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Supplemental Information

LXRs Regulate ER Stress and Inflammation through Dynamic Modulation of Membrane Phospholipid Composition

Xin Rong, Carolyn J. Albert, Cynthia Hong, Mark A. Duerr, Brian T. Chamberlain, Elizabeth J. Tarling, Ayaka Ito, Jie Gao, Bo Wang, Peter A. Edwards, Michael E. Jung, David A. Ford, and Peter Tontonoz

Supplemental Figure Legends

Figure S1 (Related to Figure 1). LXRs directly regulate the expression of Lpcat3.

(A) Tissue expression of Lpcat3 mRNA in mice determined by real-time PCR. Samples were pooled from 3 C57BL/6 mice. Values are means ± SD.

(B) Thioglycollate-elicited peritoneal macrophages from wild-type, *LXRα*$^{-/-}$, *LXRβ*$^{-/-}$ and *LXRαβ*$^{-/-}$ mice were treated with the LXR agonist GW3965 (1 μM) or T0901317 (1 μM) overnight, followed by gene expression analysis by real-time PCR. Results are representative of two independent experiments. Values are means ± SD.

(C) Thioglycollate-elicited peritoneal macrophages from wild-type and *LXRαβ*$^{-/-}$ mice were treated with the LXR agonists GW (1 μM) or T (1 μM) and the RXR ligand LG268 (50 nM), followed by gene expression analysis by real-time PCR. Results are representative of two independent experiments. Values are means ± SD.

(D) Hep3B cells were treated with GW3965 overnight at the indicated concentration. Lpcat3 expression was analyzed by real-time PCR. Results are representative of two independent experiments. Values are means ± SD.

(E) Thioglycollate-elicited peritoneal macrophages were treated with the LXR agonists GW (1 μM) or T (1 μM), the RXR ligand LG268 (50 nM), and/or cycloheximide (CHX, 10 μg/ml). Lpcat3 expression was analyzed by real-time PCR. Results represent independent replicate experiments. Values are means ± SD.

(F) Thioglycollate-elicited peritoneal macrophages from wild-type mice and *LXRαβ*$^{-/-}$ mice were treated with the LXR synthetic ligand GW (1 μM) or endogenous ligand 22(R)-hydroxycholesterol (2.5 μM). Lpcat3 expression was analyzed by real-time PCR. Results represent independent replicate experiments. Values are means ± SD.

(G) RAW264.7 macrophages were treated with vehicle, GW (1 μM) or 25-hydroxycholesterol (2.5 μM) for 24 h. Gene expression was analyzed by real-time PCR. Results are representative of two independent experiments. Values are means ± SD.

(H) Induction of Lpcat3 mRNA expression in tissues of mice treated with 40 mg/kg/day GW3956 by oral gavage for 3 days (n=5 per group). Gene expression was measured by real-time PCR. Values are means ± SEM. *$p<0.05$ **$p<0.01$ by Student's t-test.

(I) Lpcat3 activity in RAW 264.7 cells stably expressing control shRNA (shCtrl), or two Lpcat3-targeting shRNAs (sh2-1 and sh4-1) were quantified by acyltransferase activity assay. Thin layer chromoatogram is shown at top and Lpcat3 mRNA expression was quantified by real-time PCR (bottom). Values are means ± SD.

Figure S2 (Related to Figure 2). LXR agonist attenuates saturated fatty acid-induced ER stress.

(A) Hep3B cells were treated with the LXR ligand GW3965 at indicated concentration, followed by stimulation with 500 μM BSA-conjugated palmitic acid (PA). Gene expression was analyzed by real-time PCR. Results are representative of two independent experiments. Values are means ± SD.

(B) Primary mouse hepatocytes were treated with the LXR ligand GW3965 (1 μM), followed by treatment of 500 μM BSA-conjugated palmitic acid to induce ER stress. Expression of ER stress marker genes was analyzed by real-time PCR. Results are representative of two independent experiments. Values are means ± SD.

