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E2Fs Regulate Adipocyte Differentiation

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Summary

When preadipocytes reenter the cell cycle, *PPAR*γ expression is induced, coincident with an increase in DNA synthesis, suggesting the involvement of the E2F family of cell cycle regulators. We show here that E2F1 induces *PPAR*γ transcription during clonal expansion, whereas E2F4 represses *PPAR*γ expression during terminal adipocyte differentiation. Using a combination of in vivo experiments with knockout and chimeric animals and in vitro experiments, we demonstrate that the absence of E2F1 impairs, whereas depletion of E2F4 stimulates, adipogenesis. E2Fs hence represent the link between proliferative signaling pathways, triggering clonal expansion, and terminal adipocyte differentiation through regulation of *PPAR*γ expression. This underscores the complex role of the E2F protein family in the control of both cell proliferation and differentiation.

Introduction

Studies with preadipocyte cell lines, such as 3T3-L1 cells, have been instrumental in unraveling the molecular mechanisms controlling adipocyte differentiation (Green and Kehinde, 1975). Upon reaching confluence, proliferating preadipocytes become growth arrested by contact inhibition. Those contact inhibited preadipocytes reenter cell cycle after hormonal induction, arrest proliferation again, and, finally, undergo terminal adipocyte differentiation. Peroxisome proliferator-activated receptor γ (PPARγ) has been shown to be crucial in the control of terminal adipocyte differentiation (Fajas et al., 1998b; Rosen et al., 2000). PPARγ, upon activation by either fatty acid derivatives or antidiabetic thiazolidinediones, drives the expression of several adipocyte-specific genes, such as the fatty acid binding protein (*aP2*) (Tontonoz et al., 1994a) or lipoprotein lipase (*LPL*) (Schoonjans et al., 1996). Ectopic expression of PPARγ furthermore induces adipocyte differentiation (Tontonoz et al., 1994b). This primordial role of PPARγ in adipocyte differentiation was further highlighted by the phenotype observed in humans with mutations in the *PPAR*γ gene and by the characterization of PPARγ-deficient mice (reviewed in Fajas et al., 2001).

Whereas much effort has been directed toward the understanding of the terminal stages of adipocyte differentiation, the molecular mechanisms underlying the transition between cell proliferation and differentiation of preadipocytes remain largely elusive. Reentry into cell cycle is one of the key events taking place in early adipogenesis, since inhibition of DNA synthesis at this stage blocks differentiation (Patel and Lane, 2000; Reichert and Eick, 1999). Like in most cells, the entry of growth-arrested preadipocytes into S phase depends on the activation of the G1 cyclins/cdks and the retinoblastoma protein pRB-E2F pathway that controls the G1/S transition of the cell cycle. E2F transcription factors are the effectors of this pathway, and they control the expression of genes involved in cell cycle progression, apoptosis, and DNA synthesis (for review see Helin, 1998; Sardet et al., 1997; Trimarchi and Lees, 2002).

E2F activity is the result of the heterodimerization of two proteins belonging to the E2F family (E2F1–6) and the DP family (DP1 and 2), respectively (Dyson, 1998; Gaubatz et al., 1998). When bound to DNA, this heterodimeric complex exists either as free E2F/DP or forms a larger complex that contains a member of the retinoblastoma protein family (pRB, p107, or p130). pRB associates with all E2Fs except for E2F5 and E2F6, whereas p130 associates specifically with E2F4 and E2F5, and p107 complexes associate exclusively with E2F4 (Cobrinik et al., 1993; Sardet et al., 1995). E2F complexes can activate (free heterodimers) or repress (large complexes) the transcription of E2F-responsive genes. Such repression is mediated through the recruitment of histone deacetylases, which interact with proteins of the pRB family (reviewed in Harbour and Dean, 2000). Information about the role of the individual E2F family members has been derived from both overexpression studies and the analysis of E2F-deficient mice and cells (reviewed in Dyson, 1998; Trimarchi and Lees, 2002). These studies show that E2F1–3 play a key role in the activation of E2F-responsive genes and, therefore, the induction of cellular proliferation. In contrast, E2F4 and E2F5 appear to be primarily involved in the repression of target genes and are particularly relevant for the transition from cell proliferation to differentiation (Gaubatz et al., 2000; Humbert et al., 2000a; Rempel et al., 2000).

The E2F and pRB family members appear to participate in the regulation of cell cycle events that are required for adipogenesis. In growth-arrested preadipocytes, E2F4 and E2F5 are complexed with p130, leading to repression of its target genes (for review, see Dyson, 1998). Upon reentry into cell cycle of these growth-arrested preadipocytes, p130, as well as the other members of the retinoblastoma family, is phosphorylated by the cyclin/cdk holoenzymes, releasing the E2F complex,

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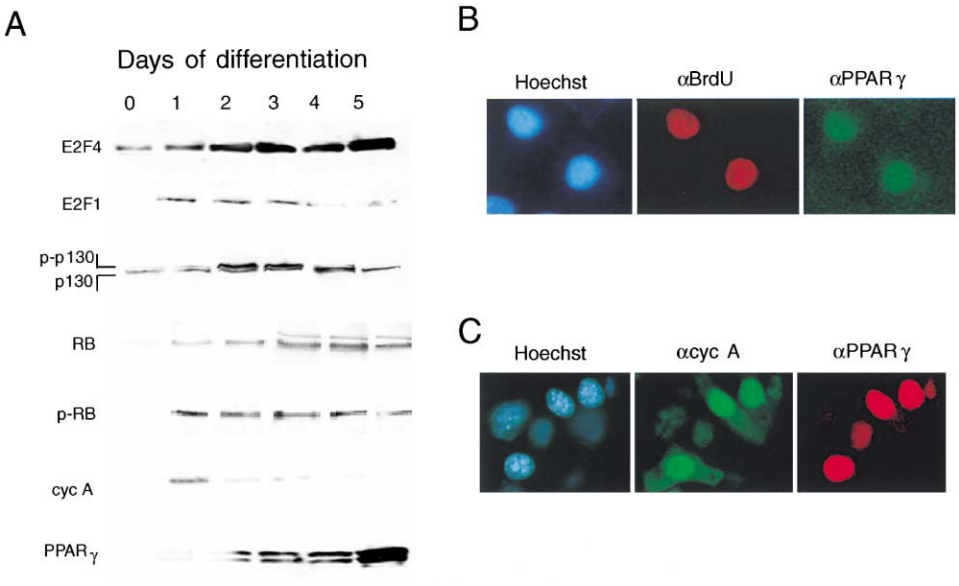

Figure 1. Expression of PPARγ and Cell Cycle-Related Proteins during 3T3-L1 Differentiation

(A) Western blot analysis of whole-cell extracts prepared at different days of adipocyte differentiation of 3T3-L1 cells. The proteins detected with specific antibodies are indicated.

(B) Analysis of PPARγ protein expression and BrdU incorporation by immunofluorescence in postconfluent 3T3-L1 cells stimulated with differentiation medium for 1 day as described under Experimental Procedures. Cells expressing PPARγ are labeled in green (FITC), whereas cells that have incorporated BrdU are labeled in red. Nuclei were stained with the Hoechst reagent (blue staining).

