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The low molecular weight protein tyrosine phosphatase promotes adipogenesis and subcutaneous adipocyte hypertrophy

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Abstract

Obesity is a major contributing factor to the pathogenesis of type 2 diabetes. Multiple human genetics studies suggest that high activity of the low molecular weight protein tyrosine phosphatase (LMPTP) promotes metabolic syndrome in obesity. We reported that LMPTP is a critical promoter of insulin resistance in obesity by regulating liver insulin receptor signaling and that inhibition of LMPTP reverses obesity-associated diabetes in mice. Since LMPTP is expressed in adipose tissue but little is known about its function, here we examined the role of LMPTP in adipocyte biology. Using conditional knockout mice, we found that selective deletion of LMPTP in adipocytes impaired obesity-induced subcutaneous adipocyte hypertrophy. We assessed the role of LMPTP in adipogenesis *in vitro*, and found that LMPTP deletion or knockdown substantially impaired differentiation of primary preadipocytes and 3T3-L1 cells into adipocytes, respectively. Inhibition of LMPTP in 3T3-L1 preadipocytes also reduced adipogenesis and expression of pro-adipogenic transcription factors peroxisome proliferator activated receptor gamma (PPARγ) and CCAAT/enhancer-binding protein alpha. Inhibition of LMPTP increased basal phosphorylation of platelet-derived growth factor receptor α on activation motif residue Y849 in 3T3-L1, resulting in increased activation of the mitogen-associated protein kinases p38 and c-Jun N-terminal kinase and increased PPARγ phosphorylation on inhibitory residue S82. Analysis of the metabolome of differentiating 3T3-L1 cells suggested that LMPTP inhibition decreased cell glucose utilization while enhancing mitochondrial respiration and nucleotide synthesis. In summary, we report a

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Conflict of interest statement

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novel role for LMPTP as a key driver of adipocyte differentiation via control of PDGFRα signaling.

Keywords

LMPTP; protein tyrosine phosphatase; adipogenesis; adipocyte; phosphorylation

Introduction

Obesity is often accompanied by metabolic syndrome -metabolic abnormalities such as insulin resistance, hypertriglyceridemia and hypercholesterolemia that frequently occur together and increase risk of obesity-related complications and lethality(Chooi, 2019; Moller, 2005). As the prevalence of obesity has substantially risen in developed countries, understanding the mechanisms underlying this condition is important for the development of novel treatment strategies.

The low molecular weight protein tyrosine phosphatase (LMPTP) is a small (18 kDa), ubiquitously expressed PTP encoded by the *ACP1* gene(Wo, 1992). LMPTP and the recently reclassified SSU72 are the only members of the class II subfamily of PTPs(Alonso, 2016). Genetic evidence in humans suggests LMPTP associates with several features of metabolic syndrome. *ACP1* genotypes encoding low LMPTP enzymatic activity confer protection against hyperglycemia and dyslipidemia in obese subjects and associate with clinical variability in obesity(E. Bottini, 1990; N. Bottini, 2002; Lucarini, 1997; Lucarini, 1990; Paggi, 1991).

LMPTP is considered an inhibitor of receptor tyrosine kinases such as the insulin receptor (IR) through dephosphorylation of phospho-tyrosine residues in the kinase activation motif(Caselli, 2016; S. K. Pandey, 2007). In mice, LMPTP knockdown using antisense oligonucleotides (ASO) decreases insulin resistance in diet-induced obese (DIO) C57BL/6 (B6) mice and enhances IR phosphorylation in liver and adipose tissue (AT)(S. K. Pandey, 2007). We reported that LMPTP knockout (KO) or inhibition using a small-molecule inhibitor improves glucose tolerance and lowers fasting insulin levels in DIO mice, without affecting body weight(Stanford, 2017). Liver-specific LMPTP deletion using an albumin promoter-driven Cre recombinase recapitulates the effects on glucose tolerance and insulin levels observed in DIO LMPTP KO mice, while no effects on glucose tolerance or insulin levels are observed in DIO mice carrying deletion of LMPTP in adipocytes using an adiponectin promoter-driven cre(Stanford, 2017). We also observed that treatment with an LMPTP inhibitor or LMPTP deletion in the liver enhances insulin-induced liver IR phosphorylation levels(Stanford, 2017).

In addition to its role in IR signaling, LMPTP reportedly regulates platelet-derived growth factor receptor (PDGFR) activation in fibroblasts. NIH-3T3 cells overexpressing LMPTP show impaired proliferative responses to FBS or PDGF stimulation(Berti, 1994). Catalytically inactive LMPTP-C12S mutant precipitates with PDGFR when overexpressed in NIH-3T3 cells(Cirri, 1998) and in pull-down assays(Chiarugi, 2002). Overexpression of

LMPTP-C12S enhances PDGF-induced PDGFR phosphorylation and kinase activity in NIH-3T3 cells(Chiarugi, 2002).

Multiple reports demonstrate that PDGFR signaling exerts an inhibitory effect on white adipose tissue (WAT) development and adipocyte differentiation. Knock-in of constitutively-active PDGFRα-D842V mutant in Myf5-expressing cells severely disrupts WAT organogenesis, while knock-in of weakly hyperactive PDGFRα-V561D mutant in AT using Sox2-Cre leads to WAT lipodystrophy(Sun, 2017). WAT from newborn Sox2-Cre PDGFRα-V561D mice displays reduced adipocytes and increased fibroblasts(Sun, 2017). Primary adipocyte precursors isolated from mice carrying PDGFRα-D842V show enhanced proliferation *in vitro* and impaired adipogenesis and expression of PPARγ in response to an adipogenic cocktail(Accili, 1991). PDGF inhibits *in vitro* adipogenesis of 3T3-L1(Artemenko, 2005) and 3T3-F442A(Hayashi, 1981) fibroblasts, and PDGFR-blocking antibodies promote adipogenesis and PPARγ expression in mesenchymal stem cell cultures(Fitter, 2012).

Given that LMPTP is expressed in AT yet its role is virtually unknown, we sought to understand the function of LMPTP in adipocyte differentiation and growth. Here, using adipocyte-specific deletion of LMPTP, combined with cell signaling and metabolomic assays of differentiating adipocytes, we identified a novel role for LMPTP as a promoter of obesity-induced hypertrophy in subcutaneous (SubQ) AT and of adipocyte differentiation by shifting the metabolic profile of differentiating adipocytes downstream PDGFRα signaling.

Materials and Methods

Additional materials and methods are described in the Supporting Information.

