1 **Inducible Liver-Specific Knockdown of Protein Tyrosine Phosphatase 1B Improves**

2 **Glucose and Lipid Homeostasis in Adult Mice**

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4 C. Owen[1*], E. K. Lees[1*], L. Grant[1], D. J. Zimmer[2], N. Mody[1], K. K. Bence[2†] and M.

5 Delibegović[1†]. [*]Joint first author.

6

7 [1]Institute of Medical Sciences, School of Medical Sciences, University of Aberdeen,

8 Aberdeen, UK. [2]Department of Animal Biology, School of Veterinary Medicine, University

9 of Pennsylvania, Philadelphia, USA.

10

11 [†]Corresponding authors:

12 Dr. Mirela Delibegović

13 **Address:** Institute of Medical Sciences, School of Medical Sciences, University of Aberdeen,

14 Foresterhill, Aberdeen, AB25 2ZD, UK.

15 **Tel:** +44 1224 437587

16 **Fax:** +44 1224 437465

17 **Email:** m.delibegovic@abdn.ac.uk

18 Dr. Kendra Bence

19 **Address:** Department of Animal Biology, School of Veterinary Medicine, University of

20 Pennsylvania, Philadelphia, USA.

21 **Tel:** +215 746 2998

22 **Fax:** +215 573 5186

23 **Email:** kbence@vet.upenn.edu

24

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28 **Abstract**

29 **AIMS/HYPOTHESIS:** Protein-tyrosine phosphatase 1B (PTP1B) is a key negative regulator

30 of insulin signalling. Hepatic PTP1B deficiency, using the *Alb-Cre* promoter to drive *Ptp1b*

31 deletion from birth, improves glucose homeostasis, insulin sensitivity and lipid metabolism.

32 The aim of this study was to investigate the therapeutic potential of decreasing liver-PTP1B

33 levels in obese and insulin resistant adult mice.

34 **METHODS:** To investigate this, inducible-*Ptp1b* liver-specific knockout mice were

35 generated using *SA-CRE-ER^{T2}* mice crossed with *Ptp1b* floxed (*Ptp1b*$^{fl/fl}$) mice. Mice were

36 fed a high-fat diet (HFD) for 12 weeks to induce obesity and insulin resistance. Tamoxifen

37 was administered within HFD to induce liver-specific deletion of *Ptp1b* (*SA-Ptp1b*$^{-/-}$ mice).

38 Body weight, glucose homeostasis, lipid homeostasis, serum adipokines, insulin signalling

39 and ER stress were examined.

40 **RESULTS:** Despite no significant change in body weight relative to HFD-fed *Ptp1b*$^{fl/fl}$

41 control mice, HFD-fed *SA-Ptp1b*$^{-/-}$ mice exhibited a reversal of glucose intolerance as

42 determined by improved glucose and pyruvate tolerance tests, decreased fed and fasted blood

43 glucose and insulin levels, lower HOMA-IR, circulating leptin, serum and liver triglycerides,

44 serum free fatty acids and decreased HFD-induced ER stress. This was associated with

45 decreased glycogen synthase, PERK, eIF2α and JNK2 phosphorylation and decreased

46 expression of *Pepck*.

47 **CONCLUSIONS/INTERPRETATION:** Inducible liver-specific *Ptp1b* knockdown

48 reverses glucose intolerance and improves lipid homeostasis in HFD-fed obese and insulin

49 resistant adult mice. This suggests that knockdown of liver-PTP1B in subjects that are

50 already obese/insulin resistant may have relatively rapid, beneficial therapeutic effects.

51 **KEY WORDS:** Liver, PTP1B, Phosphatase, NAFLD, Disease, Glucose, Lipid, Insulin,

52 Leptin, ER Stress.

Abbreviations

ER   Endoplasmic reticulum

GTT   Glucose tolerance test

HFD   High-fat diet

HOMA-IR   Homeostatic model assessment of insulin resistance

IR   Insulin receptor

JAK2   Janus kinase 2

NAFLD   Non-alcoholic fatty liver disease

PTP1B   Protein tyrosine phosphatase 1B

Introduction

Caloric excess and low physical activity are key drivers of rising obesity levels in Western society. Insulin resistance and obesity are associated with the development of cardiovascular disease, type 2 diabetes and cancer [1]. Insulin resistance leads to hyperglycaemia, caused by a decrease in insulin-dependent glucose uptake into peripheral tissues and diminished ability of insulin to suppress hepatic glucose production [2]. Insulin resistance is also associated with dyslipidemia [3] and non-alcoholic fatty liver disease (NAFLD), which is the most common liver disease across the world (20-35% of the population) [4]. It is distinguished by excess hepatic fat stores, in the absence of alcohol consumption [4]. Lifestyle factors such as nutrition and exercise can influence whether NAFLD is likely to progress to non-alcoholic steatohepatitis [4], which carries an increased risk of mortality [5]. This rising burden of metabolic disease requires the development of new therapeutic strategies [6].

Protein tyrosine phosphatise 1B (PTP1B) is a non-receptor tyrosine phosphatase which is ubiquitously expressed in all insulin-responsive tissues [7]. PTP1B is a negative regulator of both insulin and leptin signalling, through its actions on the insulin receptor (IR) and Janus

78 kinase 2 (JAK2) [7]. Whole body *Ptp1b*^{-/-} mice have enhanced insulin sensitivity, increased

79 phosphorylation of tyrosine residues on the IR and reduced adiposity on high-fat diet (HFD)

80 [8, 9]. PTP1B antisense oligonucleotides were shown to effectively lower PTP1B levels in

81 liver and fat, enhance insulin signalling, as well as decrease adiposity in *ob/ob* and *db/db*

82 mice [10, 11]. Lipogenic genes were down-regulated in fat and liver, including diminished

83 *Pparγ* gene expression in adipose tissue [12]. However, whether these positive effects were

84 due to loss of PTP1B in the liver and/or adipose tissue or any other tissue(s) was unclear.