(C) Analysis of cyclin A and PPARγ coexpression by immunofluorescence in 3T3-L1 cells induced to differentiate for 1 day. PPARγ-expressing cells, red; cyclin A-expressing cells, green. Nuclei were visualized with Hoechst staining.

resulting in the activation of the E2F target genes (Richon et al., 1997). After several rounds of DNA synthesis, the cyclin-dependent kinase inhibitors, such as *p21*, *p27*, and *p18*, are induced, and they mediate cell cycle exit and maintain the irreversible growth arrest characteristic of terminal adipocyte differentiation (Morrison and Farmer, 1999). PPARγ and C/EBPα have been shown to contribute to this permanent cell cycle exit by inhibiting the E2F DNA binding activity and upregulating the levels of *p21*, respectively (Altiok et al., 1997; Porse et al., 2001; Timchenko et al., 1996). There is also evidence that pRB plays a positive role in adipocyte differentiation through association and activation of C/EBPα (Chen et al., 1996).

In this study, we show that the E2F proteins play a direct role in the regulation of early adipocyte differentiation. E2F1 and 3 trigger the expression of *PPARγ* during the early stages of adipogenesis, whereas E2F4 represses expression of *PPARγ* at the terminal stage of adipocyte differentiation.

Results

PPARγ Expression Coincides with Reentry into Cell Cycle

When hormonally stimulated, confluent 3T3-L1 preadipocytes reenter cell cycle before they undergo differentiation (Green and Kehinde, 1975). We correlated the expression of some cell cycle regulators and known E2F target genes with the expression of PPARγ during differentiation of 3T3-L1 preadipocytes. Protein levels of cyclin A, E2F1, and E2F4 were increased after 1 day of differentiation (Figure 1A). Whereas levels of cyclin A and E2F1 were not detected during the later stages of adipocyte differentiation, E2F4 protein levels remained elevated for at least 5 days after induction of differentiation. At this time point, adipogenic markers, such as the PPARγ protein, were strongly expressed (Figure 1A, bottom panel). Interestingly, PPARγ expression was switched on at the same time point as cyclin A and E2F1. Consistent with reentry into cell cycle, p130 became and remained phosphorylated during clonal expansion (Figure 1A). At a later stage during differentiation, p130 became hypophosphorylated and, thus, active (Figure 1A). Total pRB protein levels remained almost stable from day 1 of differentiation onward (Figure 1A). Most of the pRB protein was hyperphosphorylated (Figure 1A).

Next, 3T3-L1 cells at day 1 of differentiation were incubated with BrdU to evaluate DNA synthesis. Visualization of the cells by fluorescence microscopy demonstrated that cells which had incorporated BrdU also stained positive for PPARγ protein (Figure 2B), indicating that PPARγ expression coincided with active DNA synthesis in these differentiating 3T3-L1 cells. A similar experiment was performed using an anti-cyclin A antibody. Again, PPARγ was coexpressed in the nucleus with cyclin A, a marker of the S phase of the cell cycle (Figure 2C). These results suggest that expression of *PPARγ* might be under the control of the same factors that induce reentry into the cell cycle, i.e., the E2F family of transcription factors.

E2Fs Bind In Vitro to the *PPARγ1* Promoter

Computer-assisted sequence analysis of the regulatory regions of the human *PPARγ* gene demonstrated the presence of a consensus E2F binding site at position

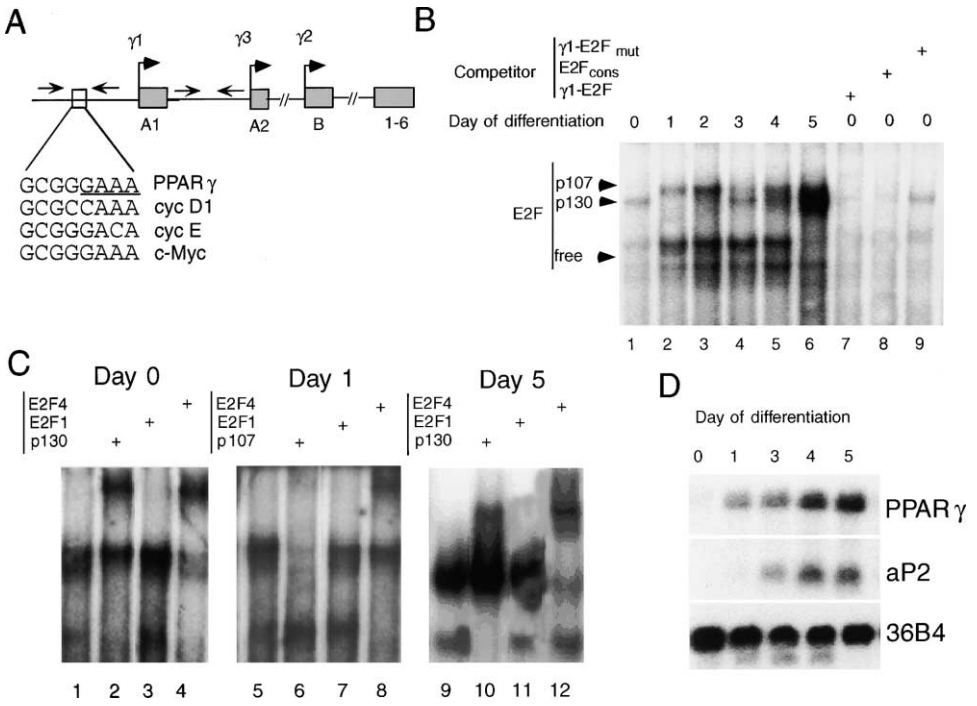

Figure 2. In Vitro Binding of E2F to the *PPARγ1* Promoter

(A) Schematic representation of the genomic structure of the 5′ region of the human *PPARγ* gene. Transcription initiation sites are indicated by arrows and labeled γ1, γ2, and γ3. Shaded boxes indicate exons. The E2F binding site in the *PPARγ1* promoter is depicted as a white box. Comparison of this PPARγ E2F site with the E2F binding site of classical E2F target genes is illustrated. Arrows indicate the DNA fragments amplified in ChIP analysis.

(B) In vitro binding of E2F to the *PPARγ1* promoter. EMSA analysis of the radiolabeled E2F DNA binding site of the PPARγ1 promoter incubated with nuclear extracts of 3T3-L1 cells at the indicated times of differentiation. Composition of the different complexes is indicated. Double-stranded cold oligonucleotides, representing either the PPARγ1-E2F site (γ1-E2F), the mutated PPARγ1-E2F site ($\gamma1\text{-E2F}_{mut}$), or the E2F consensus binding site from the adenoviral E2 promoter ($E2F_{cons}$), were included in the competition assays (lanes 7–9). Only the retarded complexes are shown.

