Supplemental Material

Expanded Materials and Methods

Animal studies

Wild-type (WT) male C57BL/6 mice were obtained from the Animal Facility of the Faculty of Health Sciences of University of Macau. The GSK3β-floxed exon 2 allele ($GSK3\beta^{fl/fl}$) was generated by the Woodgett lab and adiponectin-Cre mice were purchased from the Jackson Laboratory (Maine, USA). GSK3α floxed mice were generated and shared by the Woodgett lab. GSK3β or GSK3α adipocyte specific knockout mice were generated by crossing adiponectin-cre mice on the C57BL/6 background with flexed mice on the C57BL/6 background in our study. The mice were maintained under a 12-h light/dark cycle with free access to drinking water and food. Mice were fed a normal chow diet (NCD, 10 kcal% fat) or high-fat diet (HFD) containing 60 kcal% fat (D12491, Research Diets Inc., New Brunswick, NJ) to induce obese mice starting at 6 weeks of age for indicated weeks. PT2385 (from Selleck Chemicals, S8352) was used for animal injection at 30 mg/kg/mice, every other day, I.P. for 2 weeks. Compound C (from Selleck Chemicals, S7840) was used for animal injection at 10mg/kg every other day, I.P. for 2 weeks. SB216763 (from Selleck Chemicals, S1075) was used for animal injection at 10mg/kg every other day, I.P. for 2 weeks. Both male and female mice were used, and the mice were individually labeled using an ear tag labeling system and subsequently allocated to different experimental groups through a random selection process facilitated by a computer-based random number generator. All mice with correct genotype were included in the study. No mouse was excluded. The number of animals for each study was determined by referencing well-documented, comparable experiments in the literature. Further information about the animal samples can be found in the main resource table. The animal experiments were conducted by researchers who were blinded to the genotype and treatment assignments. All animal experiments were approved by the University's Animal Ethics Committee.

Insulin tolerance tests (ITT) and glucose intolerance test (GTT) test

GTT and ITT were performed to evaluate glucose metabolism and insulin sensitivity, respectively [35]. For the GTT, mice were fasted for 16 hours before the test. A glucose solution (2.5 g/kg body weight) was orally administered to the mice. Blood samples were collected from the tail vein at designated time points for glucose measurement using a glucometer. In the ITT, mice were fasted for 4-6 hours before the test. Regular insulin was injected intraperitoneally at a calculated dose (0.75 U/kg body weight). Blood samples were collected from the tail vein at specific time intervals for glucose measurement. Glucose levels were determined using a glucometer.

Whole-Body Metabolism Analysis

Oxygen consumption, energy expenditure, and locomotor activity of live animals were monitored by the Oxymax Lab Animal Monitoring System. Before commencing the measurement, the animals were ensured to have unrestricted access to food and water. The Oxymax system and gas analyzers were calibrated in accordance with the manufacturer's instructions. The animals were placed individually in the metabolic chambers, ensuring proper sealing and connection to the Oxymax system. The data acquisition software was started, and the desired measurement parameters were configured. The animals were allowed to acclimate to the chambers for a specified period of 12 hours to minimize potential stress effects. The data recording process was initiated, and oxygen consumption (VO2), carbon dioxide production (VCO2), and locomotor activity were recorded for the desired experimental duration (3 days).

Adipose Tissue Oxygen Consumption Rate (OCR) Analysis

XFe 24 Extracellular Flux Analyzer (Agilent Seahorse Biosciences) was used to determine the bioenergetic profile of adipose tissue including oxygen consumption rate (OCR), and extracellular acidification rate (ECAR). Briefly, freshly isolated adipose tissue was rinsed with Seahorse XF-DMEM containing 25 mM HEPES (Thermo Fisher Scientific, 15630106) and cut into pieces (~10 mg). Then one piece of tissue was placed in each well of a Seahorse XF24 Islet Capture Microplate and covered with the islet capture screen. Added 500 µl Seahorse XF Assay Medium (Agilent, 102353-100) to cover the tissue. The prepared microplate containing the tissue samples and assay medium, was loaded into the XFe 24 Extracellular Flux Analyzer. This instrument was capable of real-time measurement of OCR and ECAR value. The analyzer conducted sequential measurements, recording the OCR and ECAR values from the adipose tissue samples. OCR was used to represent the rate at which the tissue consumes oxygen, while ECAR indicated the rate of extracellular acidification. The obtained OCR and ECAR values were normalized using the actual cell count or protein concentration, which was determined immediately after the OCR and ECAR recordings. Mito Stress Test (Agilent, 103015-100) drugs were delivered to final concentrations of: oligomycin (Oligo, 2 µg/mL), FCCP (1.5 µM), and rotenone (3 µM). Optimal drug concentrations were determined in preliminary experiments.

Cell Culture

Mouse 3T3-L1 preadipocytes (ATCC, CL-173) were cultured in DMEM (Thermo Fisher Scientific, 11995065) supplemented with 10% FBS (Thermo Fisher Scientific, 10270106) and 1% penicillin/streptomycin (Thermo Fisher Scientific, 15070063) in a 5% CO2 environment. To induce preadipocyte differentiation, cells at 100% confluency were stimulated with MDI medium containing 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich, I5879), 5 µg/ml insulin (Sigma-Aldrich, I3536), and 1 µM dexamethasone (Sigma-Aldrich, 265005) in DMEM [81]. After 3 days, the cells were switched to a maintenance medium (DMEM, 10% FBS, 1% penicillin/streptomycin, 5 µg/ml insulin) and the medium was changed daily for an additional 5-6 days. Mature 3T3-L1 adipocytes were cultured under both normoxia (21% O2) and hypoxia (1% O2) to mimic the hypoxic environment in obese adipose tissue. The cells were cultured for the specified durations as indicated in the figures. The relevant culture medium was collected as conditioned medium (CM) for culturing 3B-11 cells for tube formation assay or for ELISA test. Mature adipocyte were treated with CHIR99021 (SML1046) (10 µM), and AKT inhibitor Triciribine (T3830) (10 µM) purchased from Sigma-Aldrich, ERK inhibitor SCH772984 (S7101) (200 nM) and AMPK inhibitor Compound C (S7840) (10 µM) purchased from Selleck Chemicals, at the indicated time points as shown in the figures.

Tube Formation Assay

3B-11 (ATCC, CRL-2160) and EOMA mouse endothelial cells (ATCC, CRL-2586) were cultured in DMEM supplemented with 10% FBS in a 5% CO2 environment. Endothelial cell tube formation assay was also performed as described [82]. Briefly, the 96-well plate and tips were placed in the refrigerator or on ice for 20-30 minutes before loading Matrigel (Growth Factor Reduced Basement Membrane Matrix, Corning, CLS354230) into the plate. Then, 50 µl of Matrigel was pipetted into each well. The plate was incubated at 37 °C and 5% CO2 for 30 minutes to allow the Matrigel to solidify. The conditioned media or media with drug treatment was mixed with $1x10^5$ endothelial cells in a 200 µl volume. The number of cells could be adjusted depending on the specific endothelial cell type used. Tube formation began within 4-6 hours. After peak tube formation, the cells were stained with Calcein AM (Sigma-Aldrich, 206700) for 30 minutes. The media was carefully removed from the well to avoid disrupting the tube network on the Matrigel. Each well was washed with PBS, and fresh PBS was added for immediate visualization using a fluorescence/confocal microscope. Alternatively, to fix the tube network for later visualization, 4% paraformaldehyde (PFA, Boster, AR1068) was added to each well, incubated for 15 minutes at room temperature, the PFA was removed, each well was washed twice with PBS, and fresh PBS was added to each well. All cells stained with Calcein AM were imaged using a Nikon A1R Confocal microscope to observe the tube network.

Vessel perfusion

Vessel perfusion was analyzed by intravenous injections of labeled Tomato Lectin (50µg/100µl/mouse, Vector Labs, DL-1177-1) to assess microvasculature perfusion before euthanasia. The adipose tissues were collected and immersed in 4% PFA for overnight fixation at 4 °C. After fixation, the tissues were washed with PBS and subsequently sectioned for Lectin visualization or for additional staining purposes, such as staining for other proteins like CD31 (BD Biosciences, 553370) using the whole-mount immunofluorescence staining method (please refer to the procedures outlined in the "Histology and Whole-mount Immunofluorescence Staining" section). The stained sections were then mounted using VECTASHIELD® Antifade Mounting Medium from Vector Laboratories (H-1000-10). The samples were coverslipped and imaged using the same procedure as described for whole mount staining.

Adipose tissue adipocyte and SVF cell isolation

Adipocytes and stromal vascular fraction (SVF) cells were extracted as described [35]. Briefly, adipose tissues were digested by collagenase type 2 (Thermo Fisher Scientific, 17101015) at 37°C for 40-60 min with agitation. The digested tissues were then filtered through a 100 µm filter (Falcon-352360) and washed with DMEM containing 10% FBS. Subsequently, the suspensions were centrifuged at 500g for 5 minutes to separate the floating adipocytes from the SVF pellets. The floating mature adipocytes were washed again for further application. The SVF pellets were suspended in erythrocyte lysis buffer (Thermo Fisher Scientific, 00-4333-57) for 10 minutes to eliminate erythrocytes, followed by centrifugation at 300g. The cells were then resuspended and washed in DMEM medium with 10% FBS, and the cells were spun at 300g to collect the SVF cells.

Isolated SVF cells were plated onto collagen-coated dishes and incubated at 5% CO2 in growth DMEM media, supplemented with 10% FBS, and 1%Pen/Strep. Upon reaching 100% confluency, cells were stimulated with MDI medium containing 0.5 mM 3-isobutyl-1-methylxanthine, 5 µg/ml insulin, 1 µM dexamethasone, and 1 µM rosiglitazone in DMEM. After 3 days, the cells were switched to a maintenance medium (DMEM, 10% FBS, 1% penicillin/streptomycin, 5 µg/ml insulin) and the medium was changed daily for an additional 5-6 days until harvest.

Oil Red O staining for adipogenesis

Differentiated cells were fixed in 10% formalin (Sigma-Aldrich, F1635) for 15 min at room temperature then washed twice with water. Cells were incubated in Oil Red O (Sigma-Aldrich, O0625) working solution (1.8mg/ml Oil Red O in 60% isopropanol) for 10 min to stain accumulated lipids. Cells were then washed three times with water before bright field images were acquired.

Adipose Tissue Lipolysis analysis

To assess the inhibition of adipose tissue lipolysis by insulin, we quantified glycerol levels in serum before and 30 minutes after insulin injection (0.75 U/kg). Mice were fasted in the morning for 6 hours before tests. The quantification was performed using the Glycerol Determination Kit (Sigma-Aldrich, FG0100). Briefly, mouse serum was collected and mixed with the free glycerol reagent. The mixture was then incubated for 5 minutes at 37 °C. The absorbance (A540) of the Blank, Glycerol Standard (G7793, Sigma-Aldrich), and Sample was read and recorded. Finally, the glycerol concentration of the sample was calculated.

Serum and tissue lysate parameter analysis

ELISA was used to quantify protein levels of mouse VEGF (R&D Systems, MMV001) and adiponectin (MRP300, R&D systems), insulin (80-INSMS-E01, Alpco). In brief, for a sample containing 1 g of fat tissue, a 4 ml lysis buffer (10 mM Tris-HCl (pH 7.0), 10% glycerol, 150 mM NaCl, 2 mM EDTA (Thermo Fisher Scientific, AM9260G), and 1 mM PMSF (Sigma-Aldrich, 93482), along with a protease inhibitor cocktail (Sigma-Aldrich, P8340) and a phosphatase inhibitor cocktail (Cell signaling, 5870) was prepared. The mixture was incubated for 15 minutes, then centrifuged at 3,000 g for 15 minutes. To remove the lipid layer, the supernatant was centrifuged at 15,000 g, 4°C, for 20 minutes. Finally, the resulting supernatant was aliquoted and stored at -80°C for further ELISA analysis. The tissue Triglyceride (TG) level was measured using a high sensitivity triglyceride assay kit (Sigma-Aldrich, MAK264). Homogenization of 10 mg of tissue in 100 µL of ice-cold Triglyceride Assay Buffer was done for 10 minutes on ice. The samples were then centrifuged at 12,000g for 5 minutes to remove insoluble material. The supernatant was collected, and 50 µL of each sample was transferred into a well of a 96-well plate. 2 µL of Lipase was added to each well, including the standard wells. The contents were mixed well, and the plate was incubated at 37 °C for 20 minutes. After the incubation, the high sensitivity Probe, triglyceride enzyme mix, and triglyceride developer were added to each well. The plate was incubated for an additional 30 minutes at 37 °C, while being protected from light. The fluorescence intensity was measured using an excitation wavelength (λex) of 535 nm and emission wavelength (λem) of 587 nm. Free fatty acid was measured using the FFA quantification Kit (K612-100, Biovision). Tissue harvesting and washing with cold PBS was followed by homogenization in 200 µL chloroform with 1% Triton X-100. The homogenized tissues were further processed using a micro-homogenizer. The extract was spun for 5-10 minutes after incubating on ice for 10 – 30 minutes. The lower phase was collected and air-dried at 50°C to remove chloroform. The dried parts were dissolved in 200 µL of Fatty Acid Assay Buffer. Serum samples were directly tested by adding them to the microplate wells. Reaction wells, including standard wells and sample wells, were set up. ACS Reagent was added to all standard and sample wells, mixed, and then the reaction was incubated for 30 minutes at 37°C. A reaction mix was prepared for each reaction to be performed: standards, samples, and controls. The reaction mix was added to each well and incubated at 37ºC for 30 minutes, protected from light. The plate was read on a microplate reader at OD 570 nm for the Colorimetric assay or Ex/Em= 535/587 nm for the Fluorometric assay.

Western blot and multiplex protein assays

For Western blot analysis, protein extracts were prepared from lysed cells with RIPA lysis buffer containing a protease inhibitor cocktail (Sigma-Aldrich, P8340). For phosphorylated proteins, a phosphatase inhibitor cocktail (Cell signaling, 5870) was also added to the lysis buffer. The proteins were separated on 10% SDS-PAGE gels and transferred to a Nitrocellulose Membrane (Bio-Rad, 1620115). Primary antibodies for WB and their respective dilutions were indicated in the section of “Reagents and Antibodies”. The protein bands were detected using ECL™ Western Blotting Reagents (Cytiva, RPN2232). The ChemiDoc system from Bio-Rad was utilized for gel and immunoblot data acquisition, while ImageJ was employed for image processing and semi-quantified and blots were normalized against loading controls. We selected illustrative images that best represent the average results for each group as representative images from the entire series of experiments. Quantitative measurements of changes of multiplexing immunoassays of phosphorylated kinases were performed using multiplex-based technology (48–611MAG and 48–612 MAG kits, Millipore, Billerica, MA, USA) by determining the ratio of the corresponding phosphoproteins relative to total specific proteins. To prepare 16 ml of lysis buffer for a sample with 4 g of fat tissue, the following ingredients were included: 10% glycerol, 150 mM NaCl, 2 mM EDTA, 1 mM PMSF, along with a protease inhibitor cocktail and a phosphatase inhibitor cocktail. The samples were then centrifuged at 3,000 g for 15 minutes to remove the lipid layer. Subsequently, the resulting supernatant was centrifuged at 15,000 g, 4°C for 20 minutes. The supernatant was aliquoted and stored at -80°C for future measurement.

Immunoprecipitation (IP) and In vitro protein phosphorylation assay

Adipose tissue or matured adipocytes were lysed using either lysis-buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 (Sigma-Aldrich, T8787)) supplemented with the phosphatase inhibitor (Cell Signaling, 5870) and the protease inhibitor (Sigma-Aldrich , P8340). A total of 2 mg of protein extract was precleared using control IgG (Santa Cruz Biotechnology) and protein A/G beads (Thermo Fisher Scientific, 26161). The precleared extract was then incubated with specific antibodies for HIF-1α (Abcam, ab179483) or HIF-2α (Cell Signaling Technology, 57921) (2ug) or 2 µg of rabbit IgG (Cell Signaling Technology, 3900) ass negative control, along with protein A/G beads in 1.5 mL of IP buffer (0.5% NP-40, 10 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 10% glycerol, and protease inhibitor) at 4 °C for 4-6 hours or overnight. After a brief centrifugation, the pellets were washed 4-5 times with IP buffer at 4 °C for 10 minutes. The immunoprecipitated protein complexes were then analyzed using Western blot with specific antibodies. The eluted protein samples were loaded onto an SDS-PAGE gel to isolate proteins by electrophoresis. The proteins were then transferred to a Nitrocellulose membrane. The Nitrocellulose membrane was stained with the Pro-Q Diamond phosphoprotein blot stain kit (Thermo Fisher Scientific, P33356). In brief, the membrane was immersed in 25 mL of fix solution and incubated for 10 minutes, followed by washing in dH2O for 5 minutes (repeated three times). Next, place the fixed membrane in 25 mL of blot stain and incubate for 15 minutes. After staining, the membrane was washed in 30 mL of the destain solution for 5 minutes (repeated three times). Subsequently, the membrane should be washed in 25 mL of 50 mM sodium acetate, pH 4.0, for 5 minutes (repeated three times). Dry the membrane and the stained bands were imaged using a Bio-Rad ChemiDoc Imaging System.

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (PCR)

Total RNA was isolated from the cells using TRIzol (Invitrogen, 15596018), and 2 µg of the RNA sample was reverse-transcribed using QuantiTect reverse transcription kit (Qiagen, 205311). Genomic DNA in RNA samples was eliminated using the gDNA Wipeout Buffer provided in the kit. The RNA sample was mixed with the gDNA wiping buffer and incubated for 2 minutes at 42°C, followed by incubation with the reverse-transcription master mix containing all components for cDNA synthesis for 15 minutes at 42°C. We utilized the QuantStudio 7 Flex Real-Time PCR instrument (Applied Biosystems) to conduct quantitative PCR assays with the setting including an initial stage of 10 minutes pre-denaturation at 95°C, followed by the amplification stage, which involves denaturation for 15 seconds at 95°C, and a combined annealing and extension step for 60 seconds at 95°C. The sequences of primers used in our study and amplicon size are available in Supplementary major source table. For determining the relative mRNA levels of all genes, SYBR Green-based real-time PCR was employed, with normalization to the mouse housekeeping gene S16 serving as an internal control. We conducted melting curve analysis to validate the RT-PCR products. The relative mRNA expression levels of target genes were quantified by the comparative ΔΔCT method.

