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RESEARCH ARTICLE

Transcriptional coactivator EDF-1 is required for PPARγ-stimulated adipogenesis

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Abstract Peroxisome proliferator-activated receptor-γ (PPARγ) is essential for adipogenesis. Since EDF-1 is a cofactor of PPARγ, we investigated the molecular cross-talk between EDF-1 and PPARγ in adipogenesis. While EDF-1 was not modulated during differentiation of 3T3-L1 cells, it co-immunoprecipitated with PPARγ. Silencing EDF-1 by shRNAs inhibited the differentiation in adipocytes of 3T3-L1 cells, as detected by the staining of intracellular triglycerides and the expression of the PPARγ target gene aP2. Accordingly, we found that anti-EDF-1 shRNAs decreased ligand dependent activation of PPARγ in 3T3-L1 transiently transfected with a vector expressing luciferase under the control of a PPARγ responsive consensus. To rule out that this inhibition is due to the concomitant downregulation of PPARγ levels, we overexpressed PPARγ in 3T3-L1 silencing EDF-1 and found a decrease of ligand dependent activation of PPARγ, in spite of the high amounts of PPARγ. These results demonstrate that EDF-1 is required for PPARγ transcriptional activation during 3T3-L1 differentiation.

Keywords Adipogenesis · EDF-1 · PPARγ · Transcriptional coactivator

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Introduction

Adipose tissue is composed of adipocytes that store energy in the form of triglycerides. Excessive accumulation of white adipose tissue leads to obesity, which is a risk factor of several common diseases, including type II diabetes and cardiovascular diseases. The differentiation of preadipocytes into adipocytes is regulated by an elaborate network of transcription factors that control the expression of hundreds of genes responsible for establishing the mature adipocyte phenotype [1]. The peroxisome proliferator-activated receptor-γ (PPARγ) is the main protein orchestrating the differentiation and function of adipocytes, as evidenced by the combination of in vitro studies, the analysis of mouse models, and the characterization of patients with mutations in the human PPARγ gene [2, 3]. PPARγ induces the expression of adipocyte-specific genes through the binding of PPARγ-retinoid X receptor (RXR)-heterodimers to a PPAR-response element (PPRE) [4], resulting in the promotion of intracellular fat storage. PPARγ is activated by the binding of small lipophilic ligands, mainly fatty acids, derived from nutrition or metabolic pathways, or synthetic agonists, like the anti-diabetic thiazolidinediones [3, 5, 6]. Docking of these ligands alters the conformation of PPARγ, resulting in transcriptional activation because of the release of corepressors and the recruitment of coactivators. Many corepressors and coactivators modulating PPARγ activity have been described. They regulate transcriptional activity by altering chromatin structure via enzymes such as histone deacetylases and histone acetyltransferases [7]. Among others, EDF-1 (also known as hMBF-1) has been shown to be a coactivator for PPARγ [8]. EDF-1 is a highly conserved intracellular protein of 148 amino acids which was initially isolated in our laboratory as implicated in

endothelial differentiation [9]. In endothelial cells, EDF-1 is both cytosolic and nuclear [10–12]. We have shown that cytosolic EDF-1 binds calmodulin, whereas nuclear EDF-1 interacts with the TATA box-binding protein (TBP) [10, 11]. Interestingly, EDF-1 possesses a helix-turn-helix region (residues 57–148) [13], but it has no enzymatic activity resulting in histone modification [8]. EDF-1 is also a coactivator for steroidogenic factor 1, Liver receptor homologue 1 (LRH-1), and Liver X receptor alpha (LXR) [8], and it has been shown to discriminate among bZIP proteins since it enhances the transcriptional activity of Djun, c-Jun, NRL, and ATF1, while it does not affect the transcriptional activity of Dfos, EB1 and ATF4 [14].

Although co-activators are important for transcription, their in vivo roles are mostly poorly understood. This is also the case of EDF-1. Because PPARγ is a master regulator of adipocyte differentiation and EDF-1 is one of its coactivator, we evaluated whether EDF-1 might play a role in modulating PPARγ activity in an experimental model of adipogenesis, i.e. the differentiation of 3T3-L1 cells in adipocytes.

Materials and methods

Cell culture and differentiation

Murine 3T3-L1 preadipocytes (kindly provided by E. Nisoli, Milan, Italy) were cultured, maintained, and differentiated as described previously [15]. Briefly, cells were grown for 2 days post-confluence in DMEM supplemented with 10% calf serum. Differentiation was then induced (day 0) by changing the medium to DMEM containing 10% fetal bovine serum (FBS) (Euroclone, Milan, Italy), 0,5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 10 μg/mL insulin, thereafter indicated as differentiation medium (DMI). After 48 h, cells were maintained in DMEM containing 10% fetal bovine serum. The medium was changed every 2 days.

Transient transfection and luciferase assay

Sub-confluent 3T3-L1 cells were transfected with three pGIPZ shRNAmir EDF-1 (B5, H7, C5) (0.2 μg/cm^2) alone and in combination, or non-silencing pGIPZ shRNAmir (all from Open Biosystems, Celbio, Milan, Italy), in a 1:5 ratio with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in DMEM according to the manufacturer's instructions. Additional controls were performed: cells were transfected with the vector alone or with an unrelated shRNA (generated against an unrelated gene). Because the results were identical, we only show the non-silencing pGIPZ shRNA. After 3 h incubation, an equal volume of DMEM supplemented with 20% FBS was added to the transfection medium. The day after (day 0), transfection medium was removed and the cells were cultured in DMI for different times. At the end of the experiment, the cells were washed with PBS, scraped, and lysed in Laemmli sample buffer.

To study PPARγ activity, sub-confluent 3T3-L1 cells (day 1) were co-transfected with plasmids pDR1-Luc (0.2 μg/cm^2) (kindly provided by J.M. Zingg, Switzerland), pGIPZ shRNAmir EDF-1 (0.04, 0.1, or 0.2 μg/cm^2) or non-silencing pGIPZ shRNAmir (0.2 μg/cm^2) or pcDNA4.1/His Max A EDF-1 wt (0.04, 0.1 or 0.2 μg/cm^2) in a 1:5 ratio with Lipofectamine 2000. Next, 24 h later, the cells were treated with DMI or in the presence of 15-deoxy-delta (12,14)-prostaglandin J2 (15PGJ2) (10 μM). To increase PPARγ levels in undifferentiated 3T3-L1, cells treated with 15PGJ2 were also transfected with pcDNA3.1 myc-His-A PPARγ (Addgene plasmid 8895, Cambridge, MA, USA) (0.2 μg/cm^2). The luciferase activity was measured 2, 4, and 8 days after treatment with 15PGJ2 or DMI. The transfection efficiency was normalized against a co-transfected reporter plasmid phRL-TK (5 ng/cm^2) by dividing the firefly luciferase activity by the *Renilla* luciferase activity according to the Dual-Luciferase Reporter Assay kit manual (Promega, Madison, WI). Results are shown as the mean of the ratio of relative luciferase unit (RLU) versus the controls ± SD of three separate experiments.