Mice

Animal experiments were conducted in accordance with Institutional Animal Care and Use Committee approved protocols at the La Jolla Institute for Allergy & Immunology (#AP126-NB3) and the University of California, San Diego (S16098). B6 mice were purchased from Jackson Laboratory (JAX#000664). Generation of *Acp1* floxed ($Acp1^{fl}$) mice carrying Cre expressed under the adiponectin (Adipoq) promoter was described(Stanford, 2017). LMPTP KO mice were generated by crossing $Acp1^{fl}$ mice with mice carrying Cre expressed under the cytomegalovirus promoter (JAX #006054), followed by crossing to B6 mice to remove the Cre transgene. To generate DIO mice, male littermate $Acp1^{fl/fl}$ Cre^{+} and Cre^{-} littermate mice were fed high-fat diet (HFD) chow containing 60 kcal % fat (Research Diets) for 12 months starting at 4–8 weeks of age.

Adipocyte size quantification

Subcutaneous and epididymal fat pads from male DIO Adipoq-Cre+ and Cre- littermates were fixed in formalin for 24 hr, processed, embedded in paraffin, cut and stained with H&E according to standard protocols. Cells were analyzed using the Measure and Label Macro in ImageJ software.

Adipogenesis assays

3T3-L1 preadipocytes were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% bovine calf serum (BCS), 100 U/ml penicillin, and 100 μg/ml streptomycin (BCS/DMEM). Cells were seeded in 6-well plates and grown to confluence. Undifferentiated wells remained in growth media for the duration of the experiment and media was replaced every 2 days. Differentiated cells were treated with growth media in which the BCS was replaced with 10% fetal bovine serum (FBS/DMEM). Two days post-confluence, cells were stimulated with FBS/DMEM induction media containing 1 μM dexamethasone, 170 nM insulin, 0.52 mM 3-isobutyl-1-methylxanthine (IBMX). After 2 days, media was exchanged for 10% FBS/DMEM containing 170 nM insulin. After 2 days, media was exchanged for FBS/DMEM. Cells were then maintained in FBS/DMEM, changed every 2 days, until 10 days post-confluence. For inhibition studies, cells were treated with 0.1% dimethyl sulfoxide (DMSO) or 10 μM LMPTP inhibitor starting on the day of confluence and every 2 days thereafter; for knockdown studies, cells were treated with 10 μM non-targeting ASO or LMPTP-targeted ASO starting on the day of confluence and days 2 and 4 post-confluence. Adipogenesis was quantified after addition of AdipoRed reagent according the manufacturer's instructions (Lonza). Fluorescence was measured using a Tecan microplate reader with excitation at 485 nm and emission at 572 nm.

Primary mouse wild-type (WT) or LMPTP KO preadipocytes were isolated from subcutaneous fat pads by digestion in Hank's Balanced Salt Solution (HBSS) containing 3% FBS, 1 mM $CaCL_2$, 1 mM $MgCl_2$, 10 mg/ml Collagenase II (Worthington), and 2.4 U/ml Dispase II for 30 min. Digests were filtered through a 40 uM nylon cell strainer and centrifuged at 350 g for 5 min. Red blood cells were lysed (RBC Lysis Buffer, CST) and digests resuspended and re-centrifuged. Remaining preadipocytes were plated and cultured in DMEM/F12 containing 10% FBS (FBS/DF12) and grown to confluence. Undifferentiated cells remained in FBS/DF12 for the duration of the experiment. Cells were induced to differentiate as for 3T3-L1, replacing FBS/DMEM with FBS/DF12.

Primary human visceral preadipocytes (Lonza) were grown in Preadipocyte Basal Medium-2 with PGM-2 SingleQuots supplement. Cells were induced to differentiate according to the Poietics™ Visceral Preadipocyte (Lonza) adipogenesis assay instructions in the presence of 10 μM Ctrl ASO or LMPTP-targeted ASO.

Untargeted polar and targeted nonpolar ultra-high performance liquid chromatography coupled to mass spectrometry (UHPLC-MS)

Metabolites were extracted by the modified Bligh-Dyer method in extraction buffer with butylated hydroxytoluene to preserve metabolites susceptible to oxidation(Lu, 2019; Sweeney, 2020). Immediately following extraction and just prior to analysis, polar fractions were transferred to LC-MS vials and spiked with internal standards consisting of a mixture of deuterated stable isotopes used to ensure instrument stability through monitoring retention time and ionization efficiency.

The polar untargeted metabolic analysis was performed on a Q Exactive Hybrid Quadrupole Orbitrap mass spectrometer (Thermo) coupled to a Vanquish Horizon UHPLC system

(Thermo) via an electrospray ionization source simultaneously operating in fast negative/positive ionization switching mode. For the primary analysis, to resolve redox cofactors and nucleotides, a SeQuant ZIC-HILIC 3.5 μm, 100 Å, 150×2.1 mm column (Millipore Sigma) was used(Sweeney, 2020). Following acquisition, samples were evaporated to dryness in a CentriVap refrigerated vacuum concentrator (Labconco) at 4°C, then resuspended in HPLC water. The secondary analysis, to resolve polyamines, a Kinetex C18, 2.6 μm, 100 Å, 150×2.1 mm column (Phenomenex) with the mobile phases, water + 0.2% formic acid (A) and methanol (B), was used. A gradient separation was achieved at a flow rate of 150 μl/min for with an injection volume of 5 μl, the elution of A:B was as follows: 98:2 for 4 min, 20:80 for 10 min, 2:98 for 7 min, and 98:2 for 14 min. Detection for both methods was performed in full MS with the following conditions: spray voltage, 3.0 kV; capillary temperature, 320°C; sheath gas, 45 (arbitrary units); auxiliary gas, 10 (arbitrary units); microscans, 1; AGC target, 1e6; maximum injection time, 200 ms; mass resolution, 70,000 FWHM at *m/z* range of 70–1000 (HILIC) and 50–750 (Kinetex). Nonpolar fractions were analyzed by a targeted UHPLC-MS method that allows measurement of Coenzymes Q9 and Q10 redox states with all experimental conditions(R. Pandey, 2018). Following acquisition, samples were evaporated to dryness and stored at −80°C. For each analysis, the MS detector was calibrated prior to analysis to maintain mass accuracy below 5 ppm. A pooled quality control (QC) sample was acquired every six samples to ensure consistency in data acquisition across the entire batch.

Statistical analysis

The two-way analysis of variance (ANOVA), unpaired t-test, and unpaired t-test with Welch's correction were performed using GraphPad Prism software. p-values <0.05 were considered significant. In metabolomics analyses, probabilistic quotient normalization was performed for normalization, and false discovery rate (FDR) corrected q-value was used to determine statistical significance in the MATLAB programming environment (Mathworks). Principal component analysis (PCA) was carried out in PLS-Toolbox (version 8.9, Eigenvector Research) in MATLAB after glog−9 transform. Volcano plots were completed using R 3.5.0(Team, 2019) and the *EnhancedVolcano*(Blighe K., 2020). The fold change and q-value were transformed by log2 and –log10, respectively. Significant metabolites were selected by volcano plot using log2-fold change cutoff, 1.5 and q-value cutoff, <0.05.