Immunofluorescence and Immunohistochemistry analysis

To detect inflammation in adipose tissue sections, immunofluorescence was performed using directly conjugated primary anti-F4/80 antibodies (5 µg/mL, eBioscience, 14-4801-85,). In brief, the paraffinized adipose tissue sections were deparaffinized in xylene and rehydrated through a series of graded alcohols. Subsequently, the sections were incubated with conjugated primary anti-F4/80 antibodies, followed by dehydration, clearing in xylene, and mounting with coverslips using appropriate mounting media for microscopic analysis. 3T3-L1 adipocytes were cultured on cell culture chamber slides (Nalge Nunc International, 177402). The specified treatments were administered as described either in the cell culture section or in the figure legend. After washing with PBS, the slides were treated with 4% PFA for 15 minutes, then further washed with PBS three times. Subsequently, they were treated with 5% Triton X-100 for 10 minutes and blocked with 1% BSA for 30 minutes. The cell culture slides were then incubated overnight at 4 °C with primary antibodies for HIF-1α or HIF-2α (1:100), followed

by the incubation with fluorescence-conjugated secondary antibodies (1:200, Thermo Fisher Scientific, 35552). To validate antibody specificity, isotype controls were conducted with rabbit IgG (3900, Cell Signaling technology) at the same concentrations as the primary antibodies. Secondary antibody only controls were performed to distinguish genuine target staining from background. The slides were mounted using Vector DAPI mounting medium (Vector Laboratories Inc, H-1800). The samples were examined, captured, and quantified using an Axio Observer Z1 inverted confocal microscope equipped with a Zeiss LSM 5 Live DuoScan laser scanning system (Carl Zeiss MicroImaging). We selected illustrative images that best represent the average results for each group as representative images from the entire series of experiments.

Histology and Whole-mount immunofluorescence staining

Tissues collected from terminally anesthetized mice were cut and fixed in 4% PFA for overnight. Adipose tissue was analyzed either as whole mount staining (for CD31 staining and lectin analysis), embedded in OCT (Tissue-Tek) (for DHE staining for ROS analysis) or embedded in paraffin (for other IHC staining) and cut in 5 μm sections. Liver was embedded in OCT or in paraffin for Oil Red O staining or Hematoxylin-eosin (H&E) (VectorLab, H-3502) staining, respectively. For Oil Red O (Sigma-Aldrich, O0625) staining, fresh tissues were initially embedded in OCT compound and stored at -80°C until analysis. Subsequently, frozen tissues were sectioned at 6 μm using a cryostat (Leica Biosystems, USA). The sections were air-dried for 30 minutes and then fixed in 10% formalin for 10 minutes. The slides were washed with 60% isopropanol and then stained in working Oil Red O solution (30 mL of stock solution (300mg of Oil Red O in /100mL isopropanol) mixed with 20 mL of Distilled Water) for 15 min. The slides were counterstained with hematoxylin and washed with distilled water. Hematoxylin and Eosin (H&E)-stained sections were used to visualize crown-like structures of adipose tissue and lipid accumulation in liver. For H&E staining, tissues were fixed in 4% PFA overnight at 4°C. Subsequently, the samples underwent dehydration using a series of alcohol solutions and were embedded in paraffin wax. The paraffin-embedded tissues were then sectioned at a thickness of 5 μm using a microtome and were mounted on glass slides. The tissue sections were deparaffinized in xylene and sequentially rehydrated through a descending alcohol series. Following this, the sections were stained with hematoxylin for 5 minutes, followed by a water rinse and differentiation in acid alcohol. After another water rinse, the sections were counterstained with eosin for 2 minutes. Post-staining, the sections underwent dehydration in an ascending alcohol series, clearing in xylene, and were finally mounted with a coverslip using mounting media. Whole-mount immunofluorescence staining was performed as described [83]. In details, fresh tissues were collected and then dissected into pieces measuring approximately 5 mm x 5mm x 2 mm using dissection scissors, washed with ice-cold PBS and then followed by fixation in 4% PFA at 4°C overnight. The tissue samples were then transferred to room temperature (RT) for 30 minutes and subsequently moved to a 12- or 24-well cell culture plate for faster washing using PBST for 5 minutes for three times on a shaker at RT. The tissues were then permeabilized using PBS containing 0.4% TritonX-100 and blocked using a blocking buffer containing 3% animal serum (such as goat serum) diluted in PBS for 30 minutes to 1 hour at RT. Then primary antibodies incubation (anti-CD31 diluted 1:50 in PBST with 1% goat serum) or staining for injected lectin were conducted on a shaker at 4°C overnight. On the next day, the tissues were washed three times with PBST for 5 minutes each at RT. If co-staining with CD31 and DyLight 594-conjugated Lectin (Vectorlab, DL-1177-1), secondary antibody conjugated with Alexa Fluor 488 (Thermo Fisher Scientific, A-11006) was used for CD31 primary antibody. If staining CD31 alone, secondary antibody conjugated with DyLight 594 (Thermo Fisher Scientific, A-21209) was used, diluted 1:200 in PBST and added to the wells. The plate was wrapped in aluminum foil to prevent exposure to the light and incubated on a shaker for 1 hour at RT. After the incubation with the secondary antibody, the tissues were washed twice with PBST for 5 minutes each at RT. If a neutral lipid stain is desired, or LipidTOX™ Green Neutral Lipid Stain (Invitrogen H34475) could be applied to the tissues. Then the samples were then ready for microscopy. Immunofluorescence images were taken with a Zeiss LSM710 Confocal microscope. Brightfield sections were imaged with Olympus

BX53 Upright Microscope. To capture images of whole-mount-stained tissues, conducted Z-stack imaging with a depth of 100 μm and a step size of 6 μm at the desired magnification. We selected illustrative images that best represent the average results for each group as representative images from the entire series of experiments. Immunostaining staining was quantified by a researcher blinded to the genotype.

Detection of Tissue Hypoxia

Immunochemical detection of adipose tissue was performed using Hypoxyprobe™-1 Plus Kit (Chemicon International, Temecula, CA, HP2-100Kit). Mice were injected with 60 mg/kg pimonidazole intraperitoneally, 1h before sacrifice. Adipose tissues were harvested and fixed in 10% formalin overnight, and then processed into paraffin blocks. The sections were used for pimonidazole staining to detect tissue hypoxia status. In brief, to perform hypoxyprobe staining, the tissue sections were deparaffinized using clean xylene. The sections were then hydrated through a series of ethanol solutions. Endogenous peroxidase activity was quenched by treating the sections with 3% hydrogen peroxide (H2O2). Antigen retrieval was carried out using a 10 mM Citrate buffer. After antigen retrieval, nonspecific binding was blocked using 1% BSA or 1% normal mouse serum in TBS. A FITC-conjugated mouse anti-hypoxyprobe monoclonal antibody with a concentration of 0.5 mg/ml was applied to each tissue section. The sections were incubated with the primary antibody for the recommended duration. Subsequently, the sections were incubated with a horseradish peroxidase-conjugated secondary antibody at a dilution of 1:100. For visualization, a suitable 3,3'-diaminobenzidine reagent (DAB) was applied to the sections. This enabled visualization of the staining. Brightfield sections were imaged with an Olympus BX53 Upright Microscope.

Picrosirius red staining for fibrosis quantification

Fibrosis was determined by picrosirius red staining. To perform Picrosirius Red staining, the sections were deparaffinized, if needed, and hydrated. Deparaffinized sections were incubated in picrosirius red Solution (Abcam, ab150681) for 1 hour, and the slide was quickly rinsed in an acetic acid solution (0.5%) two times before being mounted in synthetic resin. Quantification was determined as a percentage of stained red area (fibrosis)/tissue area ratio.

ROS quantification

For tissue ROS detection, frozen sections of adipose tissues were defrosted for 1h in a humidified chamber at room temperature followed by the incubation with 5μm DHE (Dihydroethidium) (Sigma-Aldrich, 309800) protected against light at 37°C for 30 min. Slides were subsequently washed for 5 min for twice in PBS. Slides were finally counterstained with Hoechst and mounted with Vectashield anti-fading medium (Vector Laboratories, H-1000). Immunofluorescence images were taken with a Zeiss LSM710 Confocal microscope.

RNA-Sequencing (RNA-Seq) Analysis

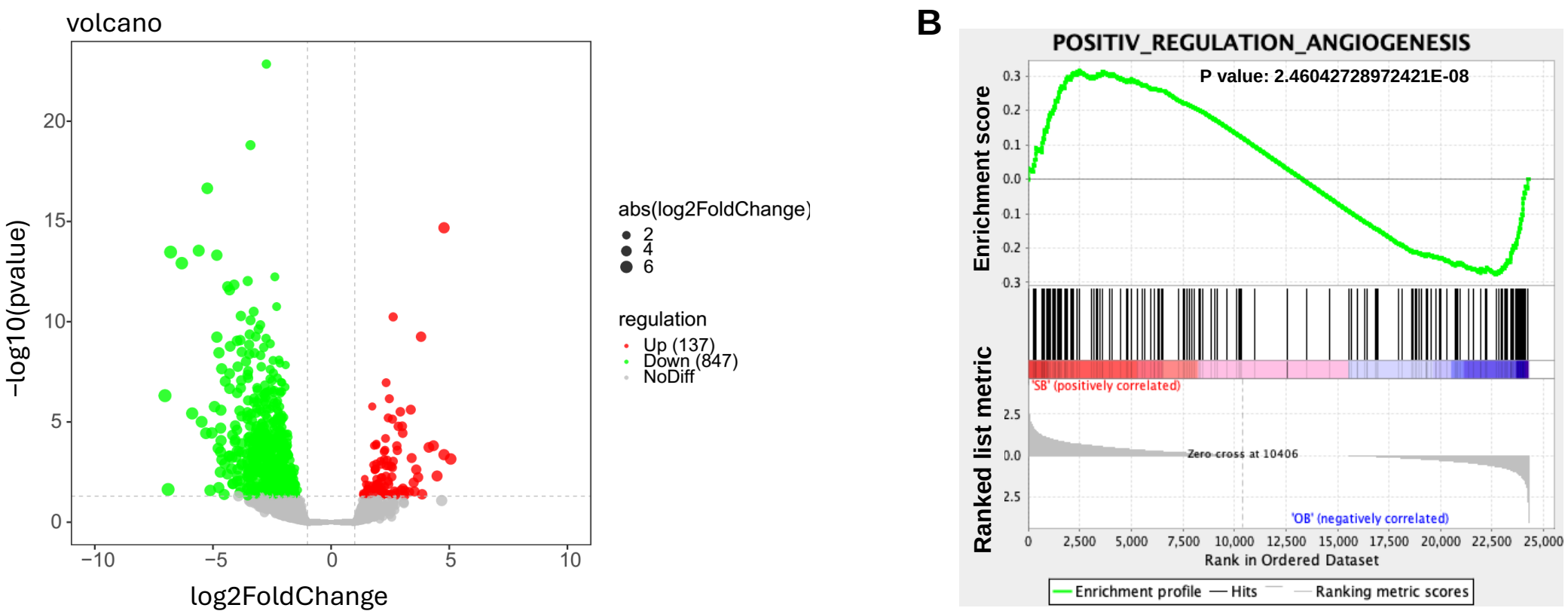
Total RNA was extracted from adipose tissues of indicated mice using the RNeasy extraction Kit (Qiagen, 74106). The Illumina NovaSeq 6000 platform utilizing Paired-End 150 (PE150) sequencing technology was employed for RNA-Seq. The sequence reads underwent rigorous quality control assessment with FastQC. Next, the RNA-seq data was aligned to the GRCm39 mouse reference genome using Bowtie2 (Version 2.5.2) with a specified trimming of 10 bases from the 5' end of the nucleotide chain. Reads were filtered to ensure a final length of at least 25 bases post-trimming. The paired-end read counts were quantified using feature Counts (Version 2.0.6). Differential expression analysis of read counts was then conducted using the DESeq2 package, and sample normalization was performed via variance stabilizing transformation (VST). Genes with a fold change greater than 2 and p-value < 0.05 were defined as differentially expressed genes. To analyze the Gene Ontology (GO) pathway of Differentially Expressed Genes (DEGs) or perform Gene Set Enrichment Analysis (GSEA), the cluster Profiler package in R was utilized. The RNA-sequencing data generated in this study

have been deposited in the National Genomics Data Center (accession number: OMIX007660). In the context of our heatmaps, gene expression was normalized using the `scale` function in the R package, which computes a Z-score on a per-gene basis by centering and scaling the data according to the standard deviation. The normalized expression values are visually represented in the heatmap using a spectrum of colors. The resulting Z-score scaling indicates higher expression levels relative to the mean are depicted with positive Z-scores (shown in red), while lower expression levels are represented with negative Z-scores (shown in green). The array of colors in the heatmap reflects changes in the expression of individual genes across the sample set while not recommended for direct comparison of expression levels between different genes due to the normalization process applied.

Statistical Analysis

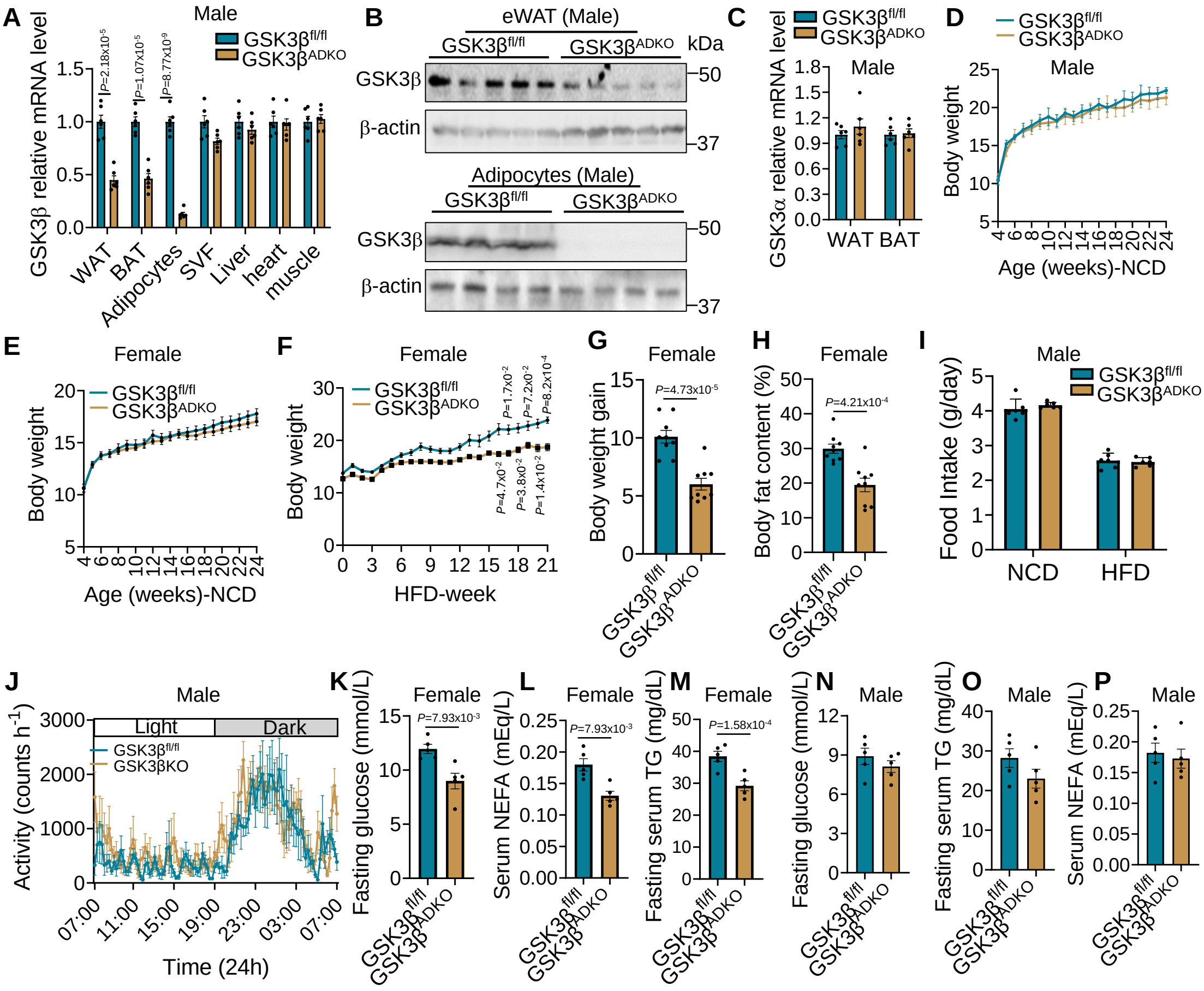
The data are presented as means ± standard error of the mean (SEM). All acquired data were analyzed using Microsoft Excel 2010 and GraphPad Prism 8.0.2. Multiple comparison corrections were only conducted within individual tests; no corrections were applied across the entirety of the study. We performed normal distribution tests using the Shapiro-Wilktest in GraphPad Prism 8.0.2. The homogeneity of variance test was conducted using either Levene's test or Bartlett's test. Comparison between two groups with a sample size of 6 or more was evaluated using an unpaired two-tailed t-test. For comparisons involving two groups with a sample size less than 6 or in cases where the data fails the normality test with a sample size of 6 or more, the non-parametric Mann-Whitney test was utilized. When comparing more than two groups with a sample size of 6 or more, one-way ANOVA followed by Tukey's test for post-hoc analysis was applied. For comparisons among groups with a sample size less than 6 or in instances where the data does not pass the normality test with a sample size of 6 or more, a non-parametric test was taken (Kruskal-Wallis test followed by post-hoc Dunn's test). For repeated measures across time (all with a sample size ≥ 6), a repeated measures ANOVA followed by Bonferroni's test for post-hoc analysis was used. The statistical analysis methods for each figure are detailed in the "Supplementary Statistics Analysis Methods" table. Statistical significance was determined at a threshold of $P<0.05$. We utilized the ROUT method ($Q=1\%$) in GraphPad Prism to identify outliers. All experiments were conducted in a randomized manner, and the reported data were derived from multiple independent biological replicates, with the exact sample sizes specified in the figure or figure legends. We selected illustrative images that best represent the average results for each group as representative images from the entire series of experiments.

