. We used as an experimental system the ATF4-expressing MIN6 cells to

277   profile quantitatively the sulfhydrated proteome. As shown in Figure 2A, ATF4-

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278   mediated signaling triggers the cellular response, which leads to increased

279   protein sulfhydration. Using the experimental plan in Figure 3A, we identified over

280   834 cysteine-containing peptides (Figure 3B and source data 1). Of these

281   peptides, 771 exhibited pair-labeling with an overall average H/L ratio 2.6, and

282   348 peptides (45%) displayed high ratios (H/L>2). These findings confirmed that

283   ATF4 drives global changes in protein sulfhydration in MIN6 cells.

284   Sulfhydrated cysteine residues in proteins may contribute to their biological

285   activities, especially when these modified cysteine residues reside within

286   functional domains. We thus queried the Uniprot database to retrieve functional

287   annotations for the aforementioned 827 peptides. The analysis revealed that 28%

288   of the peptides were localized to protein regions whose structural and functional

289   properties are known (Figure 3C, Figure 3-figure supplement 1). An additional

290   18% were found within functional regions of proteins with cysteine residues of

291   unknown significance. Our finding of sulfhydration of specific cysteine residues

292   within functional domains of proteins suggests that the cysteine modification

293   influences the activity of proteins. In contrast, 4.2% of the peptides in question

294   contained cysteine residues in experimentally proven active sites of enzymes or

295   cysteine residues involved in disulfide bond formation (Figure 3C). This

296   percentage of active cysteine-containing peptides from the BTA assay is

297   significantly larger than the 0.2% of all cysteines in the Uniprot database that

298   have been assigned to experimentally characterized active cysteines

299   (Weerapana et al., 2010). Finally, 2.4% of the annotated peptides were found in

300   known nucleotide binding domains of proteins, suggesting the potential for

301   regulation of gene expression by H_2S cysteine-modified proteins. None of these

302   proteins have been reported as targets of sulfhydration. Although not found

303   among the 827 peptides in this study, the NF-κB RelA transcription factor has

304   been shown to be sulfhydrated in the DNA-binding domain, resulting in increased

305   DNA-binding activity (Sen et al., 2012). We also found by querying the redox

306   modification databases (RedoxDB and GPS-SNO) that 11% of the 834 peptides

307   from the RedoxDB and 36% from the GPS-SNO corresponded to cysteine

308   residues that are known to be modified by nitrosylation or glutathionylation

309   (Figure 3B, Figure 3-figure supplement 2). Taken together, these data indicate

310   that protein sulfhydration not only influences the catalytic activity of enzymes like

311   GAPDH, but potentially can regulate a broad range of biological processes.

312   We noted a wide range of H/L ratios in the identified peptides, reflecting a large

313   difference in the reactivity of individual cysteines to sulfhydration in MIN6 cells

314   that overexpress ATF4 (Figure 3B-D). In addition, labeled cysteines on the same

315   protein exhibited remarkably different ratios (Figure 3-figure supplement 3). For

316   example, the protein, PHGDH, was labeled on two cysteines, Cys[281] had an H/L

317   ratio of 5.8, whereas Cys[254] displayed a ratio of 17. To identify a sulfhydration

318   motif in proteins, all cysteine-containing peptides (Figure 3B) were analysed by

319   the pLogo motif analyzer program. This analysis did not reveal conserved

320   residues surrounding the modified cysteine site (Figure 3-figure supplement 4A),

321   a result consistent with other enzyme-independent oxidative modifications of

322   cysteines (Weerapana et al., 2010) (Marino and Gladyshev, 2011). However,

323   when we selected only the peptides with high H/L ratios (>2), and searched for a

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324   sequence motif at the cysteine modification sites (Figure 3-figure supplement 4B),

325   an Arg residue was significantly enriched at the +1 position of the modified

326   cysteine (Figure 3-figure supplement 4B). Additionally, structure motif and

327   surface accessibility analysis revealed that the modified cysteine is highly

328   accessible and positioned at the N-terminal alpha-helices (Figure 3-figure

329   supplement 4C-D). This is consistent with previous reports suggesting that a

330   reactive cysteine thiolate anion is stabilized by interaction with alpha-helix dipoles

331   (Kortemme and Creighton, 1995; Weerapana et al., 2010).

332   Bioinformatics clustering with two pathway annotation programs DAVID and

333   Ingenuity Pathway Analysis (IPA) revealed an enrichment of sulfhydrated

334   proteins in glycolysis and mitochondrial oxidative metabolism (Figure 3E, Figure

335   3-figure supplement 5). Data from this analysis are in agreement with increased

336   activity of the glycolytic enzyme, GAPDH, by sulfhydration, and prompted the

337   question as to whether or not glycolytic flux is regulated by the H_2S-dependent

338   modification of enzymes involved in intermediary metabolism. Also in agreement

339   with this hypothesis, we found that ATF4-overexpressing MIN6 cells had higher

340   glycolytic rates as evaluated with a Seahorse analyzer (Figure 4-figure

341   supplement 1). Furthermore, the activity of the rate-limiting glycolytic enzyme,

342   pyruvate kinase 2 (PKM2), also was increased in ATF4 overexpressing MIN6

343   cells, in a manner dependent on CTH activity (Figure 4-figure supplement 2). We

344   therefore returned to the induction of ER stress by Tg-treatment of MIN6 cells

345   and evaluated glycolytic flux rates in the absence or presence of PAG. The

346   advantage of using PAG instead of genetic manipulation such as gene

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347　knockdown is that the inhibitor could be added at the same time as labeled

348　glucose, thus allowing assessment of the inhibitor's effect on glycolytic flux rates.

349　We directly measured changes in metabolic flux by utilizing stable isotope label

350　incorporation and mass isotopomer analyses. MIN6 cells were treated with Tg for

351　18 h; the growth media was changed to (D-glucose-$^{13}C_6$) media in the presence

352　or absence of PAG during the last 3.5 h of treatment. Tg-treatment significantly

353　augmented glycolytic flux as determined by the increase in the glycolytic

354　intermediate, 3 phosphoglyceric acid (3PG), as well as lactate and alanine

355　(Figure 4A-C, Supplementary File 1). The flux was consistent with an increase in

356　the relative concentrations of 3PG and alanine (Figure 4B, Supplementary File

357　1). However, lactate levels were decreased significantly despite the increased

358　flux. A decrease in cellular lactate levels supports the idea that there is high

359　consumption of pyruvate by the mitochondria to generate oxaloacetate (OAA). In

360　contrast to the increased glycolytic rates, flux to Tricarboxylic Acid Cycle (TCA)

361　intermediates was significantly reduced by Tg-treatment as evidenced by the low

362　^{13}C label incorporation of both acetyl-CoA and OAA moieties of citrate, fumarate,

363　and malate (Figure 4A-B, Supplementary File 1). When the cells were exposed to

364　PAG along with Tg-treatment, the increase in glycolytic flux was prevented, as

365　shown by the decrease in ^{13}C-labeling of 3PG, lactate, and alanine. In contrast,

366　TCA cycle flux was restored, as exemplified by the increase in ^{13}C-labeling of

367　both OAA and acetyl-CoA moieties of citrate, succinate, fumarate, and malate,

368　and a decrease in their concentrations, suggesting utilization. ^{13}C-labeling of

369　aspartate and glutamine also increased significantly, indicating increased

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370　cataplerosis of TCA cycle intermediates. Moreover, we determined the activity of

371　PKM2 in MIN6 cells treated with Tg in the presence or absence of PAG. Tg-

372　treatment increased PKM2 activity, but PAG addition inhibited the increase,

373　without affecting PKM2 protein levels (Figure 4-figure supplement 3). These data

374　suggest that ER stress, via H_2S-mediated signaling, promotes glycolysis and

375　decreases mitochondrial oxidative metabolism.

376

Discussion

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378　In summary, sulfhydration of specific cysteines in proteins is a key function of

379　H_2S (Kabil and Banerjee, 2010; Paul and Snyder, 2012; Szabo et al., 2013).

380　Thus, the development of tools that can quantitatively measure genome-wide

381　protein sulfhydration in physiological or pathological conditions is of central

382　importance. However, a significant challenge in studies of the biological

383　significance of protein sulfhydration is the lack of an approach to

384　selectively detect sulfhydrated cysteines from other modifications (disulfide

385　bonds, glutathionylated thiols and sulfenic acids) in complex biological samples.

386　In this study, we introduced the BTA approach that allowed the quantitative

387　assessment of changes in the sulfhydration of specific cysteines in the proteome

388　and in individual proteins. BTA is superior to other reported methodologies that

389　aimed to profile cysteine modifications, such as the most commonly used, a

390　modified biotin switch technique (BST). BST was originally designed to study

391　protein nitrosylation and postulated to differentiate free thiols and persulfides

392　(Mustafa et al., 2009). A key advantage of BTA over the existing methodologies,

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393 is that the experimental approach has steps to avoid false-positive and negative

394 results, as target proteins for sulfhydration. BST is commonly generating such

395 false targets for cysteine modifications (Forrester et al., 2009; Sen et al., 2012).

