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Poly(ADP-ribose) polymerase-2 controls adipocyte differentiation and adipose tissue function through the regulation of the activity of the retinoid X receptor/peroxisome proliferator-activated receptor- γ heterodimer.

Péter Bai, Sander M. Houten, Aline Huber, Valérie Schreiber, Mitsuhiro Watanabe, Borbála Kiss, Gilbert de Murcia, Johan Auwerx, and Josiane Ménissier-de Murcia

There was an error in the title of the article. The correct title is shown above.

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Novel binding site for Src Homology 2-containing protein-tyrosine phosphatase-1 in CD22 activated by B lymphocyte stimulation with antigen.

Chenghua Zhu, Motohiko Sato, Teruhiko Yanagisawa, Manabu Fujimoto, Takahiro Adachi, and Takeshi Tsubata

Dr. Adachi was inadvertently omitted as an author of this article. The correct authors are listed above. Dr. Adachi's affiliation is the Laboratory of Immunology, School of Biomedical Science, and the Department of Immunology, Medical Research Institute, Tokyo Medical and Dental University and Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, 113-8510 Tokyo, Japan.

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The localization and activity of sphingosine kinase 1 are coordinately regulated with actin cytoskeletal dynamics in macrophages.

David J. Kusner, Christopher R. Thompson, Natalie A. Melrose, Stuart M. Pitson, Lina M. Obeid, and Shankar S. Iyer

On Page 23157, the final sentence of the legend to Fig. 8 should read as follows: Data represent the mean \pm S.D. of duplicate determinations from a single representative experiment of a total of four identical experiments. On Page 23158, there is an error in the data in Fig. 9 (A–C), and these three panels should be retracted.

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Peroxisome Proliferator-activated Receptor (PPAR)-2 Controls Adipocyte Differentiation and Adipose Tissue Function through the Regulation of the Activity of the Retinoid X Receptor/PPAR γ Heterodimer*

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Péter Bai^{‡§1}, Sander M. Houten^{¶||}, Aline Huber[‡], Valérie Schreiber[‡], Mitsuhiro Watanabe[¶], Borbála Kiss[‡], Gilbert de Murcia[‡], Johan Auwerx^{¶***}, and Josiane Ménissier-de Murcia[‡]

From the [‡]Département Intégrité du Génome, UMR 7175, CNRS, Ecole Supérieure de Biotechnologie de Strasbourg, BP 10413, Illkirch 67412, France, the [¶]Institut de Génétique et Biologie Moléculaire et Cellulaire, 1 Rue Laurent Fries, BP 10142, Illkirch 67404, France, the [§]Department of Medical Chemistry, Medical and Health Science Center, University of Debrecen, Nagyerdei krt. 98, Pf. 7., Debrecen 4032, Hungary, the ^{||}Laboratory of Genetic Metabolic Diseases, Academic Medical Center, Amsterdam 1105 AZ, The Netherlands, and the ^{***}Institut Clinique de la Souris, 1 Rue Laurent Fries, BP 10142, Illkirch 67404, France

The peroxisome proliferator-activated receptor- γ (PPAR γ , NR1C3) in complex with the retinoid X receptor (RXR) plays a central role in white adipose tissue (WAT) differentiation and function, regulating the expression of key WAT proteins. In this report we show that poly(ADP-ribose) polymerase-2 (PARP-2), also known as an enzyme participating in the surveillance of the genome integrity, is a member of the PPAR γ /RXR transcription machinery. PARP-2^{-/-} mice accumulate less WAT, characterized by smaller adipocytes. In the WAT of PARP-2^{-/-} mice the expression of a number of PPAR γ target genes is reduced despite the fact that PPAR γ 1 and - γ 2 are expressed at normal levels. Consistent with this, PARP-2^{-/-} mouse embryonic fibroblasts fail to differentiate to adipocytes. In transient transfection assays, PARP-2 small interference RNA decreases basal activity and ligand-dependent activation of PPAR γ , whereas PARP-2 overexpression enhances the basal activity of PPAR γ , although it does not change the maximal ligand-dependent activation. In addition, we show a DNA-dependent interaction of PARP-2 and PPAR γ /RXR heterodimer by chromatin immunoprecipitation. In combination, our results suggest that PARP-2 is a novel cofactor of PPAR γ activity.

Adipose tissue is composed of adipocytes that store energy in the form of triglycerides. Excessive accumulation of white adi-

pose tissue (WAT)² leads to obesity, whereas its absence leads to lipodystrophic syndromes. The peroxisome proliferator-activated receptor- γ (PPAR γ , NR1C3) is the main protein orchestrating the differentiation and function of WAT, as evidenced by the combination of *in vitro* studies, the analysis of mouse models, and the characterization of patients with mutations in the human PPAR γ gene (1, 2). PPAR γ acts as heterodimer with the retinoid X receptor (RXR) (3). The PPAR γ /RXR receptor dimer is involved in the transcriptional control of energy, lipid, and glucose homeostasis (4, 5). The actions of PPAR γ are mediated by two protein isoforms, the widely expressed PPAR γ ₁ and adipose tissue-restricted PPAR γ ₂, both produced from a single gene by alternative splicing and differing only by an additional 28 amino acids in the N terminus of PPAR γ ₂ (3, 6).

PPAR γ is activated by binding of small lipophilic ligands, mainly fatty acids, derived from nutrition or metabolic pathways, or synthetic agonists, like the anti-diabetic thiazolidenediones (2, 7, 8). Docking of these ligands in the ligand binding pocket alters the conformation of PPAR γ , resulting in transcriptional activation subsequent to the release of corepressors and the recruitment of coactivators. Many corepressors and coactivators have been described such as the nuclear receptor corepressor and the steroid receptor coactivators, also known as p160 proteins (9–11). These corepressors and coactivators determine transcriptional activity by altering chromatin structure via enzyme such as histone deacetylases and histone acetyltransferases (CREB-binding protein/p300). Other mechanisms include DNA methylation, ATP-dependent remodeling, protein phosphorylation, sumoylation, ubiquitinylation, and poly(ADP-ribosylation).

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This work is dedicated to the memory of Josiane Ménissier-de Murcia, who passed away (July 15, 2007).

¹ To whom correspondence should be addressed. Tel.: 36-52-412-345; Fax: 36-52-412-566; E-mail: baip@dote.hu.

