Supplemental Figure Legends

Figure S1. Rosiglitazone treatment of primary adipocytes induces FGF21 expression and secretion, related to Figure 1.
Preadipocytes from the stromal vascular fraction of wild-type (WT) and FGF21-knockout (KO) mice were differentiated in vitro and treated with vehicle, GW7647 (10 nM) or rosiglitazone (rosi; 500 nM) for 48 hours.
(A) *Fgf21* mRNA was measured by RT-qPCR.
(B) FGF21 protein was measured in the media by ELISA.
Data are shown as the mean ± SEM. *b*, $p<0.01$ vs vehicle.

Figure S2. Body depot analysis in FGF21-knockout mice on the C57Bl/6 background, related to Figure 2.
(A – E) The mass of various body depots was measured in 2.5-month-old, male C57Bl/6 wild-type (WT) and FGF21-knockout (KO) mice. For **(E)**, adipose tissue mass was measured and normalized to body weight for epididymal (e), subcutaneous (sc), mesenteric (m), retroperitoneal (rp) and brown adipose tissue (BAT) depots. n=4/group. All data are shown as the mean ± SEM. *a*, $p<0.05$ vs WT.

Figure S3. FGF21 inhibits PPARγ sumoylation, related to Figure 4.
(A) Total and S112 phosphorylated PPARγ were measured by western blot analysis in either (left panel) wild-type (WT) and FGF21-knockout (KO) adipocytes treated with vehicle or FGF21 (200 ng/ml) for 4 hours prior to harvest; or, (right panel) epididymal white adipose tissue (eWAT) extracts from 2- to 3-month-old, male WT or KO mice, with each lane representing protein extract pooled from 4 mice. β-Actin was included as a loading control.
(B) Total and S273 phosphorylated PPARγ were measured in eWAT extracts from 2-month-old, male wild-type (WT) and FGF21-knockout (KO) mice. Each lane is protein from a single mouse. Quantification of the data by densitometry is shown below.
(C) Total and sumoylated PPARγ were measured in eWAT extracts from 2-month-old, male C57Bl/6 WT and FGF21-KO mice killed in the fed state. For each lane, protein extracts were pooled from 6 mice. β-Actin was included as a loading control. Quantification of data from individual mice (n=6/group) is shown in the graph. *a*, $p<0.05$ vs WT.
(D) Gene expression was measured by RT-qPCR in the eWAT of 2-month-old, male C57Bl/6 WT and FGF21-KO mice killed during the fed state (n=5/group).
(E) Flag-tagged PPARγ2, PPARγ2-K107R or PPARγ2-K395R were introduced into primary FGF21-KO adipocytes by transfection and their sumoylation measured by immunoprecipitation with a Flag antibody followed by western blot analysis with either a SUMO1 or PPARγ antibody. Input levels of Flag-tagged PPARγ and the PPARγ mutants were determined by western blot analysis with a Flag antibody. A representative experiment is shown.
(F) PPARγ expression was measured by RT-qPCR in wild-type (WT) and FGF21-knockout (KO) adipocytes transduced with lentiviruses expressing wild-type (WT) PPARγ2, PPARγ2-K107R, PPARγ2-K395R or GFP control and differentiated for 8 days.
Bar graphs are shown as the mean ± SEM. For **(D)**, *a*, $p<0.05$ vs WT.

Figure S4. Insulin-sensitizing effect of rosiglitazone is reduced in FGF21-knockout adipose tissue, related to Figure 5.

Two- to 3-month-old, male wild-type (WT) and FGF21-knockout (KO) mice were fed a high fat diet for 12 weeks. During the last two weeks, groups of mice were administered rosiglitazone (10 mg/kg) or vehicle (1% methylcellulose). Mice were injected with vehicle or insulin (10 U/kg) and killed 10 minutes later. Total and S473 phosphorylated Akt were measured by western blot, and data were quantified by densitometry. n=3/group. *a*, $p<0.05$.