396 Using mutiple validations, our data support the specificity and reliability of the

397 BTA assay for analysis of protein sulfhydration both in vitro and in vivo. With this

398 approach, we found that ATF4 is the master regulator of protein sulfhydration in

399 pancreatic β cells during ER stress, by means of its function as a transcription

400 factor. A large number of protein targets have been discovered to undergo

401 sulfhydration in β cells by the BTA approach. Almost 1,000 sulfhydrated cysteine-

402 containing peptides were present in the cells under the chronic ER stress

403 condition of treatment with Tg for 18 h. Combined with the isotopic-labeling

404 strategy, almost 820 peptides on more than 500 proteins were quantified in the

405 cells overexpressing ATF4. These data show the potential of the BTA method for

406 further systematic studies of biological events. To our knowledge, the current

407 dataset encompasses most known sulfhydrated cysteine residues in proteins in

408 any organism. Our bioinformatics analyses revealed sulfhydrated cysteine

409 residues located on a variety of structure-function domains, suggesting the

410 possibility of regulatory mechanism(s) mediated by protein sulfhydration.

411 Structure and sequence analysis revealed consensus motifs that favor

412 sulfhydration; an arginine residue and alpha-helix dipoles are both contributing to

413 stabilize sulfhydrated cysteine thiolates in the local environment.

414 Pathway analyses showed that H_2S-mediated sulfhydration of cysteine residues

415 is that part of the ISR with the highest enrichment in proteins involved in energy

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416   metabolism. The metabolic flux revealed that H_2S promotes aerobic glycolysis

417   associated with decreased oxidative phosphorylation in mitochondria during ER

418   stress in β cells. The TCA cycle revolves by the action of the respiratory chain

419   that requires oxygen to operate. In response to ER stress, mitochondrial function

420   and cellular respiration are down-regulated to limit oxygen demand and to

421   sustain mitochondria. When ATP production from the TCA cycle becomes limited

422   and glycolytic flux increases, there is a risk of accumulation of lactate from

423   pyruvate. One way to escape accumulation of lactate is the mitochondrial

424   conversion of pyruvate to oxalacetic acid (OAA) by pyruvate carboxylase. This

425   latter enzyme was found to be sulfhydrated, consistent with the notion that

426   sulfhydration is linked to metabolic reprogramming towards glycolysis.

427   The switch of energy production from mitochondria to glycolysis is known as a

428   signature of hypoxic conditions. This metabolic switch has also been observed in

429   many cancer cells characterized as the Warburg effect, which contributes to

430   tumor growth. The Warburg effect provides advantages to cancer cell survival via

431   the rapid ATP production through glycolysis, as well as the increasd conversion

432   of glucose into anabolic biomolecules (amino acid, nucleic acid and lipid

433   biosynthesis) and reducing power (NADPH) for regeneration of antioxidants.,

434   This metabolic response of tumor cells contributes to tumor growth and

435   metastasis (Vander Heiden et al., 2009). By analogy, the aerobic glycolysis

436   trigged by increased H_2S production could give β cells the capability to acquire

437   ATP and nutrients to adapt their cellular metabolism towards maintaining ATP

438   levels in the ER (Vishnu et al., 2014), increasing synthesis of

20

439 glycerolphospholipids, glycoproteins and protein (Krokowski et al., 2013b), all

440 important components of the ISR. Similar to hypoxic conditions, a phenotype

441 associated with most tumors, the decreased mitochondria function in β cells

442 during ER stress, can also be viewed as an adaptive response by limiting

443 mitochondria ROS and mitochondria-mediated apoptosis. We therefore view that

444 the H_2S-mediated increase in glycolysis is an adaptive mechanism for survival of

445 β cells to chronic ER stress, along with the improved ER function and insulin

446 production and folding, both critical factors controlling hyperglycemia in diabetes.

447 Future work should determine which are the key proteins targeted by H_2S and

448 thus contributing to metabolic reprogramming of β cells, and if and how insulin

449 synthesis and secretion is affected by sulfhydration of these proteins during ER

450 stress.

451 Abnormal H_2S metabolism has been reported to occur in various diseases,

452 mostly through the deregulation of gene expression encoding for H_2S-generating

453 enzymes (Wallace and Wang, 2015). An increase of their levels by stimulants is

454 expected to have similar effects on sulfhydration of proteins like the ATF4-

455 induced CTH under conditions of ER stress. It is the levels of H_2S under

456 oxidative conditions that influence cellular functions. In the present study, ER

457 stress in β cells induced elevated Cth levels, whereas CBS was unaffected. The

458 deregulated oxidative modification at cysteine residues by H_2S may be a major

459 contributing factor to disease development. In this case, it would provide a

460 rationale for the design of therapeutic agents that would modulate the activity of

461 the involved enzymes.

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462

463

Materials and Methods

Mouse Islets RNA isolation

Experimental protocols were approved by the Case Western Reserve University Institutional Animal Care and Use Committee. C57BL/6J and C57BL/6-$Ins2^{+/Akita}$ mice were used for experiments. Mice from the Jackson Laboratory were bred at the animal facilities at Case Western Reserve University and were fed standard lab chow (LabDiet). Mice were housed under 12:12 h light/dark cycle with free access to food and water at 23°C. Mouse pancreatic islets were isolated as described before (Krokowski et al., 2013a). Islets from 6 weeks old male C57BL/6-$Ins2^{+/Akita}$ (n=6) and age and sex matched wild type (n=6) were cultured for 2 h in RPMI 1640 media supplemented with 10% FBS and 5 mM glucose before RNA isolation. For Tg treatment (1 µM), islets from wild type mice (n=6) were combined and cultured in RPMI 1640 medium supplemented with 10% FBS in atmosphere of 5% CO_2 at 37°C for 24 h. From each group 150-200 islets were manually picked and used for RNA isolation. Islets were treated with QIAshredder (Qiagen), and RNA was purified using the RNeasy Plus Micro kit (Qiagen).

Human Islets RNA isolation

Institutional review board approval for research use of isolated human islets was obtained from the University of Michigan. Human islets were isolated from previously healthy, nondiabetic organ donors by the University of Chicago Transplant Center. The islets were divided into two groups, incubated in CMRL

23

487   medium containing either 5.5 mM glucose with or without Tg (1 μM), for 24 h.

488   The islets were frozen at -80°C before analysis. RNA was isolated as described

489   above from 200 islets/treatment.

490

491   **RT-qPCR analysis of mRNAs for MIN6 cells**

492   RNA was isolated from mouse MIN6 cells using TRIzol (Invitrogen). cDNA was

493   synthesized from total RNA isolated from islets or MIN6 cells using the

494   SuperScript III First-Strand Synthesis Super Mix (Invitrogen), and the abundance

495   of cDNA isolated from each sample was quantified by qPCR using the VeriQuest

496   SYBR Green qPCR Master Mix (Affymetrix) with the StepOnePlus Real-Time

497   PCR System (Applied Biosystems).

498

499   **Cell Culture and viral particles**

500   MIN6 cells were cultured in high glucose DMEM supplemented with 10% FBS, 2

501   mM L-glutamine, 1 mM sodium pyruvate, 55 μM β-mercaptoethanol, 100 units/ml

502   penicillin, and 100 □g/ml streptomycin at 37°C in atmosphere of 5% CO_2. β-

503   mercaptoethanol was removed from the media 12 h before experimentation. Rat

504   INS1 cells were cultured in RPMI 1640 supplemented with 11 mM glucose, 10%

505   heat inactive FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml

506   penicillin, and 100 μg/ml streptomycin at 37°C in atmosphere of 5% CO_2. Tg

507   (Sigma-Aldrich) was used at 400 nM and the Cth inhibitor - DL-propargylglycine

508   (PAG, Sigma Aldrich) at 3 mM. Lentiviral particles were prepared in HEK293T as

509   described before (Saikia et al., 2014). Lentiviral vector expressing shRNA against

24

510 *ATF4* were obtained from Sigma-Aldrich (TRCN0000301646). Adenovirus

511 mediated shRNA against mouse CTH (shRNA sequence:

512 CCGGCCATTACGATTACCCATCTTTCTCGAGAAAGATGGGTAATCGTAATGG

513 TTTTTG) was purchased from Vector Biolabs. MIN6 cells were infected in the

514 presence of 10 µg/ml polybrene and selection with 2.5 µg/ml puromycin (Life

515 Technologies) was conducted 24 h post-infection for 5 days. Adenovirus particles

516 for expression of β galactosidase (β-Gal), GFP or mouse ATF4 protein were

517 prepared in HEK293 cells and were used for infection as described before (Guan

518 et al., 2014).

