Thyrotropin and Obesity: Increased Adipose Triglyceride Content Through Glycerol-3-Phosphate Acyltransferase 3

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Supplemental Experimental Procedures

Materials

The cell culture reagents newborn calf serum (NBS) and fetal bovine serum (FBS) were purchased from Gibco BRL （Invitrogen Corporation； Carlsbad, CA, USA). Bovine TSH, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, insulin, Oil Red O, AICAR and GW9662 were purchased from Sigma (St Louis, MO, USA). Rabbit anti-phospho-Thr172-AMPK and anti-total-AMPK antibodies were purchased from Cell Signaling (Beverly, MA, USA). Rabbit anti-GPAT3 and mouse anti-β-actin antibody were purchased from Proteintech (Chicago, IL, USA). Rabbit anti-PPARγ antibody was purchased from Merck Millipore (Darmstadt, Germany). Mouse anti–GAPDH antibody was purchased from Cwbiotech (Beijing, China). Horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit antibodies were purchased from Jackson ImmunoResearch (West Grove, PA, USA).

3T3-L1 cell culture

3T3-L1 preadipocytes were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 10% (v/v) NBS, penicillin-streptomycin (100 U/mL), at 37°C in a humidified atmosphere of 95% air and 5% CO2. The medium was renewed every 2 days until confluence was reached.

After 2 days' confluence, the cells were incubated in fresh medium containing 10% FBS. The cells were then treated with IBMX (0.5 mM), dexamethasone (DEX,

2.5 μM) and insulin (8.75 μM) to initiate differentiation (designated as day 0). After exposure to MDI for 2 days, the cells were incubated in fresh medium (10% FBS) containing only insulin (8.7 μM) for 2 additional days. The cells were then fed every second day with fresh medium containing 10% FBS. Triglyceride droplets were visible on day 4, and the cells were fully differentiated into adipocytes on day 8 (over 90% of the plate). After a 16-day induction of differentiation, 3T3-L1 adipocytes exhibited the characteristics of mature adipocytes and were then used in the following experiments. The cells were typically incubated in the presence of MDI and the indicated agents for a period of only 48 h, unless otherwise indicated [1].

Oil Red O staining

Differentiated 3T3-L1 cells were stained with Oil Red O. The cells were washed three times with PBS and fixed with 4% paraformaldehyde for 10 min; the cells were then washed three times with PBS and then stained with Oil Red O solution (0.3% Oil Red O in 100% isopropanol, filtered) for 8 min. The cells were then washed with 60% ethanol and rinsed with water for 3 min. The phenotypic changes associated with lipogenesis were observed under a microscope [2].

Triglyceride assay

Intracellular and adipose tissue triglycerides were assayed using a triglyceride assay kit (GPO-POD; Applygen Technologies, Beijing, China), according to the manufacturer's recommended protocol.

H&E staining

Tissues were embedded in paraffin and sectioned into 7 μm sections. Sectioned tissues were stained with hematoxylin and eosin (H&E) and visualized under a microscope.

Adiponectin assay

Frozen mouse sera were slowly brought to room temperature and gently mixed. The adiponectin concentration was determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Invitrogen, New York, CA, USA).

Cell transfection

CA-AMPK, DN-AMPK and PPARγS112A were transfected as follows. The cells were transfected using the PolyJet™ transfection reagent (Signagen, Ijamsville, MD, USA) according to the manufacturer's instructions. Differentiated 3T3-L1 cells on 35 mm plates were freshly cultured in 1.5ml of complete DMEM for 30 min before transfection. For each plate, 1 μg of DNA was diluted in 50 μl of serum-free high-glucose DMEM. Then, 3 μl of PolyJet™ transfection reagent was diluted into 50 μl of serm-free high-glucose DMEM. The diluted PolyJet™ transfection reagent was immediately added to the diluted DNA solution all at once. The solution was incubated for 15 min at room temperature to allow PolyJet™/DNA complexes to form,

and was then diluted with DMEM to 1.5 ml before being added to the cells for transfection. After 48 h, the cells were cultured in serum–free medium and treated with or without TSH. Then, the cells were harvested for experiments.

Knockdown of *Tshr*, *PPARγ* or *GPAT3* by RNAi

Mouse TSHR and PPARγ siRNAs were synthesized by GenePharma Corporation (Shanghai, China). The primers are listed in Supplemental Table 1. Transfection of siRNA was performed using the PolyJet™ transfection reagent according to the manufacturer's recommendations (Signagen, Ijamsville, MD, USA). Differentiated 3T3-L1 adipocytes were transfected with or without 30 pmol of *Tshr*, *PPARγ* or *GPAT3* siRNA in 35-mm dishes, with a corresponding volume of transfection reagent. After 48 h, the cells were cultured in serum–free medium and treated with or without TSH. The cells were then harvested for RNA or protein extraction.

PPRE-luciferase reporter assay

After being co-transfected with 1 μg of the PPRE-luciferase reporter construct (Addgene) along with PPARγ expression vectors for 48 h, HEK293 cells were placed in serum-free medium and were subsequently incubated with GW9662 or TSH for another 24 h. pSV-40 was used to normalize the luciferase activity. The cells were then harvested, and luciferase activities were measured using a dual-luciferase reporter assay system (Promega) [3].

