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Thyroid Hormone Receptor Sumoylation Is Required for Preadipocyte Differentiation and Proliferation*

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Background: Thyroid hormone receptor (TR) sumoylation is essential for thyroid hormone regulation of gene expression.
Results: TR sumoylation mutants impair differentiation though down-regulation of C/EBPs, constitutive interaction with NCoR, interference with PPARγ signaling, and disruption of the Wnt canonical signaling pathway important for preadipocyte proliferation.
Conclusion: TR sumoylation site mutations impair preadipocyte proliferation and differentiation.
Significance: TR sumoylation is required for adipogenesis.

Thyroid hormone and thyroid hormone receptor (TR) play an essential role in metabolic regulation. However, the role of TR in adipogenesis has not been established. We reported previously that TR sumoylation is essential for TR-mediated gene regulation and that mutation of either of the two sites in TRα or any of the three sites in TRβ reduces TR sumoylation. Here, we transfected TR sumoylation site mutants into human primary preadiocytes and the mouse 3T3L1 preadipocyte cell line to determine the role of TR sumoylation in adipogenesis. Reduced sumoylation of TRα or TRβ resulted in fewer and smaller lipid droplets and reduced proliferation of preadipocytes. TR sumoylation mutations, compared with wild-type TR, results in reduced C/EBP expression and reduced PPARγ$_2$ mRNA and protein levels. TR sumoylation mutants recruited NCoR and disrupted PPARγ-mediated *perilipin1* (*Plin1*) gene expression, associated with impaired lipid droplet formation. Expression of NCoRΔID, a mutant NCoR lacking the TR interaction domain, partially "rescued" the delayed adipogenesis and restored *Plin1* gene expression and adipogenesis. TR sumoylation site mutants impaired Wnt/β-catenin signaling pathways and the proliferation of primary human preadipocytes. Expression of the TRβ K146Q sumoylation site mutant down-regulated the essential genes required for canonical Wnt signal-mediated proliferation, including Wnt ligands, *Fzds*, *β-catenin*, *LEF1*, and *CCND1*. Additionally, the TRβ K146Q mutant enhanced the canonical Wnt signaling inhibitor Dickkopf-related protein 1 (*DKK1*). Our data demonstrate that TR sumoylation is required for activation of the Wnt canonical signaling pathway during preadipocyte proliferation and enhances the PPARγ signaling that promotes differentiation.

Thyroid hormone receptor (TR)[3] and triiodothyronine (T_3) have an essential role in metabolic homeostasis. Patients with reduced or increased thyroid hormone levels experience changes in body weight and body composition due to thyroid hormone-mediated action on metabolic targets. In white adipose tissue (WAT), thyroid hormone enhances catecholamine-mediated lipolysis by stimulating expression of adrenergic receptors and enhancing sensitivity (1), which leads to activation of hormone-sensitive lipase (2). In brown adipose tissue (BAT), thyroid hormone and catecholamines stimulate UCP1 expression and promote thermogenesis and oxidative phosphorylation, which increases energy expenditure and reduces fat accumulation (3). Knock-out of the type 2 5′-deiodinase (*D2*) gene, which converts thyroxine to the T_3 active form, is associated with reduced BAT activity and glucose intolerance. *D2* knock-out mice have a defect in diet-induced thermogenesis, resulting in glucose intolerance, obesity, and enhanced hepatic steatosis (4). TR mutations alter fat deposition in several TRα mutant mouse models. TR gene point mutations that disrupt ligand binding produce a dominant-negative TR that antagonizes the wild-type TR. TRα1$^{PV/PV}$ mice have markedly reduced WAT and fat mobilization due to TRα1$^{PV/PV}$ inhibition of PPARγ-mediated transcription (5). TRα P398H mice have significantly increased visceral fat due to impaired catecholamine-stimulated lipolysis and β-oxidation in WAT (6). TRα1 R384C mice have reduced fat depots, increased lipid mobilization in WAT, and BAT activation due to increased sympathetic outflow, which normalizes when the animals are kept at thermoneutral conditions (7). TRβ$^{PV/PV}$ mice do not

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[3] The abbreviations used are: TR, thyroid hormone receptor; T_3, triiodothyronine; WAT, white adipose tissue; BAT, brown adipose tissue; PPAR, peroxisome proliferator-activated receptor; KLF, Krüpple-like factor; SUMO, small ubiquitin-like modifier; NCoR, nuclear receptor co-repressor; C/EBP, CCAAT/enhancer-binding protein; PPRE, peroxisome proliferator response element; PV, thyroid hormone receptor carboxy terminal truncation identified in a patient with Resistance to Thyroid Hormone; TZD, troglitazone; RTH, resistance to thyroid hormone; PCP, planar cell polarity; TRE, T_3 response element; qPCR, quantitative PCR.

display abnormalities in WAT but have excessive hepatic lipid deposition (8).

The network of core factors required for the regulation of adipogenesis has been described previously (9, 10). Adipocyte differentiation is primarily regulated by PPARγ. Pro-adipogenic factors, such as Krüpple-like factors (KLF) 4, 5, and 15, in concert with CCAAT/enhancer-binding proteins (C/EBPβ/δ and C/EBPα), activate *PPARγ* gene expression (11–15). In contrast, anti-adipogenic factors GATA2/3, KLF2/7, TCF/LEF, Forkhead box O (FOXO), and transforming growth factor β (TGFβ) inhibit *PPARγ* expression.

A widely used *in vitro* model used to study adipogenesis is the mouse 3T3L1 cell line. 3T3L1 cells must be grown to confluence to reach growth arrest, a prerequisite for preadipocyte differentiation. However, another model, 3T3-F422 cells, is grown in suspension and differentiates without reaching confluence (16). During *in vitro* adipogenesis, 3T3L1 cells require induction by a hormonal mixture (insulin, dexamethasone, and 3-isobutyl-1-methylxanthine) for 48–72 h. The cells undergo two rounds of clonal expansion prior to final growth arrest and differentiation. Human primary preadipocytes require a constant presence of the hormone mixture and differentiate without further cell division. Differentiated adipocytes are filled with intracellular lipid droplets before maturation. The lipid droplets are coated by perilipin1 (Plin1), a protein in which expression is largely restricted to adipose tissue and is highly induced during adipogenesis. Plin1 prevents unregulated lipolysis by hormone-sensitive lipase, facilitates lipid transfer into the lipid droplet, and enables adrenergic signal-stimulated lipolysis by allowing phosphorylated hormone-sensitive lipase to enter the lipid droplets (17–19). Unregulated lipolysis increases lipid deposition in tissues, activates inflammation, and enhances insulin resistance. Null mutations of the hormone-sensitive lipase (*HSL*) gene are associated with an increased risk of type 2 diabetes (20). Plin1-deficient mice have lipodystrophy and atherosclerosis, as lipid is deposited in tissues outside of the normal fat storage areas (21, 22). In humans, Plin1 deficiency, due to a *Plin1* frameshift mutation, induces partial lipodystrophy, severe dyslipidemia, and insulin-resistance (23).

TR-dependent gene regulation requires the covalent conjugation of a small ubiquitin-like modifier (SUMO) to TR. Previously, we identified sumoylation sites in TRα1 and TRβ1 and characterized TR sumoylation properties (24). TRα1 is sumoylated at lysines 283 and 389 and TRβ at lysines 50, 146, and 443. TRα prefers E3 ligase PIASxβ, and TRβ prefers E3 ligase PIAS1. TRα, but not TRβ, requires T_3 for sumoylation.

TR isoform specificity in gene regulation has been linked to the relative level of TR isoform expression in a specific tissue, response element configuration in the regulated gene, and intrinsic properties of the receptor. A recent genome-wide study of genes regulated by TRα and TRβ suggests that TR isoform selectivity is not due to the response element sequence but to intrinsic receptor properties that influence the interaction with coactivator or corepressor (25). SUMO modification, therefore, may have a role in TR isoform specificity. Mutation of any sumoylation site impairs TR-dependent gene repression or induction (22).

We have demonstrated previously TR-SUMO conjugation in white adipose tissue (24). Here, we mutated TR sumoylation sites to reduce the capacity for sumoylation and investigated whether TR sumoylation plays a role in adipocyte differentiation. We utilized the mouse 3T3L1 preadipocyte differentiation model for mechanistic studies and human primary preadipocytes to confirm that these mechanisms were relevant for human adipocyte differentiation. We demonstrated that TR-SUMO conjugation is necessary for preadipocyte proliferation, PPARγ-mediated gene expression, including *Plin1* expression, and differentiation. The total number of fat cells in humans is set in late adolescence, although adipocytes in adults are constantly formed from preexisting preadipocytes, and ~10% of subcutaneous adipocytes are replaced every year (26). Understanding the role of TR in adipocyte differentiation provides important insights into the regulation of adipose tissue as well as potential novel therapeutic targets for metabolic diseases.

EXPERIMENTAL PROCEDURES

Human Primary Preadipocyte Culture, Transfection, and Differentiation—Human subcutaneous primary preadipocytes purchased from Lonza Inc. (Basel, Switzerland) were grown in preadipocyte medium to 80% confluence and then collected for transfection. Transfection conditions were optimized using 4 μg of plasmid, 0.75 million cells, Nucleofector Kit V, and program V-033. Transfections were performed using a Nucleofector 2D device (Lonza). After electroporation, cells were grown in preadipocyte medium until growth arrest. The plating density after transfection was ~11,000 cells/cm^2. Cells were then differentiated in differentiation medium (Lonza) and maintained in the same medium for 9–12 days. The lipid droplets appeared after day 4 of differentiation.