Figure S1



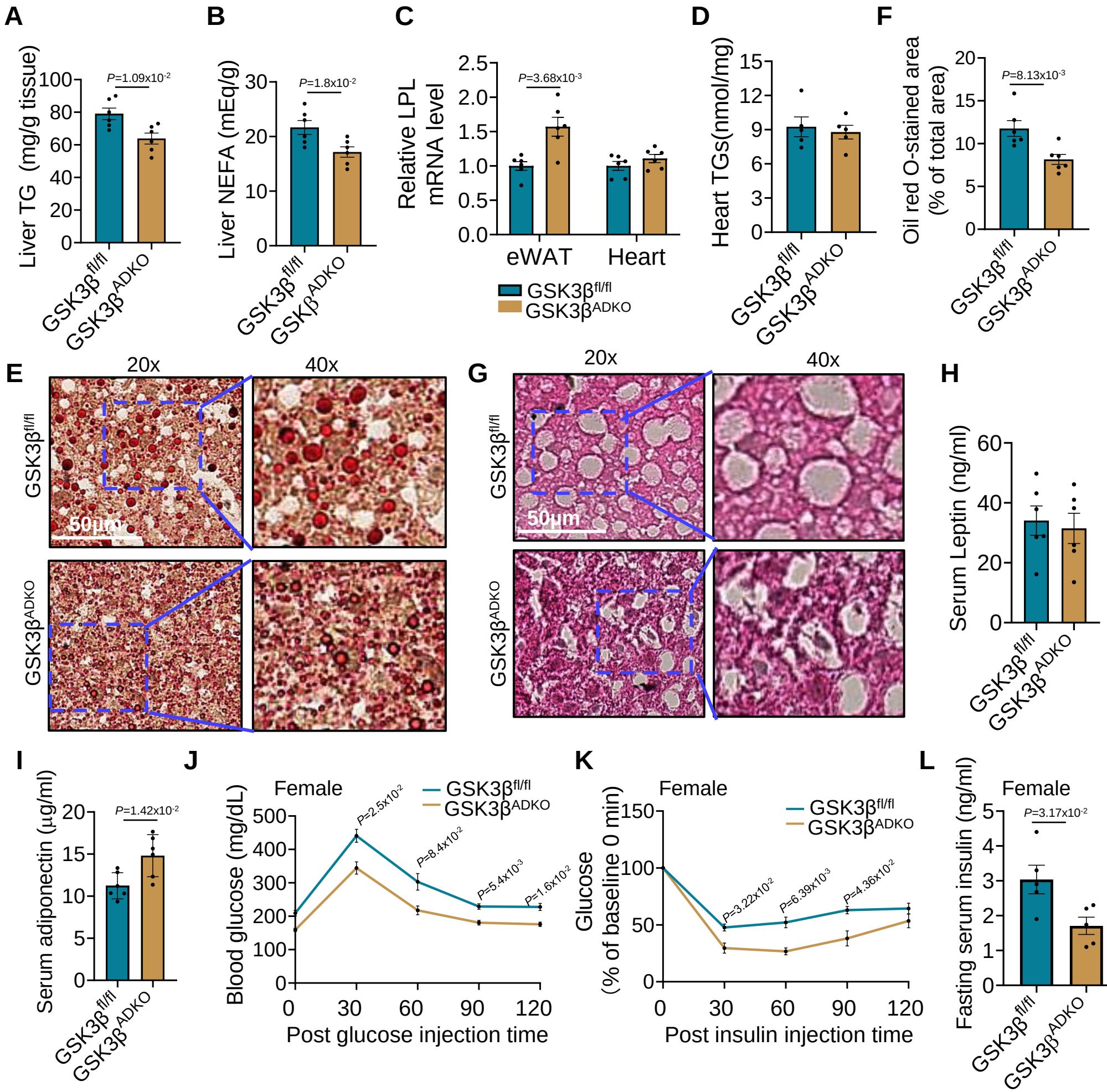
Supplementary Figure1. GSK3 inhibition stimulates angiogenesis associated program in obese AT. Male C57Bl/6J lean mice (LE) were fed a normal chow diet (NCD), whereas male obese mice (OB) were fed a high-fat diet (HFD) with or without SB216763 treatment (OB + SB). **A.** Volcano plot of the DEGs from eWAT of OB+SB vs. OB mice (n=2). **B.** GESA plot illustrates the positive regulation of the angiogenesis pathway using two sets of DEGs from OB + SB vs. OB mice. **C. H**eatmap of expression values (Z-Score) for angiogenesis-associated genes in eWAT of OB and OB+SB mice (n=2).

Figure S2



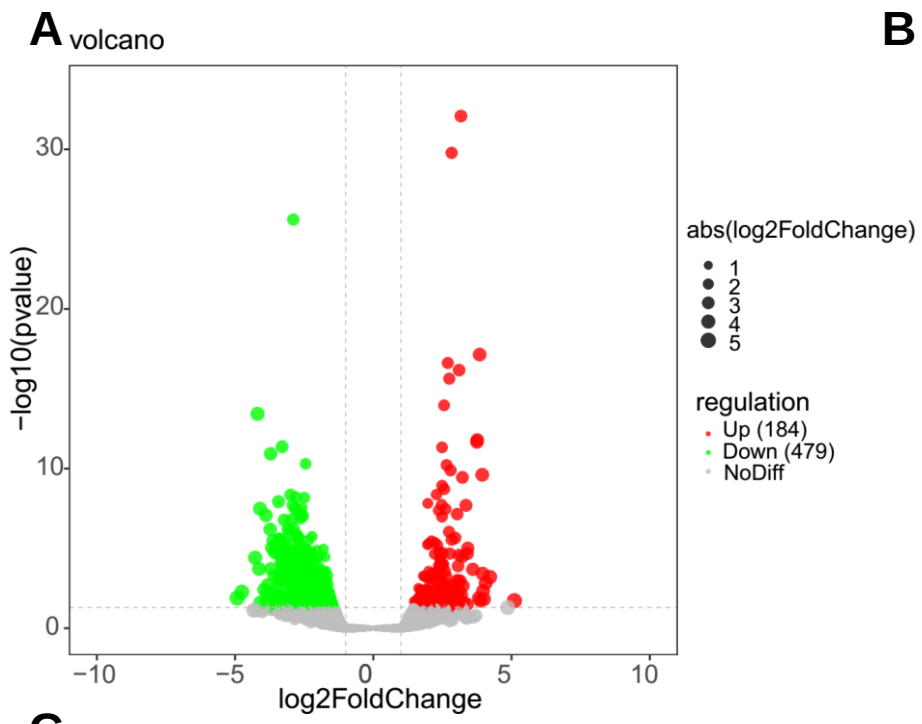
Supplementary Figure 2. The validation of GSK3β ablation in adipocytes and the effects of GSK3β deficiency in adipocytes on the metabolic health of mice fed an NCD or an HFD. A. The expression of GSK3β in multi-tissues at mRNA level (n=6). **B.** GSK3β protein analysis in eWAT and adipocytes of male GSK3β$^{fl/fl}$ and GSK3β^{ADKO} mice to confirm GSK3β deletion. **C.** GSK3α expression analysis in WAT and BAT in male GSK3β$^{fl/fl}$ and GSK3β^{ADKO} mice (n=6). **D.** Body weight of male GSK3β$^{fl/fl}$ and GSK3β^{ADKO} mice fed an NCD (n=6). **E-F.** Body weight of female GSK3β$^{fl/fl}$ and GSK3β^{ADKO} mice fed an NCD (E, n=6) or HFD (n=9). **G-H.** Comparison of body weight gain (G) and body fat content (H) of female obese GSK3β$^{fl/fl}$ and GSK3β^{ADKO} mice fed an HFD (n=9). **I.** Comparison of food intake of male GSK3β$^{fl/fl}$ and GSK3β^{ADKO} mice fed an NCD or HFD (n=6). **J.** The physical activity of male obese GSK3β$^{fl/fl}$ and GSK3β^{ADKO} mice fed an HFD (n=6). **K-M.** Comparison of fasting serum glucose (K), non-esterified fatty acid (NEFA) (L), and triglyceride (TG) levels of (M) of female obese GSK3β$^{fl/fl}$ and GSK3β^{ADKO} mice (n = 5). **N-P.** Fasting serum glucose (N), Fasting serum TG (O) and serum free fatty acids (P) from male GSK3β$^{fl/fl}$ and GSK3β^{ADKO} mice fed an NCD (n=5). A, C and G-I, Two-tailed Unpaired t test; D-F, Repeated ANOVA and Bonferroni's post hoc test; K-P, Non-parametric test.

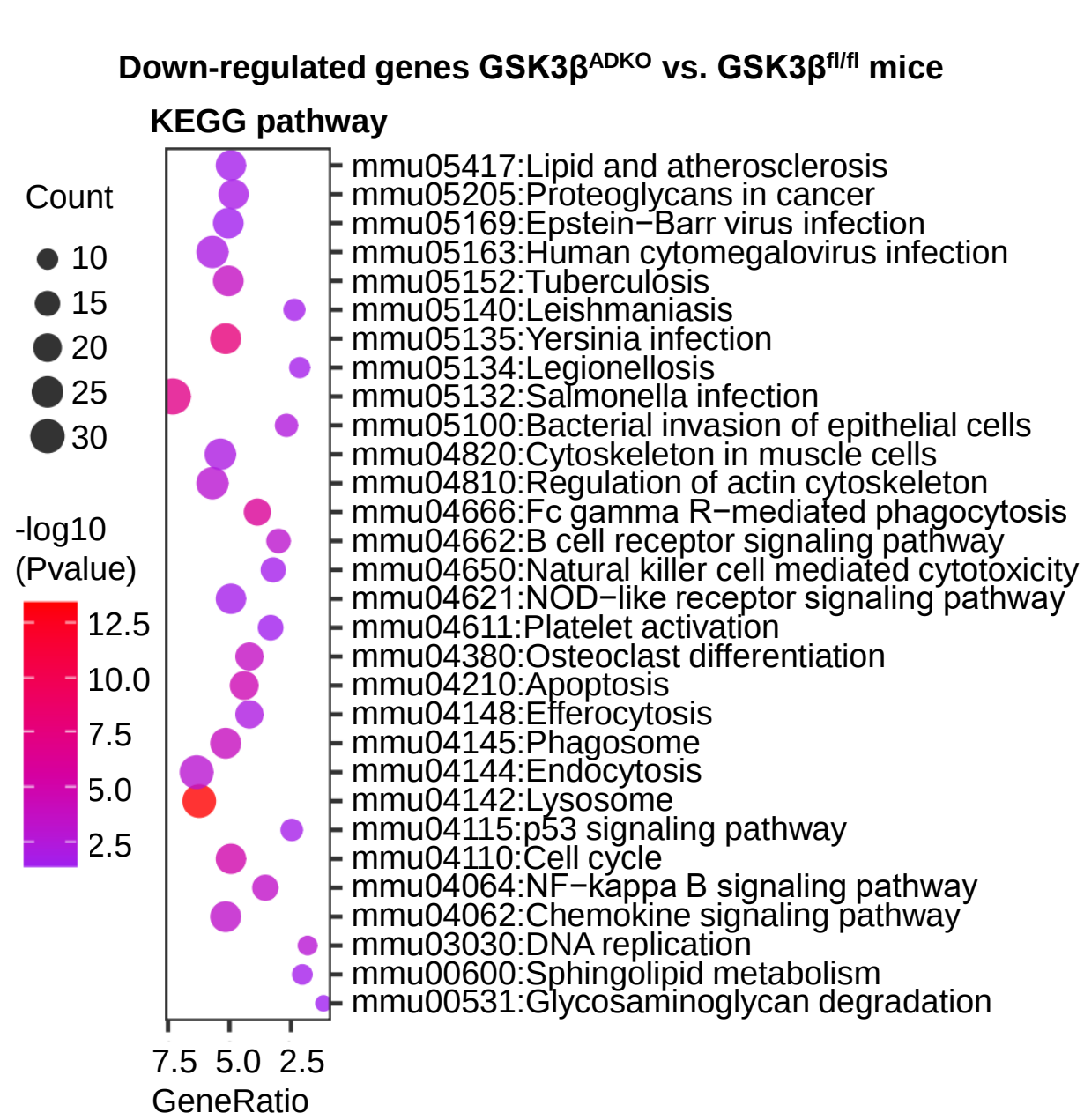
Figure S3

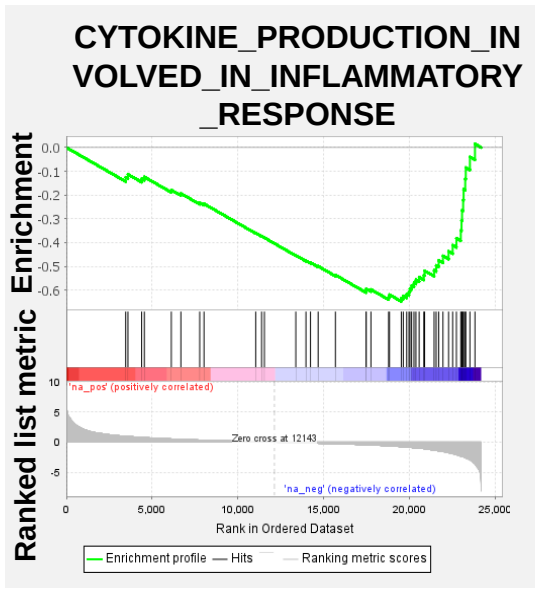


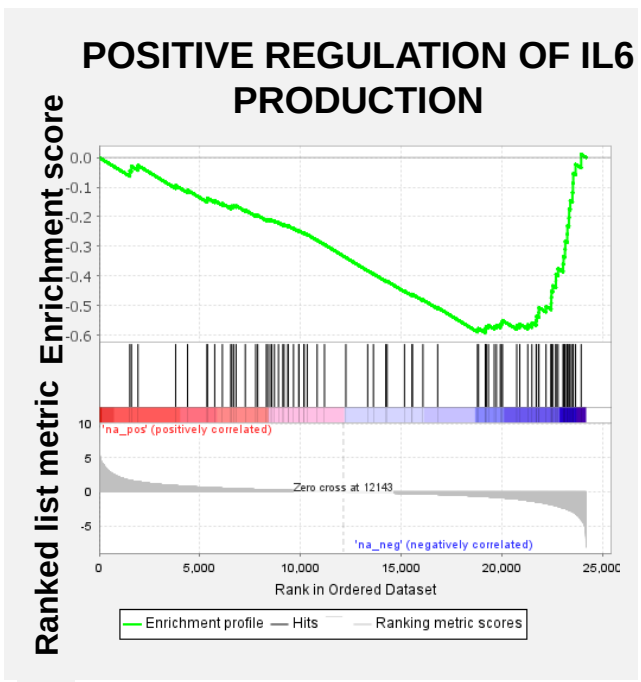
Supplementary Figure 3. The effects of the adipocyte-specific ablation of GSK3β on metabolic parameters including lipid deposition, glucose tolerance, and insulin sensitivity. A-B. Liver TG (A) and NEFA (B) were quantified in male obese GSK3β$^{fl/fl}$ and GSK3β^{ADKO} mice fed an HFD (n=6). **C.** LPL mRNA level analysis by RT-PCR in eWAT and heart of male GSK3β$^{fl/fl}$ and GSK3β^{ADKO} mice fed an HFD (n=6). **D.** TG levels in heart of male GSK3β$^{fl/fl}$ and GSK3β^{ADKO} mice fed an HFD (n=5). **E-G.** Representative images of Oil red O staining of livers (E), percentage of Oil red O-stained area was determined using an ImageJ (F) and H&E staining of livers (G) from male GSK3β$^{fl/fl}$ and GSK3β^{ADKO} mice fed an HFD (n=6). **H-I.** Serum leptin (H) and adiponectin (I) levels of male GSK3β$^{fl/fl}$ and GSK3β^{ADKO} mice fed an HFD (n=6). **J-L.** Glucose tolerance test (J), insulin tolerance test (K) and fasting serum insulin level (L) of female obese GSK3β$^{fl/fl}$ and GSK3β^{ADKO} mice fed an HFD (n = 6 for J-K; n=5 for L). Scale bar: 50μm. A-C, F, H, I and L, Two-tailed Unpaired t test; D, Non-parametric test; J-K, Repeated ANOVA and Bonferroni's post hoc test.

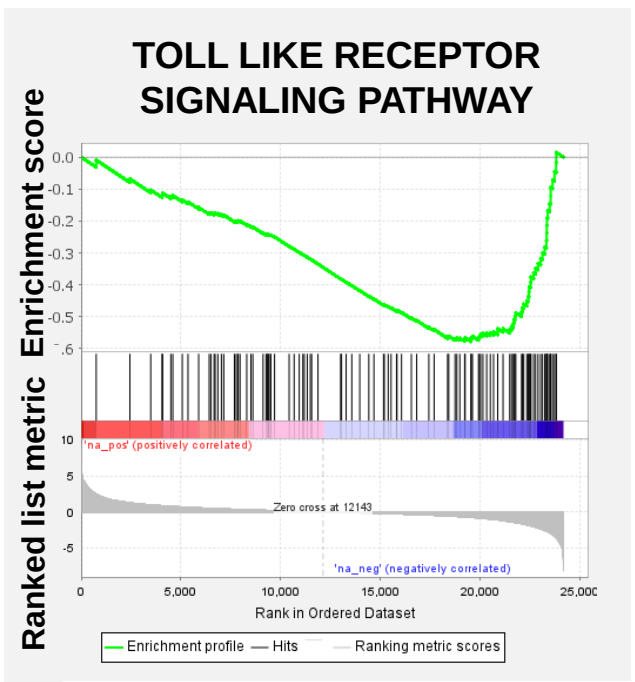
Figure S4


A
volcano
−log10(pvalue)
log2FoldChange
abs(log2FoldChange)
1
2
3
4
5
regulation
Up (184)
Down (479)
NoDiff


B
Down-regulated genes GSK3β^ADKO vs. GSK3β^fl/fl mice
KEGG pathway
Count
10
15
20
25
30
-log10
(Pvalue)
12.5
10.0
7.5
5.0
2.5
mmu05417:Lipid and atherosclerosis
mmu05205:Proteoglycans in cancer
mmu05169:Epstein−Barr virus infection
mmu05163:Human cytomegalovirus infection
mmu05152:Tuberculosis
mmu05140:Leishmaniasis
mmu05135:Yersinia infection
mmu05134:Legionellosis
mmu05132:Salmonella infection
mmu05100:Bacterial invasion of epithelial cells
mmu04820:Cytoskeleton in muscle cells
mmu04810:Regulation of actin cytoskeleton
mmu04666:Fc gamma R−mediated phagocytosis
mmu04662:B cell receptor signaling pathway
mmu04650:Natural killer cell mediated cytotoxicity
mmu04621:NOD−like receptor signaling pathway
mmu04611:Platelet activation
mmu04380:Osteoclast differentiation
mmu04210:Apoptosis
mmu04148:Efferocytosis
mmu04145:Phagosome
mmu04144:Endocytosis
mmu04142:Lysosome
mmu04115:p53 signaling pathway
mmu04110:Cell cycle
mmu04064:NF−kappa B signaling pathway
mmu04062:Chemokine signaling pathway
mmu03030:DNA replication
mmu00600:Sphingolipid metabolism
mmu00531:Glycosaminoglycan degradation
7.5 5.0 2.5
GeneRatio


C
CYTOKINE_PRODUCTION_IN
VOLVED_IN_INFLAMMATORY
_RESPONSE
Enrichment
Ranked list metric
'na_pos' (positively correlated)
Zero cross at 12143
'na_neg' (negatively correlated)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores


D
POSITIVE REGULATION OF IL6
PRODUCTION
Enrichment score
Ranked list metric
'na_pos' (positively correlated)
Zero cross at 12143
'na_neg' (negatively correlated)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores


E
TOLL LIKE RECEPTOR
SIGNALING PATHWAY
Enrichment score
Ranked list metric
'na_pos' (positively correlated)
Zero cross at 12143
'na_neg' (negatively correlated)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores

