

## Alternative Mechanisms by Which Mediator Subunit MED1/TRAP220 Regulates Peroxisome Proliferator-Activated Receptor $\gamma$ -Stimulated Adipogenesis and Target Gene Expression<sup>∇</sup>

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**Mediator is a general coactivator complex connecting transcription activators and RNA polymerase II. Recent work has shown that the nuclear receptor-interacting MED1/TRAP220 subunit of Mediator is required for peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ )-stimulated adipogenesis of mouse embryonic fibroblasts (MEFs). However, the molecular mechanisms remain undefined. Here, we show an intracellular PPAR $\gamma$ -Mediator interaction that requires the two LXXLL nuclear receptor recognition motifs on MED1/TRAP220 and, furthermore, we show that the intact LXXLL motifs are essential for optimal PPAR $\gamma$  function in a reconstituted cell-free transcription system. Surprisingly, a conserved N-terminal region of MED1/TRAP220 that lacks the LXXLL motifs but gets incorporated into Mediator fully supports PPAR $\gamma$ -stimulated adipogenesis. Moreover, in undifferentiated MEFs, MED1/TRAP220 is dispensable both for PPAR $\gamma$ -mediated target gene activation and for recruitment of Mediator to a PPAR response element on the *aP2* target gene promoter. However, PPAR $\gamma$  shows significantly reduced transcriptional activity in cells deficient for a subunit (MED24/TRAP100) important for the integrity of the Mediator complex, indicating a general Mediator requirement for PPAR $\gamma$  function. These results indicate that there is a conditional requirement for MED1/TRAP220 and that a direct interaction between PPAR $\gamma$  and Mediator through MED1/TRAP220 is not essential either for PPAR $\gamma$ -stimulated adipogenesis or for PPAR $\gamma$  target gene expression in cultured fibroblasts. As Mediator is apparently essential for PPAR $\gamma$  transcriptional activity, our data indicate the presence of alternative mechanisms for Mediator recruitment, possibly through intermediate cofactors or other cofactors that are functionally redundant with MED1/TRAP220.**

Peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) is a key regulator of transcriptional pathways important for adipogenesis (34). PPAR $\gamma^{-/-}$  mice show a total absence of both brown and white adipose tissue. Furthermore, retrovirus vector-mediated ectopic expression of PPAR $\gamma$  alone can stimulate mouse embryonic fibroblasts (MEFs) to undergo adipogenesis. In such cells, the expression of CCAAT/enhancer-binding protein  $\alpha$  (C/EBP $\alpha$ ), another key transcriptional regulator of adipogenesis, and adipogenesis markers such as *aP2*, fatty acid synthase (FAS), and adipsin are induced in a PPAR $\gamma$ -dependent manner.

PPAR $\gamma$  and other nuclear hormone receptors comprise a superfamily of DNA binding transcription factors. However, they also require various transcriptional coactivators to acti-

vate, in a ligand-dependent manner, transcription of the specific target genes important for cell growth, homeostasis, and differentiation (36). These transcription coactivators often exist as multiprotein complexes. They may act either through chromatin remodeling and histone modification, after recruitment by promoter-bound nuclear receptors, or at steps involving subsequent preinitiation complex formation or function (transcription initiation and elongation). Transcription coactivators that act at the level of chromatin include ATP-dependent chromatin remodeling complexes and factors that contain (or interact with) histone acetyl transferases and methyltransferases (40).

The Mediator coactivator complex, in contrast to chromatin-modifying factors, acts more directly to facilitate promoter recruitment and function of RNA polymerase II and cognate general transcription factors. First identified as a defined complex in yeast, Mediator is evolutionarily conserved and contains approximately 30 subunits (7, 26). It is believed to connect transcriptional activators with the RNA polymerase II transcription machinery and appears to be essential for most (17), but not necessarily all (9), RNA polymerase II transcription. The mammalian Mediator/thyroid hormone receptor-associated protein (TRAP) complex was first isolated through affinity purification of an epitope-tagged thyroid hormone receptor  $\alpha$  (TR $\alpha$ ) from HeLa cells grown in the presence of a

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TR $\alpha$  ligand (10), and the complex is similar to or identical with other more recently described complexes that include the SRB/MED-containing cofactor complex (SMCC) and the PC2, NAT, mouse mediator, ARC, CRSP, DRIP, and human mediator complexes (26). These closely related mammalian Mediator complexes have been shown to interact, through distinct subunits, with diverse transcription activators that include nuclear receptors, Sp1, SREBP, NF- $\kappa$ B, p53, VP16, and E1A (reviewed in reference 4).

The MED1/TRAP220 subunit of the Mediator complex shows ligand-dependent interactions, through a region containing two nuclear receptor recognition (LXXLL) motifs, with multiple nuclear hormone receptors that include TR $\alpha$ , vitamin D receptor, PPAR $\gamma$  and PPAR $\alpha$ , retinoic acid receptor  $\alpha$  (RAR $\alpha$ ), retinoid X receptor (RXR), farnesoid X receptor, and estrogen receptor  $\alpha$  and  $\beta$  (ER $\alpha$  and ER $\beta$ ) (10, 20, 30, 32, 43, 44). A MED1/TRAP220 LXXLL-dependent interaction between the intact Mediator complex and TR $\alpha$  has also been demonstrated (25). These results have suggested a broad role for the Mediator complex in nuclear receptor function. The mouse MED1/TRAP220 subunit was independently isolated as a PPAR $\gamma$ -interacting protein in yeast two-hybrid screens and was shown to interact, in a ligand-dependent manner, with PPAR $\gamma$ . MED1/TRAP220 modestly increased the transcriptional activity with a PPAR $\gamma$ -responsive reporter, and a fragment of MED1/TRAP220 spanning the two LXXLL motifs acted as a dominant-negative repressor, suggesting that MED1/TRAP220 is a coactivator for PPAR $\gamma$  (44).

We recently showed that the MED1/TRAP220 subunit of the Mediator complex is essential for PPAR $\gamma$ -stimulated adipogenesis and expression of adipogenesis markers in MEFs, but not for MyoD-stimulated myogenesis (11). This finding provided an example of the regulation of cell-specific transcription and differentiation events through a distinct Mediator subunit. Further biochemical analyses showed (i) that PPAR $\gamma$  interacts directly with the purified Mediator complex in a ligand-dependent manner, (ii) that Mediator functions directly as a transcriptional coactivator for PPAR $\gamma$  on a DNA template containing three copies of the DR1 PPAR $\gamma$  recognition site in an in vitro transcription system reconstituted with highly purified factors, and (iii) that MED1/TRAP220 serves as an essential bridge for the interaction between Mediator complex and PPAR $\gamma$  in vitro (11). These data suggested a potential mechanism that may account for the inability of *MED1/TRAP220*<sup>-/-</sup> MEFs to undergo PPAR $\gamma$ -stimulated adipogenesis. However, the precise molecular mechanisms underlying the roles of MED1/TRAP220 and the associated Mediator complex in PPAR $\gamma$ -stimulated adipogenesis in vivo and the mechanism by which MED1/TRAP220 and Mediator regulate PPAR $\gamma$  transcriptional activity remain unclear.

Here, structural and functional analyses of MED1/TRAP220 indicate, surprisingly, that a strong, direct interaction of PPAR $\gamma$  with Mediator through the LXXLL motifs of MED1/TRAP220 is not required for PPAR $\gamma$ -stimulated adipogenesis of cultured MEFs and, furthermore, that PPAR $\gamma$  target gene expression and recruitment of Mediator to a PPAR $\gamma$  response element on the *aP2* promoter in undifferentiated MEFs do not require MED1/TRAP220. The minimal region required for MED1/TRAP220 function in adipogenesis is mapped to an evolutionarily conserved 530-amino-acid N-terminal region

that mediates the incorporation of MED1/TRAP220 into the Mediator complex. Our data thus suggest the existence of an alternative mechanism, involving other potentially redundant cofactors or intermediate cofactors, by which MED1/TRAP220 and the associated Mediator complex regulate the expression of known PPAR $\gamma$  target genes, as well as the possibility of as-yet-undefined genes that require MED1/TRAP220 for expression in adipogenesis.

