Lanthionine synthetase C-like protein 2 (LanCL2) is important for adipogenic differentiation

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Abstract **Adipogenic differentiation is a highly regulated process that is necessary for metabolic homeostasis and nutrient sensing. The expression of PPARγ and the subsequent activation of adipogenic genes is critical for the process. In this study, we identified lanthionine synthetase C-like protein 2 (LanCL2) as a positive regulator of adipogenesis in 3T3-L1 cells. Knockdown of LanCL2, but not LanCL1, inhibited adipogenic differentiation, and this effect was not mediated through cAMP or Akt signaling pathways. The expression of early adipogenic markers CCAAT enhancer binding protein β (C/EBPβ) and C/EBPδ remained intact in LanCL2 knockdown cells, but levels of late adipogenic markers PPARγ and C/EBPα were suppressed. The addition of the naturally occurring PPARγ activator 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 or conditioned medium from differentiating cells did not restore differentiation, implying that LanCL2 may not be involved in the production of a secreted endogenous PPARγ ligand. Pull-down assays demonstrated a direct physical interaction between LanCL2 and PPARγ. Consistent with a regulatory role of LanCL2, luciferase reporter assays revealed that full transcriptional activation by PPARγ was dependent on LanCL2. Taken together, our study reveals a novel role of LanCL2 in adipogenesis, specifically involved in PPARγ-mediated transactivation of downstream adipogenic genes.**—Dutta, D., K-Y. Lai, A. Reyes-Ordoñez, J. Chen, and W. A. van der Donk. **Lanthionine synthetase C-like protein 2 (LanCL2) is important for adipogenic differentiation.** ***J. Lipid Res.*** **2018.** 59: **1433–1445.**

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The adipose tissue plays a critical role in lipid metabolism and energy homeostasis. It serves as a physiological depot for the storage of lipids and as an endocrine organ mediating the secretion of a variety of cytokines such as leptin, adiponectin, and TNF-α (1). Many of these cytokines, often released under conditions of excessive lipid storage, are involved in pathological events such as inflammation and diabetes (2). A thorough understanding of the adipogenic process is therefore crucial for developing strategies to combat such comorbidities.

For the in vitro study of adipogenesis, 3T3-L1 preadipocytes are the most widely used and best-characterized cell culture model to date (3, 4). Studies in these cells have revealed the highly complex nature of adipocyte development and outlined the role of several transcription factors important in the process. For example, hormonal stimulation of these cells induces the expression of the CCAAT enhancer binding proteins C/EBPβ and C/EBPδ, which in turn increase the expression of PPARγ (5). PPARγ then activates C/EBPα, and the two proteins are involved in a positive-feedback mechanism, reinforcing the expression of each other and the expression of several downstream adipogenic genes (6).

Among all the proteins that have been reported to be involved in adipocyte development, PPARγ remains the most critical (7). Forced expression of PPARγ is sufficient to induce adipocyte differentiation in fibroblasts (8), and PPARγ-deficient cells fail to differentiate (9). PPARγ is a nuclear receptor, and its activation requires the binding of

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Abbreviations: 15d-PGJ2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J2; ABA, abscisic acid; aP2, adipocyte protein 2; C/EBP, CCAAT enhancer binding protein; CM, conditioned medium; DEX, dexamethasone; EdU, 5-ethynyl-2'-deoxyuridine; eGFP, enhanced green fluorescent protein; KD, knockdown; LanCL, lanthionine synthetase C-like; MBP, maltose binding protein; MDI, isobutylmethylxanthine, dexamethasone, and insulin; MIX, isobutylmethylxanthine; PPRE, PPARγ response element; qPCR, quantitative PCR.

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an endogenous ligand. Ligand binding induces a conformational change in the receptor, resulting in the dissociation of repressors and the differential recruitment of coactivators. This process is followed by the transcriptional activation of PPARγ-target genes and marks the final stage of adipogenic differentiation, whereby the mRNA and protein levels for several enzymes involved in triglyceride metabolism, such as adipocyte protein 2 (aP2) and stearoyl-CoA desaturase, increase severalfold and result in the accumulation of fat (10–12).

Lanthionine synthetase C-like proteins (LanCLs) are eukaryotic homologs of bacterial LanC, a cyclase involved in the formation of lanthionine rings in lantibiotics (13). Humans have three LanCL proteins, LanCL1, LanCL2, and LanCL3, encoded on chromosomes 2, 7, and X, respectively. LanCL1 is involved in antioxidant responses in the brain—KO animals accumulate lipid, protein, and DNA damage with age and show evidence of mitochondrial dysfunction and apoptotic degeneration (14). LanCL2 has been shown to be involved in the abscisic acid (ABA) signaling pathway, acting downstream to propagate ABA-specific effects in immune and insulinoma cells (15). ABA was shown to increase PPARγ reporter activity in RAW 264.7 macrophages, and knockdown (KD) studies showed LanCL2 expression to be necessary for this activity (16). Our previous results reported LanCL2 to be a novel regulator of Akt, specifically involved in facilitating optimal phosphorylation of Akt by mammalian target of rapamycin complex 2 (mTORC2), through direct physical interaction with both the kinase and the substrate (17). LanCL2 is highly expressed in the testis and the brain, with lesser, albeit ubiquitous expression in all other tissues examined (18). Very little is known about LanCL3. In this study, we found a novel role of LanCL2 in regulating the adipogenic differentiation process, acting at the stage of PPARγ-mediated transactivation of adipogenic genes.

MATERIALS AND METHODS

Reagents

3T3-L1 cells were obtained from Zenbio (Research Triangle Park, NC). Dexamethasone (DEX), insulin, and Oil Red O were purchased from Sigma-Aldrich (St. Louis, MO). Isobutylmethylxanthine (MIX), 15d-PGJ2, and troglitazone were purchased from Cayman Chemical (Montgomery, TX). Forskolin was from MP Biomedicals (Solon, OH), and 8-Br-cAMP was from Tocris (Ellisville, MO). Polybrene was purchased from Millipore (Billerica, MA) and puromycin from Calbiochem (La Jolla, CA). All cell culture reagents were obtained from Invitrogen (Carlsbad, CA).

Antibodies

The following antibodies were obtained from Cell Signaling Technology (Beverly, MA): rabbit anti-C/EBPδ (catalog no. 2318), rabbit anti-C/EBPα (catalog no. 8178), rabbit anti-PPARγ (catalog no. 2435), rabbit anti-aP2 (catalog no. 2120), rabbit anti-FA synthase (catalog no. 3180), rabbit anti-pAkt-S473 (catalog no. 9271), rabbit anti-pAkt-T308 (catalog no. 9275), rabbit anti-Akt (catalog no. 9272), mouse anti-His-tag (catalog no. 2366), mouse anti-β-actin (catalog no. 3700), mouse anti-maltose binding protein (MBP)-tag (catalog no. 2396), and mouse anti-rabbit IgG (conformation-specific) (catalog no. 5127). Rabbit anti-C/EBPβ was from Santa Cruz Biotechnology (Santa Cruz, CA, catalog no. sc-150), and rabbit anti-LanCL1 was from Bethyl Laboratories (Montgomery, TX, catalog no. A304-482A). Goat anti-rabbit GAPDH antibody (HRP-conjugated) was from Genscript (Piscataway, NJ, catalog no. A00192-40). Anti-LanCL2 antibody was generated by Proteintech Group (Chicago, IL) using full-length recombinant mouse LanCL2 protein as the antigen. This antibody recognizes both mouse and human LanCL2. HRP-conjugated secondary antibodies were from GE-Amersham (catalog no. NA934V for anti-rabbit and NA931V for anti-mouse). Anti-FLAG M2 beads were from Sigma-Aldrich (catalog no. A2220).