Immunoprecipitation and western blot

Cells were lysed in 0.1 M Tris–HCl, 4% SDS, 20% glycerol, aprotinin 2 μg/ml, leupeptin 5 μg/ml, 1 mM PMSF. Cellular debris were pelleted by centrifugation at 13,000 rpm for 15 min at room temperature. For immunoprecipitation, cell lysates (1 mg) were incubated with 10 μg of primary antibody for 3 h at 4°C with rotation. Immunocomplexes were collected using 50 μl of protein A Sepharose, 4 Fast Flow (GE Healthcare, Milan, Italy) by rotating at 4°C overnight. After centrifugation (10,000 rpm for 1 min), immunoprecipitates were washed three times and resuspended in Laemmli sample buffer.

Western blot was performed on 3T3-L1 lysates or on nuclear and cytosolic fractions prepared as described [11, 12]. Proteins were separated on 12.5% SDS-PAGE, transferred to nitrocellulose sheets at 100 mA overnight, and probed with polyclonal anti-EDF-1 (Aviva Systems Biology, San Diego, CA), anti-PPARγ 1-2, anti-retinoid X receptor (RXR)α, anti-actin, anti-TATA binding protein (TBP) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Tebu-bio, Magenta, Italy). The purified mouse anti-6X His is from BD Pharmingen (San José, CA, USA). Secondary antibodies were labeled with horseradish peroxidase (GE Healthcare). The SuperSignal

chemiluminescence kit (Pierce, Rockford, IL) was used to detect immunoreactive proteins. All the experiments were repeated at least three times with comparable results.

Densitometry was performed by the ImageJ software on 3–5 different blots and expressed using an arbitrary value scale. Results are shown as the mean ± standard deviation.

Immunofluorescence staining

3T3-L1 cells cultured on coverslips were fixed in PBS containing 3% paraformaldehyde and 2% sucrose (pH 7.6) for 10 min at room temperature. Cells were then washed and permeabilized with HEPES–Triton X-100 buffer (20 mM Hepes, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 0,5% Triton X-100). Cells were incubated at 37°C in 2% bovine serum albumin (BSA) in PBS for 40 min prior to incubation (30 min) with antibody anti-EDF-1 (10 μg/ml). After washing, EDF-1 was detected with FITC-labelled anti-rabbit secondary antibody (Sigma Aldrich, Milan, Italy). Coverslips were observed with a fluorescence microscopy (Olympus AX70) at 20× magnification.

Staining with rabbit non-immune IgGs did not yield any significant signal.

Oil Red O staining

Oil Red O (Sigma Aldrich) staining was performed as described in [17]. The cells were photographed by a phase-contrast microscope. For a quantitative assay, Oil Red O dye was extracted with isopropanol 100% and the absorbance was measured at 500 nm by spectrophotometry.

Reverse transcription PCR

Total RNA was extracted with TRIzol® (Invitrogen) according to the manufacturer's instructions. After quantification, equivalent amounts of total RNA were assayed by first strand cDNA synthesis using SuperScript II RT (Invitrogen). Primers for aP2 are the following: (5′-TGGTCACC ATCCGGTCAGAG-3′, 5′-GCCCCGCCATC TAGGGT TAT-3′); primers for GAPDH are the following: (5′-ATC ACCATC TTCCAGGAGCGAG-3′, 5′-GTTGTCATGGAT GACCTTGGCC-3′. Primers were synthesized by Primm srl (Milan, Italy). All PCRs were performed in the linear range of cycle number for each set of primers, and the corresponding products were analyzed by 1.5% agarose gel electrophoresis and revealed with ethidium bromide.

Statistical analysis

Statistical significance was determined using the Student's t test and set at P values less than 0.05. In the figures, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Results

EDF-1 is not modulated during 3T3-L1 differentiation in adipocytes

We first investigated whether the expression of EDF-1 is modulated during the differentiation of 3T3-L1 cells. Pre-adipocytes were treated with a hormonal cocktail to induce terminal differentiation to adipocytes [15]. The total amounts of EDF-1, and in parallel the levels of PPARγ, were evaluated in the cells before (day 0) and after exposure to the hormonal cocktail for 2, 4, and 8 days. Western blot revealed that EDF-1 was not modulated (Fig. 1, middle panel), while the levels of PPARγ increased and remained elevated up to day 8 after the treatment with the differentiation cocktail (upper panel).

Since we have demonstrated in endothelial cells that EDF-1 translocates from the cytosol to the nucleus upon stimulation [10, 11], we investigated the subcellular localization of EDF-1 in 3T3-L1 cells induced to differentiate. By immunofluorescence on 3T3-L1 cells before and 4 days after exposure to the hormonal cocktail, we show that EDF-1 was always nuclear-associated (Fig. 2a). This result was confirmed by western blot on nuclear and cytosolic fractions isolated from 3T3-L1 cells before and at day 2 or 4 after the differentiative stimulus. EDF-1 was mainly nuclear and its amounts did not change during differentiation (Fig. 2b), whereas PPARγ was undetectable at the beginning of the experiment and accumulated in the nucleus of 3T3-L1 cells, 2 and 4 days after the stimulation. TBP and GAPDH were used as controls of the quality of nuclear and cytosolic extracts, respectively.

