The hepatic compensatory response to elevated systemic sulfide promotes diabetes

**Highlights**
- TST deficiency elevates sulfide, invoking exaggerated hepatic sulfide disposal
- Exaggerated sulfide disposal triggers global hepatic protein underpersulfidation
- Skewed persulfidation is associated with higher gluconeogenesis and impaired fat oxidation
- Diabetogenic hepatic metabolism dominates over apparent peripheral insulin sensitization

**Graphical abstract**

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**In brief**
Carter et al. show that mice lacking the mitochondrial sulfide oxidation pathway enzyme TST have high systemic sulfide levels that invoke an alternative hepatic sulfide disposal strategy. Consequently, hepatic metabolism is dominantly skewed toward a diabetogenic profile despite peripheral insulin sensitization. This has implications for sulfide donor therapeutic agents.
Article

The hepatic compensatory response to elevated systemic sulfide promotes diabetes

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SUMMARY

Impaired hepatic glucose and lipid metabolism are hallmarks of type 2 diabetes. Increased sulfide production or sulfide donor compounds may beneficially regulate hepatic metabolism. Disposal of sulfide through the sulfide oxidation pathway (SOP) is critical for maintaining sulfide within a safe physiological range. We show that mice lacking the liver-enriched mitochondrial SOP enzyme thiosulfate sulfurtransferase (Tst−/− mice) exhibit high circulating sulfide, increased gluconeogenesis, hypertriglyceridemia, and fatty liver. Unexpectedly, hepatic sulfide levels are normal in Tst−/− mice because of exaggerated induction of sulfide disposal, with associated suppression of global protein persulfidation and nuclear respiratory factor 2 target protein levels. Hepatic proteomic and persulfidomic profiles converge on gluconeogenesis and lipid metabolism, revealing a selective deficit in medium-chain fatty acid oxidation in Tst−/− mice. We reveal a critical role of TST in hepatic metabolism that has implications for sulfide donor strategies in the context of metabolic disease.

INTRODUCTION

The prevalence of type 2 diabetes (T2D) continues to soar in parallel with that of obesity (World Health Organization, 2016). Increased hepatic glucose production and aberrant hepatic lipid metabolism are cardinal features of T2D (Consoli et al., 1989; Lewis et al., 2002). Dysregulation of hepatic nutrient metabolism in T2D is a promising area for therapeutic intervention because it precipitates the more severe liver pathologies that manifest along the spectrum of non-alcoholic fatty liver disease (NAFLD),
steatosis, steatohepatitis, and hepatocellular carcinoma (Caron et al., 2011).

Hydrogen sulfide (hereafter referred to as sulfide), an endogenously produced gaseous signaling molecule (Abé and Kimura, 1996; Wang, 2012; Mishanina et al., 2015; Filipovic et al., 2017), has recently emerged as a modulator of nutrient metabolism (De-sai et al., 2011; Szabo, 2011; Hine et al., 2015; Carter and Morton, 2016). Enzymatic sulfide production from sulfur amino acids is catalyzed by cystathionine beta synthase (CBS), cystathionine gamma lyase (CCTH) (Chen et al., 2004; Singh et al., 2009), and 3-mercaptoppyruvate sulfurtransferase (MPST) (Shibuya et al., 2009; Mikami et al., 2011; Yadav et al., 2013). Thioredoxin-mediated reduction of cysteine persulfides on proteins also regulates free sulfide and cysteine persulfide levels (Wedemann et al., 2016). Endogenously produced and exogenously administered sulfide specifically influences hepatic glucose and lipid metabolism (Mani et al., 2014; Pichette and Gagnon, 2016). Thus, in vitro, treatment of murine hepatocytes with sodium hydrosulfide (NaHS), or overexpression of rat Cth in HepG2 liver cells increased glucose production through increased gluconeogenesis and reduced glycogen storage (Zhang et al., 2013). Conversely, glucose production was lower in hepatocytes from Cth gene knockout (Cth−/−) mice, which exhibit low sulfide production (Zhang et al., 2013). Elevation of sulfide with NaHS administration in vivo reduced cholesterol and triglyceride accumulation in the liver of high-fat diet (HFD)-fed mice (Wu et al., 2015). In contrast, inter-crossing of sulfide production-deficient Cth−/− mice with the hyperlipidemic Apoe−/− mouse strain (Cth−/−Apoe−/−) produced a phenotype of elevated plasma cholesterol following exposure to an atherogenic diet (Mani et al., 2013). Consistent with their higher cholesterol, Cth−/−Apoe−/− mice developed fatty streak lesions earlier than Apoe−/− mice, and this effect was reversed by NaHS administration (Mani et al., 2013). Sulfide may also indirectly affect hepatic nutrient metabolism through its effect on hepatic artery vasorelaxation and, thus, liver perfusion (Fiorucci et al., 2005; Distritto et al., 2008). The apparently beneficial effects of sulfide administration in multiple disease indications has led to a major drive toward development of targeted H2S donor molecules as a therapeutic approach (Whitman et al., 2011; Sestito et al., 2017).

However, an often overlooked aspect of net sulfide exposure, key to the efficacy of therapeutic H2S donors, is that it is regulated through its oxidative disposal. Thus, endogenous sulfide exposure is actively limited to prevent mitochondrial respiratory toxicity (Reifenstein, 1992; Tiranti et al., 2008; Libliad et al., 2018). Sulfide is oxidized rapidly (Hildebrandt and Grieshaber, 2008; Norris et al., 2011) through the mitochondrial sulfide oxidation pathway (SOP), consisting of sulfide quinone oxidoreductase (SQOR), persulfide dioxygenase (ETHE1/PDO), and thiosulfate sulfurtransferase (TST; also known as rhodanese) (Hildebrandt and Grieshaber, 2008; Jackson et al., 2012; Libliad et al., 2014). The liver is highly abundant in SOP enzymes and is a major organ of whole-body sulfide disposal (Norris et al., 2011). Mice lacking the Ethe1 gene (Ethe1−/−) die of fatal sulfide toxicity (Tiranti et al., 2009), consistent with its critical role in sulfide oxidation and the severe pathological consequences of unchecked sulfide buildup in tissues. However, the importance of mitochondrial TST in the SOP in vivo remains obscure. In contrast to Ethe1−/− mice, Tst−/− mice were grossly normal despite exhibiting substantially elevated blood sulfide levels, as implied by qualitative measures (Morton et al., 2016). This revealed an important but distinct role of TST in the SOP in vivo. Nevertheless, Tst−/− mice showed an apparently diabetogenic impairment of glucose tolerance (Morton et al., 2016), consistent with the concept that increased sulfide promotes hepatic glucose production (Zhang et al., 2013). Because Tst deficiency is a model of chronic but viable sulfide elevation, determining the molecular mechanisms driving the aberrant metabolic profile can provide important insights into the optimal range for therapeutic sulfide exposure, particularly in light of the current interest in developing mitochondrially targeted sulfide donors (Gerô et al., 2016; Karwi et al., 2018). To this end, we sought to define the effect of Tst deficiency on the underlying molecular pathways that affect hepatic metabolism.

RESULTS

Tst−/− mice exhibit increased hepatic gluconeogenesis and dyslipidemia despite mild peripheral insulin sensitization

TST mRNA expression is highest in the liver (http://biogps.org/#goto=genereport&id=22117; tissue hierarchy of expression was validated in our own mouse substrain; Figure S1A). We therefore hypothesized that liver TST deficiency was the principal driver of the impaired glucose tolerance observed previously in Tst−/− mice (Morton et al., 2016). Tst−/− mice exhibited higher glucose levels than C57BL/6J controls in response to pyruvate challenge, consistent with higher hepatic glucose production (Figure 1A). We next tested phosphoenolpyruvate carboxykinase (PEPCK), a key enzyme of de novo hepatic glucose synthesis, and found that it was higher in liver homogenates from Tst−/− mice (Figure 1B). Next we performed a 1-h 13C3-pyruvate metabolite pulse incorporation experiment in isolated hepatocytes cultured in 12C3-pyruvate-free medium. Hepatocytes from Tst−/− mice displayed 13C labeling consistent with increased metabolism of pyruvate to oxaloacetate, a critical early step in gluconeogenesis. Specifically, aspartate, which is derived from pyruvate via oxaloacetate, was increased significantly in Tst−/− hepatocytes (Figure 1C). A trend toward higher 13C3 malate and lower 13C2 acetyl-coenzyme A (CoA) was also observed (Figures S1B and S1C). 13C3 lactate was similar between genotypes, suggesting a similar activity of glycolytic disposal of pyruvate through lactate dehydrogenase (Figures S1B and S1C). Isotopologue distribution is shown in Figure S1C. Total pool sizes for all measured metabolites were similar between genotypes (Figure S1D). Although not a direct measure of glucose production, the data from in vitro hepatocytes suggested skewing of hepatocyte metabolism toward gluconeogenesis, and we therefore investigated this possibility. Indeed, consistent with increased endogenous glucose production in Tst−/− mice, fasting plasma glucose was higher in Tst−/− mice relative to 6J mice during the pre-clamp 3-3H glucose tracer infusion phase (60–90 min after the tracer) of euglycemic, hyperinsulinemic (EH) clamp experiments (Figure 1D; Table S1A). Higher plasma glucose levels in Tst−/− mice under these conditions was not explained by lower glucose utilization in Tst−/− mice; glycogen synthesis and glycolysis were comparable between
controls (***), whereas no effect of diet is found, but a significant effect of diet (***) and an interaction (*) were found. Post hoc analysis using Sidak’s multiple comparison test shows an effect of diet on the 6J genotype (*).

Tests revealed that the decrement of glucose from baseline 30 and 60 min after insulin was greater in

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T_{st}^{-/-} \text{ mice.} \quad \text{For (F), no main genotype effect was found, but a significant effect of diet (***) and an interaction (*) were found. Post hoc analysis using Sidak’s multiple comparison test shows an effect of diet on the 6J controls (**), whereas no effect of diet is found on } T_{st}^{-/-} \text{ mice. See also Figures S1 and S2 and Table S1.}
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A Pyruvate tolerance

B PEPCK activity

C \[^{13}\text{C}_3\] Aspartate

D Pre-clamp glucose

E Plasma \[^{3}\text{H}]\text{-glucose}

F Insulin tolerance

G Plasma triglycerides

H Liver Lipid (Oil red O)

I Liver Lipid (Oil red O)

(1) Analysis of the area of red staining (oil red O) after thresholding, using ImageJ, from ND-fed (no pattern, \(n = 3-4/\text{genotype}\)) or HFD-fed (hatched pattern, \(n = 4-5/\text{genotype}\)) C57BL/6J (white bars) and \(T_{st}^{-/-}\) (red bars) mice.

Data are represented as mean + SEM. Significance was calculated using repeated-measures ANOVA (A and F), 2-way ANOVA (I), 3-way repeated-measures ANOVA (D), or unpaired two-tailed Student’s t test (B, C, E, and G); \(*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001\). For (D), significant effects of time (***) and genotype (*) were found. For (F), the analysis was performed on absolute glucose values and demonstrated a significant effect of time (***) and an interaction between time and genotype (*). Tests revealed that the decrement of glucose from baseline 30 and 60 min after insulin was greater in \(T_{st}^{-/-}\) mice (*). For (I), no main genotype effect was found, but a significant effect of diet (***) and an interaction (*) were found. Post hoc analysis using Sidak’s multiple comparison test shows an effect of diet on the 6J controls (**), whereas no effect of diet is found on \(T_{st}^{-/-}\) mice. See also Figures S1 and S2 and Table S1.

Figure 1. \(T_{st}\) deletion results in impaired glucose and lipid metabolism

(A) Plasma glucose over 120 min, following pyruvate (i.p., 1.5 mg/g) administration in overnight-fasted C57BL/6J (black line, \(n = 9\)) and \(T_{st}^{-/-}\) (red line, \(n = 8\)) ND-fed mice.

(B) Extinction of NADH, measured by absorbance at 340 nm, coupled to PEPCK activity from liver homogenates taken from C57BL/6J (white bar, \(n = 6\)) and \(T_{st}^{-/-}\) (red bar, \(n = 6\)) ND-fed mice.

(C) Production of \(^{13}\text{C}\) aspartate generated after a 1-h pulse of 1 mM 3-carbon labeled \(^{13}\text{C}\) (M+3) pyruvate in \(^{12}\text{C}\) pyruvate-free medium, expressed as a percentage of the total amount of detected metabolite, in primary hepatocytes from C57BL/6J (white bars, \(n = 6\)) and \(T_{st}^{-/-}\) (red bars, \(n = 5\)) ND-fed mice.

(D) Blood glucose during the pre-clamp phase of the EH clamp from C57BL/6J (black lines) and \(T_{st}^{-/-}\) (red lines) mice fed a control (ND, solid lines, \(n = 3, 6\)) or high-fat diet (HFD, broken lines, \(n = 6, 7\)).

(E) Mean integrated radioactive glucose (inversely related to whole-body glucose uptake) during a EH clamp from ND-fed C57BL/6J control (white, \(n = 3\)) and \(T_{st}^{-/-}\) (red, \(n = 6\)) mice.

(F) Plasma glucose, expressed as percent of baseline glucose, over 120 min following insulin (i.p., 1 mU/g) administration in 4-h-fasted C57BL/6J (black line, \(n = 8\) and \(T_{st}^{-/-}\) (red line, \(n = 7\)) ND-fed mice.

(G) HPLC-quantified total and VLDL plasma triglyceride in 4-h-fasted C57BL/6J (white bar, \(n = 6\)) and \(T_{st}^{-/-}\) (red bar, \(n = 6\)) ND-fed mice.

(H) Representative light microscopy images of liver sections stained with oil red O from normal diet (ND)-fed or HFD-fed C57BL/6J and \(T_{st}^{-/-}\) mice. Magnification is 40X.