## MATERIALS AND METHODS

**Plasmids.** A PPAR $\gamma$  cDNA encoding the mouse PPAR $\gamma$ 2 isoform (505 amino acids) was used in this study. For recombinant retroviruses, PPAR $\gamma$  cDNA was inserted into pMSCVpuro and pMSCVhyg (Clontech). PPAR $\gamma$  cDNA with an N-terminal FLAG tag was cloned into pWZLneo to generate pWZLneo-F-PPAR $\gamma$ . FLAG-tagged and hemagglutinin (HA)-tagged full-length wild-type and mutant MED1/TRAP220 cDNAs were cloned into pWZLhygro, as described previously (11). pMSCVpuro was used to express FLAG- and HA-tagged MED1/TRAP220 truncations. For glutathione *S*-transferase (GST) pull-down assays, the use of GST-TR $\alpha$  and GST-PPAR $\gamma$  fusions has been described previously (43). For in vitro transcription/translation, MED1/TRAP220 and Myc-tagged MED7 were cloned into pGEM7. For mammalian two-hybrid assays, full-length PPAR $\gamma$  or TR $\alpha$  cDNA was cloned into pACT (Promega) to generate pVP16-PPAR $\gamma$  or pVP16-TR $\alpha$ . MED1/TRAP220 cDNA encoding amino acids 1 to 670 [MED1/TRAP220(1-670)] was cloned into pBIND (Promega) to generate pGal4-MED1/TRAP220. For immunofluorescence, cDNAs of FLAG- and HA-tagged MED1/TRAP220 truncations were placed under the control of the cytomegalovirus promoter in the pIN vector, modified from pIRESneo (Clontech). All plasmids were verified by DNA sequencing.

**Cell culture, retrovirus gene transfer, and induction of adipogenesis.** All cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum (Mediatech). The HeLaS cell line stably expressing FLAG-tagged PPAR $\gamma$  (HeLaS/F-PPAR $\gamma$ ) was established by using the retrovirus vector pWZLneo-F-PPAR $\gamma$  and following the protocol described previously (6). Retrovirus-mediated gene expression in self-immortalized wild-type and *MED1/TRAP220*<sup>-/-</sup> MEFs and the induction of adipogenesis were performed essentially as described previously (11). Briefly, 2 days after cells reached confluence, they were treated with a differentiation cocktail containing 0.5 mM 3-isobutyl-1-methyl-xanthine (Sigma), 1  $\mu$ M dexamethasone (Sigma), 5  $\mu$ g per ml insulin (Sigma), and 0.5  $\mu$ M rosiglitazone or vehicle (dimethyl sulfoxide [DMSO]) alone. Two days later, cells were changed to medium containing 5  $\mu$ g per ml insulin and 0.5  $\mu$ M rosiglitazone or DMSO. The medium was replenished at 2-day intervals. At day 8 postinduction, cells were stained for lipid droplets with Oil Red O (Sigma) to check for morphological differentiation. Total RNA was purified with Trizol (Invitrogen) for Northern blot analysis.

**Mammalian two-hybrid assay, luciferase reporter assay, and immunofluorescence.** For mammalian two-hybrid assays, 293T cells were seeded in 24-well plates at a density of  $2 \times 10^5$  cells per well. Twenty-four hours later, cells were transfected by LipofectAmine 2000. For each well, a mixture of 50 ng of the reporter plasmid pG5luc (Promega), 200 ng of pBluescript as carrier, 250 ng of pVP16-PPAR $\gamma$  or pVP16-TR $\alpha$ , and 250 ng of pGal4-MED1/TRAP220 (wild type or mutant) was used. About 16 h after the start of transfection, cells were changed to phenol red-free DMEM (Invitrogen) supplemented with 1% charcoal-stripped fetal bovine serum (HyClone) and 1  $\mu$ M T3 or rosiglitazone. Each type of transfection was done in triplicate. About 40 h after the start of transfection, cells were harvested, and cell extracts were analyzed for luciferase activity, using a dual-luciferase reporter assay system (Promega). Use of the luciferase reporter assay with *MED24/TRAP100*<sup>-/-</sup> MEF cells was described previously (19). Immunofluorescence was done as described previously (12).

**Affinity purification of PPAR $\gamma$ -associated proteins and mass spectrometry.** HeLaS cells stably expressing FLAG-tagged PPAR $\gamma$  (HeLaS/F-PPAR $\gamma$ ) or vector only (HeLaS/Vec) were grown to confluence on 400- by 15-cm dishes in DMEM containing 10% bovine serum (Invitrogen). Twelve hours before cells were harvested, 0.5  $\mu$ M rosiglitazone was added to the medium. All solutions for nuclear extract preparation and affinity purification were supplemented with 0.5  $\mu$ M rosiglitazone. Nuclear extracts were precleared with mouse immunoglobulin G (IgG) agarose and EZview Red protein A affinity gel (Sigma) in buffer A (20 mM HEPES [pH 7.9], 180 mM KCl, 0.2 mM EGTA, 1.5 mM MgCl<sub>2</sub>, 20% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.1% NP-40) supplemented with the protease inhibitors aprotinin (1  $\mu$ g/ml), leupeptin (2  $\mu$ g/ml), and pepstatin (0.7  $\mu$ g/ml) (Roche). Precleared nuclear extracts were

incubated with the anti-FLAG M2 agarose (Sigma) in buffer A at 4°C overnight. After bound proteins were washed 12 times with buffer A, they were eluted twice with 0.25 mg/ml FLAG peptide (Sigma), concentrated with a Microcon column (Millipore), and resolved with 4 to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad). Protein identification by mass spectrometry was performed essentially as described previously (6).

**Northern blotting and immunoblotting.** Northern and immunoblot analyses were performed as described previously (11). For Northern blotting, 10  $\mu$ g of total RNA isolated with Trizol was loaded into each lane. Signal intensity was measured by PhosphorImager (Fuji) and normalized to the actin signal. For immunoblotting, rabbit anti-HA (catalog no. sc-805; Santa Cruz Biotechnology) and mouse anti-PPAR $\gamma$  (catalog no. sc-7273; Santa Cruz Biotechnology) antibodies were used at 1:400 and 1:200 dilutions, respectively. Mouse anti-FLAG (catalog no. F3165; Sigma) was used at a concentration of 1  $\mu$ g ml<sup>-1</sup>. Rabbit anti-MED7, anti-MED12, anti-MED14, anti-MED17, and anti-MED21 were used at a 1:1,000 dilution as described previously (11).

**GST pull-down and ChIP assays.** GST pull-down assays of in vitro-translated [<sup>35</sup>S]methionine-labeled proteins and Mediator from nuclear extracts were conducted essentially as described previously (11). For chromatin immunoprecipitation (ChIP) assays, mouse anti-MED23/Sur2 (catalog no. 550429) and anti-PPAR $\gamma$  (catalog no. sc-7273) were from BD Bioscience and Santa Cruz Biotechnology, respectively. ChIP was performed in triplicate (as described in reference 24), with minor modifications. In brief, MEFs were fixed for 10 min at 25°C with 1% formaldehyde in the culture medium. Cross-linking was stopped by the addition of glycine to a final concentration of 0.2 M. Cells were washed twice with phosphate-buffered saline, scraped down, and harvested by centrifugation. The cell pellet was washed once again with phosphate-buffered saline, resuspended in lysis buffer (50 mM HEPES, pH 7.5, 140 mM NaCl, 1% Triton X-100, 0.1% Na deoxycholate, 1  $\mu$ g/ml each of pepstatin, leupeptin and aprotinin, and 1 mM phenylmethylsulfonyl fluoride), and vortexed for 30 min at 4°C. Whole-cell lysates were sonicated on ice with 10 cycles of 20-s pulses on and 59-s pulses off, using a microtip probe on a sonication unit (Sonic dismembrator 500; Fisher) at a power setting of 22%. Sonicated lysates were clarified by centrifugation, and the supernatant was precleared with 200  $\mu$ l of protein A-agarose (Upstate) for 2 h at 4°C. Each immunoprecipitation was performed with 3 to 5  $\mu$ g of purified antibodies and 1 to 5 mg of precleared lysate. After the culture was incubated overnight at 4°C, 60  $\mu$ l of protein A-agarose was added, and the incubation was continued for 1 h. Precipitates were sequentially washed twice in lysis buffer, twice in lysis buffer containing 500 mM NaCl, twice in wash buffer (10 mM Tris-HCl, pH 8.0, 250 mM LiCl, 0.5% NP-40, 0.5% Na deoxycholate, and 1 mM EDTA), and finally twice in Tris-EDTA. Bound complexes were eluted by incubating beads twice for 5 min at 65°C in 75  $\mu$ l of elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 1% SDS). Cross-linking of immunoprecipitates and input samples was reversed by an overnight incubation at 65°C, after which DNA was extracted with a PCR purification kit (Qiagen). Real-time PCR quantitation of the precipitated genomic DNA relative to that of the inputs was performed in triplicate using TaqMan probes on an ABI PRISM 7900HT model system. The sequences of TaqMan probes and primers are available upon request.