GPAT3-luciferase reporter assay

Mouse GPAT3 promoter-luciferase reporter plasmids were constructed by GeneChem Corporation (Shanghai, China). After being transfected with 1 μg GPAT3 promoter-luciferase reporter plasmids for 48 h, HEK293 cells were placed in serum-free medium and subsequently incubated with TSH for another 24 h. pSV-40 was used to normalize the luciferase activity. The cells were then harvested and luciferase activities were measured using a dual-luciferase reporter assay system (Promega) [4].

Western blot analysis

Samples were homogenized in RIPA lysis buffer containing protease and phosphatase inhibitors. Protein concentrations were determined using the BCA method. Proteins were resolved on SDS-PAGE gels and transferred to PVDF membranes (Millipore). Subsequently, the membranes were blocked in 5% non-fat milk for 1 hour and incubated with primary antibodies overnight at 4°C, followed by incubation with peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies for 1 h at room temperature. After washing with TBST, the bound primary antibodies were visualized with the Alpha Q and exposed to film.

Real-time quantitative PCR

Total RNA was isolated from cells and fresh tissues using an RNeasy Total

RNA Isolation kit (TaKaRa) and was reverse transcribed into cDNA (TaKaRa). SYBR Green (DBI) quantitative PCR analysis reactions were then performed using the Roche 480 detection system. The β-actin gene was simultaneously detected as a control. Relative gene expression was quantified using the $2^{-\Delta\Delta t}$ method, and the results are expressed as the fold change relative to the control. The primer sequences are listed in Supplemental Table 1.

Immunofluorescence

Differentiated 3T3-L1 adipocytes were fixed with 4% paraformaldehyde for 15 min and permeabilized in PBS containing 0.15% Triton X-100 for 5 min (PPARγ) or not (GPAT3). After blocking with 10% normal rabbit or mouse serum for 30 min, the samples were incubated with the appropriate primary antibody (1:40) at 4°C overnight. The cells were subsequently incubated with a goat anti-rabbit FITC-conjugated, a rabbit anti-mouse FITC-conjugated or a goat anti-rabbit TRITC-conjugated secondary antibody at a 1:100 dilution at 37°C for 1 h. The nuclei were stained with DAPI (Vector Laboratories, Burlingame, CA, USA), and the coverslips were sealed to the microscope slides using mounting media. Cell images were captured using a fluorescence microscope (Karl Zeiss A2 Microscopes, Wetzlar, Germany). All of the images were acquired using the same intensity and photodetector gain to allow for quantitative comparisons of the relative degrees of immunoreactivity between sections.

Supplemental Figure Legends

Supplemental FIG. 1

TSH-stimulated adipogenesis in mature adipocytes

Effects of TSH on the adipogenic parameters in (A) mouse and (B) human primary adipocytes. The time-dependent and/or dose-dependent effects of TSH on p-AMPK, total AMPK, PPARγ and GPAT3 were detected by western blotting, and the grayscale images were normalized to GAPDH to correct for loading errors.

Supplemental FIG. 2

(A) Plasma TG of the HFD-fed C57/BL6 mice were administered GW9662 or vehicle ($^{\#}p<0.05$ compared with control). (B) Plasma TG of HFD-fed *Tshr*$^{+/+}$ and *Tshr*$^{-/-}$. The results are expressed as the mean±SD ($^{\#}p<0.05$ compared with *Tshr*$^{+/+}$). All of the panels above are representative of 3 independent experiments.

Supplemental FIG. 3

Differentiated 3T3-L1 cells were transfected with CA-AMPK or DN-AMPK plasmids for 48 h and then stimulated, with or without 1 μM TSH, for another 24 h. Intracellular TG contents were detected by Oil red O staining.

Supplemental FIG. 4

(A) The change in the mRNA levels of the *Tshr* mice after their differentiation into mature adipocytes. The expression of *Tshr* mRNA was determined by quantitative

real-time PCR. The results are expressed as the mean±SD ($^{\#}p<0.05$ compared with control). (B)The effects of TSH (2 μM for 48 h) on TSHR expression in differentiated 3T3-L1 cells at the mRNA level. The expressions of *Tshr* mRNA were determined by quantitative real-time PCR. The results are expressed as the mean±SD ($^{\#}p<0.05$ compared with control). All of the above panels are representative of 3 independent experiments.

Supplemental FIG. 5

Effects of H89 on AMPK phosphorylation in C57/BL6 mice. In total, 12 male C57/BL6 mice were divided into 4 groups (n = 3 per group) and gavaged with PBS or H89 at 20 or 40 nM. After 3 or 5 days, the p-AMPK and total AMPK protein levels were detected by western blotting, and the grayscale images were normalized to GAPDH to correct for loading errors.