85 Subsequently, tissue-specific *Ptp1b* knockout mice were generated to identify the specific

86 tissues responsible for PTP1B's effects on insulin sensitivity and lipid metabolism [2, 13, 14].

87 Neuronal *Ptp1b*^{-/-} mice display decreased body mass, reduced food intake and enhanced

88 energy expenditure on a high-fat diet (HFD) [13]. This is due, at least in part, to leptin

89 hypersensitivity in these mice; PTP1B acts as a negative regulator of leptin signalling via its

90 ability to dephosphorylate JAK2 on tyrosine sites Y1007/Y1008 and altering downstream

91 STAT3 phosphorylation [15]. Adipocyte-specific *Ptp1b* deletion increases adipocyte size and

92 serum levels of glucose and leptin, without affecting body weight [6]. Muscle-specific and

93 liver-specific deletion of *Ptp1b* has no effect on body weight, but in contrast to adipocyte-

94 *Ptp1b* deletion, improves peripheral insulin sensitivity and whole body glucose homeostasis

95 [2, 14]. Liver-specific *Ptp1b* deletion also decreases serum triglyceride levels and lowers

96 lipogenic gene expression in livers of mice fed HFD (*Srebp1c*, *Srebp1a* and *Fas*). It is

97 thought that this may be due to decreased endoplasmic reticulum (ER) stress response

98 induction observed in these mice [16, 17].

99 Specifically targeting liver-PTP1B appears to be an attractive drug therapy for treatment of

100 metabolic syndrome, as it not only improves whole body insulin sensitivity and glucose

101 homeostasis but also decreases lipid deposition in the liver, thus potentially limiting the

102 development of co-morbidities such as NAFLD.

103   Previous studies have examined beneficial effects of liver PTP1B deficiency using *Alb-Cre*

104   mice which induces hepatocyte-*Ptp1b* deletion from birth [2]. The aim of this study was to

105   investigate whether inhibiting liver-PTP1B in adult mice with already established obesity and

106   insulin resistance could reverse the phenotype and therefore present a novel treatment for

107   insulin resistance and type 2 diabetes. To do this, we used a tamoxifen-dependent Cre

108   recombinase system under the control of the serum albumin promoter to target *Ptp1b*

109   specifically in liver.

110

111   **Materials and Methods**

112   **Ethics statement.** All animal procedures were approved by the University of Aberdeen

113   Ethics Review Committee Board and performed under a project license approved by the

114   Home Office under the Animals (Scientific Procedures) Act 1986 (PPL60/3951).

115   **Animal studies.** *Ptp1b*$^{fl/fl}$ mice and *SA-CRE-ER*^{T2} mice expressing *Cre-ER*^{T2} recombinase

116   under the control of the serum albumin promoter were described previously [18, 19].

117   Tamoxifen treatment of mice efficiently induces Cre-mediated recombination of LoxP

118   flanked (floxed) alleles in hepatocytes but not in other cell types or tissues [19]. *SA-Cre-ER*^{T2}

119   mice, when crossed to *Ptp1b*$^{fl/fl}$ mice, provide a tool to temporally control targeted

120   mutagenesis in hepatocytes. Tamoxifen administration induces a liver-specific deletion of

121   *Ptp1b* (hereafter termed *SA-Ptp1b*$^{-/-}$). DNA extraction and genotyping for the *Ptp1b* floxed

122   allele and the presence of *Cre-ER*^{T2} by PCR were performed as described previously [18].

123   Mice studied were age-matched littermates, which were generated on a C57BL/6

124   background. Mice were housed in groups, unless otherwise stated, and maintained at 22-24°C

125   on a 12-h light/dark cycle with free access to food and water. At weaning (~21 days), mice

126   were placed on standard 3.4% fat chow pellet diet (Rat and Mouse Breeder and Grower,

127   Special Diets Services, DBM, Scotland) or HFD (Adjusted Calories Diet, 55 % fat, Harlan

128 Teklad, USA) and weight was recorded every two weeks. The approximate fatty acid profile

129 of Adjusted Calories Diet (% total fat) was 28% saturated, 30% trans, 28% monounsaturated

130 (cis) and 14% polyunsaturated (cis), as described previously [20]. For insulin signalling

131 experiments, HFD-fed mice were fasted overnight (16 hours) and then injected

132 intraperitoneally with saline or insulin (10 mU/g body weight) for 10 minutes. Tissues were

133 dissected immediately post-cervical dislocation and frozen in liquid nitrogen.

134 **Tamoxifen administration.** To prepare tamoxifen, ethanol was added to make a 10

135 mg/100 µl suspension. Sunflower seed oil was then added to prepare a 10 mg/ml solution.

136 This was heated at 55˚C for 30 minutes. This mixture of tamoxifen, ethanol and sunflower oil

137 was then incorporated into HFD (55% fat) at 0.7 mg tamoxifen/gram of food. A control HFD

138 (55% fat) was simultaneously administered to a control group containing ethanol and

139 sunflower oil only. Mice were fed the tamoxifen or control HFD for 28 days.

140 **PTP1B activity assay.** Tissue lysates were prepared in PTP lysis buffer (130 mmol/l

141 NaCl, 20 mmol/l Tris (pH 7.5), 5 mmol/l EDTA, 1% Triton X-100 (v/v), 0.5% Nonidet P-40

142 (v/v) containing protease inhibitors. PTP1B protein was immunoprecipitated using PTP1B

143 antibody (Millipore) and protein G-sepharose beads. Beads were re-suspended in 60 µl of

144 PTP assay buffer (100 mmol/l Hepes (pH 7.6), 2 mmol/l EDTA, 1 mmol/l DTT, 150 mmol/l

145 NaCl, 0.5 mg/ml BSA) containing phosphoregulatory peptide (200 µmol/l). The reaction

146 proceeded for 30 minutes at 30°C with constant shaking. The concentration of phosphate

147 produced (µmol/l) was then measured by absorbance at 620 nm using biomol green reagent

148 (Enzo Life Sciences) and phosphate standards.