(C) Thioglycollate-elicited peritoneal macrophages were treated with the LXR ligand GW3965 (1 μM), followed by treatment of 500 μM BSA-conjugated palmitic acid to induce ER stress. Expression of ER stress marker genes was analyzed by real-time PCR. Results are representative of two independent experiments. Values are means ± SD.

(D) Microscopic images of Hep3B cells treated with GW3965 (1 μM), followed by palmitic acid (500 μM) stimulation for 18 h.

(E) Hep3B and Huh7 cells were treated with GW3965 (1 μM) for 24 h and stimulated with BSA-conjugated palmitic acid for 18 h. Caspase-3 activity was measured by colorimetric assay. Values are means ± SD. *$p<0.05$ by one way ANOVA with Bonferroni post hoc tests over veh+PA.

Figure S3 (Related to Figure 4). Lpcat3 activity modulates phosphatidylethanolamine (PE) profiles *in vivo*.

(A) *ob/ob* mice were transduced with adenoviral-expressed shRNA target Lpcat3 (shLpcat3) or control (shCtrl) for 7 days. Liver PE profile was quantified by ESI-MS/MS. N=6 per group. Values are means ± SEM.

(B) Metabolic parameters in *ob/ob* mice transduced with adenoviral-expressed shRNA targeting Lpcat3 (shLpcat3) or control (shCtrl) for 7 days. N=6 per group. Values are means ± SEM.

Statistical analysis was performed using student's t-test. *$p < 0.05$; **$p < 0.01$.

Figure S4 (Related to Figure 6). Lpcat3 activity regulates inflammatory gene expression in mouse liver.

(A) Quantitation of F4/80 expression in livers of mice transduced with adenoviral-expressed shRNA targeting Lpcat3 (shLpcat3) or control (Ctrl) for 8 days by real-time PCR. N=5 per group. Values are means ± SEM.

(B) Pro-inflammatory cytokine expression in liver of mice in (A) was quantified by real-time PCR. N=5 per group. Values are means ± SEM.

(C) Serum ASL and ALT level of ob/ob mice transduced with adenoviral-expressed shRNA targeting Lpcat3 (shLpcat3) or control (Ctrl) for 7 days. N=5 per group. Values are means ± SEM.

(D) Gene expression of livers from C57BL/6 mice transduced with adenoviral vectors expressing control (Ad-Ctrl) or Lpcat3 (Ad-Lpcat3) for 9 days. N=6 per group. Values are means ± SEM.

(E) Serum ASL and ALT level of db/db mice transduced with adenoviral-expressed Lpcat3 or control EGFP (Ctrl) for 7 days. N≥4 per group. Values are means ± SEM.