Supplemental FIG. 6

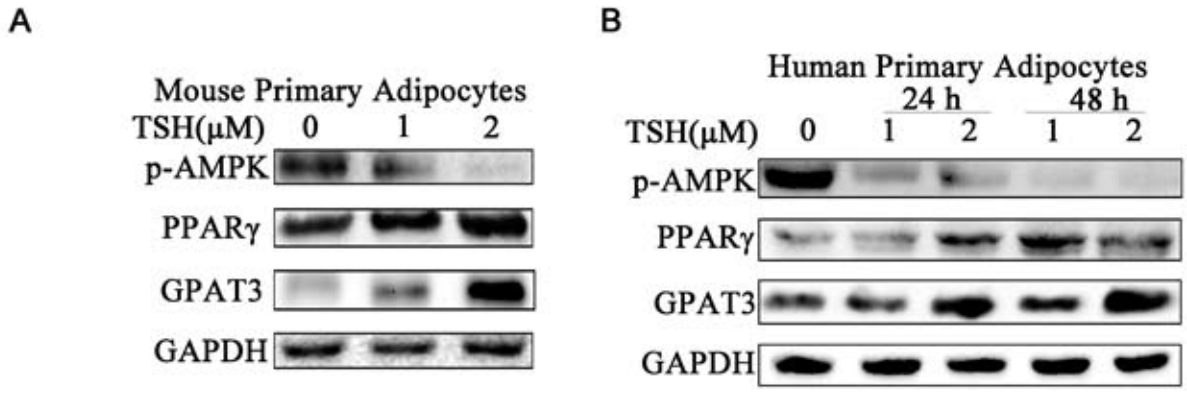
Full-length gels and blots for key data. These cropped blots are used in the main figure (Figure 2B, 3C, 3H, 4C, 5A, 5E) and these full-length blots are included in the supplemental figure 6.

1. Guo W*, et al.* Adipogenesis licensing and execution are disparately linked to cell proliferation. *Cell Res* **19**, 216-223 (2009).
2. Li F*, et al.* Protein kinase A suppresses the differentiation of 3T3-L1 preadipocytes. *Cell Res* **18**, 311-323 (2008).
3. Tamori Y, Masugi J, Nishino N, Kasuga M. Role of peroxisome

proliferator-activated receptor-gamma in maintenance of the characteristics of mature 3T3-L1 adipocytes. *Diabetes* **51**, 2045-2055 (2002).

4. Lu B*, et al.* Expression and regulation of GPAT isoforms in cultured human keratinocytes and rodent epidermis. *J Lipid Res* **51**, 3207-3216 (2010).

Supplymental figure 1



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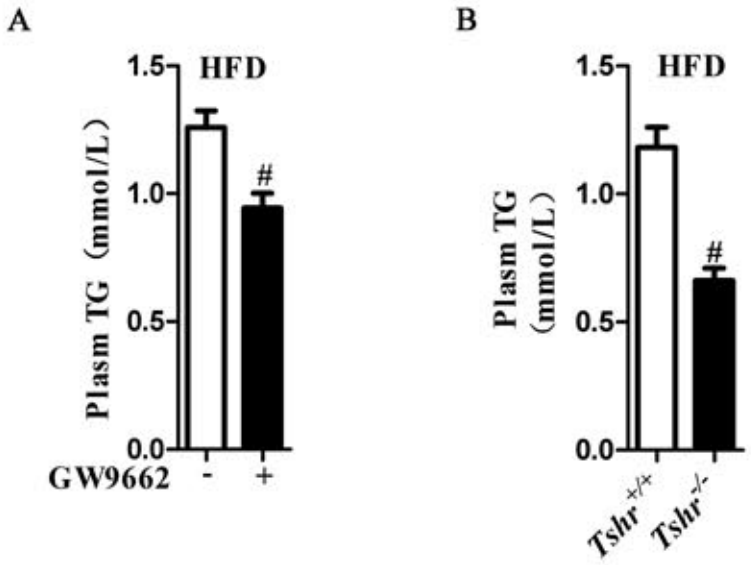