EDF-1 interacts with PPARγ during adipocyte differentiation

Because EDF-1 has been shown to bind PPARγ [8], we evaluated EDF-1/PPARγ interactions in 3T3-L1 cells by co-immunoprecipitation. We therefore immunoprecipitated

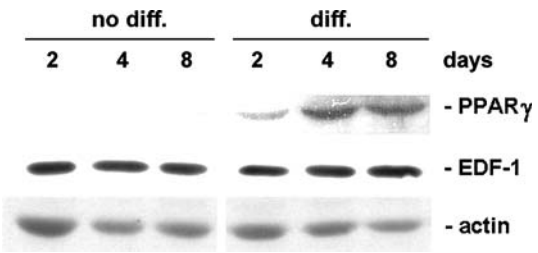


Fig. 1 EDF-1 is not modulated during 3T3-L1 differentiation in adipocytes. Cell lysates (70 μg) from 3T3-L1 cells induced to differentiate for different times were separated by SDS-PAGE and western blot was performed with antibodies against PPARγ and EDF-1. The blot was incubated with an anti-actin antibody to show that comparable amounts of protein were loaded per lane

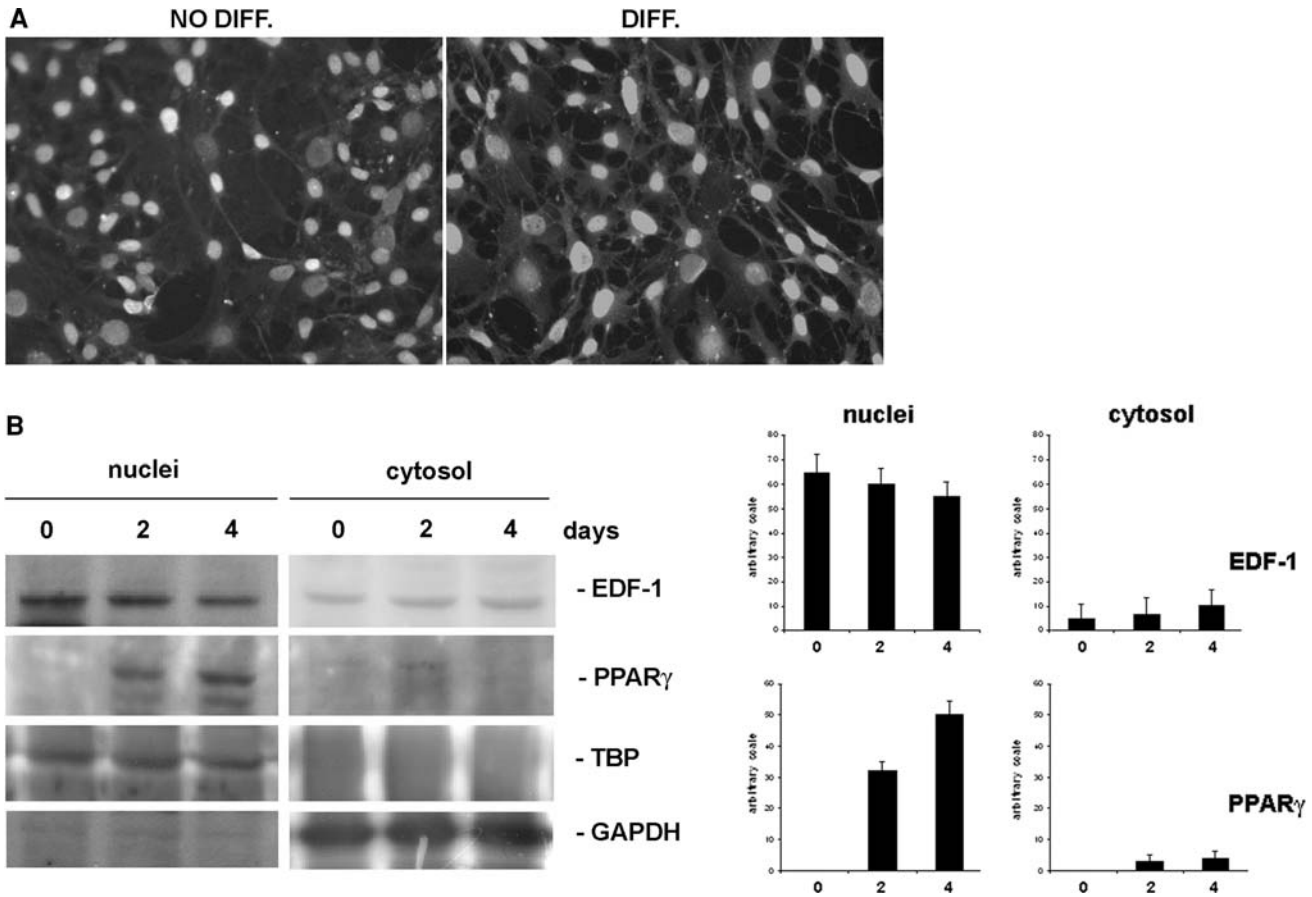


Fig. 2 Subcellular localization of EDF-1 does not change in differentiating 3T3-L1 cells. **a** 3T3-L1 cells were cultured on coverslips and induced to differentiate. After 4 days, the cells were fixed and stained with anti-EDF-1 antibodies, followed by FITC-labelled anti-rabbit IgGs (20× magnification). *NO DIFF* control, *DIFF* differentiating cells. **b** Nuclear and cytosolic extracts (60 μg) prepared from preadipocytes (day 0) and differentiated cells 2 and 4 days after the addition of the hormonal cocktail and their controls were separated on SDS-PAGE and processed for western blot with antibodies against EDF-1 and PPARγ. TBP and GAPDH were utilized as nuclear and cytosolic markers, respectively. *Right panel* shows the densitometric analysis (three different experiments): for cytosolic proteins, EDF-1/GAPDH and PPARγ/GAPDH ratio was determined; for nuclear proteins, EDF-1/TBP and PPARγ/TBP ratio was evaluated. Results are expressed as the mean ± standard deviation

PPARγ from 3T3-L1 cells treated or not with the differentiation cocktail for 2, 4, and 8 days and performed a western blot with the antibody against EDF-1. The protein complex PPARγ/EDF-1 was detectable in differentiating cells, whereas only a faint signal was visualized in undifferentiated cells (Fig. 3, upper panel). A staining with anti-PPARγ antibodies was used to measure the immunoprecipitated proteins (Fig. 3, lower panel). As expected, the amounts of immunoprecipitated PPARγ are enhanced under differentiating conditions. This event might explain why we detect the interactions between PPARγ and EDF-1 only in differentiating 3T3-L1 cells.

EDF-1 enhances PPARγ transcriptional activity in 3T3-L1 cells

To elucidate whether EDF-1 functions as a transcriptional co-activator of PPARγ in 3T3-L1 cells, we co-transfected subconfluent cells with a vector expressing luciferase under the control of a PPARγ responsive consensus (pDR1), with pcDNA3.1 myc-His-A PPARγ and with different concentrations of another vector expressing recombinant His-tagged EDF-1. After 24 h, the transfected cells were treated or not with 15PGJ2 (10 μM) for 48 h and luciferase activity was detected by fluorimetry. As shown in Fig. 4a, the ligand-dependent luciferase activity was stimulated in a dose-dependent manner by transfected His-tagged-EDF-1.

