Multi-omics Reveal that c-Src Modulates the Mitochondrial Phosphotyrosine Proteome and Metabolism According to Nutrient Availability

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Abstract
Background/Aims: Src kinase family members, including c-Src, are involved in numerous signaling pathways and have been observed inside different cellular compartments. Notably, c-Src modulates carbohydrate and fatty acid metabolism and is involved in the metabolic rewiring of cancer cells. This kinase is found within mitochondria where it targets different proteins to impact on the organelle functions and overall metabolism. Surprisingly, no global metabolic characterization of Src has been performed although c-Src knock-out mice have been available for 30 years. Considering that c-Src is sensitive to various metabolites, c-Src might represent a crucial player in metabolic adjustments induced by nutrient stress. The aim of this work was to characterize the impact of c-Src on mitochondrial activity and overall metabolism using multi-omic characterization. Methods: Src+/+ and Src−/− mice were fed ad libitum or fasted during 24h and were then analyzed using multi-omics. Results: We observed that deletion of c-Src is linked to lower phosphorylation of Y412-NDUFA8, inhibition of oxygen consumption and accumulation of metabolites involved in glycolysis, TCA cycle and amino acid metabolism in mice fed ad libitum. Finally, metabolomics and (phosphotyrosine) proteomics are differently impacted by Src according to nutrient availability. Conclusion: The findings presented here highlight that c-Src reduces mitochondrial metabolism and impacts the metabolic adjustment induced by nutrient stress.
Introduction

Carbohydrates are major macronutrients in energy metabolism. They are oxidized via glycolysis, tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS), which generate most of the cellular ATP. Excess carbohydrates can be converted into fatty acids and stored as triglycerides (TG) [1]. During nutrient deprivation, adjustments in metabolism allow to sustain ongoing energy requirements of cells. Notably, the liver breaks down glycogen into glucose, which is then released in the bloodstream to serve as fuel for other tissues. As fasting progresses, hepatic glycogen reserves deplete [2]. TG stored in adipose tissue are then hydrolyzed into free fatty acids (FFAs) and transported to the liver [3, 4], where they are oxidized into mitochondria to form acetyl coenzyme A (acetyl-CoA). FFA-derived acetyl-CoA can be redirected toward the production of ketone bodies such as acetoacetate and β-hydroxybutyrate (BHB) [5]. Ketones bodies represent essential alternative fuels for extrahepatic tissues during fasting [6]. The mitochondrial acetyl-CoA can also be oxidized by the TCA cycle to produce reducing equivalents (NADH and FADH₂) that will in turn allow ATP synthesis via OXPHOS. This process involves a series of five enzyme complexes embedded in the inner mitochondrial membrane. Electrons from NADH and FADH₂ are transferred through electron transport system (ETS) complexes (C) I, II, III and IV, allowing the reduction of O₂ into H₂O. Simultaneously, protons are pumped from the matrix to the intermembrane space at CI, III and IV to generate a proton gradient which is used by the ATP synthase to drive ATP synthesis [7]. Mitochondria are able to switch between different metabolic fuels such as carbohydrates and fatty acids, allowing the maintenance of metabolic homeostasis essential for cell survival during periods of nutrient stress.

Mitochondria are also biosynthetic hubs that produce a variety of signaling molecules such as reactive oxygen species (ROS) and TCA cycle intermediates [8–11]. For instance, α-ketoglutarate (α-KG) can bind and inhibit ATP synthase during glucose deprivation leading to reduced ATP content and decreased oxygen consumption in both Caenorhabditis elegans and mammalian cells [12]. The reduction of ATP levels activates the stress responsive metabolic regulator AMP-activated protein kinase (AMPK). In turn, AMPK inhibits the mechanistic target of rapamycin complex 1 (mTORC1) by direct phosphorylation of the tumor suppressor TSC2 or the regulatory associated protein of mTOR (known as Raptor) and lead ultimately to inhibition of cellular growth and activation of autophagy [13–15]. AMPK can also trigger mitophagy via phosphorylation of ULK1 during nutrient deprivation [16]. Simultaneously, AMPK promotes mitochondrial biogenesis via peroxisome proliferator-activated receptor γ coactivator protein -1 α (PGC-1α) dependent transcription [17]. Overall, this highlights that nutrient sensing pathways are tightly linked to mitochondrial functions to maintain metabolic homeostasis [18, 19].

The c-Src kinase (named hereafter Src) is another important signaling effector in the control of metabolism. Src promotes glycolysis by phosphorylating both hexokinase 1 and 2 (HK1 and HK2, respectively), stimulating their catalytic activities [20]. This kinase also increases flow through the pentose phosphate pathway by activating HK when intracellular glucose is abundant [20]. Overexpression of Src increases glucose transport and glycolysis in rat and chick embryonic fibroblasts [21, 22], whereas downregulation of Src in β-cells blunts glucose-dependent ATP production [23]. In endothelial cells, the activation of glucose-6-phosphate dehydrogenase in response to vascular endothelial growth factor (VEGF) is dependent on Src-mediated tyrosine-phosphorylation [24]. It has also been shown that Src resides inside mitochondria where it targets various proteins [25–28]. Intra-mitochondrial Src inactivates pyruvate dehydrogenase (PDH), thereby attenuating the flow of glycolysis-derived pyruvate into mitochondrial oxidative metabolism in cancer cells [29]. Several components of the ETS have also been identified as targets of Src. Notably, Src phosphorylates the CI subunit NDUFB10 and the CIV subunit COXII, resulting in increased activities of these complexes [27, 30]. Phosphorylation of the mitochondrial flotillin-1 by Src seems important for efficient electron transfer into complex II and the prevention of ROS production [31]. Altogether, these findings demonstrate that Src represents an important
regulator of mitochondrial metabolism. However, except for cancer cells, the role of Src in the regulation of metabolism remains scantly studied.