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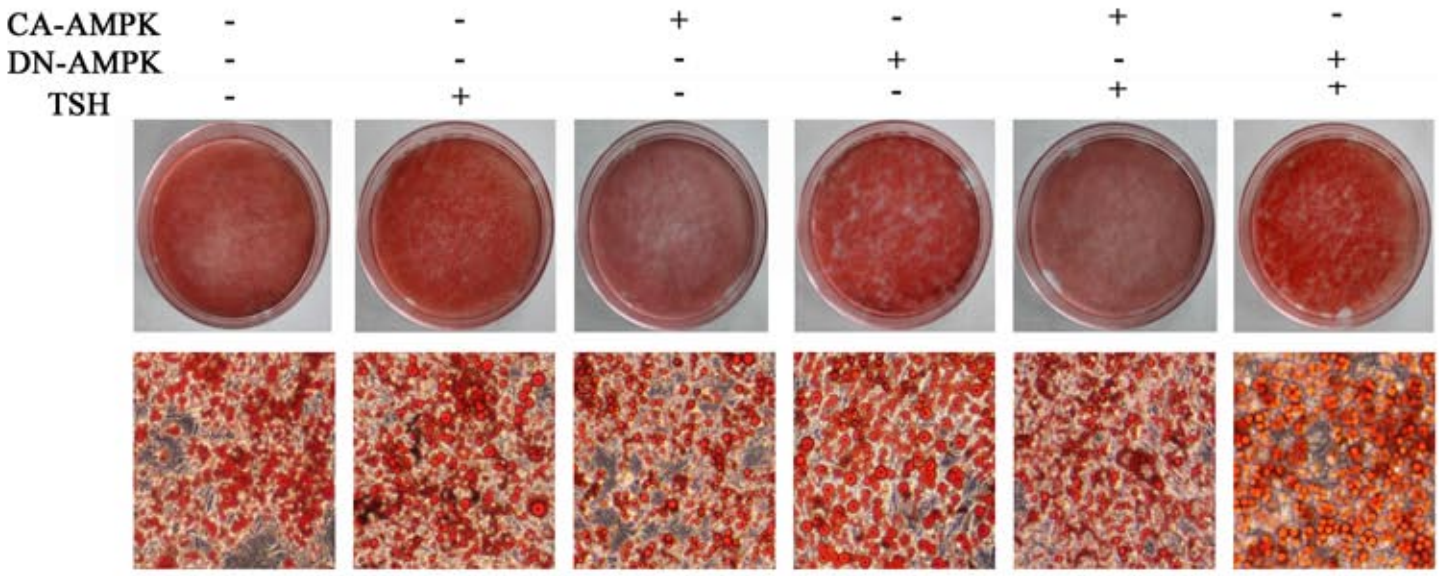

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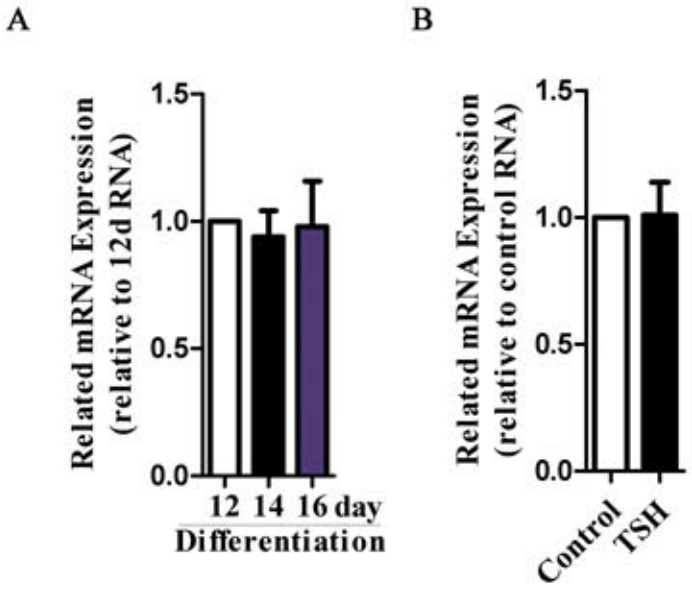

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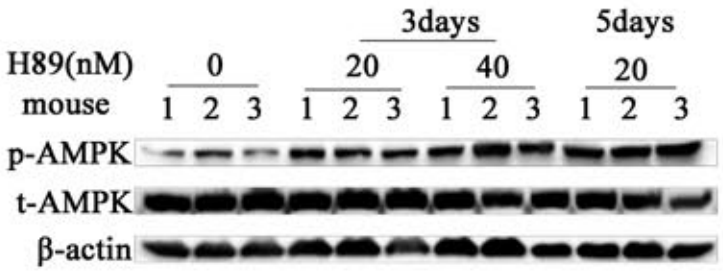