To demonstrate the relevance of EDF-1 in modulating PPARγ transcriptional activity in 3T3-L1 cells, we used a small hairpin RNA (shRNA) approach to inhibit EDF-1 synthesis. First, we transiently transfected subconfluent 3T3-L1 cells with specific shRNAs designed on three different target sequences on EDF-1 mRNA (named 1, 2 and 3) alone or in combination for 48 and 96 h. As a control, a non-silencing shRNA sequence was used. EDF-1 levels were dramatically reduced up to 96 h by the three shRNAs

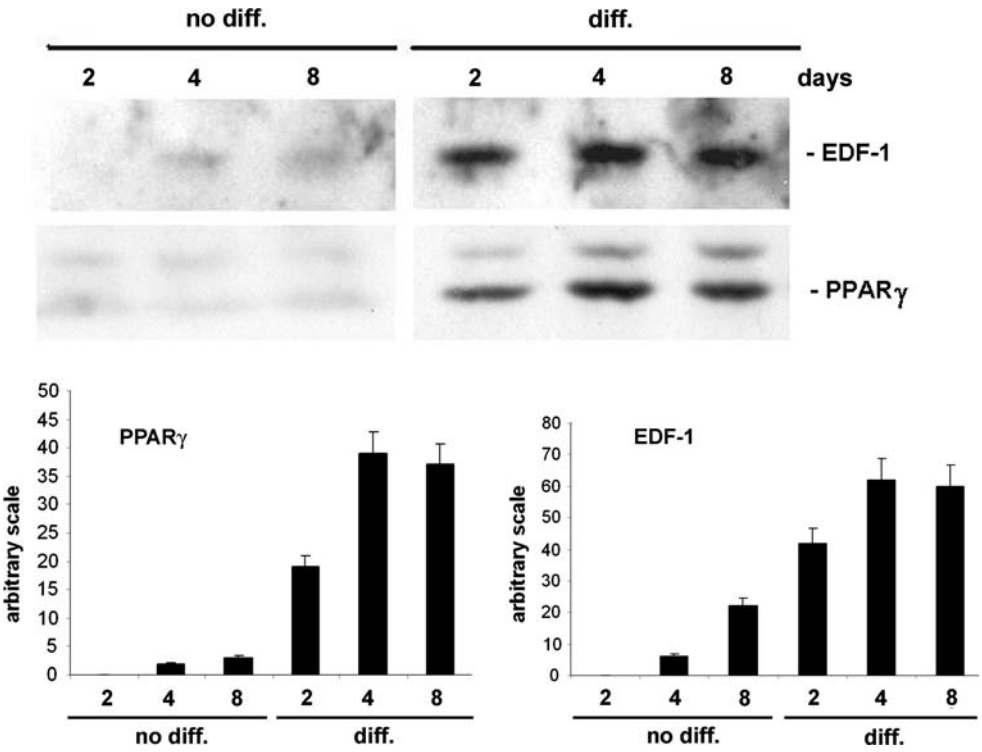


Fig. 3 EDF-1 interacts with PPARγ during adipocyte differentiation. 3T3-L1 cells were induced to differentiate for 2, 4, and 8 days. Cell extracts were immunoprecipitated with an antibody against PPARγ and western blot was performed with antibodies against EDF-1 (*upper panel*). The filter was then probed with anti-PPARγ antibodies to verify the equal amount of immunoprecipitated protein (*lower panel*). Densitometric analysis (five different experiments) was performed on the total amounts of EDF-1 and PPARγ. Results are expressed as the mean ± standard deviation

versus the control, but the higher efficiency was exhibited by the combination of the three (Fig. 4b, upper panel). To obtain the maximum silencing of EDF-1, we have therefore used the mixture of the three shRNAs in all the following experiments.

We then performed a luciferase assay in 3T3-L1 cells in the presence of shRNAs against EDF-1. We co-transfected subconfluent cells with pDR1, pcDNA3.1 myc-His-A PPARγ, and different amounts of shRNAs against EDF-1. After 24 h, the transfected cells were treated or not with 15PGJ2 for additional 48 h and luciferase activity was detected by fluorimetry. As shown in Fig. 4c, ligand-dependent luciferase induction was reduced by shRNAs in a dose-dependent fashion. These data suggest that EDF-1 is crucial to promote the transcriptional activity of PPARγ in 3T3-L1 cells.

EDF-1 is a positive regulator of adipogenesis

We then investigated whether EDF-1 plays a role in adipogenesis by co-activating PPARγ. We inhibited EDF-1 expression in differentiating 3T3-L1 by transfecting subconfluent cells with the shRNAs against EDF-1. By western blot, we show that the shRNAs against EDF-1 downregulated its levels during the differentiation up to day 4 and returned to the normal levels at day 8 (Fig. 5a). Since the most important events in differentiation take place within the first 2–4 days after the addition of the hormonal cocktail [16], this time frame of EDF-1 silencing is long enough to perform our studies. When pre-adipocytes differentiate in vitro, they accumulate lipid droplets in the cytosol which can be visualized by Oil Red O staining [17]. To explore the contribution of EDF-1 to adipogenesis, 3T3-L1 cells were transfected with anti-EDF-1 shRNAs and induced to differentiate with the hormonal cocktail up to day 8. 3T3-L1 cells transfected with a non-silencing shRNAs were used as negative controls. After staining with Oil Red O, we observed that the number of the lipid droplets was dramatically lower in differentiating cells which downregulated EDF-1 than controls (Fig. 5b) and this was confirmed by spectrophotometry (Fig. 5c).

These data indicate that EDF-1 expression is required in this model of adipogenesis.