Results

LMPTP deletion leads to reduced subcutaneous adipocyte size

To examine the role of LMPTP in adipocytes, we placed male littermate $Acp1^{fl/fl}$ Adipoq-Cre^{+} and Cre^{-} littermate mice on HFD chow for 12 months. Consistent with previous data(Stanford, 2017), no difference in body weight was observed between the DIO Cre^{+} and Cre^{-} mice (Fig. 1a), however subcutaneous (SubQ) fat pads from Cre^{+} mice were significantly lighter in weight (Fig. 1b). H&E staining of sections from SubQ fat pads revealed that SubQ adipocytes from Cre^{+} mice were significantly reduced in size compared to Cre^{-} mice (Fig. 1c–d). Size distribution analysis confirmed a skewing of the adipocyte distribution from Cre^{+} mice, with a higher frequency of smaller size cells, compared to Cre^{-} mice (Fig. 1e). In contrast, epididymal adipocytes from Cre^{+} mice displayed a slight

increase in size compared to Cre^{-} mice (Fig. S1). Taken together, these data using conditional LMPTP deletion suggest that LMPTP promotes SubQ adiposity in obesity through an adipocyte-specific mechanism.

LMPTP promotes adipocyte differentiation *in vitro*

We examined whether LMPTP promotes the ability of preadipocytes to differentiate into adipocytes. We isolated stromal cells from SubQ fat pads from littermate global LMPTP KO and WT mice and subjected them to an insulin-dependent differentiation assay and assessed intracellular lipid accumulation using AdipoRed. Cells from LMPTP KO mice showed substantially reduced signal in the AdipoRed assay (Fig. 2a), suggesting impaired ability to differentiate into adipocytes. We assessed the effect of LMPTP knockdown using cell-permeable ASO (Fig. S2) on adipogenesis of 3T3-L1 cells and primary human preadipocytes, and observed that LMPTP deficiency led to substantially reduced adipogenesis (Fig. 2b–c). To test whether the adipogenic role of LMPTP is due to the catalytic activity of the enzyme, we subjected 3T3-L1 to adipogenesis conditions in the presence of selective LMPTP inhibitors Compd. 3 and 23(Stanford, 2017). LMPTP inhibition substantially reduced adipogenesis of 3T3-L1 (Fig. 2d–e). Taken together, these data suggest LMPTP activity is essential for the intrinsic differentiation of preadipocytes into adipocytes.

LMPTP promotes expression of key adipogenic genes during the adipogenesis process

We reasoned the impaired adipogenesis observed in LMPTP-deficient or -inhibited cells could be due to reduced expression of adipogenic genes, therefore we tested expression of key adipogenic transcription factors in 3T3-L1 subjected to LMPTP inhibition. While we observed no effect on expression of the gene encoding early adipogenic transcription factor CCAAT/enhancer-binding protein β (*Cebpb*)(Lee, 2019; Rosen, 2000), we noticed substantially reduced expression of genes encoding peroxisome proliferator activated receptor γ (*Pparg*) and CCAAT/enhancer-binding protein α (*Cebpa*)(Lee, 2019), which was observable at day 4 and became more divergent over the course of the assay (Fig. 3). We examined expression of PPARγ-dependent genes(Ma, 2018) such as those encoding adiponectin (*Adipoq*) and fatty acid synthase (*Fas*), and observed significant reductions (Fig. 3). Taken together, these data suggest LMPTP promotes expression of key adipogenic transcription factors PPARγ and CEBPα, as well as downstream PPARγ-dependent genes.

LMPTP suppresses basal PDGFRα signaling in 3T3-L1

LMPTP was suggested to regulate phosphorylation of the PDGFR in fibroblasts(Chiarugi, 2002), and PDGF signaling was reported to inhibit 3T3-L1 adipogenesis(Artemenko, 2005), therefore we examined a potential role for LMPTP in regulating adipogenesis through the PDGFR. We tested PDGFR phosphorylation in serum-starved and PDGF-stimulated conditions. Interestingly, we observed significantly increased basal but not PDGF-stimulated phosphorylation of PDGFRα on activation loop Y849(Heldin, 2013) in 3T3-L1 treated with LMPTP inhibitor Compd. 23 (Fig. 4a and data not shown). We examined basal signaling downstream of PDGFRα, and observed significant increases in phosphorylation of activating residues of MAPKs p38 and c-Jun-N-terminal kinase (JNK), but not extracellular

signal-related kinase (ERK) (Fig. 4b). Taken together, these data suggest LMPTP acts as an inhibitor of basal PDGFRα signaling in preadipocytes.

LMPTP promotes adipogenesis by impairing PPARγ phosphorylation on inhibitory S82

JNK and p38 were reported to regulate the activity of PPARγ by phosphorylating PPARγ on inhibitory residue S82(Aouadi, 2006; Camp, 1999), thus we examined the role of LMPTP in regulation of PPARγ phosphorylation in 3T3-L1. Treatment of 3T3-L1 with Compd. 23 substantially increased basal phosphorylation of PPARγ-S82 (Fig. 4c). Similar results were observed in Compd. 23-treated 3T3-L1 at day 3 of the adipogenesis course (data not shown). Taken altogether, these data suggest LMPTP is a key promoter of PPARγ activation and adipogenesis, likely by suppressing a basal PDGFRα-MAPK signaling axis.

LMPTP inhibition in differentiating 3T3-L1 reduces glucose metabolism

To further characterize the role of LMPTP in adipogenesis, we performed UHPLC-MS-based analysis of the intracellular metabolome in DMSO and Compd. 23-treated 3T3-L1 at days 2, 6, and 10 of the adipogenesis course. PCA revealed striking separation between DMSO and Comp. 23-treated cells, which was evident at day 2 and became more divergent at days 6 and 10 (Fig. 5a and Fig. S3–5). The volcano plot at day 10 revealed the most biologically relevant metabolites that exhibited the greatest significance and fold change (Fig. 5b). Of those appreciably decreased in Compd. 23-treated cells, were glycolytic intermediates fructose 1,6-bisphosphate (fructose 1,6BP), phosphoenolpyruvate (PEP), and the final glycolytic product lactate (Fig. 6a). Interestingly, at days 6 and 10, glucose exhibited a significant increase in Compd. 23-treated cells (Fig. 6a). As shown in Fig. 6b, we observed a significant decrease in pentose phosphate pathway (PPP) oxidative phase intermediate 6-phosphogluconate, as well as increased cofactor nicotinamide adenine dinucleotide phosphate (NADP+). These data suggest decreased glycolytic flux within the LMPTP inhibitor-treated samples.

Given that glucose-6-P, the first product of glycolysis, is the precursor to all glycolytic intermediates and is utilized as the initial substrate in the PPP(Ge, 2020), we reasoned decreased glucose metabolism in LMPTP-inhibited cells could be attributed to inhibition of the first step in glycolysis. Hexokinase 2 (HK2), an enzyme that catalyzes phosphorylation of glucose to glucose-6-P, is transcriptionally regulated by PPARγ(Panasyuk, 2012). Thus, we examined expression levels of HK2 over the adipogenesis course, and noticed substantially reduced levels in Compd. 23-treated samples, which were clearly evident by day 6 of the adipogenesis course (Fig. 6c). Taken together, these data suggest that in differentiating adipocytes, treatment with LMPTP inhibitor impairs expression of HK2, leading to reduced glucose metabolism.