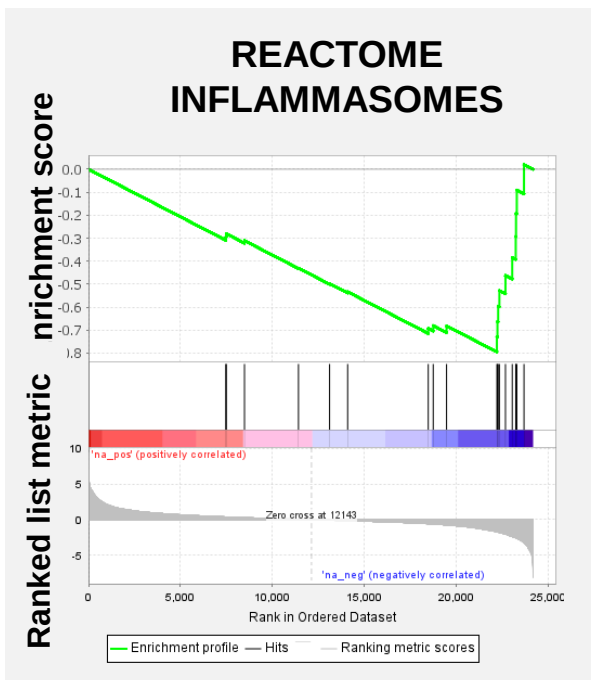

F
REACTOME
INFLAMMASOMES
nrichment score
Ranked list metric
'na_pos' (positively correlated)
Zero cross at 12143
'na_neg' (negatively correlated)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores

Figure S4 cont'd

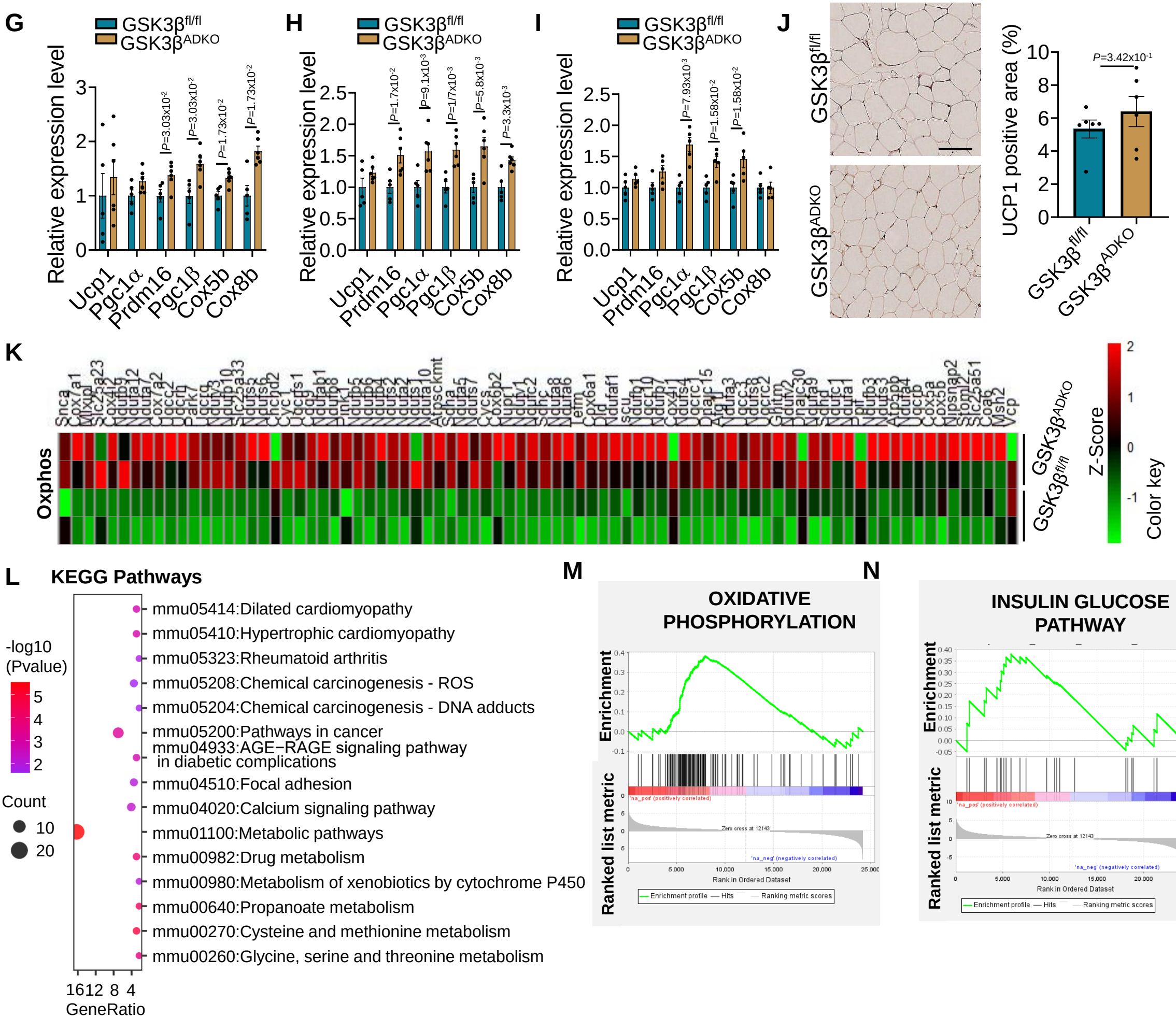
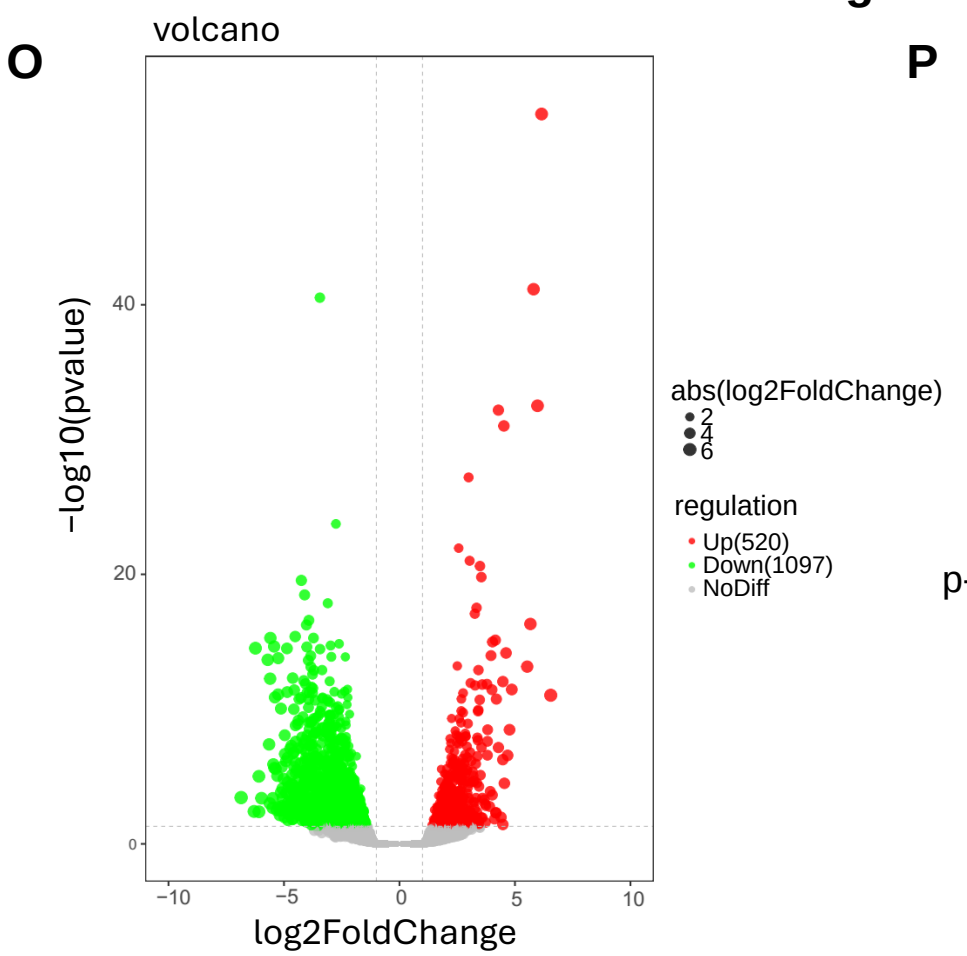
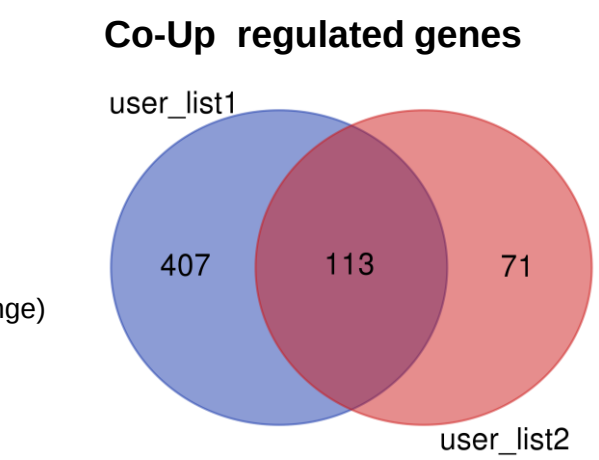
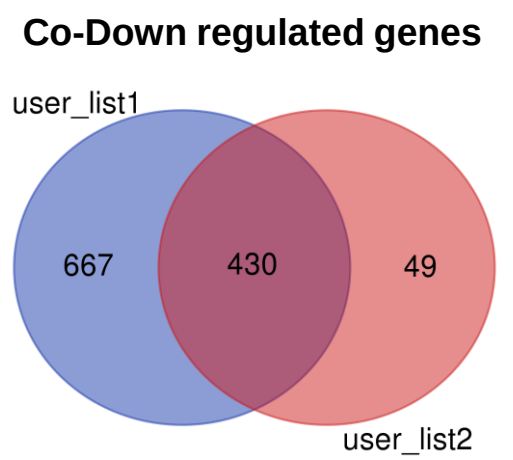


Figure S4 cont'd

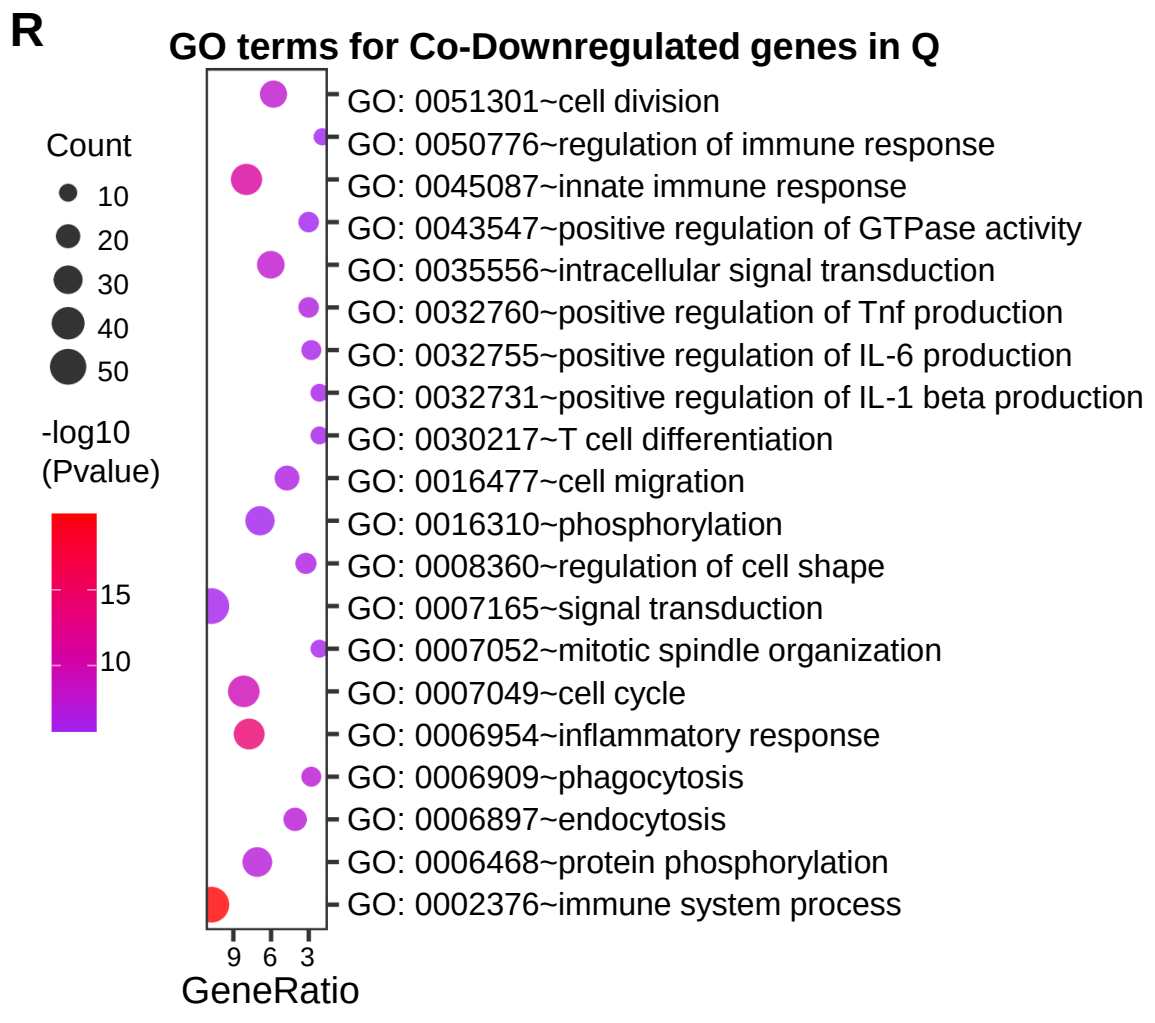


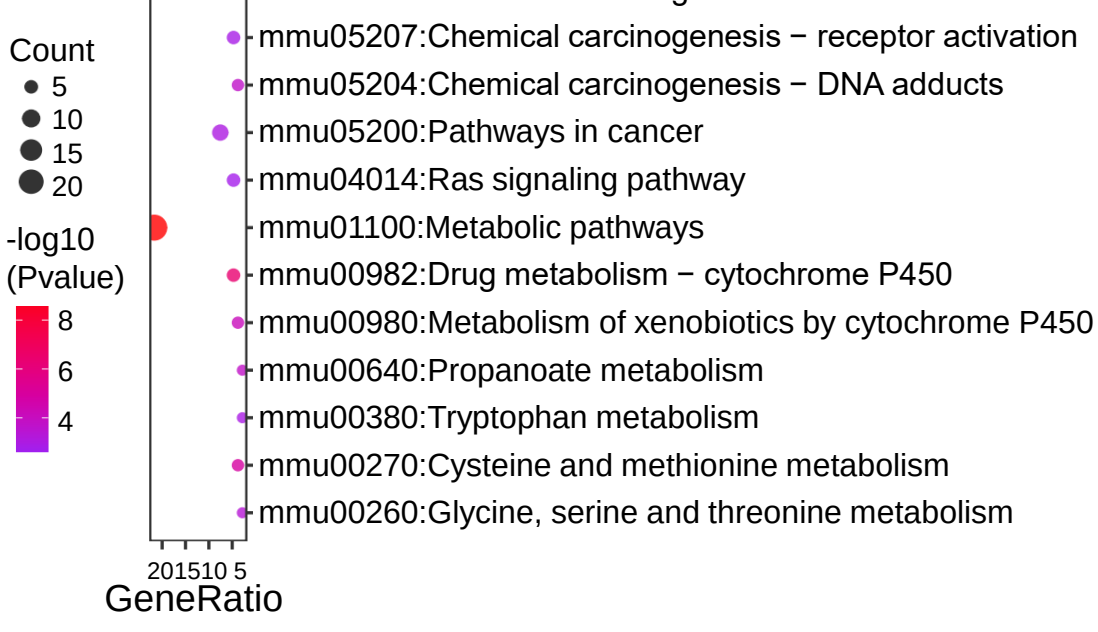


p-value < 0.05 and $\log_2$ FC > $\log_2(2)$



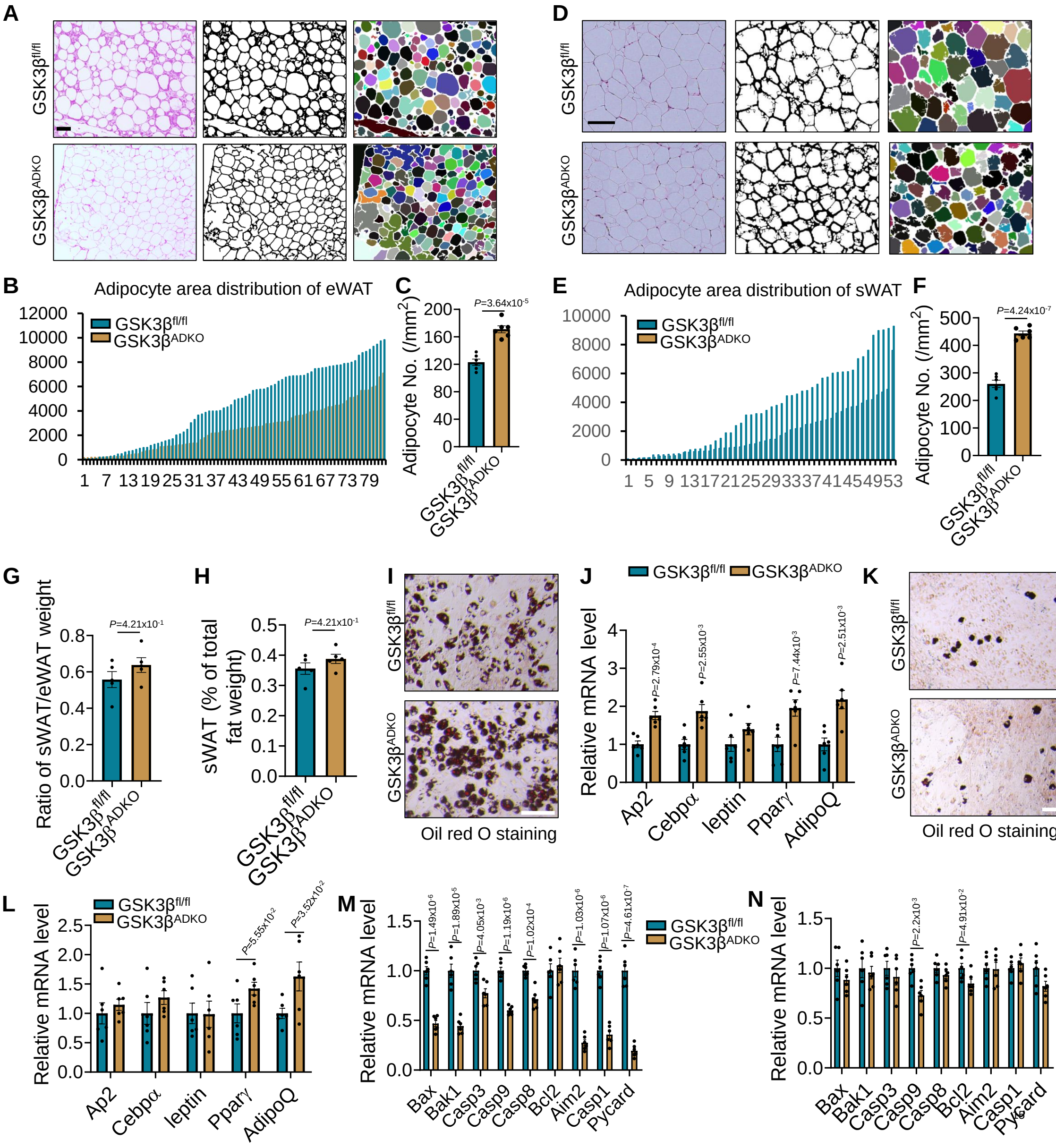
p-value < 0.05 and $\log_2$ FC > $\log_2(2)$