519

520 **Bacterial expression of wild type and Cys^{150}Ser human recombinant**

521 **GAPDH**

522 Human GST-tagged wild type or C^{150}S GAPDH mutant (Hara et al., 2005) were

523 expressed in the *E. coli* BL21 strain. Protein expression was induced by addition

524 of IPTG (100 µM). When bacterial cultures reached OD$_{600}$ of 0.8 at 37 °C, IPTG

525 was added for 4 h incubation before lysis in a buffer containing 50 mM Tris-HCl

526 (pH 7.5) and 1 mM EDTA. Lysates were centrifuged and applied on a buffer-

527 equilibrated GST-sepharose affinity spin column (Pierce). After extensive washes

528 to remove unbound protein, recombinant GAPDH was released by digestion with

529 thrombin protease (Sigma). The protein purity was determined on SDS/PAGE

530 gels stained by Coomassie blue.

531

532 **GAPDH activity assay**

25

533 The specific activity of GAPDH was determined as described before (Hara et al.,

534 2005). Recombinant protein (50 nM) was used for the in vitro activity assays. To

535 test the GAPDH activity in cell lysates, MIN6 cells were harvested in RIPA buffer

536 (150 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 0.5 % deoxycholic acid, 50 mM

537 Tris-HCl, pH 7.5), sonicated on ice and centrifuged at 4°C. 1-20 µg of protein

538 lysate was used for the activity assays. The reaction mixture contained 100 mM

539 Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 3 mM 3-phosphoglycerate, 5 units/ml of *S.*

540 *cerevisiae* 3-phosphoglycerate kinase (PGK, Sigma-Aldrich), 2 mM ATP and

541 0.25 mM NADH. Reactions were conducted in 200 µl volume at 25°C and

542 monitored spectrophotometrically at 340 nm for 3 min using a M3 microplate

543 reader (Molecular Devices).

544

545 **PKM activity assay**

546 PKM activity was tested in the cell extracts as described (Anastasiou et al.,

547 2011). MIN6 cells were collected by trypsinization and the cell pellets were

548 washed twice with cold PBS. Cells were resuspended in RIPA buffer, sonicated

549 on ice and subjected to a quick centrifugation at 4°C. Reaction mixtures

550 contained 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM ADP, 0.2

551 mM NADH, 8 units of lactate dehydrogenase (Sigma-Aldrich), and 1-10 µg of cell

552 lysate. The enzymatic reaction was initiated by the addition of PEP

553 (phosphoenolpyruvic acid, 0.5 mM) as the substrate. The oxidation of NADH was

554 monitored at 340 nm for 3 min using a M3 microplate reader (Molecular Devices).

555

26

Activity of cystine/glutamate exchanger Slc7a11

556

557 The activity of the amino acid transporter was tested as described before

558 (Krokowski et al., 2013a). Uptake of Glu was tested in the absence of Na$^+$ ions

559 (EBSS solution, NaCl replaced with choline chloride), with 100 µM Glu and 4

560 µCi/ml of ^{3}H-Glu (Parkin Elmer) for 3 min at 37°C. MIN6 cells were washed twice

561 with cold PBS then amino acids were extracted with ethanol and radioactivity

562 was counted. The specific activity was normalized to protein content that was

563 determined by the Lowery assay.

564

Intracellular glutathione content and the ratio of oxidized and reduced

565

glutathione

566

567 MIN6 cells (6×10^4 cell per well) were seeded into 96-well plates. After 48 h the

568 growth media was removed, the total glutathione contents and GSH/GSSG ratios

569 were determined with the GSH/GSSG-Glo Tm assay from Promega.

570

ROS Measurements

571

572 Total intracellular levels of ROS were quantified using dichlorofluorescein

573 diacetate (CM-H$_2$DCFDA; 10 µM). MIN6 cells were seeded into 96-well plates.

574 After 48 h, cells were washed with warm PBS and incubated with the dye in

575 phenol-red free DMEM without FBS. After 1 h, the cells were washed with PBS to

576 remove the dye and placed in phenol-red free DMEM. CM-H$_2$DCFDA

577 fluorescence was measured at excitation/emission wavelengths of 495/517 nm.

578 Cells not exposed to the probe were used to test the background fluorescence.

579 After subtraction of background fluorescence results were normalized to protein

580 content determined by the BCA assay.

581

582 **H$_2$S-production assays**

583 Frozen cell pellets were lysed in 100 mM HEPES (pH 7.4), to obtain a lysate

584 concentration of 100 mg/ml. H$_2$S production was measured as described

585 previously (Kabil et al., 2011). Briefly, reactions containing cell lysate (200 µl), 10

586 mM cysteine and 100 mM HEPES (pH 7.4) were prepared in 20-ml

587 polypropylene syringes in a total reaction volume of 400 µl. Reactions were

588 started with the addition of cysteine. Syringes were sealed and the headspace

589 was flushed with nitrogen 5 times by using a three-way stopcock, and left in

590 nitrogen in a final total volume (aqueous + gas) of 20 ml. Syringes were placed at

591 37°C in a shaker incubator (75 rpm) for 20 min. Control reactions without cell

592 lysates were prepared in parallel. Aliquots of 0.2 ml from the gas phase were

593 collected through a septum attached to the stopcock, and injected in an HP 6890

594 gas chromatograph (GC) (Hewlett Packard) equipped with a DB-1 column

595 (30 m×0.53 mm×1.0 µm). Flow rate of the carrier gas (helium) was 1 ml/min, and

596 the temperature gradient ranged from 30°C to 110°C over a 20-min period. H$_2$S

597 was detected by a 355 sulfur chemiluminescence detector (Agilent) attached to

598 the GC. H$_2$S standard gas (Cryogenic Gases, Detroit, MI) with a stock

599 concentration of 40 ppm (1.785 µM) in nitrogen was used to generate a standard

600 curve. The amount of H$_2$S in the injected volume was calculated from the peak

601 areas by using the calibration coefficient obtained from the standard curve.

28

602   Ionized H_2S concentration in the liquid phase was calculated for the pH of the

603   reaction mixture (pH 7.4) by using a pKa value of 6.8 for ionization of H_2S. The

604   resulting H_2S concentration in the total reaction volume was then used to obtain

605   the specific activity expressed as nmol H_2S per mg protein per min.

606

607   **Biotin Thiol Assay**

608   In order to detect and identify sulfhydrated proteins from MIN6 cells, cells were

609   lysed with the RIPA buffer (150 mM NaCl, 1 mM EDTA, 0.5 % Triton X-100, 0.5

610   % deoxycholic acid and 100 mM Tris-HCl (pH 7.5) containing protease and

611   phosphatase inhibitor from Roche. Cells were sonicated on ice, lysates were

612   clarified by centrifugation at 4°C and the protein concentrations were determined

613   by the BCA assay (BioRad). Equal amount (4 mg) of proteins was incubated

614   with 100 µM NM-biotin (Pierce) for 30 min with occasionally gentle mixing at

615   room temperature and subsequently precipitated by cold acetone. After

616   centrifugation, pellets of precipitated proteins were washed with 70% cold

617   acetone twice, and then suspended in buffer (0.1% SDS, 150 mM NaCl, 1 mM

618   EDTA and 0.5% Triton X-100, 50 mM Tris-HCl, pH 7.5) mixed with Streptavidin-

619   agarose resin (Thermo Scientific) and kept rotating overnight at 4°C. The beads

620   were washed 5 times with wash buffer 1 (0.5 % Triton x-100, 150 mM NaCl, 50

621   mM Tris-HCl, pH 7.5) followed by 5 washes with wash buffer 2 (0.5% Triton X-

622   100, 600 mM NaCl, 50 mM Tris-HCl, pH 7.5). Resin with bound proteins was

623   incubated with 500 µl of the elution buffer with or without 20 mM DTT for 30 min

624   at 25°C. Eluted proteins were concentrated to a final volume of 25-40 µl with

29

625 utilization of Amicon Ultracel 10K (Millipore) and used for gel electrophoresis

626 followed by either western blot or MS analysis.

627

628 **Red maleimide assay**

629 The assay was previously described (Sen et al., 2012) and modified in order to

630 adapt to our experimental needs. Purified recombinant GAPDH was next treated

631 with either 50 μM NaCl as control, 50 μM NaHS or 50 μM H_2O_2 for 45 min at

632 room temperature. After desalting through a spin column (Pierce), the samples

633 were incubated with 1 μM red malemide probe (Alexa Fluor 680 C_2 Maleimide,

634 Molecular Probes) for 20 min at room temperature. After the incubation, these

635 samples were treated with or without 10 mM β-mercaptoethanol and the reaction

636 was stopped by the addition of 100 mM iodoacetamide. Samples were

637 suspended in sample buffer for non-reducing gel electrophoresis. After

638 electrophoretic separation, the gel was scanned with the Li-COR Odyssey

639 system. The intensity of red fluorescence of GAPDH was quantified with the

640 Odyssey system software. Subsequently proteins from the gel were transferred

641 on a PVDF membrane and subjected to Western blot analysis for GAPDH.