The overall aim of this work was to characterize the role of Src in metabolism. Src is sensitive to several metabolites, such as calcium, ATP and ROS [32–35], which are dysregulated during nutrient stress [36–39]. This strongly suggests that Src represents a potent effector of signal transduction throughout the cell to adjust metabolism during nutrient stress. Therefore, we also investigated the role of Src in the metabolic adjustment induced by fasting. To address this, we characterized hepatic mitochondrial metabolism, metabolomics and (phosphotyrosine) proteomics in Src+/+ and Src−/− mice fed ad libitum or fasted for 24h. Our findings demonstrate that deletion of Src impacts on several metabolic pathways including glycolysis, TCA cycle, OXPHOS, as well as oxidation of amino acid and fatty acid. We also observed that Src modulates phosphorylation of the CI subunit NDUFA8, at least indirectly. Finally, this study demonstrates that Src-dependent regulation of hepatic mitochondrial metabolism differs according to nutrient availability. Globally, this work highlights the importance of Src in the maintenance of metabolic homeostasis.

Materials and Methods

Animals

All experimental procedures were approved by the Committee on Animal Care of Université de Moncton (authorization numbers 16-04, 16-05, 19-04 and 19-05). Src+/+ and Src−/− mice (B6.129S7-Srctm1Sor/J) were obtained from Jackson Laboratory (USA), and housed under conditions of a 12-h light/dark cycle. Experimental mice were 7 to 14 weeks old and were fed ad libitum with a standard rodent diet or fasted for 24h.

Mitochondrial isolation

All steps were performed at 4°C. After sacrifice, brain, liver and kidney were collected in 10 mL of isolation buffer [for brain mitochondria: 320 mM sucrose, 10 mM Tris, 1 mM EDTA, pH 7.6; for liver and kidney mitochondria: 300 mM sucrose, 5 mM Tris, 1 mM EGTA, pH 7.4] supplemented with 2 mM of the phosphatase inhibitor sodium orthovanadate (modified from [40]). Tissues were homogenized using a Teflon pestle. Brain homogenates were centrifuged at 1,500 g for 5 min. Supernatants were then centrifuged at 12,500 g for 10 min. This 2-step centrifugation cycle was repeated and the final pellet was resuspended in isolation buffer and layered on top of a discontinuous gradient of Ficoll (supplemented with 2 mM sodium orthovanadate), as described elsewhere [41]. Following centrifugation for 30 min at 100,000 g, mitochondrial pellets were resuspended in isolation buffer and centrifuged again for 10 min at 12,500 g. The resulting pelleted purified brain mitochondria were resuspended in isolation medium. Liver and kidney homogenates were centrifuged at 800 g for 5 min. Supernatants were then centrifuged at 8,700 g for 10 min. This 2-step centrifugation cycle was repeated, and the final pellet was resuspended in isolation buffer. Protein concentration was determined by Bradford assay [42].

Trypsin sensitivity assay

The sub-mitochondrial localization of Src was evaluated by treating mitochondria with 0.5% trypsin for 10 min, with or without swelling in 10 mM HEPES-KOH, 1 mM EDTA pH 7.4, with or without 1% (v/v) Triton X-100 during 10 min at 37 °C. The reaction was stopped with protease inhibitors (Bioshop, CA), and samples were processed for SDS-PAGE.

Mitochondrial Oxygen Consumption

Mitochondrial oxygen consumption assays were performed using the high-resolution respirometry system Oxygraph-2k (OROBOROS INSTRUMENTS, Austria). Mitochondrial respiration was performed with 50 µg of mitochondria in respiration buffer (75 mM mannitol, 25 mM sucrose, 100 mM KCl, 10 mM Tris, 10 mM Tris-phosphate and 50 µM EDTA [43]) at 30°C. A substrate-uncoupler-inhibitor-titration protocol [44] was used to evaluate mitochondrial capacity with different substrates and inhibitors to examine specific steps of ETS and OXPHOS: pyruvate/malate (5 mM/2 mM), glutamate/malate (10 mM/2 mM), succinate
(10 mM), 500 µM ADP, 0.5 µM rotenone, 2.5 µM antimycin A, N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) and ascorbate (0.5 mM and 2 mM, respectively) were added to measure complex IV dependent respiration which was corrected for chemical background after complete inhibition by potassium cyanide (1 mM).

**Enzymatic assays**

All enzymatic assays were performed using a Varioskan plate reader (Flash version 2.4.5, Thermo Fisher; MA, USA) set at 37°C.

**Complexes I+III.** Combined activity of complex I (NADH: ubiquinone oxidoreductase) and III (coenzyme Q: cytochrome c – oxidoreductase) activity was determined by adding 195 µL of assay buffer [100 mM KH2PO4, pH 8.5, 0.03% Triton X-100, 2 mM iodonitrotetrazolium chloride (INT), 0.85 mM NADH] to 15 µg of mitochondria. Activity was monitored by following INT reduction at 490 nm for 4 min (ε=15.9 mL.cm⁻¹.µmol⁻¹).

**Complex II.** Mitochondria were incubated in 25 mM potassium phosphate buffer, pH 7.5 supplemented with 2 mM EDTA, 1 mg/ml BSA, 50 µM 2, 6-dichlorophenolinophenol (DCPIP), 4 µM rotenone, 0.2 mM ATP and 10 mM succinate for 10 min at 37°C. After addition of succinate, the baseline was read at 600 nm for at least 2 min, and the change in absorbance was then monitored for 5 min after addition of 80 µM decylubiquinone. Malonate (10 mM) was finally added to inhibit the oxidation of succinate and the assay was run for another 5 min. Enzyme activity was calculated with ε=19.1 mL.cm⁻¹.µmol⁻¹ for DCPIP.

**Complex IV.** Complex IV (cytochrome c oxidase) activity was determined in isolated mitochondria, by following the oxidation of reduced cytochrome c at 550 nm (ε=18.5 mL.cm⁻¹.µmol⁻¹) for 4 min, in 50 mM potassium phosphate buffer (pH 8.0) and 80 mM reduced cytochrome c (freshly prepared before each experiment).

**Citrate synthase.** Mitochondria (15 µg) were added in a solution containing 100 mM imidazole-HCl (pH 8.0), 0.1 mM 5, 5-dithio-bis-2-nitrobenzoic acid (DTNB), 0.2 mM oxaloacetate, and 0.1 mM acetyl-CoA. The reaction of free coenzyme A with DNTB to produce TNB was followed spectrophotometrically at 412 nm for 4 min (ε=13.6 mL.cm⁻¹.µmol⁻¹).