Supplementary Figure 4. The effects of adipocyte-specific deletion of GSK3β on the expression of genes and pathways associated with inflammation and metabolic activity in AT of obese mice. Male GSK3β$^{fl/fl}$ and GSK3β^{ADKO} mice were fed an HFD to induce obesity and used for the experiments. **A.** Volcano plot of the DEGs from eWAT of obese GSK3β^{ADKO} vs obese control GSK3β$^{fl/fl}$ mice (n=2). **B.** KEGG analysis showing downregulated gene signatures from the eWAT of obese GSK3β^{ADKO} vs GSK3β$^{fl/fl}$ mice. **C-F.** GSEA plot of the inflammatory response pathways using two sets of genes from the eWAT of obese GSK3β^{ADKO} vs. obese control GSK3β$^{fl/fl}$ mice. **G-I**. The expression analysis of BAT marker genes in eWAT (G), sWAT (H) and BAT (I) by RT-PCR (n=5). **J.** UCP1 staining in sWAT of obese GSK3β$^{fl/fl}$ and GSK3β^{ADKO} mice (n=6). **K.** Heatmap of expression values (Z-Score) for OxPhos-associated genes in eWAT from the indicated obese mice (n=2). **L.** KEGG analysis showing upregulated gene signatures from the eWAT of obese GSK3β^{ADKO} vs GSK3β$^{fl/fl}$ mice. **M-N.** GESA plot of oxidative phosphorylation (M) and insulin/glucose (N) pathways using two sets of genes from eWAT of obese GSK3β^{ADKO} vs GSK3β$^{fl/fl}$ mice. **O.** Volcano plot of the DEGs from eWAT of lean GSK3β$^{fl/fl}$ group vs. obese GSK3β$^{fl/fl}$ group (n=2). **P-Q.** Venn diagrams to summarize RNA-seq data sets. Blue circles indicate the numbers of genes up- (P) or down-(Q) regulated in eWAT of lean GSK3β$^{fl/fl}$ group vs. obese GSK3β$^{fl/fl}$ group; orange circles represent the numbers of up-or down-regulated genes in eWAT of the obese GSK3β^{ADKO} group vs. obese GSK3β$^{fl/fl}$ group, red region indicated commonly up- or down-regulated genes in eWAT of both lean GSK3β$^{fl/fl}$ and obese GSK3β^{ADKO} compared to obese GSK3β$^{fl/fl}$ mice, respectively. **R.** Gene ontology terms analysis of co-downregulated gene signatures in panel Q. **S.** KEGG analysis of co-upregulated gene signatures in panel P. Scale bar: 100μm. G-I, Non-parametric test; J, Two-tailed Unpaired t test.

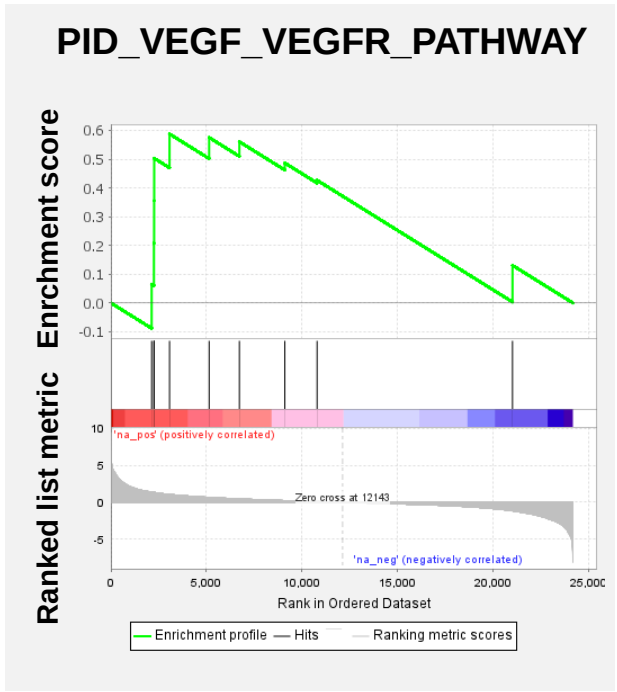
Figure S5

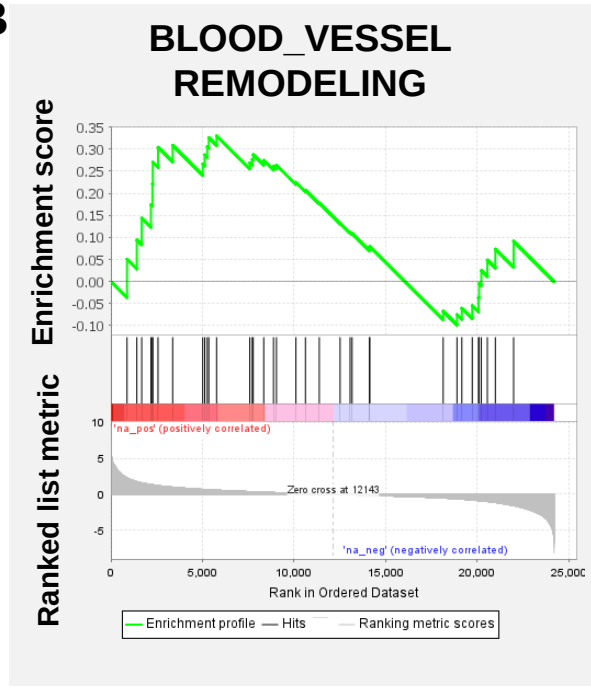

A
GSK3β^fl/fl
GSK3β^ADKO
D
GSK3β^fl/fl
GSK3β^ADKO
B
Adipocyte area distribution of eWAT
GSK3β^fl/fl
GSK3β^ADKO
12000
10000
8000
6000
4000
2000
0
1 7 13 19 25 31 37 43 49 55 61 67 73 79
C
Adipocyte No. (/mm2)
P=3.64x10-5
200
160
120
80
40
0
GSK3β^fl/fl
GSK3β^ADKO
E
Adipocyte area distribution of sWAT
GSK3β^fl/fl
GSK3β^ADKO
10000
8000
6000
4000
2000
0
1 5 9 13 17 21 25 29 33 37 41 45 49 53
F
Adipocyte No. (/mm2)
P=4.24x10-7
500
400
300
200
100
0
GSK3β^fl/fl
GSK3β^ADKO
G
Ratio of sWAT/eWAT weight
P=4.21x10-1
0.8
0.6
0.4
0.2
0.0
GSK3β^fl/fl
GSK3β^ADKO
H
sWAT (% of total fat weight)
P=4.21x10-1
0.5
0.4
0.3
0.2
0.1
0.0
GSK3β^fl/fl
GSK3β^ADKO
I
GSK3β^fl/fl
GSK3β^ADKO
Oil red O staining
J
GSK3β^fl/fl
GSK3β^ADKO
Relative mRNA level
4
3
2
1
0
P=2.79x10-4
P=2.55x10-3
P=7.44x10-3
P=2.51x10-3
Ap2
Cebpα
leptin
Pparγ
AdipoQ
K
GSK3β^fl/fl
GSK3β^ADKO
Oil red O staining
L
GSK3β^fl/fl
GSK3β^ADKO
Relative mRNA level
2.5
2.0
1.5
1.0
0.5
0.0
P=5.55x10-2
P=3.52x10-2
Ap2
Cebpα
leptin
Pparγ
AdipoQ
M
Relative mRNA level
GSK3β^fl/fl
GSK3β^ADKO
1.5
1.0
0.5
0.0
P=1.49x10-6
P=1.89x10-5
P=4.05x10-3
P=1.19x10-6
P=1.02x10-4
P=1.03x10-6
P=1.07x10-6
P=4.61x10-7
Bax
Bak1
Casp3
Casp9
Casp8
Bcl2
Aim2
Casp1
Pycard
N
Relative mRNA level
1.5
1.0
0.5
0.0
P=2.2x10-3
P=4.91x10-2
Bax
Bak1
Casp3
Casp9
Casp8
Bcl2
Aim2
Casp1
Pycard

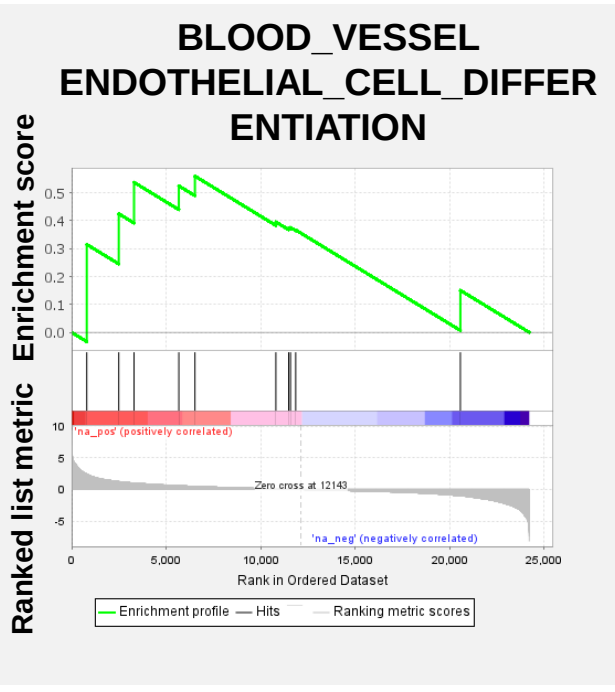
Supplementary Figure 5. The effects of GSK3β deficiency on adipocyte size, sWAT/eWAT ratio and the expression of adipogenic genes. **A-B.** Determination of adipocyte size of eWAT from male obese GSK3β$^{fl/fl}$ and GSK3β^{ADKO} mice fed an HFD. Voronoi Diagram to show sectional view of adipose tissue (A) and area distribution (B). **C.** Adipocyte number analysis of eWAT from male obese GSK3β$^{fl/fl}$ and GSK3β^{ADKO} mice (n=6). **D-E.** Determination of adipocyte size of sWAT from obese male GSK3β$^{fl/fl}$ and GSK3β^{ADKO} mice. Voronoi Diagram to show sectional view of adipose tissue (D) and area distribution (E). **F.** Adipocyte number analysis of sWAT from obese male GSK3β$^{fl/fl}$ and GSK3β^{ADKO} mice (n=6). **G-H.** The ratio of sWAT to eWAT weight (G) and the percentage of sWAT weight in total fat weight (sWAT+eWAT) (H) of male GSK3β$^{fl/fl}$ and GSK3β^{ADKO} mice fed an NCD (n = 5). **I-J.** Oil Red O staining of in vitro differentiated adipocytes (at day 8) from sWAT SVF progenitor cells of male obese GSK3β$^{fl/fl}$ and GSK3β^{ADKO} mice (I) and mRNA level analysis of key adipogenic markers in differentiated adipocytes by RT-PCR (J) (n=6). **K-L**. Oil Red O staining of in vitro differentiated adipocytes (at day 8) from eWAT SVF progenitor cells of male obese GSK3β$^{fl/fl}$ and GSK3β^{ADKO} mice (K) and mRNA level analysis of key adipogenic markers in differentiated adipocytes by RT-PCR (L) (n=6). **M-N**. mRNA level analysis of genes associated with apoptosis and inflammatory cell death in eWAT (M) and sWAT (N) of male obese GSK3β$^{fl/fl}$ and GSK3β^{ADKO} mice by RT-PCR (n=6). Scale bar: 100 μm.Scale bar: 100μm. C, F, J and L-N, Two-tailed Unpaired t test; G-H, Non-parametric test.

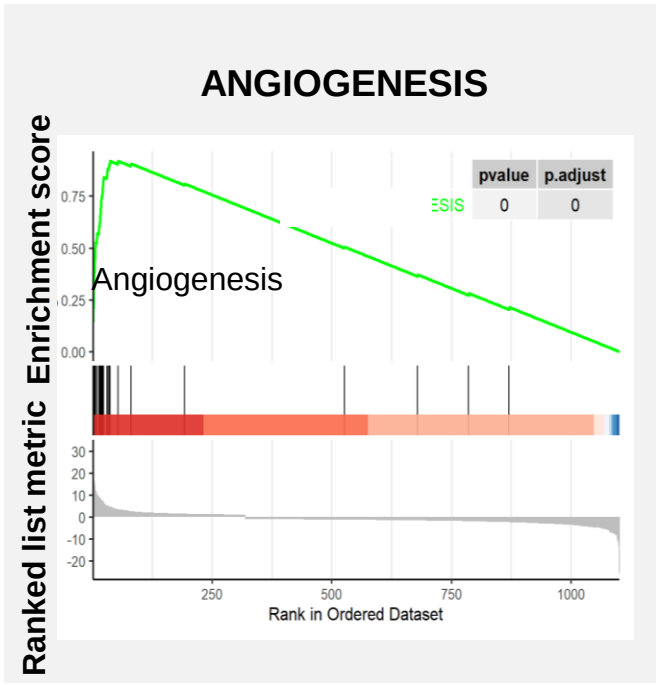
.

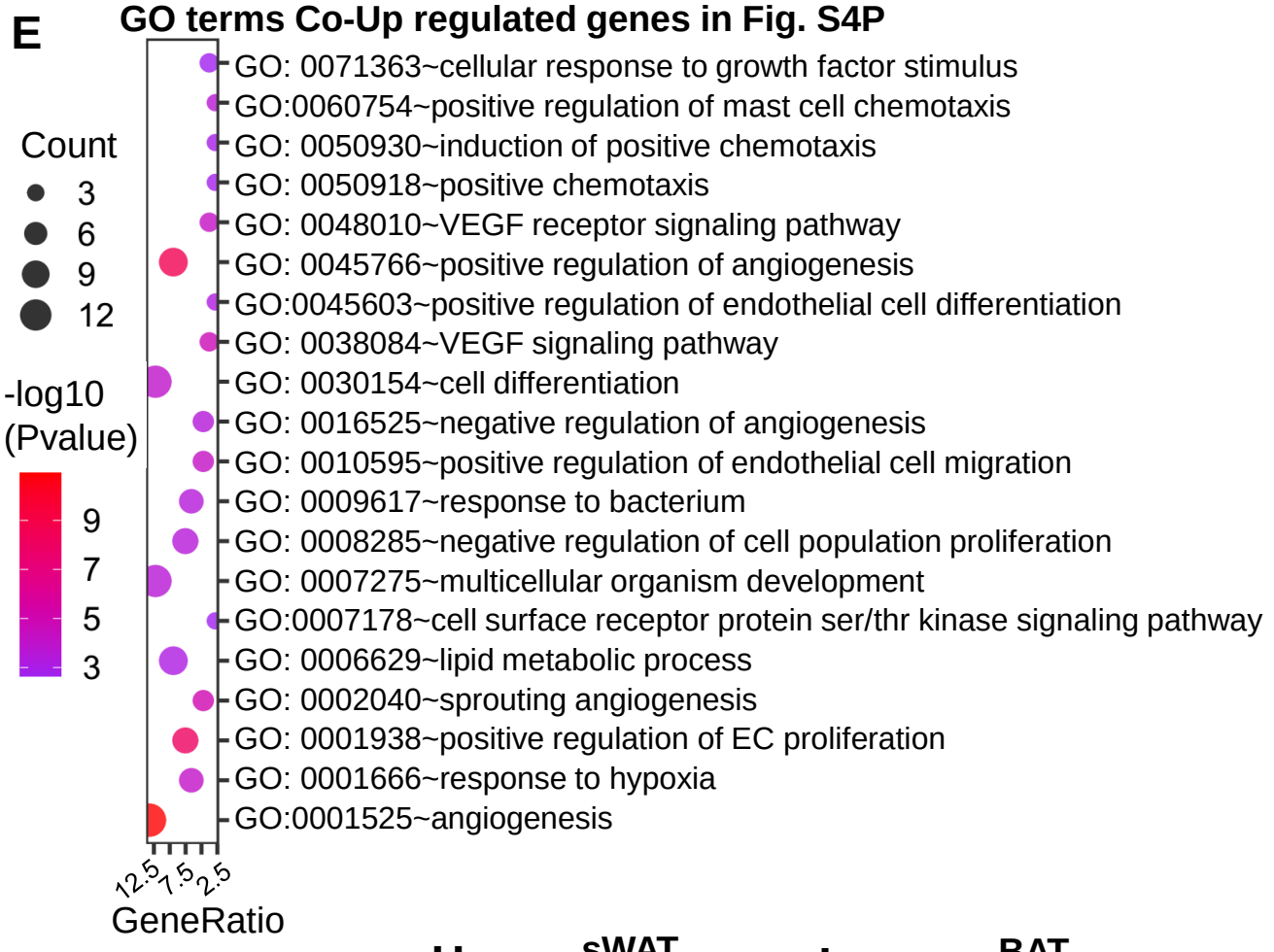
Figure S6

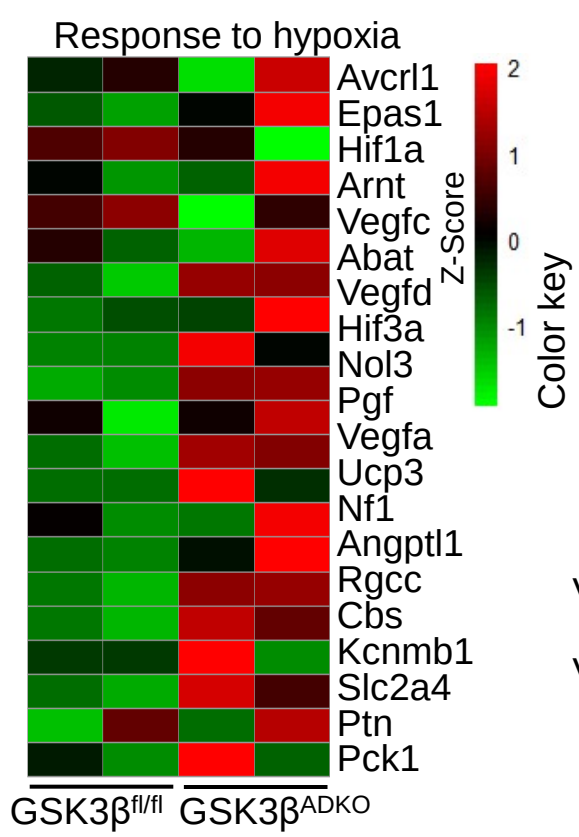

A
PID_VEGF_VEGFR_PATHWAY
Enrichment score
Ranked list metric
'na_pos' (positively correlated)
Zero cross at 12143
'na_neg' (negatively correlated)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores

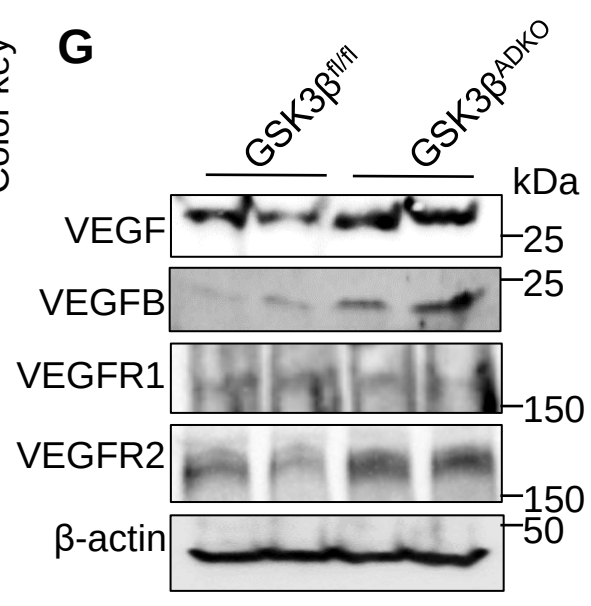

B
BLOOD_VESSEL REMODELING
Enrichment score
Ranked list metric
'na_pos' (positively correlated)
Zero cross at 12143
'na_neg' (negatively correlated)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores

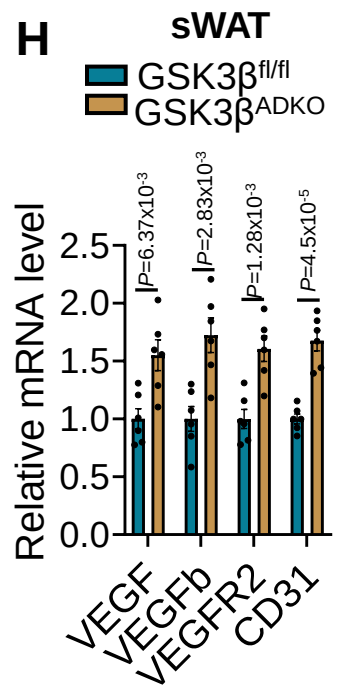

C
BLOOD_VESSEL ENDOTHELIAL_CELL_DIFFER ENTIATION
Enrichment score
Ranked list metric
'na_pos' (positively correlated)
Zero cross at 12143
'na_neg' (negatively correlated)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores


D
ANGIOGENESIS
pvalue
p.adjust
0
0
Angiogenesis
Enrichment score
Ranked list metric
Rank in Ordered Dataset


E
GO terms Co-Up regulated genes in Fig. S4P
Count
3
6
9
12
-log10 (Pvalue)
9
7
5
3
GO: 0071363~cellular response to growth factor stimulus
GO:0060754~positive regulation of mast cell chemotaxis
GO: 0050930~induction of positive chemotaxis
GO: 0050918~positive chemotaxis
GO: 0048010~VEGF receptor signaling pathway
GO: 0045766~positive regulation of angiogenesis
GO:0045603~positive regulation of endothelial cell differentiation
GO: 0038084~VEGF signaling pathway
GO: 0030154~cell differentiation
GO: 0016525~negative regulation of angiogenesis
GO: 0010595~positive regulation of endothelial cell migration
GO: 0009617~response to bacterium
GO: 0008285~negative regulation of cell population proliferation
GO: 0007275~multicellular organism development
GO:0007178~cell surface receptor protein ser/thr kinase signaling pathway
GO: 0006629~lipid metabolic process
GO: 0002040~sprouting angiogenesis
GO: 0001938~positive regulation of EC proliferation
GO: 0001666~response to hypoxia
GO:0001525~angiogenesis
12.5
7.5
2.5
GeneRatio


F
Response to hypoxia
Avcrl1
Epas1
Hif1a
Arnt
Vegfc
Abat
Vegfd
Hif3a
Nol3
Pgf
Vegfa
Ucp3
Nf1
Angptl1
Rgcc
Cbs
Kcnmb1
Slc2a4
Ptn
Pck1
Z-Score
Color key
GSK3β fl/fl
GSK3β ADKO


G
GSK3β fl/fl
GSK3β ADKO
kDa
VEGF
VEGFB
VEGFR1
VEGFR2
β-actin
25
25
150
150
50


H
sWAT
GSK3β fl/fl
GSK3β ADKO
Relative mRNA level
P=6.37x10-3
P=2.83x10-3
P=1.28x10-3
P=4.5x10-5
VEGF
VEGFb
VEGFR2
CD31

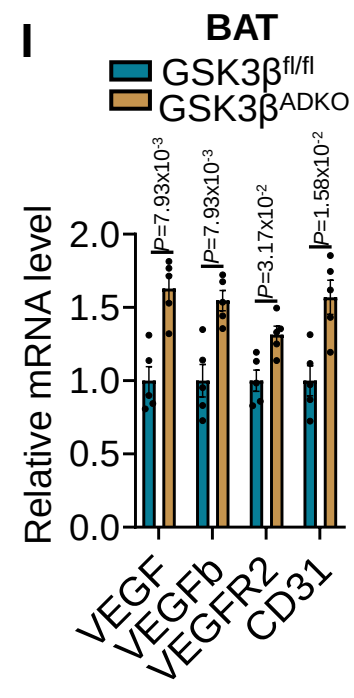
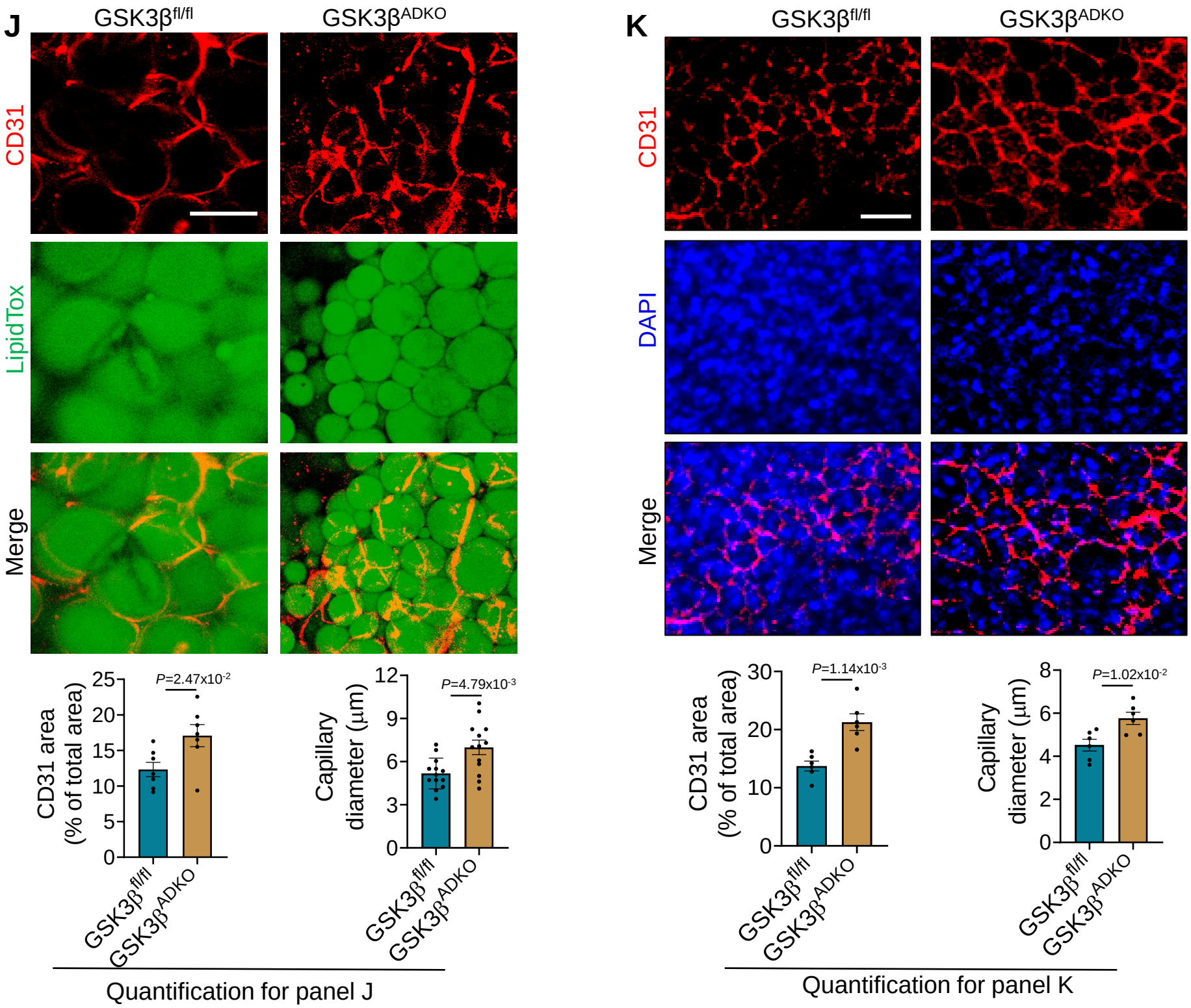

I
BAT
GSK3β fl/fl
GSK3β ADKO
Relative mRNA level
P=7.93x10-3
P=7.93x10-3
P=3.17x10-2
P=1.58x10-2
VEGF
VEGFb
VEGFR2
CD31

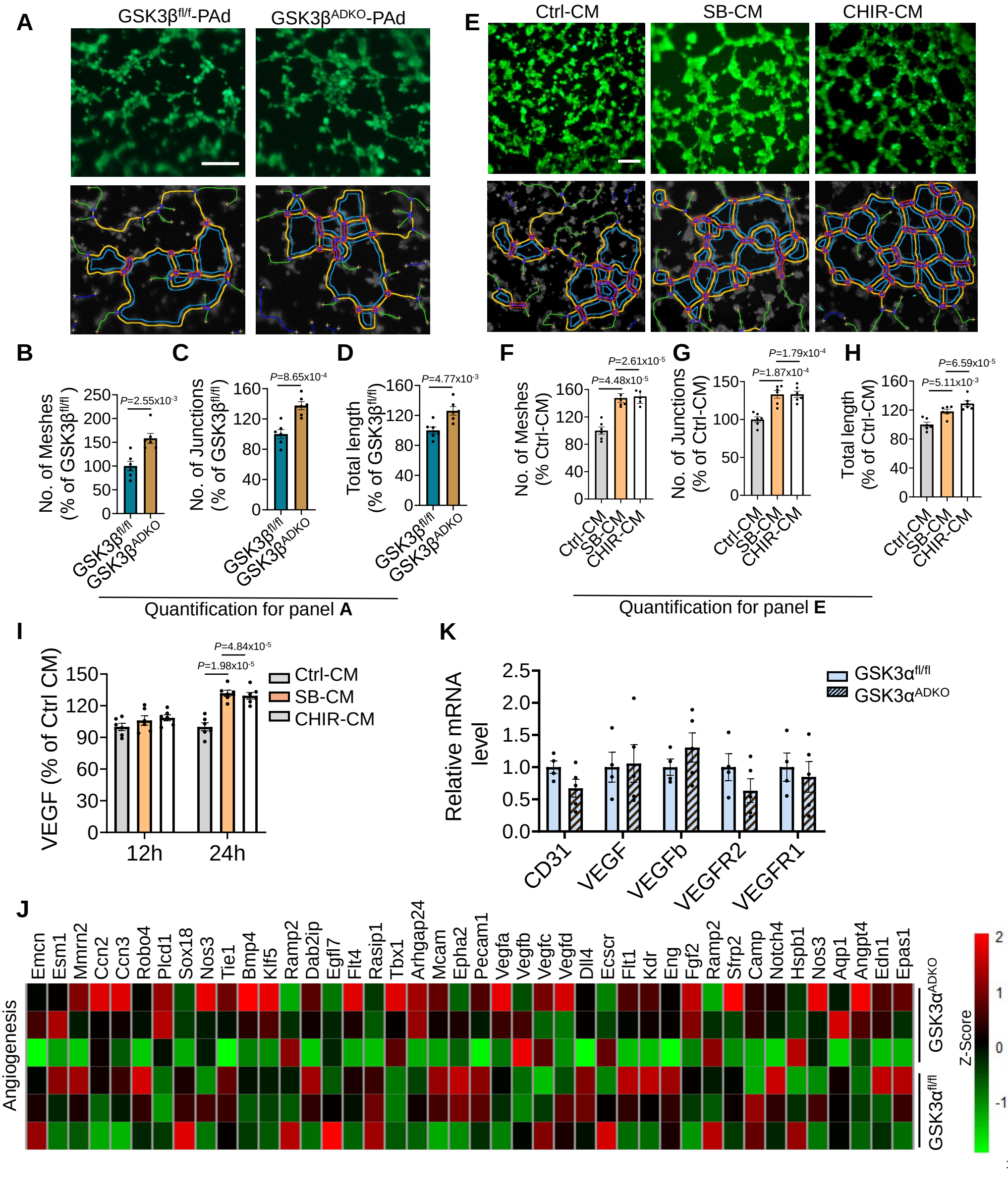
Figure S6 cont'd



Supplementary Figure 6. Adipocyte specific ablation of GSK3β stimulates the expression of angiogenic factors and positively impacts vascularization of obese adipose tissue. A-D. GESA plot of vascularization associated pathways including VEGF/VEGFR pathway (A), blood vessel remodeling (B), blood vessel endothelial cell differentiation pathways (C) and angiogenesis (D) using two sets of genes from eWAT of male obese GSK3β^{ADKO} vs GSK3β$^{fl/fl}$ mice fed an HFD (n=2). **E.** Gene ontology terms analysis showing co-upregulated gene signatures of male Lean vs obese control GSK3β$^{fl/fl}$ mice and obese GSK3β^{ADKO} vs obese control GSK3β$^{fl/fl}$ mice (Lean and GSK3β^{ADKO} co-upregulated genes from Fig. S4P). **F.** Heat map of expression values (Z-Score) for the genes associated with the response to hypoxia in eWAT from male obese GSK3β^{ADKO} and GSK3β$^{fl/fl}$ mice. **G.** Western blotting analysis of the indicated proteins in eWAT from the female obese GSK3β$^{fl/fl}$ and GSK3β^{ADKO} mice (n = 5). **H-I.** The expression analysis of some angiogenesis associated genes by RT-PCR in sWAT (H) (n=6) and in BAT (I) (n=5) from male obese GSK3β$^{fl/fl}$ and GSK3β^{ADKO} mice. **J.** Representative images of whole-mount staining of sWAT for CD31 and analysis for CD31-positive area, and capillary diameter quantified using Image J software from male obese GSK3β$^{fl/fl}$ and GSK3β^{ADKO} mice (n = 7). **K.** Representative images of whole-mount staining of BAT for CD31 and analysis of CD31 area and capillary diameter quantified using Image J software from male obese GSK3β$^{fl/fl}$ and GSK3β^{ADKO} mice (n = 6). Scale bar: 100µm. H, J and K, Two-tailed Unpaired t test; I, Non-parametric test.

.

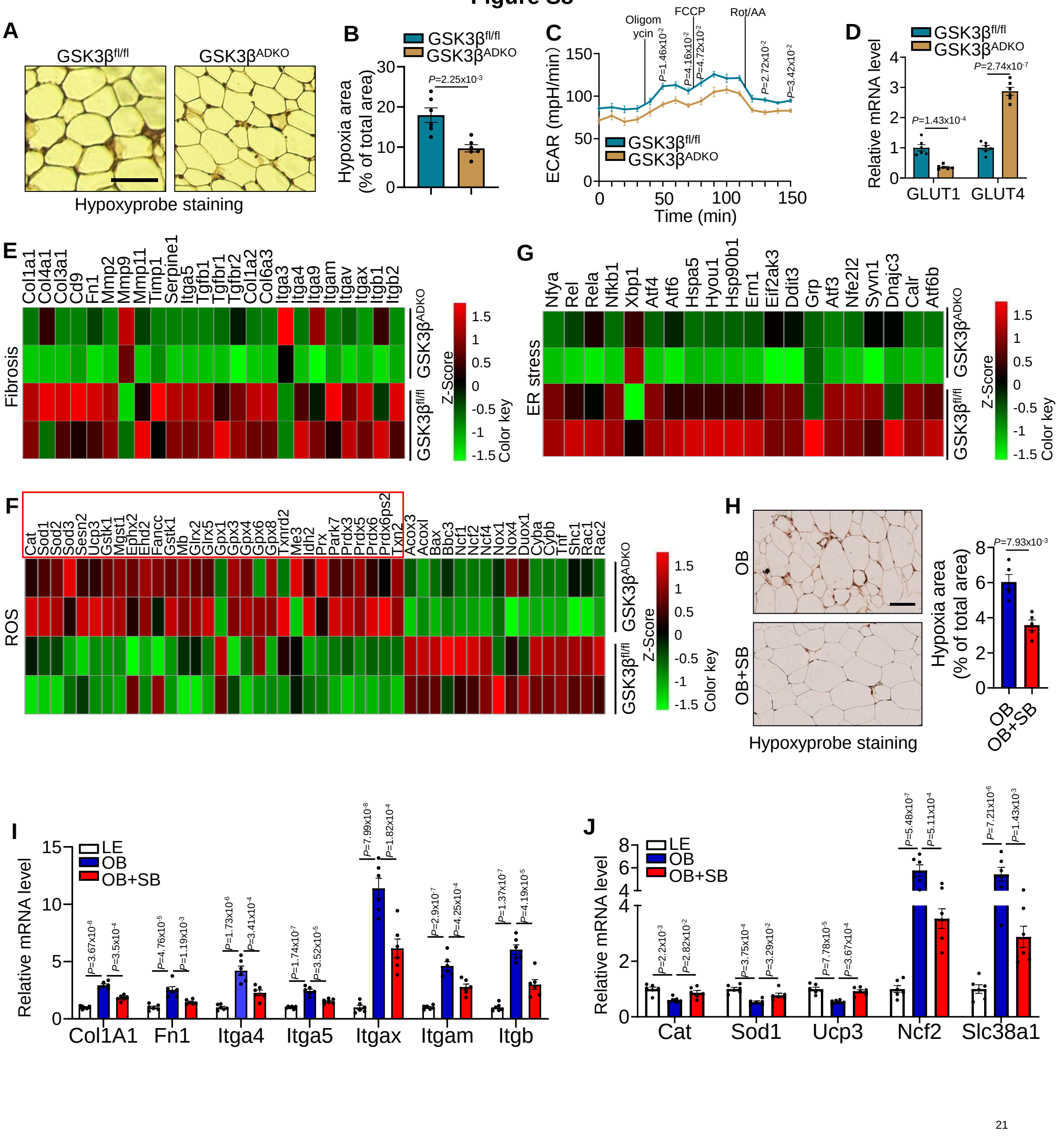
Figure S7


A
GSK3β^fl/f-PAd
GSK3β^ADKO-PAd
E
Ctrl-CM
SB-CM
CHIR-CM
B
No. of Meshes (% of GSK3β^fl/fl)
P=2.55x10^-3
GSK3β^fl/fl
GSK3β^ADKO
C
No. of Junctions (% of GSK3β^fl/fl)
P=8.65x10^-4
D
Total length (% of GSK3β^fl/fl)
P=4.77x10^-3
F
No. of Meshes (% Ctrl-CM)
P=2.61x10^-5
P=4.48x10^-5
Ctrl-CM
SB-CM
CHIR-CM
G
No. of Junctions (% of Ctrl-CM)
P=1.79x10^-4
P=1.87x10^-4
H
Total length (% of Ctrl-CM)
P=6.59x10^-5
P=5.11x10^-3
Quantification for panel A
Quantification for panel E
I
VEGF (% of Ctrl CM)
P=4.84x10^-5
P=1.98x10^-5
12h
24h
K
Relative mRNA level
GSK3α^fl/fl
GSK3α^ADKO
CD31
VEGF
VEGFb
VEGFR2
VEGFR1
J
Angiogenesis
Emcn
Esm1
Mmrn2
Ccn2
Ccn3
Robo4
Plcd1
Sox18
Nos3
Tie1
Bmp4
Klf5
Ramp2
Dab2ip
Egfl7
Flt4
Rasip1
Tbx1
Arhgap24
Mcam
Epha2
Pecam1
Vegfa
Vegfb
Vegfc
Vegfd
Dll4
Ecscr
Flt1
Kdr
Eng
Fgf2
Ramp2
Sfrp2
Camp
Notch4
Hspb1
Nos3
Aqp1
Angpt4
Edn1
Epas1
GSK3α^ADKO
GSK3α^fl/fl
Z-Score
Color key