149 **Histology.** Frozen tissues were embedded in OCT and sectioned by cryostat. Samples were

150 stained in hematoxylin and eosin. Slides were visualised using a Zeiss Axioskop microscope

151 (Carl Zeiss Microscopy, LLC, NY, USA) and imaged using AxioVision 4.8 digital image

152 processing software (Carl Zeiss Microscopy, LLC, NY, USA).

Serum analysis. Serum insulin and leptin (CrystalChem, Downers Grove, USA, Cat 90080 Insulin/90030 Leptin) were determined by ELISA, following manufacturer's instructions. TNFα, IL-6, MCP-1 and Resistin were determined by multiplex ELISA assay (MADKMAG-71K, Millipore), following manufacturer's instructions. Serum glucose (glucose oxidase, Thermo Scientific, Cat TR1503) and serum triglycerides (Sentinel Diagnostics, Milan, Italy, Cat 17628 or Sigma, Cat TR0100) were determined using appropriate kits, following manufacturer's instructions. Serum free fatty acids were determined using a non-esterified fatty acid (NEFA C) kit (Wako Chemicals, Virginia, USA, Cat 994-75409E). Alanine aminotransferase activity was determined using an alanine aminotransferase activity assay kit (BioVision, California, USA, Cat K752-100) to determine liver function and health. Glucose and insulin concentrations were used to calculate the homeostasis model assessment of insulin resistance (HOMA-IR), a reliable marker of insulin sensitivity [21], which is defined as: fasting glucose (mmol/l) X fasting insulin (mU/l)/22.5. Assays were measured with a Spectramax Plus 384 spectrophotometer (Molecular Devices, CA, USA).

Liver triglycerides. ~100 mg pieces of liver were cut and weighed using analytical scales. 1 ml PBS was added to each tube and homogenised. Samples were centrifuged for 15 seconds at room temperature. The top layer was resuspended with gentle shaking. The supernatant was transferred (without disturbing the pellet) to new 1.5 ml tubes. Triglycerides were then assayed using a kit, following manufacturer's instructions (Sentinel Diagnostics, Milan, Italy, Cat 17628).

Glycogen determination. Two ~20 mg pieces of liver were cut and placed into 2 ml tubes. 500 μl 2 mol/l HCL was added to half of the samples and 500 μl 2 mol/l NaOH (to control for free glucose) was added to the other half. All samples were heated for 2 h at 95°C. 500 μl 2 mol/l NaOH was added to each tube containing HCL and 500 μl 2 mol/l HCL was added to

178 each tube containing NaOH for neutralization. Tubes were vortexed and centrifuged for 1

179 min. Samples were diluted 1:50 in equal volumes of HCL and NaOH. 10 μl of diluted

180 samples were used for the assay. 150 μl of hexokinase reagent (Sigma) was added to each

181 well and incubated for 10 min at room temperature. The assay was measured at 340 nm with

182 a Spectramax Plus 384 spectrophotometer (Molecular Devices, CA, USA).

183 **Glucose and pyruvate tolerance tests.** For glucose tolerance tests, mice were

184 intraperitoneally injected, following a 16-hour fast, with 1.5-2 mg/g (1.5 mg for HFD-fed and

185 2 mg for mice on chow diet) body weight glucose. For pyruvate tolerance tests, mice were

186 injected with 1.5 mg/g pyruvate. Tail blood glucose values were measured using glucometers

187 (AlphaTRAK, Berkshire, UK) immediately before and at 15, 30, 60, and 90 or 120 min post-

188 injection.

189 **Immunoblotting.** Tissue lysates were prepared in RIPA buffer containing fresh sodium

190 orthovanadate and protease inhibitors, as described previously [22]. Proteins were separated

191 by 4-12% SDS-PAGE and transferred to nitrocellulose membranes. Immunoblots were

192 performed using antibodies from Cell Signaling (Cell Signaling by NEB, Hitchin, UK)

193 (unless stated otherwise) against PTP1B (Millipore), Beta-Actin (Thermo Scientific), SHP2

194 (Santa Cruz), pGS S641, pGSK3α/β S21/S9, pIR Y1162/63 (Invitrogen), pIR Y1158

195 (Invitrogen), pAkt/PKB S473, pERK1/2 MAPK T202/Y204, pAkt/PKB T308, p-FRAP/p-

196 mTOR S2448 (Santa Cruz), pS6 ribosomal protein S235/236, pPERK T980, peIF2α S51,

197 pSAPK/JNK T183/Y185, ERK2 and total Akt/PKB (Santa Cruz). Immunoblots were

198 developed using horseradish peroxidase-conjugated secondary antibodies, visualized using

199 enhanced chemiluminescence, and quantified by densitometry scanning with Image J or

200 Bio1D software (PeqLab, Fareham, UK).

201 **Gene expression analysis.** Total RNA was isolated from mouse liver and epididymal

202 adipose tissue using TRI Reagent (Ambion, Warrington UK), according to the

manufacturer's protocol. First strand cDNA was synthesized from 1 μg of total RNA employing the Bioline Bioscript™ Pre-amplification System and oligo(dT)$_{12-18}$. Two (2) μl of diluted cDNA (1:10) was used to amplify target genes by real-time RT-PCR (10 μl), using GoTaq qPCR Master Mix (Promega, Southampton, UK). The Roche LightCycler® 480 System (Roche Diagnostics, Burgess Hill, UK) was used for analysis. Relative gene expression was calculated using the comparative Ct (2−ΔΔCt) method. A geometric mean of three commonly used reference genes; hypoxanthine-guanine phosphoribosyltransferase (*Hprt*), 18S ribosomal RNA (*18S*) and Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) was used to normalise data. A geometric mean of the relative copy numbers of mouse PCRs were followed by melting curves (70-95°C).