Plasmids

The following plasmids were obtained from Addgene (Cambridge, MA): PPARγ response element (PPRE)-X3-TK- firefly luciferase (plasmid no. 1015) (19); pIS1, a derivative of pRL-TK containing a renilla luciferase reporter driven by the HSV TK promoter (plasmid no. 12179; gift from David Bartel); pcDNA FLAG-PPARγ (plasmid no. 8895) (20, 21); and pcDNA-enhanced green fluorescent protein (pcDNA eGFP; plasmid no. 13031; gift from Doug Golenbock). For LanCL2 overexpression studies, human LanCL2 cDNA was subcloned into p3×FLAG-CMV-14 vector (Sigma-Aldrich), with the 3×FLAG tag fused to the C terminus of LanCL2, as reported previously (17). For bacterial expression of LanCL2-His, human LanCL2 cDNA was subcloned into a pET-Duet-1 vector using *Nco*I and *Not*I cloning sites (Novagen, Madison, WI) in multiple cloning site I. A DNA sequence encoding a His_6-tag was incorporated into the reverse primer of human LanCL2 cDNA. For bacterial expression of MBP-PPARγ, mouse PPARγ2 cDNA from FLAG-PPARγ2 plasmid was subcloned into a pMAL-p2x vector (New England Biolabs, Beverly, MA) using *Bam*HI and *Hin*dIII restriction sites. For localization experiments, human LanCL2 cDNA was cloned into the pcDNA-eGFP backbone using *Xho*I and *Xba*I cloning sites, with the eGFP tag fused to the C terminal end of LanCL2 generating hLanCL2-eGFP. A list of primers used for generating all the constructs is provided in supplemental Table S1.

Cell culture, differentiation, and nucleofection

The 3T3-L1 cells were maintained in DMEM with 10% FCS and 1× penicillin/streptomycin, at 37°C in the presence of 5% CO_2. For adipogenic differentiation, 2 days after the cultures were confluent, cells were incubated with DMEM containing 10% FBS, 0.5 mM MIX, 1 μM DEX, and 1 μg/ml insulin (collectively called MDI). This time point was considered day 0. On day 2, the induction medium was replaced by DMEM with 10% FBS containing 1 μg/ml insulin, and cells were subsequently fed every 2 days with the same medium. At the time points indicated, cells were either harvested for Western blot analysis or fixed in 10% formalin and stained with Oil Red O. For differentiation experiments with cAMP activators, the indicated concentrations of forskolin or 8-Br-cAMP were added to the MDI medium during the first 2 days of differentiation. For differentiation experiments with PPARγ ligands, 3 μM 15d-PGJ2 or 5 μM troglitazone was added to the MDI medium during the first 2 days of differentiation. HEK293 or HEK293T cells were maintained in DMEM with 10% FBS and 1× penicillin/streptomycin. For LanCL2 overexpression in 3T3-L1 cells, 3×FLAG-tagged LanCL2 plasmid was nucleofected in these cells using a Nucleofector II/2b device (Lonza, Walkersville, MD), and nucleofected cells were subjected to selection with 700 μg/ml G418 for 2 days and subsequently plated for differentiation.

Oil Red O staining

3T3-L1 adipocytes were washed with Dulbecco's PBS (DPBS), fixed in 10% formalin for 30 min, and subsequently rinsed in

deionized water. Cells were then incubated with 60% isopropanol for 5 min, followed by staining with 0.2% (wt/vol) Oil Red O solution in 60% isopropanol for 10 min. Cells were subsequently rinsed to remove nonspecific binding of Oil Red O and imaged. To quantify lipid content, Oil Red O was extracted with 100% isopropanol, and absorbance was measured at 500 nm. Oil Red O values from undifferentiated cells in parallel cultures served as blanks.

Lentivirus packaging and transduction

LanCL1 and LanCL2 lentiviral shRNA constructs in pLKO vector were purchased from the RNAi Consortium library of Sigma-Aldrich. Their clone IDs are as follows: mouse shLanCL2#1, TRCN0000076161; mouse shLanCL2#2, TRCN0000076158; mouse shLanCL1 #1, TRCN0000028861; mouse shLanCL1#2, TTRCN0000028930; human shLanCL2#1, TRCN0000045406; and human shLanCL2#2, TRCN0000076161. Lentivirus packaging was performed by cotransfecting pLKO-shRNA, pCMV-dR8.91, and pCMV-VSV-G into HEK293T cells using TransIT-LT1 (Mirus Bio, Madison, WI). Medium was replaced with DMEM containing 30% FBS 24 h after transfection, and virus-containing supernatants were collected a further 24 h later and filtered through a 0.45 μm filter. The 3T3-L1 preadipocytes or HEK293 cells at 40% confluency were transduced with the viral supernatants in the presence of 8 μg/ml polybrene for 24 h, followed by selection with 3 μg/ml puromycin for 3 days (for 3T3-L1 cells) or 1 μg/ml puromycin for 2 days (for HEK293 cells). The cells were subsequently plated for differentiation.

The 5-ethynyl-2'-deoxyuridine staining

Two-day confluent shScr and shLanCL2#1 cells were treated with the adipogenic cocktail. After 16 h, 5-ethynyl-2'-deoxyuridine (EdU, Berkshire, UK) was added to the medium at a final concentration of 10 μM, for an additional 2 h. After the 2-h pulse, cells were rinsed twice with PBS and fixed in 10% formalin solution for 15 min. Cells were subsequently incubated with PBS buffer containing 40 μM FAM (fluorescein) azide 5-isomer (Lumiprobe, Hunt Valley, MD), 1 mM $CuSO_4$, and 100 mM ascorbic acid for 30 min, protected from light. The cells were then washed with PBS containing 0.5% Triton X-100 and stained with 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI, Thermo Scientific, Waltham, MA) for 30 min. Cells were finally washed with PBS, and images were taken using a Zeiss Axiovert 200M microscope. EdU- and DAPI-positive cells were counted using ImageJ software (National Institutes of Health).

Western blotting

Cells were lysed using RIPA buffer (Thermo Scientific) in the presence of 1× protease inhibitor cocktail (Thermo Scientific) and centrifuged at 14,000 *g* for 5 min at 4°C. Protein concentrations were determined using Dc assay (Bio-Rad, Hercules, CA), and equal amounts of protein for each sample were mixed with Laemmli sample buffer (Bio-Rad) and boiled for 5 min at 95°C. Proteins were resolved using polyacrylamide gels (Bio-Rad) and subsequently transferred to nitrocellulose membranes (GE-Healthcare, Piscataway, PA). Membranes were then blocked for 1 h in 5% nonfat dry milk, followed by overnight incubation with the appropriate primary antibodies. Membranes were subsequently washed with TBS with 0.05% Tween 20 and incubated with secondary antibodies. Chemiluminescent signals were developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific), and signals were captured using MyECL Imager (Thermo Scientific).

cAMP assay

cAMP concentrations in 3T3-L1 cells were measured by using a Cyclic AMP XP assay kit from Cell Signaling Technology. The 3T3-L1 cells plated in 12-well plates were treated with 1 μM forskolin, in the presence of 0.5 mM MIX for 30 min at 37°C. Cells were subsequently lysed, and cAMP concentrations were measured following the manufacturer's recommendations. The absolute cAMP concentrations were calculated from a standard curve generated using cAMP standards provided in the kit.

Supernatant replacement assay

Two-day postconfluent shScr 3T3-L1 cells were treated with the adipogenic cocktail for 2 days, after which the conditioned medium (CM) was removed and added to scramble and LanCL2 KD cells in another plate. Fresh medium containing insulin was added to the first plate, and this medium was again added to the scramble and LanCL2 KD cells 2 days later. This protocol was continued until the end point of the experiment.

Real-time quantitative PCR

Total RNA was extracted from 3T3-L1 cells using the E.N.Z.A Total RNA kit (Omega Bio-tek, Norcross, GA). A total of 800 ng of RNA from each sample was reverse-transcribed into cDNA using Protoscript II reverse transcriptase (New England BioLabs) using oligo-dT primers. Quantitative PCR (qPCR) was performed on a Roche LightCycler 480 system using the iTaq Universal SYBR Green Supermix (Bio-Rad), following manufacturer's instructions. Gene expression was normalized to 36B4, which encodes for a ribosomal phosphoprotein and the expression of which remained unchanged during the course of differentiation (data not shown). The primers for PPARγ and aP2 were purchased from Sigma-Aldrich (Kiqstart SYBR Green predesigned primers). The sequences for other primer pairs are as follows: C/EBPα forward (F): 5′-TGTTGGGGATTTGAGTCTGTG-3′; C/EBPα reverse (R): 5′-GGAAACCTGGCCTGTTGTAAG-3′; 36B4 F: 5′-GTCACTGT-GCCAGCTCAGAA-3′; 36B4 R: 5′-TCAATGGTGCCTCTGGA-GAT-3′. The efficiencies for each primer pair were calculated using serial dilutions of cDNA and were found to lie within a 5% range of the housekeeping gene (36B4, data not shown).

