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## The Hepatic Compensatory Response to Elevated Systemic Sulfide Promotes Diabetes

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49 **SUMMARY**

50 Impaired hepatic glucose and lipid metabolism are hallmarks of type-2 diabetes.  
51 Increased sulfide production or sulfide-donor compounds may beneficially regulate  
52 hepatic metabolism. Disposal of sulfide through the sulfide oxidation pathway (SOP) is  
53 critical for maintaining sulfide within a safe physiological range. We show that mice  
54 lacking the liver-enriched mitochondrial SOP enzyme thiosulfate sulfur-transferase  
55 (*Tst*<sup>-/-</sup> mice) exhibit high circulating sulfide, increased gluconeogenesis,  
56 hypertriglyceridemia and fatty liver. Unexpectedly, hepatic sulfide levels are normal in  
57 *Tst*<sup>-/-</sup> mice due to exaggerated induction of sulfide disposal, with an associated  
58 suppression of global protein persulfidation and nuclear respiratory factor-2 target protein  
59 levels. Hepatic proteomic and persulfidomic profiles converge on gluconeogenesis and lipid  
60 metabolism, revealing a selective deficit in medium-chain fatty acid oxidation in *Tst*<sup>-/-</sup> mice.  
61 We reveal a critical role for TST in hepatic metabolism that has implications for sulfide-donor  
62 strategies in the context of metabolic disease.

63

## 64 INTRODUCTION

65 The prevalence of Type 2 diabetes (T2D) continues to soar in parallel with that of obesity  
66 (OMS, 2014). Increased hepatic glucose production and aberrant hepatic lipid metabolism are  
67 cardinal features of T2D (Consoli *et al.*, 1989; Lewis *et al.*, 2002). Dysregulation of hepatic  
68 nutrient metabolism in T2D is a promising area for therapeutic intervention because it  
69 precipitates the more severe liver pathologies that manifest along the spectrum of non-  
70 alcoholic fatty liver disease (NAFLD), steatosis, steatohepatitis and hepatocellular carcinoma  
71 (Caron *et al.*, 2011).

72 Hydrogen sulfide (hereafter referred to as sulfide), an endogenously produced gaseous  
73 signalling molecule (Abe and Kimura, 1996; Wang, 2012; Mishanina, Libiad and Banerjee,  
74 2015; Filipovic *et al.*, 2017), has recently emerged as a modulator of nutrient metabolism  
75 (Desai *et al.*, 2011; Szabo, 2011; Hine *et al.*, 2015; Carter and Morton, 2016). Enzymatic sulfide  
76 production from sulfur amino acids is catalysed by cystathionine beta-synthase; CBS, and  
77 cystathionine gamma lyase; CTH (Chen, Jhee and Kruger, 2004; Singh *et al.*, 2009) and by 3-  
78 mercaptopyruvate sulfur transferase; MPST (Shibuya *et al.*, 2009; Mikami *et al.*, 2011; Yadav  
79 *et al.*, 2013). Thioredoxin-mediated reduction of cysteine persulfides on proteins also  
80 regulates free sulfide and cysteine persulfide levels (Wedmann *et al.*, 2016). Endogenously  
81 produced and exogenously administered sulfide specifically influences hepatic glucose and  
82 lipid metabolism (Mani *et al.*, 2014; Pichette and Gagnon, 2016). Thus, *in vitro*, treatment of  
83 murine hepatocytes with NaHS, or overexpression of rat *Cth* in HepG2 liver cells, increased  
84 glucose production through increased gluconeogenesis and reduced glycogen storage (Zhang  
85 *et al.*, 2013). Conversely, glucose production was lower in hepatocytes from *Cth* gene  
86 knockout mice (*Cth*<sup>-/-</sup> mice) that exhibit low sulfide production (Zhang *et al.*, 2013). Elevation  
87 of sulfide with NaHS administration *in vivo* reduced cholesterol and triglyceride accumulation

88 in the liver of high fat diet (HFD)–fed mice (Wu *et al.*, 2015). In contrast, inter-cross of sulfide  
89 production–deficient *Cth*<sup>−/−</sup> mice with the hyperlipidemic *ApoE*<sup>−/−</sup> mouse strain (*Cth*<sup>−/−</sup>  
90 *ApoE*<sup>−/−</sup> mice) produced a phenotype of elevated plasma cholesterol following exposure to  
91 an atherogenic diet (Mani *et al.*, 2013). Consistent with their higher cholesterol, *Cth*<sup>−/−</sup>*ApoE*<sup>−</sup>  
92 <sup>−/−</sup> mice developed fatty streak lesions earlier than *ApoE*<sup>−/−</sup> mice, and this effect was reversed  
93 by NaHS administration (Mani *et al.*, 2013). Sulfide may also indirectly impact hepatic nutrient  
94 metabolism through its effect on hepatic artery vasorelaxation and thus liver perfusion  
95 (Fiorucci *et al.*, 2005; Distrutti *et al.*, 2008). The apparently beneficial effects of sulfide  
96 administration in multiple disease indications has led to a major drive towards development  
97 of targeted H<sub>2</sub>S–donor molecules as a therapeutic approach (Whiteman *et al.*, 2011; Sestito  
98 *et al.*, 2017). However, an often-overlooked aspect of net sulfide exposure, key to the efficacy  
99 of therapeutic H<sub>2</sub>S–donors, is that it is regulated through its oxidative disposal. Thus,  
100 endogenous sulfide exposure is actively limited to prevent mitochondrial respiratory toxicity  
101 (Reiffenstein, 1992; Tiranti *et al.*, 2009; Libiad *et al.*, 2018). Sulfide is rapidly oxidized  
102 (Hildebrandt and Grieshaber, 2008; Norris *et al.*, 2011) through the mitochondrial sulfide  
103 oxidation pathway (SOP), consisting of sulfide quinone oxidoreductase (SQOR), persulfide  
104 dioxygenase (ETHE1/PDO) and thiosulfate sulfurtransferase (TST, also known as rhodanese)  
105 (Hildebrandt and Grieshaber, 2008; Jackson, Melideo and Jorns, 2012; Libiad *et al.*, 2014). The  
106 liver is highly abundant in SOP enzymes, and is a major organ of whole body sulfide disposal  
107 (Norris *et al.*, 2011). Mice lacking the *Ethe1* gene (*Ethe1*<sup>−/−</sup> mice) die of fatal sulfide toxicity  
108 (Tiranti *et al.*, 2009), consistent with its critical role in sulfide oxidation and the severe  
109 pathological consequences of unchecked sulfide build-up in tissues. However, the importance  
110 of mitochondrial TST in the SOP *in vivo* remains obscure. In contrast to *Ethe1*<sup>−/−</sup> mice, *Tst*<sup>−</sup>  
111 <sup>−/−</sup> mice were grossly normal despite exhibiting substantially elevated blood sulfide levels, as

112 implied by qualitative measures (Morton *et al.*, 2016). This revealed an important yet distinct  
113 role for TST in the SOP *in vivo*. Nevertheless, *Tst*<sup>-/-</sup> mice showed an apparently diabetogenic  
114 impairment of glucose tolerance (Morton *et al.*, 2016), consistent with the concept that  
115 increased sulfide promotes hepatic glucose production (Zhang *et al.*, 2013). As *Tst* deficiency  
116 represents a model of chronic but viable sulfide elevation, determining the molecular  
117 mechanisms driving their aberrant metabolic profile can provide important insights into the  
118 optimal range for therapeutic sulfide exposure, particularly in light of the current interest in  
119 developing mitochondrially-targeted sulfide-donors (Gero, Domokos Torregrossa *et al.*,  
120 2016; Karwi, Bice and Baxter, 2018). To this end we sought to define the impact of *Tst*  
121 deficiency on the underlying molecular pathways that impact hepatic metabolism.

122

## 123 RESULTS

124 *Tst*<sup>-/-</sup> mice exhibit increased hepatic gluconeogenesis and dyslipidaemia despite mild  
125 peripheral insulin sensitisation.

126 TST mRNA expression is highest in the liver, <http://biogps.org/#goto=genereport&id=22117>;  
127 tissue hierarchy of expression was validated in our own mouse substrain (Figure S1A). We  
128 therefore hypothesised that liver TST deficiency was the principal driver of the impaired  
129 glucose tolerance previously observed in *Tst*<sup>-/-</sup> mice (Morton *et al.*, 2016). *Tst*<sup>-/-</sup> mice  
130 exhibited higher glucose levels than C57BL/6J controls in response to a pyruvate challenge,  
131 consistent with higher hepatic glucose production (Figure 1A). We next tested  
132 phosphoenolpyruvate carboxykinase (PEPCK) activity, a key enzyme of *de-novo* hepatic  
133 glucose synthesis, and found it was higher in liver homogenates from *Tst*<sup>-/-</sup> mice (Figure 1B).  
134 Next, we performed a 1 hour <sup>13</sup>C<sub>3</sub>-pyruvate metabolite-pulse incorporation experiment in  
135 isolated hepatocytes cultured in <sup>12</sup>C<sub>3</sub>-pyruvate-free medium. Hepatocytes from *Tst*<sup>-/-</sup> mice  
136 displayed <sup>13</sup>C labelling consistent with increased metabolism of pyruvate to oxaloacetate – a  
137 critical early step in gluconeogenesis. Specifically, aspartate, which is derived from pyruvate  
138 via oxaloacetate was significantly increased in the *Tst*<sup>-/-</sup> (Figure 1C). A trend towards higher  
139 <sup>13</sup>C<sub>3</sub> malate, and lower <sup>13</sup>C<sub>2</sub> acetyl-CoA was also observed (Figure S1B and S1C). <sup>13</sup>C<sub>3</sub> Lactate  
140 was similar between genotypes, suggesting a similar activity of glycolytic disposal of pyruvate  
141 through lactate dehydrogenase (Figure S1B and S1C). Isotopologue distribution is shown in  
142 Fig S1C. Total pool sizes for all measured metabolites were similar between genotypes (Figure  
143 S1D). Although not a direct measure of glucose production, the data from *in vitro* hepatocytes  
144 suggested skewing of hepatocyte metabolism towards gluconeogenesis, and we therefore  
145 investigated this possibility by further means. Indeed, consistent with increased endogenous



146 glucose production in the  $Tst^{-/-}$  mice, fasting plasma glucose was higher in  $Tst^{-/-}$  mice  
147 relative to 6J mice during the pre-clamp  $3\text{-}^3\text{H}$  glucose tracer infusion phase (60-90 minutes  
148 post tracer) of euglycemic, hyperinsulinemic (EH) clamp experiments (Figure 1D, Table S1A).  
149 Higher plasma glucose levels in  $Tst^{-/-}$  mice under these conditions was not explained by  
150 lower glucose utilization in  $Tst^{-/-}$  mice; glycogen synthesis and glycolysis were comparable  
151 between genotypes across 60–90 minutes (Table S1A). Glucose turnover – a derived  
152 parameter used to infer glucose production – was also comparable between genotypes (Table  
153 S1A). However, derivation of glucose turnover requires that glucose levels are stable during  
154 the period in which it is calculated. In our pre-clamp baseline period, a highly significant effect  
155 of time (Figure 1D) indicated that this assumption was not met, and thus true endogenous  
156 glucose production cannot be inferred from the glucose turnover parameter in this instance.  
157 Combined with the pyruvate tolerance, PEPCK activity and  $^{13}\text{C}_3$ -pyruvate pulse data, higher  
158 fasting glucose levels in the  $Tst^{-/-}$  mice, given comparable glucose utilization, is most likely  
159 due to higher endogenous glucose production in the  $Tst^{-/-}$  mice.

160 We next wished to explore whether the changes to glucose metabolism were driven by insulin  
161 resistance. Liver glycogen, a marker of long-term carbohydrate storage typically impaired  
162 with insulin resistance, was comparable between  $Tst^{-/-}$  and C57BL/6J control mice (Figure  
163 S2A). Despite unchanged steady-state markers of hepatic insulin sensitivity, impaired glucose  
164 tolerance, previously described in the  $Tst^{-/-}$  mice ([Morton et al., 2016](#)), suggested that whole  
165 body, and usually hepatic, insulin-resistance was present. We investigated this using the  
166 euglycaemic clamp where, unexpectedly, we observed whole-body insulin sensitisation  
167 under these short-term steady-state conditions. During the clamp, when insulin was high,  
168 and blood glucose levels were maintained constant, the glucose infusion rate was comparable  
169 between genotypes (Table S1B). However, an increase in whole-body glucose uptake

170 (integral glucose) by tissues in the  $Tst^{-/-}$  mice was apparent (Figure 1E, Table S1B),  
171 supporting increased peripheral insulin-sensitivity, with a directionally consistent trend for  
172 increased glucose uptake into several tissues. We confirmed this finding using standard  
173 insulin tolerance tests, where the glucose decrement in response to insulin was greater in  
174  $Tst^{-/-}$  mice (Figure 1F, Figure S2B). Together these data demonstrate a net increase in  
175 dynamic whole-body insulin sensitivity, despite increased hepatic glucose output in  $Tst^{-/-}$   
176 mice. Finally, we assessed whole-body glucose homeostasis with the EH-clamp method after  
177 chronic HFD feeding. Under these conditions,  $Tst^{-/-}$  mice maintained increased hepatic  
178 glucose output (Figure 1D) but showed convergence of the insulin-sensitivity profile to that  
179 of the insulin-resistant C57BL/6J mice.

180 We also assessed whether  $Tst$  deficiency was associated with impaired lipid metabolism,  
181 another hallmark of diabetes. Fast protein liquid chromatography analysis of triglyceride  
182 levels and their lipoprotein distribution revealed significantly higher total plasma triglycerides  
183 in  $Tst^{-/-}$  mice (Figure 1G). The higher triglyceride was selectively associated with an  
184 increased VLDL triglyceride fraction (Figure 1G), consistent with a dominant liver-driven  
185 impairment in lipid metabolism (Mason, 1998). Total and distinct lipoprotein fraction plasma  
186 cholesterol levels were similar between genotypes (Figure S2C and S2D), suggestive of a  
187 triglyceride-selective effect of  $Tst$  deficiency on hepatic lipid efflux. HFD significantly  
188 increased liver lipid content of C57BL/6J mice but did not further increase the elevated lipid  
189 levels in the liver of  $Tst^{-/-}$  mice (Figure 1H-1I).

190 *TST deficiency elicits compensatory hepatic sulfide disposal mechanisms that drive reduced*  
191 *global protein persulfidation.*

192 A role for TST in the disposal of sulfide was suggested by its participation in the SOP  
193 (Hildebrandt and Grieshaber, 2008; Libiad *et al.*, 2014) and supported *in vivo* by the  
194 qualitatively higher blood sulfide of *Tst*<sup>-/-</sup> mice (Morton *et al.*, 2016), schematically shown in  
195 Figure 2A. Here, we quantified circulating sulfide, showing approximately 10-fold elevation  
196 in the blood and plasma of *Tst*<sup>-/-</sup> mice (Table 1A and 1B). Thiosulfate, an oxidised metabolite  
197 of sulfide (Vitvitsky *et al.*, 2015, 2017) and a TST substrate (Banerjee *et al.*, 2015), was  
198 approximately 20-fold higher in plasma (Table 1B), and profoundly higher (450-fold) in urine  
199 (Table 1C) of *Tst*<sup>-/-</sup> mice compared to C57Bl/6J mice. Reduced glutathione (GSH) levels were  
200 ~2-fold higher in the plasma of *Tst*<sup>-/-</sup> mice (Table 1B). To determine any direct hepatic  
201 contribution to the elevated systemic sulfide *in vivo*, whole blood was sampled from the  
202 inferior vena cava (IVC) (Table 1D). IVC sulfide levels tended to be higher in the *Tst*<sup>-/-</sup> mice,  
203 but the magnitude of the increase (~3-fold) did not parallel that in trunk blood (~10-fold),  
204 suggesting liver was not a major source of the elevated circulating sulfide. Surprisingly, liver  
205 homogenate sulfide, thiosulfate, cysteine and GSH levels were similar between *Tst*<sup>-/-</sup> and  
206 C57BL/6J mice (Table 1E). Further, cultured hepatocytes from *Tst*<sup>-/-</sup> and C57BL/6J mice  
207 exhibited similar intracellular sulfide levels, as estimated using P3, a sulfide-selective  
208 fluorescent probe (Singha *et al.*, 2015) (Table 1F). Mitochondrial sulfide levels in liver reported  
209 by MitoA/MitoN (Arndt *et al.*, 2017) were similarly unchanged between genotypes (Table 1G).  
210 The apparently unaltered hepatic steady-state sulfide levels, despite higher circulating  
211 sulfide, suggested a profound homeostatic mechanism was invoked in the liver of *Tst*<sup>-/-</sup>  
212 mice. We assessed respiratory sulfide disposal (antimycin-sensitive) and found this was  
213 markedly increased in hepatocytes from *Tst*<sup>-/-</sup> mice, whereas antimycin-insensitive sulfide  
214 disposal was relatively reduced compared to hepatocytes from C57BL/6J mice (Table S2).  
215 Isolated liver mitochondria from *Tst*<sup>-/-</sup> hepatocytes also exhibited a higher sulfide disposal

216 rate (Table S2). In addition, cysteine and GSH was excreted at higher levels from *Tst*<sup>-/-</sup>  
217 hepatocytes under basal conditions and after stimulation of sulfur amino acid metabolism by  
218 addition of methionine (Figure 2B and 2C). Consistent with higher GSH turnover, hepatocytes  
219 from *Tst*<sup>-/-</sup> mice showed resistance to exogenous H<sub>2</sub>O<sub>2</sub>-mediated mitochondrial ROS  
220 production (Figure S3). We next determined the global hepatic protein persulfidation profile,  
221 the major post-translational modification mediated by sulfide (Krishnan *et al.*, 2011; Kabil,  
222 Motl and Banerjee, 2014; Koike, Nishimoto and Ogasawara, 2017). Mass spectrometry  
223 analysis of maleimide-labelled liver peptides revealed a greater abundance of peptides with  
224 a lower persulfidation level (under-persulfidated) in the liver of *Tst*<sup>-/-</sup> mice (Figure 2D). We  
225 confirmed this using semi-quantitative western-blot analysis on pulled down maleimide-  
226 labelled proteins (Figure 2E). Gene Ontology (GO) analysis of under-persulfidated peptides  
227 (20 GO categories; Table 2) showed enrichment for “*FAD-binding, methyl transferase,*  
228 *peroxisome, acyl-CoA dehydrogenase activity and transaminase*”. Over-persulfidated  
229 peptides (8 GO categories, Table 2) were predominantly “*Nicotinamide metabolism*”.  
230 Pathway-specific peptide analysis showed a bias for over-persulfidation in gluconeogenesis  
231 proteins (Figure S4A) and a significantly higher magnitude of change (independent of  
232 direction of change) in persulfidation compared to global persulfidomic changes between  
233 C57BL/6J and *Tst*<sup>-/-</sup> mice (Figure S4B).

234 *The hepatic proteome of Tst*<sup>-/-</sup> *mice reveals a distinct molecular signature of altered sulfur*  
235 *and mitochondrial nutrient metabolism.*

236 To gain molecular insight into the mechanisms underlying the apparently diabetogenic  
237 phenotype in *Tst*<sup>-/-</sup> mice we compared hepatic proteomes of normal diet (ND)-fed mice.  
238 Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis revealed 4 up-regulated

239 pathways in liver of *Tst*<sup>-/-</sup> mice related to amino acid metabolism, including sulfur amino  
240 acids, and sulfur metabolism (Table 3A). GO analysis revealed 95 significantly up-regulated  
241 categories in liver of *Tst*<sup>-/-</sup> mice (Table S3A). Among the top categories, 7 referred to amino  
242 acid metabolism and 1 referred to the organellar term '*mitochondrion*'. KEGG analysis  
243 revealed 27 down-regulated pathways in the liver of *Tst*<sup>-/-</sup> mice (Table 3B) including Phase  
244 1 and 2 detoxification pathways (Cytochrome P450s, Glutathione and Glucuronidation) and  
245 '*Lysosome*', '*Protein processing in the Endoplasmic reticulum*' organellar terms. 213 GO terms  
246 were significantly down-regulated in *Tst*<sup>-/-</sup> mice (Table S4B). Among the most significant  
247 down-regulated terms were phase 2 detoxification '*glutathione binding*', '*glutathione*  
248 '*transferase activity*' and '*endoplasmic reticulum*' categories. We validated broadly consistent  
249 direction of change in a representative subset of proteins (Figures S5A and S5D). The most  
250 robust change we observed was increased MPST protein in whole liver (Figure S5A and S5D)  
251 and mitochondrial sub-fractions (Figure S5B and S5D). This change was remarkable as mRNA  
252 levels for *Mpst* were lower in *Tst*<sup>-/-</sup> mice (Figure S5C), likely as a result of loss of proximal  
253 *Mpst* promoter function; *Mpst* is a paralog of *Tst* (Nagahara, 2011) juxtaposed approximately  
254 1kb from the *Tst* gene. Protein levels for other sulfide producing and disposal enzymes were  
255 comparable between genotypes (Table S4). A focussed comparison of canonical proteins in  
256 glucose and lipid metabolism pathways (Table S5) revealed four GO categories that were  
257 down-regulated in *Tst*<sup>-/-</sup> mice; '*Lipid metabolic process*', '*fatty acid beta-oxidation*', '*Acyl-CoA*  
258 '*dehydrogenase activity*' and '*Acyl-CoA hydrolase activity*' (Table S5). Canonical insulin-  
259 regulated proteins were largely comparable between genotypes (Table S6).

260 *Hepatic protein expression in Tst*<sup>-/-</sup> *mice is consistent with lower NRF2 activation.*

261 We performed a transcription factor binding site (TFBS) enrichment analysis in the promoters  
262 of proteins that were up-regulated in the liver of *Tst*<sup>-/-</sup> mice to look for potential hub  
263 transcriptional drivers of the proteome profile (Figure S6A). This revealed a statistically  
264 significant under-representation of TFBS for the sulfide-responsive (Yang *et al.*, 2013; Xie *et*  
265 *al.*, 2016) NRF2 transcription factor (Figure S6A). Consistent with reduced hepatic NRF2  
266 activation, 10 out of 47 known NRF2-regulated proteins were lower in the liver of ND-fed *Tst*<sup>-</sup>  
267 <sup>-</sup> mice compared to C57BL/6J (Figure S6B).

268 *Proteome of TST deficiency versus HFD response in C57BL/6J mice reveals distinct regulation*  
269 *of lipid metabolism, sulfide metabolism and detoxification pathways.*

270 We examined mechanistic commonalities between the diabetogenic hepatic phenotype of  
271 *Tst*<sup>-/-</sup> mice and that induced by the diabetogenic HFD-feeding regimen in C57BL/6J mice.  
272 Note, ND-fed *Tst*<sup>-/-</sup> mice were in a pre-existing diabetogenic state (Figure 1) that does not  
273 further worsen with HFD (Figure 1H-I, Table S1), suggesting gross phenotypic convergence of  
274 the two genotypes after HFD. We compared the identity and direction of change of the 188  
275 proteins differentially expressed in ND-fed *Tst*<sup>-/-</sup> mice (versus ND-fed C57BL/6J mice; Figure  
276 3A) to proteins that were differentially expressed in response to HFD in C57BL/6J mice (432  
277 proteins; Figure 3A). There was a striking 67% overlap in individual proteins (126) in this  
278 comparison (Figure 3A). When we analysed these two protein signatures for directionally  
279 shared pathways, one up-regulated KEGG pathway ‘*Glycine, serine and threonine metabolism*’  
280 (Table S7A) and 12 down-regulated KEGG pathways, including ‘*drug metabolism*’ and  
281 ‘*endoplasmic reticulum*’ (Table S8B) were common to the liver of the ND-fed *Tst*<sup>-/-</sup> and HFD-  
282 fed C57BL/6J mice. Consistent with a pre-existing HFD-like proteome, the dynamic response  
283 to HFD in the liver of *Tst*<sup>-/-</sup> mice was muted, relative to that observed in C57BL6J mice (106

284 proteins, a 4-fold lower response; Figure 3B). Focussing on the sulfide pathway, MPST and  
285 SUOX were increased by HFD in C57BL/6J and *Tst*<sup>-/-</sup> mice (Table S8). The HFD-induced  
286 increase in MPST was less pronounced in the liver of *Tst*<sup>-/-</sup> mice, likely a reflection that it is  
287 already elevated in ND-fed *Tst*<sup>-/-</sup> mice. We then considered contrasting, rather than  
288 congruent, proteomic responses arising from TST deficiency versus HFD responses in  
289 C57BL/6J mice to illuminate potential novel pathways underlying the otherwise functionally  
290 similar diabetogenic hepatic *Tst*<sup>-/-</sup> phenotype. 5 KEGG pathways (Table S9A) and 4 GO terms  
291 (Table S9B) were oppositely regulated in this comparison. Strikingly, the GO terms were all  
292 related to lipid metabolism, which were up-regulated in the HFD response but down-  
293 regulated with TST deficiency (Table S9A-B). An organelle-focused protein analysis showed  
294 shared up-regulation of mitochondrial and endoplasmic reticulum pathways between TST  
295 deficiency (Figure 3C upper row) and C57BL/6J HFD-responses (Figure 3C lower row), but a  
296 striking discordance in peroxisomal protein pathways (up-regulated by HFD, down-regulated  
297 with TST deficiency) and nuclear proteins (down-regulated by HFD, up-regulated with TST  
298 deficiency; Figure 3C).