Supplemental Experimental Procedures

Animal Experiments

Seventeen hour PPAR agonist treatments were performed in 2-month-old lean wild-type mice. Mice were gavaged at 5 pm and 8 am the next day with 10 mg/kg of either GW7647 (a gift from GlaxoSmithKline), rosiglitazone (Cayman Chemical) or vehicle (1% methylcellulose, Sigma) and were killed at 10 am, 4 hours into the light cycle. For DIO studies, mice were 2-3 months old at the beginning of the study and were individually caged. After 8 weeks on the high fat diet, mice were gavaged daily for 14 days, while being maintained on the high fat diet, with 10 mg/kg rosiglitazone or vehicle (1% methylcellulose). Food entrainment studies were performed in 8- to 10-week-old mice by reversing the 12 hour light-dark cycles and allowing the mice one week to acclimate before starting the food entrainment. Mice were given access to chow for a 4 hour period beginning 4 hour after the onset of the dark cycle and this feeding schedule was maintained for two weeks before tissues were collected every 4 hours for 24 hours. All tissues were snap frozen in liquid nitrogen and stored at –80˚C. Oral glucose tolerance tests were performed on groups of DIO mice treated with rosiglitazone or vehicle for 1 week. Mice were fasted for 8 hours, beginning at 6 am, and given an oral glucose load of 2 mg/g body weight. One week later, after being maintained on the high fat and rosiglitazone regimen, insulin tolerance tests were performed on these same groups of mice. Mice were fasted for 4 hours, beginning at 10 am, and injected IP with 0.75 U/kg insulin (Sigma). Tail blood was collected and assayed for glucose.

Body Composition Analysis

Body composition was measured using a Bruker Minispec mq10 NMR. Body fat mass, lean mass and fluid mass were measured between 2 pm and 3 pm in conscious mice with ad libitum access to food and water.

Metabolite and Hormone Measurements

Plasma triglycerides, cholesterol and non-esterified free fatty acids were measured using the L-type TGH Triglyceride kit, Cholesterol E kit and NEFA C kit, respectively (Wako Chemicals Inc.). For hepatic triglyceride and cholesterol measurements, total lipid was extracted from ~50 mg liver as previously described (Folch et al., 1957). ELISA kits were used to measure plasma FGF21 (Biovendor), insulin (Crystal Chem Inc.) and adiponectin (Millipore). For ELISA measurements of tissue FGF21, epididymal WAT from fresh-frozen samples was homogenized in buffer containing 150 mM NaCl, 10 mM HEPES (pH 7.4), and 0.5% Triton X-100 with antiprotease cocktail (Roche). Homogenates were snap frozen, thawed on ice and cleared by centrifugation at 4ºC. For the FGF21 ELISA, protein lysates were diluted 1:2 for WAT and 1:50 for liver using the diluent buffer supplied in the kit.

Morphometric Analysis of WAT

Epididymal white adipose tissue was fixed in Bouin's fixative for 2 days and transferred to 70% ethanol. Sections were stained with hematoxylin and eosin and images were acquired using Nikon ACT-1 (Version 2.63) software on a Nikon Eclipse TS100 microscope. Cell area was determined using ImageJ software package (Version 1.44p) with >150 adipocytes per group.

Recombinant FGF21 Production

Recombinant mouse FGF21 (amino acids 33-209) was produced in *E. coli*, refolded in vitro and purified to homogeneity by sequential affinity, ion exchange and size exclusion chromatography.

Primary Adipocyte Differentiation Assays

Primary preadipocytes were isolated from P4 wild-type and FGF21-KO mice. Briefly, pups were anesthetized on ice, decapitated, and inguinal adipose pads were isolated. Adipose pads were incubated with shaking at 37˚C in collagenase-buffer containing 12.5 mM HEPES (pH 7.4), 120 mM NaCl, 6 mM Na_2HPO_4, 2.5 mM D-glucose, 1.2 mM $MgSO_4$, 0.4 mM NaH_2PO_4, 2% bovine serum albumin and 0.1% type 2 collagenase. Adipocytes were separated and removed from the stromal vascular layer, which contains the preadipocyte population, by centrifugation and aspiration. Cells were washed once in MEF media containing 10% heat inactivated FBS, 20 mM HEPES (pH 7.3), 1X non-essential amino acids, 1X glutamax, 0.1 mM β-mercaptoethanol and high glucose DMEM to remove residual collagenase before plating in MEF media. Cells were grown to confluency and after 2 days differentiation was induced with high glucose DMEM containing 10% FBS, 10 µg/ml insulin, 0.5 mM isobutylmethyxanthine and 0.25 µM dexamethasone. Media was replaced every other day for 6 more days with high glucose DMEM containing 10% FBS and 10 µg/ml insulin. Cells were treated with vehicle (DMSO), 0.5 µM rosiglitazone and 100 ng/ml FGF21 as indicated in the figure legends. Oil red O staining was performed as described (Ramirez-Zacarias et al., 1992). Quantification of lipid staining was performed by adding 100% isopropanol to the stained cells, and absorbance measurements were taken using the Molecular Devices SpectraMax 384 plate reader at 510 nm.