LanCL2-His and MBP-PPARγ expression and purification

For expression of LanCL2-His, human LanCL2 cDNA was cloned into pETDuet-1 vector and expressed in Rosetta 2 *Escherichia coli* cells. Cells were induced with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 18°C for 18 h and were subsequently lysed in buffer containing 20 mM Tris, pH 7.5, 0.5 M NaCl, 1 mM tris(2-carboxyethyl)phosphine, 5 mM imidazole, and 10% glycerol. LanCL2 protein was purified by using a HisTrap column (GE Healthcare) and desalted using a PD 10 column (GE Healthcare). Purified LanCL2 protein was used for pulldown assays. For expression of MBP-PPARγ, *E. coli* BL21 (DE3) competent cells were transformed with pMAL-p2X vector encoding mouse PPARγ and induced with 1 mM IPTG for 18 h at 18°C. PPARγ was expressed as a fusion protein with MBP and was further purified using amylose resin (New England Biolabs).

Pulldown assays

For LanCL2-His pulldown of PPARγ in HEK293 cells, FLAG-PPARγ was overexpressed for 48 h, and the cells were lysed in His pulldown buffer (20 mM Tris-Cl, pH 8.0, 150 mM NaCl, 25 mM NaF, 25 mM β-glycerolphosphate, 0.1 mM $NaVO_3$, 20 mM imidazole, and 0.3% Triton X-100) containing 1× protease inhibitor cocktail (Roche, Indianapolis, IN). Cell lysates were centrifuged at 14,100 *g*, and supernatants were incubated with 50 μg of human LanCL2-His protein for 10 h at 4°C. For LanCL2-His pulldown of PPARγ in 3T3-L1 cells, the cells were differentiated for 3 days, and lysates were subsequently collected and incubated with 150 μg of recombinant human LanCL2-His protein for 10 h at

4°C. The cell lysates from both HEK293 and 3T3-L1 cells that had been incubated with LanCL2-His were subsequently treated with His-Pur cobalt resin (Thermo Scientific) for 2 h. The beads were washed three times with His pulldown buffer and boiled in 2× SDS buffer for 10 min. The samples were subsequently used for Western blotting. FLAG-PPARγ from HEK293 cells and endogenous PPARγ from differentiated 3T3-L1 cells were detected by anti-PPARγ antibody. LanCL2-His was detected by anti-His-tag antibody.

For pulldown of PPARγ using FLAG antibody in HEK293 cells, FLAG-PPARγ was overexpressed in HEK293 cells for 48 h, and the cells were lysed in IP lysis buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 5% glycerol, 1× protease inhibitor cocktail). Cell lysates were centrifuged at 14,100 *g* for 15 min, and the supernatants were incubated with anti-FLAG-M2 beads (Sigma-Aldrich) at 4°C for 12 h. The beads were washed three times with IP lysis buffer for 5 min each and boiled in 2× SDS buffer for 10 min. The samples were subsequently used for Western blotting. FLAG-PPARγ was detected by anti-PPARγ antibody, and LanCL2 was detected by homemade anti-LanCL2 antibody.

To determine whether PPARγ and LanCL2 have a direct physical interaction, equimolar amounts (500 nM) of purified MBP or MBP-PPARγ and LanCL2-His were incubated together at 4°C for 30 min with gentle shaking. LanCL2-His was subsequently pulled down by incubating with cobalt beads for 10 min. The beads were washed three times with His-pulldown buffer for 5 min each and boiled in 2× SDS for 10 min. The samples were subsequently used for Western blotting. The LanCL2-His protein was detected by anti-His tag antibody, and MBP or MBP-PPARγ protein were detected by anti-MBP tag antibody.

Confocal and fluorescence microscopy

For LanCL2 localization experiments, 3T3-L1 or HEK293 cells were plated in 35 mm glass-bottom dishes (MatTek Corporation, Ashland, MA) and transfected with hLanCL2-eGFP construct using Polyfect reagent (Qiagen, Valencia, CA). Twenty-four hours later, nuclei were stained with 5 μg/ml Hoechst 33342 (Molecular Probes, Eugene, OR) for 15 min at 37°C, and cells were imaged using a Zeiss LSM 700 confocal microscope. Images were processed using Zenlite software (Carl Zeiss). For fluorescence imaging of 3T3-L1 cell counts, scramble or LanCL2 KD cells were plated in 35 mm glass-bottom dishes and allowed to grow for 2 days postconfluency. Cells were subsequently stained with 5 μg/ml Hoechst 33342 for 15 min at 37°C, washed with DPBS, and imaged using a Zeiss Axiovert 200M microscope. Stained nuclei were quantified using ImageJ.

Luciferase assay

HEK293 cells were transduced with lentiviral vectors expressing shRNAs against human LanCL2 or scramble control. Transduced cells were selected with 1 μg/ml puromycin and subsequently transfected with PPRE-X3-TK-firefly luciferase plasmid, pIS1 renilla luciferase construct, and pcDNA-FLAG-PPARγ plasmid using TransIT-LT1. Twenty-four hours later, 3 μM 15d-PGJ2 or 5 μM troglitazone was added. After a subsequent 24 h, cells were lysed, and luciferase assays were performed using the Dual Glo Luciferase Assay kit from Promega (Madison, WI). For overexpression studies, hLanCL2-3X-FLAG was cotransfected along with the other constructs in HEK293 cells without KD. The readings from firefly luciferase were normalized to that of renilla to account for differences in cell number and transfection efficiencies. To determine whether LanCL2 KD in HEK293 cells decreases the levels of exogenously expressed PPARγ, lysates were collected from scramble or LanCL2 KD cells overexpressing FLAG-PPARγ for 24 h, and lysates were subsequently processed for Western blotting.

Statistics

All statistical analyses were performed using GraphPad Prism 4 (GraphPad Software, Inc., San Diego, CA). For statistical analysis, Student's *t*-test and one-way ANOVA were performed, wherever applicable. Statistical significance was set to $P < 0.05$, and the data are presented as mean ± SE.

RESULTS

KD of LanCL2 impairs 3T3-L1 differentiation

The 3T3-L1 preadipocytes were induced to differentiate by the addition of MDI. Accumulation of lipid droplets was observed by day 3, with complete differentiation evident by day 8. We first probed the expression of LanCL2 during the differentiation process and noted a constant level of expression throughout (**Fig. 1A**). To explore any potential function of LanCL2 in adipogenic differentiation, we knocked it down using lentiviral transduction of two different shRNAs directed against mouse LanCL2. An shRNA with a scrambled hairpin sequence was used as a negative control for all experiments. Efficient KD of LanCL2 was confirmed by Western blotting (Fig. 1B). LanCL2 KD strongly decreased differentiation of 3T3-L1 cells, as determined by Oil Red O staining (Fig. 1C) and spectrophotometric quantification of triglyceride accumulation (Fig. 1D). KD of LanCL1, on the other hand, did not affect differentiation of 3T3-L1 cells, implying that the inhibitory effect on adipocyte differentiation is LanCL2-specific (Fig. 1E–G). Overexpression of LanCL2 in 3T3-L1 cells (Fig. 1H) did not increase the differentiation potential of these cells further (Fig. 1I, J), suggesting that endogenous levels of LanCL2 may be sufficient for mediating full differentiation of these cells to adipocytes.