299 *The Tst*<sup>-/-</sup> liver proteome and persulfidome converge on transamination and lipid oxidation  
300 pathways.

301 To assess whether conservation of changes at protein and post-translational modification  
302 levels can illuminate key regulatory hubs driving the hepatic phenotype we ran a congruence  
303 analysis of the proteome and persulfidome. We found that GO categories 'amino acid', 'lipid  
304 metabolism' and 'peroxisome' were significantly regulated at both protein abundance and  
305 persulfidation levels in *Tst*<sup>-/-</sup> mice (Table 3C).

306 *Tst*<sup>-/-</sup> hepatocytes exhibit elevated mitochondrial respiration and a defect in medium-chain  
307 fatty acid oxidation.

308 Enhanced respiratory sulfide disposal was found from *Tst*<sup>-/-</sup> hepatocytes, and enrichment of  
309 mitochondrial proteins was suggested from the liver proteome of the *Tst*<sup>-/-</sup> mice (Table S4).  
310 We therefore sought to determine if TST deficiency affected respiratory function and  
311 substrate utilisation of the hepatocyte. Analysis of electron micrographs prepared from the  
312 liver of ND-fed *Tst*<sup>-/-</sup> mice and C57BL/6J controls showed morphologically normal  
313 mitochondria (Figure 4A). Basal respiration, comprising ATP-linked and leak respiration, was  
314 significantly higher in hepatocytes from *Tst*<sup>-/-</sup> mice (Figure 4B–4D). Maximal hepatocyte  
315 respiratory capacity and non-respiratory oxygen consumption was similar between  
316 genotypes (Figures S7A and S7B). In line with phenotypic convergence following HFD,  
317 hepatocyte respiration was comparable between genotypes from HFD-fed mice (Figures S7C-  
318 S7H). A unique feature of the liver from *Tst*<sup>-/-</sup> mice was a decrease in proteins and  
319 persulfidation levels of proteins in lipid oxidation pathways. We therefore investigated  
320 hepatocyte respiration of lipids. Using a low pyruvate (100 μM) medium to reveal respiratory  
321 dependency on other substrates, we showed that CPT1A-mediated mitochondrial oxidation  
322 of endogenous long chain fatty acids (LCFA; etomoxir-inhibited) was similar between  
323 genotypes (Figure 4E). Next, we by-passed CPT1A-mediated LCFA transfer, and revealed a  
324 marked deficit in respiration stimulated by the medium chain fatty acid octanoate in  
325 hepatocytes from *Tst*<sup>-/-</sup> mice (Figure 4F). A similar experiment adding back pyruvate  
326 revealed comparable stimulation of respiration between genotypes (Figure S7I). In amino acid  
327 free media, combined glutamine-, aspartate- and alanine-stimulated hepatocyte respiration  
328 was comparable between genotypes (Figure S7J).

329



## 330 **DISCUSSION**

331 Elevated TST expression in adipose tissue was recently identified as a genetic mechanism  
332 driving metabolically protective leanness in mice (Morton *et al.*, 2016). Conversely, mice with  
333 genetic deletion of *Tst* (*Tst*<sup>-/-</sup> mice) exhibited impaired glucose tolerance (Morton *et al.*,  
334 2016). However, *Tst*<sup>-/-</sup> mice had a subtle adipose tissue phenotype, suggesting a non-  
335 adipose origin for impaired glucose homeostasis. We found increased gluconeogenesis,  
336 steatosis and elevated plasma VLDL triglycerides consistent with a predominately hepatic  
337 origin for the diabetogenic phenotype. We cannot rule out a contribution of renal  
338 gluconeogenesis to the phenotype, and future work will address this limitation.  
339 Unexpectedly, and in spite of the markedly increased circulating sulfide levels (10-fold),  
340 steady-state sulfide level was normal in the liver of *Tst*<sup>-/-</sup> mice. Moreover, we found evidence  
341 for multiple mechanisms for increased hepatic sulfide disposal, reduced downstream sulfide  
342 signalling and associated underlying molecular links to an apparently diabetogenic  
343 phenotype. Our data suggest that the liver of *Tst*<sup>-/-</sup> mice has over-shot in its attempt to  
344 maximise hepatic sulfide removal, leading indirectly to detrimental metabolic consequences.  
345 This involves a combination of distinct compartmentalised cellular responses including  
346 increased respiratory sulfide disposal and export of cysteine and GSH. Notably, up-regulation  
347 of translation and recruitment of MPST to the mitochondria of *Tst*<sup>-/-</sup> mice is observed. This  
348 response, in face of reduced transcription of *Mpst*, suggests a powerful post-transcriptional  
349 cellular sulfide sensing mechanism. Interestingly, if MPST is compensating for TST-mediated  
350 sulfide disposal in this context, it implies a subversion of normal MPST function away from  
351 sulfide production (Módos *et al.*, 2013; Szabo *et al.*, 2014; Kimura *et al.*, 2017; Nagahara,  
352 2018). Alternatively this is a response to a perceived lower sulfide environment. TST levels

353 were also elevated in the liver of *Mpst*<sup>-/-</sup> mice, providing further support for a reciprocal  
354 compensatory mechanism between these two enzymes (Nagahara *et al.*, 2019).

355 The unexpected finding of normal hepatic sulfide levels in the *Tst*<sup>-/-</sup> mice led us to discover  
356 that the metabolic phenotype we observed was driven by the very mechanisms invoked to  
357 maintain sulfide within a normal range rather than sulfide excess *per se*. Several observations  
358 were consistent with this. For example, the major amino acid pathways increased in the liver  
359 of *Tst*<sup>-/-</sup> mice were transaminases involved in metabolism of GSH that support increased  
360 export of sulfur equivalents as GSH (and cysteine). These same transaminases support  
361 gluconeogenesis by redirecting Krebs cycle intermediates (Rui, 2014; Qian *et al.*, 2015;  
362 Sookoian *et al.*, 2016). Re-programming of amino acid metabolism for sulfide disposal with  
363 knock-on effects to drive hepatic glucose production are suggested, rather than any change  
364 to amino acid-linked mitochondrial respiration in hepatocytes. This is supported by the shift  
365 in hepatocyte pyruvate metabolism towards aspartate. In addition, glutathione-S-  
366 transferases (GST) that inhibit gluconeogenesis (Ghosh Dastidar *et al.*, 2018) were lower in  
367 the liver of *Tst*<sup>-/-</sup> mice. Further, activation of NRF2, which represses gluconeogenesis  
368 (Slocum *et al.*, 2016) appears lower in liver of *Tst*<sup>-/-</sup> mice. The involvement of NRF2 in the  
369 *Tst*<sup>-/-</sup> liver phenotype is further supported by the phenotype of *Nrf2*<sup>-/-</sup> mice that similarly  
370 exhibited steatohepatitis in the absence of insulin resistance (Meakin *et al.*, 2014). However,  
371 we note that NRF2 signalling can be complex and dependent upon dietary context; *Nrf2*<sup>-/-</sup>  
372 mice showed improved glucose tolerance after a high fat diet (Zhang *et al.*, 2012) suggesting  
373 any contribution of a NRF2 signalling deficit in the liver of the *Tst*<sup>-/-</sup> mice changes upon high  
374 fat feeding. Beyond altered pyruvate flux, we also showed that the hepatocytes of *Tst*<sup>-/-</sup>  
375 mice exhibited defective lipid metabolism. Specifically, medium-chain fatty acid (MCFA)  
376 oxidation was impaired, associated with selective reduction of both protein and

377 persulfidation levels of lipid catabolic enzymes. This represents a mechanism linking altered  
378 sulfide metabolism to lipid oxidation, hepatic lipid accumulation and dyslipidaemia.  
379 Consistent with impaired MCFA oxidation defects as one driver of the phenotype, steatosis is  
380 observed in medium-chain acyl-CoA dehydrogenase (*Mcad*)<sup>-/-</sup> mice (Tolwani *et al.*, 2005)  
381 and dyslipidemia is found in MCADD deficient humans (Onkenhout *et al.*, 1995). The data we  
382 present adds to a growing understanding of the link between sulfide regulating genes and  
383 nutrient metabolism that has hitherto focussed on the enzymes of sulfide production.  
384 Specifically, we provide support for the importance of the sulfide oxidising pathway as a  
385 regulator of cellular sulfide exposure. Unexpectedly, the data reveal cellular mechanisms that  
386 are engaged to homeostatically regulate sulfide disposal and that can impact upon cell  
387 energetics and nutrient metabolism.

388 Our findings may have implications for potentially unexpected side effects of sulfide donor  
389 therapeutics. In normal mice, *in vivo* sulfide administration for 4 weeks post-HFD partially  
390 reversed hepatic lipid accumulation invoked by chronic (16 weeks) HFD (Wu *et al.*, 2015). No  
391 evidence was provided for whether sulfide disposal mechanisms were altered (Wu *et al.*,  
392 2015). This efficacious sub-chronic sulfide administration regimen contrasts with our genetic  
393 model of chronic sulfide elevation as a driver of dysregulated metabolism and NAFLD. Clearly,  
394 the normal mice in the Na<sub>2</sub>S administration studies had a fully functional SOP, suggesting the  
395 presence of TST is required to achieve the beneficial metabolic effects of Na<sub>2</sub>S administration.  
396 This is also consistent with the apparently low sulfide signalling status (evidenced by lower  
397 persulfidation, NRF2 target protein abundance) in the liver of the *Tst*<sup>-/-</sup> mice. The benefits  
398 of elevated sulfide cannot be realised perhaps because a major mediator of those effects is  
399 missing and the alternate mechanisms invoked do not fully compensate (e.g. MPST), or  
400 actively drive aberrant nutrient metabolism. Comparable studies of glucose and lipid

401 metabolism after manipulation of other sulfide regulating genes are limited. However in a  
402 contrasting model of reduced sulfide production (*Cth*<sup>-/-</sup> mice), plasma triglycerides were  
403 lowered ([Mani et al., 2013](#)), opposite to what we observed with the *Tst*<sup>-/-</sup> mice. The hepatic  
404 sulfide disposal status of the *Cth*<sup>-/-</sup> mouse model is unknown, but our findings predict a  
405 suppression of the SOP to spare the limited endogenous sulfide produced. Intriguingly they  
406 also predict a knock-on effect on nutrient homeostasis due to reduced metabolic demand of  
407 the TST/SOP axis. A more direct model informing on the effects of impairment of the sulfide  
408 disposal pathway is deficiency of the key mitochondrial SOP enzyme ETHE1. *Ethe1*<sup>-/-</sup> mice  
409 suffer fatal sulfide toxicity ([Tiranti et al., 2009](#)) and therefore comparable metabolic studies  
410 are lacking. However, one notable observation is that *Ethe1*<sup>-/-</sup> mice have an apparently 10-  
411 fold higher liver sulfide exposure than control mice ([Tiranti et al., 2009](#)), in contrast to the  
412 normalised hepatic sulfide levels of *Tst*<sup>-/-</sup> mice. Circulating sulfide levels were not reported  
413 for comparison, but the presumably relatively lower systemic sulfide levels of *Tst*<sup>-/-</sup> mice  
414 appear to have permitted an effective homeostatic sulfide disposal response in the liver to  
415 avoid toxicity, albeit with a metabolic cost. Consequently, the liver of *Tst*<sup>-/-</sup> mice has a  
416 distinct functional and proteomic profile to that of the *Ethe1*<sup>-/-</sup> mice. For example, in the  
417 liver of *Tst*<sup>-/-</sup> and *Ethe1*<sup>-/-</sup> mice ([Hildebrandt et al., 2013](#)), proteins of the glutathione-S-  
418 transferase Mu type (GSTM) and peroxiredoxin (PRDX) families were altered, but sometimes  
419 in the opposite direction or with alteration of distinct protein sub-classes. A notable  
420 difference is also observed in amino acid metabolism. The liver of *Ethe1*<sup>-/-</sup> mice exhibited  
421 increased expression of enzymes of branched chain amino acid metabolism ([Hildebrandt et](#)  
422 [al., 2013](#)), distinct from the predominantly glutathione-related amino acid pathways that are  
423 increased in liver of *Tst*<sup>-/-</sup> mice. Beyond sulfide, TST may also have distinct cellular roles that  
424 affect metabolism such as mitoribosomal synthesis, ROS attenuation and modulation of

425 mitochondrial iron-sulfur clusters (Bonomi *et al.*, 1977; Pagani and Galante, 1983; Nandi and  
426 Westley, 1998; Nandi, Horowitz and Westley, 2000; Smirnov *et al.*, 2010).

427 Given the pro-diabetogenic liver phenotype in *Tst*<sup>-/-</sup> mice, its was surprising that insulin  
428 signalling in the liver appeared normal and peripheral insulin sensitivity was increased. There  
429 are precedents for increased hepatic glucose production independent of insulin resistance, as  
430 found in the *Nrf2*<sup>-/-</sup> mice (Meakin *et al.*, 2014) and as driven by the transcription factor  
431 ChREBP (Uyeda and Repa, 2006; Kim *et al.*, 2016). There is also evidence to support insulin-  
432 sensitising effects of sulfide administration *in vivo* in mice and rats (Feng *et al.*, 2009; Geng *et*  
433 *al.*, 2013; Xue *et al.*, 2013), consistent with sulfide-mediated insulin-sensitisation of non-  
434 hepatic tissues in *Tst*<sup>-/-</sup> mice. Higher circulating GSH in *Tst*<sup>-/-</sup> mice may also promote  
435 peripheral insulin-sensitisation (Jain *et al.*, 2014; Lutchmansingh *et al.*, 2018). Clearly, the net  
436 balance of glucose production from the liver and its peripheral disposal remains abnormal in  
437 *Tst*<sup>-/-</sup> mice. Indeed, the baseline metabolic phenotype of *Tst*<sup>-/-</sup> mice resembles in many  
438 ways that of a normal mouse fed a HFD and we showed some overlapping pro-diabetogenic  
439 signatures between the liver proteome of *Tst*<sup>-/-</sup> mice and that of HFD-fed C57BL/6J mice.  
440 However, we also found distinct lipid metabolism and peroxisomal protein changes in *Tst*<sup>-/-</sup>  
441 mice. Unlike a HFD state, which is associated with dominant hepatic insulin resistance, the  
442 increased hepatic glucose production in ND-fed *Tst*<sup>-/-</sup> mice occurs despite normal hepatic  
443 insulin sensitivity. The significant changes in persulfidation of transaminase and  
444 gluconeogenesis proteins suggests coordinated cross-talk across metabolic pathways  
445 underlies this atypical metabolic phenotype.

446 Sulfide donor therapeutics were proposed as a clinical strategy for improving cardiovascular  
447 health (Szabó *et al.*, 2011; Whiteman *et al.*, 2011; Zhang *et al.*, 2018). Elevated endogenous  
448 sulfide was also implicated in the beneficial metabolic effects of caloric restriction (Miller *et*

449 *al.*, 2005; Hine *et al.*, 2015, 2017, 2018; Shimokawa *et al.*, 2015; Lee, Kaya and Gladyshev,  
450 2016). Our results suggest that chronic sulfide elevation may have unintended detrimental  
451 consequences, driving liver glucose production and fat accumulation to undesirable levels.  
452 This caveat may be fortunately limited to cases where SOP proteins are compromised through  
453 rare genetic effects – such as TST variants (Billaut-Laden *et al.*, 2006; Libiad, Sriraman and  
454 Banerjee, 2015). More broadly, a number of drugs or supplements are known to increase  
455 cyanide, which may dominantly inhibit TST activity and result in secondary sulfide  
456 overexposure. These include nitroprusside (Morris *et al.*, 2017) and amygdalin (Bromley *et*  
457 *al.*, 2005; O'brien, Quigg and Leong, 2005). Indeed, the TST metabolite thiosulfate is  
458 commonly co-administered with nitroprusside to prevent cyanide toxicity (Curry, Carlton and  
459 Raschke, 1997). Furthermore, dietary and environmental exposure to cyanogenic compounds  
460 (Petrova, 2004), e.g. smoking (Vinnakota *et al.*, 2012) or cyanogenic diets (Kashala-Abotnes  
461 *et al.*, 2019) may interfere with normal TST function and could lead to increased sensitivity to  
462 sulfide therapeutics. In contrast, we have shown that administration of the TST substrate  
463 thiosulfate can ameliorate diabetes (Morton *et al.*, 2016) further underlining the potential  
464 utility of targeting the SOP in metabolic disease. As with all therapeutic strategies, a careful  
465 cost-benefit analysis is required. A comparable case of relevance are the statins, one of the  
466 most potent and widely used drugs to prevent atherosclerosis, which also carry a higher risk  
467 for diabetes (Swerdlow *et al.*, 2015). The full impact of TST manipulation on opposing  
468 metabolic pathways requires further study. Our current study sheds light on the underlying  
469 hepatic mechanisms invoked for sulfide disposal that are relevant to current sulfide–donor  
470 strategies and may inform on routes to reduce their potential metabolic side-effects.

471

## 472 **Limitations of the Study**

473 Whilst liver is the site of most (~60%) post-absorptive gluconeogenesis in normal animals  
474 within physiological fasting ranges, renal/small intestinal gluconeogenesis begins to  
475 substantially contribute to circulating glucose with prolonged fasting/starvation (Sasaki et al.,  
476 2017, Mutel et al., 2011, Mithieux et al., 2003, Stumvoll 1998, Owen 1969). We cannot rule  
477 out a role for renal or intestinal gluconeogenesis in the diabetogenic phenotype of *Tst*<sup>-/-</sup> mice.  
478 This will be an important area of future work, although we note that liver TST is at least >3-  
479 fold that of kidney and small intestinal TST is very low (BioGPS, Figure S1A).

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484 (Notre Dame) for advice establishing sulfide measurement by probe.

## 485 **AUTHOR CONTRIBUTIONS**

486 N.M.M. and R.N.C conceived experiments. R.N.C., M.T.G.G, M. B.-L., A. M.-C., M.L., V.V., B.E.,  
487 T. LeB., M.B., S.H., S.G.D., N.Z.M.H., C. Mc F., A.T., N. F., T.G., performed experiments. R.N.C.,  
488 M. B.-L., P.F., V.V., T. LeB., N.Z.M.H., T.S., F.B., T.G., R.C.H., B.S., G.G., A.J.F., C.S., R.B. and  
489 N.M.M. analysed and interpreted data and commented on the manuscript. K.H.A., S.S.  
490 generated reagents. C.McM. and R.C.H. generated reagents. R.N.C. and N.M.M. wrote the  
491 manuscript.

## 492 **Declaration of Interests**

493 The authors have declared that no conflict of interest exists.

494

496 **Figure 1. *Tst* deletion results in impaired glucose and lipid metabolism.** (A) Plasma glucose over 120  
 497 minutes, following pyruvate (i.p., 1.5mg/g) administration in overnight fasted C57Bl/6J (black line, n =  
 498 9) and *Tst*<sup>-/-</sup> (red line, n = 8) normal diet-fed mice. (B) Extinction of NADH measured by absorbance  
 499 at 340nm, coupled to PEPC activity from liver homogenates taken from C57Bl/6J (white bar, n = 6)  
 500 and *Tst*<sup>-/-</sup> (red bar, n = 6) normal diet-fed mice. (C) Production of <sup>13</sup>C (M+3) aspartate generated after  
 501 a 1 hour pulse of 1mM 3-carbon labelled <sup>13</sup>C (M+3) pyruvate in <sup>12</sup>C pyruvate free media, expressed as  
 502 a percentage of the total amount of detected metabolite, in primary hepatocytes from C57Bl/6J (white  
 503 bars, n = 6) and *Tst*<sup>-/-</sup> (red bars, n = 5) normal diet-fed mice. (D) Blood glucose during the pre-clamp  
 504 phase of the hyperinsulinemic, euglycemic clamp from C57Bl/6J (black lines), and *Tst*<sup>-/-</sup> (red lines) fed  
 505 a control (ND, solid lines, n = 3, 6) or high fat diet (HFD, broken lines, n = 6, 7). (E) Mean integrated  
 506 radioactive glucose (inversely related to whole body glucose uptake) during a hyperinsulinemic,  
 507 euglycaemic clamp from normal diet fed C57Bl/6J control (white, n = 3), and *Tst*<sup>-/-</sup> (red, n = 6) mice.  
 508 (F) Plasma glucose expressed as % of baseline glucose, over 120 minutes following insulin (i.p., 1mU/g)  
 509 administration in 4 hour fasted C57Bl/6J (black line, n = 8) and *Tst*<sup>-/-</sup> (red line, n = 7) normal diet-fed  
 510 mice. (G) HPLC quantified total and VLDL plasma triglyceride in 4 hour fasted C57Bl/6J (white bar, n =  
 511 6), and *Tst*<sup>-/-</sup> (red bar, n = 6) normal diet-fed mice. (H) Representative light microscopic images of  
 512 fixed liver stained with Oil-Red O from normal diet-fed (ND) or high fat diet-fed (HFD) C57Bl/6J and  
 513 *Tst*<sup>-/-</sup> mice. (I) Analysis of the area of red staining (Oil Red O) after thresholding, using Image J, from  
 514 normal diet-fed (no pattern, n = 3-4/genotype) or high fat diet-fed (hatched pattern, n = 4-5/genotype)  
 515 C57Bl/6J (white bars) and *Tst*<sup>-/-</sup> (red bars) mice. Data are represented as mean ±SEM. Significance  
 516 was calculated using repeated measures ANOVA (A,F) 2-WAY ANOVA (I), 3-WAY repeated measures  
 517 ANOVA (D) or unpaired two-tailed student's t-test (B,C,E,G) \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001, \*\*\*\*  
 518 P < 0.0001. For (D) significant effects of time (\*\*\*\*), diet (\*) and genotype (\*) were found. For (F) the  
 519 analysis was performed on absolute glucose values and demonstrated a significant effect of time  
 520 (\*\*\*\*) and an interaction between time and genotype (\*). T-tests revealed that the decrement of  
 521 glucose from baseline at 30 and 60 minutes after insulin was greater in the *Tst*<sup>-/-</sup> (\*). For (I) no main  
 522 genotype effect was found, but a significant effect of diet (\*\*\*), and an interaction (\*) were found.  
 523 Post Hoc analysis using Sidaks' multiple comparison test show an effect of diet on the 6J controls (\*\*\*),  
 524 whereas no effect of diet is found on the *Tst*<sup>-/-</sup>. See also Fig S1, S2 and Table S1.

525  
 526 **Figure 2. *Tst* deletion results in increased hepatic sulfur excretion and a reduction of protein**  
 527 **persulfidation.** (A) Schematic showing mammalian metabolism of hydrogen sulfide. The canonical  
 528 production enzymes are shown in the cytosol; **MPST** (mercaptopyruvate suflur transferase), **CBS**  
 529 (cystathionine beta synthase), and **CTH** (cystathionine gammalyase). Mitochondrial oxidation and  
 530 disposal of hydrogen sulfide occurs through the 'sulfide oxidation pathway', through the actions of  
 531 **SQOR** (sulfide quinone oxidoreductase), **ETHE1** (persulfide dioxygenase), **TST** (thiosulfate  
 532 sulfurtransferase), and **SUOX** (sulphite oxidase). These seven enzymes are widely accepted as major  
 533 contributors to intracellular sulfide (and other inorganic sulfur) metabolism. For simplicity, the  
 534 diagram does not include sulfide production which can occur within the mitochondria, or disposal  
 535 pathways in cytosol. The identity of oxidised sulfur species produced by **SQOR** remain disputed. The  
 536 precise role of **TST** and other enzymes shown here remains under investigation. (B) Cysteine  
 537 concentrations (MBB-HPLC) in the media incubated with primary hepatocytes in the presence  
 538 (hatched pattern) or absence (no pattern) of 1mM methionine, from C57Bl/6J (white bars, n =  
 539 4/treatment) and *Tst*<sup>-/-</sup> (red bars, n = 4/treatment) mice. (C) Glutathione concentrations (MBB-HPLC)  
 540 in the media incubated with primary hepatocytes in the presence (hatched pattern) or absence (no  
 541 pattern) of 1mM methionine, from C57Bl/6J (white bars, n = 4/treatment) and *Tst*<sup>-/-</sup> (red bars, n =  
 542 4/treatment) mice. (D) Pie chart depicting the proportion of liver peptides that are significantly higher  
 543 (82 peptides, purple space) or lower (311 peptides, yellow space), in their persulfidation rate in the  
 544 *Tst*<sup>-/-</sup> (n = 3) relative to C57Bl/6J (n = 3) mice. (E) Total DTT-released cysteine-persulfidated liver



545 protein as measured by REVERT total protein stain following western blotting, normalised to the total  
546 input protein of the sample from *Tst*<sup>-/-</sup> (red bar, n = 4) and C57Bl/6J (white bar, n = 4) mice. Data with  
547 error bars are represented as mean ±SEM. Significance was calculated using 2-WAY ANOVA (B, C) or  
548 student's t-test (E), \* P < 0.05, \*\* P < 0.01. For (B) and (C) the 2-WAY ANOVA reveals a main effect of  
549 genotype, indicated by \* or \*\* on the histogram. A significant effect of methionine was also found for  
550 both (B) and (C) not indicated on the histogram. For (D) peptides were selected as being significant at  
551 a P-diff of 0.95 or greater. See also Fig S3 and Table S2.