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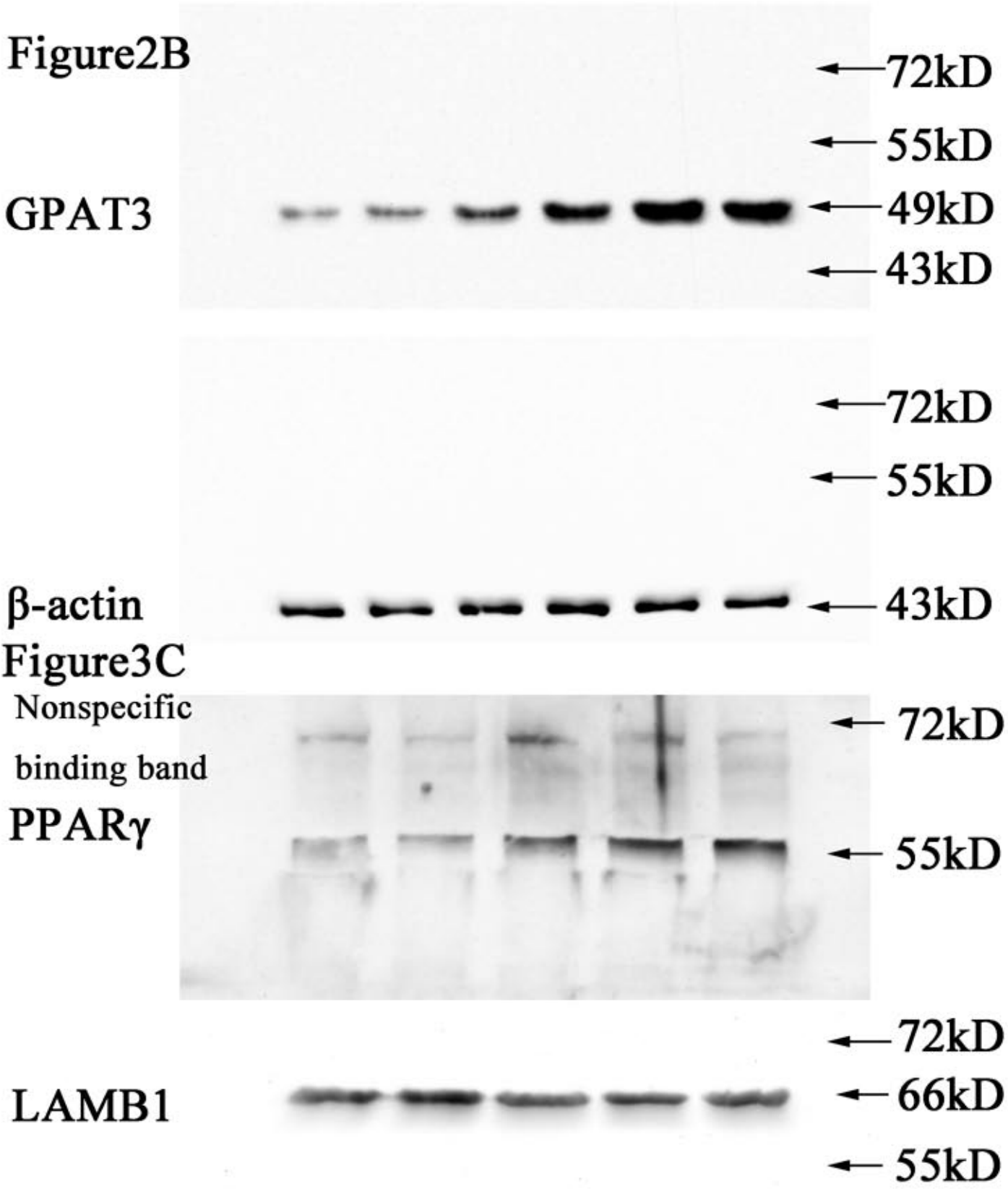