**Mitochondrial H2O2 production**

Mitochondrial H2O2 production was measured by the Amplex Red-horseradish peroxidase (HRP) method. Mitochondria (12.5 µg) were resuspended in respiration buffer containing 5 mM glutamate with 2.5 mM malate for brain mitochondria or 2.5 mM pyruvate with 2.5 mM malate for liver and kidney mitochondria, 10 mM succinate and 500 µM ADP. Fluorescence was followed using a Varioskan plate reader (Flash version 2.4.5, Thermo Fisher; MA, USA) with excitation wavelength set at 560 nm and emission wavelength set at 587 nm. The increased fluorescence over time was converted to the rate of H2O2 production using a standard curve with known H2O2 concentrations. All assays were performed at 37°C, in black 96-well plates.

**Western blot analysis**

Samples were analyzed by western blot analysis using conventional methods. Samples were diluted in SDS-PAGE sample buffer [62.5 mM Tris pH 6.8, 10 % (v/v) glycerol, 2 % (w/v) sodium dodecylsulfate (SDS), 0.5 % Bromophenol blue, 2.5 % (v/v) β-mercapto-ethanol] and boiled at 95°C during 5 min. Samples were then separated using 7, 10 or 12 % SDS-polyacrylamide mini-gel at 300 V during 30 min. Proteins were blotted to polyvinylidene difluoride membranes and detected using specific antibodies against Src (Cell signaling; 2108S), phosphoY416-Src (Cell signaling; 6943S), phosphoryrosine (Cell signaling; 9411S), SDHA (Abcam; ab14715), SMAC (Cell signaling; 15108S), TOM20 (Santa Cruz; sc-17764), SOD2 (Cell signaling; 13194S), ATP5a (Abcam; ab14748), NDUF9a (Abcam; ab14713), cytochrome c (Abcam; ab133504). The blots were visualized by chemiluminescence and band intensities were quantified by densitometric analysis using Image Lab software (BioRad).

**Blood glucose monitoring**

Blood samples were collected from mice after cervical dislocation without anesthesia. Glucose levels were measured using a glucometer ACCU-CHECK (Roche Diagnostics, Japan). All measurements were performed following the manufacturer's instructions.
Liver glycogen content measurement

Liver samples were snap-frozen in liquid nitrogen and stored at ~80 °C. The glycogen content was measured using a fluorometric Glycogen Assay Kit (Ref. 700480, Cayman Chemical, USA). All measurements were performed by following the manufacturer’s instructions.

Metabolomic analysis by GC-MS

GC-MS experiments were performed at the metabolomics core facility at the Rosalind and Morris Goodman Cancer center (McGill University, Canada). Liver samples were pulverized on dry ice and were weighed in the range of 10 mg ± 1 mg. 1 mL volume of 80 % (v/v) methanol/water and three pre-chilled 2.8 mm ceramic beads were added to each sample. Samples were subjected to three rounds of bead beating for 2 min at 30 Hz using a Qiagen Tissue lyser (Eppendorf, Canada). Samples were then centrifuged for 10 min at 15,000 rpm (4°C). Supernatants were transferred to new pre-chilled tubes containing 1 µL of 800 ng/µL -H_{27}-Myristic in pyridine. Samples were then dried by vacuum centrifugation operating at a sample temperature of -4°C.

After drying, samples were subjected to a two-step derivatization for GC-MS analysis. Following GC/MS scan and single ion monitoring (SIM) data acquisition, samples were diluted using 30. % methoxyamine hydrochloride and 70 % N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide 1:14 and data were acquired in scan mode. An Agilent 5975C GC-MS equipped with a DB-5MS+DG (30 m x 250 µm x 0.25 µm) capillary column (Agilent J&W, Santa Clara, CA, USA) was used for all GC-MS experiments, and data were collected by electron impact set at 70 eV both in scan (50-1000 m/z) and single ion monitoring (SIM) modes. Sample data were acquired in scan mode (50-1000 m/z) or in SIM with a 5 ms dwell time where the M-57 [M^+-C4H9] fragment was used for relative quantitation (area under the curve) in both modes of data acquisition. The spectra and retention times of all metabolites reported were confirmed by methoxyamine–tert-butyldimethylsilylated authentic standards. Area under the curve was normalized to tissue weight.

(Phosphotyrosine) proteomic analyses by LC-MS/MS

Sample preparation. Protein digestion, peptide labeling, and mass spectrometry analyses were performed by the proteomics platform of the CHU de Quebec research center (Canada). Liver mitochondrial lysates suspended in 50 mM ammonium bicarbonate (ABC) / 0.5 % Deoxycholate (DOC) were vortexed and sonicated on ice. Extracts were centrifuged at 13,000 rpm during 15 min. The obtained supernatant was precipitated in acetone and centrifuged again. The resulting pellet was resuspended in 50 mM ABC with 1.0 % DOC. Then, 400 µg proteins were reduced, alkylated, digested with trypsin (ratio 1:50) and incubated overnight at 37 °C. Samples were acidified with 1% formic acid and desalted using an HLB column (Waters, USA).

Phosphotyrosine enrichment. Phosphotyrosine peptides were enriched using PTMScan Phospho-Tyrosine antibody (P-Tyr-1000; Cell Signaling; 8803). Peptides were incubated with beads for 2 h at 4 °C with gentle agitation. To remove non-specifically bound peptides, beads were washed with ice-cold Immunoaffinity Purification buffer (IAP) twice and with ice-cold water three times. Elution of phosphopeptides was carried out at room temperature using 0.15 % trifluoroacetic acid (TFA).

Mass spectrometry. Samples (global or phosphopeptide samples) were analyzed using an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific) connected to a Dionex UltiMate 3000 nanoRSLC chromatography system (Thermo Fisher Scientific, CA, USA). Peptides were eluted on a Pepmap Acclaim 50 cm x 75µm internal diameter separation column (ThermoFisher) with a linear gradient from 4-32 % acetonitrile in 0.1% formic acid during 90 min (phosphopeptide) or 240 min (global), at 300 nL/min. Mass spectra were acquired using a data-dependent acquisition mode using Thermo XCalibur software (v 4.1.50).