## RESULTS

**PPAR $\gamma$  associates with the Mediator complex in cells.** A HeLaS cell line that stably expresses full-length mouse PPAR $\gamma$  with an amino-terminal FLAG epitope tag (F-PPAR $\gamma$ ) was established using retrovirus-mediated gene transduction. Nuclear extracts were prepared and incubated with anti-FLAG M2 agarose in the presence of 0.5  $\mu$ M rosiglitazone, a synthetic PPAR $\gamma$  ligand. After bound proteins were washed extensively, they were eluted with FLAG peptide, resolved on SDS-PAGE, and identified by mass spectrometry. As shown in Fig. 1, anti-FLAG M2 agarose immunoprecipitated multiple proteins from F-PPAR $\gamma$ -expressing but not from vector-expressing cells. Mass spectrometry analyses of the readily visible bands indicated that the majority were subunits of the Mediator complex. These subunits included MED1/TRAP220, MED12/TRAP230, MED13/TRAP240, MED13L/THRAP2, MED14/TRAP170, MED15/PCQAP, MED23/TRAP150 $\beta$ , and MED24/TRAP100, with sequence coverage of 41.5%, 25.9%, 20.7%,

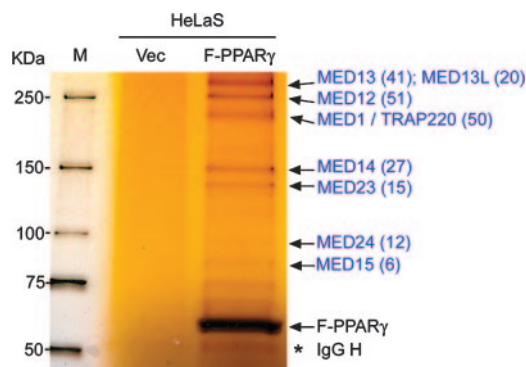


FIG. 1. PPAR $\gamma$  associates with the Mediator complex in cells. Anti-FLAG M2 agarose was used to immunoprecipitate nuclear extracts of HeLaS cells stably expressing either FLAG-tagged PPAR $\gamma$  (F-PPAR $\gamma$ ) or vector only (Vec). Immunoprecipitates were resolved on an SDS-PAGE gel and stained with silver. Mediator subunits identified by mass spectrometry are labeled in blue, with the number of matching tryptic peptides indicated in parentheses. M, protein molecular mass marker. The IgG heavy chain (IgG H) is marked with an asterisk.

13.9%, 22.6%, 9.8%, 12.6%, and 17.0%, respectively (data not shown). An immunoblot analysis also confirmed the intracellular association of F-PPAR $\gamma$  with other Mediator subunits as well (data not shown). These results are consistent with our previous demonstration of an in vitro interaction between PPAR $\gamma$  and the Mediator complex (11).

**A demonstrable physical interaction between PPAR $\gamma$  and Mediator requires the two LXXLL nuclear receptor recognition motifs on MED1/TRAP220.** MED1/TRAP220 has two closely spaced LXXLL nuclear receptor recognition motifs (residue regions 604 to 608 and 645 to 649) in a central region that mediates ligand-dependent interactions between MED1/TRAP220 and the ligand binding domains of nuclear receptors. Ligand-dependent interactions of MED1/TRAP220 with PPAR $\gamma$  and (as a control) TR $\alpha$  in cells were confirmed by a mammalian two-hybrid assay (Fig. 2A). These interactions were significantly decreased by mutation of either of the MED1/TRAP220 LXXLL motifs to LXXAA and were essentially eliminated by the joint mutation of both motifs to LXXAA (Fig. 2A). Consistent with these results, a GST-PPAR $\gamma$  pull-down assay with in vitro-translated MED1/TRAP220 confirmed that the two LXXLL motifs are required for a demonstrable interaction between MED1/TRAP220 and PPAR $\gamma$  (data not shown).

MED1/TRAP220 serves as an essential bridge for a demonstrable physical interaction between PPAR $\gamma$  and the complete Mediator complex (11). To test whether the two LXXLL motifs carried on MED1/TRAP220 are also required for the interaction between the Mediator complex and PPAR $\gamma$ , GST-PPAR $\gamma$  was incubated with nuclear extracts from MED1/TRAP220<sup>-/-</sup> cells expressing MED1/TRAP220(1-670) with either the intact LXXLL motifs or the LXXAA mutations in both motifs. These derivatives are both efficiently incorporated into the Mediator complex (25). As shown in Fig. 2B, GST-PPAR $\gamma$  could bind only the endogenous Mediator complex from MED1/TRAP220<sup>-/-</sup> cells expressing the wild-type MED1/TRAP220(1-670). This result indicates that the PPAR $\gamma$  interaction with the Mediator complex requires the two

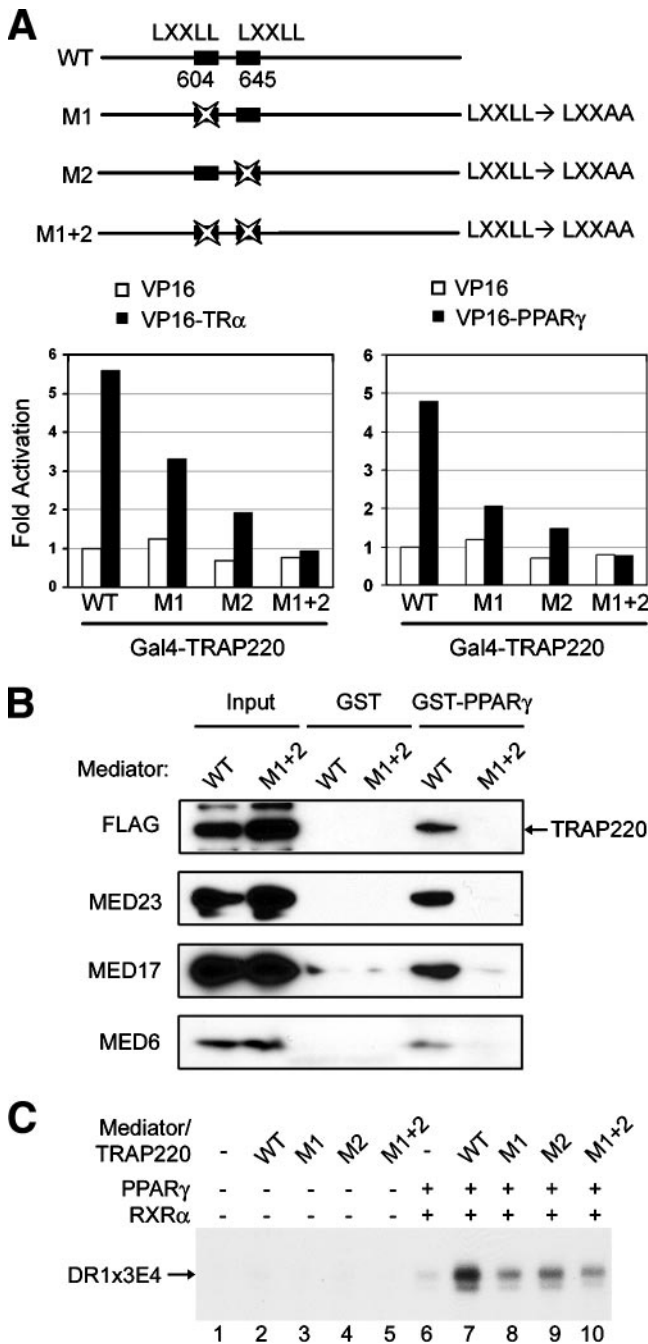


FIG. 2. Physical interaction between PPAR $\gamma$  and Mediator requires the two LXXLL motifs in MED1/TRAP220. (A) Physical interaction between PPAR $\gamma$  and MED1/TRAP220 requires both of the LXXLL motifs in MED1/TRAP220. Panel A top shows a schematic representation of the wild-type MED1/TRAP220 protein (WT) and the derived mutants with mutations in the two LXXLL motifs. The positions of the two LXXLL motifs are indicated. Panel A lower panels show mammalian two-hybrid assays. Plasmids expressing pVP16-PPAR $\gamma$  and MED1/TRAP220(1-670) fused to the Gal4 DNA binding domain (Gal4-TRAP220) were cotransfected with the reporter plasmid pG5luc into 293T cells. Interaction between TR $\alpha$  and the MED1/TRAP220 subunit is shown as a control. (B) PPAR $\gamma$  interaction with the Mediator complex requires the two LXXLL motifs in MED1/TRAP220. GST-PPAR $\gamma$  was incubated with nuclear extracts from MED1/TRAP220<sup>-/-</sup> cells expressing MED1/TRAP220(1-670), either with intact LXXLL motifs (WT) or with LXXAA mutations in

LXXLL motifs on MED1/TRAP220. In an extension of previous studies showing that Mediator is required for optimal PPAR $\gamma$  function in a purified cell-free system (11), we analyzed Mediator complexes containing MED1/TRAP220(1-670) with the intact LXXLL motifs or with the mutated motifs (LXXAA). As shown in Fig. 2C, PPAR $\gamma$  (with RXR $\alpha$ ) mediates only a minimal activation in the absence of Mediator (Fig. 2C, lane 6 versus lane 7). In contrast, Mediator with wild-type MED1/TRAP220(1-670) mediates a strong activation by PPAR $\gamma$ , as reported previously, whereas Mediator with a single mutation or joint LXXAA mutations in MED1/TRAP220(1-670) shows only a modest (severalfold reduced) activation by PPAR $\gamma$  (Fig. 2C, lanes 8 to 10 versus lane 7). Hence, while the LXXLL motifs are not absolutely required for significant Mediator-dependent activity in this purified system, which lacks many other nuclear cofactors, these motifs are required for optimal Mediator-dependent activity with PPAR $\gamma$ .