552

553 **Figure 3. *Tst* deletion engenders a high fat feeding-like hepatic proteome with a distinct organellar**  
554 **signature. (A)** Venn diagram representing the number of proteins significantly different (at P < 0.01)  
555 between normal diet-fed *Tst*<sup>-/-</sup> and C57Bl/6J (Red circle), and the number of regulated proteins  
556 between high fat fed and normal diet-fed C57Bl/6J (Green circle). The overlap (brown) represents  
557 those proteins regulated in the same direction by both comparisons (n= 4/genotype). (B) Number of  
558 proteins significantly different (at p < 0.01) between 58% high fat and normal diet in either C57Bl/6J  
559 (white bar), or *Tst*<sup>-/-</sup> mice (Red bar), (n= 4/genotype). (C) Pie charts depicting the proportion of  
560 individual liver proteins that are upregulated (Blue space) compared to downregulated (yellow space)  
561 after GO term categorisation according to subcellular location. Upper row; normal diet-fed *Tst*<sup>-/-</sup>  
562 relative to normal diet-fed C57Bl/6J. Lower row, high fat-fed C57Bl/6J relative to normal diet-fed  
563 C57Bl/6J. See also Table 3, Figure S5, S6.

564

565 **Figure 4. *Tst* deletion results in increased hepatocyte respiration but impaired medium-chain fat**  
566 **respiration. (A)** Electron microscope images of liver, visualising mitochondria from normal diet-fed  
567 C57Bl/6J (n = 4) or *Tst*<sup>-/-</sup> (n = 4) mice. (B) Seahorse trace representing the mean oxygen consumption  
568 rate (OCR), normalised to protein, by hepatocytes from normal diet-fed C57Bl/6J (n = 6) or *Tst*<sup>-/-</sup> (n  
569 = 6) mice during a mitochondrial stress test. (C) Respiratory OCR linked to ATP production (oligomycin  
570 sensitive) by hepatocytes from normal diet-fed C57Bl/6J (n = 6) or *Tst*<sup>-/-</sup> (n = 6) mice, calculated from  
571 Figure 4B. (D) Respiratory OCR relating to proton leak (oligomycin insensitive) by hepatocytes from  
572 normal diet-fed C57Bl/6J (n = 6) or *Tst*<sup>-/-</sup> (n = 6) mice, calculated from Figure 4B. (E) Reduction of  
573 maximal uncoupled respiration following inhibition of long chain fatty acid mitochondrial import using  
574 etomoxir (8 µM), from normal diet-fed C57Bl/6J (n = 4) or *Tst*<sup>-/-</sup> (n = 4) mice. (F) Stimulation of  
575 maximal uncoupled respiration following addition of medium chain fatty acid octanoate (250 µM),  
576 from normal diet-fed C57Bl/6J (n = 4) or *Tst*<sup>-/-</sup> (n = 4) mice. Data are represented as mean ±SEM.  
577 Significance was calculated using an unpaired two tailed, student's t-test (C, D, E, F), \* P < 0.05. See  
578 also Figure S7.

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	C57Bl/6J	<i>Tst</i> <sup>-/-</sup>	<i>Tst</i> <sup>-/-</sup> /6J ratio	Significance
<b>A Trunk Blood (Micromolar)</b>				
MBB-S (Sulfide)	2.28 +/- 0.43	22.18 +/- 0.85	9.73	****
MBB-SSO3 (Thiosulfate)	n.d.	6.25 +/- 3.17	n.c.	ns
<b>B Trunk Plasma (Micromolar)</b>				
MBB-S (Sulfide)	1.88 +/- 0.64	24.50 +/- 2.02	13.03	****
MBB-SSO3 (Thiosulfate)	3.99 +/- 0.99	80.29 +/- 13.6	20.12	**
MBB-GSH (reduced Glutathione)	48.0 +/- 1.15	86.25 +/- 6.27	1.80	***
<b>C Urine (Micromoles/creatinine/24hrs)</b>				
MBB-SSO3 (Thiosulfate)	4.99 +/- 2.6	2374 +/- 319	475.75	****
<b>D Inferior Vena Cava (Micromolar)</b>				
MBB-S (Sulfide)	1.22 +/- 0.20	3.58 +/- 0.87	2.93	ns (0.08)
MBB-SSO3 (Thiosulfate)	6.58 +/- 4.51	88.3 +/- 13.0	13.42	*
<b>E Liver (μmoles/kg wet liver)</b>				
MBB-S (Sulfide)	13 +/- 1	17 +/- 3	1.31	ns
MBB-SSO3 (Thiosulfate)	4 +/- 1	15 +/- 7	3.75	ns
DNFB-GSH (reduced Glutathione)	6470 +/- 380	6850 +/- 30	1.04	ns
DNFB-Cysteine (Cysteine)	82 +/- 13	67 +/- 11	0.82	ns
<b>F Sulfide P3 fluorescence (A510nm/protein)</b>				
Hepatocyte	7.22 +/- 1.00	7.89 +/- 0.80	1.09	ns
<b>G Mitochondrial sulfide (MitoA)</b>				
Liver	0.78 +/- 0.16	1.14 +/- 0.45	1.46	ns

\* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001 \*\*\*\* P < 0.0001

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589 **Table 1. *Tst* deletion results in altered sulfur metabolites in blood and liver.** (A) Sulfide dibimane,  
590 thiosulfate-MBB, measured by fluorescence detection following HPLC, from whole blood taken from  
591 trunk blood of ND-fed C57Bl/6J (n = 4) and *Tst*<sup>-/-</sup> (n = 4) mice. (B) Sulfide dibimane, thiosulfate-MBB,  
592 and rGSH-MBB, measured by fluorescence detection following HPLC, from EDTA-Plasma of ND-fed  
593 C57Bl/6J (n = 4) and *Tst*<sup>-/-</sup> (n = 4) mice. (C) Thiosulfate-MBB corrected for creatinine from 24 hour  
594 urine samples, taken from ND-fed C57Bl/6J (n = 4) and *Tst*<sup>-/-</sup> (n = 5) mice. (D) Sulfide dibimane, and  
595 thiosulfate-MBB, from whole blood taken from the inferior vena cava downstream of the hepatic vein  
596 of ND-fed C57Bl/6J (n = 3) and *Tst*<sup>-/-</sup> (n = 3) mice. (E) Sulfide dibimane, thiosulfate-MBB, rGSH-MBB,  
597 and cysteine-MBB from whole liver (n=4/genotype) of ND-fed C57Bl/6J (n = 4) and *Tst*<sup>-/-</sup> (n = 4) mice.  
598 (F) Fluorescence from cultured hepatocytes following incubation with P3 (sulfide reactive probe) from  
599 ND-fed C57Bl/6J (n = 4) and *Tst*<sup>-/-</sup> (n = 4) mice. (G) Ratio of Mito N/MitoA from the liver of ND-fed  
600 C57Bl/6J (n = 5) and *Tst*<sup>-/-</sup> (n = 5) mice. Data are represented as mean ±SEM. Significance was  
601 calculated using unpaired two-tailed student's t-test. \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001, \*\*\*\* P <  
602 0.0001  
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**Table 2. GO Terms persulfidation rate (*Tst*<sup>-/-</sup> vs C57Bl/6J liver, ND-fed)**

GO-ID	Name	Direction ( <i>Tst</i> <sup>-/-</sup> vs 6J)	Genes
<b>GO terms Identified by log fold change</b>			
0050660	FAD binding	Decreased	12
0008168	Methyltransferase activity	Decreased	9
0016741	Transferase activity, transferring one-carbon groups	Decreased	9
0008565	Protein transporter activity	Decreased	8
0008238	Exopeptidase activity	Decreased	7
0005777	Peroxisome	Decreased	7
0042579	Microbody	Decreased	7
0003995	Acyl-CoA dehydrogenase activity	Decreased	6
0008483	Transaminase activity	Decreased	6
0016769	Transferase activity, transferring nitrogenous groups	Decreased	6
0008757	S-adenosylmethionine-dependent methyltransferase activity	Decreased	6
0016655	Oxidoreductase activity, acting on NADH/NADPH, quinone	Decreased	5
0004177	Aminopeptidase activity	Decreased	5
0000059	Protein import into nucleus, docking	Decreased	3
0005643	Nuclear pore	Decreased	3
0031965	Nuclear membrane	Decreased	3
0044453	Nuclear membrane part	Decreased	3
0046930	Pore complex	Decreased	3
0015629	Actin cytoskeleton	Decreased	3
0016652	Oxidoreductase activity, NADH/NADPH, NAD/NADP acceptor	Decreased	3
0050662	Coenzyme binding	Increased	5
0016651	Oxidoreductase activity, NADH/NADPH,	Increased	5
0003954	NADH dehydrogenase activity	Increased	4
0008137	NADH dehydrogenase (ubiquinone) activity	Increased	4
0050136	NADH dehydrogenase (quinone) activity	Increased	4
0006739	NADP metabolism	Increased	3
0006769	Nicotinamide metabolism	Increased	3
0006733	Oxidoreduction coenzyme metabolism	Increased	3

604

605 **Table 2. *Tst* deletion results in differential persulfidation rate of liver proteins.** Significant GO terms  
606 represented by peptides with different persulfidation rates in the ND-fed *Tst*<sup>-/-</sup> liver relative to  
607 C57Bl/6J. ‘**Direction**’ indicates whether the persulfidation is decreased or increased in *Tst*<sup>-/-</sup> relative  
608 to C57Bl/6J. ‘**Genes**’ indicates the number of genes in the *Tst*<sup>-/-</sup> that represent the changes driving  
609 the GO term.

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**Table 3. KEGG and GO changes (*Tst*<sup>-/-</sup> vs C57Bl/6J liver, ND-fed)**

Entry	Name	Genes	Significance
<b>A. KEGG pathways Increased in ND <i>Tst</i><sup>-/-</sup> liver</b>			
00250	Alanine, aspartate and glutamate metabolism	6	***
00260	Glycine, serine and threonine metabolism	5	**
00270	Cysteine and methionine metabolism	4	*
04122	Sulfur relay system	2	*
<b>B. KEGG Pathways Reduced in ND <i>Tst</i><sup>-/-</sup> liver</b>			
00980	Metabolism of xenobiotics by cytochrome P450	12	****
00982	Drug metabolism – cytochrome P450	12	****
05204	Chemical carcinogenesis	12	****
00480	Glutathione metabolism	8	***
00040	Pentose and glucuronate interconversions	5	**
04142	Lysosome	6	**
04390	Hippo signalling pathway	4	**
00500	Starch and sucrose metabolism	5	**
05215	Prostate cancer	3	**
04024	cAMP signalling pathway	4	*
04141	Protein processing in ER	9	*
05211	Renal cell carcinoma	3	*
00830	Retinol metabolism	6	*
00053	Ascorbate and aldarate metabolism	4	*
00860	Porphyryn and chlorophyll metabolism	4	*
04722	Neurotrophin signalling pathway	3	*
04670	Leukocyte transendothelial migration	4	*
04010	MAPK signalling pathway	4	*
04720	Long-term potentiation	2	*
04914	Progesterone-mediated oocyte maturation	2	*
04062	Chemokine signalling pathway	3	*
04110	Cell cycle	3	*
04015	Rap1 signaling pathway	4	*
00983	Drug metabolism - other enzymes	5	*
04918	Thyroid hormone synthesis	3	*
04612	Antigen processing and presentation	3	*
05203	Viral carcinogenesis	5	*
<b>C. GO terms common to Persulfidome and Proteome in ND <i>Tst</i><sup>-/-</sup> liver</b>			
GO-ID	Name of GO term	Persulfidation ( <i>Tst</i> <sup>-/-</sup> vs 6J)	Abundance ( <i>Tst</i> <sup>-/-</sup> vs 6J)
0008483	Transaminase activity	Decreased	Increased
0016769	Transferase activity, transferring nitrogenous groups	Decreased	Increased
0003995	Acyl-CoA dehydrogenase activity	Decreased	Decreased
0005777	Peroxisome	Decreased	Decreased
0042579	Microbody	Decreased	Decreased
<b>* P &lt; 0.05, ** P &lt; 0.01, *** P &lt; 0.001 **** P &lt; 0.0001</b>			

617 **Table 3. Protein abundance and persulfidation in ND-fed *Tst*<sup>-/-</sup> liver.** (A) Significant KEGG pathway  
618 terms represented by proteins that are more abundant in the liver of ND-fed *Tst*<sup>-/-</sup> compared with  
619 ND-fed C57Bl/6J. (B) Significant KEGG pathway terms represented by proteins that are less abundant  
620 in the liver of ND-fed *Tst*<sup>-/-</sup> compared with ND-fed C57Bl/6J. 'Genes' indicates the number of genes  
621 in the *Tst*<sup>-/-</sup> that represent the changes driving the KEGG pathway. (C) GO terms that are significantly  
622 regulated at *both* the level of cysteine persulfidation and protein abundance in liver of ND-fed *Tst*<sup>-/-</sup>  
623 compared with ND-fed C57Bl/6J.

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644 **STAR ★ METHODS**

645 **RESOURCE AVAILABILITY**

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647 **Lead contact**

648 Further information and requests for resources and reagents should be directed to and will  
649 be fulfilled by the lead contact, Nicholas M. Morton ([nik.morton@ed.ac.uk](mailto:nik.morton@ed.ac.uk)).

650

651 **Materials availability**

652 No other new unique reagents were generated for the production of the data in this paper.

653

654 **Data and code availability**

655 Proteomics and persulfidomics root data from the iTraQ and persulfidated peptide mass  
656 spectrometry experiments have been deposited to the ProteomeXchange Consortium via the  
657 PRIDE ([Perez-Riverol et al., 2019](#)) partner repository with the dataset identifier PXD028909.

658 This paper does not report original code.

659 Any additional information required to reanalyse the data reported in this paper is available  
660 from the lead contact upon request.

661

662 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

663 ***Experimental animals.*** All experiments were performed according to guidelines set out by  
664 the ethical committees of The University of Edinburgh and Physiogenex S.A.S, Prologue  
665 Biotech, Labège, FRANCE. Experiments were carried out within the framework of the Animals  
666 (Scientific Procedures) Act (1986) of the United Kingdom Home Office or related laws from  
667 the European Union (France). In all studies, animals within genotype cohorts were randomly  
668 assigned to diet or intervention groups. All animals were maintained in standard housing with  
669 12 hour light and 12 hour dark cycles (7 a.m. to 7 p.m.) and *ad libitum* access to the  
670 appropriate diet. For in vivo experiments (pyruvate tolerance test, insulin tolerance test,

671 euglycaemic clamps), operators and animal handlers were blinded to the data, which was  
672 generated by a second individual who was blinded to the treatment regimen until the code  
673 was broken. All of the studies used male mice housed in cages of 3-6 individual littermates  
674 until intervention. The mice for this study originated from C57Bl/6N *Tst*<sup>-/-</sup> mice (Morton *et*  
675 *al.*, 2016) backcrossed onto the C57Bl/6J genetic background for >10 generations. Mice were  
676 placed onto high fat diet D12331, (58% calories from fat, Research Diets, New Brunswick,  
677 USA) from between 6-8 weeks of age, for 6-7 weeks prior to testing, and compared to mice  
678 maintained on standard low fat diets, RM1 or D12383 (low-fat high-cornstarch, Research  
679 Diets, New Brunswick, USA).

680 **Hepatocyte preparations.** Mice were killed by CO<sub>2</sub> asphyxiation, followed by cervical  
681 dislocation. The chest cavity was opened, the portal vein was cut and the thoracic vena cava  
682 was cannulated via the right atrium. The liver was perfused with (37°C) perfusion media (140  
683 mM NaCl, 2.6 mM KCl, 0.28 mM Na<sub>2</sub>HPO<sub>4</sub>, 5 mM glucose, 10 mM HEPES, 0.5 mM EGTA, pH  
684 7.4), 6 mls/min for 10 min. The liver was then perfused with digestion media (perfusion media,  
685 without EGTA, including 5 mM CaCl<sub>2</sub>, and 100 U/ml collagenase type 1) for 5-7 min. Finally,  
686 the liver was perfused with perfusion media for a further 10 min. Cells were extruded from  
687 liver into DMEM medium (DMEM, 5.5 mM glucose, 10% FCS, 7 mM glutamine, and  
688 penicillin/streptomycin antibiotics), and then passed through a 40 micron filter. Cells were  
689 spun twice and washed with medium, at 500 rpm (47 g) for 5 min. Cells were spun through a  
690 50% Percoll pH 8.5-9.5/DMEM solution at 1000 rpm (190 g) for 15 min to remove dead cells  
691 and non hepatocytic liver cell types. Hepatocytes collected in the pellet fractions were  
692 resuspended in medium and spun twice with washing at 500 rpm (47 g) 5 minutes. Yields and  
693 viability were assessed by counting using a haemocytometer, and proportion of trypan blue  
694 exclusion respectively. Yields ranged from between  $2 \times 10^6$  –  $1.5 \times 10^7$  viable cells, and viability

695 was above 85%. Unless otherwise stated, hepatocytes were seeded onto collagen coated  
696 tissue culture plastic (collagen from rat tails, Sigma), and maintained in DMEM with 5.5 mM  
697 glucose, 10% FCS, 7 mM glutamine, and antibiotics).

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## 699 **METHOD DETAILS**

700

701 ***Pyruvate tolerance test.*** Blood glucose was measured from 16 hour fasted mice before (0  
702 time) and following bolus sodium pyruvate administration (i.p. 1.5 mg/g bodyweight). Blood  
703 was collected following tail venesection at 0, 15, 30, 60 and 120 minutes after injection.  
704 Glucose was measured from blood using a Glucometer (*OneTouch*, Lifescan, Milpitas, USA or  
705 *Accu-Chek*, Performa nano, Roche).

706 ***PEPCK activity assay.*** Activity of phosphoenolpyruvate carboxykinase was measured from  
707 cytosol samples obtained from frozen liver. Samples were homogenised in 250 mM sucrose,  
708 5 mM HEPES, pH 7.4. and centrifuged at 4°C, 12,000 rpm (17,390 g) for 15 min. Supernatants  
709 were ultracentrifuged at 4° C, 60,000 rpm (289,000 g) for 30 min. Activity of PEPCK from  
710 cytosolic fractions was inferred in this assay from NADH extinction, linked to the conversion  
711 of phosphoenol pyruvate into oxaloacetate in the presence of carbonate, dGDP and MnCl<sub>2</sub>,  
712 and the subsequent conversion of oxaloacetate into malate by adding malate dehydrogenase.  
713 Baseline measurements at 340 nM (NADH) were taken for 20 min before adding phosphoenol  
714 pyruvate, and the reaction proper was initiated with dGDP. The reaction was then measured  
715 for a further 40 min.

716 ***<sup>13</sup>C Pyruvate metabolite tracing.*** After overnight culture on collagen coated 6-well tissue  
717 culture plates, hepatocytes were incubated with 1 mM <sup>13</sup>C<sub>3</sub> labelled pyruvate in serum free  
718 DMEM for 60 min. Metabolites were extracted by washing individual wells with ice-cold PBS



719 and addition of cold extraction buffer (50% methanol, 30% acetonitrile, 20% water solution  
720 at -20°C or lower). Extracts were clarified and stored at -80°C until required. LC-MS was  
721 carried out using a 100 mm x 4.6 mm ZIC-pHILIC column (Merck-Millipore) using a Thermo  
722 Ultimate 3000 HPLC inline with a Q Exactive mass spectrometer. A 32 min gradient was  
723 developed over the column from 10% buffer A (20 mM ammonium carbonate), 90% buffer B  
724 (acetonitrile) to 95% buffer A, 5% buffer B. 10 µl of metabolite extract was applied to the  
725 column equilibrated in 5% buffer A, 95% buffer B. Q Exactive data were acquired with polarity  
726 switching and standard ESI source and spectrometer settings were applied (typical scan range  
727 75-1050). Metabolites were identified based upon m/z values and retention time matching  
728 to standards.

729 **Plasma lipid analysis.** Mice were fasted with free access to water for 4 hours prior to cull by  
730 decapitation or pentobarbital euthanasia. Trunk blood (decapitation) was collected directly  
731 into Sarstedt Microvette CB 300 K2E EGTA containing plasma sample tubes (Sarstedt,  
732 Nümbrecht, Germany). Venous blood from the abdominal vena cava (post euthanasia) was  
733 collected into a BD Plastipak 1 ml syringe (BD, Madrid, Spain). This was then transferred to  
734 Sarstedt EGTA containing sample tubes for centrifugation. Blood samples obtained by either  
735 method were centrifuged at 20°C and 5000 rpm (2655 g) for 5 min to obtain plasma samples.  
736 Plasma samples were analysed for cholesterol and triglyceride content by as previously  
737 described (Peters *et al.*, 1997). Briefly, samples were subjected to gel filtration  
738 chromatography using an integrated Alliance HPLC separations module (e2695, Waters,  
739 Milford, US) to separate lipoproteins based on size. Effluent was immediately and  
740 continuously mixed with either triglyceride (Infinity Triglyceride, Thermo Scientific,  
741 Loughborough, UK) or cholesterol (Infinity Cholesterol, Thermo Scientific, Loughborough, UK)  
742 enzymatic colourmetric detection kits at the correct conditions for reaction (as specified in

743 manufacturer's guidance). The optical density was then recorded using a spectrophotometer  
744 at the appropriate wavelength and the signal turned into a continuous trace i.e. a lipid profile.  
745 By identification of the lipoprotein peaks (based on their time of emergence from the  
746 chromatograph) the concentration for each could be calculated.

747 ***Oil Red-O lipid analysis of liver.*** 5 µm cryostat cut frozen sections of liver were collected onto  
748 Superfrost slides (Thermo), and rinsed with 60% isopropanol. Slides were incubated in freshly  
749 prepared staining solution (2.1 mg/ml Oil Red O in 40% isopropanol/water) for 10 – 30 min  
750 and rinsed with 60% isopropanol. Slides for representative images were counterstained for  
751 nuclei in haematoxylin (Harris) for 1 minute. For image analysis, slides were not  
752 counterstained. All slides were then rinsed in running tap water for 2 min, before mounting.  
753 Sections were captured using an AxioScan Z1 slide scanner at ×20 magnification and analysis  
754 of the proportional area of Oil Red O staining (area of stain/unit area of section) was  
755 performed using ImageJ software (National Institutes of Health), assessed by a blinded  
756 assessor.

757 ***Liver Glycogen measurement.*** Frozen liver samples (between 30-90 mg) were heated to  
758 100°C in an Eppendorf tube with 0.3 mls of 30% KOH for 30 min with vigorous shaking at 10-  
759 min intervals. Samples were heated for a further 2-3 min after addition of 0.1 ml 1M Na<sub>2</sub>SO<sub>4</sub>  
760 and 0.8 ml ethanol. Samples were then centrifuged at 4°C at 1011 g for 5 minutes. The  
761 supernatant was removed, and the pellet resuspended in distilled H<sub>2</sub>O before 0.1 ml 1M  
762 Na<sub>2</sub>SO<sub>4</sub> and 0.8 ml ethanol were again added, and samples boiled at 100°C for 5 min before  
763 centrifugation. This was repeated a final time to wash the sample. The pellet was resuspended  
764 in a 10 mg/ml (~1200 U/ml) amyloglucosidase enzyme in 0.3 M sodium acetate (pH 4.8).  
765 Samples were then incubated at 50°C for 2 hours. Quantification of samples was then  
766 performed using a standard hexokinase based glucose assay (Glucose (HK) Assay Kit, Sigma,

767 GAHK20). The assay was performed following manufacturer's instructions and values  
768 calculated by extrapolation from a standard curve after measuring absorbance using a plate  
769 spectrophotometer (Molecular Devices OPTImax microplate reader and software, Molecular  
770 Devices, Wokingham, UK).