Database searching and Label-Free Quantification. Spectra were searched against the Uniprot Ref Mus musculus database (61364 entries) using the Andromeda module of MaxQuant software v. 1.6.0.16 [45]. Trypsin/P enzyme parameter was selected with two possible missed cleavages. Carbamidomethylation of cysteines was set as fixed modification while methionine oxidation and phosphorylation of serine, threonine, and tyrosine were set as variable modifications (for the phosphopeptides). Mass search tolerances was 5 ppm and 0.5 Da for MS and MS/MS respectively. For protein validation, a maximum False Discovery Rate of 1 % at peptide and protein level was used based on a target/decoy search. MaxQuant was also used for Label-Free Quantification. RStudio (v 1.1.383) was used for data processing. A normalization step was performed using the median of intensities of each condition. Missing values were replaced by a noise value
corresponding to 1% percentile of the normalized value for each condition. A peptide was considered as quantifiable only if at least three intensity values in one of the two samples were present or not more than one value missing per condition. For the global analysis, at least 2 peptides per protein were required for quantification.

Statistical analyses

Student’s T test and two-way ANOVA followed by Tukey’s post-hoc test, were performed using GraphPad Prism 8 (v 8.2.1), as appropriate. For metabolomic and (phosphotyrosine) proteomic analyses, partial least squares discriminant analyses (PLS-DA) were performed using MetaboAnalyst 3.0 [46, 47]. Normalization (by log transformation) was performed in order to minimize possible differences in concentration between samples. All data were mean centered and scaled to unit variance. PLS-DA identified the metabolites driving the separation and/or clustering among experimental groups by ascribing a variable importance of projection score (VIP). Significance was set at p< 0.05.

Results

Deletion of Src impairs mitochondrial metabolism in brain, liver and kidney

We first evaluated the levels of Src in brain, liver, and kidney isolated mitochondria. Results showed that Src levels are significantly higher in brain than in liver and kidney (Fig. 1a and Supplementary Fig. S1a – for all supplemental material see www.cellphysiolbiochem.com). Trypsin sensitivity assays confirmed that mitochondrial Src mostly resides inside the organelle (Fig. 1b) as previously shown [27, 48]. These results also suggest that Src is also localized in the mitochondrial matrix considering it is resistant to trypsin upon swelling of liver mitochondria.

The functional impact of Src was then evaluated by measuring the oxygen consumption of brain, liver and kidney enriched-mitochondrial fractions isolated from Src+/+ and Src−/− mice. Deletion of Src significantly decreased oxygen consumption of brain, liver and kidney mitochondria in the presence of (i) glutamate with malate (for brain mitochondria), or pyruvate with malate (for liver and kidney mitochondria), (ii) with the addition of ADP, and (iii) with further addition of succinate (Fig. 1c–e). No difference in respiration was observed when inhibitors of CI and III (rotenone and antimycin A, respectively) were added, or when maximum capacity of CIV was measured in presence of TMPD and ascorbate (Fig. 1c–e). These results indicate that Src is required to maintain mitochondrial respiration. We then measured the enzymatic activity of CI+III, II and IV. Deletion of Src significantly decreased the activity of CI+III and II in brain, liver, and kidney mitochondria (Fig. 1f and g). Deletion of Src also decreased CIV activity in brain mitochondria, had no effect in liver mitochondria and increased CIV activity in kidney mitochondria (Fig. 1h). Citrate synthase (CS) activity was decreased in liver mitochondria but unchanged in brain and kidney mitochondria upon deletion of Src (Fig. 1i). These findings indicate that Src has tissue-specific impact on CIV and CS.

Immunoblotting assays showed that levels of the mitochondrial proteins ATP5a, SDHa, NDUF9A, SOD2 and TOM20 are similar in brain, liver, and kidney mitochondria isolated from Src+/+ and Src−/− mice (Fig. 1j and Supplementary Fig. S1b). These findings demonstrate that the decreased mitochondrial respiration observed in Src−/− mice is not due to decreased mitochondrial content. Mitochondrial H2O2 production was not altered by deletion of Src (Fig. 1k). Overall, these results suggest that Src targets ETS components to regulate mitochondrial metabolism, likely via tyrosine-phosphorylation.
Fig. 1. Deletion of Src impairs mitochondrial metabolism in brain, liver and kidney. (a) Representative immunoblots (n=4) of Src and SDHa (as loading control) in brain, liver and kidney mitochondria isolated from Src+/+ and Src−/− mice (fed ad libitum). See Fig. S1a for quantification. (b) Representative immunoblots (n=3) of Src, the inner mitochondrial membrane protein SDHa, the mitochondrial intermembrane space protein SMAC and the outer membrane mitochondrial protein TOM20 in liver mitochondria treated as indicated, showing that Src is mostly located inside mitochondria. (c-e) Oxygen consumption rates of brain (c), liver (d) and kidney (e) mitochondria isolated from Src+/+ and Src−/− mice (fed ad libitum) in the presence of different substrates and inhibitors, as indicated (n=4). GM: Glutamate/Malate, PM: Pyruvate/Malate. (f-i) Enzymatic activities of complexes I+III (f), II (g) and IV (h), and citrate synthase (i) in brain, liver and kidney mitochondria isolated from Src+/+ and Src−/− mice fed ad libitum. (j) Representative immunoblots (n=4) of Src, SDHa, NDUFA9, TOM20, SOD2 and ATP5a in brain, liver and kidney mitochondria isolated from Src+/+ and Src−/− mice fed ad libitum. See Fig. S1b for quantification. (k) H2O2 production by brain, liver and kidney mitochondria isolated from Src+/+ and Src−/− mice fed ad libitum (n=4). Data are presented as mean ± s.e.m. *p<0.05, **p<0.01, ***p<0.001 determined by Student’s T test.
Effect of Src on hepatic mitochondrial activity according to nutrient availability