We next wished to explore whether the changes to glucose metabolism were driven by insulin resistance. Liver glycogen, a marker of long-term carbohydrate storage typically impaired with insulin resistance, was comparable between \(T_{st}^{-/-}\) and C57BL/6J control mice (Figure S2A). Despite unchanged steady-state markers of hepatic insulin sensitivity, impaired glucose tolerance, described previously in \(T_{st}^{-/-}\) mice (Morton et al., 2016), suggested that whole-body, and usually hepatic, insulin resistance was present. We investigated this using the euglycemic clamp, where, unexpectedly, we observed whole-body insulin sensitization under these short-term steady-state conditions. During the clamp, when insulin was high and blood glucose levels were maintained constant, the glucose infusion rate was comparable between genotypes (Table S1B). However, an increase in whole-body glucose uptake (integral glucose) by tissues in \(T_{st}^{-/-}\) mice was apparent (Figure 1F; Table S1B), supporting increased peripheral insulin sensitivity, with a directionally consistent trend for increased glucose uptake into several tissues. We confirmed this finding using standard insulin tolerance tests, where the glucose decrement in response to insulin was greater in \(T_{st}^{-/-}\) mice (Figure 1F; Figure S2B). These data demonstrate a net increase in dynamic whole-body insulin sensitivity despite increased hepatic glucose output in \(T_{st}^{-/-}\) mice. Finally, we assessed whole-body glucose homeostasis with the EH clamp method after chronic HFD feeding. Under these conditions, glucose output was comparable between genotypes across 60–90 min (Table S1A). Glucose turnover, a derived parameter used to infer glucose production, was also comparable between genotypes (Table S1A). However, derivation of glucose turnover requires that glucose levels are stable during the period in which it is calculated. In our pre-clamp baseline period, a highly significant effect of time (Figure 1D) indicated that this assumption was not met; thus, true endogenous glucose production cannot be inferred from the glucose turnover parameter in this instance. Combined with the pyruvate tolerance, PEPCK activity, and \(^{13}\text{C}_3\)-pyruvate pulse data, higher fasting glucose levels in \(T_{st}^{-/-}\) mice, given comparable glucose utilization, are most likely due to higher endogenous glucose production.
conditions, Tst<sup>−/−</sup> mice maintained increased hepatic glucose output (Figure 1D) but showed convergence of the insulin sensitivity profile with that of insulin-resistant C57BL/6J mice.

We also assessed whether Tst deficiency was associated with impaired lipid metabolism, another hallmark of diabetes. Fast protein liquid chromatography analysis of triglyceride levels and their lipoprotein distribution revealed significantly higher protein liquid chromatography analysis of triglyceride levels in Tst<sup>−/−</sup> mice (Figure 1G). The higher triglyceride was selectively associated with an increased very low density lipoprotein (VLDL) triglyceride fraction (Figure 1G), consistent with a dominant liver-driven impairment in lipid metabolism (Mason, 1998). Total and distinct lipoprotein fraction plasma cholesterol levels were similar between genotypes (Figures S2C and S2D), suggestive of a triglyceride-selective effect of Tst deficiency on hepatic lipid efflux. HFD feeding significantly increased the liver lipid content of C57BL/6J mice but did not further increase the elevated lipid levels in the liver of Tst<sup>−/−</sup> mice (Figures 1H and 1I).

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

A role of TST in disposal of sulfide has been suggested by its participation in the SOP (Hildebrandt and Grieshaber, 2008; Libiad et al., 2014) and supported in vivo by the qualitatively higher blood sulfide of Tst<sup>−/−</sup> mice (Morton et al., 2016), shown schematically in Figure 2A. Here we quantified circulating sulfide, showing an approximately 10-fold elevation in the blood and plasma of Tst<sup>−/−</sup> mice (Table 1). Thiosulfate, an oxidized metabolite of sulfide (Vitvitsky et al., 2015, 2017) and a TST substrate (Banerjee et al., 2015), was approximately 20-fold higher in the plasma of Tst<sup>−/−</sup> mice compared with C57BL/6J mice. Reduced glutathione (rGSH) levels were ~2-fold higher in the plasma of Tst<sup>−/−</sup> mice (Table 1). To determine any direct hepatic contribution to the elevated systemic sulfide in vivo, whole blood was sampled from the inferior vena cava (IVC) (Table 1). IVC sulfide levels tended to be higher in Tst<sup>−/−</sup> mice, but the magnitude of the increase (~3-fold) did not parallel that in trunk blood (~10-fold), suggesting that the liver was not a major source of the elevated circulating sulfide. Surprisingly, liver homogenate sulfide, thiosulfate, cysteine, and GSH levels were similar between Tst<sup>−/−</sup> and C57BL/6J mice (Table 1). Further, cultured hepatocytes from Tst<sup>−/−</sup> and C57BL/6J mice exhibited similar intracellular sulfide levels, as estimated using P3, a sulfide-selective fluorescent probe (Singha et al., 2015; Table 1). Mitochondrial sulfide levels in the liver, reported by MitoA/MitoN (Arndt et al., 2017), were similarly unchanged between genotypes (Table 1). The apparently unaltered hepatic steady-state sulfide levels, despite higher circulating sulfide, suggested that a profound homeostatic mechanism was invoked in the liver of Tst<sup>−/−</sup> mice. We assessed respiratory sulfide disposal (antimycin sensitive) and found that this was increased markedly in hepatocytes from Tst<sup>−/−</sup> mice, whereas antimycin-insensitive sulfide disposal was relatively reduced compared with hepatocytes from C57BL/6J mice (Table S2). Isolated liver mitochondria from Tst<sup>−/−</sup> hepatocytes also exhibited a higher sulfide disposal rate (Table S2). In addition, cysteine and GSH were excreted at higher
Liver 0.78 ± 0.16 1.14 ± 0.45 1.46 ± 0.20

Table 1. Sulfur species in blood, urine, tissue, and cells

|                          | C57BL/6J | Tst+/− | Tst+/−/6J ratio | Significance |
|--------------------------|----------|--------|-----------------|--------------|
| Trunk blood (micromolar)a |          |        |                 |              |
| 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           |
| 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 GSH)    | 48.0 ± 1.15 | 86.25 ± 6.27 | 1.80           | ***          |
| Urine (micromoles/creatinine/24 h)c |      |      |                 |              |
| MBB-SSO3 (thiosulfate)   | 4.99 ± 2.6  | 2374 ± 319   | 475.75         | ****         |
| Liver (micromoles/kg wet liver)d |   |      |                 |              |
| MBB-S (sulfide)          | 13 ± 1    | 17 ± 3    | 1.31           | ns           |
| MBB-SSO3 (thiosulfate)   | 4 ± 1     | 15 ± 7    | 3.75           | ns           |
| DNFB-GSH (reduced GSH)   | 6,470 ± 380 | 6,850 ± 30 | 1.04           | ns           |
| DNFB-cysteine (cysteine) | 82 ± 13   | 67 ± 11   | 0.82           | ns           |
| Sulfide P3 fluorescence (A510 nm/protein)f |     |      |                 |              |
| Hepatocyte               | 7.22 ± 1.00 | 7.89 ± 0.80 | 1.09           | ns           |
| Mitochondrial sulfide (MitoA)g |     |      |                 |              |
| Liver                    | 0.78 ± 0.16 | 1.14 ± 0.45 | 1.46 ± 0.20    | ns           |

Tst deletion results in altered sulfur metabolites in blood and liver. Data are represented as mean ± SEM. Significance was calculated using unpaired two-tailed Student’s t test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. MMB; monobromobimane.

aSulfide dibimane and thiosulfate-MBB, measured by fluorescence detection following HPLC, from whole blood taken from trunk blood of ND-fed C57BL/6J (n = 4) and Tst+/− (n = 4) mice.
bSulfide dibimane, thiosulfate-MBB, and rGSH-MBB, measured by fluorescence detection following HPLC, from EDTA-plasma of ND-fed C57BL/6J (n = 4) and Tst+/− (n = 4) mice.
cThiosulfate-MBB corrected for creatinine from 24-h urine samples, taken from ND-fed C57BL/6J (n = 4) and Tst+/− (n = 5) mice.
dSulfide dibimane and thiosulfate-MBB from whole blood taken from the IVC downstream of the hepatic vein of ND-fed C57BL/6J (n = 3) and Tst+/− (n = 3) mice.
eSulfide dibimane, thiosulfate-MBB, rGSH-MBB, and cysteine-MBB from whole liver (n = 4/genotype) of ND-fed C57BL/6J (n = 4) and Tst+/− (n = 4) mice.
fFluorescence from cultured hepatocytes following incubation with P3 (sulfide reactive probe) from ND-fed C57BL/6J (n = 4) and Tst+/− (n = 4) mice.
gRatio of MitoN/MitoA from the liver of ND-fed C57BL/6J (n = 5) and Tst+/− (n = 5) mice.

levels from Tst+/− hepatocytes under basal conditions and after stimulation of sulfur amino acid metabolism by addition of methionine (Figures 2B and 2C). Consistent with higher GSH turnover, hepatocytes from Tst+/− mice showed resistance to exogenous H2O2-mediated mitochondrial reactive oxygen species (ROS) production (Figure S3). We next determined the global hepatic protein persulfidation profile, the major post-translational modification mediated by sulfide (Krischan et al., 2011; Kabil et al., 2014; Koike et al., 2017). Mass spectrometry analysis of maleimide-labeled liver peptides revealed a greater abundance of peptides with a lower persulfidation level (underpersulfidated) in the liver of Tst+/− mice (Figure 2D). We confirmed this using semiquantitative western blot analysis on pulled down maleimide-labeled proteins (Figure 2E). Gene Ontology (GO) analysis of underpersulfidated peptides (20 GO categories; Table 2) showed enrichment for “FAD-binding, methyl transferase, peroxisome, acyl-CoA dehydrogenase activity, and transam.” Overpersulfidated peptides (8 GO categories; Table 2) were predominantly “nicotinamide metabolism.” Pathway-specific peptide analysis showed a bias for over-persulfidation in gluconeogenesis proteins (Figure S4A) and a significantly higher magnitude of change (independent of direction of change) in persulfidation compared with global persulfidomic changes between C57BL/6J and Tst+/− mice (Figure S4B).

The hepatic proteome of Tst+/− mice reveals a distinct molecular signature of altered sulfur and mitochondrial nutrient metabolism

To gain molecular insight into the mechanisms underlying the apparently diabetogenic phenotype in Tst+/− mice, we compared hepatic proteomes of normal diet (ND)-fed mice. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis revealed 4 up-regulated pathways in the liver of Tst+/− mice related to amino acid metabolism, including sulfur amino acids, and
Table 2. Tst deletion results in differential persulfidation rate of liver proteins

| GO ID      | Name                                           | Direction (Tst⁺⁻⁻ versus 6J) | Genes |
|------------|------------------------------------------------|-----------------------------|-------|
| 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     |
| 004453     | 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     |

Shown are significant GO terms represented by peptides with different persulfidation rates in ND-fed Tst⁺⁻⁻ mouse liver relative to C57BL/6J mice. “Direction” indicates whether persulfidation is decreased or increased in Tst⁺⁻⁻ relative to C57BL/6J mice. “Genes” indicates the number of genes in Tst⁺⁻⁻ mice that represent the changes driving the GO term.

sulfur metabolism (Table 3). GO analysis revealed 95 significantly up-regulated categories in the liver of Tst⁺⁻⁻ mice (Table S3A). Among the top categories, 7 referred to amino acid metabolism and 1 referred to the organellar term “mitochondrion.” KEGG analysis revealed 27 down-regulated pathways in the liver of Tst⁺⁻⁻ mice (Table 3), including phase 1 and 2 detoxification pathways (cytochrome P450s, GSH, and glucuronidation) and “lysosome” and “protein processing in the endoplasmic reticulum” organellar terms. 213 GO terms were significantly down-regulated in Tst⁺⁻⁻ mice (Table S4B). Among the most significant down-regulated terms were phase 2 detoxification “glutathione binding,” “glutathione transferase activity,” and “endoplasmic
Table 3. Protein abundance and persulfidation in ND-fed Tst<sup>−/−</sup> liver

| Entry | Name                          | Genes | Significance |
|-------|-------------------------------|-------|--------------|
|       | **KEGG pathways increased in ND Tst<sup>−/−</sup> liver** |       |              |
| 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     | *            |
|       | **KEGG pathways reduced in ND Tst<sup>−/−</sup> liver** |       |              |
| 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 signaling pathway | 4     | **           |
| 00500 | starch and sucrose metabolism | 5     | **           |
| 05215 | prostate cancer | 3     | **           |
| 04024 | cAMP signaling pathway | 4     | *            |
| 04141 | protein processing in ER | 9     | *            |
| 05211 | renal cell carcinoma | 3     | *            |
| 00830 | retinoic metabolism | 6     | *            |
| 00053 | ascorbate and aldarate metabolism | 4     | *            |
| 00860 | porphyrin and chlorophyll metabolism | 4     | *            |
| 04722 | neurotrophin signaling pathway | 3     | *            |
| 04670 | leukocyte transendothelial migration | 4     | *            |
| 04010 | MAPK signaling pathway | 4     | *            |
| 04720 | long-term potentiation | 2     | *            |
| 04914 | progesterone-mediated oocyte maturation | 2     | *            |
| 04062 | chemokine signaling 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     | *            |

**GO terms common to persulfidome and proteome in ND Tst<sup>−/−</sup> liver**

| GO ID     | GO term                          | Persulfidation (Tst<sup>−/−</sup> versus 6J) | Abundance (Tst<sup>−/−</sup> versus 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                              |

*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

<sup>a</sup>Significant KEGG pathway terms represented by proteins that are more abundant in the liver of ND-fed Tst<sup>−/−</sup> compared with ND-fed C57BL/6J mice.

<sup>b</sup>Significant KEGG pathway terms represented by proteins that are less abundant in the liver of ND-fed Tst<sup>−/−</sup> compared with ND-fed C57BL/6J mice.