Figure3H

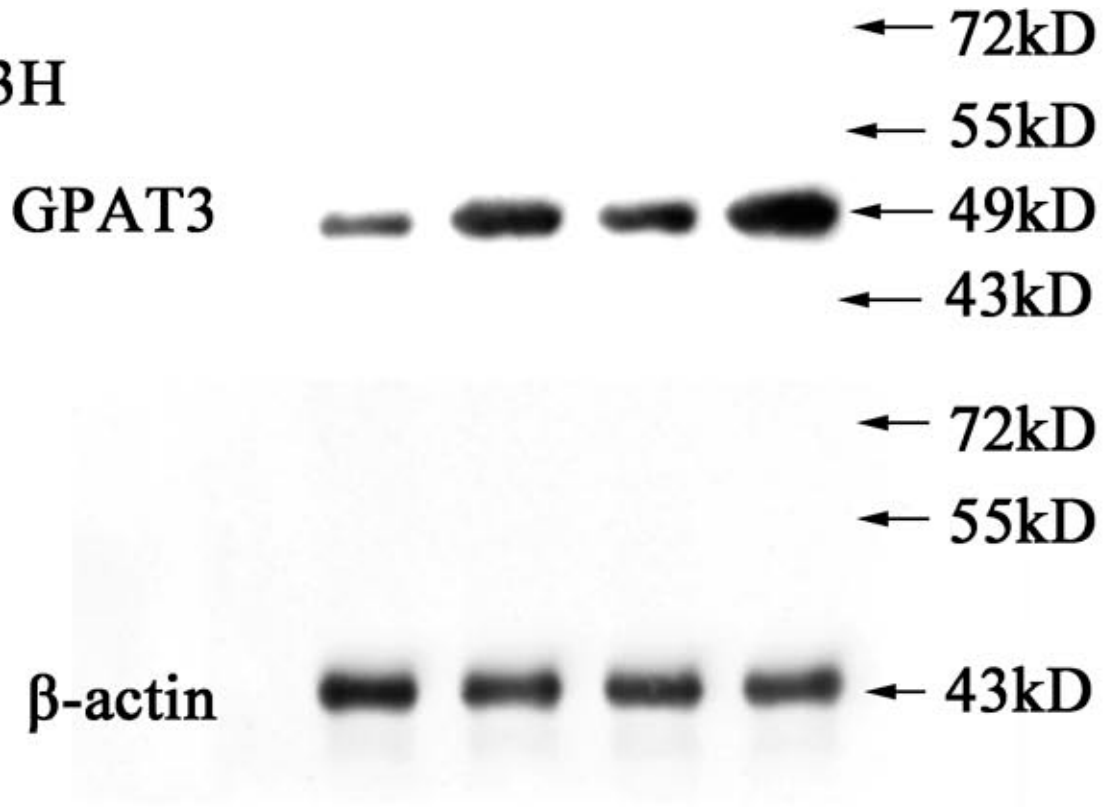


Figure4C

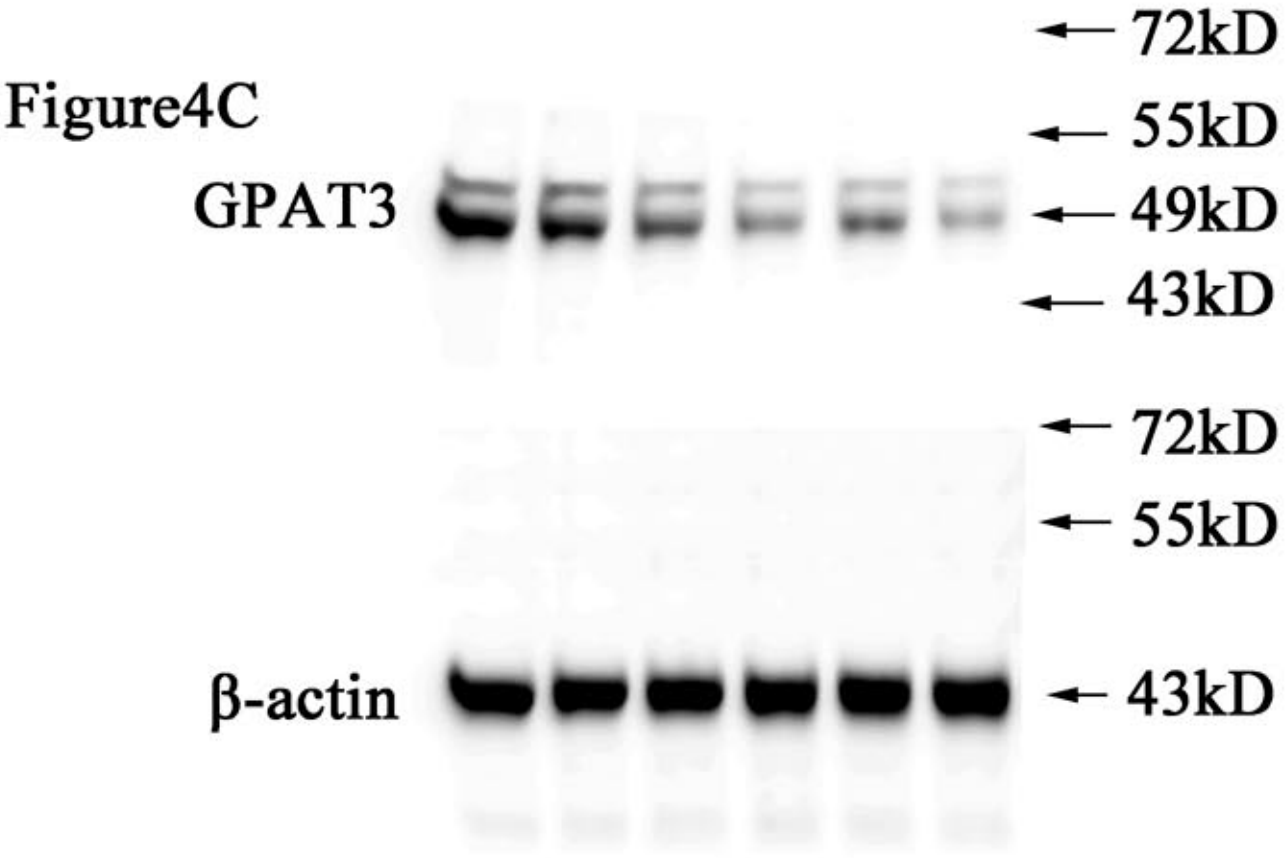