² The abbreviations used are: WAT, white adipose tissue; PPAR, peroxisome proliferator-activated receptor; PARP-1 and -2, poly(ADP-ribose) polymerase-1 and -2; TTF1, thyroid transcription factor-1; WT, wild type; RT-qPCR, reverse transcription-coupled quantitative PCR; aP2, adipocyte fatty acid-binding protein 2; ER β , estrogen receptor β ; K19, keratin-19; ChIP, chromatin immunoprecipitation; RXR, retinoid X receptor; HEK 293, human embryonic kidney 293; TNF α , tumor necrosis factor α ; CREB, cAMP-response element-binding protein; DMEM, Dulbecco's modified Eagle's medium; MEF, mouse embryonic fibroblast; siRNA, small interference RNA; BES, 2[bis(2-hydroxyethyl)amino]ethanesulfonic acid.

TABLE 1

Oligonucleotides used to generate pSuper-siPARP-2 and pSuper-scrPARP-2

The interfering sequences are in bold.

Name	Sequence (5'-3')	Structure
siPARP-2 sense	GATCT AAGATGATGCCAGAGGA ACTTTCAGAGAA AGTTCTCTGGGCATCATCTT TTTTTA	BglII/sense/loop/antisense/T(5)/HindIII
siPARP-2 antisense	AGCTTAAAA AAGATGATGCCAGAGGA ACTTCTCTGAA AGTTCTCTGGGCATCATCTT A	HindIII/T(5)/antisense/loop/sense/BglII
scrPARP-2 sense	GATCT TTCGGGGAACAACGTGCA ACTTTCAGAGAA GTGCACGTTTGTCCCGAA TTTTTA	BglII/sense/loop/antisense/T(5)/HindIII
scrPARP-2 antisense	AGCTTAAAA TTCGGGGAACAACGTGCA ACTTCTCTGAA GTGCACGTTTGTTC CCGAAA	HindIII/T(5)/antisense/loop/sense/BglII

Poly(ADP-ribose) polymerase-2 (PARP-2) was described by Ame *et al.* (12) in 1999 as a 66.2-kDa nuclear protein with poly-(ADP-ribosyl)ating activity. Through its DNA-binding domain in the N terminus (amino acids 1–62), PARP-2 can bind to DNase I-treated DNA and to aberrant DNA forms, and its subsequent activation results in poly(ADP-ribose) polymer formation (12). According to the general scheme of PARP activation, the active enzyme catalyzes the polymerization of poly(ADP-ribose) polymer onto different acceptor proteins and itself using NAD⁺ as a substrate (13). PARP-2 shares a similar catalytic domain (amino acid 202–593) as poly(ADP-ribose) polymerase-1 (PARP-1) (14), the founding member of the PARP family, though PARP-2 has a smaller reaction velocity compared with PARP-1 (12).

PARP-2 has multiple *in vivo* functions comprising DNA surveillance and DNA repair processes (reviewed in Ref. 15), spermatogenesis (16, 17), inflammation, and oxidative injury (18–20). Most of these functions are accomplished through protein-protein interactions. In PARP-2, the interaction platforms can be mapped to the DNA-binding domain and to the domain E (amino acids 63–202) (21–25). A role for PARP-2 in the regulation of transcription has already been described. In lung epithelial cells PARP-2 interacts with thyroid transcription factor-1 (TTF1). TTF1 is a homeodomain-containing transcription factor of the Nkx-2 family. In these cells, PARP-2 regulates the expression of the surfactant protein-B by affecting TTF1 activity (25). In this study we show that PARP-2 affects the transcriptional activity of PPAR γ both *in vitro* and *in vivo*.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were from Sigma-Aldrich unless stated otherwise.

Animals—PARP-2^{-/-} mice and their wild-type (WT) littermates (26) coming from heterozygous crossings were used. Mice were housed separately, had *ad libitum* access to water and chow, and were kept under a 12-h dark-light cycle. The animals were killed at the age of 7 months by cervical dislocation after 4 h of fasting, and tissues were collected.

Cell Culture—3T3-L1 cells were maintained in DMEM (Invitrogen), 10% newborn calf serum (Invitrogen), Gentamicin (Invitrogen), and HEK, and mouse embryonic fibroblasts (MEFs) were maintained in DMEM, 10% fetal calf serum (Adgenix, Voisins le Bretonneux, France), and Gentamicin (Invitrogen). The 3T3-L1 cells were maintained subconfluent.

MEF Preparation and Differentiation—MEFs were prepared from embryos as described elsewhere (26). For the differentiation studies 4 × 10⁵ MEFs were seeded in 12-well plates and maintained in DMEM, 10% fetal calf serum. The medium was changed every 2 days until confluence. The cells were maintained at confluency for 2 days. Cells were then differentiated in

DMEM, 10% newborn calf serum, 5 μ M troglitazone, 5 μ M dexamethasone, 500 μ M isobutylmethylxanthine, and 10 μ g/ml insulin (later defined as differentiation mix), while the control cells received DMEM, 10% fetal calf serum, and Me₂SO as vehicle. The medium with the differentiation mix was replaced every 2 days, and the cells were differentiated for 8 days. Control cells after confluency were cultured in DMEM plus 10% fetal calf serum containing only vehicle (Me₂SO, 0.21%).

DNA Constructs—To create an siRNA-expressing construct, double stranded oligonucleotides were cloned into the pSuper vector (for sequences see Table 1) (27). The oligonucleotides siPARP-2sense and siPARP-2antisense (containing the siRNA sequence), as well as the control scrPARP-2sense and scrPARP-2antisense (scrambled version of the siRNA sequence), respectively, were annealed in annealing buffer (150 mM NaCl, 1 mM EDTA, 50 mM Hepes, pH. 8.0). The resulting duplexes carried BglII and HindIII sites and were cloned into pSuper using these sites resulting in pSuper-siPARP-2 (oligonucleotides siPARP-2sense plus siPARP-2antisense) and pSuper-scrPARP-2 (oligonucleotides scrPARP-2sense plus scrPARP-2antisense). An EcoRV/SmaI fragment encoding mouse PARP-2 was isolated from pBC-mPARP-2 (23) and inserted into the SmaI site of pBABEpuro (Addgene, Cambridge, MA), giving the pBAbE-mPARP-2 vector. All other constructs pGL3-(J_{wt})₃TKLuc reporter construct (28), pSG-PPAR γ ₂ (3), pSG5-PPAR α (29), pSG5-PPAR β (30), pCMX-ER β , and vitellogeninA2-ERE-TKLuc (ER-luc) (31) were described before. The pCMV- β Gal construct was used to control the transfection efficiency.

Transfections—Transfections were performed either by the BES-buffered saline method (26) or by JetPei (Polyplus Transfections, Illkirch, France).