<sup>c</sup>“Genes” indicates the number of genes in Tst<sup>−/−</sup> mice that represent the changes driving the KEGG pathway.

<sup>d</sup>“GO terms that are significantly regulated at the level of cysteine persulfidation and protein abundance in the liver of ND-fed Tst<sup>−/−</sup> compared with ND-fed C57BL/6J mice.
The proteome of TST deficiency versus HFD response in C57BL/6J mice was consistent with lower NRF2 activation

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

The proteome of TST deficiency versus HFD response in C57BL/6J mice reveals distinct regulation of lipid metabolism, sulfide metabolism, and detoxification pathways

We examined mechanistic commonalities between the diabetogenic hepatic phenotype of Tst\(^{-/-}\) mice and that induced by the diabetogenic HFD feeding regimen in C57BL/6J mice. ND-fed Tst\(^{-/-}\) mice were in a pre-existing diabetogenic state (Figure 1) that does not worsen with HFD feeding (Figures 1H and 1I; Table S1), suggesting gross phenotypic convergence of the two genotypes after HFD feeding. We compared the identity and direction of change of the 188 proteins differentially expressed in ND-fed Tst\(^{-/-}\) mice (versus ND-fed C57BL/6J mice; Figure 3A) with proteins that were differentially expressed in response to HFD feeding in C57BL/6J mice (432 proteins; Figure 3A). There was a striking 67% overlap in individual proteins (126) in this comparison (Figure 3A). When we analyzed these two protein signatures for directionally shared pathways, one upregulated KEGG pathway, “glycine, serine and threonine metabolism” (Table S7A), and 12 downregulated KEGG pathways, including “drug metabolism” and “endoplasmic reticulum” (Table S8B), were common to the liver of ND-fed Tst\(^{-/-}\) and HFD-fed C57BL/6J mice. Consistent with a pre-existing HFD-like proteome, the dynamic response to HFD in the liver of Tst\(^{-/-}\) mice was muted relative to that observed in C57BL6J mice (106 proteins, a 4-fold lower response; Figure 3B). Focusing on the sulfide pathway, MPST and sulfite oxidase (SOX) were increased by HFD feeding in C57BL/6J and Tst\(^{-/-}\) mice (Table S8). The HFD-induced increase in MPST was less pronounced in the liver of Tst\(^{-/-}\) mice, likely reflecting that it is already elevated in ND-fed Tst\(^{-/-}\) mice. We then considered contrasting rather than congruent proteomics responses arising from TST deficiency versus HFD responses in C57BL/6J mice to illuminate potential novel pathways underlying the otherwise functionally similar diabetogenic hepatic Tst\(^{-/-}\) phenotype. 5 KEGG pathways (Table S9A) and 4 GO terms (Table S9B) were regulated oppositely in this comparison. Strikingly, the GO terms were all related to lipid metabolism, which was up-regulated in the HFD response but down-regulated with TST deficiency (Tables S9A and S9B). An organelle-focused protein analysis showed shared upregulation of mitochondrial and endoplasmic reticulum pathways between TST deficiency (Figure 3C, top row) and C57BL/6J HFD responses (Figure 3C, bottom row) but a striking discordance in peroxisomal protein pathways (upregulated by HFD feeding.
**DISCUSSION**

Elevated TST expression in adipose tissue has been identified as a genetic mechanism driving metabolically protective leanness in mice (Morton et al., 2016). Conversely, Tst−/− mice exhibited impaired glucose tolerance (Morton et al., 2016). However, Tst−/− mice had a subtle adipose tissue phenotype, suggesting a non-adipose origin for impaired glucose homeostasis. We found increased gluconeogenesis, steatosis, and elevated plasma VLDL triglycerides consistent with a predominantly hepatic origin for the diabetogenic phenotype. We cannot rule out a contribution of renal gluconeogenesis to the phenotype, and future work will address this limitation. Unexpectedly, and despite the markedly increased circulating sulfide levels (10-fold), the steady-state sulfide level was normal in the liver of Tst−/− mice. Moreover, we found evidence of multiple mechanisms for increased hepatic sulfide disposal, reduced downstream sulfide signaling, and associated underlying molecular links to an apparently diabetogenic phenotype. Our data suggest that the liver of Tst−/− mice has overshot in its attempt to...
maximize hepatic sulfide removal, leading indirectly to detrimental metabolic consequences. This involves a combination of distinct compartmentalized cellular responses, including increased respiratory sulfide disposal and export of cysteine and GSH. Uregulation of translation and recruitment of MPST to mitochondria of Tst−/− mice is observed. This response, in the face of reduced transcription of Mps1, suggests a powerful post-transcriptional cellular sulfide-sensing mechanism. Interestingly, if MPST is compensating for TST-mediated sulfide disposal in this context, then it implies a subversion of normal MPST function away from sulfide production (Mödis et al., 2013; Szabo et al., 2014; Kimura et al., 2017; Nagahara, 2018). Alternatively, this is a response to a perceived lower-sulfide environment. TST levels were also elevated in the liver of Mps1−/− mice, providing further support for a reciprocal compensatory mechanism between these two enzymes (Nagahara et al., 2019).

The unexpected finding of normal hepatic sulfide levels in Tst−/− mice led us to discover that the metabolic phenotype we observed was driven by the very mechanisms invoked to maintain sulfide within a normal range rather than sulfide excess per se. Several observations were consistent with this. For example, the major amino acid pathways increased in the liver of Tst−/− mice were transaminases involved in metabolism of GSH that support increased export of sulfur equivalents as GSH (and cysteine). These same transaminases support gluconeogenesis by redirecting Krebs cycle intermediates (Rui, 2014; Qian et al., 2015; Sookoian et al., 2016). Reprogramming of amino acid metabolism for sulfide disposal with knockon effects to drive hepatic glucose production are suggested, rather than any change to amino acid-linked mitochondrial respiration in hepatocytes. This is supported by the shift in hepatocyte pyruvate metabolism toward aspartate. In addition, glutathione S-transferases (GST) that inhibit gluconeogenesis (Ghosh Dastidar et al., 2018) were lower in the liver of Tst−/− mice. Further, activation of NRF2, which represses gluconeogenesis (Slocum et al., 2016) appears to be lower in the liver of Tst−/− mice. Involvement of NRF2 in the Tst−/− liver phenotype is further supported by the phenotype of Nrf2−/− mice that similarly exhibited steatohepatitis in the absence of insulin resistance (Meakin et al., 2014). However, Nrf2 signaling can be complex and dependent on dietary context; Nrf2−/− mice showed improved glucose tolerance after HFD feeding (Zhang et al., 2012), suggesting that any contribution of a Nrf2 signaling deficit in the liver of the Tst−/− mice changes upon HFD feeding. Beyond altered pyruvate flux, we also showed that hepatocytes of Tst−/− mice exhibited defective lipid metabolism. Specifically, medium-chain fatty acid (MCFA) oxidation was impaired, associated with selective reduction of the protein and peroxidation levels of lipid catabolic enzymes. This represents a mechanism linking altered sulfide metabolism to lipid oxidation, hepatic lipid accumulation, and dyslipidemia. Consistent with impaired MCFA oxidation defects as one driver of the phenotype, steatosis is observed in medium-chain acyl-CoA dehydrogenase (Mcaad)−/− mice (Tolvani et al., 2005), and dyslipidemia is found in MCADD-deficient humans (Onkenhout et al., 1995). The data we present add to a growing understanding of the link between sulfide regulating genes and nutrient metabolism that has so far focused on the enzymes of sulfide production. Specifically, we provide support for the importance of the sulfide oxidizing pathway as a regulator of cellular sulfide exposure. Unexpectedly, the data reveal cellular mechanisms that are engaged to homeostatically regulate sulfide disposal and can affect cell energetics and nutrient metabolism.

Our findings may have implications for potentially unexpected side effects of sulfide donor therapeutic agents. In normal mice, in vivo sulfide administration for 4 weeks after HFD feeding partially reversed hepatic lipid accumulation invoked by chronic (16 weeks) HFD feeding (Wu et al., 2015). No evidence was provided regarding whether sulfide disposal mechanisms were altered (Wu et al., 2015). This efficacious subchronic sulfide administration regimen contrasts with our genetic model of chronic sulfide elevation as a driver of dysregulated metabolism and NAFLD. Clearly, the normal mice in the Na2S administration studies had a fully functional SOP, suggesting that the presence of TST is required to achieve the beneficial metabolic effects of Na2S administration. This is also consistent with the apparently low sulfide signaling status (evidenced by lower persulfidation and NRF2 target protein abundance) in the liver of the Tst−/− mice. The benefits of elevated sulfide cannot be realized, perhaps because a major mediator of those effects is missing, and the alternate mechanisms invoked do not fully compensate (e.g., MPST) or actively drive aberrant nutrient metabolism. Comparable studies of glucose and lipid metabolism after manipulation of other sulfide-regulating genes are limited. However, in a contrasting model of reduced sulfide production (Cth−/− mice), plasma triglycerides were lowered (Mani et al., 2013), opposite to what we observed with Tst−/− mice. The hepatic sulfide disposal status of the Cth−/− mouse model is unknown, but our findings predict suppression of the SOP to spare the limited endogenous sulfide produced. Intriguingly, they also predict a knockon effect on nutrient homeostasis because of reduced metabolic demand of the TST/SOP axis. A more direct model informing on the effects of impairment of the sulfide disposal pathway is deficiency of the key mitochondrial SOP enzyme ETHE1. Eth1−/− mice suffer fatal sulfide toxicity (Tiranti et al., 2009); therefore, comparable metabolic studies are lacking. However, one notable observation is that Eth1−/− mice have apparently 10-fold higher liver sulfide exposure than control mice (Tiranti et al., 2009), in contrast to the normalized hepatic sulfide levels of Tst−/− mice. Circulating sulfide levels were not reported for comparison, but the presumably relatively lower systemic sulfide levels of Tst−/− mice appear to have permitted an effective homeostatic sulfide disposal response in the liver to avoid toxicity, albeit with a metabolic cost. Consequently, the liver of Tst−/− mice has a functional and proteomics profile distinct from that of Eth1−/− mice. For example, in the liver of Tst−/− and Eth1−/− mice (Hildebrandt et al., 2013), proteins of the GST Mu type (GSTM) and peroxiredoxin (PRDX) families were altered, but sometimes in the opposite direction or with alteration of distinct protein subclasses. A notable difference is also observed in amino acid metabolism. The liver of Eth1−/− mice exhibits increased expression of enzymes of branched-chain amino acid metabolism (Hildebrandt et al., 2013), distinct from the predominantly GSH-related amino acid pathways that are increased in the liver of Tst−/− mice. Beyond sulfide, TST may also have distinct cellular roles that affect metabolism,
such as mitoribosomal synthesis, ROS attenuation, and modulation of mitochondrial iron-sulfur clusters (Bonomi et al., 1977; Paganini and Galante, 1983; Nandi and Westley, 1998; Nandi et al., 2000; Smirnov et al., 2010).

Given the pro-diabetogenic liver phenotype in Tst⁻/⁻ mice, it was surprising that insulin signaling in the liver appeared normal and peripheral insulin sensitivity was increased. There are precedents for increased hepatic glucose production independent of insulin resistance, as found in the Nr2f²⁻/⁻ mice (Meakin et al., 2014) and as driven by the transcription factor carbohydrate-response element-binding protein (ChREBP) (Uyeda and Repa, 2006; Kim et al., 2016). There is also evidence to support insulin-sensitizing effects of sulfide administration in vivo in mice and rats (Feng et al., 2009; Geng et al., 2013; Xue et al., 2013), consistent with sulfide-mediated insulin sensitization of non-hepatic tissues in Tst⁻/⁻ mice. Higher circulating GSH in Tst⁻/⁻ mice may also promote peripheral insulin sensitization (Jain et al., 2014; Lutchmansingh et al., 2018). Clearly, the net balance of glucose production from the liver and its peripheral disposal remain abnormal in Tst⁻/⁻ mice. Indeed, the baseline metabolic phenotype of Tst⁻/⁻ mice resembles in many ways that of a normal mouse fed a HFD, and we showed some overlapping pro-diabetogenic signatures between the liver proteome of Tst⁻/⁻ mice and that of HFD-fed C57BL/6J mice. However, we also found distinct lipid metabolism and peroxisomal protein changes in Tst⁻/⁻ mice. Unlike a HFD state, which is associated with dominant hepatic insulin resistance, the increased hepatic glucose production in ND-fed Tst⁻/⁻ mice occurs despite normal hepatic insulin sensitivity. The significant changes in persulfidation of transaminase and gluconeogenesis proteins suggest that coordinated cross-talk across metabolic pathways underlies this atypical metabolic phenotype.

Sulfide donor therapeutic agents have been proposed as a clinical strategy for improving cardiovascular health (Szabó et al., 2011; Whiteman et al., 2011; Zhang et al., 2016). Elevated endogenous sulfide has also been implicated in the beneficial metabolic effects of caloric restriction (Miller et al., 2005; Hine et al., 2015, 2017, 2018; Shimokawa et al., 2015; Lee et al., 2016). Our results suggest that chronic sulfide elevation may have unintended detrimental consequences, driving liver glucose production and fat accumulation to undesirable levels. Fortunately, this may be limited to cases where SOP proteins are compromised through rare genetic effects, such as TST variants (Billaut-Laden et al., 2006; Libiad et al., 2015). More broadly, a number of drugs or supplements are known to increase cyanide, which may dominantly inhibit TST activity and result in secondary sulfide overexposure. These include nitroprusside (Morris et al., 2017) and amygdalin (Bromley et al., 2005; O’Brien et al., 2005). Indeed, the TST metabolite thiosulfate is commonly co-administered with nitroprusside to prevent cyanide toxicity (Curry et al., 1997). Furthermore, dietary and environmental exposure to cyanogenic compounds (Simeonova et al., 2004), e.g., smoking (Vinnakota et al., 2012) or cyanogenic diets (Kashala-Abotnes et al., 2019), may interfere with normal TST function and could lead to increased sensitivity to sulfide therapeutic agents. In contrast, we have shown that administration of the TST substrate thiosulfate can ameliorate diabetes (Morton et al., 2016), further underlining the potential utility of targeting the SOP in metabolic disease. As with all therapeutic strategies, a careful cost-benefit analysis is required. A comparable case of relevance are the statins, one of the most potent and widely used drugs to prevent atherosclerosis, which also carry a higher risk for diabetes (Swerdlov et al., 2015). The full effect of TST manipulation on opposing metabolic pathways requires further study. Our current study sheds light on the underlying hepatic mechanisms invoked for sulfide disposal that are relevant to current sulfide donor strategies and may inform on routes to reduce their potential metabolic side effects.