Figure5A

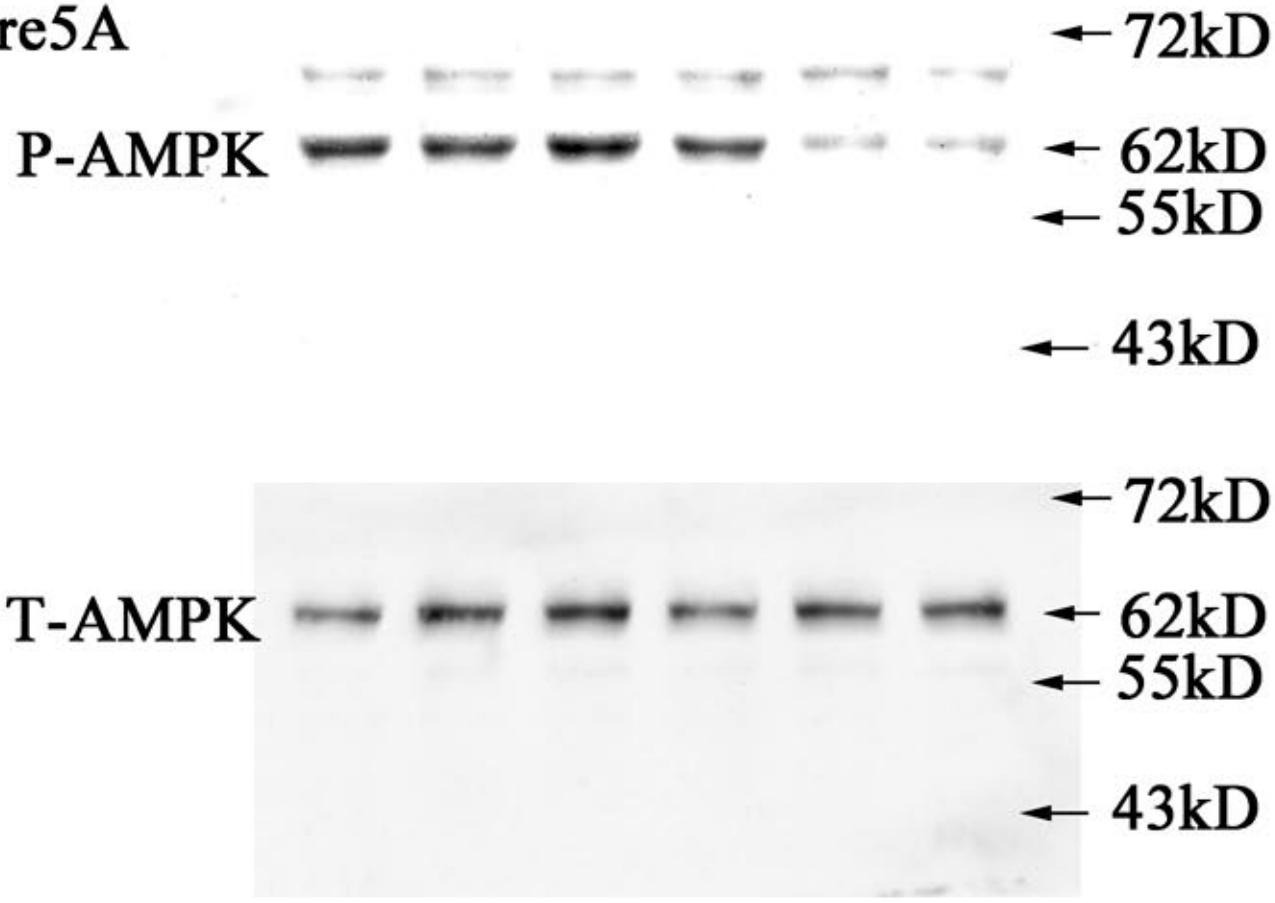
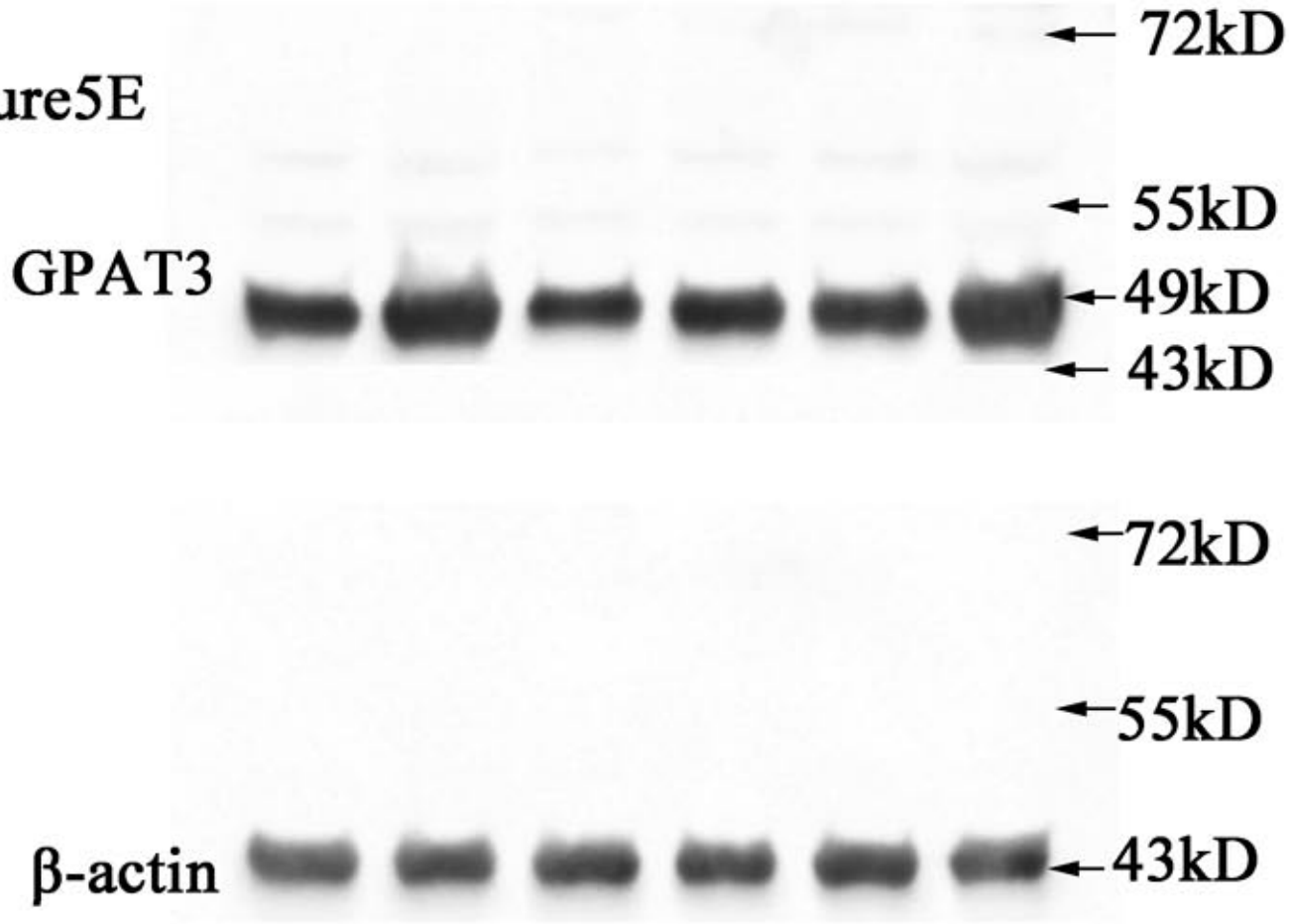