(C) EMSA demonstrating the composition of the retarded complexes in nuclear extracts of 3T3-L1 cells at day 0 (left panel, lanes 1–4), day 1 (middle panel; lanes 5–8), or day 5 (right panel; lanes 9–12) of induction of differentiation. The antibodies used for the gel supershift are indicated.

(D) Northern blot analysis of *PPARγ*, *aP2*, and *36B4* mRNA expression at different times after induction of differentiation of 3T3-L1 preadipocytes.

−215 in the *PPARγ1* promoter. The sequence of the E2F site in the *PPARγ1* promoter was identical to the E2F binding site of the c-Myc promoter and highly similar to the binding sites in the cyclins E and D1 or the adenoviral E2 promoters (Figure 2A). Binding of E2F to the *PPARγ1* promoter was tested by electrophoretic mobility shift assay (EMSA). Typical E2F complexes were bound to the radiolabeled PPARγ-E2F site when whole-cell extracts prepared at different times of the adipocyte differentiation process were used. At day 0, the predominating DNA binding complex (Figure 2B, day 0) was composed of E2F4 and p130 (Figure 2C, lanes 1–4). One day after induction of differentiation, two different complexes were detected: a slower-migrating band and a faster-migrating double complex (Figure 2B, day 1). Faster-migrating complexes contained the different free E2F species (mainly E2F4), whereas E2F4, p107, and cyclin A/cdk2 formed the slow-migrating complex (Figure 2C, lanes 5–8 and data not shown). The pattern of E2F DNA binding observed at this time suggested that most of the cells have already reentered cell cycle. Between days 2 and 4 of differentiation, free E2F remained predominant, whereas the composition of the slow-migrating E2F complex changed from E2F/p107 to E2F/p130 (Figure 2B, days 2–5). At day 5 after induction of differentiation, the free E2F complex decreased in intensity, but, now, the predominant complex was again composed of E2F4 and p130 (Figures 2B and 2C, lanes 9–12). These results hence suggest that E2F is able to bind in vitro to the *PPARγ1* promoter. Furthermore, Northern blot analysis showed a good correlation between the presence of free E2F complexes bound to the PPARγ1 E2F response element and *PPARγ* mRNA levels during adipocyte differentiation of 3T3-L1 cells (Figure 2D). This suggests that E2F could be responsible for triggering *PPARγ* expression early during adipogenesis.

E2Fs Bind In Vivo to the *PPARγ1* Promoter and Transactivate Its Expression

In order to demonstrate the occupancy of the *PPARγ1* promoter by E2F proteins in vivo in human cells, chromatin immunoprecipitation assays (ChIP) were performed using either specific antibodies (anti-E2F4, anti-E2F1, or an anti-acetylated H4 antibody) or preimmune serum. Whereas no amplification of the *PPARγ1* promoter was observed when nonspecific antibodies were used (mock; Figure 3A, lanes 5 and 10), a specific fragment,

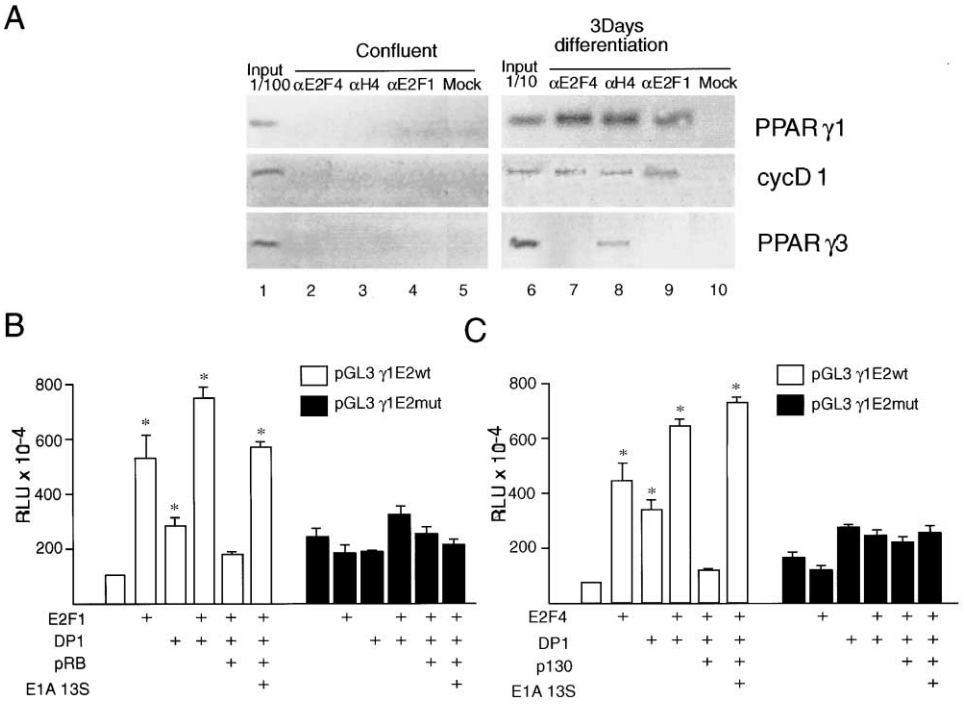

Figure 3. In Vivo Binding of E2F4 to the *PPARγ1* Promoter and Transactivation Assays

(A) Chromatin immunoprecipitation (ChIP) assays demonstrating binding of E2F1 and E2F4 to the *PPARγ1* promoter. Cross-linked chromatin from either confluent (left panel) or human primary preadipocytes differentiated during 3 days (right panel) was incubated with antibodies against E2F4 (lanes 2 and 7), E2F1 (lanes 4 and 9), acetylated histone H4 (lanes 3 and 8), or without any antibody (mock, lanes 5 and 10). Immunoprecipitates were analyzed by PCR using primers specific for the indicated promoters (see Figure 2A). As a control, a sample representing 1% of the total chromatin was included in the PCR (input, lanes 1 and 6).

(B–C) E2Fs modulate the *PPARγ1* promoter. Relative luciferase activity as determined after transfection of NIH 3T3 cells with the reporter constructs pGL3γ1E2$_{wt}$ and pGL3γ1E2$_{mut}$. Cells were either transfected with an empty expression vector, with expression vectors for E2F1, E2F4, DP1, p130, pRB, or E1A 13S, or with combinations of these vectors, as indicated. Values are the mean of three independent experiments. An asterisk depicts statistically significant differences.

corresponding to the *PPARγ1* promoter, was amplified when chromatin of cells that were stimulated to differentiate was immunoprecipitated with either the anti-E2F4 (Figure 3A, lane 7) or the anti-E2F1 (Figure 3A, lane 9) antibody. No amplification product was observed when immunoprecipitated chromatin from confluent nondifferentiated human primary preadipocytes was used as the template (Figure 3A, lanes 2 and 4). Strikingly, the same pattern of E2F1 and E2F4 binding was observed when oligonucleotides corresponding to the *cyclin D1* promoter were used to amplify the immunoprecipitated chromatin (Figure 3A, lanes 2, 4, 7, and 9). A different fragment from the *PPARγ* gene, covering the proximal *PPARγ3* promoter (Fajas et al., 1998a), could not be amplified, either from confluent or differentiated cells, when the anti-E2F1 or the anti-E2F4 antibody was used (Figure 3A, lanes 2, 4, 7, and 9). The results of these ChIP assays hence prove that E2F1 and E2F4 specifically bind to the *PPARγ1* promoter in differentiating human adipocytes. ChIP assay also demonstrated the presence of acetylated histone H4 on the *PPARγ1* and *cyclin D1* promoters (Figure 3A, lane 8), suggesting that the binding of E2Fs results in the activation of these promoters. Interestingly, when using an anti-acetylated histone H4 antibody, the *PPARγ3* promoter could also be amplified, indicating that, at this stage of differentiation, the *PPARγ3* promoter was also activated, although not directly by binding of E2F (Figure 3A, lane 8).