Luciferase Activity Measurement—3 × 10⁵ HEK cells were seeded in 6-well plates and were transfected with pSuper-siPARP-2, pSuper-scrPARP-2, pBabe, or pBabe-PARP-2 using the BES-buffered saline method. Two days later the cells were once more transfected with the constructs mentioned above. Cells were transfected 24 h later with 0.6 μ g of pSuper-siPARP-2/pSuper-scrPARP-2/pBabe/pBabe-PARP-2, 0.4 μ g of β -galactosidase expression plasmid, 1 μ g of pSG-PPAR α /pSG-PPAR β /pSG-PPAR γ ₂/pCMX-ER β expression vector, and 1 μ g of PPAR-/ER-responsive construct. Six hours after transfection, cells were scraped, and luciferase activity was determined. For the determination of PPAR activity, just before transfection, cells were washed in serum-free DMEM medium, and the transfection was carried out in DMEM plus 10% fat-free serum. As ligand we used, fenofibrate (50 μ M), monoethylhexyl phthalate (100 μ M), troglitazone (5 μ M), and β -estradiol (10 μ M). After 6 h of transfection, cells were washed with phosphate-buffered saline, scraped, and stored at -80 °C. Luciferase assay was car-

TABLE 2

qPCR primers

FAS, fatty acid synthase; LPL, lipoprotein lipase; HSL, hormone-sensitive lipase.

Name	Sequence (5'-3')	Accession number
Adiponectin	F 5'-AAG AAG GAC AAG GCC GTT CTC TT-3' (652-674) R 5'-GCT ATG GGT AGT TGC AGT CAG TT-3' (875-853)	NM_009605.4
aP2	F 5'-TGC CAC AAG GAA AGT GGC AG-3' (132-151) R 5'-CTT CAC CTT CCT GTC GTC TG-3' (294-275)	BC054426
CD36	F 5'-GAT GTG GAA CCC ATA ACT GGA TTC AC-3' (1378-1403) R 5'-GGT CCC AGT CTC AAT TAG CCA CAG TA-3' (1527-1502)	NM_007643
Cyclophilin B	F 5'-TGG AGA GCA CCA AGA CAG ACA-3' (561-581) R 5'-TGC CGG AGT CGA CAA TGA T-3' (626-608)	M60456
FAS	F 5'-GCT GCG GAA ACT TCA GGA AAT-3' (6612-6632) R 5'-AGA GAC GTG TCA CTC CTG GAC TT-3' (6695-6673)	BC046513
LPL	F 5'-AGG ACC CCT GAA GAC AC-3' (317-333) R 5'-GGC ACC CAA CTC TCA TA-3' (465-449)	BC003305
Leptin	F 5'-GAC ACC AAA ACC CTC AT-3' (147-163) R 5'-CAG AGT CTG GTC CAT CT-3' (296-280)	NM_008493
Perilipin	F 5'-GCT TCT TCC GGC CCA GC-3' (1511-1527) R 5'-CTC TTC TTG CGC AGC TGG CT-3' (1580-1561)	NM_175640
PPAR γ_1	F 5'-CCA CCA ACT TCG GAA TCA GCT-3' (158-178) R 3'-TTT GTG GAT CCG GCA GTT AAG A-3' (591-570)	NM_011146
PPAR γ_2	F 5'-ATG GGTG AAA CTC TGG GAG ATT CT-3' (46-69) R 5'-CTT GGA GCT TCA GGT CAT ATT TGT A-3' (346-322)	AY243585
HSL	F 5'-CCT CAT GGC TCA ACT CC-3' (1633/2075-1649/2091) R 5'-GGT TCT TGA CTA TGG GTG A-3' (2067/2509-2049/2491)	NM_001039507.1/NM_010719.5
TNF α	F 5'-GCC ACC ACG CTC TTC TG-3' (286-302) R 3'-GGT GTG GGT GAG GAG CA-3' (627-611)	NM_013693.2

TABLE 3

Chip primers

Name	Sequence	Reference
aP2	F 5'-CCC AGC AGG AAT CAG GTA GC-3' R 5'-AGA GGG CGG AGC AGT TCA TC-3'	52
CD36	F 5'-TTT GCT GGG ACA GAC CAA TC-3' R 5'-GCC ATG TTC CCA TCC AAG TA-3'	39
K19	F 5'-AAG GGT GGA GGT GTC TTG GT-3' R 5'-GCT TCT TTA CAC TCC TGC T AAA-3'	AF237661

ried out by standard procedures. Luciferase activity was expressed as luciferase activity/ β -galactosidase activity.

Nile Red Flow Cytometry—To assess the extent of MEF differentiation, cytosolic triglyceride content was assessed by determining Nile red uptake (modified from Ref. 32) followed by flow cytometry using a FACSCalibur machine (BD Biosciences). Cells were harvested by adding trypsin/EDTA, and the detached cells were stained with Nile red (20 μ g/ml, 5 min). Cells were subjected to flow cytometric analysis with 10,000 events collected for each sample; each measurement point was repeated in 4 parallel replicates. Samples for each cell line were normalized against the non-differentiated cells of the same line. The rate of differentiation was expressed as the percentage of the differentiated cells *versus* total number of cells.

SDS-PAGE and Western Blotting—Cells were lysed in lysis buffer (50 mM Tris, 500 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor mixture, pH 8.0). Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. For the detection of PARP-2, a polyclonal rabbit antibody was used 1:2,000, Alexis, Lausen, Switzerland), and actin was as detected using a rabbit polyclonal antibody (Sigma, 1:200). The secondary antibody was IgG-peroxidase conjugate (Sigma, 1:10,000). Reactions were developed by enhanced chemiluminescence (Amersham Biosciences, Little Chalfont, UK).

Total RNA Preparation, Reverse Transcription, and qPCR—Total RNA was prepared using TRIzol (Invitrogen) according to the manufacturer's instructions. RNA was treated with DNase, and 2 μ g of RNA was used for reverse transcription (RT). cDNA was purified on QIAquick PCR cleanup columns (Qiagen, Valencia, CA). 50 \times diluted cDNA was used for quantitative PCR (qPCR) reactions. The qPCR reactions were preformed using the Light-Cycler system (Roche Applied Science) and a qPCR Supermix (Qiagen) with the primers summarized in Table 2.

Chromatin Immunoprecipitation—Chromatin immunoprecipitation was performed according to a previous study (33) on

3T3-L1 cells using α -PARP-2, α -PPAR γ_2 (Alexis), and α -matrix metalloproteinase-9 (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. We used also a no antibody control. The chromatin fragments collected upon precipitation with the above antibodies were amplified using promoter-specific primers by qPCR. For the analysis of the coding sequence the same qPCR primer set was used as the one for the quantitation of the given gene. The respective primers are listed in Tables 2 and 3. The results were normalized for the signal of the input and were expressed as a percentage of the aP2 signal with the PARP-2 antibody.

