Peroxisome Proliferator-activated Receptor γ Regulates Genes Involved in Insulin/Insulin-like Growth Factor Signaling and Lipid Metabolism during Adipogenesis through Functionally Distinct Enhancer Classes*

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Background: The transcription factor PPARγ is required for adipocyte differentiation.

Results: PPARγ activates genes involved in lipid metabolism and in insulin/IGF signaling through two distinct functional classes of transcriptional enhancers.

Conclusion: Both broadly active and cell type-specific enhancers are involved in PPARγ-mediated transcriptional regulations during adipogenesis.

Significance: Broadly active enhancers are used by cell type-specific transcription factors to finely tune conserved biological processes/pathways.

The nuclear receptor peroxisome proliferator-activated receptor (PPAR) γ is a transcription factor whose expression is induced during adipogenesis and that is required for the acquisition and control of mature adipocyte functions. Indeed, PPARγ induces the expression of genes involved in lipid synthesis and storage through enhancers activated during adipocyte differentiation. Here, we show that PPARγ also binds to enhancers already active in preadipocytes as evidenced by an active chromatin state including lower DNA methylation levels despite higher CpG content. These constitutive enhancers are linked to genes involved in the insulin/insulin-like growth factor signaling pathway that are transcriptionally induced during adipogenesis but to a lower extent than lipid metabolism genes, because of stronger basal expression levels in preadipocytes. This is consistent with the sequential involvement of hormonal sensitivity and lipid handling during adipocyte maturation and correlates with the chromatin structure dynamics at constitutive and activated enhancers. Interestingly, constitutive enhancers are evolutionarily conserved and can be activated in other tissues, in contrast to enhancers controlling lipid handling genes whose activation is more restricted to adipocytes. Thus, PPARγ utilizes both broadly active and cell type-specific enhancers to modulate the dynamic range of activation of genes involved in the adipogenic process.

White adipose tissue (WAT)§§ is an endocrine and metabolic organ exerting key functions in glucose and lipid homeostasis (1, 2). WAT adapts to energy supply by releasing or storing lipids in response to nutrients and hormonal stimuli. The requirement for supplemental storage capacity is provided by both hyperplastic and hypertrophic responses, a process referred to as WAT expandability (3). Moreover, adipocytes are continuously renewed throughout life (4, 5), showing that adipocyte differentiation is central to WAT development, maintenance, and physiological functions (6).

White adipogenesis proceeds from the commitment of mesenchymal stem cells to the adipocyte lineage, followed by differentiation of preadipocytes into adipocytes (7, 8). The successive activation of gene networks controlled by, among others, the Wingless-type MMTV integration site, bone morphogenetic protein, and Sonic Hedgehog pathways leads to the formation of preadipocytes. Subsequently, the adipocyte differentiation process results from sequential expression and activation of several transcription factors, such as CCAAT/enhancer binding proteins (C/EBP), which trigger the expression of the nuclear receptor peroxisome proliferator-activated receptor (PPAR) γ required to reach the mature adipocyte phe-

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§§ The abbreviations used are: WAT, white adipose tissue; PPAR, peroxisome proliferator-activated receptor; IGF, insulin-like growth factor; C/EBP, CCAAT/enhancer binding proteins; FAIRE, formaldehyde-assisted isolation of regulatory elements; MeDIP, 5-methylcytosine; hMeDIP, 5-hydroxymethylcytosine; qPCR, quantitative real time PCR; Myog, myogenin; DHS, DNase I-hypersensitive site.
notype (7, 8). This process also requires PPARγ collaboration with another member of the PPAR family, i.e., PPARβ/δ (9).

Recent functional genomics studies, using the murine 3T3-L1 preadipocyte cell line as a model, have improved our understanding of how PPARγ promotes differentiation of preadipocytes (10–15). The formation of lipid-storing white adipocytes is linked to PPARγ binding to thousands of transcriptional regulatory elements distinct from gene promoters called enhancers (10–15). These enhancers are found in the vicinity of PPARγ target genes, which could be activated through chromatin looping between enhancers and promoters as shown for other nuclear receptor family members (16, 17). These studies revealed that PPARγ genomic binding in adipocytes is enriched near genes involved in glucose and lipid metabolism (11, 13).

PPARγ-recruiting enhancers in mature adipocytes are characterized by the typical chromatin structure and epigenetic signature of active enhancers (12, 14, 15). For instance, these enhancers are found in open chromatin regions showing high sensitivity to DNase I digestion (10) and enrichment by formaldehyde-assisted isolation of regulatory elements (FAIRE) (14). Moreover, they harbor mono- and dimethylated histone H3 lysine 4 (H3K4me1/2) and acetylated H3K27 (H3K27ac) (15). These active enhancer chromatin features have been shown to be established during adipocyte differentiation through sequential recruitment of transcription factors (10, 18, 19). In this model, early binding of C/EBPα initiates chromatin remodeling events that set the ground for subsequent recruitment of PPARγ upon adipocyte differentiation (10, 18, 19).