The expression of PPARγ is partially dependent on EDF-1

The expression of PPARγ is sustained by several positive feedback mechanisms in differentiation processes [1–4]. We therefore examined the possibility that a reduction of the transcriptional activity of PPARγ due to a loss of EDF-1 could trigger also a dowregulation of PPARγ expression. We analyzed the level of PPARγ by western blot in differentiating 3T3-L1 at day 0, 2, 4 and 8 from the exposure to the hormonal cocktail, in the presence of shRNAs against EDF-1 or a non silencing shRNA. As shown in Fig. 5d, the shRNA-dependent inhibition of EDF-1

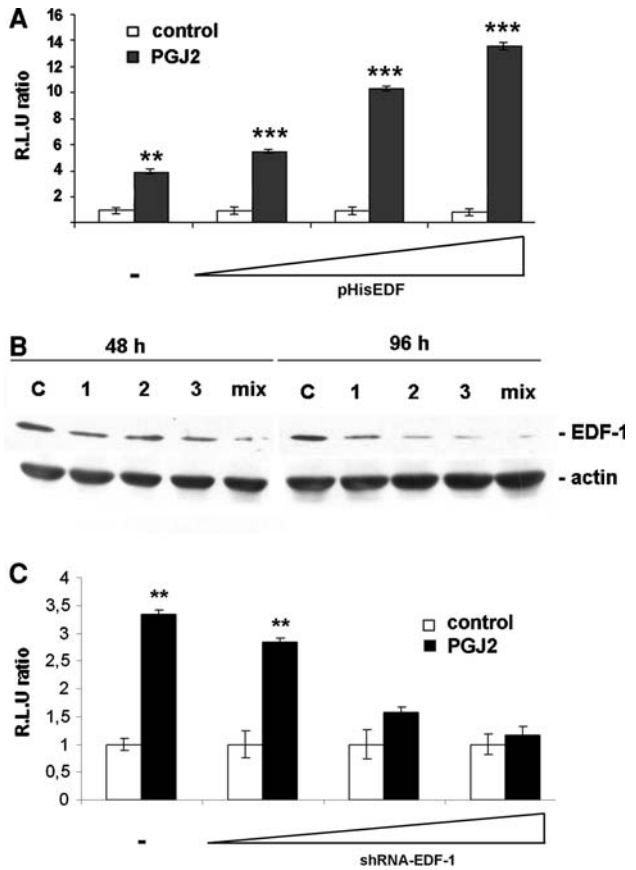


Fig. 4 EDF-1 enhances PPARγ transcriptional activity in 3T3-L1 cells. **a** Subconfluent 3T3-L1 cells were co-transfected with pDR1, with pcDNA-PPARγ (both 0.2 μg/cm^2) and with another vector expressing recombinant His-tagged EDF-1(0.2, 0.1 or 0.04 μg/cm^2). After 24 h, the transfected cells were treated or not with 15-dPGJ2 for 48 h and luciferase activity was detected by fluorimetry. Results are shown as the mean ± standard deviation (**$P < 0.01$, ***$P < 0.001$). **b** 3T3-L1 cells were transiently transfected with shRNAs (0.2 μg/cm^2) designed on three different target sequences on EDF-1 mRNA (named 1, 2, and 3) alone or in combination (mix) for 48 and 96 h. A non-silencing shRNA sequence was used as a control (C). Western blot was performed on cell extracts (70 μg) using anti-EDF-1 antibodies. The filter was then probed for actin to show that comparable amounts of protein were loaded per lane. **c** Luciferase assay was performed as described in cells incubated with different concentrations of the combination of the three shRNAs against EDF-1(0.04, 0.1 or 0.2 μg/cm^2) or a non-silencing shRNA (0.2 μg/cm^2) in the presence or in the absence of 15-dPGJ2. Results are shown as the mean ± standard deviation (**$P < 0.01$)

synthesis correlated with the down regulation of PPARγ, suggesting that EDF-1 has a role in regulating the amounts of PPARγ.

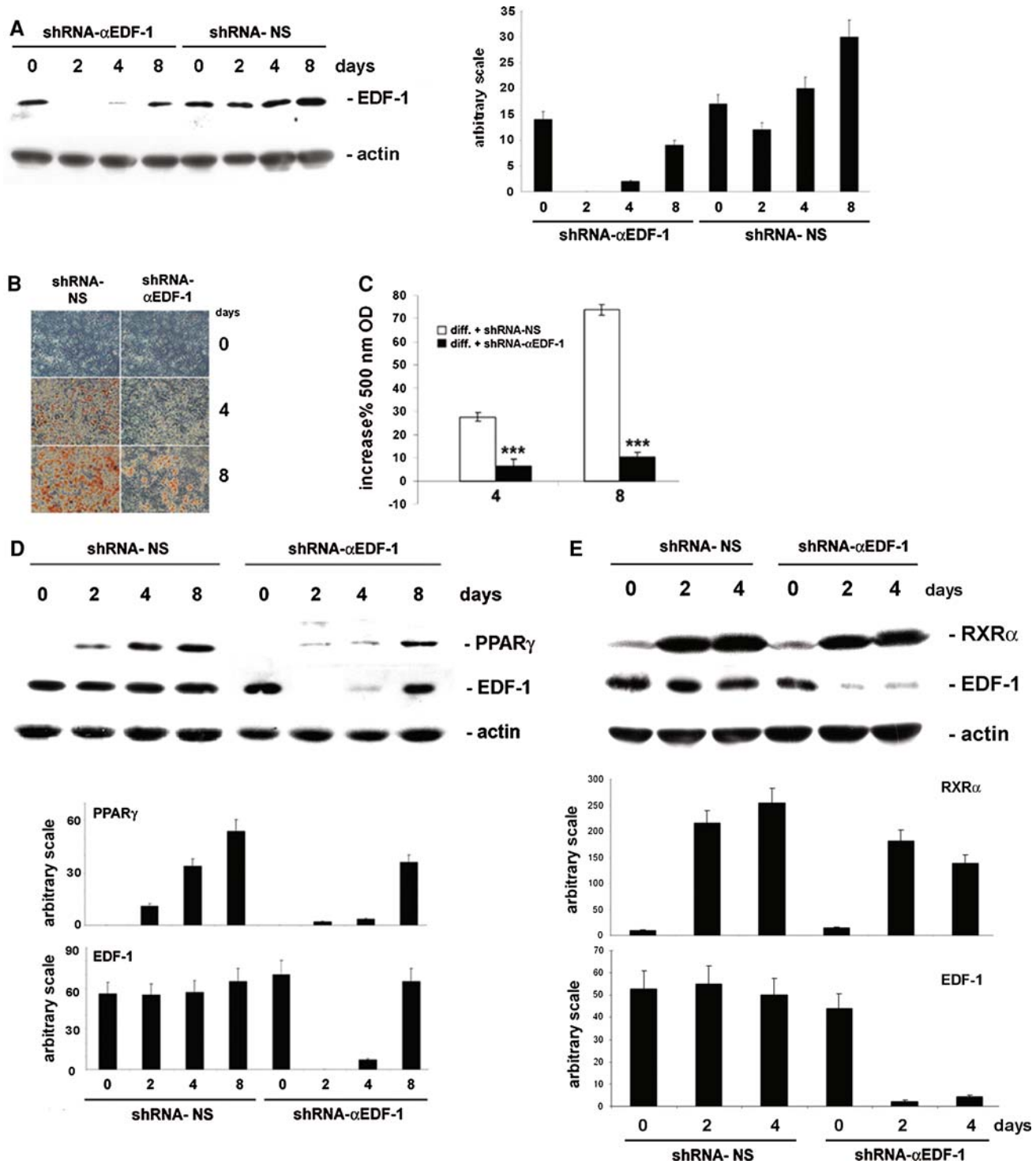
Because (1) PPARγ forms heterodimers with RXRα [18], which also interacts with EDF1 [8] and (2) RXRα is expressed in the adipose tissue and is essential for lipogenesis [18], we evaluated whether silencing EDF-1 might impact on the amounts of RXRα. We show that the total amounts of RXRα are similar in cells silencing EDF-1 and their controls and that RXRα is induced upon 3T3-L1 differentiation at day 2 and 4 from the exposure to the hormonal cocktail independently from the expression of EDF-1 (Fig. 5e).