Figure5E



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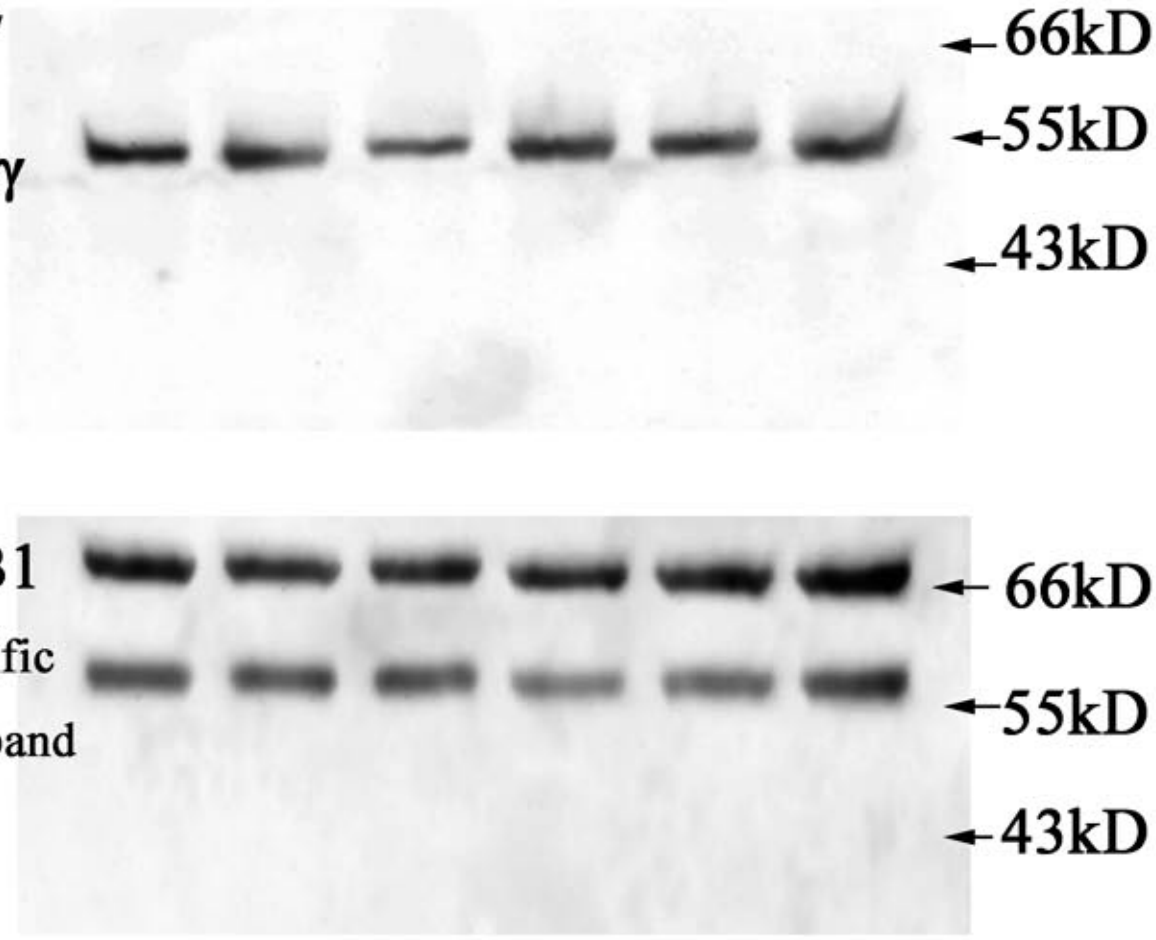
PPARγ
66kD
55kD
43kD
LAMB1
Nonspecific
binding band
66kD
55kD
43kD