To assess whether E2Fs can directly regulate the human *PPARγ1* promoter activity, we cotransfected expression plasmids coding for E2F1, E2F4, DP1, pRB, or p130 together with the pGL3γ1E2$_{wt}$ luciferase reporter vector, which contains the human *PPARγ1* promoter (Fajas et al., 1997). When NIH-3T3 cells were cotransfected with either E2F1 (Figure 3B) or E2F4 (Figure 3C), luciferase activity increased 5- and 4-fold, respectively, relative to the basal activity of the *hPPARγ1* promoter. This induction was substantially enhanced (up to 8-fold) upon cotransfection with the heterodimeric partner of E2F, DP1. As expected for an E2F target gene, when a constitutively active form of pRB or p130 was cotransfected with E2F1/DP1, the stimulatory effect on the *PPARγ1* promoter was abrogated (Figures 3B and 3C). This repressive effect of E2Fs of the *PPARγ1* promoter was clearly dependent on the presence of the pocket proteins pRB and p130, since cotransfection of the adenoviral protein E1A 13S resulted in the abrogation of the repression (Figures 3B and 3C). Similar results were observed when an E2F3 expression vector was used (data not shown). Finally, to demonstrate that it is

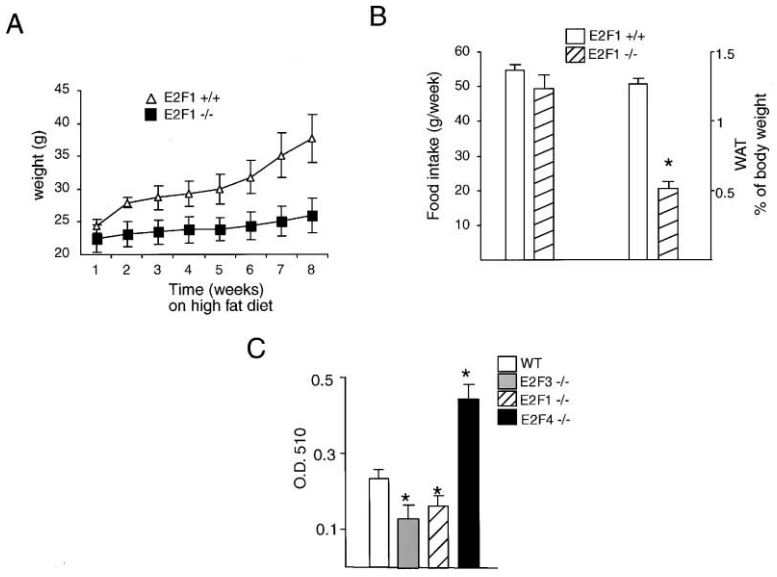

Figure 4. E2F1–3 Stimulate Adipogenesis

(A) Weight gain curves of *E2F1*$^{-/-}$ (black squares) and *E2F1*$^{+/+}$ (black triangles) mice fed with a high-fat diet. Each group was composed of ten animals. Animals were weighed every week for a period of 8 weeks.

(B) Food intake and weight of the epididymal fat pads (expressed as % of total body weight) of the animals used in the experiment described in A. Statistically significant differences ($p < 0.05$) are indicated by an asterisk.

(C) Quantification of lipid incorporation by measuring the intensity of Oil red O staining of wild-type (WT), *E2F1*$^{-/-}$, *E2F3*$^{-/-}$, or *E2F4*$^{-/-}$ MEFs stimulated to differentiate into adipocytes. An asterisk indicates statistically significant differences ($p < 0.05$).

through binding to the PPARγ1-E2F site that E2Fs modulate the activity of the *hPPARγ1* promoter, we substituted four bases in the PPARγ1-E2F site (from GCG GGAAA to GCGGCCGC) to generate the pGL3γ1E2$_{mut}$ reporter plasmid. Cotransfected E2F/DP1 was unable to stimulate the mutated pGL3γ1E2$_{mut}$ reporter vector in NIH-3T3 cells (Figure 3B, black bars). In the same experiment, the wild-type promoter was induced (data not shown).

E2F1 and E2F3 Positively Regulate Adipogenesis

We have shown that E2F1 and PPARγ1 are expressed at the same time in the differentiation process and that E2F1 binds and activates the *PPARγ1* promoter. To determine whether the activating E2Fs, E2F1 and E2F3, play a positive role in adipogenesis, we analyzed the effects of *E2F1* or *E2F3* depletion either in mice or in primary mouse embryonic fibroblasts (MEFs). Consistent with a role for E2F1 in triggering *PPARγ* expression and adipogenesis, *E2F1*$^{-/-}$ mice were resistant to obesity induced by feeding them a high-fat diet for eight weeks, whereas *E2F1*$^{+/+}$ mice increased their weight significantly upon this high-fat diet (Figure 4A). The difference in weight could be entirely attributed to differences in fat mass (Figure 4B). No significant differences were observed in food intake between the *E2F1*$^{-/-}$ and the *E2F1*$^{+/+}$ mice (Figure 4B). The phenotype of the *E2F1*$^{-/-}$ animals suggested a problem in adipose tissue homeostasis. To test this notion, we compared the capacity of MEFs deficient in the various *E2F*s to differentiate into adipocytes in vitro in response to hormone stimulation. Adipocytes were scored using Oil red O staining to detect lipid droplets. Hormonally stimulated *E2F1*$^{-/-}$ and *E2F3*$^{-/-}$ MEFs showed a slightly, but consistently, reduced capacity to differentiate into adipocytes in vitro compared to wild-type MEFs (Figure 4C). Taken together, these data suggest that E2F1–3 stimulate adipogenesis through activation of PPARγ.