642

643 **Purification of H_2S-modified cysteine-containing peptides from MIN6 cells**

644 In order to identify H_2S-modified cysteine-containing peptides from cell lysates,

645 proteins were extracted and biotinylated as described above. Biotinylated

646 proteins were precipitated with ice cold acetone, resuspended in denaturation

647 buffer (30 mM Tris-HCl (pH 7.5), 8 M urea and 1 mM $MgSO_4$) as described

30

648 (Morisse et al., 2014), diluted with 10 volumes of the buffer (30 mM Tris-HCl (pH

649 7.5), 1 mM EDTA and 200 mM NaCl) and incubated with modified porcine trypsin

650 (Promega) with occasionally mixing for 18 h at 30°C. The ratio of the enzyme to

651 substrate was 1:80 (w/w). After digestion, trypsin was inactivated by incubation at

652 95°C for 10 min then reactions were mixed with the streptavidin-agarose beads

653 (500 µl) and incubated at 4°C for 18 h following extensive washes in the

654 presence of 0.1% SDS as described above. Peptides were eluted with 20 mM

655 ammonium bicarbonate supplemented with 10 mM DTT after 25 min incubation

656 at room temperature. DTT was removed with utilization of a C-18 column

657 (Pierce). Peptides were eluted from the desalting column with acetonitrile, dried

658 under vacuum and suspended in buffer (30 mM Tris-HCl (pH 7.5), 1mM EDTA

659 and 150 mM NaCl). Free -SH groups were alkylated by NEM (either deuterium or

660 hydrogen containing) at final concentrations of 40 mM. The alkylated peptides

661 were concentrated with a C-18 column (Pierce) for LC-MS/MS analysis.

662

663 **Liquid-chromatography-mass-spectrometry (LC-MS/MS) analysis**

664 LC-MS/MS analysis was performed on an LTQ-Orbitrap Elite mass spectrometer

665 (Thermo-Fisher) coupled to an Ultimate 3000 high-performance liquid

666 chromatography system. Protein digests were loaded onto a 75 µm desalting

667 column packed with 2 cm of Acclaim PepMap C18 reverse phase resin (Dionex).

668 The peptides were then eluted onto a Dionex 15 cm x 75 µm id Acclaim Pepmap

669 C18, 2µm, 100 Å reversed- phase capillary chromatography column using a

670 gradient of 2–80% buffer B in buffer A (buffer A: 0.1% formic acid; buffer B: 5%

31

671 water, 95% acetonitrile, 0.1% formic acid). The peptides were then eluted from

672 the C18 column into the mass spectrometer at a flow rate of 300 nl/ min and the

673 spray voltage was set to 1.9 kV. One full MS scan (FTMS) (300–2,000 MW) was

674 followed by 20 data dependent scans (ITMS) of the nth most intense ions with

675 dynamic exclusion enabled.

676

677 **Peptide identification**

678 Peak lists were extracted from Xcaliber RAW files using Proteome Discoverer

679 1.4. These peak lists were searched Sequest HT and Mascot (2.3) search

680 engines. The data was searched against the mouse reference sequence

681 database which contains 77807 entries using a precursor ion tolerance of 10

682 ppm and a fragment ion tolerance of 0.6 Da. These searches included

683 differential modification of +125.047679 and +130.079062 on cysteine to account

684 for NEM and d_5-NEM alkylation and +15.994915 Da to account for oxidation on

685 methionine residues. Peptide identification was validated with the Percolator

686 node on the basis of q-values which are estimated from target-decoy

687 searches. The false discovery rate (FDR) for these searches was set to 1% at

688 the peptide level. In addition, peptides were also required to be fully tryptic and

689 have Xcorr scores > 1.5 (+1), 2.0 (+2), 2.25 (+3), and 2.5 (+4).

690

691 **Ratio quantification**

692 Quantification of light/heavy ratios (d_5-NEM/NEM) was performed using two

693 algorithms of Proteome Discoverer, the event detector and Precursor Ions

32

694   Quantifier. The event detector applied a 2 ppm mass variability and 0.2 minute

695   chromatographic window for the generation of extracted ion

696   chromatograms. The Peptide ratio was calculated from the summed extracted

697   ion chromatograms of all isotopes for the NEM and d_5-NEM containing

698   peptides. All missing ions were assigned a value equivalent to the minimum

699   intensity, only unique peptides were quantified, and since this included

700   quantitation at the peptide level, single channel was used. The H/L ratios of

701   approximately 25% of the quantified peptides were manually validated.

702

703   **Bioinformatics analysis of cysteine-containing peptides**

704   For functional annotation: Protein sequences from the FTP site of the Uniprot

705   Protein database for mouse (Proteome_ID/Tax_ID: UP000000589/10090), rat

706   (Proteome_ID/Tax_ID: UP000002494/10116) and human (Proteome_ID/Tax_ID:

707   UP000005640/9606) release current as of May 23 2015. Sequence annotation

708   in the feature fields (ACT_SITE, BINDING, CA_BIND, DISULFID, DNA_BIND,

709   DOMAIN, METAL, MOD_RES, MOTIF, NP_BIND, SITE, ZN_FING) of the

710   Uniprot entry was searched and any annotation corresponding to the labeled

711   cysteine peptides was collected.

712   For redox cysteine annotation: each peptide identified by MS, all exact matches

713   in any of the RedoxDB databases on any oxidative modification cysteine

714   sequences (fasta or additional_fasta) were collected. For motif search: The lager

715   data set of putative modification cysteine sites and their vicinity sequences were

33

716   submitted to the pLogo program (www.plogo.uconn.edu, version v1.2.0) (O'Shea

717   et al., 2013) to identify linear motif.

718   For prediction of candidate peptides for nitrosylation: the peptide sequences with

719   H/L ratio >2 were submitted for use in predicting nitrosylation sites under the

720   medium threshold condition using the batch prediction tool of the GPS-SNO 1.0

721   software (Xue et al., 2010). The predicted nitrosylation sites of sequences were

722   extracted for further analysis.

723   For determination of surface accessibility and secondary structural motif: we

724   turned to DSSP's (Kabsch and Sander, 1983; Touw et al., 2015) annotations of

725   the PDB (Berman et al., 2000). We downloaded a total of 108355 DSSP-

726   annotated PDB files from (rsync.cmbi.ru.nl/dssp/) on Sep. 9th 2015. Each

727   peptide with H/L greater than 2-fold was aligned on all matching DSSP profiles,

728   from which the 10-state structural context and accessibility were extracted. When

729   an exact match is not found, then all matches with 1 mismatch are considered.

730   The structural context of a peptide is defined as the context that reoccur most

731   frequently among the hits, while the accessibility is the average across the hits,

732   log_2-normalized by the median of accessibility considering the amino acid type;

733   positive log_2 values means that the amino acid embedded in the 3D structure of

734   the protein is more accessible that the mode (median).

735   For pathway annotation: the canonical pathways were scored based on the total

736   sulfhydrated peptides and the peptides with H/L ratios greater than 2 by using

737   DAVID   (www.david.ncifcrf.gov)   and   Ingenuity   Pathway   Analysis   (IPA,

738   www.qiagen.com/ingenuity) programs. Statistical significant of pathways are

34

739 calculated and pathways are ranked by the *p*-values based on those tests. The

740 tests measure the likelihood that the association between proteins measured in

741 our experiments and a pathway is due to random chance. The smaller the *p*-

742 value the less likely that the association is due to random chance. Top scoring

743 pathways are presented.

744

745 **Metabolic labeling of MIN6 cells**

746 MIN6 cells were plated onto 10-cm plates in triplicates and cultured in the cell

747 growth medium. After 48 h treatment with Tg, metabolic labeling was performed.

748 Cells were washed with warm PBS and incubated for 3 h in the DMEM medium

749 containing 10% heat inactive FBS, 2 mM glutamine, and 25 mM glucose

750 consisting of a mixture of 12.5 mM D-glucose plus 12.5 mM of D-[U-^{13}C] glucose.

751 After incubation, cells were washed with PBS, followed by trypsinization. Cells

752 were pelleted by centrifuging at 4°C for 5 min at 650 x *g*, and pellets were stored

753 at -80°C until extraction of metabolites.

754

755 **Assay of media [U-^{13}C] glucose enrichment**

756 Glucose isotopic enrichment was determined following (van Dijk et al., 2001) with

757 modifications. Briefly, glucose was extracted by the addition of 500 µl of ice-cold

758 ethanol to 50 µl of media. Samples were mixed and incubated on ice for 30 min.

759 Samples were centrifuged at 4°C for 10 min at 14,000 rpm and ethanol was

760 transferred to a GC/MS vial and evaporated to dryness in a SpeedVac

761 evaporator. Glucose was converted to its pentaacetate derivative by the reaction

35

762   with 150 µl of acetic anhydride in pyridine (2:1, v/v) at 60°C for 30 min. Samples

763   were evaporated to dryness and glucose derivatives were reconstituted in 80 µl

764   of ethyl acetate and transferred to GC/MS inserts. Samples were injected in

765   duplicate and masses 331-337, containing M0…M+5 isotopomers were

766   monitored. Enrichment was determined as a ratio of $(M+5) / (\Sigma_{M0-M5})$.