Plasmids, 3T3L1 Cell Culture, Lentiviral shRNA Knockdown, Stable Transfection, and Differentiation—Expression vectors for sumoylation site mutants (hTRβ K50Q, K146Q, and K443Q and hTRα K283Q/K288R and K389Q) were cloned by site-directed mutagenesis into pCMVTnT and/or pCMV6 entry. 3T3L1 preadipocytes were maintained in DMEM containing 10% bovine calf serum. Cells were transduced with lentiviral shRNA-TRβ to silence mouse TRβ gene expression. Cells (1 million) were then transfected using Nucleofector Kit V, program T-028, with plasmids (4 μg each) expressing empty vector, hTRβ, hTRα, or sumoylation site mutations hTRβ K50Q, hTRβ K146Q, hTRβ K443Q or hTRα K283Q/K288R. After G418 selection, for 7 days, cells were grown to confluence followed by hormone mixture-induced differentiation. Stable transfection (without knockdown of endogenous TR) of 3T3L1 cells with TR and TR sumoylation site mutant experiments were conducted using nucleofection as described above. After 7 days of G418 selection, cells were grown to confluence and differentiated using the standard protocol.

C/EBP Reporter Assay—A C/EBP luciferase reporter vector carrying six C/EBP-binding elements (CCAAT) was purchased from Qiagen. Stably transfected 3T3L1 cells, as described above, were plated to 96 wells and grown to confluence. After 48 h of confluence, cells were treated with the adipocyte hormonal mixture to induce differentiation. After 24 h of induc-

tion, cells were transfected with the luciferase reporter using Attractene transfection reagent. Luciferase activity was recorded in a Multimode plate reader (Promega). The data represent the mean value of six replicate reporter assays.

Cell Proliferation Assay—Cells were transfected as described above. After selection, cells were plated in a 96-well dish and grown for 36 h (~80% confluence for control cells). Proliferation of the cells was analyzed (Cell Titer 96® aqueous kit, Promega). The absorbance at 490 nm was recorded in a Multimode plate reader (Promega). Proliferation was calculated as the mean value ± S.E. of 10 duplicates and presented as percent control (cells transfected with empty vector).

Lipid Droplet Fluorescent Imaging—Lipid droplets were imaged using standard protocols (27). In brief, cells were grown and differentiated in chamber slides fixed with 3% paraformaldehyde for 15 min at room temperature, incubated in 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-*s*-indacene (BODIPY 493/503, Invitrogen) (1 μg/ml in 0.15 M NaCl in PBS) for 15 min at room temperature and then mounted with DAPI-containing Prolong Gold (Invitrogen). BODIPY 493/503 stains neutral lipids. Imaging was performed using Zeiss 70 confocal laser-scanning microscopy.

Oil Red O Staining—Cells were fixed with 4% paraformaldehyde for 30 min and washed twice with PBS followed by one wash with 60% isopropanol. Cells were then incubated with Oil Red O for 5 min, washed with H_2O and photographed.

Free Fatty Acid Assay—Free fatty acids were determined using a kit (catalog No. ab65341, Abcam) that detects octanoate and longer chain free fatty acids by an enzyme-based method. Cells were grown in a 96-well dish. After differentiation for 9 days, free fatty acids in the media were quantitated following the instructions with the kit.

Co-immunoprecipitation and Western Blot—3T3L1 cells were transfected with NCoR-FLAG and pCMV-TNT TRβ expressing vectors using Nucleofector Kit V, program T-028. The FLAG immunoprecipitation kit, FLAGPT1 (Sigma-Aldrich), was used following the manufacturer's instructions. In brief, cells were lysed in lysis buffer. After centrifugation to remove the debris, the protein concentration in each sample was adjusted for uniformity. Each sample (1.0 ml of lysate) was incubated with anti-FLAG resin (60 μl) overnight. Immunoprecipitated proteins were boiled with 2× SDS sample buffer (20 μl) and run on a 10% SDS-PAGE. The antibodies used for the Western blot were anti-TRβ (1:500, ThermoFisher Scientific Inc., MA1-216) and anti-NCoR (1:500, Abcam, ab3482). Cells were transfected with empty vector for the co-immunoprecipitation control. Cell lysate (1.0 ml) was incubated with the anti-FLAG antibody. For input, cell lysate was immunoprecipitated using anti-TRβ. One-half of the immunoprecipitated protein was used in the Western blot as TRβ input.

PPARγ expression was measured in 3T3L1 cells after 9 days of differentiation and lysis with immunoprecipitation lysis buffer (Pierce). Protein from the crude lysate (30 μg) was loaded in 10% SDS-PAGE. The antibodies used were anti-PPARγ$_2$ (1:500, rabbit IgG, Santa Cruz Biotechnology) and anti-GAPDH, which recognizes a ~35 kDa protein (1:1,000, mouse IgG, Santa Cruz Biotechnology). GAPDH was used as a loading control, and the Western blot was imaged using the BioSpectrum 310 system.

Chromatin Immunoprecipitation (ChIP) Assay—A ChIP assay was performed using EZ-ChIP™ (Millipore) following the manufacturer's instructions. In brief, 3T3L1 cells were transfected and treated either with T_3 or troglitazone (TZD) for 4 h. Cells were then incubated with 1% formaldehyde (final concentration) to cross-link chromatin. The antibodies used in the ChIP assay were anti-TRβ (1:50), anti-PPARγ (1:50, ThermoFisher Scientific, PA3-821A), and anti-NCoR (1:50, Millipore, MABE570). After the cross-linking was reversed, DNA was purified and then used for PCR amplification. The primers used were: for mouse *Plin1* PRE (−2174/−1901), F, 5′-CCCCCTGTGGTGTTGGCGAAC-3′, and R, 5′-CAGAGGCCACTTTACACGCTC-3′ (GenBank accession No. NC_000073.6); for mouse aP2 PPRE (28, 29), F, 5′-AAT GTC AGG CAT CTG GGA AC-3′, and R, 5′-GAC AAA GGC AGA AAT GCA CA-3′ (AC_000025, region 10244127–10244226); for mouse adipoQ PPRE (30), F, 5′-CACTCAGAA ACATGCTGA ATT-3′, and R, 5′-GGAAGCCAA GTAGAGCTT-3′ (accession No. AC_000038, region 23704632–23704746).

Real-time PCR Profiling Wnt Signaling Pathway and QuantiTech Primer Analysis of Gene Expression—Human primary subcutaneous preadipocytes were transfected using Nucleofector as described above. Cells were then plated in a 6-cm dish and grown for 3 days (control cells were grown to ~80% confluence). RNA was isolated and used for a Wnt signaling profiling array (Qiagen). The values were presented as the mean value from three separate experiments. The data were analyzed using Qiagen software. RNA was isolated from cells and reverse-transcribed using a Qiagen RT^2 first-strand synthesis kit to measure expression of *PPARγ$_2$*, *CEBPβ*, *CEBPα*, *Plin1*, *adipoQ*, and *aP2*. QuantiTech primers (Qiagen) were used. The PCR was done with triplicates, and the data presented are a mean value from two separate experiments.

ChIP Enrichment—In brief, TRβ-BioChIP cells were generated by stable transfection of HepG2 cells with TRβ and BiRA (biotin ligase) expression vectors. Stably transfected TR-BioChIP cells were then transduced with lentiviral shRNA SUMO1 to knock down SUMO1 mRNA. Cells were grown in Eagle's minimum essential medium with 10% serum replacement (Invitrogen) for 24 h and then treated with or without T_3 (50 nM) for 4 h. Cells were cross-linked by formaldehyde at a final concentration of 1%. Chromatin was fragmented using a Bioruptor sonicator (Diagenode Inc.). Biotinylated TRβ-associated DNA fragments were precipitated using a streptavidin-based purification system. After ChIP-seq analysis, peak regions were identified, and ChIP enrichment at the peak region was analyzed using qPCR.

Statistical Analysis—Statistical analysis for two group comparisons was performed using a two-tailed Student's *t* test. A value of $p < 0.05$ was taken as statistically significant. The *p* value was analyzed using the Qiagen software for the array data.