Fig. 5 shRNAs against EDF-1 inhibits 3T3-L1 differentiation in adipocytes and PPARγ expression. **a** 3T3-L1 cells were transiently transfected with the combination of the three shRNAs against EDF-1 (shRNA-αEDF-1) or with a non silencing shRNA (shRNA-NS) (0.2 μg/cm^2) and induced to differentiate up to 8 days. Western blot was performed on cell extracts (70 μg) using anti-EDF-1 antibodies. The filter was then probed with antibodies anti-actin to show that comparable amounts of protein were loaded per lane. *Right panel* EDF-1/actin ratio (four different experiments) was determined by densitometry. Results are expressed as the mean ± standard deviation. **b** 3T3-L1 cells transfected as in (**a**) were stained with Oil Red O after 4 and 8 days of differentiation and photographed (20× magnification). **c** Oil Red O was extracted with isopropanol and the absorbance measured at 500 nm. Data are shown as the mean ± standard deviation (***$P < 0.001$). **d** 3T3-L1 cells were induced to differentiate by exposure to the hormonal cocktail in the presence of the three shRNAs against EDF-1 or a non-silencing shRNA (0.2 μg/cm^2). Cell extracts were prepared at day 0, 2, 4, and 8 and utilized for western blot with antibodies against PPARγ and EDF-1. Actin was used as a control of loading. Densitometry was performed to determine EDF-1/actin (*upper panel*) and PPARγ/actin ratio (*lower panel*) (three different experiments). Results are expressed as the mean ± standard deviation. **e** 3T3-L1 cells were induced to differentiate as described above in the presence of shRNAs against EDF-1 or a non-silencing shRNA. Cell extracts were prepared at day 0, 2, and 4 and utilized for western blot with antibodies against RXRα. Actin was used as a control of loading. Densitometry was performed to determine RXRα/actin (*upper panel*) and EDF-1/actin ratio (*lower panel*) (three different experiments). Results are expressed as the mean ± standard deviation

The transcriptional activity of PPARγ is decreased in differentiating 3T3-L1 cells lacking EDF-1

Since (1) the transcriptional activity of PPARγ on target genes is crucial in adipocyte differentiation and (2) EDF-1 is a transcriptional coactivator for PPARγ, we investigated whether EDF-1 has a role in adipogenesis by acting as a transcriptional coactivator of PPARγ. We co-transfected subconfluent 3T3-L1 cells with pDR1 and with shRNAs against EDF-1 or non-silencing shRNA. After 12 h, we induced differentiation and measured the luciferase activity by fluorimetry at day 0 and 2, 4, and 8. Silencing EDF-1 significantly reduced the transcriptional activity of PPARγ when 3T3-L1 cells differentiate (Fig. 6a). This is evident until day 4. At day 8, the transcriptional activity is comparable in transfected cells and their controls because, as shown above (Fig. 5a), EDF-1 is not silenced any longer.

Peroxisome proliferator-activated receptor-γ promotes adipogenesis activating several genes involved in the differentiation process at the transcriptional level. We therefore analyzed whether an EDF-1-dependent reduction of PPARγ transcriptional activity affected the activation of downstream target genes. A semi-quantitative RT-PCR to detect aP2, a PPARγ target gene, was performed on 3T3-L1 cells



differentiated in the presence of shRNA against EDF-1 or non silencing shRNA. In cells silencing EDF-1, the transcriptional activation of aP2 gene was inhibited (Fig. 6b), suggesting that EDF-1 regulates adipogenesis by acting on the transcriptional activation of PPARγ-dependent genes.

The inhibition of adipogenesis in 3T3-L1 silencing EDF-1 could be due to the downregulation of PPARγ (Fig. 5d). To investigate this possibility, we overexpressed PPARγ in EDF-1-knocked down 3T3-L1 cells during the differentiation. As shown in Fig. 6c, the absence of EDF-1 also inhibits PPARγ transcriptional activity in cells overexpressing PPARγ. The overexpression of PPARγ in EDF-1-knocked down 3T3-L1 cells was demonstrated by western blot using anti-His antibodies (Fig. 6d).