**The two LXXLL motifs within MED1/TRAP220 are dispensable for PPAR $\gamma$ -stimulated adipogenesis.** We previously showed that MED1/TRAP220<sup>-/-</sup> MEF cells are refractory to PPAR $\gamma$ -stimulated adipogenesis and that this defect can be rescued by the expression of exogenous full-length MED1/TRAP220 (11). To determine the role of the two MED1/TRAP220 LXXLL motifs in PPAR $\gamma$ -stimulated adipogenesis, we tested the ability of MED1/TRAP220 with the LXXAA mutations in either or both of the two LXXLL motifs to rescue the adipogenesis defect in MED1/TRAP220<sup>-/-</sup> cells. Consistent with our previous report (11), ectopic expression of the wild-type MED1/TRAP220 subunit restored PPAR $\gamma$ -stimulated adipogenesis in MED1/TRAP220<sup>-/-</sup> cells, while the vector alone did not. Surprisingly, ectopic expression of MED1/TRAP220 with the LXXAA mutations in either (M1 or M2) or both (M1 plus M2) of the two LXXLL motifs also rescued the defect in MED1/TRAP220<sup>-/-</sup> cells (Fig. 3A). Consistent with the morphological differentiation, adipogenesis marker genes, including endogenous PPAR $\gamma$ , adipsin, *aP2*, and *FAS*, were induced at levels that were similar in both the mutant MED1/TRAP220-expressing cells and the wild-type MED1/TRAP220-expressing cells (Fig. 3C). Similarly, deletion of either one or both of the two LXXLL motifs or deletion of short adjacent regions had little effect on the ability of exogenous MED1/TRAP220 to rescue the adipogenesis defect in MED1/TRAP220<sup>-/-</sup> cells (data not shown), indicating that the two LXXLL motifs and the adjacent regions on MED1/TRAP220 are not essential for PPAR $\gamma$ -stimulated adipogenesis of MEFs. As the two LXXLL motifs mediate the observed physical interaction between PPAR $\gamma$  and MED1/TRAP220 (Fig. 2A and data not shown),

both motifs (M1 + 2), in the presence of 1  $\mu$ M rosiglitazone. Bound Mediator was monitored by immunoblotting with antibodies to indicated subunits. (C) Optimal PPAR $\gamma$  function in a purified transcription system requires both LXXLL motifs in MED1/TRAP220. Reactions with a PPRE-containing DNA template and with purified RNA polymerase II, general initiation factors, PC4, and Mediator were as described previously (11). Mediator complexes containing MED1/TRAP220(1-670) with mutations LXXLL to LXXAA in the first (M1), second (M2), or both (M1 + 2) LXXLL motifs were isolated as described previously (25).

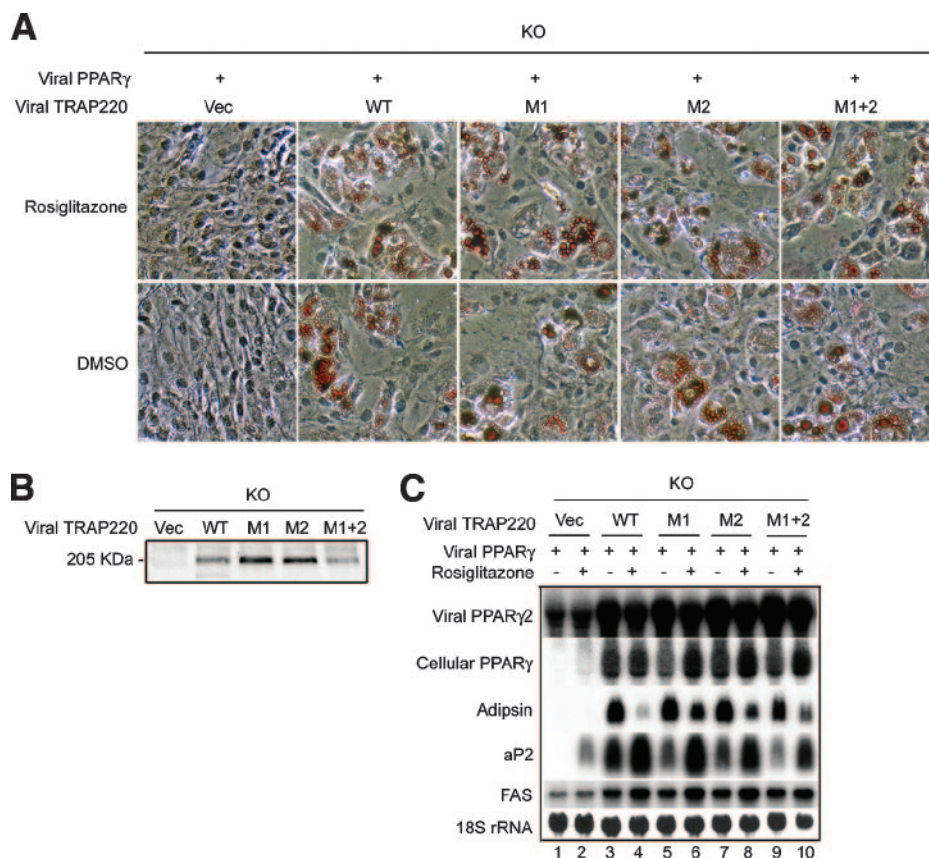


FIG. 3. The two LXXLL motifs in MED1/TRAP220 are dispensable for PPAR $\gamma$ -stimulated adipogenesis. *MED1/TRAP220*<sup>-/-</sup> (knockout [KO]) cells were infected with retroviruses expressing HA-tagged full-length wild-type (WT) or MED1/TRAP220 mutants, as shown in Fig. 2A. Cells were subsequently infected with MSCVpuro-PPAR $\gamma$  retroviruses and induced to differentiate for 8 days in the presence or absence of 0.5  $\mu$ M rosiglitazone. (A) Morphological differentiation (Oil Red O staining). Vec, vector; M, motif. (B) Immunoblot analysis of ectopic MED1/TRAP220 expression by anti-HA antibody. (C) Northern blot analysis of adipogenesis markers.

these data indicate that any PPAR $\gamma$ -MED1/TRAP220 interaction due to these residues is dispensable for PPAR $\gamma$ -stimulated adipogenesis. As MED1/TRAP220 serves as an essential bridge for strong in vitro interactions between PPAR $\gamma$  and the Mediator complex (11) and as the demonstrated interaction between PPAR $\gamma$  and the Mediator complex requires the two LXXLL motifs in MED1/TRAP220 (Fig. 2B), these data also suggest that the physical interaction between PPAR $\gamma$  and Mediator through the LXXLL motifs is not required for PPAR $\gamma$ -stimulated adipogenesis of cultured MEFs.

**The conserved amino-terminal region (residues 1 to 530) of MED1/TRAP220 is sufficient to rescue the adipogenesis defect in *MED1/TRAP220*<sup>-/-</sup> cells.** Human and *Drosophila melanogaster* MED1/TRAP220 subunits share significant homology only at their N termini (Fig. 4A). To map the functionally critical region(s) within MED1/TRAP220 and to determine whether the demonstrated physical interaction between PPAR $\gamma$  and Mediator is required for PPAR $\gamma$ -stimulated adipogenesis, a series of HA-tagged MED1/TRAP220 truncations were introduced via retroviruses into *MED1/TRAP220*<sup>-/-</sup> cells that stably express ectopic PPAR $\gamma$ . An immunoblot confirmed the expression of MED1/TRAP220 truncations and similar levels of viral PPAR $\gamma$  expression in these cells (Fig. 4C and E and data not shown). These *MED1/TRAP220*<sup>-/-</sup>-derived

PPAR $\gamma$ -expressing cells were subjected to the adipogenesis assay, and the results are shown in Fig. 4B. Interestingly, the expression of an N-terminal fragment, MED1/TRAP220(1-689), that contains the two LXXLL motifs was sufficient to rescue the adipogenesis defect in *MED1/TRAP220*<sup>-/-</sup> cells, suggesting that the C-terminal amino acids 690 to 1581 of MED1/TRAP220 are not required for PPAR $\gamma$ -stimulated adipogenesis (Fig. 4C, lower panel). Remarkably, an N-terminal fragment MED1/TRAP220(1-530) that contains only the evolutionarily conserved N-terminal region and is devoid of both LXXLL motifs was also sufficient to rescue the adipogenesis defect in *MED1/TRAP220*<sup>-/-</sup> cells (Fig. 4C, lower panel). Consistent with the morphological differentiation and with the LXXLL mutation analysis shown in Fig. 3C, the induction of adipogenesis marker genes was fully restored by expression of MED1/TRAP220(1-530) in *MED1/TRAP220*<sup>-/-</sup> cells (Fig. 4D). Similar results were obtained when such assays were repeated with MED1/TRAP220 fragments that contained residues 1 to 620, 1 to 597, and 1 to 560 and thus lacked either one or both of the two LXXLL motifs (Fig. 4B).