In addition to these enhancers whose activities are induced during adipocyte differentiation (hereafter called “activated”), PPARγ is also recruited to enhancers that are already in an active state in preadipocytes (hereafter called “constitutive”) (15). However, because they were positively associated with strong transcriptional gene induction, only activated PPARγ-recruiting enhancers were deemed to be functionally relevant PPARγ-bound regulatory sites during adipogenesis (15). Here, we thoroughly studied the features and functional involvement of these two classes of PPARγ-recruiting enhancers in adipogenesis.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The 3T3-L1 preadipocyte and C2C12 myoblast cell lines were grown and differentiated under standard and therefore comparable conditions in experiments reported here or reanalyzed from previous reports (see “Bioinformatical Analyses” below). A detailed description of the differentiation procedures can be found in Ref. 20 for 3T3-L1 cells and in Ref. 21 for C2C12 cells. Myotubes and adipocytes relate to cells that have undergone at least 3 or 6 days of differentiation, respectively.

**Hydroxy)methylated DNA Immunoprecipitation**—Genomic DNA was prepared using a DNA extraction kit (DNeasy blood and tissue kit; Qiagen) with a supplementary step of RNase digestion. Genomic DNA was then sonicated using a Bioruptor (Diagenode) to produce fragments ranging in size from 200 to 500 bp. 4 and 20 μg of fragmented, heat-denatured (10 min at 95 °C) DNA were incubated overnight at 4 °C with either 2 μg of a mouse monoclonal antibody directed against 5-methylcytosine (MeDIP) (Diagenode, Mab-081-100) or 2 μg of a rabbit polyclonal antibody directed against 5-hydroxymethylcytosine (hMeDIP) (Diagenode, CS-HMC-100), respectively. Reactions were performed in a final volume of 500 μl of IP buffer (10 mM sodium phosphate, pH 7.0, 140 mM NaCl, 0.05% Triton X-100). Immunocomplexes were precipitated by incubation with 40 μl of BSA-coated protein A-Sepharose beads (50% slurry) for 2 h at 4 °C. Beads were subsequently washed three times with 1 ml of IP buffer and finally incubated with proteinase K (280 μg/ml) overnight at 55 °C in a buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.5% SDS. (Hydroxy)methylated DNA was recovered by phenol-chloroform extraction followed by ethanol precipitation.

**Chromatin Immunoprecipitation**—ChIP assays were performed as in Ref. 22 except that magnetic beads (Dynabeads) were used. The antibody against H3K4me2 was from Millipore (antibody 07-030).

**Real Time PCR**—Quantitative real time PCR (qPCR) were performed using SyberGreen-based chemistry. DNA (hydroxy)methylation and H3K4me2 levels were analyzed by qPCR using

### TABLE 1

| Primer sequence | Targeted regions |
|-----------------|------------------|
| CCAATCTTCAGAGGTCGAC | Constitutive PPARγ enhancers 1 |
| GCATCCAGTCGATGCCG | Constitutive PPARγ enhancers 2 |
| ATGACAAACCTGGGCCAAGTCT | Constitutive PPARγ enhancers 3 |
| TTATCTGTCGAGCCTTCTA | Constitutive PPARγ enhancers 4 |
| TTGTCATGCGCCAGCCTGTA | Constitutive PPARγ enhancers 5 |
| TCTAATGCTGAAAGAAGTGG | Constitutive PPARγ enhancers 6 |
| AACTTCCTGCACAACTTCC | Constitutive PPARγ enhancers 7 |
| TAGGGTCATTTTCAAAACAA | Constitutive PPARγ enhancers 8 |
| GAGAGCGGAAGACGATGTCCT | Constitutive PPARγ enhancers 9 |
| CATAGGGTGAGGAGAGCAG | Constitutive PPARγ enhancers 10 |
| CAAGGGATCAATCTGCAATG | Constitutive PPARγ enhancers 11 |
| GCCAAGTCCTGCAACAGTA | Constitutive PPARγ enhancers 12 |
| CCTCATCCCCAGTAAACAA | Constitutive PPARγ enhancers 13 |
| ATACCCCCCTTTGTCGAAAT | Activated PPARγ enhancers 1 |
| CGTCCATGAGGAATCCAGT | Activated PPARγ enhancers 2 |
| GGGTCCTTGGAGTGTCCTTC | Activated PPARγ enhancers 3 |
| CCTACGTCCGAGCGCATAGG | Activated PPARγ enhancers 4 |
| CGAGGAGCGATTTTGAAGG | Activated PPARγ enhancers 5 |
| CGTGCAGTACACTTCCACC | Activated PPARγ enhancers 6 |
| CATTGCAGCTCCTTGTGAATA | Activated PPARγ enhancers 7 |
| TCGAGGTCATGATTCACTGGA | Activated PPARγ enhancers 8 |
| AGCCCTGGTCTGGCAGCATCTGT | Activated PPARγ enhancers 9 |
| GCCACAGATTGTGGCAACTA | Activated PPARγ enhancers 10 |
| CCCCTACAGAACACAGACGCTT | Activated PPARγ enhancers 11 |
| CCTCTTGGAGAGGAATGAGA | Activated PPARγ enhancers 12 |
| CTGCTCAGCTGCTCCACGTT | Activated PPARγ enhancers 13 |
| TCCACGCTGAACCTGCTGTA | Activated PPARγ enhancers 14 |
| AGTGAAGGAATCTGGAAACCT | Activated PPARγ enhancers 15 |
| ACCAAACACCACAATTCCCA | ChIP negative control |
| CGTCCTCCTGCTCTCTCTT | ChIP negative control |
1 μl of input DNA and 5 μl of immunoprecipitated DNA. All
primers used are listed in Table 1.