We observed LanCL2 KD cells to have slightly reduced proliferation rates in comparison to use of the scrambled hairpin. Hoechst 33342 staining of scramble and LanCL2 KD cells 2 days postconfluency showed slightly reduced cell count for LanCL2 KD cells (supplemental Fig. S1A, B). A slower proliferation rate in LanCL2 KD cells indicated that LanCL2 KD cells may spend reduced time in contact inhibition. Since contact inhibition during confluency is critical for adipogenic differentiation of 3T3-L1 cells (22, 23), we investigated the possibility that the decreased differentiation potential of LanCL2 KD cells resulted from reduced time spent in contact inhibition. LanCL2 KD cells were plated at a higher density such that they reached confluency before the scramble control cells (supplemental Fig. S1C). However, the higher cell count did not rescue differentiation in LanCL2 KD cells, ruling out a role of decreased proliferation rate or reduced contact inhibition in mediating the inhibited differentiation phenotype (supplemental Fig. S1D). We next checked whether LanCL2 KD cells could be defective in clonal expansion, a necessary step for differentiation mediated by the addition of MDI. EdU staining in scramble and LanCL2 KD cells showed similar proliferation rates after the addition of MDI (supplemental Fig. S2), suggesting that there is no defect in LanCL2 KD cells in clonal expansion.

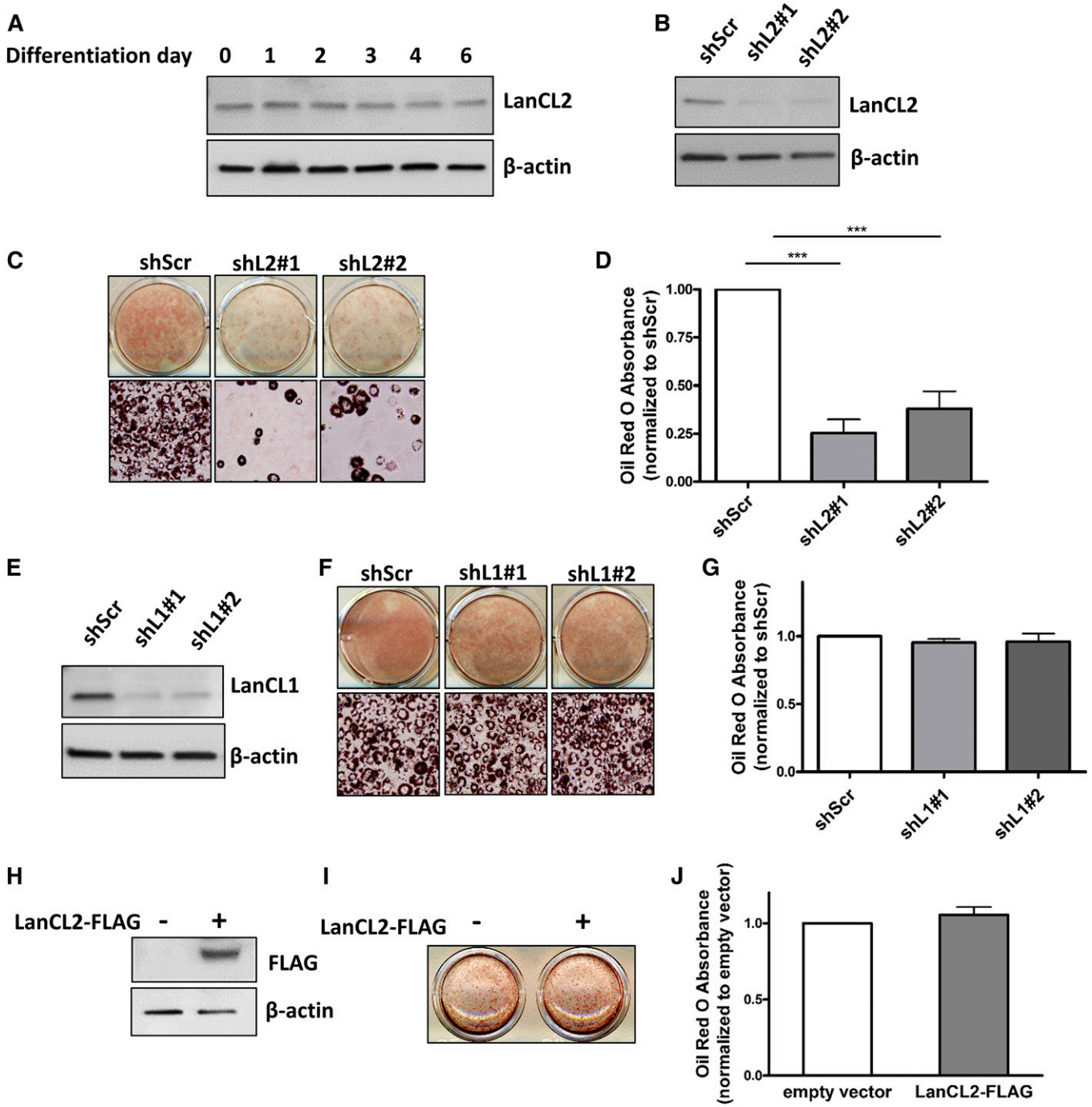

Fig. 1. KD of LanCL2 in 3T3-L1 cells impairs adipogenic differentiation capability. A: Expression of LanCL2 in 3T3-L1 cells during the course of differentiation. B: Confirmation of LanCL2 KD after lentiviral transduction of shRNAs against LanCL2. C: Oil Red O staining of differentiated scramble and LanCL2 KD cells. The upper panel indicates whole well images, and the lower panel represents magnified images from each well. D: Quantification of Oil Red O staining of differentiated scramble and LanCL2 KD cells in C. Data represent the average of four independent experiments. E: Confirmation of LanCL1 KD after lentiviral transduction of shRNAs against LanCL1. F: Oil Red O staining of differentiated scramble and LanCL1 KD cells. The upper panel indicates whole well images, and the lower panel represents magnified images from each well. G: Quantification of Oil Red O staining of differentiated scramble and LanCL1 KD cells in F. H: Confirmation of LanCL2 overexpression in 3T3-L1 cells after nucleofection with FLAG-tagged LanCL2. I: Oil Red O staining of 3T3-L1 cells expressing empty vector or FLAG-tagged-LanCL2. J: Quantification of Oil Red O staining of differentiated 3T3-L1 cells in I. Data represent the average of three independent experiments. One-way-ANOVA was used to compare each data point to scramble control. *** $P < 0.001$.

LanCL2 KD does not affect cAMP in 3T3-L1 cells

Recent reports have implicated LanCL2 to act downstream of the hormone ABA. The functional aspects of ABA are mediated by increasing the cellular concentrations of cAMP (15). An increase in cAMP concentration is essential during early stages of 3T3-L1 differentiation, acting through Epac (24). We therefore sought to determine whether the inhibition of differentiation in LanCL2 KD cells is the

result of decreased cAMP signaling. The increase in cAMP concentration in 3T3-L1 cells is mediated by MIX (25), included in the adipogenic cocktail. Our analysis of cAMP concentrations revealed no difference in scramble and LanCL2 KD cells after the addition of MDI (**Fig. 2A**). To further rule out a role of cAMP signaling in the adipogenic function of LanCL2, we included forskolin in the differentiation cocktail during the first 2 days of differentiation. A known cAMP inducer (26), forskolin resulted in a robust increase in cellular cAMP concentrations in both scramble and LanCL2 KD cells (Fig. 2B). However, addition of forskolin did not rescue differentiation in LanCL2 KD cells (Fig. 2C, D). Similar observations were made in cells treated with 8-Br-cAMP (Fig. 2E, F), a cell-permeable analog of cAMP that is resistant to degradation and is known to elicit similar effects as cAMP (27). Therefore, LanCL2 does not appear to function upstream of cAMP in adipogenic differentiation.

Akt phosphorylation is not affected by LanCL2 KD

We have previously shown that LanCL2 positively regulates Akt phosphorylation in HepG2 liver cells (17). Akt activation involves the phosphorylation of Ser473 in the C-terminal hydrophobic motif and of Thr308 in the activation loop of the protein (28, 29). Because activation of Akt is required for the transcriptional activity of PPARγ (30), we probed whether impaired Akt signaling might be responsible for reduced differentiation in LanCL2 KD cells. Immunoblotting for phospho-Ser473 and phospho-Thr308 during differentiation did not show any difference between scramble and LanCL2 KD cells (supplemental Fig. S3), implying that this pathway is not responsible for the impaired adipogenesis observed here.