For the testing of the K19 primer set we used non-confluent 3T3-L1 cells transfected with pCMX-ER β . Chromatin immunoprecipitation was performed using the α -ER β (Santa Cruz Biotechnology), and as controls we used an α -MRE11 (Santa Cruz Biotechnology) and a no antibody control. The chromatin fragments collected upon precipitation with the above antibodies were amplified using K19 promoter-specific primers by qPCR.

Microscopy—Formaldehyde-fixed, paraffin-embedded sections (7 μ m) were made from WAT samples and were stained with hematoxylin and eosine. The same sections were stained with a biotin-conjugated F4/80 antibody (Sero-tec, Raleigh, NC, 1:100 dilution), and the bound primary antibodies were detected using streptavidin-peroxidase (Vector ABC kit) and diaminobenzidine as chromogenic substrate. Terminally differentiated MEFs were stained by Oil red O as described elsewhere.

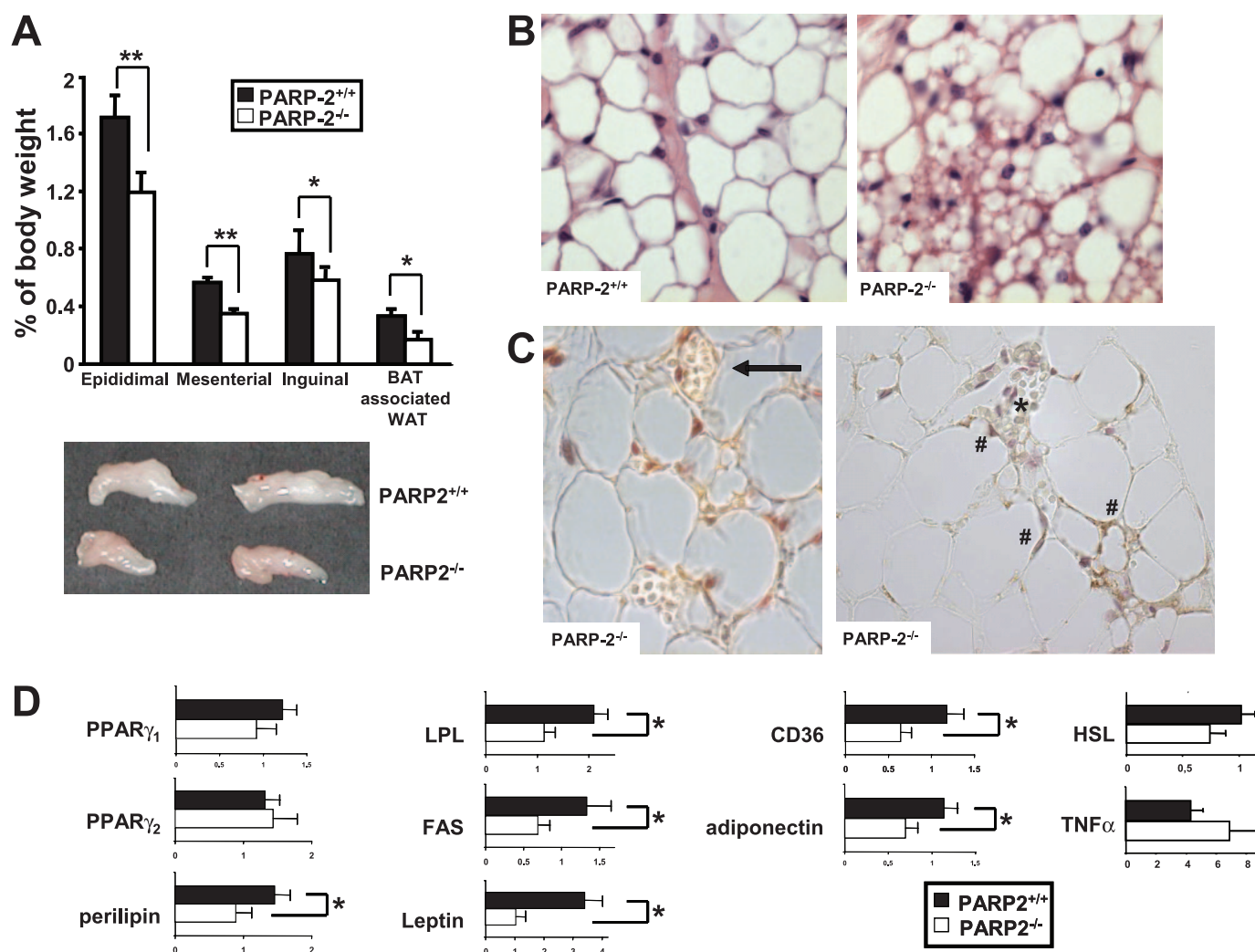


FIGURE 1. Abnormal WAT function in PARP-2^{-/-} mice. A, weight and macroscopic view of different adipose tissue depots in PARP-2^{+/+} and PARP-2^{-/-} mice (age of 7 months). In the PARP-2^{-/-} mice there is a significant reduction of the different fat depots. Error bars represent \pm S.E. *, $p < 0.05$; **, $p < 0.01$. B, the epididymal WAT stained with H&E (100 \times magnification). C, the arrow points toward a dilated capillary in the PARP-2^{-/-} epididymal WAT (100 \times magnification, H&E). Staining with the F4/80 antibody detects macrophages (marked by #) in the vicinity of the dilated capillaries (*). D, gene expression in epididymal WAT. *, $p < 0.05$.

Triglyceride Measurement—The triglyceride content of the MEFs was determined using a commercially available Sigma kit according to the manufacturer's instructions.

Statistical Analysis—Significance was analyzed by Student's *t* test. Error bars represent \pm S.E., unless noted otherwise.

RESULTS

In Vivo Dysfunction of the PPAR γ /RXR Heterodimer in the WAT of PARP-2^{-/-} Mice—The different fat depots (epididymal, mesenteric, and inguinal) and the interscapular brown adipose tissue-associated WAT were measured in 7-month-old PARP-2^{-/-} mice and their wild-type littermates. A proportional loss of the weight of all adipose tissue depots was observed in the PARP-2^{-/-} mice (Fig. 1A).

Histological examination of the PARP-2^{-/-} epididymal WAT showed adipocytes with reduced and irregular size. This tissue contained diluted capillaries, indicative of inflammation, which was confirmed by a faint staining with the macrophage-specific F4/80 antibody in the PARP-2^{-/-} (Fig. 1, B and C) and

the macroscopic appearance of the WAT (Fig. 1A). The F4/80-positive cells were present in the vicinity of the blood vessels.

To identify the molecular changes that contribute to the decreased fat accumulation and abnormal adipocyte morphology, we determined the expression of the PPAR γ target genes, TNF α , and hormone-sensitive lipase by RT-qPCR in the epididymal WAT.

