Supplementary Material (ESI) for Molecular BioSystems

Supplementary Information

A metabolomics strategy for detecting protein-metabolite interactions to identify natural nuclear receptor ligands

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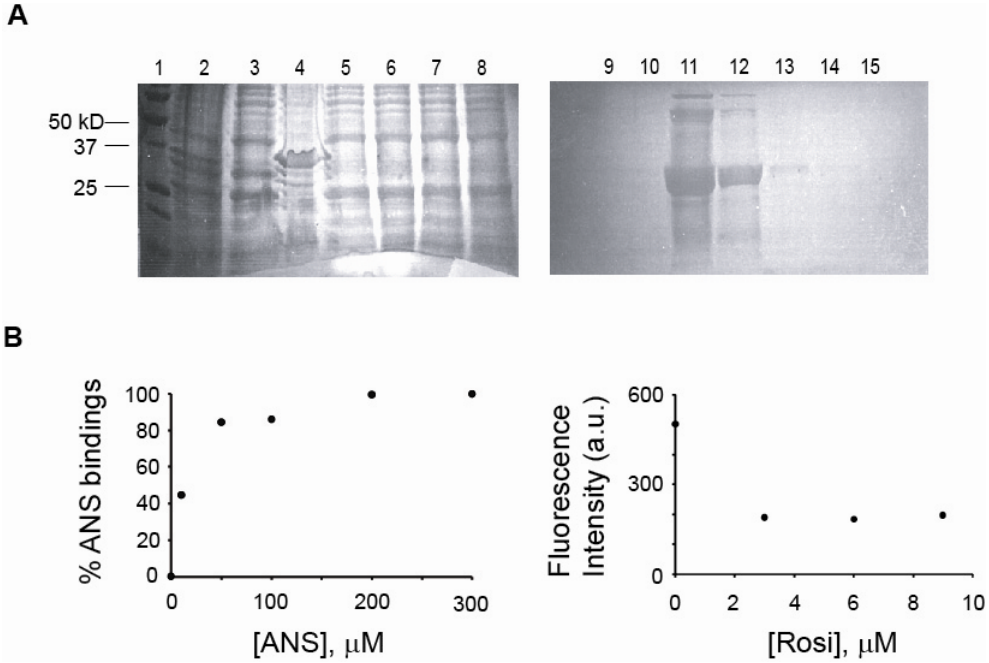
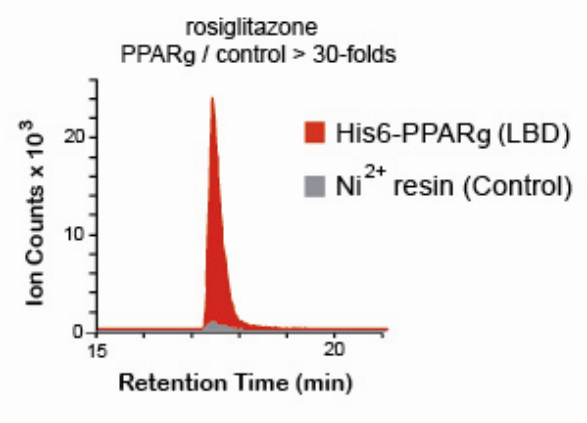
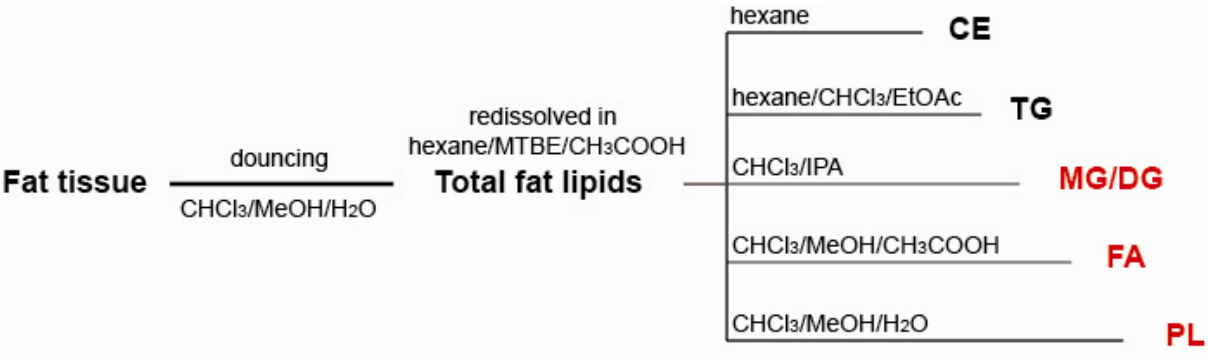
Figures