**High Throughput Sequencing**—Library preparation and high
throughput sequencing (single-read, 36 base pairs) for MeDIP-
seq was performed on Genome Analyzer II using reagents and
standard protocols from the manufacturer (Illumina).

**MeDIP-seq Data Processing**—The Casava 1.8 Analysis Pipeline
was used to process the MeDIP-seq raw data and to map reads to
the mouse genome (Mm9). Duplicated reads were removed using
Picard giving rise to 9,961,110 reads that were further processed
using MEDIP (23). First, the signal was defined as reads/million
with a resolution of 25 bp after extending reads up to 200 bp
(length of sequenced DNA fragments). Subsequently, a saturation
analysis was performed to validate the reproducibility of the DNA
methylation profile compared with another independent library of
similar size. This experiment resulted in genome-wide saturation
coverage of 0.84 on a 0–1 scale. Finally, a calibration curve was
established to evaluate the dependence between local signal inten-
sities and local CpG densities. A linear regression issued from this
analysis was obtained and used to compute the relative DNA
methylation score (rms) corresponding to the reads/million signal
normalized to local CpG densities. MeDIP-seq data have been
deposited into the Gene Expression Omnibus database under
accession number GSE50992.

**Bioinformatical Analyses**—Public functional genomic data
(10, 11, 14, 21, 24–26) used in this study were downloaded from
Gene Expression Omnibus or the UCSC Genome Browser (27)
and are listed in Table 2. ReSeq gene coordinates as well as

| Public data | Reference | Cell /Tissue | Experiment | Biological samples | Accession number | database |
|-------------|-----------|--------------|------------|--------------------|------------------|----------|
| Spandanour AA et al., Nucleic Acid Res, 2012 | 3T3-L1 | hmeDIP-seq | Preadipocytes | GSM941666 | | |
| Siersback R et al., EMBO J, 2011 | 3T3-L1 | DHS-seq | Preadipocytes | GSM686964 | | |
| Siersback MS et al., Mol Cell Biol, 2012 | 3T3-L1 | eWAT | Preadipocytes | GSM686909 | | |
| Asp P et al., Proc Natl Acad Sci USA, 2011 | C2C12 | ChIP-seq H3K4me1 | Myotubes | GSM721289 | | |
| Mouse ENCODE project | C2C12 | ChIP-seq C/EBPβ | Myotubes | GSM1015100 | | |
| Waki H et al., PLoS Genet, 2011 | 3T3-L1 | FAARE-seq | Preadipocytes | DRX000633 | | |
| Schupp M et al., J Biol Chem, 2009 | 3T3-L1 | Gene expression profiling by microarray | Preadipocytes | GSM351690 | | |
| Su AI et al., Proc Natl Acad Sci USA, 2004 | Mouse tissues and cells | Gene expression profiling by microarray | Preadipocytes | GSE1133 | | |
Two Classes of PPARγ-recruiting Enhancers in Adipogenesis

FIGURE 1. Identification of constitutive and activated PPARγ-recruiting enhancers during 3T3-L1 adipocyte differentiation. A, PPARγ-recruiting potential enhancers from 3T3-L1 adipocytes were compared with genomic regions showing significant enrichment for both H3K4me1 and H3K27ac in 3T3-L1 preadipocytes and adipocytes. Activated PPARγ-recruiting enhancers are PPARγ binding sites overlapping with H3K4me1/H3K27ac enriched regions only in adipocytes. Constitutive PPARγ-recruiting enhancers are vertebrate PhyloP (28) and PhasCons (29) conservation scores were downloaded from the UCSC Genome Browser (27). H3K4me1, H3K27ac, PPARγ, and C/EBPβ significantly enriched regions identified in 3T3-L1 cells in Refs. 10 and 15 and myogenin (Myog) binding sites identified in C2C12 cells by the ENCODE consortium (26) were used. To specifically analyze PPARγ-recruiting potential enhancers, PPARγ binding sites that lie within 2.5 kb of a RefSeq gene transcriptional start site were discarded. Peak overlaps were determined using the Cistrome analysis platform (30).

Wig files used to define signal intensity or conservation were generated at 25-bp resolution from raw data (and were normaliz-
ed to the total number of uniquely mapped sequenced reads extended to 200 bp for ChiP-seq data). Average signal plots as well as CpG density plots were generated as described in Ref. 22. First, the center of enhancers was determined, and regions of interest were defined as spanning 2.5 kb in each direction around the center. These regions were then split into 80 windows where the average signal from all enhancers was determined and plotted. ChiP-seq data were visualized using wig files and the Integrated Genome Browser (31).