LanCL2 KD cells have decreased expression of late adipogenic markers

To identify the step at which adipogenesis is inhibited in LanCL2 KD cells, we monitored the expression of early and late adipogenic markers. Expression analysis of C/EBPβ, C/EBPδ, C/EBPα, PPARγ, and aP2 from day 0 through 6 of differentiation showed very low expression of these proteins on day 0 (supplemental Fig. S4), as expected (31, 32). We therefore focused on the expression of these proteins during differentiation; i.e., from day 1 onward. Levels of early adipogenic markers C/EBPβ and C/EBPδ did not differ in scramble and LanCL2 KD cells at any time during the differentiation process (**Fig. 3A**). However, the expression of late adipogenic markers PPARγ, C/EBPα, and aP2 on days 4 and 6 was decreased in cells with LanCL2 KD. RT-qPCR analysis further revealed that the mRNA levels encoding PPARγ, C/EBPα, and aP2 were significantly reduced in LanCL2 KD cells on days 3 and 6 of differentiation (Fig. 3B).

15-deoxy-$\Delta^{12,14}$-Prostaglandin J2, or CM from control cells, cannot rescue differentiation in LanCL2 KD cells

Because sustained expression of PPARγ during adipogenesis requires the presence and binding of a ligand, we probed whether LanCL2 KD cells might have a defect at this stage. An endogenous ligand for PPARγ has been proposed to be produced and secreted by actively differentiating cells (33), and the addition of CM from differentiating 3T3-L1 cells can augment the transcriptional activity of PPARγ, resulting in increased differentiation (19). If LanCL2 KD cells are defective in the generation of an endogenous ligand, the presence of such a ligand in the CM of normally differentiating cells could restore differentiation in the KD cells. We added CM from differentiating scramble cells to scramble or LanCL2 KD cells, every 2 days, until the endpoint of the experiment. This resulted in normal differentiation in CM-treated scramble cells, but no rescue of differentiation was observed in LanCL2 KD cells (**Fig. 4A**). Likewise, addition of CM from LanCL2 KD cells induced differentiation in scramble cells, but not in LanCL2 KD cells (Fig. 4A). These results suggest that LanCL2 may not regulate differentiation through the production of a secreted PPARγ ligand.

We then explored the possibility that LanCL2 KD cells could be defective in signal propagation after the binding of a PPARγ ligand. We tested whether addition of 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (15d-PGJ2), an endogenously occurring but relatively weak PPARγ agonist (34), to the differentiation medium could rescue differentiation. As shown in Fig. 4B, C, 15d-PGJ2 could not restore differentiation in LanCL2 KD cells. Western blot and RT-qPCR analysis further showed that the protein and mRNA expression levels of late adipogenic genes encoding PPARγ, C/EBPα, fatty acid synthase, and aP2 remained low in LanCL2 KD cells grown in the presence of 15d-PGJ2 (Fig. 4D and supplemental Fig. S5).

Taken together, these results demonstrate that in the absence of LanCL2, endogenously occurring PPARγ activators such as 15d-PGJ2, or ligand(s) potentially secreted into the medium of differentiating cells, could not induce differentiation. These observations suggest that LanCL2 is not involved in the production of a secreted ligand and that LanCL2 KD may interfere with the transactivation of adipogenic genes by PPARγ.

LanCL2 interacts with PPARγ

If LanCL2 is involved in the transactivation activity of PPARγ, the simplest mechanism would be via a direct or indirect physical interaction. PPARγ has been widely reported to reside in the nucleus, consistent with its role as a transcriptional activator (35). Immunofluorescence imaging of transiently expressed hLanCL2-eGFP revealed nuclear localization of LanCL2 in both 3T3-L1 and HEK293 cells (**Fig. 5A** and supplemental Fig. S6). Because PPARγ and LanCL2 localized to the same cellular compartment, we further explored the possibility of a physical interaction. We performed pulldown assays with lysates from day 3 differentiated 3T3-L1 cells, because the expression of endogenous PPARγ is relatively high at this point (Fig. 3A), whereas lipid accumulation remains relatively low. We found that at this stage, LanCL2-His could pull down endogenous PPARγ2, the isoform that is important for adipogenesis (8, 36) (Fig. 5B and supplemental Fig. S7). To determine whether this interaction also occurred in non-adipogenic cells, we repeated the pulldown in HEK293 human embryonic kidney cells. Purified LanCL2-His pulled down transiently expressed PPARγ in HEK293 cells

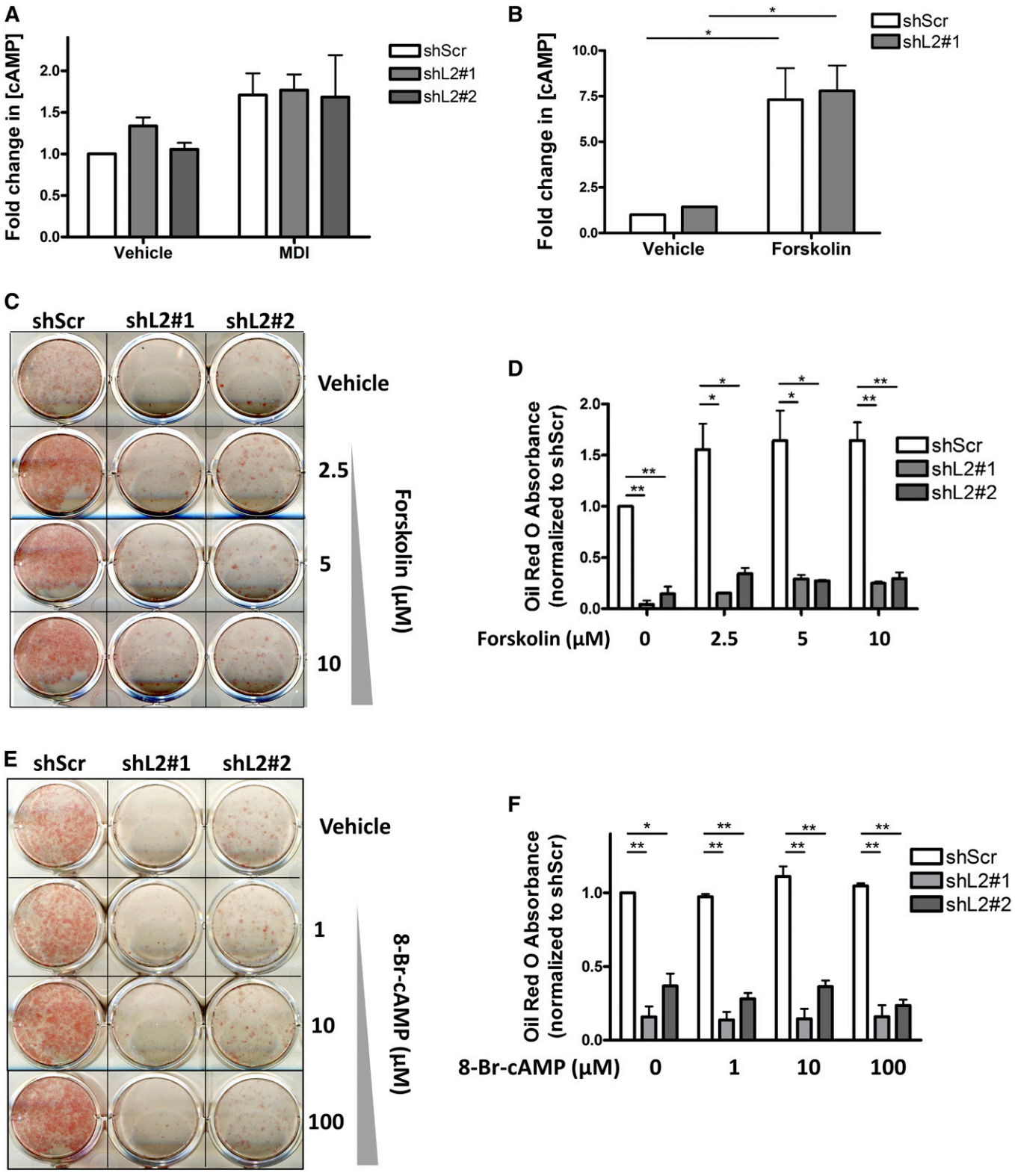

Fig. 2. Restoration of cAMP levels does not induce differentiation in LanCL2 KD cells. A: Cellular cAMP levels in scramble and LanCL2 KD cells before and after the addition of MDI. B: Increase in cAMP concentrations in 3T3-L1 cells by treatment with forskolin. C: Differentiation of scramble and LanCL2 KD cells in the presence of increasing concentrations of forskolin. D: Quantification of Oil Red O staining of differentiated scramble and LanCL2 KD cells in C. Data represent the average of two independent experiments. E: Differentiation of scramble and LanCL2 KD cells in the presence of increasing concentrations of 8-Br-cAMP. F: Quantification of Oil Red O staining of differentiated scramble and LanCL2 KD cells in E. Data represent the average of two independent experiments. One-way-ANOVA was used to compare each data point to scramble control. * $P < 0.05$; ** $P < 0.01$.