The liver plays a crucial role in maintaining energy balance and metabolic homeostasis during starvation. Src activity is sensitive to metabolites such as ATP and ROS [33, 34, 49, 50], which are affected during nutrient stress. Therefore, we sought to determine the role of Src in the adjustment of hepatic metabolism during nutrient stress. To address this, we analyzed Src+/+ and Src−/− mice either fed ad libitum or fasted during 24h. Fasting decreased blood glucose and hepatic glycogen in both genotypes (Fig. 2a and b). We then examined different respiration rates of Src+/+ and Src−/− liver enriched-mitochondrial fractions using (i) pyruvate, malate and ADP, to follow respiration via CI+II+III+IV, (ii) pyruvate, malate, succinate and ADP to follow CI+II+III+IV-dependent respiration, and (iii) TMPD and ascorbate to follow CIV-dependent respiration after inhibition of complexes I and III (Fig. 2c-e). Deletion of Src significantly decreased CI+II+III+IV and CI+II+III+IV-dependent respiration in mice fed ad libitum but not in fasted mice (Fig. 2c and d). Respiration rates were also lower in fasted Src+/+ and Src−/− mice compared to Src+/+ control mice (Fig. 2c and d). CIV-dependent respiration rate was not statistically different among experimental groups (Fig. 2e). Similar to CI+III+IV and CI+II+III+IV respiration rates, enzymatic activity of CI+II was significantly lower in Src−/− than in Src+/+ liver mitochondria from fed mice, whereas fasting reduced CI+III activity only in Src+/+ liver mitochondria (Fig. 2f). Liver CII activity was decreased by deletion of Src in mice fed ad libitum (Fig. 2g). Surprisingly, fasting increased liver CII activity in both Src+/+ and Src−/− mice (Fig. 2g), suggesting CII does not play a major role in the observed fasting-dependent inhibition of respiration rates. Liver CIV catalytic activity was only significantly decreased by fasting in Src+/+ mice (Fig. 2h). CS activity was lower upon deletion of Src in mice fed ad libitum, whereas this reduction was not observed in fasted mice (Fig. 2i). Similar to CII, this finding suggests that CS is not involved in Src-dependent regulation of respiration during fasting. Immunoblottings of SDHa, ATP5a, NDUFA9, SOD2, TOM20, and cyt c showed that mitochondrial protein levels are similar among groups (Fig. 2j and Supplementary Fig. S1c). No change in mitochondrial H2O2 production was observed after fasting (Fig. 2k). Our findings suggest that the observed Src-dependent alteration of mitochondrial oxygen consumption in liver is mediated by post-translational modification (i.e., tyrosine-phosphorylation) of CI and/or CII and depends on nutrient availability.

Effect of Src on hepatic metabolomics according to nutrient availability

To further characterize the role of Src in metabolism, we analyzed Src+/+ and Src−/− mice fed ad libitum and fasted for 24h using untargeted GC-MS metabolomics. We identified 41 metabolites involved in various pathways related to metabolism of glucose, fatty acids, amino acids, ketone bodies and nucleotides (Fig. 3 and Supplementary Fig. S2). Metabolomes were analyzed using multivariate PLS-DA [46, 47] to evaluate how they were affected by Src during fasting (Fig. 3a). The two main PLS components, PC1 and PC2, explained 16.3% and 28.1% of the total variance, respectively. Interestingly, two-dimensional score plots showed that the metabolomes of Src+/+ and Src−/− fasted mice cluster with Src+/+ mice fed ad libitum (Fig. 3a), further confirming that the effect of Src on metabolism depends on nutrient availability. Variable importance of projection (VIP) scores identified 12 metabolites significantly driving (i.e., metabolites with VIP score > 1) the specific metabolomic signature among experimental groups (Fig. 3b). Among these key metabolites, TCA cycle intermediates succinate, fumarate, and malate were all significantly increased after deletion of Src in mice fed ad libitum whereas fumarate and malate were decreased by fasting only in Src−/− mice (Fig. 3c). Glycolytic intermediates DHAP and 3-phosphoglycerate were significantly decreased upon fasting only in Src−/− mice (Fig. 3d), whereas pyruvate was significantly decreased by deletion of Src in fasted mice (Fig. 3d). Levels of amino acids aspartate, glutamate, glutamine and histidine were higher in Src−/− mice fed ad libitum, whereas their levels decreased upon fasting only in Src+/+ mice (Fig. 3e). Levels of taurine were significantly increased by fasting in both Src+/+ and Src−/− mice (Fig. 3e). Similarly, levels of the ketone body 3-hydroxybutyrate was significantly increased for both Src+/+ and Src−/− mice in response to fasting (Fig. 3f). Several other metabolites involved in different metabolic pathways did not vary among experimental
Fig. 2. Modulation of mitochondrial metabolism by Src according to nutrient availability. (a, b) Levels of blood glucose (a) and liver glycogen (b) in fed and fasted Src\textsuperscript{+/+} and Src\textsuperscript{-/-} mice (n=3). (c-e) Oxygen consumption rates of liver mitochondria obtained from fed and fasted Src\textsuperscript{+/+} and Src\textsuperscript{-/-} mice (n=7) in presence of specific substrates and inhibitors to evaluate complex CI+III+IV-dependent respiration (c), CI+II+III+IV-dependent respiration (d), and CIV-dependent respiration (e). (f-i) Enzymatic activities of complexes I+III (f), complex II (g), complex IV (h) and citrate synthase (i) in liver of fed and fasted Src\textsuperscript{+/+} and Src\textsuperscript{-/-} mice (n=4). (j) Representative immunoblot (n=4) of Src, SDHa, ATP5a, NDUFA9, SOD2, TOM20 and Cytc in liver of fed and fasted Src\textsuperscript{+/+} and Src\textsuperscript{-/-} mice. See Fig. S1c for quantification. (k) H\textsubscript{2}O\textsubscript{2} production by liver mitochondria isolated from fed and fasted Src\textsuperscript{+/+} and Src\textsuperscript{-/-} mice (n=5). Data are presented as mean ± s.e.m. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 determined by a two-way ANOVA followed by post-hoc Tukey’s test.
Fig. 3. Modulation of metabolomics by Src according to nutrient availability. (a) PLS-DA score plots of metabolites in fed or fasted Src+/+ and Src−/− mice. Ellipses represent 95% confidence intervals for each individual group on PLS-DA plots with the variance proportion represented by principal components 1 and 2 (PC1 & PC2, respectively). (b) Variable importance of projection (VIP) scores of PLS-DA, which identify the key metabolites driving the metabolomic signature for fed and fasted Src+/+ and Src−/− mice. (c-f) Quantitative analysis of intermediates of TCA cycle (c) and glycolysis/neoglucogenesis (d), amino acids (e) and ketone bodies (f) identified as drivers of the metabolomic signature of Src+/+ and Src−/− mice fed ad libitum or fasted. See also Fig. S2 for metabolites not significantly different among experimental groups. Data are presented as mean ± s.e.m. (n = 7). *p<0.05, **p<0.01, ***p<0.001 determined by a two-way ANOVA followed by post-hoc Tukey’s test.
groups (Supplementary Fig. S2). Globally, these results suggest that ETS defects induced by deletion of Src in fed mice result in accumulation of intermediates of TCA cycle and amino acid metabolism. However, this accumulation is no longer observed in fasted mice, as the levels of metabolites were mostly similar between Src+/+ and Src−/− mice.