771 **Western blotting for protein abundance.** Frozen liver samples (stored -80°C) from mice were  
772 homogenized in protein lysis buffer (50 mM Tris, 270 mM sucrose, 50 mM NaF, 1 mM EDTA,  
773 1 mM EGTA, 1% Triton X-100, 10 mM B-glycerophosphate, 5 mM Na Pyrophosphate, 1 mM  
774 orthovanadate, 0.1%  $\beta$ -Mercaptoethanol, 1 tablet protease inhibitor cocktail inhibitor, pH  
775 7.4, all Sigma Aldrich). Samples were then centrifuged at 13,200 rpm (18500 g) for 15 min at  
776 4°C and the supernatants aliquoted and stored at -80°C. Protein samples were loaded onto  
777 10% acrylamide/bis-acrylamide gels (30% acrylamide, Sigma Aldrich) and separated by  
778 electrophoresis. A coloured molecular weight marker was also run on all gels (Full range  
779 rainbow molecular weight markers, GE Healthcare). Gels were transferred overnight using a  
780 Bio Rad wet transfer system onto Amersham Hybond – P membranes (GE Healthcare). After  
781 transfer, for normalisation of specific targets to total protein, membranes were stained using  
782 the REVERT total protein stain (LICOR), according to manufacturers' instruction. Following  
783 stain and wash, lanes of each sample were analysed using a LI-COR Odyssey scanner (700nm  
784 channel). For blots using a house keeping protein for normalisation, the total protein stain  
785 was not performed, and membranes were transferred directly to blocking. All membranes  
786 were blocked in Tris buffered saline with 0.01% tween (TBST, containing 5% skimmed milk  
787 powder (Marvel skimmed milk powder) for 1 hour and then rinsed in TBST. Blocked  
788 membranes were then incubated with the appropriate primary antibody in TBST containing  
789 5% BSA (Sigma Aldrich) overnight at 4°C. Following three 5 min washes with TBST, secondary  
790 antibody incubation for all blots was with an appropriate green or red fluorescent antibody,

791 incubated at room temperature for 2 hours in TBST containing 5% BSA. Membranes were  
792 washed three times in TBST then scanned using the LI-COR Odyssey scanner. Odyssey  
793 software (LI-COR Biosciences) was used to quantify band intensity. For normalization to a  
794 house keeping protein, the individual band intensity of B-actin was used for each sample.  
795 Primary antibodies used were; TST, Rabbit, GeneTex, GTX114858, MPST, Rabbit, Abcam,  
796 Ab224043, GOT1, Rabbit, Abcam, ab170950, GSTT1, Rabbit, Proteintech, 15838-1-AP,  
797 MAT1A, Rabbit, Abcam, ab129176, BHMT Rabbit, Proteintech, 15965-1-AP, CSAD, Rabbit,  
798 Abcam, ab91016, PPCS Rabbit, Atlas Antibodies, HPA031361. Secondary antibodies used  
799 were; IRDye800CW Goat anti-Rabbit, Li-Cor, 926-32211, IRDye 680RD Donkey anti-Mouse, Li-  
800 Cor, 926-68072. For normalisation B-Actin, Mouse, Abcam, ab8226 was used for whole tissue,  
801 and Cox IV (mitochondrial loading control), Abcam, ab16056 was used for mitochondrial  
802 fractions.

803 ***Insulin tolerance test.*** Male C57BL/6J or *Tst*<sup>-/-</sup> mice were maintained on standard chow  
804 (RM1). Mice were fasted for 4 hours prior to injection i.p. of insulin (1 mU/g bodyweight,  
805 NovoRapid 100U/ml, Novo Nordisk). Tail venesection blood samples were taken prior to, and  
806 15, 30, 60 and 120 minutes post injection. Blood glucose was measured from samples using a  
807 Glucometer (Accu-Check, Performa Nano, Roche). Blood glucose was plotted across time to  
808 evaluate net glucose accumulation in blood.

809 ***Euglycemic hyperinsulinemic clamps.*** Male C57BL/6J or *Tst*<sup>-/-</sup> mice were maintained on  
810 standard diet (RM1 (E) 801492, SDS) or high fat diet for 6 weeks (58% fat, D12331, Research  
811 Diets). Prior to performing the hyperinsulinemic euglycemic clamp an indwelling catheter was  
812 placed into the femoral vein under isoflurane anesthesia, sealed under the back skin, and  
813 glued onto the top of the skull. Clamps were performed 5-6 days after recovery from  
814 catheterization. Mice were fasted 6 hours prior to a basal blood sample was taken for glucose

815 and insulin. Mice then received a bolus of D-[3-3H] glucose (30  $\mu$ Ci) and perfused with 3H-  
816 glucose (30  $\mu$ Ci/kg/min at 2  $\mu$ l/min) for 210 min (which covers the basal phase and  
817 hyperinsulinemic clamp). At steady state (60 min after start of perfusion), 5  $\mu$ l of blood was  
818 collected and glycemia measured from tail tip every 10 min over 30 min for  $^3$ H-radioactivity  
819 analysis for determination of whole body glucose turnover glycolysis and glycogen synthesis  
820 rate in the basal state. 90 min after start of perfusion, the hyperinsulinemic clamp starts by  
821 co-perfusion with insulin 8 mU/kg/min for the clamped phase over 120 min. Blood glucose  
822 was assessed every 10 minutes, and glucose infusion adjusted until steady state blood glucose  
823 (120 mg/dl +/- 10 mg/dl) was achieved. 5  $\mu$ l of blood was collected from, tail tip every 10 min  
824 for  $^3$ H- radioactivity analysis. At 150 min after the start of perfusion, a bolus of  $^{14}$ C-2-  
825 deoxyglucose (25  $\mu$ Ci) was perfused to evaluate tissue specific uptake. At the end of the  
826 perfusion (210 min), blood is collected from the retro-orbital sinus to measure plasma insulin  
827 and mice sacrificed by i.v. injection of pentobarbital and cervical dislocation. Tissues (Inguinal  
828 WAT, Epididymal WAT, Soleus muscle, Extensor digitorum longus muscle, Vastus lateralis  
829 muscle, Tibialis anterior muscle, Heart apex, Liver) were removed by dissection and flash  
830 frozen in liquid nitrogen (stored -80°C until measured). Tracers were used to calculate various  
831 aspects of glucose metabolism (Altszuler *et al.*, 1956; Carter and Morton, 2016). Parameters  
832 measured or calculated include body weight, glucose infusion rate, whole body turnover,  
833 hepatic glucose production, whole body glycolytic rate, whole body glycogen synthesis rate,  
834 and tissue glucose utilization.

835 ***MBB derivatization of whole blood and plasma.*** Whole blood was taken after cull of mice,  
836 from trunk (following decapitation), or portal vein (following CO<sub>2</sub> euthanasia). EDTA-plasma  
837 was obtained from trunk blood following decapitation and collected onto ice. Blood for  
838 plasma was centrifuged within 15 min of collection for 5 min at 5000 rpm (2655 g) at 4°C.

839 Blood and plasma samples (15-50  $\mu$ l) were derivatized with monobromobimane by addition  
840 of 200  $\mu$ L of 80 mM EPPS (4-(2-Hydroxyethyl)-1-piperazine propanesulfonic acid, 8 mM DTPA  
841 (diethylenetriaminepentaacetic acid) pH 8.0, 50% acetonitrile, 2.3 mM monobromobimane.  
842 Reaction vials were capped tightly and vortexed for 1 minute and incubated protected from  
843 light at room temperature for 30 min. 1 mL ethyl acetate was added, the tube capped and  
844 vortexed for 1 min and incubated protected from light for 10 min. The reaction vials were  
845 centrifuged at 1800 rpm (350 g) for 7 min to separate aqueous and organic layers. The organic  
846 layer was collected from each extraction, transferred to a 1.5 mL brown glass vial and the  
847 solvent was evaporated completely under a nitrogen stream. Acetonitrile (200  $\mu$ L) was added  
848 to each vial, and the solvent was again evaporated to remove any traces of ethyl acetate.  
849 Dried MBB-derivatives were stored at -20°C until analysed.

850 ***Fluorometric quantification of MBB-sulfur species.*** MBB-sulfur species (sulfide, thiosulfate,  
851 reduced glutathione, and cysteine) in samples was quantified by HPLC separation and  
852 detection with a fluorescence detector. The dried MBB derivatives were re-suspended in 50  
853  $\mu$ L of Buffer A (10 mM tetrabutylammonium phosphate aqueous, 10% methanol, 45 mM  
854 acetic acid adjusted to pH 3.4). The entire sample was transferred to an HPLC autosampler  
855 vial with a 200  $\mu$ L glass sample insert, and the vial was closed with a penetrable cap. 20  $\mu$ L of  
856 the sample was injected onto a C8 reverse-phase column (LiChrospher 60 RP-select B 5  $\mu$ m  
857 4.0  $\times$  125 mm LiChroCART 125-4, Merck KGaA) and a guard column (LiChroCART 10-2,  
858 Superspher 60 RP-select B cartridge) on an Ultimate 3000 UHPLC+ focused system (Thermo  
859 Scientific). MBB derivatives were eluted with a linear gradient from 10% buffer B (10 mM  
860 tetrabutylammonium phosphate in methanol, 10% water, 45 mM acetic acid) to 100% buffer  
861 B over 30 min. The eluent was analysed by fluorescence ( $\lambda_{ex}$  = 380 nm,  $\lambda_{em}$  = 480 nm).

862 ***Sulfur metabolite analysis from liver.*** Livers from mice were removed promptly following  
863 decapitation (within 2 min), and frozen on powdered dry ice. Frozen tissue was pulverized  
864 and derivatized with either 2,4-dinitrofluorobenzene for detecting GSH or  
865 monobromobimane for detecting sulfide and thiosulfate as described previously (Mosharov,  
866 Cranford and Banerjee, 2000; Vitvitsky *et al.*, 2006, 2015).

867 ***P3 fluorescence detection of sulfide in hepatocytes.*** Hepatocytes were seeded in glass  
868 bottomed, collagen coated wells (0.75 cm<sup>2</sup>, 12,500 hepatocytes per well) and cultured in  
869 DMEM with 5.5 mM glucose, 10% FCS, 4 mM glutamax or 7 mM glutamine, and antibiotics  
870 overnight. P3 H<sub>2</sub>S reactive probe (Singha *et al.*, 2015) was added to wells at 10 μM in serum  
871 free DMEM for 30 min, prior to gentle washing with Krebs phosphate buffered saline (pH 7.4).  
872 Plates were measured using the TECAN fluorescence plate reader, following excitation at 375  
873 nm and detection at 510 nm. No-cell control wells were used for subtracting from the cell  
874 containing values. Corrected fluorescence emission data was normalised to protein as  
875 estimated by sulforhodamine dye. Briefly, after the run cells were fixed with 10%  
876 trichloroacetic acid overnight at 4°C. Cells were washed 9 times with tap water, and air dried.  
877 Cells were incubated with 200 μl of 0.4% Sulforhodamine dye/1% acetic acid for 1 hour at  
878 room temperature. Stain was removed, and washed 4 times with 1% acetic acid, and then air  
879 dried. Stain was dissolved in 200 μl of 10 mM Tris pH 10.5 for 30 min, and 100 μl was measured  
880 by colorimetric absorbance spectroscopy at 540 nm. After subtracting a baseline absorbance  
881 from blank controls, the absorbance was used to normalise the fluorescence data from each  
882 well.

883 ***Quantification of hydrogen sulfide levels using MitoA in vivo exomarker.*** MitoA and MitoN  
884 were quantified in mouse blood using LC-MS/MS. Mice received a tail vein IV injection of 50  
885 nM MitoA in 0.9% saline (100 μL). MitoA was given 1.5 hr to distribute into mitochondria.

886 Mice were culled by decapitation 90 minutes after administration. Liver was excised and flash  
887 frozen in liquid nitrogen. MitoA and MitoN were extracted from tissue by homogenization of  
888 liver (50 mg) enriched with 5 pg d15-MitoN (95% ACN, 210  $\mu$ L) which was used as an internal  
889 standard (IS). Homogenates were centrifuged (16,000 g, 10 min, RT) and the supernatant was  
890 transferred to a clean tube and stored on ice. The pellet was re-extracted (95% CAN, 210  $\mu$ L),  
891 spun down again (16,000 g, 10 min, rT) and the supernatants were combined and incubated  
892 at 4°C for 30 mins. Calibration standards comprise MitoA and MitoN standards ranging from  
893 0.01 to 10 pg in 500  $\mu$ L 95% ACN. 500  $\mu$ L of the supernatants and calibration standards were  
894 loaded onto an ISOLUTE PLD+ protein and phospholipid removal plate (Biotage, Sweden).  
895 Samples and standards were pulled through the plate under vacuum into a 2 mL deep-well  
896 96-well plate. Wells were dried completely at 40°C under N<sub>2</sub> and resuspended in 100  $\mu$ L 20%  
897 ACN, 0.1% FA. The plate was shaken at (250 rpm, 20 min) to ensure reconstitution. Liquid  
898 chromatography-Mass Spectrometry was performed on an I-class Acquity LC system-Xevo  
899 TQS triple quadrupole mass spectrometer (Waters, Warrington, UK). Samples were kept at  
900 10°C and injected onto an Acquity UPLC BEH C18 column fitted with a 0.2  $\mu$ m filter (1 x 50  
901 mm, 1.7  $\mu$ m, Waters). Chromatographic separation of MitoA and MitoN was achieved using  
902 mobile phase A composition: water:ACN, (95:5, 0.1% FA), mobile phase B: ACN:water (90:10,  
903 0.1% FA). LC mobile phases were infused at 200  $\mu$ L/min using the gradient: 0– 0.3 min, 5% B;  
904 0.3–3 min, 5–100% B; 3– 4 min, 100% B, 4.0– 4.10, 100–5% B; 4.10– 4.60 min, 5% B. MS/MS  
905 analysis was performed under positive ion mode (Source spray voltage, 3.2 kV; cone voltage,  
906 125 V; ion source temperature, 100 °C). Curtain and collision gas were nitrogen and argon,  
907 respectively. Analytes were detected by multiple reaction monitoring (MRM). MitoA  
908 undergoes neutral loss of N<sub>2</sub> to a nitrene (precursor ion). For quantification the following  
909 transitions were used: MitoA,  $m/z$  437.2  $\rightarrow$  183.1; MitoN,  $m/z$  439.2  $\rightarrow$  120.0; d15-MitoN,



910 454.2 → 177.1 m/z. MassLynx 4.1 software was used to integrate the peak area of the  
911 analytes MitoA, MitoN and the d15-MitoN internal standard. Response was calculated by  
912 normalizing sample peak areas to the IS peak area. By comparison of sample responses to  
913 calibration standard responses the mass of each analyte in the tissue sample was calculated.  
914 The mass of analyte was normalised to the mass of tissue homogenizer and MitoN/MitoA  
915 ratio was calculated.

916 **Preparation of hepatic mitochondria.** Fresh liver was taken from mice, and homogenised in  
917 250 mM sucrose, 10 mM HEPES, 1 mM EGTA. 0.5% fatty acid free bovine serum albumin (BSA)  
918 pH 7.4 at 4°C, with seven passes of a loose glass Dounce homogeniser (Type A). Homogenates  
919 were centrifuged in glass tubes at 2900 rpm (1000 g) for 10 min in a pre-chilled 4°C Beckman  
920 centrifuge (JA-20 Fixed angle rotor). The supernatant was then centrifuged in glass tubes at  
921 8500 rpm (8700 g) for 10 min at 4°C. The supernatant was aspirated and any visible lipid was  
922 carefully removed from the sides of the tubes. The pellet was washed with 5 ml of mIR-05  
923 buffer (0.5 mM EGTA, 3 mM MgCl<sub>2</sub>, 20 mM taurine, 10 mM KH<sub>2</sub>PO<sub>4</sub>, 20 mM HEPES, 110 mM  
924 sucrose, 1 mg/ml fatty acid free BSA, pH 7.2), and centrifuged at 8500 rpm (8700 g) for 10 min  
925 at 4°C. After aspiration and removal of visible lipid, the pellet was suspended in 1ml of mIR-  
926 05 buffer and kept on ice until used. All measurements were taken within two hours of  
927 preparation. Protein concentration was determined using the DC-Protein Assay (BioRad) as  
928 per manufacturers instruction.

929 **Amperometric analysis of sulfide disposal.** Hepatocytes were prepared as described, and  
930 kept at room temperature in DMEM with 5.5 mM glucose, 10% FCS, 4 mM glutamax or 7 mM  
931 glutamine, and antibiotics at a concentration of 4 x 10<sup>6</sup> per ml. Mitochondria were prepared  
932 as described, and maintained on ice in mIR-05 buffer until use. All samples were analysed  
933 within 4 hours of preparation. Sulfide (H<sub>2</sub>S<sub>(g)</sub>) was measured (with and without samples) in a

934 2ml volume plastic chamber, to which an amperometric sensor was inserted, sealed with a  
935 rubber O-ring. Voltage measurements from the sensor (linear relationship to  $\text{H}_2\text{S}_{(g)}$   
936 concentration) were recorded using a TBR4100 Gas radical analyser (World Precision  
937 Instruments). A gas permeable membrane covered the sensor, and the outer glass sensor  
938 compartment was filled with  $\text{H}_2\text{S}$  detection fluid (World Precision Instruments). All  
939 measurements of  $\text{H}_2\text{S}_{(g)}$  from standards and samples were recorded as voltage by the  
940 amperometric sensor at ambient temperature. Mitochondrial measurements (and standards)  
941 were taken in serum free mIR-05 buffer. Hepatocyte measurements (and standards) were  
942 taken in serum-free, bicarbonate-free DMEM, buffered with 25 mM HEPES (pH 7.4), with 5  
943 mM glucose, 2 mM glutamax and 2 mM pyruvate. Sulfide was added to buffer in the form of  
944  $\text{Na}_2\text{S}$ , predicted to equilibriate according to its  $\text{Pka}$  at this pH to about 1/3 of sulfide as  $\text{H}_2\text{S}_{(g)}$   
945 2/3 as  $\text{HS}^-$ . The probes selectivity to  $\text{H}_2\text{S}_{(g)}$  (vs  $\text{HS}^-$ ) was confirmed with standards by  
946 demonstrating predicted signal amplification to a maximum following acidification of media  
947 to  $\text{pH} < 5$  (approx. 100%  $\text{H}_2\text{S}_{(g)}$ /0%  $\text{HS}^-$ ), and signal compression to a minimum following  
948 alkalisation of standard to  $\text{pH} > 10$  (Approx 0%  $\text{H}_2\text{S}_{(g)}$ /100%  $\text{HS}^-/\text{S}^{2-}$ ). A final re-acidification  
949 recovered the signal to near maximal levels. Standard curves for calculating experimental  
950 measurements were prepared using freshly made  $\text{Na}_2\text{S}$  solutions ranging from 0.25 – 20  $\mu\text{M}$   
951 (corresponding to approximately 170 nm – 6.7  $\mu\text{M}$   $\text{H}_2\text{S}_{(g)}$ ).  $\text{H}_2\text{S}_{(g)}$  disposal was measured by  
952 recording the extrapolated  $\text{H}_2\text{S}_{(g)}$  concentration after 10 min incubation with samples. A  
953 baseline without sample was taken for 5 min, and then after sample addition (400,000  
954 hepatocytes, or 1.6 – 2.0 mg of mitochondrial prep), another 5 min baseline with sample was  
955 taken. In all experiments, no detectable increase in signal (limit of detection 0.25  $\mu\text{M}$   $\text{Na}_2\text{S}$ )  
956 was observed during incubation of hepatocyte or mitochondrial samples from either  
957 genotype. Following addition of 10  $\mu\text{M}$   $\text{Na}_2\text{S}$  the (voltage) signal was recorded over a period

958 of 10 min. Disposal rates were calculated after subtraction of a baseline disposal rate in media  
959 alone, over a 10 min period, performed each day of experimentation. Sample disposal rates  
960 were in the range of 5-20 higher than baseline disposal rate confirming good signal to noise.  
961 To determine the rate of disposal that is dependent upon respiration, a fresh aliquot of the  
962 same sample was prepared as before, but 5 min after addition of sample to chamber,  
963 Antimycin A (2.5  $\mu$ M, dose titrated) was added. After a further 5 minutes, 10  $\mu$ M Na<sub>2</sub>S was  
964 added and a disposal rate (after subtraction to sample free baseline rate) was again  
965 calculated. The respiratory (Antimycin sensitive/complex III dependent) sulfide disposal rate  
966 of samples was calculated as the difference between the naive sample rate and the Antimycin  
967 inhibited rate. After each measurement, the sample was removed, and centrifuged to collect  
968 cells or mitochondria for a final protein assessment (DC-Protein Assay, Bio-Rad) for the  
969 purposes of normalisation.

970 ***Mitochondrial ROS (MitoSOX) measurement in H<sub>2</sub>O<sub>2</sub> treated hepatocytes.*** Hepatocytes were  
971 seeded overnight onto 96-well collagen coated plates. Cells were exposed to a range of  
972 concentrations of H<sub>2</sub>O<sub>2</sub> (0.125 - 8  $\mu$ M) for 2 hours. Following 3 washes with PBS, cells were  
973 incubated with MitoSOX Red mitochondrial superoxide indicator (Thermo Fisher) for 10 mins  
974 prior to three further washes. Measurement of fluorescence was carried out in a fluorescence  
975 detector plate reader (TECAN), using 510 nm for excitation and 580 nm for emission  
976 detection. Data from each well was normalised to sulforhodamine dye protein stain.

977 ***Persulfidation Mass Spec and GO term analysis.*** Livers from mice were removed promptly  
978 following decapitation (within 2 min), and snap frozen in liquid nitrogen. The persulfide  
979 proteome analysis using the BTA method was conducted as described previously (Gao *et al.*,  
980 2015). Briefly, 100-150 mg of frozen liver tissue was pulverized and lysed on ice in RIPA buffer  
981 (100 mM Tris, pH 7.5, 150 mM NaCl, 2mM EDTA, 1% Triton X-100, 25 mM deoxycholic acid, 2

982 tablets/ 100 ml of cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (Roche)). The  
983 lysates were centrifuged at 14,000 g for 10 min at 4°C and protein concentrations were  
984 determined using the Bradford reagent (BioRad). Supernatant containing 6 mg of protein was  
985 incubated with 100 μM NEM-biotin (Pierce) for 60 min at room temperature after which the  
986 proteins were precipitated with cold acetone (1:4 v/v) for 1 h at -20°C, followed by a  
987 centrifugation at 14,000 g for 10 min at 4°C. The precipitated protein was re-suspended in a  
988 denaturing buffer containing 7 M urea, 1% SDS, 150 mM NaCl, 100 mM Tris, pH 7.5. Then, the  
989 samples were diluted 10-fold with trypsin reaction buffer (1 mM CaCl<sub>2</sub>, 100 mM Tris pH 7.5)  
990 and incubated overnight with sequencing grade modified trypsin (1:50 trypsin:protein)  
991 (Promega) at 30 °C. The digestion products were mixed with streptavidin-agarose beads  
992 (ThermoScientific) and incubated at 4° overnight, followed by ten washes with the wash  
993 buffer (0.1 % SDS, 100 mM Tris, pH 7.5, 600 mM NaCl, 1 mM EDTA, 1% Triton X-100). The  
994 streptavidin-agarose bound peptides were incubated with elution buffer (100 mM Tris, pH  
995 7.5, 150 mM NaCl, 1 mM EDTA, 30 mM DTT) for 1 hr at room temperature. Persulfidated  
996 peptides were eluted by centrifugation and derivatized with 40 mM iodoacetamide for 2 hrs  
997 at room temperature in the dark. The samples were then passed through a desalting column  
998 (Pierce). LC-MS/MS analysis was carried out using an LTQ-Orbitrap Elite mass spectrometer  
999 (Thermo-Fisher) coupled to an Ultimate 3000 high-performance liquid chromatography  
1000 system. The alkylated peptides were loaded onto a 75 μm desalting column, C18 reverse  
1001 phase resin (Dionex), and eluted onto a Dionex 15 cm x 75 μm id Acclaim Pepmap C18, 2 μm,  
1002 100 Å reverse-phase chromatography column using a gradient of 2–80% buffer B (5% water,  
1003 95% acetonitrile, 0.1% formic acid) in buffer A (0.1% formic acid). The peptides were eluted  
1004 onto the mass spectrometer at a flow rate of 300 nl/min and the spray voltage was set to 1.9  
1005 kV.