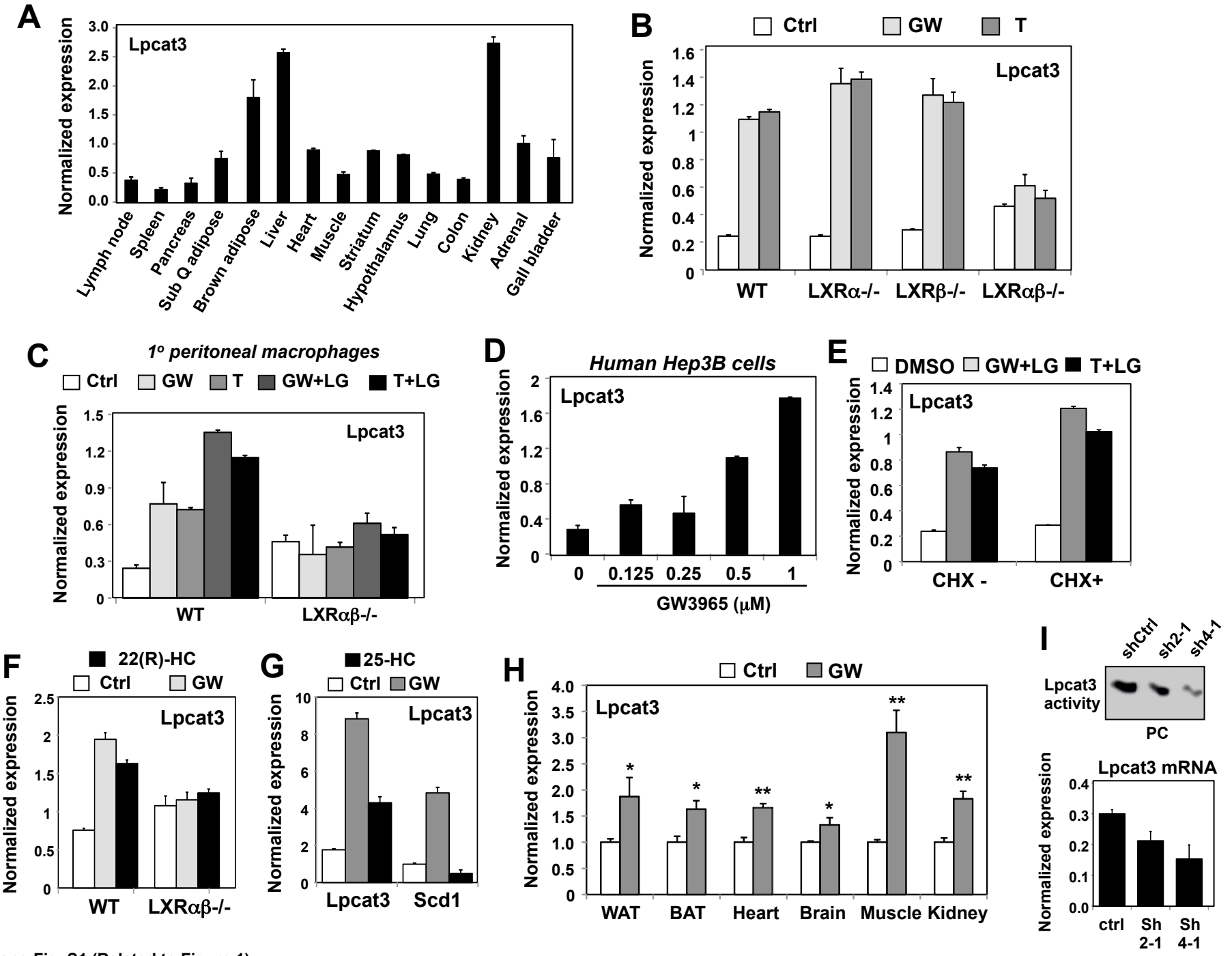
(F) Inflammatory gene expression in livers of mice transduced with adenoviral vectors expressing GFP (Ad-Ctrl) or Lpcat3 (Ad-Lpcat3) for 7 days was quantified by real-time PCR. N≥4 per group. Values are means ± SEM.

Statistical analysis was performed using student's t-test. *$p < 0.05$; **$p < 0.01$.