767

768   **Metabolite extraction**

769   Metabolites were extracted following (Yang et al., 2008) with modifications.

770   Briefly, cellular pellets in Eppendorf tubes were homogenized and frozen in 600

771   µl of Folch solution (chloroform:methanol, 2:1, vol./vol.) on dry ice. After addition

772   of 0.4 volumes of ice-cold water, cells were homogenized again and let sit on ice

773   for 30 min. Homogenates were centrifuged at 4°C for 10 min at 14,000 rpm. The

774   upper methanol/water layer was removed to GC/MS vial. To the bottom

775   chloroform layer, 120 µl of water and 200 µl of methanol were added and

776   extraction steps from above were repeated. Combined methanol/water layers

777   were evaporated to dryness in Speedvac evaporator at 4°C. Metabolites were

778   derivatized using two-step derivatization. First, keto- and aldehyde groups were

779   protected by the reaction with MOX (methoxylamine-HCl in pyridine, 1:2)

780   overnight at room temperature. Then excess derivatizing agent was evaporated

781   and dry residue was converted to MOX-TMS (trimethylsilyl) derivative by reacting

782   with bis(trimethylsilyl) trifluoroacetamide with 10% trimethylchlorosilane (Regisil)

783   at 60°C for 20 min. Resulting MOX-TMS derivatives were run in GC-MS.

784

GC-MS conditions

Analyses were carried out on an Agilent 5973 mass spectrometer equipped with 6890 Gas Chromatograph. A DB17-MS capillary column (30 m × 0.25 mm × 0.25 µm) was used in all assays with a helium flow of 1 ml/min. Samples were analyzed in Selected Ion Monitoring (SIM) mode using electron impact ionization (EI). Ion dwell time was set to 10 msec. The following metabolites were monitored: Glycerol 3 phosphate (G3P), 3 Phosphoglycerate (3PG), Lactate, Alanine, Citrate, Succinate, Fumarate, Malate, Glycine, and Serine.

Mitochondrial oxidative phosphorylation and glycolysis

MIN6 cells were diluted to 80 000 cells/well in a Seahorse tissue culture system in the presence of either *GFP* or *ATF4* overexpression. Cells were plated 2 days prior to experimentation. The cells were washed with warm PBS and then incubated for 30 min at 37°C and ambient CO_2 in HCO_3-free DMEM containing 25 mM glucose, 2 mM glutamine, 1 mM pyruvate (pH 7.4). Cells were then treated sequentially with oligomycin (0.2 µg/ml), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, 1 µM) and rotenone (1 µM). The rates of mitochondrial respiration and cellular acidification were determined by using the Seahorse extracellular flux analyzer (Seahorse Bioscience, North Billerica, MA). Corrected oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) values were normalized to cell number.

Other methods

37

808 MIN6 cells extracts for protein immunodetection were obtained after cell lysis in

809 RIPA buffer as described before (Krokowski et al., 2013a). Protein content was

810 determined by the BCA assay (BioRad). Mouse islet protein extracts were

811 extracted in RIPA buffer. From approximately 100 islets the same amount of

812 extracts determined by measuring DNA content with the Quant-iT dsDNA assay

813 kit (Molecular Probes) and equal DNA amount was used for immunodetection.

814 Western blotting was performed as described before (Krokowski et al., 2013a).

815 Anti-Actin (ab 3280) antibodies were from Abcam. Anti-Cth (H00001491) and

816 anti-CBS (H00000875) were from Abnova. Antibodies against: PERK (3192),

817 phospho PERK (3179) and PKM2 (4053) were purchased from Cell Signaling.

818 Anti-ATF4 (sc-200), anti-GAPDH (sc-32233), anti-eIF2α (sc-133227) and XBP1

819 (sc-7160) were from Santa Cruz Biotechnology. Antibodies against

820 phosphorylated eIF2α (NB 110-56949) and Ero1α (NB 100-2525) were obtained

821 from Novus and anti-tubulin (T9026) serum was from Sigma-Aldrich.

822

38

840

39

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Figure titles and legends

988 **Figure 1**. **ER stress induces protein sulfhydration, a reversible cysteine-**

989 **based post-translational modification.** (**A**) Schematic overview of protein

990 sulfhydration, which requires synthesis of H_2S and low ROS levels. Pancreatic β

991 cells (MIN6) were treated with thapsigargin (Tg) for the indicated times, and the

992 cellular levels of ROS (**B**), total levels of GSH and GSH/GSSG ratios (**C**) and H_2S

993 levels (**E**) were evaluated. (**D**) RT-qPCR analysis of the mRNA levels for the H_2S-

994 producing enzyme CTH and the cystine/glutamate exchanger Slc7a11 in MIN6

995 cells treated with Tg or pancreatic islets as indicated. (**F**) Evaluation of GAPDH

996 activity in cell extracts from MIN6 cells treated with Tg at the indicated times

997 (top), and GAPDH protein levels by Western blot analysis (bottom). (**G**). Time-

998 dependent measurements of human recombinant GAPDH activities after

999 exposure to H_2O_2 (50 μM, □□□e) or H_2O_2 together with the H_2S donor, NaHS

1000 (50 μM, red). □**H**) In vitro evaluation of the reversal of the inhibitory effect of

1001 glutathionylation on the activity of recombinant GAPDH treated for 15 min with

1002 either NaHS (20 mM), DTT (20 mM) or NaCl (20 mM). (**I**). Evaluation of GAPDH

1003 activities in MIN6 cell extracts either untreated or treated with Tg (18 h) with or

1004 without the Cth inhibitor, PAG (3 mM) (top). PAG was included for the last 3.5 h

1005 of Tg-treatment. GAPDH protein levels were evaluated by Western blotting

1006 (bottom). All quantifications are presented as mean $\pm$ S.E.M. of three

1007 independent experiments.

1008

1009   **Figure 2**. **ATF4-mediated transcriptional reprogramming during ER stress**

1010   **increases expression of a gene cohort involved in H$_2$S synthesis and**

1011   **protein sulfhydration.**   (**A**) Schematic representation of the ATF4-induced

1012   cohort of genes leading to sulfhydration of proteins during ER stress. (**B**)

1013   Evaluation of intracellular H$_2$S levels (top) or ATF4 and Cth protein levels

1014   (bottom) in MIN6 cells infected with either control shRNA or shRNA against

1015   ATF4. Cells were untreated or treated with Tg for 18 h. (**C**) Western blot analysis

1016   for the indicated proteins in MIN6 cells infected (for 48 h) with either adenovirus

1017   mediated lacZ-expression as control, or ATF4-expression, at increasing viral

1018   particle concentrations. MIN6 cells infected with either control adenovirus or

1019   ATF4-expressing adenovirus were used to measure (**D**) H$_2$S levels (**E**) GAPDH

1020   relative activities, (**F**) GSH levels and (**G**) Glutamate (Glu) uptake by the

1021   cystine/glutamate exchanger. (**H**) Schematic representation of the novel Biotin-

1022   Thiol-Assay (BTA), an experimental approach for the identification of

1023   sulfhydrated proteins in cell extracts. Highly reactive cysteine residues or

1024   sulfhydrated cysteine residues in proteins under native conditions were alkylated

1025   with low concentrations of maleimide-PEG2-biotin (NM-Biotin). Subsequent

1026   avidin column purification and elution with DTT, which cleaved the disulfide

1027   bonds, leaving the biotin tag bound to the column, produced an eluate that was

1028   further analyzed either by western blotting or coupled with LC-MS/MS. (**I**)

1029   Identification via the BTA of sulfhydrated GAPDH in MIN6 cell extracts from Tg-

1030   treated cells in the presence or absence of ATF4. (**J**) Identification of

1031   sulfhydrated GAPDH in MIN6 cells overexpressing ATF4. (**K**) Determination of

46

1032   the effect of PAG on sulfhydrated GAPDH levels in MIN6 cells overexpressing

1033   ATF4 in the presence or absence of PAG.

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1035   **Figure 3. Quantitative and pathway analysis of sulfhydrated peptides in**

1036   **MIN6 cells overexpressing the transcription factor ATF4.** (**A**) Schematic

1037   representation of the BTA experimental approach combined with alkylation of

1038   free SH groups by either the stable isotope-labeled (D_5, heavy) or normal (H_5,

1039   light) maleimide. The relative levels of H_5 and D_5 labeled peptides were

1040   quantified by the LC-MS/MS method. (**B**) Distribution of peptides containing

1041   sulfhydrated cysteine residues relative to their H/L ratios as determined by the

1042   BTA analysis of cell extracts isolated from MIN6 cells overexpressing ATF4

1043   (indicated in **A**). Values of H/L ratios are plotted against the number of identified

1044   peptides. The red line marks the H/L ratio >2, consisting of cysteine-containing

1045   peptides in proteins that exhibited higher reactivity with H_2S under ATF4

1046   overexpression. The black dots show redox sensitive cysteine peptides, which

1047   are common between the ones found in the RedoxDB database, and by the BTA

1048   assay. (**C**) Pie chart illustrating the percentage of cysteine-containing peptides

1049   (from **A**) that belong to known functional domains of proteins in the Uniprot

1050   database. (**D**) Heat map of H/L values obtained from experimental data in (**A**),

1051   illustrating the profound differences in the reactivity with H_2S of cysteine residues

1052   in different proteins. (**E**) Gene ontology biological pathways for peptides with H/L

1053   ratio >2. H/L values were obtained from the experimental data in (**A**).