Data analysis. Data are expressed as mean ± SEM and *n* represents the number of mice or biological replicates. Statistical analyses were performed using one-way ANOVA with Tukey's multiple comparison post-tests, repeated measures two-way ANOVA with Bonferroni multiple comparisons post-tests, one tailed t-tests or two-tailed Student's t-tests, as appropriate. The critical alpha level (*P*) was set at 0.05. $P < 0.05$ was considered statistically significant. GraphPad Prism 5 statistical software was used for analyses.

Results

Inducible liver-specific *Ptp1b* knockdown improves glucose homeostasis. Body weight (Figure 1) was comparable between HFD-fed *SA-Ptp1b*$^{-/-}$ and HFD-fed *Ptp1b*$^{fl/fl}$ control mice throughout the study and ~50% decrease in PTP1B levels was achieved at 4- and 12-weeks post-tamoxifen treatment in HFD-fed *SA-Ptp1b*$^{-/-}$ mice (Figure 1b and c, respectively). PTP1B activity was also ~20% lower in HFD-fed *SA-Ptp1b*$^{-/-}$ mice compared with HFD-fed *Ptp1b*$^{fl/fl}$ control mice (Figure 1d). Considering that PTP1B activity was ~40% lower in *Alb-Ptp1b*$^{-/-}$ mice (which have *Ptp1b* deletion in the liver from birth) (Figure 1d), this would be

228   consistent with 50% deletion at the protein level. As expected from other studies, tamoxifen

229   treatment caused ~20% body weight loss in both groups of mice, which returned to pre-

230   tamoxifen levels on HFD (Figure 1). An outline of the experimental design is shown in

231   Figure 1. As expected, HFD-fed SA-$Ptp1b^{-/-}$ mice displayed no differences in glucose

232   tolerance in comparison to $Ptp1b^{fl/fl}$ mice prior to tamoxifen treatment, on chow or HFD

233   (Figure 2a and b). Importantly, HFD-feeding induced glucose intolerance in both groups of

234   mice, as evidenced by increased area under the curve in both groups (Figure 2d). After

235   tamoxifen treatment, which induced PTP1B knockdown in SA-$Ptp1b^{-/-}$ mice only (Figure 1b

236   and c), HFD-fed SA-$Ptp1b^{-/-}$ mice displayed a significantly improved response to glucose

237   challenge and a reversal of glucose intolerance to chow-diet feeding levels (Figure 2a, c and

238   d). Furthermore, fed and fasted serum glucose levels were lower in HFD-fed SA-$Ptp1b^{-/-}$ mice

239   compared with HFD-fed $Ptp1b^{fl/fl}$ control mice, at 4- and 12-weeks post-tamoxifen treatment

240   (Figure 2f and g). Fed serum insulin levels tended to be lower in SA-$Ptp1b^{-/-}$ mice, although

241   this did not reach significance (Figure 2h). However, fasting serum insulin levels were

242   significantly lower at 4-weeks post-tamoxifen treatment in HFD-fed SA-$Ptp1b^{-/-}$ mice

243   compared with HFD-fed $Ptp1b^{fl/fl}$ controls (Figure 2i). Importantly, HOMA-IR, which

244   represents an index of insulin resistance, was significantly lower in HFD-fed SA-$Ptp1b^{-/-}$

245   mice, at 4- and 12-weeks post-tamoxifen treatment (Figure 2j).

246   **Inducible liver-specific *Ptp1b* knockdown improves lipid homeostasis.** Hematoxylin

247   and eosin staining revealed a high level of inter-animal variability when examining lipid

248   deposition in livers of HFD-fed SA-$Ptp1b^{-/-}$ and $Ptp1b^{fl/fl}$ mice (Figure 3a). We therefore also

249   assessed total liver triglyceride levels, which revealed that liver triglycerides were

250   significantly lower in the livers of HFD-fed SA-$Ptp1b^{-/-}$ mice compared with HFD-fed

251    $Ptp1b^{fl/fl}$ controls (Figure 3b). There were no significant differences between groups in

252   alanine aminotransferase activity (Figure 3c). Fed serum free-fatty acids were significantly

253 decreased at 4- and 12-weeks post-tamoxifen treatment in HFD-fed SA-$Ptp1b^{-/-}$ mice

254 compared with $Ptp1b^{fl/fl}$ controls (Figure 3d). HFD-fed SA-$Ptp1b^{-/-}$ mice also displayed

255 significantly lower fasting serum free-fatty acids at 12-weeks post-tamoxifen treatment

256 (Figure 3e). Fed and fasted serum triglycerides were significantly decreased at both 4- and

257 12-week post-tamoxifen treatment compared with the starting pre-tamoxifen levels in HFD-

258 fed SA-$Ptp1b^{-/-}$ mice only (Figure 3f and g). Moreover, both fed and fasted serum leptin

259 levels were significantly lower at 4 weeks post-tamoxifen treatment in HFD-fed SA-$Ptp1b^{-/-}$

260 mice compared with HFD-fed $Ptp1b^{fl/fl}$ control mice (Figure 3h and i). Furthermore, IL-6,

261 MCP-1, Resistin and TNFα were measured in $Ptp1b^{fl/fl}$ control and SA-$Ptp1b^{-/-}$ mice in the

262 fasted state (Table 2). SA-$Ptp1b^{-/-}$ mice displayed lower IL-6 at 12-weeks post-tamoxifen

263 compared with $Ptp1b^{fl/fl}$ control mice ($P = 0.059$; two-tailed t-test), whilst MCP-1 was

264 increased in SA-$Ptp1b^{-/-}$ mice at 4-weeks post-tamoxifen compared with $Ptp1b^{fl/fl}$ control

265 mice. Resistin was not different between groups at any time point (Table 2) and TNFα

266 concentrations were below the level of detection of the mouse multiplex ELISA (data not

267 shown).