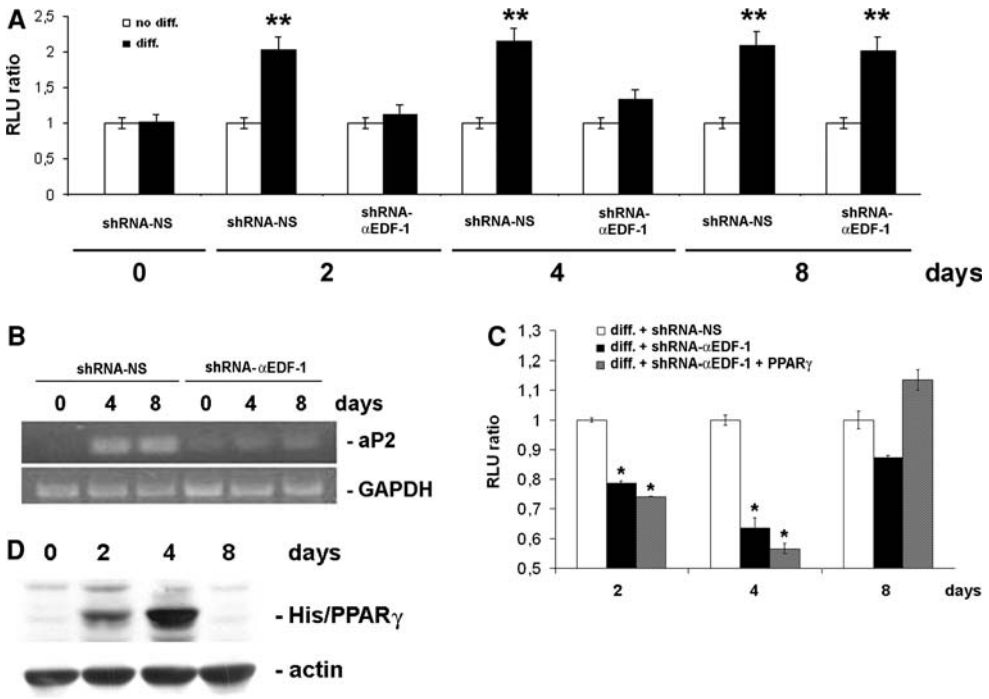


Fig. 6 shRNA against EDF-1 inhibits PPARγ trancriptional activity in 3T3-L1 cells induced to differentiate. **a** Subconfluent 3T3-L1 cells were co-transfected with pDR1 (0.2 μg/cm^2) and with the three shRNAs against EDF-1 or a non-silencing shRNA (both 0.2 μg/cm^2). After 12 h, differentiation was induced and the luciferase activity was measured by fluorimetry at day 0, 2, 4 and 8. Data are shown as the mean ± standard deviation (**$P < 0.01$). **b** RNA was extracted from 3T3-L1 cells transfected with the three shRNAs against EDF-1 or a non-silencing shRNA. aP2 expression was evaluated by RT-PCR. GAPDH was utilized to control the loading. **c** 3T3-L1 cells were co-transfected with pcDNA myc-His PPARγ and the three shRNAs against EDF-1 or a non-silencing shRNA. Luciferase activity was measured by fluorimetry 2, 4, and 8 days of differentiation. Data are shown as the mean ± standard deviation (*$P < 0.05$). **d** Extracts were prepared 2, 4, and 8 days after co-transfection with pGIPZ shRNAmir EDF-1 and pcDNA myc-His PPARγ. Western analysis using anti-His antibodies was performed. The blot was incubated with an anti-actin antibody to show that comparable amounts of protein were loaded per lane

EDF-1 could therefore promote adipogenesis by maintaining high levels of PPARγ expression through a positive feedback mechanism and by enhancing PPARγ transcriptional activity on adipogenic genes.

Discussion

The function of EDF-1 as a transcriptional coactivator was first described in *Bombyx mori* in which EDF-1 induces the activity of the Ftz-F1 nuclear receptor [19]. Since then, EDF-1 has been shown to specifically interact through its central domain (amino acids 37–113) with several transcription factors, among which some nuclear receptors implicated in lipid metabolism, including PPARγ. In particular, EDF-1 interacts with the DE domain of PPARγ [8]. PPARγ is necessary for adipocyte differentiation from preadipocytes [1, 2]. Indeed, forced expression of PPARγ and/or administration of potent PPARγ agonists promote adipose conversion of fibroblasts and myoblasts [20, 21]. We therefore anticipated a possible role of EDF-1 in modulating PPARγ activity and, consequently, in regulating adipogenesis.

We first evaluated whether the differentiation of 3T3-L1 cells was associated with the modulation of EDF-1 levels. The amounts of EDF-1 did not vary when 3T3-L1 were induced to differentiate, while, as previously demonstrated [22], PPARγ was markedly upregulated. Accordingly, we did not observe any modulation of EDF-1 in the differentiation of other cell types, such as PC12 into neural cells, NT2D1 into teratocarcinoma cells, and L6 into myocytes, as well as in U937 cell differentiation (Mariotti, unpublished results). At the moment, the modulation of EDF-1 expression seems to be specific for endothelial differentiation [9].

Another difference between endothelial and 3T3-L1 cells resides in the different subcellular localization of EDF-1. We found that EDF-1 was mainly nuclear in 3T3-L1 cells, whether differentiated or not. On the contrary, in endothelial cells, EDF-1 was both in the cytosol, where it interacts with calmodulin, and in the nucleus, where it exerts its function as a transcriptional coactivator [10–12]. Interestingly, in these cells, EDF-1 was promptly nuclear translocated upon activation of the protein kinases C and A [10, 11].

EDF-1, which is not importantly modulated in the differentiation of 3T3-L1 cells, interacts with PPARγ in these cells, and this interaction is increased during differentiation, probably because of the availability of higher amounts of PPARγ. The biological impact of this binding is underscored by the evidence that EDF-1 stimulated ligand-dependent PPARγ transcriptional activity in a dose-dependent fashion. Indeed, silencing EDF-1 inhibited ligand-dependent transcriptional activity in cells induced to differentiate. These results indicate that EDF-1 has a role in promoting PPARγ transcriptional activity in 3T3-L1 cells. Interestingly, silencing EDF-1 correlates with the reduction of PPARγ levels, an event which might explain why PPARγ transcriptional activity is inhibited in EDF-1 negative cells. To this purpose, it should be underscored that EDF-1-dependent regulation of PPARγ levels seems to be specific for 3T3-L1, since in other cell types, including endothelial cells silencing EDF-1, the total amounts of PPARγ are maintained constant (Leidi, submitted). Because EDF-1 does not induce transcription by itself when tethered to a promoter, we can exclude a direct role of EDF-1 in modulating PPARγ amounts [8, and data not shown]. However, since EDF-1 interacts with different bZIP DNA-binding proteins [14], it is likely that silencing EDF-1 might impair the activity of some transcription factors involved in PPARγ regulation. Alternatively, it should be recalled that PPARγ and the CCAAT/enhancer-binding protein (C/EBPα) are central in the network of factors which orchestrate the many functions associated with the mature adipocyte [1]. Indeed, PPARγ activates EBPα which, in turn, induces PPARγ by a positive feedback [23]. It is therefore possible that the silencing of EDF-1 ultimately might lead to an impairment of this molecular crosstalk. It is also possible that, in the absence of EDF-1, the stability of PPARγ is reduced. More experiments are required to understand the mechanisms involved in PPARγ downregulation in our experimental model of adipogenesis.

Because PPARγ forms heterodimers with RXRα which is also induced during adipocyte differentiation [24], it is noteworthy that no modulation of RXRα has been observed in differentiating 3T3-L1 cells whether in the presence or not of shRNAs against EDF-1.