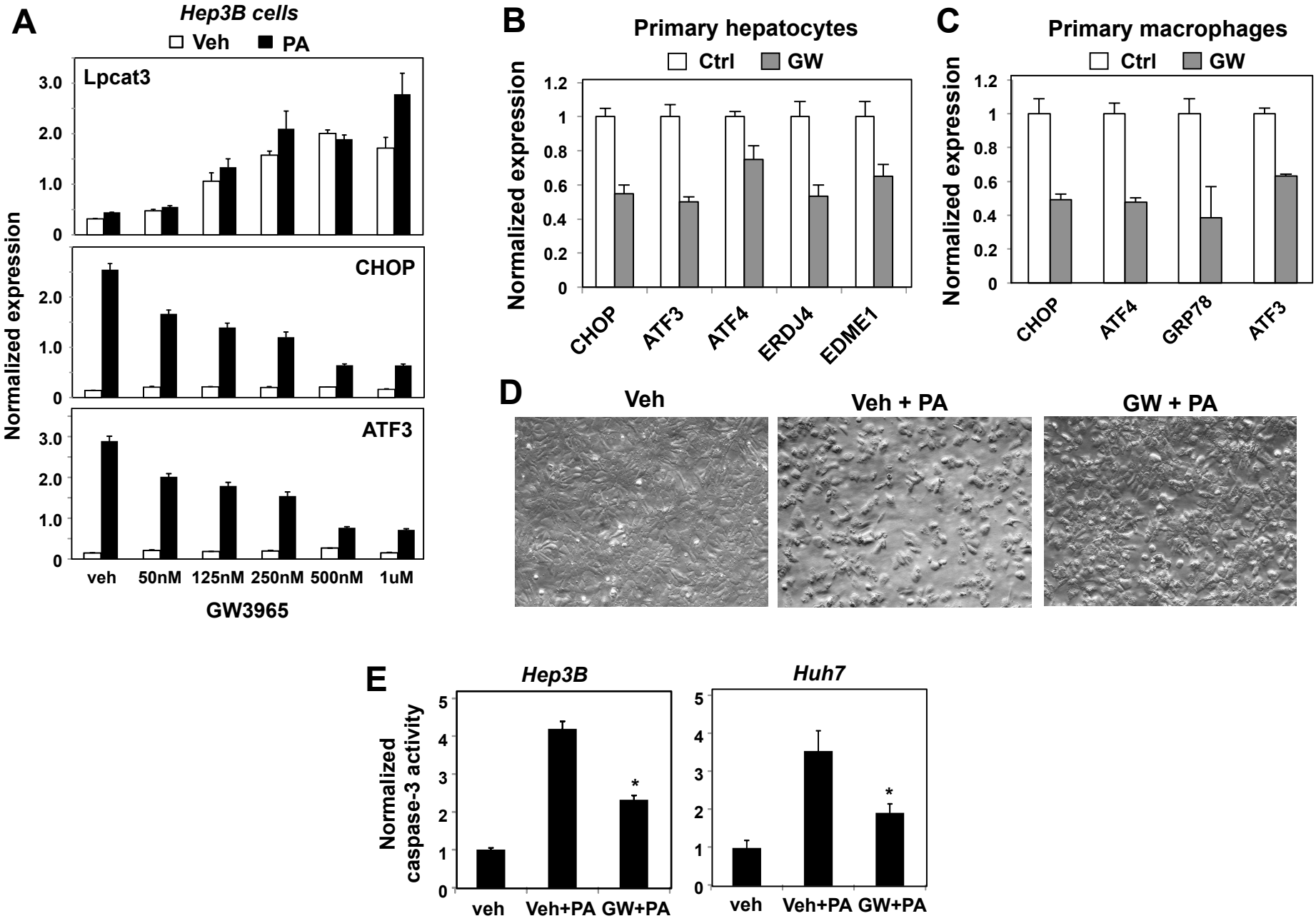
Figure S5 (Related to Figure 7). LXR activation of Lpcat3 suppresses inflammation in primary macrophages.

(A) Primary macrophages were transfected with siRNA targeting Lpcat3 (siLpcat3) or control (siCtrl) and treated with GW3965 (1 μM). Inflammatory responses were induced by LPS at 10 ng/ml. Gene expression was analyzed by real-time PCR. Values are means ± SD.

(B) Primary mouse macrophages were treated with the LXR ligand GW3965 (1 μM) and stimulated with 10 ng/ml LPS. Prostaglandin E_2 (PGE) secretion in the medium was measured by ELISA. *$p < 0.05$ by two-way ANOVA with Bonferroni post-hoc tests. Values are means ± SD.