LMPTP inhibition in differentiating 3T3-L1 leads to increased mitochondrial respiration

The observed decrease in glucose utilization in Compd. 23-treated cells led us to examine whether these cells undergo increased mitochondrial respiration as an alternative energy source. We assessed levels of electron carriers needed for activity through the mitochondrial electron transport chain (mETC). We noticed strikingly decreased ratios of reduced:oxidized forms of nicotinamide adenine dinucleotide (NADH:NAD+) and Coenzyme Q_9

(CoQ_9H_2:CoQ_9), redox cofactors needed for activity through the mETC (Fig. 7a). We also observed significantly increased levels of the total pool of mETC cofactor flavin adenine dinucleotide (FAD + $FADH_2$; Fig. 7a). Concomitant with increased levels of NAD+ -an essential cofactor for multiple steps of the tricarboxylic acid cycle (TCA)- we noticed significantly increased levels of most TCA enzyme substrates at day 10, including citrate, isocitrate, α-ketoglutarate, fumarate, and malate (Fig. 7b), suggesting increased utilization of this pathway. Since 3T3-L1 exhibit increased levels of oxidative phosphorylation during proliferation(Yao, 2019), and during proliferation cells upregulate nucleotide synthesis to meet demands of DNA replication and RNA transcription(Lane, 2015), we compared the levels of various nucleotides in Compd. 23- vs DMSO-treated cells by day 10 of the adipogenesis course. We observed significant increases in all nucleotides detected in Compd. 23-treated cells (Fig. 7c).

Taken together, we find that inhibition of LMPTP activity alters the metabolic phenotype of differentiating adipocytes, decreasing glucose utilization yet enhancing energy production through oxidative phosphorylation and enhancing nucleotide levels.

Discussion

We report the first investigation of the role of LMPTP in adipocyte growth and differentiation. The *ACP1* gene associates with variability in clinical complications in obesity (E. Bottini, 1990; N. Bottini, 2002; Lucarini, 1997; Lucarini, 1990; Paggi, 1991) and LMPTP is considered a therapeutic target for obesity-associated diabetes. To explore the role of LMPTP in obesity-induced adipocyte hypertrophy, we utilized the adiponectin-Cre strain to selectively delete LMPTP in adipocytes. Consistent with our previous report on the lack of effect of LMPTP KO on DIO mouse body weight(Stanford, 2017), we observed no difference in body weight in this study. However, DIO mice lacking adipocyte-expressed LMPTP displayed reduced SubQ adipocyte hypertrophy. To understand the role of LMPTP in adipocyte formation, we subjected preadipocytes lacking LMPTP expression or activity to adipogenesis assays. LMPTP promotes pro-adipogenic gene expression and adipocyte differentiation through an action dependent on its phosphatase activity. LMPTP inhibition enhances basal PDGFRα phosphorylation on activation motif residue Y849 and increases basal phosphorylation of MAPKs p38 and JNK on activating residues. Concomitant with increased activation of p38/JNK, we observed enhanced phosphorylation of PPARγ on inhibitory S82. We propose a model (Fig. 8) in which LMPTP promotes adipogenesis through basal inhibition of PDGFRα and p38/JNK signaling, relieving suppression of PPARγ by MAPK-mediated S82 phosphorylation and priming the cell to undergo a pro-adipogenic transcriptional program in response to adipogenic stimuli or obesity. We do not exclude the possibility of additional pro-adipogenic mechanisms downstream of PDGFRα and/or p38/JNK. For example, basal changes in PDGFRα phosphorylation could lead to modifications of the epigenome that poise the cells to differentiate. Other potential mechanisms underlying the pro-adipogenic role of LMPTP warrant further investigation.

Several lines of evidence support our conclusions that LMPTP inhibits adipocyte differentiation through basal PDGFRα inhibition. First, we find that LMPTP inhibition in 3T3-L1 leads to enhanced PDGFRα-Y849 phosphorylation in resting cells. Additionally,

LMPTP was reported to regulate PDGFR tyrosine phosphorylation in fibroblasts(Berti, 1994; Chiarugi, 2002; Cirri, 1998), a cell type that acts as a major adipocyte precursor *in vivo*(Cattaneo, 2020). LMPTP co-precipitates with -and *in vitro* dephosphorylates- the PDGFR(Chiarugi, 1996), and PDGFR tyrosine phosphorylation is reduced in mouse fibroblasts overexpressing LMPTP(Chiarugi, 2001). Our findings that loss of LMPTP in adiponectin-expressing cells leads to smaller adipocytes in obese mice are consistent with the role of PDGFRα in WAT development(Bluher, 2004; Sun, 2017). Constitutive PDGFRα overactivation in cells that give rise to adipocytes severely impairs WAT formation in mice, while weak PDGFRα hyperactivation promotes adipocyte precursor proliferation over adipocyte differentiation and leads to WAT lipodystrophy(Sun, 2017). Microarray analysis showed larger adipocytes have reduced PDGFR expression compared to smaller adipocytes(Bluher, 2004), which is consistent with a model in which LMPTP promotes adipocyte hypertrophy through inhibition of PDGFR signaling. Finally, our results that LMPTP promotes adipogenesis are consistent with reports on the anti-adipogenic role of PDGFR signaling in fibroblasts, primary adipocyte precursors, and mesenchymal stem cells(Accili, 1991; Artemenko, 2005; Fitter, 2012; Hayashi, 1981; Sun, 2017).

Concomitant with an anti-adipogenic role for the PDGFR, expression of PDGFRα and PDGFRβ is dramatically reduced upon induction of adipogenesis in 3T3-L1, and differentiated 3T3-L1 adipocytes show impaired responsiveness to PDGF stimulation(Vaziri, 1996). Since PDGFRs are downregulated early after addition of adipogenic induction media, loss of PDGFRs likely serves to remove the anti-adipogenic effect of PDGF signaling.

We used metabolomics to investigate the effects of LMPTP inhibition on cellular physiology during adipogenesis. To the best of our knowledge, this is the first unbiased assessment on the whole metabolome in response to a lack of LMPTP activity. We observed evidence of substantially increased oxidative phosphorylation in LMPTP inhibitor-treated cells compared to DMSO-treated cells. It was recently demonstrated that 3T3-L1 display enhanced oxidative phosphorylation during proliferation(Yao, 2019). In line with this, LMPTP overexpression inhibits PDGF-induced proliferation of NIH-3T3 fibroblasts(Berti, 1994; Ruggiero, 1993), while overexpression of catalytically inactive LMPTP-C12S enhances their proliferation. We observed significantly enhanced levels of all nucleotides detected in the LMPTP-treated samples, providing further evidence of cells undergoing energy-intensive processes(Lane, 2015). Altogether, these findings are consistent with LMPTP inhibition facilitating maintenance of a precursor-like cell state rather than transition to an adipocyte phenotype. While the molecular pathways connecting LMPTP-inhibited PDGFRα signaling to abnormalities in cell metabolism remain to be clarified, we surmise they likely involve changes in expression and/or activity of metabolic enzymes through alterations in transcription factor functions, epigenetic regulation of gene expression, or cell redox state.