1006 **GO enrichment analysis.** In order to identify differentially persulfidated proteins between the  
1007 C57Bl/6J and  $Tst^{-/-}$  samples, we compared the abundances of persulfidated fragments in  
1008 appropriately treated mass spectrometry data sets to the estimated overall abundance of the  
1009 corresponding parent proteins in standard label-free quantitation experiments. For each  
1010 observed persulfidated fragment in each experimental replicate, we calculated the  
1011 persulfidation rate as the  $\log_2$  ratio of the count of that persulfidated fragment to the median  
1012 count of that fragment across all experimental replicates. The observed counts for the  $Tst^{-/-}$   
1013 replicates were additionally scaled (prior to log transformation) by the ratio of abundances of  
1014 the parent protein between the C57Bl/6J and  $Tst^{-/-}$  cells to normalize for differential protein  
1015 abundance across conditions. For each peptide we then assigned an approximate average  
1016  $\log_2$  fold change in persulfidation rate between the C57Bl/6J and  $Tst^{-/-}$  conditions. If a  
1017 persulfidated peptide was identified in at least two biological replicates of one condition and  
1018 none in the other, we assigned a  $\log_2$  fold change of +/- 5.0 as placeholder values indicating a  
1019 high confidence change; peptides with only one observation in one condition and none in the  
1020 other were omitted from our analysis. Having thus obtained estimates for the magnitude of  
1021 changes in persulfidation rate of each detectable peptide, we then performed gene ontology  
1022 term enrichment analysis using the estimated  $\log_2$  fold changes. We consolidated the peptide-  
1023 level data to protein-level data by taking the largest magnitude change in persulfidation levels  
1024 across all peptides from a given protein, and then used the iPAGE program  
1025 [<http://dx.doi.org/10.1016/j.molcel.2009.11.016>] to identify GO terms with significant  
1026 mutual information with the profile of persulfidation rates. Arguments to iPAGE were “—  
1027 max\_p=0.1 —minr=0.3 —ebins=9 —exptype=continuous”, indicated that the data were  
1028 discretized into nine equally populated bins prior to analysis, and that default hypergeometric  
1029 p-value and information content thresholds were relaxed to maximize sensitivity.

1030 **Focussed analysis of persulfidation in gluconeogenesis proteins.** The gluconeogenesis  
1031 pathway was selected for a focussed analysis of the persulfidation rate of all cysteine sites  
1032 detected in the mass spectrometry data as described above. All peptides from proteins  
1033 present in the persulfidation data set used for GO enrichment analysis, that are defined by  
1034 the GO term gluconeogenesis (GO 0006094) were included, these were Pgk1, Gpi1, Fbp1 and  
1035 Tpi1 (22 peptides). The  $\log_2$  rate ratio of persulfidation ( $Tst^{-/-}$  /6J) of all of these 22 peptides  
1036 was compared first to the entire mass spectrometry dataset for  $\log_2$  rate ratio of  
1037 persulfidation (1245 peptides after removal of ambiguous peptides, peptides with a P-diff of  
1038 0, and the 22 gluconeogenesis peptides). A Mann-Whitney non parametric T-test was used to  
1039 detect significance. A second analysis was performed with the gluconeogenesis pathway. For  
1040 this analysis, all  $\log_2$  rate ratio's were given a positive sign to indicate the magnitude of change  
1041 in the  $Tst^{-/-}$  relative to 6J, independent to the direction of change. A Mann-Whitney non  
1042 parametric T-test was then performed to determine if the magnitude of change in  
1043 persulfidation in the gluconeogenesis pathway was significantly higher than that of the overall  
1044 the data set.

1045 **Persulfidation labelling and western blotting from frozen liver.** 80-120 mg of frozen liver  
1046 samples were homogenized on ice using a 2 ml glass Dounce homogenizer (Kimble), in 500  $\mu$ l  
1047 buffer (7 M urea, 100 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1%  
1048 deoxycholic acid; supplemented with cOmplete Protease Inhibitor Cocktail (Roche)). Initial  
1049 disruption of tissue was achieved with three passes, using the loose (Type A) pestle, and after  
1050 5 min incubation on ice; complete homogenization was achieved with 9 passes using the tight  
1051 fit (Type B) pestle. Homogenates were centrifuged at 5000 rpm (2655 g) for 5 min at 4°C.  
1052 Protein concentrations of supernatants were determined using the DC BCA protein assay (Bio-  
1053 Rad). Protein (6 mg) from each sample, was made up to 1 ml with phosphate buffered saline

1054 (pH 8.0). Freshly prepared EZ-link Maleimide PEG Biotin EZ-linker (Thermo Fisher 21902BID),  
1055 was added to samples to 100  $\mu$ M, and incubated for 1 hour at room temperature with gentle  
1056 mixing. Excess maleimide linker was removed from samples by acetone precipitation (3  
1057 volumes) at -20°C for 1 h, followed by centrifugation at 12000 rpm (17390 g) for 10 min at  
1058 4°C. Protein pellets were washed with ice-cold acetone and then dissolved in 250  $\mu$ l of 50 mM  
1059 Tris pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 % SDS. To each sample, 750  $\mu$ l of RIPA buffer (100  
1060 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% deoxycholic acid) was then  
1061 added. An aliquot (20  $\mu$ l) was taken from each sample for estimation of total input protein for  
1062 normalization (described below). The remainder of the samples were split into duplicates and  
1063 incubated with gentle mixing, overnight at 4°C with 320  $\mu$ l of pre-washed streptavidin agarose  
1064 beads (Thermo Fisher 20347). Beads were washed 10 times with 0.8 ml washing buffer (30  
1065 mM Tris pH 7.5, 600 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS), followed by one wash  
1066 with phosphate buffered saline (pH 7.4). Beads were then centrifuged for 1 min at 1000 rpm  
1067 (106 g) to dry. Elution of the duplicate samples for western blot analysis was performed by  
1068 adding 300  $\mu$ l of elution buffer (30 mM Tris pH 7.5, 150 mM NaCl). For each sample pair, one  
1069 duplicate was eluted in buffer supplemented with 10 mM DTT and the other without DTT.  
1070 The beads were incubated with the elution buffer for 1 hour at RT; and centrifuged for 1 min  
1071 at 1000 rpm (106 g) to collect the eluate. Each eluted sample was concentrated to 20  $\mu$ l using  
1072 an Ultra-0.5 Centrifugal Filter Device, 10 K cut-off (Amicon), as per the manufacturer's  
1073 instructions. Eluted samples, and input protein samples were loaded in their entirety onto  
1074 SDS PAGE gels and transferred overnight at 4°C by Western blotting onto PVDF membrane.  
1075 Total protein from each lane on the membrane was estimated after staining with REVERT  
1076 total protein stain (LICOR) according to the manufacturer's instructions. Briefly, following  
1077 overnight transfer and after rinsing the blot with water, the membranes were incubated with

1078 REVERT total protein stain for 5 min, and rinsed twice with wash solution. Blots were then  
1079 imaged in the 700 nm channel with an Odyssey imaging system (LICOR). Each lane was  
1080 measured for its total integrated fluorescence intensity to obtain an estimate of the total  
1081 protein in each lane. Measurements from no-DTT eluted sample lanes were subtracted from  
1082 DTT eluted sample lanes. Similar fluorescence measurements of input total protein lanes  
1083 were used to normalise the eluted sample measurements, and this was used as a measure of  
1084 relative protein-persulfidation rate.

1085 **Mass spec analysis of liver protein.** *Sample preparation;* *Tst*<sup>-/-</sup> and wild type (C57Bl/6J)  
1086 mouse strains were fed either high-fat (58% fat) or normal (low fat) diet. Livers from mice  
1087 were removed following decapitation, and snap frozen in liquid nitrogen. Four biological  
1088 replicates from the 4 conditions were used to isolated proteins and performed protein  
1089 quantitation using iTRAQ 8plex. Liver tissue was homogenized using 1 ml of 8 M urea with  
1090 HEPES buffer pH 8.0. The protein concentration was determined using the Bio-Rad RC DC  
1091 protein assay kit (Bio-Rad, Hercules, CA, USA). One hundred micro grams of protein from each  
1092 of the samples were reduced with THP (Tris(hydroxypropyl)phosphine), alkylated with MMTS  
1093 (methyl methanethiosulfonate) in 500 mM triethylammonium bicarbonate (TEAB, pH 8.5),  
1094 trypsin digested and subsequently label with iTRAQ 8plex accordingly to the manufacturer's  
1095 instructions. *Electrostatic Repulsion-Hydrophilic Interaction Chromatography (ERLIC) Peptide*  
1096 *fractionation;* Peptide fractionation was performed using a pH gradient. Labeled peptides  
1097 were dissolved in 100 µL of buffer A (100 mM formic acid, 25% acetonitrile, pH 3.0), followed  
1098 by fractionation in a 2.6 × 200 mm, 5 µm, 200 Å PolySulfoethyl A column (Poly LC Inc.,  
1099 Columbia, MD), using an Ultimate 3000 UHPLC+ focused (Thermo-Fisher Scientific) system,  
1100 operating at a flow rate of 0.2 ml/min. Twenty minutes of isocratic buffer A were followed by  
1101 a linear gradient from 0% to 100% buffer B (100 mM ammonium formate, 25% acetonitrile,



1102 pH 6.0) over 20 min and then a final linear gradient from 0% to 100% buffer C (600 mM  
1103 ammonium acetate, 25% acetonitrile, pH 6.0) over 10 min. A total of 22 fractions (1-min  
1104 intervals) were collected. All fractions were lyophilized and stored at -20 °C. *Nanoflow Liquid*  
1105 *Chromatography Tandem Mass Spectrometry*; NanoLC MS/MS analysis was performed using  
1106 an on-line system consisting of a nano-pump UltiMate™ 3000 UHPLC binary HPLC system  
1107 (Dionex, ThermoFisher) coupled with Q-Exactive mass spectrometer (ThermoFisher, San Jose,  
1108 CA. iTRAQ-labeled peptides were resuspended in 2% ACN, 0.1% formic acid (20 µL) and 6 µL  
1109 injected into a pre-column 300 µm×5 mm (Acclaim PepMap, 5 µm particle size). After loading,  
1110 peptides were eluted to a capillary column 75 µm×50 cm (Acclaim Pepmap, 3 µm particle  
1111 size). Peptides were eluted into the MS, at a flow rate of 300 nL/min, using a 90 min gradient  
1112 from 0% to 35% mobile phase B. Mobile phase A was 2.5% acetonitrile with 0.1% formic acid  
1113 in H<sub>2</sub>O and mobile phase B was 90% acetonitrile with 0.025% trifluoroacetic acid and 0.1%  
1114 formic acid. The mass spectrometer was operated in data-dependent mode, with a single MS  
1115 scan in the orbitrap (400-2000 m/z at 70 000 resolution at 200 m/z in profile mode); automatic  
1116 gain control (AGC) was set to accumulate  $4 \times 10^5$  ions, with a maximum injection time of 50  
1117 ms. MS/MS scans were performed in the orbitrap at 17 500 resolution. Ions selected for  
1118 MS/MS scan were fragmented using higher energy collision dissociation (HCD) at normalized  
1119 collision energy of 38% with an isolation window of 0.7 m/z. MS2 spectra were acquired with  
1120 a fixed first m/z of 100. The intensity threshold for fragmentation was set to 50 000 and  
1121 included charge states 2+ to 7+. A dynamic exclusion of 60 s was applied with a mass tolerance  
1122 of 10 ppm. *Data Analysis*; Raw files were converted to MGF files and searched against the  
1123 mouse UniProt database (81033 sequences, released on March 2014) using MASCOT Version  
1124 2.4 (Matrix Science Ltd, UK). Search parameters were peptide mass tolerance of 10 ppm, and  
1125 MS/MS tolerance of 0.05 amu allowing 2 missed cleavage. iTRAQ8plex (N-term) and

1126 iTRAQ8plex (K) were set as fixed modification, and acetyl (Protein N-term), Methylthio (C) and  
1127 Oxidation (M) were allowed as variable modification. Peptide assignments with ion score cut-  
1128 off of 20 and a significance threshold of  $p < 0.05$  were exported to Excel for further analysis.  
1129 Data are available from the ProteomeXchange with identifier PXD028909.

1130 **GO and KEGG enrichment analysis of proteome data.** The data generated from the initial  
1131 mass spectrometric analysis of iTRAQ labelled peptides from the 16 liver samples was  
1132 analysed by FIOS. A total of 16 samples were QC analysed using the arrayQualityMetrics  
1133 Bioconductor package to identify sub-standard and/or outlier samples. No samples were  
1134 identified as outliers. All samples passed the manual and automated quality control based on  
1135 three metrics (MAplot, Boxplot and Heatmap). The exploratory analysis using PCA showed  
1136 that the samples clustered perfectly into four groups based on the factor Group (representing  
1137 four genotype-diet combinations). The first PC captures the main source of variation in the  
1138 dataset and is showing a separation of the samples based on diet, where high-fat diet and  
1139 control diet samples separate. The second PC shows a separation between genotypes (Tst KO  
1140 and WT). The hierarchical clustering and PCA plot both show a clear separation based on the  
1141 iTRAQ labels. This is expected as the iTRAQ labels are confounded with the Groups. While the  
1142 observed separation of the samples into groups is most likely due to the underlying biological  
1143 differences, any technical variations (potentially introduced during the wet lab processing)  
1144 could be masked. The log<sub>2</sub> ratio data were subsequently normalised within arrays using loess,  
1145 followed by normalisation between samples using the Gquantile method. A total of 4 single  
1146 and/or multi-factor comparisons, using statistical approaches, were performed. The contrast  
1147 "Tst KO vs WT mice (High-fat diet)" was analysed at a cut-off (unadjusted) p-value  $< 0.01$ . Due  
1148 to the known bias in fold-change magnitudes of the iTRAQ technology, no fold-change cut-off  
1149 was applied to the significant differentially abundant proteins. With this threshold 551

1150 proteins were differentially abundant in at least one of the comparisons. The contrast "High-  
1151 fat diet vs Control diet (WT)" had the most DAPs (432) while the contrast "  $Tst^{-/-}$  vs 6J mice  
1152 (High-fat diet)" had the least DAPs (83). Noticeably, the TST protein showed the strongest  
1153 down-regulation for both of the contrasts comparing  $Tst^{-/-}$  to 6J mice, consistent with gene  
1154 deficiency and the fold change compression effect of iTRAQ. The full dataset (4,322 identified  
1155 proteins) was filtered to remove proteins having less than two detected peptides (on average  
1156 across all 16 samples); leaving 1,654 proteins for downstream analysis. Exploratory analysis  
1157 using principal component analysis (PCA) showed that the dataset separated into four distinct  
1158 groups based on the genotype-diet combinations along the two first principal components  
1159 (PCs). These 1,654 proteins were used for enrichment analysis for GO terms and KEGG  
1160 pathways. Individual proteins were considered of interest if they were found significantly  
1161 different ( $P < 0.01$ ) between selected pairwise comparisons. The four comparisons were  $Tst^{-/-}$   
1162  $^{-/-}$  normal diet vs C57Bl/6J normal diet, C57Bl/6J high fat diet vs C57Bl/6J normal diet,  $Tst^{-/-}$   
1163 high fat diet vs  $Tst^{-/-}$  normal diet, and  $Tst^{-/-}$  high fat diet vs C57B/6J high fat diet.  
1164 Normalised mean abundance of proteins was expressed as Log2 fold change ratios for each  
1165 comparison.

1166 **Transcription factor enrichment analysis.** 43 up-regulated proteins were selected for analysis  
1167 of their promoter sequences (selected on basis of P-value  $< 0.05$ ; adjusted for comparison of  
1168 diet and genotype). 67 control proteins were selected from the proteome data on the basis  
1169 of their equivalence of abundance between C57Bl/6J and  $Tst^{-/-}$ . We used a QIAGEN  
1170 hosted/SABiosciences mouse database (sabiosciences.com) of promoter located  
1171 transcription factor binding sites. 34 transcription factors were chosen to analyse, based on  
1172 either their a-priori prevalence in the promoter of  $Tst^{-/-}$  up-regulated proteins (present in  
1173 the promoters of more than 50% of the up-regulated proteins) or on their links to either

1174 sulfide or nutrient metabolism. The proportion of genes containing a TFBS was calculated for  
1175 the up-regulated set (43) and the control set (67). The ratio of up-regulated to control was  
1176 then calculated. The number of genes with and without the presence of the TFBS were  
1177 analysed for establishing statistical difference (Up-regulated vs Control), using a Fisher Exact  
1178 test ( $P < 0.05$ ).

1179 ***NRF2 target identification and proteome analysis.*** NRF2 target genes of the mouse liver were  
1180 compiled from the following reviews ([Cuadrado et al., 2019](#), [Tonelli et al., 2018](#), [Rooney et al., 2018](#), [Walsh et al., 2014](#)). 106 genes were identified as target genes (upregulated at mRNA  
1181 or protein level following NRF2 activation). 47 of these target genes were represented in our  
1182 liver proteome, and each protein was checked for relative expression between *Tst*<sup>-/-</sup> and 6J  
1183 (on normal diet, threshold of  $P < 0.01$ ). 10 of the 47 target genes were lower in abundance in  
1184 the *Tst*<sup>-/-</sup> proteome, 37 unchanged, with none upregulated. To analyse whether this was  
1185 statistically significant, we compared this to the percentage of proteins upregulated, down-  
1186 regulated or unchanged in the proteome database. 5.86% of proteins were upregulated,  
1187 5.62% downregulated, and 88.6% unchanged in the full database (1654 proteins total).  
1188 Expected (mean) numbers of proteins from a hypothetical set of 47 proteins, predict rounded  
1189 values of 3 upregulated, 3 downregulated and 42 unchanged. We used these as a reference  
1190 to the actual data for NRF2 target proteins; 0 upregulated, 10 downregulated and 37  
1191 unchanged. A Freeman-Halton Fisher Exact test was used for analysis of significance, and a  
1192 significant difference between predicted and actual distribution was found ( $P_A = 0.039$ ,  $P_B =$   
1193  $0.047$ ).

1195 ***Electron micrograph imaging.*** Liver tissue for transmission electron microscopy was prepared  
1196 following immersion fixation in 0.1 M PB buffer (pH 7.4, EM-grade) containing 4%  
1197 paraformaldehyde and 2.5% glutaraldehyde. 1mm tissue blocks were post-fixed in 1%

1198 osmium tetroxide in 0.1 M PB for 45 min before dehydration through an ascending series of  
1199 ethanol solutions and propylene oxide. Tissue blocks were then embedded in Durcupan  
1200 before ultrathin sections (~60/70 nm) were cut and collected on formvar-coated grids (Agar  
1201 Scientific, UK), stained with uranyl acetate and lead citrate in an LKB Ultrastainer and then  
1202 quantitatively assessed in a Philips CM12 transmission electron microscope (TEM).

1203 ***Seahorse respiratory analysis.*** Primary hepatocytes (C57Bl/6J and *Tst*<sup>-/-</sup> mice) were seeded  
1204 immediately following purification onto collagen coated V7 Seahorse 24-well cell culture  
1205 microplates (Agilent Technologies), in 200  $\mu$ l medium (DMEM, 5.5 mM glucose, 10% FCS, 7  
1206 mM glutamine, and penicillin/streptomycin antibiotics), for culture in a 5% CO<sub>2</sub> 37°C  
1207 incubator. Experiments were performed between 22-28 hours following collection from mice.  
1208 Optimisation experiments determined the optimal seeding density, which was then  
1209 standardised at 10,000/well. Optimisation for drugs and compounds used in Seahorse  
1210 experiments were performed separately with hepatocytes for each genotype and dietary  
1211 regime (normal or 58% high fat). This established the doses of drugs for respiratory  
1212 manipulation, which were the same for both genotypes and diets; oligomycin (2  $\mu$ M), FCCP  
1213 (0.5  $\mu$ M), and antimycin/rotenone (1  $\mu$ M/0.2  $\mu$ M). In all experiments, overnight media from  
1214 cells was replaced, after two washes (0.75 ml), with 525  $\mu$ l of run media and incubated for 30  
1215 mins at 37°C (without CO<sub>2</sub>), prior to entry into the Seahorse XFe24 Extracellular Flux Analyser  
1216 (Agilent). The analyser was operated using Wave software (Agilent), and all oxygen  
1217 consumption rate (OCR) data was normalised to protein using the Sulforhodamine B stain  
1218 (described above). Data from each biological replicate was averaged from between 4-10  
1219 replicate wells, to produce a single value at each measurement time for each biological  
1220 replicate. Respiratory parameters were calculated as described below for each biological  
1221 replicate, and this data was used for statistical analysis of genotype effects.

1222 **Mitochondrial stress test (MST).** Run media for the MST was Seahorse assay media (Agilent),  
1223 supplemented with 10 mM glucose, 2 mM sodium pyruvate, pH 7.35 ± 0.5 at 37°C). Most  
1224 measurements were made using 3 min mixing, 2 min wait, 3 min measure. Measurements  
1225 following addition of FCCP to hepatocytes from high fat fed mice were measured using 4 min  
1226 mix, 2 min wait, 2 min measure. Three measurements were taken basally, and three  
1227 measurements taken after injection of each drug (in sequence; oligomycin for inhibiting ATP-  
1228 linked respiration, FCCP for eliciting maximal uncoupled respiration, antimycin/rotenone for  
1229 inhibiting the respiratory electron chain). Respiratory parameters for each biological replicate  
1230 were calculated from the mean normalised OCR as follows. *Basal respiration* was calculated  
1231 by subtracting the third OCR measurement following injection of antimycin/rotenone (12<sup>th</sup>  
1232 measurement) from the third basal OCR measurement (3<sup>rd</sup> measurement). *ATP linked*  
1233 *respiration* was calculated by subtracting the third OCR measurement following the injection  
1234 of oligomycin (6<sup>th</sup> measurement) from the third basal OCR measurement (3<sup>rd</sup> measurement).  
1235 *Maximum (uncoupled) respiration* was calculated by subtracting the third OCR measurement  
1236 after injection of antimycin/rotenone (12<sup>th</sup> measurement) from the first measurement (peak  
1237 OCR) following injection of FCCP (7<sup>th</sup> measurement). *Proton leak respiration* was calculated  
1238 by subtracting the third OCR measurement after injection of antimycin/rotenone (12<sup>th</sup>  
1239 measurement) from the third measurement following the injection of oligomycin (6<sup>th</sup>  
1240 measurement). *Non-respiratory OCR* was taken from the third measurement after the  
1241 addition of antimycin/rotenone (12<sup>th</sup> measurement).

1242 **Octanoate rescue test.** To investigate lipid respiratory metabolism, hepatocytes were  
1243 prepared, seeded and cultured overnight as above. Run media for the Octanoate rescue was  
1244 Seahorse assay media (Agilent), supplemented with 5 mM glucose, 0.1 mM sodium pyruvate,  
1245 1 mM sodium lactate, and 0.5 mM carnitine pH 7.35 ± 0.5 at 37°C). All measurements were

1246 made using 3 min mixing, 2 min wait, 3 min measure. After washing cells into run media, and  
1247 30 min before entry into the analyser, half of the wells from each genotype were incubated  
1248 with 8  $\mu$ M etomoxir (or DMSO vehicle) to block carnitine dependent import of long chain fatty  
1249 acids into the mitochondria. Three basal measurements were taken prior to injection of  
1250 oligomycin, two measurements were taken prior to FCCP, two measurements were taken  
1251 prior to injection of sodium octanoate (250  $\mu$ M), three measurements taken prior to  
1252 antimycin/rotenone followed by two final measurements. Standard respiratory parameters  
1253 were calculated analogous to the above description for the standard mitochondrial stress  
1254 test, except using the second measurement following injection of drug when only 2  
1255 measurements were taken. Dependency upon endogenous fatty acids for supporting  
1256 uncoupled respiration (*Etomoxir inhibited respiration*) was calculated for each biological  
1257 replicate using the maximal respiration prior to the addition of octanoate. Maximal  
1258 respiration was calculated as the 6<sup>th</sup> measurement (peak FCCP OCR) – 12<sup>th</sup> measurement  
1259 (lowest Antimycin/Rotenone OCR). The mean maximal respiration from the etomoxir treated  
1260 wells was subtracted from the mean maximal respiration of the vehicle treated wells to  
1261 calculate the Etomoxir inhibited respiration (long chain fatty acid dependency) for that  
1262 biological replicate. Octanoate stimulation of respiration (*Octanoate stimulated respiration*),  
1263 was calculated for each vehicle well by subtracting the second OCR measurement after  
1264 injection of FCCP (7<sup>th</sup> measurement) from the third measurement after injection of octanoate  
1265 (10<sup>th</sup> measurement).