De novo motif discovery was performed using Sampling with Expectation maximization for Motif Elicitation (32), and logos were generated using enoLOGOS (33). Enrichment for known transcription factor DNA-binding motifs was determined using the SeqPos tool of the cistrome analysis platform (30).

Genomic Regions Enrichment of Annotations Tool was used to associate distal regulatory regions to genes (default parameters) and subsequently identify gene set over-representation (34). Gene sets that were more than 90% identical were merged into a single class for analyses of their expression.

Transcriptomic Data Analyses—Raw transcriptomic data from 3T3-L1 cells (15, 35) were analyzed using the GeneSpring GX software using the gene level analysis (Agilent). Back-ground-corrected raw data were Log2-transformed and quantile-normalized following the robust multichip average procedure giving rise to gene expression values centered on 0 (36).

Transcriptomic data for additional mouse tissues or cells were obtained from BioGPS (37). Normalized expression values

PPARγ binding sites overlapping with H3K4me1/H3K27ac enriched regions in both preadipocytes and adipocytes. B, average H3K4me1 levels at constitutive and activated PPARγ-recruiting enhancers in 3T3-L1 preadipocytes and adipocytes. Shown are average ChIP-seq signal intensities defined using PPARγ-recruiting constitutive (blue line) or activated (red line) enhancers. The arrowhead indicates the center of enhancers, which were aligned. A region extending 2.5 kb on each side of the center was analyzed. C, average H3K27ac levels at constitutive and activated PPARγ-recruiting enhancers in 3T3-L1 preadipocytes and adipocytes analyzed as in B. D, average H3K4me2 levels at constitutive and activated PPARγ-recruiting enhancers in 3T3-L1 preadipocytes and adipocytes analyzed as in B. E, average DNase I sensitivity of constitutive and activated PPARγ-recruiting enhancers in 3T3-L1 preadipocytes and adipocytes analyzed as in B. F, average FAIRE enrichment of constitutive and activated PPARγ-recruiting enhancers in 3T3-L1 preadipocytes and adipocytes analyzed as in B using FAIRE-seq data. G, top de novo motif enriched in constitutive and activated PPARγ-recruiting enhancers. H, average PPARγ recruitment levels at constitutive and activated PPARγ-recruiting enhancers in 3T3-L1 preadipocytes and adipocytes analyzed as in B. I, average retinoid X receptor (RXR) recruitment levels at constitutive and activated PPARγ-recruiting enhancers in 3T3-L1 preadipocytes and adipocytes analyzed as in B. J, constitutive and activated PPARγ-recruiting enhancers from 3T3-L1 cells were analyzed for PPARγ recruitment in primary mouse white adipocytes identified by ChiP-seq in Ref. 25.
FIGURE 2. DNA (hydroxyl)methylation and C/EBP recruitment to constitutive and activated PPARγ-recruiting enhancers. A, average CpG density at constitutive and activated PPARγ-recruiting enhancers. Shown are average CpG counts in PPARγ-recruiting constitutive (blue line) or activated (red line) enhancers. The arrowhead indicates the center of enhancers, which were aligned. A region extending 2.5 kb on each side of the center was analyzed. B, average DNA methylation (5mC) levels of constitutive and activated PPARγ-recruiting enhancers in 3T3-L1 preadipocytes analyzed as in Fig. 18 using MeDIP-seq data normalized using MEDIP. C, average DNA hydroxymethylation (5hmC) levels of constitutive and activated PPARγ-recruiting enhancers in 3T3-L1 preadipocytes analyzed as in Fig. 18 using hMeDIP-seq data. D, 5mC and 5hmC levels were analyzed using (h)MeDIP-qPCR at eight randomly selected constitutive and activated PPARγ-recruiting enhancers in 3T3-L1 preadipocytes and adipocytes. Whiskers plots (median, minimum to maximum values) show DNA recovery relative to input DNA. Statistically significant differences between preadipocytes and adipocytes (*, p < 0.05; **, p < 0.01; and *** , p < 0.001) as well as between constitutive and activated PPARγ-recruiting enhancers at the same differentiation stage (§, p < 0.05; and §§, p < 0.01) are indicated. E, H3K4me2 levels were analyzed using ChIP-qPCR at constitutive and activated PPARγ-recruiting enhancers used in D in 3T3-L1 preadipocytes and adipocytes. Whiskers plots (median, minimum to maximum values) show DNA fold enrichment relative to a negative control region [24]. Statistically significant differences between preadipocytes and adipocytes (*, p < 0.05) as well as between constitutive and activated PPARγ-recruiting enhancers in preadipocytes (§, p < 0.05) are indicated. F, average C/EBPβ recruitment levels at constitutive and activated PPARγ-recruiting enhancers in 3T3-L1 preadipocytes analyzed as in Fig. 18. The percentage of constitutive and activated PPARγ-recruiting enhancers overlapping with a C/EBPβ recruitment site is also indicated (% occupied enhancers).
for the genes of interest were extracted from the Mouse GNF1M Gene Atlas (Gene Expression Omnibus accession number GSE1133) (38).