Limitations of the study

Although the liver is the site of most (~60%) post-absorptive gluconeogenesis in normal animals within physiological fasting ranges, renal/small intestinal gluconeogenesis begins to substantially contribute to circulating glucose with prolonged fasting/starvation (Sasaki et al., 2017; Mutel et al., 2011; Milhieux et al., 2003; Stumvoll 1998; Owen et al., 1969). We cannot rule out a role of renal or intestinal gluconeogenesis in the diabetogenic phenotype of Tst⁻/⁻ mice. This will be an important area of future work, although liver TST is at least more than 3-fold that of the kidneys, and small intestinal TST is very low (BioGPS; Figure S1A).

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
  - Lead contact
  - Materials availability
  - Data and code availability
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - Experimental animals
  - Hepatocyte preparations
- **METHOD DETAILS**
  - Pyruvate tolerance test
  - PEPC activity assay
  - ¹³C Pyruvate metabolite tracing
  - Plasma lipid analysis
  - Oil Red-O lipid analysis of liver
  - Liver Glycogen measurement
  - Western blotting for protein abundance
  - Insulin tolerance test
  - Euglycemic hyperinsulinemic clamps
  - MBB derivatization of whole blood and plasma
  - Fluorometric quantification of MBB-sulfur species
  - Sulfur metabolite analysis from liver
  - P3 fluorescence detection of sulfide in hepatocytes
  - Quantification of hydrogen sulfide levels using MitoA in vivo exomarker
  - Preparation of hepatic mitochondria
  - Amperometric analysis of sulfide disposal
  - Mitochondrial ROS (MitoSOX) measurement in H₂O₂ treated hepatocytes
  - Persulfidation Mass Spec and GO term analysis
GO enrichment analysis
Focused analysis of persulfidation in gluconeogenesis proteins
Persulfidation labeling and western blotting from frozen liver
Mass spec analysis of liver protein
GO and KEGG enrichment analysis of proteome data
Transcription factor enrichment analysis
NRF2 target identification and proteome analysis
Electron micrograph imaging
Seahorse respiratory analysis
Mitochondrial stress test (MST)
Octanote rescue test
Real time for mRNA analysis

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2021.109958.

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AUTHOR CONTRIBUTIONS
N.M.M. and R.N.C. conceived the experiments. R.N.C., M.T.G.G., M.E.B.-L., M.L., V.V., B.E., T.L.B., M.B., H.S., S.G.D., N.Z.M.H., C.M., A.T., N.F., and T.G., performed the experiments. R.N.C., M.E.B.-L., P.L.F., T.L.B., N.Z.M.H., T.S., F.B., T.G., R.C.H., B.S., G.A.G., A.J.F., C.S., R.B., and T.G., analyzed and interpreted data and commented on the manuscript. N.M.M. and R.N.C. performed the experiments. R.C.H. and N.M.M. wrote the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Anti-TST            | GeneTex| GTX114858; RRID:AB_10620797 |
| Anti-MPST           | Abcam  | ab224043   |
| Anti-GOT1           | Abcam  | ab170950; RRID:AB_170950 |
| Anti-GSTT1          | Proteintech | 15838-1-AP; RRID:AB_2116344 |
| Anti-MAT1A          | Abcam  | ab129176; RRID:AB_11145300 |
| Anti-BHMT           | Proteintech | 15965-1-AP; RRID:AB_2290472 |
| Anti-CSAD           | Abcam  | ab91016; RRID:AB_10713222 |
| Anti-PPCS           | Atlas Antibodies | HPA031361; RRID:AB_10602150 |
| IRDye 800CW Goat anti-Rabbit | Li-Cor | 926-32211; RRID:AB_621843 |
| IRDye 680RD Donkey anti-Mouse | Li-Cor | 926-68072; RRID:AB_10953628 |
| Anti-B-Actin        | Abcam  | ab8226; RRID:AB_306371 |
| Anti-CoxIV          | Abcam  | Ab16056; RRID:AB_443304 |
| **Chemicals, peptides, and recombinant proteins** | | |
| sodium pyruvate $^{13}$C$_3$ | Sigma-Aldrich | 490717 |
| Amyloglucosidase    | Roche  | ROAMYGGL |
| 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-trifluoromethoxyphenyldrazone (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-$^{3}$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 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| 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 |
| Sulfurhodamine 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 |
| 14C-2-deoxyglucose | Perkin Elmer | NEC495A |
| 4-(2-Hydroxyethyl)-1-piperazine propanesulfonic acid | Sigma-Aldrich | 1.15230 |
| 2,4-nitrofluorobenzene | Sigma-Aldrich | D1529 |

**Critical commercial assays**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| 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 |

**Deposited data**

- Proteome: ProteomeXchange PXD028909
- Persulfidome: ProteomeXchange PXD028909

**Experimental models: Cell lines**

- Primary hepatocytes: C57BL/6J and Taf−/− mice n/a

(Continued on next page)
RESOURCE AVAILABILITY

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

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

Data and code availability

- Proteomics and persulfidomics root data from the iTRAQ and persulfidated peptide mass spectrometry experiments have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier ProteomeXchange: PXD028909.
- This paper does not report original code.
- Any additional information required to reanalyse the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Experimental animals
All experiments were performed according to guidelines set out by the ethical committees of The University of Edinburgh and Physiogenex S.A.S, Prologue Biotech, Labége, FRANCE. Experiments were carried out within the framework of the Animals (Scientific Procedures) Act (1986) of the United Kingdom Home Office or related laws from the European Union (France). In all studies, animals within genotype cohorts were randomly assigned to diet or intervention groups. All animals were maintained in standard housing with 12 hour light and 12 hour dark cycles (7 a.m. to 7 p.m.) and ad libitum access to the appropriate diet. For in vivo experiments (pyruvate tolerance...
test, insulin tolerance test, euglycaemic clamps), operators and animal handlers were blinded to the data, which was generated by a second individual who was blinded to the treatment regimen until the code was broken. All of the studies used male mice housed in cages of 3-6 individual littersmates until intervention. The mice for this study originated from C57BL/6N Tst−/− mice (Morton et al., 2016) backcrossed onto the C57BL/6U genetic background for > 10 generations. Mice were placed onto high fat diet D12331, (58% calories from fat, Research Diets, New Brunswick, USA) from between 6-8 weeks of age, for 6-7 weeks prior to testing, and compared to mice maintained on standard low fat diets, RM1 or D12383 (low-fat high-cornstarch, Research Diets, New Brunswick, USA).

**Hepatocyte preparations**

Mice were killed by CO2 asphyxiation, followed by cervical dislocation. The chest cavity was opened, the portal vein was cut and the thoracic vena cava was cannulated via the right atrium. The liver was perfused with (37 °C) perfusion media (140 mM NaCl, 2.6 mM KCl, 0.28 mM Na2HPO4, 5 mM glucose, 10 mM HEPES, 0.5 mM EGTA, pH 7.4), 6 mls/min for 10 min. The liver was then perfused with digestion media (perfusion media, without EGTA, including 5 mM CaCl2, and 100 U/ml collagenase type 1) for 5–7 min. Finally, the liver was perfused with perfusion media for a further 10 min. Cells were extruded from liver into DMEM medium (DMEM, 5.5 mM glucose, 10% FCS, 7 mM glutamine, and penicillin/streptomycin antibiotics), and then passed through a 40 micron filter. Cells were spun twice and washed with medium, at 500 rpm (47 g) for 5 min. Cells were spun through a 50% Percoll pH 8.5-9.5/DMEM solution at 1000 rpm (190 g) for 15 min to remove dead cells and non hepatocytic liver cell types. Hepatocytes collected in the pellet fractions were resuspended in medium and spun twice with washing using a haemocytometer, and proportion of trypan blue exclusion respectively. Yields ranged from between 2 × 10^6–1.5 × 10^7 viable cells, and viability was above 85%. Unless otherwise stated, hepatocytes were seeded onto collagen coated tissue culture plastic (collagen from rat tails, Sigma), and maintained in DMEM with 5.5 mM glucose, 10% FCS, 7 mM glutamine, and antibiotics).

**METHOD DETAILS**

**Pyruvate tolerance test**

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

**PEPCK activity assay**

Activity of phosphoenolpyruvate carboxykinase was measured from cytosol samples obtained from frozen liver. Samples were homogenized in 250 mM sucrose, 5 mM HEPES, pH 7.4. and centrifuged at 4 °C, 12,000 rpm (17,390 g) for 15 min. Supernatants were ultracentrifuged at 4 °C, 60,000 rpm (289,000 g) for 30 min. Activity of PEPCK from cytosolic fractions was inferred in this assay from NADH extinction, linked to the conversion of phosphoenol pyruvate into oxaloacetate in the presence of carbonate, dGDP and MnCl2, and the subsequent conversion of oxaloacetate into malate by adding malate dehydrogenase. Baseline measurements at NADH extinction, linked to the conversion of phosphoenol pyruvate into oxaloacetate in the presence of carbonate, dGDP and MnCl2, and the subsequent conversion of oxaloacetate into malate by adding malate dehydrogenase. Baseline measurements at 340 nm (NADH) were taken for 20 min before adding phosphoenol pyruvate, and the reaction proper was initiated with dGDP. The reaction was then measured for a further 40 min.

**13C Pyruvate metabolite tracing**

After overnight culture on collagen coated 6-well tissue culture plates, hepatocytes were incubated with 1 mM 13C3 labeled pyruvate in serum free DMEM for 60 min. Metabolites were extracted by washing individual wells with ice-cold PBS and addition of cold extraction buffer (50% methanol, 30% acetonitrile, 20% water solution at −20 °C or lower). Extracts were clarified and stored at −80 °C until required. LC-MS was carried out using a 100 mm × 4.6 mm ZIC-pHILIC column (Merck-Millipore) using a Thermo Ultimate 3000 HPLC inline with a Q Exactive mass spectrometer. A 32 min gradient was developed over the column from 10% buffer A (20 mM ammonium carbonate), 90% buffer B (acetonitrile) to 95% buffer A, 5% buffer B. 10 μL of metabolite extract was applied to the column equilibrated in 5% buffer A, 95% buffer B. Q Exactive data were acquired with polarity switching and standard ESI source and spectrometer settings were applied (typical scan range 75-1050). Metabolites were identified based upon m/z values and retention time matching to standards.

**Plasma lipid analysis**

Mice were fasted with free access to water for 4 hours prior to cull by decapitation or pentobarbital euthanasia. Trunk blood (decapitation) was collected directly into Sarstedt Microvette CB 300 K2E EGTA containing plasma sample tubes (Sarstedt, Numbrecht, Germany). Venous blood from the abdominal vena cava was centrifuged at 2000 rpm (2665 g) for 5 min to obtain plasma samples. Plasma samples were analyzed for cholesterol and triglyceride content by as previously described (Peters et al., 1997). Briefly, samples were subjected to gel filtration chromatography using an integrated Alliance HPLC separations module (e2695, Waters, Milford, US) to separate lipoproteins based on size. Effluent was immediately and continuously mixed with either triglyceride (Infinity Triglyceride, Thermo Scientific, Loughborough, UK) or cholesterol (Infinity Cholesterol, Thermo Scientific, Loughborough, UK) enzymatic colorimetric detection.
kits at the correct conditions for reaction (as specified in manufacturer’s guidance). The optical density was then recorded using a spectrophotometer at the appropriate wavelength and the signal turned into a continuous trace i.e., a lipid profile. By identification of the lipoprotein peaks (based on their time of emergence from the chromatograph) the concentration for each could be calculated.

**Oil Red-O lipid analysis of liver**

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

**Liver Glycogen measurement**

Frozen liver samples (between 30-90 mg) were heated to 100 °C in an Eppendorf tube with 0.3 mls of 30% KOH for 30 min with vigorous shaking at 10-min intervals. Samples were heated for a further 2-3 min after addition of 0.1 mL 1M Na2SO4 and 0.8 mL ethanol. Samples were then centrifuged at 4 °C at 1011 g for 5 minutes. The supernatant was removed, and the pellet resuspended in distilled H2O before 0.1 mL 1M Na2SO4 and 0.8 mL ethanol were again added, and samples boiled at 100 °C for 5 min before centrifugation. This was repeated a final time to wash the sample. The pellet was resuspended in a 10 mg/ml (~1200 U/ml) amyloglucosidase enzyme in 0.3 M sodium acetate (pH 4.8). Samples were then incubated at 50 °C for 2 hours. Quantification of samples was then performed using a standard hexokinase based glucose assay (Glucose (HK) Assay Kit, Sigma, GAHK20). The assay was performed following manufacturer’s instructions and values calculated by extrapolation from a standard curve after measuring absorbance using a plate spectrophotometer (Molecular Devices OPTImax microplate reader and software, Molecular Devices, Wokingham, UK).