Gene Transfer into Primary Adipocytes

For transfection studies, the p3XFLAG-CMV-10-PPARγ2 expression plasmid was mutated using the QuikChange Directed Mutagenesis kit (Stratagene) to generate the K107R, K395R and K107/395R mutants, which were confirmed by sequencing. Primary adipocytes were transfected with 3XFlag-tagged PPARγ2 or the K107R, K395R or K107/395R mutants, HA-tagged SUMO1 and pcDNA4 empty vector using FugeneHD (Roche) 1 day after inducing differentiation with high glucose DMEM containing 10% FBS, 10 µg/ml insulin, 0.5 mM isobutylmethyxanthine and 0.25 µM dexamethasone. The media was replaced the next day, and 2 days later the media was changed to high glucose DMEM with 10% FBS and 10 µg/ml insulin. After 2 days, the cells were harvested and lysates were subjected to immunoprecipitation and western blot analysis.

Lentiviral vectors were generated by subcloning the coding region of 3XFlag-tagged PPARγ2 or the K107R and K395R mutants into pLVX-IRES-ZsGreen1 (Clontech) and transfecting HEK293 cells with pVGVS, pΔ8.9 and the PPARγ2 expression vectors in a 1:1:1 ratio. After 48 hours, media was collected, filtered through a 0.45 µM filter and snap frozen in liquid nitrogen. For lentivirus infection experiments, 70% confluent preadipocytes were infected overnight with lentivirus expressing 3XFlag-tagged PPARγ2 (wild-type, K107R or K395R) or control GFP in media supplemented with 10 µg/ml polybrene. Two days after reaching confluence, infected pre-adipocyte were induced to differentiate as described above.

Immunoprecipitation and Western Blot Analysis

Frozen epididymal WAT was homogenized in tissue lysis buffer consisting of 150 mM NaCl, 50 mM NaF, 100 µM Na_3VO_4, 50 mM $Na_4P_2O_7$, 10 mM $C_3H_7Na_2O_6P$, 5 mM EDTA, 5 mM EGTA and 0.5% Triton X-100 supplemented with complete anti-protease cocktail (Roche). Lysates were cleared by centrifugation and incubated overnight at 4ºC with antibodies (1:100 dilution)

against SUMO1 (SC-5308, Cell Signaling Technology) or PPARγ (#2435, Cell Signaling Technology). 3XFlag-tagged PPARγ and HA-tagged SUMO1 were precipitated with anti-Flag M2 beads (Sigma) and monoclonal HA antibody (H9658, Sigma), respectively. Immunoprecipitates were washed twice in wash buffer A composed of 50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 0.1% Triton X-100 and 100 mM LiCl and twice in wash buffer B composed of 20 mM HEPES (pH 7.4)**,** 2 mM EGTA, 10mM $MgCl_2$ and 0.1% Triton X-100. Immunoprecipitated protein was subjected to SDS-PAGE and western blot analysis using SUMO1 or PPARγ antibodies. Blotting with a β-actin antibody (Sigma) was done to control for loading differences. Western blot analysis of S273 phosphorylation was done as described (Choi et al., 2010).