Rong Fig. S1 (Related to Figure 1)



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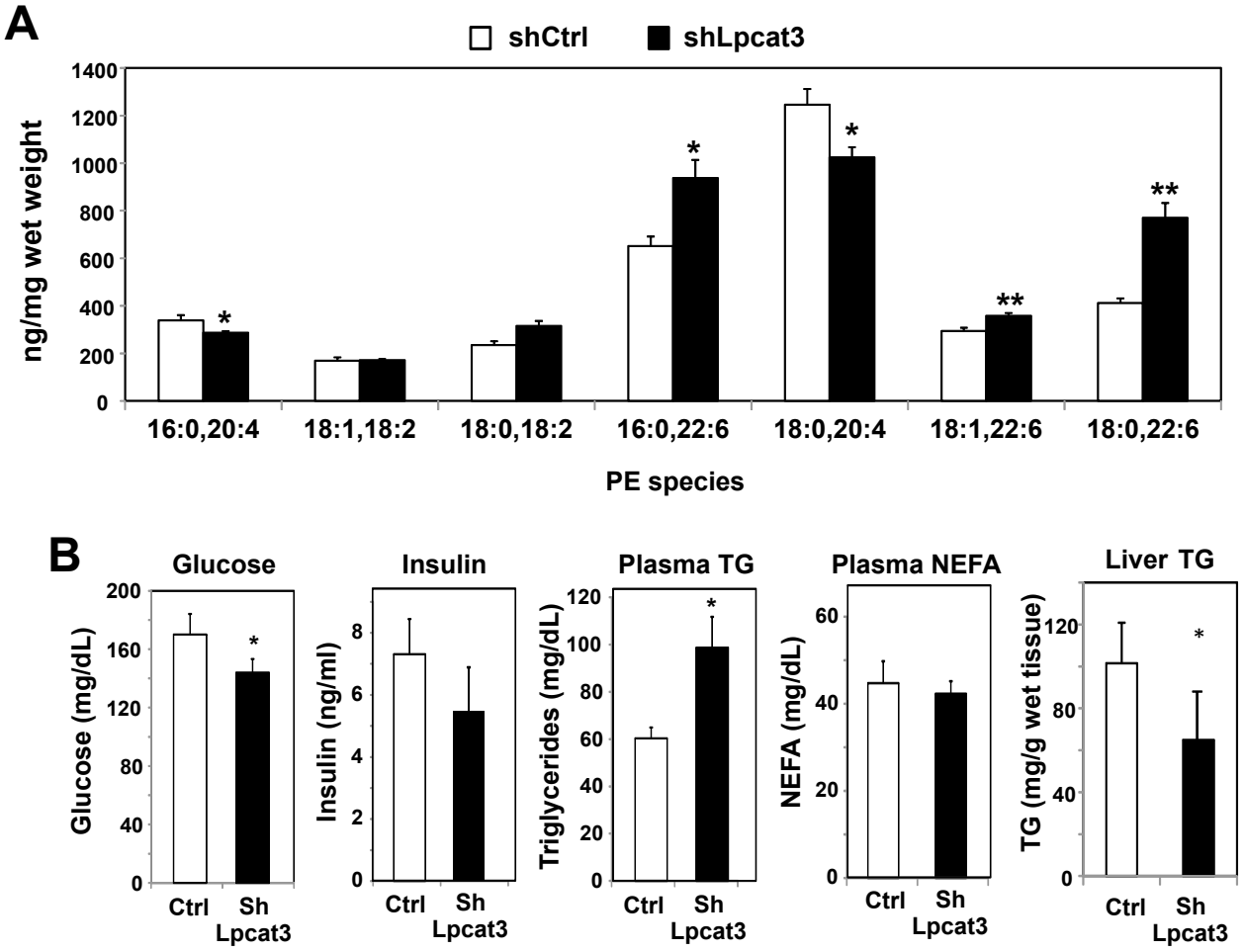