**Western blotting for protein abundance**

Frozen liver samples (stored –80 °C) from mice were homogenized in protein lysis buffer (50 mM Tris, 270 mM sucrose, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10 mM B-glycerophosphate, 5 mM Na Pyrophosphate, 1 mM orthovanadate, 0.1% β-Mercaptoethanol, 1 tablet protease inhibitor cocktail inhibitor, pH 7.4, all Sigma Aldrich). Samples were then centrifuged at 13,200 rpm (18500 g) for 15 min at 4 °C and the supernatants aliquoted and stored at –80 °C. Protein samples were loaded onto 10% acrylamide/bis-acrylamide gels (30% acrylamide, Sigma Aldrich) and separated by electrophoresis. A colored molecular weight marker was also run on all gels (Full range rainbow molecular weight markers, GE Healthcare). Gels were transferred overnight using a Bio Rad wet transfer system onto Amersham Hybond – P membranes (GE Healthcare). After transfer, for normalization of specific targets to total protein, membranes were stained using the REVERT total protein stain (LI-COR), according to manufacturers’ instruction. Following stain and wash, lanes of each sample were analyzed using a LI-COR Odyssey scanner (700nm channel). For blots using a house keeping protein for normalization, the total protein stain was not performed, and membranes were transferred directly to blocking. All membranes were blocked in Tris buffered saline with 0.01% tween (TBST, containing 5% skimmed milk powder). Membranes were washed three times in TBST then scanned using the LI-COR Odyssey scanner. Odyssey software (LI-COR Biosciences) was used to quantify band intensity. For normalization to a house keeping protein, the individual band intensity of B-actin was used for each sample. Primary antibodies used were; TST, Rabbit, GeneTex, GTX114858, MPST, Rabbit, Abcam, Ab224043, GOT1, Rabbit, Abcam, ab170950, GSTT1, Rabbit, Proteintech, 15838-1-AP, MAT1A, Rabbit, Abcam, ab129176, BHMT Rabbit, Proteintech, 15965-1-AP, CSAD, Rabbit, Abcam, ab91016, PPCS Rabbit, Atlas Antibodies, HPA031361. Secondary antibodies used were; IRDye800CW Goat anti-Rabbit, Li-Cor, 926-32211, IRDye 680RD Donkey anti-Mouse, Li-Cor, 926-68072. For normalization B-Actin, Mouse, Abcam, ab22026 was used for whole tissue, and Cox IV (mitochondrial loading control), Abcam, ab16056 was used for mitochondrial fractions.

**Insulin tolerance test**

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

**Euglycemic hyperinsulinemic clamps**

Male C57BL/6J or Tst+/− mice were maintained on standard diet (RM1 (E) 801492, SDS) or high fat diet for 6 weeks (58% fat, D12331, Research Diets). Prior to performing the hyperinsulinemic euglycemic clamp an indwelling catheter was placed into the femoral vein under isoflurane anesthesia, sealed under the back skin, and glued onto the top of the skull. Clamps were performed 5-6 days after recovery from catheterization. Mice were fasted 6 hours prior to a basal blood sample was taken for glucose and insulin. Mice then
Whole blood was taken after cull of mice, from trunk (following decapitation), or portal vein (following CO2 euthanasia). EDTA-plasma MBB derivatization of whole blood and plasma glucose utilization.

rate, whole body turnover, hepatic glucose production, whole body glycolytic rate, whole body glycogen synthesis rate, and tissue 3H- radioactivity analysis. At 150 min after the start of perfusion, a bolus of 14C-2-deoxyglucose (25 µCi) received a bolus of D-[3-3H] glucose (30 µCi/kg/min for the clamped phase over 120 min. Blood glucose was assessed every 10 minutes, and glucose infusion adjusted until steady state blood glucose (120 mg/dl ± 10 mg/dl) was achieved. 5 µl of blood was collected from, tail tip every 10 min for 3H- radioactivity analysis. At 150 min after the start of perfusion, a bolus of 14C-2-deoxyglucose (25 µCi) was perfused to evaluate tissue specific uptake. At the end of the perfusion (210 min), blood is collected from the retro-orbital sinus to measure plasma insulin and mice sacrificed by i.v. injection of pentobarbital and cervical dislocation. Tissues (inguinal WAT, Epididymal WAT, Soleus muscle, Extensor digitorum longus muscle, Vastus lateralis muscle, Tibialis anterior muscle, Heart apex, Liver) were removed by dissection and flash frozen in liquid nitrogen (stored –80°C until measured). Tracers were used to calculate various aspects of glucose metabolism (Steele et al., 1956; Carter and Morton, 2016). Parameters measured or calculated include body weight, glucose infusion rate, whole body turnover, hepatic glucose production, whole body glycolytic rate, whole body glycogen synthesis rate, and tissue glucose utilization.

MBB derivatization of whole blood and plasma

Whole blood was taken after cull of mice, from trunk (following decapitation), or portal vein (following CO2 euthanasia). EDTA-plasma was obtained from trunk blood following decapitation and collected onto ice. Blood for plasma was centrifuged within 15 min of collection for 5 min at 5000 rpm (2655 g) at 4°C. Blood and plasma samples (15-50 µl) were derivatized with monobromobimane by addition of 200 µl of 80 mM EPPS (4-(2-Hydroxyethyl)-1-piperazine propanesulfonic acid, 8 mM DTPA (diethylenetriaminepentaacetic acid) pH 8.0, 50% acetonitrile, 2.3 mM monobromobimane. Reaction vials were capped tightly and vortexed for 1 minute and incubated protected from light at room temperature for 30 min. 1 mL ethyl acetate was added, the tube capped and vortexed for 1 min and incubated protected from light for 10 min. The reaction vials were centrifuged at 1800 rpm (350 g) for 7 min to separate aqueous and organic layers. The organic layer was collected from each extraction, transferred to a 1.5 mL brown glass vial and the solvent was evaporated completely under a nitrogen stream. Acetonitrile (200 µL) was added to each vial, and the solvent was again evaporated to remove any traces of ethyl acetate. Dried MBB-derivatives were stored at –20°C until analyzed.

Fluorometric quantification of MBB-sulfur species

MBB-sulfur species (sulfide, thiosulfate, reduced glutathione, and cysteine) in samples was quantified by HPLC separation and detection with a fluorescence detector. The dried MBB derivatives were re-suspended in 50 µL of Buffer A (10 mM tetrabutylammonium phosphate aqueous, 10% methanol, 45 mM acetic acid adjusted to pH 3.4). The entire sample was transferred to an HPLC autosampler vial with a 200 µL glass sample insert, and the vial was closed with a penetrable cap. 20 µl of the sample was injected onto a C8 reverse-phase column (LiChrospher 60 RP-select B 5 µm 4.0 × 125 mm LiChroCART 125-4, Merck KGaA) and a guard column (LiChroCART 10-2, Superspher 60 RP-select B cartridge) on an Ultimate 3000 UHPLC+ focused system (Thermo Scientific). MBB derivatives were eluted with a linear gradient from 10% buffer B (10 mM tetrabutylammonium phosphate in methanol, 10% water, 45 mM acetic acid) to 100% buffer B over 30 min. The eluent was analyzed by fluorescence (λex = 380 nm, λem = 480 nm).

Sulfur metabolite analysis from liver

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

P3 fluorescence detection of hydrogen sulfide in hepatocytes

Hepatocytes were seeded in glass bottomed, collagen coated wells (0.75 cm², 12,500 hepatocytes per well) and cultured in DMEM with 5.5 mM glucose, 10% FCS, 4 mM glutamax or 7 mM glutamine, and antibiotics overnight. P3 H2S reactive probe (Singha et al., 2015) was added to wells at 10 µM in serum free DMEM for 30 min, prior to gentle washing with Krebs phosphate buffered saline (pH 7.4). Plates were measured using the TECAN fluorescence plate reader, following excitation at 375 nm and detection at 510 nm. No-cell control wells were used for subtracting from the cell containing values. Corrected fluorescence emission data was normalized to protein as estimated by sulforhodamine dye. Briefly, after the run cells were fixed with 10% trichloroacetic acid overnight at 4°C. Cells were washed 9 times with tap water, and air-dried. Cells were incubated with 200 µL of 0.4% Sulforhodamine dye/1% acetic acid for 1 hour at room temperature. Stain was removed, and washed 4 times with 1% acetic acid, and then air-dried. Stain was dissolved in 200 µl of 10 mM Tris pH 10.5 for 30 min, and 100 µL was measured by colorimetric absorbance spectroscopy at 540 nm. After subtracting a baseline absorbance from blank controls, the absorbance was used to normalize the fluorescence data from each well.

Quantification of hydrogen sulfide levels using MitoA in vivo exomarker

MitoA and MitoN were quantified in mouse blood using LC-MS/MS. Mice received a tail vein IV injection of 50 nM MitoA in 0.9% saline (100 µL). MitoA was given 1.5 hr to distribute into mitochondria. Mice were culled by decapitation 90 minutes after administration.
Liver was excised and flash frozen in liquid nitrogen. MitoA and MitoN were extracted from tissue by homogenization of liver (50 mg) enriched with 5 pg d15-MitoN (95% ACN, 210 μL) which was used as an internal standard (IS). Homogenates were centrifuged (16,000 g, 10 min, RT) and the supernatant was transferred to a clean tube and stored on ice. The pellet was re-extracted (95% CAN, 210 μL), spun down again (16,000 g, 10 min, RT), and the supernatants were combined and incubated at 4 °C for 30 min. Calibration standards comprise MitoA and MitoN standards ranging from 0.01 to 10 pg in 500 μL 95% ACN. 500 μL of the supernatants and calibration standards were loaded onto an ISOLUTE PLD+ protein and phospholipid removal plate (Biotage, Sweden). Samples and standards were pulled through the plate under vacuum into a 2 mLL deep-well 96-well plate. Wells were dried completely at 40 °C under N2 and re-suspended in 100 μL 20% ACN, 0.1% FA. The plate was shaken at (250 rpm, 20 min) to ensure reconstitution. Liquid chromatography-Mass Spectrometry was performed on an I-class Acquity LC system-Beckman TQS triple quadrupole mass spectrometer (Waters, Warrington, UK). Samples were kept at 10 °C and injected onto an Acquity UPLC BEH C18 column fitted with a 0.2 μm filter (1 × 50 mm, 1.7 μm, Waters). Chromatographic separation of MitoA and MitoN was achieved using mobile phase A composition: water:ACN, (95:5, 0.1% FA), mobile phase B: ACN:water (90:10, 0.1% FA). LC mobile phases were infused at 200 μL/min using the gradient: 0–0.3 min, 5% B; 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 analysis was performed using a positive ion mode (Source spray voltage, 3.2 kV; cone voltage, 125 V; ion source temperature, 100 °C). Curtain and collision gas were nitrogen and argon, respectively. Analytes were detected by multiple reaction monitoring (MRM). MitoA undergoes neutral loss of N2 to a nitrene (precursor ion). For quantification the following transitions were used: MitoA, m/z 437.2 → 183.1; MitoN, m/z 439.2 → 120.0; d15-MitoN, 454.2 → 177.1 m/z. MassLynx 4.1 software was used to integrate the peak area of the analytes MitoA, MitoN and the d15-MitoN internal standard. Response was calculated by normalizing sample peak areas to the IS peak area. By comparison of sample responses to calibration standard responses the mass of each analyte in the tissue sample was calculated. The mass of analyte was normalized to the mass of tissue homogenizer and MitoN/MitoA ratio was calculated.

**Preparation of hepatic mitochondria**

Fresh liver was taken from mice, and homogenized in 250 mM sucrose, 10 mM HEPES, 1 mM EGTA, 0.5% fatty acid free bovine serum albumin (BSA) pH 7.4 at 4 °C, with seven passes of a loose glass Dounce homogenizer (Type A). Homogenates were centrifuged in glass tubes at 2900 rpm (1000 g) for 10 min in a precooled 4 °C Beckman centrifuge (JA-20 Fixed angle rotor). The supernatant was then centrifuged in glass tubes at 8500 rpm (8700 g) for 10 min at 4 °C. The supernatant was aspirated and any visible lipid was carefully removed from the sides of the tubes. The pellet was washed with 5 mL of miR-05 buffer (0.5 mM EGTA, 3 mM MgCl2, 20 mM taurine, 10 mM KH2PO4, 20 mM HEPES, 110 mM sucrose, 1 mg/ml fatty acid free BSA, pH 7.2), and centrifuged at 8500 rpm (8700 g) for 10 min at 4 °C. After aspiration and removal of visible lipid, the pellet was suspended in 1 mL of miR-05 buffer and kept on ice until used. All measurements were taken within two hours of preparation. Protein concentration was determined using the DC-Protein Assay (BioRad) as per manufacturers instruction.