RESULTS

TR Sumoylation Is Required for Differentiation of Preadipocytes—TR sumoylation is essential for the regulation of thyroid hormone target genes in a range of transient transfection

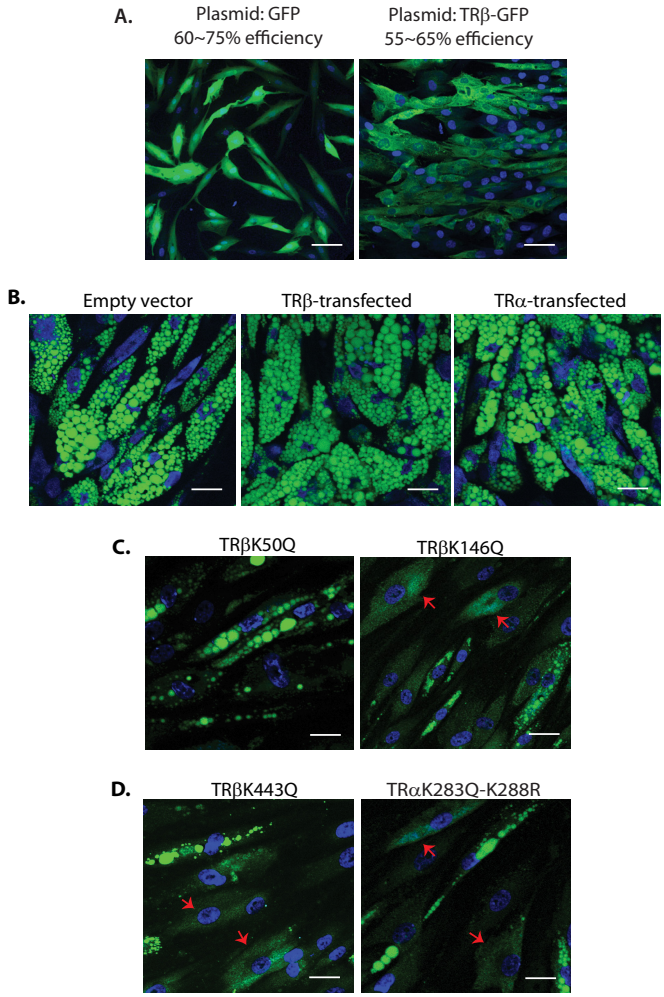

FIGURE 1. **Influence of TR sumoylation site mutations on human preadipocyte differentiation.** *A*, optimization of primary human adipocytes transection efficiency. After transfection (48 h) cells were fixed with 4% paraformaldehyde and mounted for imaging. For differentiation, primary human adipocytes were transfected and grown in chamber slides. Cells were differentiated for 9 days in differentiation medium and then fixed and stained with BODIPY 493/503 for visualization of neutral lipid. *B*, control cells transfected with empty vector, TRβ, and TRα. *C* and *D*, cells were transfected with TR sumoylation site mutants as shown. The *arrows* depict representative cells showing *green* BODIPY staining in the cytoplasm, but no lipid droplet are seen within the cells. *White scale bars* represent 50 μm.

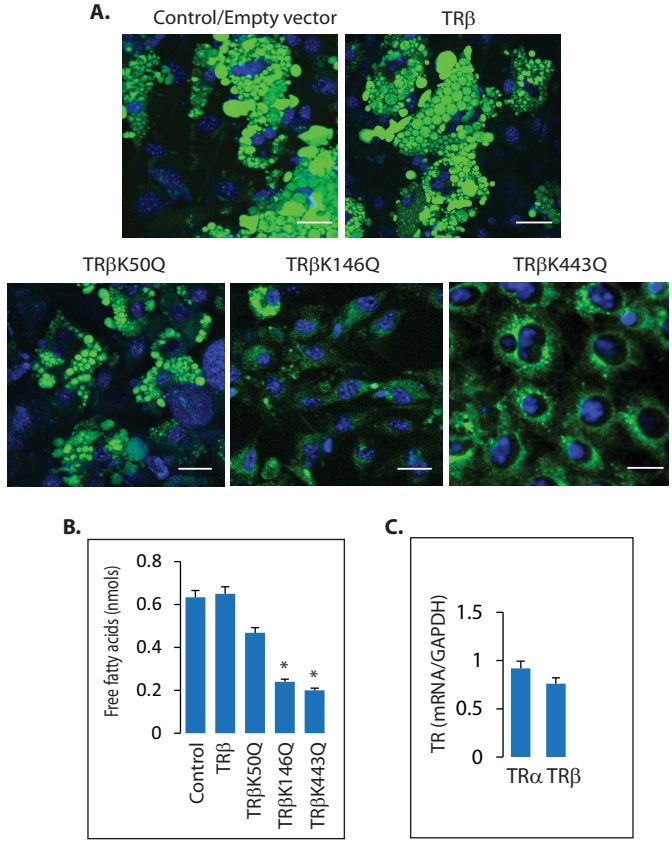

FIGURE 2. **Effects of TRβ sumoylation site mutations on mouse 3T3L1 cell differentiation.** 3T3L1 cells were transduced with lentiviral shRNA to knock down endogenous TR and then stably transfected with empty vector (*Control*), human TRβ, or TRβ sumoylation mutants K50Q, K146Q, and K443Q. *A*, cells were differentiated for 9 days in differentiation medium, fixed, and then stained with BODIPY 493/503 for visualization of neutral lipid. The *white scale bars* represent 50 μm. *B*, the medium-free fatty acid was measured using an enzyme-based assay with 10 duplicates for each sample. The mean value is presented $\pm$S.D. *, $p < 0.05$ compared with control. *C*, endogenous TRα and TRβ mRNA levels in undifferentiated control 3T3L1 cells.

models (24). The biological importance of TR sumoylation, however, has not been established. We reported previously that TR sumoylation was observed in mouse WAT (24). We therefore generated TR sumoylation site mutations in TRα and TRβ, TRα K283Q/K288R and TRβ K50Q, K146Q, and K443Q, to test the effects of TR sumoylation on adipogenesis in adipocyte differentiation models. Because Lys-283 and Lys-288 are interchangeable sites for sumoylation in TRα, a double mutation, K283Q/K288R, was generated.

To investigate the role of TR sumoylation in preadipocyte differentiation, we transfected vectors expressing TRα and TRβ with sumoylation site mutations into human primary subcutaneous preadipocyte cells. We optimized transfection conditions using cell type-specific transfection solution in combination with electroporation programs and obtained a transfection efficiency up to 75% (Fig. 1*A*). Growth arrest, a prerequisite condition for preadipocyte differentiation, was confirmed by a stable cell number at 48 h. Control cells (transfected with empty vector or with a vector expressing TRα or TRβ) reached confluence and growth arrest after 4 days of transfection. Cells expressing TR sumoylation site mutations required 6 days to reach growth arrest. The cells were then differentiated, and intracellular lipid droplets were visualized by BODIPY 495/503, which stains neutral lipids. Control cells (transfected with empty vector or a vector expressing TRα or TRβ) were filled with cytoplasmic lipid-storing vesicles after 9 days of differentiation, indicating commitment to a terminal adipocyte lineage (Fig. 1*B*). Cells expressing TRβ and TRα sumoylation site mutants, however, had significantly fewer cells committed to differentiation and fewer and smaller lipid droplets in differentiated cells (Fig. 1, *C* and *D*).

We next determined whether TR sumoylation mutants similarly disrupted preadipocyte differentiation in the well characterized mouse 3T3L1 cell line preadipocyte model. TRβ mRNA knockdown was used to reduce endogenous TRβ, and the cells were then stably transfected with TRβ sumoylation mutants. After differentiation for 9 days, cells expressing the TRβ sumoylation mutants, TRβ K146Q and K443Q, had only a few lipid droplets (Fig. 2*A*). TRβ K50Q-expressing cells had near

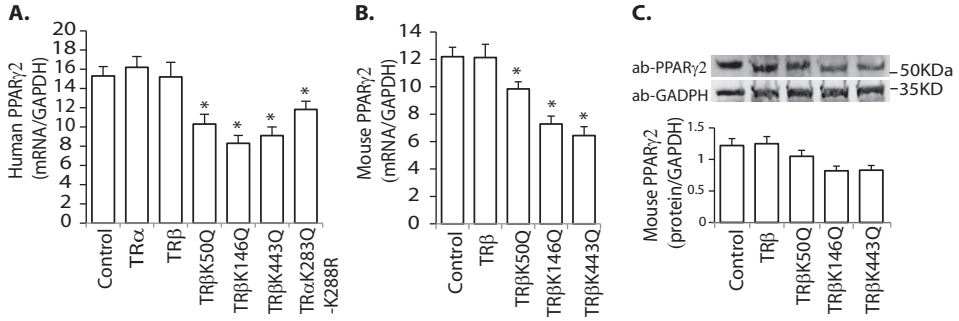

FIGURE 3. **TR sumoylation mutants repress PPARγ expression.** *A*, human primary preadipocytes were transfected with GFP (*Control*) or expression vectors for TR isoforms or TR sumoylation mutants as shown. *B*, 3T3L1 cells were transfected as described in the legend for Fig. 2. Cells were grown to confluence followed by 48 h of rest. Then, cells were differentiated for 9 days. RNA was isolated and PPARγ_2 mRNA levels quantified by real-time PCR. *C*, 3T3L1 cells were differentiated for 9 days and lysed in immunoprecipitation lysis buffer. Protein (30 μg of crude lysate) was separated by 10% SDS-PAGE. PPARγ_2 was detected by Western blot using anti-PPARγ_2 antibody (1:500). GAPDH protein was used as a loading control and detected by anti-GAPDH (1:1000). The Western blot, quantified using BioSpectrum imaging software, is shown in the *lower panel*. *, $p < 0.05$ compared with control.

normal differentiation, although the size and number of fat droplets were much less than in control cells (Fig. 2*A*). We quantitated the impact of TR sumoylation mutants on preadipocyte differentiation by measuring free fatty acids in the medium (Fig. 2*B*). TRβ K146Q-expressing cells had a 46% reduction compared with control cells and TRβ K4443Q-expressing cells a 62% reduction. TRβ K50Q-expressing cells had reduced free fatty acid levels but were not significantly different from control, consistent with the differentiation phenotype. TRα and TRβ mRNA levels were similar in 3T3L1 cells (Fig. 2*C*). These data demonstrate that TR sumoylation is important for adipocyte differentiation and fat droplet formation, although the extent of disruption of adipocyte differentiation varied among the sumoylation mutants.