Suplmentary Figure 1. (A) Expression and purification of His6-PPARγ-LBD. Lane 1, molecular weight ladder (MW); lane 2, combined suspensions; lane 3, soluble protein; lane 4, pellet; lane 5, flow-through 1; lane 6, flow-through 2; lane 7, flow-through 3; lane 8, flow-through 4; lane 9, wash 4; lane 10, wash 7; lane 11, elution 1; lane 12, elution 2; lane 13, elution 3; lane 14, elution 4; lane 15, elution 5. (B) ANS (0 ~ 300 μM) binding to His6-PPARγ-LBD (1 μM), measured as an increase in the fluorescence of ANS (ex. 400, em. 480 nm); Displacement of 100 μM ANS from 1 μM His6-PPARγ-LBD by increasing concentration of rosiglitazone (0 ~ 9 μM).



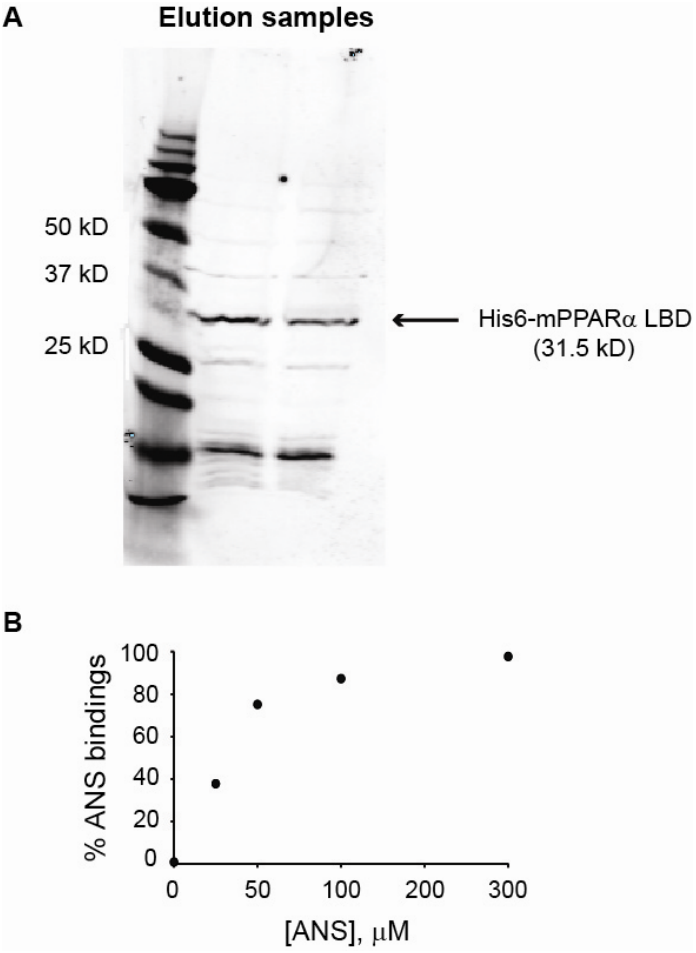
Supplementary Figure 2. The activity of the recombinant His6-PPARγ-LBD was confirmed by the enrichment of rosiglitazone from a complex mouse brain lipid extract.



Supplementary Figure 3. Highly abundant cholesteryl esters (CEs) and triglycerides (TGs) from adipose tissue were removed by using a solid phase extraction (Sep-Pak® Amino Propyl (NH_2) cartridge) protocol.

Supplementary Table 1.