**Amperometric analysis of sulfide disposal**

Hepatocytes were prepared as described, and kept at room temperature in DMEM with 5.5 mM glucose, 10% FCS, 4 mM glutamax or 7 mM glutamine, and antibiotics at a concentration of 4 mg/ml. Mitochondria were prepared as described, and kept at room temperature in DMEM with 5.5 mM glucose, 10% FCS, 4 mM glutamax and 2 mM pyruvate. Sulfide was added to buffer in the form of Na2S, enriched with 5 pg d15-MitoN (95% ACN, 210 μL) which was used as an internal standard (IS). Homogenates were centrifuged (16,000 g, 10 min, RT) and the supernatant was transferred to a clean tube and stored on ice. The pellet was re-extracted (95% CAN, 210 μL), spun down again (16,000 g, 10 min, RT) and the supernatants were combined and incubated at 4 °C for 30 min. Calibration standards comprise MitoA and MitoN standards ranging from 0.01 to 10 pg in 500 μL 95% ACN. 500 μL of the supernatants and calibration standards were loaded onto an ISOLUTE PLD+ protein and phospholipid removal plate (Biotage, Sweden). Samples and standards were pulled through the plate under vacuum into a 2 mL deep-well 96-well plate. Wells were dried completely at 40 °C under N2 and re-suspended in 100 μL 20% ACN, 0.1% FA. The plate was shaken at (250 rpm, 20 min) to ensure reconstitution. Liquid chromatography-Mass Spectrometry was performed on an I-class Acquity LC system-Beckman TQS triple quadrupole mass spectrometer (Waters, Warrington, UK). Samples were kept at 10 °C and injected onto an Acquity UPLC BEH C18 column fitted with a 0.2 μm filter (1 × 50 mm, 1.7 μm, Waters). Chromatographic separation of MitoA and MitoN was achieved using mobile phase A composition: water:ACN, (95:5, 0.1% FA), mobile phase B: ACN:water (90:10, 0.1% FA). LC mobile phases were infused at 200 μL/min using the gradient: 0–0.3 min, 5% B; 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 analysis was performed using a positive ion mode (Source spray voltage, 3.2 kV; cone voltage, 125 V; ion source temperature, 100 °C). Curtain and collision gas were nitrogen and argon, respectively. Analytes were detected by multiple reaction monitoring (MRM). MitoA undergoes neutral loss of N2 to a nitrene (precursor ion). For quantification the following transitions were used: MitoA, m/z 437.2 → 183.1; MitoN, m/z 439.2 → 120.0; d15-MitoN, 454.2 → 177.1 m/z. MassLynx 4.1 software was used to integrate the peak area of the analytes MitoA, MitoN and the d15-MitoN internal standard. Response was calculated by normalizing sample peak areas to the IS peak area. By comparison of sample responses to calibration standard responses the mass of each analyte in the tissue sample was calculated. The mass of analyte was normalized to the mass of tissue homogenizer and MitoN/MitoA ratio was calculated.
Mitochondrial ROS (MitoSOX) measurement in H₂O₂ treated hepatocytes

Hepatocytes were seeded overnight onto 96-well collagen coated plates. Cells were exposed to a range of concentrations of H₂O₂ (0.125 - 8 μM) for 2 hours. Following 3 washes with PBS, cells were incubated with MitoSOX Red mitochondrial superoxide indicator (Thermo Fisher) for 10 mins prior to three further washes. Measurement of fluorescence was carried out in a fluorescence detector plate reader (TECAN), using 510 nm for excitation and 580 nm for emission detection. Data from each well was normalized to sulfur-hodamine dye protein stain.

Persulfidation Mass Spec and GO term analysis

Livers from mice were removed promptly following decapitation (within 2 min), and snap frozen in liquid nitrogen. The persulfide proteome analysis using the BTA method was conducted as described previously (Gao et al., 2015). Briefly, 100-150 mg of frozen liver tissue was pulverized and lysed on ice in RIPA buffer (100 mM Tris, pH 7.5, 150 mM NaCl, 2mM EDTA, 1% Triton X-100, 25 mM deoxycholic acid, 2 tablets/ 100 mL of Complete, Mini, EDTA-free Protease Inhibitor Cocktail (Roche)). The lysates were centrifuged at 14,000 g for 10 min at 4°C and protein concentrations were determined using the Bradford reagent (BioRad). Supernatant containing 6 mg of protein was incubated with 100 μM NEM-biotin (Pierce) for 60 min at room temperature after which the proteins were precipitated with cold acetone (1:4 v/v) for 1 h at −20°C, followed by a centrifugation at 14,000 g for 10 min at 4°C. The precipitated protein was re-suspended in a denaturing buffer containing 7 M urea, 1% SDS, 150 mM NaCl, 100 mM Tris, pH 7.5. Then, the samples were diluted 10-fold with trypsin reaction buffer (1 mM CaCl₂, 100 mM Tris pH 7.5) and incubated overnight with sequencing grade modified trypsin (1:50 trypsin:protein) (Promega) at 30°C. The digestion products were mixed with streptavidin-agarose beads (Thermo-Scientific) and incubated at 4°C overnight, followed by ten washes with the wash buffer (0.1% SDS, 100 mM Tris, pH 7.5, 600 mM NaCl, 1 mM EDTA, 1% Triton X-100). The streptavidin-agarose bound peptides were incubated with elution buffer (100 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 30 mM DTT) for 1 hr at room temperature. Persulfidated peptides were eluted by centrifugation and derivatized with 40 mM iodoacetamide for 2 hr at room temperature in the dark. The samples were then passed through a desalting column (Pierce). LC-MS/MS analysis was carried out using an LTQ-Orbitrap Elite mass spectrometer (Thermo-Fisher) coupled to an Ultimate 3000 high-performance liquid chromatography system. The alkylated peptides were loaded onto a 75 μm desalting column, C18 reverse phase resin (Dionex), and eluted onto a Dionex 15 cm x 75 μm id Acclaim Pepmap C18, 2 μm, 100 Å reverse-phase chromatography column using a gradient of 2%–80% buffer B (5% water, 95% acetonitrile, 0.1% formic acid) in buffer A (0.1% formic acid). The peptides were eluted onto the mass spectrometer at a flow rate of 300 nl/min and the spray voltage was set to 1.9 kV.

GO enrichment analysis

In order to identify differentially persulfidated proteins between the C57BL/6J and Tst−/− samples, we compared the abundances of persulfidated fragments in appropriately treated mass spectrometry datasets to the estimated overall abundance of the corresponding parent proteins in standard label-free quantitation experiments. For each observed persulfidated fragment in each experimental replicate, we calculated the persulfidation rate as the log₂ ratio of the count of that persulfidated fragment to the median count of that fragment across all experimental replicates. The observed counts for the Tst−/− replicates were additionally scaled (prior to log transformation) by the ratio of abundances of the parent protein between the C57BL/6J and Tst−/− cells to normalize for differential protein abundance across conditions. For each peptide we then assigned an approximate average log₂ fold change in persulfidation rate between the C57BL/6J and Tst−/− conditions. If a persulfidated peptide was identified in at least two biological replicates of one condition and none in the other, we assigned a log₂ fold change of ± 5.0 as placeholder values indicating a high confidence change; peptides with only one observation in one condition and none in the other were omitted from our analysis. Having thus obtained estimates for the magnitude of changes in persulfidation rate of each detectable peptide, we then performed gene ontology term enrichment analysis using the estimated log₂ fold changes. We consolidated the peptide-level data to protein-level data by taking the largest magnitude change in persulfidation levels across all peptides from a given protein, and then used the iPAGE program [https://dx.doi.org/10.1016/j.molcel.2009.11.016] to identify GO terms with significant mutual information with the profile of persulfidation rates. Arguments to iPAGE were “—max_p=0.1 —minr=0.3 —ebins=9 —exptype=continuous,” indicated that the data were discretized into nine equally populated bins prior to analysis, and that default hypergeometric p value and information content thresholds were relaxed to maximize sensitivity.

Focused analysis of persulfidation in gluconeogenesis proteins

The gluconeogenesis pathway was selected for a focused analysis of the persulfidation rate of all cysteine sites detected in the mass spectrometry data as described above. All peptides from proteins present in the persulfidation dataset used for GO enrichment analysis, that are defined by the GO term gluconeogenesis (GO 0006094) were included, these were Pfk1, Gpi1, Fbp1 and Tpi1 (22 peptides). The log₂ rate ratio of persulfidation (Tst−/−/6J) of all of these 22 peptides was compared first to the entire mass spectrometry dataset for log₂ rate ratio of persulfidation (1245 peptides after removal of ambiguous peptides, peptides with a P-diff of 0, and the 22 gluconeogenesis peptides). A Mann-Whitney non parametric t test was used to detect significance. A second analysis was
performed with the gluconeogenesis pathway. For this analysis, all log₂ rate ratio’s were given a positive sign to indicate the magnitude of change in the Tsr⁻⁄⁻ relative to 6J, independent to the direction of change. A Mann-Whitney non parametric t test was then performed to determine if the magnitude of change in persulfidation in the gluconeogenesis pathway was significantly higher than that of the overall the dataset.

**Persulfidation labeling and western blotting from frozen liver**

80-120 mg of frozen liver samples were homogenized on ice using a 2 mL glass Dounce homogenizer (Kimble), in 500 µl buffer (7 M urea, 100 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% deoxycholic acid; supplemented with Complete Protease Inhibitor Cocktail (Roche)). Initial disruption of tissue was achieved with three passes, using the loose (Type A) pestle, and after 5 min incubation on ice; complete homogenization was achieved with 9 passes using the tight fit (Type B) pestle. Homogenates were centrifuged at 5000 rpm (2655 g) for 5 min at 4°C. Protein concentrations of supernatants were determined using the DC BCA protein assay (Bio-Rad). Protein (6 mg) from each sample, was made up to 1 mL with phosphate buffered saline (pH 8.0). Freshly prepared EZ-link Maleimide PEG Biotin EZ-linker (Thermo Fisher 21902BID), was added to samples to 100 µM, and incubated for 1 hour at room temperature with gentle mixing. Excess maleimide linker was removed from samples by acetone precipitation (3 volumes) at -20°C for 1 h, followed by centrifugation at 12000 rpm (17390 g) for 10 min at 4°C. Protein pellets were washed with ice-cold acetone and then dissolved in 250 µl of 50 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA, 1% SDS. To each sample, 750 µl of RIPA buffer (100 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% deoxycholic acid) was then added. An aliquot (20 µl) was taken from each sample for estimation of total input protein for normalization (described below). The remainder of the samples were split into duplicates and incubated with gentle mixing, overnight at 4°C with 320 µl of pre-washed streptavidin agarose beads (Thermo Fisher 20347). Beads were washed 10 times with 0.8 µl washing buffer (30 mM Tris pH 7.5, 600 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS), followed by one wash with phosphate buffered saline (pH 7.4). Beads were then centrifuged for 1 min at 1000 rpm (106 g) to dry. Elution of the duplicate samples for western blot analysis was performed by adding 300 µl of elution buffer (30 mM Tris pH 7.5, 150 mM NaCl). For each sample pair, one duplicate was eluted in buffer supplemented with 10 mM DTT and the other without DTT. The beads were incubated with the elution buffer for 1 hour at RT; and centrifuged for 1 min at 1000 rpm (106 g) to collect the eluate. Each eluted sample was concentrated to 20 µl using an Ultra-0.5 Centrifugal Filter Device, 10 K cut-off (Amicon), as per the manufacturer’s instructions. Eluted samples, and input protein samples were loaded in their entirety onto SDS-PAGE gels and transferred overnight at 4°C by western blotting onto PVDF membrane. Total protein from each lane on the membrane was estimated after staining with REVERT total protein stain (LICOR) according to the manufacturer’s instructions. Briefly, following overnight transfer and after rinsing the blot with water, the membranes were incubated with REVERT total protein stain for 5 min, and rinsed twice with wash solution. Blots were then imaged in the 700 nm channel with an Odyssey imaging system (LICOR). Each lane was measured for its total integrated fluorescence intensity to obtain an estimate of the total protein in each lane. Measurements from no-DTT eluted sample lanes were subtracted from DTT eluted sample lanes. Similar fluorescence measurements of input total protein lanes were used to normalize the eluted sample measurements, and this was used as a measure of relative protein-persulfidation rate.

**Mass spec analysis of liver protein**

**Sample preparation; Tst⁻⁻** and wild-type (C57BL/6J) mouse strains were fed either high-fat (58% fat) or normal (low fat) diet. Livers from mice were removed following decapitation, and snap frozen in liquid nitrogen. Four biological replicates from the 4 conditions were used to perform proteomic analysis and obtain protein quantitation using iTRAQ 8plex. Liver tissue was homogenized using 1 mL of 8 M urea with HEPES buffer pH 8.0. The protein concentration was determined using the Bio-Rad RC DC protein assay kit (Bio-Rad, Hercules, CA, USA). One hundred micro grams of protein from each of the samples were reduced with THP (Tris(hydroxypropyl)phosphine), alkylated with MMTS (methyl methanethiosulfonate) in 500 mM triethylammonium bicarbonate (TEAB, pH 8.5), trypsin digested and subsequently label with iTRAQ 8plex accordingly to the manufacturer’s instructions. Briefly, following overnight transfer and after rinsing the blot with water, the membranes were incubated with REVERT total protein stain for 5 min, and rinsed twice with wash solution. Blots were then imaged in the 700 nm channel with an Odyssey imaging system (LICOR). Each lane was measured for its total integrated fluorescence intensity to obtain an estimate of the total protein in each lane. Measurements from no-DTT eluted sample lanes were subtracted from DTT eluted sample lanes. Similar fluorescence measurements of input total protein lanes were used to normalize the eluted sample measurements, and this was used as a measure of relative protein-persulfidation rate.
injection time of 50 ms. MS/MS scans were performed in the orbitrap at 17 500 resolution. Ions selected for MS/MS scan were fragment-mental using higher energy collision dissociation (HCD) at normalized collision energy of 38% with an isolation window of 0.7 m/z. MS2 spectra were acquired with a fixed first m/z of 100. The intensity threshold for fragmentation was set to 50 000 and included charge states 2+ to 7+. A dynamic exclusion of 60 s was applied with a mass tolerance of 10 ppm. Data Analysis: Raw files were converted to MGF files and searched against the mouse UniProt database (81033 sequences, released on March 2014) using MASCOT Version 2.4 (Matrix Science Ltd, UK). Search parameters were peptide mass tolerance of 10 ppm, and MS/MS tolerance of 0.05 amu allowing 2 missed cleavage. iTRAQ8plex (N-term) and iTRAQ8plex (K) were set as fixed modification, and acetyl (Protein N-term), Methylthio (C) and Oxidation (M) were allowed as variable modification. Peptide assignments with ion score cut-off of 20 and a significance threshold of \( p < 0.05 \) were exported to Excel for further analysis. Data are available from the ProteomeXchange with identifier PXD028909.

