Methods

Patients and clinical samples

De-identified frozen human liposarcoma tissues were obtained from the CHTN tissue bank or collected by co-author Raphael Pollock under the approved institutional review board protocol. Liposarcoma subtypes were classified according to the surgical pathology reports.

Mice

Tumor allograft experiments were approved by the Animal Care and Use Committee of Purdue University. NRG (NOD/RAG1/2$^{-/-}$IL2Rγ$^{-/-}$) mice (2-4 months old) provided by Purdue University Biological Evaluation Shared Resource were used as recipients. Cells (1×10^6) were resuspended in 50-100 μL PBS and injected into the left or right flank. After cell injection, the mice were monitored daily, and tumor length and width were measured using a digital caliper once palpable tumors were detected and randomized assign to each group. The grafted mice were treated with vehicle, CB103 (40 mg/kg/day), or 1 mg/mL doxycycline (Dox) in water (Tokyo Chemical Industry, D4116) supplemented with 1% sucrose (Fisher Scientific, S5-500), to inhibit Notch or to activate PGC1α. The allograft tumors were dissected for downstream analysis at the end of the experiment or if the length reaches 3 cm, whichever comes first. Mice were housed under standard laboratory conditions, including a 12hr light/dark cycle, with free access to food and water.

Cell culture

Human primary WDLPS cells LPS80.2, LPS123, and LPS135 and primary DDLPS cells LPS187, LPS120, and LPS27 were established by Raphael Pollock. Human LPS246 (DDLPS) cells were provided by L. Chin (MD Anderson Cancer Center, Houston, TX, USA) [24]. Human primary liposarcoma cells and cell line were cultured in DMEM (Sigma, D5796) containing 10% FBS (Corning, 35-010-CV) and penicillin (100U/ mL)/streptomycin (100 μg/mL) (Hyclone #SV30010). The mouse liposarcoma cell lines mLPS1, mLPS1$^{\Delta NICD}$, mLPS1 stably-transfected with TetO-Empty or TetO-PGC1α^{OE} vectors were cultured in RPMI1640 (Corning, 10-040-CV) containing 10% FBS and penicillin (100U/mL)/streptomycin (100 μg/mL). Cells were certified as mycoplasma-free by RT-qPCR analysis of the mycoplasma-specific 16s rRNA gene region.

Vector construction and cell transfection

The doxycycline-inducible TetO-PGC1α^{OE} mLPS1 and empty vector control cells were generated using the Sleeping Beauty transposon system. Briefly, 2.85 μg pSBtetRP (Addgene #60497) plasmid with a 0.15 μg pCMV(CAT)T7-SB100 plasmid (Addgene #34879) was co-transfected into mLPS1 cells using 3 μL Lipofectamine 2000 (ThermoFisher #11668019), following the manufacturer's protocol. The PGC-1α cDNA sequence was subcloned from the TRIPZ-haPGC1A plasmid (kindly provided by Veronica Torrano) [25]. Transfected cells were selected with 1.2 μg/mL puromycin for 1 week, and transgene expression was induced by treatment with 1 μg/mL doxycycline (Tokyo Chemical Industry #D4116) for 24 h. mLPS1$^{\Delta NICD}$ cells were generated by clustered regularly interspaced short palindromic repeats (CRISPR) targeting, catalyzed by CRISPR-associated protein 9 (CAS9) and directed by a guide RNA (gRNA) that recognizes specific sequences of NICD. Briefly, gRNA was cloned into the pSpCas9(BB)-2A-Puro (PX459) V2.0 plasmid. Two NICD gRNAs were used to target the different regions of the NICD cassette. The sequences used were gRNA#1 (5'-GACCAAGAAGTTCCGGTTTG-3') and gRNA#2 (5'-GTGTCTTCCAGATCCTGCTC-3'). mLPS1 cells (1×10^6) were co-transfected with two gRNA plasmids (2 μg for each plasmid) using Lipofectamine 2000 transfection reagent. Single clones were selected after 1 week of puromycin selection (1.2 μg /mL). The efficiency of gene silencing or overexpression was verified using qPCR and western blotting.