E2F4 Negatively Regulates Adipogenesis

The ChIP experiments showed that, in addition to E2F1, E2F4 associated with the *PPARγ* promoter in vivo. Our transient transfection assays showed that E2F4 activates the pGL3γ1 luciferase reporter, and this is repressed by the coexpression of p130 (Figure 3C), confirming that E2F4 can regulate the *PPARγ* promoter. However, since E2F4 behaves as an activating E2F when it is overexpressed, this assay did not reveal whether the endogenous E2F4 regulates *PPARγ* expression and, therefore, adipogenesis positively (through free E2F/DP heterodimers) or negatively (through recruitments of pRB family members and associated HDACs). To address this issue, we determined how the absence of E2F4 affected adipogenesis. The *E2F4*$^{-/-}$ mice are highly susceptible to bacterial infections, and the resulting abnormal feeding pattern precludes their use for analysis of adipose tissue homeostasis in vivo (Humbert et al., 2000a; Rempel et al., 2000). As an alternative approach, we first evaluated the ability of *E2F4*$^{-/-}$ MEFs (Figures 4C and 5A) and ES cells (Figure 5B) to differentiate into adipocytes in vitro. In both cases, we saw a significant increase in the number of Oil red O-positive cells in the *E2F4*$^{-/-}$, compared to the *E2F4*$^{+/+}$, monolayers (Figures 4C, 5A, and 5B). This suggests that E2F4 exerts a negative effect on adipogenesis.

To further explore this hypothesis, *E2F4*$^{+/+}$ or *E2F4*$^{-/-}$ 129 D3 ES cells were injected into C57BL/6 blastocysts, which were then reimplanted in pseudopregnant wild-type mice in order to create chimeric progeny. Twelve mice of each genotype considered highly chimeric were sacrificed, and several organs were harvested and analyzed for the contribution of the ES cells to the composition of the tissues, using GPI as a marker. Tissue analysis revealed only a minor contribution of the injected 129 D3 *E2F4*$^{+/+}$ ES cells to the development of white adipose tissue of the chimeric mice (Figures 5C and 5D). In contrast, white adipose tissue from chimeric mice resulting from injection of 129 D3 *E2F4*$^{-/-}$ cells was composed of at least 40% of the mutant cells (Figures 5C and 5D).

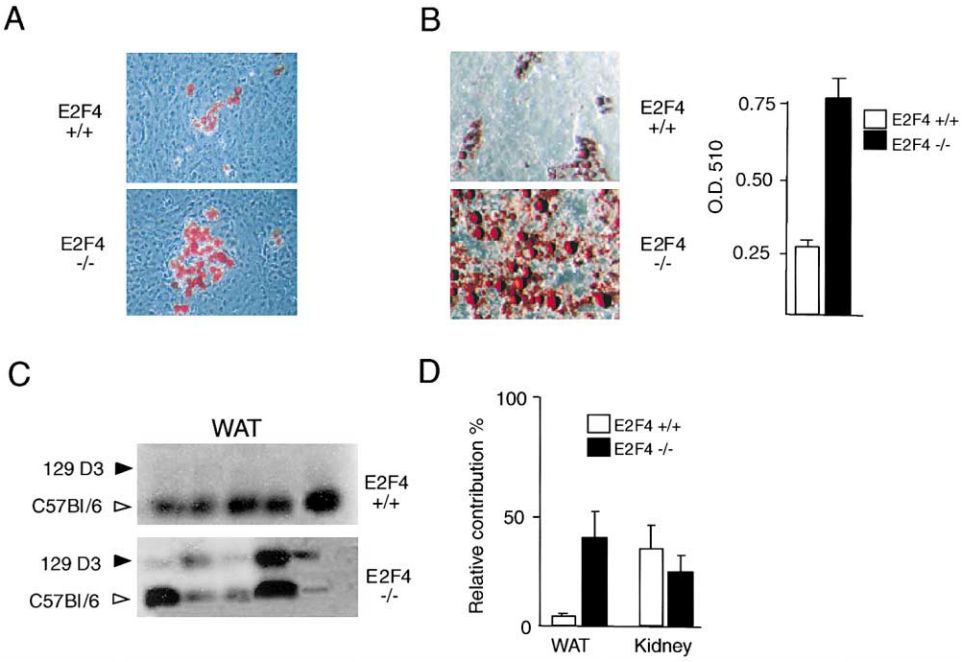

Figure 5. E2F4 Inhibits Adipogenesis

(A) Oil red O staining comparing lipid accumulation in *E2F4*$^{+/+}$ or *E2F4*$^{-/-}$ MEFs induced to differentiate into adipocytes.

(B) Oil red O staining comparing lipid accumulation in *E2F4*$^{+/+}$ or *E2F4*$^{-/-}$ ES cells induced to differentiate into adipocytes as described in Experimental Procedures. Quantification of lipid incorporation by measuring the intensity of Oil red O staining is indicated at the right.

(C) Protein electrophoresis followed by colorimetric detection of the two different GPI isoforms in adipose tissue (WAT) of chimeric mice. The lower band, indicated by an open arrowhead, corresponds to the GPI isoform contributed by recipient C57Bl/6 blastocyst cells. The upper band, indicated by a closed arrowhead, corresponds to the GPI isoform derived from microinjected *E2F4*$^{+/+}$ (upper panel) or *E2F4*$^{-/-}$ 129 D3 ES cells (bottom panel).

(D) Quantification of the relative contribution of the microinjected ES cells (WT, *E2F4*$^{-/-}$) in the development of either adipose tissue (WAT) or kidney.

Importantly, no significant differences in contribution between *E2F4*$^{+/+}$ and *E2F4*$^{-/-}$ chimeras were observed in some other tissues, such as kidney or pancreas (Figure 5D and data not shown). Together with the in vitro studies of the *E2F4*$^{-/-}$ cells, the analysis of these chimeric mice proves that E2F4 has a negative effect on adipose tissue development.

Coincidence of PPARγ and E2F1 or E2F4 Expression in the Nucleus Is Dependent on the Stage of Adipogenesis

Significantly, our analysis of the endogenous E2F complexes shows that E2F4 associates with the hypophosphorylated and, thus, active form of p130 prior to induction of adipogenesis, and the disappearance of this complex coincides with the induction of *PPAR*γ expression on day 1 of differentiation (compare Figures 2B and 1A). This suggests that E2F4 inhibits adipocyte differentiation by repressing *PPAR*γ expression through its association with p130. It has been previously shown that the free E2F4/DP complexes are predominantly nuclear, whereas association with the p130 and pRB relocalizes these complexes in the cytoplasm (Magae et al., 1996; Muller et al., 2001; Verona et al., 1997). We therefore compared the expression and subcellular localization of PPARγ, E2F1, and E2F4 in 3T3-L1 cells and human primary preadipocytes at different stages of adipogenesis by immunofluorescence microscopy (Figure 6 and data not shown). Confluent, growth-arrested 3T3-L1 preadipocytes displayed high levels of nuclear E2F4, whereas E2F1 and PPARγ were expressed at low to undetectable levels (Figures 6A and 6B, 0 hr). Twenty-four hours after induction of differentiation, a significant fraction of the cells expressed PPARγ (Figures 6A and 6B, 24 hr). Significantly, the PPARγ-positive cells all expressed high levels of nuclear E2F1 and E2F4 protein. This is consistent with the idea that E2F1 activates *PPAR*γ expression (Figure 3B). Moreover, since the p130 protein has largely dissociated from E2F4 at this time (Figure 2B), it suggests that free E2F4 may also contribute to the activation of *PPAR*γ. During the terminal differentiation phase (96 hr), nuclear PPARγ protein is expressed in most, but not all, of the cells (Figures 6A and 6B). By this stage, E2F1 is undetectable. This is consistent with the end of the clonal expansion phase and the declining expression of E2F1 (Figure 1A). In contrast, E2F4 is still expressed in all cells (Figure 6A). However, at this stage, the majority of these cells have cytoplasmic, rather than nuclear, E2F4 (Figure 6A, arrowheads). Significantly, we saw a perfect correlation between the localization of E2F4 and the expression of PPARγ: E2F4 was cytoplasmic in PPARγ-positive cells