**GO and KEGG enrichment analysis of proteome data**

The data generated from the initial mass spectrometric analysis of iTRAQ labeled peptides from the 16 liver samples was analyzed by FIOS. A total of 16 samples were QC analyzed using the arrayQualityMetrics Bioconductor package to identify sub-standard and/or outlier samples. No samples were identified as outliers. All samples passed the manual and automated quality control based on three metrics (MAplot, Boxplot and Heatmap). The exploratory analysis using PCA showed that the samples clustered perfectly into four groups based on the factor Group (representing four genotype-diet combinations). The first PC captures the main source of variation in the dataset and is showing a separation of the samples based on diet, where high-fat diet and control diet samples separate. The second PC shows a separation between genotypes (Tst KO and WT). The hierarchical clustering and PCA plot both show a clear separation based on the iTRAQ labels. This is expected as the iTRAQ labels are confounded with the Groups. While the observed separation of the samples into groups is most likely due to the underlying biological differences, any technical variations (potentially introduced during the wet lab processing) could be masked. The log2 ratio data were subsequently normalized within arrays using loess, followed by normalization between samples using the Gquantile method. A total of 4 single and/or multi-factor comparisons, using statistical approaches, were performed. The contrast “Tst KO vs WT mice (High-fat diet)” was analyzed at a cut-off (unadjusted) \( p < 0.01 \). Due to the known bias in fold-change magnitudes of the iTRAQ technology, no fold-change cut-off was applied to the significant differentially abundant proteins. With this threshold 551 proteins were differentially abundant in at least one of the comparisons. The contrast “High-fat diet vs Control diet (WT)” had the most DAPs (432) while the contrast “Tst KO vs WT mice (High-fat diet)” had the least DAPs (83). Noticeably, the TST protein showed the strongest downregulation for both of the contrasts comparing Tst KO to WT, consistent with gene deficiency and the fold change compression effect of iTRAQ. The full dataset (4,322 identified proteins) was filtered to remove proteins having less than two detected peptides (on average across all 16 samples); leaving 1,654 proteins for downstream analysis. Exploratory analysis using principal component analysis (PCA) showed that the dataset separated into four distinct groups based on the genotype-diet combinations along the two first principal components (PCs). These 1,654 proteins were used for enrichment analysis for GO terms and KEGG pathways. Individual proteins were considered of interest if they were found significantly different (\( p < 0.01 \)) between selected pairwise comparisons. The four comparisons were Tst KO normal diet versus C57BL/6J normal diet, C57BL/6J high fat diet versus C57BL/6J normal diet, Tst KO high fat diet versus Tst KO normal diet, and Tst KO high fat diet versus C57BL/6J high fat diet. Normalized mean abundance of proteins was expressed as Log2 fold change ratios for each comparison.

**Transcription factor enrichment analysis**

43 upregulated proteins were selected for analysis of their promoter sequences (selected on basis of \( P < 0.05 \); adjusted for comparison of diet and genotype). 67 control proteins were selected from the proteome data on the basis of their equivalence of abundance between C57BL/6J and Tst KO. We used a QIAGEN hosted/SABiosciences mouse database of promoter located transcription factor binding sites. 34 transcription factors were chosen to analyze, based on either their a-priori prevalence in the promoter of Tst KO upregulated proteins (present in the promoters of more than 50% of the upregulated proteins) or on their links to either sulfide or nutrient metabolism. The proportion of genes containing a TFBS was calculated for the upregulated set (43) and the control set (67). The ratio of upregulated to control was then calculated. The number of genes with and without the presence of the TFBS were analyzed for establishing statistical difference (Upregulated versus Control), using a Fisher Exact test (\( p < 0.05 \)).

**NRF2 target identification and proteome analysis**

NRF2 target genes of the mouse liver were 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 or protein level following NRF2 activation). 47 of these target genes were represented in our liver proteome, and each protein was checked for relative expression between Tst KO and WT (on normal diet, \( p < 0.01 \)). 10 of the 47 target genes were lower in abundance in the Tst KO proteome, 37 unchanged, with none upregulated. To analyze whether this was statistically significant, we compared this to the percentage of proteins upregulated, downregulated or unchanged in the proteome database. 5.86% of proteins were upregulated, 5.62% downregulated, and 88.6% unchanged in the full database (1654 proteins total). Expected (mean) numbers of proteins from a hypothetical set of 47 proteins, predict rounded values of 3 upregulated, 3 downregulated and 42 unchanged. We used these as a reference to the actual data for NRF2 target proteins; 0 upregulated, 10 downregulated and 37 unchanged. A Freeman-Halton
Mitochondrial stress test (MST)

Each biological replicate, and this data was used for statistical analysis of genotype effects.

Respiratory parameters were calculated as described below for each biological replicate using Wave software (Agilent), and all oxygen consumption rate (OCR) data was normalized to protein using the Sulforhodamine B stain (described above). Data from each biological replicate was averaged from between 4-10 replicate wells, to produce a single value at each measurement time for each biological replicate.

Octanoate rescue test

To investigate lipid respiratory metabolism, hepatocytes were prepared, seeded and cultured overnight as above. Run media for the Octanoate rescue test was Seahorse assay media (Agilent), supplemented with 5 mM glucose, 0.1 mM sodium pyruvate, 1 mM sodium lactate, and 0.5 mM carnitine pH 7.35 ± 0.5 at 37°C. All measurements were made using 3 min mixing, 2 min wait, 3 min measure. After washing cells into run media, and 30 min before entry into the analyzer, half of the wells from each genotype were incubated with 8 μM etomoxir (or DMSO vehicle) to block carnitine dependent import of long chain fatty acids into the mitochondria. Three basal measurements were taken prior to injection of oligomycin, two measurements were taken prior to FCCP, two measurements were taken prior to injection of sodium octanoate (250 μM), three measurements taken prior to antimycin/rotenone followed by two final measurements. Standard respiratory parameters were calculated analogous to the above description for the standard mitochondrial stress test, except using the second measurement following injection of drug when only 2 measurements were taken. Dependency upon endogenous fatty acids for supporting uncoupled respiration (Etomoxir inhibited respiration) was calculated for each biological replicate using the maximal respiration prior to the addition of octanoate. Maximal respiration was calculated as the 6th measurement following the injection of oligomycin (6th measurement). Non-respiratory OCR was taken from the third measurement after the addition of antimycin/rotenone (12th measurement).

Fisher Exact test was used for analysis of significance, and a significant difference between predicted and actual distribution was found (PA = 0.039, PB = 0.047).

Electron micrograph imaging

Liver tissue for transmission electron microscopy was prepared following immersion fixation in 0.1 M PB buffer (pH 7.4, EM-grade) containing 4% paraformaldehyde and 2.5% glutaraldehyde. 1mm tissue blocks were post-fixed in 1% osmium tetroxide in 0.1 M PB for 45 min before dehydration through an ascending series of ethanol solutions and propylene oxide. Tissue blocks were then embedded in Durcupan before ultrathin sections (~60/70 nm) were cut and collected on formvar-coated grids (Agar Scientific, UK), stained with uranyl acetate and lead citrate in an LKB Ultrostainer and then quantitatively assessed in a Philips CM12 transmission electron microscope (TEM).

Seahorse respiratory analysis

Primary hepatocytes (C57BL/6J and Tst−/− mice) were seeded immediately following purification onto collagen coated V7 Seahorse 24-well cell culture microplates (Agilent Technologies), in 200 μl medium (DMEM, 5.5 mM glucose, 10% FCS, 7 mM glutamine, and penicillin/streptomycin antibiotics), for culture in a 5% CO₂ 37°C incubator. Experiments were performed between 22-28 hours following collection from mice. Optimization experiments determined the optimal seeding density, which was then standardized at 10,000/well. Optimization for drugs and compounds used in Seahorse experiments were performed separately with hepatocytes for each genotype and dietary regime (normal or 58% high fat). This established the doses of drugs for respiratory manipulation, which were the same for both genotypes and diets; oligomycin (2 μM), FCCP (0.5 μM), and antimycin/rotenone (1 μM/0.2 μM). In all experiments, overnight media from cells was replaced, after two washes (0.75 ml), with 525 μL of run media and incubated for 30 mins at 37°C (without CO₂), prior to entry into the Seahorse XFe24 Extracellular Flux Analyzer (Agilent). The analyzer was operated using Wave software (Agilent), and all oxygen consumption rate (OCR) data was normalized to protein using the Sulforhodamine B stain (described above). Data from each biological replicate was averaged from between 4-10 replicate wells, to produce a single value at each measurement time for each biological replicate. Respiratory parameters were calculated as described below for each biological replicate, and this data was used for statistical analysis of genotype effects.

Mitochondrial stress test (MST)

Run media for the MST was Seahorse assay media (Agilent), supplemented with 10 mM glucose, 2 mM sodium pyruvate, pH 7.35 ± 0.5 at 37°C. Most measurements were made using 3 min mixing, 2 min wait, 3 min measure. Measurements following addition of FCCP to hepatocytes from high fat fed mice were measured using 4 min mix, 2 min wait, 2 min measure. Three measurements were taken basally, and three measurements taken after injection of each drug (in sequence; oligomycin for inhibiting ATP-linked respiration, FCCP for eliciting maximal uncoupled respiration, antimycin/rotenone for inhibiting the respiratory electron chain). Respiratory parameters for each biological replicate were calculated from the mean normalized OCR as follows. Basal respiration was calculated by subtracting the third OCR measurement following injection of antimycin/rotenone (12th measurement) from the basal OCR measurement (3rd measurement). ATP linked respiration was calculated by subtracting the third OCR measurement following the injection of oligomycin (6th measurement) from the basal OCR measurement (3rd measurement). Maximum (uncoupled) respiration was calculated by subtracting the third OCR measurement after injection of antimycin/rotenone (12th measurement) from the first measurement (peak OCR) following injection of FCCP (7th measurement). Proton leak respiration was calculated by subtracting the third OCR measurement after injection of antimycin/rotenone (12th measurement) from the third measurement following the injection of oligomycin (6th measurement). Non-respiratory OCR was taken from the third measurement after the addition of antimycin/rotenone (12th measurement).
respiration), was calculated for each vehicle well by subtracting the second OCR measurement after injection of FCCP (7th measurement) from the third measurement after injection of octanoate (10th measurement).

Real time for mRNA analysis
RNA extraction, cDNA synthesis and real-time PCR were performed as described (Morton et al., 2011; Moreno-Navarrete et al., 2013). Probes were mouse Mps1, Mm00460389_m1, Tst, Mm00726109_m1; Gapdh (internal control), Mm99999915_g1; and Tbp (internal control), Mm0000446973_m1.

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

The hepatic compensatory response
to elevated systemic sulfide promotes diabetes

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

A  TST mRNA in C57Bl/6J

B  Pathways for deriving Isotopomers from $^{13}$C$_3$ Pyruvate

C  $^{13}$C$_3$ Pyruvate pulse derived $^{13}$C Isotopologues

D  Cell normalised total pool size of metabolites
Figure S1. Tst mRNA tissue expression profile in C57BL/6J mice and the metabolic fates of $^{13}$C$_3$ pyruvate in hepatocytes from Tst$^{-/-}$ 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 the most abundant detected following pulse with $^{13}$C$_3$ pyruvate. In red is the direction of change in hepatocytes of Tst$^{-/-}$ mice, with significance or P-values (when less than 0.2) from t-tests. (C) Histogram showing isotopologues derived from $^{13}$C$_3$ pyruvate from C57BL/6J (white bars, n = 5) and Tst$^{-/-}$ (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 $^{12}$C and all detected $^{13}$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 $^{12}$C species. Data are represented as mean ± 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.

(A) Liver glycogen content

(B) Insulin tolerance

(C) Total cholesterol

(D) VLDL cholesterol

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 ±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−/− mice resist hydrogen peroxide induced mitochondrial reactive species accumulation. Related to Figure 2 and Table 1.

A

Hepatocyte mitochondrial ROS

*  

MitoSox

0  0.125  0.25  0.5  1  2  4  8

H₂O₂ (Micromolar)

Figure S3. Hepatocytes from Tst−/− 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−/− (red bars, n = 7). Cells were exposed to a range of doses of H₂O₂ prior to MitoSox incubation and fluorescent detection. Data are represented as mean ±SEM. Significance was calculated using 2-WAY ANOVA for H₂O₂ dose and genotype. A significant effect of genotype is represented above the histogram with a *. H₂O₂ 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−/− mice. Related to Figure 2 and Table 3.

(A) Persulfidation ratio in gluconeogenesis proteins

(B) Magnitude of persulfidation ratio in gluconeogenesis proteins

Figure S4. Persulfidation in the gluconeogenesis pathway is significantly different to global persulfidation patterns in the liver of the Tst−/− mice. Related to Figure 1, Figure 2 and Table 3. (A) Beeswarm plots showing the persulfidation log2 rate ratio (Tst−/− divided by 6J) for peptides in the entire data set (ALL), alongside the log2 rate ratio for peptides corresponding to proteins of gluconeogenesis (GLN). (B) Beeswarm plots showing the magnitude of the log2 rate ratio (independent to direction of change), for peptides in the entire data set (ALL), alongside the log2 rate ratios for peptides corresponding to proteins of gluconeogenesis (GLN). Data are represented as individual peptide log2 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.

(A) Protein abundance in liver

| Protein | 6J | Tst<sup>−/−</sup> |
|---------|----|-----------------|
| MPST    | 2.0| *              |
| GOT1    | 1.5|                |
| GSTT1   | 1.0|                |
| MAT1A   | 0.5|                |
| BHMT    | 0.0|                |
| CSAD    | 0.0|                |
| PPCS    | 0.0|                |

(B) MPST protein in liver mitochondria

| Protein | 6J | Tst<sup>−/−</sup> |
|---------|----|-----------------|
| MPST    | 8.0| *              |
| GOT1    | 6.0|                |
| GSTT1   | 4.0|                |
| MAT1A   | 2.0|                |
| BHMT    | 1.0|                |
| CSAD    | 0.5|                |
| PPCS    | 0.0|                |

(C) Mps1 mRNA in liver

| Protein | 6J | Tst<sup>−/−</sup> |
|---------|----|-----------------|
| GOI     | 1.5|                |
| GSTT1   | 1.0|                |
| MAT1A   | 0.5|                |
| BHMT    | 0.0|                |
| CSAD    | 0.0|                |
| PPCS    | 0.0|                |

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) Mps1 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−/− mice show underrepresentation of NRF2 promoter binding sites. Related to Figure 3 and Table 3.