1266 ***Real time for mRNA analysis.*** RNA extraction, cDNA synthesis and real-time PCR were  
1267 performed as described ([Morton et al., 2011](#), [Moreno-Navarrete et al., 2013](#)). Probes were  
1268 mouse *Mpst*, Mm00460389\_m1, *Tst*, Mm00726109\_m1; *Gapdh* (internal control),  
1269 Mm99999915\_g1; and *Tbp* (internal control), Mm0000446973\_m1.

## 1270 **QUANTIFICATION AND STATISTICAL ANALYSIS**

1271 **Quantification and Statistical Analysis.** For analytes, bioenergetics, fluorescent probes, gene  
1272 expression, and protein levels, generally group sizes of 6 were calculated to allow detection  
1273 of differences in these variable parameters to a threshold of 15% (there is sufficient power to  
1274 detect smaller differences in certain parameters with this cohort size) with a power of at least  
1275 0.8. In some studies, limitations in animal numbers, or fewer remaining samples from larger  
1276 group sizes resulting from their use for multiple end-points, precluded the desired minimum  
1277 of  $n = 6$  per group. Protein or mRNA differences in validation studies with 2 parameters (e.g.  
1278 diet with line or genotype) were analysed using 2-way ANOVA for line and diet effects  
1279 followed, where appropriate, by post-hoc Tukey tests or Holm-Sidak multiple comparison  
1280 tests using Sigmastat version 3.5 (Systat Software) or Prism (Graphpad Software). For simple  
1281 2 condition comparisons,  $t$ -test was used. For simple control versus treated (including  
1282 different treatments or concentration response curves) data were analysed by 1-way ANOVA.  
1283 For longitudinal measures (e.g. PTT, ITT, bodyweight gain) repeated measures (RM) ANOVA  
1284 was used and multiple comparisons determined. For all main *in vivo* studies, a blinding  
1285 strategy was used where the operator (e.g. for injections of glucose, or administration of drug)  
1286 was blind to the genotype of the subject during the experiment. Similarly, for analysis of  
1287 images (e.g. oil-red O staining) the scorer was blind to genotype and the data coded, with the  
1288 code broken by a second individual. Downstream analysis of e.g. tissue mRNA and protein  
1289 content was not generally blinded to allow appropriate data arrangement on e.g.  
1290 representative western blots. For clamp studies, mean  $\pm$  standard error of mean (sem) will be  
1291 presented, statistical analysis will use  $t$ -test to investigate differences of genotype on each  
1292 diet (2 independent experiments, normal diet, and high fat diet, are not compared directly to



1293 each other). Statistical significance and the number (n) of subjects or samples for analysis are  
 1294 indicated in the figure legends.

1295 **KEY RESOURCES TABLE**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Anti-TST	GeneTex	GTX114858
Anti-MPST	Abcam	ab224043
Anti-GOT1	Abcam	ab170950
Anti-GSTT1	Proteintech	15838-1-AP
Anti-MAT1A	Abcam	ab129176
Anti-BHMT	Proteintech	15965-1-AP
Anti-CSAD	Abcam	ab91016
Anti-PPCS	Atlas Antibodies	HPA031361
IRDye 800CW Goat anti-Rabbit	LiCor	926-32211
IRDye 680RD Donkey anti-Mouse	LiCor	926-68072
Anti-B-Actin	Abcam	ab8226
Anti-CoxIV	Abcam	Ab16056
<b>Chemicals, peptides, and recombinant proteins</b>		
sodium pyruvate <sup>13</sup> C <sub>3</sub>	Sigma-Aldrich	490717
Amyloglucosidase	Roche	ROAMYGLL
Antimycin A	Sigma-Aldrich	A8674
B-glycerophosphate	Sigma-Aldrich	G9422
B-mercaptoethanol	Sigma-Aldrich	444203
Bovine serum albumin, essentially fatty acid free	Sigma-Aldrich	10775835001
Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP)	Cayman Chemicals	15218
Oligomycin A	Cayman Chemicals	11342
DL-Carnitine hydrochloride	Sigma-Aldrich	C9500
Collagenase type 1	Worthington Laboratories	LS004194
cOmplete, Mini, EDTA-free Protease Inhibitor Cocktail	Roche	11836153001
Glucose, D-[3- <sup>3</sup> H]	PerkinElmer	NET331C
Deoxycholic acid	Sigma-Aldrich	D2510
2'-deoxyguanosine 5'-diphosphate, sodium salt	Sigma-Aldrich	D9250
Dithiothreitol	Sigma-Aldrich	43816
Durcupan ACM	Sigma-Aldrich	44610
(+)- Etomoxir sodium salt hydrate	Sigma-Aldrich	E1905
EZ-link Maleimide-PEG2-Biotin	Thermo Fisher Scientific	21901BID
Fetal Calf Serum, Brazilian Origin	SLS Life Science	HYC85
Prestained Protein Marker	Proteintech	PL00001
Glutamax	Thermo Fisher Scientific	35050061
L-Glutamine	Sigma-Aldrich	G7513
Glycine	Sigma-Aldrich	50046
Hematoxylin solution	Abcam	Ab220365
Iodoacetamide	Sigma-Aldrich	I1149
L-cysteine	Sigma-Aldrich	30089
Lead citrate, tribasic trihydrate	Sigma-Aldrich	15326
Malate dehydrogenase, porcine heart	Sigma-Aldrich	442610-M

Skim Milk Powder	Millipore	70166
S-Methyl methanethiosulfonate	Sigma-Aldrich	64306
MitoSOX Red Mitochondrial superoxide indicator	Thermo Fisher Scientific	M36008
NADH, Grade I disodium salt	Roche	10107735001
Pierce NEM (N-ethylmaleimide)	Thermo Fisher Scientific	23030
Sodium octanoate	Sigma-Aldrich	C5038
Oil Red O	Sigma-Aldrich	O0625
Oligomycin A	Sigma-Aldrich	75351
Penicillin Streptomycin	Thermo Fisher Scientific	15140122
PERCOLL 8.5-9.5	Sigma-Aldrich	P1644
Phosphoenol pyruvate	Roche	10108294001
cOmplete protease cocktail inhibitor	Roche	04693159001
Rat tail collagen 1	Sigma-Aldrich	08-115
REVERT total protein stain	LICOR	926-11011
Rotenone	Sigma-Aldrich	R8875
Sequencing grade modified Trypsin	Promega	V5111
Sodium fluoride	Sigma-Aldrich	S7920
Sodium L-lactate	Sigma-Aldrich	71718
Sodium orthovanadate	Sigma-Aldrich	450243
Sodium pyrophosphate	Sigma-Aldrich	221368
Sodium pyruvate	Sigma-Aldrich	P8574
Sodium sulfate	Sigma-Aldrich	S9627
Sodium thiosulfate	Sigma-Aldrich	563188
Sodium sulfide	Sigma-Aldrich	407410
Sulforhodamine B dye	Sigma-Aldrich	230162
Taurine	Sigma-Aldrich	86329
Tetrabutylammonium phosphate	Sigma-Aldrich	86833
Trichloroacetic acid	Sigma-Aldrich	T6399
Triethylammonium bicarbonate	Sigma-Aldrich	18597
Trifluoroacetic acid	Sigma-Aldrich	80457
Uranyl acetate	Electron Microscopy Sciences	22400
Urea	Sigma-Aldrich	U5128
XF Seahorse Base Media (DMEM)	Agilent	102353-100
<sup>14</sup> C-2-deoxyglucose	Perkin Elmer	NEC495A
4-(2-Hydroxyethyl)-1-piperazine propanesulfonic acid	Sigma-Aldrich	1.15230
2,4-nitrofluorobenzene	Sigma-Aldrich	D1529
<b>Critical commercial assays</b>		
Infinity Triglyceride Assay	Thermo Fisher Scientific	TR22421
Infinity Cholesterol Assay	Thermo Fisher Scientific	TR13421
Glucose Hexokinase Assay	Abcam	Ab136957
iTRAQ reagent – 8PLEX	Sigma-Aldrich	4281663
<b>Deposited data</b>		
Proteome	ProteomeXchange	PXD028909
Persulfidome	ProteomeXchange	PXD028909
<b>Experimental models: Cell lines</b>		
Primary hepatocytes	C57BL/6J and <i>Tst</i> <sup>-/-</sup> mice	n/a

Experimental models: Organisms/strains		
C57BL/6J (JAX mice strain)	Charles River	Strain code: 632
<i>Tst</i> <sup>-/-</sup> C57Bl/6N mouse (backcrossed for >10 generations at University of Edinburgh)	University California (Davis) International Mouse Knockout Project	VG13928; model <i>Tst</i> <sup>tm1(KOMP)Vlcg</sup>
Oligonucleotides		
Tst (mouse) FAM gene expression assay 4331182	Thermo Fisher	Mm00726109_m1
Mpst (mouse) FAM gene expression assay 4331182	Thermo Fisher	Mm00460389_m1
Gapdh (mouse) FAM gene expression assay 4331182	Thermo Fisher	Mm99999915_g1
Tbp (mouse) FAM gene expression assay 4331182	Thermo Fisher	Mm0000446973_m1
Software and algorithms		
Microsoft office		
Graph Pad Prism v8, 9 and 10		
Other		
Amersham Hybond P blotting membranes, PVDF	Merck	GE10600021
Microvette CB300 K2E EDTA tubes	Sarstedt	16.444.100
Ultra-0.5 centrifugal filter, 10K cut off	Millipore	UFC501096
Streptavidin-agarose beads	Thermo Scientific	20347
Formvar coated grids	Agar Scientific	AGS138
Standard rodent diet	SDS	RM1
Cornstarch diet	Research Diets	D12383
58% High fat diet	Research Diets	D12331

1296

1297 **SUPPLEMENTAL FIGURES and EXCEL TABLE; TITLES AND LEGENDS**1298 **Table S3 (Excel)**

1299 **Table S3. *Tst* Deletion results in differential hepatic protein abundance of GO terms.** Related to Table  
1300 3. (A) Significant GO terms represented by proteins that are more abundant in the ND-fed *Tst*<sup>-/-</sup> liver  
1301 compared with normal diet-fed C57Bl/6J. (B) Significant GO terms represented by proteins that are  
1302 less abundant in the ND-fed *Tst*<sup>-/-</sup> liver compared with normal diet-fed C57Bl/6J. 'Genes' indicates  
1303 the number of genes in the *Tst*<sup>-/-</sup> that represent the changes driving the GO term. The lists include  
1304 all regulated GO terms at a significance threshold of P < 0.05  
1305

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Figure 1

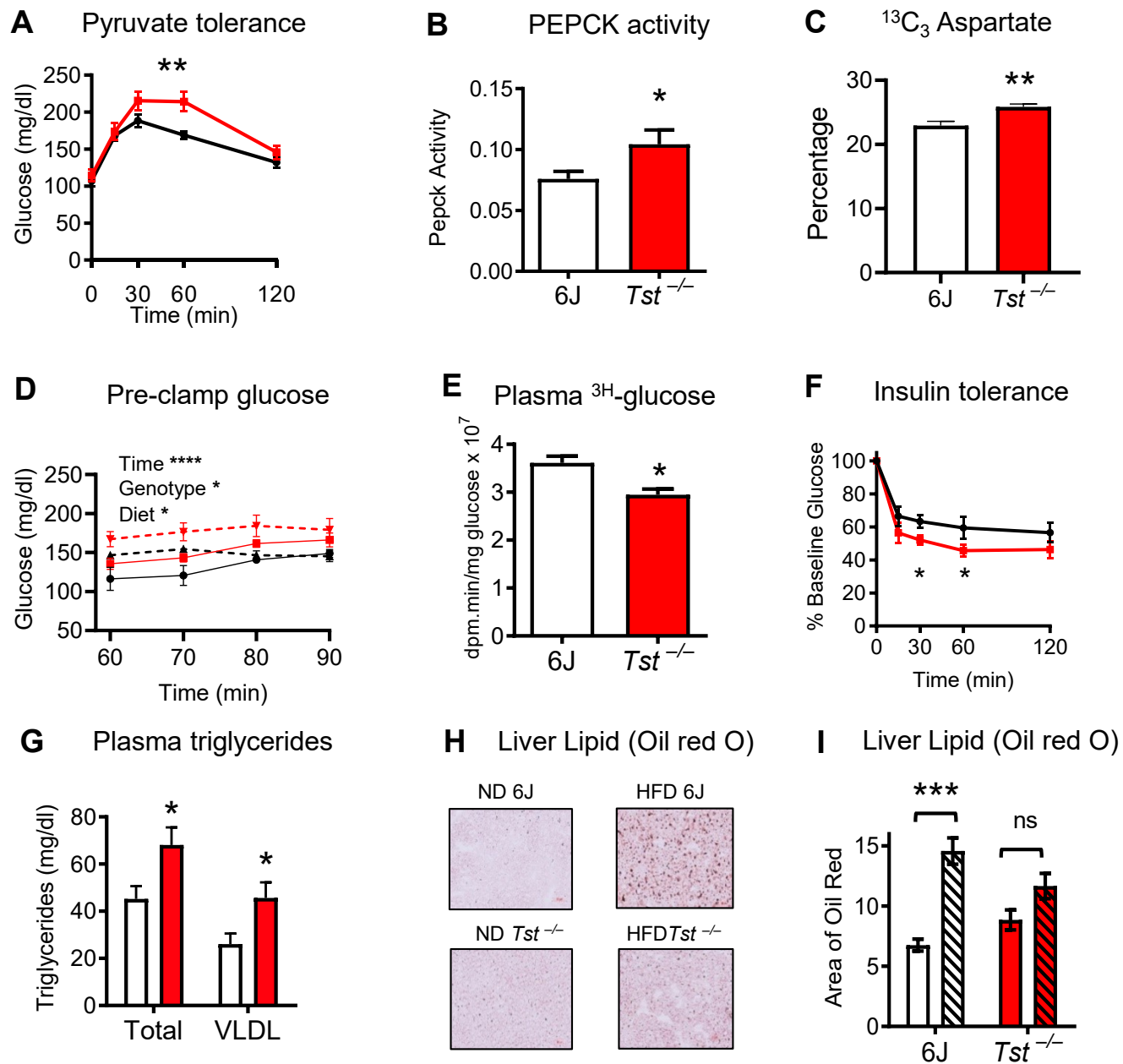


Figure 2

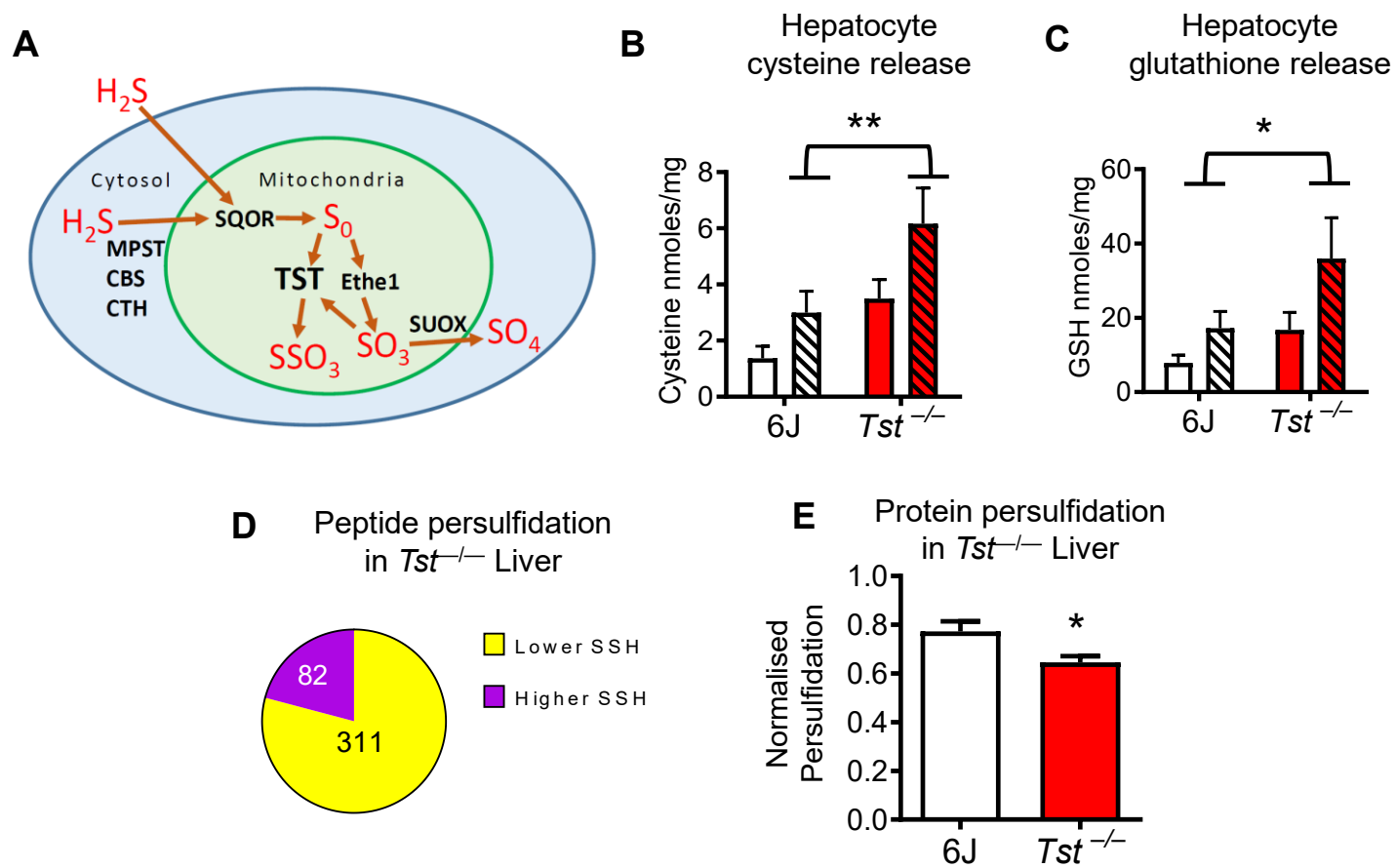
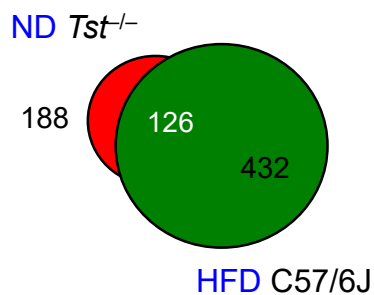
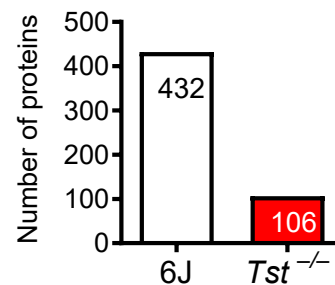


Figure 3

**A** Proteome overlap of *Tst*<sup>-/-</sup> with high fat feeding



**B** Impact of high fat feeding on proteome



Protein enrichment by organelle

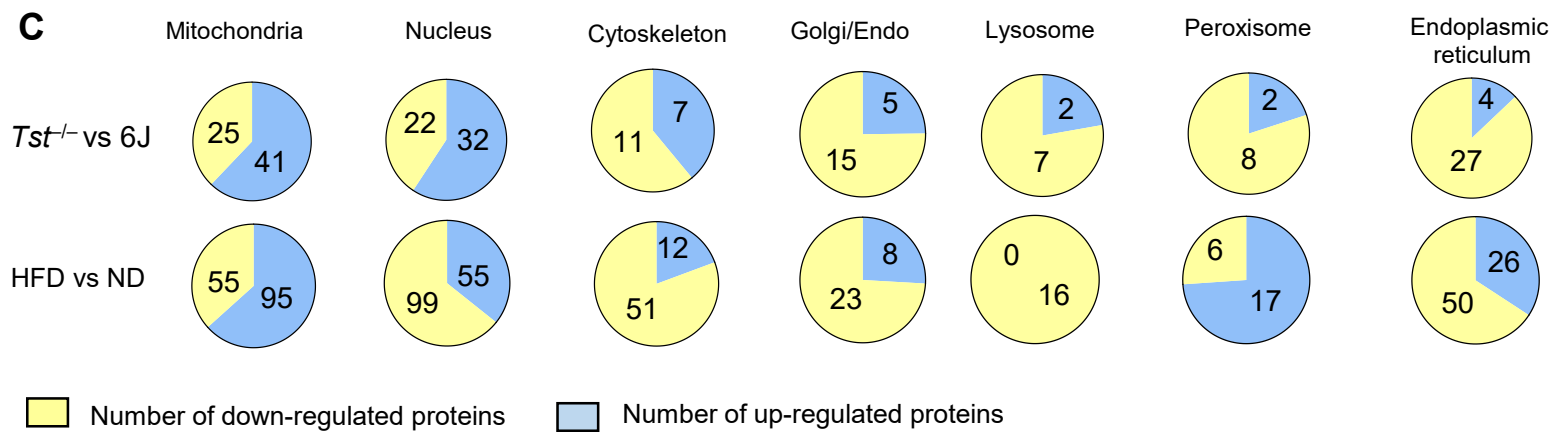
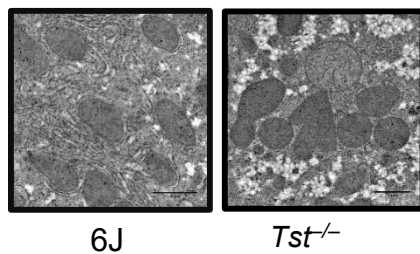
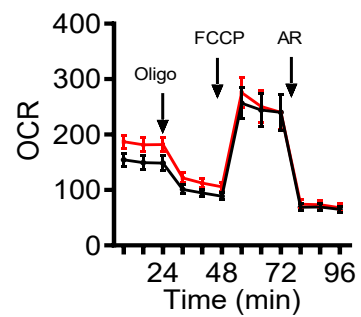