1054

48

1055 **Figure 4**. **H$_2$S synthesis during ER stress modulates metabolism in MIN6**

1056 **cells.** (**A**) Measurement of ^{13}C-glucose flux in metabolites, expressed as the

1057 molar percent enrichment [(ratio of labeled/sum (labeled + unlabeled) x 100%)],

1058 in MIN6 cells treated with Tg for 18 h or after addition of PAG for the last 3.5 h of

1059 Tg treatment. [U-^{13}C]-glucose replaced glucose in the media for the last 3.5 h of

1060 treatments. (**B**) Evaluation of the concentration of metabolites and amino acids in

1061 the same samples described in (**A**). All quantifications are presented as mean ±

1062 S.E.M. of technical duplicates and are represented four independent

1063 experiments. (**C**) Schematic representation of the major findings on metabolic

1064 flux from glucose during chronic ER stress. Chronic ER stress increased

1065 glycolytic flux and decreased forward TCA flux. Inhibition of CTH by PAG

1066 reversed the observed changes in glucose flux during ER stress.

1067

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1068   **Figure 1-figure supplement 1.**

1069   ER stress induces the levels of the H_2S-producing enzyme CTH but not CBS.

1070   Western blot analysis for the indicated proteins, of cell extracts isolated from Tg

1071   treated MIN6 cells for the indicated times.

1072

1073   **Figure 1-figure supplement 2.**

1074   **Regulation of gene expression in MIN6 cells, human and mouse islets in**

1075   **response to ER stress.** MIN6 cells (**A**) and human islets (**B**) were treated with

1076   Tg for the indicated times. Islets were isolated from WT and heterozygous Akita

1077   *Ins2* ^{c96y} six-week old mice (**C**). RNA was isolated from cells and islets and was

1078   tested by RT-qPCR for expression of the indicated genes.

1079

1080   **Figure 1-figure supplement 3.**

1081   **Activation of the Integrated Stress Response leads to increased expression**

1082   **of CTH in wild type mouse islets treated with Tg**. Western blot analysis for the

1083   indicated proteins of cell extracts isolated from Tg treated islets for 24 h.

1084

1085   **Figure1-figure supplement 4**

1086   **Glutamate uptake in MIN6 cells treated with Tg at the indicated times.**

1087

1088   **Figure 1-figure supplement 5.**

1089   **Analysis of S-glutathionylated GAPDH by LC-MS/MS**. Recombinant GAPDH

1090   (20 µg) was treated with GSSG (5 mM) for 45 min, then incubated with 50 mM of

50

1091   NEM. The sample was resolved by SDS-PAGE electrophoresis and analyzed by

1092   LC-MS/MS. Tandem mass spectrum of the active site peptide

1093   IISNASCTTNCLAPLAK of the protein with glutathionylated Cys^{150}, and Cys^{154}

1094   alkylated with NEM. Comparison with the same peptide both NEM alkylation,

1095   there are no mass changes of y series ions from y4 to y10 and b series ions from

1096   b2 to b4, but a mass shift from y11 to y16, and b8, b9 ions, which strongly

1097   suggests the modification at Cys^{150}. @ represents the neutral loss of -129Da

1098   from a glutathione adduct.

1099

1100   **Figure 2-figure supplement 1.**

1101   Expression of genes in MIN6 cells overexpressing the transcription factor ATF4.

1102   RT-qPCR analysis of RNA isolated from cells infected with control adenovirus (-)

1103   or ATF4-expressing adenovirus (+) for 48 h, for the indicated genes. The results

1104   are shown as the average of three independent determinations.

1105

1106   **Figure 2-figure supplement 2.**

1107   **Schematic representation of the predicted proteins in the eluate of the BTA**

1108   **approach as a consequence of increasing concentrations of biotin**

1109   **conjugated maleimide (NM-biotin, red)**. At low concentration (top), the high

1110   reactive -SH groups (orange) including unmodified and sulfhydrated cysteines

1111   are discriminated for alkylation, leading to elution of sulfhydrated proteins from

1112   the avidin column by DTT. At the high concentration (bottom), all -SH groups in

1113   cysteines are labeled, leading to the proteins permanently bound to the beads

1114   and unable to be eluted by DTT.

1115

1116   **Figure 2-figure supplement 3.**

1117   Increasing concentrations of NM-biotin in the BTA of cell extracts isolated from

1118   Tg treated for 18 h MIN6 cells, inhibit the elution of sulfhydrated proteins. Eluted

1119   proteins were analysed by SDS-PAGE and stained by Coomassie blue. Western

1120   blot analysis for tubulin, of equal amount of MIN6 cell extracts before binding to

1121   the avidin column.

1122

1123   **Figure 2-figure supplement 4.**

1124   **H_2S covalently modifies proteins via sulfhydration of cysteine residues.** (**A**)

1125   Mouse liver lysates were subjected to NM-biotin, then divided in 8 equal

1126   fractions, were bound on avidin columns and eluted by the addition of the

1127   indicated concentrations of DTT. The eluates were analysed by SDS-PAGE

1128   electrophoresis and stained by Coomassie blue. (**B**) Lysates from MIN6 cells

1129   treated with Tg for 18 h, were pretreated with or without 20 mM DTT for 20 min,

1130   and after passing through a NAP-5 gel filtration column, were subjected to the

1131   BTA assay followed by SDS-PAGE electrophoresis and silver staining of the

1132   gels. Equal amount of extracts before loading on the avidin columns were

1133   analysed by western blotting for tubulin. DTT treatment of extracts before the

1134   avidin column, reversed global protein sulfhydration, implying a covalent

1135   cysteine-based modification.

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1136

1137 **Figure 2-figure supplement 5.**

1138 **Assessment of the specificity of BTA to identify reactive -S-SH groups of**

1139 **proteins via the use of recombinant GAPDH.** (**A**) The purity of human

1140 recombinant GAPDH was evaluated by SDS-PAGE electrophoresis and was

1141 stained by Coomassie blue. ***-** indicates GST-tagged GAPDH. (**B**) Recombinant

1142 GAPDH (100 µg) was incubated for 45 min with 50 µM NaCl, as the control, H_2O_2

1143 (mediating oxidation of cysteines) or NaHS (mediating sulfhydration of

1144 cysteines). Following desalting, samples were subjected to the BTA assay.

1145 Eluates were analyzed by Western blotting for GAPDH. Extracts from samples

1146 before loading to the avidin column were also analyzed by Western blotting for

1147 GAPDH (input).

1148

1149 **Figure 2-figure supplement 6.**

1150 **Assessment of GAPDH sulfhydration by the red maleimide assay.**

1151 Recombinant GAPDH (20 µg) was exposed to NaHS (50 µM), or NaCl (50 µM)

1152 as the control, for 45 min. The levels of sulfhydrated GAPDH were evaluated with

1153 the red maleimide assay and measured by the decreasing fluorescence intensity

1154 after β☐mercaptoethanol (β-ME) treatment.

1155

1156 **Figure 2-figure supplement 7.**

1157 **BTA identifies sulfhydration of GAPDH at the catalytic cysteine, Cys^{150}.** (**A**)

1158 Both wild type (WT) and Cys^{150}Ser recombinant GAPDH mutant (100 µg) were

53

1159   incubated with 50 µM each, NaHS, H_2O_2 or NaCl (as the control), for 45 min.

1160   After desalting, samples were subjected to the BTA assay. Eluates were

1161   analyzed by Western blot analysis for GAPDH. Sulfhydration was observed only

1162   for the WT GAPDH. (**B**) Wild type recombinant GAPDH (20 µg) was pretreated

1163   with DTT (20 mM) for 45 min following desalting. The protein was subsequently

1164   incubated with or without NaHS (50 µM) for 45 min, followed with treatment with

1165   NEM (50 mM). The latter treatment is expected to block all free -SH groups. The

1166   samples were resolved by SDS-PAGE electrophoresis followed by LC-MS/MS

1167   analysis. The NaHS-treated GAPDH showed a mass shift of Cys^{150}

1168   corresponding to the sulfinic acid due to sulfhydration (bottom). The oxidation of

1169   the S-SH group of Cys^{150} to sulfinic acid is expected due to its high reactivity

1170   when exposed to O_2 under the aerobic conditions of the experiment.

1171

1172   **Figure 2-figure supplement 8.**

1173   **The BTA assay shows that Tg-induced ER stress in MIN6 cells promotes**

1174   **global protein sulfhydration**. (**A**) The BTA assay was performed to detect

1175   protein sulfhydration in extracts from MIN6 cells treated with Tg for 18 h. (**B**)

1176   Western blot analysis confirmed that GAPDH was sulfhydrated in MIN6 cells

1177   during Tg treatment. (**C**) GAPDH activity in cell extracts from untreated or Tg-

1178   treated MIN6 cells for 18 h. (**D**) LC-MS/MS of a subset of the sulfhydrated

1179   proteins in (**A**), including GAPDH, actin and β-tubulin (Mustafa et al., 2009).