TR Sumoylation Site Mutations Repress PPARγ Gene Expression—PPARγ gene expression is essential for adipocyte differentiation. Reduced PPARγ mRNA expression in white adipose tissue was reported previously in TRα PV mice, a TR mutation associated with resistance to thyroid hormone (RTH) (5). We analyzed PPARγ mRNA and protein levels in human preadipocytes differentiated for 9 days using real-time PCR and Western blot. PPARγ_2 mRNA expression was reduced 32% in TRβ K50Q-, 46% in TRβ K146Q-, 40% in TRβ K443Q-, and 26% in TRα K283Q/K288R-expressing cells compared with control cells (Fig. 3*A*). A similar pattern of reduced PPARγ_2 mRNA expression was seen in 3T3L1 cells transfected with TRβ sumoylation site mutants (Fig. 3*B*). The protein level of PPARγ_2 was reduced 14% in cells transfected with TRβ K50Q and 33% in cells transfected with TRβ K146Q and TRβ K443Q (Fig. 3*C*).

Down-regulation of C/EBPs in Mitotic Expansion Phase by TR Sumoylation Mutants—Initiation of 3T3L1 cells differentiation by hormonal mixture allows growth-arrested cells synchronously reentering the cell cycle to undergo mitotic clonal expansion. During this period, C/EBPβ is strongly induced, which stimulates the genes required for adipogenesis, such as *C/EBPα*, and later the induction of *PPARγ* and *C/EBPα* (31, 32). C/EBP is known to be regulated by TR (33). We hypothesized that reduced PPARγ in TR sumoylation mutant-expressing cells may be due to reduced C/EBP expression in the mitotic expansion phase. To investigate, C/EBPβ and C/EBPα mRNA

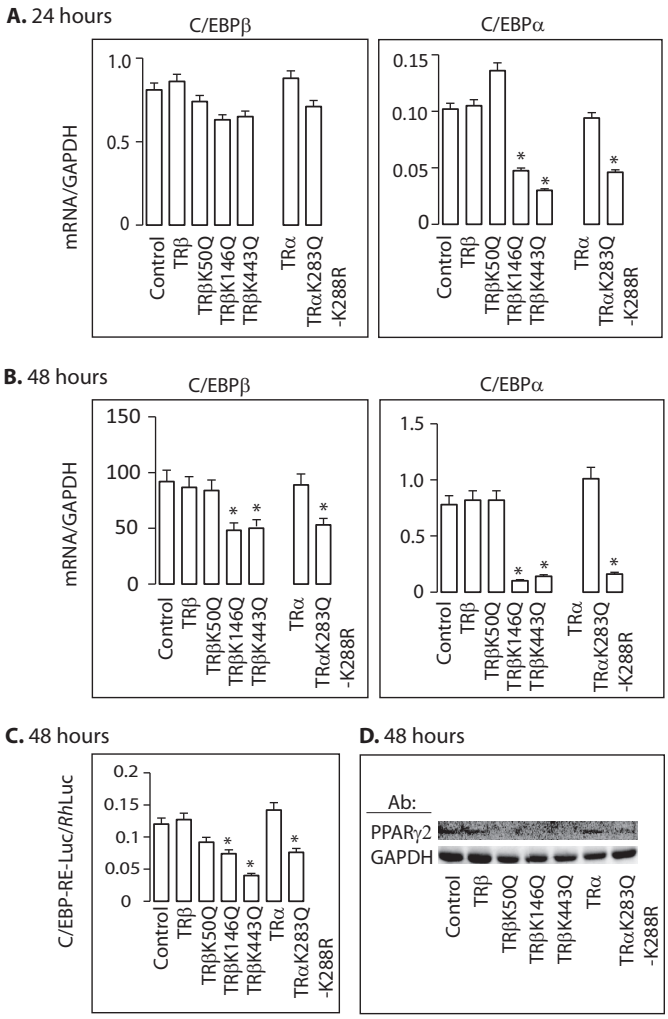

FIGURE 4. **Down-regulation of C/EBP expression in mitotic clonal expansion by TR sumoylation mutants.** *A*, 3T3L1 cells were transfected with expression vectors as shown. At 48 h post-confluence, cells were induced to differentiate with a hormone mixture, and RNA was isolated at 24 h of induction. *B*, mRNA was isolated from 3T3L1 cells after 48 h of hormonal induction. Gene expression of C/EBPα and C/EBPβ was analyzed by qPCR using the standard curve method. *C*, cells differentiated for 24 h were transfected with C/EBP luciferase reporter with 6× CCAAT sites. Reporter activity was analyzed after 48 h of differentiation. *D*, PPARγ_2 protein was detected by Western blot using anti-PPARγ_2 (1:500) at 48 h of induction. GAPDH protein level is shown as a loading control. *, $p < 0.05$ compared with control.

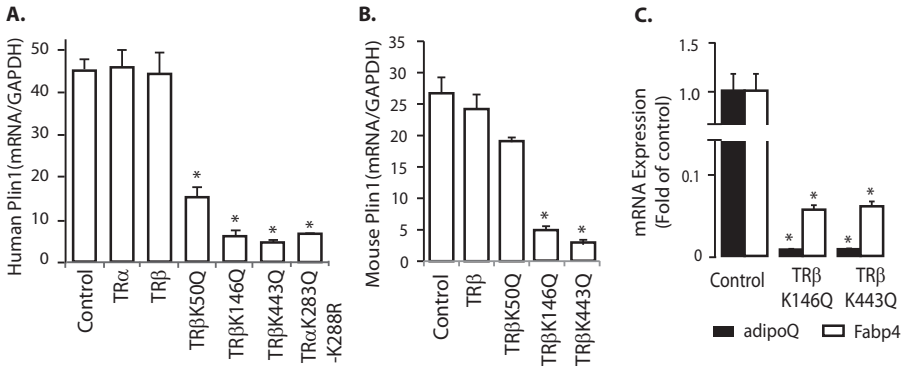

FIGURE 5. **TR sumoylation site mutations reduce *Plin1* gene expression.** *A*, human primary adipocytes were transfected with expression vectors as indicated and differentiated for 9 days. Control cells were transfected with a vector expressing GFP. RNA was isolated and used in qPCR (standard curve method) profiling *Plin1* mRNA. *B*, the studies as described in *A* were done in mouse 3T3L1 cells. *C*, endogenous expression of mRNA of the PPARγ-responsive genes, *adipoQ* and *Fabp4*, with and without TRβ sumoylation mutants was measured in 3T3L1 cells as described in *B*. The data presented are the mean value of triplicates. *, $p <$ 0.001 compared with control.

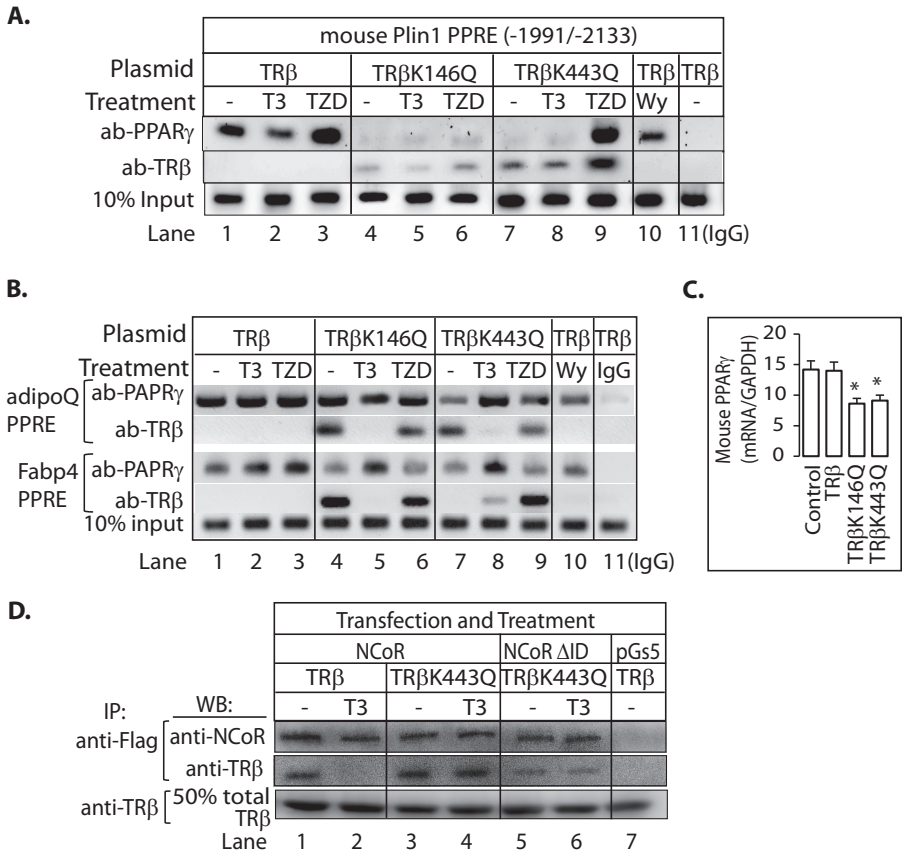

FIGURE 6. **TRβ K443Q cross-talk with PPARγ on the PPREs and the role of NCoRΔID.** *A*, analysis of the cross-talk between PPARγ and TRβK146Q or TRβK443Q on Plin1 PPRE by ChIP assay. 3T3L1 cells were transfected with expression vectors for PPARγ and TRβ or TRβ K443Q and differentiated for 4 days. Before harvesting, cells were treated with T_3 (10 nM), TZD (15 μM), or Wy14,634 (*Wy*, 15 μM) for 4 h. The chromatin was immunoprecipitated with anti-TRβ (1:50), anti-PPARγ (1:50), and mouse IgG (2 μg, as negative control, *lane 11*). The immunoprecipitated DNA was analyzed by PCR amplification for 40 cycles. *B*, PPRE occupancy by TR sumoylation mutants in adipoQ and Fabp4 promoters. Cells condition and ligand treatment were as described in *A*. *C*, qPCR (standard curve method) analysis of PPARγ$_2$ mRNA level in cells used for the ChIP assay. *D*, co-immunoprecipitation analysis of TRβ-NCoRΔID interaction. HepG2 cells were co-transfected with expression vectors as indicated and treated with or without T_3 (50 nM) for 4 h. The cell lysates (1.0 ml) were incubated with anti-FLAG resin (60 μl). In a separate reaction, total TRβ in the cells (1.0 ml of lysate) was immunoprecipitated with anti-TRβ (1:50) and 50% immunoprecipitated protein was used as input control. For Western blot, the antibodies used were anti-NCoR (1:500) and anti-TRβ (1:500).