268 **Inducible liver-specific *Ptp1b* knockdown improves suppression of hepatic**

269 **gluconeogenesis.** To assess if most of the improvements in glucose homeostasis were due to

270 improvements in suppression of hepatic gluconeogenesis, we performed a pyruvate tolerance

271 test. HFD-fed SA-$Ptp1b^{-/-}$ mice displayed a significantly improved response to pyruvate

272 challenge following overnight fasting compared with HFD-fed $Ptp1b^{fl/fl}$ controls (Figure 2e).

273 This is consistent with increased insulin-induced dephosphorylation of glycogen synthase at

274 both 4- and 12-weeks post-tamoxifen treatment (Figure 4 a, b, c and d). Liver glycogen

275 content was unaltered between SA-$Ptp1b^{-/-}$ and $Ptp1b^{fl/fl}$ control mice (5.97 ± 3.41 vs. 8.32 ±

276 2.93 µg/mg, respectively). Surprisingly, components of the classical insulin signalling

277 pathway were unchanged with liver-*Ptp1b* knockdown at either 4- or 12-week post-tamoxifen

278   (Figure 4a, b and e). Furthermore, insulin signalling in muscle and epididymal WAT was

279   comparable between *SA-Ptp1b*^{-/-} and *Ptp1b*^{fl/fl} control mice (Figure 4f and g).

280   **Inducible liver-specific *Ptp1b* knockdown is associated with decreased expression of**

281   **liver gluconeogenic genes.** To assess the mechanism(s) behind improvements in whole body

282   glucose and lipid homeostasis, we analysed expression of genes involved in gluconeogenesis

283   and lipogenesis. Consistent with the physiological data from pyruvate tolerance tests (Figure

284   2e) and signalling data (Figure 4a, b, c and d), HFD-fed *SA-Ptp1b*^{-/-} mice displayed a

285   decrease in gluconeogenic markers in comparison to HFD-fed *Ptp1b*^{fl/fl} control mice, as

286   evidenced by decreased liver gene expression levels of *Pepck* (Table 1). Moreover, *Pparγ*

287   was significantly decreased in these mice (Table 1). Furthermore, *Hmgcs1* was increased in

288   HFD-fed *SA-Ptp1b*^{-/-} mice compared to HFD-fed *Ptp1b*^{fl/fl} control mice (Table 1). HFD-fed

289   *SA-Ptp1b*^{-/-} mice exhibited unaltered lipogenic gene expression in liver or epididymal white

290   adipose tissue compared to *Ptp1b*^{fl/fl} control mice (Table 1). Lipolytic and adipokine gene

291   expression levels were unaltered between the groups in epididymal white adipose tissue

292   (Table 1).

293   **Inducible liver-specific *Ptp1b* knockdown decreases ER stress.** At the gene expression

294   level there was a significant decrease in *Grp94* in HFD-fed *SA-Ptp1b*^{-/-} mice compared to

295   HFD-fed *Ptp1b*^{fl/fl} control mice (Table 1). At the protein level, HFD-fed *SA-Ptp1b*^{-/-} mice

296   exhibited significantly lower phosphorylation of PERK, eIF2α and JNK2 when compared to

297   *Ptp1b*^{fl/fl} control mice (Figure 5a, b, c and d).

298

299   **Discussion**

300   There is a growing body of evidence to suggest that PTP1B inhibitors hold great promise for

301   treatment of type 2 diabetes as well as cancer [3, 4, 8, 16-18, 22-30]. Numerous mouse and

302   human studies have demonstrated that decreasing PTP1B in various tissues including muscle,

303   liver and the brain leads to a multitude of beneficial effects [17, 18, 22, 31]. Liver-specific

304   *Ptp1b* knockout in mice (*Alb-Ptp1b$^{-/-}$*) led to improved glucose homeostasis and decreased

305   levels of triglycerides independent of changes in body weight [17]. However, previous

306   studies investigated mice with a knockout of *Ptp1b* from birth and have therefore examined

307   the effects of *Ptp1b* deletion as a preventative of type 2 diabetes, not as a treatment in the

308   already obese and insulin resistant states. Using a tamoxifen-dependent Cre recombinase

309   system, we now demonstrate that decreasing liver-PTP1B by ~50% in obese and insulin

310   resistant adult mice, leads to a reversal of glucose intolerance and improvements in lipid

311   homeostasis, and that these effects are manifested within just a matter of weeks post hepatic-

312   *Ptp1b* knockdown.

313   As expected, and reported by others, oral tamoxifen treatment caused a transient decrease

314   in body weight in both groups of mice [32-34]. As with other mouse models of *Ptp1b*-

315   specific deletion [17, 22], body weight of the inducible liver-specific *Ptp1b* knockout mice

316   did not differ from control mice. *Ptp1b*-knockdown decreased PTP1B protein levels by ~50%

317   and PTP1B activity by ~20% in livers from *SA-Ptp1b$^{-/-}$* mice. 50% knockdown is less than

318   was observed previously in livers from *Alb-Ptp1b$^{-/-}$* mice (achieving ~80% hepatic *Ptp1b*

319   deletion and ~40% activity inhibition) [16, 17]. It has recently been reported that different

320   Cre lines display different degrees of efficiency and specificity [35]. In addition to

321   differences amongst Cre mice, different floxed gene loci were shown to display a range of

322   sensitivity to recombination when using different Cre lines [35]. However, a 50% decrease in

323   PTP1B levels is physiologically relevant, as PTP1B inhibitors would only be expected to

324   achieve approximately these levels [24].