(A) Transcription Factor Binding Sites in Promoters of Tst−/− up-regulated genes

(B) NRF2 Target Proteins in the Tst−/− Liver

Figure S6. Hepatic proteins enriched in Tst−/− mice show underrepresentation of NRF2 promoter binding sites. Related to Figure 3 and Table 3. (A) abundance in Tst−/− liver compared to a control set of proteins that are unchanged between 6J and Tst−/−. The proportion of genes containing a promoter binding site from proteins increased in Tst−/− 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−/− 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−/− mice in vitro.** Related to Figure 4.

Maximal respiration and non respiratory OCR (normal diet)

Respiratory parameters from HFD–fed (6J vs Tst−/−)

Figure S7. Hepatocyte respiration after high–fat feeding or after amino acid or pyruvate challenge is comparable between C57Bl/6J and Tst−/− mice in vitro. Related to Figure 4. (A) Maximal respiration OCR elicited by uncoupling with FCCP, by hepatocytes from normal diet-fed C57Bl/6J (n = 6) or Tst−/− (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−/− (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−/− 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−/− (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−/− (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−/− (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−/− (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−/− (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−/− (n = 4) mice. (J) Stimulation of maximal uncoupled respiration following addition of aspartate (1mM) and glutamox (1mM), by hepatocytes from normal diet-fed C57Bl/6J (n = 1) or Tst−/− (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        | 7st−/− chow | Genotype (chow) | 6J HFD         | 7st−/− HFD | Genotype (HFD) |
|----------------------------------------|----------------|-------------|-----------------|----------------|------------|----------------|
| Fasted Glucose 60 min (mg/dl)          | 116.3 ± 14.93  |             |                 | 135.72 ± 7.22  | 146.50 ± 3.89 |                |
| Glycogen synthesis (mg/kg/min)         | 11.63 ± 1.60   |             |                 | 12.9 ± 0.57     | 12.61 ± 0.62  | ns              |
| Glycolysis (mg/kg/min)                 | 21.48 ± 2.06   |             |                 | 19.02 ± 2.04    | 15.08 ± 2.76  | ns              |

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

| Parameter                              | 6J chow        | 7st−/− chow | Genotype (chow) | 6J HFD         | 7st−/− HFD | Genotype (HFD) |
|----------------------------------------|----------------|-------------|-----------------|----------------|------------|----------------|
| Glucose 160 min (mg/dl)                | 108.0 ± 5.0    |             |                 | 121.0 ± 10.1    | ns         |                 |
| Glucose 170 min (mg/dl)                | 120.3 ± 6.4    |             |                 | 129.8 ± 5.8     | ns         |                 |
| Glucose 180 min (mg/dl)                | 115.0 ± 5.5    |             |                 | 125.5 ± 2.5     | (0.08)     |                 |
| Glucose 190 min (mg/dl)                | 124.0 ± 1.0    |             |                 | 131.0 ± 4.6     | ns         |                 |
| Glucose 200 min (mg/dl)                | 121.0 ± 5.0    |             |                 | 125.3 ± 5.8     | ns         |                 |
| Glucose 210 min (mg/dl)                | 113.0 ± 7.8    |             |                 | 123.5 ± 4.6     | ns         |                 |
| Glucose IR 160-210 min (mg/kg/min)     | 84.9 ± 4.2     |             |                 | 85.0 ± 2.6      | ns         | 70.26 ± 4.83   |
| Glucose IR 160 (mg/kg/min)             | 82.3 ± 5.1     |             |                 | 87.1 ± 3.2      | ns         | 68.8 ± 4.2     |
| Glucose IR 170 (mg/kg/min)             | 85.2 ± 4.9     |             |                 | 89.0 ± 3.0      | ns         | 70.0 ± 5.1     |
| Glucose IR 180 (mg/kg/min)             | 84.7 ± 4.4     |             |                 | 83.5 ± 4.2      | ns         | 70.6 ± 5.1     |
| Glucose IR 190 (mg/kg/min)             | 85.2 ± 4.0     |             |                 | 86.1 ± 2.1      | ns         | 69.9 ± 4.8     |
| Glucose IR 200 (mg/kg/min)             | 85.2 ± 4.0     |             |                 | 84.8 ± 2.4      | ns         | 70.4 ± 4.7     |
| Glucose IR 210 (mg/kg/min)             | 85.2 ± 4.0     |             |                 | 84.5 ± 2.4      | ns         | 70.4 ± 4.7     |
| Turnover (mg/kg/min)                   | 85.97 ± 3.52   |             |                 | 94.50 ± 3.87    | ns         | 73.61 ± 5.07   |
| Hepatic Glucose Prod. (mg/kg/min)      | 1.10 ± 0.41    |             |                 | 9.92 ± 9.15     | ns         | 3.326 ± 4.03   |
| Glycolysis (mg/kg/min)                 | 45.90 ± 2.218  |             |                 | 48.37 ± 2.05    | ns         | 42.85 ± 1.48   |
| Glycogen synthesis (mg/kg/min)         | 40.06 ± 4.44   |             |                 | 46.12 ± 2.68    | ns         | 30.76 ± 5.36   |
| Integral Glucose (dpm.min/mg)          | 3.6e² ± 1.4e³  |             |                 | 2.95e² ± 1.2e³  | *          | 1.88e² ± 1.4e³  |
| IWAT glucose utilization (mg/min)      | 14.21 ± 4.05   |             |                 | 18.62 ± 2.04    | ns         | 4.53 ± 1.07    |
| EWAT glucose utilization (mg/min)      | 7.523 ± 4.39   |             |                 | 7.21 ± 2.18     | ns         | 2.94 ± 0.56    |
| VL glucose utilization (mg/min)        | 33.77 ± 2.98   |             |                 | 38.66 ± 1.70    | (0.17)     | 49.65 ± 9.19   |
| EDL glucose utilization (mg/min)       | 35.79 ± 11.09  |             |                 | 40.86 ± 10.25   | ns         | 68.79 ± 8.65   |
| Soleus glucose utilization (mg/min)    | 99.85 ± 12.38  |             |                 | 123.60 ± 13.90  | (0.13)     | 220.0 ± 24.45  |
| Tibialis glucose utilization (mg/min)  | 47.25 ± 7.22   |             |                 | 53.67 ± 4.40    | ns         | 74.39 ± 6.46   |
| Heart glucose utilization (mg/min)     | 161.40 ± 7.65  |             |                 | 198.00 ± 14.09  | (0.13)     | 262.6 ± 51.01  |
| Liver glucose utilization (mg/min)     | 3.53 ± 0.56    |             |                 | 3.57 ± 0.63     | ns         | 3.378 ± 0.39   |
| End Clamp Insulin (µU/ml)              | 133.6 ± 7.03   |             |                 | 126.4 ± 5.06    | ns         | 147.2 ± 9.4    |

* P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 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 7st−/− (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
| nmoles/min/mg protein       | C57Bl/6J               | Tst^/-       | Significance |
|-----------------------------|------------------------|--------------|--------------|
| Hepatocytes                 | 3.88 +/- 0.095         | 4.15 +/- 0.345 | ns           |
| Hepatocytes (Respiratory)   | 1.97 +/- 0.176         | 2.91 +/- 0.288 | *            |
| Hepatocytes (Non-respiratory)| 1.91 +/- 0.181         | 1.24 +/- 0.117 | *            |
| Liver Mitochondria          | 0.65 +/- 0.095         | 1.23 +/- 0.129 | *            |
| Liver Mitochondria (Respiratory) | 0.26 +/- 0.060     | 0.50 +/- 0.080 | *            |

* P < 0.05

Table S2. Hydrogen sulfide disposal by hepatocytes and mitochondria (Amperometry)

Table S2. Tst deletion results in increased respiratory H₂S disposal by hepatocytes. Related to Figure 2 and Table 1. H₂S disposal rates (measured by gas selective amperometry following addition of 10 µM Na₂S) of hepatocytes (n = 6/genotype), or isolated liver mitochondria (n = 7/genotype) of ND-fed C57Bl/6J and Tst^/-^/- mice. Rates of H₂S 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.
| Feature ID | Name                                | Fold Change | Significance |
|------------|-------------------------------------|-------------|--------------|
| Q3UW66     | MPST  | Mercaptopyruvate sulfurtrasferase   | 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−/− relative to C57Bl/6J.
Table S5. GO terms - Nutrient metabolism; reduced in ND Tst\(^{-/-}\) liver (Tst\(^{-/-}\) 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\(^{-/-}\) liver compared with ND-fed C57Bl/6J. ‘Genes’ indicates the number of genes in the Tst\(^{-/-}\) that represent the changes driving the GO term.
Table S6 Insulin regulated proteins in Tst\(^{-/-}\) and C57Bl/6J mice

(A) Abundance of peptides of insulin–induced proteins (Tst\(^{-/-}\) 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\(^{-/-}\) 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\(^{-/-}\) 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\(^{-/-}\) 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 < 0.05, ** Adjusted P < 0.05

Table S6. Proteins regulated by insulin are broadly comparable in expression between Tst\(^{-/-}\) 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−/− 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−/− relative to C57Bl/6J.
Table S7. KEGG pathways shared by high fat feeding and TST deletion

| Entry | Name                                                | Comparison          | Significance |
|-------|-----------------------------------------------------|---------------------|--------------|
|       | **A. Shared up-regulated pathways**                 |                     |              |
| 00260 | Glycine, serine and threonine metabolism            | Tst<sup>−/−</sup> vs 6J | **           |
|       |                                                     | HFD vs ND           | **           |
|       | **B. Shared down-regulated pathways**               |                     |              |
| 00980 | Metabolism of xenobiotics by cytochrome P450        | Tst<sup>−/−</sup> vs 6J | ****         |
|       |                                                     | HFD vs ND           | *            |
| 00982 | Drug metabolism – cytochrome P450                   | Tst<sup>−/−</sup> vs 6J | ****         |
|       |                                                     | HFD vs ND           | *            |
| 04142 | Lysosome                                            | Tst<sup>−/−</sup> vs 6J | **           |
|       |                                                     | HFD vs ND           | ***          |
| 04390 | Hippo signaling pathway                             | Tst<sup>−/−</sup> vs 6J | **           |
|       |                                                     | HFD vs ND           | ****         |
| 05215 | Prostate cancer                                     | Tst<sup>−/−</sup> vs 6J | **           |
|       |                                                     | HFD vs ND           | *            |
| 04024 | cAMP signaling pathway                              | Tst<sup>−/−</sup> vs 6J | *            |
|       |                                                     | HFD vs 6J           | *            |
| 04141 | Protein processing endoplasmic reticulum            | Tst<sup>−/−</sup> vs 6J | *            |
|       |                                                     | HFD vs 6J           | **           |
| 05211 | Renal cell carcinoma                                | Tst<sup>−/−</sup> vs 6J | *            |
|       |                                                     | HFD vs 6J           | *            |
| 04722 | Neurotrophin signaling pathway                      | Tst<sup>−/−</sup> vs 6J | *            |
|       |                                                     | HFD vs 6J           | *            |
| 04110 | Cell cycle                                          | Tst<sup>−/−</sup> vs 6J | *            |
|       |                                                     | HFD vs 6J           | **           |
| 04918 | Thyroid hormone synthesis                           | Tst<sup>−/−</sup> vs 6J | *            |
|       |                                                     | HFD vs 6J           | ***          |
| 04612 | Antigen processing and presentation                 | Tst<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 the sulfide pathway of C57Bl/6J and Tst−/− mice. Related to Figure 3. Protein abundances of the sulfide production and disposal pathway from the liver proteome of C57Bl/6J and Tst−/− 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.

| Feature ID | Name  | Fold change in 6J | Significance | Fold change in Tst−/− | Significance |
|------------|-------|-------------------|--------------|-----------------------|--------------|
| Q3UW66     | MPST  | 1.35              | **           | 1.15                  | *            |
| Q8R086     | SUOX  | 1.21              | **           | 1.23                  | **           |
| Q54550     | 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
| Entry | Name                                                                 | Comparison          | Direction |
|-------|----------------------------------------------------------------------|---------------------|-----------|
| A     | **KEGG Pathways**                                                     |                     |           |
| 00980 | Metabolism of xenobiotics by cytochrome P450                           | $Tst^{-/-}$ vs 6J   | Decreased |
|       |                                                                     | HFD vs ND           | Increased |
| 00983 | Drug metabolism – other enzymes                                       | $Tst^{-/-}$ vs 6J   | Decreased |
|       |                                                                     | HFD vs ND           | Increased |
| 00053 | Ascorbate and aldarate metabolism                                     | $Tst^{-/-}$ vs 6J   | Decreased |
|       |                                                                     | HFD vs ND           | Increased |
| 00040 | Pentose and glucoronate interconversions                              | $Tst^{-/-}$ vs 6J   | Decreased |
|       |                                                                     | HFD vs ND           | Increased |
| 00830 | Retinol metabolism                                                    | $Tst^{-/-}$ vs 6J   | Decreased |
|       |                                                                     | HFD vs ND           | Increased |
| B     | **GO Terms**                                                          |                     |           |
| 006629| Lipid metabolic process                                               | $Tst^{-/-}$ vs 6J   | Decreased |
|       |                                                                     | HFD vs ND           | Increased |
| 006631| Fatty acid beta-oxidation                                             | $Tst^{-/-}$ vs 6J   | Decreased |
|       |                                                                     | HFD vs ND           | Increased |
| 003995| Acyl-CoA dehydrogenase activity                                       | $Tst^{-/-}$ vs 6J   | Decreased |
|       |                                                                     | HFD vs ND           | Increased |
| 0047617| Acyl-CoA hydrolase activity                                           | $Tst^{-/-}$ 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 $Tst^{-/-}$ 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 $Tst^{-/-}$ 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.