In spite of the mechanism involved in reducing the levels of PPARγ, it is noteworthy that we could not rescue its transcriptional activity even after overexpressing it in cells silencing EDF-1. These results indicate that PPARγ requires EDF-1 to activate the transcriptional program necessary for adipogenesis. Indeed, silencing EDF-1 markedly retards 3T3-L1 cell differentiation into adipocytes, as detected by Oil Red O staining of intracellular triglycerides, changes in morphology, and the expression of the fat-specific protein aP2.

EDF-1 also stimulates PPARγ transcriptional activity. Since EDF-1 directly interacts with the TFIID complex [8], which consists of TBP and TBP-association factors [17], we hypothesize that EDF-1 might bridge between PPARγ and TFIID, thus increasing PPARγ transcriptional activity.

In conclusion, this report provides evidence that EDF-1 facilitates PPARγ-mediated differentiation and gene expression programs in 3T3-L1 cells. The identification of EDF-1 as a novel player in modulating PPARγ activity and expression might have clinical implications since PPARγ has a role in the pathogenesis of several diseases such as obesity, lipodystrophy, metabolic syndrome, and type II diabetes, which affect a large part of western population and have high costs to society.

References

1. Rosen ED, Walkey CJ, Puigserver P, Spiegelman BM (2000) Transcriptional regulation of adipogenesis. Genes Dev 14:1293–1307
2. Gurnell M (2005) Peroxisome proliferator-activated receptor gamma and the regulation of adipocyte function: lessons from human genetic studies. Best Pract Res Clin Endocrinol Metab 19:501–523
3. Knouff C, Auwerx J (2004) Peroxisome proliferator-activated receptor-gamma calls for activation in moderation: lessons from genetics and pharmacology. Endocr Rev 25:899–918
4. Evans RM, Barish GD, Wang YX (2004) PPARs and the complex journey to obesity. Nat Med 10:355–361
5. Rosen ED, Spiegelman BM (2001) PPARgamma: a nuclear regulator of metabolism, differentiation, and cell growth. J Biol Chem 276:37731–37734
6. Lehrke M, Lazar MA (2005) The many faces of PPARgamma. Cell 123:993–999
7. Rosenfeld MG, Lunyak VV, Glass CK (2005) Sensors and signals: a coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response. Genes Dev 20:1405–1428
8. Brendel C, Gelman L, Auwerx J (2002) Multiprotein bridging factor-1 (MBF-1) is a cofactor for nuclear receptors that regulate lipid metabolism. Mol Endocrinol 16:1367–1377
9. Dragoni I, Mariotti M, Consalez G, Soria M, Maier JAM (1998) EDF-1, a novel gene product downregulated in human endothelial cell differentiation. J Biol Chem 273:31119–31124
10. Mariotti M, Avon E, De Benedictis L, Maier JAM (2000) Interaction between endothelial differentiation-related factor-1 and calmodulin in vitro and in vivo. J Biol Chem 275:24047–24051
11. Ballabio E, Mariotti M, De Benedictis L, Maier JAM (2004) The dual role of EDF-1 in the cytosol and in the nucleus: modulation by PKA. Mol Cell Life Sci 61:1069–1074
12. Bernardini D, Mariotti M, Ballabio E, Maier JAM (2005) Differential expression of EDF-1 and endothelial nitric oxide synthase by proliferating, quiescent and senescent microvascular endothelial cells. Biochim Biophys Acta Mol Cell Res 1745:265–272
13. Mishima M, Ozaki J, Ikegami T, Kabe Y, Goto M, Ueda H, Hirose S, Handa H, Shirakawa M (1999) Resonance assignments, secondary structure and 15 N relaxation data of the human

transcriptional coactivator hMBF1 (57–148). J Biomol NMR 14:373–376
14. Miotto B, Struhl K (2006) Differential gene regulation by selective association of transcriptional coactivators and bZIP DNA-binding domains. Mol Cell Biol 26:5969–5982
15. Stephens JM, Lee J, Pilch PF (1997) Tumor necrosis factor-alpha-induced insulin resistance in 3T3–L1 adipocytes is accompanied by a loss of insulin receptor substrate-1 and GLUT4 expression without a loss of insulin receptor-mediated signal transduction. J Biol Chem 272:971–976
16. Tzameli I, Fang H, Ollero M, Shi H, Hamm JK, Kievit P, Hollenberg AN, Flier JS (2004) Regulated production of a peroxisome-proliferator activated receptor-γ ligand during an early phase of adipocyte differentiation in 3T3–L1 adipocyte. J Biol Chem 279:1093–1102
17. Sentinelli F, Filippi E, Cavallo MG, Romeo S, Fanelli M, Baroni MG (2006) The G972R variant of the insulin receptor substrate-1 gene impairs insulin signaling and cell differentiation in 3T3–L1 adipocytes; treatment with a PPARgamma agonist restores normal cell signaling and differentiation. J Endocrinol 188(2): 271–285
18. Metzger D, Imai T, Jiang M, Takukawa R, Desvergne B, Wahli W, Chambon P (2005) Functional role of RXRs and PPAR gamma in mature adipocytes. Prostaglandins Leukot Essent Fatty Acids 73(1):51–58
19. Takemaru K, Li FQ, Ueda H, Hirose S (1997) Multiprotein bridging factor 1 (MBF1) is an evolutionarily conserved transcriptional coactivator that connects a regulatory factor and TATA element-binding protein. Proc Natl Acad Sci USA 94:7251–7256
20. Wu Z, Xie Y, Bucher NL, Farmer SR (1995) Conditional ectopic expression of C/EBP beta in NIH-3T3 cells induces PPAR gamma and stimulates adipogenesis. Genes Dev 9:2350–2363
21. Hu E, Tontonoz P, Spiegelman BM (1995) Transdifferentiation of myoblasts by the adipogenic transcription factors PPAR gamma and C/EBP alpha. Proc Natl Acad Sci USA 92:9856–9860
22. Albright SR, Tjian R (2000) TAFs revisited: more data reveal new twists and confirm old ideas. Gene 242:1–13
23. Fox KE, Fankell DM, Erickson PF, Majka SM, Crossno JT Jr, Klemm DJ (2006) Depletion of cAMP-response element-binding protein/ATF1 inhibits adipogenic conversion of 3T3–L1 cells ectopically expressing CCAAT/enhancer-binding protein (C/EBP) alpha, C/EBP beta, or PPAR gamma 2. J Biol Chem 281:40341–40353
24. Takahashi Y, Inoue J, Kagechika H, Sato R (2009) ApoC-III gene expression is sharply increased during adipogenesis and is augmented by retinoid X receptor (RXR) agonists. FEBS Lett 583:493–497