A previous report of metabolomics of differentiating 3T3-L1 demonstrated increased glycolytic flux -as evidenced by decreased glucose and increased lactate and pyruvate- during differentiation in response to an adipogenic cocktail, which diminished by terminal differentiation(Roberts, 2009). We also observed evidence of an increase in glycolytic flux in the control cells over the course of adipogenesis comparing lactate levels at days 2, 6, and

10 (Fig. 6a). We did not observe a decrease in lactate at day 10 of our study. This discrepancy is likely due to slight differences in the adipogenic protocol and time-course in the earlier report, in which cells were treated with induction media and allowed to differentiate for longer time periods. Our data may be more comparable to data they obtained at an earlier time point, in which case the decreased glycolytic flux in LMPTP-inhibited cells is reflective of these cells exhibiting a more preadipocyte-like phenotype than DMSO-treated cells.

An open question is why reduced adipocyte hypertrophy in adipocyte-specific LMPTP KO mice is depot-specific, as we observed smaller adipocytes in SubQ but not epididymal WAT of DIO mice. A similar difference in depots was reported for mice lacking the tyrosine phosphatase PTP1B in adipocytes using adiponectin-cre (Owen, 2012). Loss of PTP1B led to larger adipocyte size in epididymal WAT of chow and HFD fed mice, while no effect of PTP1B loss was observed in SubQ adipocytes(Owen, 2012). These findings likely reflect biological differences in signaling pathways in SubQ vs other WAT depots.

LMPTP has been proposed to dephosphorylate and inhibit the IR, and we observed that LMPTP KO in the liver or LMPTP inhibition leads to enhanced responsiveness of the liver IR to insulin(Stanford, 2017). While we do not exclude the possibility of LMPTP inhibiting IR signaling in adipocytes, our findings are inconsistent with a major inhibitory effect of LMPTP on the IR in these cells. There have been several reports on the physiological effects of adipocyte-specific IR KO using adiponectin-cre; they have consistently demonstrated that adipocyte-specific IR loss leads to lipodystrophy of WAT depots, insulin resistance, and hyperglycemia(Boucher, 2016; Friesen, 2016; Qiang, 2016; Softic, 2016). Based on these findings, if LMPTP was a major inhibitor of the IR in adipocytes, we would expect loss of LMPTP in adipocytes to enhance cell size and WAT depot mass, while protecting from insulin resistance and hyperglycemia. Therefore, our findings that adipocyte-specific loss/inhibition of LMPTP impairs SubQ WAT mass and adipocyte size suggest LMPTP does not exert a major regulatory role on IR signaling in adipocytes as was observed in the liver. Consistent with this concept, adipocyte-specific LMPTP deletion did not protect from HFD-induced diabetes development(Stanford, 2017). Furthermore, our findings that loss/inhibition of LMPTP impair adipogenesis are consistent with lack of effect on the IR, as adipocyte differentiation in 3T3-L1 is impaired by partial loss of IR expression(Accili, 1991). It is possible that in adipocytes, other PTPs besides LMPTP predominate in the regulation of IR tyrosine phosphorylation. It was reported that overexpression of leukocyte common antigen-related (LAR) PTP -but not catalytically inactive C1522S mutant- in 3T3-L1 impaired IR tyrosine phosphorylation and phosphorylation of downstream signaling mediators, and inhibited the differentiation of these cells into adipocytes(Kim, 2009). Conversely, LAR knockdown dramatically increased the adipogenic differentiation of 3T3-L1(Kim, 2009).

In conclusion, using a combination of *in vivo*, *in vitro*, and metabolomics approaches, we report a novel role for LMPTP as a key regulator of obesity-induced SubQ adipocyte hypertrophy and key promoter of adipocyte differentiation. Further investigation of the functions of LMPTP in adipocyte biology is warranted in order to understand the implications of targeting LMPTP for treatment of obesity-related complications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability statement

The data that support the findings of this study are available from the corresponding author(s) upon reasonable request.

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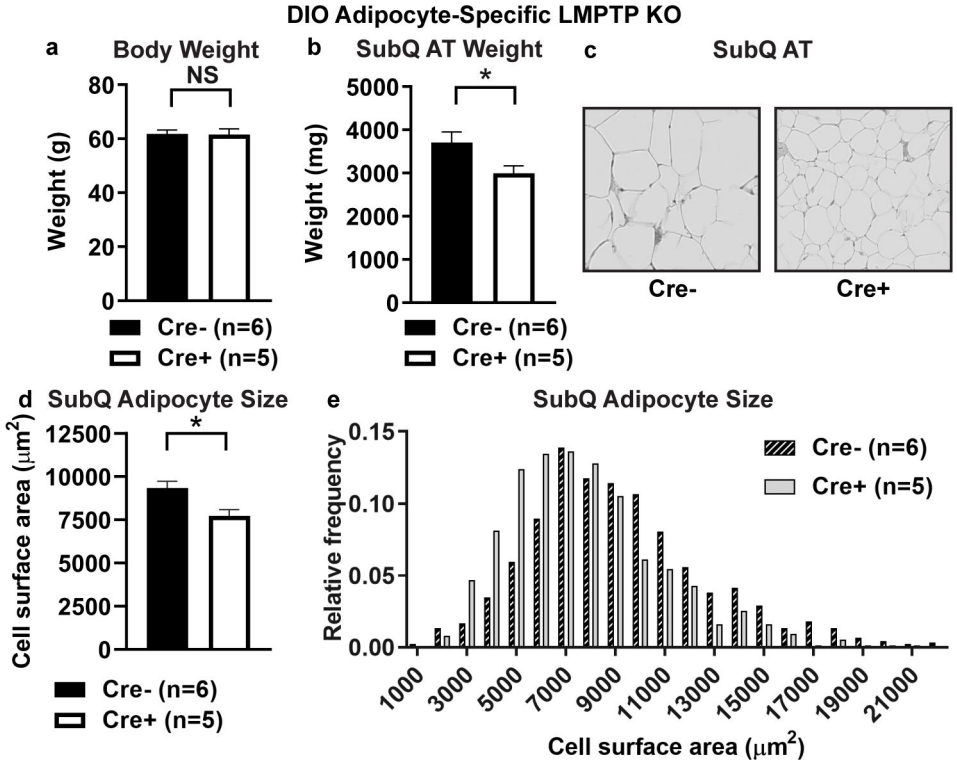

Fig. 1. Adipocyte-specific LMPTP deletion reduces SubQ adipocyte size in obese mice.