Further deletion of 40 or more amino acids from the C terminus of MED1/TRAP220(1-530) resulted in a complete loss of the ability to rescue the adipogenesis defect in *MED1/TRAP220*<sup>-/-</sup> cells (Fig. 4B and E). To understand the under-

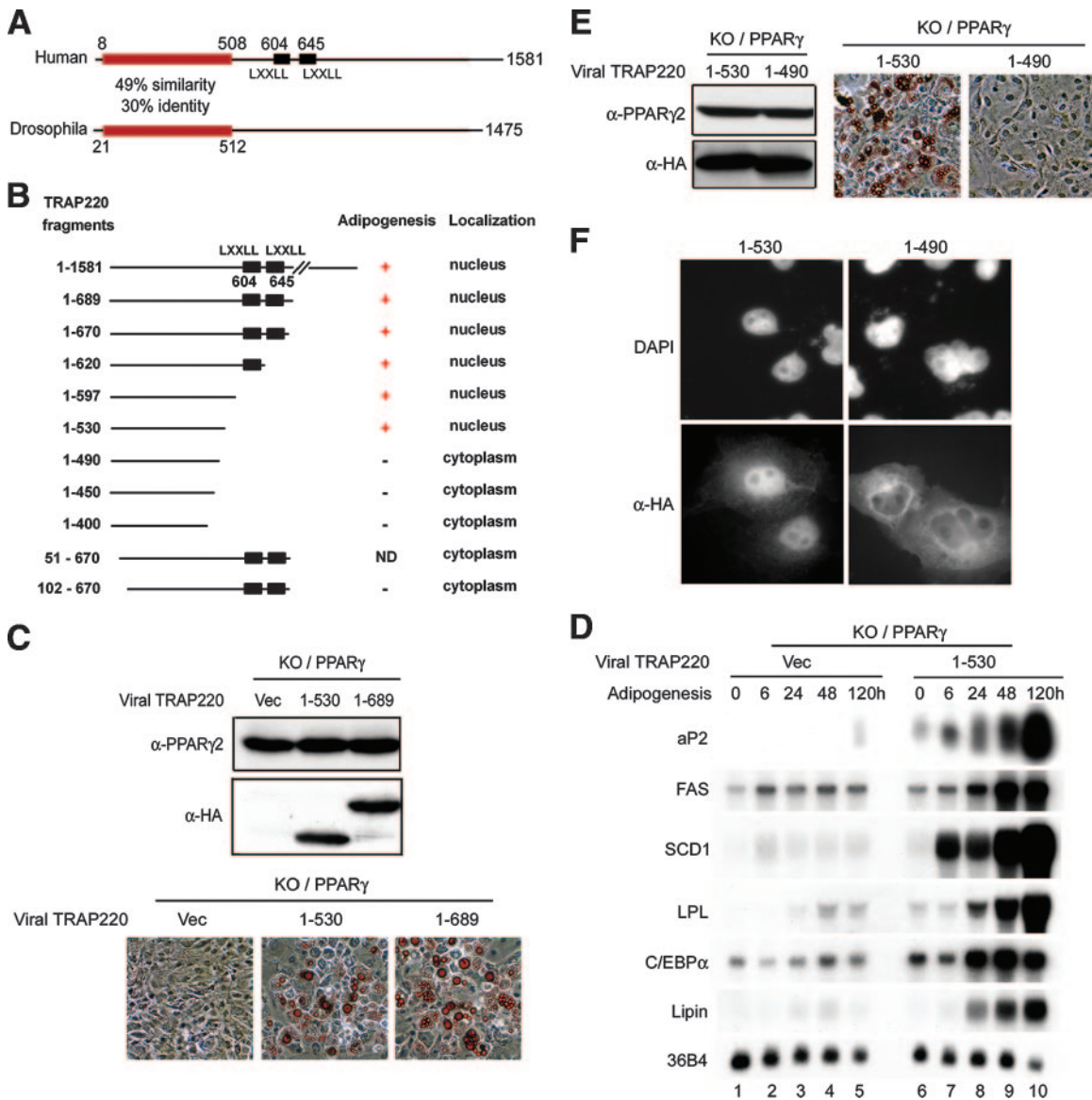


FIG. 4. Residues 1 to 530 of MED1/TRAP220 are sufficient to rescue the adipogenesis defect in *MED1/TRAP220*<sup>-/-</sup> cells. (A) Schematic representation of homology between human and *Drosophila* MED1/TRAP220 proteins. The human MED1/TRAP220 protein sequence was used with BLASTp to query the NCBI protein database. (B) Summary of the structural and functional analyses of MED1/TRAP220 in the context of PPAR $\gamma$ -stimulated adipogenesis. *MED1/TRAP220*<sup>-/-</sup> cells expressing exogenous PPAR $\gamma$  were subsequently infected either with vector only or with retroviruses expressing HA-tagged MED1/TRAP220 fragments as indicated and induced to differentiate for 8 days in the absence of rosiglitazone. Similar results were obtained when the adipogenesis assay was done in the presence of 0.5  $\mu$ M rosiglitazone (data not shown). (C) Residues 1 to 530 of MED1/TRAP220 are sufficient to rescue the adipogenesis defect in *MED1/TRAP220*<sup>-/-</sup> (knockout [KO]) cells. The experiments were done as described in the legend to panel B. (Top) Immunoblot before induction of adipogenesis. (Bottom) Morphological differentiation. Vec, vector. (D) *MED1/TRAP220*<sup>-/-</sup> cells expressing exogenous PPAR $\gamma$  were infected with retroviruses expressing MED1/TRAP220(1-530) and induced to differentiate in the absence of rosiglitazone. RNA samples collected at the indicated time points were subjected to Northern blot analysis. (E) Residues 1 to 490 of MED1/TRAP220 cannot rescue the adipogenesis defect in *MED1/TRAP220*<sup>-/-</sup> cells. The experiments were done as described in the legend to panel B. (Left) Immunoblot before induction of adipogenesis; (right) morphological differentiation. (F) Residues 1 to 490 of MED1/TRAP220 are localized to the cytoplasm. COS7 cells transfected with plasmids expressing HA-tagged MED1/TRAP220(1-530) or MED1/TRAP220(1-490) were stained with anti-HA antibody followed by Texas Red-conjugated secondary antibody. Nuclei were labeled with DAPI (4',6'-diamidino-2-phenylindole).

lying molecular mechanism, we examined the cellular localization of MED1/TRAP220 truncations. While residues MED1/TRAP220(1-530) and MED1/TRAP220(1-689) were localized almost exclusively within the nucleus, MED1/TRAP220(1-490) and shorter truncations resided predominantly in the cyto-

plasm (Fig. 4B and F). No classical nuclear localization signal was identified within MED1/TRAP220(1-530), and the addition of triplicate simian virus 40 (SV40) nuclear localization signals failed to relocate MED1/TRAP220(1-490) to the nucleus (data not shown), suggesting that the nuclear localization

of MED1/TRAP220 may be facilitated by MED1/TRAP220-interacting subunits of the Mediator complex. As both the full-length MED1/TRAP220 subunit and the Mediator complex are known to be localized and functional within the nucleus, the cytoplasmic localization could explain, at least in part, the failure of residues 1 to 490 and shorter truncations of MED1/TRAP220 to rescue the adipogenesis defect in *MED1/TRAP220*<sup>-/-</sup> cells. Thus, MED1/TRAP220 requires only its evolutionarily conserved N terminus (residues 1 to 530) to support PPAR $\gamma$ -stimulated adipogenesis. As the demonstrated interaction of PPAR $\gamma$  with the Mediator complex is mediated by the two LXXLL motifs in MED1/TRAP220 (Fig. 2B), these data indicate that the strong ligand-dependent physical interaction between PPAR $\gamma$  and Mediator is not essential for PPAR $\gamma$ -stimulated adipogenesis in the cellular assays.