**Effect of Src on the hepatic proteome according to nutrient availability**

The mitochondrial proteome of liver enriched-mitochondrial fractions derived from fed and fasted Src+/+ and Src−/− mice was then characterized by nanoLC-MS/MS. Among the identified proteins (Supplementary Table S1), 612 are mitochondrial proteins (Supplementary Table S2). Considering that the mitochondrial proteome includes approximately 1,000 proteins [51] (as compared to 14,000 proteins in the total liver proteome), we obtained a ≈5-fold enrichment of mitochondrial proteins in our liver mitochondria-enriched fractions (see also Supplementary Fig. S3a). PLS-DA of all the detected proteins showed that the hepatic proteomic signatures of fed and fasted Src+/+ mice cluster together whereas fed and fasted Src−/− mice have different proteomic signatures (Fig. 4a). Procedures to isolate mitochondria are designed to yield enriched fractions of functional mitochondria. However, they are not designed to enrich proteins from all the subcellular compartments equally and likely lead to an over- and/or under-representation of proteins from other specific compartments. We thus repeated PLS-DA only on the detected mitochondrial proteins to avoid such bias. The mitochondrial proteomic signature among experimental groups was similar to the signature observed when all the proteins identified in our mitochondria-enriched fractions were considered (Fig. 4a and b), confirming that the variance observed in our proteomic analysis is mostly explained by changes in mitochondrial proteins. Among the identified mitochondrial proteins, 83 proteins had significant impact (VIP score > 1) on the mitochondrial proteomic signature among the different groups (Fig. 4c, Table 1 and Supplementary Table S3). Interestingly, most of these proteins are involved in metabolism of fatty acid, pyruvate and amino acid. Among these 83 proteins, two-way ANOVA revealed significant genotype×diet interaction only for glycine amidinotransferase (GATM) and acyl-coenzyme A thioesterase 9 (ACOT9) (Fig. 4d and Supplementary Fig. S3b), confirming that Src-dependent modulation of metabolism does not involve major change in mitochondrial mass. Deletion of Src significantly increased the levels of GATM and ACOT9 in mice fed ad libitum but not in fasted mice (Fig. 4d), corroborating that the impact of Src differs between fed and fasted mice.

**Effect of Src on the hepatic phosphotyrosine proteome according to nutrient availability**

To understand the role of Src-mediated signaling on hepatic metabolism, we first examined the level and activity of Src in liver enriched-mitochondrial fractions. The results showed that fasting induces activation of Src (Fig. 5a and b, Supplementary Fig. S4a). To identify the mitochondrial proteins potentially targeted by Src, we quantitatively analyzed tyrosine-phosphorylated proteins among liver enriched-mitochondrial fractions derived from fed and fasted Src+/+ and Src−/− mice using immuno-enrichment of phosphotyrosine peptides and nanoLC-MS/MS. We detected 701 tyrosine-phosphorylated peptides (Supplementary Table S4), among which 79 correspond to mitochondrial proteins (Supplementary Table S5 and Fig S4b). PLS-DA using all the identified phosphopeptides showed different phosphotyrosine proteomic signatures for each individual experimental group (Fig. 5b). Again, to avoid bias due to the presence of over- or under-represented phosphopeptides from extra-mitochondrial compartments, we repeated PLS-DA using only the 79 mitochondrial phosphopeptides, and obtained similar phosphotyrosine proteomic signatures (Fig. 5c). Among the mitochondrial phosphotyrosine peptides, 13 had VIP score > 1 (Fig. 5d and Table 2) and were considered crucial to the phosphotyrosine proteomic signature among the four experimental groups. These phosphotyrosine peptides correspond to proteins with various molecular functions, including OXPHOS as well as metabolism of fatty acid and amino acid. Two-way ANOVA revealed significant genotype×diet interaction for one phosphotyrosine peptide corresponding to NDUFA8, a nuclear-encoded subunit of CI (Fig. 5e and Fig S4c). We also observed a significant decrease of phosphorylated Y142-NDUFA8 upon deletion of Src.
in mice fed *ad libitum* (Fig. 5e). Phosphorylation of Y142-NDUFA8 was however increased upon deletion of *Src* in fasted mice, suggesting that this residue might be targeted by other kinases. *In silico* analyses using the NetPhos 3.1 online tool (http://www.cbs.dtu.dk/index.php) predicted a probability of 0.456 that *Src* directly phosphorylates Y142-NDUFA8. Similar results were obtained using the GPS online tool (prediction score: 2.708; http://gps.biocuckoo.cn). Overall, phosphoproteomic analyses support the hypothesis that *Src* impacts on hepatic metabolism in a nutrient availability dependent manner.
Table 1. Mitochondrial proteins significantly driving the mitochondrial proteomic signature among Src+/+ and Src-/− mice fed ad libitum or 24h-fasted. Only proteins with VIP score > 2 are presented. See Supplementary Table S3 for all proteins with VIP score > 1.

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<td>Cbl2</td>
<td>Kynurenine-oxoglutarate transaminase</td>
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<td>Long-chain-fatty-acid–CoA ligase 3</td>
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<td>Aldh18a1</td>
<td>Delta-1-pyrroline-5-carboxylate synthase</td>
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<td>Mthfd1l</td>
<td>Monofunctional C1-tetrahydrofolate synthase</td>
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<td>Gatm</td>
<td>Glycine amidinotransferase</td>
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Table 2. Phosphorylation sites among mitochondrial proteins significantly driving the phosphotyrosine proteomic signature in liver mitochondria derived from Src+/+ and Src-/− mice fed ad libitum or 24h-fasted. Only proteins with VIP score > 1 are presented.