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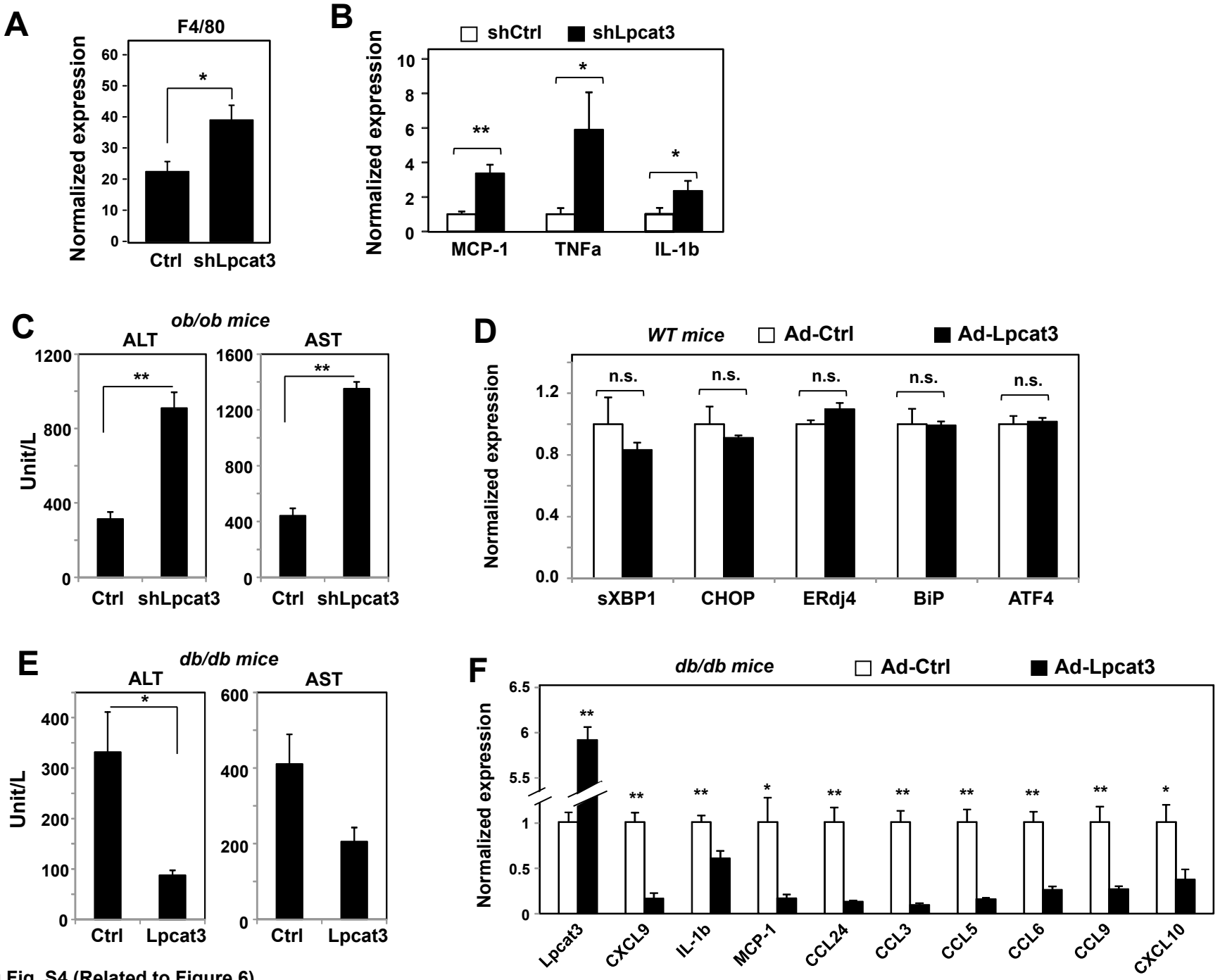