**The conserved region (residues 1 to 530) of MED1/TRAP220 is incorporated into the Mediator complex but is unable to mediate a demonstrable interaction between Mediator and PPAR $\gamma$ .** Since residues 1 to 530 of MED1/TRAP220 comprise the evolutionarily conserved region and are essential for adipogenesis and in light of our observation that MED1/TRAP220 interacts with the Mediator complex through its N-terminal 670 amino acids (25), we speculated that residues 1 to 530 may suffice for the interaction between MED1/TRAP220 and the rest of the Mediator complex. Indeed, anti-FLAG antibody immunoprecipitated the endogenous Mediator complex from nuclear extracts prepared from *MED1/TRAP220*<sup>-/-</sup> cells expressing FLAG-tagged MED1/TRAP220 (1-530) (Fig. 5A). To screen for the potential binding partner(s) of MED1/TRAP220 within the Mediator complex, in vitro-translated [<sup>35</sup>S]methionine-labeled MED1/TRAP220 (1-530) and individual in vitro-translated Myc-tagged Mediator subunits were mixed and subjected to coimmunoprecipitation with anti-Myc antibody. Of the Mediator subunits tested, MED7 bound strongly to MED1/TRAP220(1-530) (Fig. 5B and data not shown). This result is consistent with the observation that yeast homologs of MED7 and MED1/TRAP220 interact directly and that both reside in the middle module of yeast Mediator (3). These data indicate that the conserved N terminus (amino acids 1 to 530) mediates MED1/TRAP220 interaction with the rest of the Mediator complex and furthermore suggest that the MED1/TRAP220 interaction with the Mediator complex is important for its function in adipogenesis. Consistent with our observation that the PPAR $\gamma$  interaction with the Mediator complex requires the two LXXLL motifs on MED1/TRAP220 (Fig. 2B), the conserved region containing residues 1 to 530 of MED1/TRAP220 was unable to mediate a demonstrable interaction between Mediator and PPAR $\gamma$  (Fig. 5C).

**MED1/TRAP220 is not essential for PPAR $\gamma$ -dependent and ligand-dependent expression of PPAR $\gamma$  target genes in undifferentiated MEFs.** MED1/TRAP220 is important for the expression of adipogenesis markers and PPAR $\gamma$  target genes during adipogenesis (11). However, the dramatic induction of these genes during adipogenesis results from the combined action of multiple adipogenic transcription factors that include C/EBP $\alpha$ , C/EBP $\beta$ , and SREBP1 in addition to PPAR $\gamma$  (35). Also, there exists the possibility that the dramatic loss of the PPAR $\gamma$  target gene expression during adipogenesis may be an indirect consequence of the inability of *MED1/TRAP220*<sup>-/-</sup>

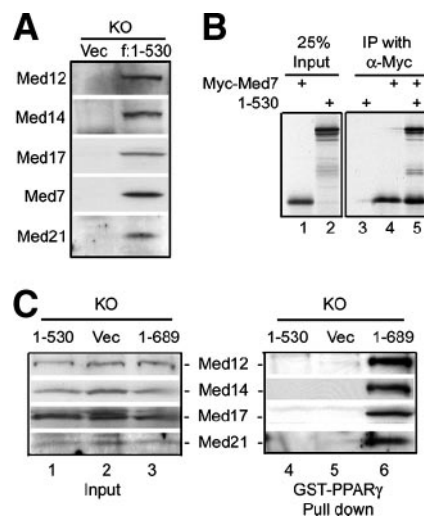


FIG. 5. Residues 1 to 530 of MED1/TRAP220 are incorporated into the Mediator complex but are unable to mediate the interaction between Mediator and PPAR $\gamma$ . (A) Anti-FLAG M2-agarose was used to immunoprecipitate from nuclear extracts of *MED1/TRAP220*<sup>-/-</sup> (knockout [KO]) cells expressing FLAG-tagged MED1/TRAP220(1-530). Bound proteins were analyzed by immunoblotting with antibodies against the indicated Mediator subunits. Vec, vector. (B) The residue sequence 1 to 530 of MED1/TRAP220 interacts with the MED7 subunit of the Mediator complex. <sup>35</sup>S-labeled in vitro-translated MED1/TRAP220(1-530) and Myc-tagged MED7 were mixed and immunoprecipitated with anti-Myc antibody. Bound proteins were washed three times with BC180/0.1% NP-40 and resolved with SDS-PAGE. (C) Mediator containing amino acids 1 to 530 of MED1/TRAP220 does not bind PPAR $\gamma$ . GST-PPAR $\gamma$  was incubated with nuclear extracts from *MED1/TRAP220*<sup>-/-</sup> (KO) cells expressing vector only, MED1/TRAP220(1-530), or MED1/TRAP220(1-689) in the presence of 1  $\mu$ M rosiglitazone. Bound Mediator was monitored by immunoblotting with antibodies to the indicated subunits.

cells to undergo adipogenesis and to express other factors important for optimal PPAR $\gamma$  target gene expression. Therefore, it is unclear from previous reports whether the MED1/TRAP220 requirement reflects a direct involvement in PPAR $\gamma$ -mediated activation of its target genes.

To begin to address this issue, we investigated the requirement of MED1/TRAP220 for PPAR $\gamma$ -induced activation of endogenous target genes in the absence of adipogenesis. Ectopic PPAR $\gamma$  was stably expressed at similar levels in wild-type cells, in *MED1/TRAP220*<sup>-/-</sup> cells, and in *MED1/TRAP220*<sup>-/-</sup> cells expressing exogenous full-length MED1/TRAP220, as described previously (11). Cells were treated with 0.5  $\mu$ M rosiglitazone for 0, 3, 6, 12, 24, or 48 h, and the induction of the PPAR $\gamma$  target genes was monitored by Northern blotting. Importantly, to prevent the potential initiation of adipogenesis, cells were plated at low density, and samples were collected before cells reached confluence. As shown in Fig. 6A, rosiglitazone significantly induced expression of the endogenous PPAR $\gamma$  target genes *aP2*, *ANGPTL4* (*PGAR/FLAF*), *C/EBP $\alpha$* , and *FAS*. Strikingly, the ligand-dependent induction of endogenous PPAR $\gamma$  target genes was completely independent of MED1/TRAP220 status, with similar levels of induction and potency in the three cell lines. The induction of endogenous PPAR $\gamma$  target genes was strictly dependent on exogenous PPAR $\gamma$  and rosiglitazone, as no induction was detected in cells

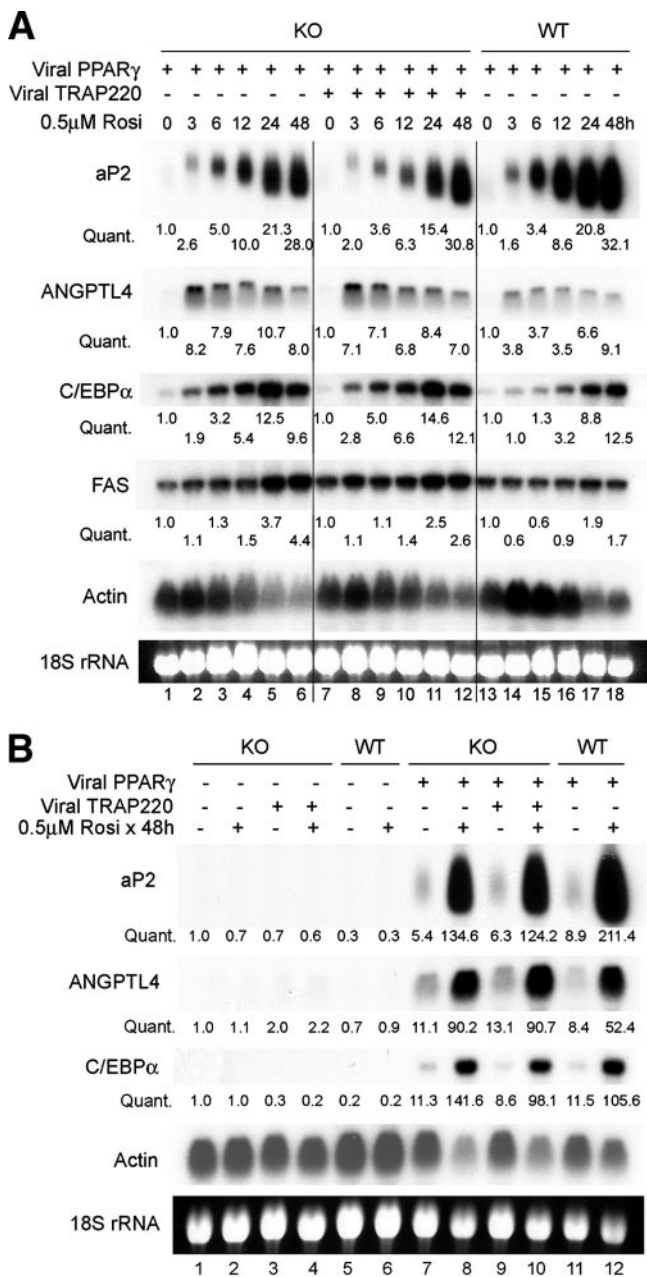


FIG. 6. MED1/TRAP220 is not essential for PPAR $\gamma$ -dependent and ligand-dependent expression of PPAR $\gamma$  target genes in undifferentiated MEFs. (A) MED1/TRAP220 is not essential for PPAR $\gamma$ -dependent and ligand-dependent expression of PPAR $\gamma$  target genes. Subconfluent undifferentiated MEFs were treated with 0.5  $\mu$ M rosiglitazone (Rosi) for 0, 3, 6, 12, 24, or 48 h. Total RNA was extracted and subjected to Northern blotting. (B) The expression of PPAR $\gamma$  target genes in subconfluent MEF cells is strictly dependent on ectopic PPAR $\gamma$  and ligand. Cells were treated with 0.5  $\mu$ M rosiglitazone for 0 or 48 h. Total RNA was extracted and subjected to Northern blotting. When total RNA was collected, cells were at ~80 to 90% confluence (to prevent initiation of adipogenesis). KO, MED1/TRAP220 $^{-/-}$  cells; WT, wild-type cells. Signal intensities after normalization to actin signal are labeled underneath.