Statistical Analyses—Statistical analyses were performed using Prism software (GraphPad, San Diego, CA.). Statistical significance was determined using a Student’s t test comparison for unpaired or paired data and was indicated as follows: *, p < 0.05; **, p < 0.01; ***, p < 0.001. Differences in the overlap of constitutive and activated PPARγ-recruiting enhancers with DNase I-hypersensitive sites (DHS) were analyzed using chi-squared tests using Holm’s adjustment when appropriate. Differences in the expression of genes associated with constitutive and activated PPARγ-recruiting enhancers between the white adipose tissue and other mouse tissues or cells were analyzed using Wilcoxon tests using FDR adjustment.

RESULTS

Identification of Activated and Constitutive PPARγ-recruiting Enhancers during Adipocyte Differentiation of 3T3-L1 Cells—Active transcriptional enhancers are best identified using a specific epigenomic signature composed of H3K4me1 and H3K27ac (39–41). Using this signature, we discriminated potential enhancers recruiting PPARγ activated during adipocyte differentiation (i.e., activated enhancers gaining H3K4me1/H3K27ac during differentiation) from enhancers already active in preadipocytes (i.e., constitutive enhancers already displaying H3K4me1/H3K27ac in preadipocytes). PPARγ, H3K4me1, and H3K27ac significantly enriched regions identified by ChIP-seq in (15) indicated that approximately one-third of PPARγ-recruiting enhancers are constitutive, whereas two-thirds are activated during 3T3-L1 differentiation (1,526 and 2,856 potential enhancers, respectively) (Fig. 1A). Average H3K4me1 and H3K27ac signals in 3T3-L1 preadipocytes and mature adipocytes verified that these marks were enriched at constitutive enhancers at both stages, whereas they were gained during differentiation at activated enhancers (Fig. 1, B and C). Interestingly, a subset of activated enhancers (29%) shows the presence of H3K4me1 already in preadipocytes giving rise to the moderate H3K4me1 enrichment observed in Fig. 1B. This subset of enhancers may therefore represent so-called “poised” enhancers, which are marked by H3K4me1 but lack H3K27ac (41–43). The constitutive and activated nature of PPARγ-recruiting enhancers was also observed when analyzing H3K4me2, DHS, and FAIRE signals in 3T3-L1 preadipocytes and mature adipocytes (Fig. 1, D–F, respectively).

A de novo motif search identified as the first hit the known PPARγ response element in both the constitutive and activated PPARγ-recruiting enhancers (Fig. 1G). Accordingly, PPARγ binding to the two classes of enhancers was associated with that of its dimerization partner retinoid X receptor (Fig. 1, H and I). Through a comparison with PPARγ genomic binding sites identified in Ref. 25 using ChIP-seq data from in vitro differentiated epididymal and inguinal white adipocytes, we found that more than 80% of both constitutive and activated enhancers also recruit PPARγ in primary mouse adipocytes (Fig. 1J). Hence, constitutive and activated PPARγ-recruiting enhancers are genuine PPARγ binding sites that display differential functionalization dynamics during 3T3-L1 adipocyte differentiation.

| Transcription Factors | DNA Binding Domain | Motif Consensus | Motif enrichment 10log(p) |
|-----------------------|-------------------|----------------|--------------------------|
| EKXR1a                | Hormone-receptor Family | TGACTCTTGC | 600 |
| EKXR1b                | Hormone-receptor Family | TGACTCTTGC | 600 |
| NR2f                  | Hormone-receptor Family | TGACTCTTGC | 600 |
| NR2f                  | Hormone-receptor Family | TGACTCTTGC | 600 |
| PPARγ                 | Hormone-receptor Family | TGACTCTTGC | 600 |
| PPARγ                 | Hormone-receptor Family | TGACTCTTGC | 600 |
| NR1B                  | Hormone-receptor Family | TGACTCTTGC | 600 |
| NR1B                  | Hormone-receptor Family | TGACTCTTGC | 600 |
| SPI                   | Betahelix-alpha finger Family | TGACTCTTGC | 600 |
| SPI                   | Betahelix-alpha finger Family | TGACTCTTGC | 600 |

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TABLE 3

Transcription factor DNA recognition motifs enriched in constitutive and activated PPARγ-recruiting enhancers