Genotyping analysis of CRISPR editing cells

PCR was performed using genomic DNA extracted from the mLPS1$^{\Delta NICD}$ cells. For Sanger sequencing, PCR amplification was performed using the forward primer 5'-AGCTCTGGTTCCCTGAGGGTTT-3' and reverse primer 5'-CAGTCTCATAGCTGCCCTCAC GG-3' to generate a 910 bp product. The PCR product was gel-purified and cloned into the pGEM-T Easy vector (Promega # A1360), and the vector was transformed into DH5α competent cells. After transformation, a single DH5α E. coli colony was selected and the purified plasmid was sequenced using the same PCR primers used for Sanger sequencing.

Cell growth assay and adipogenic differentiation assay

Cell proliferation was evaluated by counting the number of cells using a hemocytometer. Cells at a density of 1×10^4 cells/well were seeded onto 24-well plates and incubated at 37 °C in 5% CO_2 for 1-4 days. Cells were dissociated daily and the cell numbers were counted. For adipogenic differentiation of LPS cells, the cells were

seeded into 24-well culture plates at a density of 1×10^5 cells/well and cultured in RPMI-1640 medium at 37 °C and 5% CO_2 until they reached 90% confluency. To induce adipogenic differentiation, culture medium was replaced with the adipocyte differentiation medium containing 33 μM biotin, 0.5 μM human insulin, 17 μM pantothenate, 0.1 μM dexamethasone, 2 nM triiodothyronine, 500 μM IBMX (3-isobutyl-1-methylxanthine), 30 μM indomethacin, and 2% FBS for 8 days and medium were refreshed every 3 days. After 8 days in differentiation medium, culture medium was changed to adipocyte maturation medium containing 0.5 μM human insulin, 2 nM triiodothyronine, and 2% FBS for 6 days. The medium was refreshed every 2 days. Adipogenic differentiation was confirmed by staining with BODIPY (Thermo Scientific, #D6003) and Hoechst 33342 (#H3570; Invitrogen). Briefly, cells were fixed with 4% paraformaldehyde for 30 min at room temperature, followed by 3x10 min washes with PBS three times. The fixed cells were incubated with BODIPY (1:5000) and Hoechst 33342 (1:10000) diluted in PBS at 37 °C for 15 min, followed by 2x10 min washes with PBS. Images were acquired using a fluorescence microscope (Leica DMI6000B; Wetzlar, Germany).

Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay

Cells were seeded in 96-well plates at a density of 3×10^3 cells/well. Cells were treated with vehicle control, CB-103 (HY-135145, Medchemexpress) or IMR-1 (HY-100431, Medchemexpress) at various concentrations in 200 μL complete medium for 48 h. To determine the growth kinetics, 20 μL MTT (5 mg/mL, Acros Organics, Cat#158990010) was added to each well and cultured at 37 °C for 2 h, during which MTT is converted to a dark blue compound in live cells that absorbs light at 570 nm. After incubation, the culture medium was removed, DMSO was added to each well to dissolve the formazan crystals, and the number of viable cells was analyzed based on absorbance at 570 nm with a spectrophotometer (Spark 10M, TECAN).

Colony formation

Liposarcoma cancer cells at a density of 5×10^2 cells/well were placed in six-well plates and cells were treated with 20 μM CB-103 or 20 μM IMR1 for 14 days. The cells were then washed with PBS and fixed with methanol/acetic acid 3:1 (vol/vol) for 5 min at room temperature. The colonies were stained with 0.1% crystal violet (Coleman & Bell Company). Stained colonies were photographed and analyzed using the ImageJ software.

Cell death assay by TUNEL (Terminal dUTP nick end labeling)

The mLPS1 cells were treated with IMR1, CB103 treatment for 24 hr and deoxyribonuclease (Invitrogen, AM1907) were used as the positive control. After drug treatment, cells were rinsed once with PBS, fixed with 4% PFA, followed by PBS containing 0.2% Triton X-100 for 30 minutes at room temperature, and washed twice in PBS. After fixation, samples were incubated with 100 uL TUNEL Equilibration Buffer for 5 minutes and incubated with the TdT Reaction buffer for 60 minutes at room temperature. Samples were washed with PBS containing 0.1% Triton X-100 and 5 mg/ mL BSA and counterstained with DAPI. Cell apoptosis were detected with an apoptosis detection kit (Biotium) and a fluorescence microscope (Leica DMI6000B; Wetzlar, Germany).