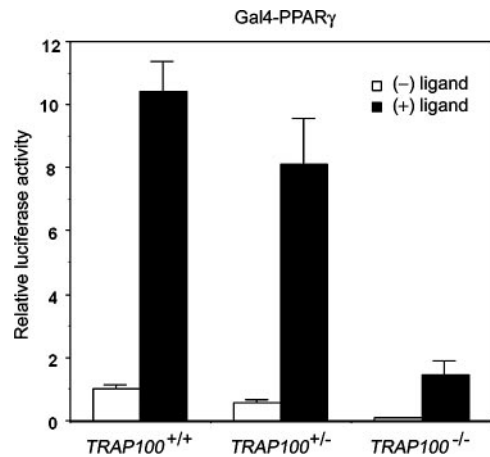


FIG. 7. Defective PPAR $\gamma$  transcriptional activity in MED24/TRAP100-deficient embryonic cells. MED24/TRAP100 $^{+/+}$  or MED24/TRAP100 $^{-/-}$  embryonic cells were cotransfected with a plasmid carrying a Gal4 DNA binding domain-PPAR $\gamma$  (Gal4-PPAR $\gamma$ ) fusion protein and a luciferase reporter with Gal4-binding sites upstream of the SV40 promoter, as described previously (19). Reporter expression levels were normalized to the Renilla luciferase (control) expression levels, which were comparable for wild-type and Med24/TRAP100-deficient cells.

expressing the vector alone (Fig. 6B). Consistent with Northern blotting results, microarray analysis (using samples shown in Fig. 6B, lanes 2, 4, 8, and 10) failed to identify a MED1/TRAP220 requirement for the activation of any of the known PPAR $\gamma$  target genes by ectopic PPAR $\gamma$  and synthetic ligand (data not shown). Thus, MED1/TRAP220 is not essential for PPAR $\gamma$  to activate its target genes in MEF cells, at least under the cell culture conditions and ectopic PPAR $\gamma$  and synthetic ligand treatments employed. As MED1/TRAP220 serves as a bridge for a strong ligand-dependent interaction between PPAR $\gamma$  and Mediator, these results also indicate that a direct interaction between PPAR $\gamma$  and Mediator through MED1/TRAP220 is not essential for ectopic PPAR $\gamma$ -stimulated target gene expression in these cultured, undifferentiated MEFs.

The Mediator complex is thought to be generally required for transcription by RNA polymerase II (17). It has been shown previously that the MED24/TRAP100 component of the Mediator complex is essential in broad transcriptional events and is important for the integrity of the Mediator complex (19). Consistent with these observations, PPAR $\gamma$  showed significantly reduced transcriptional activity in embryonic cells deficient for MED24/TRAP100 (Fig. 7), suggesting that while the MED1/TRAP220 subunit is dispensable for PPAR $\gamma$ -mediated induction of target genes in undifferentiated MEFs, the intact Mediator complex is still required.

Mediator subunits, especially MED1/TRAP220, have been shown to be recruited to the PPAR $\gamma$  response elements (PPRE) of the PPAR $\gamma$  target gene *aP2* (14, 31). To test whether MED1/TRAP220 is essential for the recruitment of Mediator to the *aP2* promoter in undifferentiated MEFs, ChIP assays were performed with MED1/TRAP220 $^{-/-}$  cells expressing exogenous PPAR $\gamma$  in the presence of 0.5  $\mu$ M rosiglitazone. As shown in Fig. 8A, MED23/Sur2, which is an integral subunit of the Mediator complex, was recruited to the PPRE-containing region on the *aP2* promoter, indicating that the Mediator



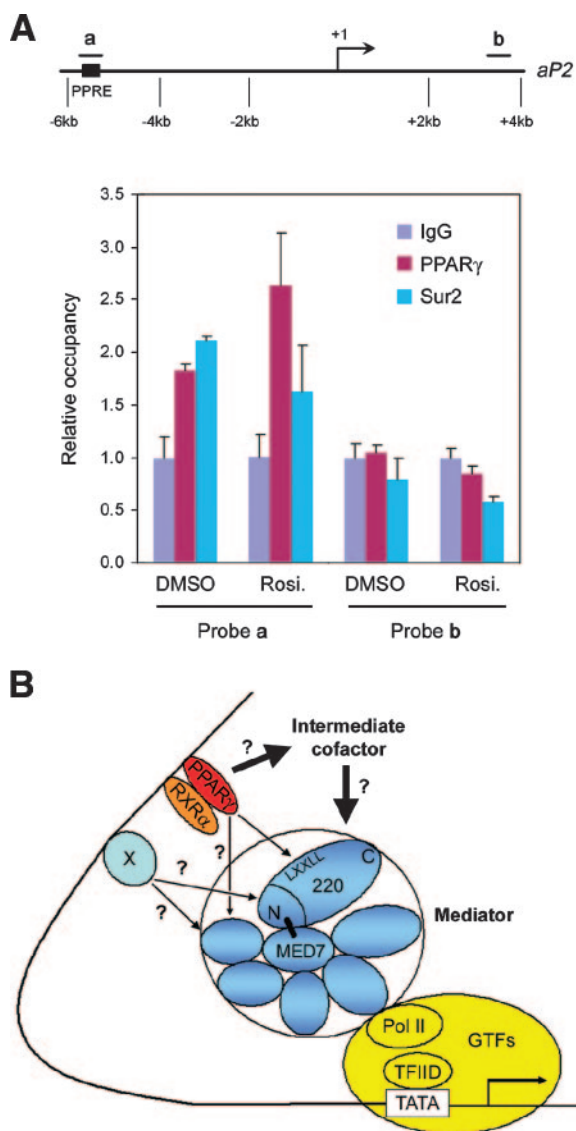


FIG. 8. PPAR $\gamma$  and subunits of the Mediator complex are recruited to the PPAR $\gamma$ -response element of the *aP2* enhancer in the absence of MED1/TRAP220. (A) ChIP assay. (Top) Schematic representation of the mouse *aP2* promoter. The positions of TaqMan probes are indicated. (Bottom) Subconfluent undifferentiated *MED1/TRAP220*<sup>-/-</sup> MEFs expressing ectopic PPAR $\gamma$  were treated with 0.5  $\mu$ M rosiglitazone (Rosi.) or vehicle (DMSO) alone for 24 h, followed by ChIP assays with the indicated antibodies. Immunoprecipitated DNA was quantified by real-time PCR with TaqMan probes a and b. (B) Model of mechanisms underlying MED1/TRAP220 and Mediator regulation of PPAR $\gamma$ -stimulated adipogenesis and gene expression.

complex can be recruited to the PPRE in the absence of a direct PPAR $\gamma$  interaction that involved MED1/TRAP220. Notably, however, PPAR $\gamma$  and Mediator are constitutively bound to the promoter in these cells. This result is consistent with the dispensability of MED1/TRAP220, which normally shows ligand-dependent interactions with PPAR $\gamma$ , and with Mediator recruitment through an alternative mechanism that could involve interactions of PPAR $\gamma$  or of other DNA-bound accessory factors with other Mediator subunits (see Discussion).

DISCUSSION

In this report, structural and functional analyses of MED1/TRAP220 have been directed toward an understanding of the mechanisms by which the Mediator complex and the MED1/TRAP220 subunit regulate PPAR $\gamma$ -stimulated adipogenesis and target gene expression. We show (i) that direct, ligand-dependent physical interaction between PPAR $\gamma$  and Mediator (11) requires the two LXXLL motifs in MED1/TRAP220, (ii) that a direct physical interaction between PPAR $\gamma$  and Mediator through these motifs is not required for PPAR $\gamma$ -stimulated adipogenesis, (iii) that the minimal region of MED1/TRAP220 required for adipogenesis lies in the evolutionarily conserved amino-terminus (amino acids 1 to 530) that is incorporated into the Mediator complex but unable to mediate a demonstrable interaction between the PPAR $\gamma$  and the Mediator complex, and (iv) that in cultured, undifferentiated MEFs, MED1/TRAP220 is required neither for ectopic PPAR $\gamma$ -mediated activation of several known target genes nor for the recruitment of Mediator to the PPRE on the *aP2* target gene. The implications of these findings for our current understanding of the Mediator function in the regulation of transcription, especially by nuclear receptors, are discussed.