Supplementary Figure 7. GSK3β negatively affects tube formation of endothelial cells in vitro, whereas GSK3α does not affect the expression of angiogenesis-associated genes. A-D. *In vitro* tube-formation assay of EOMA mouse endothelial cells cocultured with primary adipocytes (Pad, P0) isolated from the indicated male obese mice (A) and analysis for the number of meshes (B), number of junctions (C), and total segment length (D) (n = 6). **E-H.** In vitro tube formation assay of 3B-11 cells cultured in conditional medium (CM) collected from matured 3T3-L1 adipocytes that were cultured under hypoxic (1%O_2) conditions, without or with GSK3β inhibitor SB216763 (SB, 10 μM) or CHIR99021 (CHIR, 10μM) treatment for 24 hours, and analysis of tube formation from panel E, number of meshes (F), number of junctions (G), total segment length (H) (n=6). **I.** ELISA analysis of VEGF in CM used in panel E for 12 and 24 hours (n=6). **J.** Heatmap of expression values (Z-Score) showing angiogenesis associated genes in eWAT from male obese GSK3α^{ADKO} vs GSK3α$^{fl/fl}$ mice (n=3). **K.** The expression analysis of some angiogenesis associated genes by RT-PCR in eWAT of male obese GSK3α^{ADKO} mice (n=5) and GSK3α$^{fl/fl}$ mice (n=4). Scale bar: 50μm. P0: passage 0. B-D, F-H and K, Two-tailed Unpaired t test; I, one-way ANOVA and Tukey's post hoc test.

.

Figure S8


A
GSK3β^fl/fl
GSK3β^ADKO
Hypoxyprobe staining
B
GSK3β^fl/fl
GSK3β^ADKO
Hypoxia area (% of total area)
P=2.25x10-3
C
Oligomycin
FCCP
Rot/AA
P=1.46x10-2
P=4.16x10-2
P=4.72x10-2
P=2.72x10-2
P=3.42x10-2
ECAR (mpH/min)
Time (min)
D
Relative mRNA level
P=1.43x10-4
P=2.74x10-7
GLUT1
GLUT4
E
Fibrosis
Col1a1 Col4a1 Col3a1 Cd9 Fn1 Mmp2 Mmp9 Mmp11 Timp1 Serpine1 Itga5 Tgfb1 Tgfbr1 Tgfbr2 Col1a2 Col6a3 Itga3 Itga4 Itga9 Itgam Itgav Itgax Itgb1 Itgb2
Z-Score
Color key
G
ER stress
Nfya Rel Rela Nfkb1 Xbp1 Atf4 Atf6 Hspa5 Hyou1 Hsp90b1 Ern1 Eif2ak3 Ddit3 Grp Atf3 Nfe2l2 Syvn1 Dnajc3 Calr Atf6b
F
ROS
Cat Sod1 Sod2 Sod3 Sesn2 Ucp3 Gstk1 Mgst1 Ephx2 Ehd2 Fancc Gstk1 Mb Glrx2 Glrx5 Gpx1 Gpx3 Gpx4 Gpx6 Gpx8 Txnrd2 Me3 Idh2 Prx Park7 Prdx3 Prdx5 Prdx6 Prdx6ps2 Txn2 Acox3 Acoxl Bax Bbc3 Ncf1 Ncf2 Ncf4 Nox1 Nox4 Duox1 Cyba Cybb Tnf Shc1 Rac1 Rac2
H
OB
OB+SB
Hypoxyprobe staining
Hypoxia area (% of total area)
P=7.93x10-3
I
Relative mRNA level
LE
OB
OB+SB
Col1A1 Fn1 Itga4 Itga5 Itgax Itgam Itgb
J
Relative mRNA level
Cat Sod1 Ucp3 Ncf2 Slc38a1

Supplementary Figure 8. The effects of adipocyte-specific GSK3β deficiency on hypoxia, fibrosis and ROS status in obese adipose tissue. **A-B.** Representative images of Hypoxyprobe staining of sWAT (A) and quantification of staining (B) from male obese GSK3β$^{fl/fl}$ and GSK3β^{ADKO} mice (n=6). **C.** Extracellular acidification rate (ECAR) was assessed in eWAT from male obese GSK3β$^{fl/fl}$ and GSK3β^{ADKO} mice (n=7). **D.** The expression analysis of GLUT1 and GLUT4 in eWAT of male obese GSK3β$^{fl/fl}$ and GSK3β^{ADKO} mice (n=6). **E-G.** Heatmap of expression values (Z-Score) for the genes associated with fibrosis (E), ROS (F, the genes in red box are antioxidant genes and other genes are ROS production associated genes), and ER stress (G), in eWAT from male obese GSK3β$^{fl/fl}$ and GSK3β^{ADKO} mice (n=2). **H.** Hypoxyprobe staining of eWAT and quantification of staining from male obese mice (OB) without or with GSK3β inhibitor treatment (OB+SB) (n=5). **I-J**. The expression analysis of fibrosis (I), antioxidant and ROS production (J) associated genes in eWAT from male lean (LE) and obese mice (OB) without or with GSK3β inhibitor SB216763 treatment (OB+SB) (n=6). Scale bar: 100μm. B, D, I and J, Two-tailed Unpaired t test; C, Repeated ANOVA and Bonferroni's post hoc test; H, Non-parametric test.

.

Figure S9

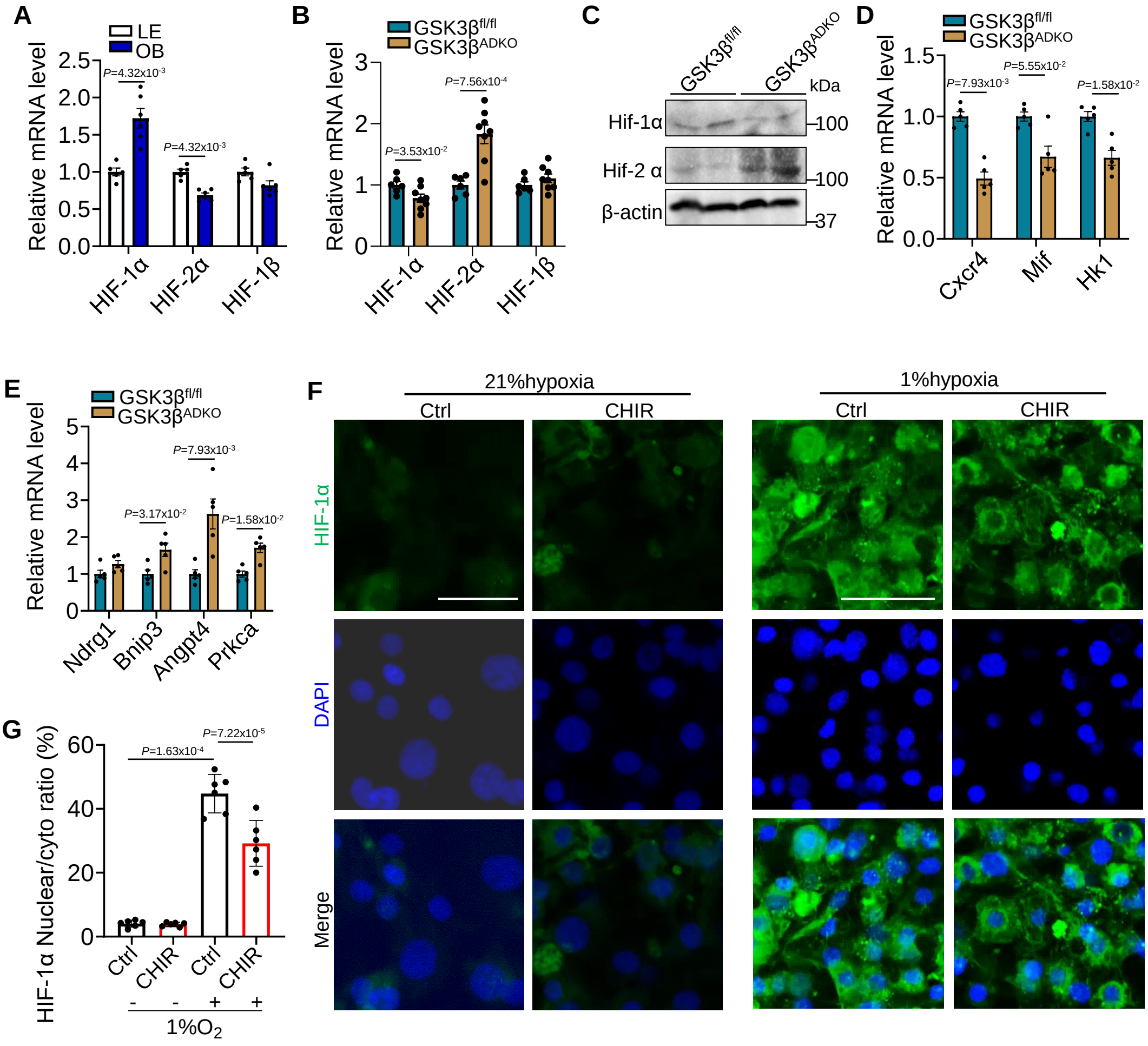
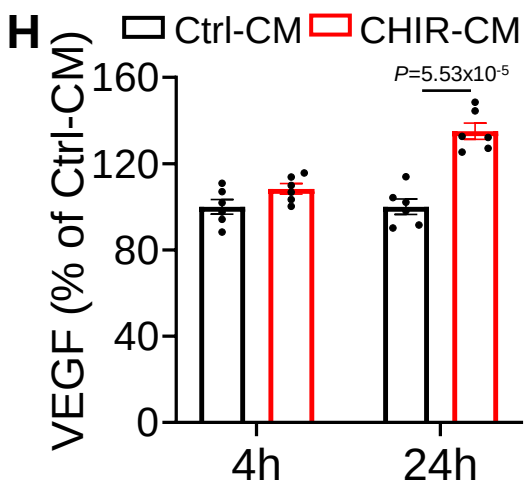
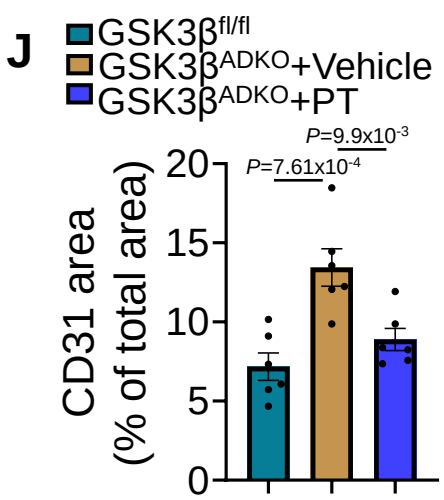
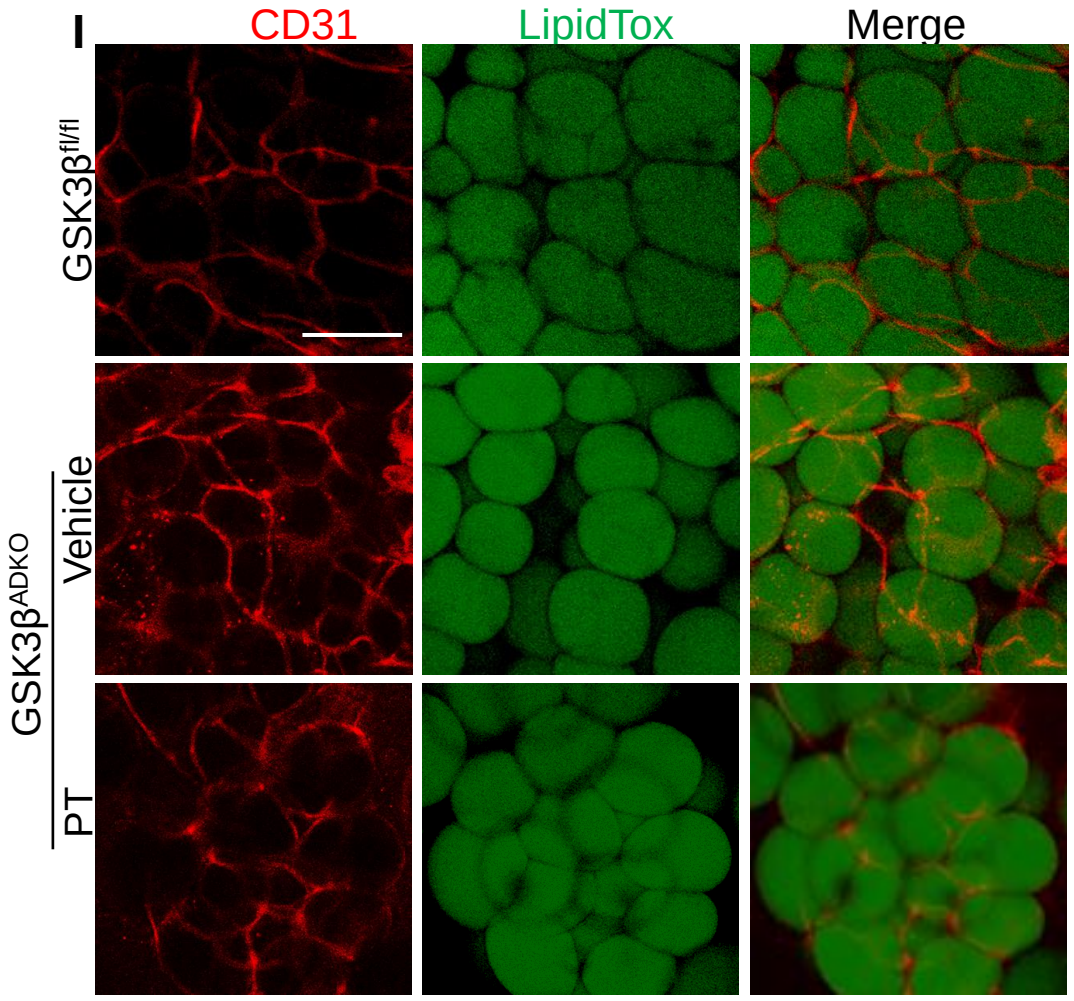
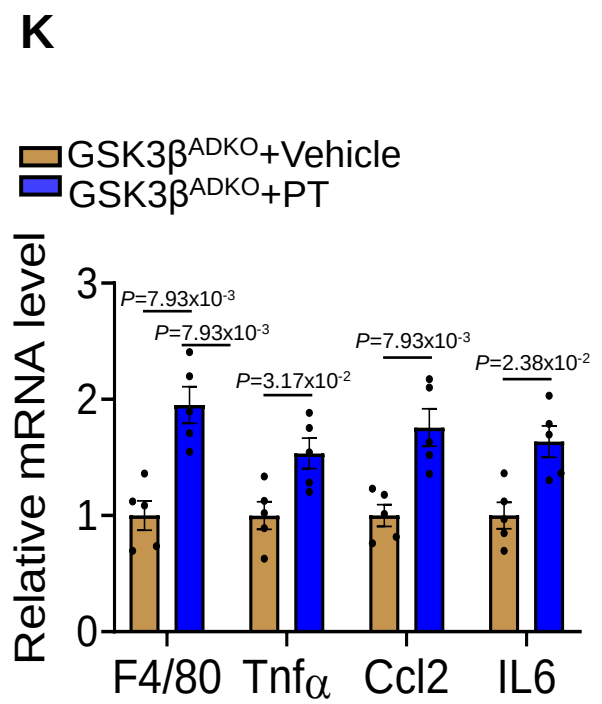

A
LE
OB
Relative mRNA level
P=4.32x10-3
P=4.32x10-3
HIF-1α
HIF-2α
HIF-1β
B
GSK3βfl/fl
GSK3βADKO
Relative mRNA level
P=3.53x10-2
P=7.56x10-4
HIF-1α
HIF-2α
HIF-1β
C
GSK3βfl/fl
GSK3βADKO
kDa
Hif-1α
Hif-2 α
β-actin
100
100
37
D
GSK3βfl/fl
GSK3βADKO
Relative mRNA level
P=7.93x10-3
P=5.55x10-2
P=1.58x10-2
Cxcr4
Mif
Hk1
E
GSK3βfl/fl
GSK3βADKO
Relative mRNA level
P=7.93x10-3
P=3.17x10-2
P=1.58x10-2
Ndrg1
Bnip3
Angpt4
Prkca
F
21%hypoxia
1%hypoxia
Ctrl
CHIR
Ctrl
CHIR
HIF-1α
DAPI
Merge
G
HIF-1α Nuclear/cyto ratio (%)
P=1.63x10-4
P=7.22x10-5
Ctrl
CHIR
Ctrl
CHIR
-
-
+
+
1%O2