325   In agreement with previous studies, glucose homeostasis is improved in *SA-Ptp1b$^{-/-}$* mice

326   compared with control mice [17]. Interestingly, glucose tolerance of *SA-Ptp1b$^{-/-}$* mice

327   returned to the responsiveness measured in these mice on chow diet, suggesting a reversal in

328   glucose intolerance that was caused by 12-weeks of HFD-feeding prior to inhibition of

329   hepatic *Ptp1b*. Furthermore, *SA-Ptp1b*^{-/-} mice exhibited significantly lower blood glucose

330   levels in response to a pyruvate bolus, suggesting an increased ability of insulin to suppress

331   hepatic gluconeogenesis. Consistent with these physiological data and our previous studies

332   using *Alb-Ptp1b*^{-/-} mice, we observed increased insulin-induced dephosphorylation of

333   glycogen synthase and decreased expression of the gluconeogenic gene *Pepck* in livers of *SA-*

334   *Ptp1b*^{-/-} mice, in the absence of changes in liver glycogen content [17]. This suggests that *SA-*

335   *Ptp1b*^{-/-} mice have an improved gluconeogenic response, efficiently shutting down hepatic

336   glucose production compared to control mice.

337   It is interesting to note that in our experiment insulin treatment of the control mice led to

338   increased phosphorylation of GS, whilst in the *SA-Ptp1b*^{-/-} mice it led to the expected

339   dephosphorylation. At the moment, it is unclear how hepatic PTP1B inhibition affects GS

340   phosphorylation independently of its effects on the insulin receptor; however, PTP1B has

341   been shown to regulate PP2A activation [36] as well as regulate hepatic *Srebp1* gene

342   expression through the PP2A axis [37], which may then affect GS hepatic phosphorylation

343   state [38]. This is currently under investigation in our lab, but is consistent with data from

344   *Ptp1b*^{-/-} immortalised cells treated with insulin, which were also shown to exhibit enhanced

345   dephosphorylation of the S641 site on GS [39].

346   Liver-*Ptp1b* deletion has previously been shown to decrease serum triglyceride with lower

347   expression of lipogenic genes [17]. This suggests that PTP1B knockdown may be a suitable

348   therapy for NAFLD, which is characterised by increased hepatic lipid accumulation and

349   insulin resistance. A recent study showed that the dietary supplement, curcumin, inhibits

350   PTP1B and prevents hepatic steatosis in fructose-fed rats, providing support behind this

351   notion [40]. Here, we demonstrate that *Ptp1b* knockdown in obese and diabetic mice results

352 in lower liver triglyceride levels associated with decreased expression of *Pparγ*, which has

353 been found to be elevated in fatty livers [41].

354 Interestingly, a paradoxical phenotype was previously observed in *Alb-Ptp1b*^{-/-} mice; they

355 displayed increased hepatic insulin signalling and decreased expression levels of hepatic

356 *Srebp1c*, *Fas* and other lipogenic markers [16, 17]. It is suggested that PTP1B may affect

357 *Srebp1* gene expression via a non-insulin signalling pathway in the liver involving effects on

358 PP2A activity [23, 37]. No differences were noted in *Srebp1a*, *Srebp1c* or *Fas* in the current

359 study; it may be that a 50% PTP1B knockdown is not sufficient to measure detectable

360 changes or may be due to the differences in timing of the *Ptp1b* deletion.

361 We have previously shown that *Alb-Ptp1b*^{-/-} mice, which delete hepatic-*Ptp1b* from birth,

362 are protected against HFD-induced hepatic ER stress [17]. Consistent with this, *SA-Ptp1b*^{-/-}

363 mice also have decreased phosphorylation levels of PERK, eIF2α and JNK2, indicating that

364 ~50% knockdown of *Ptp1b* can temporally improve ER stress. Moreover, in the absence of

365 changes in the classical IR signalling, this study suggests that improvements in lipid

366 homeostasis observed with hepatic-*Ptp1b* knockdown, may be due to decreased ER stress

367 response signalling.

368 *SA-Ptp1b*^{-/-} mice displayed significantly lower fed and fasted leptin levels 4-weeks after

369 *Ptp1b* knockdown was induced. This is the first time that *Ptp1b* knockdown in the liver has

370 been reported to affect circulating leptin levels. PTP1B has been well documented to regulate

371 leptin receptor signalling [18, 42]. However, leptin action in the liver remains inconclusive.

372 Diet-induced obese rats have been shown to exhibit decreased hepatic levels of leptin

373 receptor transcripts [43, 44]. Moreover, leptin-treatment of wild type mice led to increased

374 mRNA expression of several isoforms of the leptin receptor, including the long form of the

375 receptor (ObRb) [45], suggesting that the liver may be an important site of leptin action.

376 Furthermore, over-expression of PTP1B in the liver was shown to restrict the ability of leptin

377 to lower blood glucose levels and suppress food intake [46]. It was suggested that strategies

378 aimed at suppressing PTP1B specifically in the liver could improve both hepatic insulin and

379 leptin sensitivity [46]. Revealing the mechanism(s) behind our current observations should

380 form part of future studies.

381   Overall, tissue-specific knockout/knockdown studies of PTP1B have revealed key roles for

382 brain-, liver- and muscle-PTP1B in the regulation of global energy and glucose homeostasis.

383 We now demonstrate that liver-*Ptp1b* knockdown does not only prevent, but can reverse

384 established insulin resistance and glucose intolerance and also decrease ER stress and fat

385 accumulation in the liver in the obese and insulin resistant states. Inhibition of PTP1B

386 remains a promising potential therapy for type 2 diabetes treatment as well as a potential

387 protection against the development of NAFLD.

388

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403 NM is the recipient of a British Heart Foundation intermediate basic research fellowship.

404

405 **Duality of interest**

406 The authors declare that there is no duality of interest associated with this manuscript.

407

408 **Contribution statement**

409 CO, EKL, LG, NM, and MD contributed to acquisition of the data. CO, EKL, LG, NM and

410 MD performed the analyses. CO and EKL wrote the first draft of the paper, and LG, DJZ,

411 NM, KKB and MD contributed to the interpretation of data and critical revision of the

412 manuscript. All authors were involved in the writing of the manuscript and approved the final

413 version of the article.