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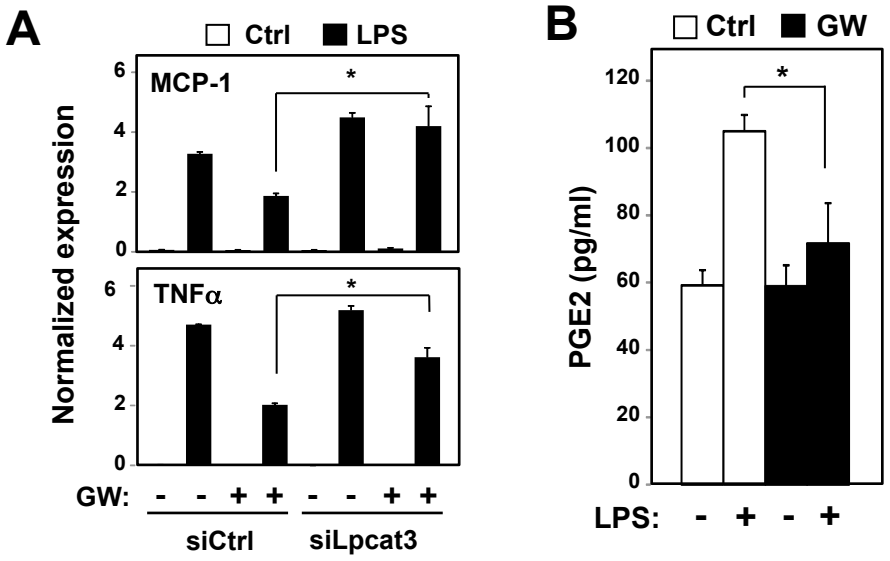

Rong Fig. S5 (Related to Figure 7)

Supplemental Experimental Procedures

Lipid Analyses–Cells or liver tissue were snap frozen in liquid nitrogen. Cell suspensions or liver homogenates were subsequently subjected to a modified Bligh-Dyer lipid extraction (Bligh and Dyer, 1959) in the presence of lipid class internal standards including eicosanoic acid, 1-0-heptadecanoyl-sn-glycero-3-phosphocholine, 1,2-dieicosanoyl-sn-glycero-3-phosphocholine, and 1,2-ditetradecanoyl-sn-glycero-3-phosphoethanolamine (Demarco et al., 2013). Fatty acids were converted to their pentafluorobenzyl esters and then were subsequently quantified using GC-MS with negative ion chemical ionization with methane as the reactant gas (Quehenberger et al., 2008). For phospholipids, lipid extracts were diluted in methanol/chloroform (4/1, v/v) and molecular species were quantified using electrospray ionization mass spectrometry on a triple quadrupole instrument (Themo Fisher Quantum Ultra) employing shotgun lipidomics methodologies (Han and Gross, 2005). Both phosphatidylcholine and lysophosphatidylcholine molecular species were quantified as lithiated adducts in the positive ion mode using neutral loss scanning for 59.1 amu (collision energy = -28eV). Phosphatidylethanolamine molecular species were quantified in the negative ion survey scan mode. Individual molecular species were quantified by comparing the ion intensities of the individual molecular species to that of the lipid class internal standard with additional corrections for type I and type II ^{13}C isotope effects (Han and Gross, 2005).

Protein Analysis–Cells were lysed in RIPA buffer (Tris-HCL, pH 7.4 50 mM, NaCL 150 mM, NP-40 1%, Sodium deoxycholate 0.5%, SDS 0.1%) supplemented with protease and phosphatase inhibitors (Roche Molecular Biochemicals). Tissues were homogenized with a dounce in the same lysis buffer. Non-dissolved components in the lysate were

cleared through centrifugation. Supernatants were run on 4%–12% Bis-Tris Gel (Invitrogen), transferred to hybond ECL membrane (GE Healthcare, Piscataway, NJ) and analyzed with anti-phospho-eIF2a (9721, Cell Signaling, Danvers, MA), anti-eIF2a (5324S, Cell Signaling), anti-phospho-PERK (3179S, Cell Signaling), anti-CHOP (sc-575, Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-JNK (9251, Cell signaling), or anti-JNK (sc-7345, Santa Cruz Biotechnology). Goat anti-mouse and Goat anti-rabbit secondary antibodies were visualized with chemiluminescence (ECL, Amersham Pharmacia Biotech).