Figure 4

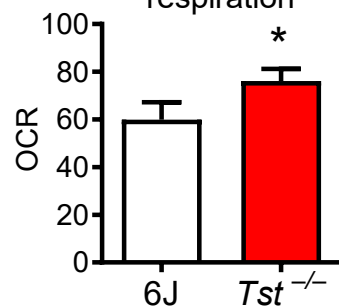
**A** Mitochondrial structure



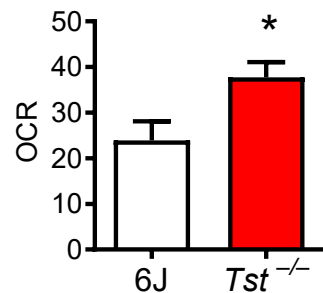
**B** Mitochondrial stress test



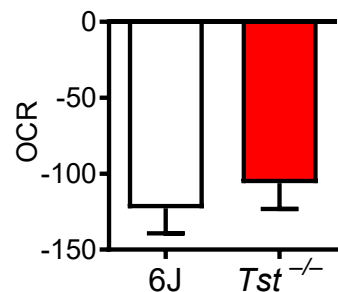
**C** ATP linked respiration



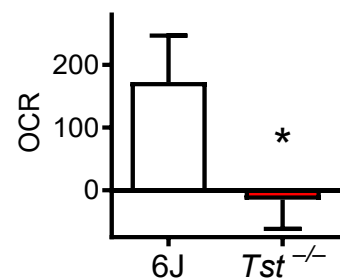
**D** Leak respiration



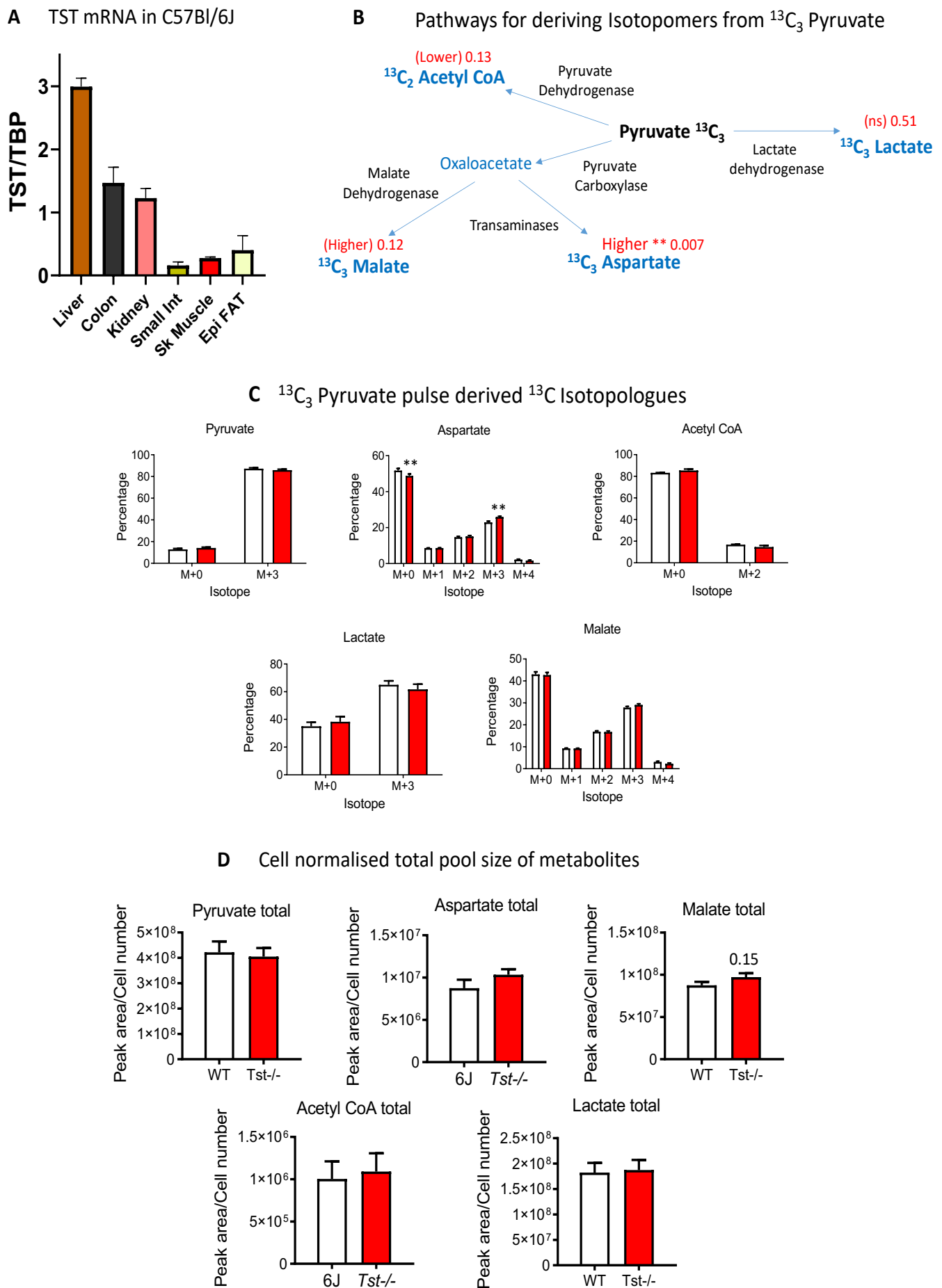
**E** Etomoxir inhibited respiration



**F** Octanoate stimulated respiration

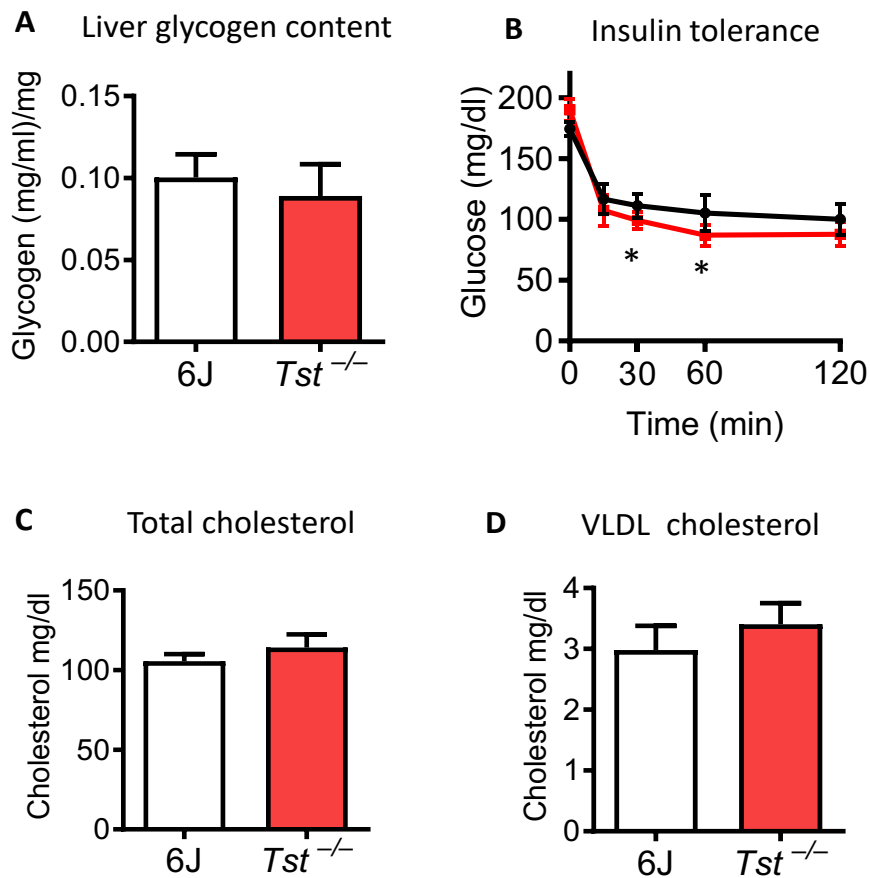


**Figure S1. *Tst* mRNA tissue expression profile in C57BL/6J mice and the metabolic fates of  $^{13}\text{C}_3$  pyruvate in hepatocytes from *Tst*<sup>-/-</sup> mice. Related to Figure 1**



**Figure S1. *Tst* mRNA tissue expression profile in C57BL/6J mice and the metabolic fates of  $^{13}\text{C}_3$  pyruvate in hepatocytes from *Tst*<sup>-/-</sup> mice.** Related to Figure 1 **(A)** Histogram showing *Tst* mRNA level across liver, colon, kidney, small intestine, skeletal muscle and epididymal fat from male C57BL/6J mice measured by realtime-PCR and normalised to *Tbp* mRNA **(B)** Diagram representing metabolites derived from pyruvate. Oxaloacetate was not detected but is indicated as an intermediate to production of malate or aspartate. The isotopologue shown on the diagram represents to most abundant detected following pulse with  $^{13}\text{C}_3$  pyruvate. In red is the direction of change in hepatocytes of *Tst*<sup>-/-</sup> mice, with significance or P-values (when less than 0.2) from t-tests. **(C)** Histogram showing isotopologues derived from  $^{13}\text{C}_3$  pyruvate from C57BL/6J (white bars, n = 5) and *Tst*<sup>-/-</sup> (red bars n = 4) cultured hepatocytes. Data represents the amount of isotopologues detected by mass spectrometry, as a percentage of the total detected metabolite (total includes unlabelled  $^{12}\text{C}$  and all detected  $^{13}\text{C}$  isotopologues). Counts were first normalised to cell number. **(D)** Histograms showing the total pool size of each metabolite. Data is cell normalised mass spec counts from all isotopologues of the given metabolite, including the relevant unlabelled  $^{12}\text{C}$  species. Data are represented as mean  $\pm$  SEM. Each metabolite was analysed using a t-test, \*\* indicates that P < 0.01. P-values less than 0.2 are also indicated for showing potential trends.

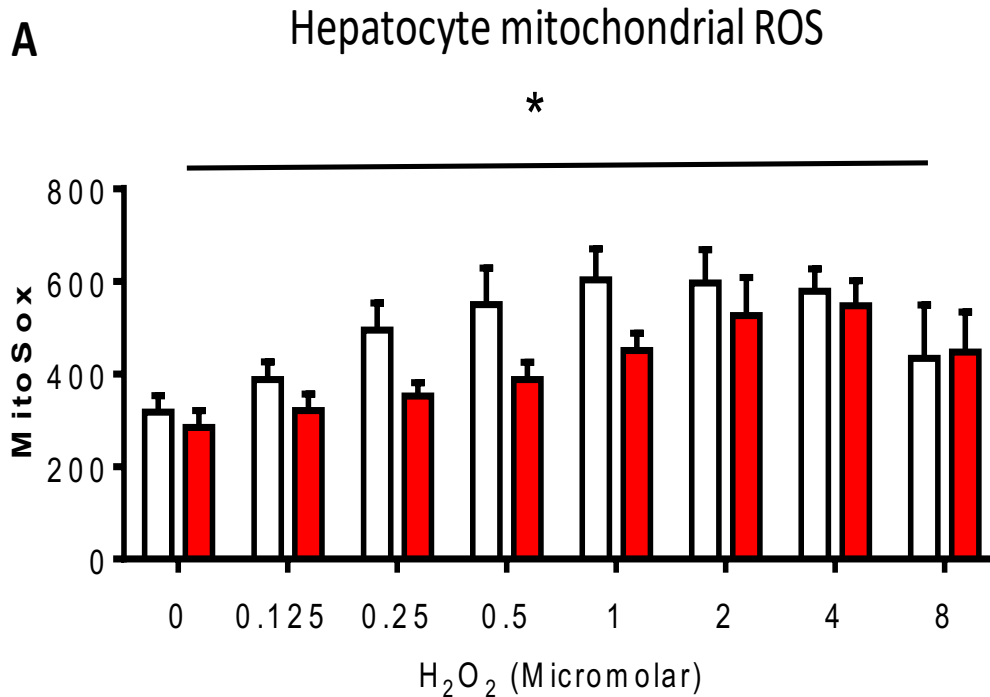
**Figure S2. Insulin-regulated metabolic parameters in liver and plasma of  $Tst^{-/-}$  mice.** Related to Figure 1.



**Figure S2. Insulin-regulated metabolic parameters in liver and plasma of  $Tst^{-/-}$  mice.** Related to Figure 1. **(A)** Glycogen measured from whole liver from normal diet-fed 4 hour fasted C57Bl/6J (6J; white bar,  $n = 5$ ), and  $Tst^{-/-}$  (red bar,  $n = 5$ ) mice. Data are represented as mean  $\pm$ SEM. **(B)** Plasma glucose (mg/dl), over 120 minutes following insulin administration (i.p., 1mU/g) in normal diet-fed 4 hour fasted C57Bl/6J (black line,  $n = 8$ ) and  $Tst^{-/-}$  (red line,  $n = 7$ ) mice. **(C)** HPLC quantified total plasma cholesterol in normal diet-fed 4 hour fasted C57Bl/6J (white bar,  $n = 6$ ) and  $Tst^{-/-}$  (red bar,  $n = 6$ ) mice. **(D)** HPLC quantified VLDL plasma cholesterol in normal diet-fed 4 hour fasted C57Bl/6J (white bar,  $n = 6$ ), and  $Tst^{-/-}$  (red bar,  $n = 6$ ) mice. For **(B)** a Repeated Measures analysis demonstrated a significant effect of time (\*\*\*\*) and an interaction between time and genotype (\*). T-tests revealed that the decrement of glucose from baseline at 30 and 60 minutes after insulin was greater in the  $Tst^{-/-}$  (\*).

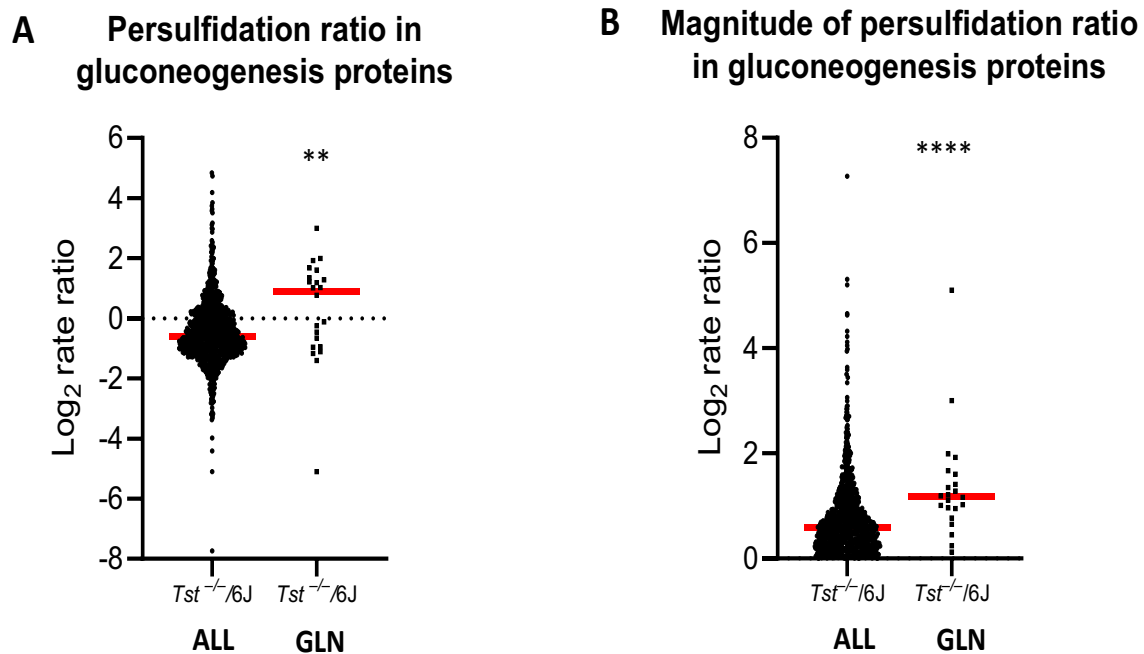


**Figure S3. Hepatocytes from *Tst*<sup>-/-</sup> mice resist hydrogen peroxide induced mitochondrial reactive species accumulation.** Related to Figure 2 and Table 1



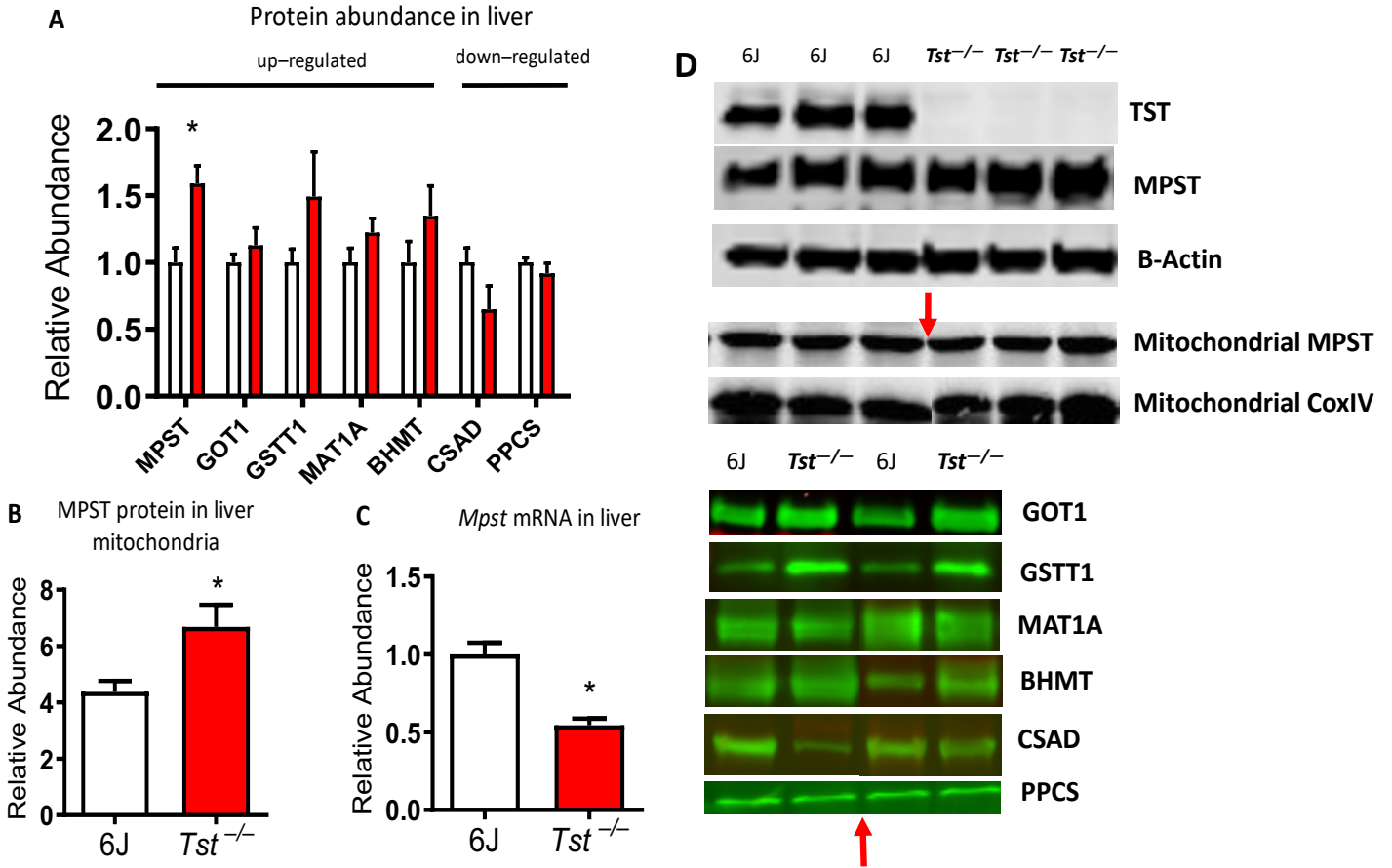
**Figure S3. Hepatocytes from *Tst*<sup>-/-</sup> mice resist hydrogen peroxide induced mitochondrial reactive species accumulation.** Related to Figure 2 and Table 1 (A) Mitochondrial reactive oxygen species measured from primary hepatocytes by MitoSox fluorescence from C57Bl/6J (white bars, n = 7) and *Tst*<sup>-/-</sup> (red bars, n = 7). Cells were exposed to a range of doses of H<sub>2</sub>O<sub>2</sub> prior to MitoSox incubation and fluorescent detection. Data are represented as mean ±SEM. Significance was calculated using 2-WAY ANOVA for H<sub>2</sub>O<sub>2</sub> dose and genotype. A significant effect of genotype is represented above the histogram with a \*. H<sub>2</sub>O<sub>2</sub> was significant to P < 0.001 (not represented on the histogram).

**Figure S4. Persulfidation in the gluconeogenesis pathway is significantly different to global persulfidation patterns in the liver of the *Tst*<sup>-/-</sup> mice.** Related to Figure 2 and Table 3.



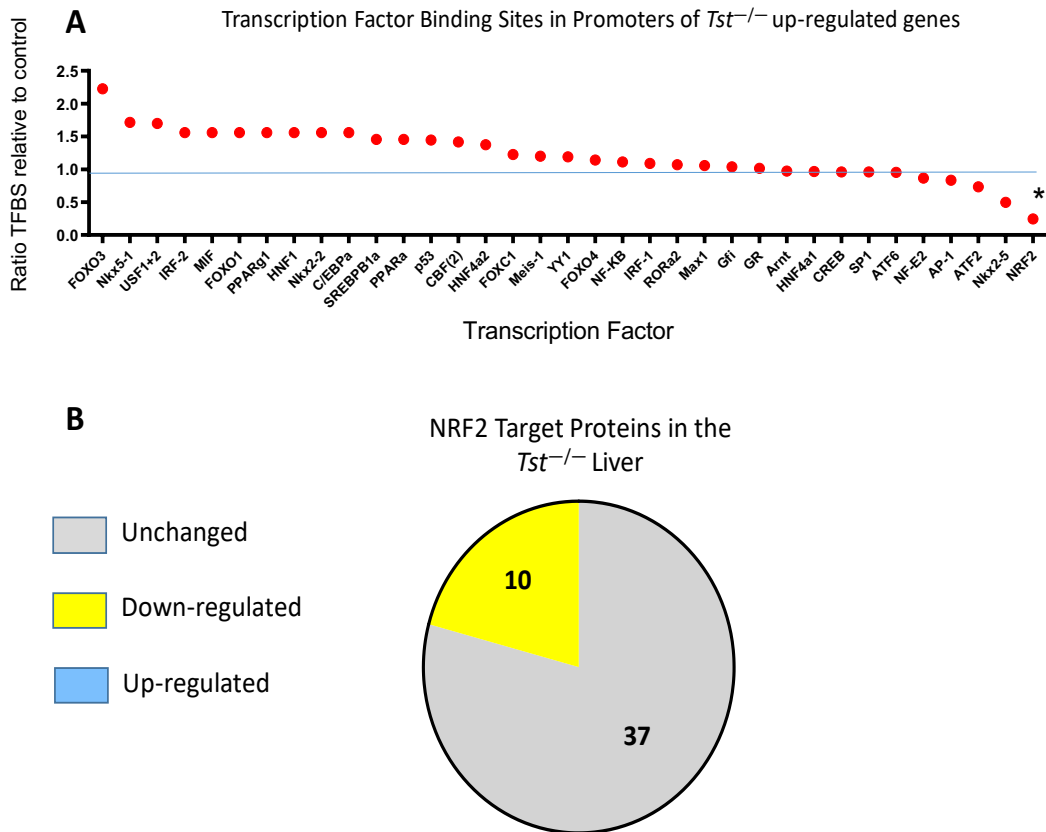
**Figure S4. Persulfidation in the gluconeogenesis pathway is significantly different to global persulfidation patterns in the liver of the *Tst*<sup>-/-</sup> mice.** Related to Figure 1, Figure 2 and Table 3. (A) Beeswarm plots showing the persulfidation log<sub>2</sub> rate ratio (*Tst*<sup>-/-</sup> divided by 6J) for peptides in the entire data set (ALL), alongside the log<sub>2</sub> rate ratio for peptides corresponding to proteins of gluconeogenesis (GLN). (B) Beeswarm plots showing the magnitude of the log<sub>2</sub> rate ratio (independent to direction of change), for peptides in the entire data set (ALL), alongside the log<sub>2</sub> rate ratios for peptides corresponding to proteins of gluconeogenesis (GLN). Data are represented as individual peptide log<sub>2</sub> rate ratio values, with the median represented as a red line. Significance was calculated using the Mann-Whitney U non parametric T-test. \*\* P < 0.01, \*\*\*\* P < 0.0001.

**Figure S5. Validation of proteomic profiles by select western blot is exemplified by increased mitochondrial MPST.** Related to Figure 3 and Table 3.



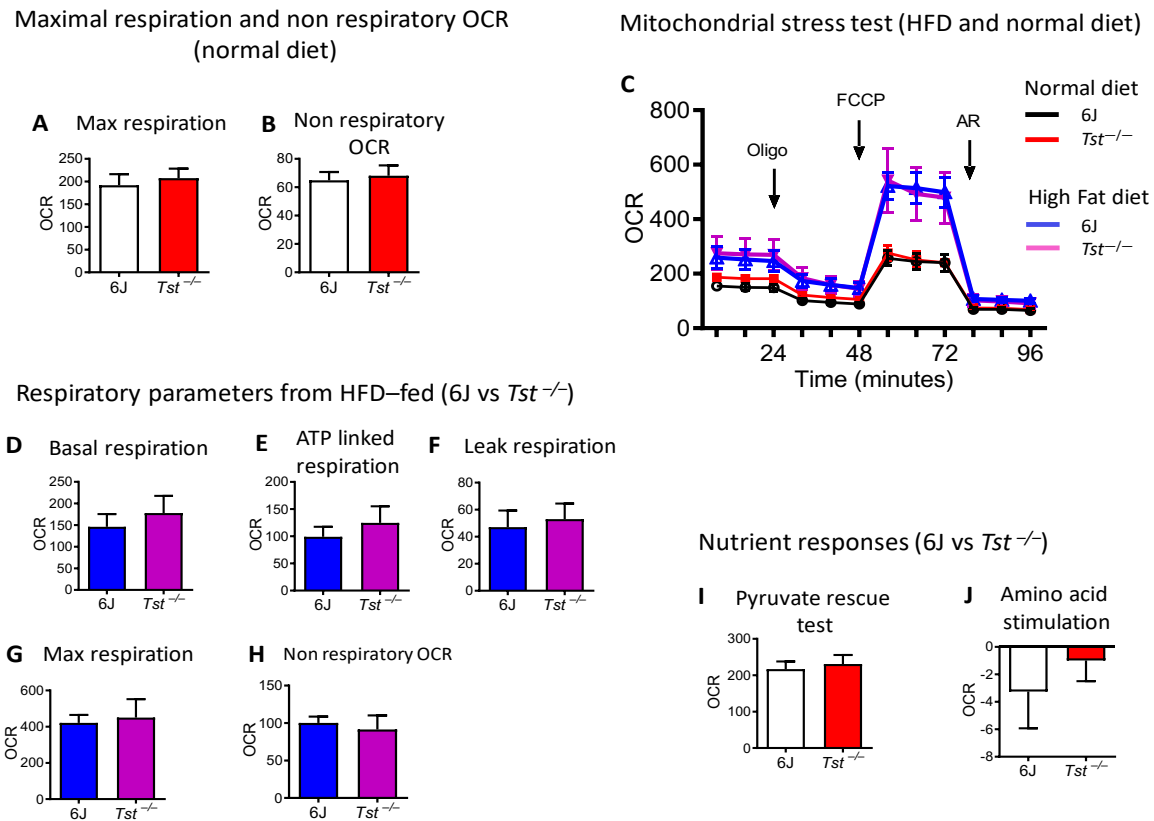
**Figure S5. Validation of proteomic profiles by select western blot is exemplified by increased mitochondrial MPST.** Related to Figure 2 and Table 3. (A) Quantification of western blots for a range of proteins found significantly up or down-regulated in the liver proteome of normal diet-fed 4 hour fasted C57Bl/6J (6J; white bar, n = 4-6) and *Tst*<sup>-/-</sup> (red bar, n = 4-6) mice. (B) Quantification of western blots for MPST from isolated liver mitochondria of normal diet-fed 4 hour fasted C57Bl/6J (white bar, n = 6), and *Tst*<sup>-/-</sup> (red bar, n = 6) mice. (C) *Mpst* mRNA quantified by real time PCR from liver of normal diet-fed C57Bl/6J (6J; white bar, n = 6) and *Tst*<sup>-/-</sup> (red bar, n = 6) mice. (D) Representative blots from LICOR imaging for the data quantified in (A). Red arrow indicates where superfluous lanes have been removed to simplify visualisation of genotype comparisons (GOT1, MAT1A, BHMT CSAD, mitochondrial MPST and COXIV). Data are represented as mean ± SEM. Significance was calculated using un-paired two-tailed student's t-test. \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001.