414

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532

533   Table 1. Lipid and glucose metabolism gene expression in liver and epididymal WAT.

Parameter	$Ptp1b^{fl/fl}$ (n = 6-7)	$SA\text{-}Ptp1b^{-/-}$ (n = 6-8)	P value
Liver metabolism			
Fas	1.0 ± 0.06	1.0 ± 0.23	0.983
Srebp1c	1.0 ± 0.11	1.4 ± 0.24	0.155
Srebp1a	1.0 ± 0.22	1.3 ± 0.22	0.380
Srebp2	1.0 ± 0.20	1.1 ± 0.23	0.751
Hmgcs1	1.0 ± 0.14	1.9 ± 0.32*	0.023
Pparγ	1.0 ± 0.12	0.6 ± 0.08*	0.023
Pparα	1.0 ± 0.14	0.8 ± 0.12	0.307
Pgc1α	1.0 ± 0.13	0.7 ± 0.07	0.056
Pepck	1.0 ± 0.14	0.6 ± 0.05*	0.018
G6p	1.0 ± 0.26	0.7 ± 0.15	0.289
Liver ER stress			
Bip	1.0 ± 0.08	0.9 ± 0.08	0.317
Grp94	1.0 ± 0.12	0.6 ± 0.10*	0.043
Chop	1.0 ± 0.05	1.1 ± 0.22	0.667
Xbp Spliced	1.0 ± 0.13	1.3 ± 0.16	0.241
Xbp Total	1.0 ± 0.08	1.2 ± 0.15	0.268
Atf4	1.0 ± 0.06	1.1 ± 0.09	0.296
Adipose tissue metabolism			
Fas	1.0 ± 0.11	0.8 ± 0.14	0.303
Srebp1c	1.0 ± 0.20	1.2 ± 0.21	0.605
Srebp1a	1.0 ± 0.15	1.1 ± 0.13	0.565
Pparγ	1.0 ± 0.10	0.9 ± 0.16	0.622
Pparα	1.0 ± 0.27	1.2 ± 0.43	0.775
Hsl	1.0 ± 0.17	0.9 ± 0.13	0.673
Atgl	1.0 ± 0.17	0.7 ± 0.11	0.150
Rbp4	1.0 ± 0.15	0.8 ± 0.19	0.461
Leptin	1.0 ± 0.27	0.9 ± 0.29	0.820
Adipoq	1.0 ± 0.15	0.6 ± 0.20	0.185
Resistin	1.0 ± 0.23	0.9 ± 0.23	0.792
Glut4	1.0 ± 0.17	0.8 ± 0.12	0.315
Pepck	1.0 ± 0.17	0.7 ± 0.19	0.272
F480	1.0 ± 0.44	0.7 ± 0.38	0.652

534   Data are presented as fold change relative to $Ptp1b^{fl/fl}$ group. Data represented as mean ±

535   SEM. Data were analyzed using two-tailed Student's t test (*$P < 0.05$).

536 Table 2. Serum glucose parameters IL-6, MCP-1 and Resistin.

Parameter	$Ptp1b^{fl/fl}$ ($n = 6$)	$SA\text{-}Ptp1b^{-/-}$ ($n = 6$)
IL-6 Pre (pmol/l)	1.04 ± 0.39	0.65 ± 0.18
IL-6 Post 4 (pmol/l)	0.31 ± 0.08	0.71 ± 0.32
IL-6 Post 12 (pmol/l)	1.43 ± 0.43	0.5 ± 0.07†
MCP-1 Pre (pmol/l)	1.53 ± 0.1	1.27 ± 0.26
MCP-1 Post 4 (pmol/l)	0.64 ± 0.26	2.82 ± 0.63*
MCP-1 Post 12 (pmol/l)	1.17 ± 0.47	1.81 ± 0.52
Resistin Pre (pmol/l)	108.13 ± 21.47	83.28 ± 15.55
Resistin Post 4 (pmol/l)	102.38 ± 14.18	81.90 ± 22.28
Resistin Post 12 (pmol/l)	77.04 ± 17.38	79.68 ± 13.53

537 Data represented as mean ± SEM. Data were analyzed using one-way ANOVA with Tukey's

538 multiple comparison post-tests (*$P < 0.05$; † represents $P = 0.059$).

539

540 **FIGURE LEGENDS**

541 Figure 1. **Body weight and *Ptp1b* knockdown/activity.** *a*: Body weight of HFD-fed *SA-*

542 *Ptp1b*$^{-/-}$ ($n = 8$) and HFD-fed *Ptp1b*$^{fl/fl}$ control mice ($n = 9$). Experimental design and timings

543 of tamoxifen treatment also displayed. *b*: PTP1B knockdown 4-weeks post-tamoxifen ($n = 4$-

544 7). *c*: PTP1B knockdown 12-weeks post-tamoxifen ($n = 4$-7). *d*: PTP1B activity 12-weeks

545 post-tamoxifen. HFD-fed *Ptp1b*$^{fl/fl}$ control mice $n = 8$, HFD-fed *SA-Ptp1b*$^{-/-}$ mice $n = 8$ and

546 HFD-fed *Alb-Ptp1b*$^{-/-}$ mice $n = 4$. Data are represented as mean ± SEM. White bars/circles,

547 *Ptp1b*$^{fl/fl}$; black bars/circles *SA-Ptp1b*$^{-/-}$; grey bars *Alb-Ptp1b*$^{-/-}$. Data were analyzed by one-

548 tailed or two-tailed Student's t test (*$P < 0.05$; **$P < 0.01$; † represents $P = 0.054$).