The top 30 known transcription factor DNA recognition motifs identified by the SeqPos tool (cistrome analysis platform) are reported. Only the most conserved nucleotides in the consensus sequences (the numbers of nondisplayed nucleotides is provided). P-values (pval) are based on motif enrichment toward the center of the PPARγ-recruiting enhancers. Enriched motifs for the nuclear receptor and C/EBP transcription factor families are highlighted in green and purple, respectively.
Constitutive PPARγ-recruiting Enhancers Are DNA-hypo-methylated and Bound by C/EBPβ in Preadipocytes—Recent studies have highlighted a role for cytosine DNA methylation (5mC) in the inhibition of enhancer activation (22, 44–46). Further oxidation of cytosines giving rise to hydroxymethylated cytosine (5hmC) could, in this context, allow DNA demethylation and ensuing enhancer activation (47, 48). Moreover, we have recently identified 5hmC at PPARγ-recruiting enhancers in 3T3-L1 adipocytes (24). Therefore, we investigated whether DNA methylation could discriminate between constitutive and activated PPARγ-recruiting enhancers. We performed genome-wide mapping of 5mC using MeDIP-seq in 3T3-L1 preadipocytes and monitored 5mC levels at the two classes of PPARγ-recruiting enhancers. We observed that, despite a higher CpG density (Fig. 2A), constitutive enhancers showed a strong DNA hypomethylation compared with activated enhancers (Fig. 2B). This was correlated with higher levels of 5hmC (Fig. 2C). The 5(h)mC dynamics during 3T3-L1 adipocyte differentiation was then analyzed using (h)MeDIP-qPCR on a randomly selected set of constitutive and activated PPARγ-recruiting enhancers. These data show that activated enhancers exhibited decreased 5mC, which correlated with increased 5hmC during adipocyte differentiation (Fig. 2D). Although this pattern was also detectable for constitutive enhancers, it was less pronounced because of already low 5mC and high 5hmC levels in preadipocytes (Fig. 2D). This pattern was consistent with that of H3K4me2 (Fig. 2E), in line with our previously observed correlative changes in H3K4me2 and 5hmC at enhancers (24). These data therefore indicate that the differential epigenomic dynamics at constitutive and activated PPARγ-recruiting enhancers during 3T3-L1 differentiation extends to DNA (hydroxy)methylation.

Because constitutive enhancers display an active chromatin state in preadipocytes, we hypothesized that transcription factors would already be recruited to these sites at that stage. Analysis of transcription factor recognition motif enrichment iden-
identified the C/EBP family member DNA-binding motif in both activated and constitutive PPARγ-recruiting enhancers (Table 3). Using ChIP-seq data for C/EBPβ in 3T3-L1 preadipocytes (10), we found that this transcription factor was bound to a subset of enhancers activated during 3T3-L1 differentiation (Fig. 2F), in line with a previous report (10). Interestingly, these data also indicated that C/EBPβ was in fact more prominently present at constitutive enhancers in preadipocytes (Fig. 2F). Hence, constitutive PPARγ-recruiting enhancers are characterized by DNA hypomethylation and binding of the C/EBPβ transcription factor in preadipocytes. In line with studies showing that the chromatin structure of enhancers is much more dynamic than that of promoters (49, 50), the differential expression pattern of genes associated with constitutive and activated PPARγ-recruiting enhancers were not associated with differential chromatin structures of their promoters (Fig. 3).

Constitutive PPARγ-recruiting Enhancers Are Linked to Genes Involved in Insulin/IGF Signaling—We next questioned the functional significance of constitutive and activated PPARγ-recruiting enhancers. Because the genomic distribution of enhancers is biased toward their target genes (51, 52), we thus performed a functional classification of genes associated with the two classes of enhancers using the Genomic Regions Enrichment of Annotations Tool (34). This analysis indicated that genes associated with activated PPARγ-recruiting enhancers were primarily the well defined PPARγ target genes involved in lipid metabolism (Fig. 4A and Table 4). However, genes associated with constitutive PPARγ-recruiting enhanc-
ers were mainly enriched within the insulin/IGF signaling pathway (Fig. 4B and Table 5). Transcriptomic analyses indicated that genes associated with constitutive enhancers showed stronger average expression levels in preadipocytes when compared with genes associated with activated enhancers (Fig. 4C). Their expression was increased in adipocytes in a PPAR/H9253-dependent manner, even though this increase was less pronounced than that observed for genes associated with activated enhancers (Fig. 4C). These distinct expression patterns therefore matched with the differential functionalization dynamics of constitutive and activated PPAR/H9253-recruiting enhancers observed earlier (Figs. 1 and 2).

To illustrate these findings, four different PPAR/H9253-regulated genes involved in the insulin/IGF signaling pathway (Growth factor receptor bound protein 2 (Grb2) and Insulin receptor substrate 2 (Irs2)) or in lipid metabolism (Monoglyceride lipase (Mgll) and Stearoyl-Coenzyme A desaturase 1 (Scd1)) were specifically analyzed (Fig. 5A). H3K4me1 and H3K27ac ChIP-seq signals from preadipocytes and adipocytes are shown across these genes in Fig. 5B. These data show that all but one PPAR/H9253-bound enhancer near or within Grb2 and Irs2 are constitutive, whereas all but one PPAR/H9253-bound enhancer near or within Mgll and Scd1 are activated. In line with results from Fig. 1 (B and C), constitutive enhancers are characterized by strong H3K4me1 levels in both preadipocytes and adipocytes and by the presence of H3K27ac in preadipocytes, which is further increased in adipocytes. In contrast, activated enhancers gain both H3K4me1 and H3K27ac upon preadipocyte differentiation into adipocytes. Hence, constitutive enhancers are linked to PPAR-γ-regulated genes involved in the insulin/IGF signaling pathway in adipocytes.