**Figure S6. Hepatic proteins enriched in *Tst*<sup>-/-</sup> mice show under-representation of NRF2 promoter binding sites.** Related to Figure 3 and Table 3.



**Figure S6. Hepatic proteins enriched in *Tst*<sup>-/-</sup> mice show under-representation of NRF2 promoter binding sites.** Related to Figure 3 and Table 3. (A) abundance in *Tst*<sup>-/-</sup> liver compared to a control set of proteins that are unchanged between 6J and *Tst*<sup>-/-</sup>. The proportion of genes containing a promoter binding site from proteins increased in *Tst*<sup>-/-</sup> was divided by the proportion of genes containing a binding site from a control set of genes. (B) Pie charts representing the number of NRF2-target proteins whose abundance is increased (blue), decreased (yellow) or unchanged (grey) in the *Tst*<sup>-/-</sup> liver. Significance of transcription factor enrichment analysis was calculated using a Fishers Exact test. \* P < 0.05. Significance for NRF2 target abundance was performed with the Freeman-Halton Fishers Exact Test.

**Figure S7. Hepatocyte respiration after high-fat feeding or after amino acid or pyruvate challenge is comparable between C57Bl/6J and *Tst*<sup>-/-</sup> mice *in vitro*.** Related to Figure 4.



**Figure S7. Hepatocyte respiration after high-fat feeding or after amino acid or pyruvate challenge is comparable between C57Bl/6J and *Tst*<sup>-/-</sup> mice *in vitro*.** Related to Figure 4. (A) Maximal respiratory OCR elicited by uncoupling with FCCP, by hepatocytes from normal diet-fed C57Bl/6J (n = 6) or *Tst*<sup>-/-</sup> (n = 6) mice, calculated from Figure 3B. (B) Non-respiratory OCR remaining following the inhibition of respiration with antimycin and rotenone, by hepatocytes from normal diet-fed C57Bl/6J (n = 6) or *Tst*<sup>-/-</sup> (n = 6) mice, calculated from Figure 3B. (C) Seahorse trace representing the mean oxygen consumption rate (OCR), normalised to protein, by hepatocytes from normal diet-fed (n = 6/genotype), and high fat diet-fed (n = 4/genotype) C57Bl/6J and *Tst*<sup>-/-</sup> mice during a mitochondrial stress test. (D) Basal respiratory OCR linked to ATP production (antimycin/rotenone sensitive) by hepatocytes from high fat diet-fed C57Bl/6J (n = 4) or *Tst*<sup>-/-</sup> (n = 4) mice, calculated from Figure S4C. (E) Respiratory OCR linked to ATP production (oligomycin sensitive) by hepatocytes from high fat diet-fed C57Bl/6J (n = 4) or *Tst*<sup>-/-</sup> (n = 4) mice, calculated from Figure S4C. (F) Respiratory OCR relating to proton leak (oligomycin insensitive) by hepatocytes from high fat diet-fed C57Bl/6J (n = 4) or *Tst*<sup>-/-</sup> (n = 4) mice, calculated from Figure S4C. (G) Maximal respiratory OCR elicited by uncoupling with FCCP, by hepatocytes from high fat diet-fed C57Bl/6J (n = 4) or *Tst*<sup>-/-</sup> (n = 4) mice, calculated from Figure S4C. (H) Non-respiratory OCR remaining following the inhibition of respiration with antimycin and rotenone, by hepatocytes high fat diet-fed C57Bl/6J (n = 4) or *Tst*<sup>-/-</sup> (n = 4) mice, calculated from Figure S4C. (I) Stimulation of maximal uncoupled respiration following addition of pyruvate (2mM), from normal diet-fed C57Bl/6J (n = 4) or *Tst*<sup>-/-</sup> (n = 4) mice. (J) Stimulation of maximal uncoupled respiration following addition of aspartate (1mM) and glutamate (1mM), by hepatocytes from normal diet-fed C57Bl/6J (n = 1) or *Tst*<sup>-/-</sup> (n = 1) mice. Data are represented as mean ± SEM. Significance was calculated using an unpaired two tailed, student's t-test. \* P < 0.05.

**Table S1. Parameters during the euglycemic hyperinsulinemic clamp****(A) Parameters during the basal (pre clamp) experiment (60-90 minutes post tracer)**

Parameter	6J chow	<i>Tst</i> <sup>-/-</sup> chow	6J HFD	<i>Tst</i> <sup>-/-</sup> HFD	Genotype	Diet
Fasted Glucose 60 min (mg/dl)	116.35± 14.93	135.72± 7.22	146.50± 3.89	167.13± 9.68	*	**
Glycolysis (mg/kg/min)	11.63 ± 1.60	11.12 ± 0.62	12.89 ± 0.57	12.61 ± 0.62	ns	ns (0.09)
Glycogen synthesis (mg/kg/min)	21.48 ± 2.06	19.02 ± 2.04	15.08 ± 2.76	16.28 ± 2.50	ns	****

**(B) Measurements and parameters during the clamp experiment (160-210 minutes post tracer)**

Parameter	6J chow	<i>Tst</i> <sup>-/-</sup> chow	Genotype (chow)	6J HFD	<i>Tst</i> <sup>-/-</sup> HFD	Genotype (HFD)
Glucose 160 min (mg/dl)	108.0 ± 5.0	121.0 ± 10.1	ns	120.1± 3.7	125.6 ± 10.3	ns
Glucose 170 min (mg/dl)	120.3 ± 6.4	129.8 ± 5.8	ns	118.1 ± 4.1	143.9 ± 14.0	ns (0.08)
Glucose 180 min (mg/dl)	115.0 ± 5.5	125.5 ± 2.5	ns (0.08)	125.9 ± 2.9	126.7 ± 6.8	ns
Glucose 190 min (mg/dl)	124.0 ± 1.0	131.0 ± 4.6	ns	121.5 ± 4.7	116.1 ± 3.7	ns
Glucose 200 min (mg/dl)	121.0 ± 5.0	125.3 ± 5.8	ns	121.9 ± 3.0	115.7 ± 5.3	ns
Glucose 210 min (mg/dl)	113.0 ± 7.8	123.5 ± 4.6	ns	119.4 ± 4.0	116.9 ± 2.7	ns
Glucose IR 160-210 min (mg/kg/min)	84.9 ± 4.2	85.0 ± 2.6	ns	70.26 ± 4.83	69.95 ± 4.96	ns
Glucose IR 160 (mg/kg/min)	82.3 ± 5.1	87.1 ± 3.2	ns	68.8 ± 4.2	68.9 ± 5.1	ns
Glucose IR 170 (mg/kg/min)	85.2 ± 4.9	89.0 ± 3.0	ns	70.0 ± 5.1	74.6 ± 4.4	ns
Glucose IR 180 (mg/kg/min)	84.7 ± 4.4	83.5 ± 4.2	ns	70.6 ± 5.1	70.7 ± 6.2	ns
Glucose IR 190 (mg/kg/min)	85.2 ± 4.0	86.1 ± 2.1	ns	69.9 ± 4.8	69.1 ± 5.7	ns
Glucose IR 200 (mg/kg/min)	85.2 ± 4.0	84.8 ± 2.4	ns	70.4 ± 4.7	71.2 ± 4.5	ns
Glucose IR 210 (mg/kg/min)	85.2 ± 4.0	84.5 ± 2.4	ns	70.4 ± 4.7	71.2 ± 4.5	ns
Turnover (mg/kg/min)	85.97 ± 3.52	94.50 ± 3.87	ns	73.61 ± 5.07	63.08 ± 7.10	ns
Hepatic Glucose Prod. (mg/kg/min)	1.10 ± 5.31	9.92 ± 9.15	ns	3.326 ± 4.03	-6.83 ± 7.93	ns
Glycolysis (mg/kg/min)	45.90 ± 2.218	48.37 ± 2.05	ns	42.85 ± 1.48	36.42 ± 4.62	ns (0.19)
Glycogen synthesis (mg/kg/min)	40.06 ± 4.44	46.12 ± 2.68	ns	30.76 ± 5.36	26.66 ± 4.49	ns
Integral Glucose (dpm.min/mg)	3.6e <sup>7</sup> ± 1.4e <sup>6</sup>	2.95e <sup>7</sup> ± 1.2e <sup>6</sup>	*	1.88e <sup>7</sup> ± 1.4e <sup>6</sup>	1.7e <sup>7</sup> ± 1.6e <sup>6</sup>	ns
IWAT glucose utilization (ng/mg.min)	14.21 ± 4.05	18.62 ± 2.04	ns	4.53 ± 1.07	6.64 ± 1.00	ns
EWAT glucose utilization (ng/mg.min)	7.523 ± 4.39	7.21 ± 2.18	ns	2.94 ± 0.56	3.64 ± 0.39	ns
VL glucose utilization (ng/mg.min)	33.77 ± 2.98	38.66 ± 1.70	ns (0.17)	49.65 ± 9.19	47.42 ± 9.19	ns
EDL glucose utilization (ng/mg.min)	35.79 ± 11.09	40.86 ± 10.25	ns	68.79 ± 8.65	61.29 ± 11.80	ns
Soleus glucose utilization (ng/mg.min)	99.85 ± 12.38	123.60 ± 13.90	ns (0.13)	220.8 ± 24.45	198.5 ± 32.92	ns
Tibialis glucose utilization (ng/mg.min)	47.25 ± 7.22	53.67 ± 4.40	ns	74.39 ± 6.46	80.16 ± 6.09	ns
Heart glucose utilization (ng/mg.min)	161.40 ± 7.65	198.00 ± 14.09	ns (0.13)	226.6 ± 51.01	262.5 ± 23.29	ns
Liver glucose utilization (ng/mg.min)	3.53 ± 0.56	3.57 ± 0.63	ns	3.378 ± 0.39	3.58 ± 0.46	ns
End Clamp Insulin (μU/ml)	133.6 ± 7.03	126.4 ± 5.06	ns	147.2 ± 9.4	124.4 ± 10.9	ns

\* P &lt; 0.05, \*\* P &lt; 0.01, \*\*\* P &lt; 0.001, \*\*\*\* P &lt; 0.0001

**Table S1. Parameters during the euglycemic hyperinsulinemic clamp.** Related to Figure 1. Metabolic parameters measured during continuous trace infusion but prior to clamp (A) and during maintenance of euglycemia and hyperinsulinemia (B) from C57Bl/6J (chow-fed, n = 3, hfd-fed, n = 8) and *Tst*<sup>-/-</sup> (chow-fed, n = 6, hfd-fed, n = 7) mice. Data are represented as mean ± SEM. Significance for the basal experiment (A) was

calculated using a 2-WAY ANOVA for *genotype* and *diet*. Significance for the clamp (**B**) was calculated for each diet separately using T-tests for *genotype*. \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001, \*\*\*\* P < 0.0001

<b>Table S2. Hydrogen sulfide disposal by hepatocytes and mitochondria (Amperometry)</b>			
<b><i>n</i>moles/min/mg protein</b>	<b>C57Bl/6J</b>	<b><i>Tst</i><sup>-/-</sup></b>	<b>Significance</b>
Hepatocytes	3.88 +/- 0.095	4.15 +/- 0.345	<b>ns</b>
Hepatocytes (Respiratory)	1.97 +/- 0.176	2.91 +/- 0.288	<b>*</b>
Hepatocytes (Non-respiratory)	1.91 +/- 0.181	1.24 +/- 0.117	<b>*</b>
Liver Mitochondria	0.65 +/- 0.095	1.23 +/- 0.129	<b>*</b>
Liver Mitochondria (Respiratory)	0.26 +/- 0.060	0.50 +/- 0.080	<b>*</b>

**\* P < 0.05**

**Table S2. *Tst* deletion results in increased respiratory H<sub>2</sub>S disposal by hepatocytes.** Related to Figure 2 and Table 1. H<sub>2</sub>S disposal rates (measured by gas selective amperometry following addition of 10 μM Na<sub>2</sub>S) of hepatocytes (n = 6/genotype), or isolated liver mitochondria (n = 7/genotype) of ND-fed C57Bl/6J and *Tst*<sup>-/-</sup> mice. Rates of H<sub>2</sub>S disposal were measured with and without respiratory inhibition following addition of Antimycin (2μM). Antimycin insensitive disposal rates are referred to as non-respiratory. The Antimycin sensitive disposal rates are referred to as respiratory. Data are represented as mean ±SEM. Significance was calculated using paired two-tailed student's t-test. \* P < 0.05.



**Table S4. Sulfide metabolism proteins in liver proteome (*Tst*<sup>-/-</sup> vs C57Bl/6J, ND-fed)**

Feature ID	Name	Fold Change	Significance
Q3UW66	MPST Mercaptopyruvate sulfurtransferase	1.27	**
Q8R086	SUOX Sulfite Oxidase	1.06	Ns
Q3UDS4	SQOR Sulfide quinone reductase-like	1.06	Ns
Q91WT9	CBS Cystathionine beta-synthase	1.03	Ns
Q9DCM0	ETHE1 Ethylmalonic encephalopathy 1	-1.01	Ns
Q8VCNS	CTH Cystathionine gamma-lyase	-1.02	Ns

\* Raw P < 0.05, \*\* Adjusted P < 0.05

**Table S4. *Tst* deletion selectively regulates MPST in the sulfide pathway of normal diet-fed mice.**

Related to Figure 3 and Table 3. Relative peptide abundance of proteins of the sulfide production and disposal pathway from the liver proteome of normal diet (ND) fed mice. 'Fold Change' indicates the relative abundance of the protein in *Tst*<sup>-/-</sup> relative to C57Bl/6J.

**Table S5. GO terms - Nutrient metabolism; reduced in ND *Tst*<sup>-/-</sup> liver (*Tst*<sup>-/-</sup> vs C57Bl/6J liver, ND-fed)**

GO-ID	Name	Genes	Significance
0006629	Lipid metabolic process	19	**
0006631	Fatty acid beta-oxidation	7	**
0003995	Acyl-CoA dehydrogenase activity	3	*
0047617	Acyl-CoA hydrolase activity	2	*

\* P < 0.05, \*\* P < 0.01

**Table S5. *Tst* Deletion results in reduction of selective fatty acid specific GO terms.** Related to Figure 3 and Table 3. Significant GO terms (glucose or lipid related) represented by proteins that are less abundant in the ND-fed *Tst*<sup>-/-</sup> liver compared with ND-fed C57Bl/6J. 'Genes' indicates the number of genes in the *Tst*<sup>-/-</sup> that represent the changes driving the GO term.

**Table S6 Insulin regulated proteins in *Tst*<sup>-/-</sup> and C57Bl/6J mice****(A) Abundance of peptides of insulin-induced proteins (*Tst*<sup>-/-</sup> vs C57Bl/6J, ND-fed)**

Feature ID	Name	Fold change	Significance
Q3UGT1	CPT1A	1.03	Ns
P19096	FASN	-1.04	Ns
Q3UDA8	CPT2	-1.05	Ns
Q3V2G1	APOA1	-1.10	Ns
Q5SVI5	GCK	-1.14	*

**(B) Abundance of peptides of insulin-suppressed proteins (*Tst*<sup>-/-</sup> vs C57Bl/6J, ND-fed)**

Feature ID	Name	Fold change	Significance
QO5421	CYP2E1	1.13	**
Q9D6M3	SLC25AA2	-1.03	Ns
Q8CI37	PCK1	-1.09	Ns
Q3UJ70	HMGCS1	-1.09	Ns
O08601	MTTP	-1.20	**

**(C) Abundance of peptides of insulin-induced proteins (*Tst*<sup>-/-</sup> vs C57Bl/6J, High Fat-fed)**

Feature ID	Name	Fold change	Significance
Q3UGT1	CPT1A	1.03	Ns
P19096	FASN	-1.04	Ns
Q3UDA8	CPT2	-1.05	Ns
Q3V2G1	APOA1	-1.1	Ns
Q5SVI5	GCK	-1.14	*

**(D) Abundance of peptides of insulin-suppressed proteins (*Tst*<sup>-/-</sup> vs C57Bl/6J, High Fat-fed)**

Feature ID	Name	Fold change	Significance
QO5421	CYP2E1	-1.05	Ns
Q9D6M3	SLC25AA2	-1.01	Ns
Q8CI37	PCK1	1.09	Ns
Q3UJ70	HMGCS1	-1.04	Ns
O08601	MTTP	1.09	*

\* Raw P &lt; 0.05, \*\* Adjusted P &lt; 0.05

**Table S6. Proteins regulated by insulin are broadly comparable in expression between *Tst*<sup>-/-</sup> and C57Bl/6J. Related to Figure 3. Relative abundance in proteins that are known to be induced (A) or suppressed**

(B) by insulin in the liver, from the liver proteome of normal diet fed mice. 'Fold Change' indicates the relative abundance of the protein in *Tst*<sup>-/-</sup> relative to C57Bl/6J. Relative abundance in proteins that are known to be induced (C) or suppressed (D) by insulin in the liver, from the liver proteome of high fat diet fed mice. 'Fold Change' indicates the relative abundance of the protein in *Tst*<sup>-/-</sup> relative to C57Bl/6J.

**Table S7. KEGG pathways shared by high fat feeding and TST deletion**

Entry	Name	Comparison	Significance
<b>A. Shared up-regulated pathways</b>			
00260	Glycine, serine and threonine metabolism	<i>Tst</i> <sup>-/-</sup> vs 6J	**
		HFD vs ND	**
<b>B. Shared down-regulated pathways</b>			
00980	Metabolism of xenobiotics by cytochrome P450	<i>Tst</i> <sup>-/-</sup> vs 6J	****
		HFD vs ND	*
00982	Drug metabolism – cytochrome P450	<i>Tst</i> <sup>-/-</sup> vs 6J	****
		HFD vs ND	*
04142	Lysosome	<i>Tst</i> <sup>-/-</sup> vs 6J	**
		HFD vs ND	****
04390	Hippo signaling pathway	<i>Tst</i> <sup>-/-</sup> vs 6J	**
		HFD vs ND	****
05215	Prostate cancer	<i>Tst</i> <sup>-/-</sup> vs 6J	**
		HFD vs ND	*
04024	cAMP signaling pathway	<i>Tst</i> <sup>-/-</sup> vs 6J	*
		HFD vs 6J	*
04141	Protein processing endoplasmic reticulum	<i>Tst</i> <sup>-/-</sup> vs 6J	*
		HFD vs 6J	**
05211	Renal cell carcinoma	<i>Tst</i> <sup>-/-</sup> vs 6J	*
		HFD vs 6J	*
04722	Neurotrophin signaling pathway	<i>Tst</i> <sup>-/-</sup> vs 6J	*
		HFD vs 6J	*
04110	Cell cycle	<i>Tst</i> <sup>-/-</sup> vs 6J	*
		HFD vs 6J	**
04918	Thyroid hormone synthesis	<i>Tst</i> <sup>-/-</sup> vs 6J	*
		HFD vs 6J	***
04612	Antigen processing and presentation	<i>Tst</i> <sup>-/-</sup> vs 6J	*
		HFD vs 6J	**

\* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001 \*\*\*\* P < 0.0001

**Table S7. KEGG Pathways shared by high fat feeding and TST deletion.** Related to Figure 3. (A) KEGG pathways that are significantly up-regulated in the same direction by both high fat diet (HFD vs ND), and *Tst* deletion (*Tst*<sup>-/-</sup> vs C57Bl/6J). (B) KEGG pathways that are significantly down-regulated in the same direction by both high fat diet (HFD vs ND), and *Tst* deletion (*Tst*<sup>-/-</sup> vs C57Bl/6J). 'Comparison' indicates the two groups being compared.

**Table S8. Effect of high fat feeding on sulfide pathway proteins (High fat diet vs ND-fed)**

Feature ID	Name	Fold change in 6J	Significance	Fold change in <i>Tst</i> <sup>-/-</sup>	Significance
Q3UW66	MPST	1.35	**	1.15	*
Q8R086	SUOX	1.21	**	1.23	**
Q545S0	TST	1.19	Ns	n/a	n/a
Q8VCNS	CTH	-1.04	Ns	-1.06	Ns
Q9DCM0	ETHE1	-1.05	Ns	1.03	Ns
Q3UDS4	SQOR	-1.08	Ns	1.01	Ns
Q91WT9	CBS	-1.10	*	-1.06	Ns

\* Raw P < 0.05, \*\* Adjusted P < 0.05

**Table S8. Effect of high fat feeding on the sulfide pathway of C57Bl/6J and *Tst*<sup>-/-</sup> mice.** Related to Figure 3. Protein abundances of the sulfide production and disposal pathway from the liver proteome of C57Bl/6J and *Tst*<sup>-/-</sup> mice. 'Fold Change' indicates the relative abundance of the protein in high fat diet fed mice relative to normal diet fed mice, shown separately for each genotype.

**Table S9. Pathways in *Tst*<sup>-/-</sup> that are regulated oppositely to high fat feeding**

Entry	Name	Comparison	Direction
<b>A</b>			
<b>KEGG Pathways</b>			
00980	Metabolism of xenobiotics by cytochrome P450	<i>Tst</i> <sup>-/-</sup> vs 6J	Decreased
		HFD vs ND	Increased
00983	Drug metabolism – other enzymes	<i>Tst</i> <sup>-/-</sup> vs 6J	Decreased
		HFD vs ND	Increased
00053	Ascorbate and aldarate metabolism	<i>Tst</i> <sup>-/-</sup> vs 6J	Decreased
		HFD vs ND	Increased
00040	Pentose and glucuronate interconversions	<i>Tst</i> <sup>-/-</sup> vs 6J	Decreased
		HFD vs ND	Increased
00830	Retinol metabolism	<i>Tst</i> <sup>-/-</sup> vs 6J	Decreased
		HFD vs ND	Increased
<b>B</b>			
<b>GO Terms</b>			
0006629	Lipid metabolic process	<i>Tst</i> <sup>-/-</sup> vs 6J	Decreased
		HFD vs ND	Increased
0006631	Fatty acid beta-oxidation	<i>Tst</i> <sup>-/-</sup> vs 6J	Decreased
		HFD vs ND	Increased
0003995	Acyl-CoA dehydrogenase activity	<i>Tst</i> <sup>-/-</sup> vs 6J	Decreased
		HFD vs ND	Increased
0047617	Acyl-CoA hydrolase activity	<i>Tst</i> <sup>-/-</sup> vs 6J	Decreased
		HFD vs ND	Increased

**Table S9. KEGG pathways and GO terms that are regulated in the opposite direction by high fat feeding compared to *Tst* deletion.** Related to Figure 3. (A) KEGG pathways that are regulated in the opposite direction by high fat diet (HFD-fed C57Bl/6J vs ND-fed C57Bl/6J), to *Tst* deletion (ND-fed *Tst*<sup>-/-</sup> vs C57Bl/6J). (B) GO terms that are regulated in the opposite direction by high fat diet (HFD-fed C57Bl/6J vs ND-fed C57Bl/6J), to *Tst* deletion (ND-fed *Tst*<sup>-/-</sup> vs C57Bl/6J). ‘Comparison’ Indicates the two groups being compared. ‘Direction’ indicates whether the protein abundance is decreased or increased in the first group relative to the second.



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