549

550 Figure 2. **Inducible liver-specific *Ptp1b* knockdown improves glucose homeostasis.** *a*:

551 Glucose tolerance test (GTT) of both groups on chow diet prior to tamoxifen treatment. *b*:

552   GTT of both groups on HFD for 8 weeks prior to tamoxifen treatment. *c*: GTT of both groups

553   on HFD at 3 weeks after tamoxifen treatment. *d*: Area under the curve of GTT's. *e*: Pyruvate

554   tolerance test of both groups on HFD 12 weeks after tamoxifen treatment. *f*: Fed serum

555   glucose levels. *g*: Fasted serum glucose levels. *h*: Fed serum insulin levels. *i*: Fasted serum

556   insulin levels. *j*: HOMA-IR. *SA-Ptp1b*$^{-/-}$ and *Ptp1b*$^{fl/fl}$ control groups are indicated in the

557   figures. For all experiments *n* = 8 for HFD-fed *SA-Ptp1b*$^{-/-}$ mice and *n* = 9 for HFD-fed

558   *Ptp1b*$^{fl/fl}$ control mice. Data are represented as mean ± SEM. White bars/circles, *Ptp1b*$^{fl/fl}$;

559   black bars/circles *SA-Ptp1b*$^{-/-}$. Data were analyzed by repeated measures two-way ANOVA

560   with Bonferroni multiple comparisons post-tests, one-way ANOVA with Tukey's multiple

561   comparison post-tests or two-tailed Student's t test, where appropriate (*$P < 0.05$).

562

563   Figure 3. **Lipid homeostasis is improved with inducible liver-specific *Ptp1b* knockdown.**

564   *a*: Hematoxylin and eosin staining of livers *b*: Liver triglyceride assay from HFD-fed *SA-*

565   *Ptp1b*$^{-/-}$ mice (SA) and HFD-fed *Ptp1b*$^{fl/fl}$ control mice (FL). *c*: Alanine aminotransferase

566   activity assay. *d*: Fed serum free fatty acid assay. *e*: Fasted serum free fatty acid assay. *f*: Fed

567   serum triglyceride assay. *g*: Fasted serum triglyceride assay. *h*: Fed circulating leptin assay. *i*:

568   Fasted circulating leptin assay. *SA-Ptp1b*$^{-/-}$ and *Ptp1b*$^{fl/fl}$ control groups are indicated in the

569   figures. For all experiments *n* = 8 for HFD-fed *SA-Ptp1b*$^{-/-}$ mice and *n* = 9 for HFD-fed

570   *Ptp1b*$^{fl/fl}$ control mice. Data are represented as mean ± SEM. White bars, *Ptp1b*$^{fl/fl}$; black bars

571   *SA-Ptp1b*$^{-/-}$. Data were analyzed by one-way ANOVA with Tukey's multiple comparison

572   post-tests or two-tailed Student's t test, where appropriate (*$P < 0.05$).

573

574   Figure 4. **Inducible liver-specific *Ptp1b* knockdown improves suppression of hepatic**

575   **gluconeogenesis.** *a*: Liver insulin signalling 4-weeks post-tamoxifen in HFD-fed *Ptp1b*$^{fl/fl}$

576   and HFD-fed *SA-Ptp1b*$^{-/-}$ mice after injection with saline or insulin (10 mU/g). *b*: Liver

577 insulin signalling 12-weeks post-tamoxifen in HFD-fed $Ptp1b^{fl/fl}$ and HFD-fed SA-$Ptp1b^{-/-}$

578 mice after injection with saline or insulin (10 mU/g). c: Quantification of glycogen synthase

579 immunoblot 4-weeks post-tamoxifen. HFD-fed $Ptp1b^{fl/fl}$ control mice $n = 7$ (3 saline/4

580 insulin) and HFD-fed SA-$Ptp1b^{-/-}$ $n = 10$ (3 saline/7 insulin). d: Quantification of glycogen

581 synthase immunoblot 12-weeks post-tamoxifen. HFD-fed $Ptp1b^{-fl/fl}$ control mice $n = 7$ (3

582 saline/4 insulin) and HFD-fed SA-$Ptp1b^{-/-}$ $n = 10$ (3 saline/7 insulin). e: Liver IR

583 phosphorylation by immunoprecipitation 12-weeks post-tamoxifen. HFD-fed $Ptp1b^{fl/fl}$ control

584 mice $n = 6$ (2 saline/4 insulin) and HFD-fed SA-$Ptp1b^{-/-}$ $n = 6$ (2 saline/4 insulin). f Muscle

585 insulin signalling 12-weeks post-tamoxifen. g: Epididymal WAT insulin signalling 12-weeks

586 post-tamoxifen. HFD-fed $Ptp1b^{fl/fl}$ control mice $n = 7$ (3 saline/4 insulin) and HFD-fed SA-

587 $Ptp1b^{-/-}$ $n = 7$ (3 saline/4 insulin). Data are represented as mean ± SEM. White bars, $Ptp1b^{fl/fl}$;

588 black bars SA-$Ptp1b^{-/-}$. Data were analyzed by one-way ANOVA with Tukey's multiple

589 comparison post-tests or two-tailed Student's t test, where appropriate (*$P < 0.05$).

590

591 Figure 5. **Inducible liver-specific $Ptp1b$ knockdown reduces ER stress.** a: Representative

592 blot of liver ER stress signalling 12-weeks post-tamoxifen in HFD-fed $Ptp1b^{fl/fl}$ and HFD-fed

593 SA-$Ptp1b^{-/-}$ mice. b-e: Quantification of pPERK, peIF2α, pJNK2 and pJNK1 immunoblots.

594 HFD-fed $Ptp1b^{fl/fl}$ control mice $n = 7$ and HFD-fed SA-$Ptp1b^{-/-}$ $n = 10$. Data are represented

595 as mean ± SEM. White bars, $Ptp1b^{fl/fl}$; black bars SA-$Ptp1b^{-/-}$. Data were analyzed by two-

596 tailed Student's t test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).