Source	Lipid	Control (avg. ion counts, n=3)	PPARγ (avg. ion counts, n=3)	PPARγ+Rosi mix (avg. ion counts, n=3)	Fold Change (PPARγ/control)
Brain	Arachidonic acid	3.17E+04	2.74E+05	1.05E+05	8.6*
	Lysophosphatidic acid (18:1 LPA)	1.37E+04	5.98E+04	N.D	4.4*
Liver	Lysophosphatidic acid (18:1 LPA)	1.77E+04	1.21E+05	3.77E+04	6.8*
Fat	Arachidonic acid	1.78E+04	3.22E+05	1.77E+04	18.1*
	Linoleic acid	3.15E+05	5.03E+06	2.61E+05	16.0*
	Oleic acid	3.11E+05	1.95E+06	3.02E+05	6.3*
3T3-L1 cells (Day2, DMI)	Arachidonic acid	5.04E+03	2.55E+04	4.92E+03	5.1*
	Oleic acid	1.97E+04	6.69E+04	1.80E+04	3.4*
	Linoleic acid	5.69E+03	1.37E+04	5.16E+03	2.4*
	Palmitoleic acid	1.89E+04	4.24E+04	1.43E+04	2.2*



Supplementary Figure 4. (A) Detection of His6-PPARα-LBD *via* Western Blot. (B) ANS (0 ~ 300 μM) binding to His6-PPARα-LBD (1 μM), measured as an increase in the fluorescence of ANS (ex. 400, em. 480 nm)

Experimental Section

Recombinant His6-PPARγ-LBD expression. A plasmid containing the mouse PPARγ LBD gene (Leu^{202}-Tyr^{475}) was purchased (Open Biosystems, AL, USA) and the LBD domain was subcloned into the BamHI and SalI site of the pET200 D-TOPO vector (Invitrogen, CA, USA) to afford the plasmid pET200-mPPARγ LBD. The final construct contains a his6-tagged protein fused to the coding sequence of mPPARγ LBD. This vector was transformed into BL21 cells. The overnight cultured BL21 cells (6 mL) were transferred into 1 L media and then the protein expression was induced by 1 mL of IPTG (50 mg/mL) addition to the BL21 cells (OD = 0.3~0.6) containing pET200-mPPARγ LBD. After 6 hr at 18 °C, the cells were harvested and lysed and the His6-PPARγ-LBD proteins were purified by metal ion (Ni^{2+})-affinity chromatography using IMAC Sepharose 6 Fast Flow (GE Healthcare Life Science, NJ, USA) resin and size-exclusion chromatography using Superdex 200 10/300 GL (GE Healthcare Life Science, NJ, USA).

Binding activity assay of His6-PPARγ-LBD or PPARα-LBD with 1-anilinonaphthalene-8-sulfonate (ANS). The binding of ANS to His6-PPARγ-LBD or PPARα-LBD was monitored as the fluorescence enhancement of ANS at 480 nm upon excitation at 400 nm using a Spectramax fluorescence plate reader (Molecular Devices, Sunnyvale, CA). The protein (1 μM) and ANS (0 to 300 μM) were mixed together to a final volume of 150 μL across six wells. The ANS fluorescence showed a non-linear increase indicating a binding equilibrium.

ANS displacement assay of His6-PPARγ-LBD with rosiglitazone. Having observed the binding of ANS, we checked for binding of rosiglitazone to His6-PPARγ-LBD by monitoring the displacement of His6-PPARγ-LBD-bound ANS by rosiglitazone. The decrease in ANS fluorescence upon addition of rosiglitazone was measured using a Spectramax fluorescence plate reader. Rosiglitazone in DMSO was added to a solution of protein and ANS in a pH 8 buffer containing 50 mM Tris, 150 mM NaCl, and 0.5 mM EDTA. The final volume was 150 μL. Final concentrations of protein, ANS, and DMSO were 1 μM, 100 μM, and 4 % (v/v), respectively. The rosiglitazone concentration varied from 0 to 9 μM across four wells.