### Constitutive PPAR-γ-recruiting Enhancers Are More Conserved and Ubiquitously Active, Whereas Activated PPAR-γ-recruiting Enhancers Exhibit Stronger Cell Type Specificity—Activated PPAR-γ-recruiting enhancers mainly regulate genes that characterize adipocyte functions (i.e., lipid metabolism), whereas constitutive enhancers mainly regulate genes with more ubiquitous functional roles (i.e., insulin/IGF signaling). Moreover, the DNA sequence of constitutive enhancers showed a greater evolutionary conservation compared with activated enhancers as judged by their stronger average PhyloP and PhasCons vertebrate conservation scores (Fig. 6A). Therefore, we reasoned that constitutive enhancers might exhibit a more widespread activity. To test that hypothesis, we used DHS data obtained by the ENCODE consortium in various mouse tissues and cells as an indicator of enhancer activity (26). Interestingly, we found that the pattern of overlap with DHS was significantly different for activated and constitutive enhancers (p < 2.10^-14). Indeed, ~50–60% of activated PPAR-γ-recruiting enhancers overlapped with DHS from fat tissues, which was significantly higher than the overlap obtained with DHS from other cells or tissues (Fig. 6B). Conversely, constitutive PPAR-γ-recruiting enhancers overlapped a high number of DHS from both the fat pads and other tissues such as the heart and skeletal muscle or cells including fibroblasts and NIH3T3 (Fig. 6B). Accordingly, this was correlated with a much stronger average FAIRE signal from NIH3T3 cells (14) at constitutive compared with activated PPAR-γ-recruiting enhancers (data not shown). Moreover, genes associated with activated enhancers showed strong expression preferentially in adipose tissues, whereas

### TABLE 4

| Genes involved in lipid metabolism linked to PPAR-γ-recruiting activated enhancers identified using GREAT |
|-------------------------------------------------|
| Protein | Gene Name |
|--------|-----------|
| A4ggl | Alms1 |
| Aacc | Accnsp |
| Abc1a | Apoc1 |
| Abcg1 | Apob |
| Abhd5 | Arntl |
| Abca1b | Atp1a1 |
| Abca | Atp2a2 |
| Abca8 | Bcl2 |
| Abca9 | Bdkh1 |
| Abcc4 | Btg2 |
| Abcc5 | Cacybp |
| Abcc7 | Cend2 |
| Abcl1 | Col1a1 |
| Abf1 | Dlc1 |
| Abf2 | Egf2 |
| Abf2 | Ereg |
| Abf2 | Fads1 |
| Abf2 | Fox01 |
| Abf2 | Fox06 |
| Abf2 | Fox51 |
| Abf2 | Gfpt1 |

### TABLE 5

| Genes involved in insulin/IGF signaling linked to PPAR-γ-recruiting constitutive enhancers identified using GREAT |
|-------------------------------------------------|
| Protein | Gene Name |
|--------|-----------|
| Adm | Ghsr |
| Akap1 | Gja1 |
| Anxa1 | Gpam |
| Ar | Grb14 |
| Atf2a2 | Grb2 |
| Bcl2 | Hif1a |
| Bdkh1 | I10 |
| Btg2 | Socs7 |
| Cacybp | Sorbs1 |
| Cend2 | Srf2 |
| Col1a1 | Stat3 |
| Dlc1 | Sxebpa3 |
| Dlc1 | Timp4 |
| Fox51 | Nkx6-1 |
| Fzd1 | Pik3r1 |
| Fzd1 | Por |
| Gfpt1 | Ppar |
| Gfpt1 | Prkce |
| Gfpt1 | Pip1 |
| Gfpt1 | Sh2b |
| Gfpt1 | Sik2 |
| Gfpt1 | Slec2a8 |
| Gfpt1 | Socs7 |
| Gfpt1 | Sorbs1 |
| Gfpt1 | Srf3 |
| Gfpt1 | Stat3 |
| Gfpt1 | Sxebpa3 |
| Gfpt1 | Timp4 |
| Gfpt1 | Nkx6-1 |
| Gfpt1 | Pik3r1 |
| Gfpt1 | Por |
FIGURE 5. Specific examples of PPARγ-regulated genes involved in lipid metabolism and insulin/IGF signaling. A, robust multichip average normalized expression levels of the indicated genes in 3T3-L1 preadipocytes or 3T3-L1 adipocytes transfected with a control siRNA (siCtrl, nontargeting siRNA from Dharmacon) or a siRNA targeting PPARγ (siPPARγ, CAACAGGCCUCAUGAAGAAUU) (35). The data are means ± S.E. from triplicates. Statistically significant differences in gene expression levels are indicated (*, p < 0.05; and ***, p < 0.001). B, the Integrated Genome Browser (31) was used to visualize H3K4me1 and H3K27ac ChIP-seq signal intensities from 3T3-L1 preadipocytes (Pread.) and adipocytes (Ad.) at the indicated genes. The scale was conserved identical for all ChIP-seq data across the four genes. PPARγ-recruiting enhancers are indicated by blue (constitutive) and red (activated) bars above the tracks showing H3K4me1 and H3K27ac ChIP-seq signal intensities.
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A

PhyloP

Constitutive

Activated

PhasCons

B

Activated

Constitutive

PPARγ-recruiting enhancers in adipocytes

C

Activated

Constitutive

Genes associated with PPARγ-recruiting enhancers

D

C2C12 myotubes

Constitutive

Activated

H3K4me1

H3K9ac

E

C2C12 myotubes

Myogenin

% occupied enhancers

19.7

6.3
genes associated with constitutive enhancers showed more even expression across mouse tissues and cell types (Fig. 6C).