Membrane Preparation and *in vitro* Acyltransferase Activity Assay–The membrane preparation and *in vitro* acyltransferase activity assay were performed as described (Zhao et al., 2008). For membrane preparation, cells were harvested into ice-cold buffer (20 mM Tris-HCl pH 7.4, 250 mM sucrose, 1mM EDTA, and protease inhibitors), and lysed by passing through a 25 gauge needle. Total cell lysates were subjected to an initial centrifugation at 10,000g for 10 min at 4 °C. Supernatants were then centrifuged at 100,000g for 1 hour at 4°C to obtain a membrane fraction, which was suspended in the same buffer and stored at -80 °C. For activity assay, 200μM 16:0 lysophosphatidylcholine, 10 μCi H^3 arachidonate-CoA and 5ug of membrane protein were mixed in the reaction buffer (5 mM Tris-HCl pH 7.4, 1 mg/ml fatty acid-free bovine serum albumin) and incubated for 30 min. Reactions were stopped by adding 1 ml of chloroform/methanol (2:1, v/v). Lipids were extracted from the reaction mixture and separated by thin layer chromatography (chloroform:ethanol:water:triethylamine 30:35:7:35). Acyltransferase activity was determined by the formation of radiolabeled PC.

Fatty Acid and Phospholipid Treatment–Fatty acid free, low endotoxin BSA was purchased from Sigma (Sigma-Aldrich, St. Louis, MO) and dissolved in 1X phosphate buffered saline. Palmitic acids were dissolved in ethanol at 70°C and added to BSA solution at 37°C slowly with vortexing to make the final concentration of 10mM PA and 6:1 PA/BSA mole ratio. BSA-conjugated PA was filtered and added to cells at a final concentration of 500μM PA for 6 hours, unless otherwise indicated. Phospholipids dissolved in chloroform were purchased from Avanti Polar Lipids (Avanti Polar Lipids, Alabaster, AL). 1 μmol phospholipids solutions were evaporated under a stream of nitrogen gas and thoroughly dried by rotary vacuum dryer. Dry phospholipids were then hydrated in 1ml PBS at 55 °C for 16:0 18:0 PC and 25 °C for 16:0 20:4 PC with rigorous shaking, followed by sonication until the mixture became almost clear. PLs were added to cells at the indicated concentration(s).

Glucose tolerance tests (GTTs)–Mice were fasted for 6 hr prior to an i.p. injection of glucose (1 g/kg of body weight). Blood glucose was measured by tail vein bleeding and analyzed by Bayer Contour next EZ blood glucose monitoring system at 0, 15, 30, 60, 90 and 120 min after glucose injection.

Detergent-Resistant Membrane Isolation–The method for the isolation of detergent-resistant lipid rafts has been described (Lingwood and Simons, 2007). Briefly, cells were washed and harvested in TNE buffer (150 mM NaCl, 2 mM EDTA, 50 mM Tris-HCl, pH7.4), lysed by passing through a 25 gauge needle, and solubilized with 1% TritonX-100 at 4ºC for 30 min. Lysates were mixed with Optiprep to form a 40% iodixanol bottom layer in the centrifuge tube, overlaid with a 30% iodixanol layer and a 0% (TNE

buffer) layer, and centrifuged for 2 hours at 260,000 g in TLA100.2 rotor. Four fractions were collect

Prostaglandin E_2 secretion–Primary mouse macrophages were treated with the LXR ligand GW3965 (1 μM) overnight. Then cells were washed with cold PBS twice and incubated in serum-free DMEM medium with 10 ng/ml LPS and GW3965 (1 μM). Culture medium was harvested after 8 hours and centrifuged to remove cellular debris. PGE_2 concentration in the medium was measured by Prostaglandin E2 EIA Kit Cayman Chemical, Ann Arbor).

ed from the top of the gradient and subjected to immunoblot analysis.