Recombinant His6-PPARα-LBD expression. A plasmid containing the mouse PPARα LBD gene (Glu^{192}-Tyr^{468}) was purchased (Open Biosystems, AL, USA) and the LBD domain was sub-cloned into the pET200 D-TOPO vector (Invitrogen, CA, USA) to afford the plasmid pET200-mPPARα LBD. The final construct contains a his6-tagged protein fused to the coding sequence of mPPARα LBD. This vector was transformed into BL21 cells. After overnight cultured BL21 cells (6 mL, 37 °C) were transferred into 1 L media, the protein expression was induced by adding 1 mL of IPTG (50 mg/mL) to the pET200-mPPARα LBD-containing BL21 cells at OD = 0.3-0.6. After 6 hr at 18 °C, the cells were harvested and lysed and the His6-PPARα-LBD proteins were purified by metal ion (Ni^{2+})-affinity chromatography using IMAC Sepharose 6 Fast Flow (GE Healthcare Life Science, NJ, USA) resin and size-exclusion chromatography using Superdex 200 10/300 GL (GE Healthcare Life Science, NJ, USA).

Detection of His6-PPARα-LBD *via* Western Blot. After running the elution sample

through a 15% Tris-HCl gel (Bio-Rad) for 1h at 150V, the Western transfer step was run in a 4 °C room for 1.5 hr at 50 V. The membrane (Millipore) was then washed twice for 5 min with room-temperature Tris Buffered Saline + 0.1% Tween-20 by agitation. The blocking step was completed by gentle agitation at room temperature in 25 mL blocking buffer (Rockland) for 1hr. The membrane was then incubated with gentle shaking in 15 mL primary antibody for 1hr at room temperature (1/2000 anti-His(rabbit) Rockland in Odyssey blocking buffer), followed by incubation/shaking in 15 mL secondary antibody for 1hr at room temperature (1/2000 anti-Rabbit IRDye700DX in Odyssey blocking buffer). The membrane was protected from light during the latter step. Before and after each antibody step, the membrane was washed 2 times for 5 min in TBS-tween at room temperature by gentle rocking. Finally, the tagged protein was visualized through the 700 channel of the Odyssey system.

Lipid extraction from tissues. Each frozen mouse brain (452 mg, pel-freez biologicals), liver (498 mg, pel-freez biological, AR, USA) and rat fat (469 mg, pel-freez biological) was dounced in 1× PBS buffer (2 mL) by using tissue grinders. The homogenized solution was transferred into the $CHCl_3$/MeOH/H_2O solution. A 2:1:1 solution of $CHCl_3$/MeOH/H_2O (8 mL per tissue) was prepared for tissue extraction. Each sample was then centrifuged at 2,200 ×g for 6 min at 4 °C in a glass vial and then, the organic layer (bottom) was carefully transferred to another glass vial. The organic layer was dried under a stream of nitrogen and redissolved in DMSO prior to use in enrichment experiments.

Removal of cholesteryl ester (CE) and triglyceride (TG) from rat fat. After the lipid extraction from rat fat, the high abundant CEs and TGs were selectively removed by the method of Ågren *et al.*[1]. Briefly, the extracted lipids from rat fat were redissolved with 500 μL of hexane-methyl tert-butylether-acetic acid (100:3:0.3) and then loaded to Sep-Pak® Amino Propyl (NH_2) cartridge (Waters, MA, USA) that pre-washed with 3 mL of acetone/H_2O (7:1) and 6 mL of hexane. The cartridge was washed with 2 mL of hexane/methyl tert-butylether/acetic acid (100:3:0.3). First, cholestetryl esters were eluted by 5 mL of hexane and triglycerides were eluted with 6 mL of hexane/$CHCl_3$/ethyl acetate (100:5:5). Mono- and diglycerides were fractionated with $CHCl_3$/2-propanol (2:1) and then the free fatty acids were eluted with $CHCl_3$/MeOH/acetic acid (100:2:2). The residual phospholipids were finally eluted by $CHCl_3$/MeOH/water (5:10:4). For further PPARγ pulldown assay using rat fat, mono- and diglycerides, free fatty acids and phospholipids were only used.