1180

1181   **Figure 2-figure supplement 9.**

1182 **LC-MS/MS spectrum of H₂S-modified peptides purified by the BTA**

1183 **technique from MIN6 cells treated with Tg for 18 h.**

1184

1185 **Figure 2-figure supplement 10.**

1186 **Western blot analysis of eluates from the BTA of sulfhydrated GAPDH and**

1187 **PHGDH in MIN6 cells treated with Tg in a time-dependent manner**.

1188

1189 **Figure 2-figure supplement 11.**

1190 Western blot analysis for CTH protein levels from MIN6 cells infected with either

1191 adenovirus-mediated expression of GFP as control, or shRNA against the CTH

1192 mRNA in a dose- (**A**) and time- (**B**) dependent manner. (**C**) Western blot analysis

1193 of the indicated proteins from cell extracts before applying to the column (input)

1194 or after the column (eluate). The effects of CTH knockdown by shRNA on

1195 sulfhydrated GAPDH and PHGDH levels in MIN6 cells treated with or without Tg

1196 for 18h, is shown.

1197

1198 **Figure 3-figure supplement 1.**

1199 **Sulfhydrated cysteine-containing peptides are enriched in functional**

1200 **residues.** Distribution of peptides containing sulfhydrated cysteines relative to

1201 their H/L ratios with functional annotations from the Uniprot database where

1202 active sites (ACT_SITE), binding sites (BINDING), calcium binding sites

1203 (CA_BIND), disulfide bonds (DISULFID), DNA binding sites (DNA_BIND),

1204 specific domains (DOMAIN), metal binding sites (METAL), modified residues

1205 (MOD_RES), motifs with biological activity (MOTIF), nucleotide binding sites

1206 (NP_BIND) and zinc finger domains (ZN_FING) are shown as black dots.

1207

1208 **Figure 3-figure supplement 2.**

1209 **Sulfhydrated proteins are potential targets for nitrosylation**. A total of 827

1210 H_2S-modified and pair-labeled peptides were scored for their nitrosylation

1211 potential via the use of the GPS-SNO algorithm. We identified 303 peptides with

1212 predicted S-nitrosylation sites. Among them, only 5 peptides (red dots) had an

1213 H/L ratio below 2 (1.5% of the scored peptides for S-nitrosylation).

1214

1215 **Figure 3-figure supplement 3.**

1216 **Quantitative profiling of proteins containing cysteines with different**

1217 **reactivity to H_2S from ATF4 overexpressing MIN6 cells**. MS1 profiles for

1218 multiple cysteine-containing peptides from GDI1, PHGDH, VCP and LAP3, only

1219 one of which exhibits the highest reactivity with H_2S, as shown by the H/L ratios.

1220

1221 **Figure 3-figure supplement 4.**

1222 (**A**-**B**) Sulfhydrated peptides do not reveal a consensus sequence motif, but the

1223 modified cysteine residue is significantly accessible and preferentially positioned

1224 at the N-terminal of alpha helix. (**A**) A total of 739 pair-labeled peptide sequences

1225 were used to show that no primary sequence motif could be detected using the

1226 pLogo program. (**B**) A total of 333 peptides with H/L ratios over 2-fold showed

1227 significant enrichment for Arg residue found next to the modified cysteine. (**C**-**D**)

56

1228   the surface accessibility and secondary structure of the modified cysteine

1229   residues in peptides were annotated by comparing those peptides with H/L ratios

1230   greater than 2-fold and proteins with known structures in the PDB database (172

1231   protein structures were employed). In the secondary structure motif, H= alpha

1232   helix; G=3_{10}-helix; E=beta sheet; T= helix turn; S=bend (high curvature).

1233

1234   **Figure 3-figure supplement 5.**

1235   **Gene ontology biological pathways enriched among all pair-labeled**

1236   **peptides in ATF4 overexpressed MIN6 cells.** 739 pair-labeled cysteine-

1237   containing peptides were subjected to a pathway analysis carried by the Davide

1238   and IPA programs (**A**, **B**). (**C**-**D**) the proteins associated with the H/L greater

1239   than 2-fold were selected and subjected for both pathway annotation

1240   calculations. Top 5 pathways are shown with their statistical significance

1241   (Bonferroni correction for N=17 tests).

1242

1243   **Figure 3-figure supplement 6.**

1244   **MS analysis of the full proteome from MIN6 cells treated with Tg for 18h.**

1245   **Cell extracts were resolved by reducing SDS-PAGE and stained with**

1246   **Coomassie blue**. The entire gel lane was cut into 11 fractions for in-gel

1247   digestions. The peptides from each fraction were combined as indicated and

1248   submitted for LC-MS/MS analysis. A total of 2,244 proteins were quantified by

1249   label-free, semi-quantitative MS approaches.

1250

1251   **Figure 3-supplement figure 7.**

1252   **Protein sulfhydration does not correlate with their protein abundance**. The

1253   relative abundance of the proteins in the full proteome was determined based on

1254   the peptide spectral counts, which were corrected by normalizing to both the total

1255   number of spectra and the length of the protein, and this value was expressed as

1256   normal spectra abundance factor (NSAF). A comparison of the full protein

1257   abundance and sulfhydrome from Tg-treated (**A**), or ATF4 overexpressing MIN6

1258   (**B**). (**C**) A comparison of sulfhydrated protein datasets for the full proteome

1259   reveals that a large fraction of medium abundant proteins are mainly targeted by

1260 H_2S. (**D**) A comparison of sulfhydrated proteins with H/L ratios greater than 2-fold

1261   and the full proteome.

1262

1263   **Figure 4-figure supplement 1.**

1264   **ATF4 -overexpressing MIN6 cells exhibit significantly high glycolytic rates**.

1265   The rates of extracellular acidification (ECAR) were determined by using the

58

1266 Seahorse analyzer from ATF4 expressed MIN6 cells, or GFP as the control after

1267 48h of adenovirus infection. ECAR was normalized to cell numbers. The results

1268 are shown as a mean of four independent determinations.

1269

1270 **Figure 4-figure supplement 2.**

1271 **Pharmacological inhibition of CTH activity represses PKM2 activation in**

1272 **ATF4 overexpressed MIN6 cells**. Determination of the effect of PAG on the

1273 activity of PKM2 in MIN6 cells infected with either GFP as the control, or ATF4

1274 adenovirus for 48h. The activities are shown as a mean of four independent

1275 determinations.

1276

1277 **Figure 4-figure supplement 3.**

1278 **PKM2 activation dependents on CTH activity during ER stress in MIN6**

1279 **cells**. (**A**) ER stress does not affect PKM2 protein levels. MIN6 cells were

1280 incubated with Tg at the indicated times. The protein expression was evaluated

1281 by Western blot analysis. (**B**) Western blot analysis of the expression of PKM2 in

1282 mouse islets. (**C**) Time-dependent increase of PKM2 activity in MIN6 cells treated

1283 with Tg at the indicated times. (**D**) Determination of the effect of PAG on the

1284 activity of PKM2 in MIN6 cells treated with Tg in the presence or absence of PAG

1285 (top) and on protein levels evaluated by Western blot analysis (bottom). For

1286 panels **C** and **D** the results are shown as a mean of four independent

1287 determinations.

1288 **Supplementary File 1. Metabolite flux and relative concentrations of**

1289 **metabolites from control (CON) and Tg-treated MIN6 cells for 18 h.** PAG was

1290 added for the last 3.5 h of Tg-treatment in the indicated experimental samples.

1291 **Notes:** the reported serine M+2 labeling reflects the 2 carbon atoms in the GC-

1292 MS fragment ion that was quantitated, and is expected to be predominantly

1293 derived from fully-labeled (M+3) serine.

1294

1295 **Figure 2-source data 1**

1296 Sulfhydrated proteins from MIN6 cells treated with Tg for 18h

1297

1298 **Figure 3-source data 1**

1299 Sulfhydrated proteins from ATF4 overexpressed MIN6 cells

1300

1301 **Figure 3- source data 2**

1302 **R**elative abundance of proteins in MIN6 cells treated with Tg for 18h

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1305

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1307

1308

1309 **Primers used for RT-qPCR**

1310 **Mouse**

1311

60

1312	*Cbs*	5' CCTATGGTCAGAATCAACAAGAT
1313		5' TGATAGTGTCTCCAGGCTTCAA
1314		
1315	*Cth*	5' GGGATGGCGGTGGCTCGTTT
1316		5' AGCCCGAGCACTGGCGTTTG
1317		
1318	*Chop*	5' CAGGGTCAAGAGTAGTGAAGGT
1319		5' CTGGAAGCCTGGTATGAGGAT
1320		
1321	*Ero1α*	5' CCTGGACGACTGTACCTGTG
1322		5' CAGAATGGCAGGGTTTGACG
1323		
1324	*Gadd34*	5' TACCCCTGTCTCTGGTAACCT
1325		5' TGGCTTTGCATTGTACTCATCA
1326		
1327	*Gapdh*	5' CGCCTGGAGAAACCTGCCAAGTATG
1328		5' GGTGGAAGAATGGGAGTTGCTGTTG
1329		
1330	*Slc7a5*	5' CTGCTACAGCGTAAAGGC
1331		5' AACACAATGTTCCCCACGTC
1332		
1333	*Slc7a11*	5' TGTCCACAAGCACACTCCTC
1334		5' CTGCCAGCCCCATAAAAAGC