were analyzed after 24 and 48 h of hormonal induction. After 24 h of hormonal induction *C/EBPβ* was reduced, but this was not statistically significant compared with control cells (Fig. 4*A*). After 48 h of induction, the C/EBPβ mRNA level was dramatically increased in control cells, whereas *C/EBPβ* was reduced to 58% ($p < 0.05$) of control in TRβ K146Q-transfected cells and 60 and 65% of control ($p < 0.05$) in TRβ K443Q- and TRα K283Q/K288R-transfected cells, respectively (Fig. 4*B*). *C/EBPα* was significantly down-regulated although expressed at a low level after 24 h. After 48 h C/EBPα mRNA was further reduced to 13, 20, and 21% of control level in cells expressing TRβ K146Q, TRβ K443Q, and TRα K283W/288R (Fig. 4, *A* and

B). In a complementary study, we measured functional C/EBP in a reporter assay and saw a significant reduction of C/EBP-RE reporter expression with co-transfection of TR sumoylation mutants compared with the control (Fig. 4*C*). Only a trace of PPARγ_2 protein was detectable after 48 h of induction; however, the levels were lower in TR sumoylation mutant-expressing cells compared with control (Fig. 4*C*). These data demonstrate that TR sumoylation mutants inhibit *C/EBPβ* and *C/EBPα*, which likely contributes to reduced *PPARγ* expression.

TR Sumoylation Site Mutations Attenuate PPARγ-mediated Plin1 Gene Expression—The marked decrease in cytoplasmic lipid droplets observed with the TR sumoylation site mutations suggested a deficit in lipid droplet formation (34). Plin1 is the primary protein that coats the fat droplet. The actions of Plin1 include facilitating cytoplasm fatty acid transfer into fat droplets and regulating fatty acid release from adipose tissue (18). We analyzed *Plin1* expression in differentiated human primary adipocytes and 3T3L1 adipocytes. Plin1 mRNA was expressed at a high level in control cells, but expression was significantly reduced in cells expressing TR sumoylation site mutants. In human primary adipocytes, *Plin1* was expressed at 37.7% of control levels in TRβ K50Q-expressing cells, 14.6% in TRβ K146Q-expressing cells, 11.1% in TRβ K443Q-expressing cells, and 15.3% in TRα K283Q/K288R-expressing cells (Fig. 5*A*). In 3T3L1 adipocytes, *Plin1* was expressed at 15.4 and 9.8% of control levels in cells expressing TRβ K146Q and TRβ K443Q (Fig. 5*B*). In cells transfected with TRβ K50Q, *Plin1* was expressed at 71% of control level ($p < 0.058$), which was not significantly different from control, consistent with the near normal cell morphology. These results indicate that TR sumoylation is important for *Plin1* gene regulation, although mutation of the TRβ K50Q sumoylation site did not significantly reduce *Plin1* expression.

We next determined whether *Plin1* was directly regulated by TR. We identified two potential TR binding sites in the 5′-distal region of the *Plin1* gene by sequence inspection and analyzed them with luciferase reporter assays. We found that both TR and the TR sumoylation site mutant had only a modest effect on reporter gene expression, either in the presence or absence of T3.

It has been reported that PPARγ regulates *Plin1* gene transcription via a PPRE in the *Plin1* 5′-flanking region (35, 36). Similar patterns of reduced mRNA expression were also observed in the PPARγ-regulated genes *FABP4* and *adipoQ* (Fig. 5*C*). The reduced PPARγ protein levels we observed in the presence of TR sumoylation mutants (Fig. 3) likely contribute to reduced PPARγ-regulated gene expression. The reduction in PPARγ-regulated gene expression, such as *Plin1*, however, is much greater than the reduction in the PPARγ protein level, indicating that additional mechanisms leading to reduced PPARγ signaling are likely important.

Because TR-PPARγ cross-talk has been demonstrated for several genes (5, 37, 38), we considered the possibility that TR sumoylation site mutants interfere with PPARγ signaling by binding to the *Plin1* gene PPRE. To test this hypothesis, we performed ChIP assays in 3T3L1 cells transfected with expression vector expressing TRβ or TRβ with sumoylation site mutations (Fig. 6*A*). As expected, PPARγ binding to the PPRE was detected and was enhanced by the addition of the PPARγ ligand TZD, and TRβ did not bind (Fig. 6*A*, *lanes 1–3*). In cells expressing TRβ K146Q or TRβK443Q, PPARγ binding to the PPRE was reduced and TR binding was augmented (Fig. 6*A*, *lanes 4–9*), except that in TRβ K443Q-expressing cells TZD enhanced binding of PPARγ and TR to the PPRE (*lane 9*). Similar to what we observed with the TR sumoylation mutants, TRα PV mutants have been reported to heterodimerize with PPARγ on the acyl-CoA promoter PPRE and block PPARγ-mediated acyl-CoA transcription (5).

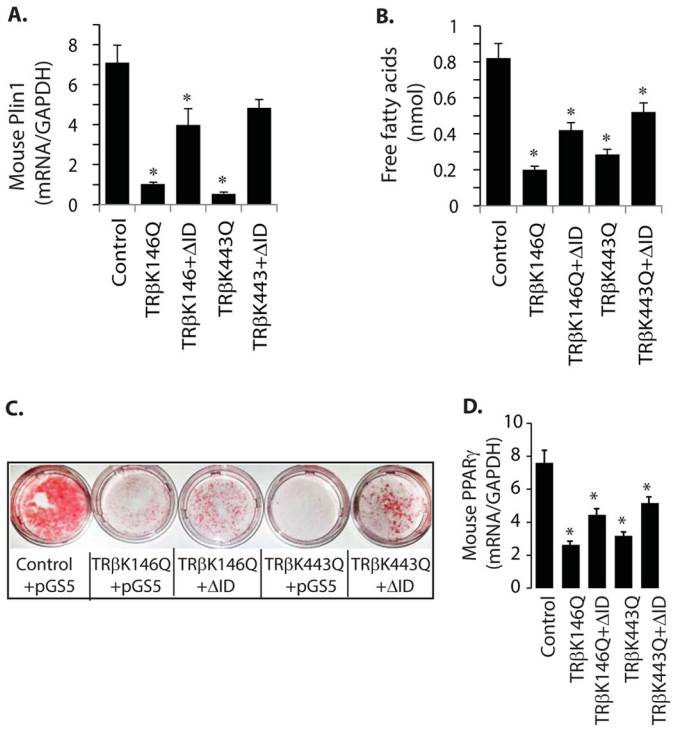

FIGURE 7. **NCoRΔID partially rescued impaired *Plin1* mRNA expression and adipocyte differentiation.** *A*, 3T3L1 cells expressing NCoRΔID and/or TRβ mutant were differentiated for 9 days. *Plin1* mRNA level was determined by real-time PCR. *B*, medium-free fatty acid levels were analyzed as described previously (see Fig. 2 legend). *C*, the neutral triglyceride and lipid in differentiated cells were stained by Oil Red O. *D*, PPARγ mRNA was determined in 3T3L1 cells with co-transfected TRβ sumoylation mutants as shown. *, $p < 0.05$ compared with control. **Δ***ID*, NCoRΔID; *pGS5*, empty vector

We further analyzed the occupancy of TRβ sumoylation site mutants on the PPRE of aP2 and adipoQ promoters. In the presence of wild-type TR, PPARγ, but not TRβ, bound the PPRE of both promoters (Fig. 6*B*, *lanes 1–3*). TRβ sumoylation mutants, however, bound to the aP2 and adipoQ PPREs in some ligand treatments (Fig. 6*B*, *lanes 4*, *6*, and *9*), and PPARγ binding was not consistently reduced (Fig. 6*B*, *lanes 4–9*). The mRNA level of PPARγ_2 in the cells used for the promoter occupancy assay showed reduced mRNA expression with transfection of TR sumoylation mutants, similar to that previously shown (Fig. 3*C*). We concluded that TR sumoylation site mutations enhance the binding of the mutant TR to the PPRE associated with reduced PPARγ-mediated gene expression.