TNF α expression was undetectable in 8 of the 22 mice used for this study (4 out of 14 PARP-2^{+/+} and 4 out of 8 PARP-2^{-/-}). In the TNF α -positive mice, expression levels were not different, ruling out a major role for inflammation in the adipose tissue dysfunction in PARP-2^{-/-} mice. The expression level of hormone-sensitive lipase, which is responsible for lipolysis, was also not different between the two genotypes. The expression of several PPAR γ target genes, however, was markedly decreased. These include genes involved in chylomicron and very low density lipoprotein triglyceride hydrolysis (lipoprotein lipase), free fatty acid uptake (CD36), *de novo* fatty acid synthesis, and endocrine signaling (leptin and adiponectin) (Fig. 1D). Interestingly,

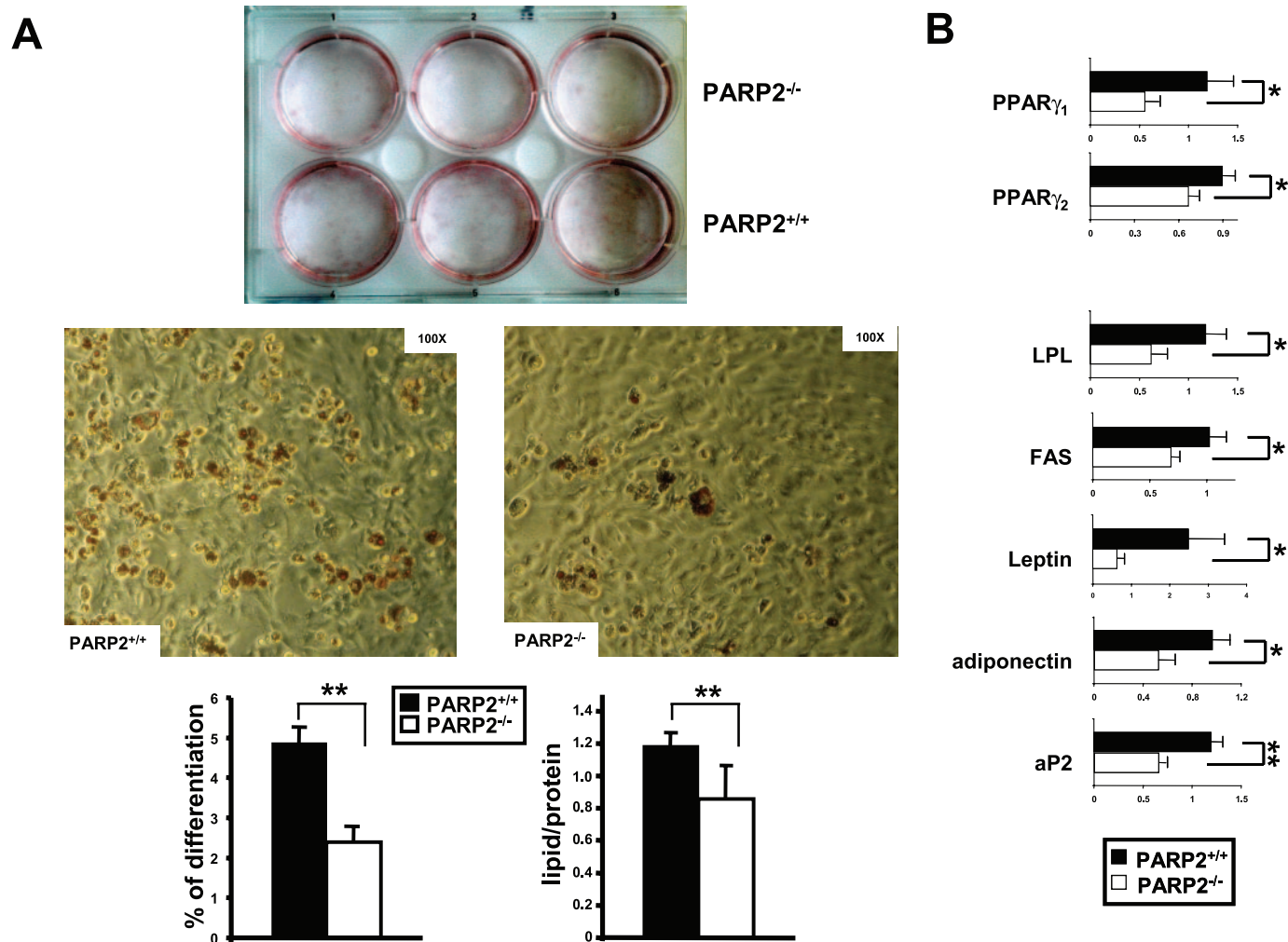


FIGURE 2. Effect of PARP-2 on MEF differentiation into adipocytes. A, MEFs were differentiated into adipocytes and stained with Oil red O. On the terminally differentiated MEFs, Nile red fluorescence-activated cell sorting analysis and lipid measurements were performed. The *left histogram* shows the percentage of differentiation as measured with Nile red, and the *right histogram* shows the accumulation of lipids in the culture. *, $p < 0.05$; **, $p < 0.01$. B, expression of selected marker genes of adipocyte differentiation as measured by RT-qPCR on MEF cDNA samples (*, $p < 0.05$; **, $p < 0.01$).

no difference was detected in PPAR γ_1 and PPAR γ_2 mRNA levels between the different genotypes.

MEF Differentiation Is Affected by PARP-2 Ablation—We next aimed to determine whether MEFs differentiation toward adipocytes was affected by the PARP-2 deletion. Differentiation of PARP-2^{-/-} MEFs into adipocytes was decreased as judged by Oil red O staining, determination of lipid content, and Nile red staining followed by fluorescence-activated cell sorting analysis (Fig. 2A).

The expression of genes involved in adipocyte differentiation and function such as PPAR γ_1 and PPAR γ_2 were decreased in the PARP-2^{-/-} MEFs (34). Because the PPAR γ transcripts are primarily present in the differentiated cells, these data confirm that PARP-2^{-/-} cells differentiate less into adipocytes. The expression of PPAR γ target genes, such as lipoprotein lipase, fatty acid synthase, leptin, adiponectin, and adipocyte fatty acid-binding protein 2 (aP2), were decreased in parallel (Fig. 2B).