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<td>Stxbp1</td>
<td>Syntaxin-binding protein 1</td>
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<td>Short chain L-3-hydroxyacyl-CoA dehydrogenase</td>
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<td>Ass1</td>
<td>Argininosuccinate synthase</td>
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<td>Got2</td>
<td>Aspartate aminotransferase</td>
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<td>Fgr</td>
<td>Tyrosine-protein kinase Fgr</td>
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<td>Hcls1</td>
<td>Hematopoietic lineage cell-specific protein</td>
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<td>Glycogen synthase kinase-3 beta</td>
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<td>Q8VCF0</td>
<td>Mavs</td>
<td>Mitochondrial antiviral-signaling protein</td>
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<td>Aldh6a1</td>
<td>Methylmalonate-semialdehyde dehydrogenase</td>
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<td>Octd1</td>
<td>OCIA domain-containing protein 1</td>
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Fig. 5. The impact of Src on mitochondrial phosphotyrosine proteomics according to nutrient availability. (a) Top. Representative immunoblots (n=4) of pY416-Src, total Src and SDHa in liver mitochondria isolated from Src+/+ and Src−/− mice fed ad libitum or fasted during 24h. Note that the faint pY416-Src signal in Src−/− mice is likely due to other Src kinases present in liver mitochondria. Bottom. Quantification of Src activity (labeling of pY416-Src normalized to Src labeling), indicating that Src activity is higher upon fasting. (b, c) PLS-DA plots showing phosphotyrosine proteomic signatures of Src+/+ and Src−/− mice fed ad libitum or fasted when all phosphotyrosine peptides (b) or only mitochondrial phosphotyrosine peptides (c) detected by nanoLC-MS/MS were considered. (d) Variable importance of projection (VIP) scores of PLS-DA which identify the key proteins driving the phosphotyrosine proteomic signatures of fed and fasted Src+/+ and Src−/− mice. (e) Quantitative analysis of phosphopeptides driving the phosphotyrosine proteomic signature of Src+/+ and Src−/− mice fed ad libitum or fasted. See also Fig. S5 for key phosphotyrosine proteins not significantly different among experimental groups. Data are mean ± s.e.m. (n = 3). *p<0.05, **p<0.01, ***p<0.001 determined by a two-way ANOVA followed by post-hoc Tukey’s test.
Discussion

Src is involved in multiple signaling pathways maintaining cellular homeostasis during metabolic stress. Notably, Src directly targets mitochondrial proteins to impact on mitochondrial functions [29, 30, 49]. Although metabolic stress can lead to dysregulation of Src activity and mitochondrial metabolism [36–39], the impact of Src on metabolic homeostasis during challenging nutrient conditions remains poorly understood. The aim of our study was thus to characterize the role of Src in metabolism. Our findings suggest that deletion of Src inhibits hepatic OXPHOS. Using omics, we also showed that deletion of Src alters TCA cycle, amino acid and fatty acid metabolism as well as phosphorylation of Y142-NDUFA8 (Fig. 6). Our findings highlight the role of Src as a central player in mitochondrial metabolism and its impacts on the metabolic adjustment to nutrient stress.

This work confirms that Src can target OXPHOS components since it is present inside mitochondria, as previously described [26, 27]. Intra-mitochondrial Src was previously shown to be associated to IMM using immunogold labeling [26, 27]. Here, we showed that mitochondrial Src is also resistant to degradation by trypsin after swelling of isolated organelles. This finding thus suggests that Src is also localized in matrix, and that it could have other targets than only IMM proteins facing the IMS.

We identified 13 phosphotyrosine residues that were linked to the overall phosphotyrosine proteomic signature of Src+/+ and Src−/− mice fed or fasted, including Y142-NDUFA8, Y136-CYB5A and Y109-NDUFC2. The only protein with lower tyrosine-phosphorylation in Src−/− mice is the CI subunit NDUFA8. Phosphorylation of Y142-NDUFA8 was ≈7-fold lower upon deletion of Src in mice fed ad libitum. NDUFA8 subunit is part of the proton pumping P-module of CI [52]. This subunit is located at the proximal end of the membrane arm and faces the intermembrane space, which is consistent with our findings and previous observations showing that Src is located within mitochondria [53]. NDUFA8 is considered as an accessory subunit essential for the proper assembly of the quinone binding Q module [54]. Interestingly, the alteration of Y142-NDUFA8 phosphorylation induced by the anaesthetic propofol is associated with reduced levels of several CI subunits in HT22 cells [55]. It is therefore possible that phosphorylation status of Y142-NDUFA8 would impact assembly and stability of CI in Src−/− mice. Our data however suggest that phosphorylation status of Y142-NDUFA8 is not sufficient to explain functional changes of mitochondrial metabolism since levels of phosphorylated Y142-NDUFA8 peptides do not match respiration rates among Src+/+ and Src−/− mice fed ad libitum or fasted during 24h. Also, our findings do not allow to conclude that Src phosphorylates NDUFA8 directly or indirectly. First, in silico analyses revealed significant probability that Src targets directly Y142-NDUFA8. Phosphorylation of this protein however increased in Src−/− mice upon fasting, suggesting that other kinases could target this residue. It is thus possible that Y142-NDUFA8 is mainly phosphorylated by Src when mice are fed ad libitum and targeted by other kinases in fasted Src+/+ mice. Future studies should examine whether Src can directly phosphorylate this residue. Src could also target (directly or not) other proteins inside hepatic mitochondria for which phosphotyrosine residue(s) could have been lost during the experimental procedure. Notably, enzymatic activities of CII and CS were decreased in liver mitochondria after deletion of Src although these proteins were not detected in our phosphoproteomic characterization. Several previously identified targets of mitochondrial Src, such as NDUFB10 and COXII [27, 30], were also not detected in the present study. It is possible that such phosphorylation events appear in a cell type-specific manner. For instance, over-expression of Src specifically in mitochondria did not alter phosphorylation of COXII in breast cancer cells MDA-MB-231 [56].