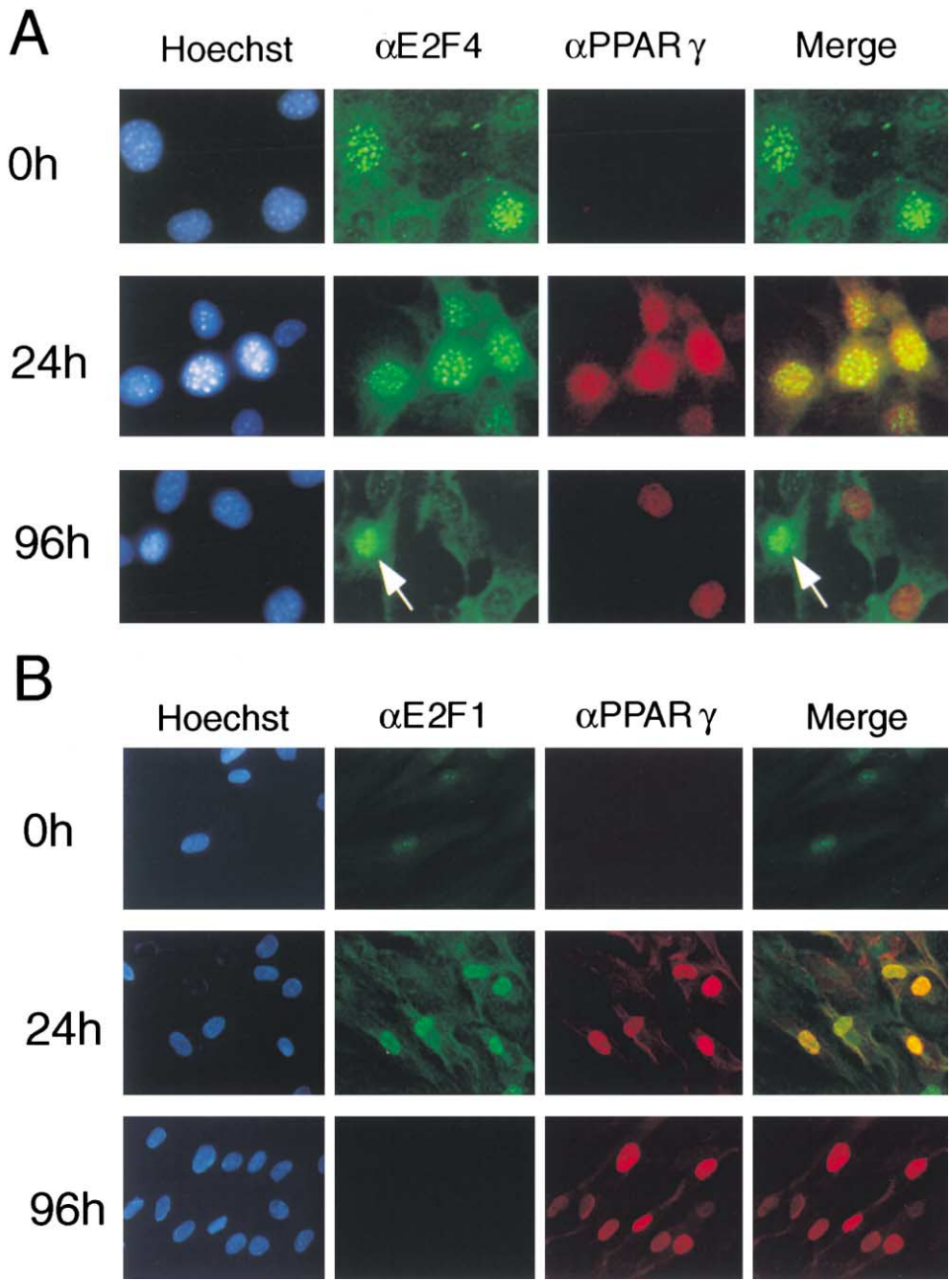

Figure 6. Comparative Analysis of PPARγ, E2F4, and E2F1 Expression by Immunofluorescence at Different Times of the Adipocyte Differentiation Program

Confluent 3T3-L1 cells (A) or human primary preadipocytes (B) were stimulated to differentiate as described. At the indicated time points of differentiation, cells were fixed and incubated with both anti-PPARγ and anti-E2F4 (A) or anti-E2F1 (B) antibodies. PPARγ protein was detected with a Texas red-labeled secondary antibody (red labeling), whereas E2F4 and E2F1 proteins were detected with an FITC-labeled secondary antibody (green labeling). Nuclear localization was assessed by Hoechst staining of the nuclei. The white arrow in panel (A) highlights the fact that E2F4 nuclear localization in differentiating cells coincides with the absence of PPARγ expression.

and nuclear in PPARγ-negative cells (Figure 6A, arrowhead). Thus, at the late stage of differentiation, expression of PPARγ can occur in the complete absence of either free E2F1 or free E2F4 complexes. Moreover, the presence of nuclear E2F4 complexes correlates with the repression of this responsive gene.

Discussion

Differentiation of preadipocytes into adipocytes requires that growth-arrested preadipocytes reenter the cell cycle before undergoing terminal differentiation. This particular situation is underscored by the fact that

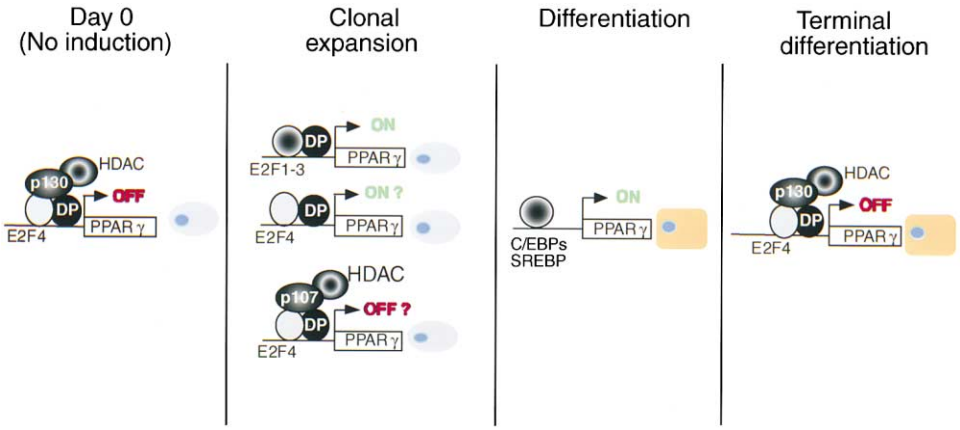

Figure 7. Model of *PPARγ* Regulation by E2Fs during Adipogenesis in the Presence or Absence of E2F4