Constitutive PPARγ-recruiting enhancers showed stronger overlap with DHS from mouse skeletal muscle (Fig. 6B). Hence, we used ChIP-seq data obtained in C2C12 myotubes (21, 26) to analyze the functionality of adipocyte constitutive and activated PPARγ-recruiting enhancers in a different cellular context. We observed a strong enrichment for H3K4me1 and for the active histone mark H3K9ac at constitutive, but not at adipocyte-activated enhancers in C2C12 myotubes (Fig. 6D).

Interestingly, constitutive enhancers also recruited the myogenic transcription factor Myog in C2C12 myotubes (Fig. 6E), suggesting that these conserved sites could recruit different cell type-specific transcription factors, such as PPARγ and Myog, in different cellular lineages.

**DISCUSSION**

The nuclear receptor PPARγ is a central regulator of adipogenesis (7, 8) operating mainly through binding to enhancers (10–15). PPARγ is required to induce genes instrumental to white adipocyte functions such as lipid metabolism and storage (11). PPARγ-mediated transcriptional regulations have been suggested to rely on enhancer activation during differentiation of preadipocytes into adipocytes (10, 15). Indeed, we have here...
defined PPARγ-recruiting enhancers activated during differentiation that fulfill this function. These enhancers exhibit preferential activity in fat tissues and exhibit strong dynamic epigenomic remodeling during differentiation (Fig. 7). In addition to chromatin opening and active histone marks (10, 14, 15), we show that inactivation of these enhancers in preadipocytes is linked to DNA methylation, which is lost upon adipocyte differentiation (Fig. 7). This is most probably directly functionally correlated with the sharp increase in DNA hydroxymethylation and therefore further emphasizes a role for DNA modifications in the control of enhancer activity during adipogenesis (24).

Importantly, in addition to PPARγ-recruiting enhancers activated during differentiation, we provide here evidence for a functional role exerted by PPARγ recruitment to enhancers already active in preadipocytes (Fig. 7). These enhancers had been suggested to lack functional significance in adipogenesis because they are not associated with typical, strongly induced PPARγ target genes (15). We found that these constitutive enhancers are more evolutionarily conserved and display a relaxed tissue specificity in mouse compared with activated enhancers. This is in line with their association with genes involved in the prevalent insulin/IGF signaling pathway, whose functionality is instrumental for the control of both adipogenesis and mature adipocyte functions (8, 53). Although already expressed in preadipocytes, the expression of these genes is further increased at late stages of adipogenesis, in line with the observed additional increase in chromatin opening and active histone mark levels and the decrease in DNA methylation at constitutive enhancers. Our study therefore extends previous findings (54, 55) indicating an often overlooked role for PPARγ in the transcriptional control of genes involved in insulin/IGF signaling and, most importantly, provides the mechanistic basis of this control by uncovering the involvement of a specific class of PPARγ-recruiting enhancers.

In contrast to enhancers, modulation of gene transcription is not associated with dramatic chromatin remodeling of promoters during adipogenesis. These findings, which are in line with previous studies (49, 50), do not rule out chromatin remodeling at a limited number of promoters and do not exclude that more subtle or specific chromatin remodeling occurs at promoters of regulated genes. For instance, genes up-regulated during myogenesis already harbor active histone modification marks in myoblasts but lose H3K27me3 during differentiation (21). Alternatively, instead of relying on chromatin structure, the regulation of promoter activities may involve alternative recruitment of specific general transcription factors and specific functional cooperation with enhancers (56, 57). C/EBP transcription factors are important for PPARγ expression and activities during adipogenesis (7, 8, 18, 19). Indeed, a subset of activated PPARγ-recruiting enhancers are made competent through the recruitment of C/EBPβ in preadipocytes and subsequent recruitment of C/EBPα in mature adipocytes (10, 13). Although our data are consistent with a role for C/EBPβ at activated enhancers, we report that this factor mainly binds in preadipocytes to constitutive PPARγ-recruiting enhancers. This therefore identifies a potential novel functional link between C/EBPβ and PPARγ in the control of adipogenesis and adipocyte functions.

In this study, we have uncovered that PPARγ regulates genes involved in different biological processes important for adipocyte differentiation and functions through usage of two distinct transcriptional enhancer classes, which are either constitutively active or activated during differentiation. Our study indicates that transcription factors involved in terminal differentiation exert their functions not only through activated cell type-specific enhancers (58) but also in part by modulating activities of more promiscuous, constitutively active ones (Fig. 7). This is illustrated in our study by the observation that constitutive PPARγ-recruiting enhancers in adipocytes are also active and can recruit the myogenic transcription factor Myog in myotubes.

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