To generate diet-induced obese (DIO) mice, male Acp1$^{fl/fl}$ adiponectin-Cre+ (n=5) and Cre- (n=6) littermates were placed on high-fat diet for 12 months. **(a)** Mean±SEM mouse body weight. **(b)** Mean±SEM SubQ fat pad weight. **(c-e)** Subcutaneous (SubQ) adipose tissue (AT) was harvested and stained with H&E and adipocyte surface area calculated in ImageJ. **(c)** Representative H&E-stained images. **(d)** Mean±SEM adipocyte surface area. **(e)** Frequency distribution of SubQ adipocyte size. **(a, b, d)** *, $p < 0.05$; NS, non-significant: unpaired t-test.

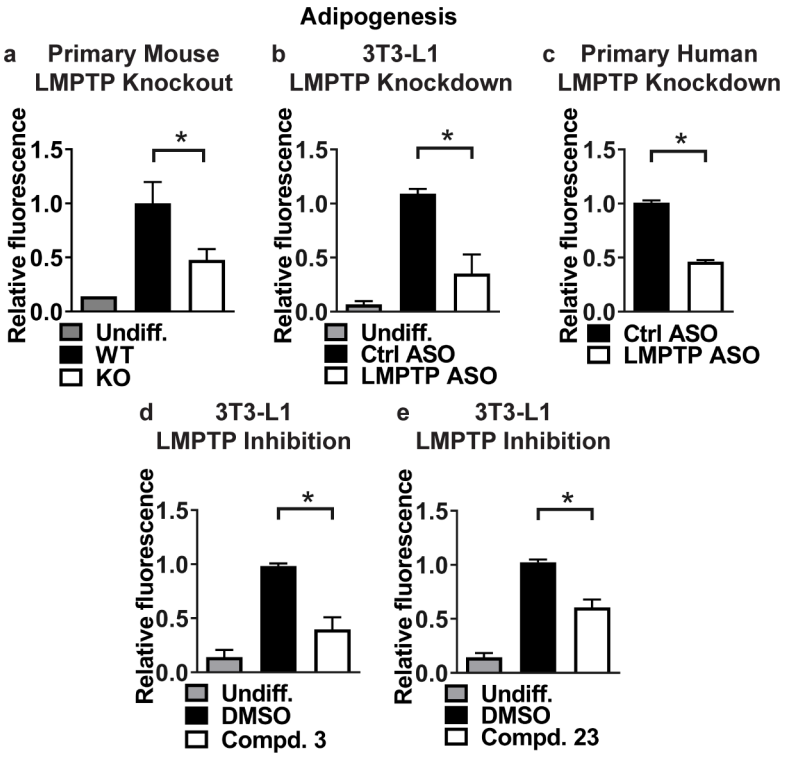

Fig. 2. LMPTP promotes adipogenesis through its catalytic activity.

(a) Primary preadipocytes were isolated from the SubQ fat pad of wild type (WT; n=3) and LMPTP knockout (KO; n=4) mice and were subjected to an adipogenesis assay. Cells from 1 WT mouse were used as an undifferentiated (Undiff.) control. Mean±SEM is shown. **(b)** 3T3-L1 preadipocytes were subjected to an adipogenesis assay in the presence of 10 μM mouse LMPTP-targeting antisense oligonucleotide (ASO) or control non-targeting (Ctrl) ASO. Mean±SEM from 3 independent experiments is shown. **(c)** Primary human visceral preadipocytes were subjected to an adipogenesis assay in the presence of 10 μM human LMPTP-targeting ASO or Ctrl ASO. Mean±range from 2 independent experiments is shown. **(d-e)** 3T3-L1 were subjected to an adipogenesis assay in the presence of 10 μM LMPTP inhibitor Compd. 3 **(d)** or Compd. 23 **(e)** or dimethyl sulfoxide (DMSO). Mean ±SEM from **(d)** 4 and **(e)** 14 independent experiments is shown. **(a-e)** Cells were stained with AdipoRed reagent and fluorescence was measured. Fluorescence relative to the control sample in each experiment is shown. Undiff, undifferentiated; *, $p < 0.05$: **(a-b)** unpaired t-test or **(c-e)** unpaired t-test with Welch's correction.

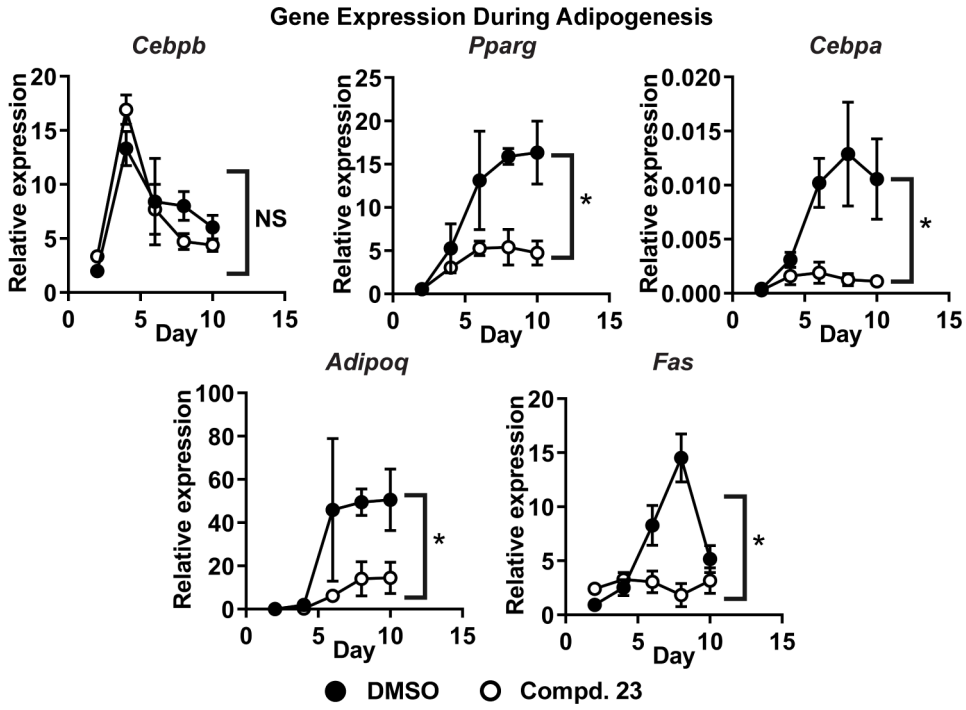

Fig. 3. LMPTP promotes expression of pro-adipogenic transcription factors.