In growth-arrested preadipocytes (day 0), the *PPARγ* gene is silenced by E2F4-p130-HDAC complex. During the clonal expansion phase, at least two pools of cells can be distinguished: cells in which E2F1–3 and possibly E2F4 trigger the expression of *PPARγ* and cells in which E2F4 is associated with a p107-HDAC repressor complex. In cells that are actively differentiating (Differentiation), E2F4 is exported to the cytoplasm, and E2F1 is not expressed. This allows that other transcription factors, such as C/EBPs or ADD1/SREBP1, sustain the expression of *PPARγ*, resulting in the differentiation of the cells into adipocytes. When cells are terminally differentiated (Terminal differentiation), E2F4 represses the expression of *PPARγ*, through association with p130/p107, which recruits HDACs to the *PPARγ* promoter. Undifferentiated preadipocytes, gray; differentiated adipocytes, orange.

adipocyte differentiation is essentially triggered by proliferative stimuli, such as insulin or cAMP. In this study, we elucidate the molecular mechanisms operating at the transition between cell proliferation and adipocyte differentiation. Consistent with the previous observation that a block of DNA synthesis during the clonal expansion phase of adipocyte differentiation inhibits adipogenesis (Patel and Lane, 2000; Reichert and Eick, 1999), we have demonstrated that clonal expansion is accompanied by changes in the E2F complexes. Within 1 day of hormonal induction, the suppressive E2F4/p130 complex is completely lost, and there is a major induction of the expression of the activating E2Fs, particularly E2F1. These changes correlated closely with the induction of E2F-responsive genes, like *cyclin A*, that are known to be required for cellular proliferation. Significantly, the master regulator of adipogenesis, *PPARγ*, is induced at the same time, and our data strongly suggest that this is a bona fide E2F target gene. First, the E2F proteins bind to a consensus E2F DNA binding site in the *PPARγ1* promoter both in vitro (EMSA) and in vivo (ChIP). Second, reporter assays confirm that the E2Fs can regulate transcription from the *PPARγ1* promoter in a manner that is entirely dependent upon the identified E2F binding site. Consistent with the hypothesis that the E2F proteins play a direct role in the regulation of *PPARγ* expression, we also present genetic evidence that the individual E2F proteins have profound effects upon adipocyte differentiation in vitro and in vivo. E2F1- and E2F3-deficient cells have a reduced capacity to differentiate into adipocytes, and *E2F1*$^{-/-}$ mice have limited fat accretion upon high-fat feeding. Thus, these activating E2Fs play a positive role in adipogenesis. In contrast, E2F4 has a negative influence on the differentiation process. E2F4-deficient MEFs and ES cells have an increased propensity to undergo adipogenesis. Moreover, *E2F4*$^{-/-}$ ES cells contribute at a significantly higher level to adipose than to other tissues of chimeric mice.

Based on the appearance/disappearance and subcellular localization of the individual E2F complexes during differentiation and the phenotypes of the E2F-deficient mice and cells, we propose the following model of E2F action (Figure 7). In confluent preadipocytes, the E2F4/p130 complex acts to repress the transcription of E2F-responsive genes, including *PPARγ*. Hormonal stimulation results in the loss of this repressive complex and the induction of E2F1. This leads to the coordinate expression of the classic E2F target genes, the activation of clonal expansion, the expression of *PPARγ*, and, thereby, activation of differentiation. Our data strongly suggest that free E2F1/DP is involved in the transcriptional activation of *PPARγ*. During the late stages of differentiation, the expression of *PPARγ* clearly occurs in the absence of nuclear E2F species. This is entirely consistent with previous observations that *PPARγ* expression is regulated by other transcription factors, including C/EBPs and SREBP (Fajas et al., 1999; Saladin et al., 1999). Importantly, at this stage, the E2F4/p130 complex reappears, and the presence of nuclear E2F4 is clearly incompatible with *PPARγ* expression. This raises the possibility that the reformation of E2F4/p130 might facilitate the "switching off" of *PPARγ* that occurs in terminally differentiated adipocytes. Significantly, the opposing roles of E2F1–3 and E2F4 in the regulation of *PPARγ* expression and, therefore, adipogenesis are entirely consistent with the prevailing view that these proteins are involved in the activation or repression of classic E2F-responsive genes, respectively (reviewed in Trimarchi and Lees, 2002). Moreover, our model fits with the previous demonstration that adipogenesis is promoted by the combined loss of p107 and p130, the major E2F4-associated proteins (Classon et al., 2000). However, a number of issues remain to be resolved. First, since E2F4 remains in the nucleus during clonal expansion, we cannot rule out the possibility that free E2F4/DP complexes actually contribute to the activation of *PPARγ*. At least in transient transfection assays, E2F4

can transactivate the *PPARγ* promoter in the absence of p130. The phenotype of the E2F4-deficient mice and cells simply shows that E2F4's repressive activity is dominant over any role it might play in activation. Second, the E2F and pocket proteins have now been shown to regulate adipogenesis through a number of different mechanisms that appear to affect cell cycle regulation and/or the differentiation process. For example, pRB appears to be essential for both cell cycle exit and cooperation with the C/EBP family members in the activation of key transcriptional targets (Chen et al., 1996; Classon et al., 2000; Hansen et al., 1999; Higgins et al., 1996). Thus, it will be important to establish the relative importance of these various mechanisms and to determine how they are coordinated in vivo. Finally, it is currently unclear why *PPARγ* is not expressed along with other E2F-responsive genes in actively cycling cells. However, we envisage several possibilities. Chromatin remodeling could account for the silencing of the *PPARγ* gene in proliferating cells, as has been shown for other genes (Krebs et al., 2000; Krebs et al., 1999). In addition, other repressive protein complexes may specifically inhibit the expression of the *PPARγ* gene in cycling cells. GATA-2, GATA-3, and AEBP1 are all good candidates for such a role (Tong et al., 2000; He et al., 1995). Alternatively, signaling through the Wnt-10b and Pref-1 (Notch-like) pathway could render the *PPARγ* gene refractory to induction by E2Fs (Ross et al., 2000; Smas and Sul, 1993). Interestingly, the induction of *PPARγ* expression has been reported in a number of proliferative disorders, such as colon cancer (DuBois et al., 1998), prostate adenocarcinoma (Mueller et al., 2000), and breast cancer cells (Mueller et al., 1998; Samid et al., 2000). It is therefore tempting to speculate that the mechanism that normally blocks *PPARγ* expression in cycling cells is somehow abrogated.