Wound-healing assay

The mLPS1 TetO-Vector and mLPS1 TetO-PGC1α^{OE} cells were seeded into six-well plates in 80% confluence. Upon cell attachment (~6 h), 1 ug/mL doxycycline was added into the medium. After cells reached 100% confluence as a monolayer, a scratch in the monolayer of cells was generated using a 100 µl plastic pipette tip, and the dislodged cells were removed by PBS wash. Then, the cells were replenished with fresh medium with 1 ug/mL of doxycycline. Images were captured with a light Leica microscope at 0, 6, and 20 h. ImageJ software was used to determine the relative migration in each group.

Western blot

Cells were lysed on ice in NP-40 buffer containing phosphatase inhibitor cocktail (Sigma, 23228). Protein concentrations were determined using bicinchoninic acid (BCA) assay (Thermo Scientific, 23228). Samples were run on denaturing conditions of Tris-Glycine gels and transferred onto 0.2 µm PVDF membranes. Membranes were blocked with 5% non-fat milk for 1 h at room temperature, followed by overnight incubation at 4 °C with the following specific primary antibodies: β-actin (A5441, 1:5000; Sigma-Aldrich), NICD (MAB5352, 1:1000; Sigma-Aldrich), HES1 (AB5702, 1:1000; Sigma-Aldrich), MDM2 (OP46, 1:1000; Calbiochem), and PGC-1α (ST1202, 1:1000; Sigma-Aldrich). HRP-conjugated secondary antibodies were incubated for 1 hour at room temperature. Membranes were developed using the western blot luminol reagent (Santa Cruz Biotechnology) and imaged using the FluorChem R image system (ProteinSimple).

Real-time quantitative PCR and mitochondrial DNA (mtDNA) content determination

Total RNA was extracted using TriReagent (Sigma-Aldrich #T9424) and converted into cDNA using M-MLV reverse transcriptase (Invitrogen #28025–021). All reactions were run in triplicate and normalized using 18s as the internal control gene. To determine mtDNA content, total DNA was extracted from the cell samples using phenol/chloroform. qPCR was run to quantify the mtDNA copy number. MT-ND1 was used as the standard for mitochondrial DNA (mtDNA), and HK2 was used as the nuclear DNA (nDNA) normalizer to calculate the mtDNA/nDNA ratio. Quantitative PCR was performed on a LightCycler 96 real-time PCR system (Roche). Fold changes in gene expression were calculated using the comparative Ct method (ΔΔCt). The mouse-specific primer sequences are listed in **Supplementary Table 1**.

MitoTracker Staining

Cells were incubated with MitoTracker Deep Red FM reagent (Thermo Fisher Scientific, M36008) 1:10000 in PBS (Invitrogen, M46753) at 37 °C for 30 min. After staining was complete, cells were gently washed once with PBS. The samples were analyzed by flow cytometry using a BD FACS Aria III flow cytometer at the Purdue Flow Cytometry and Cell Separation Facility. The data were analyzed using FlowJo version 10.4.

Glucose uptake, lactate production, and ATP assay

For glucose uptake, mLPS1 and mLPS1$^{\Delta NICD}$ cells were seeded in 24-well plates at a density of 1×10^5 cells/well and co-cultured in RPMI 1640 medium supplemented with 10% FBS for 16 h. The cells were then washed with PBS, and cultured for 6 in fresh RPMI 1640 medium. The culture medium was collected, and glucose uptake was analyzed using a OneTouch Ultra 2 Blood Glucose Meter (Lifescan). Glucose readings were normalized to the number of cells per well. For the lactate production and ATP assays, mLPS1 and mLPS1$^{\Delta NICD}$ cells were seeded in 96-well plates at a density of 1×10^4 cells/well and co-cultured in RPMI 1640 medium supplemented with 10% FBS for 4 h. The cells were then washed with PBS and cultured for 16 h in fresh RPMI 1640 medium. The culture medium was collected, and lactate production was analyzed using the L-Lactate Assay Kit II (Eton Bioscience) according to the manufacturer's instructions. Simultaneously, cell lysates were collected to measure ATP production using an ATP Detection Assay Kit (Cayman, 700410), following the manufacturer's instructions.