PARP-2 Expression Modulates Transactivation of PPARs—To measure whether changes in PARP-2 expression affect PPAR transactivation, we used HEK 293 cells transfected with a PPAR γ_2 expression vector and a PPAR γ -responsive luciferase

construct. In these experiments we modulated the expression of PARP-2 expression by overexpression and siRNA depletion. For the siRNA depletion of PARP-2 we used the pSuper-siPARP-2 construct, whereas for PARP-2 overexpression we used the pBabe-PARP-2. The pSuper-scrPARP-2 and the empty pBabe vector served as the respective controls. PARP-2 levels were assessed by Western blotting using a PARP-2-specific antibody. For both constructs, the cells were transfected twice, on day 0 and on day 2. On day 3, the specific siRNA decreased PARP-2 protein levels significantly, whereas the scrambled PARP-2 siRNA did not alter the PARP-2 levels. A strong increase in PARP-2 protein was observed on day 3 of the overexpression experiment (Fig. 3).

PARP-2 depletion diminished the basal PPAR γ activity and abrogated receptor activation by its synthetic ligand, troglitazone. Conversely, PARP-2 overexpression induced by 3-fold the basal PPAR γ activity, although it does not significantly change the ligand-dependent activation by troglitazone (Fig. 4A). To verify whether this effect of PARP-2 was specific for PPAR γ , we performed similar experiments for the related nuclear receptors PPAR α (NR1C1) and PPAR β (NR1C2), and

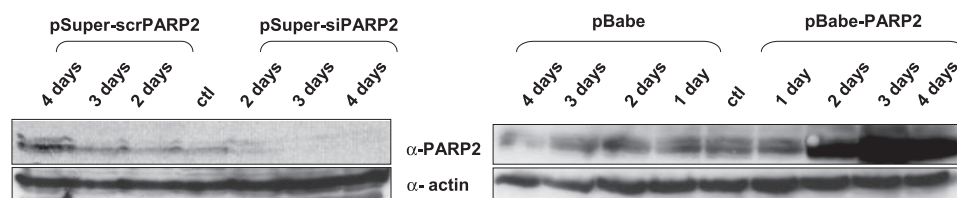


FIGURE 3. **Characterization of the pSuper-scrPARP-2 and the pSuper-siPARP-2 constructs.** 3×10^7 HEK cells were plated in Petri dishes and were BES-buffered saline transfected on day 0 and on day 2. Cells were scraped from day 2 daily. These samples were analyzed by Western blotting. PARP-2 was depleted by the pSuper-siPARP-2 construct, but was unmodified by the pSuper-scrPARP-2 construct. Whereas the transfection with pBabe-PARP-2 resulted on day 3 and day 4 in a robust induction of PARP-2 expression, the transfection with pBabe alone did not modify PARP-2 expression.

the unrelated estrogen receptor β (ER β , NR3A2). Interestingly, siRNA depletion of PARP-2 increased the basal activity of both PPAR α and $-\beta$ (Fig. 4, B and C). PARP-2 overexpression did not affect PPAR β but increased PPAR α activity. The activation of PPAR α and $-\beta$ with fenofibrate and monoethylhexyl phthalate, respectively, was not modified by the modulation of PARP-2 expression. In addition, neither PARP-2 depletion, nor PARP-2 overexpression had an effect on the basal or ligand-induced activity of ER β (Fig. 4D). Combined these results indicate specificity of the PARP-2-dependent effect on PPAR γ .

PARP-2 Is the Member of the RXR/PPAR γ Transcription Complex—To demonstrate an interaction between PPAR γ and PARP-2 we used ChIP assays. To precipitate chromatin from undifferentiated 3T3-L1 cells we used antibodies against PARP-2 and PPAR γ_2 . An anti-matrix metalloproteinase-9 antibody and a sample without antibody served as negative controls. We used qPCR to amplify the promoters of the aP2 (6) and CD36 (35) as promoters driven by PPAR γ , and keratin-19 (K19), as a non-related, ER β -regulated promoter (36). PARP-2 and PPAR γ gave a strong signal on PPAR γ -regulated promoters. These signals were significantly higher compared with the signal from the K19 promoter (Fig. 5A). We also performed qPCR reactions to cover the coding sequences of aP2 using the chromatin fragments obtained in the ChIP experiments. The signal of PARP-2 and PPAR γ coding sequences in the immunoprecipitates was strongly decreased compared with the signal of the corresponding promoter. Apparently, both PARP-2 and PPAR γ are present on the PPAR γ -driven promoters but not in the coding sequence (Fig. 5B). In addition, our results suggest that PARP-2 possesses specificity toward the PPAR γ -driven promoters, because the signal from ER β -driven K19 promoter was significantly lower than that from PPAR γ -driven promoters.

Despite the huge difference in the signal of the specific promoters and the nonspecific regions (K19 promoter, coding sequence) we observed some background signal from the non-specific region. It is likely that this represents the real presence of PARP-2 in these regions, which is probably linked to the formaldehyde-induced DNA damage.

To provide proof that the interaction of ER β with the K19 promoter is basically detectable we complemented 3T3-L1 cells with ER β , and we performed ChIP probing with the K19 primer set. To precipitate chromatin from ER β -complemented 3T3-L1 cells we used an antibody against ER β , an anti-MRE11 antibody and a sample with no antibody served as negative controls. The precipitate of the ER β -specific antibody gave signif-

icantly higher signal than the non-specific MRE11 (2.7-fold increase) as well as with the non-antibody control (6.1-fold increase) proving that the K19 primer pair is capable of detecting the K19 promoter if present in the precipitate (Fig. 5C).

DISCUSSION

PPAR γ plays an important role in adipose tissue differentiation and function. In PARP-2 knock-out mice we have identified a defect of adipose tissue function and a decrease of adipocyte differentiation. *In vivo*, the adipose tissue depots had smaller weight and histologically showed an adipodegenerative phenotype.

We have detected a mild inflammation in the WAT of the PARP-2 $^{-/-}$ mice. The capillaries were dilated, and we have detected F4/80-positive cells in the vicinity of the capillaries suggesting the presence of macrophages. The areas more distant from the capillaries are devoid of staining. Similar coloration was not observed in the WAT of the wild-type mice. Activated macrophages and adipocytes may secrete pro-inflammatory cytokines, such as TNF α that may induce adipocyte cell death (37). Because TNF α expression was not detectable in many mice and, if it was detected, its expression was not significantly increased by the absence of PARP-2, it is less likely that inflammation is a leading cause of the adipodegeneration in the PARP-2 $^{-/-}$ mice. It is also unlikely that increased lipolysis may contribute to the phenotype in the PARP-2 $^{-/-}$ mice, because there was no difference in the expression of hormone-sensitive lipase between the wild-type and PARP-2 $^{-/-}$ mice.

We did observe decreased expression of multiple PPAR γ target genes involved in adipocyte function. Expression of both PPAR γ isoforms was normal, suggesting effects on PPAR γ /RXR transactivation. *In vitro*, the differentiation of the PARP-2 $^{-/-}$ MEFs into adipocytes was delayed when compared with the differentiation of wild-type MEFs. At the end of the differentiation the expression of both PPAR γ_1 and PPAR γ_2 was decreased in the PARP-2 $^{-/-}$ cells indicating the lack of differentiation. Similarly, the expression of the PPAR γ target genes was decreased.