Role of NCoR in TR Sumoylation-mediated Inhibition of Plin1 Expression—Sumoylation influences the activity of transcription factors by conformational changes that modulate the inter-

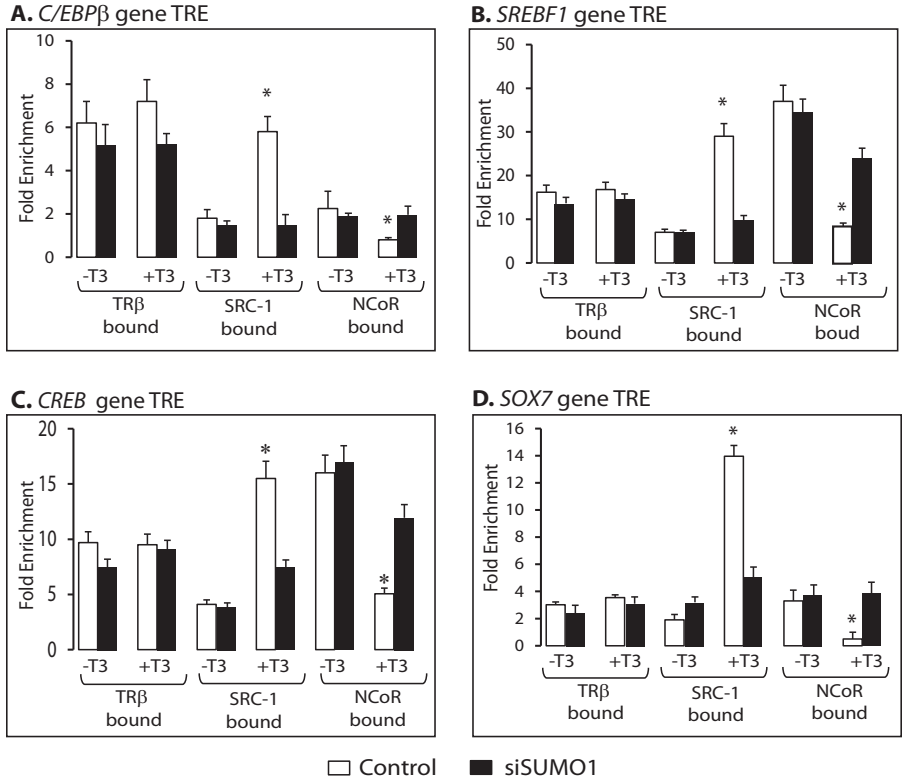

FIGURE 8. **Effects of SUMO1 knockdown on TRβ co-factor recruitment profile.** HepG2 cells stably expressing TRβ-BLRP (biotin ligase recognition peptide)-FLAG were transfected with shRNA SUMO1 and selected for 10 days. The cells were treated with or without T_3 (50 nM) for 4 h prior to fixation with 1% formaldehyde and subsequent ChIP using anti-FLAG M2 resin (67). The relative enrichment of the TRβ-bound region in gene-proximal regions including promoter and 5′-UTR regions was analyzed by qPCR with primers as described under "Experimental Procedures." *A*, binding of TRβ, SRC-1, and NCoR to C/EBPβ TRE. *B*, binding of TRβ, SRC-1, and NCoR to SREBF1 (sterol regulatory element-binding transcription factor 1 TRE). *C*, binding of TRβ, SRC-1, and NCoR to CREB (cyclic AMP response element-binding protein TRE). *D*, binding of TRβ, SRC-1, and NCoR to SOX7 (SRY (sex-determining region Y)-box7 TRE). Data are shown without (*open bars*) and with (*filled bars*) SUMO1 knockdown. *, $p < 0.05$ compared with without T_3 in the same group.

action with ligand, substrate, or other proteins. Unliganded TR binds the corepressor NCoR, and the binding is disrupted by the addition of ligand. TR dominant-negative mutations associated with RTH bind to corepressor NCoR, but the addition of ligand does not disrupt the binding, leading to tonic gene repression (39). To investigate whether inhibition by TR sumoylation site mutations may also be linked to interaction with NCoR, we utilized an NCoR mutation, NCoRΔID, which lacks the interacting domains (N2 and N3) and does not bind to TR (40–42). This mutation, when co-transfected or expressed in an animal model with an RTH-associated TR mutant, "rescues" the abnormal phenotype. We performed a co-immunoprecipitation assay in cells transfected with TRβ K443Q and NCoR or NCoRΔID and measured the TRβ K443Q-NCoR complex. Overexpression of NCoRΔID should limit TR sumoylation mutant interaction with NCoR and release the transcription repression. Co-immunoprecipitation analysis showed that TRβ bound NCoR only in the absence of T_3 as expected (Fig. 6*D*, *lanes 1* and *2*). TRβ K443Q mutant bound to NCoR both in the absence and presence of T_3 (Fig. 6*D*, *lanes 3* and *4*). Overexpression of NCoRΔID significantly diminished the TRβ K443Q-NCoR interaction (Fig. 6*D*, *lanes 5* and *6* compared with *3* and *4*), indicating that NCoRΔID reduced NCoR interaction with the sumoylation mutant TR.

We analyzed the Plin1 mRNA level and free fatty acid in medium of cells co-transfected with TRβ sumoylation mutations and NCoRΔID. In NCoRΔID co-transfected cells, Plin1 mRNA was increased to 53% of the control level in TRβ K146Q-expressing cells and 75% in TRβ K443Q-expressing cells (Fig. 7*A*). The medium-free fatty acid content was also increased to 52 and 60% of control level in these cells, respectively (Fig. 7*B*). Cell differentiation was significantly enhanced in the presence of NCoRΔID, with some increase in neutral triglyceride and lipid droplets as shown by Oil Red O staining (Fig. 7*C*), although it was not fully restored to the level in control cells. These data indicate that the ligand-resistant interaction of the TR sumoylation mutant TR with NCoR contributes to altered gene expression and reduced free fatty acid and that this phenotype is partially rescued by the expression of NCoRΔID.

Genome-wide Study of the Impact of TR Sumoylation on DNA Binding and Co-factor Recruitment—The importance of TR sumoylation for normal ligand-reversible interaction with NCoR is supported by our genome-wide study of TRβ DNA binding after SUMO1 knockdown. Four genes, *C/EBPβ*, *CREB*, *SOX7*, and *SREBF1*, all important for adipocyte differentiation and metabolism, were evaluated (Fig. 8). SUMO1 knockdown had no significant effect on TRβ DNA binding to any of these genes. The TR recruitment pattern of coactivators and corepressors to these genes, however, was altered. After SUMO1 knockdown, TR did not recruit coactivator SRC-1 and was not able to release NCoR in response to T_3. These data demonstrate

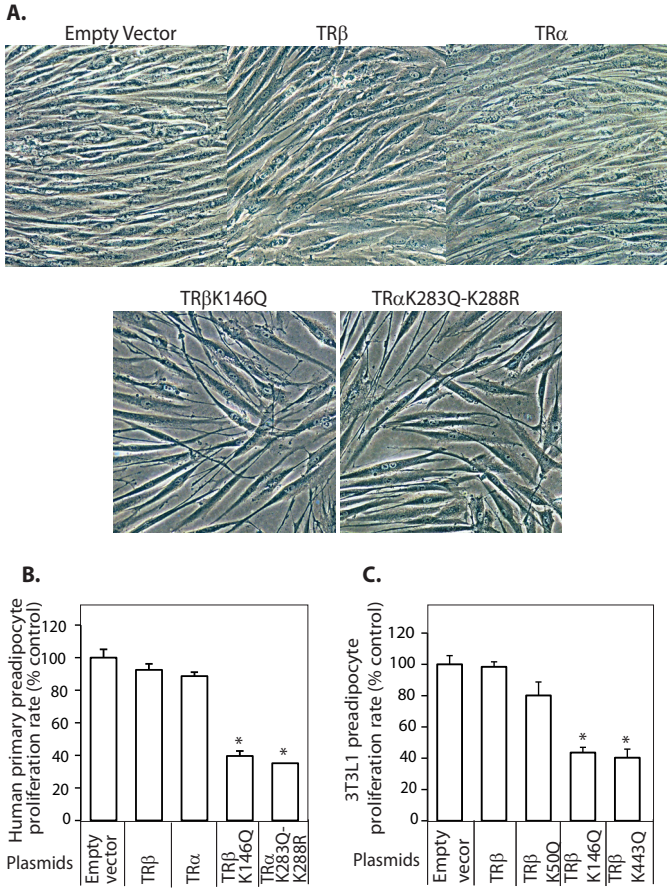

FIGURE 9. **Effects of TR sumoylation on human primary preadipocyte proliferation.** *A*, human primary preadipocytes were transfected with expression vectors as indicated and grown in 6-well dishes for 4 days for controls (empty vector, TRβ, and TRα) or for 6 days for mutant-expressing cells (TRβ K146Q and TRα K283Q/K2883R). Cells were imaged using inverted light microscopy. *B*, human preadipocytes were grown in a 96-well dish for the proliferation assay with expression of wild-type and sumoylation mutant TRs. When control cells reached ~80% confluence (36 h), the assay was performed for all samples. *C*, the same proliferation assay was repeated in 3T3L1 cells. The data presented is the mean value of 10 replicates.

that TR-SUMO conjugation is a key factor for coactivator and corepressor interaction.

Role of TR Sumoylation in Proliferation of Human Primary Preadipocytes—Human adipocytes are differentiated from preadipocytes generated from mesenchymal stem cells in adipose tissue. The number of adipocytes in WAT is dependent on the proliferation of preadiocytes (9, 26). Newly formed adipocytes are more insulin-responsive and metabolically active. In *in vitro* models of adipogenesis, the proliferation of preadipocytes is also a critical step in downstream differentiation.