Seahorse XF cell mitochondria stress and glycolysis stress analysis

The mitochondrial respiratory capacity was determined using the XF Cell Mito Stress Test Kit (Agilent Technologies, 103015–100). The cells were seeded in an XFe24 cell

culture microplate at a density of 4×10^4 cells/well. Five replicates were set up for each of the following groups: (1) mLPS1, (2) mLPS1$^{\Delta NICD}$, (3) mLPS1 TetO-Vector + Dox and (4) mLPS1 TetO-PGC1α^{OE} + Dox. Briefly, cells were seeded in a microplate for 12 h at 37 °C. The mLPS1 TetO-Vector and mLPS1 TetO-PGC1α^{OE} cells were pretreated with 1 mM doxycycline for 48 h prior to seeding into an XFe24 cell culture microplate. The Seahorse XFe24 Flux sensor cartridge was hydrated overnight in a utility plate filled with 800 ml of Seahorse Calibrant in a non-CO_2 incubator at 37 °C. The next day, the cells were incubated with a base medium containing 1 mM L-glutamine, 1 mM sodium pyruvate, and 5 mM glucose for 30 min prior to the assay. The oxygen consumption rate (OCR) was measured using an XFe24 extracellular flux analyzer with sequential injection of 30 μM oligomycin A, 30 μM FCCP, and 15 μM rotenone/antimycin A. To evaluate glycolysis, cells were exposed to Seahorse XF base medium supplemented with 1 mM L-glutamine in a non-CO_2 incubator for 30 min prior to the assay. The extracellular acidification rate (ECAR) was measured by the sequential injection of 100 mM glucose, 30 μM oligomycin, and 500 mM 2-DG. After the experiment, cells were fixed in 20% (w/v) TCA overnight at 4 °C. The fixed cells were washed four times with ddH_2O and air-dried at room temperature. Cells were stained with 0.04% sulforhodamine B (SRB) solution at room temperature for 1 h and quickly rinsed with 1% (v/v) acetic acid, followed by air drying. Cell lysates were resolved in 10 mM Tris-base solution (pH 10.5), and the absorbance was measured at 510 nm to determine cell number [26]. OCR and ECAR values were normalized to the number of cells in each well.

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RNA-seq analysis

The mLPS1 and mLPS1$^{\Delta NICD}$ cells were collected from three batches of different passage numbers. Total RNA was extracted using TriReagent (Sigma-Aldrich #T9424) and prepared for RNA-seq analysis at BGI America (Cambridge, MA, USA). The integrity of the RNA was confirmed using a Bioanalyzer 2100 (Agilent Technologies). 4 μg of column-purified RNA was ployA-selected and subsequently subjected to fragmentation and cDNA synthesis. RNA-seq libraries were constructed and sequenced using the DNBSEQ platform (DNBSEQ Technology). The clean reads were quality-checked using FastQC and subsequently aligned to the mouse reference genome (version mm10) using Salmon with default parameters [27]. Differential expression was determined using a cutoff significance level of FDR (padj) < 0.05, using the DESeq2 [28]. RStudio software was used for data visualization, and pathway analysis was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) platforms. Transcriptome data were deposited into the GSE210457 dataset.

Kaplan–Meier curve of overall survival of liposarcoma patients

Kaplan–Meier curves of disease-specific survival of patients with liposarcoma and dedifferentiated liposarcoma, stratified by tumor expression of HEY1, were generated using cBioPortal (http://www.https://cbioportal.org) from TCGA Cell, 2017 dataset. Overall survival of sarcoma patients stratified by tumor expression of PPARGC1A from TCGA database (BMC Cancer, 2014) using PROGgeneV2 platform analysis (http://gepia.cancer-pku.cn/index.html). PPARGC1A expression levels in human

liposarcoma tissues were determined using Genevestigator (https://genevestigator.com) in the HS_AFFY_U133PLUS_2-1 dataset.

Statistical analysis

All experiments were independently repeated at least thrice, and the number of replicates is provided in the figure legends. Statistical analyses and graphs were generated using Microsoft Excel and GraphPad Prism, version 9. Data are presented as the mean ± SD. Statistical analysis was performed using a two-tailed unpaired t-test with the following significance indicators: * $P < 0.05$, ** $P < 0.001$