In transfection assays, the ablation of PARP-2 results in the diminution, whereas PARP-2 overexpression raises transactivation by PPAR γ . The effect of PARP-2 seems specific for PPAR γ , because opposite or no effects were observed for the related PPAR α and PPAR β , and the non-related ER β .

PARP-2 achieves these activities, because it is part of the PPAR γ /RXR transcription complex as shown by ChIP assays, suggesting that PARP-2 could act as a PPAR γ /RXR receptor cofactor.

Both members of the PPAR γ /RXR nuclear receptor dimer might be the effector behind the phenotype of the PARP-2 $^{-/-}$ mice. If PARP-2 would directly influence RXR, all PPAR isoforms should respond the same way to the modulation of PARP-2 expression. PPAR γ was differentially regulated when compared with PPAR α and $-\beta$, suggesting that PARP-2 acts on

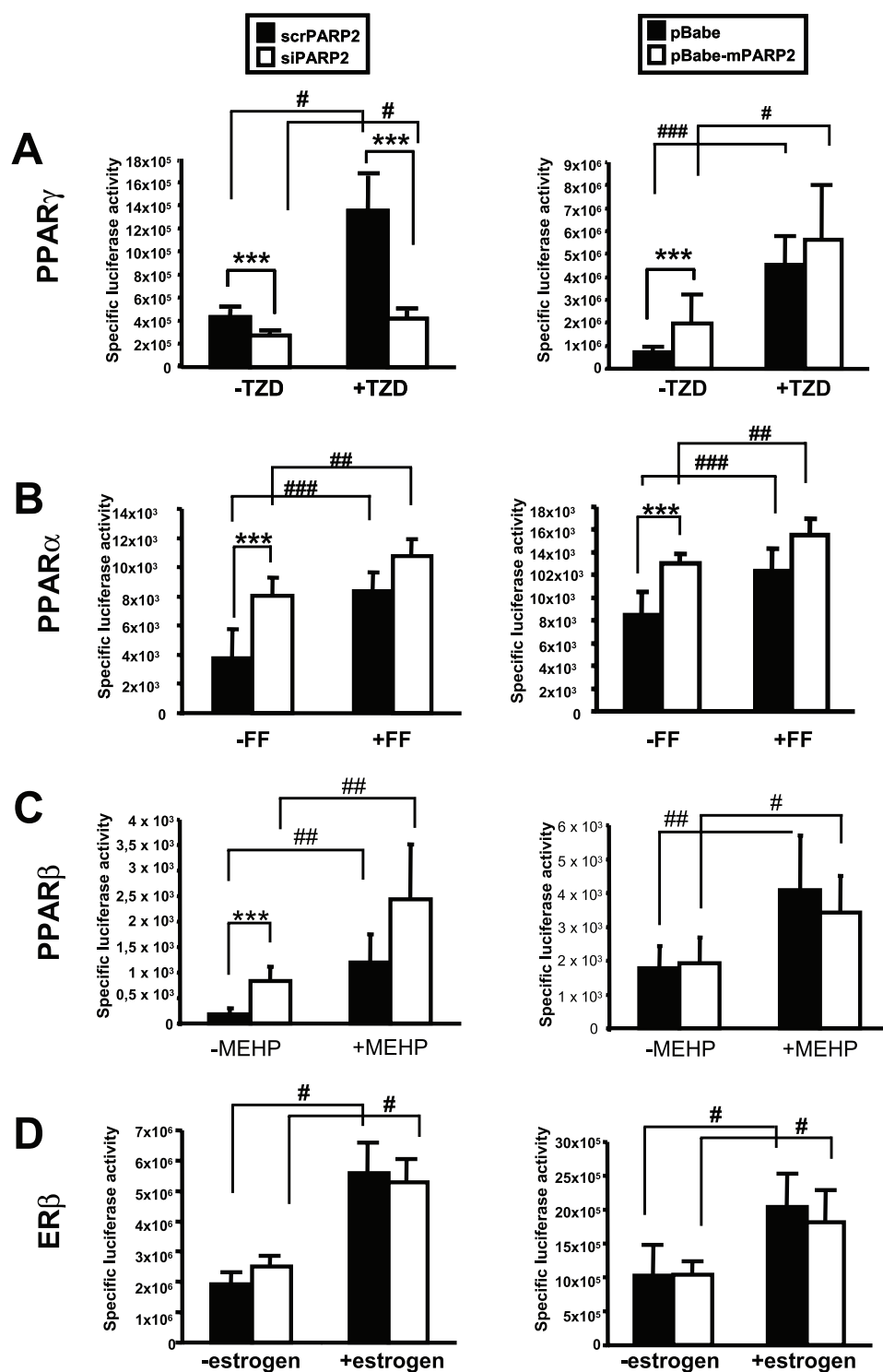


FIGURE 4. Effect of PARP-2 expression levels on PPAR γ_2 transactivation. Effect of PARP-2 depletion and overexpression on the basal activity and receptor activation of PPAR γ (A), PPAR α (B), PPAR β (C), and ER β (D) receptors. Error bars represent \pm S.D. ### and ***, $p < 0.001$; **, $p < 0.01$; #, $p < 0.05$.

PPAR γ . Our report comprises *in vitro* data and gives first time *in vivo* evidence that PARP-2 may be considered a cofactor of nuclear receptor transcription.

PARP-2 is a multidomain protein with multiple functions. These functions comprise DNA repair (reviewed in Ref. 15), spermatogenesis (16, 17), T-cell development (38), inflammation, and oxidative injury (18–20). Most of these functions are

accomplished through protein-protein interactions. The N terminus, with the following domain E are apparently important protein-protein interaction domains, serving as an interaction platform for TRF-2 (21), B23 (22), PARP-1, XRCC1, and DNA polymerase β (23), and TTF1 (25). PARP-2 also homodimerizes with itself through its domain E (23).