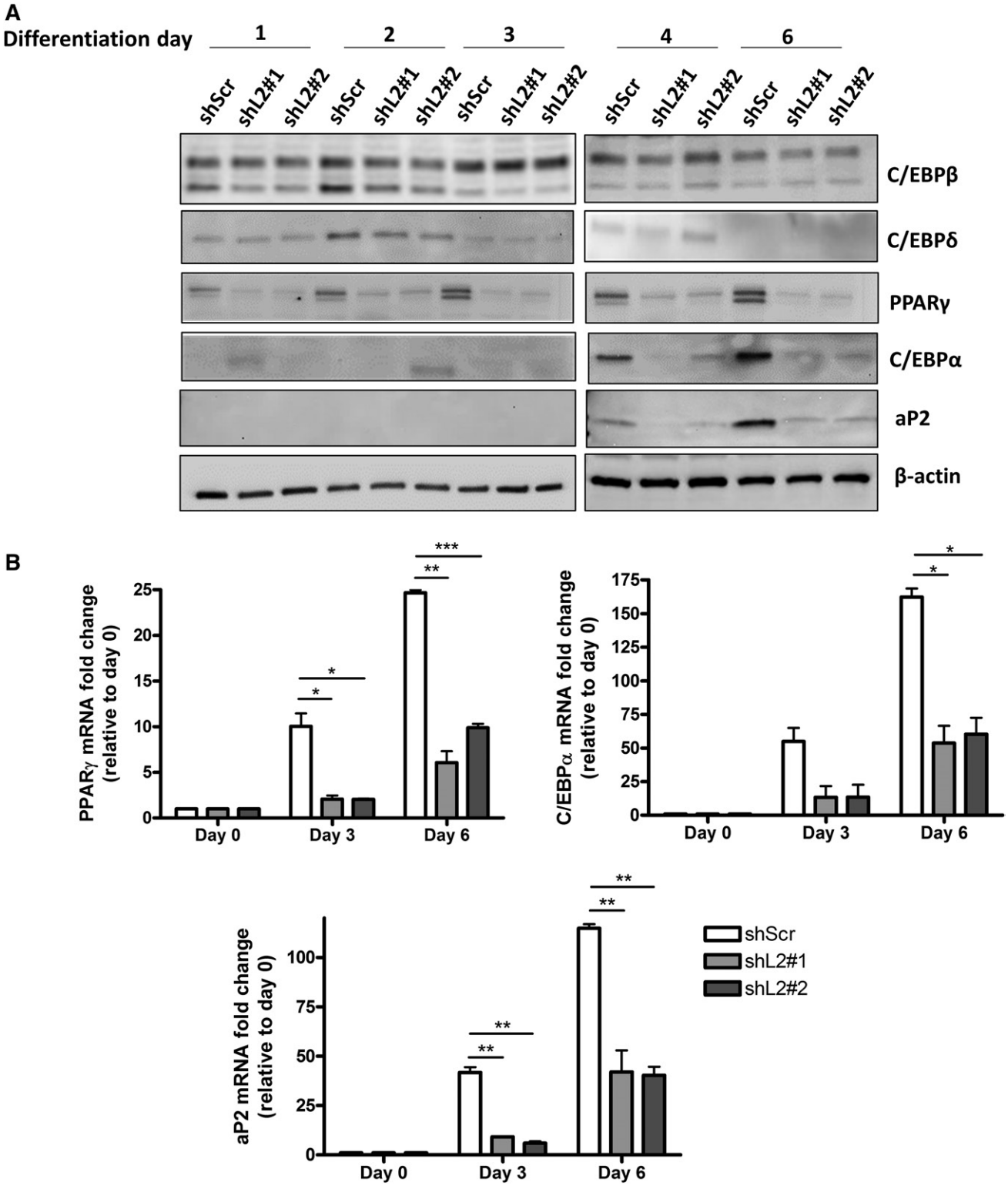

Fig. 3. LanCL2 KD cells show decreased expression of late adipogenic markers. A: Expression of adipogenic markers in scramble and LanCL2 KD cells during early (day 1–3) and late (day 4 and 6) stages of differentiation monitored by Western blot. B: mRNA expression analysis of late adipogenic genes PPARγ, C/EBPα, and aP2 in scramble and LanCL2 KD cells. Data represent the average of two independent experiments. One-way-ANOVA was used to compare each data point to day 0 undifferentiated controls. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

(Fig. 5C). Furthermore, overexpressed FLAG-PPARγ coimmunoprecipitated with endogenous LanCL2 in these cells (Fig. 5D), and in vitro pulldown assays using purified PPARγ and LanCL2 proteins demonstrated a direct physical interaction between the two proteins (Fig. 5E).

LanCL2 is important for PPARγ activity during differentiation

Given that LanCL2 physically interacts with PPARγ, we explored the possibility that LanCL2 regulates PPARγ-mediated transcriptional activation of target genes. To this