RT-qPCR Analysis

Primers were designed with Primer Express software (Applied Biosystems) based on GeneBank sequence data. Primers were selected to span exon junctions where possible. RNA extracts were subjected to DNase-treatment (Roche) and cDNA was synthesized from RNA (4 µg) using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). RT-qPCR (10 µl total) reactions contained 25 ng of cDNA, 150 nM of each primer and 5 µl of SYBR GreenER (Invitrogen). All reactions were performed in triplicate on the Applied Biosystems Prism 7900HT system and relative mRNA levels were calculated by the comparative threshold cycle method using U36B4 as an internal control (Bookout and Mangelsdorf, 2003). Primer sequences:

Gene	**Orientation**	**Sequence**
mouse *Fgf21*	forward	CCTCTAGGTTTCTTTGCCAACAG
	reverse	AAGCTGCAGGCCTCAGGAT
mouse *klb*	forward	GATGAAGAATTTCCTAAACCAGGTT
	reverse	AACCAAACACGCGGATTTC
mouse *Fgfr1c*	forward	GCCAGACAACTTGCCGTATG
	reverse	ATTTCCTTGTCGGTGGTATTAACTC
mouse *ap2*	forward	AGTGAAAACTTCGATGATTACATGAA
	reverse	GCCTGCCACTTTCCTTGTG
mouse *Cebpa*	forward	GACATCAGCGCCTACATCGA
	reverse	TCGGCTGTGCTGGAAGAG
mouse Cebpb	forward	ATTTCTATGAGAAAAGAGGCGTATGT
	reverse	AAATGTCTTCACTTTAATGCTCGAA
mouse *Cebpd*	forward	TTCCAACCCCTTCCCTGAT
	reverse	CTGGAGGGTTTGTGTTTTCTGT
mouse *Ppar*g	forward	CAAGAATACCAAAGTGCGATCAA
	reverse	GAGCTGGGTCTTTTCAGAATAATAAG
mouse *Slc25a1*	forward	GGCTGTCAGGTTGGGGATGT
	reverse	TGGGCATCCCGCATGT
mouse *Acly*	forward	GCCAGCGGGAGCACATC
	reverse	CTTTGCAGGTGCCACTTCATC
mouse *Me1*	forward	GCCGGCTCTATCCTCCTTTG

	reverse	TTTGTATGCATCTTGCACAATCTTT
mouse *Acaca*	forward	GGCAGCTCTGGAGGTGTATG
	reverse	TCCTTAAGCTGGCGGTGTT
mouse *Fasn*	forward	GCTGCGGAAACTTCAGGAAAT
	reverse	AGAGACGTGTCACTCCTGGACTT
mouse *Dgat2*	forward	CCGCAAAGGCTTTGTGAAG
	reverse	GGAATAAGTGGGAACCAGATCA
mouse *Pck1*	forward	CACCATCACCTCCTGGAAGA
	reverse	GGGTGCAGAATCTCGAGTTG

Microarray Analysis

RNA was prepared from epididymal WAT from DIO wild-type and FGF21-KO mice treated with rosiglitazone or vehicle for 14 days (2 pools of 2 mice per condition). RNA (500 ng) was reverse transcribed into cRNA and biotin-UTP labeled using the Illumina TotalPrep RNA Amplification Kit (Ambion) and hybridized to the Illumina mouseRefseq-8v2 Expression BeadChip using standard protocols. Image data were converted into unnormalized Sample Probe Profiles using the Illumina BeadStudio software and analyzed on the VAMPIRE microarray analysis framework3. Stable variance models were constructed for each of the experimental conditions (n=2). Differentially expressed probes were identified using the unpaired VAMPIRE significance test with a 2-sided, Bonferroni-corrected threshold of aBonf = 0.05. A list of 545 genes with statistically significant fold change differences was clustered using centered correlation (Cluster 3.0) and then visualized as a heat map on Java Tree View.

Supplemental References

Bookout, A.L., and Mangelsdorf, D.J. (2003). Quantitative real-time PCR protocol for analysis of nuclear receptor signaling pathways. Nucl Recept Signal *1*, e012.

Choi, J.H., Banks, A.S., Estall, J.L., Kajimura, S., Bostrom, P., Laznik, D., Ruas, J.L., Chalmers, M.J., Kamenecka, T.M., Bluher, M., Griffin, P.R., and Spiegelman, B.M. (2010). Anti-diabetic drugs inhibit obesity-linked phosphorylation of PPARgamma by Cdk5. Nature *466*, 451-456.

Folch, J., Lees, M., and Sloane Stanley, G.H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem *226*, 497-509.

Ramirez-Zacarias, J.L., Castro-Munozledo, F., and Kuri-Harcuch, W. (1992). Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with Oil red O. Histochemistry *97*, 493-497.