PARP-1 has been described as a cofactor for numerous transcription factors (reviewed in 40 and 25), including for some members of the nuclear receptor family, such as the progesterone receptor (41), RXR (42, 43), androgen receptor (44), and the thyroid receptor (42). A recent study, based on *in vitro* results, suggested that PARP-2 acts as a cofactor of a homeodomain-containing transcription factor, TTF1, which belongs to the Nkx-2 family. Binding of PARP-2 through its E domain to the C terminus of TTF1 regulates the expression of the surfactant protein-B in lung epithelial cells. TTF1-mediated transcription encompasses similar mechanisms, including chromatin modification, and involves some of the same cofactors such as the steroid receptor coactivators as described for PPAR γ -coupled transcription. This suggests that similar molecular mechanisms exist both in the case of PPAR γ - and TTF1-mediated transcription (25). Our results hence confirm the observation of Maeda and colleagues (25), that PARP-2 is a cofactor of some transcription factors, and extend these conclusions by showing that PARP-2 is involved in nuclear receptor-mediated transcriptional control *in vivo*. Recent evidence has suggested that the interaction between PARP-1 and the promoter of target gene could be

mediated via double strand breaks, which are produced by activation of a nuclear receptor followed by the unwinding of DNA by topoisomerase II (45). Our results do provide evidence that interaction with DNA is important for the interaction between PPAR γ and PARP-2. ChIP assays that depend on DNA binding show strong interaction. In contrast, immunoprecipitation experiments performed on cell

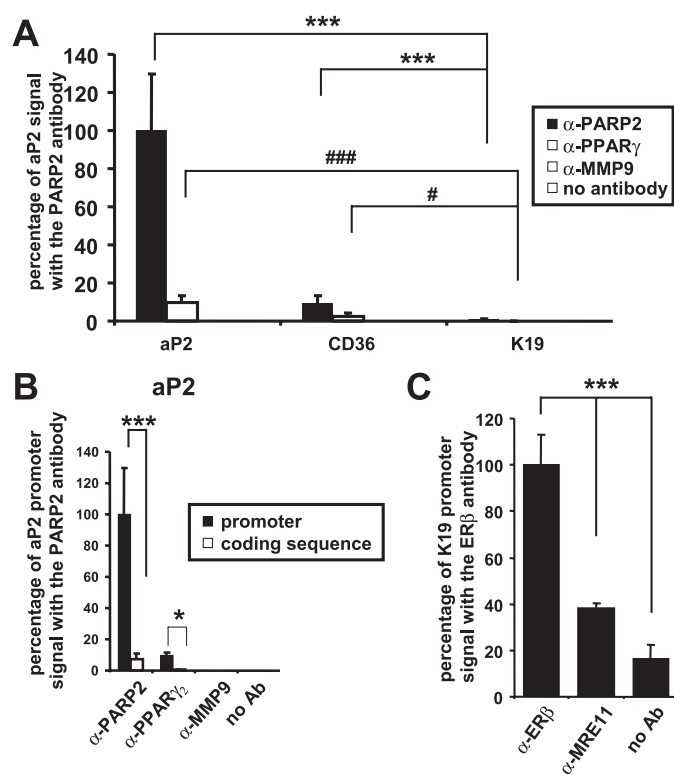


FIGURE 5. PPAR γ and PARP-2 occupy together the PPAR γ -dependent promoters. A, PARP-2 is present on PPAR γ -driven promoters (aP2 and CD36) as demonstrated in ChIP assays. The PARP-2 signal is increased in the PPAR γ -driven promoters compared with the non-related, ER β -driven K19 promoter. Error bars represent \pm S.D. (### and ***, $p < 0.001$; # and *, $p < 0.05$). B, a similar ChIP assay was performed to compare the presence of PARP-2 on the promoters versus the coding sequence of the same gene. PARP-2 is present preferentially on the promoter rather than the coding sequence of the aP2 gene. Error bars represent \pm S.D. (***, $p < 0.001$; *, $p < 0.05$). C, as a positive control, the K19 primer set was tested for the ability to detect the presence of ER β in ER β -transfected 3T3-L1 cells. The α -ER β signal was significantly higher than the signal of the negative control α -MRE11 and the control without antibody. These data confirm that the K19 primer set is capable of detecting the presence of the ER β receptor on the K19 promoter. Error bars represent S.D. (### and ***, $p < 0.001$; # and *, $p < 0.05$).

extracts showed only a weak interaction between PARP-2 and PPAR γ (data not shown), which was abrogated by low concentrations of Nonidet P-40 ($>0.1\%$). Furthermore, like in the case for PARP-1, the N terminus of PARP-2, comprising its DNA-binding domain, seems also to play the most important role in the interaction with PPAR γ . Consistent with this observation a nuclear receptor-binding consensus sequence (113 LIQLL 117) was present in the E domain of PARP-2.

Concerning the mode of action of PARP-2, it is possible that not only the physical presence but also the activity of PARP-2 is necessary for the nuclear receptor function. Poly(ADP-ribosylation) is reported to increase throughout the differentiation process of 3T3-L1 cells (46). Interestingly, this poly(ADP-ribosylation) activity is not completely inhibited by PARP-1 depletion (47), suggesting the involvement of other member(s) of the PARP family, such as PARP-2. Indeed both PARP-1 and PARP-2 are reported to poly(ADP-ribosylate) histones (13). Similarly to histone acetylation, poly(ADP-ribosylation) of the high mobility group of proteins and histones loosens chromatin structure enabling

transcription initiation (48). Consistent with this line of thinking, there is molecular and *in vivo* evidence that the enzymatic activity of PARP-1 is necessary for efficient gene transcription, and inhibition of PARP activity impairs the transcription of a number of different genes, including different chemokines and inflammation-related genes (e.g. iNOS, TNF α , ICAM-1, IL-8, MIP-1 α , and IL-12) (40, 49, 50).

PARP-2 specifically occupies the promoter of PPAR γ target genes, because it bound efficiently to the regulatory sequence, whereas binding to the corresponding coding sequences was strongly decreased. Despite this rather specific binding, we observed a background signal rising most likely from non-coding regions or from non-PPAR γ -dependent promoters, such as that of the K19 gene, which is under the control of ER β . When comparing the specific to the above mentioned nonspecific signal, it is at least 10- to 100-fold increased, which can be considered as a significant difference. It is likely that the nonspecific presence of PARP-2 on the K19 promoter and in the non-coding regions is explained by the fact that PARP-2 binds to the DNA-damage sites created by the formaldehyde treatment during the cross-linking of the cells. The cross-linking-related DNA damage is present throughout the entire genome, equally affecting coding regions and promoters, thus theoretically providing a background signal throughout the genome.

The present study indicates that PARP-2 modulates the activity of PPAR γ /RXR nuclear receptor complex, a key transcription factor involved in the pathogenesis of several important diseases such as obesity, insulin resistance, type II diabetes atherosclerosis, and lipodystrophy. Because many of these diseases affect a large part of the population and have high costs to society, our data, linking the activation of PPAR γ and PARP-2, show it is possible to modulate PPAR γ activity via PARP-2. It is therefore tempting to speculate that the various PARP inhibitors that are currently being developed and tested in clinical trials (51) could also be useful in the metabolic disease arena.

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