LC-MS analysis. LC-MS analysis was performed using an Agilent LC/MSD TOF. For the LC analysis in negative mode, a Gemini (Phenomenex, CA, USA) C18 column (5 μm, 4.6 x 100 mm) was used together with a pre-column (C18, 3.5 μm, 2 x 20 mm). Mobile phase A consisted of water/methanol (95:5) and mobile phase B was made up of isopropanol/ MeOH/water (60:35:5). Both A and B were supplemented with 0.1% ammonium hydroxide as solvent modifiers. The flow rate for each run was 0.4 mL/min for the duration of the gradient. During the sample loading, the flow path traveled beneath the waste for 12 min before starting the gradient. The gradient started at 0% B and then linearly increased to 100% B over 40 min followed by an isocratic gradient of 100% B for 8 min before equilibrating for 7 min at 0% B. The total analysis time, including 5 min at 0.1 mL/min, was 65 min. MS analysis was performed with an electrospray source ionization (ESI) interface. The capillary voltage was set to 3.0 kV and the fragmentor voltage to 100 V. The drying gas temperature was 350 °C, the drying gas flow was 10 L/min, and the nebulizer pressure was 45 psi, and

data was collected using a mass range of 100-1500 Da. All protein containing and control samples must be run on the same day to minimize day-to-day shifts in the instrument intensity to allow relative ion intensity to be used for quantitation during untargeted metabolomics.

PPARγ and PPARα pulldown assay. IMAC Sepharose 6 Fast Flow (GE Healthcare Life Science, NJ, USA) resin suspension (10 μL of a 50 % v/v in ethanol) was added to a 0.8 ml Pierce® Centrifuge Column (Pierce Scientific, IL, USA). The column was centrifuged on a mini centrifuge and the beads were washed with distilled water twice to remove any residual ethanol. To charge the resin with metal ions, 100 μL of 0.2 M Ni^{2+} sulfate was added to the washed beads and incubated for 60 min. The beads were washed with distilled water and 30 mM imidazole buffer (20 mM Na_2HPO_4, 0.75 M NaCl, 30 mM imidazole, pH 7.4) thrice. A His6-PPARγ-LBD or PPARα-LBD solution (5 nmol) was added to the washed beads and incubated for 2 hr with intermittent shaking. After incubation, unbound protein was separated from the beads by centrifugation and then protein bound beads were washed with 30 mM imidazole buffer. The lipid mixture from mouse brain, liver, rat fat and 3T3-L1 cells was applied to the beads as a solution in 1x PBS buffer (pH 7.4) containing 4 % v/v DMSO, incubated for 2 hr with intermittent shaking, and then removed by centrifugation. The beads were washed by three times addition of 30 mM imidazole buffer (100 μL) and immediately centrifuged. The protein and any protein bound metabolites were eluted by incubating the resin with 500 mM imidazol buffer (100 μL) for 10 min. This elution was replicated three times and the elution fractions were pooled, and subsequently injected to LC-MS (80 μL).

XCMS analyis. LC-MS chromatograms of eluents from the following sets of samples were obtained (n=3): (**A**) Ni^{2+} resin + lipid mixture, (**B**) His6-PPARγ-LBD + lipid mixture, (**C**) His6-PPARγ-LBD + rosiglitazone (100 μM) + lipid mixture; (**A**) Ni^{2+} resin + lipid mixture, (**B**) His6-PPARα-LBD + lipid mixture. The XCMS analysis of the LC-MS data was performed as previously described [2].

PPARγ assay. The binding constants of PPARγ ligands was directly measured by using the PolarScreen™ PPAR competitor assay kit (Invitrogen, CA, USA) following the manufacturer's protocol. Briefly, 20 μL of 2× arachidonic acid, linoleic acid and oleic acid (from 10 nM to 400 μM) was added into 384-microwell plate and then gently mixed with 20 μL of 2× PPARγ-LBD/Fluormone™ PPAR Green complex. The plate was covered to protect the reagents from light and incubated at room temperature for 2 hrs. The fluorescence polarization value of each well was measured by a fluorescence polarization plate reader.

References

[1] J. J. Ågren, A. Julkunen, and I. Penttilä, *J. Lipid Res.*, 1992, **33**, 1871-1876
[2] R. Tagore, H. R. Thomas, E. A. Homan, A. Munawar, and A. Saghatelian, *J. Am. Chem. Soc.* 2008, **130**, 14111-14113