1335

1336 **Human**

1337

1338 *β-actin*   5' TCCCTGGAGAAGAGCTACGA

1339   5' AGCACTGTGTTGGCGTACAG

1340

1341 *CTH*   5' GACTCTACATGTCCGAATGG

1342   5' AACCTGTACACTGACGCTTCA

1343

1344 *ERO1α*   5' GCCCGTTTTATGCTTGATGT

1345   5' AACTGGGTATGGTGGCAGAC

1346

1347 *GADD34*   5' CGACTGCAAAGGCGGC

1348   5' CAGGAAATGGACAGTGACCTTCT

1349

1350 *MST*   5' CCACCAGTGGCTTAGACAGG

1351   5' CCGAGACGGCATTGAACCT

1352

1353 *SLC7A5*   5' ATCGGGAAGGGTGATGTGTC

1354   5'CAGGGGCAGGTTTCTGTAGG

1355

1356 *SLC7A11*   5' TGTGGGCATAACTGTAGTGATGG

1357   5' GCGTAATACTTGAATCTCTTCCTG

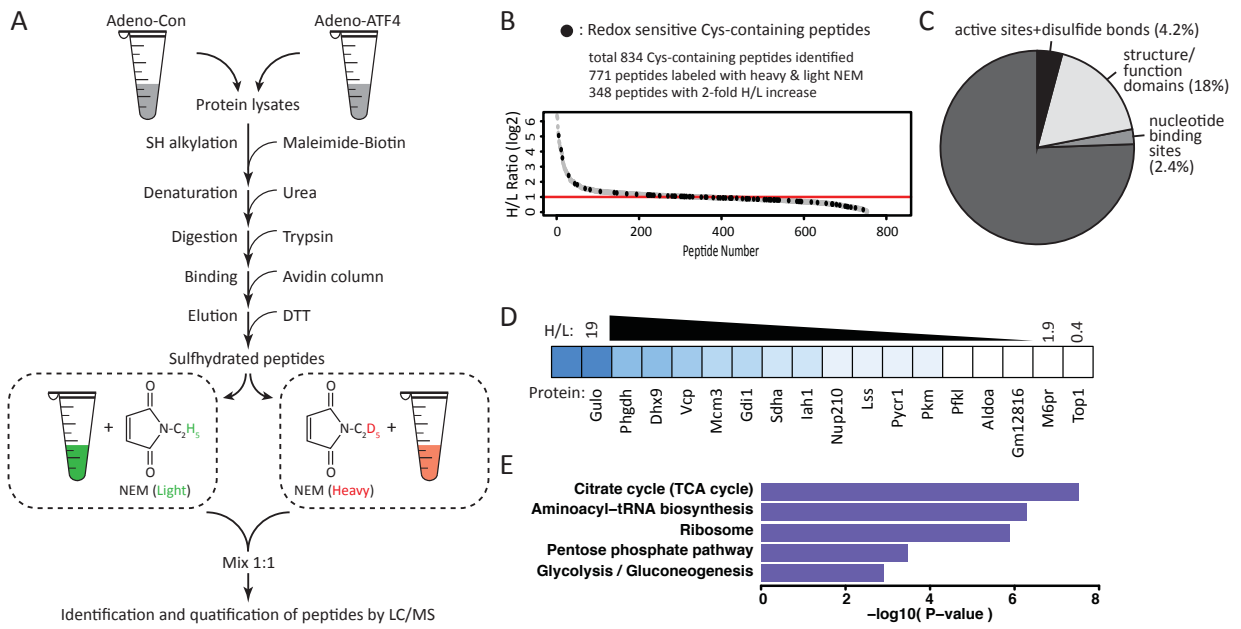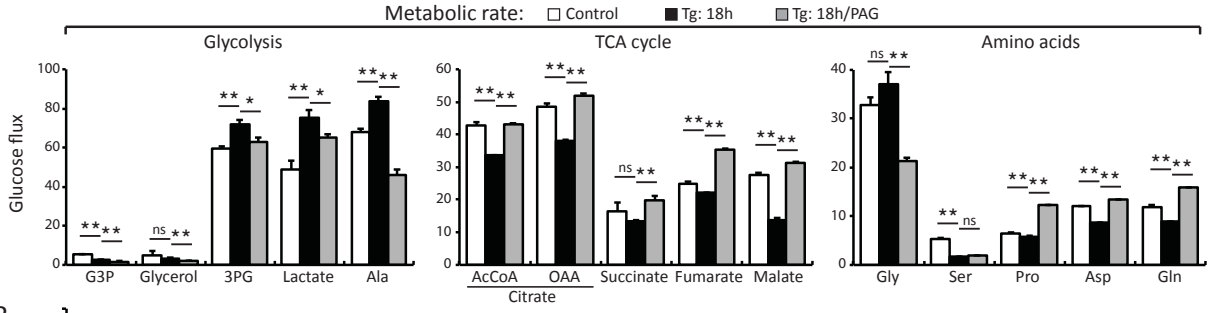1358

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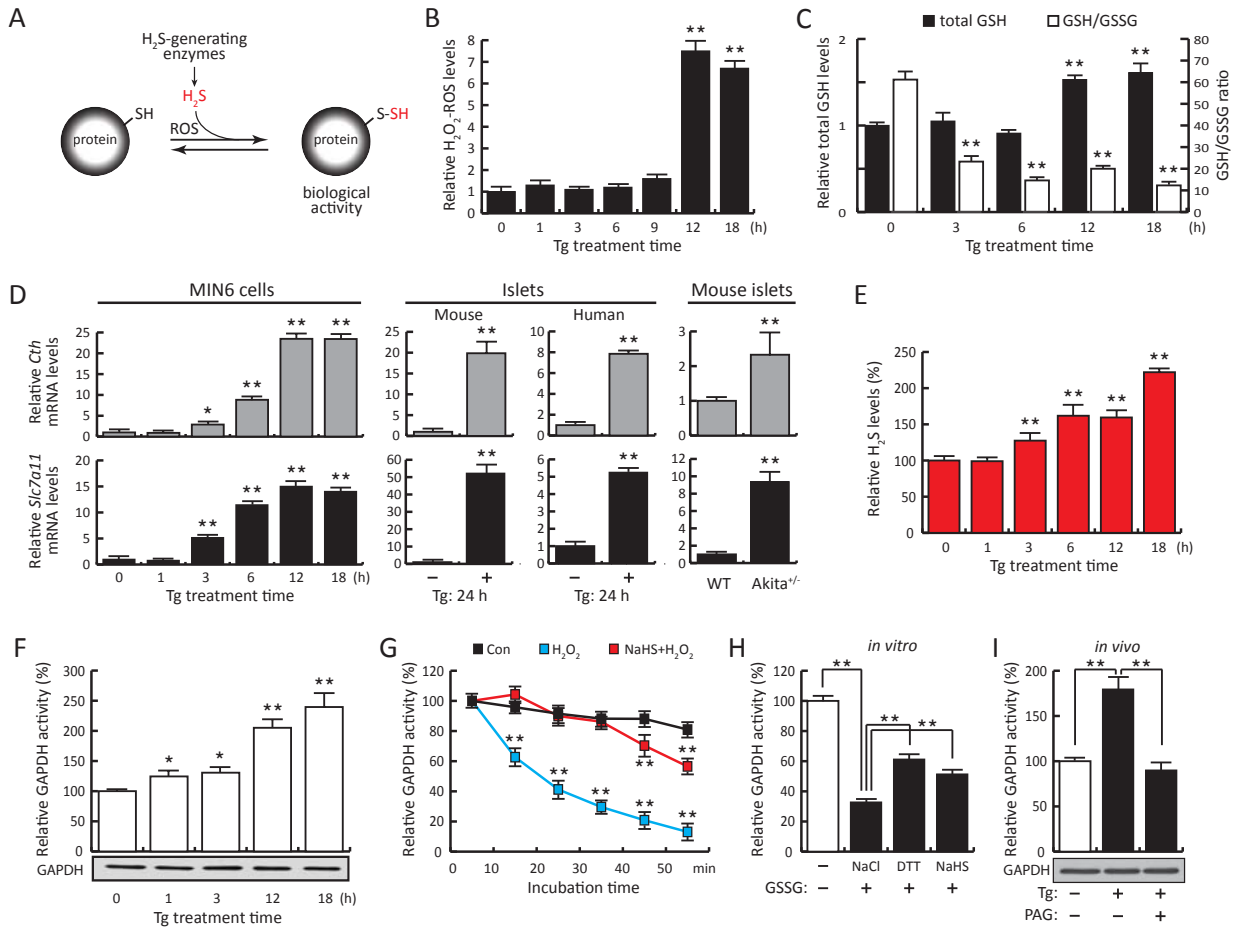
Figure 1.

Figure 2.

Figure 3.

Figure 4.