3T3-L1 were subjected to an adipogenesis assay in the presence of 10 μM Compd. 23 or DMSO. mRNA was measured at the indicated times by qPCR. Mean±SEM relative expression from 3 independent experiments following normalization to the housekeeping gene POLR2A is shown. *, $p < 0.05$; NS, non-significant: two-way ANOVA treatment effect.

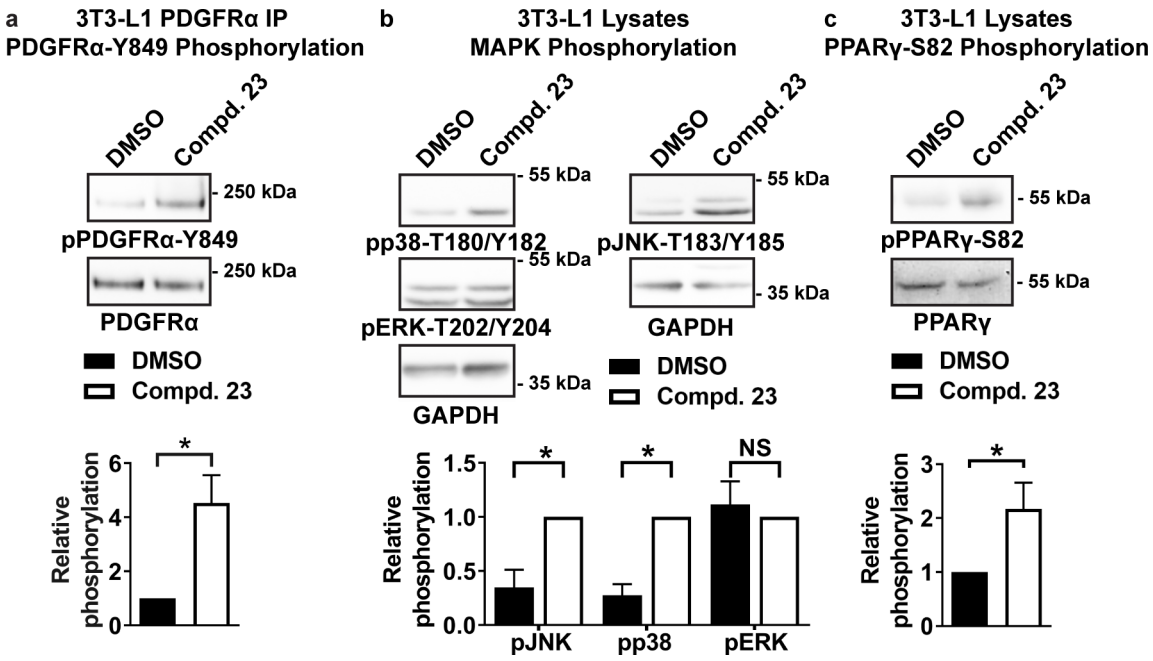

Fig. 4. LMPTP inhibits activation of PDGFRα, p38 and JNK, and promotes activation of PPARγ in preadipocytes.

3T3-L1 were serum-starved overnight and incubated with DMSO or 10 μM Compd. 23 and phospho-analytes were assessed by Western blotting. Upper panels show representative blots. Lower panels show quantification of phospho-blots. **(a)** PDGFRα was immunoprecipitated (IP) and Y849 phosphorylation (pPDGFRα-Y849) was assessed in 5 independent experiments. **(b)** MAPK activation motif phosphorylation in cell lysates was assessed in 4 independent experiments. **(c)** PPARγ-S82 phosphorylation in cell lysates was assessed in 5 independent experiments. **(a-c)** Mean±SEM relative phospho-blot signal to **(a)** PDGFRα, **(b)** GAPDH, or **(c)** PPARγ is shown. *, p <0.05; NS, non-significant: unpaired t-test with Welch's correction.

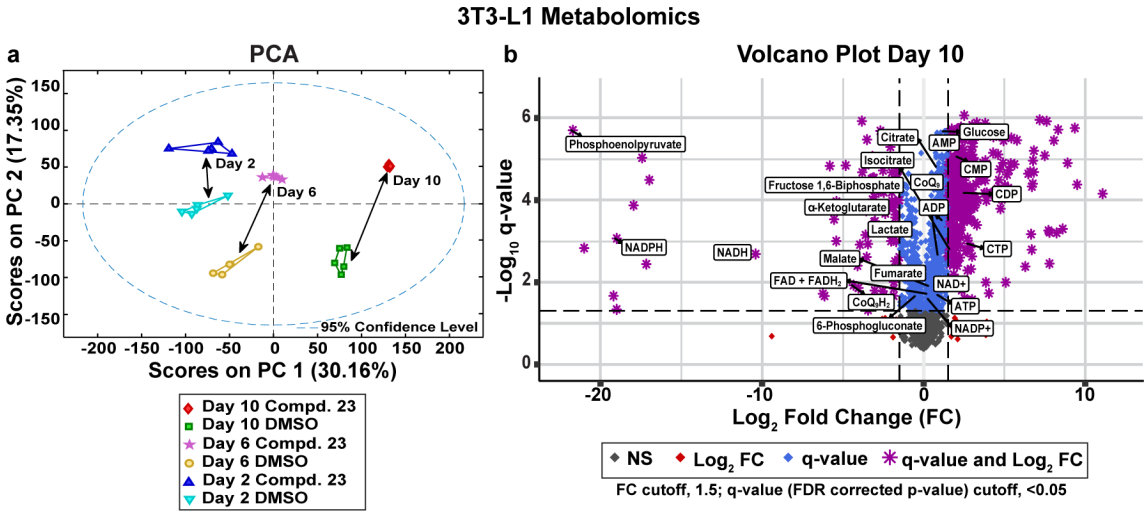

Fig. 5. LMPTP inhibition alters the metabolome of adipocytes during differentiation.

(a) PCA (PC1 vs. PC2) obtained from the metabolomic and the fatty acid analyses of 3T3-L1 treated with Compd. 23 or DMSO at days 2, 6, and 10 of the differentiation protocol. **(b)** Volcano plot comparison of features from metabolomic and fatty acid analyses at day 10. Fold change cutoff, 1.5; q-value (FDR corrected p-value) cutoff, <0.05. **(a-b)** Data from 5 experimental replicates is shown.