In summary, our data show that E2F1–3 and E2F4 have opposing effects on adipocyte differentiation that appears to be largely attributable to their differential regulation of *PPARγ* expression. This provides direct support for the idea that the E2F and pRB family members play a central role in coordinating the transition between cell proliferation and terminal differentiation. Modulation of the activity of E2Fs might open up new perspectives in the control of adipogenesis and metabolic diseases.

Experimental Procedures

Materials and Oligonucleotides

The oligonucleotides used for various experiments in this manuscript are the following in (5′ to 3′ orientation): E2 consensus, GCA TAAGTTTCGCGCCCTTTCTCAG; E2F-PPARγ1 site, GGACGCGG GAAAGCCGGTGGCTCC; E2F-PPARγ1 mutated, GGACGCGGCC GCGCCGGTGGCTCC; ChIP PPARγ1 forward, GGACGCGGGAA AGCCGGTGGCTCC; ChIP PPARγ1 reverse, GAGGGAGCGGCC CGGGCTCGG; ChIP PPARγ3 forward, GTTAAGATTTGAAAGAAGC CGACAC; ChIP PPARγ3 reverse, GGCTCTTCATGAGGCTTATTG TAGA; ChIP cyclin D1 forward, CAGGCGCGGCGCCTCAGG GATGGC; ChIP cyclin D1 reverse, GTCCGTGTGACGTTACTG TTGTTA. Rosiglitazone (BRL 49,653) was a kind gift of Dr. R. Heyman of Ligand Pharmaceuticals (San Diego, CA). All chemicals, unless stated otherwise, were purchased from Sigma (St. Louis). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) except for the anti-BrdU antibody, which was purchased from Sigma.

Plasmids and Probes

The PPARγ, aP2, and 36B4 probes are described previously (Fajas et al., 1997). For the construction of the expression vector pcDNA-E2F4, a BamHI/EcoRI fragment from pBabe-E2F4 (a kind gift of Dr. Helin) was inserted in the same sites of the pcDNA3 vector (Stratagene, La Jolla, CA). The CMV-DP1, CMV-E2F1, and p130 expression vectors were a gift from Dr. L. Le Cam. The PPARγ1, PPARγ2, and PPARγ3 promoter reporter vectors have been previously described (Fajas et al., 1997; Fajas et al., 1998a). The PPARγ1 promoter reporter vector with a mutation in the E2F binding site was constructed by site-directed mutagenesis using the indicated oligonucleotides.

Cell Culture, Protein Extracts, Retroviral Infection, Transfections, and Implantation of Preadipocytes

E2F1$^{-/-}$, *E2F3*$^{-/-}$, and *E2F4*$^{-/-}$ and wild-type MEFs were described (Humbert et al., 2000a; Humbert et al., 2000b). NIH-3T3 and 3T3-L1 cells were grown in DMEM and 10% fetal calf serum (FCS). Human primary preadipocytes were purchased from Zen-Bio (Research Triangle, NC) and maintained under the conditions specified by the provider. Cells were differentiated with DMEM, 10% serum, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 10 μg/ml insulin, and 1μM dexamethasone for 2 days. From day 3 on, cells were incubated with DMEM, 10% serum, 10 μg/ml insulin, and 1 nM BRL 49,653. Oil red O staining and quantification are described elsewhere (Ramirez-Zacarias et al., 1992). Nuclear and whole-cell extracts were prepared as described (Fajas et al., 1997). All transfections were performed using the Lipofectamine Plus reagent (GIBCO Life Technologies, Rockville, MD). Luciferase activity was measured as described (Fajas et al., 1997).

ES Cells Differentiation, Chimeric and Knockout Mice

Generation and maintenance of *E2F4*$^{+/+}$, *E2F4*$^{+/-}$, and *E2F4*$^{-/-}$ ES cells were described previously (Humbert et al., 2000a). ES cells were differentiated according to Rosen et al. (Rosen et al., 1999). For generation of chimeric mice, wild-type or *E2F4*$^{-/-}$ ES cells were injected into 4 day blastocysts from C57BL/6 mice and reimplanted into pseudopregnant wild-type mice. At 7 weeks of age, animals were sacrificed, and the organs were harvested in 50 mM Tris-HCl and 0.1% Triton X-100. All materials for glucose phosphate isomerase (GPI) analysis were purchased from Helena Labotratories (Beaumont, Texas). GPI analysis was performed as previously described (Nagy and Rossant, 1999). Briefly, cells derived from the injected 129 D3 ES cells (*E2F4*$^{+/+}$ and *E2F4*$^{-/-}$) will have a different GPI isoform with different electrophoretic mobility than the cells derived from the C57BL/6 recipient blastocysts. Quantification of the electrophoretic bands after an enzymatic colorimetric reaction will be indicative of the relative composition of the analyzed tissue. *E2F1*$^{-/-}$ and *E2F1*$^{+/+}$ mice were purchased from Jackson Laboratories (Bar Harbor, ME) and fed a high-fat diet (58% fat, 25% carbohydrates, and 16% protein) for 8 weeks.

Electrophoretic Mobility Shift Assays (EMSA) and Chromatin Immunoprecipitation (ChIP)

Cell extracts were incubated for 15 min at 21°C in a total volume of 20 μl binding buffer [10 mM Tris-HCl (pH 7.9), 40 mM KCl, 10% glycerol, 0.05% Nonidet P-40, 1 mM DTT, and 1 μg poly(dI:dC)] in the presence of 1 ng of a T4-PNK end-labeled double-stranded oligonucleotide probe. For gel supershift analysis, 2 μg of antibody were added to the reaction. DNA-protein complexes were separated by electrophoresis on a 4% polyacrylamide gel in 0.25 × TBE buffer at 21°C and 10 V/cm. Chromatin immunoprecipitation assays were performed as described previously (Takahashi et al., 2000). Briefly, proteins were formaldehyde cross-linked to DNA in confluent human primary preadipocytes before induction of differentiation or in cells induced with differentiation medium for 3 days. Proteins were then immunoprecipitated using the indicated antibodies, DNA was extracted from the immunoprecipitates, and PCR amplification was performed using promoter-specific oligonucleotide primers.

Immunofluorescence

For all immunofluorescence experiments, cells were grown on coverslips. For BrdU incorporation, cells were incubated 4 hr in the

presence of BrdU, and an additional treatment of the cells with 1.5 N HCl for 10 min at 21°C was performed. After fixation and permeabilization with methanol, cells were incubated with the indicated antibodies. Preparations were then incubated with a combination of Texas red-conjugated anti-mouse IgG and FITC-conjugated anti-rabbit IgG.

Northern and Western Blot Analysis

SDS-PAGE and electrotransfer were performed as described (Schoonjans et al., 1996). The membranes were blocked overnight in blocking buffer (20 mM Tris, 100 mM NaCl, 1% Tween-20, and 10% skim milk). Filters were first incubated 4 hr at 21°C with the indicated antibody and then for 1 hr at 21°C with a peroxidase conjugate secondary antibody. The complex was visualized with 4-chloro-1-naphtol as reagent. Northern blots were hybridized with PPARγ, aP2, and 36B4 cDNAs and visualized as described (Schoonjans et al., 1996).

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