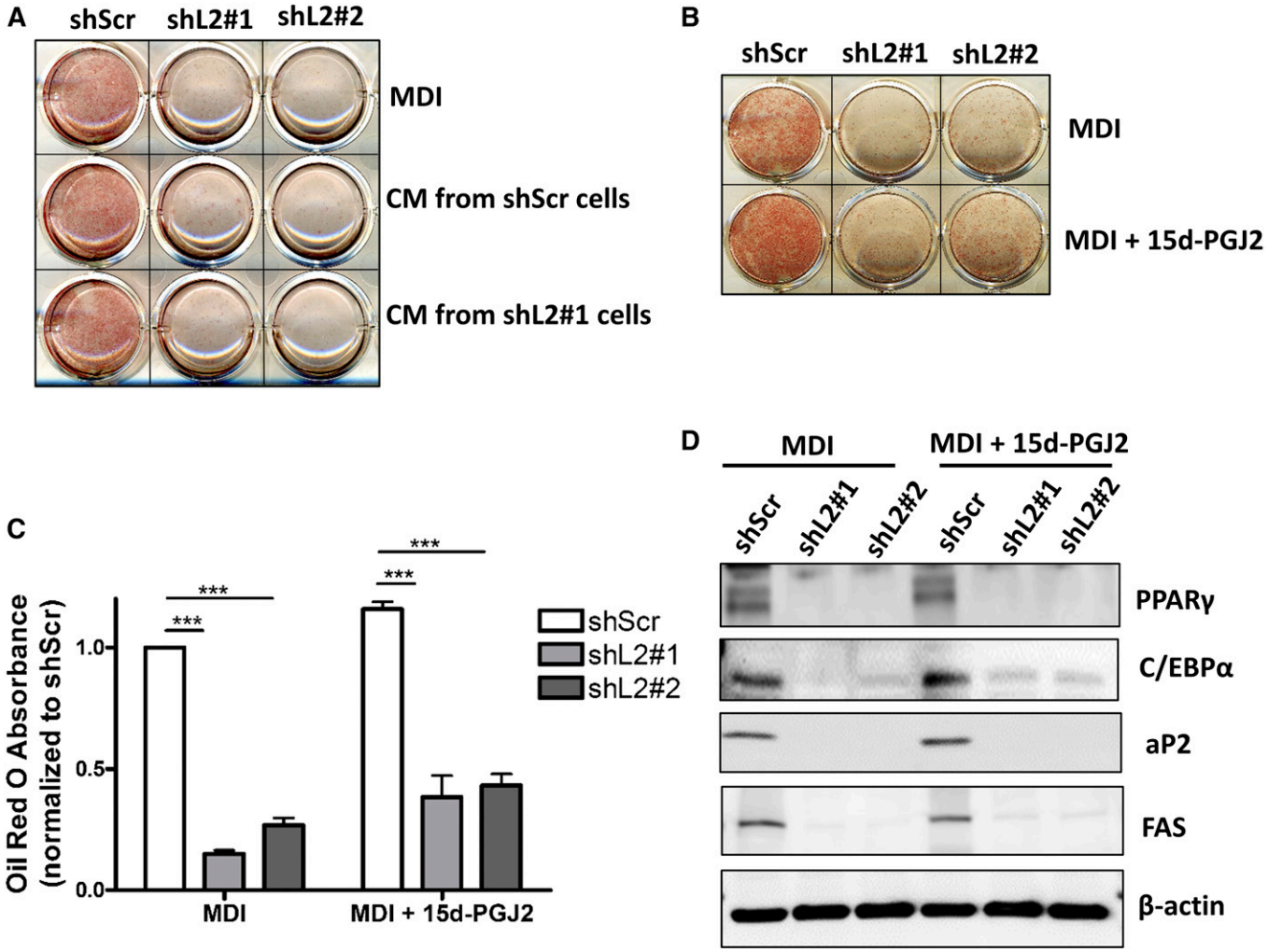

Fig. 4. Addition of CM from differentiating 3T3-L1 cells or PPARγ ligand 15d-PGJ2 does not rescue differentiation in LanCL2 KD cells. A: Oil Red O staining of scramble and LanCL2 KD cells, differentiated using CM from adipogenic cocktail-treated scramble or LanCL2 KD cells. B: Oil Red O staining of scramble and LanCL2 KD cells differentiated in the presence of 15d-PGJ2. C: Quantification of Oil Red O staining of scramble and LanCL2 KD cells in B. Data represent the average of three independent experiments. One-way-ANOVA was used to compare each data point to day 0 undifferentiated controls. *** $P < 0.001$. D: Expression of adipogenic markers in MDI and MDI plus 15d-PGJ2-treated cells, differentiated for 6 days.

end, we utilized a luciferase reporter driven by PPRE-luc (37). As expected, expression of recombinant PPARγ in HEK293 cells, which express a very low level of endogenous PPARγ (**Fig. 6A**, left inset), stimulated the activity of this reporter (Fig. 6A). The reporter activity was further enhanced by the addition of 15d-PGJ2 or troglitazone, a strong synthetic activator of PPARγ (34, 38, 39). Importantly, when LanCL2 was knocked down by lentivirus-mediated delivery of shRNAs, the reporter activity was significantly reduced under all conditions (Fig. 6A). This reduction was not caused by decreased PPARγ levels upon LanCL2 KD (supplemental Fig. S8). Overexpression of LanCL2 in HEK293 cells did not further increase PPRE-luc activity (Fig. 6B), indicating that the endogenous level of LanCL2 in these cells might be sufficient to induce full activation of PPRE. Consistent with troglitazone providing the strongest recovery of PPARγ activity upon LanCL2 KD in these reporter assays, troglitazone could also partially restore adipogenesis in 3T3-L1 cells in which LanCL2 was knocked down (supplemental Fig. S9). Taken together with the observed physical interaction between LanCL2 and PPARγ, these results strongly suggest an important role of LanCL2 in mediating the full transactivation potential of PPARγ. We tried to assess whether LanCL2 KD would exert an effect on PPARγ after completion of differentiation, but we were unable to knock down LanCL2 in already differentiated 3T3-L1 cells.

DISCUSSION

Our study establishes LanCL2 as a positive regulator of adipocyte differentiation, as KD of LanCL2 in 3T3-L1 cells strongly reduced triglyceride accumulation. The effect was specific for LanCL2, as KD of LanCL1 did not inhibit differentiation. We ruled out a role of cAMP or Akt signal transduction pathways in mediating the impaired adipogenic phenotype in LanCL2 KD cells. The arrest in differentiation

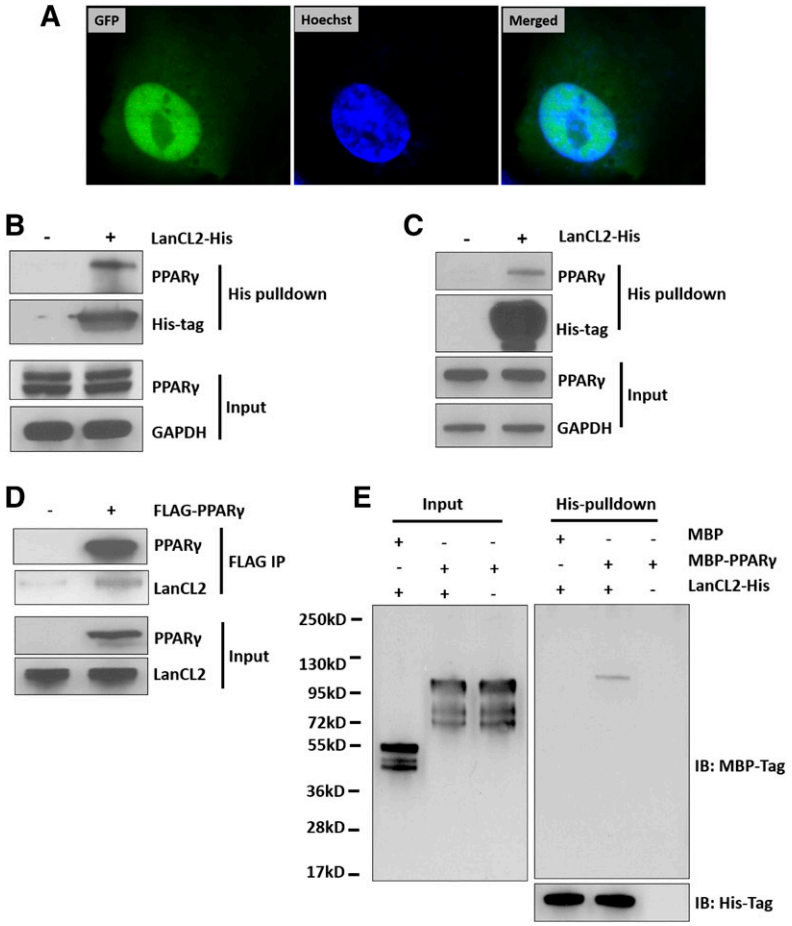

Fig. 5. LanCL2 physically interacts with PPARγ in 3T3-L1 and HEK293 cells. A: Confocal imaging of GFP fluorescence in 3T3-L1 cells transfected with the LanCL2-eGFP construct. Nuclei were stained with Hoechst. B: Lysates of 3T3-L1 cells differentiated for 3 days were incubated with purified LanCL2-His, followed by pulldown of LanCL2-His and Western blotting for endogenous PPARγ. C: Lysates of HEK293 cells overexpressing FLAG-PPARγ were incubated with purified LanCL2-His, followed by pulldown of LanCL2-His and Western blotting for recombinant PPARγ. D: FLAG-PPARγ overexpressed in HEK293 cells was immunoprecipitated, followed by Western blotting for endogenous LanCL2. E: Purified MBP-tagged PPARγ was incubated with purified His-tagged LanCL2, followed by pulldown of LanCL2-His with cobalt beads and Western blotting for MBP to detect PPARγ. MBP, 53.4 kDa; MBP-PPARγ, 103.2 kDa.

did not involve early transcription factors C/EBPβ and C/EBPδ. Instead, the blockage in adipogenesis seems to be at the stage of PPARγ-mediated transactivation of downstream adipogenic genes. Furthermore, we showed that LanCL2 physically interacts with PPARγ in different cell types, is located in the same intracellular compartment as PPARγ, and is required for the transactivation function of PPARγ. We note that a previous study on ABA action in immune cells also reported decreased PPARγ activity in reporter assays upon LanCL2 KD (16).