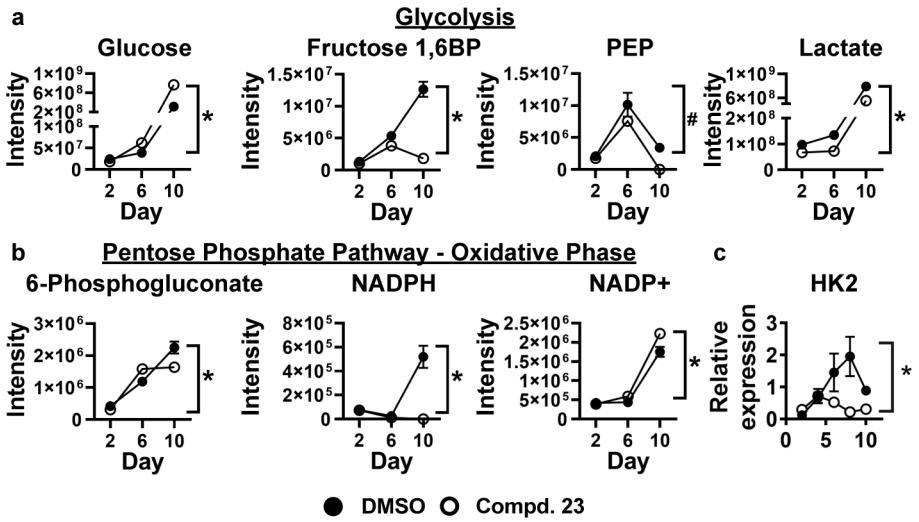

Fig. 6. LMPTP inhibition reduces glycolysis in adipocytes during differentiation.

(a-b) The cellular metabolome was assessed by HPLC-MS in differentiating 3T3-L1 treated with 10 μM Compd. 23 or DMSO. **(a)** Intensity of glucose, glycolytic intermediates, and lactate. **(b)** Intensity of 6-phosphogluconate, NADP+, and NADPH. **(c)** Hexokinase 2 (HK2) expression was assessed by qPCR in differentiating 3T3-L1 treated with 10 μM Compd. 23 or DMSO. **(a, b)** Data from 5 experimental replicates is shown. **(c)** Relative expression from 3 independent experiments following normalization to the housekeeping gene POLR2A is shown. **(a-c)** Mean±SEM is shown. *, $p < 0.05$: two-way ANOVA interaction effect; #, $p < 0.05$: two-way ANOVA treatment effect.

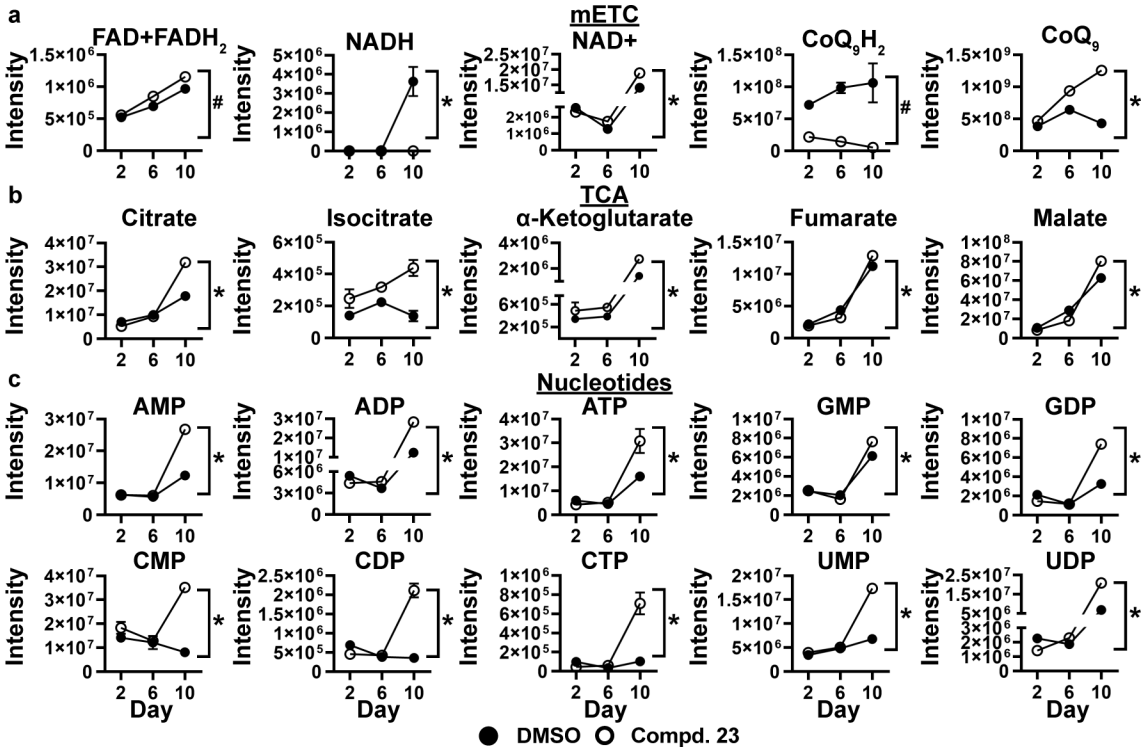

Fig. 7. LMPTP inhibition enhances mitochondrial electron transport chain activity in adipocytes during differentiation.

The cellular metabolome was assessed by HPLC-MS in differentiating 3T3-L1 treated with 10 μM Compd. 23 or DMSO. **(a)** Intensity of mitochondrial electron transport chain (mETC) electron carriers. **(b)** Intensity of tricarboxylic acid cycle (TCA) substrates. **(c)** Intensity of nucleotides. **(a-c)** Mean±SEM is shown. Data from 5 experimental replicates is shown. *, $p < 0.05$: two-way ANOVA interaction effect; #, $p < 0.05$: two-way ANOVA treatment effect.

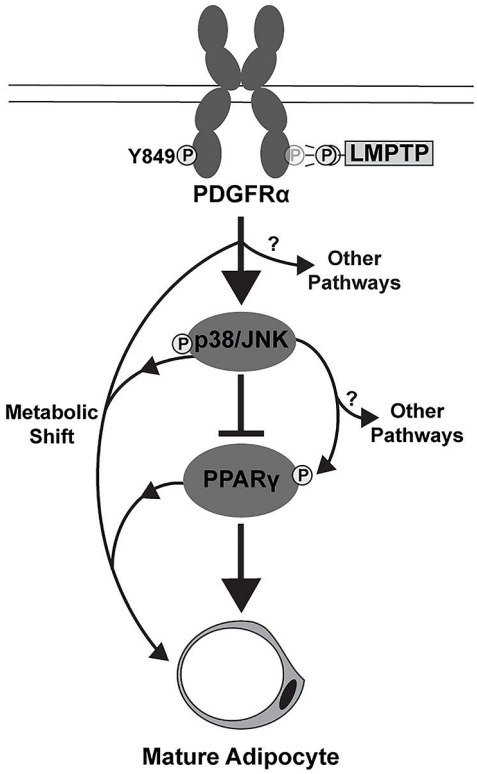

Fig. 8. Proposed role for LMPTP in adipogenesis.

Our model suggests LMPTP dephosphorylates PDGFRα-Y849 in preadipocytes, reducing basal PDGFRα activity and leading to decreased p38/JNK activation. This enhances PPARγ activation by decreasing phosphorylation on inhibitory residue S82. In the dephosphorylated state, PPARγ is free to promote expression of genes associated with the differentiation of 3T3-L1 into mature adipocytes. Additional mechanisms downstream of PDGFRα and/or p38/JNK are also possible.