We explored the role of TR sumoylation in regulating the proliferation of preadipocytes using human primary preadipocytes. Control cells (transfected with empty vector, TRβ, or TRα) were grown to confluence in 4 days. In the same period, cells expressing the TR sumoylation site mutants TRβ K146Q and TRα K283Q/K288R were only ~40% confluent (Fig. 9*A*). The proliferation rate was similar in control cells transfected with empty vector, TRβ, or TRα. The proliferation rate for TRβ K146Q-expressing cells was 40.5% ($p < 0.001$) of that of control cells transfected with empty vector and for TRα K283Q/K288R-expressing cells 37.3% ($p < 0.03$) (Fig. 9*B*). In a parallel study, we used 3T3L1 preadipocytes stably transfected with TRβ sumoylation mutants (K50Q, K146Q, and K443Q). A similar reduction in the rate of proliferation was seen. The proliferation rate for TRβ K50Q-expressing cells was 81.5% ($p < 0.03$) of control cells, for K146Q-expressing cells 43.6% ($p < 0.045$), and for K443Q-expressing cells 40.3% ($p < 0.05$) (Fig. 9*C*).

Differential Effects of TRα and TRβ Sumoylation Site Mutants on the Wnt Signaling Pathway—Wnt signaling is inhibited during differentiation of mesenchymal cell to precommitted preadipocytes and during terminal differentiation of preadipocytes (43). *In vitro* differentiation requires proliferation of precommitted preadipocytes until growth is rested. The proliferation of precommitted preadipocytes is via cell cycle regulation, which is partly mediated though the canonical Wnt/β-catenin signaling pathway. Activation of this pathway leads to increased transcription of the genes controlling G_1/S transition, such as cyclin D1, c-Jun, and c-Myc (41). Disassembling of the β-catenin-LEF/TCF complexes leads to the G_2/M phase in cell cycle progression (44). We noticed premature proliferation in human primary preadipocytes. To investigate the influence of TR sumoylation on Wnt/β-catenin signaling pathway-mediated proliferation, we used human primary preadipocyte cells transfected with TRα K283Q/K288R or TRβ K146Q, compared with those transfected with wild-type TRα and TRβ, and analyzed the cells with the Wnt signaling profiling array. Compared with control cells, the mRNA of six genes were significantly down-regulated in the presence of either TRα or TRβ sumoylation site mutants. These down-regulated genes included, *frizzled 9* (*Fzd9*), *frizzled-related protein* (*Frzb or SFRP3*), *matrilysin* (*MMP7*), *C-terminal-binding protein 1* (*CTBP1*), and *casein kinase-1α1* and *-2α1* (*CSNK1A1* and *CSNK2A1*) (Table 1 and Fig. 8). FRZB is known to antagonize Fzd by directly binding to Wnt and inhibits both canonical and noncanonical Wnt signaling pathways (45). CSNK proteins interact with AXIN1, a component of the β-catenin disruption complex. *CTBP1* regulates tumor suppressor and promotes cellular proliferation in cancer cells (46) *MMP7* is the β-catenin/TCF/LEF target (47). The reduction in these genes suggested altered canonical Wnt activity. Besides these effects seen with either TRα or TRβ, sumoylation site mutants had effects on Wnt ligands, Wnt receptors, and Wnt signaling inhibitors that differed between TRα and TRβ (Table 2 and Fig. 10). TRβ K146Q expression was associated with significant down-regulation of the genes involved in the canonical Wnt signaling pathway, including *CTNNB1* (3.4-fold) coding for β-catenin, *LEF* (2.9-fold), *CCND1* (3.3-fold), which codes for cyclin D1, Wnt3 (3.17-fold), and Wnt8A (4.58-fold). FZD8, involved in both canonical and noncanonical Wnt signaling (48, 49), was reduced 2.85-fold. TRβ K146Q expression was associated with up-regulation of two genes (*Wnt10A and DKK1*) that are inhibitors of adipogenesis. Wnt10A stimulates osteoblastogenesis and suppresses adipogenesis (50). Dickkopf-related protein 1 (DKK1) binds to LPR5/6 and prevent formation of the Wnt-FZD-LPR5/6 complex, which lead to cytoplasmic β-catenin degradation. In contrast to TRβ K146Q mutant, TRα K283Q/K288R showed greater influence on the noncanonical Wnt signaling pathway. *DKK3*, *Wnt5A*, *Princkle1*, and *Vangl2* gene

TABLE 1
Gene expression altered by both TRβK146Q and TRαK283Q/K288R during human primary preadipocyte differentiation

Function	Gene symbol	TRβK146Q *vs.* control		TRαK283Q/K288R *vs.* control	
		-fold	*p* value[a]	-fold	*p* value[a]
Binding to axin	*CSNK1A1*	−3.72	0.016	−4.53	0.01
Binding to axin	*CSNAK2A1*	−4.57	0.018	−5.56	0.015
Transcription repressor	*CTBP1*	−3.85	0.011	−3.04	0.015
Mitrolysin	*MMP7*	−8.39	0.046	−3.98	0.049
Wnt receptor	*FZD9*	−5.32	0.001	−2.52	0.003
Wnt inhibitor	*FRZB*	−8.3	0.018	−4.35	0.027

[a] $p < 0.05$ indicates statistical significance.

TABLE 2
Differential effects of TRβ and TRα sumoylation site mutants on Wnt signaling in human primary preadipocytes

Function	Gene symbol	TRβK146Q *vs.* control		TRαK283Q/K288R *vs.* control	
		-fold	*p* value[a]	-fold	*p* value[a]
β-Catenin for canonical Wnt singling	*CTNNB1*	−3.43	0.006	−1.14	0.167
Transcription factor	*LEF1*	−2.92	0.001	−1.44	0.054
Proliferation	*CCND1*	−3.34	0.004	−1.62	0.068
Wnt receptor	*FZD1*	−1.25	0.247	−3.04	0.006
	FZD8	−2.85	0.048	−1.91	0.106
Wnt ligand	*WNT3*	−3.17	0.003	1.92	0.095
	WNT8A	−4.58	0.042	−2.21	0.097
	WNT10A	2.53	0.017	1.99	0.053
	WNT6	−2.12	0.07	2.87	0.001
Wnt signaling inhibitor	*DKK1*	2.63	0.048	1.62	0.14
	DKK3	−1.47	0.063	23.35	0.003
Wnt/Planar cell pathway	*PRICKLE1*	−1.3	0.954	4.22	0.028
	VAGL2	1.08	0.67	4.04	0.001
	WNT5A	1.09	0.618	2.06	0.022

[a] $p < 0.05$ indicates statistical significance.

expression was significantly stimulated in cells transfected with TRα K283Q/K288R (Table 2 and Fig. 8). These genes are involved in Wnt/planar cell polarity (PCP) pathways. The upstream core component genes in the PCP pathway are *Wnt5A*, *Fzd*, *Dishevelled* (*Dvl*), *Vangl2*, and *Prickle*. Wnt5A is a PCP-specific Wnt signal (51). Up-regulation of *Wnt5A* in cells induces conversion of the canonical to the noncanonical pathway in hematopoietic stem cells and reduced self-renewable capacity (52). The specificity of the Fzd family in PCP is not clear. *Dvl* had no significant change in cells expressing TRα K283Q/K288R. Three of the five core component genes were significantly stimulated, suggesting that expression of TRα K283Q/K288R in preadipocytes induced a PCP tendency. Interestingly, *DKK3* was stimulated 23.4-fold, which is widely considered to be a tumor suppressor. Reduced DKK3 protein and RNA levels are directly associated with activation of Wnt/β-catenin pathway-mediated tumor growth in most cancer cells, including papillary thyroid cancer (53–55). These data indicate that sumoylation of TRα and TRβ is important for preadipocyte proliferation by interactions with the Wnt signaling pathway.

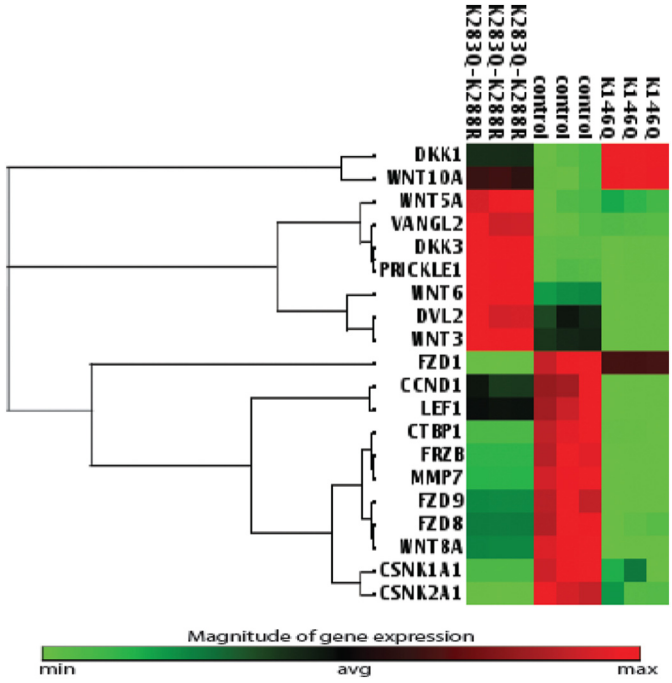

FIGURE 10. **Clustergram showing the influence of TR sumoylation site mutations on Wnt signaling pathways.** Human primary preadipocytes were transfected with empty vector, TRβ K146Q, and TRα K283Q/K288R mutants. Cells were grown for 3 days. RNA was isolated and analyzed by real-time PCR analysis using the Wnt signaling pathway array. Data were analyzed using Qiagen software. The magnitude of gene expression is shown in the *horizontal bar*.