PPARγ-mediated transcriptional activation of adipogenic genes is an elaborate process: Ligand binding induces a conformational change in the nuclear receptor, resulting in the dissociation of transcriptional corepressors, such as NCor/SMRT, and the recruitment of several coactivators, such as CREB binding protein and p300 (40–42). Such coactivators facilitate gene transcription by remodeling the chromatin to an open configuration, through their intrinsic histone acetyltransferase (HAT) activity (43, 44). A second group of coactivators that forms a large multiprotein complex in itself, without any intrinsic enzymatic activity, is named the TRAP/DRIP/ARC/Mediator complex (44). Comprising 15–20 proteins, members of this complex serve as molecular bridges between coactivator proteins with HAT activity and the basal transcriptional machinery, such as the RNA polymerase II preinitiation complex (43). For instance, TRAP220 is a pivotal member of the TRAP/Mediator complex; it physically interacts with PPARγ, and its specific deletion abrogates PPARγ-mediated transcriptional activity (45). Because no enzymatic activity of LanCL2 has been reported to date, it is possible that LanCL2 belongs to this growing class of docking/scaffolding proteins, necessary for PPARγ-mediated transactivation. Indeed, our studies show LanCL2 to directly interact with PPARγ in vitro, as well as pull down PPARγ on day 3 of differentiation—i.e., at a time when the transcriptional activation of downstream adipogenic genes is underway. In addition, a scaffolding function of LanCL2 has been suggested before: in orienting Akt for optimal phosphorylation by the kinase complex mTORC2 (17). It should, however, be noted that the expression of LanCL2 is ubiquitous (18, 46), and it is possible that LanCL2 is involved in a general pathway of transcriptional activation, as opposed to specific roles in the adipose tissue. In support of this notion, a role of LanCL2 in regulating gene transcription in a human uterine carcinoma cell line has been proposed by Park and James (47), where it is involved in regulating the expression of multidrug resistance protein-1.

Overexpression of LanCL2 in 3T3-L1 preadipocytes did not enhance their differentiation. These results are in line with our observations from luciferase assays that did not show further enhancement of PPARγ transactivation potential when LanCL2 was overexpressed. A similar observation has been made with adipogenic activators SRC-2 and SRC-3, which are critical components of the basal adipogenic machinery (48). Their single or double KD decreased

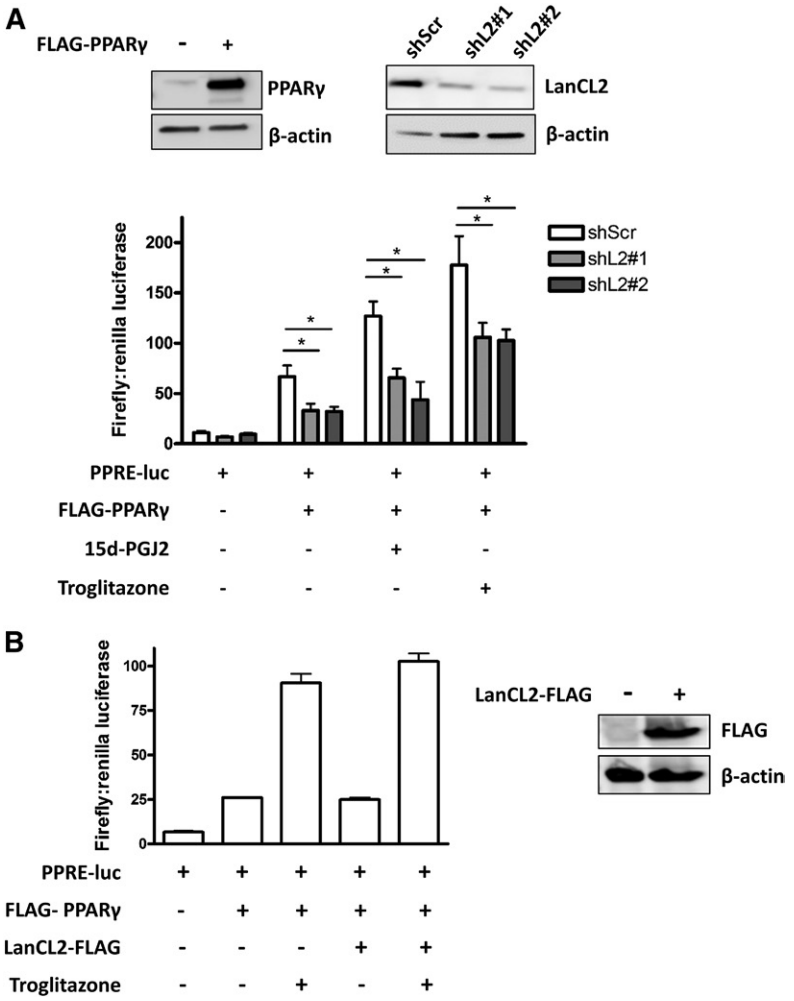

Fig. 6. Transactivation potential of PPARγ in the presence of 15d-PGJ2 or troglitazone is decreased in LanCL2 KD cells. A: Luciferase assay measuring the activation of PPRE-luc with 15d-PGJ2 or troglitazone. Left upper shows overexpression of PPARγ by transient transfection; right upper shows confirmation of KD of LanCL2 in HEK293 cells. Results indicate the average of three independent experiments. One-way-ANOVA was used for analysis. * $P < 0.05$. B: Luciferase assay measuring the activation of PPRE-luc with LanCL2 overexpression. Right: Confirmation of LanCL2 overexpression in HEK293 cells. Data represent the average of two independent experiments.

adipogenesis in 3T3-L1 cells, but their overexpression showed little to no effect on the enhancement of adipogenesis (48). These findings suggest that the equilibrium levels of such proteins are sufficient to induce adipogenesis. Consistent with this hypothesis, we observed that the levels of LanCL2 remain constant throughout differentiation.

Both C/EBPβ and C/EBPδ are known to regulate the expression of PPARγ and C/EBPα at the early stages of adipogenesis (49). Although our results do not completely rule out a role of LanCL2 at this stage, our luciferase assays in HEK293 cells directly assessed the role of LanCL2 on PPARγ-mediated transactivation in the absence of C/EBPβ and C/EBPδ (50). The results show a clear effect of LanCL2 KD on PPARγ signaling, and hence we believe it is unlikely that LanCL2 affects C/EBPβ and C/EBPδ activity.

Our differentiation experiments in LanCL2 KD cells suggested that an endogenously occurring, natural ligand of PPARγ, 15d-PGJ2, or CM from normally differentiating control cells was unable to induce lipid accumulation in the absence of LanCL2, whereas a strong, synthetic PPARγ agonist such as troglitazone can partially rescue differentiation. Indeed, the thiazolidinedione class of ligands (such as troglitazone and rosiglitazone) consists of robust activators of PPARγ with an affinity of <2 nM, and they are very strong inducers of adipogenesis in cell culture models (19). On the other hand, all endogenously occurring putative ligands, such as 15d-PGJ2, 9-hydroxyoctadecadienoic acid (9-HODE), 13-HODE, and linoleic acid, bind PPARγ with a much lower affinity (K_d 2–50 μM) and are considered to be relatively weak, albeit natural, activators of PPARγ (34, 51–53). Binding of a strong (or full) PPARγ agonist induces a conformational change different than that of a weak (or partial) agonist (54). Partial agonists are known to associate with different structural domains inside the PPARγ ligand binding domain, resulting in the recruitment of a different set of coactivators and subsequent differences in the expression of target genes (54). Hence, one possible explanation of our observations is that LanCL2 is important for mediating the activation of PPARγ by an endogenous ligand, but a strong synthetic ligand like troglitazone can partially overcome this need by inducing a potent but nonphysiological mode of transcriptional activation.

In summary, our study identifies LanCL2 as a novel regulator of adipogenesis in 3T3-L1 cells, involved in PPARγ-mediated activation of downstream adipogenic genes. We ruled out that this effect is caused by decreasing cAMP levels or Akt phosphorylation, and our data demonstrate that LanCL is not involved in the production of a secreted ligand. Instead, we show that LanCL2 is engaged in a physical interaction with PPARγ, which we propose to be important for its full transactivation potential.

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