**Physical and functional interaction of PPAR $\gamma$  with mediator through the MED1/TRAP220 LXXLL motifs.** Given its critical role in activator-driven transcription of most (17), but not necessarily all (9), genes, a critical question concerns the mechanism by which Mediator is recruited to target promoters. Biochemical and genetic evidence strongly suggest a mechanism in which different (promoter-bound) activators recruit Mediator through interactions with distinct Mediator subunits (4, 21, 26). Relevant to the present study, many nuclear receptors have been shown to interact, in a ligand-dependent manner, with the Mediator MED1/TRAP220 subunit, through interactions between the liganded AF2 receptor domain and the MED1/TRAP220 LXXLL motifs (8, 20, 30, 32, 33, 38, 39, 43, 44), and studies of mutated Mediator complexes have established a key role for MED1/TRAP220 and resident LXXLL domains in strong interactions between TR $\alpha$  and the complete Mediator complex (25).

In the case of PPAR $\gamma$ , our previous studies have shown a direct ligand-dependent interaction between PPAR $\gamma$  and Mediator that is dependent upon MED1/TRAP220 (11). Similar to our earlier demonstration of an intracellular TR $\alpha$ -Mediator complex (10), we now have shown an intracellular PPAR $\gamma$ -Mediator interaction and, furthermore, that this interaction depends upon the LXXLL motifs in MED1/TRAP220. Beyond the physical interaction assays, transcription assays with mutant Mediator complexes in a cell-free system reconstituted with purified factors have shown that the intact MED1/TRAP220 LXXLL motifs are essential for optimal Mediator-dependent PPAR $\gamma$  function. These results clearly establish the potential for MED1/TRAP220 LXXLL-dependent Mediator function but do not eliminate the possibility that other cellular cofactors (not present in the purified system) might bypass the LXXLL motif requirement (see further discussion below).

**In vitro differentiation of MEFs into adipocytes in the absence of the MED1/TRAP220 LXXLL motifs.** PPAR $\gamma$  plays a key role in adipogenesis, both in vivo and in vitro (cell culture) (reviewed in reference 34), and the in vitro MEF differentia-

tion systems have provided powerful tools for analyzing PPAR $\gamma$  gene activation mechanisms through various cofactors (42). As a follow-up to our previous study demonstrating a key role for MED1/TRAP220 in PPAR $\gamma$ -dependent adipogenesis (11), we have analyzed the structure-function relationships of MED1/TRAP220. Remarkably, in the in vitro MEF differentiation assay, an N-terminal fragment (residues 1 to 530) that lacks both of the LXXLL motifs proved sufficient for normal levels of adipogenesis and for attendant PPAR $\gamma$  target gene expression. This fragment corresponds to the only phylogenetically conserved part of MED1 and, since it is incorporated normally into the Mediator complex, likely functions through the Mediator complex. MED1/TRAP220(1-530)-mediated adipogenesis is apparent in both the presence and the absence of an ectopic PPAR $\gamma$  ligand (rosiglitazone), such that this surprising result cannot be attributed to an abnormally (hyper-) activated PPAR $\gamma$ . From a mechanistic view, these results indicate that, at least in cultured cells, a strong PPAR $\gamma$ -Mediator interaction mediated by the MED1/TRAP220 LXXLL motifs is not required for the PPAR $\gamma$  functions important for adipogenesis. As discussed below, there may be alternate, possibly redundant, mechanisms for Mediator recruitment. Should this be the case and should these be completely independent of MED1/TRAP220, a key unanswered question concerns the role of the MED1/TRAP220(1-530) fragment that is essential for adipogenesis. Finally, while the MED1/TRAP220 LXXLL motifs are clearly dispensable for in vitro differentiation of MEFs to adipocytes, it remains to be determined if this is true in vivo.

**PPAR $\gamma$  target gene activation and mediator recruitment in the absence of MED1/TRAP220.** Since the apparent MED1/TRAP220 subunit requirement for PPAR $\gamma$  target gene expression in adipogenesis might be indirect, a role for MED1/TRAP220 in the ligand-dependent function of ectopic PPAR $\gamma$  in subconfluent undifferentiated MEFs was explored. Remarkably, ligand- and ectopic PPAR $\gamma$ -dependent expression of a number of PPAR $\gamma$  target genes was found to be comparable in wild-type and *MED1/TRAP220*<sup>-/-</sup> cells. While these results do not rule out a direct role for MED1/TRAP220 in PPAR $\gamma$  target gene activation during adipogenesis, they clearly indicate the potential for MED1/TRAP220-independent PPAR $\gamma$  functions that might be relevant to endogenous PPAR $\gamma$  function in certain responses. Despite the lack of the MED1/TRAP220 requirement for ectopic PPAR $\gamma$  function in this assay, ChIP assays indicated Mediator recruitment, consistent with Mediator function, to the PPRE of the *aP2* PPAR $\gamma$  target gene. The ligand-independent nature of this recruitment is consistent with a mechanism that does not involve MED1/TRAP220.

While the present results do not rule out Mediator recruitment and function through MED1/TRAP220 when this cofactor is present, they do suggest alternative, potentially redundant, pathways for Mediator recruitment and/or PPAR $\gamma$  function. Thus, as indicated in Fig. 8B, intermediate cofactors that are recruited directly by PPAR $\gamma$  may interact with and recruit Mediator. Such a model is also consistent with the recent report that both the SWI/SNF and the Mediator complexes can be targeted to a TR target gene through p300, which itself is recruited through interaction with SRC nuclear receptor coactivators (18). Similarly, others have reported interac-

tion between Mediator and a group of factors that includes the nuclear receptor coactivator NCOA6/PRIP/ASC-2 (22, 37), which is a component of a histone methyltransferase complex (6, 13, 23). The demonstration of physical and functional interactions of Mediator with PPAR $\gamma$  coactivator  $\alpha$  (PGC-1 $\alpha$ ) provides another example of a mechanism for indirect recruitment of Mediator (41). Significantly, however, and in agreement with the observed ligand-independent recruitment of Mediator to an RAR-activated promoter (29), Mediator recruitment to the rosiglitazone-activated *aP2* promoter in undifferentiated MEFs is ligand-independent. This suggests recruitment via a PPAR $\gamma$  AF2 domain-independent mechanism. Potentially relevant, glucocorticoid receptor has been shown to interact in a ligand-independent manner with the MED14/TRAP170 component of Mediator (16), and recent studies indicate that MED1/TRAP220 and MED14/TRAP170 are differentially required for different GR target genes (5).

Hence, there exists the possibility of as-yet-undescribed interactions between PPAR $\gamma$  and other Mediator subunits. Such interactions might be substantially weaker than the ligand-dependent PPAR $\gamma$ -MED1/TRAP220 interaction and demonstrable only in the context of multicomponent receptor-cofactor complexes on the promoter. As indicated in the Fig. 8B model and given that most promoters employ multiple DNA-binding factors, the Mediator might be recruited by another constitutively bound factor but remain poised until other ligand-induced events occur (28, 29). Finally, recent demonstrations of Mediator-dependent basal (activator-independent) transcription and Mediator interactions with components of the general transcription machinery indicate the potential for activator-independent recruitment of the Mediator with target genes (1, 2, 27). MED1/TRAP220- and MED1/TRAP220 LXXLL motif-independent PPAR $\gamma$  target gene activation mechanisms, and the identification of relevant cofactors, must be the subject of future investigation.

**Specialized roles for MED1/TRAP220 LXXLL domains?** The short LXXLL sequence has been identified as a signature motif that mediates the binding of a number of coactivators to the ligand binding domains of nuclear receptors (15), and there is detailed structural and functional information about the role of these motifs in nuclear receptor function in various (mostly cell-based) assays. In view of the nearly universal function of the Mediator complex in gene activation events in yeast (17), and presumably metazoans, it is notable that MED1/TRAP220 is the only Mediator subunit known to bind to the ligand-induced AF2 domains of nuclear receptors and that various in vitro assays have established the importance of the LXXLL domain for optimal receptor function. Nonetheless, as emphasized by the current study, the in vivo significance of the direct interactions between the PPAR $\gamma$  and the Mediator through the MED1/TRAP220 LXXLL motifs remains unclear. Such an interaction may be important for the expression of select (as-yet-unidentified) PPAR $\gamma$  target genes in a ligand- and tissue-specific manner or for the expression of a broader set of target genes when the activities of alternate, potentially redundant cofactors are limiting. The resolution of this question will depend upon more complete information on all cofactors involved in PPAR $\gamma$  function and effects of combinatorial knockouts and (mutant) knockins of these factors.

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