DISCUSSION

We have demonstrated that TR sumoylation plays an important role in preadipocyte proliferation and differentiation. Mutation of sumoylation sites in either TRα or TRβ showed a similar effect on adipocyte differentiation, including fewer and smaller lipid droplets compared with control cells and reduced PPARγ-mediated gene expression. There was, however, some

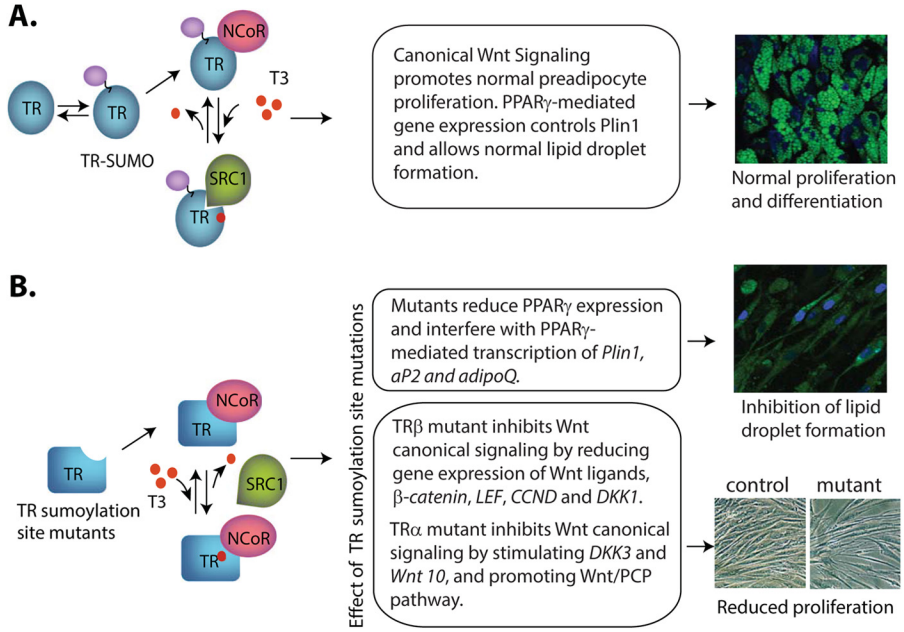

FIGURE 11. **Summary of the influence of TR sumoylation on preadipocyte proliferation and differentiation.** *A*, under normal conditions, TR-SUMO conjugation is dynamic. TR-SUMO promotes recruitment of either the coactivator or corepressor depending on the promoter configuration, ligand availability, and cellular signals. Predipocytes undergo normal proliferation and growth arrest. When induced, cells are differentiated into adipocytes filled with lipid droplets. The differentiation is driven by PPARγ-mediated gene expression. *B*, TR sumoylation site mutants alter protein-protein interface and/or TR conformation, resulting in increased recruitment of NCoR resistant to disruption by T_3 and a diminished ability to recruit the coactivator SRC1. Additionally, TR mutants may lose or reduce the capacity to interact with factors carrying SUMO interaction motifs. Our data show that TR sumoylation site mutants inhibit *Plin1* expression by interfering with PPARγ expression and PPARγ signaling, leading to impaired lipid droplet formation and adipogenesis. In the proliferation phase, TR sumoylation site mutants reduce preadipocyte proliferation by inhibiting Wnt canonical signaling. *Green* fluorescence (BODIPY) depicts neutral triglycerides and lipids in adipocytes. *Blue* fluorescence (DAPI) depicts nuclei.

variation in the disruption of preadipocyte differentiation, depending on the TR isoform and specific sumoylation site. Mutation of TRβ at sumoylation site Lys-146 inhibited the canonical Wnt signaling pathway, including down-regulation of *CTNNB1* (coding for β-catenin), *LEF1*, and several Wnt ligands and Wnt receptors. In addition, it stimulated *DKK1*, which inhibits Wnt contact with the low density lipoprotein receptor-related protein (LRP) and inhibits the canonical signaling pathway. Mutation of TRα at sumoylation site K283Q/K288R stimulated the core components of the Wnt noncanonical PCP pathway and *DKK3* gene expression, a potent inhibitor of the Wnt canonical pathway (Fig. 11).

Expression of C/EBPs during mitotic clonal expansion is important for stimulation of pro-adipogenic genes. TR sumoylation mutants down-regulated *C/EBPβ* and *C/EBPα*, resulting in reduced *PPARγ2* expression. In addition to the C/EBPs, other factors have been reported to regulate *PPARγ* expression and influence adipogenesis, including GATA (56), KLFs (11, 12), FOXO1 (57), SREBP (58, 59), and TNFα/NFκB (60). Whether these factors are additionally influenced by TR sumoylation mutants is not known.

TR isoform-specific actions have been reported in heart, brain, pituitary, liver, bone, and testes (3). These isoform-specific actions are thought to be related to the distribution of TR isoforms in tissue as well as to their intrinsic properties. We showed some TR isoform preference for the disruption of some adipogenic pathways, but the sumoylation mutants in the context of TRα or TRβ had similar effects.

It has been speculated that there is a TRβ and TRα selectivity for the T_3 response element (TRE) contained in the gene regulatory region. A recent genome-wide ChIP-seq that analyzed TR isoform binding sites concluded that receptor isoform-selective DNA binding is not observed in neural cells, although receptor-selective response is observed (25). This finding indicates that other properties, such as posttranslational modification, may confer TR isoform specificity. TR sumoylation may be one of the mechanisms for TR isoform-selective regulation.

TRα and TRβ have a different number of sumoylation motifs. SUMO conjugation modifies the surface of TR, resulting in general conformational change and may result in alterations of protein-protein interactions such as TR interactions with co-repressors. These changes are dynamic, as sumoylation conjugation is rapid and reversible. A further level of regulation may be related to the multiple TR sumoylation motifs and may be influenced by whether TR is fully or selectively conjugated. Although both mono- and polysumoylation have been observed in TR (24), there is no evidence so far for selective sumoylation, because it is very difficult to isolate the TR with site-specific SUMO conjugation.

There are similarities between TR sumoylation mutants and RTH-associated TR mutants with respect to their action on PPARγ signaling, as both reduce PPARγ protein and disrupt PPARγ signaling. TR-SUMO conjugation plays an important role in *Plin1* gene expression. Mutations of the TRβ sumoylation site markedly diminished *Plin1* expression due to crosstalk with the PPARγ signaling pathway. As shown by ChIP assay, TRβ K443Q bound strongly to the *Plin1* PPRE (Fig. 6*A*). The DNA-binding complex is either a TR/TR homodimer or a TR/RXR heterodimer. The addition of TZD stimulated the

binding of both PPARγ and TRβ K443Q to the PPRE, suggesting that TRβ K443Q interacted directly with PPARγ. Mutants associated with RTH, TRα PV and TRα P398H, have been shown to influence PPAR signaling by binding to a PPRE. Wild-type TR is also capable of cross-talk with PPAR, although it does not bind the PPRE but competes for the common heterodimer partner, RXR.

The interference of TRβ sumoylation mutant K443Q with PPARγ-mediated *Plin* gene expression was due, in part, to constitutive interaction with NCoR. Utilizing NCoRΔID to disrupt TR-NCoR interaction partially reversed the inhibition by the mutant TR, consistent with the mutation at Lys-443 influencing the interface with NCoR. A recent study reported that the altered adipogenesis seen in TRαPV mice was completely rescued by expression of NCoRΔID (61). Although the model system and the TR mutation differ from the TR sumoylation mutants that we studied, the mechanism is similar. These similarities emphasize the central role of TR co-regulator interactions and the various ways that it can be disrupted by mutations that influence SUMO conjugation or ligand binding.

TR-SUMO conjugation, however, likely has regulatory roles in addition to co-regulator interaction. The addition of NCoRΔID only partially reversed the inhibition associated with expression of TR sumoylation site mutants, with partial recovery of *Plin1* gene expression and adipocyte differentiation. TR sumoylation site mutations likely have effects beyond cross-talk with PPAR and constitutive interaction with NCoR. We have identified two sumoylation motifs in TRα and three in TRβ. It is possible that monosumoylation in TR promotes interaction with factors in addition to NCoR. Moreover, polysumo chains have been found in many SUMO substrates (62, 63) including TR in mouse liver, heart, and adipose tissue (24). Polysumoylation may create additional binding sites for factors containing SUMO interaction motif (Fig. 8), which have been demonstrated in transcription regulation of HIF-1α by SUMO polymers. The biological relevance of the polymeric SUMO signal has been studied predominantly in yeast. It is related to DNA repair and sporulation efficiency (64, 65). Polysumoylation may function, for example, as a signal for ubiquitination, which would lead to selective removal of specific proteins (66, 67). Therefore, mutations at the SUMO conjugation site of a SUMO acceptor (TR) may lead to protein accumulation and alter signaling pathways. Further characterization of the TR protein interactions influenced by mono- and polysumoylation in adipogenesis will provide potential targets for metabolic regulation.

TR sumoylation is required for normal adipocyte differentiation and proliferation. We have identified key pathways influenced by TR sumoylation including PPARγ protein levels and signaling, C/EBPs expression, NCoR interactions with TR, and Wnt signaling (Fig. 11). The relative roles of these various actions may vary depending on the tissue studied, as well as the integration of other nutritional signals. The use of primary cells and cell lines showed largely complementary results and supports the relevance of TR sumoylation in the preadipocyte to adipocyte model. The relative roles of the various mechanisms studied, however, and identification of therapeutic targets relevant to metabolic diseases will need to be studied in whole animal models.

Note Added in Proof—Fig. 1 was incomplete in the version of the article that was published as a Paper in Press on January 8, 2015. The correct image is now shown.

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