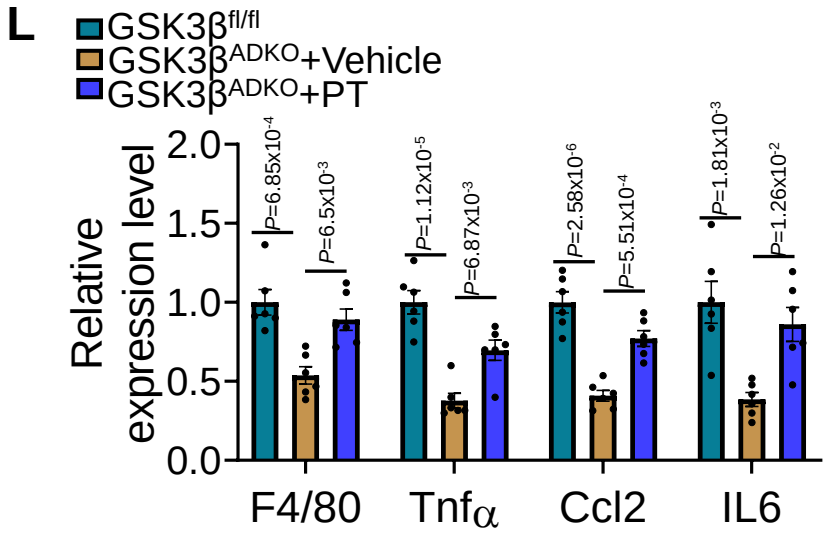
Figure S9 cont'd

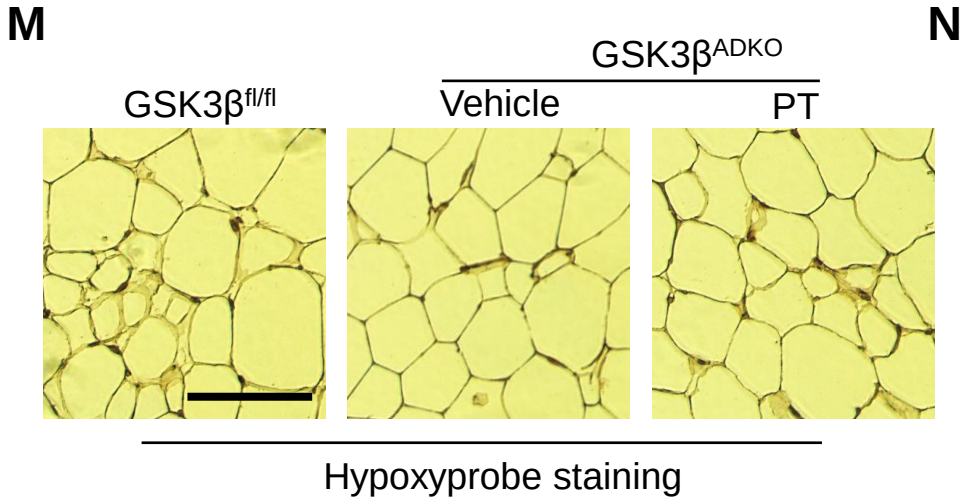


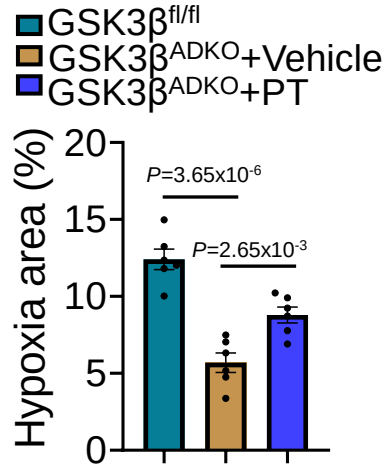












Supplementary Figure 9. The expression analysis of HIF-1α and HIF-2α and the validation that HIF-2α, not HIF-1α, mediates the effects of GSK3β deficiency on vasculature and hypoxia status in obese AT. A. The expression levels of HIF-1α, HIF-2α and HIF-1β in eWAT of male LE (n=5) and OB mice (n=6) at the mRNA level using RT-PCR. **B.** Expression analysis of HIF-1α and HIF-2α at the mRNA level using RT-PCR in eWAT from male obese GSK3β$^{fl/fl}$ (n=6) and GSK3β^{ADKO} mice (n=8). **C.** The protein levels of HIF-1α and HIF-2α were analysed in the eWAT of female male obese GSK3β$^{fl/fl}$ and GSK3β^{ADKO} mice using Western blotting (n = 5). **D-E.** The expression analysis of HIF-1α target genes (D) and HIF-2α target genes (E) in eWAT of male obese GSK3β$^{fl/fl}$ and GSK3β^{ADKO} mice (n=5). **F-G.** Nuclear accumulation of HIF-1α in matured 3T3-L1 adipocytes with GSK3 inhibitor (CHIR99021 (CHIR, 10 μM)) treatment under normoxia (21%O2) and hypoxia (1%O2) for 4 hours and analysis of nuclear/cytoplasmic ratios (G) (n=6). **H.** ELISA for determining the VEGF levels in the culture medium (CM) of matured 3T3-L1 adipocytes, with or without GSK3 inhibition, for 4 hours and 24 hours under hypoxic conditions (n=6). **I-J.** Whole-mount CD31 staining (I) and CD31-positive area quantification (J) of eWAT sections from female obese GSK3β$^{fl/fl}$ and GSK3β^{ADKO} mice without or with PT treatment (n=6). **K.** Inflammatory marker gene expression levels in eWAT from male obese GSK3β^{ADKO} mice without or with PT treatment using RT-PCR (n=5). **L.** Inflammatory marker gene expression levels in eWAT from female obese GSK3β$^{fl/fl}$ and GSK3β^{ADKO} mice without or with PT treatment using RT-PCR (n=6). **M-N.** Representative images of Hypoxyprobe staining of eWAT (M) and quantification of staining (N) from female obese GSK3β$^{fl/fl}$ and GSK3β^{ADKO} mice without or with PT treatment (n=6). CHIR: CHIR99021, GSK3 inhibitor. PT: PT2385 (30mg/kg), HIF-2α inhibitor. Scale bar: 100μm. A, D, E and K, Non-parametric test; B,H, Two-tailed Unpaired t test; G, J, L and N, one-way ANOVA and Tukey's post hoc test.

.

Figure S10

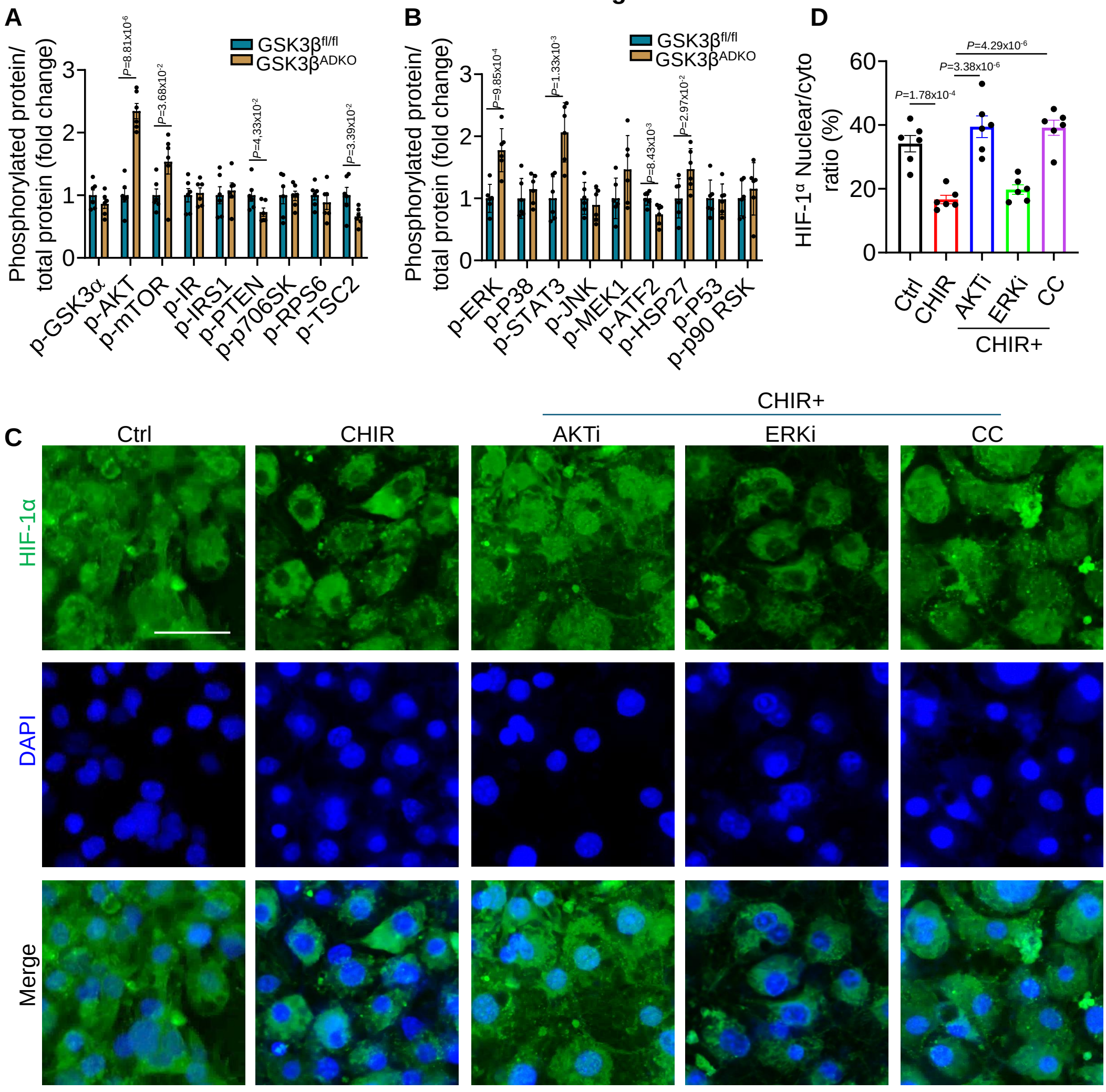
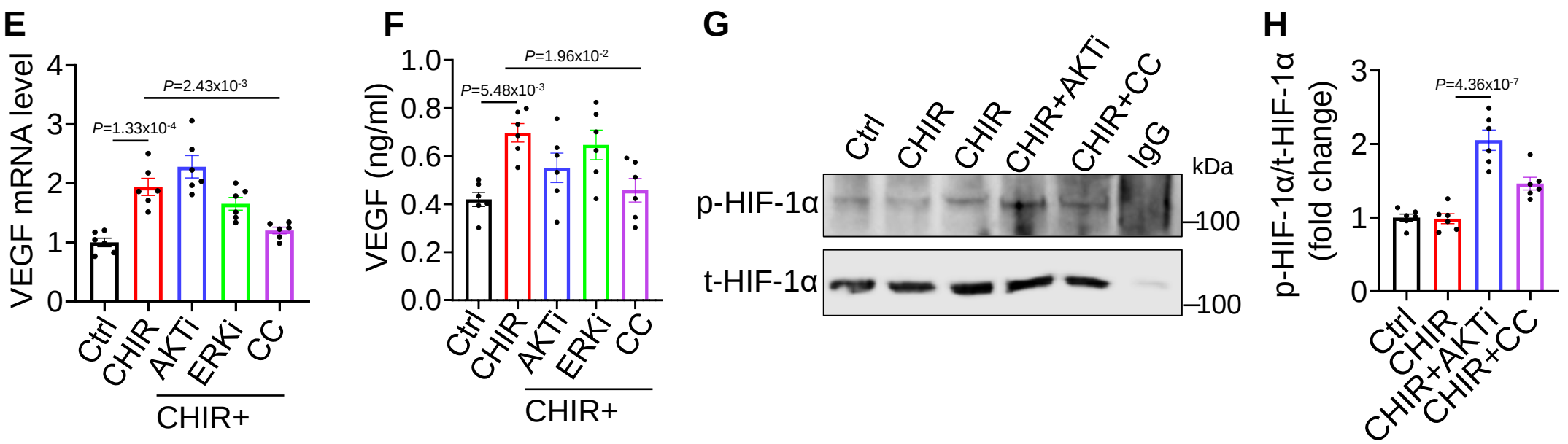

A
Phosphorylated protein/ total protein (fold change)
GSK3β^fl/fl
GSK3β^ADKO
P=8.81x10-6
P=3.68x10-2
P=4,33x10-2
P=3.39x10-2
p-GSK3α
p-AKT
p-mTOR
p-IR
p-IRS1
p-PTEN
p-p706SK
p-RPS6
p-TSC2
B
Phosphorylated protein/ total protein (fold change)
GSK3β^fl/fl
GSK3β^ADKO
P=9.85x10-4
P=1.33x10-3
P=8.43x10-3
P=2.97x10-2
p-ERK
p-P38
p-STAT3
p-JNK
p-MEK1
p-ATF2
p-HSP27
p-P53
p-p90 RSK
D
HIF-1α Nuclear/cyto ratio (%)
P=1.78x10-4
P=3.38x10-6
P=4.29x10-6
Ctrl
CHIR
AKTi
ERKi
CC
CHIR+
C
CHIR+
Ctrl
CHIR
AKTi
ERKi
CC
HIF-1α
DAPI
Merge

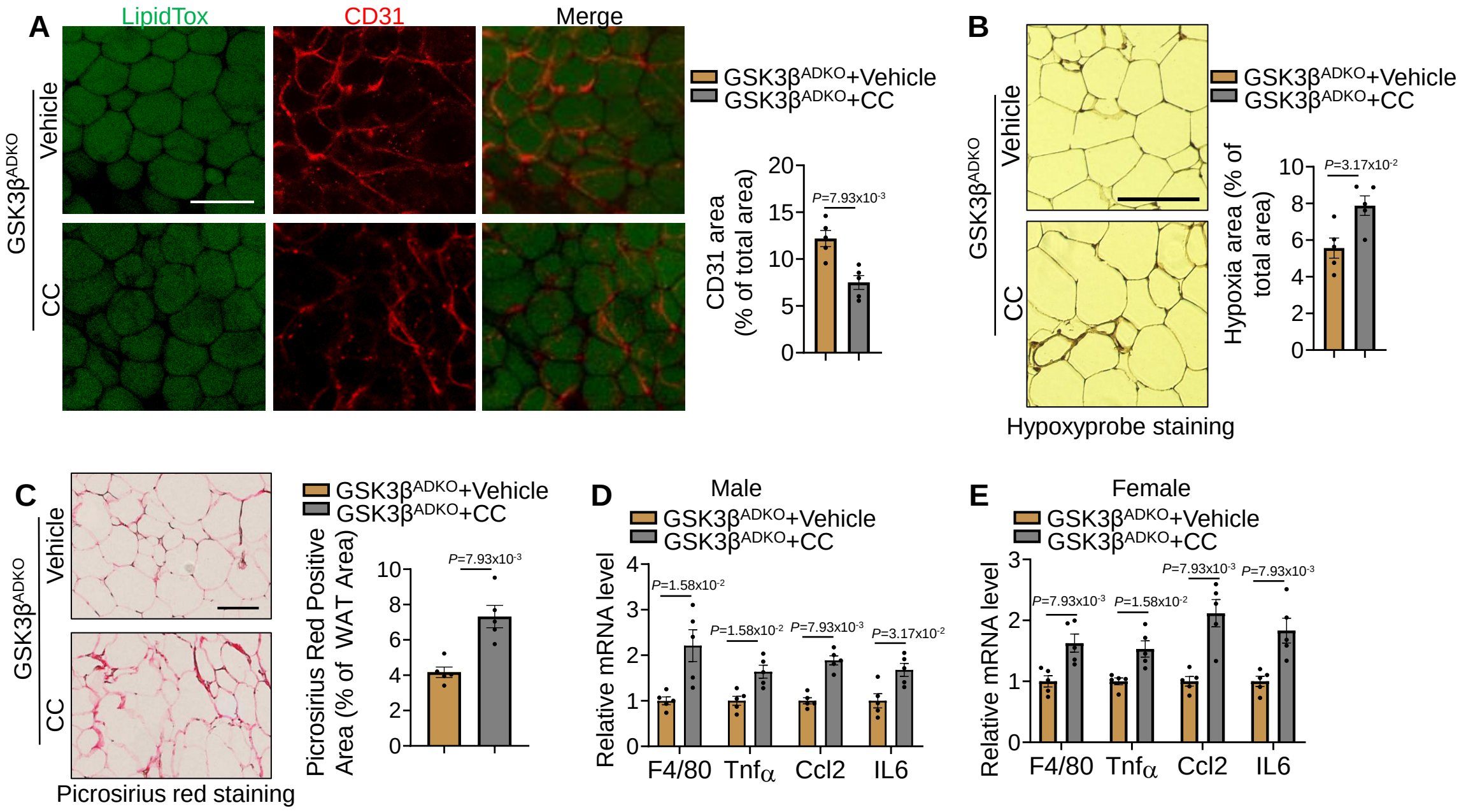
Figure S10 cont'd



Supplementary Figure 10. Impact of adipocyte-Specific GSK3β ablation on kinase phosphorylation, VEGF expression, and HIF-1α phosphorylation and nuclear accumulation . A-B. Relative levels of multiple phosphorylated kinases versus total kinases in the eWAT of male obese GSK3β^{ADKO} mice vs. GSK3β$^{fl/fl}$ mice (n=6). **C-D.** Nuclear accumulation of HIF-1α in matured 3T3-L1 adipocytes with GSK3β inhibition (CHIR, 10μM) and without or with kinase inhibitors treatment (AKT inhibitor (AKTi, 10μM), ERK inhibitor (ERKi, 300nM) and AMPK inhibitor (CC, 10μM) under hypoxia (1%) (C) for 4 hours and analysis of nuclear/cytoplasmic ratios (D) (n=6). **E-F.** Analysis of *VEGF* expression at the mRNA level using RT-PCR in 3T3-LI adipocytes (E) and at the protein level using ELISA (F) in the culture medium of 3T3-L1 adipocytes with the indicated treatment for 24 h (n=6). **G-H.** HIF-1α phosphorylation analysis (G) in 3T3-L1 adipocytes with the indicated treatment for 4 h and quantification (H) using ImageJ software (n=6). Scale bar: 100μm. A, B, Two-tailed Unpaired t test; D-F and H, one-way ANOVA and Tukey's post hoc test.

.

Figure S11



Supplementary Figure 11. AMPK mediates the effects of GSK3β deficiency on vasculature, hypoxia and fibrosis status in obese AT. A. Whole-mount CD31 staining and CD31-positive area quantification from female obese GSK3β^{ADKO} mice without or with AMPK inhibitor Compound C (GSK3β^{ADKO} + CC (10mg/kg)) treatment (n=5). **B.** Representative images of Hypoxyprobe staining of eWAT from female obese GSK3β^{ADKO} mice without or with CC treatment and quantification of staining (n = 5). **C.** Representative picrosirius red staining and quantification for the fibrosis analysis of eWAT from male obese GSK3β^{ADKO} mice without or with CC treatment (n = 5). **D-E.** Analysis of the expression of inflammation marker genes in eWAT from male (D) and female (E) obese GSK3β^{ADKO} mice without or with CC treatment by qPCR (n=5). Scale bar: 100µm. A-E, Non-parametric test.