Hundreds of phosphorylation sites have been previously identified among mitochondrial proteins using various approaches and tissues [57]. Phosphotyrosines usually represent less than 1-10% of phosphorylation events in global proteomic studies: 35 phosphotyrosine residues among 594 phosphorylation sites were recently reported in rat liver mitochondria [58] whereas 16 phosphotyrosines among 155 phosphorylation sites were reported in human
skeletal mitochondria [59]. Here, we identified 79 tyrosine-phosphorylated mitochondrial proteins upon immuno-enrichment of phosphotyrosine. These proteins are located in different mitochondrial compartments and involved in several processes, such as OXPHOS (including the CIV subunit NDUF4A and the CIV assembly factor COX20), metabolism of fatty and amino acids (including acyl-coenzyme A synthetase 1 and aspartate aminotransferase) as well as mitochondrial dynamics (including MICOS complex subunit 19 and prohibitin). Interestingly, similar tyrosine-phosphorylation of ANT1 and ANT2 was previously observed [60]. It will be important to compare levels of dephosphorylated and phosphorylated peptides to examine the functional impact of these individual phosphorylation sites in future studies. Various tyrosine-phosphorylated mitochondrial proteins previously identified were however not observed in the present study. These discrepancies suggest that tyrosine-phosphorylation is a fine-tuned process rather than a unspecific event. For instance, rat liver mitochondria are enriched in phosphoproteins involved in amino acid and fatty acid metabolism, whereas heart and skeletal muscle mitochondria are enriched in phosphoproteins involved in TCA cycle and OXPHOS [58]. Also, a significant fraction of phosphotyrosines reported in the literature were observed in cancer tissue and cells (see www.phosphosite.org), in which several tyrosine kinases, including Src, are over-activated [61]. It is therefore possible that phosphotyrosine levels are lower in non-cancer tissue. Our technical procedures could have nevertheless removed several phosphosites. For instance, freezing-thawing between isolation of mitochondrial fractions and phosphoproteomics could have resulted in loss of phosphorylation sites.

Although they have been available for decades, no metabolic characterization of Src<sup>−/−</sup> mice has been performed. Our findings demonstrate that deletion of Src induces major metabolic shifts in liver (Fig. 6). In various types of tumors, overexpression or overactivation of this kinase stimulates glucose uptake [62], hexokinase activity and production of lactate [20], whereas it inhibits pyruvate dehydrogenase [29] and OXPHOS [56]. Here, Src<sup>−/−</sup> mice showed accumulation of the TCA cycle intermediates succinate, fumarate and malate despite similar glycogen and blood glucose availability. It is thus possible that inhibition of ETS complexes resulted in accumulation of metabolites upstream of ETS. Several glycolytic and other TCA cycle metabolites were however not increased in Src<sup>−/−</sup> mice. Fatty acid oxidation and catabolism of amino acid could have also been increased to compensate the inhibition of ETS induced by deletion of Src. For instance, we observed increased levels of GATM and catabolism of amino acid could have resulted from higher production of succinyl-CoA by increased levels of ACOT9. Here, accumulation of succinate, fumarate and malate observed in Src<sup>−/−</sup> mice could thus result from higher production of succinyl-CoA by increased levels of ACOT9. We therefore suggest that Src is central to maintain equilibrium among metabolism of carbohydrate, fatty acid and amino acid.

Multivariate analysis of metabolomics and (phosphotyrosine) proteomics demonstrated specific phenotypic signatures for control and Src<sup>−/−</sup> mice, indicating that Src is important for the hepatic metabolic adjustment induced by nutrient stress. Src activity was increased in liver mitochondria-enriched fractions upon fasting, likely to allow adjustment of metabolism to nutrient deprivation. Activation of Src kinases is a common phenomenon observed in various types of metabolic stress. For instance, ATP levels are maintained by upregulated fatty acid oxidation in triple negative breast cancer cells, leading to auto-activation of Src and higher metastasis potential [67]. Similarly, Fyn activity is increased in macrophage from mice fed a high-fat diet [68], suggesting that the higher activation of Src observed in liver...
mitochondria-enriched fractions from Src−/− fasted mice could have been induced by higher fatty acid oxidation. In this study, pyruvate level was decreased in Src−/− fasted mice despite similar glycogen and blood glucose availability. PDH activity is inhibited by fasting in skeletal muscle [69] and by Src kinase [29, 69], suggesting that glycolysis is not the main pathway fueling oxidative metabolism in Src−/− fasted mice.

Fig. 6. Deletion of Src induces shifts in hepatic metabolism. Representation of metabolic alterations observed in liver of Src−/− mice. Major metabolic pathways are indicated (in purple): glycolysis, urea cycle, β-oxidation, tricarboxylic (TCA) cycle and oxidative phosphorylation (OXPHOS). Oxidation of amino acids are also indicated. Previous works suggested that Src is present in IMS whereas our findings indicate that Src is also localized in the matrix. Deletion of Src increased levels of proteins GTAM (glycine amidinotransferase) and Acyl-CoA thioesterase 9 (ACOT9), amino acids aspartate (Asp), glutamate (Glu), glutamine (Gln) and of TCA cycle intermediates malate, fumarate and succinate (indicated in green) in liver of mice fed ad libitum. These changes were associated with lower activities of citrate synthase (CS), and of electron transport system complexes I, II and III (indicated by red arrows) in liver of Src−/− mice fed ad libitum. Phosphotyrosine proteomic analyses showed lower levels of phosphorylated NDUFA8 in liver of Src−/− mice fed ad libitum, suggesting that Src target (directly or indirectly) this CI subunit. Arg: Arginine; Asn: Asparagine; Asp: Aspartate; Cyt c: Cytochrome c; e−: electron; Gln: glutamine; Glu: glutamate; FFA: Free Fratty Acid; IMM: Inner Mitochondrial Membrane; IMS: Intermembrane Space; Leu: Leucine; Lys: Lysine; Orn: Ornithine; Phe: Phenylalanine; Q: Ubiquinone; Tyr: Tyrosine.
Conclusion

In conclusion, our work demonstrates that Src has a significant impact on metabolism in multiple tissues, including brain, kidney and liver. Deletion of Src induces inhibition of mitochondrial respiration and alters intermediates and proteins involved in metabolism of carbohydrates, amino acid and fatty acid. The present study also reports several tyrosine-phosphorylated mitochondrial proteins involved in various functions, ranging from OXPHOS, metabolism of fatty acid or amino acid, to mitochondrial dynamics. Future experiments will be necessary to examine how Src modulates oxidation of amino acid and fatty acid, test whether Src can directly target Y142-NDUFA8 and evaluate the functional impacts of the reported phosphotyrosine sites including Y142-NDUFA8.

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Author Contributions

HG, NP and EHC designed the study. HG, MCS, SJ and MADJ performed experiments. HG, MCS, NP and EHC analyzed data. HG, NP and EHC wrote and edited the manuscript. All authors approved the final version of the manuscript.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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